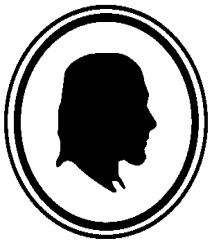
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J098: DNA ligation

1.0 Version Number: 07

2.0 Other documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\Imsnw3 jenner server\jenner\hill group\Safety\COSHHassessments\Manufacturers material safety data sheets
J084 DNA band excision from agarose and DNA extraction
J089 Quantification of DNA using nanodrop
J065 Agarose Gel Electrophoresis and Gel Imaging

3.0 Definitions

DNA deoxyribonucleic acid
TAE Tris-acetate EDTA
SAP Shrimp-alkaline phosphatase
CIP Calf intestinal phosphatase

4.0 Objective

This protocol describes methods using both NEB T4 DNA ligase and Invitrogen T4 DNA ligase. Methods for the NEB enzyme are shown in black text, those for Invitrogen are shown in blue text.

The insertion of DNA into a vector is achieved by prior digestion of both species with restriction enzymes. Ideally, identical or compatible enzymes are used to create cohesive ends, which will anneal specifically. Alternatively, blunt-cutting enzymes are used or the overhangs filled-in to allow ligation of non-complementary DNA ends.

Preparation of DNA for ligation.
Restriction enzyme digest (and filling in of ends where necessary) should be followed with purification of the digested DNA from other DNA species and/or the enzymic mix and the removal of 5' phosphate groups from one of the fragments.

Notes:
Generally the 5' phosphate groups are removed from the vector backbone as this prevents potential self-ligation of a molecule containing antibiotic resistance.

Self-ligation of the plasmid can occur where a single enzyme has been used, or if one of the enzymes in a double-digest has not cut to completion.

DNA Ligase
T4 DNA ligase is unstable on ice and should be stored at -20°C until immediately before it is required. It should then be placed in a labtop cooler while out of the freezer.

The buffer contains ATP and therefore is stored as single-use aliquots to avoid freeze/thawing. The buffer should be vortexed before use to resuspend any precipitated matter and any residual buffer discarded.

Vector:insert ratios.
A vector: insert ratio of 1:1 to 1:3 works well in most cases. DNA can be quantified by running on an agarose gel with molecular weight standards of known concentration.

Alternatively, running a known volume on a gel will allow the concentration of the two products to be assessed in relation to each other. To calculate the ratio, the mass of the fragments must be known, for equivalent fluorescence when stained with SYBRsafe, under uv light, a 900bp fragment will contain 3x lower moles of DNA than a 300 bp fragment.

In practice, volume ratios of 1:1, 1:3 and 1:10 vector: insert are used. Obviously, if one of the fragments is a lot fainter when run on a gel this should be compensated for accordingly.

Control reactions
• **Uncut DNA** added to a reaction without digested plasmid or insert is used to verify that the bacteria used for transformation at the end of the reaction are viable.

• **No ligase** added will show if there is any uncut plasmid DNA present in the reaction once bacterial cells are transformed with the ligation-reaction products.

• **No insert** demonstrates the presence of plasmid DNA that is capable of self-ligation in the presence of ligase once bacterial cells are transformed with the ligation reaction products.

5.0 Reagents

Sterile distilled water (RNAse & DNAse Free)	Sigma	W4502
400 U / µl T4 DNA ligase + 10 x reaction buffer*	NEB	M0202
5U/µl T4 DNA ligase + 5 x buffer*	Invitrogen	15224-041

*the units are not equivalent.

6.0 Equipment

Gilson Pipettes – P1000, P200, P20, P10
Pipette tips – P1000, P200, P20, P10

7.0 Method

1) Reagent mix when using NEB DNA ligase:

Prepare ligation reactions, omitting no. 4, according to the following table (assuming plasmid and insert DNA are of similar intensity when stained with SYBRGold and viewed under uv light). If the concentration of the insert DNA is low, include reaction no. 4:

Reagent	1	2	3	4	5	6
distilled water	16 µl	15 µl	13	6 µl	16 µl	14 µl
10x ligation buffer	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
Plasmid DNA	1 µl uncut	1 µl	1 µl	1 µl	1 µl	1 µl
Insert DNA	-	1 µl	3 µl	10 µl	-	3 µl
T4 DNA ligase	1 µl	1 µl	1 µl	1 µl	1 µl	-

2) Reagent mix when using Invitrogen DNA ligase to ligate cohesive ends:

Reagent	1	2	3	4
distilled water	Up to 20 µl	Up to 20 µl	Up to 20 µl	Up to 20 µl
5 x ligation buffer	4 µl	4 µl	4 µl	4 µl
Plasmid DNA	30 fmol uncut	30 fmol	30 fmol	30 fmol
Insert DNA	-	90 fmol	-	90 fmol
T4 DNA ligase (1U final)	0.2 µl	0.2 µl	0.2 µl	-

Incubate at RT for 1 hour (or overnight at 14°C)

3) Reagent mix when using Invitrogen DNA ligase to ligate blunt ends:

Reagent	1	2	3	4
distilled water	Up to 20 µl	Up to 20 µl	Up to 20 µl	Up to 20 µl
5 x ligation buffer	4 µl	4 µl	4 µl	4 µl
Plasmid DNA	60 fmol uncut	60 fmol	60 fmol	60 fmol
Insert DNA	-	180 fmol	-	180 fmol
T4 DNA ligase (1U final)	0.2 µl	0.2 µl	0.2 µl	-

Incubate overnight at 14 °C.
Refer to manufacturer's manuals for alternative incubation times.

Dilute the reactions 1:5 with water (>10 ng of DNA is toxic to cells).
Transform bacterial cells with 3 µl of the reaction as per J062.

Notes:
• Once complete, ligation reactions can be stored at 4ºC until transformation. **DO NOT FREEZE REACTIONS.**
• Glycerol content of the reactions should not exceed 10%, therefore T4 DNA ligase (50% glycerol) should not exceed 20% of the total volume.

Calculation for amount of DNA

For blunt ligation,		**For cohesive ends:**	
	60 fmol vector DNA		30 fmol vector DNA
	180 fmol insert DNA		90 fmol insert DNA
Vector DNA			
1000 bp	60 fmol = 20 ng	1000 bp	30 fmol = 10 ng
2000 bp	60 fmol = 40 ng	2000 bp	30 fmol = 20 ng
3000 bp	60 fmol = 60 ng	3000 bp	30 fmol = 30ng
4000 bp	60 fmol = 80 ng	4000 bp	30 fmol = 40 ng
5000 bp	60 fmol = 100 ng	5000 bp	30 fmol = 50 ng
6000 bp	60 fmol = 120 ng	6000 bp	30 fmol = 60 ng
7000 bp	60 fmol = 140 ng	7000 bp	30 fmol = 70 ng
8000 bp	60 fmol = 160 ng	8000 bp	30 fmol = 80 ng
Insert DNA			
100 bp	180 fmol = 6 ng	100 bp	90 fmol = 3 ng
200 bp	180 fmol = 12 ng	200 bp	90 fmol = 6 ng
300 bp	180 fmol = 18 ng	300 bp	90 fmol = 9 ng
400 bp	180 fmol = 24 ng	400 bp	90 fmol = 12 ng
500 bp	180 fmol = 30 ng	500 bp	90 fmol = 15 ng
600 bp	180 fmol = 36 ng	600 bp	90 fmol = 18 ng
700 bp	180 fmol = 42 ng	700 bp	90 fmol = 21 ng
800 bp	180 fmol = 48 ng	800 bp	90 fmol = 24 ng
900 bp	180 fmol = 54 ng	900 bp	90 fmol = 27 ng
1000 bp	180 fmol = 60 ng	1000 bp	90 fmol = 30 ng

RE digest and gel extraction yields approx 60% expected recovery at worst, 90% at best.

Therefore, if the plasmid is 8 kb and your insert is 1 kb, the insert forms 12.5% of the total DNA.

The maximum yield for 1 µg is 125 ng. For a blunt ligation you require 60 ng for a 1:3 molar ratio as described above. This would be achieved with a 50% recovery.
Remember to digest extra DNA to be able to prepare control ligation reactions (+ ligase, - ligase etc).

8.0 Revision History

Version Number	Why it Changed	Who Changed it	Date changed
1.0		Matt Dicks	
2.0		A.Turner	
3.0	Changed to include overnight incubation recommendation.	A.Turner	
4.0	To include reaction volumes for Invitrogen enzyme	Ali Turner	
5	Change of Invitrogen ligase method	Ali Turner	17th Nov 2010
6	Addition of example amounts of DNA for correct concentration in ligation reactions	Ali Turner	6th December 2010
7	Changed Invitrogen table to read that water is used to make total volume up to 20 µl	Ali Turner	13th Jan 2011

J039: High Fidelity PCR using Phusion polymerase

9.0 Version Number: 05

10.0 Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:\\Imsnw3 jenner server\jenner\hill group\Safety\COSHHassessments \Manufacturers material safety data sheets

J086 Qiagen PCR purification kit
J065 Agarose Gel Electrophoresis and Gel Imaging
J085 DNA band excision from agarose and DNA extraction
J056 DNA restriction digest
J084 TOPO blunt cloning

11.0 Definitions

PCR polymerase chain reaction
RE Restriction enzyme
DNA deoxyribonucleic acid
cDNA complementary DNA
TOPO Topoisomerase

12.0 Objective

This protocol describes high fidelity PCR of antigen sequences as required for cloning or sequencing DNA. **Note**: The Phusion polymerase generates blunt-ended PCR products that are only suitable for TOPO blunt cloning (J084). Further information about the polymerase enzyme can be found at: http://www.neb.com/nebecomm/products/categories.asp

A PCR reaction is set up using primers that bind either side of the antigen sequence of interest, amplifying the whole insert from template DNA. A water negative control should also be included to ensure that the PCR reagents are not contaminated, leading to false positive result. Filter tips and extreme care should be exercised to avoid cross-contamination. See Jenner Pox Virus document or individual SOPs for details of specific primers and antigens.

The number of cycles in the PCR is kept to a minimum to reduce the possibility of errors (although the polymerase has an extremely high fidelity (error rate of 4.4 x 10e7)).
Phusion High-Fidelity DNA Polymerase brings together a novel *Pyrococcus*-like enzyme with a processivity-enhancing domain. This results in a high speed of amplification, allowing elongation times of as little as 15 – 30 sec per kb).

NOTE: Tm of primers should be calculated using the Finnzymes calculator and not Primer select. We have found it to give a very different value.

Such PCR products are purified from the polymerase enzyme and primers by one of two methods depending on their destination. In preparation for restriction enzyme digest, a Qiagen PCR purification kit can be used (J086). If the PCR product is to be TOPO cloned, purify by DNA band excision and gel purification (J085) and ligate into TOPO vector (J084).

13.0 Reagents

Template DNA (1 pg – 10 ng low complexity DNA (plasmid, lambda or BAC DNA) or 50 – 250 ng genomic DNA per 50 µl reaction)		
Sterile distilled water (RNAse & DNAse Free)	Sigma	W4502
Phusion High-Fidelity Master Mix with buffer	NEB	F-531L
10 µM forward primer	MWG	
10 µM reverse primer	MWG	

14.0 Equipment

Plate, thermowell PCR M (plus cover)	AppletonWoods	TA308
Adhesive plate covers		
Pipettes		
Pipette filter tips		
Thermal cycler	MJ Research	DNA Tetrad engine

15.0 Method

A typical PCR reaction can be set up as follows:

Reagent	**Volume**
forward primer (10 µM)	0.5 µl
reverse primer (10 µM)	0.5 µl
Phusion HF mastermix	25.0µl
Sample template (conc as appropriate*)	5.0 µl*
Sterile water	to 50ul final vol.
Final reaction volume	**50µl**

*1 pg – 10 ng low complexity DNA (plasmid, lambda or BAC DNA) or 50 – 250 ng genomic DNA per 50 µl reaction.

Typical PCR program:

Initial denaturation	98°C	2 min	
Denaturation	98°C	15 sec	} 25 cycles***
Annealing	52-72°C**	30 sec	} 25 cycles***

Extension	72°C	15-30 sec/kb***
Final extension	72°C	10 min
End		

** Annealing temperature is recommended to be **Tm + 3°C** of the lowest Tm primer.

*** cycle number and extension time may need to be adjusted depending on the size of PCR product.

Thaw all PCR reagents on ice.

If performing multiple PCR reactions, a "master mix" lacking only the template DNA can be made up using multiples of the volumes in the guideline table above.
Transfer PCR mix to a new 96 well PCR plate or 200 µl PCR tubes.
Add template DNA and place a clean PCT plate lid over the plate or close tube lids.
Transfer plate to PCR machine and set thermal cycling program running selecting the calculated method and heated lid option.
At end of PCR program, plate may be stored at -20˚ until needed.

Optimisation strategies for non-ideal template/primers

Increase of $MgCl^{2+}$ concentration
If the template is AT-rich and primers are consequently of low Tm, the increase of the Mg^{2+} concentration to 4 mM can serve to increase the stability of the primer-template interaction and increase the success of the PCR

16.0 Revision History

Version Number	What Changed	Who Changed it	Date changed
01	First written	A.Turner	
02	More info added	A.Turner	
03	Optimisation using Mg2+ added	A.Turner	
04	Note about primer Tm added	Ali Turner	18.05.10
05	Cycle number decreased from 30-35 cycles to 25 cycles.	Jake Matthews	11.11.10



J160: Gateway LR Clonase reaction using pAd/PL-DEST™

1.0 Version Number 1.0

2.0 Other documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\Imsnw3_jenner_server\jenner\hill_group\Safety\COSHHassessments\Manufacturers material safety data sheets

Manual	pAd/CMV/V5-DEST and pAd/PL-DEST Gateway Vectors Manual-Invitrogen
J004	Transfection of 293 cells with Adenoviral DNA
J065	Agarose Gel Electrophoresis and Gel Imaging
J062	Transformation and culture of E-coli
J115	PCR screening of transformed bacterial colonies
J103	Pouring LB agar plates containing antibiotic

3.0 Objective

Gateway® is a universal cloning technology that takes advantage of the site specific recombination properties of bacteriophage lambda to provide a rapid and highly efficient way to move your DNA sequence of interest into multiple vector systems. To express your gene of interest in mammalian cells
using the Gateway® Technology, simply:

1. Clone your gene of interest into a Gateway® entry vector of choice to create an entry clone (**Note:** If you are using pAd/PL-DEST™, your insert will need to include a promoter of choice, the gene or sequence of interest, and a polyadenylation signal) then **2.** Generate an expression clone by performing an LR recombination reaction between the entry clone and a Gateway® destination vector (*e.g.* pAd/PLDEST™ or pAd/CMV/V5-DEST™).

4.0 Equipment

Purified plasmid DNA of entry clone (150 ng/µl)
pAd/PL-DEST™ vector (150 ng/µl)
Gateway® LR Clonase™ II Enzyme Mix
TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
2 ug/µl Proteinase K solution (supplied with the LR Clonase™ II enzyme mix)
pENTR™-gus positive control (supplied with the LR Clonase™ II enzyme mix)
Competent *E. coli* host and growth media for expression

S.O.C. Medium
LB agar plates containing 100 µg/ml ampicillin to select for expression clones
LB agar plates containing 30 µg/ml chloramphenicol to counter select

5.0 Method

1) Clone the gene of interest into a Gateway® entry vector of choice to create an entry clone.
2) Perform an LR recombination reaction to transfer the gene of interest into the pAd/PL-DEST™ vector.
3) Add the following components to microcentrifuge tubes at room temperature and mix.

Component	Sample	Positive Control	Negative Control
Entry vector (50-150ng/rxn)	1-7 µl	/	1-7µl
pENTRgus (50ng/µl)	/	2 µl	/
Destination vector (300ng/µl)	1 µl	1 µl	1 µl
TE buffer pH8.0	to 8µl	5µl	to 8µl
LR Clonase™ II enzyme mix	2 µl	2 µl	/

4) Remove the LR Clonase™ II enzyme mix from –20 °C and thaw on ice (~ 2 minutes).
5) Vortex the LR Clonase enzyme mix briefly twice (2 seconds each time).
6) To each sample above, add 2µl of LR Clonase™ II enzyme mix. Mix well by pipetting up and down. **Return LR Clonase enzyme mix to –20°C immediately after use.**
7) Incubate reactions at 25°C for 1 hour.
8) Add 1µl of the Proteinase K solution to each reaction and incubate for 10 minutes at 37°C.
9) Transform 2-3µl of the LR recombination reaction into a suitable *E. coli* host**. Excessive manipulations can shear the DNA, do not vortex or pipette up and down.**
10) Select for expression clones (True expression clones will be ampicillin-resistant and chloramphenicol-sensitive).
11) Carry out a plasmid prep on an overnight culture (type depends on the yield of DNA required).

Confirming the expression clone

The *ccd*B gene mutates at a low frequency, resulting in a low number of false positives. True expression clones will be ampicillin/carbenicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccdB* gene will be ampicillin/carbenicillin- and chloramphenicol-resistant. To check your putative expression clone, test for growth on LB plates containing 30 ug/ml

chloramphenicol. A true expression clone should not grow in the presence of chloramphenicol.

Linearization by digestion with PacI

Digest the expression clone with Pac I to expose the viral inverted terminal repeats (ITRs). See Jenner Laboratory protocol: Transfection of 293 wells with Adenoviral DNA in T25 flasks (J004 v09).

6.0 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
01	First Written	N/A	Aisling Vaughan



J004: Transfection of HEK293 or 293TREx cells with Adenoviral DNA in T25 flasks and preparation of premaster seed stock of virus.

1.0 Version Number 12

2.0 Other documents

MSDS refer to MSDS for the relevant safety information on the individual reagents: \\Imsnw3_jenner_server\jenner\hill_group\Safety\COSHHassessments\Manufacturers material safety data sheets

R002	Adenovirus
R004	GMO RA appendix
J008	Infecting 293 cells with adenovirus
J011	Passaging 293 cells
J199	Passaging TREx cells
J173	Preparation of template DNA using DNA Releasy
J175	Virus ID and Purity PCR using KAPA 2G robust polymerase

3.0 Definitions

DMEM	Dulbecco's Modified Eagle's Media
FBS	Foetal Bovine Serum
RNAse	Ribonuclease
DNAse	Deoxyribonuclease
BSA	Bovine serum albumin
CPE	Cytopathic effects

4.0 Objective

To transfect HEK293 or 293TREx cells with recombinant adenoviral plasmids to generate a new virus stock. It is recommended to perform a control transfection with serotype-matched GFP or mCherry-expressing virus at same time (p2026: pAdc63-GFPTIP-nil; p2108: pAd5-mCherry). This will help to separate problems due to poor transfection efficiency from problems related to virus growth/antigen toxicity. It may also be useful to perform a mock transfection with Lipofectamine alone to enable any toxic effects of the transfection reagent to be observed.

This protocol describes the transfection and also the subsequent passaging of transfected cells. The cells are passaged into larger tissue-culture flasks once or twice as they reach 90% confluency as this maintains the expression of the E1 genes by the cells, such expression is required for virus rescue. Usually, rescued virus is apparent (by CPE) on day 7 or 8 (i.e ~5 days after subculture into a T75 flask). However, in some instances the virus has only rescued after a further passage into a T150. The important factor is to keep the cell culture healthy and sub-culture when required. We

would not routinely continue with a virus longer than 14 days but would attempt a new transfection with freshly prepared plasmid DNA. If this failed we would discuss how to continue with virus requestor.

Note:

1) When using T-REx-293 cells, blasticidin **MUST** be present in the media at all stages to retain the repression of antigen expression. This includes post-transfection culture where the media does not contain Pen/Strep.
2) HEK293A cells and T-REx-293 cells are immortalized lines of primary human embryonic kidney cells transformed by sheared human Ad5 DNA. The cells harbor the E1A and E1B region of the adenoviral genome, that complement, in trans, the deletion of the E1 region in the recombinant adenovirus. The cells constitutively express the adenoviral E1 proteins (E1A and E1B) while they are dividing. When they reach confluency, total protein expression, including E1, is reduced. During their natural lifecycle, adenoviruses express the E1 proteins at all stages (see figure 3) and the proteins are therefore extremely important for successful virus rescue. It is therefore important that cells do not reach confluency during routine passage and also that seeding densities in preparation for transfection are suitably low.

5.0 Reagents

1 x T25 per transfection being performed of HEK293 or 293TREx cells at 90-95% confluency in 5 ml antibiotic-free 10% DMEM (for seeding densities see below).

DMEM (high glucose, with pyruvate)	Sigma	D6546
OptiMEM (Reduced serum medium, with Glutime and HEPES, without Phenol red)	Invitrogen	11058-21
TrypLE™ Express	Invitrogen	12605010
Phosphate Buffered Saline (without Ca^{2+} and Mg^{2+})	Sigma	D8537
200 mM Glutamine	Sigma	G7513
100x Penicillin/Streptomycin solution (10,000 Units penicillin-G and 10mg strep per ml)	Sigma	P0781
Foetal Bovine Serum	Sigma	F2442
Lipofectamine™ 2000 transfection reagent	Invitrogen	11668-019
10 mg/ml Blasticidin S-HCl	Melford Labs	B1105
5 μg recombinant destination plasmid created using Gateway Technology (linearised by digesting with PacI or PmeI as appropriate- see below)		
Molecular biology grade water (RNase/DNase free)	Sigma	W4502

10x Buffer 1	NEB	
100 x BSA*	NEB	
10 U/µl PacI	NEB	R0547S
10U/µl PmeI	NEB	R0560S
6x DNA loading dye (15% w/v Ficoll 400, 0.4% w/v orange G, 0.05M EDTA, 10 mM Tris pH 8.0)	In-house reagent	

Complete 10% FCS DMEM+Blasticidin (D10B)

50 ml FCS	(~10% final conc)
5 ml Pen/strep	(100 U Penicillin, ~0.1 mg strep ml^{-1} final conc)
10 ml L-glutamine	(~4 mM final conc)
250 µl Blasticidin	**(~5ug/ml final conc)**

Complete 10% FCS DMEM+**Blasticidin** without Pen/strep (D10B-P/S)

50 ml FCS	(~10% final conc)
10 ml L-glutamine	(~4 mM final conc)
250 µl Blasticidin	**(~5ug/ml final conc)**

*BSA supplied by NEB is certified to be of USA origin, a copy of the certificate can be found in the following location: S:\Vector Core Facility\projects\100927 pre-GMP MVA production\ C of O BSA general - sent from NEB

6.0 Equipment

15ml Falcon Tubes		
50 ml Falcon Tubes		
Heating block		
Class II BioSafety Cabinet	Scanlaf Ltd	
Microscope	Leica	DMIL
Centrifuge	Beckman	
2 ml screw-capped tubes		
T25 tissue culture plates		
1.5 ml microcentrifuge tubes (autoclaved)	TREFF	
Sterile, serological pipettes		
Sterile filter tips	Rainin	

7.0 Method

Digestion of Adenoviral plasmid

The RE digestion and heat inactivation are performed in the molecular biology laboratory. Ensure that the working area is cleaned down thoroughly prior to commencing as the lab space is also used for large scale preparation of bacterial cultures. The material that is digested will be added to cell culture and therefore it is extremely important that this is free of bacterial contamination.

The adenovirus shuttle plasmid is large (~35 kb) and as such is fragile. Pipette very gently to prevent shearing the DNA.

The quantities described here are sufficient for transfection of 1 x T25 flask

Reaction mix
6 µg pAd shuttle plasmid
20 U PacI (if Ad Hu5) or PmeI (if Ad Ch63 in buffer 4 with BSA)
1 µL 100x BSA
10 µL 10x Buffer 1
Make up to 100 µL total in mol biol grade water

Incubate at 37°C for 2-3 hours.

Heat the reaction (25 min at 65°C) to inactivate the restriction enzyme.

Remove 15 µl of the reaction to a separate 1.5 ml microcentrifuge tube; add 5 µl water and 4 µl 6x loading dye.

Run the sample on a 1% agarose gel to check plasmid was linearised by RE digest.

The digest should release a fragment of ~2000bp and the Ad backbone which is about 35 kb. Save and print the image file, add to production record and record in your lab book.

Use the remaining 85 µl for transfection.

Transfection Procedure

Day 1 Cell Seeding

The cells are provided by the person performing routine cell culture. It is important to make sure that you request T25 flasks at the relevant densities and also the anti-biotic-free media should be used (either completely antibiotic free for HEK293A cells, or D10B-P/S for TREx293 cells.
Invitrogen recommend that cell density is 90-95% for transfection using Liopfectamine2000. However, for adenovirus rescue, the cells need to be dividing (to express E1 proteins and support viral replication)

Prepare sufficient T25 flasks for the following:

1 x T25 for untransfected control
1 x T25 to be transfected with mcherry control DNA
1 x T25 to be mock-transfected with Lipofectamine alone
? x T25 for experimental transfection.

Seed T25 flasks at the following cell densities – two sets of flasks are set up such that those reaching 80-90% can be selected.

a) *Seed 2 x 10^6 cells in 5 ml D10B-P/S media in one set of flasks*

b) *Seed 2.5 x 10^6 cells in 5 ml D10B-P/S media in a second set of flasks*

Incubate overnight at 37°C, 5% CO_2.

Day 2 Cell transfection

Warm an aliquot of OptiMEM for the preparation of DNA-Lipofectamine complexes to ROOM TEMPERATURE – (not 37°C).

Warm an aliquot of OptiMEM for cell washing/media replacement to 37°C.

Warm 10% FCS DMEM without antibiotics to 37°C.

Note: The adenovirus shuttle plasmid is large (~35 kb) and as such is fragile. Pipette very gently to prevent shearing the DNA.

Prepare:
1 x sterile 2 ml screw-capped tube containing 85 μl restriction enzyme mix + 215 μl OptiMEM without serum.

1 x sterile 2 ml screw-capped tube containing 30 μl lipofectamine 2000 + 270 μl OptiMEM without serum.

Mix tubes gently by flicking.

Incubate at room temperature for 5 min.

Mix the contents of the 2 tubes. Add the DNA to the lipofectamine, not vice versa. Flick tubes gently to mix, and incubate at room temperature for at least 20 min (complexes are stable for 6 h at Room Temp).

Remove the media from the flasks of cells and wash twice with OptiMEM (no additions)

Add 1.4 ml of OptiMEM, to each flask.

Add the 600 μl of DNA/lipid complexes to the flasks and mix by tilting the flask gently to ensure that the transfection mix covers all the cells.

Place the cells at 37°C, 5% CO_2 for ~4 hours. 293 cells can be left overnight but for T-REX cells the transfection mix **must be removed after 4 hrs** to prevent toxicity
Replace the media with 5 ml of D10B-Ab.

Place the cells at 37°C, 5% CO_2.

Day 4 Passaging cells to maintain cell viability and promote virus rescue.

Inspect cells using microscope, they should be confluent and beginning to appear crowded.

Check for mcherry protein expression to assess transfection efficiency.

Warm TrypLE Express, PBS and 10% FCS DMEM without antibiotics to 37°C. The cells are fragile at this stage and must be treated very gently.

Transfer media from the T25 to a fresh T75.

Wash cells carefully with 5 ml PBS (aim fluids along the top of the flask to prevent dislodging the cells).

Add 2 ml TrypLE Express and place flask at 37°C for approx 2 min to detach cells.
Add 10 ml D10 to the flask and transfer to the T75.

Place at 37°C, 5% CO_2. The cells will take approximately 48 hr to reach confluency and then monitor for the appearance of CPE, this should begin 48-72 hr after cell passage.

Continue with either harvest at day 7-11 or a further round of cell passage.

Day 7 – 11 Harvesting of adenovirus-infected cells

293 cells showing a CPE should detach from the flask with gentle tapping. Transfer cells and media to a sterile, 50 ml tube. This stage is called the transfection stock (TF). Freeze/thaw 3 times to release virus from the cells. Centrifuge (2000 rpm for 10 min), transfer the supernatant to a fresh tube and store at -20°C.

Day 7-11 Passaging cells into T150 if there are no signs of CPE

Inspect cells using microscope, they should be confluent and beginning to appear crowded.

Warm TrypLE Express, PBS and 10% FCS DMEM without antibiotics to 37°C. The cells are fragile at this stage and must be treated very gently.

Transfer media from the T75 to a fresh T150.

Wash cells carefully with 10 ml PBS (aim fluids along the top of the flask to prevent dislodging the cells).

Add 5 ml TrypLE Express and place flask at 37°C for approx 2 min to detach cells.

Add 15 ml 10% FCS DMEM to the flask and transfer to the T75.

Place at 37°C, 5% CO_2 and monitor for the appearance of CPE, this should begin 48-72 hr after cell passage. It is important to be able to distinguish between gaps between the cells where they have not reached confluency and genuine CPE. The positive

control virus should be observed in parallel to clarify cell morphology under the two different conditions.

Premaster infection of cells with rescued adenovirus

The aim of the production of a premaster stock of adenovirus is to try and achieve samples that contain a relatively standard amount of virus such that the hyperflasks can be infected at an almost optimal MOI. We do not have capacity to titrate all of the viruses we produce at this stage and therefore this is a means of trying to standardize the viral preps.
The amount of virus produced from the transfection/rescue stage will vary greatly. In order to achieve a sample of virus from optimally infected cells, a six-well plate is prepared that is infected with a serial dilution of the cell lysate from the previous step (If the cells round up by the following day, this is not true CPE but is due to the effects of the excess penton capsomere that is produced during virus growth).

The well that displays almost complete and uniform CPE after 72 hours is selected and harvested to form the premaster stock for both hyperflask infection and archiving.

1) Prepare a 6-well plate with either HEK293 or HEK293TREx cells the day before required (see J011 and J199).
2) On the day of infection, replace media with 2 ml fresh D10 (this also serves to remove non-adhered cells to which adenovirus would otherwise attach).
3) Add 1 ml cell lysate supernatant to the first well and rock plate backward, forwards and side to side to evenly distribute the virus.
4) Take 200 µl of media from the first well, add to the second well and repeat the mixing step.
5) Repeat step 9.2.35 for the remaining 4 wells
6) Place plate at 37°C, 5% CO_2 for ~72 hours.
7) Harvest the cells and media from the well that shows uniform CPE using a cell lifter and P1000 pipette.
8) Remove a 100 µl sample to a 1.5 ml microcentrifuge tube to provide material for PCR QC testing (J173 and J175).
9) Freeze/thaw the ~2 ml sample x 3 and then centrifuge (2500 rpm, RT, 5 min), transfer the supernatant to a fresh tube and store at -80°C until bulk prep.
10) Infect 1 x HF with 300 µl cell lysate.
11) Continue with infection of hyperflask as described in J008.

8.0 Revision History

Version Number	What Changed	Who Changed it	Date changed
02	Not obvious that need to double the amount of plasmid to be digested if setting up transfections in duplicate	Nicky Green	
02	Only needs 4h	Nicky Green	
02	Cells showing a CPE should bang off easily	Nicky Green	
02	To release virus	Nicky Green	
03	To fill gap in protocols	Fionnadh	

	To reflect what is done in practice, media removed and replaced with fresh.		
04	Changed to include heat inactivation of RE and also to change method to 6-well plates		
05	Minor changes to method as per Matt Dicks	Sandy Douglas	
06	Alternative method using 1 x T25 flask per transfection. Comments regarding TREX cells added	Nicky Green	
07	Using microcentrifuge tubes to preparation of DNA/lipid mix Addition of images in figures	Ali Turner	
08	Alteration of scale of cultures post virus rescue. Altered to T75 and 1 x HF	Ali Turner	
09	Note about temperature to warm Opti-MEM to	Ali Turner	12th Jan 2011
10	Image of example CPE added, protocol changed to 6-well plate for premaster	Ali Turner	17th Jan 2011
12	Detail added to protocol	Ali Turner	1st Mar 2011



J005: Adenovirus purification using discontinuous and isopycnic CsCl gradients

1.0 Version Number 10

2.0 Title: Other documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\Imsnw3 jenner server\jenner\hill group\Safety\COSHHassessments\Manufacturers material safety data sheets
R002 Adenovirus
R004 GMO RA appendix
J008 Infecting 293 cells with adenovirus
J011 Passaging 293 cells
J104 Adenovirus Titre Immunoassay
J005 App I Annotated images of CsCl centrifugation steps for troubleshooting.

3.0 Definitions

Ad Adenovirus
CsCl Caesium chloride
CPE Cytopathic effect
PBS Phosphate buffered saline
HF Hyperflasks

4.0 Objective

The purification of recombinant adenovirus by two different methods of density centrifugation using Caesium chloride solutions as solvents. The first step involves a form of rate zonal centrifugation whereby the component particles are separated in a manner based upon mass (larger particles will sediment faster). The second centrifugation step is isopycnic centrifugation whereby the particles are separated according to density.
This method describes purification of recombinant adenovirus from a single hyperflask – scale up or down as necessary.
Note concerning incubation time for benzonase treatment of harvested, infected 293 cells:

The optimum incubation temperature for benzonase is 37°C. However, as a compromise between virus stability and enzyme activity, the incubation is performed at RT.

Note about CsCl solutions

In January 2011 we had an issue with bands not forming on two separate viruses, one on first banding and one on second banding (lab book VC27 5th Jan 2001; Production records VC261 And VC296). The virus that failed the first banding – one tube was correct and the other had a diffuse band where the empty and complete capsids should have banded. Both tubes were pooled and the viruses banded perfectly during the overnight centrifugation. The second virus banded perfectly during the 2 hr spin but was diffuse after the overnight centrifugation. To solve this issue, 1) the centrifuge was cleaned thoroughly and all of the o-rings and gaskets were greased. 2) Nicky suggested that she has previously seen CsCl settling out during prolonged storage and as a consequence loaded CsCl of incorrect density.
The virus that didn't band on the overnight centrifugation step was re-banded o/n with thoroughly mixed CsCl and worked perfectly.
Therefore – ensure all CsCl solutions are thoroughly mixed both prior to aliquoting and prior to adding to centrifugation tubes.

General virus handling:

Ensure only one virus is handled at any one time in the hood.
Wipe hood thoroughly with Microsol solution between viruses and change gloves.
Soak forceps in 10% Microsol solution for 10 mins after use with each virus as these come into very close contact with the virus preparations. Use a fresh pair of forceps if more than one virus is being purified simultaneously. Ensure that the virus is not exposed to Microsol.

This protocol should not be performed without prior training as some of the procedures are complex and demonstration of the techniques is required.

5.0 Reagents

Hyperflasks containing adenovirus-infected HEK293A or TREx293 cells.
Cells should be showing a cytopathic effect CPE (round grapelike clusters), but the majority should still be attached to the flask.

18.2 MΩ water		
1M Trizma-HCl pH7.8	Sigma	T-2569-100ml
Caesium Chloride	Sigma	203025
PBS containing Ca & Mg	PAA	H15-001
250U/μl Benzonase	Sigma	E8263-25Ku
1M $MgCl_2$	Sigma	63069-100ML
Sucrose*	Fluka	84097

*it is important that this material is low in endotoxins, do not substitute this supplier

Solution preparation:

Pre-rinse Duran bottles with 18 MΩ-water. Add solids to a Duran bottle and add some 18.2 MΩ-water, then add liquids. Add a clean magnetic stir bar and place on a stir plate until solids have dissolved. Transfer to a volumetric flask and top up with water.
Filter sterilise the solution into 500 ml sterile tissue culture bottles.

For each new solution record lot number of Trizma base and Sucrose used.

1.25 g/ml density CsCl (density = 1.25 g/mL = 1.98 M) in 10 mM Tris pH 7.8.
166.89 g CsCl
5 ml 1 M Tris pH 7.8
Add 18.2 MΩ water to 500 ml using volumetric flask
Filter sterilise.

1.35 g/ml density CsCl (density = 1.35 g/mL = 2.78 M) in 10 mM Tris pH 7.8.
233.65 g CsCl
5 ml 1 M Tris pH 7.8
18.2 MΩ water to 500 ml using volumetric flask.
Filter sterilise.

Lysis buffer (10 mM Tris, 1 mM $MgCl_2$. pH 7.8)
5 ml 1 M Tris-HCl pH 7.8
0.5 ml 1 M $MgCl_2$
Make up to 500 ml with 18.2 MΩ water to 500 ml using a measuring cylinder
Filter sterilise.

Storage buffer (10 mM Tris, 7.5% w/v sucrose. pH 7.8)
Weigh 75 g sucrose and transfer to a 1 litre measuring cylinder.
Add 18.2 MΩ water up to ~800 ml.
Add 10 ml 1 M Tris-HCl pH 7.8
Seal top of measuring cylinder with parafilm and invert cylinder repeatedly until sucrose has full dissolved.
Adjust volume to 1000 ml.
Filter sterilise and store either at 4°C in the virus lab or on the shelf in mol biol.

6.0 Equipment

Class II BioSafety Cabinet	Scanlaf	Mars
Benchtop Centrifuge	Beckman Coulter	Allegra X-12R
CO_2 incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope	Leica	DMIL
IMS	Fisher	M/4400/17
Corning conical tubes, 250 ml PP Centrifuge tube, plug seal cap	Fisher	CFT-900-021Y
Bottle-top filters	Fisher	TKV-238-050Y
Volumetric flasks		
500 ml and 1 l sterile Duran bottles		
5 ml syringes	Fisher	SZR-150-170T19G
Needles	nu-care products	M1500
Ultraclear centrifuge tubes 14.0 ml	Beckman Coulter	344060
Dialysis cassettes 3-12 ml & 0.5-3ml	Thermo-Fisher	66453 & 66455
Slide-A-Lyzer Buoys for 3-12 ml and 0.5-3 ml dialysis cassettes		66432 & 66430
250 ml polypropylene bottles		

Dry ice in suitable container
Beckman ultracentrifuge Beckman Coulter Optima L-80 XP
SW40Ti rotor & buckets Beckman Coulter
Balance
Forceps
Tube holder

7.0 Method

Harvest infected cells - The time of harvest is critical for optimum virus yield. Monitor the cells every day for CPE and harvest the cells when the majority of the cells are rounded up but still adhered to the surface of the flask. This should be within 72 – 96 hours.

Note:
Cells detaching overnight are a sign of toxicity due to high concentration of penton protein present in the inoculum and the HF should be discarded. Repeat using a lower volume inoculum.

Any HF inoculated with insufficient virus such that complete CPE is not obtained after 96 hr should be discarded. After this time, we generally achieve very poor virus yields. We think that proteases may have have been released from lysed cells by this time and these degrade any virus produced. Repeat using a higher volume of inoculum.

Detach adenovirus-infected cells by gently banging the flasks with the palm of your hand until the cells detach from the flasks.
Pour the media into 250 ml polypropylene tubes.

If using hyperflasks (HF), after detaching adenovirus-infected 293 cells by gently banging the HF with the palm of your hand until the cells detach from the flasks and pouring the media into 250 ml bottles, wash out the HF with ~250 ml PBS. Tip the HF so that the PBS is evenly distributed across the layers and agitate to remove any remaining adherent cells. Pool the PBS wash with the harvested media.

Centrifuge for 5 min. at 1500 x g to pellet the cell debris (with virus associated) and aspirate supernatant.
Resuspend the cell pellets in a total volume of 10 ml lysis buffer and either freeze on dry ice or place at -20°C.

The protocol may be stopped at this point by storing the resuspended cell pellet at -20°C.

Thaw the cell pellet and add 250 U of Benzonase per ml of cell lysate.
Incubate for 1 hour at RT.

Freeze/ thaw (dry ice/37°C water bath) the cell pellet a further two times. Whilst thawing leave at 37°C for the shortest time possible.

Centrifuge at 1500 x g for 5 minutes at 4°C and transfer the supernatant to a fresh tube.

Make the total volume of the supernatant up to ~10 ml using lysis buffer.

At this stage, check that the ultracentrifuge buckets are clean, place buckets in the biological safety cabinet and spray with 70% IMS to sterilise. Wipe off excess IMS with blue roll and allow the remaining liquid to evaporate whilst the samples are prepared.

If preparing two viruses at the same time ensure that they are kept completely separate and that tubes containing different viruses are NEVER open at the same time in the hood. Wipe down all surfaces carefully between handling different viruses and change gloves.

Mix aliquots of CsCl thoroughly before use.

Using 5 ml pipettes, set up relevant number of Caesium Chloride gradients in 14 ml Beckman Ultraclear tubes.

Add 3.5 ml 1.25 g/ml CsCl solution to the tubes using a 5 ml pipette.

Take up 4 ml of 1.35 g/ml CsCl solution into a 5 ml pipette and use this to underlay the 1.25 g/ml CsCl solution with 3.5 ml 1.35 g/ml CsCl solution. Pipette very slowly and do not add the last 0.5 ml, in this way, no air bubbles are added to the tube which may disrupt the interface.

Ensure that the interface between the two solutions is visible.
This is absolutely crucial to this centrifugation method as the interface remains intact during centrifugation and is the limit to which the adenovirus migrates.

Using either a 5 ml pipette or Pasteur pipette, carefully add ~6 ml of virus supernatant to each of the gradients (do not disturb the interface whilst adding the sample. This can be avoided by adding the sample slowly down the side of the tube). Top up the tubes if required with lysis buffer. It is important to fill the tubes to within 3 mm of the top to prevent tube failure.

Place centrifuge tubes in the buckets for the SW40 Ti rotor and balance them using the scales.

Centrifuge for 2 hours at **110 000 x g** @ 4˚C.

NOTE: use all 6 buckets correctly sealed and mounted in the rotor in their numbered positions as this is a balanced set.

Once the centrifuge run is complete, remove rotor from the ultracentrifuge, transfer the buckets to the holder and move to the biological safety hood.

Unscrew the bucket lids and remove the tubes using forceps.

Place the tube in a clamp stand above a beaker of Microsol. two virus bands should be visible close to the centre of the tube; the upper band is incomplete virus and the lower band intact virus (see figure 1).

Using a 19G needle and 5 ml syringe, pierce the tube approximately 5 mm below the band by gently twisting and pushing the needle through the wall of the tube. *Be careful not to hold your other hand on the other side of the tube whilst doing this for risk of injury.*

Once the needle is inside the tube gently pull on the syringe with the bevel pointing upwards to extract the lower band and store in a TREFF tube on ice.

Discard the used centrifuge tube into the beaker of Microsol. Wipe up any spills carefully with Microsol wipes or Microsol dampened tissue. Decontaminate the buckets using Microsol, rinse with water and ensure they are clean and dry.

Decide how many tubes are required for the second centrifugation step:
This is based on experience. The major concern is that placing too much virus in each tube will lead to aggregation of the virus particles.
*Dilute virus in lysis buffer, you need approx 7 ml of virus per gradient, this allows space for the tubes to be balanced with lysis buffer**

**The actual density gradient that forms during the isopycnic centrifugation depends on the total CsCl concentration present in the tube. This will vary depending on the volume that the adenoviral band is drawn off in after the first centrifugation step. The adenovirus from the 1st banding will be in CsCl at ~1.35 g/ml. The lysis buffer that is added to make up the volume is at ~1 g/ml.*
Consequently, the position of the band after the 2nd ultracentrifugation step will vary. This does not matter as only complete virus will band together and any empty capsid will accumulate higher in the tube.

Set up the second caesium gradients in 14 ml Beckman ultraclear tubes:
Mix aliquots of CsCl thoroughly before use.

Using 10 ml serological pipettes, add 6 ml of 1.35 g/ml CsCl to each tube
Using 10 ml serological pipettes or a Pasteur pipette, add ~7 ml of virus supernatant to each of the gradients, maintenance of the interface is not crucial for this centrifugation method as a concentration gradient forms completely during the >16 hr centrifuge run. Top up the tubes if required with lysis buffer. It is important to fill the tubes to within 3 mm of the top to prevent tube failure.

Balance the tubes with lysis buffer and place in the buckets for the SW40 Ti rotor and centrifuge at **160000 x g for 16-18 hr** (i.e. overnight).

Once the centrifuge run has finished, remove rotor from the centrifuge, transfer the buckets to the holder and move to the biological safety hood.

Remove the centrifuge tube using forceps and place in the retort clamp. Two bands may be visible. However, the upper band may not be present if the virus added onto the 2nd gradient was very pure. Remove the lower band using a 19G needle and 5 ml

syringe. This can be placed safely to one side to be directly loaded into the dialysis cassette.

At this stage, mark a dialysis cassette with the name of the virus and pre-wet with pre-chilled storage buffer.

Discard the used centrifuge tubes into the beaker of Microsol. Wipe up any spills carefully with Microsol wipes or Microsol dampened tissue. Decontaminate the buckets using Microsol, rinse with water and ensure they are clean and dry.

Add the purified virus band to a dialysis cassette using a needle and syringe:

Transfer the wetted dialysis cassette to the hood.

Insert cassette into the buoy to function as a holder.

Place a needle on a 5 ml syringe and insert the needle so that just the bevel of the needle in inside the cassette. Insert a small amount of air to the cassette so that it is slightly inflated which makes it easier to add the virus without piercing the membrane.

Add virus solution

Tilt cassette and remove air such that the cassette is full with no air bubble.

Place the cassette back into the beaker of 500 ml of cold storage buffer, cover with foil and place on a magnetic stir plate. Dialyse for 90 minutes changing the buffer every 30 mins.

Remove virus from dialysis cassette, tilting the cassette ensure all of the solution is removed, and aliquot.

Ensure there are at least 10 x 10 µl aliquots for QC

Label virus clearly with stock number. Store at -80°C.

Enter in the File Maker Pro Virus database the details and location of the new virus.

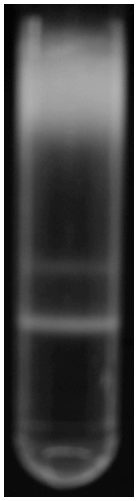


Figure 1. Discontinuous gradient centrifugation of adenovirus preparation.

Viral banding pattern typically formed following 2 hr centrifugation step.
Lower band is correctly formed adenovirus.
Upper band contains viral capsids lacking DNA

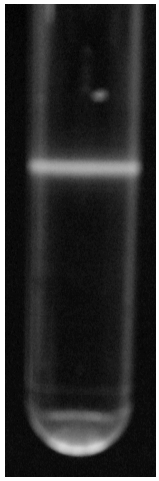


Figure 2. Isopycnic centrifugation of adenovirus preparation.

Viral banding pattern typically formed following 16 hr centrifugation step.

Band contains correctly formed adenovirus.

8.0 Revision History

Version Number	What Changed	Who Changed it	Date changed
2	Amount of Benzonase added	Nicky Green	
2	2nd gradient preparation, dilution of band from first gradient and only using heavy CsCl	Nicky Green	
3	Amount of Benzonase added	Nicky Green	
3	Dialysis step added between bandings 2 x 2 step gradients	Nicky Green	
4	Buffer recipes to include ready-made stocks of Tris buffer and $MgCl_2$. Removal of dialysis step between bandings. Second, optimised, isopycnic centrifugation procedure added instead of step gradient.	Ali turner	
5	Modified for new ultracentrifuge	Nicky Green	
6	Slight modifications to format. Addition of note about preparing two viruses at the same time.	Ali Turner	
7	Changed to single HF	Ali	
8	Centrifugation speeds corrected – RCF is entered as RCF (max) from rotor tables	Ali	
9	Note about CsCl settling and relevant change to protocol	Ali	07.01.11
10	Method clarification and images added	Ali	07.03.11

J010: Adenovirus purification using Puresyn's Adenopure™ Kit

1.0 Materials

Adenopure™ kit (lot #: AK-26 oct05).

Examination gloves (Microflex, Cat# EN 455/ASTM D3578, lot # 5068225209)
-80^{o}C Freezer for keeping unpurified virus (Lab Impex Research, model # L1U1786W12, serial # V16D-171674-VD)

Autoclaves (BMM Weston, Model #s V6902 and V6903, serial #s DH12528 and DH12529 respectively)

37°C water bath (Grant instruments Ltd, Cambridge, Type JB1, serial #:600210013)

Vortex – 2 Geneic (Scientific instrument Inc., model #: G-560E, serial #:2-77246)

Centrifuge, Buckets and holders (Beckman GS-6K Centrifuge Model # 362126, serial # GCY95L03, CH 3.8 Bucket, holder 345369)

Class II Microbiological safety cabinet (Walker safety cabinets Ltd, Serial # SO000271)

Nalgene filtration unit, 500ml, 0.2um pore size (Fisher scientific cat # 569-0020, lot # 548280)

Pump (Charles Austen pumps Ltd, Model# DA7C, serial # DT8391)

25ml disposable plastic pipettes (Appleton woods, cat # CC116, lot# 000000062)

10ml disposable plastic pipettes (Corning Inc, cat # 4101, lot# 34605012).

Dry ice (BOC industrial)

-80^{o}C Freezer for keeping Dry ice (Lab Impex Research, model # L1U1786W12, serial # X25C-149975-XC)

Absolute Ethanol (Riedel- de Haën, cat # 24103, lot # 52300)

50ml Centrifuge tubes (Corning Inc., cat# 430829, lot# E01606004)

15ml Centrifuge tubes (Greiner bio-one Cell star cat# 188271, lot# 06070197)

Stand and clips (Stand Fisher brand and clips Fisons

Dispo- Safe Jars (The microbiological supply company, BA 04).

Beaker 1lit (Pyrex)

Microsol[3], 5 liter conc. (Anachem, Cat# MIC-003, lot# 51048).

2ml Freezer vials PP (Sarstedt, Cat# D-51588, lot # 5073801).

Pipettes 0.5-10ul, 2-20ul, 20-200ul and 100-1000ul (Nichipet, NICHIRYO, model # 5000DG)

0.1-10ul filter tips (Starlab, Cat# S1121-3810, lot # U122419N)

1-20ul filter tips (Starlab, Cat# S1120-1810, lot # U121787L)

1-200ul filter tips (Starlab, Cat# S1121-8810, lot # U121767L)

101-1000ul filter tips (Starlab, Cat# S1126-7810, lot # T116198G)

Freezer box

Fridge (Lec larder, serial # 9A001831)

-20^{o}C Freezer (Lec, serial # 9A003475)

-80^{o}C Freezer for keeping Adenovirus stocks (Sanyo Electric co Ltd, VIP series, model # MDF-U50V, serial # 00201393)

Autoclaved1.5ml polypropylene tubes (Anachem Scotlab, cat# 96.7811.9.05, lot # 33052011) in Autoclave Bag 140X50X330 (Laboratoires, F, 444)

Spectrophotometer (Beckman DU640, serial # 4317030)

Cuvette, precision cell made of Quartz Suprasil (Hellma Quarzglas, Type # 105.020-QS, light path= 10mm)

Distilled water

2.0 ADENOPURE™ KIT'S INTRODUCTION AND BACKGROUND

Puresyn's Adenopure™ Kit couples membrane adsorber technology, a simple sample loading mechanism and proprietary buffer formulations to provide rapid and simple isolation of highly purified adenovirus preparations suitable for *in vitro* biological assays.

Membrane adsorber ion exchange modules are based on a technology that attaches ion exchange functional groups to the inner surface of synthetic microporous membranes in a syringe-filter format, with high binding capacity at high flow rates, and scaleability.

The current size Adenopump™ Adenovirus Purification Units are capable of purifying up to 5 x 10^{12} viral particles in less than two hours.

3.0 Method:

4-5 DAY INFECTED CELL SUSPENSION SAMPLE PREPARATION

1. Thaw the frozen cell suspension in water bath at 37^{o}C.

2. Pellet cell debris by centrifugation at 1500xg for 5 minutes at room temperature.

3. Filter the supernatant using Nalgene filtration unit, 500ml, 0.2um pore size, attached to pump with 25ml disposable plastic pipette.

4. Vortex the cell pellets, freeze on ethanol / dry ice and thaw in water bath at 37^{o}C. Repeat this step two times.

Note: Additional freeze-thaw cycles are not recommended as this may lead to adenovirus degradation.

5. Pellet cell debris by centrifugation at 1500xg for 5 minutes at room temperature.

6. Filter this supernatant using the above Nalgene filtration unit, with 10ml disposable plastic pipettes.

7. Add 100µl of 25U/µl Benzonase® to the filtered lysate and mix by gently swirling. Incubate the mixture at 37°C for 30 minutes in water bath.

Note: Do not exceed 30 minutes incubation as this may lead to adenovirus degradation.

8. Dilute: Add 27ml of 10X Dilution Buffer to the Benzonase®-treated filtered lysate and mix by gentle swirling. Determine the amount of 10X Dilution Buffer required using the following formula:

Volume of filtered cell lysate (250ml) ÷ 9 = Volume of 10X Dilution Buffer required (27ml)

Note: Accurate sample volume determination is critical, as incorrect volume addition of 10X Dilution Buffer will lead to poor binding of adenovirus to the *virus-binding module* of the syringe-check valve purification unit.

9. Proceed to adenovirus purification.

ADENOVIRUS PURIFICATION

1. Mount the Adenopump™ in a clamp attached to a ring-stand and make sure all the connections of the Adenopump™ are tight.

2. Fully depress the syringe plunger.

3. Fill one of the supplied 5cc syringes with 3ml of Elution Buffer and 2ml of air. Set aside for the elution step.

4. Equilibrate: Place the *feed tube* into a 50ml centrifuge tube containing 30ml of Equilibration-Wash Buffer to be passed over the *virus-binding module*.

5. Pull up on the syringe plunger drawing the solution through the *oneway dual check valve T-fitting* into the syringe. Push down on the syringe plunger to pass the solution back through the *one-way dual check valve T-fitting* and over the *virus-binding module*.

Notes: a) Avoid pushing air through the *virus-binding module* as this can prevent even distribution of solution across the membranes in the module. Leaving a small amount of Equilibration-Wash Buffer in the syringe will not interfere with efficient adenovirus purification; b) Do not place the feed tube directly into any of the supplied buffer containers because cross contamination may occur between adenovirus purification procedures. When purification steps require it, pour the appropriate amount of supplied buffer into a clean feed reservoir.

6. Load: Load the diluted-filtered lysate onto the *virus-binding module* using the Adenopump™. Repeat the loading step as many times as required to pass the entire sample over the *virus-binding module*. Try not to push air through the *virus-binding module* as this can prevent even distribution of solution across the membranes in the module.

Note: In general, a slower flow rate will increase the efficiency of binding of adenovirus to the *virus-binding module* with a flow rate of 10ml/minute being suitable. A flow rate of 10ml/minute may be approximated if you can "count the drops" as they come out of the *virusbinding module*. A flow rate that results in a stream of material coming out of the *virus-binding module* is too fast.

7. Wash: Wash the adenovirus bound to the *virus-binding module* by passing 50ml of Equilibration-Wash buffer over the module using the Adenopump™.

Notes: a) Avoid pushing air through the *virus-binding module* as this can prevent even distribution of solution across the membranes in the module; b) Do not place the feed tube directly into any of the supplied buffer containers because cross contamination may occur between adenovirus purification procedures. When purification steps require it, pour the appropriate amount of supplied buffer into a clean feed reservoir.

8. Elute: Detach the *virus-binding module* from the *one-way dual check valve T-fitting* of the Adenopump™, and reattach the module to the 5cc syringe containing Elution Buffer prepared in step 3. Collect all material at this step as one fraction as it contains the purified adenovirus in 15ml tube. Pass 15 drops of Elution Buffer through the *virus-binding module*. Incubate the *virus-binding module* for 5 minutes at room temperature before continuing. Pass the remainder of the Elution Buffer through the *virus-binding module* and push the air in the syringe through as well to expel as much Elution Buffer as possible from the module (~4ml).

Note: During the 5 minute incubation, the syringe *virus-binding module* may be laid on its side or held vertically.

9. Aliquots: Pipette 100ul of the virus in freezer vials

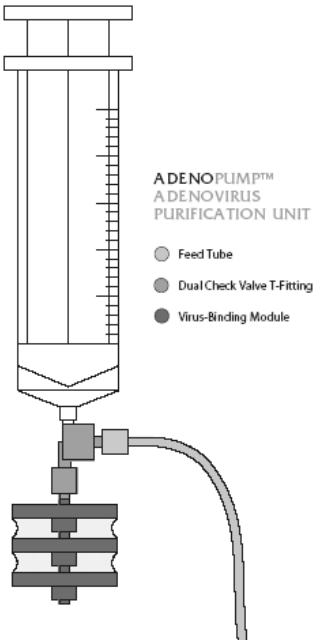
10. Quantitation of adenovirus:

• Dilute the virus in 1.5ml centrifuge tubes
For 1/100: 2ul of adenovirus and 198ul of DW.
For 1/50: 4ul of adenovirus and 196ul of DW.
• Take the diluted virus tubes, cuvette, 20-200ul pipette, tips and 50ml tube (for waste) to the spectrophotometer. Put on the monitor and click on the Nucleic Acids and both UV and VIS on.
• Take the blank reading, then 1/100 and then 1/50 reading at 260nm
• Calculate the DNA concentration.

11. Enter the new virus stock number in the virus stocks folder.

12. Keep all the vials in a freezer box labeled and freeze in -80^{o}C freezer.

13. Keep one vial in -20oC freezer to isolate the genomic DNA.



POST-PURIFICATION GUIDELINES
Adenopure™ purified adenovirus preparations may be stored at 4°C for short-term use (up to one week), or at -20°C/ -80°C for long-term storage. Repeated freeze-thaw cycles are not recommended.



J106: Quantification of virus particles using Beckman Coulter DU 730 spectrophotometer

1.0 Version Number: 03

2.0 Other documents

R002	Adenovirus
R004	GMO RA appendix

3.0 Definitions

dH_2O	distilled water
SDS	sodium dodecyl sulphate

4.0 Objective

NOTE: Filtration appears to increase absorption at 260nm and 280nm, possibly due to filter residue entering filtered material. Filtered virus stocks, storage buffer and PBS all give significant readings (A260nm ~0.100) when blanked against the equivalent unfiltered solution. It is suggested that either the virus stocks are not filtered, or that absorption values are measured before filtering.

This protocol describes the method for checking the quality of an adenovirus stock and for measuring the concentration of viral particles per ml, using UV spectrophotometry at wavelengths of 260, 280 and 320 nm.

The concentration of viral particles may be measured directly by UV-spectroscopy, since in an adenoviral particle there is a constant ratio of DNA:protein (approx 87% protein and 13% DNA) and DNA has an absorbance maximum at 260 nm.

In addition, intact, monomeric viral particles have a high organization state that causes light scattering which can bias the UV-measurement. In this assay, the 0.1% SDS reduces loosely associated virus to a monodispese solution. The UV-absorbance of the virus in SDS at 320 nm provides a measurement of more stable viral aggregation, whilst the readings at 260 nm and 280nm account for viral DNA content and protein content respectively. The viral particle concentration is calculated using a method described by Maizel *et al*. In this method, an absorbance of 1.00 (AU, 1 cm pathlength) at 260 nm corresponds to 1.1 x 1012 viral particles/mL.

If the virus has been through a high concentration step (we estimate approx 10E13 vp/ml) at any point in the purification i.e. first or second CsCl centrifugation, it is likely to form stable aggregates (possibly as small as dimmers/trimers) which are then non-infectious. The OD320 in this instance will be high (0.01 – 0.1). If this is due to aggregation, we have found that diluting the virus in 1% SDS fully dissociates such

viruses and gives a true OD260 and OD280 reading in addition to proving that stable aggregates are present in the virus preparation. As at 6th May 2010, Vector core are attempting to clarify this situation and devise a robust production method to minimise virus aggregation at all steps.

The Beckman Coulter DU 730 has an adapter for the 0.1 ml cuvette which is screwed in to the base. The cuvette is placed in the adapter and is 'blanked' using Jenner storage buffer with 0.1% SDS. The cuvette should not be moved for duration of the session. Virus is diluted in Jenner storage buffer with 0.1% SDS. The spectrophotometer programme 'Adeno' should be used. Three separate 1:10 dilutions should be prepared and the readings at 260 nm, 280 nm and 320 nm taken, recorded and entered in the Excel spreadsheet 'adenovirus production chart'.

The linear range at which the UV spectrophotometer can provide accurate readings of VP/ml is for absorbance values between 0.1and 1. The virus should be diluted further with Jenner storage buffer with 0.1% SDS until all absorbance readings for a given virus fall within this range.

5.0 Reagents

3 x 10 µl viral samples		
10% SDS solution in water	Sigma/Fluka	71736
Jenner storage buffer with 0.1% SDS		
Sterile distilled water (RNAse & DNAse Free)	Sigma	W4502

6.0 Equipment

Gilson Pipettes
Pipette tips
Beckman Coulter DU 730 Spectrophotometer with small cuvette holder
1.5 ml microcentrifuge tubes

7.0 Method

Sample preparation in virus hood

1) Dilute 3 x virus samples 1:10 in Jenner storage Buffer with 0.1% SDS, (10 µl virus and 90 µl Buffer).

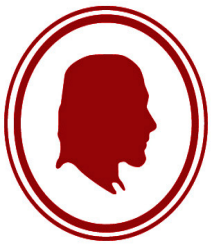
Virus quantification

2) Ensure the insert for the 0.1 ml cuvette is in place. Clean the cuvette with a kim wipe to dry and remove any fingerprints that may interfere with the passing of light through sample. The cuvette should be positioned so the red dot is positioned in the bottom left corner. Do not remove the cuvette for duration of session.
3) Switch on the spectrophotometer, close sliding lid and allow self checks to run.
4) Select 'User programmes' from menu
5) Select 'Adeno' programme
6) Select 'Blank' (even with no cuvette in place) which switches on the uv lamp and allows it to warm up. Allow to warm up for approx. 10 min.

7) Add 100 µl of buffer (0.1% SDS in Jenner storage buffer) to cuvette. Close the sliding door of the spectrophotometer.
8) Press 'blank' on the menu screen.
9) When blank has been performed, open the door and remove the buffer from the cuvette with a pipette.
10) Load 100 µl sample, ensuring no bubbles.
11) Press 'Read' on the menu screen. Record the 260 nm, 280 nm and the 320 nm values.
12) Read the remaining two replicates in an identical manner.
13) If doing more than one virus, remove the liquid from the cuvette, add 100 µl buffer, pipette up and down twice and remove. Fill with a fresh 100 µl of buffer and read. Check that the reading is 0.
14) Remove the cuvette, clean it and place in a 50 ml tube of 10% Microsol.
15) Remove the insert that holds the cuvette.
16) Turn off the spectrophometer.

8.0 Revision History

Version Number	What Changed	Who Changed it	Date changed
01	Original	N/A	
02	0.1% SDS is used instead of 1%. Also, OD320 values are to be recorded. To remove ambiguity over SDS concentration and to measure aggregation.	Jake Matthews	
03	Method to include taking readings at all wavelengths. Also introduction to explain aggregation.	Ali Turner	06.05.10
04	Note added about the effect of filtration on absorption readings.	Jake Matthews	05.11.10



J006: Plaque forming unit (pfu) assay

1.0 Version Number 02

2.0 Other documents

J011 Passaging 293 cells
R002 Adenovirus
R004 GMO RA appendix

3.0 Definitions

Definitions of abbreviations

4.0 Objective

To calculate the infectious viral titre by visualizing plaque forming unit*s*

5.0 Reagents

Dulbecco's Modified Eagles Media (DMEM) - high glucose with 4500 mg/L glucose, sodium pyruvate, and sodium bicarbonate, without L-glutamine (Sigma, product # D6546-500ML)

L-Glutamine (Sigma, product # 8540)

Penicillin/Streptomycin solution (Sigma # 4333)

Foetal Bovine Serum (FBS) (Sigma, product # F6178-500 ml)

2% low melting point agarose (Sigma, product # A9414)

6 well plates containing confluent monolayer of 293 cells (set up the day before infection, see protocol J011 for passaging 293 cells)

6.0 Equipment

List equipment needed and if appropriate specify where it is in the Jenner labs

7.0 Method

ALL work should be carried out in the Biosafety Cabinet. The cabinet should swabbed with Microsol before use. Gloves and a lab coat should be worn at all times.

1. Warm media (DMEM with no additions) to 37°C in a water bath for 10 minutes.

2. Prepare work space in hood by swabbing with microsol.
Before placing bottles in the hood they should also be wiped with microsol taking particular care around the bottle necks.

3. Once warmed aliquot media into a series of 15 ml tubes or 1.5 ml Treff tubes according to the table shown below.

Tube Number	Media (ml)	Virus (µl)	Dilution
1	1	1	-3
2	1	1	-6
3	0.9	100	-7
4	0.9	100	-8
5	0.9	100	-9
6	0.9	100	-10
7	0.9	100	-11

4. Add 1 µl of pure virus to tube 1 containing 1 ml of media. Mix well by pipeting or inverting the tube.

5. Add 1 µl of diluted virus from tube 1 to tube 2 and mix well by pipeting or inverting the tube. . Continue to serially dilute the virus as detailed in the table above. For example the subsequent dilution (-7) will be created by adding 100 µl of diluted virus from tube 2 to 900 µl of media in tube 3.

6. Aspirate media from 6 well plates prepared the previous day.
Take care not to touch the cell monolayer with the pipette. This can be avoided by tilting the plate so that the media flows to the corner of the well.

7. Add 200µl of each dilution per well, gently tilt plate so that that the media covers the monolayer, Incubate at 37° C for 2 hours rocking the plates every 15 mins to ensure that the cells do not dry out. Usually a dilution range of -6 to -11 (in duplicate) is sufficient to determine viral titre.

8. Warm 2X complete media in a 37 °C water bath for 30 minutes.

9. Heat 2% agarose in microwave for 3 minutes or until it boils, allow to cool at 37°C water bath for approximately 20 minutes. Ensure that the agarose is not above 40 °C when added as this may cause damage to the cells.

10. Prepare work space in hood by swabbing with microsol. Before placing bottles in the hood they should also be wiped with microsol taking particular care around the bottle necks.

9. Mix media and agarose together in a 1:1 ratio in a pre-warmed tube, allow 3mls per well

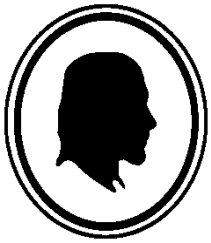
10. Aspirate media from 6 well plates and gently add 3 ml of complete media/agarose mix to each well using a pre-warmed serological pipette. Allow to solidify in laminar flow hood for approx 10 minutes before placing in 37°C incubator.

11. After 3 days overlay with 2 ml of 2 xDMEM/2% agarose
An additional overlay is usually required 9 days post infection if the agarose appears yellow.

12. 10 days post infection stain living cells using MTT (5mg per ml PBS, add 100µl per ml of overlay) and count the number of plaques.
Alternatively if the adenoviral construct contains GFP, plaques can be counted using the inverted microscope.

8.0 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
02	Volume of media cells infected in	Should be as small volume as possible	Nicky Green
02	Timing of agarose overlay, after 2 hours NOT overnight	Standard protocol	Nicky Green
02	Use of pre-warmed tubes and pipettes	To prevent agarose from setting	Nicky Green



J013: CEF culture

1.0 Version Number: 05

2.0 Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\Imsnw3 jenner server\jenner\hill group\Safety\COSHHassessments\Manufacturers material safety data sheets
Note: **Trypan blue solution** is a possible **carcinogen** and should be handled using the appropriate PPE
C030 Culture of primary cells and cell lines, including freezing and reviving
J114 Counting cells

3.0 Definitions

CEF	Chicken embryo fibroblasts
FCS	foetal calf serum
DMEM	Dulbecco's modified Eagle's media
PPE	Personal protective equipment

4.0 Objective

To receive CEF and culture cells in preparation for poxvirus infection.

5.0 Reagents

Primary CEF	Institute for Animal Health, Compton,	
DMEM	Sigma	D6546
100x Pen/Strep solution (10,000 units penicillin-G and 10 mg strep per ml)	Sigma	P0781
200 mM Glutamine	Sigma	G7513
FCS	Sigma	F2442
TrypLE Express	Gibco	I2605
Trypan blue	Sigma	T8154
PBS	Sigma	D8537

Complete 2% **(10%)** FCS DMEM:
500 ml DMEM
10 **(50 ml)** ml FCS (2% **(10%)** final conc)
5 ml Pen/strep (100 U Penicillin, 0.1 mg strep ml^{-1} final conc)
10 ml L-glutamine(4 mM final conc)

6.0 Equipment

Class II BioSafety Cabinet	Scanlaf	Mars

CO_2 incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope	Leica	DMIL
Disposable haemocytometer		
Culture flasks (T150 or T75 or T25)	Corning	
TC plates (6 or 24 or 96 wells)	Nunc	

7.0 Method

Background information: confluent cell numbers and sub-culturing seeding densities.

T150

Confluent:	~2 x 10^7 CEF / flask		
Seed	1 x 10^7 CEF/flask	→	confluent after 3 days

6-well plate

Confluent	~1 x 10^6 CEF/well		
Seed	1 x 10^6 CEF/well	→	confluent overnight
Seed	5 x 10^5 CEF/well	→	confluent after 2 days

Culturing primary CEF in T150 Culture flasks

1 Receive vial of CEF from Compton (Wednesday).
2 Assign a batch number if relevant (CEF Dddmm)*
3 Perform cell count as described below and in J114 – note viable and non-viable cell counts in record book.
4 Add 1 x 10^7 viable cells to T150 flasks in a total of 20 ml of 10% FCS DMEM (Cell concentration is shown on the vial).
5 Seed any additional culture vessels required. Incubate at 37°C with 5% CO_2 until cells are 90% confluent (~ 2 days).

*D"deliviered" day day month month

Subculturing CEF in T150 flasks.

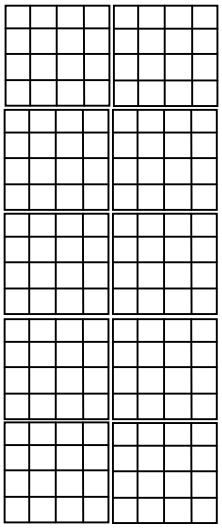
1. When cells are 90% confluent (Friday), aspirate the culture media from the flasks, rinse with 5 ml PBS and then add 4-5 ml TrypLE Express (warmed to Room Temperature).
2. Incubate at 37°C until 90% of the cells are detached (3 – 5 min).
3. Add 11 ml of 10% FCS DMEM (Total 15ml/flask).
4. Count the cells and seed flasks as required.
5. Incubate the flasks at 37°C with 5% CO_2 until they are 90% confluent (70-75 hours, weekend).

Counting cells.

1. When counting **P0 CEF** received from IAH Compton, dilute cells **1:10** in trypan blue stain.
2. For CEF plated in flasks, detach cells from flask using TrypLE as above, resuspend in 15 ml 10% FCS DMEM.

3. Add one drop of cell suspension into a 96-well, round bottomed plate (stored in tissue culture lab, next to microscope)
4. Add 20 µl **(90 µl)** Trypan blue solution to a separate well of the 96-well plate.
5. Transfer 20 µl **(10 µl)** cell suspension to the trypan blue stain, mix thoroughly and transfer to a chamber of a haemocytometer.
6. Count the cells in 16 squares (see figure below).
7. Number of cells/ml=$\frac{\text{Total number of cells counted x } 10^4}{}$
8. Dilution factor

9. If this dilution results in too numerous cells to count, dilute the cells further and repeat method.



Fastread counting chamber
Ten 4x4 counting squares
The volume of each 4x4 square is 10^{-4} ml

1.0 Revision History

Version Number	What Changed	Who Changed it	Date Changed
01	First written	Turner	
02	To accommodate counting of P0 CEF from IAH Compton	Ali Turner	
03	Counting chamber image altered and batch number assigning details	Ali Turner	
5	Reference to C030 added	Ali Turner	17.05.10

J014: Infection and transfection of CEF with MVA and shuttle vector

1.0 Version Number: 07 (Replaced by J176)

2.0 Title: Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:

\\Imsnw3 jenner server\jenner\hill group\Safety\COSHHassessments\Manufacturers material safety data sheets

Note: **Trypan blue solution** is a possible **carcinogen** and should be handled using the appropriate PPE

R003 Poxvirus Risk Assessment
\\Imsnw3 jenner server\jenner\hill group\Safety\GMO RA\R003 poxvirus.doc

J017 Linearisation of plasmids
J015 Cell sorting following infection-transfection and first screen
J013 CEF culture

3.0 Definitions

CEF Chicken embryo fibroblasts
FCS foetal calf serum
DMEM Dulbecco's modified Eagle's media
MVA Modified vaccinia Ankara

4.0 Objective

To infect, and subsequently transfect, CEF with parental MVA and shuttle vector respectively. Recombination occurs at a frequency of approximately 0.1%. After the recombination has been performed and incubated for 48 hr, the cells are examined for the relevant fluorescence and then harvested by scraping. T150 flacks containing CEF are then infected with 10 μl of the cell lysate and MoFlo sorted into 96-well plates after approximately 72 hr culture. This length of time allows the recombinant virus to proliferate and infect sufficient cells for sorting.
Ensure cells transfected with different shuttle vectors are handled separately in the biological safety cabinet, wiping the surface down and changing gloves between different vectors.

5.0 Reagents

6-well plates containing ~60-80% confluent **PRIMARY** CEF (i.e. non-passaged). These are seeded at 3 x 10^6 cells/well in 1 ml D10 media. Due to the primary nature of the cells, they adhere to a greater or lesser extent and this seeding density, in our hands, results in sufficient levels of infection and transfection and subsequent recombination.

DMEM	Sigma	D6546
100x Pen/Strep solution (10,000 units penicillin-G and 10 mg strep per ml)	Sigma	P0781
200 mM Glutamine	Sigma	G7513
FCS	Sigma	F2442
TrypLE Express	Gibco	I2605

Complete 2% FCS DMEM:
500 ml DMEM
10 ml FCS (2% final conc)
5 ml Pen/strep (100 U Penicillin, 0.1 mg strep ml^{-1} final conc)
10 ml L-glutamine(4 mM final conc)

Effectene Transfection Reagent Kit	Qiagen	301425

MVA.RFP
4 μg of linearised shuttle vector DNA

6.0 Equipment

Class II BioSafety Cabinet	Scanlaf Mars	
CO_2 incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope	Leica	DMIL

6-well tissue culture plates
Disposable haemocytometer
15 ml centrifuge tubes

7.0 Method

Ideally, this protocol is performed on the day of delivery of the primary CEF. Cells are seeded as soon as they arrive and left for 2 – 4 hrs to adhere to the 6-well plate. Infection is then performed followed by transfection 90 min later. Effectene is non-toxic to CEF and can therefore be left in the media.
Alternatively, if the cells have not attached in a reasonable timeframe, infection and transfection can be performed early the next day.

Day 1:

Prepare separate 6 well plates with 3 x 10^6 primary CEF / well (2 wells in separate plates per recombination to be performed) in 10% FCS DMEM.

Leave for ~4 hours to allow cells to attach to the culture surface. If cells arrive late they can be left overnight and recombined early the next day.

Check confluencey levels and general cell health.

Transfer plates to incubator of the Virus lab.

Suggested plate layout:

Experimental wells:

CEF MVA.RFP Shuttle vector (1)		
CEF MVA.RFP Shuttle vector (1)		

Dilute MVA.RFP virus to 1 x 10^6 pfu/ml in a total volume of 1 ml D2 media.

Aspirate the media from those wells requiring MVA.RFP

Add 0.5 ml of diluted virus to each well.

Replace plates in the incubator.

Incubate plates at 37°C with 5% CO_2 for 90 minutes.

Approx. 15 mins prior to the end of the incubation, prepare transfection reagents:

Add 4 μg DNA to DMEM (no additives) to a final volume of 350 μl in 1.5 ml Eppendorf tubes.

Add 40 μl of Superfect reagent to the above mix, 80 minutes after infecting the cells.

Vortex all the tubes well.

Incubate at RT for 10 minutes.

Aspirate media in experimental wells and replace with of 1.5 ml 2% FCS DMEM.
Add 190 μl of the transfection mix dropwise to the each of the experimental wells
Incubate (37°C, 5% CO_2).

Replace the media in the experimental wells either after 3 hours (if protocol started am) or the next day (2ml of 2% FCS-DMEM /well).

Briefly check that the cells still look healthy under low power microscopy.

Follow the pre-sorting protocol (J015) on the day of sorting (two days after infection).

8.0 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
V01	First written	N/A	Ali Turner
V02	Method altered	Changed to accommodate only having one virus in the hood at one time.	Ali Turner
V03	Method altered	Changed to remove incorrect step of looking for recombinant virus on day of recombination	Ali Turner
V04	Method Altered	All media should be removed an hour and 30minutes after the initial virus infection (in both experimental and control wells) to allow comparison of the infection rate of CEF cells between experimental and control wells to be measured.	Ciara McCarthy
V06	Method altered	MoFlo controls removed as this is now performed after a further round of CEF infection.	Ali Turner



J176: Recombination of either MVA or FP9 with shuttle vector using Effectene Transfection reagent on Primary CEF

1.0 Version Number: 04

2.0 Other documents

J013 CEF culture
J017 Linearisation of plasmids
J015 Cell sorting following infection-transfection and first screen
MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\Imsnw3 jenner server\jenner\hill group\Safety\COSHHassessments\Manufacturers material safety data sheets
Note: **Trypan blue solution** is a possible **carcinogen** and should be handled using the appropriate PPE
R003 Poxvirus Risk Assessment
\\Imsnw3 jenner server\jenner\hill group\Safety\GMO RA\R003 poxvirus.doc
R004 GM RA appendix

3.0 Definitions

CEF Chick Embryonic Fibroblast
DMEM Dulbecco's Modified Eagles Medium
FCS Foetal Calf Serum

4.0 Objective

To recombine an FP9 or MVA shuttle vector with FP9-mCherry or MVA-RFP parental virus respectively. This protocol should result in the production of recombinant, GFP positive viruses for subsequent MoFlo sorting.

This protocol refers to the use of linearised plasmid DNA. However, if marker-less virus is being prepared using the transient/dominant selection system, the plasmid should NOT be linearised.

5.0 Equipment/materials

6-well plates containing ~100% confluent **PRIMARY** CEF (i.e. non-passaged).
These are seeded at 3 x 10^6 cells/well in 2 ml D10 media. Due to the primary nature of the cells, they adhere to a greater or lesser extent. However, this seeding density, in our hands, results in sufficient levels of infection and transfection and subsequent recombination.

DMEM	Sigma	D6546
100x Pen/Strep solution (10,000 units penicillin-G and 10 mg strep per ml)	Sigma	P0781
200 mM Glutamine	Sigma	G7513
FCS	Sigma	F2442
TrypLE Express	Gibco	I2605

Complete 2% FCS DMEM:
500 ml DMEM
10 ml FCS (2% final conc)
5 ml Pen/strep (100 U Penicillin, 0.1 mg strep ml^{-1} final conc)
10 ml L-glutamine (4 mM final conc)

Effectene Transfection Reagent Kit	Qiagen	301425
MVA.RFP/FP9-mcherry 1 μg of linearised shuttle vector DNA		

Class II BioSafety Cabinet	Scanlaf Mars	
CO_2 incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope	Leica	DMIL
6-well tissue culture plates		
Disposable haemocytometer		
1.5 ml microcentrifuge tube (heat sterilised)		
Aspirator	Integra BioSciences	Vacusafe
sterile 2 ml screw-capped tubes		
Cell lifter	Fisher	FB55160

6.0 Method

1.1 Upon arrival from IAH Compton (usually ~ 12:30 pm), seed P0 CEF at 3 x 10^6 in 1 ml in one well of a 6 well plate. Seed a single well in separate plates for each recombination to be performed.

1.2 Leave for ~4 hours to allow cells to attach to the culture surface. If cells arrive late they can be left overnight and the protocol performed early the next day.

1.3 Aspirate the media from wells.

1.4 Infect CEF with FP9-mCherry or MVA-RFP at an MOI of 5. Assuming cells are ~100% confluent, this should be approx. 1 x 10^6 cells/well. Therefore, infect with 5 x 10^6 total PFU, in 1 ml D2 media

1.5 Incubate at 37°C; 5% CO_2 for 90 minutes.

1.6 ~ 75 minutes into the above incubation period, begin preparing transfection complexes in a sterile 1.5 ml microcentrifuge tube: 1 μg linearised DNA is added per well. Give the DNA a brief vortex (1 sec) and then flick tube to collect contents towards the base of the tube. DNA used should be high quality plasmid DNA, purified using a Qiagen purification kit.

DNA	Volume of Enhancer	Volume of Buffer EC
1.5 µg	**3 µl**	**Make total volume up to 112.5 µl**

1.7 Incubate the reaction mix at room temperature for 5 min and pulse centrifuge for a few seconds to remove all droplets from the lid.

1.8 Add 37.5 µl of Effectene Transfection reagent to the DNA-Enhancer mixture and vortex for 10 sec, flick tube to collect contents towards the base of the tube.

1.9 Incubate at room temperature for 10 min to allow transfection complex formation.

1.10 During incubation, aspirate media from the well of CEF to be transfected and replace with 2 ml fresh DMEM + 2% FCS.

1.11 Make final volume of transfection mix up to 0.75 ml using DMEM + 2% FCS and triturate 10 times to ensure the complexes are thoroughly resuspended.

1.12 Add 0.5 ml transfection complex mix to each well and ensure equal distribution by swirling the plate in a figure-of-eight pattern.

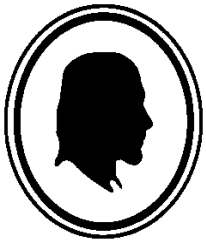
1.13 Incubate 48 hours at 37°C; 5% CO_2.

1.14 Harvest cells and media by scraping (using a cell lifter) and transfer to a sterile 2 ml screw-capped tube.

1.15 Store at -80°C until required.

7.0 Revision History

Version Number	Why it changed/ What Changed	Who Changed it	Date Changed
1	First written	Chris	30.06.10
2	Volumes adjusted to account for the use of only 1 well rather than 2.	Chris	28.07.10
3	To account for both MVA and Fp9 shuttle vectors	Chris	01.10.10
4	Method clarified	Ali	08.12.10



J015: Cell sorting following infection-transfection

1.0 Version Number: 05 (Replaced by J131)

2.0 Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\Imsnw3 jenner server\jenner\hill group\Safety\COSHHassessments\Manufacturers material safety data sheets
R003 Poxvirus Risk Assessment
\\Imsnw3 jenner server\jenner\hill group\Safety\GMO RA\R003 poxvirus.doc
R004 GMO RA appendix
J014 Infection and transfection of CEF with MVA and shuttle vector
J016 Infection of CEF after cell sorting

3.0 Definitions

CEF Chicken embryo fibroblasts
FCS Foetal calf serum
DMEM Dulbecco's modified Eagle's media
PBS Phosphate buffered saline
RFP Red fluorescent protein
GFP Green fluorescent protein

4.0 Objective

To prepare MVA-infected/shuttle vector-transfected CEF for MoFlo sorting.
Control CEF: uninfected control, CEF infected with MVA.RFP, CEF infected with MVA.GFP. Control virus-infected cells are trypsinised first to allow Drew to set up the MoFlo. When this is almost complete, the experimental virus-infected CEF are prepared, one at a time. It takes approximately ½ hour to sort the two independent plaques of each virus during which time the next one can be prepared.

It is important to keep the cells as healthy as possible for sorting handle as gently as possible. In addition, cells should not be prepared too far in advance as they may settle and clump in the 15 ml tubes.

5.0 Reagents

Infected/transfected CEF
Prepared in-house

FCS	Sigma	F2442
TrypLE Express	Gibco	I2605
PBS (without Ca^{2+} and Mg^{2+})	SIGMA	D8537

Complete 2% FCS DMEM:

500 ml DMEM
10 ml FCS (2% final conc)
5 ml Pen/strep (100 U Penicillin, 0.1 mg strep ml^{-1} final conc)
10 ml L-glutamine (4 mM final conc)

CEF for addition to plates after MoFlo (5×10^6 cells required in 20 ml D2 per plate).
Sterile water

6.0 Equipment

Class II BioSafety Cabinet	Scanlaf	Mars
Benchtop Centrifuge	Beckman Coulter	Allegra X-12R
37°C CO_2 incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6

15 ml and 50 ml Falcon tubes
Qiagen box for sample transportation to the Flow cytometry room.
96-flat bottom well plate

7.0 Method

1.16 Add 50 µl sterile water to each well of an appropriate number of 96-well flat-bottomed plates (2 per virus – one for "A" and one for "B").
1.17 Per virus, prepare 50 ml fresh sterile 2% FCS in **PBS** in a Falcon tube (1 ml FCS+ 49 ml PBS) and **warm to 37°C**.
1.18 Pre-warm some PBS and TrypLE to 37°C.
1.19 Aspirate the media from one plate of the control CEF.
1.20 Wash cells once with ~5 ml of PBS.
1.21 Add 0.5 ml of TrypLE per well and incubate at 37°C until 80% of the cells are detached – monitor frequently under microscope.
1.22 Add 5 ml of 2% FCS in PBS to each well then take cells up and down gently and transfer to 15 ml Falcon tubes.
1.23 Rinse wells with an additional 5 ml of 2% FCS in PBS and transfer to the 15 ml Falcon tube.
1.24 Centrifuge at 900 rpm for 3 minutes at RT.
1.25 Aspirate the supernatant.
1.26 Resuspend the cells gently by tapping the 15 ml tube.
1.27 Add 10 ml 2% FCS/PBS.
1.28 Centrifuge at 900 rpm for 3 minutes at RT.
1.29 Aspirate the supernatant and then flick the tube gently in the residual liquid to get a single cell suspension. Add ~200 µl 2% FCS/PBS.
1.30 Place all the samples in a Virus carrying box. (Wipe it with Microsol before use).
1.31 Prepare all control CEF and take them to Drew to set up the MoFlo.
1.32 Repeat process to prepare experimental viruses.
1.33 96-well plates are returned to the virus room and CEF added: 50 000 per well in 200 µl. (5×10^6 cells in 20 ml per plate). Alternatively, the 96-well plates can be placed at -20°C and the cells added later.

1.34 Place plate in incubator (37°C, 5% CO_2) for 2-4 days until recombinant virus can be observed.

1.35 The entire well is then harvested, frozen/thawed and titrated as described in J018.

8.0 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
01	First written	N/A	A.Turner
02	Method altered	Altered to include only handling one recombinant virus in the hood at any one time	Ali Turner
03	Method altered	Altered for clarification of some points	Ali Turner
04	Extra wash steps and MoFlo sorting added	To try and improve resolution from parental MVA	Ali Turner
05	Few improvements to method	To try and improve resolution from parental MVA	Ali Turner



J131: 96-well plate MoFlo sort of cells infected with recombinant poxviruses

1.0 Version Number 6

2.0 Other documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\Imsnw3 jenner server\jenner\hill group\Safety\COSHHassessments\Manufacturers material safety data sheets
R003 Poxvirus
J013 CEF culture
J176 Recombination of Poxvirus with shuttle vector using Effectene Transfection reagent
J018 Purification of recombinant MVA by plaque picking in 6 well plates
J173 Preparation of template DNA using DNA Releasy
J175 Virus ID and Purity PCR using KAPA2G robust polymerase

3.0 Definitions

CEF Chicken embryo fibroblasts
FCS Foetal calf serum
MEM Minimum essential media
RFP Red fluorescent protein.
GFP Green fluorescent protein.
DMEM Dulbecco's modified Eagle's media

4.0 Objective

Purification of fluorescent, recombinant poxviruses can be accelerated by a MoFlo sort whereby infected cells are single-cell sorted into a 96-well plate based on their fluorescence. This method is superior to that of plaque picking as none of the cells surrounding the plaque are co-transferred to the well. The resultant single cells are lysed by sorting them into wells containing sterile water and then CEF added to the wells to permit virus propagation. Wells containing appropriate virus are identified by microscopy and the entire well is harvested, screened by PCR and cell lysates from the relevant well titrated following J018.
This method is performed on CEF from a T150 flask, infected with a small volume of cell lysate harvested from the recombination between Poxvirus and shuttle vector. It is important that the cell culture does not contain a large amount of virus, although the cells are sorted based on fluorescence, if there are virus particles attached to the outside of the cell membrane, they will be co-transferred to the wells where they can then replicate.

The number of wells in which virus replicates following MoFlo sorting is often quite low. We have not managed to improve this to date. However, we usually recover at least 6 wells that have been independently sorted and harvest the "dump well" in addition to these. The lysate from the dump well is considerably enriched for recombinant virus but usually not pure. However, it serves as a useful backup.

5.0 Reagents

CEF	Institute for Animal Health, Compton.	
DMEM	Sigma	D6546
100x Pen/Strep solution (10,000 units penicillin-G and 10 mg strep per ml)	Sigma	P0781
200 mM Glutamine	Sigma	G7513
FCS	Sigma	F2442
TrypLE Express	Gibco	I2605
2% LMP agarose in dH_20 (heat sterilized)	Sigma	A9414
Trypan blue stain	Sigma	T8154

Complete 2% **(10%)** FCS DMEM:

500 ml DMEM
10 **(50 ml)** ml FCS (2% **(10%)** final conc)
5 ml Pen/strep (100 U Penicillin, 0.1 mg strep ml^{-1} final conc)
10 ml L-glutamine(4 mM final conc)

6.0 Equipment

Class II BioSafety Cabinet	Scanlaf Mars	
CO_2 incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Disposable haemocytometer		
-80°C freezer		
Dry ice		
96 well tissue culture plates	Fisher	TKT-520-030T
Gilson pipettes and filter tips		

7.0 Method

Day 1:

Seed a 6 well plate with CEFs at $1x10^6$ per well for MVA **($3x10^6$ primary CEF for FP9)** in 10% FBS DMEM for MoFlo controls (2 wells RFP; 2 wells GFP; 2 wells uninfected for MVA **(2 wells mCherry; 2 wells GFP; 2 wells uninfected on primary CEFs for FP9).**
Prepare one T150 flask per virus (Seed at 1.5×10^7 CEFs for MVA **(3×10^7 primary CEFs for FP9)** in 10% FBS DMEM).
Incubate at 37°C with 5% CO_2 overnight.

Day 2:

Transfer the 6 well plate and T150 flasks to virus lab.
Place plate/flasks in incubator (37°C, 5% CO_2) until required.
Freeze/thaw the cell lysate(s) to be MoFlo sorted 3x and vortex for 15 sec.
Aliquot media into Falcon tubes:
3 ml D2 media into each of 3x15ml Falcon tubes (for controls)
15 ml D2 media into 50ml tubes for each test sample.

To prepare controls, add 30 µl of either MVA-RFP and MVA-GFP or **FP9-mCherry and FP9-GFP** cell lysate to respective 3 ml aliquots of D2 media and vortex thoroughly. Leave one of the 3 ml aliquots of D2 to add to uninfected controls. Aspirate media from all wells and add 1 ml of each control to 2 wells.

To prepare viruses, thoroughly clean cabinet, taking particular care to clean Gilson pipettes, and change gloves. Add 10 µl of thoroughly vortexed test sample cell lysate to 15 ml D2 media and vortex (10 sec).

Aspirate the D10 media from the T150 and replace with 15 ml of virus/D2 solution.

Incubate MVA-infected cells for 3 days at 37°C with 5% CO_2; incubate FP9-infected cells for 2 days at 37°C with 5% CO_2.

Day 4/5:

Observe the plate/flasks using the fluorescence microscope to check for GFP positive recombinant virus. Also use red fluorescence to check RFP/mCherry is expressing.

Prepare one 96-well plate per virus to be sorted by adding 50 µl sterile water to each well.

Prepare fresh sterile 2% FCS in sterile **PBS** in 50 ml Falcon tubes (1 ml FCS+ 49 ml PBS) – 1 tube for controls and **separate tubes for each test sample.**

Pre-warm some PBS and TrypLE to 37°C.

Aspirate the media from the control CEF.

Wash cells once with ~5 ml of PBS **(use ~10ml to wash cells in T150s).**

Add 0.5 ml of TrypLE per well **(5 ml per T150)** and incubate at 37°C until 80% of the cells are detached – monitor frequently under microscope.

Add 5 ml of 2% FCS in PBS to each well **(10 ml to a T150)** then take cells up and down gently and transfer to 15 ml **(50 ml)** Falcon tubes.

Rinse wells with an additional 5 ml of 2% FCS in PBS **(10 ml for T150)** and transfer to the 15 ml (50 ml) Falcon tube.

Centrifuge at 190 x g (900 rpm) for 3 minutes at RT.

Aspirate the supernatant.

Resuspend the cells gently by tapping the tube.

Add 10 ml **(30 ml for T150)** 2% FCS/PBS.

Centrifuge at 190 x g (900 rpm) for 3 minutes at RT.

Aspirate the supernatant and then flick the tube gently in the residual liquid to get a single cell suspension. Add ~200 µl 2% FCS/PBS.

Place all the samples in a Virus carrying box. (Wipe it with Microsol before use).

Prepare all control CEF and take them to Drew to set up the MoFlo.

Repeat process to prepare experimental viruses.

For MVA sorts, 96-well plates are returned to the virus room and CEF added: 50 000 per well in 200 µl. (5 x 10^6 cells in 20 ml per plate). Alternatively, the 96-well plates can be placed at -20°C and the cells added later. For FP9 sorts, the plates must be frozen and 1x10^5 **primary** CEFs added per well.

Place plate in incubator (37°C, 5% CO_2) for 2-4 days until recombinant virus can be observed.

The entire wells containing recombinant virus (as visualized by fluorescence) are then harvested. At least 6 wells should be harvested in the first instance + the dump well. Wells to be harvested are scraped with a P1000 tip, and 100 µl

placed into a 2 ml Starstedt tube and frozen/thawed 3x. The residual volume from the well should be transferred into an eppendorf, pelleted(13000g for 1min on microcentrifuge) and the supernatant removed. These small pellets are then DNA extracted as per J173 and PCR screened with reference to J175. If additional fluorescent plaques are present in the 96-well plates, they are marked and placed at -20°C in case further plaques are required to be screened.

8.0 Revision History

Version Number	What Changed	Who Changed it	Date Changed
01	First written	Ali Turner	
02	Infecting T150 flasks with virus instead of 6 well plates	Chris Schultz	
03	Testing of dump well virus by PCR screen	Ali Turner	
04	Made applicable to both MVA and Fp9 sorts	Chris Schultz	
05	Method clarified	Ali Turner	15th December 2010

J017: Linearisation of MVA-shuttle vectors with restriction enzyme for recombination

1.0 Version Number: 02

2.0 Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\Imsnw3 jenner server\jenner\hill group\Safety\COSHHassessments\Manufacturers material safety data sheets
J065 Agarose Gel Electrophoresis and Gel Imaging
J014 Infection and transfection of CEF with MVA and shuttle vector
J089 Quantification of DNA using nanodrop.

3.0 Definitions

PCR	polymerase chain reaction
BSA	Bovine serum albumin
dH_2O	Distilled water
RE	Restriction enzyme
RNAse	Ribonuclease
DNAse	Deoxyribonuclease
MVA	Modified vaccinia Ankara

4.0 Objective

To linearise a shuttle vector in preparation for recombination with MVA. Linearisation efficiency is confirmed by electrophoresis on an agarose gel.

5.0 Reagents

10x RE buffer (depending on the enzyme used)
100x BSA
3 µl of Restriction Enzyme

QIAquick PCR Purification Kit	Qiagen	
Sterile distilled water (RNAse & DNAse Free)	Sigma	W4502
5 µg of the shuttle vector	In-house reagent	

6.0 Equipment

Vortex
Microfuge
37°C water bath
1.5 ml microcentrifuge tubes

7.0 Method

Check the shuttle vector maps for the restriction enzyme to linearise it. RE site(s) should be outside the TKR-antigen-TKL stretch. (AatII, XmnI or HindIII can be used for most of the vectors, but Sph1 or other REs can also be used)

Prepare the following reaction mix:

5 µg shuttle vector
5 µl 10x buffer (depending on the enzyme used)
0.5 µl 100x BSA (depending on enzyme used)
3 µl Restriction Enzyme
→ 50 µl sterile distilled water

Vortex and centrifuge briefly to ensure contents are at the base of the tube.

Incubate at 37°C for one - two hours.

After digestion, proceed with either Qiagen PCR purification method or, If you want to ensure linearised DNA is separated from non-linearised DNA, use the Qiagen gel extraction procedure following manufacturer's instructions.

It is not essential that all DNA is linearised as this procedure is to assist the formation of the recombinant virus during recombination. A trace of non-linearised DNA is not a problem for this procedure.

Take out 5 µl from the digestion mix and add 1µl of 6X loading dye and run it on 1% agarose gel.

Quantify the DNA using the nanodrop.

Freeze the linearised DNA until required for infection-transfection.

8.0 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
01	First written	N/A	A.Turner
02	Method updated	To include using gel extraction method and DNA quantification	Ali Turner



J016: Infection of CEF with recombinant MVA after cell sorting.

1.0 Version Number: 02

2.0 Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\Imsnw3 jenner server\jenner\hill group\Safety\COSHHassessments\Manufacturers material safety data sheets
R003 Poxvirus Risk Assessment
\\Imsnw3 jenner server\jenner\hill group\Safety\GMO RA\R003 poxvirus.doc
J013 CEF culture
J014 Infection and transfection of CEF with MVA and shuttle vector
J015 Preparation of CEF infected with recombinant MVA for cell sorting
J018 Purification of recombinant MVA by plaque picking in 6 well plates

3.0 Definitions

CEF Chicken embryo fibroblasts
FCS Foetal calf serum
DMEM Dulbecco's modified Eagle's media
MVA Modified vaccinia Ankara

4.0 Objective

To infect CEF with a lysate of previously sorted CEF following infection and transfection with MVA and shuttle vector respectively.
Ensure cells infected with different viruses are handled separately in the biological safety cabinet, wiping the surface down and changing gloves between different viruses.

5.0 Reagents

90% confluent CEF		
DMEM	Sigma	D6546
100x Pen/Strep solution		
(10,000 units penicillin-G and 10 mg strep per ml)	Sigma	P0781
200 mM Glutamine	Sigma	G7513
FCS	Sigma	F2442
MoFlo-sorted viruses		
Either snap frozen on day of MoFlo-sort		
or snap-frozen and stored at -80°C	In-house reagent	
2% LMP agarose in dH_20 (heat sterilized)	Sigma	A9414

2xMEM without phenol red	Gibco	21935

Complete 2% **(10%)** FCS DMEM:

500 ml DMEM	
10 **(50 ml)** ml FCS	(2% **(10%)** final conc)
5 ml Pen/strep	(100 U Penicillin, 0.1 mg strep ml^{-1} final conc)
10 ml L-glutamine	(4 mM final conc)

6.0 Equipment

Class II BioSafety Cabinet	Scanlaf	Mars
CO_2 incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope	Leica	DMIL
6-well tissue culture plates		
Disposable haemocytometer		
Vortex		
Sterile 1.5 ml microcentrifuge tubes		

7.0 Method

Infection
Prepare one 6 well plate of CEF in 10% FCS DMEM per virus required:

Plate cells at 1 x 10^6 CEF in 2 ml per well if required the following day

Plate cells at 0.5 x 10^6 cells in 2 ml per well if required 48 hours late

Incubate (37°C, 5% CO_2) until 90% confluent.4.Transfer 6-well plates to virus lab.

Thaw the sorted tubes containing the virus-infected CEF, add 1 ml of 2% FCS

DMEM in each tube.

Vortex the tubes (this will bring out all the enclosed viral particles from the cells).

Label sufficient sterile 1.5 ml microcentrifuge tubes for the following dilutions,



		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Virus (μl)	neat	100	100	100	100	100
2% DMEM (μl)	-	900	900	900	900	900

Add the required volume of 2% FCS DMEM to each microcentrifuge tube.

Thaw the virus (es) to be titrated and add 100 μl of the stock to the first tube of the series. Continue to prepare the dilution series as shown in the table above.
N.B. vortex every preparation of the new dilution for 3 seconds and change pipette tips for every dilution.

Aspirate the media from the first plate.

Pipette the virus-containing medium from the tubes into the relevant wells of the 6 well plates.

Incubate for 1hour (37°C, 5% CO_2).

Proceed with either 9.2 or 9.3

Agarose Overlay

Heat the 2% Low melting point agarose (solid) in the microwave, to melt (2-3 minutes on high power) and keep it in water bath at 37°C for at least 15 minutes.
Place 4% FCS MEM in 37°C water bath for at least 15 minutes.

Remove the culture medium from the well containing **neat lysate** and transfer to a 2 ml tube – store at -80°C in case there is any problem with the protocol.

Aspirate the media from the remaining wells of the 6 well plates.

Pour 4% FCS MEM into an equal volume of 2% low melting point agarose in 50 ml Falcon tube (note: add FCS MEM to the agarose to prevent agarose setting in the 50 ml Falcon tube as it is poured).

Pipette 2 ml of this mixture in the wells of six well plates, as quick as possible to prevent re-solidification of the agarose.

Incubate (37°C, 5% CO_2) for 2-4 days.

CMC overlay:

Mix sufficient D2 media and CMC in a ratio 2/3 D2:1/3 CMC for 2 ml per well required.

Aspirate media containing virus.

Replace with 2 ml D2/CMC overlay per well.

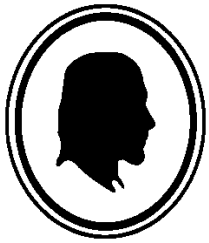
Incubate for 2-4 days

Checking for recombinant virus production

After two days check the 6-well plates under fluorescence microscope and mark the required virus plaques. Continue as per J018.

8.0 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
01	First written	N/A	Turner
02	Various points in method	Updated for CMC overlay	Ali Turner



J018: Purification of recombinant Poxvirus by plaque picking in 6 well plates

1.0 Version Number: 04

2.0 Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\Imsnw3 jenner server\jenner\hill group\Safety\COSHHassessments\Manufacturers material safetly data sheets
Note: **Trypan blue solution** is a possible **carcinogen** and should be handled using the appropriate PPE
R003 Poxvirus Risk Assessment
\\Imsnw3_jenner_server\jenner\hill_group\Safety\GMO RA\R003 poxvirus.doc
J013 CEF culture
J019 Bulking up poxviruses
J020 Jenner poxvirus PCR Document

3.0 Definitions

CEF	Chicken embryo fibroblasts
FCS	Foetal calf serum
MEM	Minimum essential media
RFP	Red fluorescent protein.
GFP	Green fluorescent protein.
DMEM	Dulbecco's modified Eagle's media

4.0 Objective

To purify recombinant poxvirus from parental virus by several rounds of plaque picking followed by infection of CEF with a serial dilution of plaque lysate. When no parental poxvirus (MVA-RFP or FP9-mcherry positive) can be observed by microscopy, ID and purity PCR are performed. After two rounds of picking have been confirmed as free of parental virus by PCR, a bulk stock of the recombinant virus is prepared.
Ensure cells infected with different viruses are handled separately in the biological safety cabinet, wiping the surface down and changing gloves between different vectors.

5.0 Reagents

CEF	Institute for Animal Health, Compton.	
DMEM	Sigma	D6546
2xMEM without phenol red	Gibco	21935
100x Pen/Strep solution		

(10,000 units penicillin-G and 10 mg strep per ml)	Sigma	P0781
200 mM Glutamine	Sigma	G7513
FCS	Sigma	F2442
TrypLE Express	Gibco	I2605
2% LMP agarose in dH_20 (heat sterilized)	Sigma	A9414
Trypan blue stain	Sigma	T8154
CMC high viscosity	BDH#	279294T

Complete 2% **(10%)** FCS DMEM:

500 ml DMEM	
10 **(50 ml)** ml FCS	(2% **(10%)** final conc)
5 ml Pen/strep	(100 U Penicillin, 0.1 mg strep ml^{-1} final conc)
10 ml L-glutamine	(4 mM final conc)

do not substitute for alternative supplier. This product has been found to be lower in endotoxins levels than those from other suppliers.

6.0 Equipment

Class II BioSafety Cabinet	Scanlaf Mars	
CO_2 incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope with objective marker attachment	Leica	DMIL
Disposable haemocytometer		
-80°C freezer		
Dry ice		
6 well tissue culture plates	Fisher	TKT-520-030T
Gilson pipettes and filter tips		

7.0 Method

Plaque picking.

Mark the desired number of recombinant poxvirus plaques using a fluorescent microscope and object marker attachment (at least two per virus).

Replace the plates in the incubator (37°C, 5% CO_2).

Pipette 100 µl of 10 mM Tris [pH9] in a 2 ml screw-capped tube for each plaque.

Clean round the microscope and spray with 70% ethanol.

Place the 6-well plate under the microscope such that the required plaque is visible.

Take a P10 pipette, set to 10 µl, and sterile tip in the safety cabinet and depress such that it is ready to aspirate a sample.

Using the microscope, carefully remove the lid of the 6-well plate and circle the plaque with the pipette tip in order to disrupt the cell monolayer around the plaque.

Carefully scrape the cells in the plaque to release them from the plastic.

Aspirate the cells into the tip.

Return to the safety cabinet and add the cells to the prepared tubes.

Place the tubes on dry ice to freeze; thaw in 37°C water bath and vortex very hard. Repeat freeze, thaw and vortex twice more for each tube.

(Virus may be frozen at -80°C for storage).

Infection of CEF.

Prepare one 6 well plate of CEF per virus (use only primary CEF when working with FP9).

Incubate at 37°C with 5% CO_2 until ~90% confluent.

Transfer the 6 well plates to virus lab.

Prepare the dilution series below in 1.5 ml sterile microcentrifuge tubes:

	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Virus (μl)	10	100	100	100	100	100
2% DMEM (μl)	990	900	900	900	900	900

Aspirate the media from the 6-well plate.

Transfer 900 μl of virus suspension from the microcentrifuge to relevant wells of the 6-well plate.

Incubate for 1 hour at 37°C with 5% CO_2.

Mix sufficient D2 media and CMC in a ratio 2/3 D2:1/3 CMC for 2 ml per well required.

Aspirate media containing virus.

Replace with 2 ml D2/CMC overlay per well.

Incubate for 2-4 days.

Pick plaques as required.

Recombinant virus purity

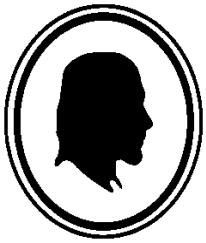
Once the recombinant plaques appear free from contamination by parental poxvirus (MVA-RFP or FP9-mcherry positive) by microscopy , harvest the cells and media from the well containing the most concentrated virus, take a 100 µl aliquot, pellet and use material for ID and purity PCR.

Two rounds of plaque picking should be confirmed free of parental virus to ensure purity.

Use 50 µl of the lysate harvested from the relevant plaque to prepare a 1 x T150 premaster poxvirus culture.

8.0 Revision History

Version Number	What Changed	Who Changed it	Date changed
01	First written	A.Turner	
02	Altered to contain CMC method variation	A.Turner	
03	Method altered	Ali Turner	
04	Agarose method removed	Ali Turner	15th Dec 2010



J019: Bulking up and purifying poxviruses

9.0 Version Number: 06

10.0 Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\Imsnw3_jenner_server\jenner\hill_group\Safety\COSHHassessments\Manufacturers material safety data sheets
Note: **Trypan blue solution** is a possible **carcinogen** and should be handled using the appropriate PPE

Risk assessments covering the use of poxviruses within the Jenner Institute are listed below and can be found in the safety folder on the s: drive or hard copies are available in the office area. These must be read and understood prior to commencing work using these viruses. In addition, the appendix to the GMO RA (R004) documents all of the antigens might be encoded by the poxviruses. These should be read and understood as relevant to the work being undertaken.

GMO RA R003 poxvirus
GMO RA R004 GM RA app
C052 use of sonicator

J018	plaque picking
J208	preparation of 36% sucrose
J209	preparation of 10mM Tris for MVA production

11.0 Definitions

CEF	Chicken embryo fibroblasts
CPE	Cytopathic effects
FCS	Foetal calf serum
MVA	Modified vaccinia Ankara
FP9	a plaque-purified, high passage attenuated, European strain of Fowlpox virus
DMEM	Dulbecco's modified Eagle's media
GMO	Genetically modified organism
RA	Risk assessment

12.0 Objective

Large-scale preparation of MVA/FP9 requires the infection of CEF, the extraction of virus from the cell lysate and crude purification to remove cell debris from the sample. This protocol describes the preparation of a premaster stock of virus,

infection of CEF with premaster lysate to form a master seed virus prep and the purification through a sucrose cushion. Sucrose cushion purification separates the viral particles from the cell lysate. The potency, ID, purity and sterility of the virus are then assayed using standard methods.

Ensure cells infected with different viruses are handled separately in the biological safety cabinet, wiping the surface down and changing gloves between different vectors.

13.0 Reagents

CEF		
DMEM	Sigma	D6546
Pen/Strep	Sigma	
Glutamine	Sigma	
FCS	Sigma	F2442
Trypan blue stain	Sigma	T8154
TrypLE Express	Gibco	I2605
10 mM Tris [pH9]		
10 mM Tris pH9, sterile		
From 1M Tris pH 9 stock solution T2819-100ml	SIGMA	
36% w/v sucrose solution (in 10mM Tris pH9), (filter sterilised)		
It is important that this material is low in endotoxins		
	Fluka	84097

Premaster cell/media lysate

Cells and media from a T150 flask infected with material confirmed free from parental poxvirus by PCR. If this is the first time the virus has been prepared, the premaster will have been prepared from the lysate of a 6-well plate infected with a plaque pick that has been confirmed free of parental virus for two rounds of purification. If this is a subsequent bulk prep, the premaster is prepared by infection of a T150 CEF with cell lysate from the original premaster.

14.0 Equipment

Class II BioSafety Cabinet	Scanlaf Mars	
Benchtop Centrifuge	Beckman Coulter	Allegra X-12R
CO_2 incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope	Leica	DMIL
Industrial methylated spirit (IMS)	Fischer	M/4400/17
2 ml screw cap tubes.		
Tube, Thinwall, Ultra-Clear™, 14 ml, 14 x 95 mm		
These actually have a max capacity of 13 ml	Beckman Coulter	344060
Corning conical tubes, 250 ml PP Centrifuge tube, plug seal cap CFT-900-021Y		Fisher
Bottle, Polyallomer, 250 mL, 60 x 120 mm	Beckman Coulter	355627
Disposable haemocytometer		
Cup-horn Sonicator	Sonics and Materials	
Sterile transfer pipette (pastette)		

-80°C freezer	New Brunswick Scientific	U57085
25 cm cell scrapers		
Storage bottle disposable with plug seal cap sterile	Fisher Scientific	tkv-185-030f
50ml centrifuge tubes		
15 ml centrifuge tubes		
Dry ice		
Optima L-80 XP Ultracentrifuge	Beckman Coulter	
Type 19 fixed-angle centrifuge rotor	Beckman Coulter	

15.0 Method

Preparing and infecting CEF with virus in T150 flasks (Premaster).

Prepare one T150 flask per virus with 1 x 10^7 CEF for each set of 10 x T150 flasks required.

Incubate (37°C, 5% CO_2) until 90% confluent.

Transfer T150 flask to the virus lab

For each flask, pipette 100 µl of prepared virus (three times freeze, thaw and vortexed) into 15 ml of 2% FCS DMEM in a 15 ml falcon tube and vortex thoroughly.

Aspirate the 10% DMEM and replace with the 2% media containing the virus.
Incubate (37°C, 5% CO_2) and monitor appearance of CPE (~100% infected cells should be evident after approximately 3 days).

Scrape off the virus-infected CEF from T150 flask into 50 ml falcon tubes. *(Cells and supernatant may be frozen for storage at this stage).*

Place the 50 ml falcon tubes with virus infected CEF on dry ice to freeze, thaw in 37°C water bath and vortex very hard.

Repeat freeze/thaw twice more. *(Cells and supernatant may be frozen for storage at this stage).*

Preparing and infecting CEF with virus in T150 flasks (Master seed virus).

Prepare 10 (or 20 flasks) of T150 flasks for each virus (the number of flasks is such that the supernatant fits into the centrifuge bottles at step 9.3.3).
Incubate (37°C, 5% CO_2) until ~90% confluent.

Transfer T150 flasks to virus lab.

Prepare D2 media containing virus lysate by pipetting 150 ml D2 media per 10xT150 flask prep into an empty 500 ml TC bottle and adding 14 ml of the premaster cell/virus lysate

The remaining 1 ml of lysate should be transferred to 1.5 ml O-ring tube and stored at -80^{0}C as 'premaster MVA stock'.

Aspirate the 10% FCS DMEM and replace with 15 ml of 2% FCS DMEM/virus.

(Incubation of the cells in 15 ml media per flask allows the cell harvest from each 10-flask preparation to fit into a single 250 ml conical tube for centrifugation and streamline production).

Incubate (37°C, 5% CO_2) until all cells are infected and beginning to show signs of CPE (4-5 days).

Harvesting virus.

Scrape off the virus-infected CEF with the cell scraper and transfer everything, using 25 ml pipette, into 250 ml conical bottomed tubes.

Centrifuge the 250 ml conical tubes at 670 x g for 10 minutes to pellet cell debris.

Transfer the supernatants to the autoclaved 250 ml flat-bottom Ultracentrifuge tubes (Maximum volume ~200 ml). Retain cell pellet.

Centrifuge the tubes (33 000 x g, 60 min, 4°C.) to pellet the virus particles in Beckman Coulter Optima L-80 XP Ultracentrifuge using the Type 19 fixed-angle centrifuge rotor

During the 60 min centrifugation, process the cell debris from step 9.3.2.

Add 3 ml of 10 mM Tris [pH9] to the pelleted cell debris, resuspend thoroughly and transfer to 15 ml Falcon tube.

Freeze the pellets on dry ice, thaw in 37°C water bath and vortex.

Repeat freeze/thaw/vortex twice more and place tubes on ice until the 60 min centrifugation of the supernatants has been completed.

Following the 60 min centrifugation of the supernatants (step 9.3.4), aspirate the supernatant from the 250 ml bottles, leaving a virus-rich pellet.

Centrifuge the 15 ml Falcon tubes from step 9.3.8 at 670 x g for 5 minutes.

Carefully remove the supernatant using a pipette and transfer to the 250 ml bottle containing the virus-rich pellet, store this material on ice while continuing with the processing of the cell pellet.

Resuspend the cell pellet in the 15 ml tubes by adding 5 ml 10 mM Tris [pH9].

Sonicate for 1 minute, 57 Hz. *(Switch on the sonicator, connect the lower pipe of the tube holder to the cold tap water and drain the upper pipe just in the sink, keep the*

tube in the holder and push the start button. Leave the lab, until it stops. After finishing disconnect everything and put the sonicator off)

Centrifuge the 15 ml tube (1700 rpm, 5 min) and pool the supernatant with that obtained previously in the 250 ml bottle.

Discard the cell debris pellets.

Transfer virus suspension to 15 ml Falcon tubes and make up to approximately 10 ml with 10 mM Tris pH9.

This material can be stored at -20°C or -80°C.

Purification through 36% w/v sucrose cushion.

If previously frozen: thaw the impure virus in 37°C water bath and vortex.

Ensure no clumps are present in the virus suspension – centrifuge samples (950 x g, 3 min, 4°C)

For each 10 x T150 flask prep, use two ~7.5 ml sucrose cushions.

Prepare the ultracentrifuge buckets by placing in the biological safety cabinet, Wiping the bucket lids thoroughly with 10% Microsol solution. Remove the Microsol completely by spraying with 70% IMS and wiping with blue roll. Note: traces of Microsol in contact with the virus will inactivate the virus. Spray the internal surfaces of buckets of the SW40 rotor with 70% IMS, turn upside-down to drain and then place inside the virus lab biological safety cabinet to allow residual ethanol to evaporate.

Add 7.5 ml of 36% sucrose (in 10 mM Tris [pH9]) to a 13 ml thin-walled ultracentrifuge tube.

Layer the virus solution (max. volume of 5.5 ml for this size centrifuge tube) onto the top of the sucrose, slowly and carefully with a sterile transfer pipette without disturbing the sucrose layer.

If preparing two viruses, seal the first tube, wipe down the surfaces and change gloves before loading the second virus.

Place the ultracentrifuge tubes inside the buckets and balance using the balance scale.

Centrifuge at 30 000 x g, 4°C for 2 hours using the Optima L-80 XP Ultracentrifuge.

Remove and discard the supernatant carefully using the aspirator – remove the top of the upper layer first to ensure this does not contact the purified pellet at the base of the tube.

Re-suspend the pellet in 10 mM Tris [pH9] in a total volume of 800 µl if from ~10 T150 flasks or ~1800 µl if from ~20 flasks.

Transfer the virus to a 2 ml screw cap tube (2 tubes if from 26 flasks).

Wash each of the centrifuge tubes with 200 µl of 10 mM Tris [pH9] and transfer this to the screw cap tube.

Sonicate the tube (1 min, 57 Hz)

Vortex the tube for 10 sec.

Transfer 4 x 10 µl into two 2 ml screw-capped vials for virus titration and store at -20°C until required for titration.

Transfer 1 x 10 µl into a screw capped tube for DNA extraction and ID and purity PCR and store at -20°C.

Transfer 2 x 10 µl into two screw capped tubes for sterility testing.

Aliquot the remainder into 200 µl aliquots and note the number of aliquots for entry into the virus database

Freeze the remaining sample in freezer vial with new virus stock number at -80°C.

Immerse the centrifuge buckets and tubes in a container containing 10% Microsol for a maximum of 10 min., rinse thoroughly with tap water and then 15MΩ Elga water.

The disposable storage bottles with plug seal caps should be placed in autoclave bags (unassembled) and sent for autoclaving.

Centrifuge buckets are allowed to air-dry – do not put llids on the buckets, leave them loose on the holder to allow and residual water to evaporate.

Database maintenance
Virus storage (s:Reagent storage info/virus storage/virus stock database)
Account name: user
Password: virus
Add new record and fill in details.
MVA production chart: (s:Technical group meetings/vector core meetings/MVA production chart).

Add virus stock number to relevant entry

16.0 Revision History

Version Number	What changed and Why it Changed	Who Changed it	Date changed
01	N/A	Ali Turner	
02	Altered to specify one virus in the hood at any one time. Final resuspension volume changed	Ali Turner	

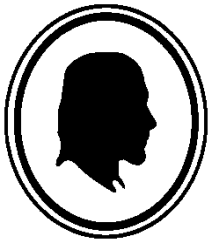
	Premaster stock changed from 1 x T75 to 1 x T150		
03		Ali Turner	
04	Addition of references to GMO RA Alterations for new ultracentrifuge	Ali Turner	
05	Centrifugation of sample prior to loading on sucrose cushion. Sonication of sample prior to aliquoting	Ali Turner	18th Oct 2010
06	Reference to risk assessment for sonicator Method clarification	Ali Turner	15th Dec 2010

Notes:

MVA IMV and EEV can also be purified using CsCl gradients (IMV and EEV sediment at 1.27 and 1.23 g/ml respectively). However, sucrose cushion is much less expensive.

We perform only a single round of purification through sucrose (a single 36% w/v sucrose centrifugation). This generates a pellet that contains both virus and cell debris.

A further purification step is possible using a sucrose gradient (40%, 36%, 32%, 28% and 24%) that is left for 2.5 hr to diffuse and then overlaid with virus from the first purification step. Centrifugation is performed (19 000 x g, 50 min, 4°C)



J082: Poxvirus titration using CEF in 6-well plates.

1.0 Version Number: 05

2.0 Other Documents

MSDS refer to MSDS for the relevant safety information on the individual reagents:
\\Imsnw3 jenner server\jenner\hill group\Safety\COSHHassessments\Manufacturers material safetly data sheets

R003	Poxvirus Risk Assessment
\\Imsnw3 jenner server\jenner\hill group\Safety\GMO RA\R003 poxvirus.doc	
J013	CEF culture
J019	Bulking up poxviruses
C009	MVA Immunostaining Titre
C010	MVA X-Gal Liquid Stain Titre

3.0 Definitions

CEF	Chicken embryonic fibroblasts
FCS	foetal calf serum
DMEM	Dulbecco's modified Eagle's media
MVA	Modified vaccinia Ankara
LMP	Low melting point
CMC	Carboxymethylcellulose
x-gal	5-bromo-4-chloro-3-indoyl-beta-D-galactoside
DAB	3,3'-Diaminobenzidine

4.0 Objective

To titrate a preparation of recombinant MVA using CEF in 6-well plates. Method is given for screening by microscopy for fluorescent marker- expressing recombinant poxvirus. Each virus preparation should be tested in quadruplicate: Two different CEF preps are used at different passage number (i.e. seed 6-well plates from fresh CEF and the previous week's delivery). Two separate serial dilutions are titrated on each cell batch. A single concentration of a reference virus should be titred at the same time. Therefore, plate one 6-well plate of each cell batch for the virus titration and on a separate plates seed two wells with each cell batch for the reference virus.

5.0 Reagents

1 x 6 well plate containing 90% confluent CEF per virus to be titred

DMEM	Sigma	D6546
100x Pen/Strep solution		

(10,000 units penicillin-G and 10 mg strep per ml)	Sigma	P0781
200 mM Glutamine	Sigma	G7513
FCS	Sigma	F2442
2 x MEM without phenol red	Gibco	21935
2% LMP agarose in dH_20 (heat sterilized)	Sigma	A9414
CMC high viscosity	BDH#	279294T

0.2% Triton-X 100 in PBS			
low gelling temperature agarose	(Type VII)	Sigma	A-4018

ImmPACT DAB (3,3'-Diaminobenzidine) + Liquid Chromogen Stain & Diluent	Vector Labs	SK-4105
Pre-Sept tablet	ASP	SPR25
Primary antibody: Rabbit anti-Vaccinia Virus	ISL	126-1063
Secondary antibody: Anti-Rabbit HRP Conjugated (from Donkey)	GE Healthcare	NA934V

Reagents stored in molecular biology lab

16% Paraformaldehyde	Alfa Aesar	43368
10x PBS	Sigma	P-44170.5M
$K_4Fe(CN)_6.3H_2O$	Sigma	P9387
0.5M $K_3Fe(CN)_6$	Sigma	P8131
1M $MgCl_2$	Sigma	M2393
18.2MΩ/cm ultrapure water	Elga	

do not substitute for alternative supplier. This product has been found to be lower in endotoxins levels than those from other suppliers.

Reagent Recipes:

Complete 2% (10%) FCS DMEM:

500 ml DMEM	
10 (**50 ml)** ml FCS	(2% **(10%)** final conc)
5 ml Pen/strep	(100 U Penicillin, 0.1 mg strep ml^{-1} final conc)
10 ml L-glutamine	(4 mM final conc)

4% FCS 2xMEM:

250 ml 2xMEM	
10 ml FCS	(4% final conc)
5 ml Pen/strep	(200 U Penicillin, 0.2 mg strep ml^{-1} final conc)
10 ml L-glutamine	(8 mM final conc)

4% Paraformaldehyde in PBS:
In the fume hood dilute 16% paraformaldehyde solution 1:4 in sterile PBS. Break the ampoule open with care and dispose of glass in a sharps bin.

0.2% Triton X-100 in PBS:

Disolve 100ul Triton X-100 in 50ml sterile PBS. Cut of end of pipette tip to dispense this viscous detergent.

X-Gal Liquid Staining Solution:
Stock solutions (prepare and store in mol biol lab) :
$K_3Fe(CN)_6$ 0.5M: dissolve 1.64g in 10 ml H_20
$K_4FE(CN)_6.3H_2O$ 0.5M: dissolve 2.112g in 10 ml H_20
$MgCl_2$ 1M: dissolve 2.033g $MgCl_2.6$ H_20 in 10 ml H_20
[These solutions are stable at room temp for at least 6 months if protected from light.]

Per plate (make fresh – each plate requires 6 ml solution) – 9.73 ml H_20, 0.1 ml 0.5 M $K_3Fe(CN)_6$, 0.1 ml 0.5M $K_4FE(CN)_6$. $3H_2O$, 20 µl 1M $MgCl_2$, 50µl 50mg/ml X-Gal in DMF.

[Note: X-Gal concentration may be increased 2-3 fold for better plaque visualisation if required]

6.0 Equipment

Class II BioSafety Cabinet	Scanlaf	Mars
CO_2 incubator	RS Biotech	Galaxy R
37°C water bath	Grant	SUB6
Microscope	Leica	DMIL
6-well tissue culture plates		
Benchtop Centrifuge	Beckman Coulter	Allegra X-12R
Disposable haemocytometer		
Multipette repeat pipettor	Eppendorf	Stream
10 ml pipette tips	Eppendorf	
1.5 ml microcentrifuge tubes (heat sterilised)		
10 µl filter tips		
200 µl filter tips		
1000 µl filter tips		
Vacuum aspirator		
Aspirator pipettes		
5, 10 and 25 ml serological pipettes		
Vortex		
1000mL waste bottle & lid (Duran) marked for Formaldehyde waste		

7.0 Method

Prepare a 6 well plate per virus with 1 x 10^6 CEF per well in 2 ml 10% FCS DMEM for use the next day or 0.5 x 10^6 CEF per well in 2 ml 10% FCS DMEM for use 2 days later.
Incubate (37°C, 5% CO_2) overnight.
Aspirate the 10% FCS DMEM using a 200 µl aspirating tip and replace with 1 ml of 2% FCS DMEM. Incubate (37°C, 5% CO_2) until required.
Label sufficient sterile, 1.5 ml microcentrifuge tubes for the following dilutions, in duplicate for each virus:



	Virus stock	10^{-2}	10^{-4}	10^{-6}	10^{-7}	10^{-8}
Virus (µl)	Stock	10	10	10	100	100
2% DMEM (µl)	-	990	990	990	900	900

Add the required volume of 2% FCS DMEM to each tube using the multipipettor. Thaw the virus (es) to be titred and add 10 µl of the stock to the first tube of the series. Continue to prepare the dilution series as shown in the table above.

N.B. vortex every preparation of the new dilution for 3 seconds and change pipette tips for every dilution.

Prior to addition of virus dilutions to wells, re-vortex those samples that will be added to the plates. Virus particles will settle of the tubes are not used immediately.

Add 100 µl of the appropriate viral dilutions to the relevant wells of the 6-well plate as shown below:

Series 1: 10^{-6}	Series 1: 10^{-7}	Series 1: 10^{-8}
Series 2:10^{-6}	Series 2:10^{-7}	Series 2: 10^{-8}

Ref at 5^{-5}	Ref at 10^{-6}	Ref at 5 x 10^{-6}
Ref at 5^{-5}	Ref at 10^{-6}	Ref at 5 x 10^{-6}

Once 100 µl of sample has been added, mix evenly in well by repeated movements left/right and forward/backwards. **DO NOT** swirl plates, this will lead to plaques concentrated around the sides of the wells.

Incubate at 37ºC (+/- 2ºC) + 5% CO_2 for 1 hour.

Proceed with either Agarose or CMC overlay:

Agarose overlay:

During the incubation, take the bottle of 2% LMP agarose (stored in the tissue culture lab) to melt (2-3 minutes on high power in the microwave in the mol. biol. lab). Transfer required amount of agarose to 50 ml Falcon tubes in the tissue culture lab biological safety cabinet. Pass the Falcon tubes through to the virus lab and place in the water bath at 37°C for at least 15 minutes.

Place the 4% FCS MEM in 37°C water bath in the virus lab.

Aspirate the culture medium from the wells (do not allow cells to dry out).

Pour 4% FCS MEM into an equal volume of 2% low melting point agarose in 50 ml Falcon tube *(note: add FCS MEM to the agarose to prevent agarose setting in the 50 ml Falcon tube as it is poured).*

Quickly pipette 2 ml per well of this mixture (to prevent the agarose solidifying).

Incubate for 2-4 days and count the plaques under fluorescent microscope.

CMC overlay:

Mix sufficient D2 media and CMC in a ratio 2/3 D2:1/3 CMC for 2 ml per well required.
Aspirate media containing virus using a 200 µl aspirating tip.
Replace with 2 ml D2/CMC overlay per well.
Incubate for 2-4 days and count the plaques as below:

GFP-expressing virus
Count the number of plaques in duplicate wells containing 50 - 100 plaques per well.

β-galactosidase-expressing virus:
Remove the fluid from the wells with the Vacusafe aspirator and 200 µl aspirating tips.

Fix the cells with 1 ml per well of paraformaldehyde (4% in PBS) for 5 min at room temperature.

Remove the fixative with a pipette to the Formaldehyde waste bottle.

Wash once with approximately 2 ml per well of PBS and remove using a pipette and place in formaldehyde waste bottle.

Add 1 ml X-Gal liquid staining solution per well. Leave for 1 hour.

Aspirate staining solution and wash cells with approx 2 ml per well PBS, then aspirate using Vacusafe pipetter/aspirator.

Plates may be inverted over a light background for counting blue plaques by eye (circle plaques with a marker pen on wells with between approximately 50 - 100 plaques for an accurate count)

If there is any uncertainty about whether a plaque is independent or a merge of two, this can be checked under a microscope (Note: plates may be photographed at this stage if required) Record results.

Dispose of plates as for normal virus contaminated waste.

Immunostaining

Remove the fluid from the wells with the Vacusafe aspirator

Fix the cells with approximately 1 ml per well of paraformaldehyde (4% in PBS) for 5 min at room temperature.

Remove the fixative with a pipette to the formaldehyde waste bottle.

Wash once with 2 ml per well of PBS. Again remove waste with a pipette to formaldehyde waste bottle.

Permeabilise cells with 0.2% Triton X-100 in PBS. Add 1ml per well for 5 min at room temperature. Aspirate waste

Wash cells twice with 2 ml per well PBS using Vacusafe aspirator.

Block with 1 ml per well PBS + 3% FCS and incubate for 1 hr at room temperature
Remove PBS + 3% FCS using Vacusafe aspirator.

Prepare 1ml per well of primary antibody diluted 1:1000 in PBS + 3% FCS

Add 1 ml of the primary antibody per well and incubate for 1 hr at room temperature.
Remove antibody solution and wash twice with approximately 2 ml of PBS per well.
Remove using Vacusafe aspirator

Prepare the secondary HRP-conjugated antibody by diluting 1:300 in PBS + 3% FCS.
Add 1 ml of secondary antibody solution per well. Incubate for 45 minutes.

Remove the antibody solution and wash twice with approximately 2 ml of PBS per well. Remove using Vacusafe aspirator

Make up the DAB Liquid Chromagen developing solution: (allow 1ml per well) add one drop of Chromogen liquid to every 1 ml of DAB Liquid buffer solution that is required and mix well. (Always make up this solution fresh)

Add 1 ml of developing solution per well. Plaques will stain a distinct brown colour. Leave for ~10 min.

Discard solution into a glass bottle (marked DAB waste) containing one Pre-Sept tablet (leave bottle over night and discard contents with plenty of water down sink the next day).

Wash plates gently with approximately 2 ml tap water per well.

Allow plates to dry for half hour in air. Ideally leave upside down on a paper towel.
Count the number of plaques in duplicate wells containing 50 - 100 plaques per well.

Determination of Titre (pfu/ml)

Count the number of plaques in duplicate wells containing 50 - 100 spots per well.
Use the following formula to determine the titre in pfu/ml:

"*average number of spots"* x "*serial dilution"* x "*dilution factor"* = "*x"* pfu/ml

e.g. $\frac{(36+40)}{2}$ = 38 x ($1x10^7$) x 10 = 3.8 x 10^9 pfu/ml

Database maintenance

Virus storage (s:Reagent storage info/virus storage/virus stock database)

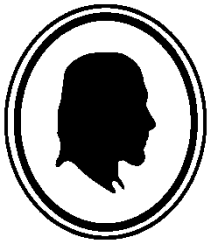
Account name: user
Password: virus
Add new record and fill titre details.

MVA production chart: (s:Technical group meetings/vector core meetings/MVA production chart).

Add virus titre to relevant entry

8.0 Revision History

Version Number	What Changed	Who Changed it	Date changed
01	First written	A.Turner	
02	Risk assessment referred to and CMC overlay option added	A.Turner	
03	Reference dilutions and dilution mixing clarified	S. Elias	
04	Changed to include FP9	Ali Turner	
05	FP9 removed to separate protocol. Counting using fluorescence microscope, X-gal staining and immunostaining merged to one protocol.	Ali Turner	23.08.10



J079: ID and Purity PCR for recombinant MVA

1.0 Version Number: 01

2.0 Other Documents

J065 Agarose Gel Electrophoresis and Gel Imaging
J014 Infection and transfection of CEF with MVA and shuttle vector
J002 DNA extraction from Adenovirus and MVA

3.0 Definitions

PCR	polymerase chain reaction
BSA	Bovine serum albumin
dH_2O	Distilled water
RE	Restriction enzyme
RNAse	Ribonuclease
DNAse	Deoxyribonuclease
MVA	Modified vaccinia Ankara

4.0 Objective

This procedure confirms, using specific primers, the insertion of various antigens into the thymidine kinase locus of MVA, and hence the identity of the MVA virus sample. Using parental virus-specific primers the presence of contaminating parental virus can be determined in the test samples.

5.0 Reagents

DNA extracted from MVA as per J002
Positive control plasmid DNA
AmpliTaq Gold with Geneamp (10x PCR buffer II & $MgCl_2$) Applied Biosystems 4311818
- 5U/µl AmpliTaq DNA polymerase
- 10x PCR buffer (100 mM Tris-HCl, pH8.3, 500 mM KCl)
- 25 mM $MgCl_2$

8 mM equal mix dNTPs
Sterile distilled water (RNAse & DNAse Free) Sigma W4502
10 µM DNA primers (see primers database) MWG
- e.g. for MVA.Red primers: Redvirus 3 and TKL
- for specific insert: specific primer + TKL

6.0 Equipment

Microfuge
PCR machine MJ Research DNA engine Tetrad

96-well PCR plate and adhesive lid Corning
Pipette tips with hydrophobic filters
Gilson pipettes

7.0 Method

Prepare two master mixes on a thoroughly clean bench in 1.5 ml microcentrifuge tubes, labeled 'Purity (containing MVA.Red3 and TKL primers)' and 'ID (containing specific primer + TKL)', as appropriate. Prepare sufficient for each virus test sample to be analysed plus four additional samples (MVA.wt, MVA.Red, reference material (plasmid), sterile water and one extra for pipetting error). The amount of each master mix required for a single sample is as follows:

0.5 μg of template DNA is recommended by Applied Biosystems for the Amplitaq enzyme.

Refer to the Amplitaq instruction manual for any further information required.

Prepare the following master mix for the required number of reactions + allow 1 rxn volume for pipetting error:

1 x PCR mix

4 μl	sterile water		
1 μl	10x AmpliTaq buffer	final conc	1x
0.6 μl	25 mM $MgCl_2$	final conc.	1.5 mM
1 μl	10 μM forward primer	final conc	1 μM
1 μl	10 μM reverse primer	final conc	1 μM
1 μl	8 mM equal dNTP mix	final conc	200 μM each
0.4 μl	5U/μl AmpliTaq polymerase	final conc	2 U
1 μl	DNA sample		

	Final Conc.	Vol. per 1 reaction (μl)	Vol. per 2 reactions (μl)	Vol. per 3 reactions (μl)	Vol. per 4 reactions (μl)	Vol. per 5 reactions (μl)	Vol. per 6 reactions (μl)
Sterile H_2O	-	4	8	12	16	20	24
Amplitaq Buffer (10x)	(1x)	1	2	3	4	5	6
25 mM $MgCl_2$	150 μM	0.6	1.2	1.8	2.4	3.0	3.6
Forward primer (10 μM)	1 μM	1	2	3	4	5	6
Reverse primer (10 μM)	1 μM	1	2	3	4	5	6
Equal mix dNTP's (8mM)	*200 μM*	*1*	*2*	*3*	*4*	*5*	*6*
AmpliTaq Polymerase (5U/μl)	0.2 Unit/μl	0.4	0.8	1.2	1.6	2.0	2.4

Total		9	18	27	36	45	54

Aliquot 9 µl of the master mix into wells of the 96-well plate. Add 1 µl of the sample to be tested, plasmid positive control or sterile water to 9 µl of Master Mix.

PCR Conditions

Primary Denature	95°C	1min	
Denature	94°C	10sec	x 38 Cycles
Annealing	52°C	30sec	
Extension	72°C	1 min	
Final Extension	72°C	3 min	
Finish & Hold	4°C	forever	

Fix the adhesive lid into place on the 96 well plate and place the plate in the PCR machine.
Start the relevant PCR run, using the heated lid option.
Completed reactions are analyzed by standard agarose gel electrophoresis.
Samples may be stored at -20°C until analysis by electrophoresis.

8.0 Revision History

Version Number	What Changed	Why it Changed	Who Changed it

J144: Maintenance and Culture of Non-Infected *Anopheles stephensi*

1.0 Version Number 1.1

2.0 Other documents

J150 – Working in the Jenner Insectary

3.0 Definitions

4.0 Objective

The culture and Maintenance of *An stephensi* throughout all of the mosquitoes life cycle

5.0 Materials

Equipment

Item	Supplier
Plastic larvae bowls	-
Crystallizing basins (100 or 300 mL)	Fisher
Whatman filter paper	-
1 ml syringes	Jenner Stock
26G needles	Jenner Stock
Mosquito cages	
Pump for collecting pupae	

Reagents

Item	Supplier
Fructose	Sigma (#F0127-500G)
P-aminobenzoic acid (PABA)	Sigma (#A9878-25G)
Fish Pellets (e.g.)	
Liquifry	
Minadex vitamin solution	Boots Chemist
Ketaset anesthetic (100 mg/ml ketamine)	Oxford University Veterinary Services
Rompun anesthetic (2% w/v Xylazine)	Oxford University Veterinary Services
PBS	Sigma

Solutions to be made up

Fructose/PABA solution
For 1 litre, add 80g of fructose and 0.5g of PABA to a 1L bottle. Make up to 1L with distilled water. Filter sterilize or autoclave. Autoclaving will caramelize the fructose and turn the solution a brown colour – this is not a problem. Add 2-3 drops of Minadex vitamin solution to the bottle and store at 4C.

Anesthetic
Mix 1 mL of 2% Rompun solution (Bayer) with 2 mL of Ketaset (100 mg/ml ketamine) and 3 mL of PBS. This will keep for two weeks at 4C. After two weeks discard and make up a fresh stock solution.

6.0 Method

This is an ongoing and routine procedure for the maintenance of *A. stephensi* mosquitoes and production of eggs for future generations.

Lifecycle of the Mosquito

The lifecycle of the mosquito has both an aquatic and non aquatic stage. Eggs are laid from adult mosquitoes that have had a blood meal in water where they hatch after two days. From here the mosquito larvae stage begins which is highly dependent upon the temperature and density in the bowls taking approximately 10-14 days to get to the non feeding pupae stage which is the stage the adult mosquito emerges from. The adult mosquito will then live for about 28 days on provided sugar solutions and blood when further generations of mosquitoes are needed.

The mosquito colony requires daily maintenance. The aim is to have 1-2 stock cages which house mosquitoes that are fed with non-infected blood in order to produce eggs, plus a number of experimental cages each week which house 3-7 day old mosquitoes which are removed and infected with *Plasmodium* parasites.

The daily breakdown of activities is:

Monday
-Remove egg bowls from stock cages. Pour hatched larvae into a fresh larvae bowl, provide some fish pellets and place in the incubators.
-Collect any adult mosquitoes that have emerged in the larvae bowls over the weekend using the electric pooter. Put these in the stock cages.
-Collect pupae from the larvae bowls using the electric vacuum pump. Put pupae in a pyrex bowl in either an experimental cage if desired, or in the stock cages.
-Ensure all larvae bowls have fresh water, fish pellets and are about the right density (approx. 3-400 larvae per bowl)

Tuesday
-Feed the stock cages blood (see section 8.1)
-Collect pupae and place in an experimental cage
-Feed fish pellets to larvae if needed (not necessary if food is still visible in the bowls)

Wednesday + Thursday
-Collect pupae and place in an experimental cage

-Feed fish pellets to larvae if needed (not necessary if food is still visible in the bowls)

Friday
- Collect pupae and place in an experimental cage
-Ensure larvae bowls have fresh water and plenty of pellets for the weekend.
-Put fresh fructose in all cages
-Place egg bowls in the stock cages – these are small pyrex bowls filled with water and a drop of liquifry. Place a filter paper cone in the bowl to allow eggs to be laid.

Generally, more mosquitoes will emerge in the experimental cages than are needed. Therefore, a good system is to replace one stock cage each week with the surplus experimental cage. This can be done on Monday after the egg bowls have been removed, and is done as follows:

1) Judge which stock cage has the least mosquitoes in it. Remove as many as possible using the pooter and place in the other stock cage.
2) Put the now-nearly-empty stock cage in the fridge for a couple of days to euthanize any remaining live insects. This can now be disassembled and cleaned.
3) Re-label the experimental cage as stock.
4) Assemble a fresh experimental cage for the coming week.

Blood Feeding of Stock Cages
Female TO mice are kept in the FGF. At least two mice will be needed per week to provide blood for the stock cages although more may be needed depending on the size of the mosquito colony.

The night before the blood feed, remove fructose from the cage(s) to be fed and replace with water.

Mice are collected by arrangement with Carol Williams in the FGF and then walked through the goods entrance to the insectary (see J150).

Anaesthetize mice with 50 µl of anesthetic (see section 7.3.2) given *i.m.* Ensure mice are non-responsive through pinch reflex of the foot.

Place mice on top of the cage. Ensure they are spread out as much as possible to maximize the surface area available for the mosquitoes to feed. Feeding is optimal in the dark. Turn out lights and place the 'mosquito feed in progress' sign on the door of the insectary. After 15-20 minutes remove mice and cull by cervical dislocation. Place mice in a plastic bag and put in freezer. Replace fructose in stock cage.

7.0 Revision History

Version Number	What Changed	Why it Changed	Who Changed it
1.0	**Created**		
1.1	**Revised and Clarified**		**Andrew Williams**



J146: Infecting Anopheles stephensi with *Plasmodium yoelii*

1.0 Version Number 1

2.0 Objective

Infection of female *Anopheles stephensi* mosquitoes with the rodent malaria parasite *Plasmodium yoelii.*

3.0 Reagents

Anaesthetic (Rompun:Ketaset:PBS 1:2:3)
Glucose (10%)
Giemsa (20%)
Heparin
Methanol
PBS
Phenylhydrazine (6mg/ml)
RPMI

4.0 Equipment

Scissors
1mL syringe
26G 1 ½ needle (Brown)
30G 1 ½ (Yellow)
Microscope Slides
Coverslips (18x18)
Pots
Storage Containers
Electric Pooter
Cooled Incubators
Phase contrast light microscope
Dissecting microscope

5.0 Method

Day 0 – Starting *P.yoelii* Infection
Take a vial of frozen *P.yoelii* cells on ice to the Experimental Psychology BMS unit on F level.

Allow *P.yoelii* to thaw out before injecting intra-peritoneal (*i.p.*) 150µl into a naïve TO mouse.

Mice to be used in the feed (1 mouse/mosquito pot) are to be given 200µl *i.p* of phenylhydrazine (6mg/ml) n.b using 5 mice is recommended for the mosquito feed.

Only use frozen P.yoelii to passage number 8 freeze down fresh P0's from vaccine challenge naives.

Day 3 - Checking Parasitemia and Passaging infected blood into PH treated mice.

The initial naïve mouse injected with the frozen stock of *P.yoelii* is checked for infection.

Obtain a drop of blood by Tail snipping the initial naïve mouse this is carried out by using a pair of scissors to snip the end of the tail.

A drop of blood is placed on to a microscope slide and a thin smear is produced.

Allow the smear to air dry before fixing the smear in 100% methanol for 1 minute this is then allowed to dry.

The fixed slide is then immersed in 20% Giemsa (diluted in water) for 10-20 minutes after which the slide is rinsed in tap water and air dried before examination under a light microscope.

If succesfully infected the parasitemia is counted and if the infection is found to be suitable between 1-5% the blood will be ready to be passaged into the PH treated mice.

The blood is collected from the mouse through cardiac puncture using a needle (26G) and syringe after anaesthetizing with a mixture of Rompun, Ketaset and PBS (1:2:3 ratios).

First inject the mouse with 150µl of anaesthetic given intra-muscular (*i.m*).

Once the mouse is non responsive, the blood is collected in a 1ml syringe with a 26-guage needle (brown) containing 50µl of heparin to prevent clotting.

After collecting the required amount of blood the mouse is culled by cervical dislocation.

130µl of collected blood will be injected into each of the naive PH treated mice by *i.p* using a 30-guage needle (yellow) instead of the 26-guage (brown) needle.

Day 5 - Collection of Mosquitoes from Imperial College.
Make up the pots in which the mosquitoes are to be stored. These are 16oz food containers that have a hole cut in the side for delivery of the mosquitoes. The lid is made of netting which is secured in place by a cut out 16oz lid.

Mosquitoes are collected from a cage containing male and female mosquitoes by placing a heat source alongside one the cages side.

The female mosquitoes will move towards the heat source and probe from here they can be collected using an electric pooter and placed into the made up pots (100 mosquitoes/pot).

The pots are then placed into a storage container to allow greater protection for travel.

In the storage containers there is damp blue tissue (deionised water) around the bottom to keep humidity levels up so that it's not detrimental to the mosquitoes survival.
They are then transported in an insulated bag back to the insectary at the Peter Medawar, Oxford and starved overnight in preparation of maximising the number of mosquitoes that feed the next day.

Day 6 - The mosquito Feed.

The feed must be carried out early in the morning between 6 and 8 o'clock to reduce the amount of people that you come in contact with when walking from the animal unit to the insectary.

Set up the mosquitoes in the central lab area of the insectary.

Take a transport box over to the BMS unit in Experimental Psychology on F level and check the parasitemia and exflagellation of each mouse to be used in the mosquito feed.

Parasitemia is checked as previously mentioned in the protocol through a thin smear with 15-25% being the acceptable range.

Exflagellation is checked by placing a drop of blood in a drop of RPMI and covering it by using a coverslip. The slide is left for ten minutes and checked under phase contrast for male gametocyte exflagellation (5-10 per field).

If the parasitimea and exflagellation is acceptable then the mouse is anaesthetized by giving 100µl *i.m* of Rompun, Ketaset and PBS (1:2:3 ratios).

The mice are placed in a filter box inside a transport box and walked from Experimental Psychology F level down to the insectary in the Peter Medwar Building via the linkway between the two buildings.

Once in the insectary make sure the sign is placed on the door along with the rodent barrier to inform people entering the insectary that a mosquito feed is taking place.
The anaesthetized mice are then placed onto the pots (1 mouse per pot) for 20-30 minutes before being removed and culled via cervical dislocation.

The mosquitoes pots still in the containers are then placed on their side in a cooled incubator at 24.5 ^{0}C to prevent mosquitoes from dying in the drying plasma at the bottom of the pot (temperature range of P.yoelii is 23.5-25 ^{0}C)

The mice are placed into bags and taken back to Experimental Psychology for disposal.

At the end of the day 6 the unfeds are removed by eye using the electric pooter and the pot is stood back upright.

The pot is fed with a covered cotton wool ball soaked with a 10% glucose solution placed on top of the pot. This will be renewed every 1 ½ days with fresh glucose solution.

The blue tissue at the bottom of the container will also be renewed with distilled water to keep humidity levels up in the pots.

Day 14 - Oocyst Count

Five mosquitoes are removed from each pot using the electric pooter and then placed on ice to knock-them down. The midguts are dissected out and checked for oocysts, if present the oocyst number is determined.

Midgut dissections are carried out in a drop of PBS on a microscope slide by placing a mosquito on its side and grasping the lower abdominal part with forceps or dissection needles and the lower part of the thorax/upper abdomen using a needle and gently pulling them apart to leave the midgut behind. The needle can then be used to separate away other internal parts of the mosquito such as the foregut and malpighian tubules to leave the midgut on its own. This can then be covered with a coverslip and looked at under phase contrast on a light microscope for oocysts.

Day 20 - Sporozoite check.

Ten mosquitoes are removed from each pot using the electric pooter and then placed on ice to knock-them down. The salivary glands (two sets of tri lobed organs) are then dissected out and checked for sporozoites, infectivity is assessed by number of sporozoites seen in individual mosquitoes and in how many of the ten are infected.

Salivary gland dissections are carried out by placing a mosquito on its side in a drop of PBS on a microscope slide and inserting a dissection needle through the upper thorax to keep it in place. Another needle is then placed on the neck of the mosquito and carefully pulled apart from the thorax so that the head is eventually separate. The salivary glands should come with the head and can be removed and covered with a coverslip after removing the body and head of the mosquito. If the salivary glands are not connected to the head after being separated they can be teased out of the thorax using the needle by gently pulling at the neck of the thorax.

Day 21 - Delivery of Mosquitoes for Challenge

Mosquitoes are delivered from the Peter Medawar building to the Old college road campus building in a insulated bag on the morning of the challenge between 7:30 and 8:30 for dissections on that day.