**WHAT WILL WE BE DOING?**

We will be using the yeast Golden Gate (yGG) method developed by Neta Agmon and Leslie Mitchell in Jef Boeke’s lab to assemble a functional yeast gene with protein-coding and regulatory sequences. First, we will assemble a single gene whose activity is easy to measure (e.g. an auxotrophic marker, antibiotic resistance gene, LacZ, etc.) just to get the system up and running in your lab. Once you do, you can apply the methodology to any gene(s) of interest (i.e. it does not have to be a yeast gene). You can also use a variation of this method to string together *multiple* genes (via VEGAS assembly) into a single plasmid to study metabolic pathways.

Before proceeding, we should define some terms and establish “rules” to define the elements comprising our gene assemblies (adapted from Agmon, et al):

1. **Transcription units (TUs)** are the “building blocks” of neochromosomes. One TU consists of three parts:
2. **Promoter (PRO):** DNA sequence indicating where transcription begins. We will assume that yeast promoters are located either within 500 base pairs upstream of a gene’s start codon, or within the sequence between a gene’s start codon and an adjacent gene’ stop codon (as it lies along a chromosome), whichever length is shorter.
3. **Coding sequence (CDS):** Protein-codingportion of a gene from the start to stop codon. Also, although very few yeast genes contain introns, you should check for any in your CDS before proceeding!
4. **Terminators (TER):** DNA sequence indicating where transcription ends and a polyA-tail will be added. We will assume that the terminators of yeast genes lie within 200 base pairs downstream of the stop codon, or up to the nearest gene’s start codon, whichever length is shorter.
5. Note that the above definitions of promoters and terminators are imperfect but are based on information about “average” yeast genes. However, these simple definitions provide a convenient guide about how much of a gene fragment to amplify from chromosomal DNA.

**MATERIALS NEEDED FOR NEOCHROMOSOME ASSEMBLY**

1. PCR reagents:
2. High fidelity, proofreading polymerase, such as Phusion or Q5, from New England Biolabs (NEB) for amplifying PRO, CDS and TER “parts”
3. 2xOneTaq mix (or equivalent, or just regular Taq polymerase) for colony PCR reactions
4. dNTPs
5. Type IIS restriction enzyme BsaI (and/or BsmBI, depending on the experimental design) from NEB
6. System for cloning blunt-ended PCR products, such as the ZeroBlunt Topo II cloning kit from ThermoFisher. These kits are very expensive, but you can also make a homemade version – see Motohaszhi reference.
7. Yeast Golden Gate Acceptor vector, such as pAV116. Each three-part transcription unit (TU) will be assembled into this vector. The Boeke lab generated many variations of these vectors with different selectable markers (and other features) and made them available from AddGene. See the Agmon et al. reference for more information.
8. 1 kb plus DNA ladder (or equivalent)
9. High-concentration T4 DNA ligase (2,000,000 U/mL) from NEB
10. X-GAL
11. IPTG
12. Primers for amplifying your PRO, CDS and TER parts and adding specific sequences to the ends (see below)

**NOTE ABOUT THE WORKFLOW BELOW**

Different workflows are possible for this experiment, depending on the learning outcomes of your course/project. The first nine steps below describe how to construct individual, separate plasmids containing PRO, CDS or TER fragments with “Golden Gate-compatible ends.” The PRO, CDS and TER can then be “snapped” together in a precise order by yeast Golden Gate assembly to generate a complete TU. Designing and generating these genetic parts involves basic molecular biology techniques and could fit into a standard semester-long course. However, this section may work better for you as an “independent study” where individual students generate “pre-made” genetic parts that could be used in a course or other project later.

If TU “part” construction is *not* appropriate for your course, you could certainly start the workflow at the Golden Gate assembly step (step 10), assuming you have previously-generated PRO, CDS and TER parts in hand. We can also share parts with each another to make “chimeric,” mix-and-match TUs with different PRO, TER and CDS sequences. Alternatively, you can even skip the entire cloning section (i.e. steps 4-9) and directly use PCR products in the Golden Gate assembly, although there are certain advantages to subcloning the parts into plasmids first.

**CONSTRUCTING A SINGLE TRANSCRIPTION UNIT BY YEAST GOLDEN GATE ASSEMBLY**

*In this experiment, you will use Golden Gate assembly to construct a single yeast transcription unit (TU) consisting of a promoter (PRO), protein-coding sequence (CDS), and transcriptional terminator (TER). Before proceeding, it is helpful to describe the activity of Type IIS restriction enzymes, since they are key to this procedure.*



Recall that “typical” restriction enzymes cut DNA *within* their specific recognition sequences. For example, the enzyme BamHI recognizes the palindromic 6-base sequence GGTACC (shown in the rectangle in the figure at right) and cuts between the adjacent “G” nucleotides on each strand.

Type IIS restriction enzymes, however, cleave DNA ***outside*** their recognition sequences and do so some number of nucleotides away. They also usually cut in a “staggered” manner that leaves an unequal number of bases on the two resulting DNA strands. For instance, look at the activity of the enzyme BsaI depicted in the diagram at left. BsaI recognizes the specific 6-base pair sequence GGTCTC (shown in the rectangle), but then cuts the top strand one nucleotide away and the bottom strand five nucleotides away, thus leaving 4-base overhangs.

Golden Gate assembly takes advantage of these 4-base overhangs! By designing specific nucleotide sequences *adjacent* *to* BsaI recognition sites, you can generate DNA fragments with compatible/ complementary overhangs that “fit” together in a precise order and direction with other DNA fragments (e.g. “DNA fragment II” in the diagram.” There are many more details about this below!

*WE’RE READY TO DESIGN AND ASSEMBLE A SINGLE TRANSCRIPTION UNIT!*

****

The best way to figure out how the Golden Gate assembly works is to design and assemble your own TU. The workflow consists of three general sections (**each with multiple steps!**), and are described in more detail below:

1. Section 1: PCR. Amplify the TU fragments (i.e. PRO, CDS, and TER) separately using primers that add properly oriented BsaI sites and appropriate 4 bp overhangs to each.
2. Section 2: Blunt-end ligations.Ligateblunt PCR products containing the PRO, CDS and TER sequences individually into plasmids with KANR gene.
3. Section 3: Golden Gate assembly.Mix the PRO, CDS and TER-containing plasmids with an “**acceptor vector**” (with an AMPR gene) in a Golden Gate assembly reaction that simultaneously liberates the BsaI-flanked TU fragments and ligates them (in a precise order) into the “acceptor” vector. Then, transform products into bacteria.

*MORE DETAILS ABOUT EACH OF THE THREE STEPS IN THE TU ASSEMBLY WORKFLOW:*

**• SECTION I -- PCR:**

The figure above shows the design of the PRO, CDS and TER parts with compatible 4-base overhangs that can “snap” together in a precise order. You can see that each part has unique 4-base sequences on the left (5’ end) and right (3’ end) that are complementary to those of the adjacent part that it will ligate to. For example, in the middle of the diagram (“section 2”) you can see that the 4-base overhang on the 5’ end of the CDS (AATG) is complementary to the 4-base overhang on the 3’ end of the PRO (TTAC). In turn, the 4-base overhang on the 5’ end of the PRO (CAGT) is complementary to the 4-base overhang on the 3’ end of the acceptor vector (GTCA) that all three parts will be assembled into, etc. You can also see that the start (ATG) and stop (TGA) codons are built into the 4-base sequences on the 5’ and 3’ ends of the CDS, respectively.

Incorporating these specific features into each TU part requires careful PCR primer design so each PRO, CDS and TER contains a properly oriented BsaI site, and the sequences for generating the appropriate 4-base overhangs. This can be easily done using thedesign strategy that Agmon et al. devised for each TU part, as depicted in the diagram above. Conforming to these sequences is useful, since you can generate “standardized” and “swappable” PRO, CDS and TER parts. In other words, since every PRO will have the same, specific 4bp overhangs (as will every CDS and TER) you will be able to ligate *any* PRO to *any* CDS, or *any* CDS to *any* TER, etc.

***“Universal” primer design for amplifying PRO, CDS, and TER parts***

Several features must be added to the PCR primers to add the properly oriented BsaI sites and appropriate 4bp overhangs**.** These arehighlighted in the sequences below with the following scheme:

* Capital letters that are NOT underlined indicate the BsaI recognition sequence
* The lower case “a” is a single nucleotide “spacer” needed because BsaI cuts the top DNA strand one nucleotide away from the 3’ end of the recognition sequence. Any nucleotide suffices here (i.e. it does not need to be an “a,” so if you prefer “c, g or t”, then go for it!)
* The bold, underlined, capital letters indicate the 4-base overhang generated after digesting the PCR product with BsaI
* \*NOTE: If performing a Golden Gate assembly directly with PCR products (without subcloning them into plasmids), then add 3 random nucleotides to the 5’ end of each primer to ensure that BsaI cuts efficiently at the end of the DNA fragment.

***Step 1: Designing primers to amplify any promoter (PRO):***

Forward PRO primer:

 5’-GGTCTCa**CAGT**…(insert forward PRO-specific priming sequence)…

Reverse PRO primer:Note that the diagram shows an upside down, reversed BsaI site on the right side of the PRO, indicating that the sequence “reads” from 5’ to 3’ (i.e. right to left) on the bottom DNA strand.

5’-GGTCTCa**CATT**…(insert reverse PRO-specific priming sequence)…

***Designing primers to amplify any coding sequence (CDS)***

Forward CDS primer:

 5’-GGTCTCa**AATG**…(insert forward CDS-specific priming sequence starting with the ***second*** codon, since the ATG start codon is built into the primer/overhang)…

Reverse CDS primer:

5’-GGTCTCa**CTCA**…(insert reverse CDS-specific priming sequence starting with the ***last amino acid-specifying*** codon, since a stop codon is built into this primer/overhang)…

***Designing primers to amplify any terminator (TER):***

Forward TER primer:

 5’-GGTCTCa**TGAG**…(insert forward TER-specific priming sequence)…

Reverse CDS primer:

5’-GGTCTCa**AAAA**…(insert reverse TER-specific priming sequence)

***Step 2: Setting up the PCR reactions to amplify your PRO, CDS and TER parts while adding properly oriented BsaI sites and appropriate 4-base overhangs***

*Be sure to use a high fidelity, proofreading polymerase that leaves blunt ends on the PCR product. It is convenient to use pre-made 2x polymerase mixes that include dNTPs, buffer, Mg2+, etc. (e.g. 2x Q5 polymerase mix from NEB), but “homemade” reagents are just fine.*

1. Add the components below *in order* to a TINY PCR tube:

|  |  |
| --- | --- |
| **Ingredient** | **Volume** |
|  H20 | 2 µL |
| Template DNA (plasmid (~1 ng/µL) or yeast gDNA (prepared by protocol in appendix) | 4 µL  |
| 5 µM Forward Primer | 2 µL  |
| 5 µM Reverse Primer | 2 µL |
| 2x Q5 polymerase (or other mix with proofreading polymerase) | 10 µL |
| **Total volume** | **20 µL** |

\*If you are trying to amplify a GC-rich sequence (i.e. >50% G-C), replace 1 µL of H2O with 1 µL of DMSO and increase the denaturing times below from 10 to 30 sec.

1. Here are “standard” reaction conditions for Q5 polymerase

Step 1: Initial denature: 98°C for 30 sec

Step 2: Denature: 98°C for 10 sec

Step 3: Anneal: 55°C for 30 sec

Step 4: Extension: 72°C (30 sec per kilobase of product)

Step 5: Go to Step 2 for 29 more cycles

Step 6: Final extension: 72°C for 3 min

1. But, if it is easy to program the thermocycler, I have more c onsistent success using a “touchdown” protocol that utilizes progressively lower annealing temperatures during the first set of cycles:

Step 1: Initial denature: 98°C for 30 sec

Step 2: Denature: 98°C for 10 sec

Step 3: Anneal: 62°C for 30 sec

Step 4: Extension: 72°C (30 sec per kilobase of product)

Step 5: Go to Step 2 for 6 more cycles and decrease the annealing temperature 1°C/cycle.

Step 6: Initial denature: 98°C for 30 sec

Step 7: Denature: 98°C for 10 sec

Step 8: Anneal: 55°C for 30 sec

Step 9: Extension: 72°C (30 sec per kilobase of product)

Step 10: Go to Step 6 for 24 more cycles

Step 11: Final extension: 72°C for 3 min

***Step 3: Analyze a fraction of your PCR products on an agarose gel to determine if the PCR reaction(s) worked.***

1. Pour/cast an agarose gel according to the protocol in the appendix
2. *Prepare 1 kb plus ladder*:

|  |  |
| --- | --- |
| **Ingredient** | **Volume** |
| 1kb plus ladder (diluted 10-fold) | 5 µL |
| 6x blue/purple loading dye | 2 µL |
| H2O | 5 µL  |
| AMOUNT TO LOAD ON GEL  | 12 µL  |

1. *Prepare your PCR samples*

|  |  |
| --- | --- |
| **Ingredient** | **Volume** |
| H2O | 8 µL |
| PCR product | 2 µL |
| 6x blue/purple loading dye | 2 µL  |
| AMOUNT TO LOAD ON GEL | 12 µL |

1. Load your gel as follows:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sample** | 1kb plusladder | PCR sample #1 | PCR sample #2 | etc.  |

1. Run gel at ~100-110 volts until the dye front is ~1 cm from bottom (~45 minutes).
2. Put on protective face shield. Then, turn off lights and place gel on UV transilluminator. Takea picture of the gel with your phone.
3. If you see a nice, single PCR product of the desired size, then great! If not, then the PCR conditions above may not compatible with your template/primer set. Don't worry, this is a common occurrence! See the appendix for tips about optimizing unsuccessful PCRs.

**\*\*\*\*At this point, you can proceed with the workflow in one of two ways:**

1. Clone the PCR products into a plasmid with a kanamycin-resistance (go to steps 4-9).
2. Gel purify the PCR products according to the procedure in the APPENDIX called “Extracting and purifying DNA fragments from agarose gel slices using Qiagen gel extraction kit.” Then, run a fraction of each final, purified product on a gel (to assess purity and concentration), and skip ahead to the Golden Gate assembly (step 10).

That said, there are advantages to cloning the PCR products first (via steps 4-9), as discussed below.

***Step 4: Blunt-end ligations into kanamycin-resistant plasmid***

*It is useful to incorporate the PCR products into kanamycin-resistant plasmids here to decrease unwanted “background” later in the workflow (details to follow). Commercially available kits, such as the Zero Blunt PCR cloning kit, work well here, but they are very expensive! You can use ¼ the amount of vector recommended in the manufacturer’s instructions to maximize the number of reactions you can get from the limited material provided in the kit. If you do not have a commercially available kit, you can make a “homemade” blunt PCR cloning system using a vector from the Motohashi paper in the references section – it is available from AddGene for a nominal cost. Once you get this up and running, you can freeze a large amount of pre-cut, purified plasmid for these reactions.*

1. First, why is it useful to clone the PCR products into kanamycin-resistant plasmids (as opposed to just using the PCR products) before the Golden Gate assembly?
2. Although a PCR product may appear to be “correct” based on its size on a gel, keep in mind that a band on a gel actually consists of a mixed population of many individual molecules, and some may possess unwanted mutations! Also, there are likely to be different-sized (unwanted) products in the mix as well (that may appear as fainter bands or smears on your gel). Unfortunately, we cannot use tiny tweezers to physically separate the DNA molecules from one another and pick out the perfect one. Even if we could, it would take years to sort through the millions of fragments!

Instead, we can take advantage of **cloning**, which effectively separates this mixed population of DNA molecules into individuals, and enables you to “sort through” them to identify those with the desired, full-length DNA sequences. Then, when you proceed to the next step, you can be confident you are using perfect “ingredients.”

1. Cloning also enables you to generate a permanent, renewable stock of each plasmid with the desired TU fragment. Since plasmids are equipped with features that enable them to be replicated in bacteria, making more of the DNA just requires growing more bacteria, and the cells will then serve as “factories” for making more perfect plasmid for you.
2. Procedure for blunt end ligation using the ThermoFisher Zero Blunt PCR cloning kit:
3. *I prepare a master mix containing the kit components (i.e. the vector, ligase and buffer), aliquot 3.5 µL into single-use tiny PCR tubes, and distribute on ice to students who then add 1.5 µL of their PCR products.*
4. Set up ligation reactions as follows:

|  |  |
| --- | --- |
| **Ingredient** | **Volume** |
| PCR product (added by student) | 1.5 µL |
| H2O | 2.25 µL |
| T4 DNA ligase | 0.5 µL |
| 10x Ligase buffer | 0.5 µL |
| pCR-Blunt vector | 0.25 µL  |
| TOTAL VOLUME | 5 µL |

Prepare a mix of these

ingredients

1. Incubate reaction at room temperature for 30 minutes.
2. Proceed to the transformation step!
3. If using a “homemade” blunt-cloning vector:
4. *It is most convenient to dilute the linearized, blunt-ended cloning vector to 50 ng/µL and prepare a “master” mix containing the vector, ligase and ligase buffer*
5. Set up ligation reaction as follows:

|  |  |
| --- | --- |
| **Ingredient** | **Volume** |
| PCR product at 5x molar ratio over the vector | 5 µL (you may need to add water to bring to this volume, depending on the approximate concentration of your PCR product) |
| 50 ng EcoRV- or SmaI-\_ digested vector | 1 µL |
| 10x T4 ligase buffer | 1 µL |
| T4 ligase | 1 µL (400 U) |
| H2O | Enough to bring volume to 10 µL |
| TOTAL VOLUME | 10 µL |

1. Incubate the reaction for 30 minutes at 16°C (or room temperature).
2. Proceed to the transformation step (Step 5) below!

***Step 5: Transform ligation products into bacteria***

The ligation products are heterogeneous. During the ligation reaction, some percentage of the KANR plasmids will have simply re-circularized without incorporating a PCR product. Other plasmids WILL have incorporated PCR products, but these “inserts” may not be “full-length” fragments that encode our desired, complete PRO, CDS and TER sequences. Somehow, we must sort through this mixed pool of ligation products to find the ones we want.

Here is how the transformation will help us to do so! We will assume that any individual bacterial cell takes up just one plasmid molecule, and that as the cell replicates, it will pass along this plasmid to all its progeny. At the end of the transformation procedure, we spread the cells out on a plate, which physically separates them from one another. Again, we could not use tiny tweezers to separate the plasmid DNA molecules from one another, but we can easily separate the cells harboring these plasmids (by simply spreading them out on a plate!). Each cell will then divide and divide to form a colony with millions of cells (all harboring the identical plasmid!). We can then simply “pick” each individual colony and analyze their plasmids to determine which one(s) do, indeed, contain full-length PCR, CDS or TER PCR products.

1. The detailed bacterial transformation protocol is found in the appendix, but here are a few important notes:
2. You must use a **ccdB-sensitive bacterial** strain, such as Top10 or DH5alpha. Certain other strains are ccdB-resistant, including JM109 and XL1-Blue.
3. The plasmids are **kanamycin-resistant** – be sure to add 50 µg/mL **kanamycin** to your LB plates!
4. You will also be performing a blue-white screen to identify plasmids that have actually taken up a PCR product (instead of just re-circularizing), so you will need to add supplements to your LB + kanamycin plates:
5. Spread 40 µL of X-Gal (20 mg/mL, dissolved in DMSO) onto each plate. If using TOP10 cells, then the plates are ready!
6. For cell types other than TOP10, you may need to spread 120 µL of IPTG (20 mg/mL, dissolved in H2O) on each plate as well
7. After transforming, incubate the plates overnight at 37°C. If the blue color is not well-developed, then keep incubating them at 37°C until the blue is distinct (could take 24 hrs).

***Step 6*: *Colony PCR to identify bacteria harboring plasmids with full-length PRO, CDS and TER PCR products.***

The good news is that you now (hopefully) have bacterial colonies growing on your plates! The bad news is that only certain bacteria will actually be harboring the “desired” PCR products! Some plasmids may simply have re-circularized without taking up a plasmid (and somehow survived the ccdB selection), others may have incorporated truncated PCR products, etc. We need to identify the “correct” ones via colony PCR.

In the map below, you can see the location where PCR products should incorporate into the plasmid (between the two EcoRI restriction sites). You can also see the positions of the M13 reverse and T7 priming sites, which we will use to amplify the intervening region and determine which plasmids have the desired PRO, CDS and TER sequences.

Keep in mind that we performed a blue-white screen and are only interested in the white (or light blue) colonies (i.e. NOT the dark blue ones), which are those harboring plasmids that actually incorporated PRO, CDS and TER PCR products.

*COLONY PCR PROCEDURE*

1. Inoculate small cultures.
2. Aliquot 100 µL LB + 50 µg/mL kanamycin into appropriate wells of a sterile 96-well plate (with lid).
3. Using sterile toothpicks or pipet tips, pick 6 white (or light blue) colonies from each transformation plate (making sure that the colonies are well isolated from their neighbors) and “wriggle” the bacteria into the medium in the wells.
4. Grow the cultures overnight at 37C for 18-24 hours (shaking is not necessary).
5. The next day, set up colony PCR reactions as follows.
6. First, prepare a PCR reaction “master mix.” The reagent volumes below are amounts to include in ONE reaction, so multiply these volumes by the number of samples (+2, to account for pipetting error). We use 2xOneTaq mix from NEB.

|  |  |
| --- | --- |
| **Ingredient** | **Volume** |
| H2O | 5 µL |
| M13Rev primer (5 µM) | 2 µL |
| T7 primer (5 µM) | 2 µL |
| 2x OneTaq | 10 µL  |
| **TOTAL VOLUME** | 19 µL |

1. Aliquot 19 µL of the reaction master mix into each tiny PCR tube.
2. Add 1µL bacteria to each tube. Put tubes into thermocycler and set it for the following “standard” program:

Step 1: Initial denature: 94°C for 30 sec

Step 2: Denature: 94°C for 30 sec

Step 3: Anneal: 55°C for 30 sec

Step 4: Extension: 68°C (1 min sec per kilobase of product)

 Step 5: Go to Step 2 for 29 more cycles

 Step 6: Final extension: 72°C for 3 min

1. **IMPORTANT**: KEEP your 96-well plate with the small bacterial cultures! Put your initials on the plate, wrap it in plastic wrap, and keep it in the fridge/cold room!

***Step 7: Agarose gel to identify “positive” clones from the colony PCR that have incorporated a PRO, CDS or TER PCR product***

*Just to reiterate, just because a colony is white (i.e. NOT blue) does not necessarily mean that it harbors the desired DNA fragment. We are now “sorting through” numerous colonies to identify the correct ones. Note that the “desired” PCR product will be ~221bp larger than expected, since the M13Rev and T7 priming sites are some distance away from the location where the PCR products are incorporated into the plasmid.*

1. Pour an agarose gel according the instructions in the appendix.
2. Analyze a fraction of your PCR product to determine which plasmids contain DNA fragments of the desired size:
3. *Prepare 1 kb plus ladder*:

|  |  |
| --- | --- |
| **Ingredient** | **Volume** |
| 1kb plus ladder (diluted 10-fold) | 5 µL |
| 6x blue/purple loading dye | 2 µL |
| H2O | 5 µL  |
| AMOUNT TO LOAD ON GEL  | 12 µL  |

1. *Prepare your PCR samples*

|  |  |
| --- | --- |
| **Ingredient** | **Volume** |
| H2O | 8 µL |
| PCR product | 2 µL |
| 6x blue/purple loading dye | 2 µL  |
| AMOUNT TO LOAD ON GEL | 12 µL |

1. Load your gel as follows:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sample** | 1kb plusladder | PCR sample #1 | PCR sample #2 | etc.  |

1. Run gel at ~100-110 volts until the dye front is near the bottom (~45 minutes).
2. Put on protective face shield, turn off lights, place gel on UV transilluminator, and takea picture of the gel with your phone. You may find PCR products of unusual/unexpected sizes, but you will hopefully find the right thing!

***Step 8: Grow “winning” colonies that harbor the desired plasmid***

If you identified any bacteria with the correct length PRO, CDS, or TER PCR products incorporated into their plasmids, then you need propagate them further, and purify plasmid DNA from them! To do so…

1. Go back to the 96-well plate containing your bacterial cultures and identify those that, based on your colony PCR results, harbor the desired plasmids (two positive clones of each type should be fine).
2. Inoculate 3 mL LB + 50 µg/mL kanamycin with 3 µL of each bacterial culture (or, just dip a sterile stick into the small culture and then wriggle it into the fresh medium) and grow overnight with shaking at 37°C.
3. Wrap your 96-well plate again with plastic warp and store in fridge (4°C), just in case.

***• Step 9: Plasmid minipreps to purify plasmids from your bacterial cultures.***

*Now, you will need to purify the plasmids that you (and the bacteria) worked so hard to construct! Again, these plasmids contain your PRO, CDS or TER parts ligated into them.*

1. You will need ~1.5 mL of bacteria for each plasmid miniprep. We use DNA purification columns purchased from BioBasic along with homemade solutions (see APPENDIX for the miniprep procedure, recipes for homemade solutions, and ordering information).
2. Quantify plasmid DNA concentrations (in ng/µL) using a spectrophotometer
3. You should have the “parts” sequenced if you want to archive them permanently. Again, the M13Rev and T7 priming sites are useful for this purpose.
4. Make a permanent freezer stock of your bacteria (using careful aseptic technique)
5. Label the side of a “cryovial” with your TU part name, the primers used to amplify it, your initials, and the date. Each part will also be assigned a number you should write on the top of the cap
6. Add 500 µL of your bacterial culture into the cryovial.
7. Then, add 500 µL of sterile 30% glycerol to the cryovial, and mix by vortexing or pipetting up and down.
8. The cryovial should be placed into the appropriate box in the -80°C freezer.

**• Step 10: Golden Gate assembly.**

Here is a link to a video that provides a good overview of how the Golden Gate assembly works:https://www.youtube.com/watch?v=piyc2ONyV1o

At this point, we constructed kanamycin-resistant plasmids containing our PRO, CDS and TER parts with properly oriented BsaI sites and unique 4-base flanking sequences. We are now ready to precisely “snap” the TU parts together by Golden Gate assembly.

Before getting into the details of the Golden Gate assembly reaction, we must introduce one more ingredient – the Golden Gate “acceptor vector.” The TU parts (PRO, CDS and TER) will be cut out of their current plasmids (using BsaI) and assembled, in a precise order, into the acceptor vector (also cut with BsaI). Agmon *et al.* constructed many variations of acceptor vectors (with different selectable markers, etc.), but we will use one called pAV116, which is depicted schematically in the diagram at right.

For the Golden Gate assembly reaction, we will combine the three TU part-containing plasmids (i.e. those containing the PRO, CDS and TER DNA fragments) with the acceptor vector (e.g. pAV116) and add a “Golden Gate assembly mix” containing two enzymes:

1. **BsaI** will cut the PRO, CDS and TER fragments out of their current KanR plasmids, and “open up” the acceptor vector (releasing the RFP gene). It will also generate the specific 4-base overhangs on the ends of each DNA fragment that will enable them to fit together in a precise order.
2. **T4 DNA ligase** will ligate together the BsaI-digested fragments.

The Golden Gate reaction consists of a “cycle” of alternating 37°C and 16°C steps where the BsaI and T4 DNA ligase are active, respectively**.** To maximize the success of this step of the procedure, Agmon et al. incorporated additional features into TUs and acceptor vectors:

1. The TU-containing plasmids contain a kanamycin-resistance gene, whereas the acceptor vector has an ampicillin-resistance gene. Eventually, the Golden Gate reaction products will be transformed into bacteria and plated on medium containing *ampicillin*. Therefore, we will only select for bacteria that take up the desired ampicillin-resistant acceptor vector and not any undigested or re-circularized TU part-containing plasmids.



1. The RFP gene inserted between the acceptor vector’s BsaI restriction sites enables us to do a “red-white” screen to select for plasmids that have actually taken up the assembled PRO, CDS and TER parts. Here is how this works. Bacteria harboring the original, RFP gene-containing plasmids appear red. However, if the RFP gene is cut from the acceptor vector by BsaI and replaced with TU parts, then bacteria taking up this vector will be white. Using this simple color-based screen, we can easily eliminate bacteria that have taken up incompletely digested or re-circularized acceptor vectors).
2. BsaI sites in different orientations flanking the RFP gene. The diagrams below show a somewhat more detailed view of how the orientation of the BsaI sites in the acceptor vector and an “insert” generates the 4bp overhangs needed to precisely ligate the fragments during the Golden Gate assembly reaction.



The first diagram (at left) shows the BsaI sites (in boxes) relative to the RFP gene in the acceptor vector. On the left side of the RFP gene, the BsaI recognition site GGTCTC is on the bottom strand and oriented from right to left (i.e. 5’ to 3’). However, on the right side of the RFP gene, the BsaI recognition site is on the top strand and oriented from left to right. In the second panel, you can see that during the BsaI digestion, the RFP gene and BsaI sites are removed, generating the desired 4-base overhangs on the vector backbone. The acceptor vector is now poised to receive an insert with complementary overhangs.

Similarly, the BsaI sites on the insert are oriented such that they will generate 4-base overhangs complementary to above, enabling it to be ligated in a precise orientation. Once an insert is ligated to the acceptor vector, there are no more BsaI sites present in the new plasmid construct, and the insert cannot be excised during continued cycles of the Golden Gate assembly reaction. However, should an RFP gene re-ligate to the original acceptor vector or an insert re-ligate to the kanamycin-resistant blunt cloning vector, they will simply be re-cut, since the BsaI sites will be back in place. Finally, any unwanted plasmids will be selected against during the transformation, since you will select white colonies (that have lost the RFP gene and must have inserts) on ampicillin plates (thereby killing off any bacteria that took up an intact kanamycin-resistant plasmid).

**Procedure for the Golden Gate assembly reaction:**

It is important to quantify the concentrations of each plasmid before putting them into the Golden Gate reaction. The reaction should contain 100 ng of the acceptor vector (e.g. pAV116) and equimolar amounts of each TU part-containing plasmid. The molar ratio of TU-containing plasmids to acceptor vector should be ~2:1. The equations and table below can help with calculating the amount of each plasmid to use.

Let’s work through the example shown in the table below. If you are using 100 ng of the pAV116 acceptor vector in the reaction, then you can use Equation 1 (below) to determine that this equals 0.026 pmols of DNA. So, you would want to add 2x that number of pmols (or, 0.052 pmol) of each of the three TU part-containing plasmids. To calculate the number of ng of each TU part-containing plasmid, use Equation 2.

Equation 1: pmols = (weight in ng) x 1000 / (base pairs x 650 daltons)

Equation 2: ng = (pmols) X (base pairs x 650 daltons) / 1000

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Plasmid** | **bp in plasmid backbone\*** | **bp insert\*** | **Total bp in the plasmid** | **ng** | **pmol** |
| pAV116 acceptor vector | 5806 | N/A | 5806 | 100 | 0.026 |
| pCR Blunt (from Thermo Fisher cloning kit)  | 3519 | 500 bp (~ size of PRO) | 4019 | 131 | 0.052 |
| pCR Blunt (from Thermo Fisher cloning kit)  | 3519 | 250 bp (~size of TER) | 3769 | 122 | 0.052 |
| pCR Blunt (from Thermo Fisher cloning kit)  | 3519 | 1500 bp (~size of CDS) | 5019 | 163 | 0.052 |

*\*you should insert the correct bp for your own inserts, of course!*

*Now that you have determined the ng/pmol of each plasmid to use, you can set up the Golden Gate reaction by adding the following to a tiny PCR tube:*

|  |  |
| --- | --- |
| **Ingredient** | **Volume** |
| PRO plasmid | TBD |
| TER plasmid | TBD |
| CDS plasmid | TBD |
| pAV116 acceptor vector (100ng) | TBD |
| 10 mg/mL Bovine serum albumin (BSA) | 0.15 µL  |
| T4 DNA ligase (600 units)\* | 0.3 µL (if 2,000,000 units/mL stock) |
| BsaI-HFv2 (10 units) | 0.5 µL |
| 10x T4 ligase buffer | 1.5 µL |
| H2O | Enough to bring volume to 15 µL |
| TOTAL VOLUME | 15 µL |

\*\*It is best to use a high concentration ligase to minimize the amount of glycerol in the Golden Gate reaction, which could inhibit the enzymes. For example, you can use T4 DNA ligase from NEB (cat# M0202T, 2,000,000 units/mL $64.00 for 20000 units). If you have a lower concentration ligase (e.g. 400,000 units /mL), then you will need to scale up the reaction volume to keep the glycerol concentration less than 5%. The two enzymes are both dissolved in 50% glycerol, so the total volume of enzyme added must be less than 10% of the reaction volume (so, no more than 2 µL of enzyme in a 20 µL reaction)..

Program a thermocycler with the following conditions:

Step 1: 37°C for 3 min

Step 2: 16°C for 4 min

Step 3: Go to Step 1 for 24 more cycles

Step 4: 50°C for 5 min

Step 5: 80°C for 5 min

**• Step 11: Transform** Golden Gate assembly reaction into competent bacteria and select on LB + ampicillin.

**• Step 12: Analyze clones.** Grow 6 white (not red colonies) colonies for minipreps (as before) and analyze by restriction digest and/or PCR to confirm which have incorporated the TU parts correctly.

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**APPENDIX**

**PLASMID MINIPREPS FROM BACTERIA**

We use EZ-10 Spin Column from BioBasic

|  |
| --- |
| BioBasic |
| 4160 Bailey Avenue |
| Amherst, NY, USA, 14226 |
| Phone: (800) 313-7224 ext.234 |
| Fax: (905) 474-5794 |
| Email: info@biobasic.com |

Catalog # SD5005

EZ-10 Spin Column, pk of 100 costs $24.15

**Prepare homemade solutions:**

**Buffer P1**

50mM Tris (pH 8.0), 10mMEDTA, RNAseA (100µg/mL)

**Buffer P2**

0.2M NaOH, 1% SDS

**Buffer N3**

4.2M Gu-HCl, 0.9M KAc (use acetic acid to adjust to pH 4.8)

**Buffer PE**

10mM Tris, (pH 8.0), 80% EtOH

**Plasmid miniprep procedure:**

1. The night before: grow overnight bacterial culture of bacteria in LB + antibiotic with shaking at 37°C.
2. Label one microfuge tube for each plasmid prep.
3. Fill a microfuge tube with bacteria, either by pouring or pipetting. The tubes fit ~1.5 mL.
4. Spin in microfuge for 30 seconds at max speed.
5. Remove supernatant, either by aspirating in hood (but be careful not to suck up the pellet!) or by removing liquid with pipetman.
6. Resuspend the pellet in 250 µL P1/RNAse (stored at 4°C in cold room) by vortexing or pipetting up and down. Be sure the pellet is completely resuspended – it could take some elbow grease!
7. Add 250 µL P2 (stored at RT). Gently mix by inverting tube 5-6 times.
8. Add 350 µL N3 (stored at RT). Set vortexer to medium and vortex using 3 x 1 second pulses. A fluffy white precipitate should form.
9. Spin 10 minutes at max speed in microfuge.
10. Apply clear supernatant (you may need to “spool” the pipet tip to avoid/minimize sucking up the precipitate) to the spin column.
11. Spin 1 min at max speed in microfuge.
12. Wash by adding 750 µL buffer PE to column. Spin 1 min at max speed in microfuge.
13. Pour out flow through into sink (the DNA is bound to the column).
14. Spin an additional minute at max speed to remove excess liquid from column.
15. Place column into clean microfuge tube. Add 50 µL H2O to center of column, incubate 1 min, then spin 1 min at max speed in microfuge.
16. Voila! You have your DNA! Be sure to label the microfuge tube with sufficient detail that you know what it is! Do not just write “DNA” or “1.” Label the top of the tube and use white labeling tape wrapped around the side of the tube. Be sure to include the date so you can cross reference the tube to an entry in your lab notebook.
17. Store the plasmid preps in the freezer (-20°C).

**TRANSFORMING PLASMID DNA INTO BACTERIA.**

1. Obtain LB plates containing appropriate antibiotic, depending on the step of the process you are using:
2. When transforming the TU part-containing PCR products (i.e. the PRO, CDS and TER parts) incorporated into the blunt-ended vector (e.g. TOPO vector), use LB + 50 µg/mL kanamycin
3. When transforming the Golden Gate assembly reaction, use LB + 100 µg/mL ampicillin
4. When transforming the TU part/kanamycin-resistant plasmid, you will need to do a blue-white screen. This requires that you add the following supplements to the LB + Kan plates:
5. Spread 40 µL of X-Gal (20mg/mL, dissolved in DMSO) onto each plate. If using TOP10 cells, then the plates are ready!
6. For cell types other than TOP10, you may need to spread 120 µL of IPTG (20 mg/mL, dissolved in H2O) on each plate as well
7. Prepare an ice bucket. Then, obtain one tube of competent bacteria for each transformation. Place the tube on ice. ***For optimum results, keep the cells ice-cold as much as possible!*** Label the top of each tube (you may need to wipe it with a paper towel to remove condensation).
8. Thaw bacterial on ice – you can monitor this by occasional flicking the tube gently to see whether the cells are still frozen. Label the tube.
9. Transfer 5 µl of ligation product to cells. Mix by flicking the tube gently.
10. Incubate on ice for ~10-15 minutes.
11. Heat shock: bring ice bucket containing tube over to the 42°C water bath and place tube in 42°C water bath for 45 seconds. Put tube back on ice and carry bucket back to your table.
12. Add 125 µL of LB to the tube. Place in the 37°C incubator for 30-60 minutes.
13. Pipet the entire mixture onto the center of an LB plate with appropriate antibiotic. Spread cells evenly over surface of medium using sterile spreader OR 4-5 sterile glass beads.
14. Put plates upside down in the 37°C incubator – be sure to label plates with your initials, the name of the TU “part” (and/or the primers used to amplify it), and the date. Incubate the plate overnight at 37°C.

**AGAROSE GEL ELECTROPHORESIS**

1. Weigh out 0.5 g of agarose using the plastic weigh boat labeled “agarose.” Pour into a 250 mL Ehrlenmeyer flask.
2. Add 50 mL of 1x TAE\* (from the large container)
3. Place in microwave and set for 2 minutes on HIGH. Every 20 seconds or so, use oven mitt to swirl flask to assure the agarose is melting evenly. When agarose is completely melted, place into container of water for a few minutes to cool.
4. Assemble gel apparatus
5. Wet rubber gaskets on ends of gel trays by running under water in sink.
6. Push into gel box.
7. Place 2x10-well combs into the slot – one comb at one end and the other in the middle. Be sure that the thicker side is facing down.
8. When the gel has cooled to ~50°C (so you can hold it with a gloved hand), add 50 µL of 1000x GelRed dye, swirl, and pour into the gel tray. Let the gel harden for ~10-15 minutes. No need to rinse the Ehrlenmeyer flask – just put it back into box near balance.
9. Turn gel tray 90 degrees so that the wells are facing lengthwise within box. Wriggle combs out, rinse combs and then leave by side of sink.
10. Using spatula, cut gel in half. Wrap bottom half in plastic wrap with a moist paper towel, write your initials on the outside and store in the “Agarose gels” section of cold room.
11. Cut out a section of gel with enough lanes for you (leave a couple of extra just in case!). Wrap up the rest as before and store in cold room.
12. Place the gel (on gel tray) you are using into the gel box
13. Add 1x TAE to the gel box such that the buffer is at the fill line marked on side of box (the level of buffer should be just a little bit above the surface of the gel and both sides of the gel box and the wells should be filled).
14. Prepare DNA samples with loading dye and load into wells. Run the gel at 100-110 V until the dye front is about 1 cm from the bottom of the gel.
15. Shut off power supply, remove plastic tray containing gel and place into Tupperware for carrying around. Leave the buffer in the tank – it can be used multiple times.
16. Put on protective face shield. Then, turn off lights and place gel on UV transilluminator. Takea picture of the gel with your phone.
17. In Powerpoint (or equivalent), crop excess from picture, label it and write a brief description of what you see. These pictures will be figures for your lab report! See example below:



If printing, INVERT IMAGE SO IT IS BLACK ON WHITE (saves on toner!)

\*1x TAE is prepared from 50x TAE (which is the concentrated stock). Fill a 2-liter graduated cylinder with 1960 mL of H20 (the deionized water from the machine above the sink in the third floor prep room) and add 40 mL 50x TAE.

**EXTRACTING AND PURIFYING DNA FRAGMENTS FROM AGAROSE GEL SLICES USING QIAGEN GEL EXTRACTION KIT**

*(I have used cheaper ones from BioBasic that work well also – although the procedure is slightly different and the buffers have different names)*

1. Cut out gel slice containing desired DNA fragment and place into microfuge tube. Try to cut as close as possible to the DNA band (i.e. try not to have excess agarose).
2. Weigh gel slice (the microfuge tubes weight almost exactly 1 gram).
3. Add 3 volumes of Buffer QG (the yellow buffer) – so, if your gel slice is 100 mg, then add 300 µL Buffer QG.
4. Incubate tube at 42°C for ~10 minutes, or until gel slice dissolves.
5. Add 1 volume of isopropanol to the dissolved gel. So, if the gel slice weighed 100 mg, then add 100 µL of isopropanol.
6. Mix by inverting the tube several times.
7. Apply up to of 750µL to the purple column (you can spin the remaining liquid later). Spin at max speed for 1 minute.
8. Dump out flow through (the DNA is now bound to the column). If there is still more yellow buffer QG/DNA mix, then keep applying to column and spinning as before.
9. Wash by adding 750µL of PE to the column.
10. Spin 1 minute at max speed.
11. Discard flow through into sink.
12. Spin one additional minute at max speed to collect any excess liquid.
13. Transfer column to new clean microfuge tube.
14. Add 30µL H20 to column and let sit for 1 minute.
15. Spin 1 minute at max speed.
16. Your DNA is purified!
17. Run 1/10 of purified DNA on gel to make sure that the purification worked:

|  |  |
| --- | --- |
| **Item** | **Volume** |
| Gel-purified DNA | 3 µL |
| H2O | 7 µL |
| 6x blue/purple loading dye | 2 µL |