Eukaryotic Expression Facility at University of Bristol

(June 2016 edition)

European Commission | wellcome trust | University of BRISTOL

BERGER GROUP

Blue/white screening & transfection

Day 1

Use the competent cells DH10EMBacY. Transform them with the plasmid:

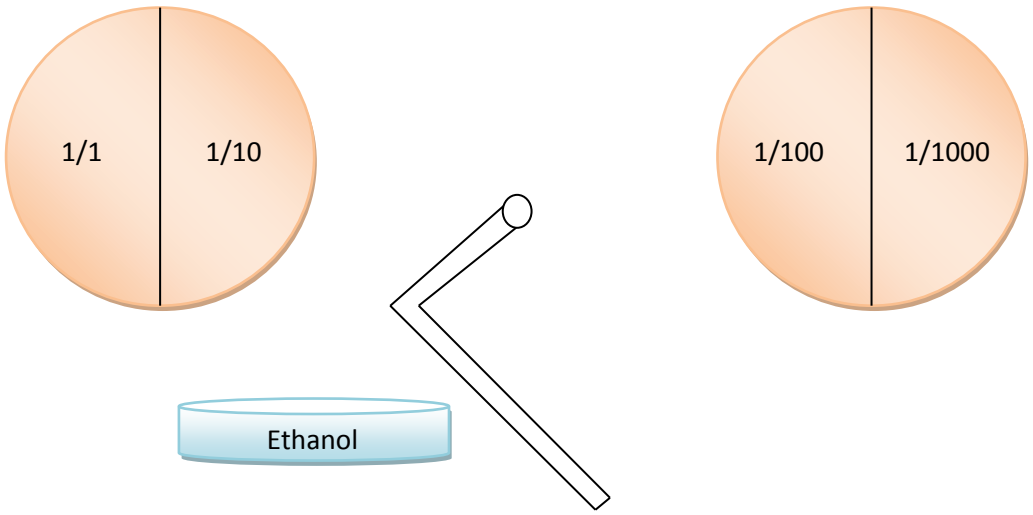
- Take one tube of DH10EMBacY out of -80°C and **put immediately on ice!**
- Add ~1 µg of plasmid (**sterile**!)
- Leave on ice 20 min
- Heat-shock 45 sec at 42°C
- Leave on ice 2 min
- Add 400 µL of LB (w/o antibiotic) (**sterile**!)
- Incubate O/N shaking at 37 °C.

Day 2

Streak out the transformed cells on Kanamycin/Tetracycline/Gentamycin/IPTG/BluOGal plates in dilution series as followed:

1/1 ⟶ 1/10 ⟶ 1/100⟶ 1/1000 (135 µL of medium and 15 µL of cells)

Put 135 µL of each dilution on plates as followed (keep the rest of the 1/1 undiluted cells at 4°C).



Incubate the plates O/N at 37°C.

Day 3

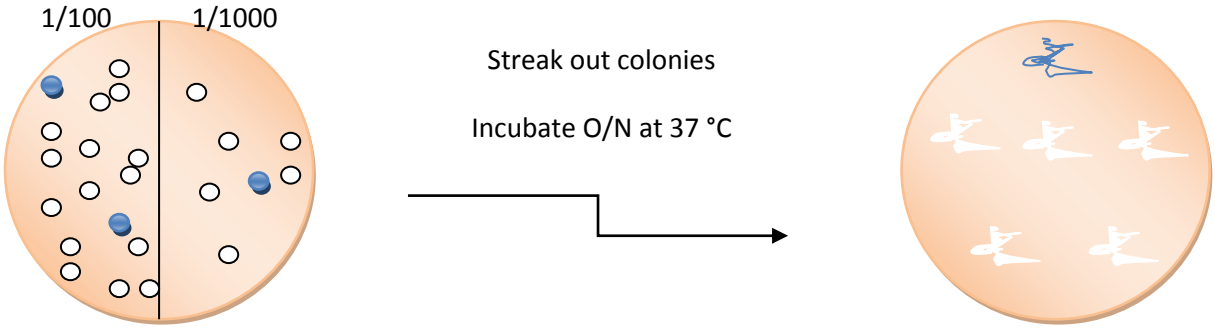
You should obtain white and blue colonies after >24 hrs.

Both types of colonies now have the plasmid and will be resistant to Gentamycin.

The blue colonies are still able to process the BluOGal (via the betaGalactosidase enzyme), thereby producing a blue compound.

The white colonies owe their color to the loss of the betaGalactosidase activity, following the transposition of the gene of interest into their baculoviral genome and the disruption of the lacZ gene (betaGalactosidase gene).

Pick 5 white colonies on Kan/Tet/Gent/BluOGal/IPTG to confirm the phenotype and 1 blue colony as a positive control from the plate.



Day 4

For each plate, choose 2 white clones and launch 2 mL of LB cultures for each with Kan/Tet/Gent shaking at 37°C O/N.

Day 5

ON THE BENCH

1) Centrifuge the 2mL cultures 10 min at 2900 rcf. Discard the supernatant (freeze at -20°C if prep is scheduled later).
2) Add 300 µL of Buffer P 1 (home made or Qiagen MiniPrep Kit) to the pellet, pipet up & down to resuspend and transfer to a 1.5 mL tube.
3) Add 300 µL of Buffer P2 (MiniPrep Kit). If Buffer P1 contains Lysis Blue, your sample will turn blue. **GENTLY** invert the tube until the blue color is homogenous. Do not incubate > 5 mins.
4) Add 300µL of Buffer N3 (MiniPrep Kit). A white precipitate will now appear. **GENTLY** invert the tube until the blue colour has completely disappeared.

   Centrifuge 10 mins at max speed.

5) Transfer the supernatant into a new tube in **one s**hot, using P1000.
Centrifuge 5 mins at max speed.

6) Transfer the supernatant into a new tube as previously.
Add 700 µL of 100% Isopropanol (ideally you want to use 0.7 volume isopropanol to have roughly 40% final concentration of isopropanol).
Invert **GENTLY** until isopropanol and the aqueous portion are homogeneously mixed.
Centrifuge 10 mins at max speed.

7) Take off most of the supernatant with p1000 and then the last amount with p200. You might not see any pellet (bacmid pellet should be clear). A white pellet might be due to residual white precipitate (eg. RNA).
Add CAREFULLY (drop by drop on the opposite side of the pellet, holding the tube horizontally) 200 µL of 70% Ethanol.
Centrifuge 5 mins at max speed.

8) Take off Ethanol with p200.
Add CAREFULLY (drop by drop on the opposite side of the pellet, holding the tube horizontally) 50 µL of 70% Ethanol. (Should not sit in ethanol longer than 30mins)

UNDER A STERILE HOOD (EEF)

1) Take care of your DNA

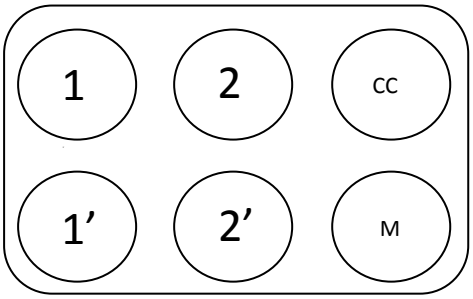
Remove all the Ethanol with p200.
Let pellet dry (open eppendorf) during 10 mins (DO NOT OVERDRY).
Meanwhile do step 2.
Add **20 µL of filter sterilized water**.
Resuspend pellet by tapping 10 times on the hood bench.
Add **200 µL of medium** in each tube.

2) Take care of your cells

Count your cells. You need to have between 0.5 and $1*10^6$ cells/well in a total volume of 3 mL.
Note 1: you will transfect with DNA from 2 different clones you have picked from your plate for each plasmid.
Note 2: you will set up duplicates of each clone in case one gets contaminated during the experiment. Therefore for each plasmid (construct) you will set up 2*2 = **4 wells**
You will also need one well for cell control and one for media control.

First, pipet the appropriate volume of medium (2mL/well, 3mL for the media control), then the cells, drop by drop (using "S" setting on pipetboy) and evenly (1mL/well).

Leave ~ 15 mins at 27°C (RT).



CC = Cell control (uninfected cells) ; M = Medium only

3) Take care of the transfection reagent

For X tube(s) of samples, prepare ONE tube with:

X00µL of medium

+ X0µL of XtremeGene Transfection Reagent

4) Mix DNA and transfection reagent

Add 100 µL of the XtremeGene mixture to EACH of your DNA containing tubes.

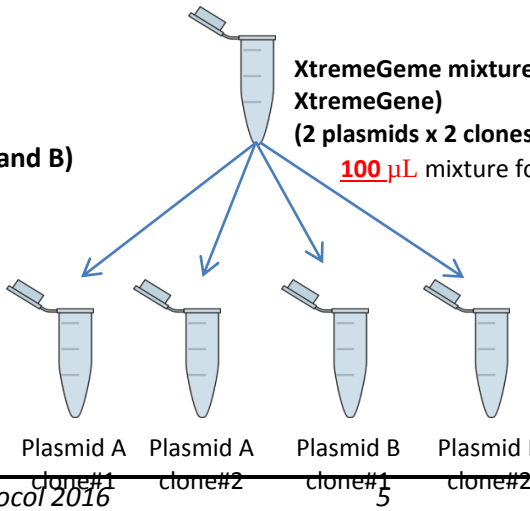
5) Add the transfection cocktail to your cells

From each DNA+XtremeGene cocktail, take 2x 150µL that you add to each of the two dedicated (duplicate) wells.

Use a p200 and be careful not to pass your hand over the uncovered wells; slowly rotate the plate each time. Pour the cocktail drop by drop.

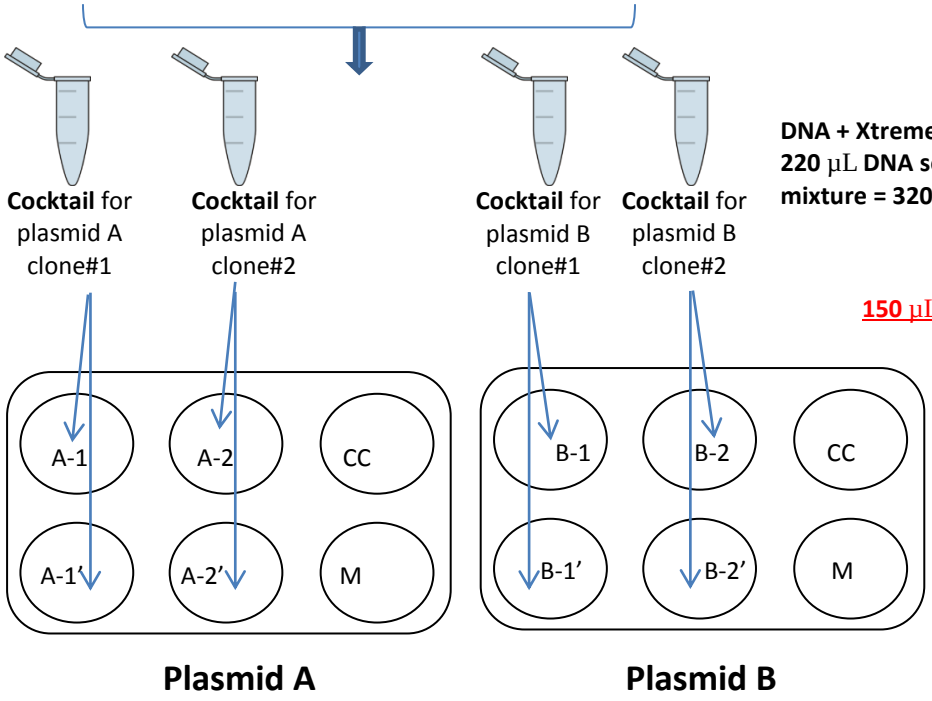
Example

(2 plasmids A and B)



XtremeGeme mixture (for 4 tubes : 400 µL medium + 40 µL XtremeGene)
(2 plasmids x 2 clones = 4 tubes with DNA)
100 µL mixture for each tube

DNA = (bacmid + plasmid of interest)
(20 µL DNA + 200uL medium = 220 µL in each tube)



DNA + XtremeGene cocktail now contains: 220 µL DNA solution + 100 µL XtremeGene mixture = 320 µL in each

150 µL cocktail for each well

Wait for 48-60 hours. Check every day to see if everything is clear (no contamination, cells still alive, etc). Infected cells will appear larger, irregularly shaped, and not confluent compared to control cells.

Then, remove supernatant from 6 wells plates. **This is the V_0.**

Tilt the plate so that you can put the pipet against a side and collect the entire medium. You can combine the Vo for each duplicate (eg. 1 and 1'). You will now have ~6 mL virus for each clone; put it in a 15mL Falcon tube, seal with parafilm, wrap in aluminum foil and store at 4°C.

Once you have harvested Vo, add 3 mL of fresh medium to the 6 well plate (gently, drop by drop and on the side in order not to disrupt the cell layer).

Wait for 48-60 hours and harvest the cells (process with the "**protein expression test on 6-wellplate**" protocol).

Note 3: you may already start the V1 amplification at this stage, if you have some pre-adapted flasks of 25 mL culture at a density of 0.5 to $0.8*10^6$ cells/mL. Add 3 mL of V0 per 25 mL of culture.

Protein Expression Test experiment on 6-well plate

Since the number of cells in the 6-well plate in not known, one has to be aware that this is a qualitative test.

96-120 hrs after the transfection / 48-60 hrs after the harvesting of V0, remove the supernatant

At this step 6-well plate can be stored at -20 °C.

Resuspend both duplicate wells corresponding to each clone with 500 µL of 1X PBS by pipetting up and down. Transfer in a fresh 1.5 mL tube.
Then, follow the "**Processing the sample for SDS-PAGE and YFP measurement**" protocol, starting from "Sonicate at medium setting" step.

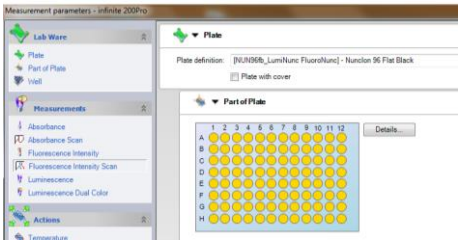
Error! Objects cannot be created from editing field codes.

Note well: if your analysis includes several Western blot runs, take > 50 µL *SNP and SN.*

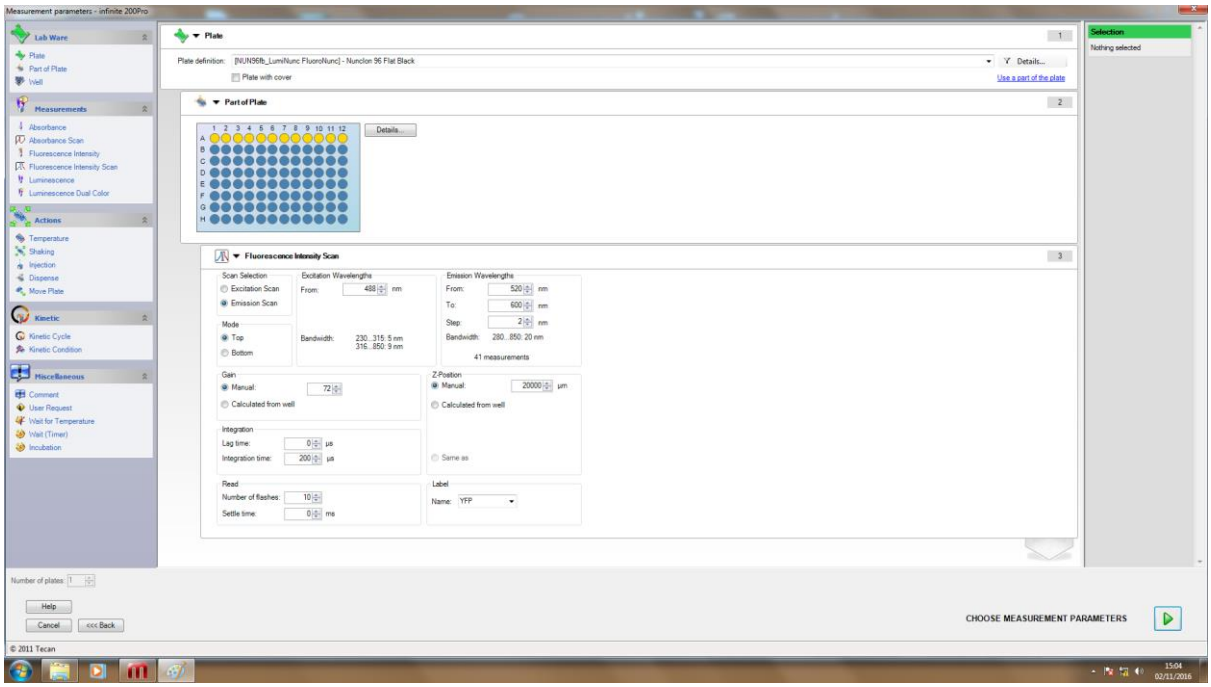
Freeze the SNP/SN aliquots at -20°C. Load 100 µL of each remaining SN sample onto a black 96 well BD Falcon micro-plate. Also include 100 µL of PBS and YFP standard in two of your wells.

- Turn on the machine with the main switch located at the back as well as the computer located below the taken robot (if you are not the first user it should be already on).
- Double click the following icon on the desktop. The compartment for plate will automatically open, when the program starts. Insert the plate in the correct orientation (A1 in upper Left).

- A window will pop up; select “start measurement” and click on the start icon (green play button).
- Another window will pop up; select “Obtain Raw Data” and click on the start icon (green play button).
- A new window will open. Select the **plate definition** “[NUN96fb_LumiNunc FluroNunc]-Nunclon 96 Flat Black” from dropdown window as shown in below image (make sure that the box labeled “plate with cover” is unchecked)
- Select the sample positions by dragging the mouse or with ctrl button, as shown below. (Selected position are shown in yellow color).



- Double click on “Fluorescence Intensity Scan” from Measurement panel on Left side. New sub-window will pop up and the window will look like as below



- Change the setting in “Fluorescence Intensity Scan” tab as shown above. In the ‘Label’ option, you can type the name as you wish.

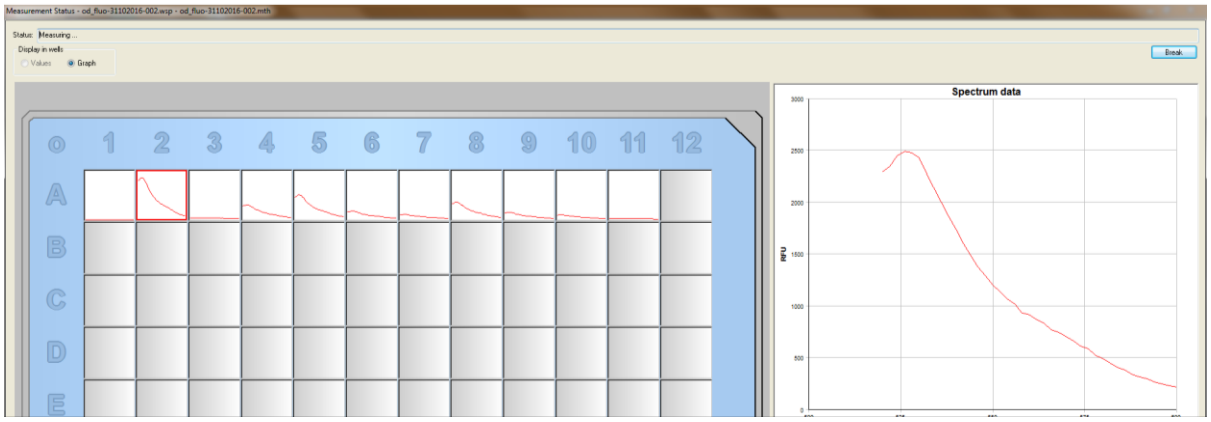
What is the difference between a “detection scan” and a “single read”?

Detection scan *will result in data output including the full spectrum graph and will take longer. A detection scan is usually used to check for DPA because the shape of the curve will tell us whether there is YFP or not. Also the gain on the detection scan can be adjusted to your own needs to become more sensitive (i.e. changing from 72 to 90 for example).*

Single read *will only provide one data point at the peak of the spectrum. This is a quick way of checking your large scale expression for example, or following a sample that already has a detectable YFP value.*

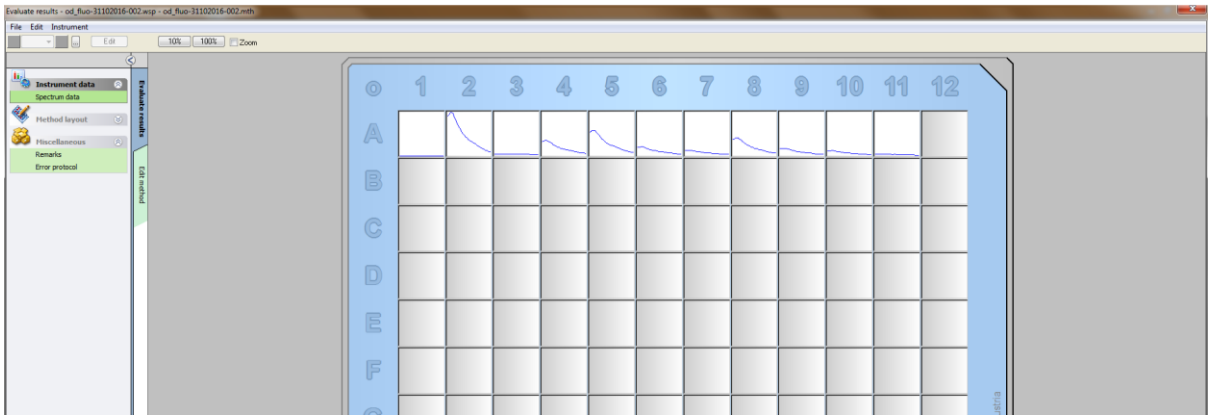
- Choose selected parameters by clicking on the *Start* icon (green play button).
- A new window will pop-up. Don’t change anything and start the measurement by clicking on the *Start* icon (green play button).

During the measurement following screen will be shown.



When the measurement is finished, following window will automatically appear on the screen.

The PBS should give no reading and there should be no curve present. The YFP standard value should be roughly around 2500.



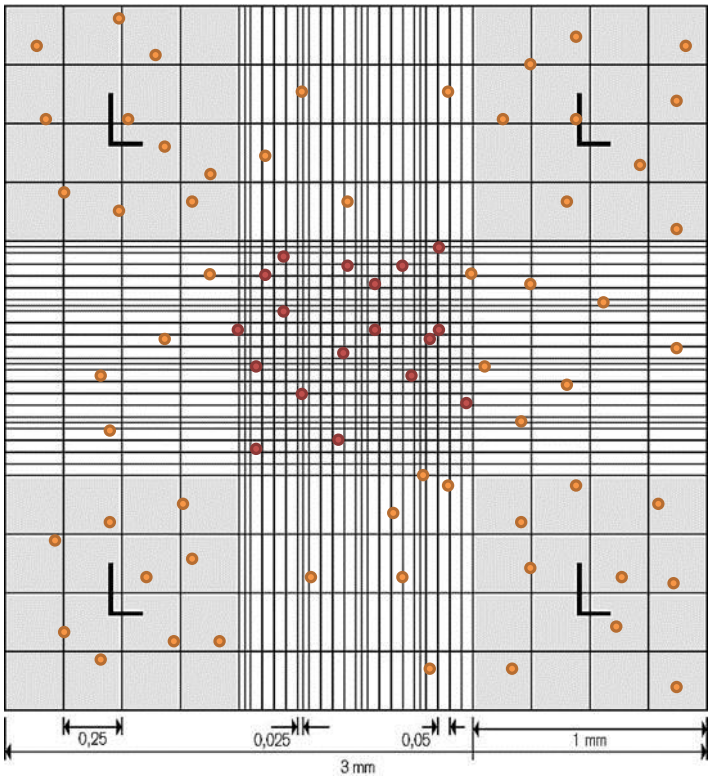
- Click on the Edit tab and export the data as excel file and save it.
- Take out you plate from the machine.
- Then by clicking the *Start* icon (green play button) keep going to the next windows (Don't save the data) and exit from the last window by clicking on exit Magellan.

- To transfer data:
  Double click on "Transfer files to remote desktop" icon on desktop.
  Login with your UoB credential and transfer the data to your desktop.

How do we count cells?

Fill a Neubauer's cell with 20 µL of suspension.

Count all insect cells present inside the 16 large squares (see red cells below).



H

ere one can count 18 cells.
This is the number of cells per mL with a 10^4 factor.

Here we have $18*10^4$ cells/mL or $0.18*10^6$ cells per mL.

<u>Information:</u>

The population of cells is doubling every 24 hrs.

The "healthy" concentration of cells is between 0.5 and $2*10^6$cells/mL. Therefore, one has to split the cells one time a day around the same time.

Virus Amplification & Protein Expression Test experiment

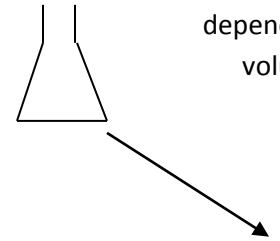
Infect shaker flasks containing 25mL at $0.5*10^6$ cells/mL with 3 mL of V_0. Count cells every day.

If the concentration is < $1*10^6$ cells/mL, then we don't need to dilute because cells have stopped dividing (they are still growing but the population is not doubling in 24 hrs).

If the concentration is > $1*10^6$ cells/mL, then we need to dilute to $0.5*10^6$ cells/mL, because the cells are still in division.

DPA

(Day after Proliferation Arrest)



If your cells have stopped dividing, depending on concentration – retrieve a volume which contains **$1*10^6$cells** *(i.e. 1.1 mL at density 0.9)*

Transfer cell suspension in Eppendorf tube labelled "DPA"

Centrifuge 2 min at 13000 rpm

Discard supernatant

*

DPA+24

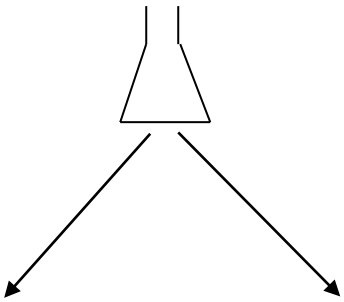
Let's say that you have diluted your culture once and its volume is 50 mL.

Centrifuge culture gently 3 min at 800 rpm in a 50mL Falcon tube.

Take supernatant (V_1 Virus) and put it in a fresh sterile and properly labelled 50mL Falcon tube.

Gently resuspend the cell pellet with 50 ml of fresh medium.

Transfer resuspended cells back into the same shaker flask for expression test.



Count cells and retrieve volume that contains **$1*10^6$cells** in Eppendorf tube labelled "dpa+24"

Centrifuge 2min at 13000rpm

Discard supernatant

*

* **Continue with protocol "Sample Processing" and measure YFP. This sampling must be performed every 24 hrs until the YFP value reaches a plateau. Then harvest cells (800 rpm, 3 min), discard supernatant and keep the pellet at -20 °C.**

Small scale expression protocol

Preparation of cells:

Prepare many flasks with 50mL of culture at $0.5*10^6$cells/mL.

Infection:

We need to find the optimal volume of **V_1 virus** which leads to the proliferation arrest.

If cells stop their proliferation immediately after adding of V_1, restart the experiment by decreasing the volume of V_1.

If cells don't stop dividing, increase the volume of V_1.

Counting the cells:

Count cells every 24 hrs. If they are still dividing, maintain a concentration < $1*10^6$ cells/mL.

Wait for DPA (Day after Proliferation Arrest).

Monitoring YFP:

When cells stop doubling, monitor YFP every 24hrs (keep probes for gel , cf. "**Processing the sample for SDS-PAGE and YFP measurement**" protocol).

Monitor YFP until it reaches a plateau.

Then, harvest cells, discard supernatant, keep the pellet at -20°C and/or make a protein expression in large volume.

If the expression of protein is good, you may use this V1 pellet to start a small scale purification test.

Freezing and thawing insect cells

1) <u>Freezing cells</u>

- count cells, make sure that you have enough cells for preparing 2-4 vials (see table)
- prepare cryovials on ice
- centrifuge the cells at 400-600g for 10 mins at RT
- remove supernatant

(For High Five cells, keep the conditioned medium for making freezing medium)

- resuspend cells in the given density in the right medium
- transfer 1mL to sterile cryovial
- place at -20 °C for 1h, then store at -80 °C for 24-48h
- transfer in liquid N2 for long term storage.

Cell line	Freezing medium	Density
Sf21	60% Sf900 medium 30% FBS 10% DMSO	$1*10^{7}$ cells/mL
High Five	42.5% conditioned Express 5 medium 42.5% fresh Express 5 medium 5% FBS 10% DMSO	$3*10^{6}$ cells/mL

2) <u>Thawing cells from frozen stock</u>

- Remove vials from liquid nitrogen and place it in a water bath at 37°C
- Thaw rapidly with gentle agitation until cells are <u>almost</u> thawed and remove the cells from the water bath **(Leaving cells at 37°C after they have thawed will result in cell death)**
- Quickly decontaminate the outside of the vial by treating with 70% ethanol, dry the vial and place on ice
- Pre-wet a 25 cm2 flask by coating the adherent surface with 4 ml medium
- Transfer the 1 ml cell suspension directly into the 4 ml of medium
- Transfer flask to a 27°C incubator and allow cells to attach for 30-45 minutes
- After the cells are attached, gently remove the medium (as soon as possible to remove the DMSO from the freezing medium)
- feed cells with 5 ml of fresh medium
- after 24h, change the medium
- leave the cells grow until confluence and then start monolayer or suspension culture.

Freezing Baculovirus-infected insect cells (BIIC) stocks

Here is a home-made protocol from the paper in press "The titerless infected-cells preservation and scale-up (TIPS) method for large scale production of NO-sensitive human soluble guanylate cyclase (sGC) form insect cells infected with recombinant baculovirus" from D.J. Wasilko *et al.*

Freezing BIIC (250mL of culture for 25 aliquots of 1mL of BIIC)

1. Grow 250 mL culture of SF21 cells until $1*10^6$ cells/mL
2. Infect cells with Virus (volume should be checked according to "Small Scale Expression" protocol)
3. Maintain a concentration of $1*10^6$ cells/mL until Day after Proliferation Arrest (DPA)
   You will be able to just barely detect YFP on the fluorimeter
4. Centrifuge 800rpm for 5-10 mins. Use 5 falcon tubes to split the volume
5. Prepare a 50mL solution containing:
   45mL = 90% of medium (Hyclone)
   0.5g = 10g/L of BSA
   } Sterile Filtered (0.22 µm)
   5mL = 10% of DMSO – already sterile and for insect cells only
6. When centrifuge is done, remove supernatant
7. Resuspend cells gently to a final density of $1*10^7$ cells/mL (use 25mL of the above solution)
8. Aliquot 1mL into each cryovial
9. Place at -20°C for 1 hr
10. Store at -80°C for 24-48 hrs
11. Store in liquid nitrogen.

Infection after thawing a BIIC (1mL of BIIC for 800mL of expression culture)

1. Pre-adapt 375 mL cultures of uninfected SF21 cells at ~$1* 10^6$ cells/mL
2. Quickly thaw one vial in your hands (use paper towels to protect your skin)
3. Dilute the vial in 50 mL of medium (Hyclone)
   You obtain 50 mL at $2*10^5$cells/mL
4. Add 25 mL of this solution in each of the 375 mL flasks
   You should obtain 400 mL of cells at slightly more than 1 10^6cells/mL (the concentration increases by 2.7% only)*
5. Maintain the cells at $1*10^6$ cells/mL until you observe the proliferation arrest
6. Monitor the YFP until it reaches a plateau
7. Harvest the cells.

Stock concentrations of reagents in the 103 lab

Antibiotics 1000X:

*Amp: 100mg/mL

*Chl: 30mg/mL (in absolute Ethanol)

*Kan: 50mg/mL

*Tet: 10mg/mL (in absolute Ethanol)

*Gent: 10mg/mL

*Spec: 50mg/mL

Others:

*IPTG: 1M (1000X)

*BluOGal: 100mg/mL (500X) Light sensitive!!!

Cleaning Flasks

Dispose cells and/or media into a glass bottle (bring to autoclave when full)

Add 35-50 ml 10% acetic acid to flask

Brush flask (make sure to remove the cell ring, you can use the same acid for each flasks)

Dispose the 10% acetic acid in an acid waste container (provided by Pierre or Annie)

Rinse flasks with distilled water several times and fill with distilled water to a level above where the cell ring was formed

Bring flasks with your name and room number on autoclave tape to Annie/Virginie/Audrey for EMBL and Pierre/Mireille for CIIB, they will:

~Autoclave once with water inside

~Autoclave a second time without water (dry)

Bacmid prep checking

Running an agarose gel (10uL of Bacmid needed)

Place spacer in each space allocated (top and bottom)

Place comb over the red tape

Prepare 1X TBE

Mix 0.8g agarose with 100mL 1X TBE

Put in microwave around 4 min at 750 watts (until it dissolve)

Cool down under stirring (take around 30 min)

Put agarose in the tank up to the wells of the comb

Add 2.5uL of EB (mix with the tip)

Wait that the gel had polymerized and take off the comb and spacer

Fill the tank up to above the gel

Dilute your Bacmid to 1/10 with water

Mix 2uL of Bacmid 1/10 + 8uL of water + 2uL of 6X DNA gel loading buffer (use up all volume for loading)

Mix 1uL of Marker 1Kb Neb + 7uL of water + 2 of 6X DNA gel loading buffer

(use up all volume for loading)

Place the lid (Negative close to DNA on the top, DNA will run to the positive side)

Run with constant Voltage 80V and with maximum Amps

(run can take around 30-50 minutes)

Take picture in the dark room

Throw the gel away in the allocated bin for EB

Estimation of the Bacmid concentration using nanodrop device

Open the software ND-1000

Clic on nucleic acid

Clean up the nanodrop with 2uL water and tissue

Initialize the instrument by pressing OK

Blank the system by using the buffer of your sample (in Bacmid case use water)

Wipe

Put 2uL of sample (name your sample in “sample ID” window)

Clic on measure

Save your value

Clean with water

Close the lid of the nanodrop