Timeline-making p53 mutant & checking activity

Version 1.5 6/28/07 – Sam Danziger

Day 1: E

Design and order oligos

Use Mutant_Design_proc.xls on a windows computer to design the mutants.

Day 2: M

Mix oligos to make the template

Make 1:10 dilution of a 1:10 dilution of each oligo (total 1:100 dilution, or 1 uM).

Template (1:10, 100nM)		
Name	**Amount**	**Notes**
Oligo X (1:100 dilution)	5 uL of each	Mix 5 uL of each oligo in the fragment. e.g. Mix wild-type pF 1-1,1-2,…,1-10,1-13,1-14 with mutant **pF 1-11 and pF 1-12**.
ddH_2O	45 uL * #oligos	For Example: fragment 1 has 14 oligos. That means 70 uL of 1:100 oligos and 630 uL of ddH_2O
Total		pF1=700uL, pF2=500uL, pF3=300uL, pF4=400uL, pF5=500uL, pF6=400uL

Regulation: For each primary fragment, mix this much ddH_2O with the .5 uL of each of the 100 uM oligos: PF1: 3 uL, PF2: 5 uL, PF3: 9 uL, PF 4: 6 uL, PF5: 5 uL, PF 6: 6 uL. This results in a 10uM Solution.
Store in -20 degrees C.

PCR to combine oligos into a fragment

PCR recipe		
Name	**Vol (uL)**	**Notes**
template (1:10, 100nM)	8 4 uL	Total = 800 nM template. Regulation: 40 uM
dNTPs (2.5mM)	4	The 4 components in dNTPs are at 2.5 mM ea.
3' primer (1:10, 10 uM)	1	Use the first oligo for this fragment (Ex Pf1-1) Total = 10 uM
5' primer (1:10, 10 uM)	1	Use the last oligo for this fragment (Ex Pf1-14) Total = 10 uM
Mg 50 mM	.5	For PFx only. Other PFUs don't need.
PFx buffer (10x)	5	
ddH_20	29.5	This will fill to 50 uL
Pfx	1	Add this after 5-10 minute hot start for PFx. Do NOT add less than .75 uL
Total	50	

Use a `95C, 10min` hot start, add the pfx, then `Repeat [95C,30sec → 54C,30sec → 72C,30sec] 35x → 72C,5min → 4C,hold.` . (Program p53co on PCR #1)
Store the PCR product overnight in PCR machine at 4 degrees, or at -20 degrees.
REGULATION: `95C, 10min if hot start OR 1min → Repeat [95C,20sec → 54C,30sec → 72C,30sec*] 30x → 72C,5min → 4C,hold.`
*72C time calculation: PFx is 1 kb/min, Pfu Ultra II is 4 kb/min.
NOTE: If using Platinum PFx: **No hot start**. `94C, 2min → Repeat [94C,15sec → 55C,30sec → 68C,30sec*] 25-35x → 68C,5min → 4C,hold.` *1 kb/min
NOTE: Fragments are 159-318bp so 20sec at 68C/72C should be sufficient.

Why is Regulation different than the listed procedure? The PCR works with $1/10^{th}$ the regulation oligos.

Day 3: H

Check PCR on gel

Use 5-10uL of 100 bp ladder.
Mix ~3 uL of 10-20x loading buffer with 5 uL of PCR product.
Run at 100 V for about 30 minutes (high quality) or 120 V for 20 minutes (low quality).
Fragments should have the following lengths: pf1=303bp, pf2=223bp, pf3=145bp, pf4=181bp, pf5=246bp, pf6=196bp. If you use primers with the Xho I and Spe I restriction sites, pf1=318bp, pf2=237bp, pf3=159bp, pf4=195bp, pf5=261bp, pf6=210bp

Purify PCR products

QIAquick PCR Purification kit: Make certain you add ethanol to the correct buffer or you will lose your DNA. In the final step, use 20 uL ddH20* and let it soak for 1 minute before centrifuging to get a high concentration. *Buffer EB should also be fine.

OR

MinElute PCR Cleanup kit: Regulation. $441/250 column or $97/50 column.

1) Add 5 volumes of Buffer PB (or PBI) to 1 vol of PCR reaction mix. (225 uL PB for 45 uL PCR).
2) BIND sample to MinElute column by centrifuging (~ 13,000 RPM) & discard flow-through.
3) WASH column with 750 uL Buffer PE. Centrifuge 1 min & discard flow-through.
4) DRY column by centrifuging 1 additional minute & discarding flow-through.
5) ELUTE by placing column in 1.5mL eppendorf tube. Add 10 uL Elution Buffer, let stand for 1 minute, and then centrifuge for 1 minute.

Be certain to use Qiagen reagents.

Check concentration of PCR product using 1.4 uL (as per Neuhausen lab instructions) in Neuhausen Nano-drop. Regulation is 1.0 uL. If you elute with elution buffer, reblank Nanodrop with elution buffer.

Dilute PCR Products

Dilute Fragments so that 1 uL of each is required during "Assemble p53 Core Domain".

Fragment Dilution		
Name	**Target Concentration (ng/uL)**	**Notes**
Fragment 1	20.99	1 pMol
Fragment 2	15.64	1 pMol
Fragment 3	10.49	1 pMol
Fragment 4	12.87	1 pMol
Fragment 5	15.05	1 pMol
Fragment 6	13.86	1 pMol

REGULATION: Use "Assembly_blank_file.xls" to determine the volume of each fragment to add.

Assemble p53 Open Reading Frame (PCR to combine fragments)

Create the full length p53 Open Reading Frame (p53 ORF). Use either mutant or wild-type fragments as appropriate.

Assembly PCR recipe		
Name	**Vol (uL)**	**Notes**
Fragments 1-6	1 of each	Total = 6uL, 1 pMol of each fragment
dNTPs (2.5 mM)	4	Total = 10 mM
PF1-FORWARD (1:10, 10uM)	1	Primer. Stock is 100uM, need 10 uM This contains the XhoI restriction site.
PF6-REVERSE (1:10, 10uM)	1	Primer. Stock is 100uM, need 10 uM This contains the SpeI restriction site.
Mg 50 mM	.5	Only for pfx
pfx buffer	5	
ddH_20	31.5	Dilute to 50 uL.

Pfx	1	Add this after 5-10 minute hot start
Total	50	

`95C,5min if hot start OR 2min → Repeat [95C,30sec → 62C,30sec → 72C,90sec*]30x → 72C,10min → 4C,hold.` (Program called P53ASSEM on PCR machine)
*PFx is 1 Kb/min at 68C, and the p53 ORF is 951 bp.
*PFu Ultra II is 2 Kb/min at 68C, and the p53 ORF is 951 bp.
Store the PCR product overnight in PCR machine at 4 degrees, or at -20 degrees.

Purify PCR products: If possible get concentrations > 190 ng/ul**.**
QIAquick PCR Purification kit: Make certain you add ethanol to the correct buffer or you will lose your DNA. In the final step, use 20 uL ddH20* and let it soak for 1 minute before centrifuging to get a high concentration. *Buffer EB should also be fine.
OR
MinElute PCR Cleanup kit: Regulation. $441/250 column or $97/50 column.

Check concentration of PCR product using 1.4 uL (as per Neuhausen lab instructions) in Neuhausen nano-drop. Regulation is 1.0 uL.

Digest p53-ORF to create sticky ends

Backbone Digestion Recipe – Purified DNA from CODA		
Name	**Vol (uL)**	**Notes**
P53 ORF (from assembly)	12	Want ~130 ng/ul after 12/17.5 dilution for ligation.
10x BSA	1.75	Or 1uL of 100x BSA
XhoI	1	
SpeI	1	
N.E.B. Buffer 2	1.75	Use NEB Catalog to determine best buffer
Total	17.5	

Run at 37 C for 1-3 hours. Inactive enzymes with 20 minutes at 65 C
NOTE: The PCR machine will not respond to the pause button if changing temperature.

Replicate PCR Wild-type Fragments (if we need more for assembly)

***PCR Wild-type Fragments From Storage (if we run out)**		
Name	**Vol (uL)**	**Notes**
Fragment X	1	1 pM
dNTPs (2.5mM)	4	The 4 components in dNTPs are at 2.5 mM ea.
3' Primer (1:10, 10 uM)	1	Use the first oligo for this fragment (Ex Pf1-1) Total = 10 uM
5' Primer (1:10, 10 uM)	1	Use the last oligo for this fragment (Ex Pf1-14) Total = 10 uM
Mg 50 mM	.5	Only needed for pfx
pfx buffer	5	
ddH_20	36.5	This will fill to 50 uL
Pfx	1	Add this after 5-10 minute hot start Do NOT add less than .75 uL
Total	50	

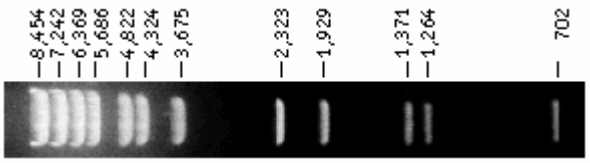
`95C,5min if hot start OR 2min → Repeat [95C,30sec → 54C,30sec → 68C,30sec*]30x → 72C,10min → 4C,hold.`
*PFx is 1 Kb/min at 68C, Cloned pfu is ~.5-1 Kb/min @ 72C. The longest p53 fragment is 318 bp.
Store the PCR product overnight in PCR machine at 4 degrees, or at -20 degrees.

Digest p53-CODA-OUT(+1) backbone (if we need more backbone)

Backbone Digestion Recipe – Purified DNA from CODA		
Name	**Vol (uL)**	**Notes**
P53 CODA-OUT(+1) DNA	12	Need 5000 ng. 422 ng/ul*12 = 5064.
10x BSA	6	Or 1uL of 100x BSA
XhoI	1.5	
SpeI	1.5	
N.E.B. Buffer 2	6	
ddH_20	33	Fill to 60uL
Total	60	

Run at 37 C for 8-16 hours. Heat inactivate enzyme for 20 min at 65 C.
NOTE: The PCR machine will not respond to the pause button if changing temperature.

Purify backbone (if we need more backbone)
1) Create a gel as in 1.5% gel recipe, except, use TAE not TTE. Run at 85 V for ~ 1 ½ hours.
(REGULATION: 1.0% TAE agarose)
2) Use a thick gel or tape several wells together on a comb to fit all 60 uL in a well.
3) Run gel using BstEII digest of lambda DNA (BSTE II). Backbone is ~5000 bp.



BSTE II, 1.0% agarose, etbr

4) Cut out gel using (blue) Nitrile gloves, lab coat, UV face shield, two razor blades and the UV plate. Turn out the lights and close the door, then slowly increase the UV brightness until you can just see the band (too much UV will damage the DNA).
5) Use QIAquick Gel Extraction Kit.
6) Check concentration using 1.4uL with nano-drop. Dilute concentration to 50ng/uL.

Day 4: M

Set up ligation (p53 CODA-OUT(+1) Protocol)

Ligation Recipe		
Name	**Vol (uL)**	**Notes**
Digested p53 ORF	7	Want 900 ng
p53 CODA-OUT(+1) Backbone	1	Need 50 ng. 50 ng/uL * 1 uL = 50 ng.
10x T4 Ligase Buffer	1	
ddH20	0	Add enough so that total volume = 10 uL
T4 DNA Ligase	1	
Total	10	

Incubate 16 degrees overnight OR 1-3 hours at room temperature.

***E. coli* transformation (pCODA-OUT (+1) Protocol)**
Use 3 uL of ligation with ultra competent E. coli cells. (DH5alpha if available).
Use Brachmann Lab Protocol: **Transformation of "ultra-competent" E. coli**

Transformation Recipe		
Name	**Vol (uL)**	**Notes**
Ligation Product	5	REGULATION: 3 uL
"ultra competent" E. Coli	100	Stored in -80. 1) **Gently** thaw on ice. 2) Mix with Ligation Product in pre-**chilled** tubes. 3) Chill on ice for 30 minutes 4) Heat Shock in 42 C for 30s

		5) Chill on ice for 2-5 minutes
S.O.C. media	400	6) Add to tubes, 10 ml S.O.C. aliquots in -20 C 7) Roll for 1 hr in 37 C incubator
Total	505	Actual volume is not critical.

1) Pre-warm LB-chloramphenicol (LB-cam) (5 black) plates by putting in 37 C incubator for one hour while the S.O.C. media rolls.
2) Make one 1:10 dilution and 1 undiluted plate. We want plates with 50-100 colonies.
REGULATION: Make 2-3 plates with undiluted 505 uL. For aprox. 50 colonies / plate.
3) Grow overnight in 37 degrees

Day 5: L

1) Choose 8 colonies from the LB-cam plate and using a grid, plate product onto LB-cam (5 black) and **then** LB-amp (1 black) plates in exactly the same place. **Ultimately we will take < 8 colonies if most sequence correctly**.
2) Grow overnight in 37 degrees

Day 6: L

Choose colonies that grow on both LB-camp and LB-amp plates.

PCR with taq to Check for Insert

PCR w' Low Fidelity taq Polymerase		
Name	**Vol (uL)**	**Notes**
Bacteria Template	-	¼ - ½ patch w' glass rod Use 1 uL of purified ORF (from the assembly or digestion step) as a positive control
taq Buffer	2	
dNTPs (2.5mM)	2	The 4 components in dNTPs are at 2.5 mM ea.
3' Primer. Fragment 1-1 (1:10, 10 uM)	1	Total = 10 uM
5' Primer. Fragment 6-10 (1:10, 10 uM)	1	Total = 10 uM
ddH_20	14	This will fill to 20 uL
Taq	1	Add this after 5-10 minute hot start
Total	20	

`95C,5min → Repeat [95C,30sec → 54C,30sec → 72C,70sec*] 30x → 72C,10min → 4C,hold.`
*taq is ~1 Kb/min. ORF is ~ 1Kb

Run a gel to see if insert was replicated
Load supernatant (not pellet) from taq polymerase.
Use 5-10uL of BSTE II and 100bp.

Inoculate for miniprep
1) If necessary, prepare a stock solution of LB+Chloramphenicol (cam). Stock cam is 1000x. Dilute to 1x in LB stock.
2) Take 5mL of LB+cam for each bacteria colony and add to a glass tube.
3) Scrape each colony using a long wooden rod and place in the LB+cam tube.
4) Roll overnight in 37 C

Day 7: H

Mini prep to isolate E.Coli Plasmid With p53 ORF
1) If inoculation is not cloudy, redo inoculation.
2) Pour cloudy inoculation product into 1.5 mL eppendorf tube until full.

2) Spin at 13,000 RPM for 1 minute. Pour off excess liquid down drain but do not disturb pellet. Place tubes joint side up so pellet is in the correct place.Repeat step (1) 3-4x until all inoculation product is used up. **OR** spin large tubes containing all liquid for 10 minutes at 3,000 RPM.
4) Pipette out any remaining liquid.
5) Use QIAGen mini-prep kit.
6) May reduce ddH_20 in final step to increase concentration as in PCR purification.

REGULATION: Use Fermentas kit with 50 uL elution buffer and check 1.0uL with nanodrop.

Check miniprep concentration
Use 1.4 uL with nanodrop to confirm concentration >100 ng/uL (ng/uL). If concentration is <100 ng/uL, redo the miniprep as the product will not sequence well.

Digest with Xho1 / Spe1 and run gel to check for insert.

Quick XhoI/SpeI digestion.		
Name	**Vol (uL)**	**Notes**
PCR Product (DNA oligo)	2	
NEB Buffer 2 (10x)	1	
BSA (100x)	0.5	Would 1 uL of 10x work?
XhoI	0.5	
SpeI	0.5	
ddH_20	5.5	This will fill to 10 uL
Total	10	

`37C,20+ minutes. No need for 65C, 20 minutes to deactivate.`

Run a gel to see if insert was replicated
Mix digestion product with ~3.3 uL 5-10x loading buffer. Load 5-10uL of BSTE II and 100bp as ref. Looking for 5 kb plasmid and 951 bp insert.

Set up sequencing reaction
Send DNA out in 48 or 96 well plates to be sequenced ($2.5-$5 @ Agilent or GeneWiz).
Genewiz: Prepare two tubes for each mutant, one will use forward, the other reverse primer.

Genewiz Sequencing Reaction		
Name	**Vol (uL)**	**Notes**
PCR Product (DNA Oligo)	2?	Need 500 ng. 2 ul * 250 ng/uL = 500 ng.
10 uM Primer	1	pSalect-for / pSalect-rev
ddH_20	9	Fill to 12 uL (8 uL if using Genewiz primer)
Total	12	

OR
See "**APPENDIX 1: In-House Sequencing Protocol**" at the end of this document.

Day 8: **H**

Check sequencing results
Use sequencer or other software to compare sequencing results with wild-type to make sure that the sequence shows exactly what we want.

Make a Perm for each mutant.

PCR to replicate the "p53 Open Reading Frame (p53 ORF)"
Modified from Brachmann Protocol "Colony PCR" and **Assembly PCR recipe**

$$Molarity(nM) = \frac{Concentration(ng/uL)}{660 * Length(bp)}$$

p53 ORF PCR recipe		
Name	**Vol (uL)**	**Notes**
E.Coli Plasmid With p53 ORF (Miniprep Out)	10	1000 ng?. Plasmid + ORF should be ~6000bp. Concentration = 100 ng/uL → .25 pM
dNTPs (2.5mM)	4	The 4 components in dNTPs are at 2.5 mM ea.
Pf1-1 (1:10, 10 uM)	1	3' Primer. Need 10 uM Does **NOT** include the XhoI restriction site.
Pf6-8 (1:10, 10 uM)	1	5' Primer. Need 10 uM Does **NOT** include the SpeI restriction site.
Mg 50 mM	.5	
pfx buffer	5	
ddH_20	27.5	This will fill to 50 uL
Pfx	1	Add this after 5-10 minute hot start Do NOT add less than .75 uL
Total	50	

`95C,10min if hot start OR 2min → Repeat [95C,20sec → 55C,30sec → 68C,60sec*] 30x → 72C,5min → 4C,hold.`

*PFx is 1 Kb/min at 68C, and the p53 ORF is 951 bp.

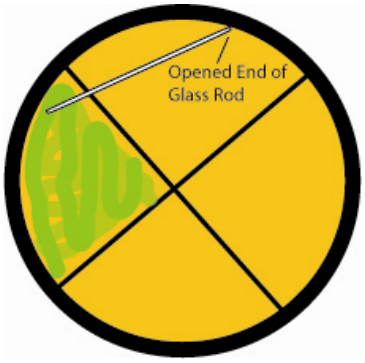
Store the PCR product overnight in PCR machine at 4 degrees, or at -20 degrees.

NOTE: If the CBRL/CODA produces the mutants, the Brachmann lab will receive 1) pCodaOut oligo with p53 ORF. 2) PCR Product containing ORF. 3) LB+cam plate growing e.coli containing oligo with ORF.

Transform yeast plasmid into e.coli to mass produce it. (If we need more gapped plasmid)

Transformation Recipe		
Name	**Vol (uL)**	**Notes**
STET Product	3-5	Use STET 3296* (Sam's 3296-14,15 are best)
"ultra competent" E. Coli. NOTE: 3296 is toxic DH5ά use JM109.	100	Stored in -80. 1) **Gently** thaw on ice. 2) Mix with Ligation Product in pre-**chilled** tubes. 3) Chill on ice for 30 minutes 4) Heat Shock in 42 C for 30s 5) Chill on ice for 2-5 minutes
S.O.C. media	400	6) Add to tubes, 10 ml S.O.C. aliquots in -20 C 7) Roll for 1 hr in 37 C incubator
Total	505	Actual volume is not critical.

1) Plate onto LB+Amp plates at 1,1:10, 1:100, 1:1000 dilutions.
2) Grow Overnight at 37 C.
3) Patch out 8-12 independent colonies onto two plates for STET prep protocol using a sealed sterile capillary pipette (glass rod).
4) Grow Overnight at 37 C.
5) Scrape up each 1/4 to 1/6 of the e.coli plate with a sealed pipette and resuspend in 500 uL STET buffer in a 1.5 mL Eppendorf tube.
6) Place the tubes in the Labnet multi-tube shaker and thaw a tube of lysozyme (Stored at -20 C) on lab bench. (Run the Eppendorf tube along holder to aid resuspension if necessary).
7) Add 25 uL of lysozyme to each tube and invert 3x / tube (no vortex).
8) Boil immediately in 100 C block for 3 minutes.



9) Microcentrifuge for 15 minutes at 14,000 RPM.
10) Remove pellet with toothpick.
11) Add 500uL of isopropanol and invert each tube three times.
12) Rinse pellet with 70% Ethanol using wash bottle.
13) Dry pellet over drying rack covered with paper towels until mostly dry.
14) Resuspend remainder in 100 uL ddH20 (or TE).

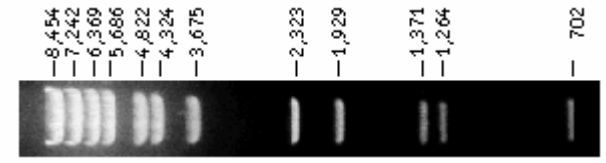
*See appendix for more information about the plasmid.

Digest yeast plasmid create the "Gapped Plasmid" for ORF. (If we need more gapped plasmid).

Gapped Plasmid Backbone Digestion Recipe		
Name	**Vol (uL)**	**Notes**
p53 Yeast Plasmid	3-15	This depends on how bright the bands are (how much DNA there is).
AgeI	1.5	
StuI	.75	The StuI is 2x concentration as AgeI
N.E.B. Buffer 1	4	
ddH_20	18.75- 30.75	Fill to 40uL
Total	40	

1) 37 C for 20 minutes for a quick digest to check band strength.
2) Digest 8 hours or over night for best results digesting the backbone.
2) Run at 65 C for 20 minutes to deactivate the enzymes.

Purify Gapped Plasmid (if we need more gapped plasmid)
1) Create a 1.0% TAE agarose gel. Run at 85 V for ~ 1 ½ hours.
2) Use a thick gel or tape several wells together on a comb to fit all 60 uL in a well.
3) Run gel using 100 bp ladded and BstEII digest of lambda DNA (BSTE II). Backbone is ~5000 bp.



BSTE II, 1.0% agarose, etbr

4) Cut out gel using (blue) Nitrile gloves, lab coat, UV face shield, two razor blades and the UV plate. Turn out the lights and close the door, then slowly increase the UV brightness until you can just see the band (too much UV will damage the DNA).
5) Use QIAquick Gel Extraction Kit or other Gel Extraction Kit.
6) Check concentration using 1.4uL with nano-drop and run a gel to check for plasmid. Good concentrations will be > 30 ng/uL.

Freeze yeast cultures in the -80 C for easy transforms
1) Make Competent Yeast: Inoculate yeast RBy #379 in 10ml medium (YPD) overnight on 30C roller.
2) Spin at 1500 rpm for 5 min., pour off liquid and vortex with 10ml ddH_20.
3) Resuspend in 15% Glycerol and freeze in -80 C

Yeast Transformation (Homologous Recombination)

See "'Turbo' yeast transformation" and "High-Efficiency yeast transformation" and "Gap Repair Protocol for p53 Suppressor Mutations"

Controls: Also plate out 1) No DNA control, 2) Gapped Plasmid 3) Undigested Plasmid 4) WT.

1) Thaw -80C stock of RBy #379 at R.T. or in hands. Each vial is good for 10 transforms.
2) Prepare ~7 mL 1xTE / 0.1M LiAc for every 10 transforms
3) Prepare ~6 mL 1xTE / 0.1M LiAc / 40% PEG 3350 (1:1:8 from stock) for every 10 transforms
4) Spin RBy #379 at 1500 rpm for 5 min., pour off liquid and vortex with 5ml 1xTE/0.1M LiAc.
5) Spin at 1500 rpm for 5 min., pour off liquid and suspend in 100uL to 1ml 1xTE/0.1M LiAc (a 1 mL culture is enough for 10 transformations)

Yeast Transformation Recipe		
Name	**Vol (uL)**	**Notes**
p53 ORF from PCR	0.5-1	Need ~20 (2-200)ng. (~.38 or .038-3.8 pM)
Gapped Plasmid	3	Need 150+ng of yeast plasmid(~.038 pM?)
Competent Yeast	100	
Single Stranded Carrier DNA, 2mg/mL	5-10	Boil (100 C) for 5 mins, or 10 mins directly out of -20 C
PEG Solution	500	Prepare according to the following ration: 8ml 50% PEG 3350, 1mL 1M LiAc, 1mL 10x TE. PEG Solution can be stored up to 1 week at RT.
Total	600+	Actual volume is not critical.

6) Mix above **IN ORDER** and vortex well. Prepare SC-His plates and warm to 30 C.
7) Incubate at 30 C for 1-4 hours or overnight at room temperature.
8) Vortex, then heat shock in 42 C for 18 minutes.
9) Spin for 5 sec at (6,000-8,000 RPM).
10) Aspirate Liquid and add 200 uL ddH_20.
11) Resuspend GENTLY with 1mL pipette. Plate on to SC-HIS.
12) Colonies will be large enough after 2 days.

NOTE: AFTER THIS METHOD WE WERE UNABLE TO RESCUE THE PLASMIDS FROM ANY OF THE ACTIVE MUTANTS. INSTEAD USE PETER KAISER'S METHOD.

Day 9/10

Wait for the colonies to show up.

Try to plan the experiments carefully so day 9/10 would be the weekend.

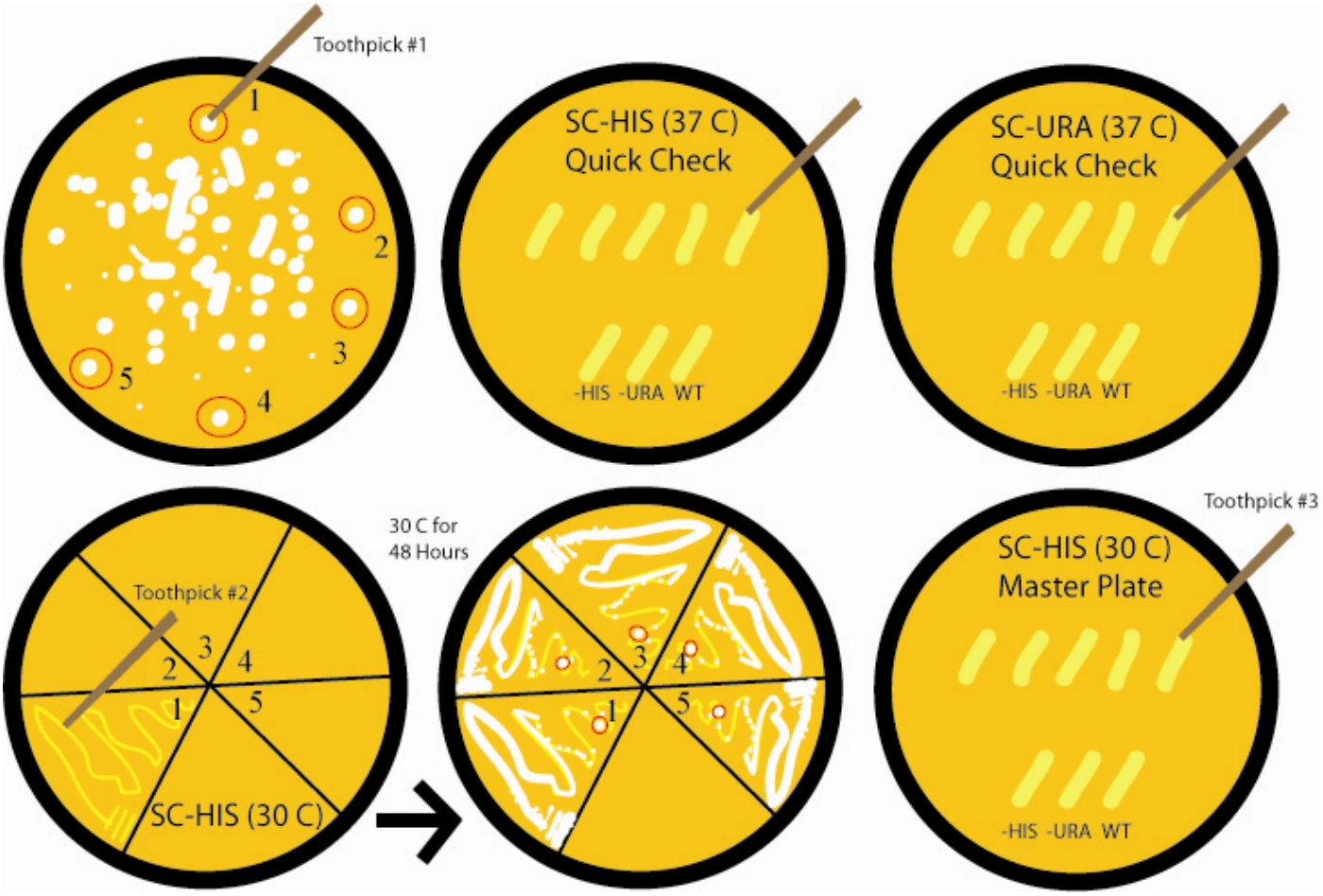
Day 11

Perform a Quick Check using 5 colonies / mutant and prepare Single Colony Purification.

Controls: Also plate the C+, His-, and Ura- yeast strains onto the His and Ura plates.

1) Section off an SC-His and an SC-Ura plate into a grid for a Quick Check.
2) Divide a SC-His plate into six parts (hexants) for Single Colony Purification
3) Touch a tooth pick to each colony and scrape into the same square on SC-His and SC-Ura,
4) Use the same toothpick, make 3 scrapes onto the SC-His Single Colony Plate.
5) With a new toothpick, touch part of the third scrape and spread it into the hextant. (see diagram)
6) Repeat 3) until 5 colonies are plated.
7) Grow Quick Check plates over night in 37 C.
8) Grow Single Colony Purification for 24-48 hours at 30 C.

His+/Ura+ mutants are "Active", His+/Ura- mutants are "Inactive."



Day 12

Make a master plate on SC-His using purified Single Colonies.

Day 13

Check Phenotype using purified single colonies and SC-Ura plates pre-warmed to 30C and 37C.
Grow overnight at 30C and 37C respectively.
Use replica plating!

Check Phenotype by plating onto .1% FOA plate.
The URA gene in the will make the acid on the FOA plate toxic. The FOA check at 30 C should yield the same results as –URA at 37 C. A true "Active" mutant will be His+/Ura+/FOA sensitive.
Use replica plating!

Start a -80 C Perm
1) Plate the yeast onto ¼ - 1/8 of a SC-His plate. Make sure to keep the yeasts separate.
2) Grow yeast overnight at 30 C.

Day 14

Make a -80 C Perm
1) Plate the yeast onto ¼ - 1/8 of a SC-His plate. Make sure to keep the yeasts separate.
2) Grow yeast overnight at 30 C.
3) Use a glass rod to take up two match head size globs of yeast and suspend in 1.5mL 15% glycerol.
4) Label each vial very well and enter # into database. Put yeasts in -80 C

Perform Plasmid Rescue on "Active" mutants and send to sequencing.
Perhaps use "A 10-Minutes DNA Preparation from Yeast" in Methods in Yeast Genetics, Burke et al.

Kaiser Lab Yeast Rescue Protocol

1) Grow the yeast O/N either on a plate or in 1.5 mL of the appropriate both.
2) Spin down broth for 5 minutes at 5000 rpm OR scrape plate to get a pellet.
3) Resuspend yeast pellet in 100 uL STET and an equal volume of glass beads in a screw top tube.

Kaiser STET Buffer (10 mL)		
Name	**Vol (uL)**	**Notes**
5 M NaCl	200	0.1 M NaCl
1 M Tris pH 8.0	100	10 mM TrisCl pH 8.0
0.5 M EDTA pH 8.0	20	1 mM EDTA pH 8.0
10 Triton X-100	5,000 (5 mL)	5% Triton X-100
H_20	4,680 (4.68 mL)	To reach 10 mL.
Total	10,000 (10 mL)	

4) Fast-prep cells for 80s (The Fast-prep machine is in the cold room. It vigorously shakes tubes)
5) Poke a hole in the bottom of the screw top tube and place the tube in a regular 1.5 mL tube. Spin these at 5000 rpm for 1 minutes to draw the liquid (not the glass beads) into the 1.5 mL tube.
6) Add 100 uL of STET buffer to the 1.5 mL tube.
7) Boil for 3 minutes at 72 or 100 C.
8) Cool on ice for 1-2 minutes.
9) Spin at 14000 rpm for 10 minutes in the cold room (4 C).
10) Transfer 100 uL supernatant to a new tube and add 50 uL of 7.5 M NH_4Ac.
11) Incubate for 1 hour at -20 C.
12) Spin at 14000 rpm for 10 minutes in the cold room (4 C).
13) Transfer 100 uL supernatant to a new tube.
14) Add 200 ul 100% EtOH.
15) Incubate for 1 hour at -20 C.
16) Spin for 15 minutes in the cold room (4 C).
17) Wash 1X 70% EtOH.
18) Re-suspend pellet in 40 uL TE. (Or ddH_20).
19) Transform Rescued Plasmid into e.coli as shown in Day 4 instructions.

APPENDIX 1: In-House Sequencing Protocol

Use Brachmann modified protocol **Sequencing using ABI BigDye Kit I**

Sequencing Recipe Part 1		
Name	**Vol (uL)**	**Notes**
Template	5	500ng. 100ng/uL * 5uL = 500ng
2.5x Buffer	7	
Sequencing Ready Reaction Mix	1	
Primer	.4	20 pmoles. Stock is 100uM. Run two reactions, one with 5' fragment, one with 3' fragment.
ddH20	6.6	Fill to 20 uL
Total	20	

1) Prepare one 5' and one 3' version of each colony as per **Sequencing Recipe Part 1**.
2) Program TW1 -- Cycle 50 times as follows: (1)92 C, 30s; (2) 50 C, 15s; (3) 60 C, 240s. Hold at 4 C forever.

Sequencing Recipe Part 2		
Name	**Vol (uL)**	**Notes**
Result from above	20	
3M sodium acetate	2	pH 4.6
95% Ethanol	50	
Total	72	Mix in an Eppendorf tube

3) Vortex results from **Sequencing Recipe Part 2** and then let sit at room temperature from 15 minutes.
4) Set the tube in the centrifuge with the joint upwards so the solid will collect towards the top and won't be scraped off during aspiration. Spin for 20 minutes at 14,000 rpm. Carefully aspirate the supernatant and discard.
5) Rinse pellet with 250uL 70% Ethanol
6) Vortex briefly and spin for 5 minutes at 14,000 rpm. Carefully aspirate the supernatant and discard.
7) Dry pellet in vacuum centrifuge for 10-15 min or until dry. Do not overdry.

We use two sequencing facilities:

I. For upstairs lab (DNA core facility, 3rd floor Sprague hall. 824-1870. Leave samples in fridge with plastic shark.

Login into ftp://dnacore.biochem.uci.edu:570 login:user0070 password: iuirwq8837. Under the folder RBLab, make a subfolder with the date (e.g. 02/05/07)

They sequence on Tuesday and Thursday and are more expensive.

II. Next door

Suspend sample in 10 uL buffer HDF (Hi-Di-Formaldehyde) in a 96 well plate. Cover with film and denature at 95 C for 5 minutes. They run samples in batches of 16, so ask around to get to this number. Talk with Ding to find out when he has time to sequence. Ask him for the plate back after the run so we can reuse unused wells.

We should get results back by the next day or sooner. Use a memory stick to get the results.

APPENDIX 2: General Recipes

1.5% Gel recipe		
Name	**Amount**	**Notes**
100 mL 1x TTE	100 uL	Use Graduated Cylinder to measure
Agarose	1.5 g	1) Use wax paper to measure into TTE. 2) Microwave for about 1 ½ mins. Swirl to mix. Another 20s, swirl, 20s, swirl (repeat until liquid is clear). 3) Wait until the liquid is cool enough to hold.
Ethidium Bromide (Etbr)	6 uL	Add **carcinogenic** Etbr **CAREFULLY.**

DNTPs recipe (2.5mM)		
Name	**Amount**	**Notes**
dCTP	10 uL	Stored in -80C. Stock is 100 mM
dATP	10 uL	Stored in -80C. Stock is 100 mM
dTTP	10 uL	Stored in -80C. Stock is 100 mM
dGTP	10 uL	Stored in -80C. Stock is 100 mM
ddH_20	360 uL	Dilutes to 2.5 mM of each
Total	400 uL	Makes 5 80uL aliquots

STET Buffer (1 Liter)		
Name	**Amount**	**Notes**
8% Sucrose	80 g	
5% Triton X-100	50 mL	
50mM Na_2EDTA	18.6 g	Fisher BP120-500
50mM Tris Base	6.05 g	Tris ultraPURE, GibcoRL # 15504-038
ddH_20	~800 mL	1) Fill to aprox 900 mL. 2) Adjust pH to 8.0!!
ddH_20	~100 mL	Fill to 1 Liter and stir until clear.
Total	1000 mL	DO NOT AUTOCLAVE