A Study of Linkage in Haploid Budding Yeast by Random Spore Analysis

One way to determine linkage and map distance in diploid organisms is to cross a heterozygous parent with a homozygous recessive parent. In this way it is possible, in effect, to determine the genotypes of the gametes produced by the heterozygous parent and assess whether two genes are linked (part of the same chromosome) and the relative distance between them if they are linked. If two genes are linked (not independently assorting), we expect more gametes of parental genotype than gametes of recombinant genotypes. Whereas, if two genes are unlinked (independently assorting), we expect 50% parental gametes and 50% recombinant gametes. Consider the results of the following cross:

<table>
<thead>
<tr>
<th>PARENTS:</th>
<th>AaBb</th>
<th>X</th>
<th>aabb</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAMETES:</td>
<td>AB</td>
<td>+</td>
<td>ab</td>
</tr>
<tr>
<td></td>
<td>40%</td>
<td></td>
<td>40%</td>
</tr>
<tr>
<td>PROGENY:</td>
<td>AaBb</td>
<td>aabb</td>
<td>Aabb</td>
</tr>
<tr>
<td></td>
<td>40%</td>
<td>40%</td>
<td>10%</td>
</tr>
<tr>
<td>PHENOTYPES OF PROGENY:</td>
<td>AB</td>
<td>ab</td>
<td>Ab</td>
</tr>
<tr>
<td></td>
<td>40%</td>
<td>40%</td>
<td>10%</td>
</tr>
</tbody>
</table>

We see from the phenotypes of the progeny and, therefore, the gametes of the heterozygous parent, that only 20% (10% Ab and 10% aB) are recombinant. Eighty percent (AB and ab) are nonrecombinant. This indicates that genes A and B are part of the same chromosome (linked) and are about 20cM apart.

In an organism, such as budding yeast (Saccharomyces cerevisiae), which can exist as a haploid, linkage and map distance can be found without test crossing diploid parents. A protocol called meiotic mapping allows determination of the genotype of organisms equivalent to the gametes of the diploid organism in the above cross.

Wild-type yeast can grow on minimal medium without being supplemented with any amino acids. Mutant yeast strains are often identified by their inability to grow on minimal media. A mutant strain often simply has an abnormal allele of a gene encoding an enzyme required to make a particular amino acid. For example, the LEU2 gene encodes the enzyme ß-isopropylamalate dehydrogenase. This enzyme catalyzes an essential step in the metabolic pathway for leucine synthesis. Thus, in a leu2 haploid strain, functional enzyme is not made, and the cells must be supplemented with leucine in the medium in order to grow. A leu2 mutant can be recognized, then, by its inability to grow on a plate lacking leucine; such a strain is said to be auxotrophic for leucine.

To perform a meiotic mapping study in yeast, two haploid cells are mated to produce a diploid zygote. This diploid will continue to undergo mitosis as long as it is cultured on a nourishing medium. Plating the diploid on special sporulation medium (starvation conditions) induces the cells to undergo meiosis and sporulation. The four meiotic products are packaged inside thick cell walls and become spores. A group of four spores is held together in an ascus or tetrad. A sporulated culture contains a mixture of unsporulated diploid cells and asci. The spores will not grow or divide on sporulation medium, and may be stored in the refrigerator for months without loss of viability.
To determine if various genes are linked and the approximate distance between any that are, we will perform **random spore analysis**. The first step in random spore analysis is to treat the cells of a sporulated culture with a snail enzyme, glusulase, which digests the ascus wall. The four spores from each ascus are then separated from each other by vigorous agitation, and the culture is diluted and plated out for single colonies on a rich medium, YPD (yeast extract, peptone, dextrose).

Since the population of diploid cells does not sporulate completely, the haploid spores must be separated from the unsporulated diploid cells. The red adenine marker \((\text{ade}2)\) is frequently used as a convenient means to distinguish haploids from unsporulated diploids. The red color of the spores results from an accumulation of a pigmented by-product of the deficient pathway for adenine synthesis. The original cross in this exercise is set up so that the diploids will be heterozygous \(\text{ADE2}/\text{ade}2\). The diploid cells will therefore produce white colonies. The sporulated digested culture is plated on rich medium, and the plates are then incubated until the \(\text{ade}2\) colonies turn red. Almost all of the red colonies are haploids. What could cause a diploid cell to turn red? The organisms you will use come from a sporulating culture of a diploid made by crossing two haploid strains as shown here:

\[
\text{CH560 } \alpha \text{ ura3-52 } X \text{ CH859 } \alpha \text{ ade2 his4-519 leu2 lys2}
\]

The spore colonies are transferred to a YPD master plate. Phenotypes of the transferred spores are determined by replicating the master plates onto appropriate selective media. Recombination frequencies between different genes can be determined by analyzing the recombinant spores.

### Symbols employed in yeast genetics

Yeast geneticists make use of certain symbols in cataloging their strains. **Lack of mention of a gene means it is wild type.** When a wild type gene is specified, it is denoted in italicized uppercase letters (meaning it is dominant). Notation for a mutant gene is lowercase and italics (i.e., \(\text{leu2}\)). Italics are used when discussing genotype. When discussing phenotype: the italics are dropped, the first letter is capitalized and the superscript is employed (i.e., \(\text{Leu}^-, \text{His}^+\)). Each strain has a number assigned to it which may not be the same from one research lab to another. (The superscripts "+" and "-" are commonly employed in genetics to connote "wild type" and "mutant", respectively. In yeast genetics the superscripts are usually not employed when discussing genotype.)

![Symbols employed in yeast genetics diagram](image)
Part 1: RANDOM SPORE ISOLATION AND MICROSCOPIC OBSERVATIONS

NOTE: It is important that you use sterile technique. Tubes and plates should be kept covered except when you are adding to them or removing something from them.

1. Label and inoculate a sterile microfuge tube containing 200 µl of 2% glusulase with the sporulated cells. Using a sterile toothpick, pick up cells from the sporulation plate (about a matchhead's worth) and transfer to the glusulase tube. Vortex the tube for about 30 seconds.

2. Put the tube in a 30°C water bath for two hours and vortex every 20 minutes or so, to allow the cell walls to be digested by the glusulase.

3. At the end of 2 hours, add 800 µl sterile water to the glusulase treated yeast and vortex.

4. In order to compensate for varying numbers of yeast cells in everyone’s samples, you will set up two dilutions at this point of your sample, a 1:100 and a 1:1000 dilution. Label your tubes carefully.

5. You will need isolated colonies that developed from a single cell to determine the genotype next week; therefore you need to spread the yeast cells for isolation onto YPD plates. Label the bottom of the plates, not the lids, with your initials, the date, lab section and dilution factor. Place 100 µl of sterile water and 10 µl of the appropriate diluted yeast sample (be sure to vortex your samples again right before plating) onto a plate. Pour out ~8-10 sterile glass beads onto the plate and shake (with the lid on) in order to spread the liquid evenly over the surface of the media (It’s all in the wrist!). Make a total of 3 plates for each dilution, the 1:100 and the 1:1000.

6. Stack your plates and tape them together. Invert the plates and incubate at 25°C for 2 or 3 days. Gremlins will take your plates out and place them on the lab bench for you to complete part 2. About five days after your lab day you need to arrange a time with your partner(s) to come in and set up Part 2. Arey Rm 1 will be unlocked and materials available for you to complete Part 2, allow about 30 minutes.
Part 2

PLATE ~50 SINGLE HAPLOID COLONIES

1. Using sterile toothpicks, pick ~50 of the well-isolated red colonies (ade2) and make masters on YPD plates using the grid provided at the end of the handout and similar to that below. It does not matter which plate you pick from as long as the colony is isolated and red (or pink as the case may be). The two different dilutions are used merely to account for differences in the amount of yeast spores isolated.

Transfer approximately 25 colonies to each YPD plate from your spore isolation plates; you don't want to overcrowd the growing colonies. This actual-size template is intended to illustrate that you should leave adequate space between colonies. Be sure to use about 50 Ade⁻ colonies total if you can successfully transfer them from the spore isolation plates.

Note:
- The larger the sample size the better. You should transfer 25 colonies per plate. Don't plate them too close together -- you want to allow them enough room to grow without touching each other.
- Make sure that you number on the bottom of the plates the location of haploid #1 (and the orientation of your numbering of the spores).
- Be careful to pick up only one colony at a time and use a new toothpick each time. Do not gouge the plate, but swipe gently over the surface of the agar media.
- Only well-isolated red (haploid) colonies should be transferred to the YPD masters. Although it's best to transfer a total of 50 colonies, it should not be at the expense of picking up a bit of a neighboring white (possibly diploid) colony by accident.
- Avoid those colonies that appear half-red and half-white.

2. Label your plates and tape them together.

3. Invert the plates and incubate at 25˚C. Gremlins will take care of your plates until lab at this point.
**Part 3**

**STREAK ONTO ‘DROPOUT PLATES’**

1. To determine the genotype of each strain, each colony from the YPD master plates will be grown on dropout plates and to YPD media (listed below). Which plate serves as the negative control and which as the positive control?

   - min his leu lys ura ("ade")
   - min ade leu lys ura ("his")
   - min his ade lys ura ("leu")
   - min his ade leu ura ("lys")
   - min his ade leu lys ("ura")
   - min his ade leu lys ura ("YPD")

Using a single sterile toothpick, touch it to the yeast colony from the “master” plate made 2 or 3 days ago and then swipe it gently onto each of the 6 plates above in the order listed. As you are working, keep the lids over the plates to avoid contaminants falling onto your media from above. Be sure to grid and label your colonies clearly using the template provided as a guide.

2. Tape your plates together and incubate them again, inverted, at 25˚C. As before, they will be taken out of the incubators for you when ready, ~ 1-2 days and then stored at room temperature or 4˚C until lab next week.

**Part 4**

**SCORE GROWTH ON DROPOUT PLATES AND ANALYZE DATA**

1. Identify by number the first and last colonies in each row of each plate. Be sure to use the same numerical designation within each set of plates.

2. Score the growth of each of the colonies on the various replicated plates (not just the total number of His’, etc.). Results are to be tabulated by scoring growth as a "1", nongrowth as a "0". See Collection and Analysis of data section below.

3. Determine which genes are on different chromosomes and which show linkage. To do this, you must consider the genes two at a time (e.g. leu2 and his4) and figure out the proportion of recombinant progeny for each of the pairs of genes. Given the number of data points in the class data, using excel for your calculations will be essential. What is the map distance between the linked genes? See Collection and Analysis of data section below.

4. Discard your plates in an autoclave bag. Make sure that they're taped together.

5. Your data needs to be entered into the Excel spreadsheet in lab before you leave. This file will be available to download from the BI279 Genetics Home Page by Friday and provides the class data to analyze.
1. Miscellaneous notes:

- Remember that each spore is a single haploid meiotic product. The scored results from the six media plates will yield the phenotype of each individual spore (see figure). Growth or non-growth reveals the amino acids (or nucleic acid in the case of adenine and uracil) for which the spore is auxotrophic.

- All of the spore-derived colonies should grow on YPD plates.

- There should be no growth on the ade' plates since ade2 haploids were selected from the sporulation plates. Any colonies that grow on ade' plates should be disregarded for the analysis; the probable explanation is that a bit of a neighboring diploid colony was transferred from the sporulation plate.

- Don't worry about the color of the colonies on the dropout plates. A colony may not be red because if it is starved for something else other than adenine (say, histidine), the adenine-synthesis machinery may never be turned on in the first place, thus there is no accumulation of red pigment. However, if the YPD plate and the Master YPD plate are both white for a single colony it would be best to disregard that data as it is potentially a diploid colony.

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**Figure 3.** Each spore is analyzed individually to derive its phenotype.
2. How to Organize Your Data

Organize your data into a table such as this:

<table>
<thead>
<tr>
<th>Spore #</th>
<th>ade-</th>
<th>lys-</th>
<th>leu-</th>
<th>his-</th>
<th>ura-</th>
<th>YPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

and so on...

3. Linkage and Mapping – Converting phenotype to genotype

From this data we have determined that lack of growth, shown by the 0, is produced when the genotype is mutant at that allele while a 1, growth, could only occur if that particular yeast strain had a functioning copy of the allele, and so presumably the wild type copy of the allele. As an example, looking at spore #2 from the plates depicted above the genotype of this strain is:

Phenotype: 0 0 1 1 1
Genotype: ade2 lys2 LEU2 HIS4 URA3

(The growth, indicated as a 1, on YPD media is a control to show that the colony could grow if provided with all the nutrients but is otherwise not used in the data analysis.)

Remember the genotypes of the original cross were:

ura3-52 ADE2 HIS4 LEU2 LYS2 X URA3 ade2 his4-519 leu2 lys2

(CH560 α) (CH859 a)

Each pair of segregating alleles must be considered in turn for the entire population of spores to ascertain the total numbers of individuals that exhibit parental and recombinant genotypes. In the example presented above: when considering the LYS2 and HIS4 genes, we see that lys2, HIS4 is a recombinant combination of alleles. That is, it is not found in either of the parents (which are LYS2, HIS4 and lys2, his4 respectively). However, when looking at the gene pair LYS2 and URA3, spore 2 has a genotype of lys2 and URA3 which is the same genotype as the CH859 a parent which would count as a parental combination. The random spore analysis is accomplished by tallying the pairwise combinations of alleles into two classes: parentals and nonparentals. From this we can arrive at our recombination frequency, RF, or the % recombination (RF X 100) by employing the formula:

\[
RF = \frac{\text{# of recombinant organisms}}{\text{total # of organisms}}
\]

What does the RF for each pair of genes mean? What do you expect the RF to be if the two genes are linked compared to if they are not linked? Which, if any, of the genes (HIS4, LEU2, LYS2, URA3) are linked? What is the proposed map distance between linked genes? Of course, in order to get here you need to devise a way to have excel count the number of parentals and nonparentals from each pairwise combination. Set up your spreadsheet carefully as copy and paste functions are huge timesavers here. If you know exactly what you want to do to start with, the whole data table can be finished in less than 30 minutes.
4. Using Statistics: The $X^2$ Test

Individually you would have a rather small population sample of < 50 colonies plated and would expect to see wide deviations from the expected results. To avoid this we have collected class data and will use chi squared analysis ($X^2$) to see if there are any significant differences from what is expected, that the gene pair is not linked. Review the application of this test in your book if you need.

We begin the analysis by assuming the null hypothesis that none of the genes are linked. Therefore we would expect to see the 50:50 ratio of parental to recombinant classes characteristic of independently assorting genes (RF = 0.5).

Our degrees of freedom (df) = 1 since there are 2 classes (parental and nonparental) and the total number of strains is known (so a constant). Once one of the classes has been defined (the variable) the other class is just the total minus the first class and therefore no longer a variable. Degrees of Freedom always equal the number of classes minus 1. Traditionally the null hypothesis (that the genes in question are unlinked) is accepted in all cases where the $p$ value is > 0.05 (5%). If it is < 0.05, it is rejected (therefore linkage would normally be assumed). In the case where the $p$ value is >0.05 and the null hypothesis is accepted, it indicates that the differences in the data set are small enough as to be explained by being merely due to chance alone, and so parental type = nonparentals (recombinants). However, when the $p$ value is below 0.05 (or whatever value you have chosen as being appropriate) it indicates that the data set does differs from what was expected and is less likely to be due to chance. The smaller the $p$ value, the greater the differences from expected were seen and thus unlikely to be caused by chance alone. In our case this would normally mean that the gene pair was linked. However, look at the data carefully and be sure that this is consistent with what you would expect then from linked genes (i.e. the number of parental types>> recombinants). It is always possible that other unexpected phenomena are skewing the data from what is expected and thus giving a lower $p$ value.

The calculation for $X^2$ is $\sum \frac{(O_{\text{observed}} - O_{\text{expected}})^2}{O_{\text{expected}}}$ = $(O_{\text{parental}} - E_{\text{parental}})^2/E_{\text{parental}} + (O_{\text{nonparental}} - E_{\text{nonparental}})^2/E_{\text{nonparental}}$. From here you would plug the $X^2$ value into a Chi square distribution table and find the $p$ value given the df in your experiment. However it will be much simpler for us to use Excel to calculate the exact $p$ value based on the calculated chi square value and df (CHIDIST function).

Remember, we consider the genes in pairs. Consider this example:

- **HIS4; LEU2**
  - parents: 14
  - recombinants: 18
  - RF: 0.563
  - $p$ value = 0.48

- **HIS4; LYS2**
  - parents: 18
  - recombinants: 14
  - RF: 0.438
  - $p$ value = 0.48

- **LYS2; URA3**
  - parents: 26
  - recombinants: 6
  - RF: 0.19
  - $p$ value = 4.1x10^{-4}
  - [MU = 19 cM]

In the first two cases the null hypothesis is accepted:

- **HIS4; LEU2**  
  - $p = 0.5$ - 0.1

- **HIS4; LYS2**  
  - $p = 0.5$ - 0.1

This means that the $p$ values lie between 10-50%. This implies that if our null hypothesis is correct, a deviation at least as great as that observed is expected between 10-50% of the time. This is an acceptable $p$ value; the null hypothesis is accepted.
In the third case the null hypothesis is rejected:

\[ \text{LYS2; URA3} \]
\[ p = < 0.005 \]

The \( p \) value is less than 0.05 (5%). It is very unlikely that this deviation from independent segregation is due merely to chance. LYS2 and URA3 genes are probably linked which would account for the low number of recombinants seen. From this example, since the RF is 0.19, the proposed map distance between LYS2 and URA3 in \( S. \text{cerevisiae} \) is 19 cM (yes, you still want to consider significant figures when reporting your values).

5. Temperature analysis

Temperature affects how quickly the yeast cell grows, essentially its cellular metabolism. The higher the temperature, the faster the yeast grows, the faster its metabolism and the less time a cell potentially has to obtain nutrients necessary for growth. In order to look at the effect of temperature on the growth of the isolated colonies previous classmates have collected large data sets at both 30\( ^\circ \)C and 37\( ^\circ \)C. You will be provided with an excel spreadsheet which includes all the data collected over the years with each temperature’s data in a separate tab on the spreadsheet. The format is the same for each data set so with only a few adjustments you should be able to copy and paste your calculations from one data set to the others – just double check that you have kept everything in the same position relative to the data set and that you adjust your range in the formulas as needed. When looking at RF values, does the same general trend in the pairs occur across all three temperatures? What is the trend? What does this tell you about the colonies growing on the plates that you isolated and scored? How else can you use the data collected to look at the effect of temperature on the growth of the yeast besides looking at RF values of the pairs? (Hint: Think about growth on a particular type of media and consider the effect of mutant alleles versus wildtype alleles in an individual isolated strain.)

Assignment:

Write a results and discussion section, including appropriate scientific figure(s)/table(s) (or both) with a proper figure/table legend(s) showing the linkage analysis of the gene loci for ADE2, LEU2, LYS2, HIS4, and URA3 at 25\( ^\circ \)C, 30\( ^\circ \)C, and 37\( ^\circ \)C. Be sure to include map distance for any linked genes from the class data set. Also try to determine what effect, if any, temperature has on the experiment. What questions arise from these analysis? (Hint: Look carefully at ADE2 and URA3 results.) What possible explanations are there and how may you test the different possibilities? Try to go beyond the most obvious explanation, human error, which is actually unlikely given the amount of data collected and the consistency of the results. You want to think hard about how the experiment was actually set up and the data collected in addition to what effect changing the temperature might have on the outcome of the experiment (essentially yeast grow faster at 37\( ^\circ \)C, slower at 30\( ^\circ \)C, and even slower at 25\( ^\circ \)C). There is no “correct” answer here, try to come up with possibilities that have some logic behind them when you present your ideas. The main point of this is to really analyze the data you have, try to figure out what it is telling you, and then coming up with ways you might test your possible explanations.

(This lab exercise was derived from a Harvard University Biological Teaching Laboratory Exercise by Maureen Whalen, Spring 1992. We thank Jack Howard of Harvard for his assistance. Revised 9/7/98)