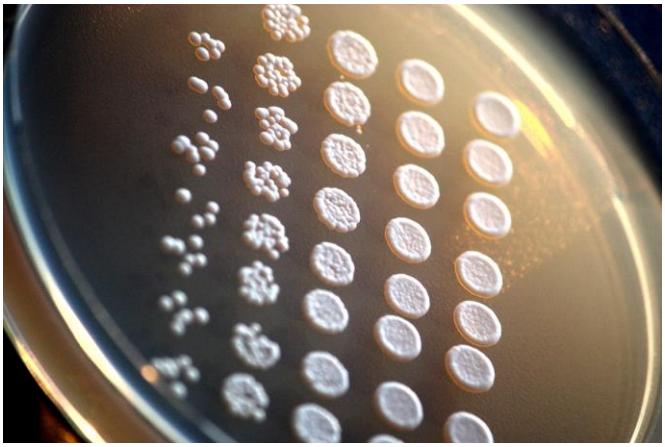

The Synthetic Yeast Project Sc2.0 developed synthetic yeast chromosomes with LoxPsym sites scattered throughout the chromosomes. By inducing the expression of Cre recombinase, new chromosome configurations can be generated in yeast cells. This population can then be screened for enhanced or altered phenotypes.

This laboratory manual includes protocols for SCRaMbling of yeast cells as well as phenotypic characterization. Appropriate student exercises are also included.

SCRaMbLE

Synthetic Chromosome Recombination and Modification by LoxP-mediated Evolution

Mark Jones and Lisa Scheifele
The Build-a-Genome Network

Build a Genome

Contents

Yeast Transformation

The plasmid DNA which induces the Scramble system will be transformed into competent yeast cells. This plasmid will be selected using a selectable (auxotophic) marker on the plasmid. Yeast cells containing the selectable marker will be able to grow on media lacking an amino acid. [Note to instructors: We can provide you with the yeast strains containing this plasmid already if you want to skip the yeast transformation step].

1. Obtain one tube of yeast cells (10^8 cells). Spin these down in the microcentrifuge for 1 min at full speed.

2. Remove the liquid (called the supernatant; you can toss it with the pipet tip into the biohazard trash. Resuspend the cells in 1 ml of sterile water. Spin cells down in the microcentrifuge for 1 min at full speed.

3. Remove the liquid (you can toss it with the pipet tip into the biohazard trash. Resuspend the cells in 1 ml of 0.1M LiAc (lithium acetate). Spin cells down in the microcentrifuge for 1 min at full speed.

4. Remove the liquid (you can toss it with the pipet tip into the biohazard trash. Resuspend the cells in 1 ml of 0.1M LiAc (lithium acetate).

5. Obtain four sterile microcentrifuge tubes (one for the assembly transformation, one for the positive control, and one for the negative control). Add 100 ul of yeast competent cells from step 4 to each tube. Label these tubes “CRE”, “NO CRE”, and “NC”.

6. Centrifuge the yeast cells at full speed for 1 minute. Remove the liquid (called the supernatant; you can toss it with the pipet tip into the biohazard trash.

7. Prepare a yeast transformation master mix. You have a tube containing 960 ul of 50% polyethylene glycol (PEG). To this tube add 144 ul of 1.0M lithium acetate (LiAc) and 40 ul of single-stranded herring sperm DNA. Mix very well by vortexing for 10 seconds.

8. Aliquot (distribute) 286 uL of yeast transformation mix into each of the three microcentrifuge tubes containing the yeast cell pellets from step 6. Pipet up and down very well to mix the yeast cells.

9. To the “NC” tube, add 25 uL of sterile water.

10. To the “CRE” tube, add 25 ul of provided plasmid DNA (plasmid pLM161; 500 ng).

11. To the “NO CRE” tube, add 25 ul of provided plasmid DNA (plasmid pRS416; 500 ng).

12. Vortex the tubes for 10 seconds to thoroughly mix the DNA with the transformation mix and yeast cells.

13. Incubate in a 42°C water bath for 20 minutes. During this time, obtain 3 SC-Ura dropout plates. Label one “CRE”, one “NO CRE”, and one “NC”.

14. After the heat shock, centrifuge the tubes at top speed for 30 seconds.

15. Remove the supernatant with a P1000 pipette set to 1000 ul.

16. Add 300 µL of sterile water to each tube. Gently pipette to resuspend the pellet.

17. From each tube, transfer 250 ul of the transformation product onto your appropriately labeled SC-Ura dropout plates. Add ~5-7 sterile large glass beads to each plate, shake the plates and toss the glass beads into the waste container.

18. Once the plates are no longer wet, incubate the plates at 30°C for 2 days (remember to turn the plates upside down).

19. Pick 2 new SC-Ura plates, label them “CRE” and “NO CRE” and with your initials. Parafilm and leave in the incubator with your transformation plates

20. After 2 days, select a single colony from the CRE plate and streak it on the new SC-Ura plate to isolate a single colony and remove any background (non-transformed) cells. Repeat for the NO CRE sample, streaking a single colony onto a new SC-Ura plate

Scramble: Inducing the Scramble system

Your instructor has inoculated a liquid culture (in 200 ml SC-Ura media) of your yeast strain containing the Cre plasmid (pRS413-CreEBD). The plasmid contains the Cre gene (remember that Cre induces the Scramble system) under the control of the estradiol promoter. In this experiment, you will add estradiol to the cells to induce the expression of Cre and therefore the Scramble system.

1. Measure the optical density of the yeast culture, which gives you an estimate of the cell density.

    a. Transfer 1 ml of media to a cuvette. Place in the spectrophotometer and blank the instrument (this only needs to be done once for the class).

    b. Transfer 1 ml of your yeast culture to a cuvette. Place in spectrophotometer and record the optical density. If the density is greater than OD=1, dilute the cells and repeat the measurement. Remember to account for the dilution when entering the OD into the formula below

2. Use the formula $C_1V_1=C_2V_2$ to determine the volume of cells equal to an OD of 0.1

    (Concentration (OD as measured on spec)(Volume to be determined) = (0.1) (25 ml)

    Volume to be determined = _____ ml

3. Into a sterile flask, add:
    a. The volume of cells that you calculated above
    b. 25 ml of SC-Ura liquid media
    c. 5 ul of beta-estradiol (1 uM final concentration).

4. Incubate the culture with shaking for 2-2.5 hours. (The cells are Scrambling now!)

5. Transfer 100 ul of the cell culture to a microcentrifuge tube.

6. Spin down the cells for 1 min at full speed. Remove the supernatant. Resuspend the culture in 1 ml sterile water.

7. Repeat step 7 two more times, ending with the cells resuspended in 1 ml sterile water.

8. Onto two selective plates, add 100 ul water. Into this drop, add 10 ul of cells from step 7. Spread the cells on the plates with glass beads.

9. Discard beads and when the liquid has absorbed into the plates, incubate the plates upside down at 30C for 2 days.

Scramble Assignment

1. Fill in the table below with the results of your yeast transformation:

Reaction	Positive control	Negative control	Cre plasmid
Number of colonies			

2. The yeast strain we are using has the genotype his3delta1 leu2delta0 met15delta0 ura3delta0. Go to: http://wiki.yeastgenome.org/index.php/Commonly_used_auxotrophic_markers and look up each of the four alleles and then complete the table below (you need to scroll all the way to the bottom of the page for the last 3 markers)

Allele	Is this marker a complete deletion of the gene?	If not, what portion of the gene is missing?
his3delta1		
leu2delta		
met15delta		
ura3delta		

3. Each allele contains a hyperlink to the Saccharomyces Genome Database. Each of these alleles is an auxotrophic marker, meaning that yeast containing the allele requires a particular nutrient. Under "Name Description", determine what nutrient yeast containing each of the alleles requires.

Allele	Name of gene	Nutrient Required
his3delta1		
leu2delta		
met15delta		
ura3delta		

4. For this experiment, the Cre gene was cloned into a plasmid containing the URA3 gene as its selectable marker, transformed into yeast, and selected on SC-Ura medium. Based on your results above, what three other genes (selectable markers) could have been used instead of *URA3* and what medium would you use for selection?

5. Why is it harder to introduce DNA into yeast cells, causing the heat shock step of transformation take 20 minutes for yeast cells but only 45 seconds for *E. coli* bacteria?

6. Yeast share many advantages with bacteria for lab work. What are three advantages to using yeast as a eukaryotic system? The section on "Why Study Yeast?" At this site may be helpful: http://wiki.yeastgenome.org/index.php/What_are_yeast%3F

7. After transformation we incubated our bacterial plates at 37 degrees and our yeast plates at 30 degrees. Why have yeast and bacteria evolved to have different optimal growth temperatures (think about the natural habitat of yeast, which is plant leaves, fruits, and soil, versus the natural habitat of *E. coli* bacteria, which is the human body)?

8. What are PCR Tags? Where are they located relative to the genes (ORFs; open reading frames) of the yeast genome?

9. PCR tags are created by recoding the yeast DNA. When changing or recoding the DNA what are two properties of the DNA that we need to keep in mind and which might restrict our ability to change the nucleotides (remember that changes to the DNA also change the RNA!)?

10. Where are the LoxP sites located in the yeast genome? What function do LoxP sites confer to the genome (in the presence of the Cre protein)

11. When we look for the presence of PCR tags by PCR, we use one set of primers that binds to the PCR tags (if present) and one set that binds to the wild-type genome. We are looking for either the presence or absence of each of these sequences. Why is it important to always use both sets of primers? (ie, if we used only one set of primers and saw no amplification, would there be alternate interpretations for the data?)

12. The yeast genome was selected as the first synthetic genome because of the simplicity of the yeast genome. Think about the differences between the yeast and human genomes. What features or aspects of the human genome would make it much more complicated to implement the Scramble system and the process of Scrambling chromosomes?

Measuring growth rates by serial dilution and plating

Up to now we have Scrambled the synthetic yeast chromosomes and selected for our phenotype of interest. We selected this phenotype based on the size of colonies on the initial selection plates (high temperature, ethanol, or caffeine). Now we need to confirm that these larger colonies really do grow better on the selective conditions. We will do this by:

1. Selecting 11 colonies that you believe show increased growth in the selective conditions
2. Transferring an equal number of cells from each of the 11 colonies to one well of a cell culture dish. You also transfer cells that have not been Scrambled into one well of the cell culture dish.
3. Performing serial dilutions of those cells. This means that in each subsequent well of the cell culture dish, you will have progressively fewer cells
4. Transfer the diluted cells from each well onto a Petri dish containing media with the selective condition
5. Incubate the plates for 2 days and then assess the growth of the diluted cells (both the size and the number of colonies) relative to the non-Scrambled strains.

An example of serial dilutions of yeast cells to compare growth rate on selective conditions (size and number of cells)

Procedure

1. Obtain your yeast cells on the selective plate from last week. Also obtain a plate with non-scrambled cells.

2. Obtain a new 96-well plate. When working with the plate, keep the lid on as much as possible; remove the lid to add or pipette the yeast cells, but put the lid right back on when you're done.

3. Transfer 200 ul of sterile water to each of the wells in the first row of a 96-well plate.

4. Using a sterile toothpick, select a colony from the non-scrambled cells. Swirl the toothpick into the water in the first well of water in the 96-well plate to disperse all the yeast cells into the liquid.

5. Using a sterile toothpick, select one of the largest colonies from the scrambled cells on the selective plate. Swirl the toothpick into the water in the first well of water in the 96-well plate to disperse all the yeast cells into the liquid.

6. Repeat step 5 for 10 more colonies, resuspending each in a new well.

7. Determine the OD of the cells in each well by taking a reading on the plate reader at an absorbance of 600 nm.

8. For each sample, calculate the volume of cells needed to prepare 200 ul of culture at an OD of 0.5 by using the formula C1V1=C2V2. Use the formula below and then complete the table below it.

   (0.5 OD) (200 ul) = (OD measured from plate reader)(Volume of cells needed).

Well	1	2	3	4	5	6	7	8	9	10	11	12
Volume of cells needed (calculated above)												
Volume of water needed to achieve a total volume of 200 ul												

9. Obtain a new 96-well plate. Into the first row, transfer the volume of cells calculated above for each well (ie, you are transferring the calculate volume from well A1 on plate 1 to well A1 on plate 2, etc.)

10. To these cells, add the volume of water calculated above to make a total volume of 200 ul.

11. Into each of the other wells in the dish, add 200 ul of water.

12. Using a P200 pipette set to 40 ul, pipette up and down 3-4X in well A1 and then transfer 40 ul of cells to the row beneath it (well B1). Pipette up and down 3-4X in well B1 and transfer 40 ul to well C1. Repeat all the way down to row H1.

13. Repeat step 12 for each column of cells (ie, transferring from A2 to B2 to C2…..)

14. Obtain 2 rectangular petri dishes with media. One should have rich media (YPD) and the other should have your media with your selective condition. Label the back of these plates with your initials and dates.

15. Obtain two paper templates containing the layouts of each plate. These will show you where on the smooth surface of the agar plate to place your cells. Place these under each of your dishes.

16. You should now have: your 96-well plate with diluted cells, your YPD plate, and your selective plate. Take 2 ul from the first well (A1) of diluted cells and pipette the liquid onto the

corresponding area of the YPD plate (A1). Take another 2 ul from the first well (A1) of diluted cells and pipette the liquid onto the corresponding area of the selective plate (A1).

17. Repeat step 16 for all wells of the 96-well plate.

18. Incubate the cells at 30C (or 40C if that is your selective condition) for 2 days.

Measuring growth rates by creating growth curves

Last week, you compared the growth rates of your scrambled and non-scrambled yeast strains by serial dilution and plating. This is a good way of comparing the growth characteristics of a large number of clones or strains. However, to get a better look at the growth dynamics of these clones we will need to monitor the throughout a defined period of time. This is typically done in liquid, rather than solid, media and growth is approximated by measuring optical density (OD) at 600 nm.

Yeast, like most microbes, has a typical growth pattern in liquid media. When it is first inoculated a yeast culture will be in **lag phase** – a period of time where the yeast cells are acclimating to this new environment. This period is variable and depends on the number of cells added to a culture. Eventually the yeast will transition into **log phase** – a period of time where the number of cells increases exponentially. This is the period during which we want to measure the yeast, with the intention of collecting enough data to calculate the **doubling time**.

Procedure

1. Your yeast cultures have already been inoculated. They are growing in a 30°C shaker in the neighboring lab
2. To take a timepoint, go next door and grab your yeast culture out of the shaker and bring it into the lab. You'll want to work quickly to get the yeast back in the shaker. Your instructor will stagger the times for each group so that the shaker and plate reader are not so crowded.
3. For each time point, pipette 200 µL each into **two wells** so that you can check the OD of two aliquots at each time point
4. Additionally, pipette two 200 µL aliquots of wild type (non-scrambled) yeast for comparison, your instructor has two flasks of WT yeast growing in caffeine or ethanol
5. Return the flask to the shaker to the same place you got it
6. Bring your 96-well plate (with lid on) to the plate reader and measure absorbance at 600 nm (OD600) in all four wells for the given time point
7. Ensure that the wells you want to measure are highlighted (green) on the plate reader. Be sure to record your data
8. Repeat step 2-6 every 30 mins for 2 hours for a total of four timepoints.

Yeast	Time	OD600_1	OD600_2

DNA Extraction from Yeast

Now that we've Scrambled the yeast DNA and selected our phenotype of interest, we need to see which gene or region of the chromosome is responsible for our phenotype. To do this, we must extract the DNA from the yeast and amplify the PCR Tags. This will allow us to determine, for each section of the chromosome, whether the wild-type or synthetic DNA is present. Today we will extract the DNA, and next week, we will amplify the PCR tags. The following week, we will run our PCR reactions on agarose gels.

1. Transfer 1ml of yeast cell culture into a 1.5ml microcentrifuge tube.

2. Centrifuge at 13,000–16,000 *× g* for 2 minutes to pellet the cells. Remove the supernatant.

3. Resuspend the cells thoroughly in 293µl of 50mM EDTA.

4. Add 7.5µl of Zymolyase enzyme, and gently pipet 4 times to mix.

5. Incubate the sample at 37°C for 60 minutes. During this time, the Zymolyase will digest the yeast cell wall. Cool to room temperature.

6. Centrifuge the sample at 13,000–16,000 *× g* for 2 minutes and then remove the supernatant.

7. Add 300µl of Nuclei Lysis Solution to the cell pellet and gently pipet to mix.

8. Add 100µl of Protein Precipitation Solution and vortex vigorously at high speed for 20 seconds.

9. Let the sample sit on ice for 5 minutes.

10. Centrifuge at 13,000–16,000 *× g* for 3 minutes.

11. Transfer the supernatant containing the DNA to a clean 1.5ml microcentrifuge tube.

> **Note:** Some supernatant may remain in the original tube containing the protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein.

12. Add 300µl of room temperature isopropanol and gently mix by inversion. You may see thread-like strands of DNA forming a visible mass.

13. Centrifuge at 13,000–16,000 *× g* for 2 minutes.

14. Carefully remove the supernatant with a P1000 pipet. Add 300µl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA pellet.

15. Centrifuge at 13,000–16,000 *× g* for 2 minutes. Carefully remove the supernatant with a P1000 pipet.

16. Centrifuge at 13,000–16,000 *× g* for 30 sec. Carefully remove the supernatant with a P200 pipet. Allow the pellet to air-dry for 10–15 minutes by leaving the tube sitting open in a rack.

17. Add 50µl of DNA Rehydration Solution.

18. Add 1.5µl of RNase Solution to the purified DNA sample. Vortex the sample for 1 second. Centrifuge briefly in a microcentrifuge for 5 seconds to collect the liquid and incubate at 37°C for 15 minutes.

19. Nanodrop the samples to determine the concentration of DNA. Turn on the machine, use DNA Rehydration Solution to "blank" the reading, then add 1 ul of your DNA solution to the machine and collect the reading.

Concentration: _____ ng/ul 260/280 : _____

Amplification of PCR Tags and Genome Databases Assignment

Once a genome is sequenced, that data must be deposited in a databank and then annotated. Annotation provides functional information about the location of genes and regulatory elements. This information is cataloged along with data relating to phenotypes and interactions between genes. For yeast, this information is curated at the Saccharomyces Genome Database (SGD). Go to the Saccharomyces Genome Database (http://yeastgenome.org/).

DNA alignment

The first thing that we would like to do is to determine what sequence in the yeast genome your DNA sequence corresponds to. This can be accomplished by performing a DNA alignment. There are two methods by which sequences can be aligned, global alignment (which seeks to align the sequence over its entire length) or local alignment (which seeks to align subsequences). We will use the BLAST algorithm (Basic Local Alignment Search Tool) to quickly find the sequence(s) that closely match our query sequence (your DNA).

At the top of the SGD homepage, under "Analyze", click BLAST. Under "Query comment", type in the name of your DNA. Paste your DNA sequence into the text box. Scroll down and click "Run WU-BLAST". You should get a colored graph representing the quality of alignment generated by BLAST which ranges from dark blue (poor alignment) to red (very good alignment).

1. BLAST provides statistics to help determine the quality of your alignment. The e-value (the expect value) represents the probability that the alignment you produced could occur by random chance.

DNA name		
How many hits does your query retrieve?		
For your top hit, what is the probability the alignment would occur by random chance (the e-value)?		
Scroll down to look at the aligned sequences. What is the percent sequence identity between the search result and your query sequence?		
What chromosome is your sequence on, and what are the sequence coordinates (Subject) of your DNA?		
Go back and shorten your query sequence so that it is only one line long. What does this do to the e-value of your BLAST hit(s) and why? **(Be sure to undo this change before continuing!)**		

Location of the sequence in the genome

From your top alignment, click on "Genome Browser". This will give you a visual representation of the chromosome, including the sequence that your building block corresponds to. The yellow vertical line corresponds to the boundaries of your query sequence. Genes (open reading frames or ORFs) are colored red. There are many other genomic features that are indicated as well. Click on the ORF that corresponds to your DNA sequence. This will give you information about this gene.

Click on the name of the gene, which will take you to the gene page where there is even more information, including functional information, links to information on gene and protein interactions, and perhaps a summary paragraph about the gene. Complete the table below for both of your DNA sequences.

What is the name of this ORF?		
What is the length of this gene?		
What is the systematic name (ID) of this ORF?		
Does this gene make a protein? If so, what is its length in amino acids? About how many copies of this protein exist in the cell?		
Is this gene essential in the yeast cell? (You can tell this by looking in the Phenotype section; next to "null", it should say either that the null mutation (deletion) is viable or inviable).		
What is the function of this gene?		
Tell me something cool about this gene!		

1. Describe what you expect to see when using wild-type DNA with each of the following primer sets:

	Expected results
YEL071W gene, wild-type primers	
YEL071W gene, synthetic primers	
YER188W gene, wild-type primers	
YER188W gene, synthetic primers	

What could you do to alter the experiment if you saw the wild-type DNA amplify with synthetic primers? Remember that it's hard and time-consuming to remake chromosomes, but easy and cheap to remake primers!

2. Why is it important to use both the synthetic strain prior to Scrambling as well as your synthetic Scrambled strain for PCR. What problem with data interpretation would we run into if we only ran the Scrambled DNA?

3. We are using one set (out of 349 sets) of PCR Tags on chromosome V. If you see a change in the size of the DNA amplified by this PCR Tag, does that mean that the gene in this region must be directly responsible for causing your phenotype?

4. Our yeast strain contains synthetic copies of chromosomes V and X. For some traits (caffeine and ethanol tolerance), we seemed to get resistant colonies very easily. For high-temperature tolerance, it seemed to be much harder. What does this mean about the chromosomal location or number of genes that could confer temperature tolerance in yeast?

5. Interpret the results of your PCR screening. Do you see any changes that are indicative of alterations to the chromosomes following Scrambling?

Sample	DNA template	Primers	Expected size of PCR product	Actual size of PCR product	Interpretation (was it what you expected?)
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					

6. How did your PCR reactions turn out? Describe any alterations to the protocol that you would make next time.