Harneet91 29 June 2012 (PDT)

- transformations worked. Put plates in Will's plate area

Harneet91 28 June 2012 (PDT)

- transformed 609/611-615 into TG1 (integration vectors with NotI linearization site)

Harneet91 22 June 2012 (PDT)

- Check yHSR005-008 transformation plates. Colonies very small so left in incubator
- Discussed primer design for Golden Gate cloning scheme

Harneet91 21 June 2012 (PDT)

- met with Cinvestav team in morning. A collaboration doesn't seem to make sense currently. Here are their project details. Cinvestav Notes

Harneet91 20 June 2012 (PDT)

- digest pHSR0002 using NotI-NsiI in buffer 3
- backdiluted and prepped yHSR002 for transformations
- did yHSR005-008 transformations and plated on SD-LU plates

Strain Name	Description	Parental Strain	Plasmids Used	Marker
yHSR005	(RPL18B-SEC63-mKate) + (RPL18B-SEC63-Venus)	yHSR002	pHSR0002	LEU (URA)
yHSR006	(RPL18B-SEC63-mKate) + (RPL18B-VacMbrn-Venus)	yHSR002	pVY004	LEU (URA)
yHSR007	(RPL18B-SEC63-mKate) + (RPL18B-Actin-Venus)	yHSR002	pTC004	LEU (URA)
yHSR008	(RPL18B-SEC63-mKate) + (RPL18B-NuclearPer-Venus)	yHSR002	pRC001	LEU (URA)

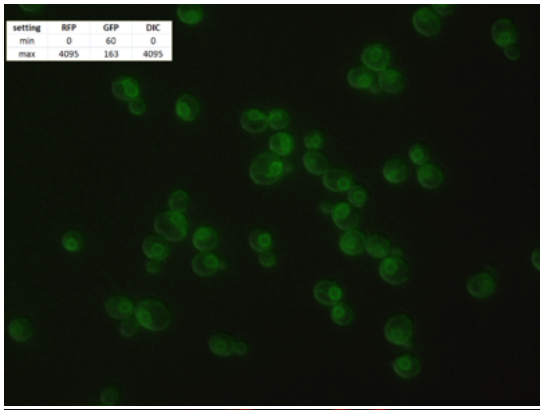
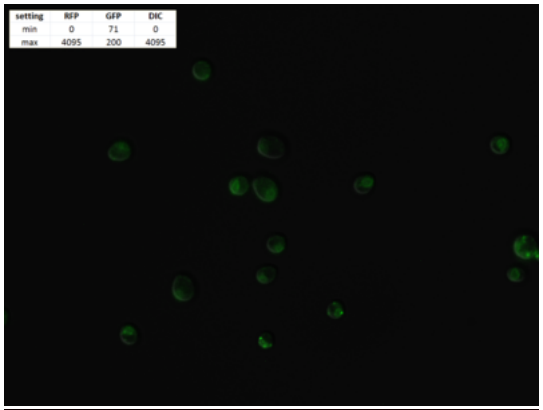
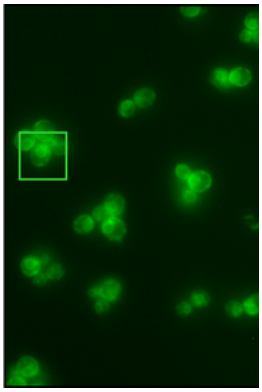
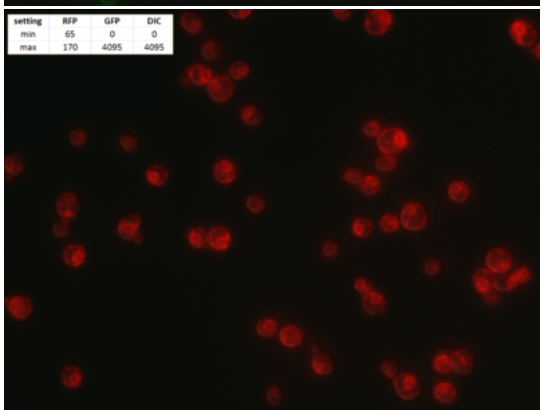
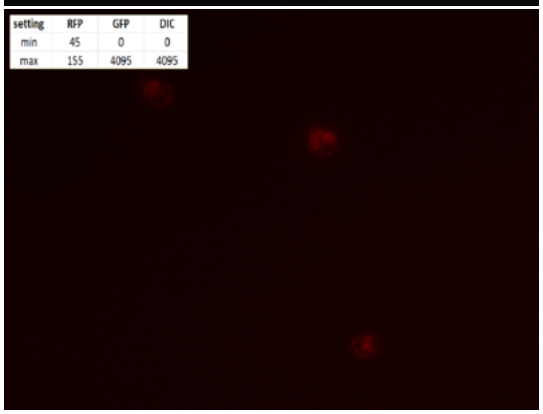
Harneet91 19 June 2012 (PDT)

- backdiluted saturated yHSR001/002 cultures too OD 0.2 and let the saturated cultures growing
- took pics of saturated and midlog (OD~0.6) samples of yHSR001/002
- picked yHSR002 into YPD for transformations tomorrow

strain/cond	setting	RFP	GFP	DIC
yHSR001m	min	0	60	0
	max	4095	163	4095
yHSR001s	min	0	71	0
	max	4095	200	4095
yHSR002m	min	65	0	0
	max	170	4095	4095
yHSR002s	min	45	0	0
	max	155	4095	4095

strain/condition **midlog** **saturated** **UCSF**

yHSR001

yHSR002				N/A
				

Harneet91 18 June 2012 (PDT)

- sequencing for pHSR0002 was good
- backdiluted yHSR002 culture and did following digestions

Plasmid	Renzymes	Buffers
pTC003	NotI-NsiI	B3
pHSR002	NotI-NsiI	B3
pVY004	NotI-SalI	B3

- Didn't do transformations because we decided on a different transformation strategy according to the meeting <br - We decided to look at the strains (both mKate/Venus) under log and saturated conditions. Picked yHSR001/002 into selective media

Harneet91 15 June 2012 (PDT)

- miniprepped more pHSR0002 and pVY004 and sent for sequencing using S16 (for) and O44 (rev)

Harneet91 14 June 2012 (PDT)

- Want to do colocalization transformation today to see if both fluorophores are targeted to locations equally well. We need this for downstream application to make sure that there is no variability in targeted location based on fluorophore
- back diluted yHSR002/004 into 5 mL YPD culture at OD 0.15
- started SacII-NotI digest of pHSR002/004 for integration into yHSR002/004
- purified digests
- we added more transformations to check colocalization

Venus plasmid/mKate strain	Actin	VM	ER
Actin	Y	Y	Y

VM	Y	Y	Y
ER	Y	Y	Y

- picked pHSR0002 (colony 3 from original transformation plate) into LB+Amp media. Also did plasmid transformation in case colony didn't grow up (wasn't that much left on plate to pick)
- new cloning scheme:

Strain	Description	Parental Strain	Plasmids Used
yHSR005	(RPL18B-SEC63-mKate) + (RPL18B-SEC63-Venus)	yHSR002	pHSR0002
yHSR006	(RPL18B-SEC63-mKate) + (RPL18B-VacMbrn-Venus)	yHSR002	pVY004
yHSR007	(RPL18B-SEC63-mKate) + (RPL18B-Actin-Venus)	yHSR002	pTC003

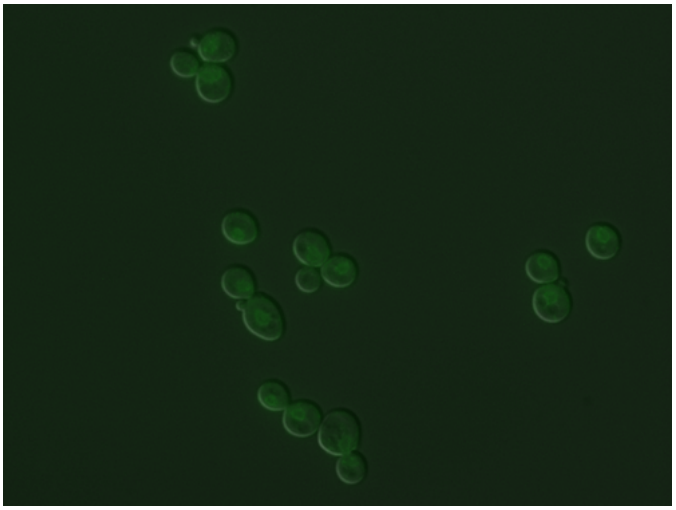
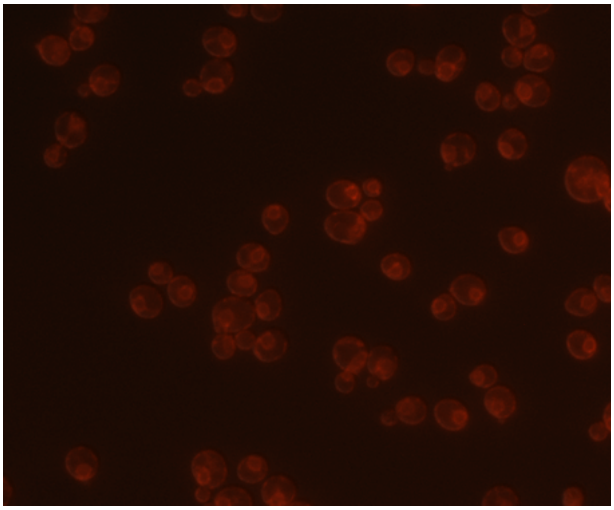
- did yHSR005-007 transformations and plated on SD-LU plates

Harneet91 13 June 2012 (PDT)

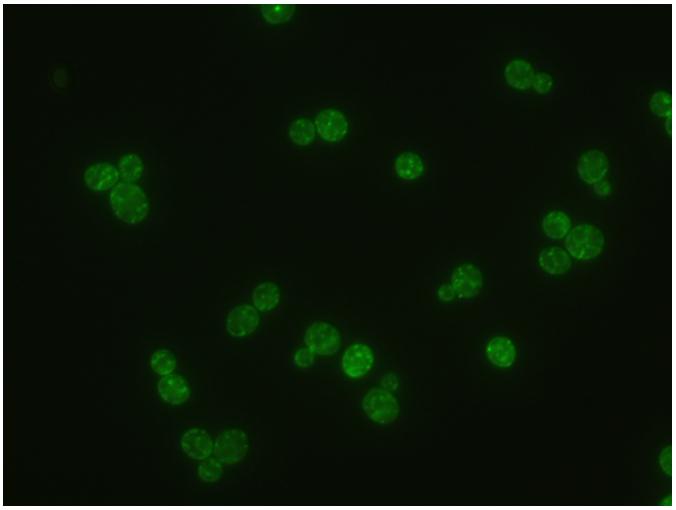
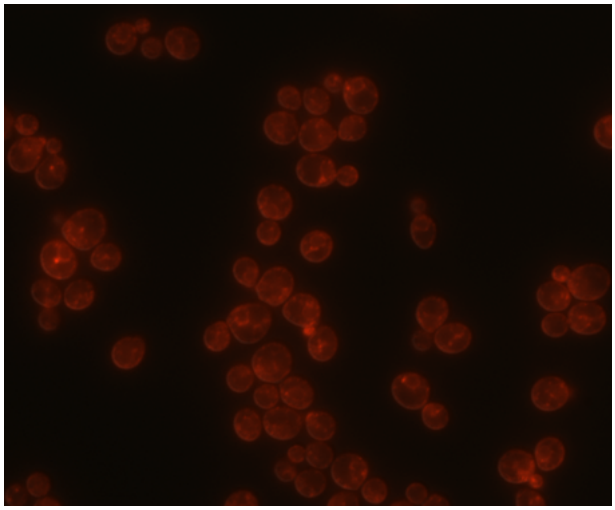
- Robert started cultures of yHSR001-004 for transformation

Harneet91 12 June 2012 (PDT)

- Took pictures of yHSR001-004 again. This time we backdiluted (OD~0.2) a saturated culture and grew up to mid log OD (OD~0.6-0.8) and then took pictures. This afforded consistency across samples and gave better pictures.
- Will showed us how to use Fiji which is basically ImageJ

organelle/fluorophore	Venus	mKate
ER		

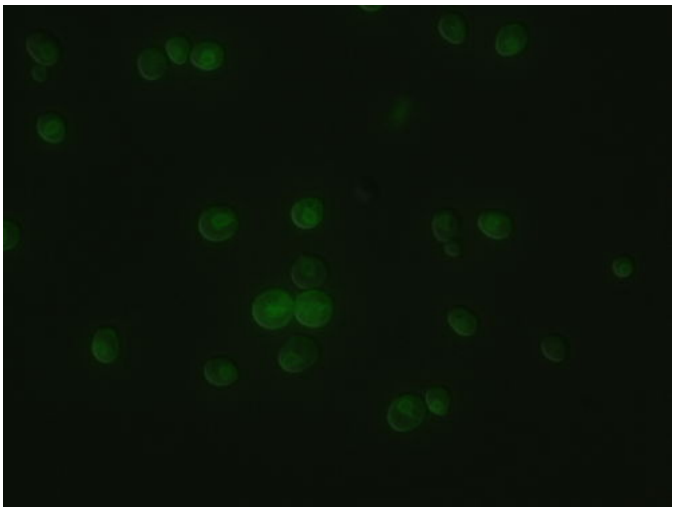
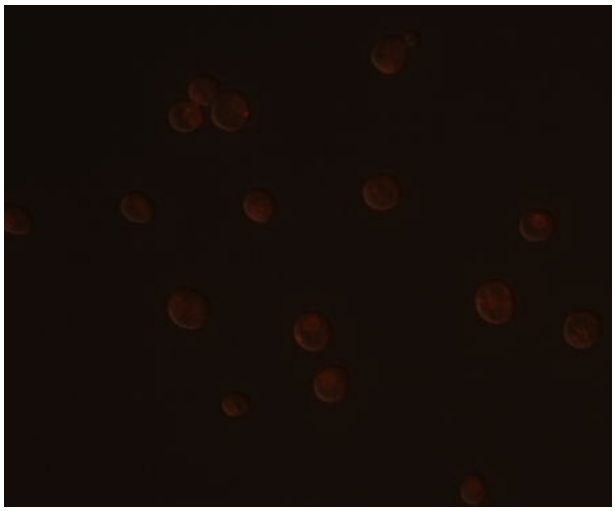
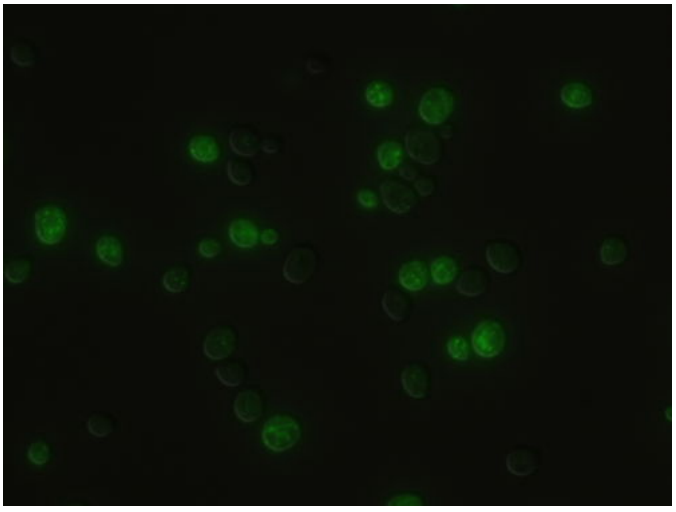
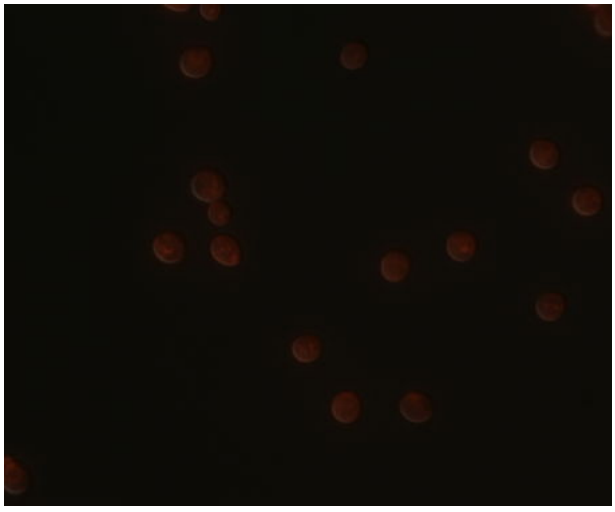
cER

- ER targeting looks good for both mKate and Venus (central ring and ring around periphery). cER targeting looks poor again with punctates present

Harneet91 11 June 2012 (PDT)

- transformations for yHSR001-004 worked
- checked on microscope

organelle/fluorophore	Venus	mKate
ER		
cER		

- targeting overall doesn't look that great. ER-Venus (yHSR001) looks good, but yHSR002-004 show poor targetting. cER protein didn't seem to target well

Harneet91 8 June 2012 (PDT)

- Sent off following for sequencing

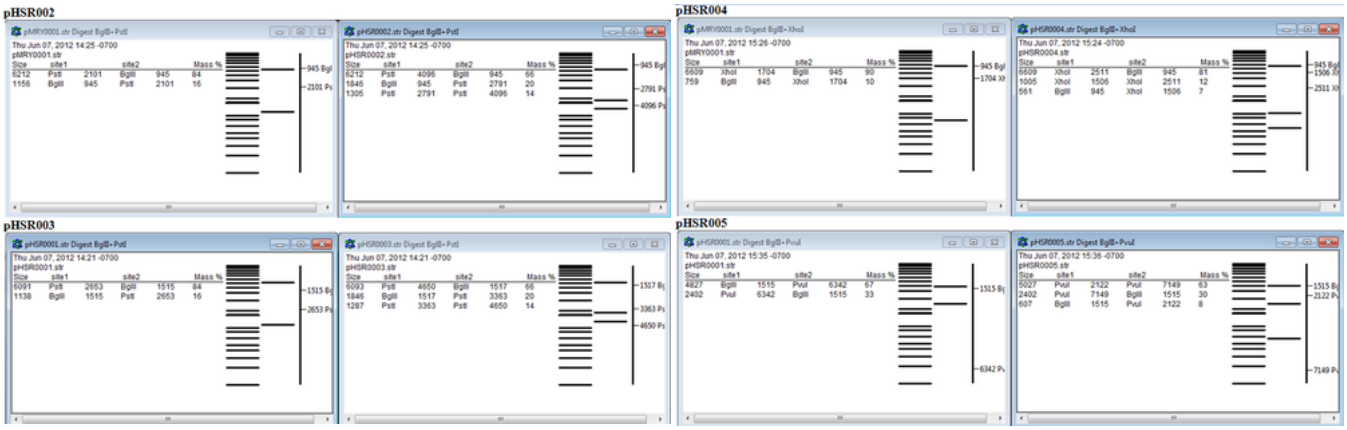
Sequence ID	Plasmid	Clone	Primer
sIGEM035	pHSR0002	3	S16
sIGEM036	pHSR0002	3	AA30
sIGEM037	pHSR0003	2	S16
sIGEM038	pHSR0003	2	AF47
sIGEM039	pHSR0004	1	S16
sIGEM040	pHSR0004	1	AA30
sIGEM041	pHSR0005	1	S16
sIGEM042	pHSR0005	1	AF47

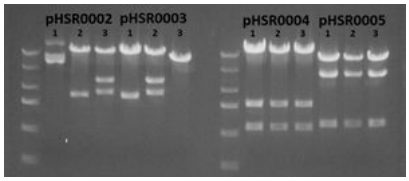
- started SacII-NotI digests for pHSR0002.3, pHSR0003.2, pHSR0004.1, pHSR0005.1
- purified digestion product and transformed into yJD001 (yHSR001-004)

Harneet91 7 June 2012 (PDT)

- transformations for pHSR0002-0005 worked quite well with ratios >2.5:1 based off of negative controls. Picked 3 colonies from each transformation plate into LB+Amp media and put in shaker. Looks like the commercial electrocomp cells did the trick
- miniprepped pHSR0002-0005 clones (1-3)
- Did test digests for pHSR0002-0005

Plasmid	RE1	RE2
pHSR0002	BglII	PstI
pHSR0003	BglII	PstI
pHSR0004	BglII	XhoI
pHSR0005	BglII	PvuI





- looks like pHSR0002.3, pHSR0003.2, pHSR004.1, pHSR0005.1 worked the best so I will send those for sequencing

Harneet91 6 June 2012 (PDT)

- electrocomp transformations didn't work. Didn't have any colonies on positive or neg control either. Since pos ctrl didn't have colonies, it means comp cell prep didn't work. Will suggested using the commercial cells
- transformed pHSR0002-0005 again using commercial cells

Harneet91 5 June 2012 (PDT)

- One of the two 25mL cultures for TG1 hadn't grown, while the other one had
- Backdiluted seed culture into 2 25 mL cultures to OD 0.1
- Apparently the electrocomp cell prep is very quick: grow to midlog, 3-4 glycerol washes, aliquot
- Transformed Gibson products (ER-m, ER-V, cER-m, cER-V) into TG1 electrocompetent cells and let recover in LB media
- Celia and Austin also transformed their gibson product plasmids that had worked so we'll find out on thursday if those transformations are successful

- mKate integrations: URA, Venus integrations: LEU

Harneet91 4 June 2012 (PDT)

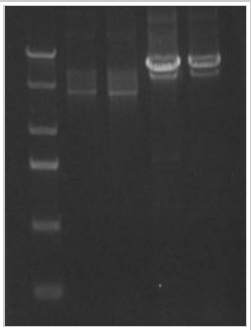
- the transformations with the electrocomp cells worked with good ratios (Note: our plates were left in the incubator a bit long so they had some satellite colonies), but overall ratios were good
- so looks like gibsons may have worked well and we just need to work on increasing transformation efficiency
- grow TG1 cells to midlog in 25 mL culture of LB by pick colony into each
- also none of the combinations for optimizing the pcr for vector linearization worked; however, we don't need this because the electroporation transformations worked
- Since we were running low on pMRY0001, I did plasmid transformation of pMRY0001 into TG1 cells and plated on LB+Amp. Can pick tomorrow along with 2 colonies from pHSR0001

Harneet91 1 June 2012 (PDT)

- Didn't get any colonies on my pHSR0002-005 plates
- We tried to troubleshoot the Gibson:

- pcr vectors from before and after BglII site: ran AD59 as reverse primer for both reactions and for vectors did AU60 (Venus) and AU62 (mKate)

Primer	Sequence	Description
AD59.oML182	TTTGTTTTTTGTTTTCTT	RPL18B LCR probe complement
AU60.iGEM0001	gcatAGATCTggttctggtagtggcagcggcagcggtagtatgtctaaaggtgaagaatt	cloning of link_venus from pWCD0421, forward
AU62.iGEM0003	CGATAGATCTggttctggtagtggcagcggcagcggtagtatggtgagcgagctgattaa	External forward cloning of mKate with GS linker for soeing



2012-06-01 (1-2) pMRY001 (AU60-AD59), pHSR0001 (AU62-AD59) phusion65 pcr

- Did electroporation transformation of reactions: 2 NCs, 2 Venus mixes and 2 mKate mixes

Reaction	Buffer	Temp (*C)
pMRY0001	HF	55
	GC	55
pHSR0001	HF	55
	GC	55

Notes
electroporation gives orders of magnitude higher transformation efficiency than heat shock
- try 2 NCs, 2 Venus mixes (dim, bright band pcr), 2 mKate (dim, bright band pcr) and electroporate those
- cells grow slowly

we need to linearize for Gibson, but BglII cut may religate so if we pcr we can get product that

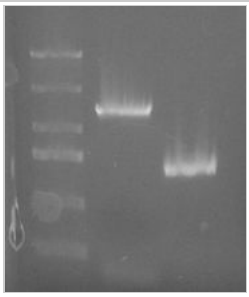
Mung Bean

Harneet91 31 May 2012 (PDT)

- Checked sequencing for pHSR0001. Both forward and reverse sequencing reactions went well. Reverse sequencing run had one silent mutation (GTG->GTT); codon usage for both about the same
- Set up PCRs for ER and cER targeting proteins off of yJD001 gDNA template

Reaction	Organelle	Primer F	Primer R	Product (bp)
1	ER	AU80	AU81	2041
2	cER	AV01	AV02	853

- My pcr didn't work the first time (Phusion 65*C) so I tried it again lowering the annealing temperature to 55*C and it worked with correct products

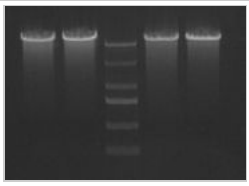


2012-05-31 ER (AU80-81), cER (AV01-02) organelle targetting gibson insert assembly

- I purified the gel fragments using zymo gel purification and as a comparison also purified the original PCR reaction using PCR zymo cleanup

Construct	Purification Type	Nanodrop (ng/uL)
ER	gel pure	13.9
ER	zymo	26.3
cER	gel pure	15.6
cER	zymo	27.4

- I will use the zymo pure products as my insert for gibson since they are at a higher concentration
- Also digested pMRY0001 and pHSR0001 with BglII to linearize vector for Gibson assembly



2012-05-31 BglII digest of pMRY001 (1-2) and pHSR001 (4-5)

- Started gibson assembly by first heating vector/insert for each reaction to 50*C for ~10 minutes and then adding in Gibson master mix
- I transformed each of the Gibson reactions into TG1 cells; I kept ~3.5 uL of each reaction as a backup in case the transformation failed. Also did a negative control Gibson reaction and transformation.

Plasmid Name	Organelle	Description
pHSR0002	ER	GibA-RPL18B-SEC63-6XGSlinker-Venus-TT-GibB
pHSR0003	ER	GibA-RPL18B-SEC63-6XGSlinker-mKate-TT-GibB
pHSR0004	cER	GibA-RPL18B-DPM1-6XGSlinker-Venus-TT-GibB
pHSR0005	cER	GibA-RPL18B-DPM1-6XGSlinker-mKate-TT-GibB

Notes

- put digested vector in thermocycler to inc temp to 55 before adding to mix
- primer dimers

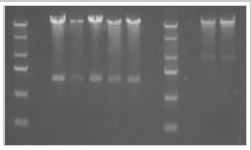
- hard to predict
- there prob is some type of homology
- the fact that on that gel shows that they're all the same length means that they're primer dimmers

- GC buffer (for high GC primers/templates)

- has more magnesium
- GC buffer at 55 and 65 and HF buffer at 55
- can also switch polymerases since phusion has more nonspecific products so can use expand which can give

Harneet91 30 May 2012 (PDT)

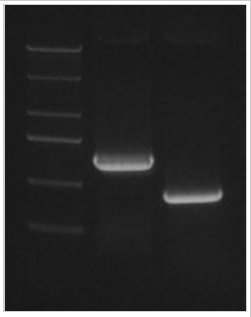
- transformations worked w/ good ratios (>3:1), so picked 2 colonies into LB+Amp media
- miniprepped clones and test digested using SpeI/BamHI. Expect: backbone(5791) and product dropout(1438). Parent (pWCD0480) would give: backbone (5791) and dropout(3472)



2012-05-30 pWCD421-Venus BglXho (1-5) and pWCD480-mKate SpeBam (6-7)

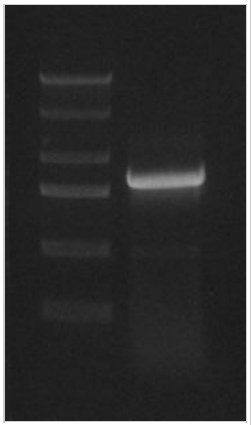
Harneet91 29 May 2012 (PDT)

- check indiv soeing pcr reactions from Friday. Expected ~440 bp from A, and ~760 bp from B.



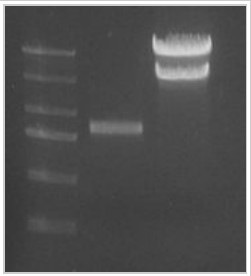
2012-05-29 pWCD480 AU62-65, AU64-63 soeing pcr products

- purified frags A and B together and used as template for final soeing step using external primers (AU62, AU63)
- final soeing pcr reaction worked w/ correct expected size of ~1150 bp



2012-05-29 pWCD480 final soeing product AU62-63

- digested pWCD0480 (vector) and soeing product (insert) with BglII-PstI

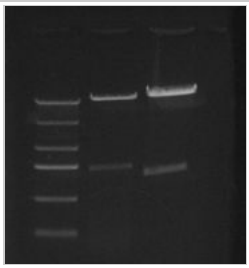


2012-05-29 pWCD480 final soeing product, vector digest BglII-PstI

- purified digest fragments, did ligation and negative control and plated on LB+Amp plates

Harneet91 25 May 2012 (PDT)

- test digested pWCD0504 and parent plasmid (pWCD0463) with BglII/XhoI. Parent dropout ~900 while PAGFP dropout should be ~720 which is what we see



2012-05-25 pWCD0463 (parent), pWCD0504 (PAGFP) BglII-XhoI test digest

- sent pWCD0504 off for sequencing using reverse primer O44
- Designed primers for gibson assembly of gene parts for phusions. The primers I designed (AU80-AU83) are for ER localized protein (Sec63p) and cortical ER localized protein (Dpm1p). Here is the work for designing the primers: 2012-05-25 Gibson gene assemblies primer design
Here's the general format for the gibson primers:

Forward:
(20bp prom end hom)-BglII-(20bp gene front hom)
(20bp prom end hom)-AGATCT-(20bp gene front hom)

Reverse:
(20bp gene end hom w/o stop)-scar-5Xlinkerhom (take reverse complement)
(20bp gene end hom w/o stop)-GGATCT-5Xlinkerhom (take reverse complement)

- The oligos for making the linker-XFP constructs in pWCD0421 and pWCD0480 came in, so started those PCR reactions. Set up the following PCR reactions:

Reaction	Product	Primers	Description
A	759	AU62	External forward cloning of mKate with GS linker for soeing
		AU65	Internal reverse cloning of mKate with GS linker for soeing
B	437	AU64	Internal forward cloning of mKate with GS linker for soeing
		AU63	External reverse cloning of mKate with GS linker for soeing

Harneet91 24 May 2012 (PDT)

- FAGFP transformation only had one colony, so we picked it into LB+Amp media. Miniprepped after culture had grown to saturation.
- Will gave us vectors for Leu and Ura integration into which we'll add in our genes to target mKate and Venus. We'll be using a GS linker to . Here's the primer design for the Venus vector: 2012-05-24 mKate, Venus vector and insert design (note: this document has been minor updated since upload)
- Need to design primers for Gibson assembly which will combine pcr product (gene) with the vectors (linearized using BglII). We want the final construct should be promoter-BglII-gene-6XGSlinker-XFP
- For finding proteins to use we will each find two proteins that localize to the specific locations. I'm looking for ER and cortical ER proteins

Harneet91 24 May 2012 (PDT)

- The yeastGFP database uses colocalization of RFP/GFP to double check that the hits they get for localization are actually correct
- If you select ORF then you can get table which shows genes that are targetted to the organelles. Also get checks w/ additional letters->don't want that. only use checks.
- I'm looking up ER and cortical ER localization

Harneet91 23 May 2012 (PDT)

- Here's what I have so far for targeting: Targeting notes for cortical ER, nucleolus, microtubule

'	Initials	Description	full protein sequence (retrieve sequences from S.c. Reference strain S288C)	or signal sequence	N/C terminus?	shown to work with FP?	comments about specificity to organelle	picture of fluorescent protein in yeast cell	Source
							yeast cortical ER closely apposed to		

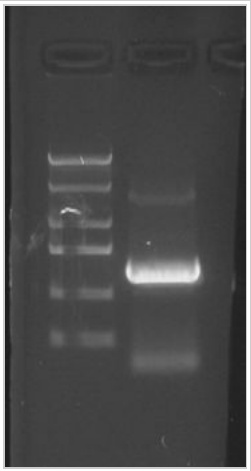
Cortical ER	HSR	cortical sorting signal (CSS) of Ist2. CSS is C-terminal region. CSS is 69AA long	Ist2 (integral membrane protein); ~2800 bp		C	Yes. mCherry-Ist2	PM and forms contacts w/ distance of 30 nm or less->very diff to distinguish between localization at cortical ER and PM	Fig1 from source	http://jcs.biologists.org/content/122/5/625.long
	HSR	Dpm1p (resident ER protein) showed localization at cortical ER->acts as ER marker protein	Dpm1 gene (804 bp)		N	Yes. Dpm1p-CFP	localizes to cortical ER and use as ER marker protein	Fig1Aa from source 1	http://ac.els-cdn.com/S0960982204000910/1-s2.0-S0960982204000910-m _tid=6a28d8311f0122146149da67380cc724&acdnat=1337765627_89082
microtubule	HSR	N-terminally fuse (X)FP to tubulin	TUB1 (alpha-tubulin) gene: ~1460bp		C	Yes. GFP-tubulin	not deletrious to microtubule formation (spindling event)	Fig 3BC, Fig 4 from source 2	http://cshprotocols.cshlp.org/content/2010/9/pdb.top85.full.pdf
Nucleolus	HSR	SV40-S6A(74–135)->62 AA w/ 11 positive charges	S6A (yeast ribosomal protein), SV40 (large T antigen)	SV40-S6A(74–135)	N	indirect immune staining	localize to nucelolus	Fig 1B from source	http://www.sciencedirect.com/science/article/pii/S0006291X05012428
	HSR	S6A(174–213)->40 AA	S6A (yeast ribosomal protein)	S6A(174–213)	N	indirect immune staining	localize to nucelolus	Fig 2A from source	http://www.sciencedirect.com/science/article/pii/S0006291X05012428

Work
- Masaki found a yeast database where researchers from UCSF had targeted every protein in yeast to a certain organelles/locations. Proteins were tagged with GFP. site: http://yeastgfp.yeastgenome.org/ Can find info about oligos, methods, constructs, localization images under info and/or faqs
- Will gave a new vector to use for ligation. The one we had digested the way before was only for practice. Did gel purification for PAGFP insert.
- Ligated vector+inst along w/ neg.ctrl lig vector+H20. Transformed using LB+Amp plates, TG1 cells

Notes
- vacuole size may vary from cell to cell. but may be able to control cell growth conditions to make sure they grow to about same size
- for locatiosn that may be tough to distinguish, just pick one location to always be paired with one FP->decs library size for each location b/c u have less FPs to work with but incs overall library size b/c u retain more locations
- high throughput for large library to easily identify genotype. easier screening for linked library
- each case we have micode and experimental phenotype and have to link those together by having cloned micode with that genotype together or randomly to sequence library to see which ones were linked
- figure out gene. get nucleotide seq from sgd. use that to design primers. for cloning

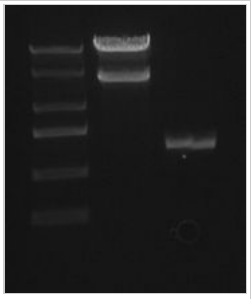
Harneet91 17:32, 22 May 2012 (PDT)

- designed primers for amplifying PAGFP off of PAGFP plasmid (pRSETA-PAGFP; plasmid 11911) and checked with Will's oligo page
- amplified PAGFP from plasmid 11911 using primers AT59/AT60 (phusion pcr)



2012-05-22 pRSETA-PAGFP AT59-AT60 phusion pcr

- gel ran well (expected product ~ 720bp). Gel purified.
- did BglII/XhoI digest of pWCD0436, PAGFP pcr product



2012-05-22 pWCD0436 BgII-Xho digest, PAGFP pcr prdct

- cut out bands and left in ADB buffer for storage

Notes

MiCodes
- put barcodes into yeast so that when u look at microscope you can tell genotype
- target fluorescent proteins to random organelles
- look at each channel individually
- have way of matching speficic localization pattern in cell to the cell's genotype->link genotype to something else ur trying to measure
- on/off (2), 3 fluorescent proteins, 6 organelles->library size = (2^3)^6
- applications
1. protein-protein interactions
2. protein shells: use as tool to find optimal expression level of genes
- importing
1. nucleus: consensus seq tag
2. some orgz well conserved/characterized
- targeting
1. mitochondria
2. Plasma membrane
   - 3 diff proteins embedded in layer.
3. nucleus
4. Golgi
5. ER
6. cortical ER
7. Actin
8. Bud scar
9. Endosomes
10. vacuoles (mbrn, lumin)
11. microtubule
12. nucleolus
13. other (vesicles, other punctuates)
14. peroxisomes
- expression off of plasmid much noisier than genomic integration
- pH sensitivity might come into play, but if we get low signal then we can increase exposure
- controls
1. antibodies
2. dyes

- each person takes 4 and make list of top 3 or 4 targeting methods for each organelle
- also make sure list is comprehensive
- we only care about getting targeting to location of organelle
- for big organelles can think about splitting it up into two (like inside and outside)

6, 12, 5, 11

Nucleolus http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2658561/pdf/embor200914.pdf

ER http://mic.sgmjournals.org/content/145/5/991.full.pdf+html

Cortical ER http://onlinelibrary.wiley.com/doi/10.1111/j.1600-0854.2009.00926.x/pdf (cortisol sorting signal) http://www.zmbh.uni-heidelberg.de/seedorf/default.shtml (coupling of ER and PM) - found cortical sorting signal for a certain protein that is targeted Microtubule http://pubs.acs.org/doi/pdf/10.1021/bi00113a008

- found proteins that associate w/ microtubules and found what part of these proteins are responsible for binding

Ideal properties of targeting sequences
- small/short
- stay localized
- previously used to target fluorescent protein
- length
- comments
- N/C terminal tags
- initials who found it

Harneet91 19:23, 21 May 2012 (PDT)

- talked about project.
- assigned and did presentations on cloning technologies

- BglBrick/Biobrick
- Gibson Assembly
- Golden Gate cloning
- Polymerase Cycling Assembly
- DNA Assembler Technology
- Yeast Genomic Integrations

- I did my presentation on DNA Assembler technology
- We also discussed potential locations to target organelles to and were assigned organelles/locations to which . See google doc for assignments

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