Amgen Protocol: Introduction and a few comments:

The following is a shortened version of the "Amgen Lab". This series of labs involves the creation of a recombinant plasmid, subsequent transformation of a strain of *E. coli* with the expression of a red recombinant protein (RRP). This product can be purified using a hydrophobic interaction chromatography (HIC) column, (as was the case with the pGLO produced protein green fluorescent protein (GFP) in the biology 107 lab). GFP will NOT be purified in this lab series and instead, the bacteria will be grown out for the purpose of isolating the plasmid and purifying it using a commercial plasmid purification kit available from QIAGEN. The following are some notes and modified protocols based on the Amgen lab series. A complete copy of this lab series is available free to students, and it is recommended that students download and review this (http://www.bwbiotechprogram.com/), as it is an excellent review of the crucial concepts and steps inherent in the creation and purification of a recombinant engineered protein.

While the Amgen lab series has been in existence for well over 20 years, and has reached literally hundreds of thousands of students, we have modified it some to increase the chances of success. When an outreach lab is developed, the protocols are tested and re-tested. Supplies, equipment and even training are provided by an extensive network of dedicated individuals. Given the enormity of the project and the cost of the supplies, the materials that are provided (free of charge) are close to the limit of what will work for any given protocols. The amounts and concentrations will work, but are very minimal and dilute. The slightest mistake from a simple pipeting error, too much time spent in a water bath and the process may suffer. In an effort to increase student success as well as streamline elements of the lab, the following modifications have been written into this adapted protocol. Please note: you are sharing the supplies with other students, as you will all be taking your reagents from "stock" bottles. While this process is more like an actual lab (as opposed to having EVERY reagent pre-aliquoted to the exact required amount, labeled, etc.), it can pose a problem if students do not pipet correctly. Be sure to review pipeting principles and realize the amounts provided are just enough for the group to achieve success. Also, while it may be tempting, do not take more of any one reagent, as the amounts and ratios are very precise, and any alteration of the protocol could compromise your results. So, relax and enjoy this premier lab exercise from one of the pre-eminent leaders in biotechnology: AMGEN.

Digestion of pARA and pKAN plasmids:

Keep all agents on ice unless told to do other wise

a. Obtain from the instructor the box of reagents: **Recall that this will be shared by three groups total!**

b. Label 4, 1.5 ml microfuges tubes: A-- / A + / K-- / K +

c. Follow the below chart and add the necessary reagents as needed.

Tube	Water	10 X Buffer	pARA plasmid	Bam/Hind III mix	Total Volume
A--	4 µl	1 µl	5 µl	0 µl	10 µl
A+	6 µl	2 µl	10 µl	2 µl	20 µl
Tube	Water	10 X Buffer	pARA plasmid	Bam/Hind III mix	Total Volume
K--	4 µl	1 µl	5 µl	0 µl	10 µl
K+	6 µl	2 µl	10 µl	2 µl	20 µl

Note: the + samples are called the cut or "restricted" samples FYI.

d. Incubate tubes @ 37 ^{o}C for one hour.

e. Quickly spin the samples. Gently mix the **restricted samples**, and split into two tubes (10 µl per tube). Save one set of tubes for agarose gel analysis. **The second tube will be used for the ligation step.**

f. Heat the second tubes of A+ (10 µl of restricted samples) and K + (10 µl of restricted sample), at 70 ^{o}C for 30 minutes (to deactivate the enzymes).

g. Go to the stock box of reagents and get the 5X ligation buffer and the **ligase** (keep **ligase** always on ice as it is notoriously sensitive). Pre-label new microfuge tube as the "ligase tube".

h. After 30 minutes of heat treatment, into your labeled ligase tube add the following:

- 2 µl of **Ligase** enzyme
- 10 µl of the heat deactivated and CUT A+ sample
- 10 µl of the heat deactivated and CUT K+ sample
- 6 µl of 5X ligation buffer
- 2 µl of water

Total volume should equal 30 µl.

Remember to spin in centrifuge for a minute to collect all reagents at the bottom of the tube.

i. Place your "Ligase" tube at room temperature for 24 hours and after that, @ 4^{o}C until needed. The sample and other saved samples should be boxed up and kept in refrigerator until needed for later labs on transformation and gel conformation.

Discard all left over reagents EXCEPT for: A-- / K---/ A+ / A--/ and ligated samples. There should be 5 tubes per lab team FYI.

Conformation Gel of Restriction Digest and Ligation Reaction:

1. Using a horizontal gel box and rig with at least 6 wells, prepare a 0.8% agarose gel using 1 % TAE. Do not forget to add cyber safe or some other DNA dye to the gel
2. Prepare gel box, power supply, etc. Pick up previous 5 samples from last lab.
3. Prepare samples with 5X loading dye as noted:

Tube	Water	5X loading dye	K +	K--	A+	A--	Ligation	Total Vol.
A--	6 ul	4 ul				10ul		20 ul
A+	6 ul	4 ul			10ul			20 ul
K--	6 ul	4 ul		10ul				20 ul
K+	6 ul	4 ul	10ul					20 ul
Ligation	6 ul	4 ul					10ul	20 ul

4. Load all of the 20ul samples to the gel. Remember to load in order. Run gel for 1 hour @ 100 volts.
5. Analyze gel via gel document software and place the gel image in your lab notebook. Remember to note: agarose %, run time, voltage and buffer type and concentration.

Transformation with recombinant plasmid.

Depending on protocol variations, the bacteria provided may be either a single colony on a plate, or a liquid culture. Furthermore, as part of the ongoing refinement aspect of the lab, some of the class may do the protocol with plated bacteria and others with the liquid culture.

Please ask the instructor which protocol you will be using:

1. Label 2, 1.5 ml microfuge tubes with either a P+ or P--.

DO EITHER STEP 2A OR STEP 2B AS DIRECTED BY YOUR INSTRUCTOR.

2A. For plate protocol: add 100 ul of cold calcium chloride to both the P+ and P— tubes. Your instructor will have a "streak to colony" plate of the LMG strain of *E. coli.* for you to use. Add one colony to each tube. Carefully use the loop to scrape a colony off the plate and ensure it gets into the liquid calcium chloride. Spin the loop while immersed to help ensure bacteria get into the liquid.

2B: For liquid culture, add 100 ul of the liquid, competent *E. coli* culture. Your instructor will have the liquid culture on ice.

3. Add 10 ul of the ligated plasmid to the ***P+ tube ONLY***.

4. Incubate both the P+ and P—tubes on ice for 15 minutes.

5. While cells are incubating, obtain 2, 100 mm Petri plates of the following media:

- LB (Luria Broth agar/black stripe),
- LB/Amp (Luria Broth agar with ampicillin/black, red stripe)
- LB/Amp/Ara (Luria Broth agar with ampicillin/arabinose/black, red, blue stripe).

So, 6 plates total, 3 different types.

On the plate bottom, around the edge, write the needed information (LMG-*E. coli*, date, P+ or P-, initials, date and any other information that the instructors deems necessary).

6. After 15 minutes on ice, heat shock both P tubes for EXACTLY 45 seconds @ 42^{o}C.

7. Add 150 ul of Luria broth to both tubes, mix by inverting a few times and let them recover for 5 minutes.

8. To the LB and LB/amp plates, spread 50 ul of the liquid culture onto the correct plate. Use the loop to gently spread it evenly as possible. Leave plate upright (with lid closed for 5 minutes to allow liquid culture to absorb into the plate).

Spread 100 ul of the remaining liquid cultures to the LB/amp/ara plates. Spread as noted above.

9. Invert all plates, stack into one set of 6, and use a piece of tape to secure together. Place in 37 ^{o}C incubator for about 24-48 hours.

Overnight Culture Grow-Out and Plate Assessment:

1. After 24-48 hours of grow-out, remove plates and observe.

2. Plate results should be reviewed as a class. Only those plates sets that have results the completely agree with expected results would be used in later phases of experimentation. As your transformation allows the bacteria to grow out a red protein, it is pretty obvious that the process was or was not successful.

3. Using the P+, LB/Amp/Ara, identify a pRED positive colony. Using a sterile loop, transfer the colony to a 5 ml liquid culture in a bacteria culture tube with LB/amp/ara broth. Be sure to label the tube with your groups' information.

4. Samples will be cultured overnight @ 37^{o}C, 200 RPM. This will encourage quick grow out and cultures with lots of plasmids.

5. Next lab, these cultures will be use to help isolate a plasmid via a QIAGEN plasmid isolation kit. Please see your instructor for a copy of the protocol for this procedure.

6. Before discarding plates, check with instructor to ensure plates are not needed for another protocol, etc.