IMAGING

MICROSCOPY

Day 1

Set up 5ml O/N

Day 2

Dilute overnight to reach an O.D. of 0.6 the next day

Day 3

1. Melt 1% agarose in 18% SW.
   First add 600 µl 30% SW to an Eppendorf tube and place in a heat block at 65-70°C to pre-heat.

2. Melt 250 mg agarose in 10 ml dH_2O in the microwave.
   While solution is hot, use P1000 to pipette several times (to pre-heat the tip, for accurate pipetting).
   Then immediately pipette 400 µl of molten agarose into 600 µl hot 30% SW and mix thoroughly.

   The 1% agarose solution lasts 1-2 hours at 65-70°C before salt precipitation becomes evident

   Adjust agarose concentration accordingly, but ensure final concentration of 18% SW

3. Clean glass slide (not gelatinsed if using GFP) with ethanol and Kimwipes, dry and place on flat surface

4. Working quickly, pipette ~100 µL of molten agarose onto centre of slide

   More agarose can be added if needed, but pipette slowly to avoid air bubbles.

   Wait 30 min for the pad to cure. Agarose pads should only be prepared once samples are ready and stained

MICROSCOPY CONTINUED...

5. Once cultures are OD=0.6, spin 1ml at 6000 rpm for 8 minutes
   Resuspend in 18% SW

6. For Hoescht 33258 staining, pipette 500µl of culture in a fresh eppendorf
   Add 25µl of 0.1mg/mL Hoescht 33258

7. Leave at room temperature for 10 minutes in the dark
   Spin again and resuspend cells in equal volume of 18% SW

8. Very gently spot 10µL of stained cells directly onto the pad
   Place a #1.5 coverslip on top

   Wait 1 minute before placing the coverslip, helps cells adsorb into pad and improve cell density

   Clumping, however, must be avoided, especially if cells are to be quantitatively analysed

9. Image with light microscope equipped with a 100x oil-immersion lens

   Slides dry out quickly so this should be done as soon as possible

Note: For image analysis studies, it is important to randomise fields to avoid bias

   Do this essentially blindly, only stopping to focus the field

   Also take images of many cells, typically 100's or 1000's, from different slides and biological replicates

SPLIT-GFP

C-GFP Mev^R \ N-GFP Nov^R	pTA2170	pTA2171
pTA2172	N-GFP-*gene* C-GFP-*gene*	*gene*-N-GFP C-GFP-*gene*
pTA2173	N-GFP-*gene* *gene*-C-GFP	*gene*-N-GFP *gene*-C-GFP

- Clone gene into 4 split-GFP plasmids pTA2170-2173
  - Passage through *dam- E. coli*, unless using *Δmrr H. volcanii*
- Transform *H. volcanii* H26 as usual with both plasmids
  - Plate at 10^0 and 10^{-1} on Hv Ca+uracil containing 2 µg/ml Mevinolin (70 µl at 10 mg/ml in ethanol) and 0.2 µg/ml Novobiocin (67 µl at 1 mg/ml in dH_2O)
- Restreak, then confirm presence of both plasmids using colony PCR
- Set up 5 ml O/N in Hv-Ca+uracil+Mevinolin+Novobiocin, at 37 °C
  - Repeat 5ml O/N at 37°C, then grow a third 5ml O/N at 30°C (for correct GFP folding)
- Pellet 2 ml of O/N at 6000 rpm for 10 minutes
  - Resuspend in 1ml 18% SW and spin again, then resuspend in 500µl 18% SW
- Pipette 300 µL of cells/well into a 96-well plate and scan using Typhoon
  - Analyse fluorescence using Cy2 channel on Typhoon, use ≥3 replicates
    - Calculate relative fluorescence (rf) using the following formula:

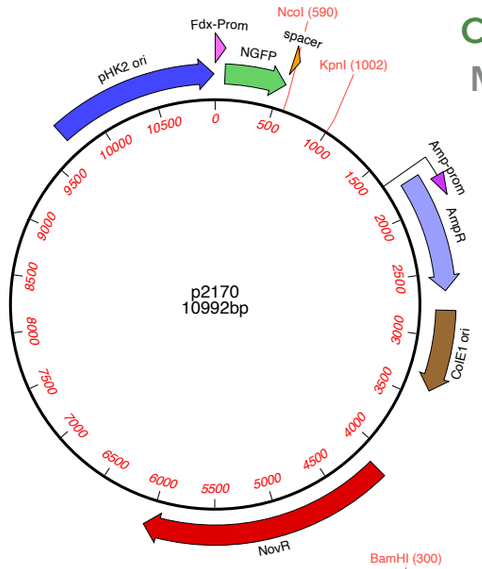
rf = (transformant − untransformed WT) / untransformed WT

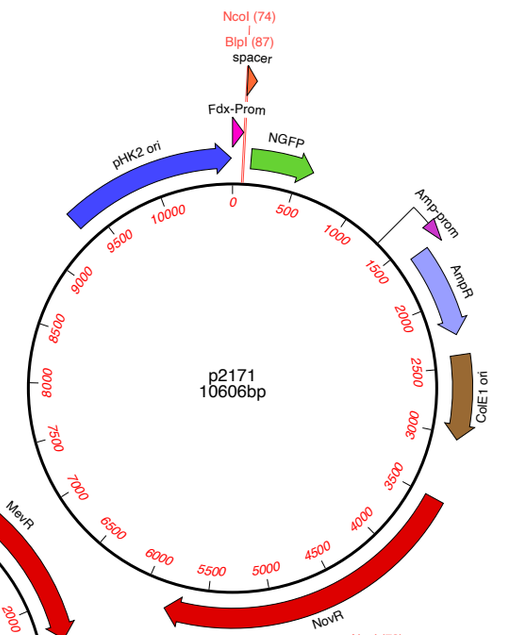
Winter et al (2018)

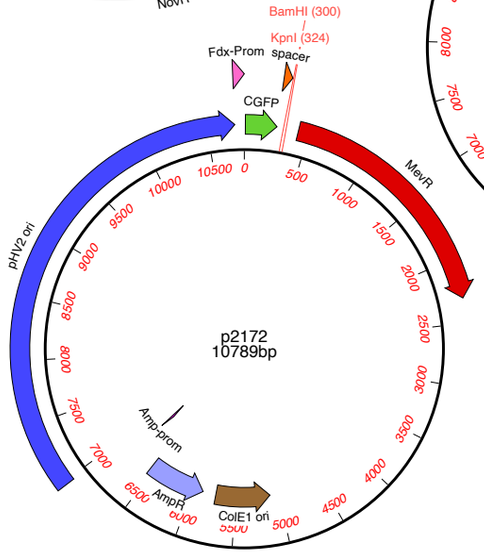
PLASMIDS FOR SPLIT GFP

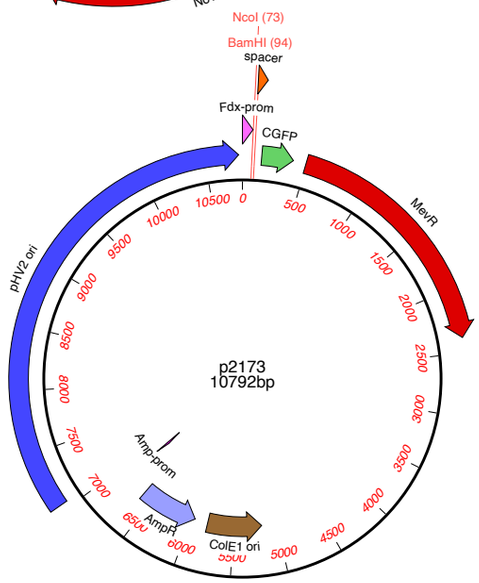
- Clone desired gene into the four split GFP episomes:
  - pTA2170 (pJAS-NGFP-Nterm): N-GFP-*gene* NovR
    - Incorporate gene of interest at NcoI/KpnI sites
      - Forward primer: ensure ATG start codon is within NcoI-compatible site (C | CATGG), will be in-frame with GFP
      - Reverse primer: KpnI site directly after Stop codon
  - pTA2171 (pJAS-NGFP-Cterm): *gene*-N-GFP NovR
    - Incorporate gene of interest at NcoI/BlpI sites
      - Forward primer as for pTA2170
      - Reverse primer: replace Stop codon with BlpI-compatible site (GC | TNAGC), ensure in-frame with GFP
  - pTA2172 (pWL-CGFP-Nterm): C-GFP-*gene* MevR
    - Incorporate gene of interest at BamHI/KpnI sites
      - Forward primer: ensure ATG start codon is directly after BamHI site (G | GATCGC), will be in-frame with GFP
      - Reverse primer as for pTA2170
  - pTA2173 (pWL-CGFP-Cterm): *gene*-C-GFP MevR
    - Incorporate gene of interest at NcoI/BamHI sites
      - Forward primer as for pTA2170
      - Reverse primer: replace Stop codon with BamHI-compatible site (G | GATCGC), ensure in-frame with GFP

C-GFP MevR \ N-GFP NovR	pTA2170	pTA2171
pTA2172	N-GFP-*gene* C-GFP-*gene*	*gene*-N-GFP C-GFP-*gene*
pTA2173	N-GFP-*gene* *gene*-C-GFP	*gene*-N-GFP *gene*-C-GFP









Winter et al (2018)

COLONY PCR: ONETAQ

DNA prep:

- Touch DNA with yellow tip (the less cells the better)
- Pipette cells up and down in 100µl SDW
- Boil at 100°C for 10mins
- Leave on ice for 10mins

PCR mix per Reaction	
DNA	1
Forward primer	0.4
Reverse primer	0.4
dNTPs	4
GC buffer (5X)	4
OneTaq	0.1
SDW	10.1
	20µl

PCR cycles	Temperature /°C	Time	
Initial D	94	30s	
D	94	30s	x 30
A	x	30s	
E	68	y (1min per kb)	
Final E	68	5mins	

Can use Q5 hotstart polymerase instead, but costs more

MICROSCOPY
Excitation/emission wavelengths

Signal	Excitation	Emission
Hoescht 33258	352nm	461nm
GFP	470nm	550nm
mCherry	587nm	610nm
Ypet	517nm	530nm
mTurq	434nm	474nm
mScarlet	569nm	593nm