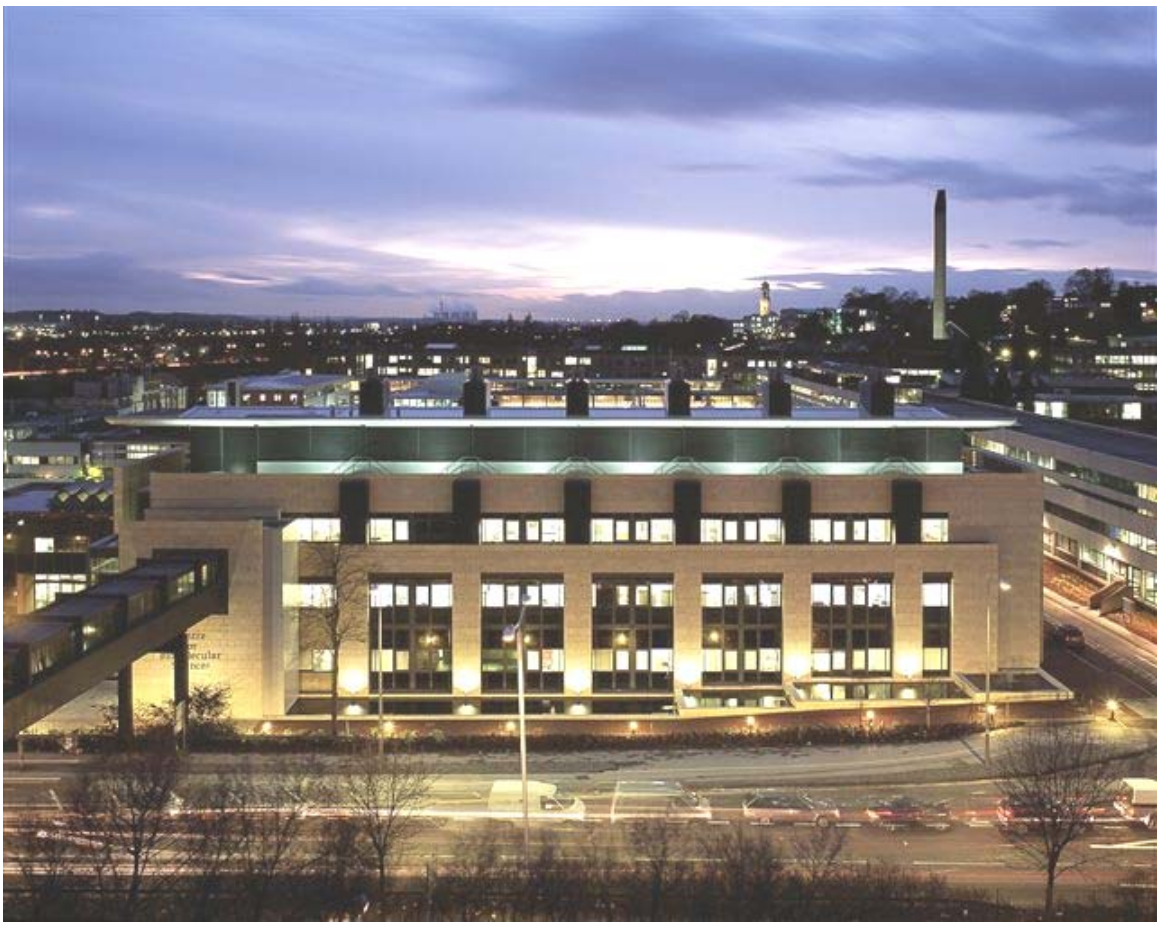
Marie Curie Workshop Laboratory Manual
Clostridial Gene Knockout using pMTL007

12th – 15th September 2006

The Minton laboratory
Institute of Infection, Immunity and Inflammation, School of Molecular Medical Sciences, Centre for Biomolecular Sciences,



Event 2

Marie Curie Training Conferences and Workshops on Clostridia

Contract: CLOSTRIDIA (SCF-CT-2005-029958)

CLOSTRIDIA is an EU Marie Curie-funded series of Conferences and Workshops which seeks to educate and inform young European scientists on the biology of members of the genus *Clostridium* and the diseases they cause. The series of Events are designed to encourage young researchers to combine the knowledge from molecular microbiology and genomics with human and veterinary clinical aspects. The goal is to:

- elevate European clostridial research to the international forefront;
- establish long-term research synergies within the EU;
- encourage young scientists to enter the field, and;
- increase mobility & improve the career opportunities.

Further information and links to conferences at www.clostridia.net

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FOREWORD

It is with great pleasure that I welcome you all to the University of Nottingham for the Workshop on Gene Transfer Techniques for Clostridia. In attendance are 13 Group 1 eligible young scientists from seven different member states (Belgium, Finland, France, Germany, Italy, The Netherlands and the UK) together with 3 Group 2 young scientists from Greece, Poland and Portugal. This wide ranging national balance should create a fertile environment for forging new transnational collaborations.

I am particularly grateful to our two Expert Tutors, Dr Peter Mullany (Eastman Dental Institute, University College London) and Professor Mike Young (Institute of Biological Sciences, University of Wales, Aberystwyth) who have generously given up their time to help run this training course. Both are leaders in the field, and have pioneered many of the developments made to date within the arena of clostridial gene systems.

I am also indebted to the Clostridia Research Group at the University of Nottingham, without whom this event would not be taking place. Special thanks are reserved for Stephen Cartman, but most of all to John Heap for his tireless efforts in bringing this workshop to fruition.

Nigel P Minton
University of Nottingham
9th September 2006

Contents

Programme

Monday 11th September – Arrival, Hugh Stewart Hall of Residence

20.00 – 24.00 Welcome Get Together – *University Staff Club*

Tuesday 12th September

07.00 – 07.45 Breakfast – *Hugh Stewart Hall*

08.00 – 08.05 Welcome – *Nigel Minton, Room B02, CBS*
08.05 – 09.00 Safety Induction – *Room B02, CBS*
09.00 – 10.30 Practical Course – *Room B39, CBS*
10.30 – 10.50 Coffee Break – *Room B02, CBS*
10.50 – 13.00 Practical Course – *Room B39, CBS*

13.00 – 14.00 Lunch – *Room B02, CBS*

14.00 – 15.30 Practical Course – *Room B39, CBS*
15.30 – 15.50 Coffee Break – *Room B02, CBS*
14.00 – 18.00 Practical Course – *Room B39, CBS*

20.00 – late Dinner – *Red Hot Buffet Shack, Nottingham City Centre*

Wednesday 13th September

08.00 – 08.45 Breakfast – *Hugh Stewart Hall*

09.00 – 11.00 Practical Course – *Room B39, CBS*
11.00 – 11.20 Coffee Break – *Room B02, CBS*
11.20 – 13.00 Introductory Lecture and primer design tutorial
Nigel P Minton & John Heap – Room B02, CBS

13.00 – 14.00 Lunch – *Room B02, CBS*

14.00 – 14.45 Seminar – "Conjugative gene transfer in saccharolytic Clostridia" – *Mike Young – Room B02, CBS*
14.45 – 15.30 Seminar – "Mobile genetic elements from *C. difficile*" *Peter Mullany – Room B02, CBS*
15.30 – 15.50 Coffee Break – *Room B02, CBS*
15.50 – 17.00 Practical Course – *Room B39, CBS*

20.00 – late Dinner – *Zizzi's, Nottingham City Centre*

Thursday 14th September

08.00 – 08.45	Breakfast – *Hugh Stewart Hall*
09.00 – 11.00	Practical Course – *Room B39, CBS*
11.00 – 11.30	Coffee Break – *Room, CBS*
11.30 – 12.30	Seminar – "Use of conjugative transposons for the genetic manipulation of *C. difficile" – Peter Mullany – Room B02, CBS*
12.30 – 13.30	Lunch – *Room B02, CBS*
13.30 – 14.30	Practical Course – *Room B39, CBS*
14.30 – 15.30	Seminar – "How is Spo0A phorphorylated in clostridia?" *Mike Young – Room B02, CBS*
15.30 – 16.00	Coffee Break – *Room B02, CBS*
16.00 – 18.00	Practical Course – *Room B39, CBS*
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Friday 15th September

08.00 – 08.45	Breakfast – *Hugh Stewart Hall*
09.00 – 11.00	Practical Course – *Room B39, CBS*
11.00 – 11.30	Coffee Break – *Room B02, CBS*
11.30 – 13.00	Practical Course – *Room B39, CBS*
13.00 – 14.00	Lunch – *Room B02, CBS*
14.00 – 15.30	Practical Course – *Room B39, CBS*
15.30 – 16.00	Coffee Break – *Room B02, CBS*
16.00 – 17.00	'Wash-up' (General Discussion. Future Developments) *Nigel Minton – Room B02, CBS*
20.00 – late	Farewell Party – *Scruffy's, Nottingham City Centre*

Saturday 16th September Departure

Overview of practical sessions

During this workshop, full instruction in the use of pMTL007 to generate clostridial gene knock-outs will be provided. All the major laboratory procedures will be taught in practical sessions (described in this manual). The minor computational elements of the process are described in lectures or in this manual.

Clostridial gene knock-out using the pMTL007 plasmid is typically completed in 10 laboratory days. Only a few of these require a full day of laboratory work, and on many there are only a few minutes of laboratory work to perform.

The instructions in this manual are adapted from the 'Protocol for Clostridial Gene Knockout using pMTL007', which details in full how to perform clostridial gene knock-out using the pMTL007 plasmid. A copy of this Protocol is provided, and should be kept to hand in the laboratory during the practical sessions.
This manual refers to Days 1, 4, 5, 6, 7 and 10 of the Protocol.

Tuesday 12th September
- Day 1 – Re-target pMTL007.
- Day 4 – Prepare to conjugate target organisms.

Wednesday 13th September
- Day 5 – Conjugate target organisms (*Clostridium difficile* and *Clostridium sporogenes*).
- Day 6 – Start overnight cultures of transconjugants and transformants.

Thursday 14th September
- Day 7 – Induce target gene knockout.

Friday 15th September
- Day 10 – Confirm knockout of target genes by PCR screening.
- Inspect plates and interpret results.

Rich complex media are suitable for all stages of the gene knockout protocol using pMTL007. The following media will be used throughout the workshop:

Strain		Media
Clostridium difficile	–	BHI
Clostridium sporogenes	–	TYG
Clostridium acetobutylicum	–	CGM

Tuesday 12th September

TODAY

Perform the entire **Day 1** protocol.

From the **Day 4** protocol – generate conjugation donor strains for *Clostridium difficile* and *Clostridium sporogenes* (the demonstrators will start overnight cultures of the appropriate conjugation recipient strains).

Day 1 – Re-target pMTL007

Perform Re-targeting PCR

1. Assemble four-primer mixture

A pre-prepared four-primer mixture will be provided including the appropriate IBS, EBS1d and EBS2 primers to re-target the intron to insert in the antisense orientation at position 178/179 of the *spo0A* gene in *Clostridium difficile* 630ΔErm. See the 'Protocol for Clostridial Gene Knockout using pMTL007' for details.

EBS Universal:
CGAAATTAGAAACTTGCGTTCAGTAAAC

Cd-spo0A-178a-IBS:
AAAAAAGCTTATAATTATCCTTATTATTCCATCTAGTGCGCCCAGATAGGGTG

Cd-spo0A-178a-EBS1d:
CAGATTGTACAAATGTGGTGATAACAGATAAGTCCATCTAGTTAACTTACCTTTCTTTGT

Cd-spo0A-178a-EBS2:
TGAACGCAAGTTTCTAATTTCGGTTAATAATCGATAGAGGAAAGTGTCT

2. Assemble PCR reaction

Assemble the PCR reaction on ice as follows:

25 µl	JumpStart REDTaq ReadyMix (provided pre-aliquotted into a PCR tube)
23 µl	Water
1 µl	Intron PCR Template
1 µl	Four-primer mix for Cdi-spo0A-178a targeting-region
50 µl	**Total reaction volume**

3. Perform PCR using the following cycling conditions:

Denature	94°C for 30 seconds	
Denature	94°C for 15 seconds	30 cycles
Anneal	55°C for 30 seconds	
Extend	72°C for 30 seconds	
Extend	72°C for 2 minutes	
Pause	4°C	

A lab thermocycler is already programmed with these cycling conditions.

4. Visualise PCR products using agarose gel electrophoresis

Add 10µl of 6x loading dye to the PCR tube and mix.
A lane in a 1% (w/v) agarose gel will be provided.
Load 25µl of your PCR product into one gel lane as directed by the demonstrators.

5. Purify the ~350bp PCR product

Purify the desired PCR product DNA using the Qiagen QIAquick Gel Extraction Kit:

1. **Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.**
2. **Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).**
   For example, add 300 µl of Buffer QG to each 100 mg of gel. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.
3. **Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.**
   IMPORTANT: Solubilize agarose completely.
4. **Add 1 gel volume of isopropanol to the sample and mix.**
   For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. Do not centrifuge the sample at this stage.
5. **Place a QIAquick spin column in a provided 2 ml collection tube.**
6. **To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.**
   The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
7. **Discard flow-through and place QIAquick column back in the same collection tube.**
8. **Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.**
   This step will remove all traces of agarose.
9. **To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.**
10. **Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 13,000 rpm (~17,900 x g).**
11. **Place QIAquick column into a clean 1.5 ml microcentrifuge tube.**
12. **To elute DNA, add 30 µl of Buffer EB to the centre of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.**

Label the tube containing the purified PCR product, and place it on ice.

Restriction digestion of the PCR product and pMTL007 plasmid DNA

1. Digest PCR product and pMTL007 with *Hin*dIII and *Bsr*GI

You only need to digest your gel-purified PCR product (you will be provided with a pre-prepared aliquot of pMTL007 linearised with *Hin*dIII and *Bsr*GI).

Assemble the restriction digestion reaction mixture as follows, adding the restriction enzymes last:

8 µl	Purified PCR product DNA
2 µl	10x Restriction Enzyme Buffer
1 µl	*Hin*dIII (20U/µl)
1 µl	*Bsr*GI (10U/µl)
8 µl	Water
20 µl	**Total reaction volume**

Incubate the reaction at 37°C for 30 minutes then 60°C for 30 minutes.

2. Analyse pMTL007 restriction digestion using agarose gel electrophoresis

This step has already been performed by the demonstrators.

3. Purify the digested PCR product

Purify the new targeting region from the digestion of the PCR product using the Qiagen QIAquick Gel Extraction Kit:

1. **Add 5 volumes of Buffer PB to 1 volume of the reaction mixture and mix.** For example, add 100 µl of Buffer PB to 20 µl reaction mixture sample.
2. **Place a QIAquick spin column in a provided 2 ml collection tube.**
3. **To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.**
4. **Discard flow-through and place QIAquick column back in the same collection tube.**
5. **To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.**
6. **Discard the flow-through and and place the QIAquick column back in the same tube. Centrifuge the QIAquick column for an additional 1 min at 13,000 rpm (~17,900 x g).**
7. **Place QIAquick column into a clean 1.5 ml microcentrifuge tube.**
8. **To elute DNA, add 30 µl of Buffer EB to the centre of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.**

Ligation of the digested PCR product into pMTL007

1. Assemble the ligation reaction mixtures

Set up a ligation reaction and a vector-only control ligation reaction.
Assemble the ligation reaction mixtures as follows, adding the ligase enzyme last:

1 µl	Digested pMTL007 vector
5 µl	Digested PCR product or water
1 µl	10x ligase buffer (New England Biolabs)
0.5 µl	Ligase (New England Biolabs)
2.5 µl	Water
10 µl	**Total reaction volume**

2. Incubate the reaction mixtures to allow ligation to occur

Incubate the ligation reactions at room temperature for 30 minutes.

NOTE – the **Day 1** and **Day 4** transformations should be performed at the same time

Transform ligation reactions into *E. coli* cloning strain

1. Transform competent *E. coli* TOP10 cells by electroporation

1. Dialyse the ligation reaction mixtures against distilled water for 15-30 minutes to remove salt.
2. Add all of each dialysed ligation reaction mixture to an aliquot (~50µl) of electro-competent cells, and stir gently with the pipette tip.
3. Incubate the mixtures on ice for 5 minutes.
4. Transfer the mixtures into chilled 0.2cm gap electroporation cuvettes.
5. Electroporate the cells using a BioRad Micropulser set to program 'Ec2'.
6. Immediately add 250µl room-temperature 2xYT recovery medium.
7. Transfer the suspension into 1.5ml microcentrifuge tube.
8. Incubate the transformation mixture at 37°C and 200rpm shaking for one hour to allow expression of the antibiotic resistance gene.

2. Select for transformed cells using chloramphenicol

Spread 100µl of neat and 10-fold diluted ligation reaction transformants onto 2xYT plates supplemented with 25 µg/ml chloramphenicol. Spread 100µl of neat vector-only control ligation reaction transformants onto another plate of the same type. The demonstrators will incubate the plates at 37°C overnight.

Day 4 – Prepare to conjugate target organisms

You will generate conjugal donor strains to conjugate *Clostridium difficile* 630ΔErm and *Clostridium sporogenes* 10696.

Re-transform plasmid DNA into an appropriate *E. coli* conjugation donor strain such as CA434 and inoculate an overnight culture with the transformants

You will be provided with one (not both) of the following re-targeted pMTL007 plasmids which have already been verified by sequencing:

pMTL007::Cdi-spo0A-178a (Targets the *spo0A* gene in *Clostridium difficile* 630ΔErm)

pMTL007::Csp-spo0A-249s (Targets the *spo0A* gene in *Clostridium sporogenes* 10696)

The plasmids must be transformed into *E. coli* CA434 cells to generate conjugal donor strains which can be used to transfer the re-targeted pMTL007 plasmids into the target clostridial strains by conjugation.

Each group will only generate one conjugal donor strain, but other groups will generate the other conjugal donor strain, so cultures of both required strains will be available on Wednesday morning.

1. Transform competent *E. coli* CA434 cells by electroporation

Transform the re-targeted pMTL007 plasmid into an aliquot of electro-competent *E. coli* CA434 cells. Use the electroporation protocol as described on the previous page, except:

> Add 1µl of the plasmid DNA solution to the aliquot of electro-competent *E. coli* CA434 cells.
>
> Dialysis of the plasmid DNA is not required.

2. Inoculate the transformed cells directly into an overnight culture

After the one hour recovery period, add the transformation mixture directly into 5 ml of LB broth supplemented with 12.5 µg/ml chloramphenicol. The demonstrators will incubate the culture at 37°C and 200rpm shaking overnight.

Wednesday 13th September

TODAY

Perform the entire **Day 5** protocol for *Clostridium difficile* and *Clostridium sporogenes*. Due to time and space constraints, the demonstrators will perform the **Day 5** protocol for *Clostridium acetobutylicum*.

From the **Day 6** protocol – start overnight cultures of pMTL007 transconjugants or transformants of *Clostridium sporogenes* and *Clostridium acetobutylicum*.

Day 5 – Conjugate target organisms (*Clostridium difficile* and *Clostridium sporogenes*)

Mix conjugal donors and recipients and incubate on a plate for 4-8hrs to allow transfer of pMTL007 by conjugation

1. Pellet and wash conjugal donor cells

Pipette 1ml of each of the stationary overnight cultures of the two conjugation donor strains into 1.5ml microcentrifuge tubes. Pellet by centrifugation at 8000rpm for 1 minute.

The two conjugation donor strains are *E. coli* CA434 cells containing:

pMTL007::Cdi-spo0A-178a (Targets the *spo0A*gene in *Clostridium difficile* 630ΔErm)
or
pMTL007::Csp-spo0A-249s (Targets the *spo0A* gene in *Clostridium sporogenes* 10696)

Discard the supernatant, then wash the pellets by re-suspending each in 0.5ml of sterile PBS. Centrifuge as before and discard the supernatant.

With a demonstrator, take the two microcentrifuge tubes containing the washed conjugation donor strain cell pellets into an anaerobic cabinet.

2. Mix conjugal donors and recipients

Re-suspend the conjugal donor pellets in 200µl of a stationary overnight culture of the appropriate conjugal recipient cells:

pMTL007::Cdi-spo0A-178a	–	*Clostridium difficile* 630ΔErm
pMTL007::Csp-spo0A-249s	–	*Clostridium sporogenes* 10696

3. 'Spot' and incubate conjugation mixture

Pipette the entire of each conjugation mixture onto a single non-selective plate containing an appropriate anaerobic solid growth medium in discrete drops or 'spots'.

Do not invert the plate. Incubate the plate at in the anaerobic cabinet for 4-8hrs to allow conjugal transfer of the re-targeted pMTL007 plasmid from the *E. coli* donor to the clostridial recipient.

During the incubation step:	
11:00 – 11:20	Coffee break
11:20 – 13:00	Introductory lecture and primer design tutorial
13:00 – 14:00	Lunch
14:00 – 14:45	Seminar
14:45 – 15:30	Seminar
15:30 – 15:50	Coffee break

Plate transconjugants onto selective growth medium and incubate overnight

1. Wash conjugation mixture off conjugation plate

Pipette 1ml of anaerobic sterile PBS onto the conjugation plate. Using a sterile spreader, scrape the layer of cells off the plate and re-suspend them in the PBS.

2. Spread conjugation slurry onto selective plates and incubate

Using a pipette, aspirate as much of the conjugation slurry as possible into a fresh microtube. Spread 100µl of the neat and 100µl of the 10-fold diluted slurry onto fresh plates containing an appropriate anaerobic solid growth medium, supplemented with 250µg/ml cycloserine to select against the *E. coli* conjugal donor and 15µg/ml thiamphenicol to select for the re-targeted pMTL007 plasmid.

Invert the plates and incubate in the anaerobic cabinet for 1-3 days.

Day 6 – Start overnight cultures of transconjugants and transformants

Due to limited time available during this workshop, we cannot wait for the transformants and transconjugants you have generated to grow. For this reason the demonstrators have prepared the appropriate transformants and transconjugants for you.

Inoculate overnight cultures of *Clostridium sporogenes* transconjugants and *Clostridium acetobutylicum* transformants

Inoculate overnight cultures in 1ml of an appropriate anaerobic liquid growth medium supplemented with 7.5µg/ml thiamphenicol using transformants from the plates provided:

Clostridium sporogenes 10696 containing pMTL007::Csp-spo0A-249s
and
Clostridium acetobutylicum ATCC824 containing pMTL007::Cac-spo0A-242a

The thiamphenicol will select for the re-targeted pMTL007 plasmid.
Incubate the cultures incubate in the anaerobic cabinet overnight.

Note:
In some organisms (such as *Clostridium difficile*) pMTL007 is very unstable, and cannot easily be maintained in liquid culture. For such organisms, an alternative protocol for Day 7 is available, and an overnight culture is not required.

Thursday 14th September

TODAY

Perform the entire **Day 7** protocol for *Clostridium difficile*, *Clostridium sporogenes* and *Clostridium acetobutylicum*.

Day 7 – Induce target gene knockout

Inoculate fresh selective broth with transconjugants and transformants

Inoculate 1ml broths of appropriate anaerobic liquid growth media (supplemented with 7.5µg/ml thiamphenicol to select for the re-targeted pMTL007 plasmid) with the transformants and transconjugants as shown:

Strain	Inoculum	Medium
Clostridium sporogenes 10696 containing pMTL007::Csp-spo0A-249s	100µl overnight culture	TYG + 7.5µg/ml thiamphenicol
Clostridium acetobutylicum ATCC824 containing pMTL007::Cac-spo0A-242a	100µl overnight culture	CGM + 7.5µg/ml thiamphenicol
Clostridium difficile 630ΔErm containing pMTL007:: Cdi-spo0A-178a	1 or 2 loops of cells	BHI + 7.5µg/ml thiamphenicol

Normally, these cultures are incubated at 37°C under anaerobic conditions until there is visible growth, indicating the culture is in exponential phase (typically after incubation for 1hr). However, to save time, we will omit this non-essential step during the workshop.

Induce intron expression with IPTG

Add 10µl of 100mM IPTG stock to each culture, resulting in final IPTG concentrations of 1mM. Incubate the cultures in the anaerobic cabinet for approximately 3hrs.

During the IPTG induction step:

11:00 – 11:30	Coffee break
11:30 – 12:30	Seminar
12:30 – 13:30	Lunch

Wash cells and allow recovery period

Pellet the cells by centrifugation at 8000rpm for 1 minute. Discard the supernatants, then wash the cells by re-suspending them in 0.5ml of sterile PBS. Centrifuge as before and discard the supernatants.

Re-suspend each pellet in 1ml of an appropriate anaerobic liquid growth media, unsupplemented with antibiotics, and incubate in the anaerobic cabinet for approximately 3hrs.

During the recovery incubation step:	
14:30 – 15:30	Seminar
15:30 – 16:00	Coffee break

Plate integrants onto selective growth medium and incubate overnight

Plate the integration mixtures onto fresh plates containing an appropriate anaerobic solid growth medium supplemented with 2.5µg/ml erythromycin to select for presence of the spliced ErmRAM, which indicates intron integration. Spread three plates with 100µl of neat, 100µl of 100-fold diluted and 100µl of 5-fold concentrated integration mixture.

To determine the frequency of the integration event, serially-dilute each integration mixture to 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} in 1.5ml tubes. Pipette 3x20µl drops of each of the 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions onto one quarter of an appropriate plate unsupplemented with antibiotics. **Do not invert the plates**.

Strain	Selective medium	Non-selective medium
Clostridium sporogenes 10696 *spo0A* knock-outs	TYG + 2.5µg/ml erythromycin	TYG
Clostridium acetobutylicum ATCC824 *spo0A* knock-outs	CGM + 2.5µg/ml erythromycin	CGM
Clostridium difficile 630ΔErm *spo0A* knock-outs	BHI + 2.5µg/ml erythromycin	BHI

Incubate the plates in the anaerobic cabinet until the end of the workshop.

Friday 15th September

TODAY

Perform the PCR screening from the **Day 10** protocol for the *Clostridium acetobutylicum* ATCC824 *spo0A* knock-out strain.

Day 10 – Confirm knockout of target genes by PCR screening

Prepare genomic DNA from overnight cultures

The demonstrators have pre-prepared genomic DNA from the *Clostridium acetobutylicum* ATCC824 *spo0A* knock-out strain.

Use PCR to screen colonies for the desired integration event

PCRs using several different combinations of primers can be used to characterise the intron integants, as shown in figure 1.

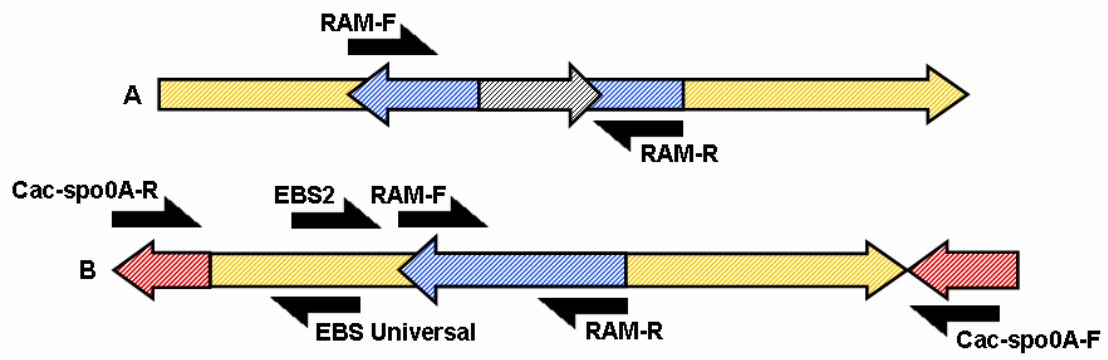

Figure 1 PCR screening for desired integrants
The yellow arrows represent the Ll.LtrB group II intron, the blue arrows represent the ermB-derived marker, the grey arrow represents the group I intron and the red arrows represent the disrupted *Clostridium acetobutylicum* ATCC824 *spo0A* gene. PCR primers are shown as black half-arrows.
(A) represents the pMTL007::Cac-spo0A-242a plasmid DNA
(B) represents chromosomal DNA in which the target gene has been disrupted by the insertion of an intron in the sense orientation relative to the target gene.

We will demonstrate intron insertion and disruption of the desired target gene using two diagnostic PCR screens:

PCR screen	Primers	Amplifies
R	ErmRAM-F + ErmRAM-R	Spliced RAM in integrated intron and/or full-length RAM from pMTL007
X	EBS Universal + Cac-spo0A-R	Across intron-exon junction only if the is intron inserted into the *spo0A* gene

Genomic DNA from wild-type *Clostridium acetobutylicum* ATCC824 and pMTL007::Cac-spo0A-242a plasmid DNA will be used as control templates.

1. Label PCR tubes

Label 8 PCR tubes with the numbers 1-8 and your group's letter.
For example, A1, A2, A3… A8.

2. Assemble PCR reaction master mixes

Assemble the PCR reaction 'R' master mix in tube number 1 on ice as follows, adding the Failsafe enzyme last:

50 µl	2x Failsafe Premix buffer E
43 µl	Water
1 µl	ErmRAM-F primer (100µM)
1 µl	ErmRAM-R primer (100µM)
1 µl	Failsafe enzyme
96 µl	**Total master mix volume**

Assemble the PCR reaction 'X' master mix in tube number 5 on ice as follows, adding the Failsafe enzyme last:

50 µl	2x Failsafe Premix buffer E
43 µl	Water
1 µl	EBS Universal primer (100µM)
1 µl	Cac-spo0A-R primer (100µM)
1 µl	Failsafe enzyme
96 µl	**Total master mix volume**

3. Divide PCR reaction master mixes

Mix the PCR reaction master mixes by gently pipetting up and down.
Divide master mix 'R' among tubes 1-4 (24µl in each tube).
Divide master mix 'X' among tubes 5-8 (24µl in each tube).
Return all tubes to ice.

4. Add PCR templates

Add 1µl of PCR templates to the tubes as follows:

Template	**Tubes**
Water	1 + 5
Genomic DNA from wild-type *Clostridium acetobutylicum* ATCC824	2 + 6
pMTL007::Cac-spo0A-242a plasmid DNA	3 + 7
Genomic DNA from the *Clostridium acetobutylicum* ATCC824 *spo0A* knock-out	4 + 8

5. Perform PCR using the following cycling conditions:

Denature	94°C for 30 seconds	
Denature	94°C for 30 seconds	30 cycles
Anneal	50°C for 30 seconds	
Extend	72°C for 1 minute and 20 seconds	
Extend	72°C for 7 minutes	
Pause	4°C	

A lab thermocycler is already programmed with these cycling conditions.

During the PCR thermocycling, inspect plates from experiments on previous days.

6. Visualise PCR products using agarose gel electrophoresis

Add 5µl of 6x loading dye to each PCR tube and mix.
Lanes in a 1% (w/v) agarose gel will be provided.
Load 15µl of each of the PCR products into gel lanes as directed by the demonstrators, in sequence 1-8.

During the gel electrophoresis, inspect plates from experiments on previous days.

7. Interpret PCR screen 'R'

Refer to figure 1 to help you interpret PCR screen 'R'.

The spliced RAM in the integrated intron is approximately 900bp in length. A PCR product of this size indicates the presence of integrated intron DNA.

The full-length RAM from pMTL007 is approximately 1300bp in length. A PCR product of this size indicates the presence of pMTL007 plasmid DNA.

8. Interpret PCR screen 'X'

Refer to figure 1 to help you interpret PCR screen 'X'.

The EBS Universal primer anneals 219bp from the 5' end of the intron on the reverse strand. The Cac-spo0A-R primer anneals at the end of the *spo0A* ORF, 604bp downstream of the intron insertion site. This pair of primers should therefore amplify a PCR product across the intron-exon junction of approximately 823bp in length, only if the intron has inserted into the desired site in the *spo0A* gene.

Inspect plates and interpret results

Re-targeted pMTL007 ligation plates

At the bench, inspect the re-targeted pMTL007 ligation plates and vector-only control ligation plate.

There should be numerous colonies on the re-targeted pMTL007 ligation plates and very few or ideally no colonies on the vector-only control plate.

Any colonies on the vector-only control plate must have been transformed with re-circularised partially-digested pMTL007 vector. A similar number of colonies containing re-circularised vector should be expected on the corresponding re-targeted pMTL007 ligation plate.

Note:
The linearised pMTL007 vector was purified by gel-extraction, but was not treated with alkaline phosphatase. Alkaline phosphatase treatment will greatly reduce re-circularisation of partially-digested pMTL007 vector.

Clostridium difficile and *Clostridium sporogenes* conjugation plates and *Clostridium acetobutylicum* transformation plates

In the anaerobic cabinet, inspect the *Clostridium difficile* and *Clostridium sporogenes* conjugation plates and *Clostridium acetobutylicum* transformation plates.

The *Clostridium sporogenes* transconjugants should have formed numerous large colonies by now.

The *Clostridium difficile* transconjugants may be visible, but any colonies are likely to be small after two days' incubation. The fewer colonies and their smaller size than the equivalent *Clostridium sporogenes* transconjugants reflects the lower stability of this plasmid in *Clostridium difficile*.

The *Clostridium acetobutylicum* transformants may be visible, but any colonies are likely to be small after two days' incubation. Transformation of *Clostridium acetobutylicum* is an inefficient process, and only a small number of colonies can be expected.

Clostridium difficile, *Clostridium sporogenes* and *Clostridium acetobutylicum* integrant (knock-out mutant) plates and controls

In the anaerobic cabinet, inspect the *Clostridium difficile*, *Clostridium sporogenes* and *Clostridium acetobutylicum* integrant (knock-out mutant) plates and controls.

Count or estimate the numbers of colonies on the erythromycin-supplemented plates. Colonies of *Clostridium difficile* and *Clostridium acetobutylicum* integrants should be visible, but colonies of *Clostridium sporogenes* integrants will probably not be visible after less than 24hrs incubation. Calculate the number of erythromycin-resistant cfu/ml that were in the integration mixture.

For each integration experiment:
Identify which dilution of the integration mixture gave between 5 and 50 colonies per 20µl drop on the unsupplemented control plates. Count the number of colonies in each drop, and calculate the average number per drop. Multiply the average number of colonies per 20µl drop by 50 and by the appropriate dilution factor to determine the number of cfu/ml that were in the integration mixture.

Calculate the intron integration frequencies:

$$\text{Frequency as \%} = \frac{\text{erythromycin-resistant cfu/ml}}{\text{total cfu/ml}} \times 100$$

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Centre for Biomolecular Sciences

Marie Curie Workshop

September, 2006

Health and Safety Information

Introduction

The Centre for Biomolecular Sciences is a multi-disciplinary centre, bringing together researchers from a wide range of different Schools and disciplines. A fundamental reason for this venture is to create new collaborations between these disciplines and groups, so that they can work together to take biomolecular research into exciting and fruitful new directions. An essential part of this process is ensuring the health and safety of all of the staff, students and visitors working in the CBS. This guide provides you with the basic health and safety information which you will need during your course at CBS. Specific health and safety information for the laboratories and techniques which you will be using will be provided by the course tutors.

Smoking

Smoking is not permitted anywhere in the CBS

Access to CBS

You will normally be allowed access to CBS **under the supervision of a Course Tutor** between:

0700 to 1900

Access through the front door will be provided by the receptionist or a Course Tutor. You will be required to sign in and out of the building at Reception.

When judged necessary by the Course Tutor, you will be allowed to remain in the building under their supervision up to

2145

The building alarms activate automatically and the doors are locked at

2200

You must leave the building before that time.

You will be issued with a key code to allow access to B floor

Do not allow anyone else who is not known to you as authorised to enter CBS to go into the building with you.

Public and circulation areas

Members of the public and visitors will use the atrium area, passenger lift and main stairwell. It is important that they are not put at risk. For that reason, hazardous substances and general laboratory equipment must not be brought into the areas, even in transit. Laboratory coats and other personal protective equipment should also not be worn when you are in these areas.

Staff and students moving between laboratories on different floors should use the stairway at the northern end of the building and the goods lift for moving materials and equipment between floors.

The movement of hazardous materials between laboratories and floors should be avoided whenever possible. When this is necessary, the items must be securely contained.

Evacuation Alarm

This is a single, continuous tone

[The alarm is tested on Wednesday morning at 1140 for about one minute. If it lasts longer than this, you should evacuate the building]

In the event of a fire

If you discover a fire:

- Go to a fire alarm point and activate the alarm system
- If practicable, dial **8888** on the nearest internal telephone and inform Security of the location of the fire.
- Walk out in an orderly manner via the nearest exit or fire exit, closing (not locking) all doors behind you. Do not stop to collect personal belongings.
- **Do not use lifts**.
- Move away from the entrance to the building and assemble at the assembly point in front of the Boots Building.

If you hear the fire/evacuation alarm:

Begin the evacuation

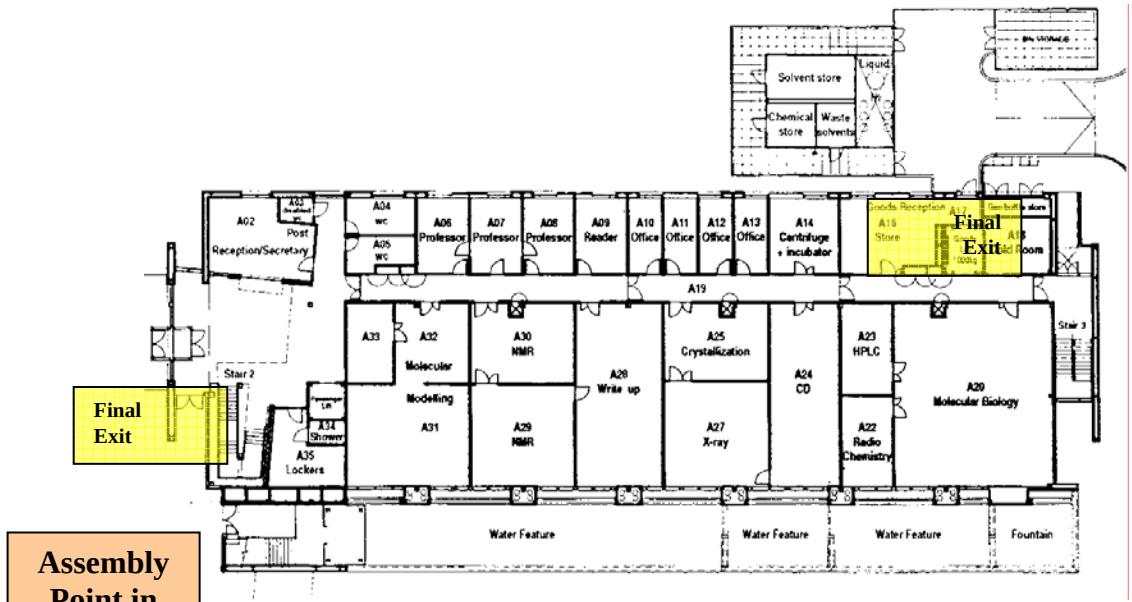
- Do not stop to collect personal belongings.
- Do not use lifts.
- Move away from the entrance to the building and assemble at the assembly point in front of the Boots Building.

If practicable,

- Switch off gas taps and live electrical devices and shut all windows as you leave,
- Pull down the sash on fume hoods.

In all cases,

- Do not do anything that will hinder the work of the Fire Service or other emergency services.
- Do not attempt to return to the building until instructed that it is safe to do so by the evacuation coordinator, identified by a high-visibility yellow jacket.



Assembly Point in Front of Boots Science Building

First Aid

- In the case of injury inform a Course Tutor or other member of staff, who will then call for a First Aider or **8888** for an ambulance, if needed.

- First Aid boxes are prominently located in each corridor. Beside these are listed the names of trained First Aiders, their internal phone numbers and room locations. This information is also given below:

Authorised First Aiders	**Room**	**Extension**
Trevor Wigham	C16	68542
Darren Furniss	B12	67955
Alan Cockayne	B13	67956
Saara Qazzi	B27	67963
Di Mitchell	B7	67950
Carole White	C17	13416
Lee Hibbett	C17	13416
Panos Soultanas	A13	13525

Good Laboratory Practice

Within the laboratories of the CBS there is a wide range of chemical, biological, radiation and equipment-related hazards. Although a number of these will require very specific precautions, for many of them adhering to the following principles of good laboratory practice will provide good protection against the risks.

You should always:

1. Carry out a ***risk assessment*** or read the assessment provided **before** you start the work and make sure you use the appropriate safety precautions identified by the assessment.

2. Follow the appropriate written Standard Operating Procedure (SOP) for the work.

3. Wear any personal protective equipment (e.g. lab coats, safety glasses, gloves) which is required for the work you are doing

4. Tie long hair back securely at all times ,since it can be a hazard when using both chemicals and equipment. Do not use hair gels and lacquers when in the laboratory, since they are often flammable

5. Wear suitable foot wear, which encloses the whole foot, not sandals or open-toed shoes.

6. Avoid wearing short skirts or shorts in laboratories using hazardous chemicals

7. Keep the work area clean at all times and free from chemicals and apparatus which are not required

8. Return all equipment not in use to its proper storage place

9. Clean up after each stage of an experiment

10. Keep the laboratory floor free from all obstructions; these form tripping hazards and can cause serious accidents

11. Clean any spillage of chemicals and biological materials up immediately, using only the appropriate procedures for the spilled material

12. Clearly and securely label all containers of chemicals and biological materials, including experimental glass and plastic ware with their contents and appropriate hazard warnings. Avoid using felt-tip pens directly onto glass; this is easily removed by a wide range of common solvents

13 Follow the following rules to reduce the risk of contaminating your body with hazardous substances

- **Never** take food and drink into the laboratory
- **Never** apply cosmetics, creams or ointments in the laboratory.
- **Never** inhale, taste or swallow any chemical
- **Never** pipette liquids by mouth. Use a mechanical filler instead.
- **Wash** your hands regularly, especially before you leave the laboratory to eat or drink or go to the toilet.
- **Before** leaving the lab on completion of your work remove your lab coat and hang on the hooks provided, **then** wash your hands. Do not hang your lab coat on top of others.

14. Do not bring outdoor coats and personal bags into the laboratory;

15. Hang lab coats which are not being used on the hooks provided; not on the backs of chairs.

16. Dispose of waste chemicals and biological materials carefully, following the appropriate procedures

Waste Disposal

All hazardous biological waste must be inactivated by validated methods, such as autoclaving or disinfection, before being sent for incineration.

Never put contaminate materials in the domestic waste bags or put contaminated media down sinks

The general procedures in place for CBS are given overleaf

Contaminated Biological Waste

Waste Category	Examples	Disposal route
Solid waste		
Contaminated small plastics	tips, tubes and spreaders	'sweetie' jars, when full close, label with autoclave tape, place in autoclave bags and take to media prep for autoclaving.
Contaminated large plastics	falcon tubes	autoclave bags, when full close, label with autoclave tape, place in autoclave bags and take to media prep for autoclaving.
Low-level contamination	gloves, tissues	yellow bags. When full place in the large yellow skip outside the media prep room
Solid growth media	Petri dishes	autoclave tins. When 2/3 full take to media prep for autoclaving. **Do not overfill**
Liquid growth media		
Small volumes	Eppendorfs	sweetie jars. when full close, label with autoclave tape, place in autoclave bags and take to media prep for autoclaving.
Larger volumes	Sterilins, falcon tubes	500ml Duran bottles. When 2/3 full. **Do not put chemical disinfectants (Trigene, etc) into these containers as they can produce hazardous vapours when autoclaved**
	Glass Universals or Erlenmeyer flasks	place in dedicated boxes in lab. Cover open flasks with aluminium foil and secure with autoclave tape. Take full boxes to media prep for autoclaving **Do not put chemical disinfectants (Trigene, etc) into these containers as they can produce hazardous vapours when autoclaved**

Mixed biological and chemical waste

Waste Category	Disposal route
Contaminated plastics	sterilise chemically using Trigene or appropriate disinfectant. Put into yellow bags for collection and incineration. **DO NOT** put mixed waste in autoclave bags due to the potential danger to the autoclave operative from chemical vapours.
Contaminated glassware	chemical sterilisation by immersion overnight in 1: 100 Trigene solutions prior to washing. Inform the technical staff in the wash-up area of the nature of the mixed biological/chemical hazard as they receive the glassware.
Low-level contamination (gloves, tissues)	yellow bags for collection and incineration
Liquid growth media	speak to local safety monitors or the CSO for specific guidance on these routes.
	small volumes in an Eppendorf tube can be put into burn bins for incineration. It is always a good idea to put some form of absorbent material in burn bins that are expected to contain liquid waste
	Larger volumes (aspirants to traps, waste contained in a multi-well dish) should be separated from non-contaminated chemical waste and processed following locally arranged routes considered in risk assessments. These should take account of methods to immobilise the chemical, the survival of the biological substance and the need to inform others involved in the waste-processing route.

Contaminated glass and metal equipment

Waste Category	Examples	Disposal route
Contaminated glassware		autoclave prior to wash-up; or chemical sterilisation by immersion overnight in 1: 100 Trigene solution.
Contaminated metal equipment	dissecting instruments and mesh screens	autoclave, or chemical sterilisation by immersion overnight in 1: 10 Trigene solution.
Contaminated sharps	needles, scalpel blades, microscope slides	yellow "contaminated sharps" waste bins for incineration. To avoid accidental stabbing, **Never** replace the needle guard

Note: Certain work areas may have specialised needs regarding waste-handling and disposal. In such cases, local guidelines must be produce, which tailored to meet the specific needs. Providing this information should form part of worker induction within that work area.