DISCOVERING GENETIC INTERACTIONS OF THE DEAD-BOX PROTEIN, DBP1

By

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Abstract:

Medulloblastoma is the single most common form of malignant brain cancer in children. A molecular understanding of the cellular processes that leads to its formation is imperative in order to design treatments or a cure to this deadly cancer. A recent genomic screening of medulloblastoma patients showed high mutation rates of the gene DDX3X indicating a possible connection to the cancer. Thus, we investigated a yeast ortholog of DDX3X, Dbp1, to gain some insight of the function of the protein within the cell and its role in medulloblastoma formation. In order to gain deeper understanding of this mostly uncharacterized DEAD-box RNA helicase, previous lab members performed a synthetic lethal screen in *Saccharomyces cerevisiae* with *dbp1-null* cells. We found that a wild type copy of *SHM2*, was found to rescue sectoring in one of these synthetic lethal yeast strains. However, it has been documented that *shm2* mutations show synthetic lethal interactions with *ade3* mutations, so we are unable to conclude that *SHM2* has genetic interaction with *DBP1*. However, this finding can be used to rule out *SHM2* as the cause of loss of sectoring phenotype in other *dbp1* synthetic lethal mutant strains.
Introduction:

The DEAD-box protein family is a group of highly conserved RNA helicases identified among a broad phylogeny of both prokaryotic and eukaryotic organisms. They are functionally characterized as RNA binding proteins that unwind RNA helices in an ATP-dependent manner (Linder et al. 1989). Though they can be highly similar in sequence, each DEAD-box protein has its own specific role within the cell. These roles encompass almost every step in the RNA life cycle, including: transcription, splicing, nuclear export, translation, subcellular localization, and decay. The necessity of the DEAD-box proteins in these steps comes from the need for a highly specific secondary structure of the RNA and tertiary structure of ribonucleoprotein complexes (RNPs) as well as effective transitioning to different specific conformations at certain times in each cellular process (Jarmoskaite and Russell 2016).

One such DEAD-box protein, DEAD box helicase 3 (DDX3), has been found to play a role in tumor progression of many cancer types in humans (Zhao, et al. 2016). Some recent studies involving the full genome analysis of numerous patients with medulloblastoma, the most common malignant brain cancer in children, revealed a link between mutations in the DDX3X (X-linked) gene and formation of the cancer (Jones 2012, Robinson 2012, Kool 2012, Bish and Vogel 2016, Zhao et al. 2016). Although we could study the molecular functions of DDX3X directly from patient tissue, with current technology genetically modifying human somatic cells is impractical and inefficient. Thus, to investigate this cancer, we have worked to characterize the function of this gene in an organism with an orthologous gene (high nucleotide or functional similarity to DDX3X) that would be easier to genetically manipulate than human cells.
Saccharomyces cerevisiae has proven an effective organism for modeling highly conserved biological processes of eukaryotic organisms due to the ease that they can be cultured in high quantities in a short amount of time within the lab. Furthermore, manipulating the genome of these single-celled organisms is relatively simple due to their compatibility with inserted extrachromosomal circular DNA (plasmids) (Møller 2015). The yeast orthologs of DDX3X were identified as both \textit{DED1} and \textit{DBP1}, which are thought to have arisen as a result of a whole genome duplication event of an ancient lineage (Byrne 2005). Though Ded1 was found to function as an RNP remodeling enzyme important for translation initiation, the function of Dbp1 is not well understood.

In this experiment a synthetic lethal mutant screen was conducted to gain insight into the function of Dbp1. Typically, the simplest way to identify a gene’s function is to “knock-out” or make the protein product non-functional through mutation to observe a phenotypic defect that offers hints to its role in the cell. However, although \textit{ded1} mutations cause cell lethality, \textit{dbp1} defects appear to be nonessential for cell viability (Jamieson and Beggs 1991), making our observations of the protein’s activity within single knock-out strains more difficult. Synthetic lethal mutants are strains of cells that have induced loss of cell viability from two or more mutated genes that do not alone result in mortality. Since \textit{dbp1} mutants alone show no visible phenotype, synthetic lethal screens can be useful to identify a well documented gene that genetically interacts with Dbp1. This knowledge would allow for conceptual gene mapping and the design of further experiments to grasp the function Dbp1 in the yeast cell and quite possibly shed light on its role in medulloblastoma development in humans.
Yiran (Kate) Li, a former member of the Bolger lab, performed random mutagenesis on
*dbp1* mutant yeasts strains that contained a plasmid containing a wild type copy of *DBP1* and an
*ADE3* nutrient marker that causes the yeast to turn red. Typically the yeast cells in these colonies
tend to lose this plasmid as they grow outward, causing the appearance of small whitish (normal
color) sections, a phenotype termed colony sectoring. Kate performed a screen for genes
exhibiting synthetic lethality with *dbp1* by identifying colonies containing the wild type *DBP1*
plasmid that underwent random mutagenesis and as a result no longer showed colony sectoring
(exhibited only the red phenotype throughout). Cells in these synthetic lethal red colonies die
when they lose the *DBP1/ADE3* plasmid, thus they no longer show white sectoring. Since the
cells survive, the unknown mutation does not cause lethality alone, and since the cells showed
sectoring prior to mutagenesis, the *dbp1* mutation does not alone cause lethality. We can thus
conclude that Kate’s red-streaking colonies contain a mutation that exhibits synthetic lethality
with *dbp1*. These “hits” are the focus of my thesis: identifying genes that have genetic
interactions with *DBP1* to help characterize the function of Dbp1 within a cell. Ultimately, we
hope that our findings may help elucidate the mechanism by which DDX3X mutations causes
medulloblastoma in human cells.

**Results:**

To identify the unknown mutation created through random mutagenesis that presumably
exhibited genetic interaction with *dbp1*, we transformed the synthetic lethal mutants with an
amplified yeast plasmid library and screened for a restoration of colony color sectoring -- cells that could grow without their DBP1/ADE3 plasmid. We were successful after a number of library transformations (Figure 1, right). Following the rescuing of lethality in one synthetic lethal mutant, labeled *1, we faced the challenge of isolating the rescuing plasmid.

Figure 1: Left - Synthetic lethal *1 mutant strain with no colony sectoring. Right - Restreaking of synthetic lethal colonies with restored sectoring results in both red and white colored yeast throughout the plate.

The first extraction technique attempted was a Qiagen miniprep kit normally intended for plasmid extraction from bacterial cells. With this extract, we attempted a chemical transformation on chemically competent E. coli using the miniprep products which involved heat shocking the cells in a water bath so they open up their membrane and uptake the plasmids. Unfortunately, no colonies grew from this. We then attempted an electroporation procedure
which involved shocking the cells with a jolt of electricity to open the pores of the cells’ membranes to uptake the plasmids. This was also unsuccessful after a number of tries.

Since both procedures showed no results, we changed our approach by altering the yeast extraction procedure instead of the bacterial transformation procedure. Instead of using the Qiagen miniprep kit, we attempted phenol-chloroform extraction which involved breaking open the yeast cells, denaturing the proteins, and separating them from cellular DNA. However, this process produced no DNA that could be detected on a spectrometer or gel electrophoresis. Then, we attempted plasmid recovery which involved extraction with various chemicals. Using this method, bands of DNA were visible on a gel. We attempted two gel extraction techniques: a simple Qiagen gel extraction kit and tombstoning. Using the products from both the gel extraction kit and the tombstoning, we attempted to transform *E. coli* with both the electroporation procedure and chemical transformation procedure. Both extraction techniques with both transformation procedures failed to yield colonies.

At this point, we obtained Zymoprep miniprep kits specifically designed for yeast plasmid extraction. Additionally, special high-efficiency chemically competent cells were ordered for this particular chemical transformation in hopes that we could achieve at least one successful transformation. We mini-prepped the *1 rescued hit using the Zymoprep protocol and proceeded to transform the DH5α high efficiency chemically competent *E. coli* via chemical transformation. This process yielded fifteen *E. coli* colonies on LB+Ampicillin plates that could be mini-prepped individually using the Qiagen miniprep kit.

The products of the *E. coli* extractions were then individually transformed into the original *1 strain to screen for which isolated plasmid carried the gene fragment that rescued the
cells from synthetic lethality. Out of 15 bacteria minipreps, one restored colony sectoring in *1 yeast and thus was confirmed as the rescuing plasmid. Finally, we sent the *E. coli* miniprep product to a lab for nucleotide sequencing. We compared the sequenced library plasmid using the yeastgenome.org database and found that the genome fragment was derived from the base pairs 255889 to 265405 (9516bp) on chromosome twelve and consisted of the genes: *SHM2, REX2, FRS1, RPL22A, BUD28* (a dubious gene), and *BMT6* (Figure 2). Thus, we successfully identified a length of the yeast genome containing six genes, one of which was presumed to contain the synthetic lethal mutation in the yeast.

![Figure 2: Visualization of library plasmid fragment that restores colony sectoring in *1 yeast. Image taken from yeastgenome.org genome browser (Chr XII 255889..265405).](image)

To find which one of these genes rescued sectoring in the *1* synthetic lethal strain, we designed oligonucleotide primers that could amplify the individual genes using polymerase chain reactions to be ligated into plasmid vectors and transformed into the original *1* colonies.

Following DNA amplification via PCR, restriction digests were performed on the genes and the plasmid vector to be used for ligation. In order to enhance the DNA concentration of
*REX2* and *FRS1* to adequate ligation amounts, we altered the PCR procedure by first using different DNA polymerase, then we redesigned the primers to have a higher GC ratio so that they anneal more tightly to the complementary strand, and finally we changed the PCR program to have longer elongation periods. After this we could visualize bright *REX2* bands on a gel, but *FRS1* showed no visible band whatsoever, so we decided to proceed with the experiment using only *REX2*.

At this point we struggled to extract the DNA from the gel. First we used the Qiagen gel extraction kit on the gel fragments, but the nanodrop spectrometer showed that there was little to no DNA yielded from this. Desperate for results, we attempted to extract the DNA by manually squeezing the fluid from thawing gel pieces placed in between a piece of folded parafilm. Again, negligible DNA amounts were detected on the nanodrop. So finally, we attempted tombstoning and moved forward with the ligation. They were transformed into *E. coli* using chemical transformations, along with the vector as a control group. We mini-prepped the six ligation colonies that grew, performed a restriction digest using BamHI and Sall, and electrophoresed them on a gel. Two of them showed two bands in the correct locations for both the Rex2 insert and the plasmid vector (Figure 3, lanes 4 and 6).
At last, with a successfully ligated gene and plasmid, we could transform them into the original *1 synthetic lethal yeast strain to look for the restoration of colony sectoring. In addition to the *REX2* plasmid, we received a *SHM2* plasmid, generously provided by Dr. Thomas Meier, a professor at the Albert Einstein College of Medicine. We transformed both *REX2* and *SHM2* into *1* colonies to check for colony sectoring restoration. Transforming the *SHM2* plasmid showed a restoration of colony sectoring. Therefore, we could conclusively affirm that a wild-type *SHM2* was the gene rescuing the *1* synthetic lethal strain, and thus the unknown mutation causing synthetic lethality in the *1* strain was in *shm2*. However, a previous report (Nigavekar and Cannon 2002) suggested that *shm2* mutations exhibit synthetic lethality in *ade3* mutant strains, thus we can not confirm that *SHM2* has genetic interactions with *DBP1*. 

Figure 3: Visualization of the six digested plasmid constructs on gel (Left) compared next to the plasmid vector (Middle) and the Rex2 PCR product (Right).
Discussion:

The first portion of the experiment, screening for rescuing of colony sectoring by transforming library plasmids into synthetic lethal mutants, was a lengthy process. Overall we predicted roughly ~50,000 colonies were transformed before colony sectoring could be restored in the *1 mutant strain. It seems like an excessive amount of transformations, but there are a number of features to this screen that can explain why the odds of finding a rescuing hit are incredibly small per transformation. There are twelve million base pairs in the yeast genome, and the plasmid fragment that rescued the unknown mutation was 9.5kb with the identified mutated gene, SHM2, being only 1.4kb long, so the odds of finding the hit were incredibly small per transformation, even considering a high yield of 100 colonies per transformation plate. Additionally, the nucleotide sequences of the different plasmid library fragments can overlap with each other, making the desired sequence less likely to be found due to the uneven coverage of the yeast genome in this amplified plasmid library. Finally, the fragment has to contain the entire sequence of the gene in order to rescue the mutation, so even partial coverage of the gene results in no rescuing of colony sectoring, making precision an important factor in this process.

Because of the long time spent and many colonies transformed before we found the rescuing plasmid, this process was seemingly inefficient. An alternative method could be performing a whole genome sequencing and identifying each site of mutation. This would produce quick, reliable results, but it would be extremely expensive to sequence the entire genome, especially if we were to repeat this with Kate’s other synthetic lethal mutants. Moreover, there are an unknown number of mutated genes produced from random mutagenesis
and the follow-up procedure of inserting each mutated gene individually could be a lengthier process than the six genes identified in our library plasmid. Overall, although time-consuming, plasmid library transformation was a cheap and effective method to identify the unknown mutation.

Following transformation, we had to keep in mind that the yeast colony containing the rescuing plasmid hit likely contained more than one plasmid. So following extraction of the yeast plasmids, we also had to transform them into *E. coli* cells which can only carry one plasmid at a time in order to isolate the specific plasmid rescuing the *1* mutants. This created a tricky step where either the extraction process or the transformation into *E. coli* could be going wrong. The only results to be observed were the presence or absence of *E. coli* colonies on the LB+Amp selective media plates. To troubleshoot, we used the Nanodrop to measure DNA concentrations after using the miniprep kit. Since the spectrometer measured the presence of DNA, we went with the assumption that the miniprep kit was functional and attempted two different transformation techniques, chemical transformation and electroporation.

Failing to find colony growth on both, we doubted the accuracy of the Nanodrop and altered our yeast plasmid extraction approach by attempting phenol extraction and plasmid recovery. In addition, we looked towards visualizing the success of the extraction by running the products on a gel via electrophoresis and looking for bands that match the plasmid length. Plasmid recovery produced visible bands, but we ran into the other issue of extracting the DNA from gels. The products of both the gel extraction Qiagen kit and tombstoning failed to successfully transform into bacterial cells. While looking towards other alternatives and asking other yeast labs in the building, we found the Zymogen yeast extraction kit online. Using this kit
worked remarkably well, yielding 15 colonies of transformed bacteria when paired with chemical transformations. Although the protocol for both miniprep kits were similar, one theory is that the zymolyase exclusive to the yeast miniprep kit was successfully able to break the yeast cell walls and thus resulted in successful plasmid extraction. Another possibility is that the DH5α high efficiency chemically competent bacteria cells we ordered were much more effective at uptaking the small concentration of plasmid yield from the extraction processes. Ultimately, it could have been a combination of both that allowed us to overcome this large step in the experiment, and future lab efforts to miniprep yeast plasmids are vastly simplified with this validated method.

After we isolated and sequenced the rescuing plasmid, we used CLC Sequence Viewer to visualize regions 1kb forward and behind the genes Rex2 and Frs1 and designed PCR primers to cover these regions. However, when we noticed that the bands were very faint or in some cases not visible at all, we attributed this to the PCR step (if digest step wasn’t working, bands would still be visible). We ran out of Ultra-AD DNA polymerase, so I was using native Pfu until the new stock arrived. There were minor successes with using a different polymerase, and the bands were a little more visible than before, but the DNA concentrations were still not high enough to perform ligations. We ordered new primers after multiple failures that were designed to have a greater GC content and more complementary nucleotides to bind to the template plasmid. We also extended the elongation time on the PCR program in hopes that we could produce more DNA. When we performed PCR with the new primers and ran these on the gel, we noticed some severely skewed bands that even included the DNA ladder. In response we used a new gel apparatus, and the REX2 band could now be visualized in high amounts. One uncertain
presumption is that the failure to amplify the \textit{FRS}1 fragment was because it was twice the length of \textit{REX}2 (1788bp vs 810bp of Rex2) and we were using the same PCR program for both genes. So whereas the program was optimal for \textit{REX}2, such was not the case for \textit{FRS}1. Since we had DNA to work with and no strong theories to be used as the basis for adjusting the method, we decided to proceed with the experiment without using \textit{FRS}1 in order to produce results in a timely manner.

The next hurdle was finding a reliable gel extraction technique. I first used the gel extraction kit on the brightest \textit{REX}2 bands I could produce, but the nanodrop spectrometer showed negative results with multiple attempts (about -30ng/μL). One explanation to this impossible result is that some salt-based contaminant in the kit was interfering with the readings. Therefore, I attempted a manual squeezing method that I used with success during my research in the Czech Republic, because the product DNA would only be mixed with 1x TAE buffer used to make the gel. The Nanodrop also showed similar negative results, which suggested that the Nanodrop itself was unreliable in its measurements of our extracts. Finally, I decided to perform a tombstone extraction on the digested \textit{REX}2 and plasmid vector due to the. Six colonies grew from transforming the ligation reaction into \textit{E. coli}, whereas three grew on the control vector-only transformation plate. This was consistent with our findings where only two out of six colonies contained the \textit{REX}2 insert.

Due to the generosity of Dr. Thomas Meier, we received a wild type \textit{SHM}2 in the form of a plasmid with a nutrient marker and ampicillin resistance gene. We specifically searched for and requested the use of this plasmid due to some papers suggesting the genetic interaction of \textit{SHM}2 and \textit{ADE}3 evidenced by synthetic lethality in double knockout yeast (Nigavekar and Cannon
2002). Due to this, we could not conclude any genetic interaction of \textit{SHM2} and \textit{DBP1}. However, because of the results of this experiment, future screenings with the other synthetic lethal hits can be transformed with the \textit{SHM2} plasmid to make sure the unknown mutations have genetic interaction with Dbp1 and not with Shm2. Ultimately, we hope this will help accelerate future attempts to identify the function of Dbp1 as we screen more synthetic lethal \textit{dbp1} mutants. With any luck, insight into the function of this undocumented gene will bring us closer to understanding the role of DDX3X in human brain cancer development.

\textbf{Methods:}

\textbf{Yeast Strains:}

The yeast used in this experiment are strains created by Kate Li performing random mutagenesis on a \textit{dbp1} null mutant to create synthetic lethal mutants (mutations initially unknown). Within them are a plasmid containing: a \textit{DBP1} wild-type copy and an \textit{ADE3} nutrient marker that colors the cells red. The specific strains used for my experiment are labeled \textit{*1, *2, and *5}. All of them grow properly incubating at 30C in YPD.

\textbf{Yeast plasmid library:}

Each plasmid contains about 10kb fragments of the wild-type yeast genome and have a LEU selective marker in each plasmid to select for transformed yeast in LEU- media. Additionally they contain AMP, an ampicillin resistance gene, to select for transformed bacteria in LB+AMP.
Transformation of yeast strains:

Foreign DNA in the form of plasmids was inserted into the yeast through the use of a lithium acetate-based procedure. A single colony of the *1 strain was taken from a YPD plate culture with a sterile wooden stick and transferred to 5mL YPD liquid media for overnight growth. In the morning, an amount of this liquid culture was transferred to a beaker, diluted based on a logistic growth curve model, and YPD liquid media was added until the final volume was 10mL per planned transformation. These yeast were grown on a shaker to mid-log phase which was measured using a spectrometer and YPD liquid media as a blank. Then the liquid colony was transferred to a sterile 50mL capped plastic tube and placed in a large centrifuge. The cells were then pelleted by spinning for 5 minutes at 3000rpm. The supernatant was aspirated and 5mL of sterile Lithium Acetate-TE (50mL 1X TE, 50mL 1 M LiAc, pH 7.5 with acetic acid, 400mL ddH₂O and filter sterilized) solution was added to resuspend the pellet via pipetting. The cells were then pelleted again by spinning in the centrifuge for 5 minutes at 3000rpm. The supernatant was again aspirated and the pellet was resuspended in 100μl LiAc-TE solution per 10mL initial liquid culture. From here, the transformations were set up in 1.5mL eppendorf. Each transformation is: 100μL of the resuspended cells, 25μL salmon sperm carrier DNA boiled at 95°C for 5-10 minutes and put on ice to cool, and 1-2μL of miniprep or amplified plasmid library DNA. Then 0.7mL of sterile 40% PEG-4000 LiAc-TE Solution (50mL 1X TE, 50mL 1 M LiAc, pH 7.5 with acetic acid, 400mL sterile 50% PEG stock, filter sterilized) was added to each transformation and the contents were mixed by inverting them 5 times. The cells were then grown out by incubating in a 30°C water bath for 30 minutes. Immediately afterwards they were heat shocked by transferring them to a 42°C water bath for 15 minutes. The cells were then
pelleted in a microfuge for 1 minute and the supernatant is removed by aspirating. The pellet was then resuspended in 0.25mL sterile TE (10mM Tris-HCl, pH 7.5, 1mM EDTA) and centrifuged for 1 minute again. The supernatant was aspirated and the pellet was resuspended in 150μL sterile TE. Each transformation was plated using glass beads on individual Leu- plates to select for colonies that uptake the desired plasmids.

Transformation of E. Coli:

10 μl of yeast miniprep DNA or plasmid ligation was added to one (or more for additional transformations) aliquot (100μl) of XL-1 Blue chemically competent *E. coli* (cells treated with calcium chloride to allow plasmids to the cell membrane). They were allowed to incubate for 30 minutes on ice and then immediately heat shocked in a 42°C heat bath for 90 seconds. Afterwards, the cells are placed on ice for 2-3 minutes while 500μl SOB nutrient rich liquid media is added to each aliquot. The cells are then placed in a 37°C water bath and allowed to grow for 1 hour. Next, the cells are pelleted on a tabletop centrifuge for 30 seconds at 13,000K. The supernatant was then aspirated to 100μl and the cells were resuspended. These resuspended cells were then plated on LB + AMP plates to select for transformed cells and incubated at 37°C overnight.

Isolation of yeast plasmids:

Once sectoring was restored in Kate’s synthetic lethal *1 cells by transforming them with the plasmid library, the transformed yeast cells containing the desired library plasmids were grown
overnight in 1.5ml Leu- liquid media. Then we used Zymo Research’s miniprep kit: Zymoprep Yeast Plasmid Miniprep II (Catalog No D2004) to isolate the plasmids.

Isolation of E. coli plasmids:
After plasmids were transformed into E. coli cells (which could only take one plasmid at a time) and plated on LB + AMP cells, we took each colony and individually inoculated them in 5ml LB + AMP liquid media for overnight growth. Then we used Qiagen’s miniprep kit: QIAprep Spin Miniprep Kit to isolate each of the plasmids for additional transformations into yeast.

Construction of plasmid:
When the library plasmid that rescues synthetic lethality in the *1 yeast strain was isolated using E. coli transformations and miniprep, we sent it to have its nucleotide sequence identified. We identified several genes in this library fragment that could be the hit to restore synthetic lethality, so plasmids must be constructed containing individual genes to identify which one has genetic interaction with DBP1. Thus, we designed oligonucleotides for REX2, one gene identified in the isolated *1 hit plasmid, by creating PCR primers ~25bp long that matched 1000kb upstream and downstream of the gene that also contained a palindromic overhang sequence that could be digested by the restriction enzymes BamHI and SalI. We performed PCR using: the forward primer, the reverse primer, the isolated *1 sectoring-rescuing plasmid as the template, and pfu Ultra-AD DNA polymerase and the Ultra-AD 10x buffer. Then, we performed restriction digests using BamHI and SalI on both the REX2 PCR product and a plasmid vector containing an ampicillin resistance gene as well as a nutrient marker for transformation into both E. coli and
yeast. To purify the resulting product and isolate the DNA fragments, we ran the digested REX2 and plasmid vector on a gel using electrophoresis. They were isolating from the gel using the tombstoning procedure described below. Finally, REX2 and the plasmid vector were ligated using T4 DNA ligase and the according 10X buffer with both DNA components in water. After incubated at 16°C overnight, the ligation reaction (as well as the vector alone to be used as a control) was transformed into E. coli. The E. coli colonies were mini-prepped and visualized on a gel as explained below.

Primer Design:
The primers used for PCR of the genes *FRS1* and *REX2* were as follows:

*FRS1*

Forward- 5’ GATCggatcc TTGCAAATCGAAAAGTTTCACACTGA 3’
Reverse- 5’ GATCgtcgc GAAATTCTCGGTAAGATTTGTTGC 3’

*REX2*

Forward- 5’ GATCggatcc TTGATGTGTGTATGCTATTCTGCC 3’
Reverse- 5’ GATCgtcgc GTTTAAGCTGTATGCTTTGCTATG 3’

The lowercase regions detail the non-complementing overhang that are designed for the activity of restriction enzymes SalI and BamHI. The regions in the reverse primers are digested by SalI, and the regions in the forward primers are digested by BamHI. The space indicates where the overhang ends and the sequence that complements with the rescuing library plasmid begins.
Tombstoning:

After electrophoresing the DNA bands until all fragments are sufficiently separated, the gel was removed from the apparatus and buffer and the fragments were visualized using UV light. An incision that stretches about the width of one well was made into the gel directly in front of the far end (where the DNA was running towards) of the desired DNA fragment with a blade. Circular cut outs of DE-81 filter paper is then cut in half and each half was trimmed to fit within the width of the gel incision. Flat forceps were used to carefully open the slit without ripping the gel and the filter paper cutout was carefully placed inside, spanning the thickness of the gel. The gel was then placed back inside the apparatus and buffer and then electrophoresed for 15-20 minutes so that the DNA band ran into the filter paper. Afterwards, the filter paper was carefully removed from the gel with forceps and placed in a 0.6mL centrifuge tube with a slit cut open in the bottom using a blade. This tube was placed in a 1.5mL centrifuge tube and centrifuged for 30 seconds to remove excess buffer. The 0.6mL tube was then placed in a fresh 1.5mL tube. Here, the elution step was performed 4 times: 100μL of HIGH Tombstone Buffer (8mL 100% Ethanol, 4mL 10M LiCl, 400μL 1M Tris pH 7.6, 80μL 0.5M EDTA pH 8, 27.5mL ddH₂O) was added to the 0.6mL tube, allowed to incubate for 5 minutes, then centrifuged for 30 seconds. The flowthrough was collected in another fresh 1.5mL tube and the elution was repeated 3 additional times. Next, 1mL of 95% ethanol was added to the elution and it was placed at -20°C overnight. Then, it was centrifuged for 15 minutes at 4°C and the supernatant was aspirated. The pellet was rinsed with 500μL of 70% ethanol, spun for 2 minutes, aspirated, and then placed in a 55°C heat block to dry. Finally, the dry pellet was resuspended in 30μL of ddH₂O.
Visualization using electrophoresis:

To visualize the effectiveness of plasmid ligation, following the ligation, transformation, and miniprep, we used the same restriction enzymes used to construct the plasmid, BamHI and SalI, and digested the final plasmid product. Then, we poured 1g of agarose gel in a flask of 100mL 1x TAE buffer (40mM Tris, 20mM Acetic Acid, 1mM EDTA), heated it in a microwave until the agarose was completely dissolved, and added 1μl of ethidium bromide. This solution was poured into a gel casting stand positioned in an apparatus, and a comb was placed in the casting stand to create a mold for the wells. The gel was allowed to cool and harden, and then the apparatus was filled above the gel with 1x TAE buffer. The comb was removed, and the plasmid digest samples (having been mixed with ⅙ volume of loading dye) were placed in the wells. One well was filled with 1kb DNA ladder to visualize the fragment length.
References:


