The **University** *of* **Western Ontario**
BIOLOGICAL AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: July 8, 2011
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario (UWO) or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biological agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biological agents being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Electronically completed forms are to be submitted to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190 or to jstanle2@uwo.ca) for distribution to the Biohazards Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety.

Please ensure that all questions are fully and clearly answered. Failure to do so will lead to the form being returned, which will cause delays in your approval and frustration for you and your colleagues on the Committee.

If you are re-submitting this form as requested by the Biohazards Subcommittee, please make modifications to the form in bold print, highlighted in yellow. Please re-submit forms electronically.

PRINCIPAL INVESTIGATOR:	**Ray Zabulionis**
DEPARTMENT:	**Biology**
ADDRESS:	**Rm 342 NCB**
PHONE NUMBER:	**86475**
EMERGENCY PHONE NUMBER(S):	**519-473-2764**
EMAIL:	**rayzab@uwo.ca**

Location of experimental work to be carried out :

Building :	**NCB**	Room(s):	**325, 329, 330, 331**
Building :	**NCB**	Room(s):	**337**
Building :		Room(s):	

***For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 15.0, Approvals).**

FUNDING AGENCY/AGENCIES:
GRANT TITLE(S):
UNDERGRADUATE COURSE NAME(IF APPLICABLE): **Bio 2290 F/G**

List all personnel working under Principal Investigators supervision in this location:

Name	UWO E-mail Address	Date of Biosafety Training
Rob Dean	**rdean1@uwo.ca**	**July, 2005**
Irene Krajnyk	**ikrajnyk@uwo.ca**	**July, 2006**
Jeni Duro	**mduro@uwo.ca**	**May, 2005**
Liz Ross	**hross@uwo.ca**	**October, 2000**
Ray Zabulionis	**rayzab@uwo.ca**	**May, 2005**
Winona Gadapati	**wgadapa2@uwo.ca**	**May, 2005**

Patrtick McDonald	pmcdona5@uwo.ca	September, 2011
Sryia Peiris	speiris2@uwo.ca	September, 2011

Please explain how the biological agents are used in your project and how they are stored and disposed of. The BARF without this description will not be reviewed.

Bacteria, fungi, and yeast are used by Bio 2290F/G students to conduct various experiments including cell growth patterns, antibiotic resistance, exposure to herbs and spices, transduction, transformation with plasmids, conjugation, ultra-violet light exposure, and heavy metal toxicity studies.

Bacteria are stored as glycerol stocks in Eppendorf tubes at -80C while fungi and yeast are stored on sealed Petri dishes at 4C. After use, all micro-organisms are destroyed by autoclaving; following confirmation of destruction, organisms are disposed in the regular garbage.

Spinach (Spinacia oleracea) leaves are obtained from local grocery stores and used to study chloroplast function. Chloroplasts are from leaves which are discarded in the regular garbage and the chloroplasts are resuspended in dichlorophenol indophenol (DCPIP). After the studies, the material is diposed in hazardous waste.

Canola (Brassica napus) are grown from seeds in the Biology lower greenhouses and used in plant density growth studies. Plants are thrown out in the regular garbage.

Please include a ONE page research summary or teaching protocol in lay terms.
Forms with summaries more than one page will not be reviewed.

See Appendix 1.

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ☒ YES ☐ NO
(non-pathogenic and pathogenic biological agents including but not limited to bacteria and other microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)? If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? ☐ YES ☒ NO
If YES, please give the name of the species ____________________
What is the origin of the microorganism(s)? ____________________

Please describe the risk (if any) of escape and how this will be mitigated:

Please attach the CFIA permit.

Please describe any CFIA permit conditions:

1.2 Please complete the table below:

Full Scientific Name of Biological Agent(s)* (Be specific)	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3

****Please attach a Material Safety Data Sheet or equivalent from the supplier if the bacterium used is not on this link:***
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

Additional Comments: **Please see Appendix 2, 3, 4 and 5.**

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ☐ YES ☒ NO
(If NO, please proceed to Section 3.0)

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture:

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	☐ Yes ☐ No		Not applicable
Rodent	☐ Yes ☐ No		
Non-human primate	☐ Yes ☐ No		
Other (specify)	☐ Yes ☐ No		

2.3 Please indicate the type of established cells that will be grown in culture in:

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Containment Level of each cell line	Supplier / Source of cell line(s)
Human	☐ Yes ☐ No			
Rodent	☐ Yes ☐ No			
Non-human primate	☐ Yes ☐ No			
Other (specify)	☐ Yes ☐ No			

****Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)***

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required ☐ 1 ☐ 2 ☐ 2+ ☐ 3

Additional Comments: ____________________

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ☐ YES ☒ NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Infected With An Infectious Agent? YES/UNKNOWN**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Blood (fraction) or other Body Fluid		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Organs or Tissues (unpreserved)		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Organs or Tissues (preserved)		Not Applicable		Not Applicable

Additional Comments: ____________________

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? ☒ YES ☐ NO If **NO**, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? ☒ YES, complete table below ☐ NO

Bacteria Used for Cloning *	Plasmid(s) **	Source of Plasmid	Gene Transformed or Transfected	Will there be a change due to transformation of the bacteria?	Will there be a change in the pathogenicity of the bacteria after the genetic modification?	What are the consequences due to the transformation of the bacteria?
see Appendix 6 and 7						

**** Please attach a Material Safety Data Sheet or equivalent if available.***
***** Please attach a plasmid map.***
******No Material Safety Data Sheet is required for the following strains of E. coli:***
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

4.3 Will genetic modification(s) of bacteria and/or cells involving viral vectors be made?
☒ YES, complete table below ☐ NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results from transduction
P1vir: see Appendix 8	**P1vir**	**Dr. Volvano - UWO**	**tetracycline resistance gene**	**E. coli becomes tetracycline resistant.**

**** Please attach a Material Safety Data Sheet or equivalent.***

4.3.1 Will virus be replication defective? ☐ YES ☒ NO

4.3.2 Will virus be infectious to humans or animals? ☐ YES ☒ NO

4.3.3 Will this be expected to increase the containment level required? ☐ YES ☒ NO

5.0 Will genetic sequences from the following be involved?
- HIV ☒ NO ☐ YES, specify
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens ☒ NO ☐ YES, specify
- SV 40 Large T antigen ☒ NO ☐ YES
- E1A oncogene ☒ NO ☐ YES
- Known oncogenes ☒ NO ☐ YES, specify
- Other human or animal pathogen and or their toxins ☒ NO ☐ YES, specify

5.1 Is any work being conducted with prions or prion sequences? ☒ NO ☐ YES

Additional Comments: ______________________________

6.0 Human Gene Therapy Trials

6.1 Will human clinical trials be conducted involving a biological agent? ☐ YES ☒ NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 6.0

6.2 If YES, please specify which biological agent will be used:
Please attach a full description of the biological agent.

6.3 Will the biological agent be able to replicate in the host? ☐ YES ☐ NO

6.4 How will the biological agent be administered?

6.5 Please give the Health Care Facility where the clinical trial will be conducted:

6.6 Has human ethics approval been obtained? ☐ YES, number: ☐ NO ☐ PENDING

7.0 Animal Experiments

7.1 Will live animals be used? ☐ YES ☒ NO If **NO**, please proceed to section 7.0

7.2 Name of animal species to be used

7.3 AUS protocol #

7.4 Will any of the agents listed in section 4.0 be used in live animals
☐ NO ☐ YES, specify:

7.5 Will the agent(s) be shed by the animal:
☐ YES ☐ NO, please justify:

8.0 Use of Animal species with Zoonotic Hazards

8.1 Will any animals with zoonotic hazards or their organs, tissues, lavages or other body fluids including blood be used (see list below)? ☐ YES ☒ NO - If **NO**, please proceed to section 8.0

8.2 Will live animals be used? ☐ YES ☐ NO

8.3 If **YES**, please specify the animal(s) used:

- Pound source dogs ☐ YES ☐ NO
- Pound source cats ☐ YES ☐ NO
- Cattle, sheep or goats ☐ YES, species ☐ NO
- Non-human primates ☐ YES, species ☐ NO
- Wild caught animals ☐ YES, species & colony # ☐ NO
- Birds ☐ YES, species ☐ NO
- Others (wild or domestic) ☐ YES, specify ☐ NO

8.4 If no live animals are used, please specify the source of the specimens:

9.0 Biological Toxins and Hormones

9.1 Will toxins or hormones of biological origin be used? ☐ YES ☒ NO If no, please proceed to Section 9.0

9.2 If YES, please name the toxin(s) or hormones(s)
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

9.3 What is the LD_{50} (specify species) of the toxin or hormone

9.4 How much of the toxin or hormone is handled at one time*?

9.5 How much of the toxin or hormone is stored*?

9.6 Will any biological toxins or hormones be used in live animals? ☐ YES ☐ NO
If **YES**, Please provide details:

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

Additional Comments: ______________________________

10.0 Insects

10.1 Do you use insects? ☐ YES ☒ NO - If **NO**, please proceed to Section 10.0

10.2 If YES, please give the name of the species.

10.3 What is the origin of the insect?

10.4 What is the life stage of the insect?

10.5 What is your intention? ☐ Initiate and maintain colony, give location:
☐ "One-time" use, give location:

10.6 Please describe the risk (if any) of escape and how this will be mitigated:

10.7 Do you use insects that require a permit from the CFIA permit? ☐ YES ☐ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

11.0 Plants

11.1 Do you use plants? ☒ YES ☐ NO - If **NO**, please proceed to Section 11.0

11.2 If YES, please give the name of the species. **Spinacia oleracea (spinach) and Brassica napus (ca**

11.3 What is the origin of the plant? **S. oleracea - grocery store. B. napus - UWO greenh**

11.4 What is the form of the plant (seed, seedling, plant, tree…)? **leaves and whole plants**

11.5 What is your intention? ☐ Grow and maintain a crop ☒ "One-time" use

11.6 Do you do any modifications to the plant? ☐ YES ☒ NO
If yes, please describe:

11.7 Please describe the risk (if any) of loss of the material from the lab and how this will be miti

greenhouse

11.8 Is the CFIA permit attached? ☐ YES ☒ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

12.0 Import Requirements

12.1 Will any of the above agents be imported? ☐ YES, country of origin ☒ NO
If **NO**, please proceed to Section 12.0

12.2 Has an Import Permit been obtained from HC for human pathogens? ☐ YES ☐ NO

12.3 Has an import permit been obtained from CFIA for animal or plant pathogens? ☐ YES ☐ NO

12.4 Has the import permit been sent to OHS? ☐ YES, please provide permit # ☐ NO

13.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biological agents in Sections 1.0 to 9.0 have been trained.

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name **Ray Zabulionis** ***Date:*** **September 26/11**

14.0 Containment Levels

14.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. ☒ 1 ☐ 2 ☐ 2+ ☐ 3

14.2 Has the facility been certified by OHS for this level of containment?
☐ YES, location and date of most recent biosafety inspection:
☐ NO, please certify
☒ NOT REQUIRED for Level 1 containment

14.3 Please indicate permit number (not applicable for first time applicants): **Bio-UWO-0226**

15.0 Procedures to be Followed

15.1 Are additional risk reduction measures necessary beyond containment level 1, 2, 2+ or 3 measures that are unique to these agents? ☐ YES ☒ NO
If **YES** please describe:

15.2 Please outline what will be done if there is an exposure to the biological agents listed such as a needlestick injury or an accidental splash:
We do not use needles in the student labs. If a nick or cut does occur, isopropanol swab the wound and send to emergency if severe. Splash—eye wash stations are in each lab that are tested weekly. For other parts of the body, in lab shower to wash off contamination.

15.3 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.shs.uwo.ca/workplace/newposition.htm

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name **Ray Zabulionis** ***Date:*** **September 26/11**

15.4 Additional Comments: ______________________________

16.0 Approvals

1) UWO Biohazards Subcommittee: SIGNATURE: ______________________
Date: ______________________

2) Safety Officer for the University of Western Ontario
SIGNATURE: ______________________
Date: ______________________

3) Safety Officer for Institution where experiments will take place (if not UWO):
SIGNATURE: ______________________
Date: ______________________

Approval Number: ________________ Expiry Date (3 years from Approval): ________________

Special Conditions of Approval:

Appendix 1:

Teaching Protocols: 1. Overview 2. Bacteria 3. Fungi 4. Yeast 5. Plants

1. Overview:

The following organisms are used by undergraduate students in Biology 2290F/G which is a required laboratory course for many of the modules in Biology, Physiology, Kinesiology and Medical Sciences. The course is taught by 7 people: Rob Dean and Winona Gadapati (cell biology - Dean Unit), Tricia Gray and Jennifer Waugh (scientific writing – Gray Unit), Irene Krajnyk (ecology – Krajnyk Unit), and Ray Zabulionis and Patrick McDonald (genetics – Zabulionis Unit). Jeni Duro, Liz Ross, and Syria Peiris are the technicians for the course. Approximately, 1000 students pass through this course each year – the maximum number of students in any one lab is 40. Not all the organisms or experiments described below are used each term; we change them so that there is some variety to the course and discourage people coping results from previous terms. We strongly emphasize lab safety – with a large number of students, anything is possible. All organisms the students use are killed after they finish their experiments. Our stock cultures are checked for contamination routinely as any contamination will ruin experiments.

2. Bacteria:

Bacillus subtilis, Erwinia carotovora, Escherichia coli K12, *Micrococcus luteus, Pseudomonas fluorescens, Streptomyces griseoviridis, and Streptomyces griseus* are used by students in the Krajnyk Unit. These bacteria are exposed to various concentrations of antibiotics: ampicillin, chloramphenicol, kanamycin, streptomycin, or tetracycline; or to various herbs and spices: cayenne pepper, cinnamon, clove, fresh garlic, ginger, juniper, lemon, nutmeg, peppermint, rosemary, sage, or thyme to determine the sensitivity of the bacteria. Students streak the bacteria onto nutrient agar plates and then place assay discs soaked in the various chemicals in the centre of the plate. Plates are sealed, incubated, and then studied while still sealed. The sealed plates are autoclaved to kill the bacteria after use.

Escherichia coli (strains BW, BW(p600), DH5, JM101, JM101(pAMP), JM101(pKAN), JM101(pA/K), JM101(p220), JM101(pGEM), JM101(p501), JM101(pVIB), JM101(pGREEN), JM101(pBR), JM101(p600), JM101(pKYLX), JM101(pCR2), JM101(pGATA), JM101(Xeno), K12, K12tet, and SCS1) are used by students in the Zabulionis Unit for various experiments. These include antibiotic physiology, conjugation, transduction, transformation, and ultra-violet (UV) light exposure.

Antibiotic physiology: The *E. coli* cells are exposed to various levels of antibiotics (ampicillin, kanamycin, novobiocin, rifampicin, or tetracycline) to determine susceptibility differences between cell lines or efficacy of antibiotics.

Conjugation: *E. coli* BW(p600) is the cell line used in conjugation whereby the p600 plasmid is transferred to a recipient cell line [JM101(pAMP), JM101(p220), JM101(p501), JM101(pGREEN), JM101(pVIB) or JM101(pGEM)]. The cells that receive the p600 plasmid are now resistant to ampicillin and tetracycline.

Transduction: P1vir bacteriophage is used to infect *E. coli* K12tet with some of the resulting progeny of the P1vir contain the tetracycline resistance gene from K12tet. This lysate of P1 is used by the students to infect other *E. coli* cell lines to see how readily they pick up the tetracycline resistance gene. See Appendix 8 for P1vir information.

Transformation: Various plasmids (p220, p501, p600, pA/K, pAMP, pBR, pCR2, pGATA, pGEM, pGREEN, pKAN, pKYLX, pVIB, pXeno) are transformed into the various *E. coli* cells lines that are sensitive to antibiotics. The plasmids contain an antibiotic resistance gene (either ampicillin, kanamycin, or tetracycline resistance gene) which enables the transformed cells to grow on the antibiotic that the plasmid provides resistance. See Appendix 7 for plasmid maps and information.

UV light exposure: *E. coli* cell lines are exposed to various amounts of UV light to determine sensitivity and survival rates. Plasmids (as above) are exposed to UV light and then transformed into antibiotic sensitive *E. coli* cells to determine transformability of the plasmid after UV treatment.

See Appendix 3 for species information.

3. Fungi:

The following fungi are used by students in the Krajnyk Unit: *Colletotrichum graminicola, Moniliella suaveolens, Phoma sorghina, Pleurotus ostreatus, Sclerotinia sclerotiorum, Sordaria fimicola,* and *Trichoderma harzionum.* They are used in heavy metal toxicity studies whereby the species are exposed on Petri dishes to millimolar concentrations of calcium chloride, cadmium chloride, cobalt chloride, copper sulphate, magnesium chloride, nickel chloride, or zinc chloride. After the species are added, the plates are sealed, incubated, and then measured while still sealed. The plates are autoclaved and then disposed in Hazardous Waste.

See Appendix 4 for species information.

4. Yeast:

The following yeast are used in the Krajnyk Unit: *Candida hawaiiana, Cryptococcus albidus, Rhodotorula rubra, Saccharomyces cerevisiae,* and *Schizosaccharomyces pombe*. They are used in heavy metal toxicity studies whereby the species are exposed on Petri dishes to millimolar concentrations of calcium chloride, cadmium chloride, cobalt chloride, copper sulphate, magnesium chloride, nickel chloride, or zinc chloride. After the species are added, the plates are sealed, incubated, and then measured while still sealed. The plates are autoclaved and then disposed in Hazardous Waste.

Saccharomyces cerevisiae is also used in the Dean Unit for finding the number of cells/ml in liquid suspension cultures using two methods: direct sampling with a haemacytometer and an indirect method using spectrophotometers. Two methods are used to determine the "yields" in cultures, over a two day incubation period, exposed to different concentrations of a specific nutrient e.g. nitrogen, or a carbon source.

See Appendix 5 for species information.

5. Plants:

In the Dean Unit, Spinach (Spinacia oleracea) leaves are obtained from local grocery stores from which chloroplasts are isolated and used to study chloroplast function under various light conditions.

Canola (Brassica napus) are grown from seeds in the Biology lower greenhouses and brought to NCB in growth pots for plant density growth studies by the students in the Krajnyk Unit.

Appendix 2:

1.0 Microorganisms:

1.2

The following organisms are maintained for use in Bio 2290F/G:

Name of Biological agent(s)*	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
Bacteria: see Appendix 3 for species information.						
Bacillus subtilis	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
Erwinia carotovora	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
Micrococcus luteus	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
Pseudomonas fluorescens	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
Streptomyces griseoviridis	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
Streptomyces griseus	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
***Escherichia coli* strains:**						
BW	NO	NO	NO	0.2 L.	Dr. Valvano UWO	***1*** 2 2+ 3
BW(p600) tet^r	NO	NO	NO	0.2 L.	we put p600 into BW	***1*** 2 2+ 3
DH5α	NO	NO	NO	0.2 L.	Gibco/BRL	***1*** 2 2+ 3
JM101	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
JM101(p220) amp^r	NO	NO	NO	0.2 L.	we put p220 into JM101	***1*** 2 2+ 3
JM101(p501) amp^r	NO	NO	NO	0.2 L.	we put p501 into JM101	***1*** 2 2+ 3
JM101(p600) tet^r	NO	NO	NO	0.2 L.	we put p600 into JM101	***1*** 2 2+ 3
JM101(pA/K) amp^r + kan^r	NO	NO	NO	0.2 L.	we put pA/K into JM101	***1*** 2 2+ 3

JM101(pAMP) amp^r	NO	NO	NO	0.2 L.	we put pAMP into JM101	*1* 2 2+ 3
JM101(pBR) amp^r + tet^r	NO	NO	NO	0.2 L.	we put pBR into JM101	*1* 2 2+ 3
JM101(pCR2) amp^r + kan^r	NO	NO	NO	0.2 L.	we put pCR2 into JM101	*1* 2 2+ 3
JM101(pGATA) kan^r	NO	NO	NO	0.2 L.	we put pGATA into JM101	*1* 2 2+ 3
JM101(pGEM) amp^r	NO	NO	NO	0.2 L.	we put pGEM into JM101	*1* 2 2+ 3
JM101(pGREEN) amp^r	NO	NO	NO	0.2 L.	we put pGREEN into JM101	*1* 2 2+ 3
JM101(pKAN) kan^r	NO	NO	NO	0.2 L.	we put pKAN into JM101	*1* 2 2+ 3
JM101(pKYLX) tet^r	NO	NO	NO	0.2 L.	we put pKYLX into JM101	*1* 2 2+ 3
JM101(pVIB) amp^r	NO	NO	NO	0.2 L.	we put pVIB into JM101	*1* 2 2+ 3
JM101(pXeno) amp^r + kan^r	NO	NO	NO	0.2 L.	we put pXeno into JM101	*1* 2 2+ 3
K12	NO	NO	NO	0.2 L.	ATCC	*1* 2 2+ 3
K12tet (tet^r gene on chromosome)	NO	NO	NO	0.2 L.	Dr. Valvano UWO	*1* 2 2+ 3
SCS1	NO	NO	NO	0.2 L.	Stratagene	*1* 2 2+ 3
Fungi: **see Appendix 4 for species information.**						
Colletotrichum graminicola	NO	NO	NO	0.2 L.	ATCC	*1* 2 2+ 3
Moniliella suaveolens	NO	NO	NO	0.2 L.	ATCC	*1* 2 2+ 3

Phoma sorghina	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
Pleurotus ostreatus	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
Sclerotinia sclerotiorum	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
Sordaria fimicola	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
Trichoderma harzionum						***1*** 2 2+ 3
Yeast: see Appendix 5 for species information.						
Candida hawaiiana	NO	NO	NO	0.2 L.	M. A. Lachance, UWO	***1*** 2 2+ 3
Cryptococcus albidus	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
Rhodotorula rubra	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
Saccharomyces cerevisiae	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3
Schizosaccharomyces pombe	NO	NO	NO	0.2 L.	ATCC	***1*** 2 2+ 3

Canadian Food Inspection Agency / Agence canadienne d'inspection des aliments

Office of Biohazard Containment and Safety
Science Branch, CFIA
59 Camelot Drive, Ottawa, Ontario K1A 0Y9
Tel: (613) 221-7068 Fax: (613) 228-6129
Email: ImportZoopath@inspection.gc.ca

Bureau du confinement des biorisques et sécurité
Direction générale des sciences, ACIA
59 promenade Camelot, Ottawa, Ontario K1A 0Y9
Tél: (613) 221-7068 Téléc: (613) 228-6129
Courriel: ImportZoopath@inspection.gc.ca

October 20th, 2009

Ms. Shamila Survery / Mr. Michael Decosimo
Cedarlane Laboratories Ltd
4410 Paletta Court
Burlington, Ontario L7L 5R2

By Facsimile: (289) 288-0020

SUBJECT: Importation of *Escherichia coli* strains

Dear Ms. Survery / Mr. Decosimo:

Our office received your query about the importation of *Escherichia coli* from the American Type Culture Collection (ATCC) located in Manassas, Virginia, United States. The following *Escherichia coli* strains are consider to be level 1 animal pathogens:

- 5K
- 58
- 58-161
- 679
- 1532
- AB284
- AB311
- AB1157
- AB1206
- AG1
- B
- BB4
- BD792
- BL21
- BL21 (DE3)
- BM25.8
- C
- C-1a
- C-3000
- C25
- C41 (DE3)
- C43 (DE3)
- C600
- Cavalli Hfr
- CIE85
- DH1
- DH10 GOLD
- DH10B
- DH5
- DH5-alpha
- DP50
- DY145
- DY380
- E11
- EJ183
- EL250
- EMG2
- EPI 300
- EZ10
- FDA Seattle 1946
- Fusion-Blue
- H1443
- HF4714
- HB101
- HS(PFAMP)R
- Hfr3000
- Hfr3000 X74
- HMS174
- J52
- J53
- JC3272
- JC7661
- JC9387
- JF1504
- JF1508
- JF1509
- JJ055
- JM83
- JM101
- JM109
- K12
- KC8
- KA802
- KAM32
- KAM33
- KAM43
- LE450
- LE451
- LE452
- MB408
- MBX1928
- MC1061
- MC4100 (MuLac)
- MG1655
- MM294
- MS101
- NC-7
- Nissle 1917
- One Shot STBL3
- OP50
- P678
- PA309
- PK-5
- PMC103
- PR13
- Rri
- RV308
- S17-1λ -PIR
- SCS1
- SMR10
- SOLR
- SuperchargeEZ10
- SURE
- TOP10
- TG1
- U5/41
- W208
- W945
- W1485
- W3104
- W3110
- WA704
- WP2
- X1854
- X2160T
- X2541
- X2547T
- XL1-BLUE
- XL1-BLUE-MRF
- XLOLR
- Y10
- Y1090 (1090)
- YN2980
- W3110
- WG1
- WG439
- WG443
- WG445

The Office of Biohazard Containment and Safety (BCS) of the Canadian Food Inspection Agency (CFIA) only issues import permits for microorganisms that are pathogenic to animals, or parts of microorganisms that are pathogenic to animals. As the products listed above are not considered pathogenic to animals, the Office of BCS does not have any regulatory requirements for their importation.

Please note that other legislation may apply. You may wish to contact the Public Health Agency of Canada's (PHAC) Office of Laboratory Security at (613) 957-1779.

Note: Microorganisms pathogenic to animals and veterinary biologics require an import permit from the CFIA.

Sincerely,

Cinthia Labrie
Head, Animal Pathogen Importation Program
Office of Biohazard Containment & Safety

Canada

Appendix 3:

Bacteria used in Bio 2290F/G:

Bacillus subtilis (Ehrenberg) Cohn – from ATCC:

ATCC® Number:	**465™**
Organism:	*Bacillus subtilis* (Ehrenberg) Cohn
Designations:	[NRS 743]
Depositor:	NM Harris
Biosafety Level:	1
Growth Conditions:	ATCC medium 3: Nutrient agar or nutrient broth **Temperature:** 30.0°C
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
References:	5507: Science 62: 57, 1925. 5923: Smith NR, et al. Aerobic spore forming bacteria. U.S. Dep. Agric. Monogr. 16: 1-148, 1952.

Erwinia carotovora **subsp.** ***carotovora*** **(Jones) Bergey et al. –** from ATCC:

ATCC® Number: **495™**

Organism:	*Pectobacterium carotovorum* subsp. *carotovorum* (Jones) Hauben et al. deposited as *Erwinia carotovora* subsp. *carotovora* (Jones) Bergey et al.		
Designations:	[D. Dye EG23, ICMP 1380, ICPB EC208, NCPPB 2042]	Isolation:	soft rot of carrot
Depositor:	LA Rogers	History:	ATCC <<--LA Rogers<<--L. Jones (Bacillus carotovorus)
Biosafety Level:	1 Gram negative.		
Growth Conditions:	ATCC medium 3: Nutrient agar or nutrient broth **Temperature:** 26.0°C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
Comments:	DNA hybridization reference strain. Gram negative.		
References:	7228: Dye DW. A taxonomic study of the genus Erwinia. II. The "carotovora" group. N.Z. J. Sci. 12: 81-97, 1969.		

Micrococcus sp. deposited as *Micrococcus luteus* (Schroeter) Cohn – from ATCC:

ATCC® Number: **398™**

Organism:	*Micrococcus* sp. deposited as *Micrococcus luteus* (Schroeter) Cohn
Designations:	426
Depositor:	GJ Hucker
Biosafety Level:	1 Gram positive.
Growth Conditions:	ATCC medium 18: Trypticase soy agar **Temperature:** 26.0°C
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Comments:	taxonomy [8354] [9665]. Gram positive.
Applications:	produces creatininase creatinine amidohydrolase [3288]
References:	3288: Suzuki M, Saito N. Creatinine amidohydrolase and creatine amidinohydrolase and process for producing them. US Patent 4,039,384 dated Aug 2 1977 8354: Kocur M, et al. Taxonomic status of Micrococcus luteus (Schroeter 1872) Cohn 1872, and designation of the neotype strain. Int. J. Syst. Bacteriol. 22: 218-223, 1972. 9665: Rosypal S, Kocur M. The taxonomic significance of the oxidation of carbon compounds by different strains of Micrococcus luteus. Antonie van Leeuwenhoek 29: 313-318, 1963.

Pseudomonas fluorescens **Migula** – from ATCC

ATCC® Number: **12842™**

Organism:	*Pseudomonas fluorescens* Migula		
Designations:	NCIB 8865 strain CO1	Isolation:	soil
Depositor:	NCIMB	History:	ATCC <<--NCIMB<<--J. Peel

Biosafety Level: 1

Growth Conditions:	ATCC medium 3: Nutrient agar or nutrient broth **Temperature:** 26.0°C
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Comments:	Oxidizes hexanoic (caproic) acid

Streptomyces griseoviridis **Anderson et al. –** from ATCC.

ATCC® Number: **39271™**

Organism: *Streptomyces griseoviridis* Anderson et al.

Designations: 6

Isolation: peat soil, Finland

Depositor: University of Helsinki

History: ATCC <<--University of Helsinki<<--R. Tahvonen

Biosafety Level: 1

Shipped: freeze-dried

Growth Conditions: ATCC medium 196: Yeast malt extract agar
Temperature: 26.0°C

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

This material is cited in a U.S. and/or other Patent or Patent Application, and may not be used to infringe on the patent claims. ATCC is required to inform the Patent Depositor of the party to which the material was furnished.

Applications: biological control of fungal plant pathogens [3980]

References: 3980: Tahvonen R. Fungistatic method. US Patent 4,595,589 dated Jun 17 1986

***Streptomyces griseus* subsp. *griseus* (Krainsky) Waksman and Henrici** – from ATTC:

ATCC® Number: **13968™**

Organism: *Streptomyces griseus* subsp. *griseus* (Krainsky) Waksman and Henrici

Designations: A-1

Depositor: Lederle Labs.

Biosafety Level: 1 Gram positive.

Growth Conditions: ATCC medium 196: Yeast malt extract agar
Temperature: 26.0°C

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

This material is cited in a U.S. and/or other Patent or Patent Application, and may not be used to infringe on the patent claims. ATCC is required to inform the Patent Depositor of the party to which the material was furnished.

Applications: production of 2-beta-hydroxylated steroids of the pregnane series [3073]

References: 3073: Feldman LI, et al. 2-Hydroxy-9alpha-fluoro pregnenes and pregnadienes. US Patent 3,063,991 dated Nov 13 1962

Escherichia coli Strains: (all strains are gram negative)

E. coli BW (BW19851) – from M. Valvano (UWO):

BW19851 (name used in research papers)**:** it is a K12 strain (1).

Pedigree: S17-1 via BW19795 (2).

Genotype: RP4-2::Mu-1*kan*::Tn*7*/*creB510 hsdR17 endA1 zbf-5 uidA*(Δ*MLu*I)::*pir*(wt) *recA1 thi* (2).

Containment Level: 1

Comments: BW contains an integrated RP4 plasmid carrying the necessary components to allow mobilization (conjugation) of recombinant plasmids containing the *mob* region which is present in p600 plasmid (2). The students use BW(p600) in conjugation experiments.

References:

1. Valvano *et al.* J. Bact. 2000. 182(2):488-497;
2. Metcalf *et al.* Gene. 1994. 138(1-2):1-7.

E. coli **DH5α** - originally from Gibco/BRL (now owned by invitrogen):

MAX Efficiency® DH5α™ Competent Cells
Cat. No. 18258-012 Size: 1 mL
Store at -70°C.
Do not store in liquid nitrogen.

Description:
MAX Efficiency® DH5α™ Competent Cells have been prepared by a patented modification of the procedure of Hanahan (1). These cells are suitable for the construction of gene banks or for the generation of cDNA libraries using plasmid-derived vectors. The φ80d*lac*ZΔM15 marker provides α-complementation of the β-galactosidase gene from pUC or similar vectors and, therefore, can be used for blue/white screening of colonies on bacterial plates containing Bluo-gal or X-gal. DH5α™ is capable of being transformed efficiently with large plasmids, and can also serve as a host for the M13mp cloning vectors if a lawn of DH5α-FT™, DH5αF'™, DH5αF'IQ™, JM101 or JM107 is provided to allow plaque formation.

Genotype
F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k-, m_k+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1

Biosafety Level: 1

Component Amount per Vial
DH5α™ Competent Cells 200 µL
pUC19 DNA (0.01 µg/mL) 100 µL

Quality Control: MAX Efficiency® DH5α™ Competent Cells consistently yield > 1.0×10^9 transformants/µg pUC19 with non-saturating amounts (50 pg) of DNA. Saturating amounts of pUC19 (25 ng) generate > 1×10^6 ampicillin-resistant colonies in a 100-µL reaction.

Part No. 18258012.pps Rev. Date: 26 October 2006

For technical support, email tech_support@invitrogen.com. For country-specific contact information, visit ***www.invitrogen.com***.

Transformation Procedure:

A stock pUC19 solution (0.01 µg/mL) is provided as a control to determine the transformation efficiency. The stock solution of pFastBac™-gus (0.2 µg/mL), provided with pFastBac™ 1 Expression Vector (Cat. No. 10360-014), can be used as a control for the transposition frequency. To obtain maximum transformation efficiency, the experimental DNA must be free of phenol, ethanol, protein and detergents.

1. Thaw competent cells on wet ice. Place required number of 17 × 100 mm polypropylene tubes (Falcon® 2059) on ice.
2. Gently mix cells, then aliquot 100 µL of competent cells into chilled polypropylene tubes.
3. Refreeze any unused cells in the dry ice/ethanol bath for 5 minutes before returning to the -70°C freezer. Do not use liquid nitrogen.
4. To determine the transformation efficiency, add 5 µL (50 pg) pUC19 control DNA to one tube containing 100 µL competent cells. Move the pipette through the cells while dispensing. Gently tap tube to mix.
5. For DNA from ligation reactions, dilute the reactions 5-fold in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Add 1 µL of the dilution to the cells (1 to 10 ng DNA), moving the pipette through the cells while dispensing. Gently tap tubes to mix.
6. Incubate cells on ice to 30 minutes.
7. Heat-shock cells 45 seconds in a 42°C water bath; do not shake.
8. Place on ice for 2 minutes.
9. Add 0.9 mL room temperature S.O.C. Medium (Cat. No. 15544-034).
10. Shake at 225 rpm (37°C) for 1 hour.
11. Dilute the reaction containing the control plasmid DNA 1:100 with S.O.C. Medium. Spread 100 µL of this dilution on LB or YT plates with 100 µg/mL ampicillin.
12. Dilute the experimental reactions as necessary and spread 100 to 200 µL of this dilution as described in Step 11.
13. Incubate overnight at 37°C.

Growth of Transformants for Plasmid Preparations:

DH5α™ Competent Cells which have been transformed with pUC-based plasmids should be grown at 37°C overnight in TB(2). A 100-mL growth in a 500-mL baffled shake flask will yield approximately 1 mg of pUC19 DNA.

Notes:

1. For best results, each vial of cells should be thawed only once. Although the cells are refreezable, subsequent freeze-thaw cycles will lower transformation frequencies by approximately two-fold.

2. Media other than S.O.C. Medium can be used, but the transformation efficiency will be reduced. Expression in Luria Broth reduces transformation efficiency a minimum of two- to three-fold (4).

3. Transformation efficiencies will be approximately 10-fold lower for ligation of inserts to vectors than for an intact control plasmid. Ligation reactions should be diluted 5-fold prior to using the

DNA in a transformation. Only 1 µL of this dilution should be used. A standard ligation reaction (20 µL) normally contains 100-1000 ng of DNA. Therefore, the addition of 1 µL of diluted DNA will result in adding 1 to 10 ng of ligated DNA to the cells. We have observed that the cells begin to saturate with 10-50 ng of DNA (3). Also our data show that the 5-fold dilution of ligation mixtures results in more efficient transformation (3,4).

4. MAX Efficiency® DH5α™ can support the replication of M13mp vectors. However, DH5α™ is F^- and cannot support plaque formation. Therefore, log phase DH5α-FT™, DH5αF'™, DH5αF'IQ™, JM101 or JM107 cells must be added to the top agar which should contain X-gal (Cat. No. 15520-034) or Bluo-gal, final concentration 50 µg/mL, and IPTG (Cat. No. 15529-019), final concentration 1 mM. The competent cells should be added to top agar after lawn cells, IPTG and Bluo-gal or X-gal have been added. Incubation at 37°C for 1 hour is not required after addition of S.O.C. Medium.

5. Generally, transformation efficiencies will be 10- to 100-fold lower for cDNA than for an intact control plasmid such as pUC19. Approximately 50,000 transformants/5 ng cDNA may be obtained. The amount of cDNA used in a 100 µL transformation should be 1-5 ng in 5 µL or less.

6. Transformation efficiency (CFU/µg):

<u>CFU in control plate</u> × <u>1 × 106 pg</u> × dilution factor(s)

pg pUC19 used in transformation µg

For example, if 50 pg pUC19 yields 100 colonies when 100 µL of a 1:100 dilution is plated, then:

CFU/µg = <u>100 CFU</u> × <u>1 × 106pg</u> × <u>1 mL</u> × $10^2 = 2 \times 10^9$

50 pg µg 0.1 mL plated

<u>References:</u>

1. Hanahan, D. (1983) *J. Mol. Biol. 166*, 557.
2. Tartof, K. D. and Hobbs, C. A. (1987) *Focus® 9:2*, 12.
3. Jessee, J. (1984) *Focus 6:4*, 5.
4. King, P. V. and Blakesley, R. (1986) *Focus® 8:1*, 1.

E. coli **JM101** - from ATCC:

ATCC® Number: 33876

Designations: JM101

Depositors: D Vapnek

Genotype: F' traD36 proA+ proB+ lacIq delta(lacZ)M15 delta(lac-proAB) supE thi-1 lambda-

Growth Conditions: **Temperature:** 37.0°C

Biosafety Level: 1

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Applications: transformation host

Comments: Host for pMOB48 (ATCC 37107).

References:
8105: Messing J, et al. A system for shotgun DNA sequencing. Nucleic Acids Res. 9: 309-321, 1981. PubMed: 6259625
8116: Bittner M, Vapnek D. Versatile cloning vectors derived from the runaway-replication plasmid pKN402. Gene 15: 319-329, 1981. PubMed: 6277736
10266: Yanisch-Perron C, et al. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119, 1985. PubMed: 2985470

***E. coli* K12** - from ATCC:

ATCC® Number: **29425™**

Organism: *Escherichia coli* (Migula) Castellani and Chalmers

Designations: K12

Isolation: Basel, 1969 [185139]

Depositor: R Yuan

History: ATCC <<--R Yuan<<--W. Arber

Biosafety Level: 1

Growth Conditions: ATCC medium 3: Nutrient agar or nutrient broth
Temperature: 37.0°C
Duration: aerobic

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

References: 185139: R Yuan, personal communication

***E. coli* K12tet** - from M Valvano (UWO):

Species: *Escherichia coli* K12.

Strain: 21566.

Genotype: *rffE:Tn10* (Tc^r) -tetracycline resistance gene inserted into chromosome in the *rff* genes (the structural genes for UDP-GlcNAc-2-epimerase) resulting in defective ECA synthesis subsequent to the synthesis of lipid I (1).

Containment Level: 1.

References:
1. Meier-Dieter *et al.* J. Biol. Chem. 1990. 265(23):13490-13497.

E. coli **SCS1** - from Stratagene:

SCS1 Supercompetent Cells   Catalog #200231

MATERIALS PROVIDED:

Materials Provided:	**Quantity:**	**Efficiency (cfu/µL of pUC18 DNA**
SCS1 supercompetent cells (green tubes)	5 × 0.2 mL	≥1 × 10^9
pUC18 plasmid (0.1 ng/µL in TE buffer)	10 µL	----------
β-Mercaptoethanol (1.42 M)	25 µL	-----------

Storage: Supercompetent cells must be placed immediately at the bottom of a –80°C freezer directly from the dry ice shipping container. Do not store the cells in liquid nitrogen.

QUALITY CONTROL TESTING: Transformations are performed both with and without plasmid DNA using 100-µL aliquots of cells and 100 pg of pUC18 control plasmid following the protocol outlined below. Following transformation, 2.5-µL samples of the culture are plated in duplicate on LB agar plates with 100 µg/mL of ampicillin. The plates are incubated at 37°C overnight and the efficiency is calculated based on the average number of colonies per plate.

BACKGROUND:
SCS1 Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17* (*r*K^- *m*K^+) *supE44 relA1* (uncharacterized mutation improves transformation efficiency). (Genes listed signify mutant alleles.)
SCS1 cells are endonuclease (*endA*) deficient, which greatly improves the quality of miniprep DNA, and are recombination (*recA*) deficient, improving insert stability. The *hsdR* mutation prevents the cleavage of cloned DNA by the *Eco*K endonuclease system.
Note *The SCS1 strain does not contain an F′ episome and does not support blue-white color screening or single-strand rescue applications.*

BIOSAFETY LEVEL: 1

TRANSFORMATION PROTOCOL:
1. Pre-chill two 14-mL BD Falcon polypropylene round-bottom tubes on ice. (One tube is for the experimental transformation and one tube is for the pUC18 control.) Preheat SOC medium to 42°C.
2. Thaw the supercompetent cells on ice. When thawed, gently mix and aliquot 100 µL of cells into each of the two pre-chilled tubes.
3. Add 1.7 µL of β-mercaptoethanol provided with this kit to each aliquot of cells.
4. Swirl the contents of the tubes gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.
5. Add 0.1–50 ng of the experimental DNA (see *Quantity and Volume of DNA*, reverse page, for guidelines) to one aliquot of cells and add 1 µL of the pUC18 control DNA to the other aliquot. Swirl the tubes gently.
6. Incubate the tubes on ice for 30 minutes.
7. Heat-pulse the tubes in a 42°C water bath for 45 seconds. The duration of the heat pulse is **critical** for maximum efficiency.
8. Incubate the tubes on ice for 2 minutes.
9. Add 0.9 mL of preheated SOC medium and incubate the tubes at 37°C for 1 hour with shaking at 225–250 rpm.

10. Plate ≤200 μL of the transformation mixture on LB agar plates containing the appropriate antibiotic. For the pUC18 control
transformation, plate 2.5 μL of the transformation on LB–ampicillin agar plates.
Notes *Cells may be concentrated by centrifuging at 1000 rpm for 10 minutes. Resuspend the pellet in 200 μL of SOC medium.*
If plating <100 μL of cells, pipet the cells into a 200 μL pool of SOC medium and then spread the mixture with a sterile spreader.
If plating ≥100 μL, the cells can be spread on the plates directly. Tilt and tap the spreader to remove the last drop of cells.
11. Incubate the plates at 37°C overnight.
12. For the pUC18 control, expect 250 colonies (≥1 × 109 cfu/μg pUC18 DNA). For the experimental DNA, the number of colonies will vary according to the size and form of the transforming DNA, with larger and non-supercoiled DNA producing fewer colonies.

TRANSFORMATION GUIDELINES AND TROUBLESHOOTING:

Storage Conditions: Competent and supercompetent cells are very sensitive to even small variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency.

Use of 14-mL BD Falcon polypropylene round-bottom tubes: It is important that 14-mL BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059) are used for the transformation protocol, since other tubes may be degraded by β-mercaptoethanol. In addition, the duration of the heat-pulse is critical and has been optimized for these tubes.

Aliquoting Cells: Keep the cells on ice at all times during aliquoting. It is essential that the polypropylene tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into pre-chilled tubes. It is also important to use the volume of cells indicated in step 2 of the *Transformation Protocol*. Decreasing the volume will result in lower efficiencies.

Use of β-Mercaptoethanol (β-ME): β-ME has been shown to increase transformation efficiency. The β-ME provided is diluted and ready to use. A fresh 1:10 dilution (from a 14.2 M stock) may be used; however, Stratagene cannot guarantee results with β-ME from other sources.

Quantity and Volume of DNA: The greatest efficiency is obtained from the transformation of 1 μL of 0.1 ng/μL supercoiled pUC18 DNA per reaction. A greater number of colonies may be obtained by transforming up to 50 ng DNA, although the resulting efficiency (cfu/μg DNA) may be lower. The volume of the DNA solution added to the reaction may be increased to up to 10% of the reaction volume, but the transformation efficiency may be reduced.

Heat Pulse Duration: Optimal transformation efficiency is observed when cells are heat-pulsed at 42°C for 45–50 seconds. Efficiency decreases sharply when cells are heat-pulsed for <45 seconds or for >60 seconds.

PREPARATION OF MEDIA AND REAGENTS:

SOB Medium (per Litre):
20.0 g of tryptone
5.0 g of yeast extract
0.5 g of NaCl
Add deionized H2O to a final volume of 1 litre
Autoclave
Add 10 mL of filter-sterilized 1 M MgCl2 and 10 mL of filter-sterilized
1 M MgSO4 prior to use.

SOC Medium (per 100 mL):
Note This medium should be prepared immediately before use.
2 mL of filter-sterilized 20% (w/v) glucose or 1 mL of filter-sterilized
2 M glucose
SOB medium (autoclaved) to a final volume of 100 mL.

LB Agar (per Litre):
10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H2O to a final volume of 1 litre
Adjust pH to 7.0 with 5 N NaOH
Autoclave.

LB–Ampicillin Agar (per Litre):
1 litre of LB agar, autoclaved
Cool to 55°C
Add 10 mL of 10 mg/mL filter-sterilized ampicillin
Pour into petri dishes (~25 mL/100-mm plate).

LIMITED PRODUCT WARRANTY
This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Stratagene. Stratagene shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.
Stratagene Technical Services
USA/Canada (Toll-free) 800 894 1304
Europe (Toll-free) 00800 7400 7400
Email tech_services@stratagene.com
World Wide Web www.stratagene.com

Appendix 4:

Fungi used in Bio 2290F/G:

Colletotrichum graminicola (Cesati) Wilson, anamorph - from ATCC:

ATCC Advanced Catalog Search »Product Details

Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's Material Transfer Agreement or, in certain cases, an MTA specified by the depositing institution.

Customers in Europe, Australia, Canada, China, Hong Kong, India, Japan, Korea, Macau, Mexico, New Zealand, Singapore, and Taiwan, R.O.C. must contact a local distributor for pricing information and to place an order for ATCC cultures and products.

Fungi ,Yeasts and Yeast Genetic Stock

ATCC® Number:	**11870™**		
Organism:	*Colletotrichum graminicola* (Cesati) Wilson, anamorph		
Alternate State:	*Glomerella graminicola* Politis, teleomorph		
Designations:	[NRRL 13648]	Isolation:	alfalfa, Iowa
Depositors:	LH Tiffany		
Biosafety Level:	1	Shipped:	frozen
Growth Conditions:	ATCC medium 336: Potato dextrose agar (PDA) **Temperature:** 24.0°C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Related Products

Comments:	Colletotrichum capsici fide Sutton [13066]
Subcollection:	Fungi
References:	13066: Sutton BC. Colletotrichum dematium (PERS.EX FR.) Grove and C. trichellum (FR.EX FR.) Duke. Trans. Br. Mycol. Soc. 42-232: 223, 1962.

***Moniliella suaveolens* var. *nigra* (Burri et Staub) de Hoog, anamorph -** from ATTC:

ATCC® Number: **18456™**

Organism:	*Moniliella suaveolens* var. *nigra* (Burri et Staub) de Hoog, anamorph deposited as *Moniliella tomentosa* van Beyma, anamorph		
Designations:	CBS 224.32 [IMI 159917]	Isolation:	dried tobacco leaves, Nicotiana tabacum, England
Depositors:	CBS	History:	ATCC <<--CBS<<--J.W. Jollyman
Biosafety Level:	1	Shipped:	freeze-dried
Growth Conditions:	ATCC medium 336: Potato dextrose agar (PDA) **Temperature:** 26.0°C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Related Products

Type Strain:	yes(type strain of Moniliella tomentosa)
Subcollection:	Fungi
References:	16240: Stolk AC, Dakin JC. Moniliella, a new genus of Moniliales. Antonie van Leeuwenhoek 32: 399-409, 1966. PubMed: 5297390

Phoma sorghina **(Saccardo) Boerema et al., anamorph deposited as *Phoma* sp.** – from ATCC:

ATCC Advanced Catalog Search »Product Details

Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's Material Transfer Agreement or, in certain cases, an MTA specified by the depositing institution.

Customers in Europe, Australia, Canada, China, Hong Kong, India, Japan, Korea, Macau, Mexico, New Zealand, Singapore, and Taiwan, R.O.C. must contact a local distributor for pricing information and to place an order for ATCC cultures and products.

Fungi ,Yeasts and Yeast Genetic Stock

ATCC® Number: **13145™**

Organism:	*Phoma sorghina* (Saccardo) Boerema et al., anamorph deposited as *Phoma* sp.
Designations:	M-536 [IMI 109556]
Depositors:	Schering Corp.
Biosafety Level:	1 Shipped: frozen
Growth Conditions:	ATCC medium 312: Czapek's agar **Temperature:** 24.0°C
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

This material is cited in a U.S. and/or other Patent or Patent Application, and may not be used to infringe on the patent claims. ATCC is required to inform the Patent Depositor of the party to which the material was furnished.

Related Products

Applications:	production of 11-hydroxylated steroids [3054]
Subcollection:	Fungi
References:	3054: Ilavsky J, Herzog H. 11-Hydroxylation of steroids by Phoma microorganisms. US Patent 3,054,725 dated Sep 18 1962

Pleurotus ostreatus **(Jacquin : Fries) Kummer, teleomorph –** from ATCC:

ATCC Advanced Catalog Search »Product Details

Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's Material Transfer Agreement or, in certain cases, an MTA specified by the depositing institution.

Customers in Europe, Australia, Canada, China, Hong Kong, India, Japan, Korea, Macau, Mexico, New Zealand, Singapore, and Taiwan, R.O.C. must contact a local distributor for pricing information and to place an order for ATCC cultures and products.

Fungi ,Yeasts and Yeast Genetic Stock

ATCC® Number:	**38538™**		
Organism:	*Pleurotus ostreatus* (Jacquin : Fries) Kummer, teleomorph		
Designations:	F		
Depositors:	G Eger	History:	ATCC <<--G Eger<<--S.S. Block
Biosafety Level:	1	Shipped:	frozen
Growth Conditions:	ATCC medium 323: Malt agar medium **Temperature:** 24.0°C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Related Products

Comments:	Breeding potential [16984] Fruits under subtropical summer conditions [16082]
Subcollection:	Fungi
References:	16082: . . Mushroom Sci. 10: 155-169, 1978. 16984: . . Theor. Appl. Genet. 47: 155-163, 1976.

Sclerotinia sclerotiorum (Libert) - from ATCC

Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's Material Transfer Agreement or, in certain cases, an MTA specified by the depositing institution.

Customers in Europe, Australia, Canada, China, Hong Kong, India, Japan, Korea, Macau, Mexico, New Zealand, Singapore, and Taiwan, R.O.C. must contact a local distributor for pricing information and to place an order for ATCC cultures and products.

Fungi ,Yeasts and Yeast Genetic Stock

ATCC® Number: **18683™**

Organism:	*Sclerotinia sclerotiorum* (Libert) de Bary deposited as *Sclerotinia sclerotiorum* (Libert) de Bary, teleomorph		
Designations:	Ss-1 or 1980	Isolation:	bean pods, New York
Depositors:	RD Lumsden	History:	ATCC <<--RD Lumsden<<--J.J. Natti
Biosafety Level:	1	Shipped:	frozen
Growth Conditions:	ATCC medium 336: Potato dextrose agar (PDA) **Temperature:** 24.0°C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Related Products

Comments:	Whetzelinia sclerotiorum (Libert) Korf et Dumont is considered an invalid name. Genome-sequenced strain of Sclerotinia sclerotiorum
Subcollection:	Fungi

Sordaria fimicola (Roberge) Cesati et De Notaris – from ATCC:

ATCC Advanced Catalog Search »Product Details

Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's Material Transfer Agreement or, in certain cases, an MTA specified by the depositing institution.

Customers in Europe, Australia, Canada, China, Hong Kong, India, Japan, Korea, Macau, Mexico, New Zealand, Singapore, and Taiwan, R.O.C. must contact a local distributor for pricing information and to place an order for ATCC cultures and products.

Fungi ,Yeasts and Yeast Genetic Stock

ATCC® Number: **14517™**

Organism:	*Sordaria fimicola* (Roberge) Cesati et De Notaris		
Designations:	A-1, wild type g+	Isolation:	dung, New York
Depositors:	LS Olive		
Biosafety Level:	1	Shipped:	freeze-dried
Growth Conditions:	ATCC medium 310: Cornmeal, yeast, glucose agar (CMYG) **Temperature:** 26.0°C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
Subcollection:	Fungi		
References:	14310: . . Bull. Torrey Bot. Club 81: 95-97, 1954. 14433: . . Am. J. Bot. 43: 97-107, 1956.		

Trichoderma harzianum Rifai, anamorph - from ATCC:

Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's Material Transfer Agreement or, in certain cases, an MTA specified by the depositing institution.

Customers in Europe, Australia, Canada, China, Hong Kong, India, Japan, Korea, Macau, Mexico, New Zealand, Singapore, and Taiwan, R.O.C. must contact a local distributor for pricing information and to place an order for ATCC cultures and products.

Fungi ,Yeasts and Yeast Genetic Stock

ATCC® Number: **18647™**

Organism:	*Trichoderma harzianum* Rifai, anamorph		
Alternate State:	*Hypocrea lixii* Patouillard, teleomorph		
Designations:	AB 63-3 [CBS 819.68]	Isolation:	soil, South Australia
Depositors:	C Dennis		
Biosafety Level:	1	Shipped:	freeze-dried
Growth Conditions:	ATCC medium 335: Potato carrot agar **Temperature:** 26.0°C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Subcollection: Fungi

Appendix 5:

Yeast used in Bio 2290:

Candida hawaiiana M.A. Lachance & J.M. Bowles – from Yeast division of the Centraalbureau voor Schimmelcultures at Utrecht, Netherlands (http://www.cbs.knaw.nl/yeast/BioloMICS.aspx).

CBS 9146 <- more data

Price: 120 Euro (-45.83% discount for Academies, universities, education)

Taxon name:	Candida hawaiiana M.A. Lachance & J.M. Bowles
Status of the strain:	T of Candida hawaiiana M.A. Lachance & J.M. Bowles
Other collections:	UWO(PS)91-698.3;MUCL 46200
Locality:	Hawaii, Manuka Res.
Isolated by:	M.A. Lachance
Deposited by:	M.A. Lachance
Substrate of isolation:	flower of Ipomoea indica
Conditions for growth (on solid media):	On solid med.: GPYA, 25 C

André Lachance October 3, 2008:

Dear Ray:

For all I know, the specie therein has no record ever of having any medically relevant association. Given that it does not grow at 37C, it is not likely that it ever will cause any harm to humans.

Cheers.

André

attached: Lachance *et al.* 2003. FEMS Yeast Research. 3:97-103.

Cryptococcus albidus (Saito) Skinner var. *albidus*, anamorph – from ATCC:

Fungi ,Yeasts and Yeast Genetic Stock

ATCC® Number: **16721™**

Organism:	*Cryptococcus albidus* (Saito) Skinner var. *albidus*, anamorph deposited as *Cryptococcus diffluens* (Zach) Lodder et Kreger-van Rij, anamorph		
Designations:	E4A2	Isolation:	fresh water, Lake Erie
Depositors:	PF Dupont	History:	ATCC <<--PF Dupont<<--L.R. Hedrick
Biosafety Level:	1	Shipped:	freeze-dried
Growth Conditions:	ATCC medium 200: YM agar or YM broth **Temperature:** 24.0°C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
Subcollection:	Yeasts		

***Rhodotorula rubra* (Demme) Lodder, anamorph–** from ATCC:

Fungi ,Yeasts and Yeast Genetic Stock

ATCC® Number: **9449™**

Organism:	*Rhodotorula mucilaginosa* (Jorgensen) Harrison var. *mucilaginosa*, anamorph deposited as *Rhodotorula rubra* (Demme) Lodder, anamorph
Designations:	NRRL Y-1592 [CBS 17, CCRC 21667, IGC 4791, MUCL 30397, VKM Y-341]
Depositors:	NRRL
History:	ATCC <<--NRRL<<--CBS 17 <<--- G. Pollacci
Biosafety Level:	1
Shipped:	freeze-dried
Growth Conditions:	ATCC medium 200: YM agar or YM broth **Temperature:** 26.0°C
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Related Products

Type Strain:	yes(type strain of Rhodotorula rubra (Demme) Lodder)
Comments:	Available as Uniplus (Ref TM) ATCC 9449-U
Related Products:	also distributed as:ATCC 9449-U
Subcollection:	Yeasts
References:	18509: Lodder J, Kreger-van Rij MJW. The yeasts: a taxonomic study. 1st ed.Amsterdam: North-Holland; 1952. 48831: Fell JW, et al. Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. Int. J. Syst. Evol. Microbiol. 50: 1351-1371, 2000. PubMed: 10843082

Saccharomyces cerevisiae **Meyen ex E.C. Hansen** – from ATCC:

ATCC® Number: **2338™**

Organism:	*Saccharomyces cerevisiae* Meyen ex E.C. Hansen deposited as *Saccharomyces cerevisiae* Hansen, teleomorph		
Designations:	[NCTC 467, NRRL Y-129]	Isolation:	Citrus fermentation
Depositors:	FW Tanner		
Biosafety Level:	1	Shipped:	freeze-dried
Growth Conditions:	ATCC medium 200: YM agar or YM broth **Temperature:** 30.0°C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Related Products

Applications:	bioresistance testing [92589]
Subcollection:	Yeasts
References:	19035: . . Ind. Eng. Chem. 28: 1224, 1936. 92589: Standard Practice for Evaluating Water-Miscible Metalworking Fluid Bioresistance and Antimicrobial Pesticide. West Conshohocken, PA:ASTM International;ASTM Standard Test Method E 2275-03E01.

Schizosaccharomyces pombe **Lindner teleomorph –** from ATCC:

ATCC® Number: **2476™**

Organism:	*Schizosaccharomyces pombe* Lindner, teleomorph		
Designations:	[NRRL Y-164]	Isolation:	rum
Depositors:	FW Tanner	History:	ATCC <<--FW Tanner<<--CBS
Biosafety Level:	1	Shipped:	freeze-dried
Growth Conditions:	ATCC medium 200: YM agar or YM broth **Temperature:** 30.0°C		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Applications:
degrades xylulose [1514]
produces alkyl esters [11821]
produces ethyl alcohol ethanol [1514]
produces pergolide sulfoxide [57784]
transforms pergolide [57784]
transformation of pergolide to pergolide sulfoxide [57784]
produces C2-C5 alkyl esters [11821]

Subcollection: Yeasts

References:

1514: et al., Lastick SM. Simultaneous fermentation and isomerization of xylose. Appl. Microbiol. Biotechnol. 30: 574-579, 1989.
11821: Farbood MI, et al. Preparation of naturally-occurring C2-C5 alkyl esters of C4-C5 carboxylic acids by means of fermentation of C5-C6 amino acids in the presence of C2-C5 alcohols. US Patent 4,686,307 dated Aug 11 1987
57784: Smith RV, et al. Microbial transformations of pergolide to pergolide sulfoxide and pergolide sulfone. J. Pharm. Sci. 72: 733-736, 1983. PubMed: 6684155

Appendix 6: see Appendix 7 for plasmid maps and information:

4.0 Genetically Modified Organisms and Cell Lines:

4.2 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? YES

***Escherichia coli* that can be modified:** BW, DH5, JM101, K12, and SCS1:

Bacteria Used for Cloning	Plasmid	Source of Plasmid	Gene Transformed or Transfected	Will there be a change due to transformation of the bacteria?	Will there be a change in the pathogenicity of the genetic modification?	What are the consequences due to the transformation of the bacteria?
E. coli	pAMP	Carolina	amp^r gene	*E. coli* cells become resistant to ampicillin	No	*E. coli* cells become resistant to ampicillin
E. coli	pKAN	Carolina	kan^r gene	*E. coli* cells become resistant to kanamycin	No	*E. coli* cells become resistant to kanamycin
E. coli	pA/K	we cut and ligated pAMP and pKAN	amp^r + kan^r genes	*E. coli* cells become resistant to ampicillin and kanamycin	No	*E. coli* cells become resistant to ampicillin and kanamycin
E. coli	p220	Stratagene	amp^r gene	*E. coli* cells become resistant to ampicillin	No	*E. coli* cells become resistant to ampicillin
E. coli	pGEM	Promega	amp^r gene	*E. coli* cells become resistant to ampicillin	No	*E. coli* cells become resistant to ampicillin
E. coli	p501	workshop – University of Wisconsin-La Crosse	amp^r + bioluminescence genes	*E. coli* cells become resistant to ampicillin and glow when grown at 27^0C	No	*E. coli* cells become resistant to ampicillin and glow when grown at 27^0C
E. coli	pBR	Boehringer Mannheim	amp^r + tet^r genes	*E. coli* cells become resistant to ampicillin and tetracycline	No	*E. coli* cells become resistant to ampicillin and tetracycline
E. coli	p600	Dr Valvano – UWO	tet^r gene	*E. coli* cells become resistant to tetracycline	No	*E. coli* cells become resistant to tetracycline
E. coli	pKYLX	Dr Maxwell – UWO	tet^r gene	*E. coli* cells become resistant to tetracycline	No	*E. coli* cells become resistant to tetracycline

E. coli	pCR2	Dr. Damjanovski – UWO	amp^r + kan^r genes	*E. coli* cells become resistant to ampicillin and kanamycin	No	*E. coli* cells become resistant to ampicillin and kanamycin
E. coli	pGATA	Dr. Damjanovski – UWO	kan^r + neo^r genes	*E. coli* cells become resistant to kanamycin and neomycin	No	*E. coli* cells become resistant to kanamycin and neomycin
E. coli	pXeno	Dr. Damjanovski – UWO	amp^r + kan^r genes	*E. coli* cells become resistant to ampicillin and kanamycin	No	*E. coli* cells become resistant to ampicillin and kanamycin

The above *Escherichia coli* that are modified by Bio 2290 students are destroyed after they have finished studying them. On occasion, we modify the above *Escherichia coli* to replenish our stocks.

Appendix 7:

Plasmid Maps:

All but one of our plasmids has only an origin of replication gene expression promoters from *Escherichia coli*; consequently, they cannot be replicated or have their genes expressed in eukaryotic cells. The exception is pKYLX which can express cloned genes in plant cells (see pKYLX plasmid map for further information).

Plasmids used:

p220 = pBluescriptII KS (+) phagemid – from Stratagene.

p501 = pUWL501 – constructed by Tom Haffie at University of Wisconsin workshop in 1996.

p600 = pME6000 – from M. Valvano (UWO).

pA/K = pAMP/KAN – made in Bio290 from pAMP and pKAN (both from Carolina) using the following protocol.

pAMP – from Carolina.

pBR = pBR322 – from Boehringer Mannheim.

pCR2 = pCRII-TOPO – from Sash Damjanovski (UWO).

pGATA = pCMVTag3B-GATA-6 – from Sash Damjanovski (UWO).

pGEM = pGEM-3Zf(+) – from Promega.

pGREEN – from Carolina.

pKAN – from Carolina.

pKYLX = pKYLX7 from Denis Maxwell (UWO).

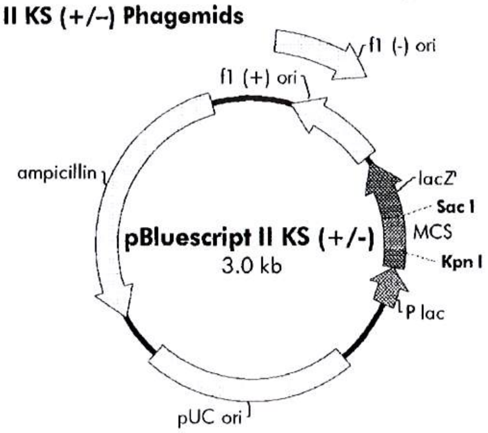
pVIB – from Carolina.

pXeno – from Sash Damjanovski (UWO).

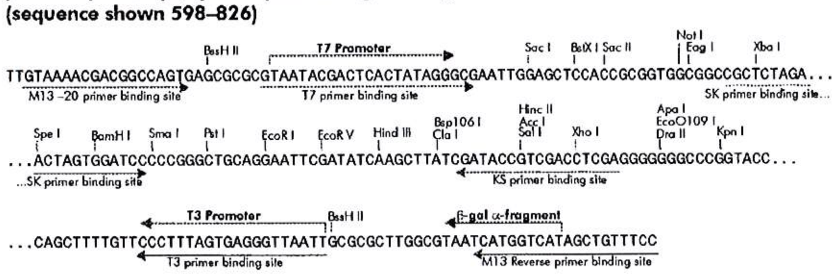
p220 = pBluescriptII KS (+) phagemid – from Stratagene:

p220 = pBluescript II KS (+)
from Stratagene

pBluescript II KS (+/–) Phagemids



pBluescript II KS (+/–) Multiple Cloning Site Region
(sequence shown 598–826)



Feature	Nucleotide Position
f1 (+) origin of ss-DNA replication (pBluescript KS (+) only)	135–441
f1 (–) origin of ss-DNA replication (pBluescript KS (–) only)	21–327
β-galactosidase α-fragment coding sequence (lacZ')	460–816
multiple cloning site	653–760
T7 promoter transcription initiation site	643
T3 promoter transcription initiation site	774
lac promoter	817–938
pUC origin of replication	1158–1825
ampicillin resistance (*bla*) ORF	1976–2833

FIGURE 2 The pBluescript II KS (+/–) phagemid vectors. The complete sequence and list of restriction sites are available at *www.stratagene.com*. Genbank® #X52327 [KS(+)] and #X52329 [KS(–)].

from: http://www.stratagene.com/manuals/212205.pdf

p501 = pUWL501 – constructed by Tom Haffie at University of Wisconsin workshop in 1996:

p501 = pUWL501

Gene Cloning: Uptake of Recombinant DNA by Bacterial Transformation

By

Michael R. Winfrey
and
Marc A. Rott

Department of Biology and Microbiology
University of Wisconsin-La Crosse

saved in "Plasmids"

Exercise 2
Gene Cloning: Uptake of Recombinant DNA by Bacterial Transformation

Objectives:
1. To become familiar with the techniques of modern molecular genetics.
2. To understand the principles of recombinant DNA technology and the role of bacterial transformation in this process.
3. To transfer a recombinant DNA molecule into competent *E. coli* cells and to select for transformants.

Introduction

Recombinant DNA technology ("genetic engineering") involves the *in vitro* enzymatic splicing of DNA from two different sources (to form a "recombinant DNA molecule") and the transfer of the recombinant DNA into an organism where it can be replicated and sometimes expressed. Once the DNA is transferred into a host bacterium and replicated, many identical copies of the recombinant DNA molecule are made (or "cloned). The two types of DNA to be recombined (the **foreign** DNA and a carrier DNA molecule or **vector**) are first digested with a type of enzyme known as a restriction endonuclease. These enzymes recognize and cleave the DNA only at specific sites and often leave short single stranded ends ("sticky ends"). These ends on the resultant DNA fragments allow them to anneal to any other end cut by the same enzyme.

Digestion of the foreign DNA (such as a bacterial chromosome) results in many different and relatively large fragments of DNA (usually several thousand base pairs long). Vector molecules, however, are generally cut only once by the restriction enzyme. The foreign DNA fragments and the digested vector DNA are mixed together, and the sticky ends will anneal forming a variety of weakly bonded recombinant DNA molecules. A large number of combinations are possible. For example, foreign DNA fragments can stick to other foreign fragments, vectors may stick to other vector molecules, or a vector molecule may stick to a foreign fragment. The enzyme DNA ligase added to the DNA mixture covalently bonds the recombinant DNA molecules together.

Bacterial plasmids and certain bacteriophage exist as extrachromosomal circular DNA molecules that have the ability to replicate in the cell. These small self-replicating molecules are ideal molecules to splice foreign DNA into and are used as vectors. Phage vectors are readily transferred into a host by packaging the recombinant DNA into a phage head that can then infect a cell by injecting the DNA into the cytoplasm. Plasmid vectors are currently more widely used, but such an easy mechanism for inserting the recombinant plasmid DNA into cells was not available when recombinant DNA technology was first developed. Thus, a method of transferring recombinant plasmids into bacterial cells was needed.

Transformation is the uptake of extracellular DNA by bacteria and would be an ideal means of transferring recombinant plasmid molecules into bacteria. However, this process only occurs naturally in a few species of bacteria and only occurs when the bacteria are in a specific physiological state (i.e. when they are "competent"). *E. coli* has long been the organism of choice for genetic studies, but it is not capable of undergoing transformation. Studies to determine if *E. coli* could be modified to allow uptake of DNA by transformation demonstrated that treatment with certain divalent cations could make the cell envelope leaky and allowed the uptake of DNA. This procedure has been refined and *E. coli* treated with calcium chloride to artificially induce competence is routinely used to take up plasmid DNA. Artificially inducing competence allows the efficient transfer of recombinant DNA molecules into *E. coli* where the plasmid DNA can be replicated and expressed.

A common type of cloning vector used in genetic engineering are the pGEM vectors because they allow easy screening of colonies that have taken up foreign DNA (transformants). The vector itself was constructed using recombinant DNA techniques and contains the origin of replication (*ori*) and a gene coding for ampicillin resistance (*amp*^{r}) from the cloning vector pBR322. pGEM also contains a gene for the lactose cleaving enzyme ß-galactosidase (*lac*Z) and a sequence known as a multiple cloning site (MCS) which is a small (69 base pairs) synthetic piece of DNA that contains restriction sites for 13 commonly used enzymes (Fig. 1). The ampicillin resistance gene allows selection of cells that have taken up the plasmid vector or a recombinant plasmid (the plasmid vector ligated to some foreign DNA). The multiple cloning site is at the beginning of the ß-galactosidase gene, and if a piece of foreign DNA is inserted into the vector at this site, it will "insertionally inactivate" the ß-galactosidase gene. This can be detected on Petri plates by including the substrate X-gal which is cleaved by ß-galactosidase to form a blue color. Thus, if a competent cell takes up the vector without any inserted DNA, it will form a blue colony. However, if it takes up a recombinant plasmid with foreign DNA inserted in the vector, ß-galactosidase will be inactivated and these cells will form a white colony. Since the media contains ampicillin, cells that do not take up any DNA will not be able to grow.

In this exercise, you will transfer a mixture of recombinant DNA molecules into *E. coli*. The recombinant DNA molecules were synthesized by ligating chromosomal DNA restriction fragments from the bioluminescent bacterium *Vibrio fischeri* with a plasmid cloning vector (pGEM-3Z). The proteins that confer bioluminescence to this organisms are coded for by seven genes (two regulatory genes and five structural genes). In *V. fischeri* all of these genes (the *lux* operon) are known to be located on a single *Sal*I restriction fragment. Both the chromosomal DNA and the plasmid have been digested by the restriction enzyme *Sal*I and ligated together by DNA ligase. This mixture of recombinant DNA molecules (or ligation mixture) will be transformed into calcium chloride treated *E. coli* cells (Fig. 1). We will then screen the transformed cells for those that have taken up a recombinant DNA plasmid that contains the *V. fischeri* bioluminescence genes (the *lux* operon) ligated into the pGEM vector. If successful, we will have cloned the *lux* genes from *V. fischeri*.

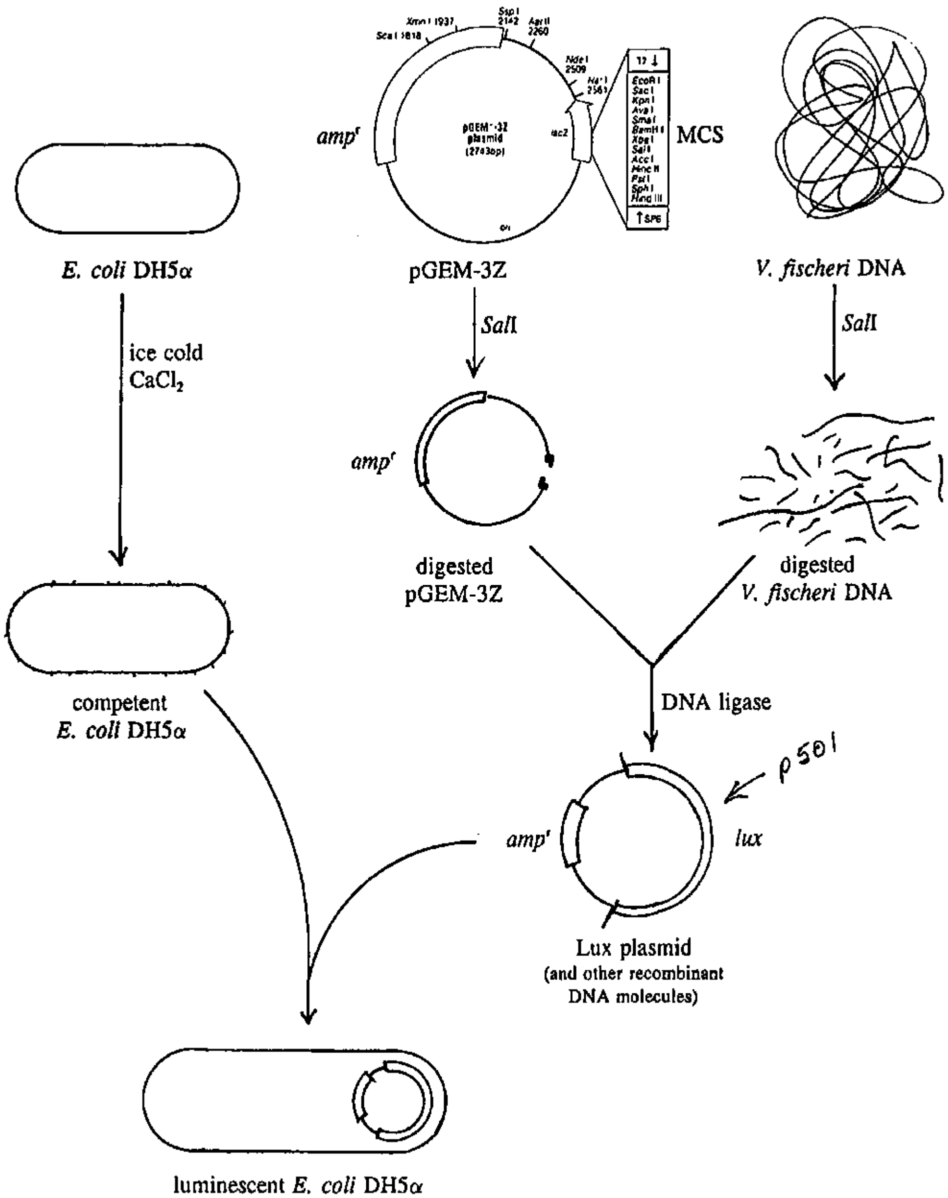


Figure 1. Transformation of a recombinant DNA plasmid into competent *E. coli*

Materials

Luria Broth (LB) culture of *E. coli* DH5α
$CaCl_2$ treated (competent) *E. coli* DH5α (on ice)
sterile TE buffer (10 mM Tris buffer [pH 8.0], 1 mM EDTA)
ligation mix
pUWL501 (0.5 μg/ml in sterile TE buffer)
pGEM-3Z (0.5 μg/ml in sterile TE buffer)
sterile LB broth (5 ml/group)
LB agar plates + 50 μg/ml ampicillin + 40 μg/ml X-gal (5/group)
sterile 15-ml disposable polypropylene culture tubes (3/group)
sterile 1-ml pipets
10 μl micropipetter
50 μl micropipetter
sterile micropipet tips
mini ice buckets
37°C shaking water bath
42°C water bath
alcohol jars
spreading triangles
spreading turntables

Procedure: (work in pairs; steps 1-4 have been done for you by your instructor prior to the lab period)

1. Inoculate a 250-ml shake flask containing 50 ml of LB broth with 1.0 ml of an overnight culture of *E. coli* DH5α and incubate on a shaker at 37°C. Grow to mid log (OD_{600} of 0.4-0.6; about 2 hours).
2. Transfer 30 ml of the mid log culture to a sterile 50-ml Oak Ridge centrifuge tube and centrifuge at 3000 X *g* (5000 rpm in a SS34 rotor) for 5 minutes at 4°C.
3. Decant and discard the supernatant. Gently resuspend the cell pellet in 15 ml (1/2 the original culture volume) of sterile ice cold 100 mM $CaCl_2$. Place the cell suspension on ice for 20 minutes.
4. Centrifuge the cells at 3000 X *g* for 5 minutes at 4°C. Discard the supernatant and resuspend in 5.0 ml (1/10 the original culture volume) of sterile ice cold 100 mM $CaCl_2$. Cells are now competent and may be used for 1-2 days after preparation if kept on ice. Cells reach their maximum level of competency about 24 hours after preparation.

Pool & make 5 ml.

Set O/N on ice.

5. Label three sterile 15-ml polypropylene culture tubes A, B, and C and place on ice. Transfer 0.2 ml of the untreated *E. coli* DH5α into tube A, and transfer 0.2 ml of the competent cells to each of tubes B and C.

Tube	Competent cells	Untreated cells	Ligation mix	TE buffer
A	--	0.2 ml	10 μl	--
B	0.2 ml	--	10 μl	--
C	0.2 ml	--	--	10 μl

6. Use the micro-pipet to add 10 μl of the recombinant DNA ligation mixture to both tubes A and B. Add 10 μl of sterile TE buffer to tube C. This will serve as a control. Your instructor will demonstrate the use of the micro-pipets.

   Your instructor will also set up a one control by adding the cloning vector (pGEM-3Z) to the competent cells and a second control with pUWL501 (a plasmid containing the *lux* operon).

7. Place all three tubes on ice for 20 minutes. This facilitates the binding of the DNA to the bacterial cells.

8. Heatshock the cells by transferring them to a 42°C water bath for exactly 90 seconds.

9. Add 1.0 ml of LB broth to each tube and incubate for 30-45 minutes in a 37°C shaking water bath. Why is this necessary?

10. Aseptically pipet the following volumes of each tube onto LB plates containing ampicillin and X-gal:

    tube A: 200 μl
    tube B: 200 μl, 50 μl, 10 μl
    tube C: 200 μl

    Spread the bacterial culture on the plates with a flame sterilized spreading triangle. To spread the 10 μl volume (tube B), first add 100 μl of sterile LB broth to the plate and add the 10 μl of the transformation mixture to the LB. Allow plates to stand upright after spreading for 5-10 minutes for the liquid to absorb into the media. Then invert and incubate at room temperature until the next period (2-3 days). Alternately, plates may be incubated at 37°C for 18-20 hours, and transferred to room temperature for 3-4 hours. Incubation at room temperature is required in order for bioluminescence to be expressed.

Results

1. Examine each of the plates for the presence or absence of colonies. Count the total number of blue and white colonies on each plate with growth, and record your results in the following table. Calculate the total number of transformants based on the fraction of the transformation mixture plated.

Tube	Blue colonies/plate	White colonies/plate	total # transformants
A			
B			
C			

2. What does the number of colonies on the control plate (A; untreated cells) tell you? What does the number of colonies on the plate from the TE transformation tell you? Can you explain these observations?

3. What does growth on media containing ampicillin tell you? Are there any colonies on the plate spread with cells from tube C? Is this what you expected?

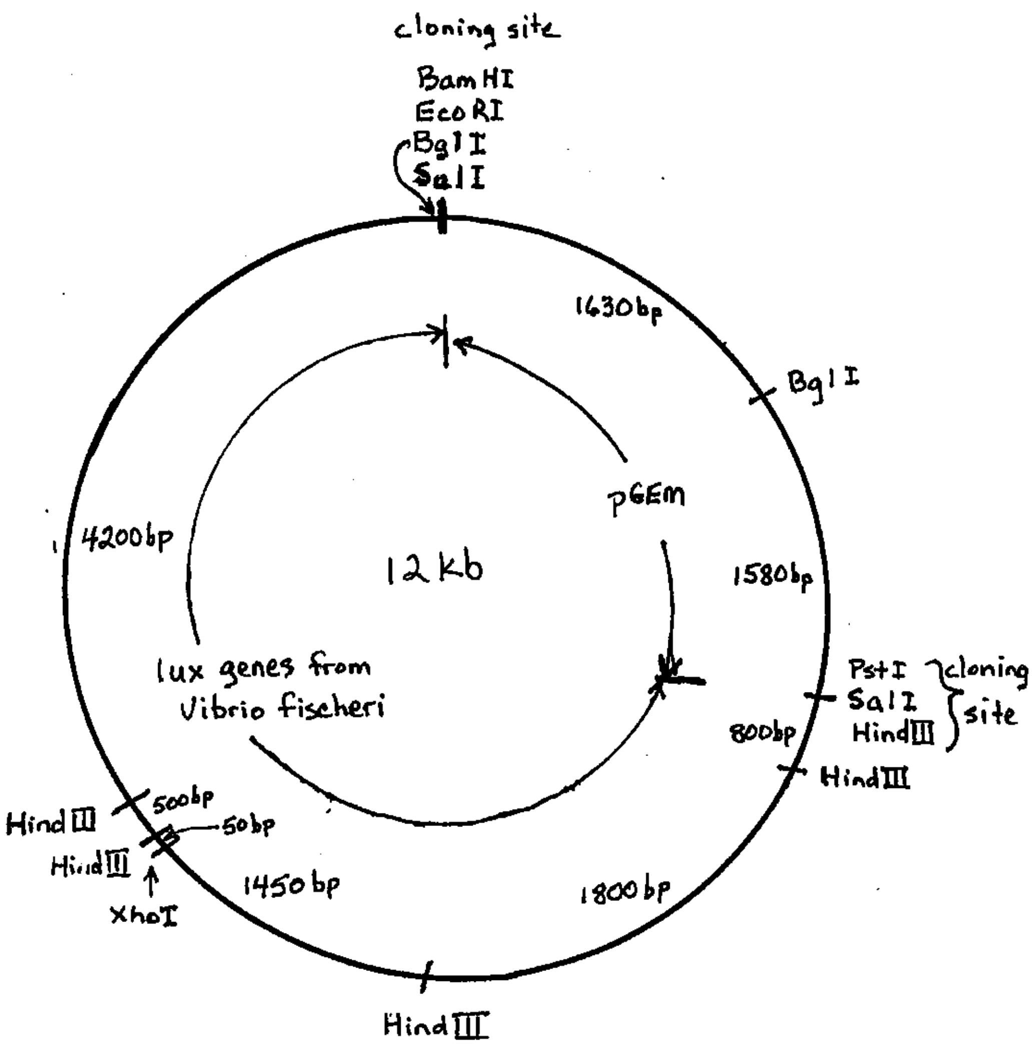
4. What proportion of the colonies on plate B are white? What does this tell you about the type of plasmid transformed into the cells? Compare your plates with the control plates set up by the instructor with cells transformed by pGEM-3Z and pUWL501. What is responsible for the different colors observed?

5. Take your plates into a dark room and examine the colonies. You may need to let your eyes dark adapt for 2-3 minutes. Are any of them bioluminescent? If so, carefully circle the luminescent colony for future purification. What does this tell you about the type of plasmid taken up by this cell?

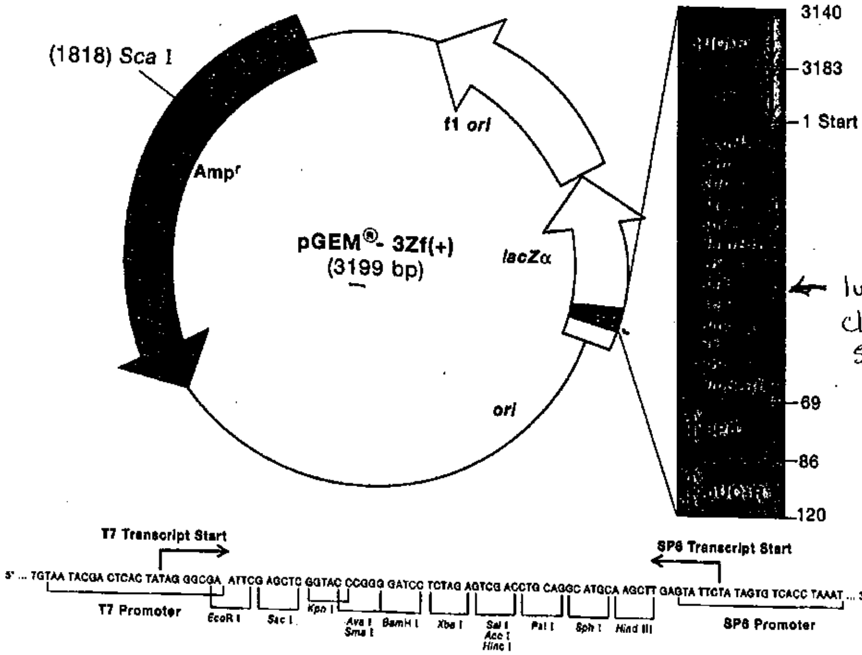
6. If you have a bioluminescent colony on your plate, carefully pick it and streak in on a LB plate containing ampicillin. Incubate at room temperature and observe after 1-2 days to verify bioluminescence.

7. Assume that there are approximately 10^8 cells per ml in the cell suspension used to spread on the plates. Calculate the transformation frequency of *E. coli* DH5α with the recombinant DNA mixture.

p501


cloning site
BamHI
EcoRI
BglI
SalI
1630bp
BglI
pGEM
12kb
4200bp
1580bp
PstI
SalI
HindIII
cloning site
800bp
HindIII
lux genes from Vibrio fischeri
HindIII
500bp
50bp
HindIII
XhoI
1450bp
1800bp
HindIII

p501 = pGEM + lux genes below in SalI site.



← lux genes cloned into SalI site

Modified from Promega Corporation with permission.

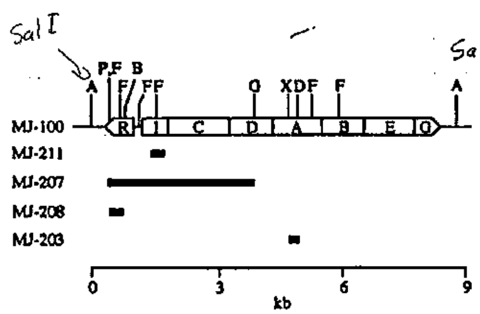


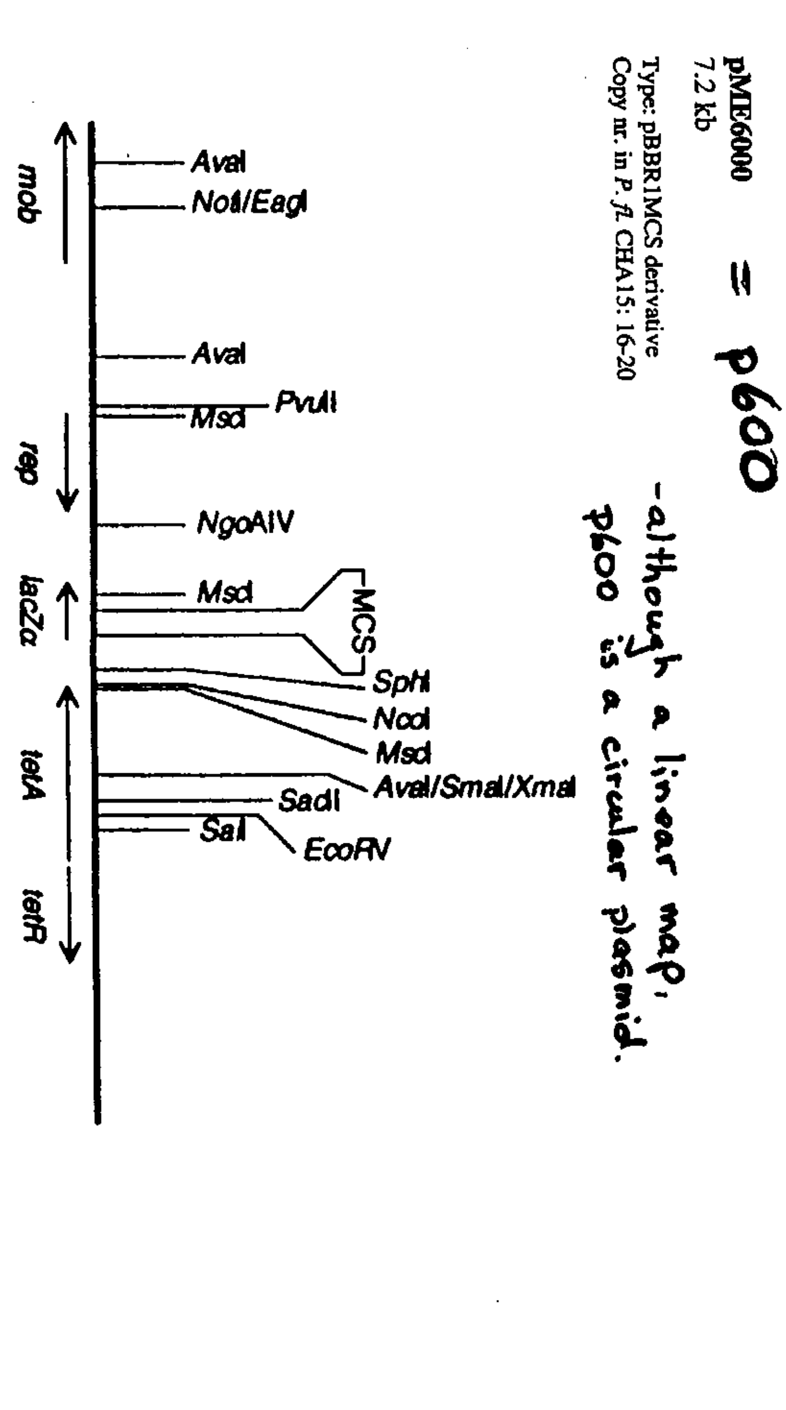
FIG. 1. The *lux* genes of *V. fischeri* and *V. fischeri lux* mutants constructed in this study. Solid bars indicate deletions. Restriction sites are denoted as follows: A, *Sal*I; P, *Pst*I; F, *Sfc*I; B, *Xba*I; G, *Bgl*II; X, *Xho*I; D, *Nhe*I. Letters inside boxes indicate *lux* genes.

from Kuo et al. 1994. J. Bacteriol. # 176(24): 7558-7565

p600 = pME6000 – from M. Valvano (UWO):

The expression vector pME6000 was constructed from pBBR1MCS plasmid by replacing the chloramphenicol resistance gene with tetracycline resistance , *tetR tetA,* from plasmid pVK100 (1). It contains a multiple cloning site within the E. coli lacZ gene (2). pBBR1MCS is a derivative of the *Bordetella bronchiseptica* plasmid pBBR1 (3). pBBR1 has 2 ORFs (open reading frames), neither of which carries virulence factors; one ORF is involved with the replication of the plasmid and the other ORF is a plasmid mobilization factor (3). In other words, pBBR1 is a cryptic plasmid (3); no functional advantage to the cell containing it. The virulence factors of *Bordetella bronchiseptica* are located on the bacterial chromosome; the bacterium is associated with infectious atrophic rhinitis in pigs and kennel cough in dogs (3).

pME6000 has a MOB gene which is required for plasmid mobilization (2). Trans acting conjugation factors from Tra1 and Tra2 regions are provided by our *E. coli* strain, BW, from the integrated RP4 plasmid (4)(5). Therefore, our p600 plasmid must be in BW in order for it to be mobilized into another *E. coli* strain. Consequently; when conducting conjugation experiments, Bio 2290 students can only transfer p600 to another *E. coli* cell line that we use. If p600 is present in JM101, for example, it cannot be transferred into another cell line and if BW contains a different plasmid, eg. p220, it can not be transferred into other strains.

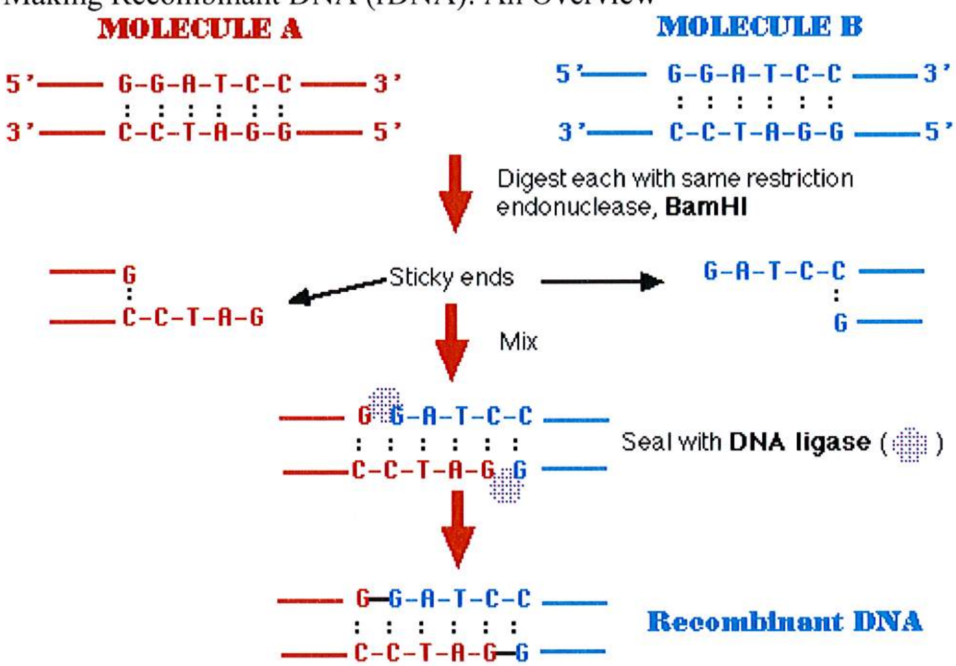


References:
1. Maurhofer *et al.* Phytopathology. 1998. 88(7):678-684.
2. Kovach *et al.* BioTechniques. 1994. 16:800-802.
3. Antoine and Locht. Mol Microbiol. 1992. 6:1785-1799.
4. Balzer *et al.* Nuc. Acids Res. 1992. 20(8):1851-1858.
5. Metcalf *et al.* Gene. 1994. 138(1-2):1-7

pA/K = pAMP/KAN – made in Bio290 from pAMP and pKAN (both from Carolina) using the following protocol:

Recombinant DNA and Gene Cloning
Recombinant DNA is DNA that has been created artificially. DNA from two or more sources is incorporated into a single recombinant molecule.

Making Recombinant DNA (rDNA): An Overview



- Treat DNA from both sources with the same restriction endonuclease (BamHI in this case).
- BamHI cuts the same site on both molecules

  5' GGATCC 3'
  3' CCTAGG 5'

- The ends of the cut have an overhanging piece of single-stranded DNA.
- These are called "sticky ends" because they are able to base pair with any DNA molecule containing the complementary sticky end.
- In this case, both DNA preparations have complementary sticky ends and thus can pair with each other when mixed.
- DNA ligase covalently links the two into a molecule of **recombinant DNA**.

To be useful, the recombinant molecule must be replicated many times to provide material for analysis, sequencing, etc. Producing many identical copies of the same recombinant molecule is called **cloning**. Cloning can be done in vitro, by a process called the **polymerase chain reaction** (**PCR**). Here, however, we shall examine how cloning is done **in vivo**.

Cloning in vivo can be done in

- unicellular microbes like **E. coli**
- unicellular eukaryotes like **yeast** and
- in mammalian cells grown in tissue culture.

In every case, the recombinant DNA must be taken up by the cell in a form in which it can be replicated and expressed. This is achieved by incorporating the DNA in a **vector**. A number of viruses (both bacterial and of mammalian cells) can serve as vectors. But here let us examine an example of cloning using **E. coli** as the host and a **plasmid** as the vector.

Plasmids

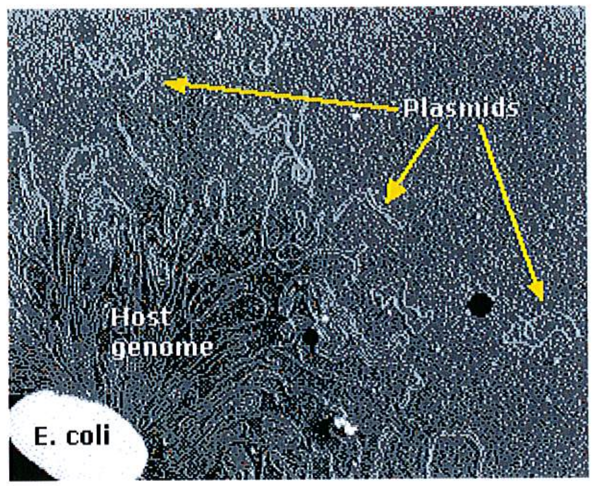
Plasmids are molecules of DNA that are found in bacteria separate from the bacterial chromosome.

They:

- are small (a few thousand base pairs)
- usually carry only one or a few genes
- are circular
- have a single **origin of replication**

Plasmids are replicated by the same machinery that replicates the bacterial chromosome. Some plasmids are copied at about the same rate as the chromosome, so a single cell is apt to have only a single copy of the plasmid. Other plasmids are copied at a high rate and a single cell may have 50 or more of them.

Genes on plasmids with high numbers of copies are usually expressed at high levels. In nature, these genes often encode proteins (e.g., enzymes) that protect the bacterium from one or more **antibiotics**.



Electron micrograph of an E. coli cell ruptured to release its DNA. The tangle is a portion of a single DNA molecule containing over 4.6 million base pairs encoding approximately 4,300 genes. The small circlets are plasmids. (Courtesy of Huntington Potter and David Dressler, Harvard Medical School.)

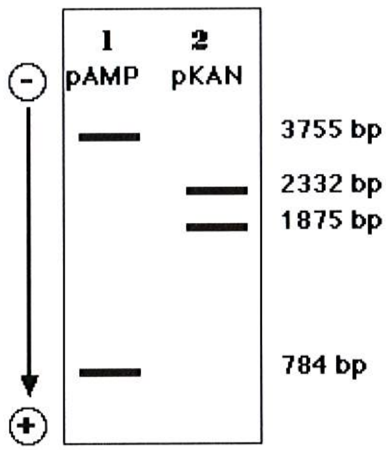
Plasmids enter the bacterial cell with relative ease. This occurs in nature and may account for the rapid spread of antibiotic resistance in hospitals and elsewhere. Plasmids can be deliberately introduced into bacteria in the laboratory transforming the cell with the incoming genes.

An Example

(courtesy of David Miklos and Greg Freyer of the Cold Spring Harbor Laboratory, who used these plasmids as the basis of a laboratory introduction to recombinant DNA technology that every serious biology student — high school or college — should experience!)

pAMP

- 4539 base pairs
- a single replication origin
- a gene (**amp**^{r})conferring resistance to the antibiotic **ampicillin** (a relative of penicillin)
- a **single** occurrence of the sequence



5' GGATCC 3'
3' CCTAGG 5'

that, as we saw above, is cut by the restriction enzyme **BamHI**

- a **single** occurrence of the sequence

5' AAGCTT 3'
3' TTCGAA 5'

that is cut by the restriction enzyme **HindIII**

Treatment of pAMP with a **mixture** of BamHI and HindIII produces:

- a fragment of **3755** base pairs carrying both the **amp**^{r} gene and the replication origin
- a fragment of **784** base pairs
- both fragments have sticky ends

pKAN

- 4207 base pairs
- a single replication origin
- a gene (**kan**^{r}) conferring resistance to the antibiotic **kanamycin**.
- a single site cut by **BamHI**
- a single site cut by **HindIII**

Treatment of pKAN with a **mixture** of BamHI and HindIII produces:

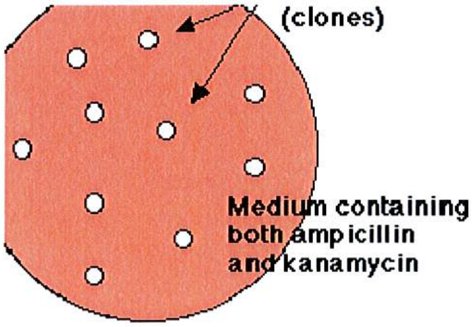
- a fragment of **2332** base pairs
- a fragment of **1875** base pairs with the **kan**^{r} gene (but no origin of replication)
- both fragments have sticky ends

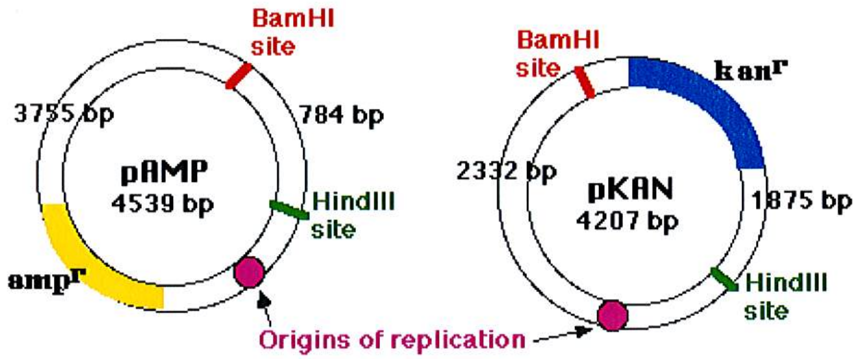
These fragments can be visualized by subjecting the digestion mixtures to electrophoresis in an agarose gel. Because of its negatively-charged phosphate groups, DNA migrates toward the positive electrode (anode) when a direct current is applied. The smaller the fragment, the farther it migrates in the gel.

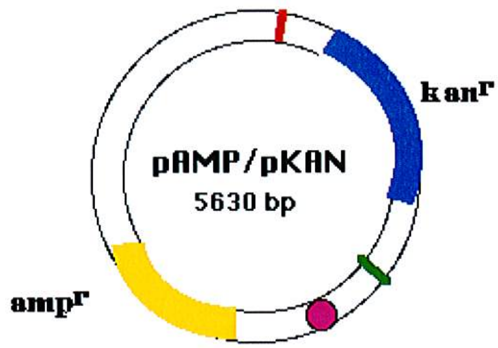
Ligation Possibilities

If you remove the two restriction enzymes and provide the conditions for DNA ligase to do its work, the pieces of these plasmids can rejoin (thanks to the complementarity of their sticky ends).

Mixing the pKAN and pAMP fragments provides several (at least 10) possibilities of rejoined molecules. Some of these will not produce functional plasmids (molecules with two or with no replication origin cannot function).







One interesting possibility is the joining of

- the 3755-bp pAMP fragment (with **amp**^{r} and a replication origin) with the
- 1875-bp pKAN fragment (with **kan**^{r})

Sealed with **DNA ligase**, these molecules are functioning plasmids that are capable of conferring resistance to **both** ampicillin and kanamycin. They are molecules of **recombinant DNA**.

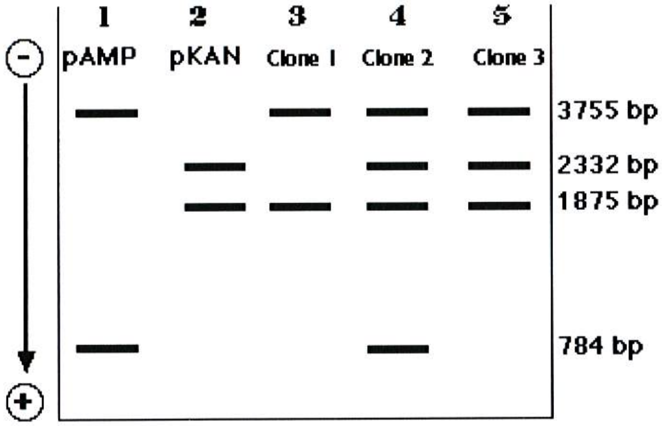
Because the replication origin, which enables the molecule to function as a plasmid, was contributed by pAMP, pAMP is called the **vector**.

Transforming E. coli
Treatment of E. coli with the mixture of religated molecules will produce some colonies that are able to grow in the presence of both ampicillin and kanamycin.

- A suspension of E. coli is treated with the mixture of religated DNA molecules.
- The suspension is spread on the surface of agar containing both ampicillin and kanamycin.
- The next day, a few cells — resistant to both antibiotics — will have grown into visible colonies containing billions of transformed cells.
- Each colony represents a **clone** of transformed cells.

However, E. coli can be simultaneously transformed by more than one plasmid, so we must demonstrate that the transformed cells have acquired the recombinant plasmid.

Electrophoresis of the DNA from doubly-resistant colonies (clones) tells the story.



- Plasmid DNA from cells that acquired their resistance from a **recombinant plasmid** only show only the **3755**-bp and **1875**-bp bands (**Clone 1**, lane 3).
- **Clone 2** (Lane 4) was simultaneous transformed by religated pAMP and pKAN. (We cannot tell if it took up the recombinant molecule as well.)
- **Clone 3** (Lane 5) was transformed by the recombinant molecule as well as by an intact pKAN.

Cloning other Genes

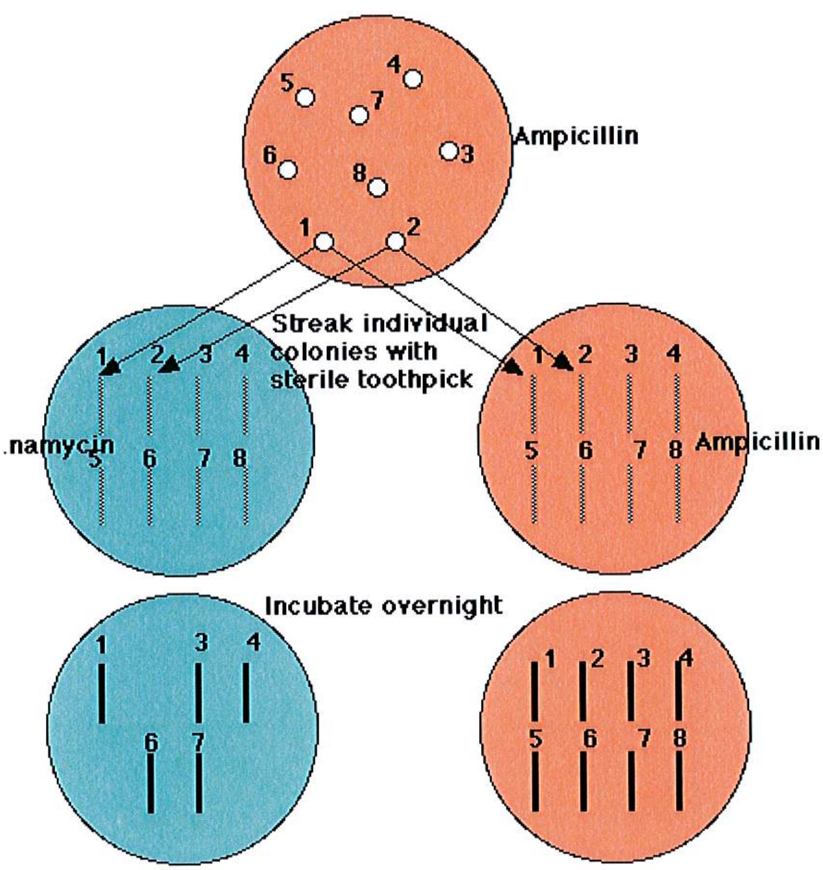
The recombinant vector described above could itself be a useful tool for cloning other genes. Let us assume that within its **kanamycin resistance gene** (**kan^r**) there is a single occurrence of the sequence

5' GAATTC 3'

3' CTTAAG 5'

This is cut by the restriction enzyme **EcoRI**, producing sticky ends.

If we treat any other sample of DNA, e.g., from human cells, with EcoRI, fragments with the same sticky ends will be formed. Mixed with EcoRI-treated plasmid and DNA ligase, a small number of the human molecules will become incorporated into the plasmid which can then be used to transform E. coli.



But how to detect those clones of E. coli that have been transformed by a plasmid carrying a piece of human DNA?

The key is that the EcoRI site is **within** the **kan^r** gene, so when a piece of human DNA is inserted there, the gene's function is destroyed.

All E. coli cells transformed by the vector, whether it carries human DNA or not, can grow in the presence of ampicillin. But E. coli cells transformed by a plasmid carrying human DNA will be unable to grow in the presence of kanamycin.

So,

- Spread a suspension of treated E. coli on agar containing ampicillin only
- grow overnight
- with a sterile toothpick transfer a small amount of each colony to an identified spot on agar containing kanamycin
- (do the same with another ampicillin plate)

- Incubate overnight

All those clones that continue to grow on ampicillin but fail to grow on kanamycin (here, **clones 2**, **5**, and **8**) have been transformed with a piece of human DNA.

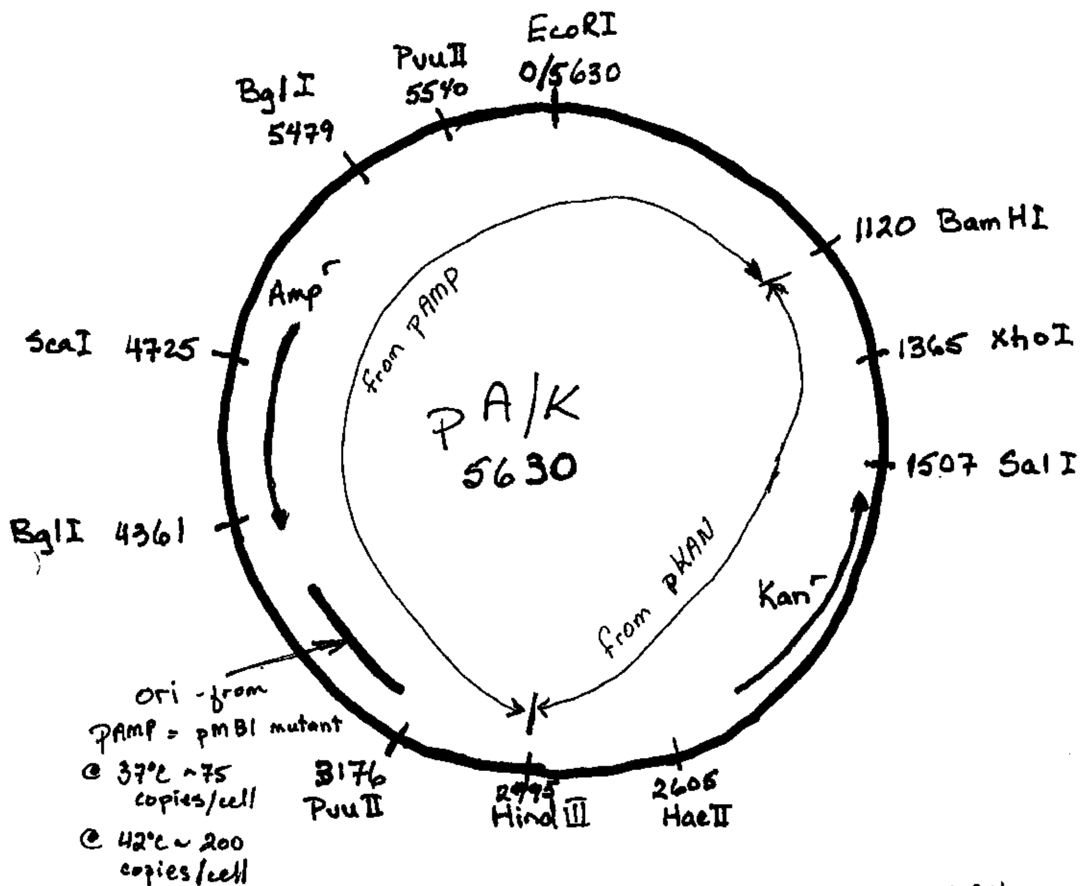
Some recombinant DNA products being used in human therapy

Using procedures like this, many human genes have been cloned in E. coli or in yeast. This has made it possible — for the first time — to produce unlimited amounts of human proteins in vitro. Cultured cells (E. coli, yeast, mammalian cells) transformed with a human gene are being used to manufacture more than 100 products for human therapy. Some examples:

- **insulin** for diabetics
- **factor VIII** for males suffering from hemophilia A
- **factor IX** for hemophilia B
- **human growth hormone (HGH)**
- **erythropoietin (EPO)** for treating anemia
- several types of **interferons**
- several **interleukins**
- **granulocyte-macrophage colony-stimulating factor (GM-CSF)** for stimulating the bone marrow after a bone marrow transplant
- **granulocyte colony-stimulating factor (G-CSF)** for stimulating neutrophil production, e.g., after chemotherapy and for mobilizing hematopoietic stem cells from the bone marrow into the blood.
- **tissue plasminogen activator (TPA)** for dissolving blood clots
- **adenosine deaminase (ADA)** for treating some forms of **severe combined immunodeficiency (SCID)**
- **parathyroid hormone**
- several **monoclonal antibodies**
- **hepatitis B surface antigen (HBsAg)** to vaccinate against the hepatitis B virus
- **C1 inhibitor (C1INH)** used to treat hereditary angioneurotic edema (HANE)

24 February 2008

PA/K



pA/k was made by Joan by cutting pAMP + pKAN with BamHI + HindIII - ligating the fragments - transforming into ~~#~~ Ecoli JM101 + selecting for transformants on LB+amp+kan plates - checked for plasmid size. from Joan's notes.

see Plasmids (12)

pAMP – from Carolina:

pAMP

CAROLINA BIOLOGICAL SUPPLY COMPANY

P.O. BOX 1059
BURLINGTON, NORTH CAROLINA 27216

1308 Rainey St.
Burlington, N.C. 27217

TEL. 800-227-1150
FAX 919-222-1926

Phone 919-226-6000
TLX 574-354

REFRIGERATE ON ARRIVAL

Plasmid pAMP DNA

21-1429
10 ug (50 ul)
0.2 ug/ul

21-1430
20 ug (100 ul)
0.2 ug/ul

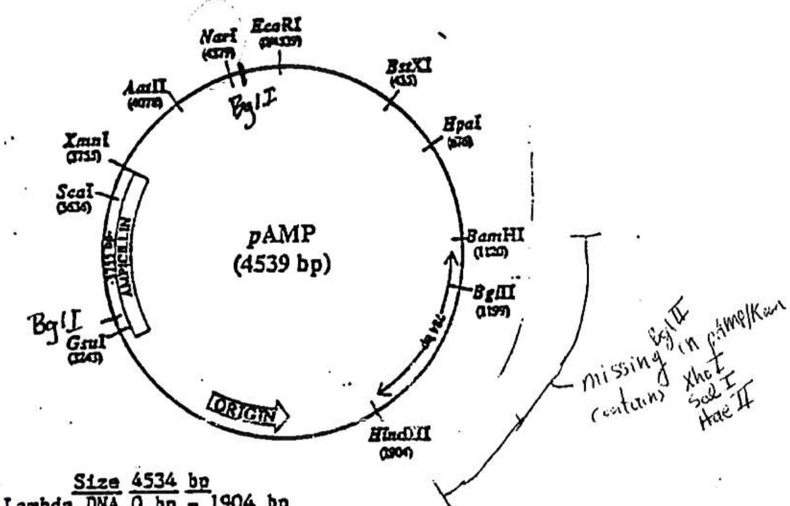
21-1431
40 ug (200 ul)
0.2 ug/ul

21-1432
20 ug (50 ul)
0.4 ug/ul

21-1433
40 ug (100 ul)
0.4 ug/ul

21-1434
10 ug (25 ul)
0.4 ug/ul

21-1438
1 ug (200 ul)
0.005 ug/ul



Size	4534 bp
Lambda DNA	0 bp - 1904 bp
pCU19	1904 bp - 4534 bp

Selected restriction sites for enzymes that cut only once.

Storage Conditions

Recommedned storage is between 2 - 5°C.

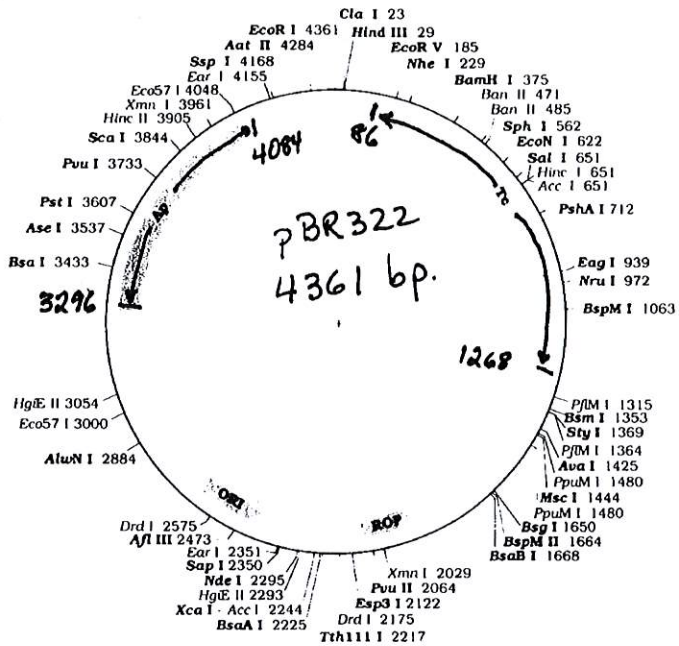
TE Buffer provided with each order.

*For diluting instructions see the reverse

pAMPKAN = 5630
Contains XhoI SalI HaeII No BglII

pBR = pBR322 – from Boehringer Mannheim:

A. pBR322



pBR322 is an *E. coli* plasmid cloning vector. Recent sequencing data from Watson (confirmed at New England Biolabs) has shown its length to be 4361 base pairs not 4363 base pairs as previously reported. pBR322 was constructed in vitro using the tetracycline resistance gene (Tc) from pSC101, the origin of DNA replication start (ORI) and *rop* gene from the ColE1 derivative pMB1, and the ampicillin resistance gene (Ap) from transposon Tn3. Numbering of the sequence begins within the unique *Eco*RI site: the first T in the sequence . . .GAATTC. . . is designated as nucleotide number 1. Numbering then continues around the molecule in the direction of Tc to Ap.

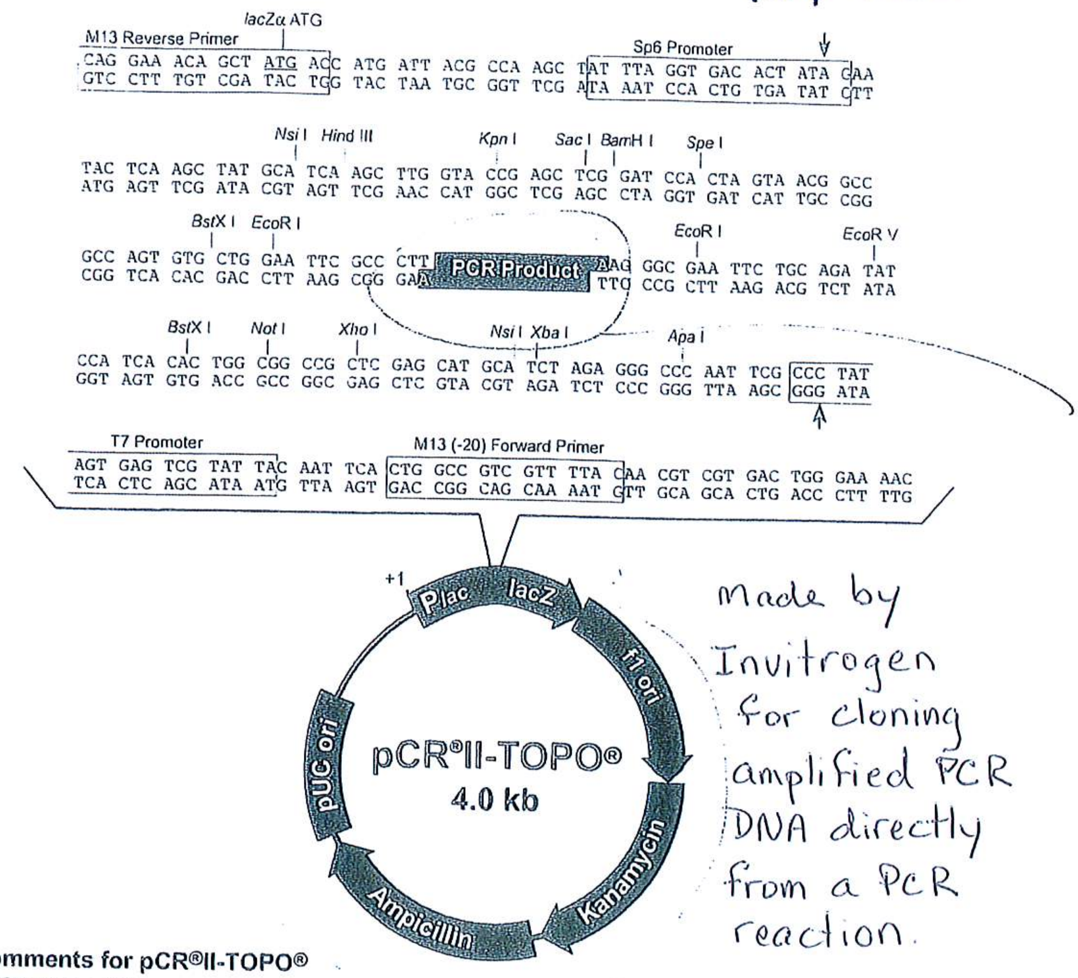
The map shows the restriction sites of those enzymes that cut the molecule once or twice; the unique sites are shown in **bold** type. The table lists the sites of those enzymes that cut a moderate number of times. The coordinates refer to the position of the 5′ base in each recognition sequence. The map also shows the relative positions of the antibiotic resistance genes, *rop* (mediates the activity of RNase I), and the origin of replication. The exact positions are: tetracycline resistance (Tc) 86–1268; β-lactamase (Ap) 3296–4084; ROP 1918–2105; origin of DNA replication start (ORI) 2535.

References

1. Bolivar, F. et al. 1977. *Gene* **2:** 95–113.
2. Sutcliffe, J.G. 1978. *Cold Spring Harbor Symp. Quant. Biol.* **43:** 77–90.
3. Sutcliffe, J.G. 1978. *Proc. Natl. Acad. Sci.* **75:** 3737–3741.
4. Peden, K.W.C. 1983. *Gene* **22:** 277–280.
5. Backman, K. and and H.W. Boyer. 1983. *Gene* **26:** 197–203.
6. Lathe, R., M.P. Kieny, S. Skory, and J.P. Lecocoq. 1984. *DNA* **3:** 173–182.
7. Heusterspreute, M. and J. Davison. 1984. *DNA* **3:** 259–264.
8. *GenBank* 1987. 50.0 VB0001.
9. Watson, N. 1988. *Gene* **70:** 399–403.

from: "A Short Course in Bacterial Genetics". Jeffrey H. Miller. 1992. Cold Spring Harbor Lab Press.

pCR2 = 4.0kb
(amp^r + kan^r)



Made by Invitrogen for cloning amplified PCR DNA directly from a PCR reaction.

amp^r + kan^r

Comments for pCR®II-TOPO®
3973 nucleotides

LacZα gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851

from Logan Walsh in Sashko Damjanovski lab.

pGATA = 6.1kb (kan^r)

Plasmid Name pCMVTag3B-GATA-6

Other Names

Constructed / Submitted by Nibbi

Vector (name and size) pCMVTag3B (4.3 Kb)

Insert (name and size) GATA-6 (1.76 Kb)

Species

Accession # 46909570

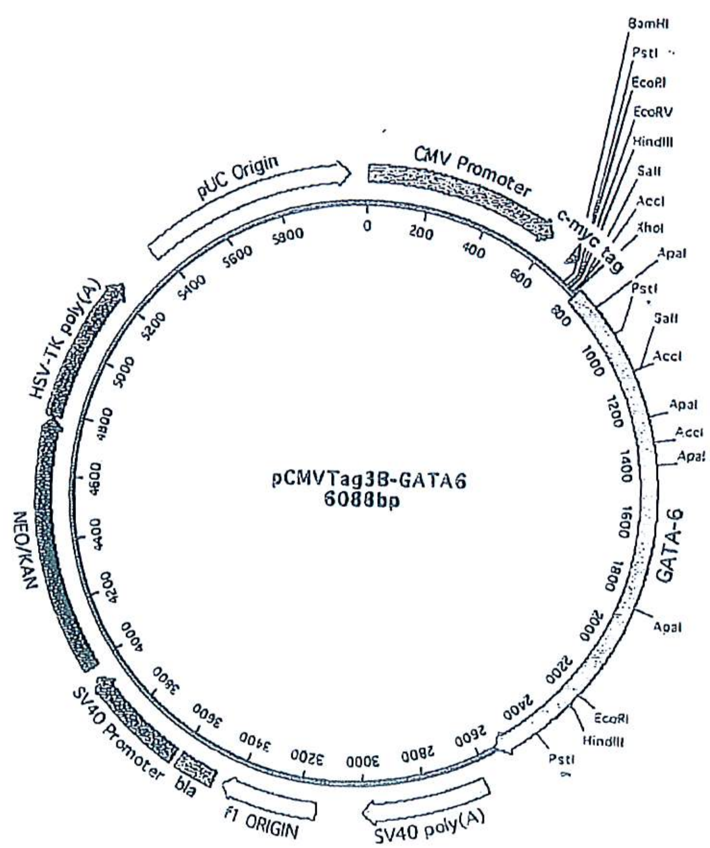
Host Strain

Selection

Verified?

Notes and Reference

GATA-6 has been cloned b/w the kpnI and XhoI sites of pCMVTag3B.



pGATA continued:

The vectors pCMV-Tag 2, **pCMV-Tag 3**, pCMV-Tag 4, and pCMVTag5 are a series of binary vectors that work in bacteria (*E. coli*) and mammalian cells with epitope tagging when expressed in mammalian cells. Inserts are ligated into the vector and selected for using *E. coli,* the plasmid (vector and insert) is amplified in *E. coli*, isolated and then transfected into mammalian cells for study. pCMV-Tag 2 is an N-terminal FLAG® tagging vector, **pCMVTag 3** is an N-terminal c-myc tagging vector, pCMV-Tag 4 is a C-terminal FLAG tagging vector and pCMV-Tag 5 is a C-terminal c-myc tagging vector. Each vector is available in three different reading frames to simplify subcloning. These reading frames, designated as A, **B**, and C, differ only by one or two bases. Thus, each pCMV-Tag vector has a reading frame that will allow cloning a gene of interest so that it is fused correctly with the epitope tag. Tagged constructs generated in the pCMV-Tag vectors can be transfected into mammalian cells and the fusion protein can be easily characterized using commercially available antibodies.

GATA-6 insert is a mouse gene from a cDNA library and is highly conserved (88% identity with human form). The gene is essential for development of extraembryonic endoderm (gives rise to the yolk sac of the developing embryo and contains signalling molecules critical for properly forming the foetus) amongst other tissues later in development. GATA-6 K/O mice die shortly after gastrulation from lack of extraembryonic endoderm tissues.

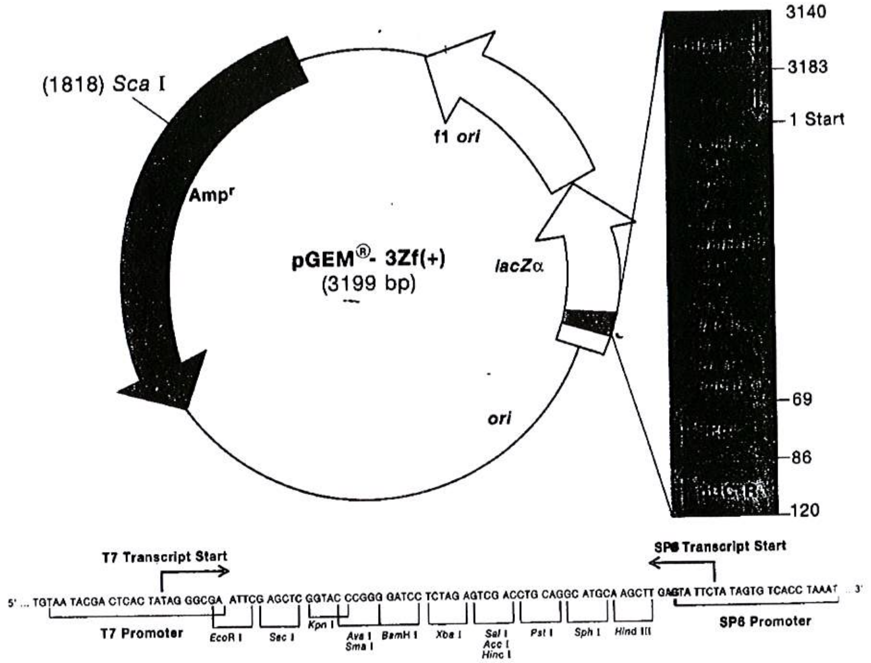
GATA proteins are transcriptional coactivators with two GATA-type zinc fingers. GATAs bind to the consensus DNA sequence (A/T) GATA (A/G) to control diverse tissue-specific programs of gene expression and morphogenesis. GATA-1, -2, -3 are selectively expressed in hematopoietic and endothelial cells, while GATA-4, -5, and -6 are expressed in developing heart and gut-derived tissues. GATA-1 and -3 exist in multiple alternatively spliced isoforms.

GATA-6 (also named GATA-GT1) is a zinc finger transcription activating protein that binds to a T/A-G-A-T-A-G/A DNA sequence motif in mesodermally and endodermally-derived tissue. There are two GATA-6 isoforms that arise from alternate start sites: a short form of 449 amino acids (aa), and a long form of 595 aa. The short form of human GATA-6 is 93%, 92% and 88% aa identical to GATA-6 in porcine, rat, and mouse, respectively.

pGEM = pGEM-3Zf(+) – from Promega:

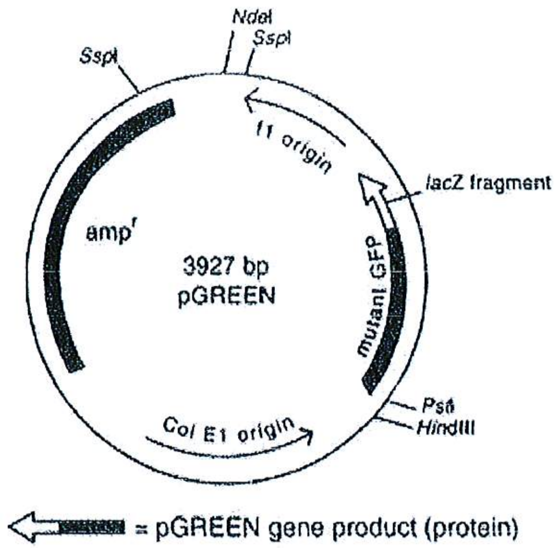
pGEM™-3Zf(+)

pGEM-3Zf(+) (see Table A11.3) is one of a series of plasmid cloning vecto oped by Promega Corporation (1991). The pGEM vectors are derivatives of pUC19 that allow α-complementation and also have bacteriophage promoters (SP6 and T7) that flank the multiple cloning site. These promoters are flanked by the pUC forward and reverse primer sites as in pUC18 and pUC19. The bacteriophage promoters allow one to synthesize an RNA copy of the cloned DNA that may be used for transcriptional studies or as hybridization probes. pGEM-3Zf(+) also contains the origin of replication of the filamentous bacteriophage f1 that allows synthesis of a single-stranded copy of the cloned DNA to use as a template for DNA sequencing.



Modified from Promega Corporation with permission.

pGREEN: (from: www. carolina.com)



GFP = green fluorescent protein
- the gene is from the jellyfish
Aequorea victoria

Green phenotype; will also glow under UV light; transformation concentration only. 1 µg (200 µL; 0.005 µg/µL).*

Transforming concentration: use 10 µL/transformation.

pKAN – from Carolina:

pKAN

CAROLINA BIOLOGICAL SUPPLY COMPANY

P.O. BOX 1059
BURLINGTON, NORTH CAROLINA 27216

1308 Rainey St.
Burlington, N.C. 27217

TEL 800-227-1150
FAX 919-222-1926

Phone 919-226-6000
TLX 574-354

REFRIGERATE ON ARRIVAL

Plasmid pKAN DNA

21-1439
10 ug (50 ul)
0.2 ug/ul

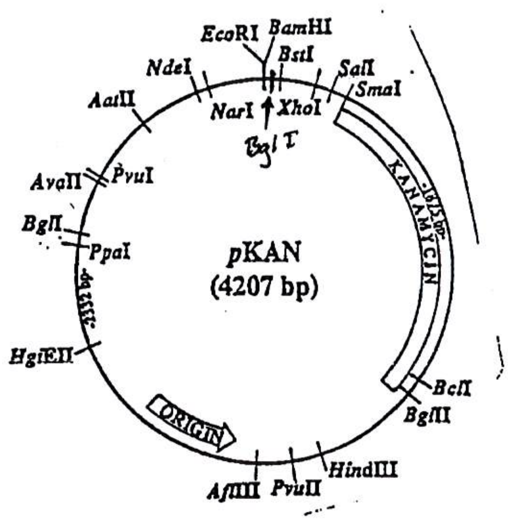
21-1440
20ug (100 ul)
0.2 ug/ul

21-1441
40 ug (200 ul)
0.2 ug/ul

21-1442.
20 ug (50 ul)
0.4 ug/ul

21-1443
40 ug (100 ul)
0.4 ug/ul

21-1444
10 ug (25 ul)
0.4 ug/ul



Selected restriction sites for enzymes that cut only once.

Storage Conditions

Recommended storage is between 2 - 5°C.

TE Buffer Provided with each order.

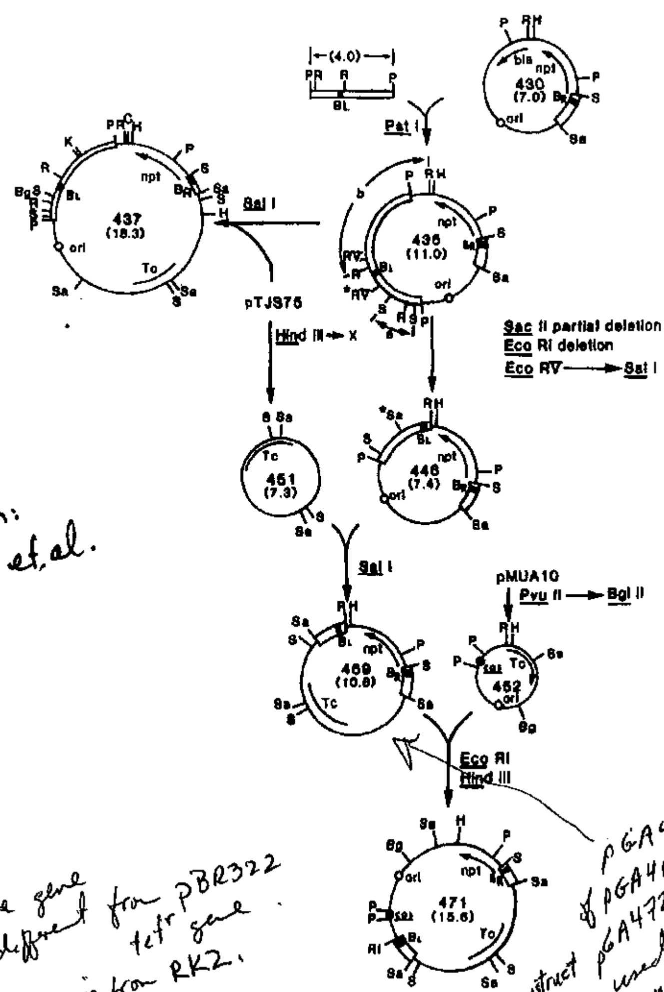
*For diluting instructions see the reverse side.

pKYLX = pKYLX7 from Denis Maxwell (UWO):

pKYLX is a binary expression vector that can be maintained in *Escherichia coli* and *Agrobacterium tumefaciens* by virtue of the RK2 origin of replication (1,2). Selection for the plasmid in prokaryotic cells is by virtue of the tetracycline resistance gene (3). Gene transfer into plant cells is *Agrobacterium tumefaciens* mediated and selected for by virtue of kanamycin resistance gene(3). In plant cells, the expression of the cloned genes in pKYLX is under control of the CaMV 35S promoter (3).

Our pKYLX does not contain any cloned genes in it; i.e. it is just the vector and is only transformed into *Escherichia coli* strains listed in the "Bacteria" Appendix.

Below are the various plasmids and protocol in the construction of pKYLX:



from:
An et. al.

tetracycline gene is different from pBR322 tetr gene ie from RK2.

pGA470 instead of pGA469 was used to construct pGA472 which was used t make pKYLX-7 (see Schardl et al 1987)

Fig. 2. Construction of shuttle vectors. pGA435 was constructed by inserting the 4.0-kb *Pst*I fragment containing the left border of pTiT37 into pGA430 (Figure 1) at the *Pst*I site of the *bla* gene. pGA437 was constructed by ligating *Sal*I-digested pGA435 into one of the *Sal*I sites of pTJS75. pTJS75 contains two *Sal*I sites; one is in the tetracycline gene and the other is located outside of the drug resistance gene and origin of replication. pGA437 was constructed by ligating pGA435 and pTJS75 at the *Sal*I site. In pGA446, the *Sac*II fragment (a) and the *Eco*RI fragment (b) were sequentially deleted from pGA435 and a *Sal*I linker weas inserted at the filled-in *Eco*RV site (*). pGA469 and pGA470 were built by inserting the *Sal*I fragment containing the *nos-npt* chimeric gene of pGA446 into the *Sal*I site of pGA451 derived from pTJS75 by filling in the unique *Hind*III with DNA polymerase large fragment. Only pGA469 is shown in the diagram. In pGA470, the direction of the *Sal*I fragment is in opposite orientation to pGA469. pGA452 was constructed by inserting a *Bgl*II linker into the *Pvu*II site of the cosmid pMUA10 (Meyerowitz *et al.*, 1980). The large *Hind*III-*Eco*RI fragment which contains the cos site was used to replace the small *Hind*III-*Eco*RI fragments of pGA469 and pGA470, to yield pGA471 and pGA472, respectively. The tetracycline gene on the wide-host replicon is from RK2 and there is no homology to the tetracycline gene residing on pGA452 which is a derivative of pBR322. The numbers in parentheses indicate the size of plasmids in kbp. The origin of the wide host replicon is located in between BR and Tc in pGA437 and in between BL and Tc in pGA469 and pGA471. Symbols: bla, ampicillin resistance gene; npt, kanamycin resistance gene of *nos-npt*; Tc, tetracycline resistance gene; BR, the right border of T-DNA; BL, the left border of T-DNA; ori, ColE1 origin of replication; *cos*, λ *cos* site; Bg, *Bgl*II; C, *Cla*I; H, *Hind*III; K, *Kpn*I; P, *Pst*I; R, *Eco*RI; RV, *Eco*RV; S, *Sac*II; Sa, *Sal*I.

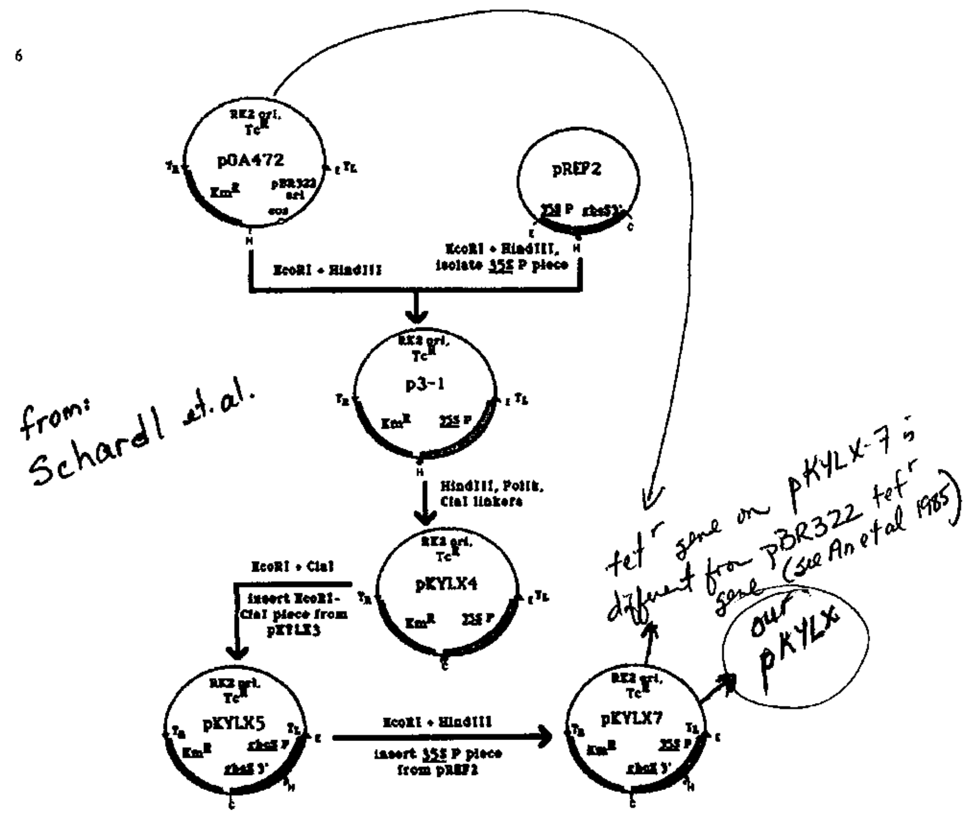
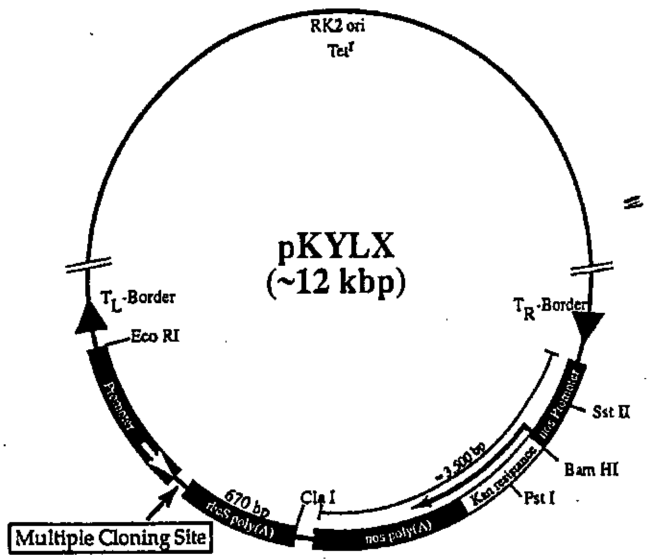


Fig. 3. Strategy for the construction of the expression/shuttle vectors pKYLX5 and pKYLX7. The parent plasmid, pGA472 (An et al., 1985) is designed for *A. tumefaciens*-mediated transformation of plant tissues. The synthetic T-region, which is transferred and integrated in the plant genome, is comprised of a right border (T_R) and left border (T_L), flanking unique *Hin*dIII and *Eco*RI sites. The chimeric *nos-Aph(3')II-nos* gene (Km^R) provides a selectable marker for plant transformation (see MATERIALS AND METHODS, section c. The 35S promoter sequence in pREF2 (see Table I) was excised as an *Hin*dIII-*Eco*RI fragment, and cloned into pGA472, to generate p3-1 (see also Fig. 1). A unique *Cla*I site was introduced into the *Hin*dIII site, to generate pKYLX4 (see RESULTS AND DISCUSSION, section b). The *Hin*dIII site was not reconstituted in the process. To produce pKYLX5, the 0.9-kb *Cla*I-*Eco*RI fragment containing the *rbcS P*-MCS-*rbcS3'* expression cassette from pKYLX3 (see Fig. 1) was introduced into pKYLX4. The *rbcS P* sequence, on an *Eco*RI-*Hin*dIII fragment, was replaced with the 35S promoter sequence contained on an *Eco*RI-*Hin*dIII of pREF2, generating the 35S *P*-MCS-*rbcS3'* expression cassette of pKYLX7. Plasmids are not drawn to scale. Both pKYLX5 and pKYLX7 are approximately 12-kb. Abbreviations are as in Fig. 1. Arrows: T-DNA borders (labelled appropriately as T_L or T_R) and orientation.

linkers (ATCGAT) were ligated to the vector and the DNA was extracted with phenol–$CHCl_3$ (1:1) and twice precipitated with ammonium acetate and ethanol. The DNA was resuspended and phosphorylated using T4 polynucleotide kinase; then the plasmid was self-annealed and a second ligation was carried out, followed by transformation of *E. coli* DH5 (Hanahan, 1983). The resulting plasmid was designated pKYLX4 (Fig. 3).

The expression cassette from pKYLX3 was cloned into the pKYLX4 binary vector, replacing the 0.9-kb *Eco*RI-*Cla*I fragment of this plasmid. The resulting vector was designated pKYLX5. A second plasmid, pKYLX7, was generated by replacing the

↑ pKYLX



= pKYLX 7. see Schardl et al.

promoter: 35S[2] (CaMV 35S promoter with a duplicated enhancer)
MCS: HindIII* - BamHI - XhoI* - PstI - SacI* - XbaI* (*-unique sites)

Constructed by the lab of A.G. Hunt at U Kentucky

Obtained from the lab of Shengyang He

from Denis Maxwell Nov 21/07

Schardl .... Hunt. 1987. Gene. 61: 1-11

An, G., et al. 1985. EMBO J. 4: 277-284

References:
1. Stalker *et al.* Mol. Gen. Genet. 1981. 181:8-12.
2. Konieczny *et al.* J. Bio. Chem. 1997. 272(32):20173-20178.
3. Schardl *et al.* Gene. 1987. 61:1-11.
4. An *et al.* EMBO J. 1985. 4(2):277-284.

pVIB (13.4kb)

FREEZE ON ARRIVAL

21-1447

Plasmid pVIB
200 µl
0.005 µg/µl

Store at 2 - 5°C

lux genes cloned into pBR322

Carolina

Teamed with Teachers

To order call:
1-800-334-5551 (US and Canada)
336-584-0381 (International)

For technical help call:
1-800-227-1150

Carolina Biological Supply Company
2700 York Road, Burlington, North Carolina 27215
www.carolina.com
e-mail: biotech@carolina.com

Introduction

Bacteria that produce light are very common in the ocean. One type of luminescent bacteria is *Vibrio fischeri*. *Vibrio fischeri* produce light through the action of the enzyme luciferase on a particular aldehyde. There are several genes involved in this light production. The luciferase enzyme itself is composed of two different subunits encoded by two different genes. Synthesis of the aldehyde also requires the action of several genes, as does the regulation. Together, all of the genes required for light production are called the lux genes.

The lux genes have been removed from *Vibrio fischeri* and placed in a plasmid. In addition to the *Vibrio fischeri* genes, pVIB also contains the gene for resistance to ampicillin. The plasmid pVIB was originally made by a team of three scientists: JoAnne Engebrecht, Kenneth Nealson, and Michael Silverman. They published a paper about their work *called Bacterial bioluminescence: isolation and genetic analysis of functions from* Vibrio fischeri, in Cell, 1983, vol. 32, pg. 773-781. In this paper, pVIB is referred to as pJE202.

Colony Transformation Applications

The solution of pVIB provided is the correct concentration for colony transformation of *E. coli* cells. Add 10 µl of pVIB to a culture tube containing *E. coli* cells in 250 µl of ice-cold calcium chloride, follow with a 42°C heat shock. For specific rapid colony transformation instructions, please review one of Carolina's rapid colony transformation kit instructions (Cat. # 21-1082, 21-1088, 21-1142, 21-1146), *DNA Science* by Micklos and Freyer, 1990, or contact the biotechnology department at 1-800-227-1150.

pVIB continued:

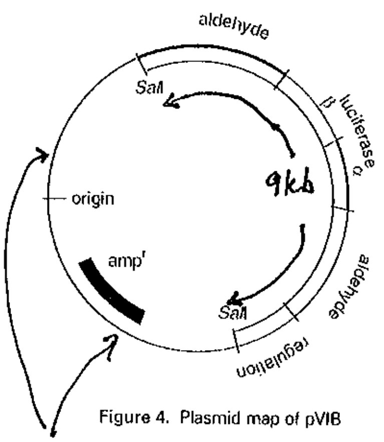


Figure 4. Plasmid map of pVIB

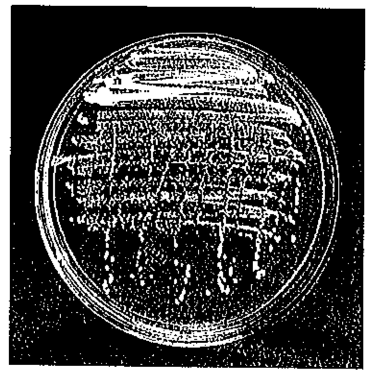


Figure 5. Photo of colonies containing pVIB

pBR322 = 4.4kb

lux genes cloned into SalI site in tetracycline resistance gene of pBR322

pVIB = 13.4Kb

Color marker genes: Lux genes

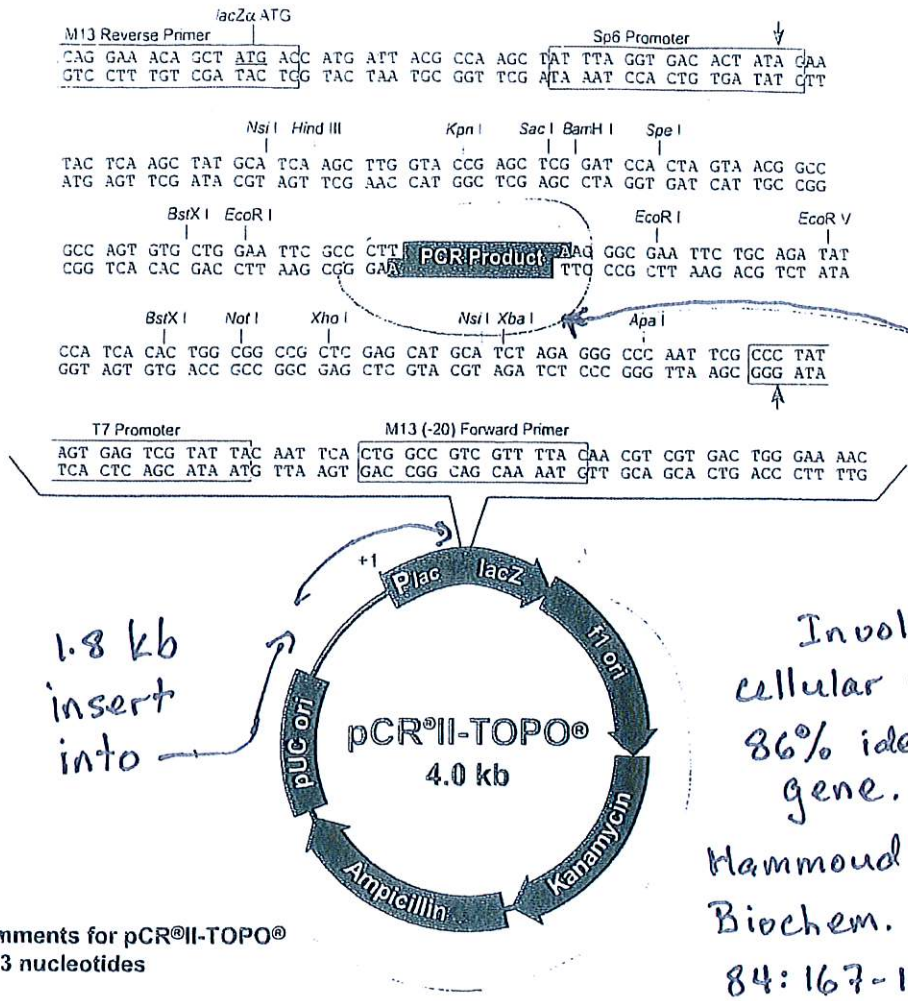
Bacteria that produce light are very common in the ocean. Some types of bacteria that produce light are free-living as plankton. Others live symbiotically with higher organisms, such as fish and squid. The fish and squid hosts use the luminescence of the bacteria for several different purposes, including attracting their prey, communicating with others of their species, and confusing predators. However, it is unknown what benefit the glowing bacteria get out of this relationship. Perhaps you and your class can develop a hypothesis to explain why these bacteria have evolved to glow. One type of luminescent bacterium is *Vibrio fischeri*. In nature, these bacteria live in the light organ of the fish *Monocentris japonicus*.

Vibrio fischeri produce light through the action of the enzyme luciferase on a particular aldehyde. Because the reaction requires a great deal of the cell's energy (10% or more!) the process is highly regulated. There are several genes involved in *V. fischeri*'s light production. The luciferase enzyme itself is composed of two different subunits encoded by two different genes. Synthesis of the aldehyde also requires the action of several genes, as does the regulation. Together, all of these genes required for the light production are called the lux genes. The group of lux genes from *Vibrio fischeri* has been placed in a plasmid.

The *Vibrio fischeri* plasmid is called pVIB. In addition to the *Vibrio fischeri* genes, pVIB also contains the gene for resistance to ampicillin. The plasmid pVIB was originally made by a team of three scientists: JoAnne Engebrecht, Kenneth Nealson, and Michael Silverman. They published a paper about their work called *Bacterial Bioluminescence: Isolation and Genetic Analysis of Functions from* Vibrio fischeri, in *Cell*, Vol. 32, pages 773–781, March, 1983.*

*In the paper, pVIB is referred to as pJE202.

pXeno = 5.8kb (amp^r + kan^r)



1.8 kb insert into

1.8kb insert of Xenopus MT3-MMP gene.

Involved in extra-cellular matrix remodeling. 86% identity to human gene.

Hammoud et.al. 2006. Biochem. Cell Biol. 84: 167-177.

Comments for pCR®II-TOPO®
3973 nucleotides

LacZα gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851

from Logan Walsh in Sashko Damjanovski lab.

Appendix 8:

Bacteriophages:

P1vir – from Miguel Valvano (UWO)
I could not find a commercial source for P1vir so I have the P1 bacteriophage from ATCC listed below and an article about P1vir for use in transduction. P1 phage can go into lysogenic or lytic cycle while P1vir only goes into the lytic cycle.

Bacteriophages

ATCC® Number:	**25404-B1™** Order this Item	Price:	**$34.00**
Preceptrol® Culture			
Host Organism:	*Escherichia coli* (Migula) Castellani and Chalmers ATCC 25404		
Designations:	P1		
Depositors:	NM Schwartz	History:	ATCC <<--NM Schwartz<<--C. Yanofsky
Biosafety Level:	1	Shipped:	frozen
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
Comments:	Transducing phage		

Sauer:P1vir phage transduction:

contributed by Sean Moore

Contents
[hide]

Background

Phage transduction is used to move selectable genetic markers from one "donor" strain to another "recipient" strain. Nat Sternberg, among others, pioneered the use of phage P1 to move genetic elements in *E. coli* and the use of the Cre/Lox system from P1 for controlled recombination. Today, phage P1 is commonly used as a transducing agent because it is a generalized tranducer (it can package random sections of the host chromosome instead of its own genome) giving rise to "transducing particles". P1*vir* is a mutant phage that enters the lytic cycle upon infection (ensuring replication and lysis). During the replication and lysis of the phage in a culture of bacteria, a small percentage of the phage particles will contain a genome segment that contains your gene of interest. P1 packages approximately 90 kb of DNA, so you can transduce genes that are linked to a selectable marker.

Once a phage population has been generated from a donor host, the phage are used to infect a recipient host. Most of the bacteria are lysed by phage that packaged P1 genomes, but a fraction of the phage inject a genome segment derived from the donor host. Homologous recombination then allows the incoming genomic segment to replace the existing homolgous segment. The infected recipient bacteria are plated on a medium that selects for the genome segment of the donor bacteria (antibiotic resistance, prototrophy, etc.)

All of this would not work if the infectivity of the phage could not be controlled. Otherwise, phage released from neighboring cells would infect and lyse the bacteria that had been infected with transducing particles. Someone really smart discovered that phage P1 requires calcium for infectivity. Therefore, you can control P1 infectivity by growing in the presence and absence of calcium. The calcium chelator citrate is usually used because it lowers the concentration of free calcium (by forming Ca-citrate) low enough to prevent P1 infection, but not so low as to starve the cells for calcium.

Lysate preparation

1. Dilute an overnight culture (LB medium) of donor strain 1:100 in fresh LB + 5 mM CaCl2 and 0.2% glucose (2.5 mL should be enough). Grow with aeration at 37 °C for 1 hr. Add 100 µL of P1 phage lysate to the culture, continue growing at 37 °C. Monitor for 1–3 hr until the culture has lysed completely.

2. Add several drops of chloroform to the lysate and vortex. Centrifuge away the debris (14,000 rpm, 1–2 min) and transfer the supernatant to a fresh tube. Add a few drops of chloroform and store at 4 °C.

Transduction

1. Grow recipient strain overnight in LB medium (2 mL culture is plenty).

2. On the next day, harvest the cells by centrifugation (6000 rpm, 2 min) and resuspend in original culture volume in fresh LB + 100 mM MgSO4 + 5 mM CaCl2. (note: 10 mM MgSO4 works fine, too, so you can use the 0.1 M MgSO4 the kitchen makes.)

3. Set up four "reactions":

A. 100 µL undiluted P1 lysate + 100 µL recipient cells B. 100 µL 1:10 diluted P1 lysate + 100 µL recipient cells C. 100 µL LB + 100 µL recipient cells D. 100 µL undiluted P1 lysate + 100 µL LB

(note for step 3: LB = LB + 100 mM MgSO4 + 5 mM CaCl2; dilute your P1 lysate in this as well)

4. Incubate tubes at 37 °C for 30 min.

5. Add 200 µL 1 M Na-Citrate (pH 5.5), then add 1 mL LB (the real thing this time) and incubate at 37 °C for 1 hr to allow expression of the antibiotic resistance marker.

6. Spin cells at 6000 rpm for 2-3 min.

7. Resuspend each in 100 µL LB + 100 mM Na-Citrate (pH 5.5) and plate all of it on an appropriate antibiotic-containing plate.

8. You should get anywhere from ~ 10 to 2000 colonies. These colonies are growing on a plate that is covered with P1 phage. If you simply pick a colony from this plate and prepare a freezer stock, you will most likely have phage contamination that will manifest when a culture is grown up in the absence of a calcium chelator. Therefore, prepare a plate spread with the selection antibiotic and 100 µL of 100 mM citrate (pH 5.5). Then, use a toothpick to touch the top of a few colonies and re-streak on the new plate for isoalted colonies.

9. Test a colony from each re-streak for the presence of the mutant gene you intended to transduce using diagnostic PCR or Southern blotting.

Anecdotes

- The chloroform used to sterilize the phage lysates, well, sterilizes. If you have visible chloroform drops in the lysate stock, don't add this to your recipient cells directly because you can kill a decent number of bacteria. Instead, aliquot your phage into microfuge tubes and incubate with the caps open at 37 ˚C for about 30 minutes to allow the chloroform to evaporate. Then add the recipient cells to the tubes with the phage.

- When preparing the donor phage lysate, there is a huge variability in the titer of phage obtained at this step which makes transduction performance unpredictable. Some donor cells are slow "wake up" from stationary phase and 3 hours will not be enough. If it is obvious that there was no culture development in the tube, let it shake overnight. The next morning, you will have a culture of cells and, perhaps, noticeable cell debris. Treating this with chloroform and preparing it as a phage lysate usually works well.

- P1 lysis is accelerated under reducing conditions (Ryland Young's Lab). Adding 1 mM DTT to the top agar allows P1 to develope better plaques. It follows that reducing agents may help the donor lysate develope and help the recipients infecected with infectious P1 to lyse before plating. If you're having trouble getting a high titer of donor phage, try β-mercaptoethanol at 1/1000 culture volume.

- P1 replicates poorly in *recA*- hosts. Moreover, RecA is required for the homologous recombination needed to integrate the donor DNA. Therefore, I have not been able to transduce into *recA* bacteria. In cases that I needed to have *recA*- bacteria following a transduction, I first transduced my desired marker into a *recA*+ recipient, then subsequently transduced *recA::kan* from a specialized *recA+* donor strain generated by Barry Wanner (BW 26,547 *recA::kan* Lambda *recA*+).

- As mentioned above, P1 packages ~90 kb of DNA. This means that genes in the vicinity of your target gene in the recipient will most likely be replaced with copies of the neighboring genes from the donor. Therefore, you can't easily transduce a marker close to an existing marker in the recipient unless you have good selection for both markers and there is enough space between the genes to allow significant recombination between them. It is a good idea to be aware of the relative distances of genes of interest in your strains when transducing to avoid accidental curing of relevent markers.

References

Reference [1] is the first report of allelic exchange by "transduction" in enteric bacteria. Ref. [2] is the earliest report I know of bacteriophage-dependent transduction in E. coli. This report is notable because it describes the K12-infective variant of P1 that has since been considered the "wild-type" P1. Ref. [3] is a beautiful and detailed experimental demonstration that the transducing particles responsible for transduction co-elute with infecting particles in ultracentrifugation, and thus are fully-formed, intact virions, but carry DNA exclusively of bacterial origin. It also is an early (first?) report of the use of virulent mutant of P1, which is defective for lysogeny (i.e. P1vir). Ref. [4] Describes transduction across species boundaries. Refs [5, 6] provide additional reading about the development of the modern protocol. Refs. [7, 8] give good overviews of the molecular biology of P1 and practical protocols for use in transduction. However, the agar plate method for preparation of phage lysates given in [8] is more laborious than the simple liquid culture method given in this protocol. Resulting phage titers are often higher with the plate based protocol, but liquid lysate preparations also given sufficient titer to effect transduction.

1. ZINDER ND and LEDERBERG J. *Genetic exchange in Salmonella.* J Bacteriol 1952 Nov; 64(5) 679-99. pmid:12999698. PubMed HubMed [1]

2. LENNOX ES. *Transduction of linked genetic characters of the host by bacteriophage P1.* Virology 1955 Jul; 1(2) 190-206. pmid:13267987. PubMed HubMed [2]

3. Ikeda H and Tomizawa JI. *Transducing fragments in generalized transduction by phage P1. 3. Studies with small phage particles.* J Mol Biol 1965 Nov; 14(1) 120-9. pmid:5883909. PubMed HubMed [3]

4. Tyler BM and Goldberg RB. *Transduction of chromosomal genes between enteric bacteria by bacteriophage P1.* J Bacteriol 1976 Mar; 125(3) 1105-11. pmid:3494. PubMed HubMed [4]

5. Goldberg RB, Bender RA, and Streicher SL. *Direct selection for P1-sensitive mutants of enteric bacteria.* J Bacteriol 1974 Jun; 118(3) 810-4. pmid:4598005. PubMed HubMed [5]

6. Wall JD and Harriman PD. *Phage P1 mutants with altered transducing abilities for Escherichia coli.* Virology 1974 Jun; 59(2) 532-44. pmid:4598709. PubMed HubMed [6]

7. Sternberg N and Hoess R. *The molecular genetics of bacteriophage P1.* Annu Rev Genet 1983; 17 123-54. doi:10.1146/annurev.ge.17.120183.001011 pmid:6364958. PubMed HubMed [7]

8. A Short Course in Bacterial Genetics

[8]

All Medline abstracts: PubMed HubMed