# May 2012 notebook

From Dueber Lab Wiki

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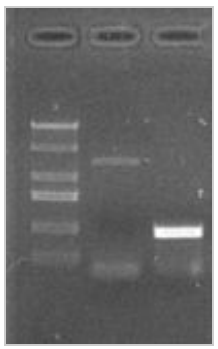
## 31 May 2012 (Th)

PCR for the genes out of Yeast Genome:

| PCR | Gene | Primers | PCR product size |
|---|---|---|---|
| 1 | YLR413W (cell periphery) | AU74 and AU75 | 2077 |
| 2 | SNA3/YJL151C (vacuole) | AU76 and AU77 | 451 |

Also just checked the protein genes for any internal restriction sites (BamHI, BglII, XhoI, Spe, Xbal). Only SNA3/YJL151C has a PstI site.

-gels for the pcr products looked good. From left to right: ladder, YLR413W (2077), SNA3 (451).



Organelle PCR product Gel

-gel purified the pcr products; now in tubes labeled "CLC gp1" and "CLC gp2". Numbering follows the table above.
-split the gel purified DNA into 4 pcr tubes for Gibson Assembly (2 plasmids for each organelle).

| PCR tube No. | Plasmid name | Organelle/Gene | Fluorescent Protein | Parent Vector name |
|---|---|---|---|---|
| 1 | pCLC001 | Cell Periphery/YLR413W | Venus | pMRY001 |
| 2 | pCLC002 | Cell Periphery/YLR413W | mKate | pHSR001 |
| 3 | pCLC003 | Vacuole/SNA3 | Venus | pMRY001 |
| 4 | pCLC004 | Vacuole/SNA3 | mKate | pHSR001 |

-transformed and plated the 4 gibson products; went into 37 incubator to grow overnight. Will pick colonies tomorrow.

# 30 May 2012 (W)

Cloning Destination Vectors:
-took plates out of incubator. Ratio of colonies on negative control to colonies on pWCD0480_mKate plate looked good. picked 2 colonies and put into lb+amp media
-cells took almost 7 hours to grow before saturating media. Miniprepped them, then took dna for test digest
-ran gels for digest. Results looked good.

PCR for genes out of Yeast Genome:
-oligos came in today. Rehydrated mine and put them into the freezer: AU74 through AU77. AU78 and AU79 (primers for Vacuolar membrane gene) used to be mine but are now given to Vincent.

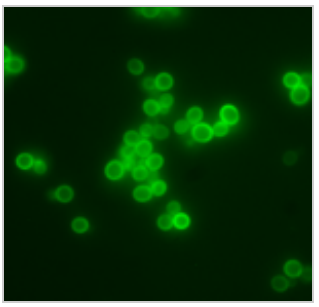
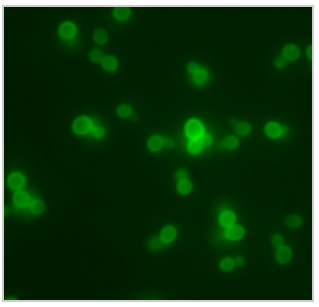
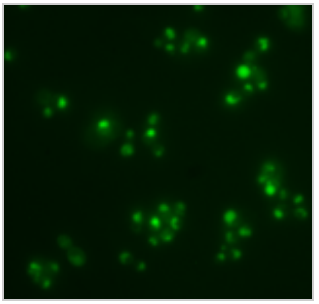
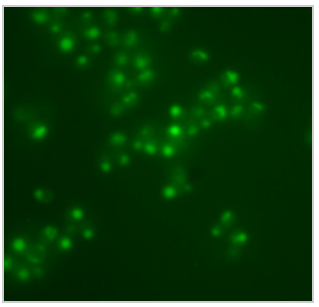
# 29 May 2012 (T)

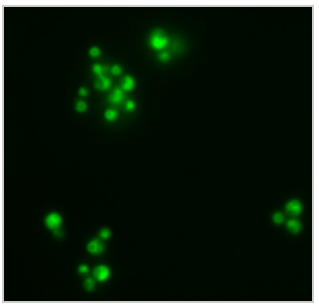
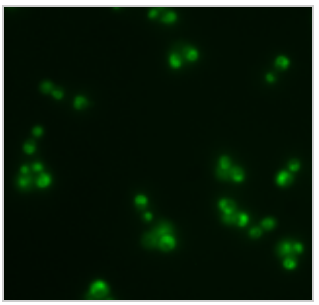
-finished the PCR soeing process for mKate; and ran gel to separate out the product. Purified gel. Fluorescent protein gene is ready to be ligated into the plasmid
-digested the parent vector (pWCD0480), and ran gel. Digested the pcr soeing product with BglII and PstI and ran gel to separate out the digested product
-ligated the digested pcr soeing product with the parent vector pWCD0480
-transformed cells with the plasmid

# 25 May 2012 (F)

Picked proteins from yeast genome, to target the cell periphery, vacuole, and vacuolar membrane. The proteins are organized in the table below.

| Organelle Name | Protein Name | Links | Picture |
|---|---|---|---|
| | YLR413W | Picture: http://yeastgfp.yeastgenome.org/displayLocImage.php?loc=6927 | |

| | | | |
|---|---|---|---|
| **Cell Periphery** | | Gene: http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=YLR413W |  YLR413W |
| | TPO3/YPR156C | Picture: http://yeastgfp.yeastgenome.org/displayLocImage.php?loc=7029 Gene: http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=YPR156C |  TPO3/YPR156C |
| **Vacuole** | SNA3/YJL151C | Picture: http://yeastgfp.yeastgenome.org/displayLocImage.php?loc=4922 Gene: http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=YJL151C |  SNA3/YJL151C |
| | KTR6/YPL053C | Picture: http://yeastgfp.yeastgenome.org/displayLocImage.php?loc=7331 Gene: http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=YPL053C |  KTR6/YPL053C |
| | VMA4/YOR332W | Picture: http://yeastgfp.yeastgenome.org/displayLocImage.php?loc=7638 Gene: http://www.yeastgenome.org/cgi-bin/locus.fpl? | |

| | | | |
|---|---|---|---|
| **Vacuolar Membrane** | | locus=YOR332W |  VMA4/YOR332W |
| | VMA10/YHR039C-A | Picture: http://yeastgfp.yeastgenome.org/displayLocImage.php?loc=7691 Gene: http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=VMA10 |  VMA10/YHR039C-A |

-designed the primers for each protein and put them onto the google doc. Mine are primers AU74.iGEM0015 through AU79.iGEM0020. Designs for the primers are here: Organelle Gene Primer Designs. Summary Table:

| Name | Description |
|---|---|
| AU74.iGEM0015 | Cell Periphery YLR413W: forward primer |
| AU75.iGEM0016 | Cell Periphery YLR413W: reverse primer |
| AU76.iGEM0017 | Vacuole SNA3/YJL151C: forward primer |
| AU77.iGEM0018 | Vacuole SNA3/YJL151C: reverse primer |
| AU78.iGEM0019 | Vacuolar Membrane VMA4/YOR332W: forward primer |
| AU79.iGEM0020 | Vacuolar Membrane VMA4/YOR332W: reverse primer |

# 24 May 2012 (Th)

**Developments:**

-compiled list of possible proteins or amino acid sequences on google doc
-found database of all proteins tagged with GFP/RFP and found their localizations in budding yeast. We will most likely use the markers from the database, and possibly other proteins that give results that are easy to read.

**First part of the project: Testing GFP and RFP on various organelles**
The purpose is to see what overall combination of organelles we can possibly use in our barcoding system. We need to express GFP or RFP on each organelle, then check the pictures in black and white to see if we can differentiate between any two organelles. This will involve tagging GFP/RFP onto the end of a protein localized at a particular organelle, or placing a signal sequence onto the GFP/RFP itself.

Details:

-Most proteins are taken from the Yeast GFP Genome Database. http://yeastgfp.yeastgenome.org/
-The markers from the database will definitely be used
-Will pick two proteins per organelle first, and maybe a signal sequence if one has been found in our literature search
-The plan is to PCR the protein gene and create plasmids that have GFP/RFP in them. We will use Gibson assembly to insert the protein gene in front of the fluorescent protein.

We will do this in several main steps:

1. First create the vectors. We are starting with 2 parent vectors as templates: pWCD0421 for GFP, pWCD0480 for RFP. Both vectors contain some unnecessary genes in addition to the actual fluorescent protein genes: Venus (GFP) and mKate(RFP). We will PCR Venus and mKate out of the plasmids, then use restriction enzymes to cut out the junk and insert the fluorescence gene. Both vectors are of the form BglII-linker-FP-BamHI-stop codon-XhoI. The linker sequence is there so that when we don't have to make different primers when PCRing the protein gene (the Gibson assembly in the later step would require different overlap regions in the primer for either mKate or Venus).
2. PCR out the protein gene. Make sure the primers have homology regions with the promoter in the parent vectors (pWCD0421 and pWCD0480 have the same promoter), or homolgy regions with the linker.
3. Cut the parent vectors with BglII and use Gibson Assembly to put the linearized parent vectors with the protein sequence.

By the end of the day:

-made 2 oligos to PCR Venus, 4 oligos for mKate. Turns out that for the RFP plasmid, we could not cut out mKate simply using BglII and XhoI, because there were multiple XhoI sites along the plasmid. So we need to use PCR soeing. One pcr product would contain mKate and another would be BamHI-stop codon-XhoI and a PstI site. The result of the PCR soeing would be: BglII-linker-mKate-BamHI-stopcodon-XhoI-...-PstI.

To Do:

-create primers for the genes we've chosen. There's 14 organelles, and we'll design primers for 2 proteins per organelle.

# 21-23 May 2012 (M-W)

**Notes from Meetings:**

Cloning Technologies to Look at:

- BglBrick/BioBrick:

- Gibson Assembly (assemble many things at the same time)
- GoldenGate Cloning
- Polymerase Cycling Assembly (synthesizing a gene from oligos)
- DNA Assembler technology
- Yeast Genomic Integrations with Homing Endonucleases
    - PMID: 21876185

Find the earliest paper that discusses it, a couple other papers to describe it.

Project:

-targeting organelles in the cell: about 11 different locations, 3 different fluorescent proteins

-First Step is to target every location possible in yeast, then see what's the easiest to differentiate
-Example is peroxisome: attaching an amino acid (SKL tag, 3 amino acids) to the RFP, and it will get exported to the location

-or attach the RFP onto something that is already there

-binding RFP to the organelles; inside or outside

-Our list of organelles: mitochondria, plasma membrane nucleus, golgi, ER, cortical ER, Actin, Bud scar, endosome, vacuole, peroxisome, microtubule
-Put two colors for each experiment, for the organelles that look similar:

-so put both RFP and GFP into each organelle. Scan, and then put together pictures

-look in black and white, test to see if we can differentiate between organelles. Colors will give answer key

-controls: antibodies and dyes that go to specific organelles.

**Research**

My research tasks: nucleus (Thomas), golgi (Robert) , endosome(Thomas), and vacuole (Robert)

- Find proteins associated with each organelles
- ways to target the organelle

Nucleus:

- loc1p protein in the nucleus only. Binds to RNA but does not leave the nucleus to cytoplasm: http://jcb.rupress.org/content/153/2/307.full.pdf+html
- a2 gene: 13- 67 amino acids attached to end of protein, and protein will get exported to yeast nucleus

http://www.sciencedirect.com/science/article/pii/0092867484900552

Golgi:

- PH (Pleckstrin Homology) domain of OSBP (Oxysterol Binding Protein) http://www.sciencedirect.com/science/article/pii/S0960982202007790. OSBP is found in humans but works in yeast. Yeast homolog is Osh1p, which works in both mammalian and yeast cells
- FYVE domain on EEA1 protein, GRIP domain on C terminus of golgin-245/p230 and golgin-97 http://www.sciencedirect.com/science/article/pii/S0960982299801663

Vacuole:

- MCH1/YDL054C protein localized to Vacuolar membrane,
- SUR1/YPL057C (lumen)
- VPS30/YPL120W (lumen) http://www.mcponline.org/content/8/2/380.full, table 1

Endosome:

- VPS38/YLR360W http://www.mcponline.org/content/8/2/380.full, table 1

Info to be put on googledoc.

Retrieved from "https://dueberlab.com/w/index.php?title=May_2012_notebook&oldid=9342"

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# June 2012 notebook

From Dueber Lab Wiki

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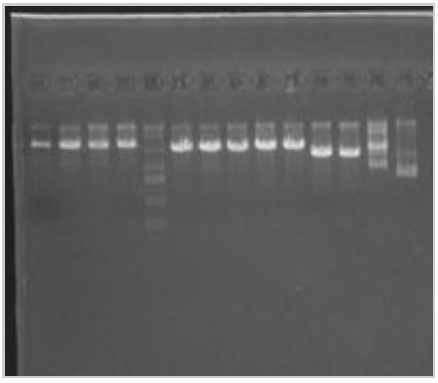
## 30 June 2012

-Notes to self on cassette design: part 1 is the integration/marker type, part 2 is the promoter, 3a is your gene, 3b is your fluorescent protein, 4 is terminator (use ADH1), 5 is the integration/marker type again, 6 is E coli origin/antibiotic.
-miniprepped the colonies for pCLC005, 6, and 7. These are the 3a parts: the targeting proteins themselves.
-KTR6 (pCLC006) was pcred entirely with expand polymerase, so I picked more colonies to make up for the decreased accuracy.
-minipreps are labeled pCLC005-1 to 4, pCLC006-1 to 5, and pCLC007-1 to 4.
-test digested with EcoRI, results looked completely wrong. Suspected that my digestion was incomplete, or I needed 2 enzymes instead of 1, to prevent it from ligating back on itself.



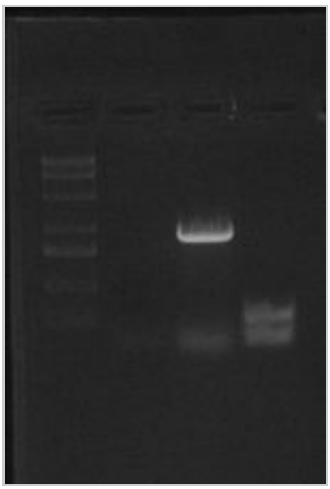
From left to right: pCLC005-1 to 4, ladder, pCLC006-1 to 5, and pCLC007-1 to 4.

# 29 June 2012

-had to redo my digest and transformations for pCLC006 and pCLC007 (KTR6 and CIS3) because I plated them on Kan plates instead of CamR plates....
-planning to pick colonies and miniprep tomorrow.

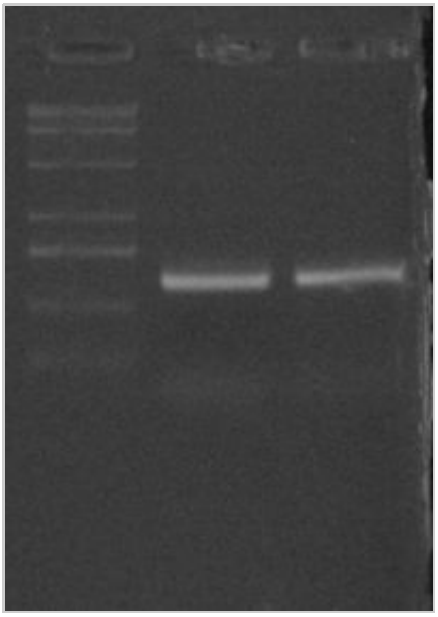
# 28 June 2012

-ran gels on expand pcrs.



Gel of Expand pcrs. Left to Right: Ladder, pcr #3, 4, 5.

-pcr #4, #5 both worked. 3 is still being stubborn.
-decided to try the pcr for #3 one last time, this time adding DMSO (4 uL, subtracted from the ddH20), using the 1K45 program. Also made new dilutions from the stock tubes, just in case there was an error.
-gels for #3 looked right this time, both with and without DMSO. That probably means there was something wrong with my dilution of the oligos, since I reused the diluted tubes.

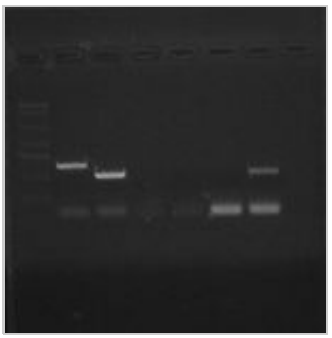


Gel for Expand pcrs of pcr #3. Left to right: ladder, Expand with DMSO, without DMSO.

**Meeting Notes:**
-growth conditions for large vacuoles (so that it is distinguishable from nucleus)
-growth conditions for vacuolar membrane to be consistent from cell to cell (unique shape that will be easy to recognize). -finished the digest of KTR6 and CIS3 (pCLC006 and pCLC007, respectively). Transformed into TG1 cells, and put onto LB+Kan plates.
-Will hopefully also be able to transform TPO3 (pCLC005) later tonight. The digest will finish at 7pm.

# 27 June 2012

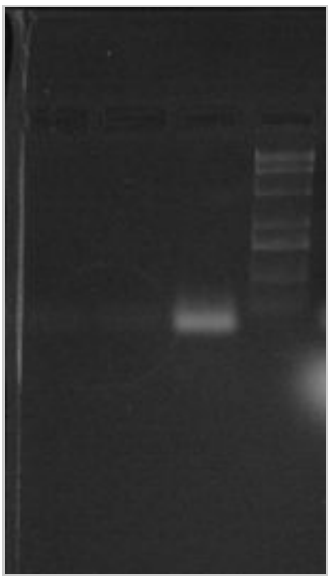
-pcrs from yesterday were done. Now gel purifying the products.
-6 wells, 1,2,6 came out right, 3, 4, and 5 showed no bands at all.



Gel of pcrs from yesterday. From left to right: Ladder, and pcr tubes 1-6, following the table below.

-redid the pcrs for the 3 that failed. Used GC buffer, same polymerase (Phusion), and lowered the annealing

temperature from 60C to 50C. Hope it works this time.
-finished 2nd pcr, ran gel again. pcrs #3 and 4 still didn't look right. pcr #5 looked like it might have worked.



2nd gel of the pcrs that failed the first time. From left to right: pcrs 3,4, 5, ladder.

-doing pcr a 3rd time, with expand polymerase, and will run gel tomorrow.

# 26 June 2012

-oligos finally came in. Organized my pcr reactions for the Golden Gate assembly.
-I'm using 3 genes: one for Cell periphery and 2 for Vacuole.

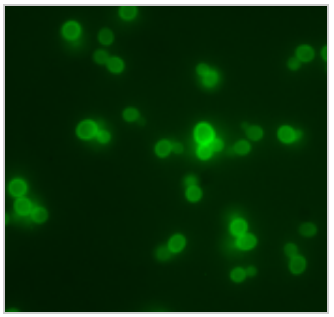
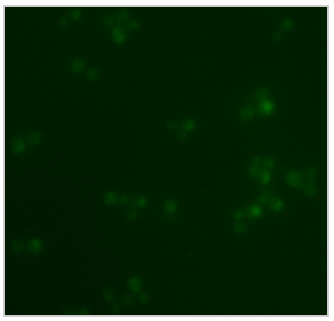
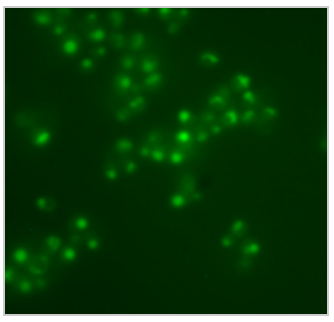
| PCR Tube No. | Gene | Primers | Oligo No. | Size |
|---|---|---|---|---|
| 1 | TPO3 | External forward, and internal reverse 1 | AX67, AX70 | 741 |
| 2 | TPO3 | Internal forward 1, Internal reverse 2 | AX69, AX72 | 521 |
| 3 | TPO3 | Internal forward 2, external reverse | AX68, AX71 | 700 |
| 4 | KTR6 | External forward, external reverse | AX73, AX74 | 1368 |
| 5 | CIS3 | External forward, internal reverse | AX75, AX78 | 228 |
| 6 | CIS3 | internal forward, external reverse | AX76, AX77 | 524 |

-pcrs put in and left overnight.

# 25 June 2012

-the new yCC006 looks very nice, plenty of colonies growing. Robert had picked a colony for me yesterday and grown it to saturation. Back diluted it this morning; we should be looking at it under the microscope in the afternoon.

-Thomas and I still need to order our oligos for the Golden Gate assembly. We'll be a day or two behind the others. We need to pcr the chosen genes out of the yeast genome and this time construct them so that they can be easily assembled using Golden Gate assembly.
-The genes I've picked: TPO3/YPR156C for the cell periphery, KTR6/YPL053C and CIS3/YJL158C for the vacuole. These were picked based on photo (how bright it looks), higher TAP abundance, and shorter gene sequences. CIS3 has the highest TAP abundance (12500) even though it looks dim in the picture.
-Table below shows pictures and links:

| Organelle Name | Protein Name | Links | Picture |
|---|---|---|---|
| **Cell Periphery** | TPO3/YPR156C | Picture: http://yeastgfp.yeastgenome.org/displayLocImage.php?loc=7029<br>Gene: http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=YPR156C |  <br>TPO3/YPR156C |
| **Vacuole** | CIS3/YJL158C | Picture: http://yeastgfp.yeastgenome.org/displayLocImage.php?loc=16290<br>Gene: http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=YJL158C |  <br>CIS3/YJL158C |
| | KTR6/YPL053C | Picture: http://yeastgfp.yeastgenome.org/displayLocImage.php?loc=7331<br>Gene: http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=YPL053C |  <br>KTR6/YPL053C |

# 22 June 2012

-Yeast plates look good. Colonies were still very tiny in the morning; will wait until later in the day to pick them and

look under the microscope?
-one plate (yCC006) was contaminated; redo the transformation. Back diluted parent strain yCC002 to 0.2 OD at noon.

# 21 June 2012

-checked on yCC004 plate; still needs to grow one more day in the incubator
-in case the transformations don't come out right tomorrow, we decided to make cultures of our yeast strains for redoing any transformations tomorrow. Picked colonies off the yCC002 and yCC004 plates and put them in shaker overnight.

# 20 June 2012

**Yeast Transformations**
-I'm doing transformations with both yCC002 (Cell periphery mKate), and yCC004 (vacuole mKate). They grew overnight and were back diluted this morning.
-I need 4 transformations for Cell periphery (yCC002), and 3 transformations for vacuole (yCC004).
-plated and made 7 new strains: yCC005 to yCC011. All are listed on the google doc.

**Yeast plates**
-yCC003 looks good (was put in incubator on Monday. Now transferred to fridge; we'll use it when we decide to do cotransformations of mKate plasmids into venus strains.
-yCC002 is still growing.

# 19 June 2012

**Yeast Cultures**
-put yCC002 and yCC003 in glycerol stocks. (yCC004 already in glycerol stocks, and yCC001 was never made because of plasmid difficulties).
-back diluted yeast culture to 0.2 OD so they will be saturated in 4 hours, and kept the saturated tubes in the shaker so we can put them onto slides once the mid log culture is ready.
-spun down 50 uL of saturated yCC004 yeast culture from culture tube, and streaked it onto plate.
-might need cell periphery and vacuole mkate strains for cotransformations tomorrow; diluted the saturated culture tube so that it will be saturated by tomorrow morning.

**Minipreps**
-back diluted the tubes. Only the newly picked colonies grew in the culture tubes. Made approximately a 1:30 ratio dilution, then realized it didn't really matter since I'm killing the cells in my miniprep anyway.
-miniprepped. Tubes are labelled pCLC002-14, pCLC002-15, pCLC003-6 and pCLC003-7. Will sequence them just to double check they are correct, even though the transformation was done using correct plasmids. pCLC003 can use just forward sequencing with S16. pCLC002 will need both S16 (forward) and Z72 (reverse).

# 18 June 2012

**Yeast Transformations**
pCLC003 plate grew up over the weekend: we have one colony. Put under microscope and it looks right: vacuole

venus. The plate that I streaked with pCLC002 last Friday also looks good; didn't put it under the microscope since we already looked at it last week.
-for cotransformations we decided to digest the venus plasmids and transform them into mkate strains. We are in charge of transforming our own mkate strains with the right set of venus plasmids, and also in charge of digesting our venus plasmids for everyone else.
-streaked a new plate of yCC003 (vacuole-venus) in case we need it in the future.

**Replan**
Because my cells for my miniprep are taking forever to saturate, need to reassess the situation to see which ones I absolutely need to miniprep, and which ones won't cause catastrophe if I can't miniprep them in time.
-pCLC004 plasmids I have plenty of, so no need to miniprep anymore of that.
-pCLC002 is an mKate strain: cell periphery. We already have a yeast strain yCC002 that has been streaked onto a plate, and there is a cell culture tube that is in the 30 room right now. Planning to catch it in mid log phase tomorrow as well as put it into glycerol stock tomorrow. Should probably make more anyway when we later decide to digest mkate plasmids and transform it into venus strains. -pCLC003 is venus vacuole. This will be need for cotransformations later in the week. Need this to be digested tomorrow.

**Minipreps**
-made new culture tubes and seeded them with a bit of culture that was placed in the fridge last Friday (the cells that grew too slowly).
-After 4 hours they're still not growing. Decided to let that grow, even if it means it oversaturates overnight, then back dilute it in new media until it saturates again. Plus will repick the colonies and put in another set of culture tubes just in case those cells never grow.
-my culture tubes will probably not be ready anytime before 8pm, so I'm coming back earlier tomorrow morning to back dilute and resaturate, so I can miniprep and digest pCLC003 and pCLC002 later in the day.

**Meeting Notes:**
-Mostly narrowed down the organelles to focus on, looked at a couple approaches to move forward on project. Still need more pictures, to standardize the way organelles come out. Will need to have a set of pictures: of yeast colonies when saturated, in mid log phase, and with the UCSF picture.
-split up a bunch of jobs; mine are to look at the yeast registry to find parts to improve on, and also to work with Harneet on standardizing image acquisition.

**To Do:**
-make more plasmids (ie miniprep pCLC002 and pCLC003) and digest venus vectors (pCLC003)
-have yeast plates of mkate strains (yCC002 we already have a plate; it's just yCC004). Both are sitting in culture tubes right now.
-look at yCC004 under the microscope again once we've back-diluted and caught it in mid log phase.
-make glycerol stocks of yCC002 and yCC003. (yCC003 is in culture tube right now as well.)

# 15 June 2012

**Yeast Transformations**
Came in this morning and noticed that the yeast culture tube I made yesterday was nowhere near saturated and hardly even cloudy. Picked more from the same colony on the plate, and put into the culture tube. Hopefully it will saturate soon enough that I can back dilute it and still be able to let it grow for 4 hours before putting it under the microscope.
-decided not to back dilute and just catch the yeast right before they saturate. Put under microscope and the

fluorescence was noisy. Added more media to the culture tube to look under the microscope a second time. Looked better under the microscope the second time. Streaked a new plate with that colony to grow over the weekend.

Also transformed pCLC003 again into a new batch of yeast cells.

**Making more plasmids for combined transformations**
-Picked 2 colonies from each plate of pCLC002, pCLC003, and pCLC004 (9:15 am). Will miniprep them later on today.
-the chemically comp TG1 cells were growing terribly slowly, probably because the shaker stopped shaking in the middle of the day. Placed the tubes in the fridge, and will seed new culture tubes on Monday.

**Sequencing Results**
Sequencing for pCLC003-2, and pClC004-2 to -6 came back today: pCLC003-2 is wrong (didn't take up the gene insert), while all the pCLC004 are correct. No need to miniprep anymore pCLC004 cells; we have plenty of those plasmids. Will still miniprep more pCLC003 and pCLC002 plasmids on Monday.

# 14 June 2012

**Transformations Update**
yCC003 again yielded no colonies; there are 2 colonies on yCC002. Will pick a colony on yCC002 once they grow a bit bigger, to put let it saturate overnight.
-picked colony, put in 3 mL of SD ura selective media, put on shaker in 30 room.
-Will do the transformations for pCLC003 into yeast one more time tomorrow along with Masaki.

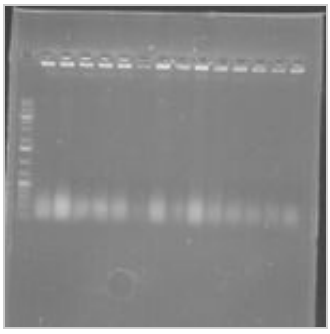
**Planning Combined Transformations** Our next step is to put combinations of 2 organelles (or 1 organelle but both venus and mKate) into a yeast strain.
-that means we need to have enough miniprepped plasmids for lots of transformations.
Inventory of my plasmids:
-pCLC001: for now we've dropped it because it never worked in Gibson reactions; parent vector always ligated back on itself without taking up the insert. -pCLC002: successful yeast transformation. One eppendorf of miniprepped plasmid in the freezer (pCLC002-5) that was verified correct from sequencing. -pCLC003: troubleshooting yeast transformation. One eppendorf of miniprepped plasmid in the freezer (pCLC003-3) that has been verified correct from sequencing. From gels on 6/5/12, colony 2 DNA (pCLC003-2) might be correct as well. Should probably sequence just to check.
-pCLC004: successful yeast transformation. Several eppendorfs of miniprepped plasmid in the freezer (pCLC004-1,2, and 4-6). Gels looked very normal from 6/5/12, and 6/1/12. I've only sequenced and used pCLC004-1 which I know is correct. Will sequence them all to double check that they are correct before we put them in yeast.

I should probably make more pCLC002; heat shocked some TG1 cells with pCLC002-5 and pCLC003-3 and pCLC004-1; then plated. Will pick colonies and miniprep them tomorrow.

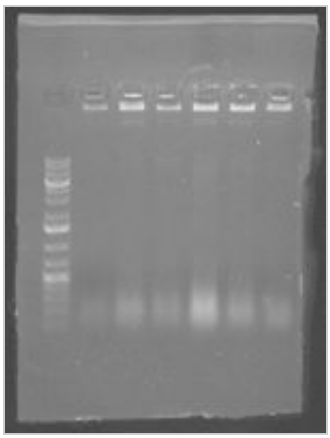
# 12 June 2012

**Sequencing Results**
pCLC001 from plate 3 was not right; gene was never inserted. Since my mKate vector did work, there shouldn't be a problem with the insert itself and the primers. Will colony pcr 24 colonies from pCLC001 plate 3.

-Did colony pcr, then ran gels to see if there were any products (should be a band at around 2000 bp).
-Still nothing on the gels (see below for pictures):



Colony pcr gel 1: ladder, colonies 1-14

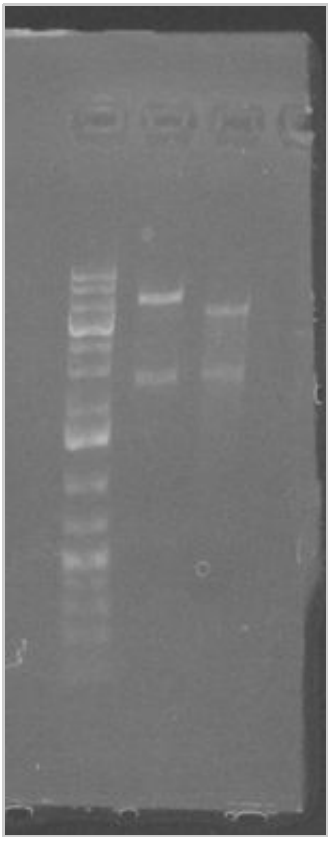


Colony pcr gel 2: ladder, colonies 15-20

-for now we'll leave this plasmid along and admit defeat... when we get to making the actual barcodes we will use a different protein (or a signal sequence) anyway.
-we still have the mKate version of this gene (cell periphery, pCLC002 is being transformed into yeast at the moment).

**Yeast Transformation**
-making a new batch of yeast culture to transform new plasmid.
-digested pCLC002-5, and pCLC003-3.
-ran gels to separate the fragment, and to check that it was digested correctly. Picture is below.

- pCLC002 should be 6874 and 2386 (6874 is the desired fragment)
- pCLC003 should be 5387 and 2386 (5387 is the desired fragment)



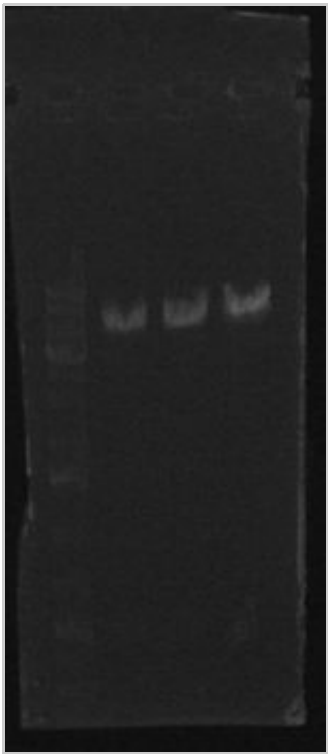
Digest for Yeast Transformation: ladder, pCLC002, pCLC003.

-transformed the plasmids into yeast and put in 30 incubator.

# 11 June 2012

**Minipreps and Test Digest**
-Plate of pCLC001 from last Friday yielded a nice amount of colonies. Picked 3 to miniprep and test digest later today (9:30am).
-Miniprepped and ran test digest with NcoI. If pCLC001 had the correct insert, there would be 3 fragments (7574, 1301, and 524). If not, then there would only be 2 fragments (6844 and 524).
-gel results came back somewhat inconclusive. Only saw one band at around the 6000-7000 bp range. The 524 fragment might have run off the gel, and the 1301 fragment was not seen. Sent in one for sequencing anyway: sigem095, with primer S16. Picture is below.

- Note: pCLC001.3-1, pCLC001.3-2, and pCLC001.3-3 is notated as pCLC001-20, pCLC001-21, pCLC001-22 respectively.



Gel of all 3 colonies of pCLC001 plate 3. Ladder, pCLC001.3-1 to pCLC001.3-3

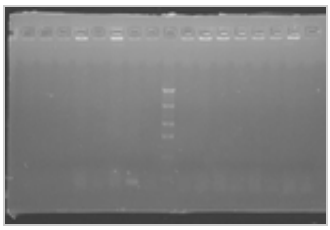
**Yeast Transformations**
Inoculated a bit of Yeast Cells, for transformations tomorrow - they need to grow for a day first. Tomorrow will transform both pCLC002, pCLC003, and hopefully pCLC001. pCLC003 yielded no yeast colonies on the first yeast plate transformation. pCLC002 was finally created correctly last Friday (sequencing results came back correct). Once I miniprep and test digest tonight, hopefully pCLC001 will also be correct.
-will transform with pCLC002-5, and pCLC003-3.

# 8 June 2012

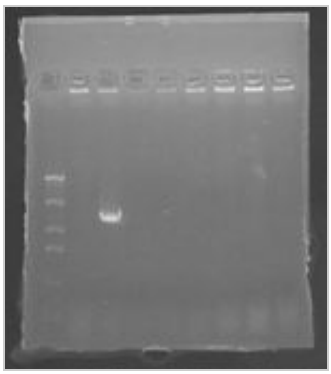
**Transformations into E Coli - pCLC001 plate 2**
I had electroporated a new batch of electrocomp TG1 cells with pCLC001 on 6/7. The plate yielded plenty more colonies than the first plate from 6/5.

**Colony PCRs**
Because the gels on the test digests all didn't work, I'll be doing colony PCRs today. My gene was 2000bp long, so I picked oligo AU75.iGEM0016 as the reverse primer - which is oligo I used to PCR the gene out of the yeast genome. For the forward primer I picked S16, which is an oligo in the promoter upstream of the gene. -picked 8 colonies from each plate: pCLC001 plate 1, pCLC001 plate 2, and pCLC002 plate. Colonies resuspended into the pcr master mix in respective pcr tubes; then took out 2 uL and put into culture tubes into plastic rack. Now on shaker at 37. (12:30pm).
-ran gels... If the gene insert is actually present in the plasmids, then we should get pcr products of 2123 bp in the gel. Labelled 1.1-1 to 1.1-8 for pCLC001 colonies plate 1, 1.2-1 to 1.2-8 for pCLC001 plate 2, and 2-1 to 2-8 for pCLC002. From Left to right in that order. pCLC002 is on it's own piece of gel. Results: everything failed...

except for one colony! pCLC002-2. Pictures below.



Colony Pcr: 1.1-1 to 8, ladder, 1.2-1 to 8. No Results.



Colony pcr: ladder, 2-1 to 8. Only 2-2 looks right.

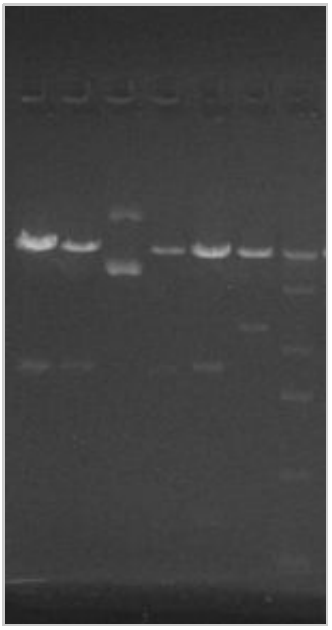
-miniprepped Colony named pCLC2-2 on the gel. *Note tube is labeled pCLC002-5 along with rest of storage in freezer. Gels labeling was for convenience, but is actually colony #5 in the long scheme of all colonies picked with this plasmid.
-Sent for sequencing. Since the insert gene is ~2000 bp, needed a forward and reverse primer. siGEM86 and siGEM87.

**pCLC001 problems**
-trying to troubleshoot why pCLC001 didn't come out correctly. Since I redid the Gibson reactions, will electroporate a new plate (plate 3) with the new Gibsons. Plate 1 and 2 were both TG1 cells electroporated with the 1st Gibson reaction.

# 7 June 2012

**Transformations into E Coli**
Plates of electroporated TG1 cells came back. pCLC002 looked good, but pCLC001 had a suspiciously low number of colonies. Picked 3 from each plate anyway (9:45am). Will electroporate a new batch with pCLC001 again today, just in case the first set comes out with the wrong sequence.
-redid the electroporating for pCLC001. Had a bit of trouble (first one heard a spark), and the transformation was not extremely efficient (time constant 2.8). Hopefully colonies will grow on the plate; plated at noon.

**Miniprep and Test Digest**
-took the colonies that we picked in the morning, and ran minipreps.
-Ran the test digest with BglII and PstI. There were internal restriction sites but they all either had multiple sites in the backbone, or they were obscure restriction enzymes that we didn't have in stock. Ran gel with all wrong results. If pCLC001 colonies had insert: fragments would be 6091, 3187; else it would be 6091, 1156. If pCLC002 colonies had insert: fragments would be 6091, 3169; else it would be 6091, 1138. Picture below:
From left to right: pCLC001-1,2,3; pCLC002-1,2,3.



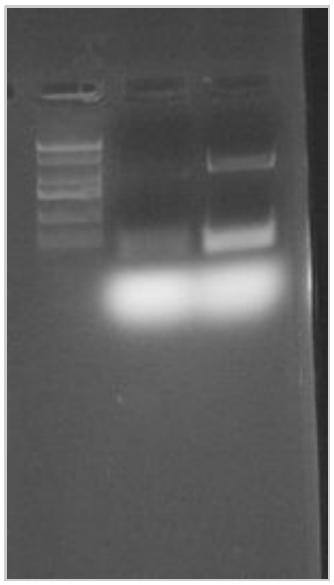
Test Digest 6/8:
pCLC001 an pCLC002.
Failed results.

# 6 June 2012

**Sequencing**
Results for pCLC003-3 came back in today. Most of it looks good, except for a very suspicious section about 80 bp into the read. There is a deletion about 20 bp into the read that is understandable; but later there is a point mutation. Turned out there was also a bug in my ApE program; had to download an older version. Other than that we suspect the point mutation is due to error in the sequencing process

**Transformations into E Coli**
-our electroporations from yesterday did not work at all (including positive and negative controls). We are now redoing the electroporations using the commercial electro-competent cells, instead of the electro-comp cells we made ourselves. This means I am redoing pCLC001 and pCLC002.
-I noticed that I needed more Gibson reactions for pCLC001-3. PCRed the genes (YLR413 and SNA3) out of the yeast genome (yJD001), and will be doing Gibson later on today.

**Gibson Reactions**
Found out that I had no more pCLC001, pCLC002, and pCLC003 Gibson reaction plasmids left. In case we need them again to transform into E Coli for whatever reason, I decided to make more. PCRed the both genes YLR413W and SNA3 out of yeast genome (yJD001). Both pCLC001 and 2 are the YLR413W gene (cell periphery) into Venus and mkate backbone (pMRY001 and pHSR001), respectively. pCL003 is the SNA3 gene (vacuole) in the venus backbone.
-the pcr product of the SNA3 gene did not run well on the gel. (see below for picture) In the end, I'll run Gibson reactions for only YLR413W. I already have plasmids pCLC003 and pCLC004 (SNA3 genes in pMRY001, pHSR001) from electroporated E Coli, that digested sequenced correctly. So technically we shouldn't need the Gibsons for SNA3 anymore. Hopefully we won't need to electroporate pCLC003 anymore.

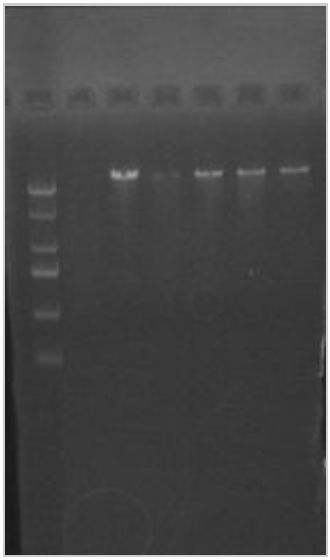


Gel of PCR products. From L to R: Ladder, SNA3, YLR413W

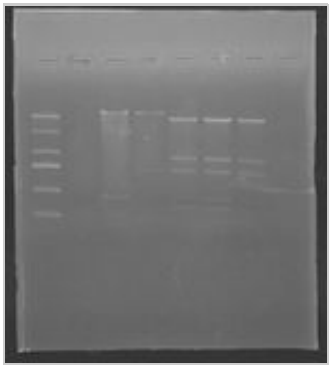
-The SNA3 gene is 451 bp, and YLR413W is 2200 bp. The SNA3 pcr looks wrong on the gel because it's below the 200 bp ladder.
-finished Gibson reactions for YLR413W. CLC gib1 and gib2 (pCLC001, pCLC002) are in freezer. Also stored the gel purified, final pcr product from YLR413W.

# 5 June 2012

**Digests and Sequencing:**
-ran test digest of the plasmids from electroporated cells: pCLC003 and pCLC004. Digested with BglII and BamHI. If the SNA3 gene was inserted correctly into the backbone, pCLC003 would digest into 6618 (backbone fragment) and 1150 (insert SNA3-venus). Otherwise it would be 6618 and 750 (insert venus without SNA3). For pCLC004 it would be 6497 and 1137 (with mKate-SNA3 in the insert), or 6497 and 759 (mKate without SNA3).
-ran gel and got strange results. Did a second test digest using XhoI and PstI. A correct pCLC004 would be cut into 5 fragments because of numerous XhoI sites in the backbone. pCLC003 would be cut into 3 fragments if SNA3 was inserted correctly (6510, 866, and 397). Otherwise it would be in 2 fragments (6971 and 397).
-the second gel looked better: From left to right: ladder, pCLC003 colonies 1-3, pCLC004 colonies 4-6.



1st gel (unsuccessful) BglII and BamHI. L to R: Ladder, pCLC003-1 to -3, pCLC-4 to 6.



2nd gel. PstI and XhoI. L to R: Ladder, pCLC003-1 to -3, pCLC-4 to 6.

-From 2nd gel: pCLC004 looks fine. In both gels pCLC003-1 didn't work. Will sequence pCLC003-3. We've already sequenced pCLC004 from last time's heat shock transformation.

**Transformations into E coli:**
-electroporated my other 2 plasmids: pCLC001 and pCLC002, plasmids directly from the Gibson Reaction.
-plated the cells onto LB+amp plates

**Transformations into Yeast:**
-since we know pCLC003 and pCLC004 worked well, we started the transformation into yeast. The first step is to take the plasmids from the miniprepped DNA (electroporated cells), and linearize. Digested the plasmid with SacII and NotI.
-finished making aliquots of yeast cells.
-did LiOAc Transformations and plated cells. Strains labelled yCLC003, yCLC004 (corresponding to plasmids).

Reminders: may need to make more Gibson products of pCLC001 to 3.

# 4 June 2012

Sequencing for pCLC004 colony #1 looked good. SNA3 gene was correctly inserted during the Gibson reaction. Supports the conclusion that low transformation efficiency is the culprit for the low bacteria growth on our plates.
-the electroporation yielded more colonies as expected. Colonies found on all 6 plates, including negative controls.

> -picked 3 colonies from each plate pCLC003 and pCLC004. In the shaker in tubes pCLC003-1 to -3 and pCLC004-4 to -6.
> -had to leave early and ran out of time; cells will still get miniprepped today (thanks to Masaki). Will run test digest and sequence tomorrow.

# 1 June 2012

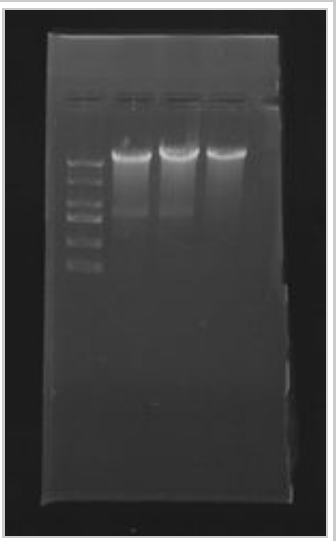
Yeast Organelle Gibson Assembly Plasmids (cont.)
-checked for colonies on plates from yesterday: pCLC001-pCLC004.
-only pCLC004 had colonies. Picked all 3 colonies.
-From everyone's result there weren't very many colonies. 2 possibilities to check for: the transformation was not efficient (most likely true), or the BglII digest of the backbone did not work well in Gibson.
-meanwhile, trying to PCR the backbone for Gibson, so we won't have to use the BglII digestion to linearize the backbone.

**Increasing transformation efficiency**
-redoing the transformations with electroporation (much higher transformation efficiency) for controls (both vectors), one yeast protein with bright band from gels, one with dim band.

-bright band is Vacuole (SNA3). pCLC003 is SNA3-venus and pCLC004 is SNA3-mKate.
-negative controls for both venus and mkate
-dim band is Endosome venus and mkate.

-transformed cells by electroporating them; plated cells.

**Testing Gibson efficiency** After growing cells from pCLC004 plate for 6 hours, miniprepped the cells, and ran a test digest.
-Test digest was done using BamHI and BglII. If the plasmids did not take up the insert, the digestion products would be 6497 (backbone) and 732 (mkate). If the plasmids did take up the insert (and Gibson was successful), then the digestion products would be 6497 and 1137 (includes mKate and SNA3 gene).
-ran gel of the test digest products. Picture is below. From left to right: Ladder, colonies 1, 2, 3.



mKate-Vacuole (pCLC004) digest

-Colonies 1 and 2 look good. Sent plasmid from colony 1 to sequencing, with primer S16.

# July 2012 notebook

From Dueber Lab Wiki

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## 31 July 2012

-took saturated cultures of yCC20, yCC21, yMRY20-25 and back diluted, hoping to grow them to 0.8 OD and then take to microscope.
-went to use the microscope at Calvin. yCC020 doesn't look right; many had GFP diffuse throughout the cell. Others you could see GFP targeted to the nucleus, but none of the other 3 organelles fluoresced. yCC021 should have been all organelles-GFP, but we only saw nucleus. Most likely nucleus brightness overpowered that of the other organelles.
-will sequence my multigene cassette plasmids, just to make sure everything is correct. It most likely is, but there's no harm checking.

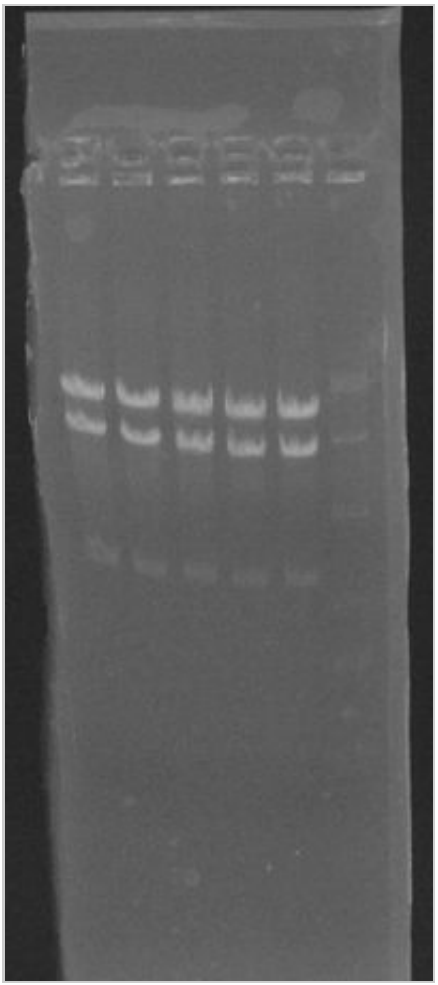
-Sequencing plans: Will sequence tomorrow... ran out of time today. For pCLC020: Z72, O44, and AW21

# 28 July 2012

-ran zymo cleanups on the linearizations from yesterday.
-transformed into yeast: pCLC020, pCLC021, pMRY51-54, pWCD581.

# 27 July 2012

-miniprepped the colonies from yesterday. Ran test digest using SacI and NotI. The expected fragments should be 7569, 5219, and 1763.
-test digest came back and looks good! 100% efficiency for all of them: 2 colonies from pCLC020 and 3 from pCLC021.



From left to right: pCLC020-2, pCLC020-3, pCLC021-1, pCLC021-2, pCLC021-4, ladder.

-digested with NotI to linearize: pCLC020-2, pCLC021-2. Linearized pMRY51, 52, 53, 54 as well and put in thermocycler; 37 degrees for 2.5 hrs.

# 26 July 2012

-checked the plates from yesterday's electroporation. There were very few, and very tiny colonies... about 4 or 5. Possibly we diluted it too much?
-picked colonies at 10:15... expecting them to take longer to grow too, since electroporated cells are slower growing than TG1 cells.

# 25 July 2012

-redid the BsmBI digestion/ligation reaction with the correct plasmids (ie pWCD615).
-electroporated the plasmids into ecoli. Had tweaked the protocol since every electroporation we had been getting a lawn of colonies: diluted the cells 10X with 10% glycerol.

# 24 July 2012

-plates of pCLC020 and 21 came back, with a lawn. Had to streak it out and let it grow for another day. Can't pick colonies or miniprep yet.
-digested 610 and 613, just in case I need for more multigene cassettes later.
-then realized that I don't need to use pWCD610 or pWCD613. Really needed only pWCD615. pWCD610 and pWCD613 had homology regions that I already had in my other plasmids.

# 23 July 2012

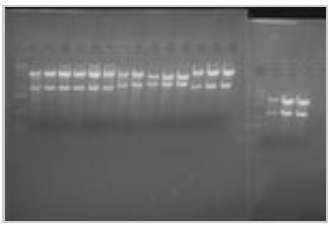
-digested pCLC014-2, pCLC015-1, pCLC016-2, pCLC017-2, pCLC018-2, pCLC019-2 with BsmBi, then ran through gel, cut out the bands I needed.
-put into BsmBI along with WCD610 and 613... as plasmids. Hopefully that won't complicate things too much. Should have digested and gel purified those two as well. -electroporated pCLC020 and pCLC021 into ecoli, on KanR plates

# 21 July 2012

-miniprepped the colonies containing plasmids pCLC014-19. Test digested:

| Plasmids | Restriction Enzymes Used | Fragments |
|---|---|---|
| pCLC014 | SacII and XhoI | 3918, 1411 |
| pCLC015 | SacII and XhoI | 3780, 1411 |
| pCLC016 | SacII and BglII | 3920, 1740 |
| pCLC017 | SacII and XhoI | 2868, 1733 |
| pCLC018 | SacII and XhoI | 3798, 1411 |
| pCLC019 | SacII and BglII | 3920, 1740 |

-16 and 19 use BglII instead of XhoI because there are two XhoI sites in the plasmids (one internal in APB1 Actin protein). -Results look great!



Test Digest. From Left to Right: pCLC014-1 to 3, pCLC015-1 to 3, pCLC016-1 to 2, pCLC017-1 to 3, pCLC018-1 to 3. Next gel, ladder, pCLC019-1 to 3.

# 20 July 2012

-ran a BsaI digest, to make cassettes for multigene next week. Will transform them into bacteria today.
-As a quick summary:

| Plasmid | Organelle-Color | Protein name | Position |
|---|---|---|---|
| pCLC014 | Cell Periphery-green | CIIC | 1st |
| pCLC015 | Vacuolar Membrane-red | ZRC1 | 2nd |
| pCLC016 | Actin-blue | APB1 | 3rd |
| pCLC017 | nucleus-green | YBL003C | 4th |
| pCLC018 | Vacuolar Membrane-green | ZRC1 | 2nd |
| pCLC019 | Actin-green | APB1 | 3rd |

-One multigene cassette will be made of pCLC014-17. Another will be of plasmids 14, 15, 18, 19.

-plates of picked colonies from yCC008 to yCC011 are now in the fridge, glycerol stocks are made.

# 19 July 2012

-getting ready to make mulitgene cassettes... as soon as we pick a protein for nucleus. Will first need to make individual cassettes with the right homology regions.
-trying to do 2 different multigene cassettes: 1 with all four proteins with GFP targeted; and 1 with CP-GFP, Nucleus-GFP, Vacuolar membrane-mKate, actin-CFP.

# 18 July 2012

-streaked the picked colonies for yCC008 to yCC011, onto new plates. Also picked colonies into culture tubes, until they're saturated, then into glycerol stocks.
-been mostly working on the iGEM Berkeley Team wiki.

# 17 July 2012

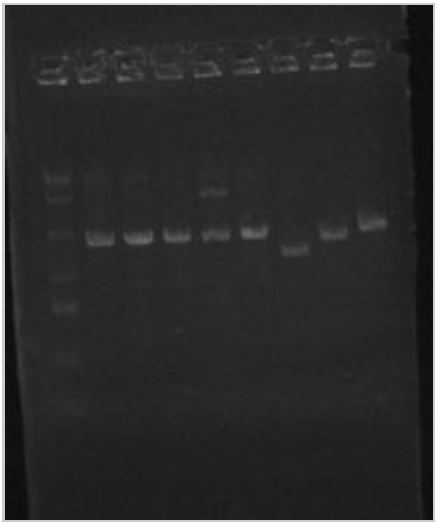
-picked colonies from the plates from yesterday. Strangely, the transformation efficiency was low for pCLC012, in that there were 4 red colonies (incorrect plasmids) and 1 white colony. Picked only that one PCLC012-1 for minipreps later today.
-miniprepped, pCLC012-1, and pCLC013-1 to 5.
-test digested with Acc651 and XhoI, same as last time. If these colonies doesn't work... not sure what to do next.

# 16 July 2012

-did a BsaI digest with pCLC007-26, to create pCLC012 (CIS3Vacuole-venus) and pCLC013 (CIS3Vacuole-mKate)
-transformed the plasmids into TG1 cells.

# 12 July 2012

**Test Digests**
-did test digests on pCLC012-1,3,4,5 and pCLC013-1,2,3,4. Digested with Acc65I and XhoI. Fragments should be 6014, 1008 for pCLC012; 5302, 990 for pCLC013.
-results don't look good; none the fragments sizes match.



From left to right: Ladder, pCLC012-1,3,4,5, pCLC013-1 to 4.

-In this case I'm planning to wait until Robert finishes the new minipreps for WCD plasmids that go into the cassette assembly. Perhaps there was an issue there.

# 11 July 2012

**Sequencing pCLC007-26**
-results came back and the gene was correctly inserted!!! The only strange thing is that the reverse primer didn't anneal, even on the forward sequencing the annealing region definitely showed up. My only guess is that there was something wrong in my dilution of the primer (AW 39).

**Minipreps**
-picked colonies from plates of TG1 made yesterday: pCLC012 and pCLC013. These are the 2 cassettes (Venus and mKate) versions with the pCLC007 gene, CIS 3 (11:30).
-miniprepped them: pCLC012-1,3,4,5 and pCLC013-1,2,3,4. pCLC012-2 didn't grow in the shaker.
-will test digest tomorrow, pick the correct plasmid, then linearize and transform into yeast.
(-seeded yeast for tomorrow.)

# 10 July 2012

-miniprepped pCLC007-26. Sent for sequencing.
-placed pCLC007 into BsaI digest for pCLC012 and pCLC013.
-transformed my digest into TG1 cells.

# 9 July 2012

**Yeast Slides**
-I got yeast colonies on all my transformations: yCC008 to yCC011.
-But under the microscope not many of them fluoresced at the right locations... see the Microscopy page for pictures.
-will look at KTR6 at mid log to see if it looks any better. Note to self: it is incredibly dim, and somehow makes the vacuoles really small sized dots.

**Post-Meeting Notes**
-I can drop yCC008 and yCC009 (Cell periphery TPO3), because Austin has found a signal sequence that works pretty well.
-I am now the person responsible for making the rest of the cassettes... once we all finish our cassettes and compare our different proteins per organelle, we'll pick on the best protein for each organelle, and I'll assemble them into the cassettes that we need.

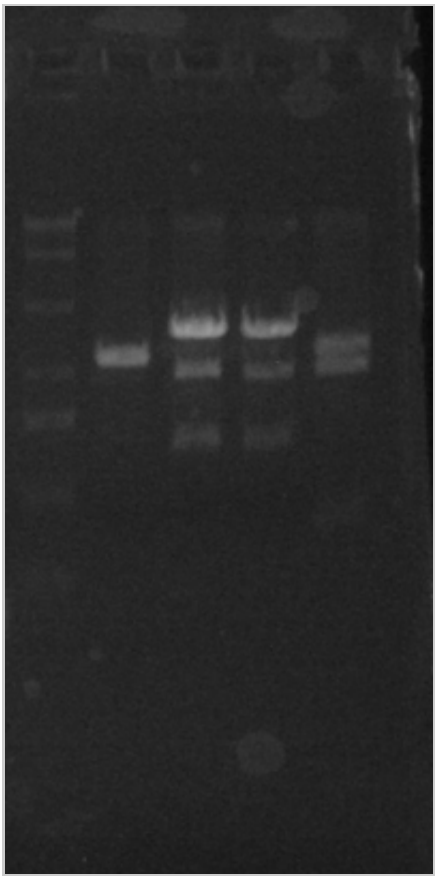
**Miniprep**
-was miniprepping pCLC007-26 today, then found out I didn't have enough culture. Seeded a new culture, in the shaker. Will miniprep tomorrow.

# 7 July 2012

**Sequencing Results for pCLC007-5**
-turned out that pCLC007-5 was still wrong: primer dimerse again! Even the test digest looked right, it's still hard to tell if gene was correctly inserted. The top band would be the same size, and only the smaller band can tell you if both halves of the gene was inserted correctly. Upon looking more carefully at my gel, the small band looks more like 290 bp (which is the pCLC007 version that is missing the first half of CIS3) than 460 bp, which is what we were looking for.
-had picked 4 more colonies from pCLC007 plate this morning, while I did the BsaI digestion thinking pCLC007-

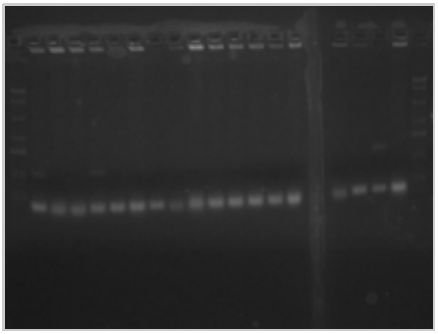
5 was correct.
-since pCLC007-5 still turned out wrong, doing minipreps of pCLC007-6 to pCLC007-9.
-also doing colony pcrs of the rest of the colonies on pCLC007 (all of them!) pCLC007-10 to pCLC007-27.

**Minipreps and Test Digests**
-for colonies pCLC007-6 to 9... this time I test digested them with enzymes with restriction sites in the first half of CIS 3 that never makes it into the plasmids. Then we have both the fragment sizes and number of bands to help us out.
-Enzymes SalI and NcoI. If the gene was inserted correctly, we'll see 1406, 588, and 351 bp fragments. If the gene is missing the first half, then we'll see only 1406 and 769 bp fragments.



From left to right: ladder, pCLC007-6 to 9.

**Colony Pcrs gel**
-ran colony pcrs and ran gels to see the products.



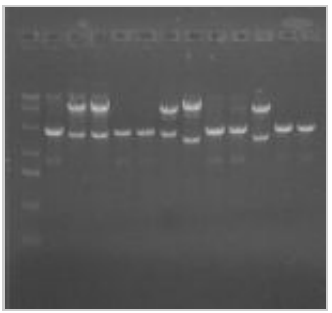
From left to right: ladder, pCLC007-10 to pCLC007-27, ladder. Note there's 2 gels.

-If the plasmid was incorrect (ie only has the the 2nd half of the gene CIS3), the pcr product will be about 540 bp. If both halves of the gene is present, the pcr product will be 725 bp long. Looking at the gel there are 2 colonies that have 540 bp pcr prdts, plenty with nothing at all, but...
-It looks like pCLC007-26 might actually be right! Will miniprep the culture (right now is saturating in a shaker box) on Monday, send for sequencing, and then do a BsaI digest using the correct plasmid.

# 6 July 2012

**Cassettes**
-ran test digests on all my minipreps: I had three of each pCLC008, pCLC009, pCLC010, and pCLC011.
-results look good: at least one colony from each plasmid digested correctly. We can now go ahead with the yeast transformations.

| | pCLC008 | pCLC009 | pCLC010 | pCLC011 |
|---|---|---|---|---|
| **Enzymes** | SacI, XhoI | SacI, XhoI | BglII, XhoI | BglII, XhoI |
| **Fragments** | 5797, 2410 | 5085, 2392 | 5609, 2070 | 4897, 2050 |

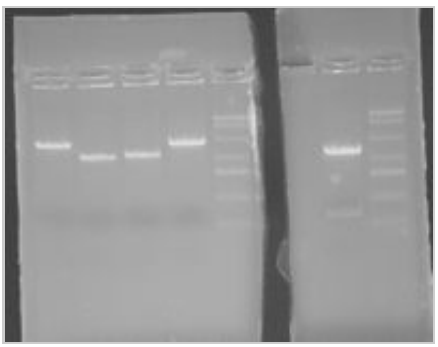


Test Digest Gel. From left to right: ladder, pCLC008-1 to 3, pCLC009-1 to 3, pCLC010-1 to 3, pCLC011-1 to 3.

**Transformations**
-linearized plasmids: pCLC008-3, pCLC009-3, pCLC010-1, pCLC011-1.

-back diluted the yeast culture this morning.

**Minipreps, TD, and Sequencing of 3a CIS3**
-picked colonies at 10am. Will miniprep and sequence later in the day.
-Once minipreps are finished will test digest the plasmids; using BglII and ScaI. ScaI site is in the vector, BglII site is in the CIS 3 gene insert.
-Correct plasmids should be linearized into fragments sized 1885 and 460.
-Results: looks like only one of them digested correctly: pCLC007-5. Sent pCLC007-5 for sequencing.

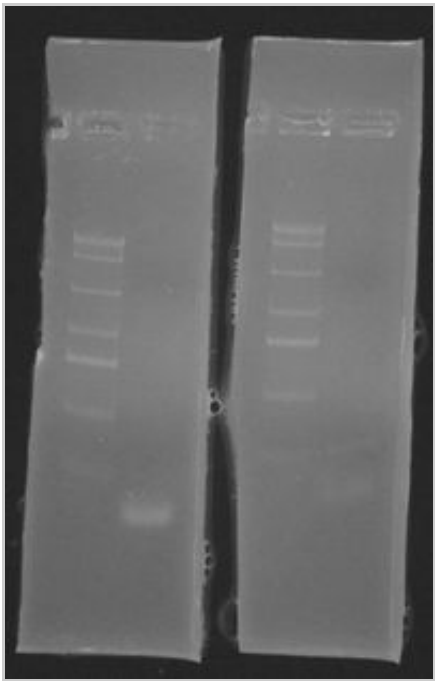


Left gel: pCLC007-1 to 4, ladder.
Right getl: pCLC007-5, ladder.

# 5 July 2012

**Cassettes**
-Picked colonies for pCLC008,9,10,11. pCLC012 and 13 had the wrong 3a part anyway.
-Did two rounds of minipreps: one in the morning for the correct colonies I had picked on Tuesday night, and another in the afternoon today.
-Finished minipreps. Will test digest tomorrow.

**Fixing 3a part (CIS 3 Vacuole) for pCLC0012,13**
-From the colony pcrs on Tuesday, I need to start from scratch in making the CIS 3. The product should be 228 bp long, which is right around where the primer dimers like to be... so I'll run the gel for a very long time until they're entirely separated. Most likely the problems I had during the first round were due to some primer dimers in my gel purifications.
-finished pcr and ran the gel to purify it. At first I couldn't see the 200 bp ladder mark, and couldn't see my pcr products either. pcr product should have been 228 bp. Followed Will's advice to soak the gel in EtBr for 15 minutes then took another picture. This time I saw both the 200 ladder and the pcr product. It worked only for Expand polymerase; phusion pcr failed.



Left gel: Phusion polymerase, Right gel: Expand. Both are ladder, pcr product.

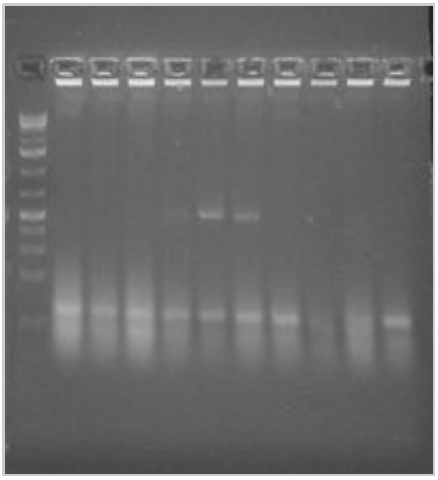
-gel purified, and put them along with CIS 3 part 2 into the BsmBI digest. Robert transformed them for me.

**Yeast Transformations**
-Hopefully test digests of the cassettes will come out right tomorrow, so then we can go ahead and transform them into yeast.
-Test digests: pick something that is in the insert. Say BglII and Xho is what Will usually picks.

# 3 July 2012

**Sequencing Results**
-pCLC005 looks good. There is a silent mutation near the end of the protein gene, and there is another mutation that looks like a bad read from sequencing.
-pCLC007 is giving me problems. The first 200 bp of the gene is missing, while the 2nd half of the gene (after the internal restriction site) is perfectly accurate. Most likely some primer dimers found its way into my gel purified DNA of the 1st half of the gene (before the internal BsaI site) and made it into the Ecoli cells.
-To make pCLC007 correctly, I have 2 choices: either colony pcr and find a colony that has the entire CIS 3 protein, or start from scratch and redo the pcr for the 1st half of CIS 3. Right now I'm doing both in parallel. If I find a correct plasmid in the colony pcr, than I can drop the other pcr.

**pCLC007 (CIS3) colony pcr results**
-If there were issues like pCLC007-1, the pcr product should be about 540 bp long. If there is a correct gene insertion, the product should be about 700 bp.
-After running gels on the pcrs, it looks like none of them are right... pCLC007-5 and pCLC007-6 are at 540, which means they're not correct either. Most likely the pcr product for the first half of CIS 3 never worked.



Gel of Colony pcr for pCLC007 CIS3. From left to right: ladder, pCLC007-1 to 10.

**Transformations**

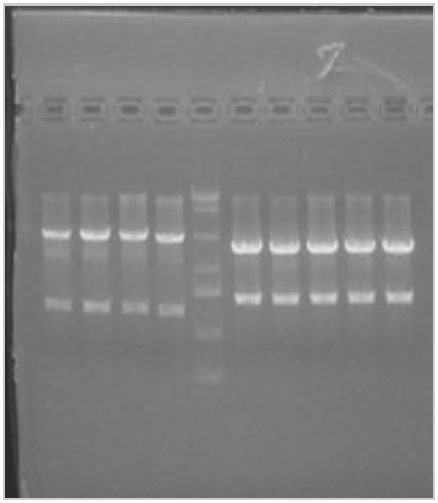
-Yesterday I had already gone ahead and digested with BsaI. This morning before sequencing results came in I transformed pCLC008, pCLC009 ... pCLC013 into ecoli cells. We now know that pCLC012 and pCLC013 will be wrong. We can keep the other 4. Even numbers are the Venus strains, odd are the mKate strains.

# 2 July 2012

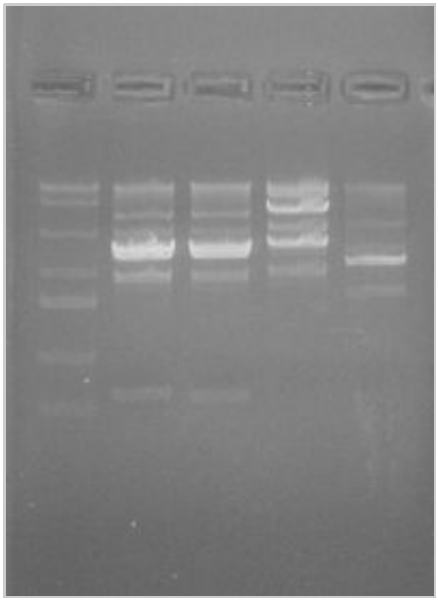
**Test Digests**

-redoing my test digest that didn't work on Saturday. This time I used 2 different enzymes for each protein, and made sure one was an internal restriction site.

| Plasmid | Enzymes | Expected fragments |
|---|---|---|
| pCLC005 | BglII, NcoI | 2811, 720 |
| pCLC006 | PstI, NcoI | 2161, 841 |
| pCLC007 | BglII, SacI | 1926, 419 |



From left to right: pCLC005-1 to 4, ladder, pCLC006-1 to 5.



From left to right: ladder, pCLC007-1 to 4.

**Sequencing**
-sequencing results looked interesting. pCLC005 forward sequencing came out with a point mutation in the oligo itself, but reverse primer never annealed to the plasmid. pCLC006 looks right both forward and reverse sequencing. pCLC007 looks completely wrong.

**Part Assembly**
-since the test digests looked better this time, will start assembling the parts together. Will use a different miniprep of pCLC005, since pCLC005-3 had a point mutation in the oligo. pCLC006-2 looked good in sequencing results, so I'll still use that. pCLC007-4 was completely wrong, and test digests looked funny. In both gels pCLC007-1 and pCLC007-2 looked exactly the same, so we suspect the enzymes didn't digest fully but the plamsids are correct (hopefully). Will sequence while I go ahead with BsaI digest on pCLC007-1. In the end I picked pCLC005-1, pCLC006-2, and pCLC007-1.



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# August 2012 notebook

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## 25 August 2012

-yCC23 troubleshooting: looking back at the test digest the plasmid looks correct. For now will transform one more time. If it still doesn't work I will redo the BsaI digest.
-this time linearized with SacII and EcoRI. Last time I had used PstI and EcoRI. We'll see how it goes this time.

## 23 August 2012

-yCC023 plate didn't yield colonies. Will try the transformation again... not sure where the error is: digestion or maybe the plasmid itself is not correct? Will troubleshoot.
-imaging the Leu and Leu+MOPS buffer yVY015 cells today.
-pH values measured:

| | **Leu** | **Leu with MOPS buffer** |
|---|---|---|
| **Without Cells (mid log)** | 6 | 7 |
| **With Cells** | 6 | 7 |

## 22 August 2012

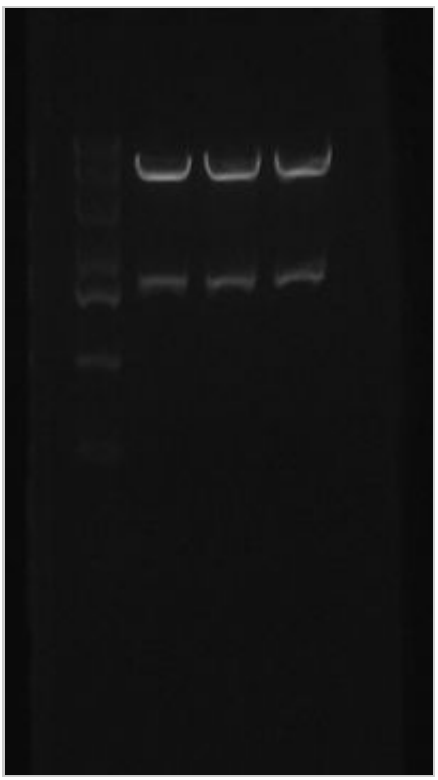
-picked yVY015 (Leu) and yCC023 (Ura) plates. To grow up in different buffers: Leu-buffered, Ura-buffered, Leu and Ura.

# 20 August 2012

-Robert transformed pCLC023. ZRC1 (VM) venus RPL18B Ura.
-will use yCC023 in vacuolar membrane growth conditions: Leu (using yVY015).

# 19 August 2012

-back diluted everything (yVY15, yAJ12, yRC27,31,37,42, yMRY09, yTC03. Will look under the microscope today.
-test digested pCLC023 with SacI and XhoI. If everything is correct it will be fragments sized 5949 and 1006.
Results:



From left to right: ladder, pCLC023-1 to 3.

-MIght??? linearize pCLC23, possibly zymo cleanup, to transform into yeast tomorrow

# 18 August 2012

-couldn't catch yRC42, yVY15, yAJ12 at 0.8 OD... already saturated by 8am. Left it to back dilute tomorrow.
-miniprepped the pCLC23 plate instead. 3 colonies.

# 17 August 2012

-picked from glycerol stocks yRC27, 31, 37 and growing up to .8 OD to look under the microscope.
-picked from plates yRC42/pRC50, yVY15/pVY09, yAJ12. Hoping to catch them tomorrow at 0.8 OD in the morning to image them.
-Note that yRC26/pRC29 did not yield any yeast colonies.

# 16 August 2012

-made glycerol stocks of pCLC021, colony #4.
-ran BsaI digest for pCLC023: which is ZRC1 (Vacuolar membrane), RPL18B, Venus, Ura. For testing vacuole size growth conditions.
-electroporated into e coli... took only 1 uL so if that doesn't work will transform the other 9uL by heat shock and see if that works...

# 14 August 2012

**Promoter Library**
-need to transform pVY009 for yVY015, and pAJ12 for yAJ012. These will be imaged along with the others on Friday. The others are already in the shaker, so they need to be backdiluted on Thursday evening, and again Friday morning to get to 0.8 OD mid log.

What I transformed today (includes some of Robert's promoter characterization things:

| Plasmid Name | Description | Strain Name | Selective Media |
|---|---|---|---|
| pVY009 | ZRC1 VM-Venus-RPL18B | yVY015 | Leu |
| pAJ012 | CIIC CP-Venus-RPL18B | yAJ012 | Leu |
| pRC029 | pTEF1-ABP1 | yRC026 | Leu |
| pRC050 | pCYC1-Venus | yRC042 | Leu |

**Imaging yCC021**
-picked colonies #1-5 again, grown to saturation and back diluted to be imaged again at mid-log.

**Vacuole Size Experiments**
-planning to play around with growth conditions that might affect vacuole size. Sometimes the vacuoles grow up to be numerous tiny dots; and we want to find out why.
-plan to make new cassettes with both Leu and Ura selectivity? or not...
-things to test: growing yeast in SD-Leu, SD-Ura, both with and without pH buffers.

# 13 August 2012

**Addressing Integration issues: pCLC021**
-we now think it might have been variability due to picking straight off the plate. So will grow colonies 1-5 to mid log and then look again. Then streak new plate and make a glycerol stock of the right one. We do know that pCLC020 worked in colony #3.

**Promoter library**
-we have most of the data for the promoters, but backgrounds don't look consistent in the pictures. Will retake the pictures.

**Microscope Issues**
-something is up with the microscope: the laser lamp looks very dim. We suspect one of the lightbulbs are out. If that is the case I should grow up anything yet until the problem gets fixed. For now will assume that everything still works.

| Organelle | Promoter 1 (Strain 1) | Promoter 2 (Strain 2) |
|---|---|---|
| Cell Periphery | TEF1 (yRC31) | RPL18B (yAJ012) |
| Nucleus | RPL18B (yMRY09) | RNR2 (yRC37) |
| Actin | RPL18B (yTC003) | RNR2 (yRC27) |
| Vacuolar Membrane | RPL18B (yVY015) | |

-we already set VM to use RPL18B. Stronger promoters are inconsistent or fill up the entire vacuole. Weaker ones are too weak to see anything.

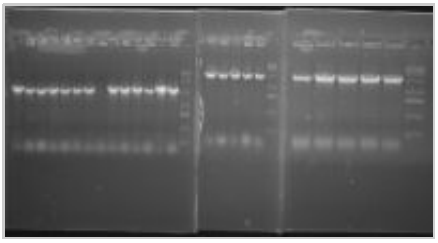
-will need to come in tomorrow to remake or find AJ12... couldn't find it in the glycerol stocks... T_T

# 6 August 2012

-going to visualize yCC021 again, this time with colony 4 and 5.
-last time (on Thursday) we looked at colony #3 for both yCC020 and 21. yCC020 finally looked right, yCC021 again looked wrong... too much GFP diffuse everywhere.

# 1 August 2012

-did a colony pcr of the yeast colonies, to see if the integration efficiency was the culprit of the strange microscopy results. pCLC020 had diffuse GFP everywhere, no organelles targeted, and only very few with nucleus targeted. pCLC021 had only nucleus targeted with GFP, though maybe nucleus was too bright and the microscope did not pick up any other fluorescence.
-colony pcr 12 colonies from each plate. From 0 (being the colony picked for visualization) to 11.
-sent plasmids pCLC020-2 and pCLC021-2 to sequencing. See the google doc for specific names of sequencing primers.
-fragments expected (used V24 and AI03 primers for colony pcr): 3269 for pCLC020, 3287 for pCLC021.
-gel came back perfect. Now it's a mystery. Maybe the multigene cassette is wrong?



From left to right: pCLC20-0 to pCLC020-11, pCLC21-0 to pCLC021-11. Not the ladder is always on the right most well of each gel.



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# September 2012 notebook

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## 2 October 2012

Running test digests on the bsmbi reactions pCLC47, 48, 52 and 53. Test digesting details:

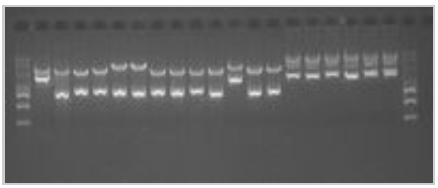
| | | |
|---|---|---|
| pCLC47 | XmaI, SacI | 7084, 5759, 1711 |
| pCLC48 | XmaI, SacI | 7084, 5759, 1711 |
| pCLC52 | EcoRI, SacI | 5526, 1711, 548 |
| pCLC53 | EcoRI, SacI | 7280, 1711, 548 |

## 28 September 2012

-picked yCC47 and yCC48 (pCLC47 and 48) off the plate and imaged them out of curiosity. The images didn't look right, so I decided to test digest the plasmids again: 24-2, 25-2, 27-2, 28-1, 32-1, 32-2, 33-1 to 33-4, 34-1, 47-1 to 47-3, 48-1 to 48-3. The enzymes and fragments are below:

| Plasmid Name | Enzymes | Fragments |
|---|---|---|
| pCLC24 | NdeI, SpeI | 3543, 2142 |
| pCLC25 | NdeI, PstI | 3373, 906 |
| pCLC27 | SacII, PstI | 3273, 1000 |
| pCLC28 | SacII, PstI | 3604, 1000 |
| pCLC32 | SacII, PstI | 4703, 1000 |
| pCLC33 | SacII, PstI | 3227, 1000 |
| pCLC34 | SpeI, PvuI | 4175, 1806 |
| pCLC40 | SacII, PstI | 3283, 1000 |
| pCLC41 | SacII, PstI | 3604, 1000 |
| pCLC47 | XmaI, SacI | 7084, 5759, 1711 |
| pCLC48 | XmaI, SacI | 7084, 5759, 1711 |

**Test Digest Results**
-adding more Gel Green fixed the problem of really faint bands. The concentration was 5X (so 10uL added to the 50mL tube)

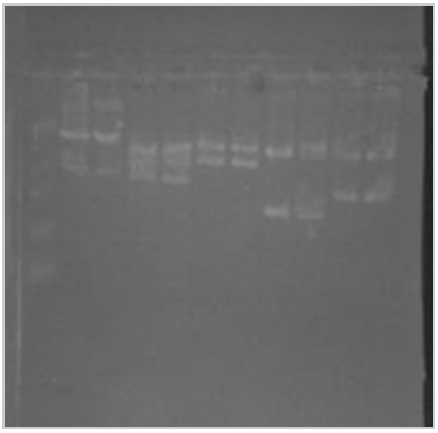


From left to right: ladder, pCLC24-2, 25-2, 27-2, 28-1, 32-1, 32-2, 33-1 to 33-4, 34-1, 47-1 to 47-3, 48-1 to 48-3, and ladder again.

Everything came out right except for the pCLC47 and 48 cassettes. so I will remake them tonight... will also remake pCLC052 and pCLC053. Recall that pCLC052 is the RNR2-nucleus-venus_CP-Venus, (pCLC32 and 33), while pCLC053 is RNR2-nucleus-venus_Actin-Venus (pCLC32+34).

# 27 September 2012

Test digest results: Gel green is making life very difficult... the DNA may be degrading? Super faint bands, but I think they were successful. I am only skeptical of pCLC33-3 and 4, so I will use the minipreps I made last time (pCLC33-2). pCLC32 also worries me, but based on both this gel and last time's gel (9/22), it might be again Gel green that is causing that strange ghost of a 3rd band.



From left to right: ladder, pCLC32-1 and 2; pCLC33-1 to 4; pCLC34-1 and 2; pCLC40-1 and 2; pCLC41-1 and 2.

- Yes they are very faint... was a bad gel because I didn't use enough Gel Green. See 9_28 for a test digest of the same minipreps.

-moving ahead to the bsmbi cassettes. To use: pCLC32-2, pCLC33-2, pCLC34-1, 40-1 and 41-1.

# 25 September 2012

-miniprepping 33, 40, 41.
-test digests for today:

| Plasmid Name | Enzymes | Fragments |
|---|---|---|
| pCLC32 | SacII, BamHI | 4283, 1420 |
| pCLC33 | PstI, BglII | 2740, 1487 |
| pCLC34 | SacII, NdeI | 3753, 2228 |
| pCLC40 | PstI, NdeI | 3376, 907 |
| pCLC41 | PstI, NdeI | 3376, 1288 |

Test digested for 20 minutes. Did not have time to run a gel will do that later (tomorrow?)

because we don't know yet if 32 and 34 actually worked. I'll probably go ahead and heat shock the new bsaI that I made last night.

# 24 September 2012

-picked colonies again for pCLC33, 40 and 41. The last tubes were in the shaker for too long.
-since the TD for 32 and 34 didn't look quite right, I redid the bsaI digest. Will heat shock tomorrow, and maybe re-test digest the old minipreps with new enzymes just in case they are right and I used bad enzymes. We would like this to be done, so we can put it onto the wiki before the wiki freeze (next Wednesday!)
-linearized pCLC47-2 and 48-1. These are nice looking PCodes that we can put in our presentation and onto the

Wiki. Masaki is doing the Yeast transformation for me.

The schedule for the next few days /general notes to self of what to do next: Tomorrow: miniprep 33/40/41... TD, heat shock 32 and 34 if necesssary. then bsmbi for nucleus exp.

Then bsmbi for the nucleus all colors experiment for thomas. Get someone else to electroporate for you in the afternoon?

Wednesday pick colonies Thursday miniprep (32,34, as well as 49 and 50). TD, linearize, transform 49-50. TD, linearize and bsmbi 32, 33, 34.

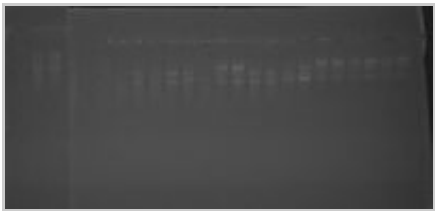
# 22 September 2012

-heat shocked pCLC33, 40-41 into tg1 cells in the morning. Will pick colonies later in the day (4pm?)
-test digested the minipreps:

| Plasmid Name | Enzymes | Fragments |
|---|---|---|
| pCLC24 | SacII, XhoI | 4274, 1411 |
| pCLC25 | SacII, XhoI | 2868, 1411 |
| pCLC27 | SacII, XhoI | 2862, 1411 |
| pCLC28 | SacII, XhoI | 2871, 1733 |
| pCLC32 | SacII, XhoI | 4292, 1411 |
| pCLC33 | PstI, BglII | 2740, 1487 |
| pCLC34 | SacII, XhoI | 4241, 1740 |
| pCLC47 | XmaI, SacI | 7084, 5759, 1711 |
| pCLC48 | XmaI, SacI | 7084, 5759, 1711 |

Note to self: pCLC47 is Pcode 2, pCLC48 is Pcode 3, pCLC49 is Pcode 1 (following the table above). pCLC49 was put on hold because one of the parts was not made yet (pCLC42).

**Test Digest Results**
Most of them look pretty good. The ladder is incredibly dim, thanks to Gel Green. But because they look consistent across the minipreps I can pretty much use the minipreps themselves in comparison with each other.



From left to right: ladder, pCLC24-1 and 2. (Next gel:) ladder, 2 colonies from each of pCLC25, pCLC27, pCLC28, pCLC32, pCLC33, pCLC34. 3 minipreps each from pCLC47 and 48.

- This gel looks terrible because I didn't use enough Gel Green. See 9_28 for a clean and visible test digest of the same minipreps.

The ones that are usable for bsmbi: 24-2, 25-2, 27-2, 28-1, 33-2. 47-2 and 48-1 are ready to be linearized and transformed into yeast. For 34 SacII and XhoI turned out to be bad enzymes to use. But since 32 and 34 are both giving me trouble, will probably put the venus-nucleus-RNR2 experiment on hold and maybe make new cassettes.

# 19 September 2012

-redoing pCLC33 BsaI digest because the transformation didn't look that successful. Also making pCLC040 and 41, which are essentially pCLC26, 29-31 with mTurquoise instead of our old CFP. Except we don't need the RPL18B versions (we already know to use RNR2 since mTurquoise is so bright).
-picked colonies for: pCLC24,25, 27,28, 32-34, and pCLC47-48 (last two are bsmbi digests).

# 14 September 2012

Checked on my cassettes: pCLC024-pCLC039. For a variety of multigene experiments:
-targeting nucleus with all colors.

-all 5 (venus, mKate, CFP, PAmCherry, PAGFP)
-without photoactivatable fluorophores (venus, mKate, CFP)

-a nice looking micode for the presentation.

The new CFP looks much better than the old one. We definitely need to use that in a new micode that looks nice enough for the presentation.

To Do: figure out the right promoters for CFP in each organelle. For the most part RPL18B should work... maybe some organelles might be too bright? Just image CFP targeted to each organelle, and analyze the image for the range of brightness. Masaki has already made new CFP targeted to peroxisome, cell periphery, vacuolar membrane. We still need to try it on nucleus (both with RPL18B and RNR2), and actin.

**Presentation micode**
We want to keep nucleus separate from cell periphery, and nucleus separate from vacuolar membrane.
So the ones we'll choose is:

| | Part 1 | Part 2 | Part 3 | Part 4 |
|---|---|---|---|---|
| Pcode1 | *CP-venus* | VM-CFP | *Actin-venus* | Nucleus-mKate |
| Pcode2 | *CP-venus* | *VM-venus* | Actin-CFP | Nucleus-mKate |
| Pcode3 | *CP-venus* | *VM-venus* | Actin-mKate | Nucleus-CFP |

Italics means I've already made it in the past. CP-venus is pCLC014, VM-venus is pCLC035, and Actin-venus is pCLC019.

So I need to make another 5: labelled pCLC042-46 in my planner.

**Nucleus multicolor** For the nucleus experiment; we want to use the new CFP. So I am redoing the BsAI digest for the CFP cassettes, before we assemble them into multigene cassettes. I am making one multigene with and one without the photoactivatable colors. These are basically pCLC040 and 41... (nucleus with the new CFP) later we will need to

Other things to do: test RNR2 with Venus, and gather data on CFP promoters.



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