

Investigating a Eukaryotic Genome: Cloning and Sequencing a Fragment of Yeast DNA

Credits:

This lab was created by Sarah C.R. Elgin and developed and written by Kathleen Weston-Hafer. Specific protocols were optimized by Kathleen Weston-Hafer and Wilhelm Cruz. This document was written and assembled by April Bednarski.

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Cloning and Sequencing a Fragment of Yeast DNA

Beginning of Instructor Pages

Purpose

The purpose of this laboratory is to introduce students to the molecular biology techniques used to clone a gene. An important part of this lab is helping students understand the set-up and analysis of control experiments. In the sequence analysis portion of the lab, students gain experience with online bioinformatics tools (BLAST and the *Saccharomyces* Genome Database). Analysis of DNA fragments recovered is designed to gain some insight into the organization of eukaryotic genomes.

Educational Context

This lab was created to accompany lecture topics in genomics, bacterial cloning, and molecular biology. The main topics covered in lecture that relate to this lab are **bacterial cloning** and **cycle sequencing**. This lab was tailored for undergraduates who are in the first semester of a three-semester introductory biology sequence. This course focuses on molecular biology, bacterial genetics, and introductory biochemistry. This lab begins to introduce students to the differences between eukaryotic genomes. The curriculum was designed for 500 students divided into lab sections of 20, but can be adapted to accommodate any number of students.

The following terms and techniques are discussed in this laboratory:

- restriction endonuclease
- plasmid
- vector
- competent cells
- ampicillin resistance gene
- restriction enzyme digest
- ligation
- transformation
- sterile plating
- plasmid isolation
- genome
- DNA gel electrophoresis
- cycle sequencing
- BLAST search

Summary

*This section contains a brief summary of the exercises contained in this lab. More thorough discussion of the materials follow in “General Materials.” The detailed protocol for each exercise is in the **Student** pages.*

For the cloning procedure, students use a pBS plasmid that contains a gene for ampicillin resistance. The plasmid is purchased in a form that has been cut with EcoRI restriction endonuclease and treated with phosphatase to prevent plasmid reclosure in the ligation reaction. The insert DNA is prepared by digesting yeast genomic DNA with EcoRI. In lab, students set up a ligation reaction to ligate a fragment from the yeast genome obtained from the EcoRI digest into the pBS plasmid. They then transform *E. coli* cells with this recombinant plasmid and plate the cells onto LB/ampicillin plates. Since the plasmid contains a gene that codes for an ampicillin resistance protein, only *E. coli* cells that have been transformed with the plasmid will be able to grow on the LB/ampicillin plates. Figures that describe the above steps are

in the **Student** pages. As a control, students also set up a ligation reaction that contains no insert. By using this control in a transformation reaction, they can get a background reading of the frequency of vector reclosure without insert by counting any resulting colonies. Since the vector is treated with phosphatase, this background is usually very low. Sample data is provided in the following section.

In the next lab session, students view their LB/ampicillin plates to determine if they obtained any colonies. Generally students have 10 – 100 colonies on their experimental plate. Instructors have grown an overnight 4 mL culture in LB/ampicillin using one of their colonies. Students then use this liquid culture to purify the recombinant plasmid. Once they have obtained pure plasmid DNA, they set up an overnight restriction digest with EcoRI. They also set up a reaction containing only plasmid DNA and buffer to use as a control. Finally, students also use their purified plasmid DNA to set up a reaction to sequence the insert.

In the next lab period, students run the restriction digest and control on an agarose gel next to a size marker to determine the presence and size of the insert. The reaction containing no EcoRI represents uncut plasmid on the gel. The students then view their sequencing results and submit the obtained sequences to a BLAST search. From the search results, students determine the identity of the cloned fragment. Approximately 50% of the yeast genome is estimated to contain genetic information, so not all students clone a gene. Many students will instead clone a segment of DNA that contains control elements or repetitive sequences. Students then use the *Saccharomyces* Genome Database to learn more about their cloned fragment.

More background information and animations are available on the following Websites:

Cloning:

<http://www.dnai.org/b/index.html>

Select “Techniques”

PCR:

<http://www.dnalc.org/ddnalc/resources/pcr.html>

Cycle sequencing:

<http://www.dnalc.org/ddnalc/resources/cycseq.html>

Sanger sequencing:

<http://www.dnalc.org/ddnalc/resources/sangerseq.html>

www.nslc.wustl.edu/elgin/genomics/

Select “Genome Sequencing Center Video Tour” in the first paragraph. This 30 min video provides a video tour of the Washington University Genome Sequencing Center with explanations and animations of each step of the sequencing process, which includes PCR and cycle sequencing.

Time Table

The table below provides a general outline for student lab time to perform the experiments. The table does not include the time it may take in lab for students to view and discuss their results or to complete their lab reports.

Technique	Student Lab Time	Notes
Ligation and Transformation	30 - 45 min	Plates incubate overnight 37°C. Instructor then starts overnight cultures from these colonies.
Plasmid Isolation	1 hr	
Restriction Digest	15 min	Overnight incubation 37°C
Sequencing	15 min	Time for results vary**
Gel Electrophoresis	1 – 1 ½ hr	
Sequence Analysis	30 min	

* Can remove and store at 4°C until students can view results in lab

** Time for results and sample preparation directions will vary depending on what facility prepares the sequencing results. Refer to the sequencing core facility you choose to use for more information.

General Materials and Equipment:

micropipettors - 0-20 μL , 20-200 μL , and 100-1000 μL
sterile tips for pipetmen
markers for labeling
vortexer
microcentrifuge
water bath
thermocycler
37 °C incubator with shaker
agarose gel electrophoresis box and power supply
-80°C freezer (for storing competent cells)
Glass spreaders (for plating cells)
Bunsen burners
ethanol (for sterilizing glass spreaders)

Materials Preparation Protocols

LB agar plates

10 g tryptone
5 g yeast extract
5 g sodium chloride
15 g agar
Dilute to 1 L. Adjust to pH 7.0 with 1 M NaOH. Autoclave, then pour into 15 mm petri dishes (25 mL per plate) before solution cools.

LB/ampicillin plates

1 liter of LB agar, autoclaved
Cool to 55 °C
Add 10 mL of 10 mg/mL filter-sterilized ampicillin
Pour into petri dishes before solution cools.

LB sterile media

10 g tryptone
5 g yeast extract
5 g sodium chloride
Dilute to 1 L. Adjust to pH 7.0 with NaOH. Autoclave and store sterile at room temperature.

LB/ampicillin sterile media

1 liter of LB sterile media
Cool to 55 °C
Add 10 mL of 10 mg/mL filter-sterilized ampicillin. Store sterile at room temperature.

1% agarose gel

0.5 g agarose

Dissolve agarose in 50 mL TBE
Microwave for 1 min. Cool for 5 min.
Add 1 μL ethidium bromide (10 mg/mL)
Pour gel.

agarose gel loading buffer

25 mg bromophenol blue
4 g sucrose
dH₂O to 10 mL

agarose gel running buffer – TBE (Tris Borate EDTA)

218 g Tris base
110 g boric acid
9.3 g EDTA
Dissolve in 1.9 L dH₂O. Bring to pH 8.3 with NaOH.

EcoRI-cut yeast genomic DNA (INSERT DNA)

40 μL Yeast genomic DNA (100 ng/ μL)
4 μL *EcoRI* (10 U/ μL)
10 μL 10x *EcoRI* buffer
46 μL dH₂O

Incubate overnight at 37°C. Remove *EcoRI* using a QIAQuick PCR Purification kit. Follow the directions in the kit. Elute with 100 μL dH₂O for 4.0 μg DNA to yield 0.04 $\mu\text{g}/\mu\text{L}$. Store cut yeast genomic DNA at -20°C.

Sequencing mix

Make a 1.6 μM stock solution of the primer.
For each reaction, add 2 μL of primer, 8 μL of BigDye

Student directions state to add 10 μL of their plasmid prep to 10 μL of the Big Dye/primer mix.

Run the samples in the thermocycler using the program:

1. 95° Denature, 3 min
2. 95° Denature, 1 min
3. 37° Anneal, 1 min
4. 72° Elongation, 1 min

Program 40 cycle of steps 2 – 4

Ordering Information

*General chemicals not listed here can be ordered from Sigma
(www.sigma-aldrich.com)*

Unless otherwise noted, the amounts given below will provide enough materials for 20 reactions, with excess.

Yeast Genomic DNA (100 μ L, 100 ng/ μ L)

Invitrogen (www.invitrogen.com)

Catalog number 40802

EcoRI restriction enzyme and buffer (5,000 U, 10 U/ μ L)

Invitrogen

Catalog number 15202-013

pBluescript[®] II XR Predigested Vector (0.02 μ g/ μ L)

Stratagene

Catalog number 212240

QIAQuick PCR Purification Kit (250)

Qiagen

Catalog number 28106

QIAprep Spin Miniprep Kit (250) (Contains solutions #1 - #3 discussed on page 6 of the student directions.)

Qiagen

Catalog number - 27106

Transformax EC 100 E. coli competent cells (10 x 50 μ L, chemically competent – order 4 sets or a total of 40 tubes of 50 μ L each for 20 reactions)

Epicentre Biotechnologies

Catalog number – CC02810

Fastlink DNA ligase kit (DNA ligase – 200 U at 2 U/ μ L, ATP – 10 mM, 200 μ L, 10X buffer)

Epicentre Biotechnologies

Catalog number – LK6201H

T7 Promoter Sequencing Primer (2 μ g, 5 μ g/mL)

Gibco BRL

Catalog number (18416-016)

Taq polymerase and buffer

Invitrogen (www.invitrogen.com)

Catalog number 18038-042

PCR-grade dH₂O

Invitrogen (www.invitrogen.com)

Catalog number 10977-015

Big Dye[®] Terminator v1.1 Cycle Sequencing Kit

Applied Biosystems (www.appliedbiosystems.com)

Product number 4336774

dNTP mix (10 mM)

Invitrogen (www.invitrogen.com)

Catalog number 18427-013

Instructor Preparation

Lab 1:Ligation and Transformation

- Prepare INSERT DNA (See EcoRI digest under **Protocols**). Each student (or group) will use 1 μL .
- Aliquot VECTOR DNA. Each student (or group) will use 2 μL .
- Set out 10 mM ATP from ligation kit on ice. Each student (or group) uses 2 μL .
- Prepare two “Ligase” tubes per student (or group). These will be their reaction tubes. They should contain 1 μL ligase, 1 μL 10x ligase buffer, and 5 μL dH₂O. Student directions state to add 1 μL of ATP (from the FastLink ligase kit), 1 μL of INSERT DNA, and 1 μL of VECTOR DNA for a final reaction volume of 10 μL .
- Need two tubes of competent *E. coli* cells (50 μL each) per student (or group). Keep at -80°C until needed.
- Place LB/ampicillin plates at 37°C. Need 2 per student (or group).
- Set water bath to 70°C.

Lab 2:Plasmid Isolation, Restriction Digest, and Sequencing Reaction

- Start overnight 4 mL liquid cultures (in LB/amp) from student cloning plates the evening before lab.
- Sequencing mix - need one BigDye tube per student (or student group)
- Companies for DNA sequencing:
<http://www.genegateway.com/>
<http://www.genomex.com/>

Lab 3:Gel Electrophoresis and Sequence Analysis

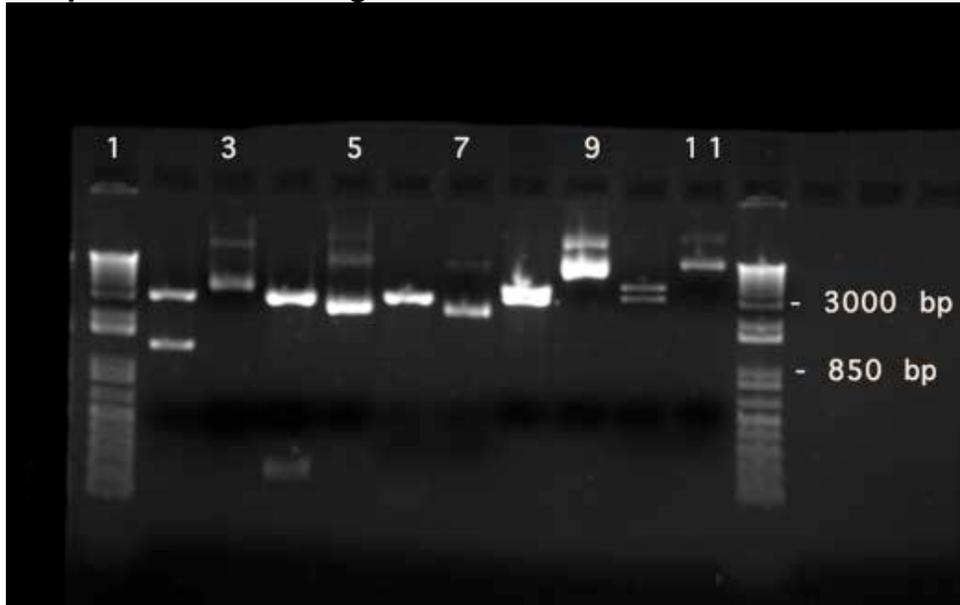
- Organize DNA sequence results on lab computers so they are easily accessible by students.

Sample Data and Results

Colonies obtained from cloning:

- + insert plate = 10 – 50 colonies
- insert plate = 0 – 2 colonies

Gel picture of EcoRI digest



Lanes 1 and 12 contain 1 kb DNA ladder.

The pBluescript vector runs at 3000 bp when cut (linear). It runs slightly faster when supercoiled (uncut).

Lanes 3, 5, 7, and 9 contain uncut recombinant plasmid.

Lanes 2, 4, 6, 8, and 10 contain the cut samples.

Lane 2 = 3 kb vector and ~1 kb insert

Lane 4 = 3 kb vector and ~200 bp insert

Lane 6 = 3 kb vector (no insert)

Lane 8 = 3 kb vector and possibly 3 kb insert (since uncut vector is running so high)

Lane 10 = 3 kb vector and ~4 kb insert

Sequence and BLAST results from www.yeastgenome.org

Insert sequence (from Lane 2 on gel above):

```
TACGGCGATAGGGTACCGGGCCCCCCTCGGCGCGCAGGCATCGATAAGCTGG
ATATCGAATTCAAGAGCTGCTAGACAATCGGATGCTCTTGAACCAGAGGTAAAGG
ATTTAAGTGAACCTACCTAGAGCTGAAAAAACCGATTTGACTAATATTTGGATTAGA
ACAGCAGCACAAGAATGAGGCATTGCTGGAAGCAAAGATATCTAAGAAAGCCAA
TAAAAGTAAGAGGGGCAAGAAGTTAAATAAAAAGGCTCTGGAAGACAACTGGC
CAACTCTATTTTCATCCATGGACAGGGATCGTTTAGTGAAGGCCTTGAATTTTACC
AATCGTCTGGACGGTAAAATTGCCAAGTCCATTTCTCGTGCCAAGTACATTCAA
ATACAAGAAAGGCTGGCTGGGATAGCACCAATGAGACTATAAAAAAAGAGCTGG
CTTTTTTGAACGGAGGGTGTCTGTGCAGGCTAAAAGTGCTAGTGAAGGTAATGC
TGAAAAGGAAGATGAGGAGATCCCAGAAGTTTTTACTCTTTAGCAGAGGATAAC
ACAGTGCAGAAGACTCCTACATATAGATTTCGGCGTCCTGCCAGACGATGTTGAA
GAATAGAATATTTTCATATGATAGGTCCTAGGAATACACGATTCTTGTACGCATTC
TTCTTTTTTCTATCTTCTTTCATTCTTTGTTTATTACATAACATGGCTTTAGCTTAGT
TTTATTTTATTTTTTATATATCTGGATGTATACTATTATTGAAAACTTCATTAATAG
TTACCAACTTTCCATATTAAGTTGATTAAGAAAAAGAAATTATTATGGGTAAGCTG
AAACCGTGTGATGCATGTCGTTTAAAGATTGTGTAATAAAGGGGACCGCAACGCT
TTCTTATATAGATTACCGCCCCACAAAGGAGTACTATGATATTCTCTGCGCATT
TTGTGGCTGGGTTTGAATCCAGCGTTGGGGAAAGGCACCTCTTGGCTATCCTAC
GCTTCATTACCGAAGATCCTCTTGGGGGCTTTTCAAGCTTCCCTATTACAAGCATT
TAACCCACACGGGACCCAGGGAAACGCCACATGAAGGCCCTAATAATGGGGC
CCAAGGGGACATTCCAGGCCGAGGCAGGACCCAGGAAAAGGGGAAGCGCGCG
AAAGGGAAGCCGCCTCGTAAGACACACTGGGAAACTCTAGGTCAAAAAGACACA
```

Blastn hit P number = 6.3×10^{-194}

Gene name = ECM 1

Lab report ANSWERS based on sample data (lanes 2 and 3 of gel shown above)

1. In the table below, indicate your results for this experiment. Count the number of colonies on each plate and record them. If there are more than 500 colonies just record '>500'; if there is a lawn record 'lawn'.

Plate	Reaction contained:	Your results
1 (-)	Cells + vector, no insert, + ligase	2
2 (+)	Cells + vector, + insert, + ligase	10

2. If this experiment worked correctly, you will see colonies on plate #2. Explain the presence of bacterial growth that you see on plate #1. Based on the presence or absence of bacterial growth on plate #1, can you say that your experiment was successful or not? Explain.

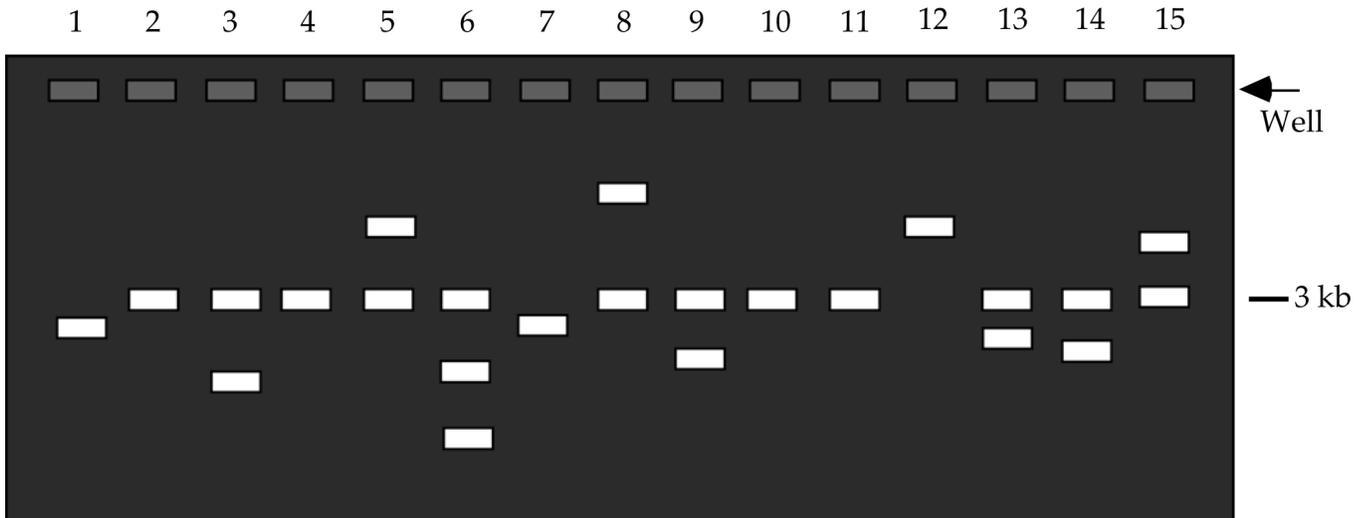
The two colonies on the (-) plate could be from several different things:

Contamination by amp-resistant bacteria.

Amp in plate may not be at a high enough concentration to inhibit growth.

Most likely, the phosphatase treatment of the vector was not 100% efficient and some vector was able to ligate in the absence of insert. This background shows that it is likely that approximately 2 colonies on the (+) plate contain vector without insert.

Here is a representation of a gel similar to the one you ran in lab:



The white bands represent ethidium stained DNA fragments.

Lane 1 = Uncut pBS vector

Lane 2 = pBS vector digested with *EcoR* I

Lanes 3 - 15 = *EcoR* I digestions of picked clones (plasmid DNA)

2. Why does the predominant band in the uncut pBS lane migrate with a different mobility compared to the linear ~3 kb *EcoR* I digested plasmid?
(Hint... electrophoretic mobility is based on both size and shape.)

The uncut vector is supercoiled, so migrates faster than linear DNA.

3. Which clones do not appear to contain inserts? Explain why you chose these clones.

Lanes 2, 4, 10, 11 Only the vector band is present.

4. Provide an explanation for the pattern seen in lane 6.

There are two inserts. The three bands are the vector at 3 kb and two inserts less than 3 kb.

Analysis of Your Gel Electrophoresis Results

These questions should be answered based on the students results, so answers will vary. The answers provided here are based on lanes 2 and 3 of the gel picture provided under the section "Sample Data."

Examine the photograph of the agarose gel and answer the following questions.

1. Were you able to isolate plasmid DNA? (Do you see bands on the gel?)

yes

2. Describe the results of your "uncut" sample (how many bands and what size(s)).

1 band approximately 4 kb. It is running a little faster.

3. Describe the results of your EcoRI digested DNA (how many bands and what size(s)).

2 bands at 3 kb and approximately 1 kb.

4. Was the plasmid you isolated a recombinant plasmid? Explain how you got your answer.

Yes, a recombinant plasmid contains DNA that has been combined from two sources. We have the vector DNA (3 kb) and a second band, which should be yeast DNA (will sequence to confirm).

Analysis of the Yeast Genome Insert Sequence

Sequence Information Form

	Sequence Cloned in Class
Sequence information:	
Name of file	Sample file (student name or initials)
P-value of top hit from the BLAST search	6.3e-194
Chromosome and coordinates (bp)	Chromosome 1 26000 to 46000 bp
Gene name	ECM1
Crick or Watson strand?	Watson
Gene product:	
What is the function of the gene product?	Unknown, interacts with MTR2 in 60S ribosomal protein subunit export
Where is the gene product found in the cell?	nucleus
Can yeast survive without this gene?	yes

1. Compare your results with results from other students. Did everyone clone a gene? What are the implications of the class results?

Not all DNA in the genome codes for a gene.

2. Which organism has more non-gene DNA, yeast or humans? (See table below.)

humans

Human Genome vs. Yeast Genome

Fact Table:

	Human	Yeast
Number of chromosomes	22 + X and Y	16
Total size (bp)	3,000,000,000	12,067,000
Total genes (estimated)	~25,000	~6,200
Repetitive elements	43%	10%
Pseudogenes	~8,000	48 (non-gene ORF's)
% of genome that is coding	~1%	~50%

The average length of a gene is ~1000 bp (although some yeast genes are much shorter and some human genes are much longer). Based on this length, what is the maximum number of genes that the human and yeast genomes could have? Which genome is closer to having its maximum number of genes?

Humans could have 3,000,000 genes, but only have 25,000. Yeast could have 12,067, but only have 6,200. Yeast are much closer to having the maximum number of genes.

Cloning and Sequencing a Fragment of Yeast DNA

Beginning of Student Pages

Cloning and Sequencing a Fragment of Yeast DNA

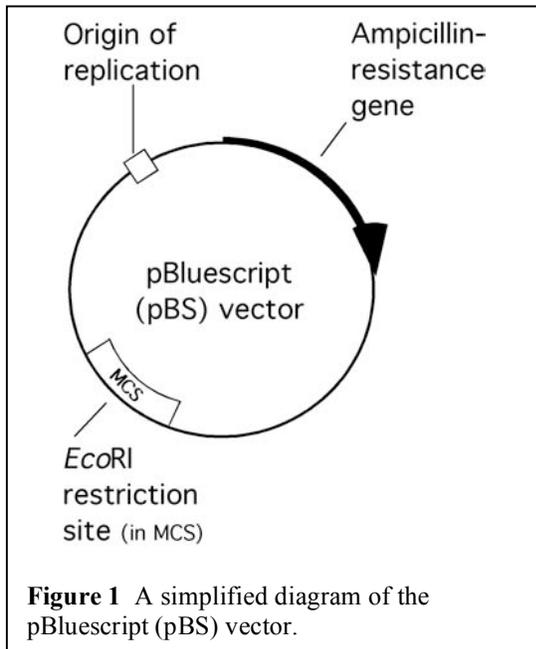
Purpose: The purpose of this lab is to clone and sequence a small fragment of the yeast genome using *Escherichia coli* bacteria then determine what gene(s) this fragment contains. The process will enable us to investigate the organization of the yeast genome.

Background:

Molecular cloning allows DNA fragments to be isolated and amplified. The basis of the technology is the *in vitro* joining of autonomously replicating vector DNA molecules with DNA insert fragments from the desired source organism. The *in vitro* joining is usually performed by a DNA ligation reaction (catalyzed by DNA ligase) between compatible restriction endonuclease-generated ends on vector and source DNA fragments. This experiment is designed to illustrate how recombinant plasmids are made and analyzed.

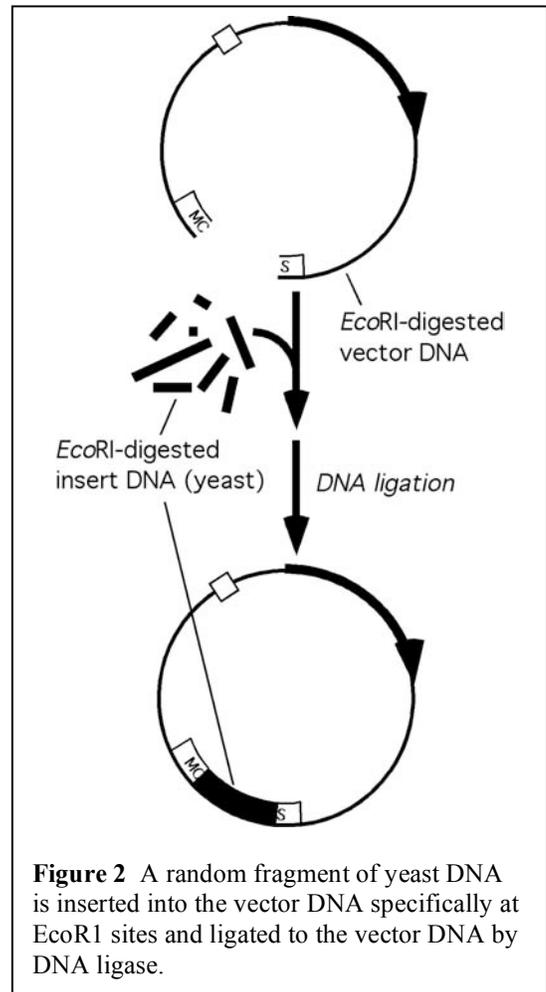
The recombinant plasmids made in this week's experiment are a combination of yeast genomic DNA and bacterial plasmid DNA. The yeast genomic DNA is referred to as the insert, and the plasmid DNA is the vector. In this kind of cloning experiment the researcher is interested in analyzing the yeast DNA, and feels the analysis will be easier if the yeast genome is separated into small parts that can be individually analyzed. The end result, if the entire yeast genome is cloned into separate chimeric plasmids, will be the creation of a yeast genomic library. This is the strategy used in big sequencing experiments, for example sequencing the entire yeast genome. The genome is first separated into manageable parts, each part is sequenced, and then the separate sequences are aligned to determine the sequence of the entire genome.

To create the recombinant plasmid, you will use a small (~3 kb) double-stranded plasmid DNA vector called pBlueScript (pBS; figure 1 below). Like all good vectors, pBS contains the necessary information to support its autonomous replication in a host (*E. coli* in this case). In addition, the plasmid carries a selectable marker (ampicillin-resistance) to allow easy identification and recovery of host cells harboring the plasmid. Finally, the plasmid has a small region, called the multiple cloning site, that contains 'unique' cutting sites for various restriction endonucleases. The pBS was cut once with the restriction endonuclease *EcoR* I to generate 5' overhangs. The cut plasmid was subsequently treated with a phosphatase which removes the 5' phosphate from the cut end, thereby preventing plasmid vector re-ligation (remember that a DNA ligase reaction requires a 5'-phosphate and a 3'-hydroxyl as substrates). The insert DNA is from the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* genomic DNA was cut with the restriction endonuclease *EcoR* I but NOT treated with phosphatase.



The source and vector DNA samples are mixed together in the presence of buffer, ATP, and DNA ligase. The result should be the formation of a collection of recombinant plasmids that each contain the vector DNA and some fragment of yeast genomic DNA (Figure 2).

In the next step, the ligation products will be mixed with *E. coli* cells that were pretreated with CaCl_2 to permeabilize their cell membranes, thereby facilitating uptake of DNA molecules from the external media. (Cells prepared in this manner are known as 'competent' cells; the process of the DNA entering the cell is called **transformation**.) At the end of the transformation reaction, the cell mixture will be spread on selective LB + ampicillin plates, allowing the bacteria that have taken up plasmid DNA to replicate while killing the bacteria that failed to take up a plasmid. The result will be the growth of colonies, each colony representing an independent 'transformation' event. This means that each colony is made of up of bacteria that all carry the same recombinant plasmid, but the bacteria in different colonies carry recombinant plasmids that differ from each other in the specific fragment of yeast genomic DNA contained in the plasmid.



Lab 1: Ligation and Transformation

Exercise 1: Ligation

1. Obtain 2 microcentrifuge tubes from your lab instructor that contain DNA ligase and reaction buffer. Label each tube with your initials. In addition, label one tube “+” and the other “-“ corresponding to whether INSERT DNA will be added to that tube or not.
2. To each tube, add 1 μ L of VECTOR DNA.
3. To the “+” tube, add 1 μ L of INSERT DNA.
4. To the “-“ tube, add 1 μ L of dH₂O.
5. To each tube, add 1 μ L of ATP to start the ligation reaction.
6. Gently tap each tube with your finger to mix. Briefly (about 20-30 seconds), centrifuge the tubes using your table-top centrifuge to collect the liquid in the bottom of the tube.
7. Incubate the tubes for 5 minutes at room temperature.
8. Incubate the tubes for 15 minutes at 70°C to inactivate the DNA ligase.
9. Briefly centrifuge the tubes and place them on ice.

Exercise 2: Transformation

1. Obtain two microcentrifuge tubes from your lab instructor that contain chemically competent *E. coli* cells and quickly place them on ice. **It is extremely important that these cells do not warm to room temperature!** Label the tubes “+” and “-.”
2. Transfer 1.5 μ L from the “+” ligation reaction tube into the “+” tube containing the *E. coli*. Carefully, tap the tube with your finger to mix the contents. Do NOT vortex.
3. Likewise, transfer 1.5 μ L from the “-“ ligation reaction tube into the “-“ tube containing the *E. coli*. Tap the tube with your finger to mix the contents.
4. Incubate for 5 minutes on ice.
5. Near the end of the 5 minute incubation, obtain two LB + ampicillin plates from your lab instructor and label them with your group’s initials and “+” or “-“ corresponding to your two tubes. *Having the plates pre-warmed to 37°C improves the success of this experiment.*

6. Using sterile technique, spread 50 μ L of the cells on the appropriately labeled pre-warmed LB+ampicillin plate.
7. Hand your plates to your lab instructor for overnight incubation at 37°C.

Lab 2: Plasmid Isolation and Restriction Digest

Today you will use with an overnight culture of bacteria that was started from one unique colony selected from your transformation plates. Using this culture, you should follow the procedure written below to isolate the plasmid DNA from those cells. As you know, an overnight bacterial culture contains approximately 10^8 cells/mL, and those cells can each contain multiple copies of the plasmid, so the plasmid DNA has already been amplified a lot.

To purify your plasmid DNA, you will use a standard 'alkaline lysis' bacterial plasmid miniprep procedure (from the QiaPrep kit) that allows isolation of plasmid DNA free of the bacterial chromosomal DNA. The protocol involves five steps. First, the bacterial cells are harvested by centrifugation. Second, the cells are resuspended in a buffered solution (Solution #1) and lysed by the addition of Solution #2 containing NaOH and sodium dodecyl sulfate (SDS, a common detergent). The NaOH also serves to denature double-stranded chromosomal and plasmid DNA. The third step is the neutralization (with Solution #3) of the alkaline lysis solution to allow double-stranded DNA to reform. The strands from each small circular plasmid DNA will remain interlocked and will quickly reanneal to reform a double-stranded plasmid. However, the complementary strands of the larger linear chromosomal DNA fragments will not reanneal properly and will become irreversibly denatured and insoluble. In addition, the potassium ions in the neutralization solution (#3) cause the detergent to precipitate with insoluble denatured proteins and improperly re-associated chromosomal DNA, carbohydrates and cellular debris. This unwanted material is pelleted by centrifugation in the fourth step, leaving a supernatant containing the desired plasmid DNA. The fifth step is the collection of the plasmid DNA using a column.

After isolating plasmid DNA from your bacteria, you will set up a restriction endonuclease digestion. A portion of your newly isolated plasmid DNA will be mixed with *EcoR* I and buffer. Your restriction digests will be incubated for several hours at 37°C and the resulting fragments size-fractionated by agarose gel electrophoresis next week in lab. Finally, you will also use the plasmid DNA to set up a sequencing reaction to sequence the insert.

Exercise 1: Plasmid DNA Isolation:

1. Transfer 1000 μ l of your overnight culture to a clean 1.5 ml microcentrifuge tube.
2. Pellet cells by spinning in a high speed microcentrifuge for 1 minute.
3. Remove the supernatant using a P-1000 pipetman (pipet supernatant into tip jar).

4. Add 250 μ l of Solution #1 to the cell pellet.
5. Vortex the tube to resuspend the cell pellet. Make sure that no pellet remains visible on the bottom of the tube.
6. Add 250 μ l of Solution #2. Close the cap and tip the tube back and forth to mix (Do not vortex!). Let the tube sit at room temperature for one minute. The mixture should become clear (loss of turbidity due to cells lysis) and viscous (long DNA polymers spill out of the broken cells).
7. Add 350 μ l of Solution #3. Close the cap and tip the tube back and forth again to mix (Do not vortex here either). You should see a white precipitate form. Continue tipping back and forth for 30 seconds.
8. Spin the tube in a high speed microcentrifuge for 10 minutes to pellet the debris (including chromosomal DNA, proteins, carbohydrates).
9. Label a clean spin column with your sample name and initials. Carefully remove the entire volume of the **supernatant** and transfer to the spin column. Be careful not to transfer any of the pellet or floating particles. Discard the tube with the precipitate.
10. Spin the DNA-binding spin column / microcentrifuge tube in the microcentrifuge on your bench for one minute. All of the liquid will be forced through the column and the DNA in the liquid will bind to the resin on the column. Discard the liquid in the microcentrifuge tube and put the column back into the same tube.
11. Transfer 700 μ l of Solution #4 onto the column. Spin the tube in the microcentrifuge on your bench for one minute. This step serves to wash any non-specifically bound material off of the column. The plasmid DNA remains bound to the column during this wash step.
12. Discard the liquid in the microcentrifuge tube and replace the column back in the same tube.
13. Spin the column / microcentrifuge tube (empty) in the high speed microcentrifuge (on the side bench) for one minute. This spin results in the removal of any residual liquid from the column. The plasmid DNA remains bound to the column.
14. Discard the microcentrifuge tube and place the column (still with your plasmid DNA) in a new, labeled microcentrifuge tube.
15. Elute (wash off) your plasmid DNA from the column by pipeting 35 μ l of dH₂O onto the column. Spin the column / microcentrifuge tube in the high speed microcentrifuge for one minute. Your plasmid DNA is now in the liquid in the

microcentrifuge tube; the column can be discarded. Use this DNA to set up both the restriction digestion and the sequencing reactions as specified below.

Exercise 2: Restriction Endonuclease Digestion

1. Transfer 5 μ l of your plasmid DNA into a clean microcentrifuge tube marked **E** for *EcoR* I digestion. Transfer another 5 μ l of your plasmid DNA into a second microcentrifuge tube marked **U** for the uncut control. Add your initials and section letter onto the side or top of the tubes.
2. Add 15 μ l of *EcoR* I restriction digestion mix (buffer + *EcoRI*) to the tube labeled 'E'. Add 15 μ l of mock restriction digestion mix (buffer but no enzyme) to the tube labeled 'U'. This mock digestion serves as a negative control to determine if the *EcoR* I digestion worked.
3. Give your tubes to your instructor for overnight incubation at 37°C. Later you will electrophorese your samples to see the result of the DNA isolation and *EcoRI* digestion.

Exercise 3: Sequencing Reaction

1. Obtain a tube containing the sequencing mix (2 μ L of the primer and 8 μ L of the BigDye Terminator mix) from your lab instructor.
2. Transfer 10 μ l of your plasmid DNA into the sequencing mix.
3. Return your tube to your instructor. Samples will be placed in a PCR machine for 40 rounds of cycle sequencing and the products will be analyzed on a DNA sequencing machine. You will receive the sequence data from the fragment you have cloned.

Agarose Gel Electrophoresis of Plasmid DNA and Computer Analysis of Yeast Genome Inserts

This lab period you will see the results of the plasmid isolation and restriction endonuclease digestion that you did previously. Recall that we are hoping to isolate recombinant plasmids that contain pBS vector sequence, and some fragment of yeast genome DNA. The two pieces of DNA were put together by ligating ends originally created by *EcoRI* digestion, so *EcoRI* recognition sites should be present at the junctions between vector and insert DNA.

For the DNA agarose gel, DNA will be loaded into the wells of the gel and a current will be applied with the positive electrode connected to the bottom of the gel. Since DNA is negatively charged, all of the DNA will run from the wells toward the bottom of the gel. The smaller pieces of DNA will be able to migrate more quickly through the agarose, and so will be farther from the wells at the end of the electrophoresis. To visualize the DNA, we will use a chemical called ethidium bromide. Two useful properties of ethidium bromide are that it naturally intercalates into DNA (thereby tagging DNA molecules), and that it fluoresces when exposed to UV light. The "glowing" spots on the gel show the position of DNA molecules in the gel. In our experiment the ethidium bromide is contained in the agarose gel, so the DNA mixes with it as it electrophoreses. Remember that **intercalating agents like ethidium bromide are mutagens**, so you must protect yourself from exposure by wearing gloves when handling gels or samples containing ethidium bromide.

While the gels are running, you will be able to analyze the sequence that was obtained of your cloned yeast DNA fragment. After viewing your sequence results, you will be able to submit the sequence to a BLAST search to look for matching sequences in the *Saccharomyces* (yeast) Genome Database (maintained at Stanford University).

Exercise 1: Electrophoresis of restriction digestions

Did I succeed in cloning a fragment of yeast DNA?

1. Add 3 μ l of 10 X loading dye to your E tube (containing your *EcoRI*-cut DNA) and to your U tube (control – uncut DNA). Spin each sample for about 30 seconds in the microcentrifuge on your bench.
2. If desired, use the 10 μ l of the practice sample to practice gel loading using the gels in petri dishes at your bench.
3. At the real gel at the front of the lab, load the entire E sample into one well and the entire U sample into another well, following the instructor's directions on specifically which wells to use.

4. The gel will run approximately 1 hour at 200 V. After electrophoresis is complete, the gel will be placed on a UV light box and photographed for analysis.

Exercise 2: Computer Analysis of Yeast DNA Inserts

Searching for information about your cloned DNA

What gene is it? Is it a gene at all?

1. Go to the Saccharomyces Genome Database at www.yeastgenome.org
2. Select the BLAST program from the links along the bar at the top of the page. When the BLAST page opens, you should see a box to enter your sequence data. You can just copy and paste the sequence results (in FASTA format) into that box. Next, change the Filter to "none." Finally, hit "Run BLAST" to scan your sequence against the yeast genome.

FASTA format and the BLAST program will be explained in class. When you submit your sequence for BLAST, the program will search the DNA sequence of the entire yeast genome to find the sequence you obtained from cloning. When it gets a match, a page will open with the results of your match and will contain links that you can use to find out more about the sequence that your clone matches (for example, gene name, location in the genome, possible function etc.)

3. On the results page, you will see some scores as well as a probability number (P-value). You can think of the P-value as the probability that the hit you found was found by random chance and is not significant (so you want the P-value to be very low!). Start filling out the Sequence Information Form as you complete the following steps.
4. It is hard to derive much information about the sequence on this page, so select "ORF Map" to see a map of the open reading frames in this area of the genome.
5. The match to your sequence is located between the red dotted lines. If there is a red or blue box in that location, you have cloned part of a gene. The key to the colors used in this diagram is directly below the diagram. The gene names are short combinations of letters and numbers near the red or blue box. If the dotted lines do not intersect with a gene, skip the next step and work on questions 10 and 11. Non-coding DNA is discussed in the lab report.
6. Click on the colored box representing the gene, and a new page will come up with information about that gene. Use this information to answer the questions about the gene product on the Sequence Information Form.

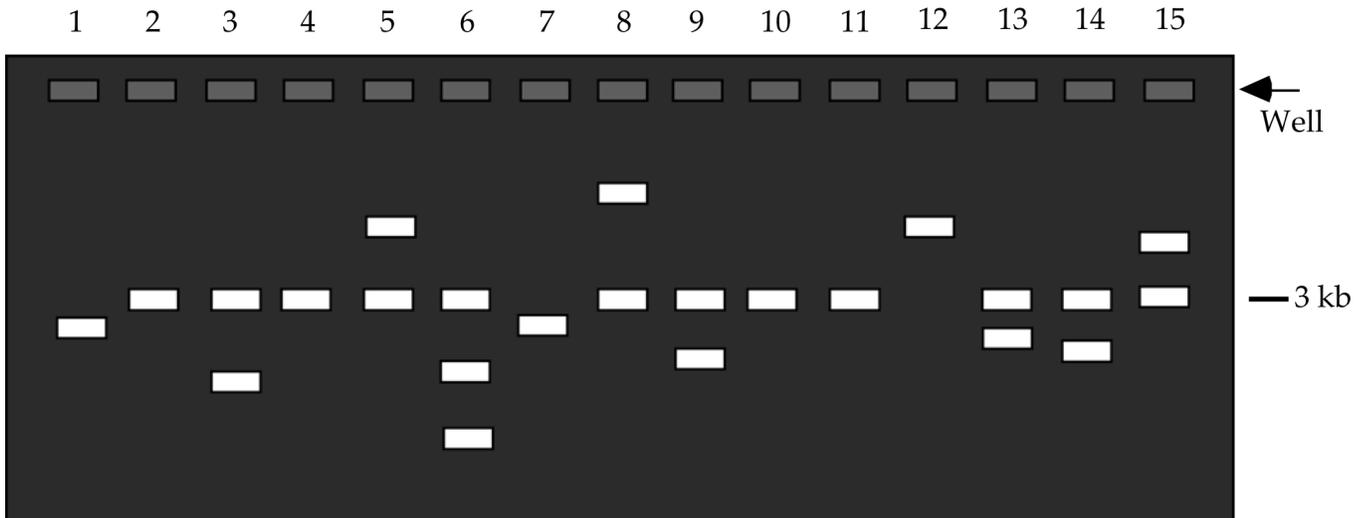
Lab Report: Molecular Cloning and Analysis of Yeast Genomic Clones

1. In the table below, indicate your results for this experiment. Count the number of colonies on each plate and record them. If there are more than 500 colonies just record '>500'; if there is a lawn record '**lawn**'.

Plate	Reaction contained:	Your results
1 (-)	Cells + vector, no insert, + ligase	
2 (+)	Cells + vector, + insert, + ligase	

2. If this experiment worked correctly, you will see colonies on Plate #2. Explain the presence of bacterial growth that you see on plates #1. Based on the presence or absence of bacterial growth on plates 1 and 2, can you say that your experiment was successful or not? Explain.

Here is a representation of a gel similar to the one you ran in lab:



The white bands represent ethidium stained DNA fragments.

Lane 1 = Uncut pBS vector

Lane 2 = pBS vector digested with *EcoR* I

Lanes 3 - 15 = *EcoR* I digestions of picked clones (plasmid DNA)

3. Why does the predominant band in the uncut pBS lane migrate with a different mobility compared to the linear ~3 kb *EcoR* I digested plasmid?
(Hint... electrophoretic mobility is based on both size and shape.)

4. Which clones do not appear to contain inserts? Explain why you chose these clones.

5. Provide an explanation for the pattern seen in lane 6.

Analysis of Your Gel Electrophoresis Results

Examine the photograph of the agarose gel and answer the following questions.

6. Were you able to isolate plasmid DNA? (Do you see bands on the gel?)

7. Describe the results of your "uncut" sample [how many bands and what size(s)].

8. Describe the results of your EcoRI digested DNA [how many bands and what size(s)].

9. Was the plasmid you isolated a recombinant plasmid? Explain your reasoning.

Analysis of the Yeast Genome Insert Sequence

Sequence Information Form

	Sequence Cloned in Class
Sequence information:	
Name of file	
P-value of top hit from the BLAST search	
Chromosome and coordinates (bp)	
Gene name	
Crick or Watson strand?	
Gene product:	
What is the function of the gene product?	
Where is the gene product found in the cell?	
Can yeast survive without this gene?	

10. Why didn't everyone clone a gene?

11. When this experiment was previously performed with 1000 cloning reactions, 500 clones contained a gene, 2 clones contained a rRNA gene, and 20 clones contained a tRNA gene. What do these results reveal about the contents of the yeast genome?

12. Which organism has more non-gene DNA, yeast or humans? (See table and questions below.)

Human Genome vs. Yeast Genome

Fact Table:

	Human	Yeast
Number of chromosomes	22 + X and Y	16
Total size (bp)	3,000,000,000	12,067,000
Total genes (estimated)	~25,000	~6,200
Repetitive elements	43%	10%
Pseudogenes	~8,000	48 (non-gene ORF's)
% of genome that is coding	~1%	~50%

The average length of a gene is ~1000 bp (although some yeast genes are much shorter and some human genes are much longer). Based on this length, what is the maximum number of genes the human and yeast genomes could have? Which genome is closer to having its maximum number of genes?