To biobrick (verb): to clone a certain DNA element (be it a promoter, CDS, tag or anything else) using a certain set of upstream and downstream restriction sites that facilitate future processes.

In our lab we will use the standard biobrick format #23 (also known as RFC 23 or the 'Silver Lab Method') conceived by Pamela Silver and colleagues at Harvard University. You can check out their specs here: Silver Biobricking Method

To start Biobricking a part, you must know enough of the sequence of your target part to construct forward and reverse primers. A major difference between biobricking and other cloning efforts is that when biobricking you should know the exact target sequence of your part. Therefore, instead of using common primer-generating programs that will examine bases around your gene of interest and find primers outside your sequence, biobricking primers will contain only regions complementary to your part. This means that although you cannot use most primer-generating programs out there, biobricking primers are much more easily generated by hand using the following method.

Once you have your target part sequence, you should design primers with 5’ extensions that will give you this PCR product:

For simplicity in this protocol, let us assume we are biobricking a part of this sequence:

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ATGCATCATCATCATCATCATCATCATGTAG
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Our forward primer will be complementary to the noncoding strand, i.e. the primer region will look identical to the 5’ region of your target part. I find that a 10bp sequence provides enough specificity for this job. So, our forward primer region will look like this:

```
ATGCATCATC = Forward Primer complementary region
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The reverse primer is a bit trickier, but still relatively simple. Because our forward primer is complementary to the non-coding strand, our reverse primer must be complementary to the coding strand. Because we know our target part's sequence, we can easily generate the reverse complement of our target part. In our example, it will look like this:

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CTACATGATGATGATGATGATGATGATGCAT
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We now take the first 10 bps of this sequence to be the complementary region of our reverse primer. If you want to generate parts for future fusions, it may be important not to include the STOP codon in this sequence

```
CTACATGATG = Reverse primer complementary region
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Now that we have the complementary regions of our primers worked out, we can add the 5’ extensions that will generate the biobrick standard restriction sites. For the Silver biobricking standard #23, these extensions look like this:

```
GCGAGAATTCGCGGCCGCTTCTAGA = Forward primer extension
CGCAGACTGCAGCGGCCGCTACTAGT = Reverse primer extension
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Now combine these sequences with the complementary primer sequences to get your final primers.

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GCGAGAATTCGCGGCCGCTTCTAGAATGCACTATC = Complete Forward Primer
CGCAGACTGCAGCGGCCGCTACTAGTCTACATGATG = Complete Reverse Primer
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Now order these primers from oligo synthesizing companies (we use IDT) and consult the rest of this protocol when you have them.

Now that you have your pair of biobricking primers you need to resuspend them. Check with your lab manager or PI before doing this because different labs have different protocols.

We will resuspend in water with 10mM Tris. We will employ a shortcut in this resuspension that will save you a lot of time trying to calculate how much water to add. If, for example, you receive 23.8 nanomoles of oligo you can simply add 238uL of liquid to get a concentration of 100uM. Easy peasy.

The 100uM primer is a stock concentration. Never use this stock when doing PCR in order to prevent contamination. Instead, dilute some of this primer stock to 1/10 its original concentration, usually 10uL stock + 90uL 10mM Tris. This gives you a 10uM concentration or primer - a good
working concentration - and if it gets contaminated there is a backup.

You're now ready for PCR! In order to see what annealing temperature you should use and to be sure that the primers are working and not generating more than one product, you should run an optimization PCR using small reaction volumes and a Tm gradient. This involves making a mastermix of the selected PCR polymerase, reagents and template (concentrations to follow) and seperating it into the PCR tubes in an 8-tube strip. This is then run in a PCR cycle (to follow) that supplies a different Tm for each tube.

**PCR Mastermix for AmpliTaq Gold 360 (for 9 10uL reactions, giving us 90uL final mastermix volume although we will only use 80uL):**

1. 9 uL 10X Amplitaq Buffer
2. 7.2 uL 25mM MgCl2
3. 7.2 uL dNTPs
4. 1.8 uL Forward Primer (at 10mM working stock concentration)
5. 1.8 uL Reverse Primer (at 10mM working stock concentration)
6. > 50ng Template DNA
7. 1 uL Amplitaq Polymerase
8. Water to 90uL

Once this PCR is run with an elongation time suitable for your target insert (~1kb/minute) and with a Tm gradient, we visualize the products on a gel. Generally speaking, for smaller parts (<1kb) we use a 1.5% agarose gel and for larger parts (>1kb) we use a 0.8% gel. The final product should look something like lanes 3-10 in this gel:

![Gel Image](https://via.placeholder.com/150)

Now you identify which lane is giving you the optimum result. Ideally, you will have a lane that is showing only one band at the size you are looking for. If there are multiple suitable Tms, choose the one with the brightest band (for example, in the above gel we would choose lane 5). You will use this Tm for a second, larger PCR reaction to use for ligating the biobrick insert into the vector. We then make another PCR reaction, this time at 50uL volume (if you’re clever you can theoretically make this reaction along with the mastermix above, but let’s take it one step at a time).

**PCR Mix for 50uL Working Reaction (with Amplitaq reagents):**

1. 5 uL 10X Amplitaq Buffer
2. 4 uL 25mM MgCl2
3. 4 uL dNTPs
4. 1 uL 10mM Forward Primer (with RFC 23 prefix)
5. 1 uL 10mM Reverse Primer (RFC 23 suffix)
6. ~50ng Template DNA (same as in the optimization, though you could use some of that PCR product if needed)
7. 0.25 uL Amplitaq Polymerase
8. Water to 50 uL

You then run this PCR reaction with the same cycle as the optimization, but only at the Tm of the lane that gave you the best results. This should give you a large quantity of insert DNA which we will clone into a Biobrick Standard Vector. Thanks to the good people at the Registry of Standard Biological Parts we have access to PCR-linearized plasmid backbone which helps ensure that the plasmids resulting from our ligation will not contain an unwanted (or absent) part. For our basic parts we will use pSB1A3, a high copy plasmid with Ampicillin resistance, and for future composite parts switch between pSB1T3 and pSB1C3 in accordance with the 3A assembly standard developed by iGEM.

To ligate the PCR product into the standar vector, first digest both the insert and vector with EcoRI and PstI. We digest 500ng of plasmid backbone at a time and use it for subsequent ligations without problem (though it is important to remember that sticky ends are very unstable), but the Insert PCR products must be digested each time. The digestion protocol we have been using is below and can be scaled up or down depending on how much DNA you are using.

**EcoRI/PstI Digestion - 10uL volume**

1. 1 uL NEBuffer 2
2. 0.1 uL 100X BSA
3. 0.2 uL EcoRI
4. 0.25 uL PstI
5. ~100 ng DNA
6. Water to 10 uL

When working with quantities on the scale of <1uL it is important to bear in mind that creating a mastermix using larger quantities will give you more exact concentrations. Incubate this digestion at 37 degrees C for 1 hour and then heat inactivate the enzymes at 80 degrees C for 20 min.

You should now have both your insert DNA and plasmid backbone with complementary sticky ends. Now it is time to ligate. One can imagine that the hardest part of a ligation is getting the right concentrations of DNA together. Because we must imagine the insert and backbone as the number of molecules rather than as nanograms, we must calculate the number of 5’ sticky ends from the number of nanograms. You do this by taking the number of base pairs of your insert (including what's left of the prefix and suffix after digestion) and multiplying it by numerical average of 660 g/mole per dinucleotide (because it's double stranded). This will give you the grams per mole of your insert. From this, it is easy to calculate the moles of insert from the weight.

Different researchers prefer different concentrations of insert to vector, generally anywhere from 1:1 to 1:20. In the Gresham Lab, we will use a 5:1 ratio of insert to vector molecules for our ligations. This means that if we have 5X10^-14 moles of insert, we would add 1X10^-14 moles of vector.

We then combine the insert and vector into a ligation reaction to form the concatenated plasmid.

**T4 DNA Ligation - 20 uL Volume**

1. Insert DNA
2. Vector DNA
3. 2 uL 10X Ligation Buffer
4. 1 uL T4 DNA Ligase
5. Water to 20 uL

This reaction occurs at room temperature for at least ten minutes.

You should now have a fully formed plasmid that is capable of being transformed and grown up in your favorite bacterial strain. Follow your lab's standard transformation protocols using 2 uL of the ligation reaction.

After transforming your cells with your new biobrick plasmid and plating them on selective medium, you should pick a few colonies to inoculate in selective liquid media. Grown these cultures overnight and miniprep them the next day to recover the plasmid. Before using these plasmids in an assembly or especially before sending them to the Registry, it is pertinent to sequence insert to be 100% certain that what you have is what you think you have. If you have used a standard biobrick plasmid backbone, then you should be able to sequence your insert using the **VF2 Sequencing Primer**.