Playing with Plasmids

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Transformation of competent *E.coli* with recombinant plasmids is a standard laboratory procedure used in many high school and college biology classes. With good reason: it convincingly demonstrates that DNA is the genetic material, is readily adaptable to student laboratory schedules, and is a common tool of the biotechnology industry. Two interesting and common plasmids are particularly suitable for student use; both are available from Carolina Biological Supply Company. One is plasmid pGreen, which causes transformants to fluoresce brightly under U.V. light. The other is pVIB, containing a set of genes from the bioluminescent bacterium *Vibrio fisheri*. Cells transformed with this plasmid 'glow in the dark'. Both plasmids contain the same gene (beta lactamase) for resistance to the antibiotic ampicillin. Each of these plasmids vividly illustrates the new property conferred upon their respective recipient cells. Once the cells have been transformed, however, don't stop there; keep going! After years of using these plasmids in genetics, microbiology, and cell/molecular biology classes, my students and I still enjoy working (and learning molecular biology) with them.

Transformation of competent cells

There are a number of techniques and strains of *E.coli* available. We normally use a strain called DH5-alpha. Strains and techniques vary as to their efficiency – that is the number of transformed cells obtained per ug of plasmid DNA. Generally speaking, the larger the size of the plasmid, the lower is the transformation efficiency. pGreen, at just about 4,000 bp in size, transforms DH5 alpha cells more readily than pVIB, which is about 13,400 bp in size. How the cells are prepared and made competent greatly affects the transformation efficiency. Commercially available ultra-competent cells are expensive, must be stored carefully and used quickly, and to be honest, who needs 10 million (or more) transformants of pGreen per ug of plasmid? There is an informal saying in the cloning business: 'it only takes one transformant'. For purified plasmids, obtained either from commercial sources or those prepared by students, the rapid and simple calcium chloride method described in the Carolina online teacher's manual is more than sufficient (2). There is also an excellent guide by Anh-Hue T. Tu, available in the Microbe Library (1). When trying to recover recombinant plasmids from ligations, however, higher transformation efficiencies are generally required. Having transformed a competent strain of *E. coli*, you now have an unlimited, expandable and storable source of a plasmid. It has been 'cloned' in the original sense of the term. This basic idea is easy to overlook; an easy-to-grow strain of bacteria is copying DNA and producing a protein using a gene obtained from another organism; that's Biotechnology!

To store clones, (as well as the untransformed E. coli strain) for future semesters and years, simply add an equivalent amount of glycerol to an overnight liquid culture (USP grade glycerol available from most drug stores is sterile and inexpensive). It is a good idea to freeze multiple (100 – 500 ul) aliquots in microfuge or plastic culture tubes that seal well. The cells will generally remain viable for six months or so in a standard (frost-free) freezer and considerably longer than that in an inexpensive chest freezer at -20 C. The continual warm/cold cycling of frost-free freezers shortens the storage life of glycerol-frozen cells, as well as most enzymes used in molecular biology. To use such frozen cultures; defrost them at room temperature and either streak on selective agar media or use the defrosted solution as an inoculum for a liquid culture. Don't forget to freeze a new batch for future use.

Phenotype

The first and obvious thing to do with transformed cells is to observe the phenotype. pGreen from Carolina will cause the transformants to appear green even under normal lighting conditions but the cells can fluoresce spectacularly under long (and short) UV wavelengths. Transformed cells grown in liquid culture can be used to 'paint' designs on agar media in Petri dishes, using sterile cotton-tipped swabs. This is often appealing for demonstrations to younger audiences. Purification and characterization of the green fluorescent protein from the transformants is a standard and useful laboratory exercise for advanced high school and college students.

pVIB-transformed cells only bioluminesce when cultured below 30 ^{o}C, however we have found that transformants are more quickly and easily obtained by overnight incubation at 37 ^{o}C. These colonies (or cultures derived from them) will exhibit bright luminescence by allowing the cultures to cool to room temp. Light production (visible to the unaided eye in a dark room) generally commences within 8 hours of the temperature change, reaching a peak at about 24 hours and gradually subsiding over the course of two or three days. The luminescence can greatly exceed that as seen on the luminescent hands of a wristwatch. Cells grown on agar media with relatively high ampicillin concentrations (up to 100 ug/ml) apparently glow more brightly, though we have not quantified this dose-dependent effect. We have never observed bioluminescence in liquid culture. "Why do you suppose that is?" is a fair question for advanced students.

Plasmid loss

Cells harbouring plasmids need to be maintained 'with selection'. In the case of pGreen and pVIB, this means growing the cells in the presence of the antibiotic ampicillin. Plasmids are extra chromosomal genetic elements that can be randomly lost by cultures if there is no need for them. Cells that contain plasmids grow a little more slowly than cells

without them; they have extra work to do, copying this extra-chromosomal DNA. In the absence of selection, cells that lose the plasmid will begin to outnumber those that have it, until it may be difficult or impossible to find a cell (a clone) with the plasmid. This is the basis of the very real concern over the use and abuse of antibiotics in both medicine and the agriculture industry. Constant use creates constant selection, forever maintaining large populations of bacteria that harbour resistance plasmids in our world. There is evidence that such plasmids 'get around' and may cause difficult-to-treat diseases due to the acquisition of these plasmids by pathogenic bacteria (4).

A useful exercise is to grow pGreen and pVIB transformed E.coli in liquid culture in the presence and absence of ampicillin. In our hands we found that pVIB is lost quite quickly in the absence of ampicillin (not too surprising as it is a fairly big plasmid) whereas we were very surprised (shocked, even!) at how long pGreen remained. Such plasmid-loss studies are simple, inexpensive, and provide ample opportunity for students to design their own experiments.

Plasmid purification and characterization

Many college and high school laboratories are equipped with everything needed to purify plasmids from the transformants, as well as to perform simple restriction enzyme digests and subsequent agarose gel electrophoresis to verify and characterize the plasmids. This is both an entertaining and practical aspect of molecular biology for students: purifying, cutting, and visualizing plasmid DNA. The 'STET' prep (5) that relies on detergent lysis of lysozyme-treated cells is an inexpensive and practical alternative to the many plasmid 'miniprep' kits that are commercially available but the latter usually produce plasmid preparations of greater yield and purity. Just like the cells harbouring the plasmids, the plasmid preps can be stored in the freezer and used in subsequent semesters/years. One should never have to buy a plasmid more than once!

Plasmids are recognized by: the phenotype they bestow upon recipient cells, their size and restriction enzyme fragment pattern, and their sequence. In the absence of actually sequencing a plasmid (still out of the scope of most high school and college laboratory exercises) it is important to characterize a plasmid by restriction enzyme analysis. A known plasmid will generate a predictable and reproducible pattern of bands in an agarose gel. The more restriction enzymes one can use, the more certain one can be of the plasmid's identity. This is particularly true of recombinant plasmids one might try to construct; a minimum of two different enzymes should be used to molecularly confirm the plasmid identity. It is also important to check for restriction enzyme activity (and presence/absence of restriction sites in a plasmid) by including enzyme–free control reactions when doing restriction digests. Uncut circular plasmids migrate differently from linear DNA molecules of the same molecular weight – often appearing as multiple bands (Figure 4, lane 2).

Restriction Enzyme Analysis of pGreen and pVIB

pGreen, as supplied by Carolina, comes with a restriction map, though the one we received was far from complete. The map indicates a single Hind III site, and shows no EcoR I site. When we digested the plasmid purified from our transformants with Hind III and EcoR I, we discovered just the opposite. Hind III does not cut the plasmid (figure 4, lane 2) and EcoRI makes a single cut, generating a single band of about 4,000 kb (figure 1, lane 1). The plasmid size indicated by Carolina is 3927 bp. The lack of a Hind III site was suggested by two observations. Firstly in an agarose gel, the appearance of the plasmid cut with Hind III was unchanged from that of the appearance of the uncut plasmid (data not shown). Secondly we were sure the Hind III enzyme was active as evidenced by the fact that the enzyme did cut other plasmids (see below). A reply from the biotech help department at Carolina, in response to our query, confirmed our observation (personal communication). However the 2012 online Carolina pGreen map still shows a single Hind III site and no EcoR I site (6). Confusingly, the plasmid map of pGreen in the Carolina teacher's manual does show both an EcoR I site and a Hind III site and gives a different plasmid size of 4528 base pairs (2).

Since our plasmid map was wrong/incomplete and we did not have the sequence of the plasmid, working with pGreen became (and has remained) a useful exercise in plasmid analysis in my classes. As we also had the enzyme Sal I, we tried to cut pGreen with that enzyme too (though there was no Sal I site indicated on either of the Carolina pGreen maps). We found that Sal-1 makes a single cut, just like EcoR I, generating an identical single band of about 4,000 bp (figure 1, lane 2). Of course it is possible to cut plasmids with more than one enzyme at a time and, fortunately for our purposes, both Sal I and EcoR I work together in the same restriction buffer. A double digest generated two bands, as one would predict from a circular plasmid cut in two places, a larger band of about 2,500 bp and a smaller band of about 1,500 bp (figure 1, lane 3). It seems likely that the pGreen plasmid sold by Carolina was made by fusing the GFP gene into the lac Z gene of one of the 'pUC' expression plasmids. This much can be deduced from the plasmid map provided by Carolina.

pVIB, does not comes from Carolina with a restriction map but rather with a reference to the original paper which reported the cloning of the bioluminescence genes (3). In that paper the plasmid is referred to as plasmid pJE202 and was produced by cloning a 9 kb Sal I fragment of genomic DNA from *V. fisheri* into the unique Sal I site of the old cloning plasmid pBR322. pBR322 is often illustrated in textbooks. Cloning into this Sal I site destroys the tetracycline-resistance gene, allowing for indirect selection of recombinant plasmids. A Sal I digest predictably cut pVIB (a.k.a. pJE202) into two fragments, a large 9 kb fragment (the bioluminescence gene cluster) and a <4,500 bp fragment that is pBR322 (it should be 4361 bp) (figure 2, lane 1). The pVIB restriction map can be pieced together from the map given in the original paper (3) and from the widely available map of pBR322 (7). There are six rather

closely spaced Hind III sites in the 9kb Sal I fragment of pVIB and a single Hind III site in pBR322 for a total of seven sites. This should generate seven fragments. When the class digested our PVIB prep with Hind III we obtained four clearly visible fragments of about 8,000, 1,700, 1,300 and 850 bp. The remaining three fragments are all less than 500 bp and are not easily seen nor separated in most gels (data not shown). Digestion with EcoRI generated a single large band on a gel of greater than 12 KB (the largest band in our DNA molecular size marker) (figure 2, lane 2). There is no EcoR1 site in the 9 kb bioluminescent gene cluster (3) and pBR322 is known to have a single EcoR I site (7), so one would predict a single band of about 13,400 bp in size.

Cutting and Pasting (or recombining recombinant plasmids)

Over the semesters we have performed some simple cloning experiments with pGreen and pVIB plasmids. First, since we recognized that both pGreen and pVIB have a single EcoR I site, we tried to join the two plasmids together at this site. It was important to be able to efficiently screen for recombinant plasmids after transforming with a ligation reaction, as we expected to see three classes of transformants: those with pGreen, those with pVIB, and those with the newly created plasmid. The initial selection for transformants was done on ampicillin media but all three of these plasmids confer ampicillin resistance. We hoped the new plasmid would have the novel and dual phenotype of GFP fluorescence and bioluminescence.

Using commercially available ligation kits and competent cells we had prepared in class, we transformed a ligation mix of EcoR I digested pGreen and pVIB and selected for ampicilln resistance. After the transformants had grown and cooled to room temperature we screened the plates visually in a dark room, with a hand-held UV light. By alternating between no light and UV light, we were able to identify three types of transformants, as we had expected. Most of the transformants were simply pGreen. Being the smallest plasmid, it transformed cells most efficiently. Most of the remaining transformants contained pVIB and exhibited bioluminescence, but we did find four transformants (from several hundred colonies) that were both bioluminescent and fluorescently green: our putative recombinant plasmid.

Plasmid preps of these transformants confirmed that we had indeed generated a large hybrid plasmid of about 17,500 bp in size. As expected, an EcoRI digest yielded two bands of 4 and <14 kb in size – the original sizes of pGreen and pVIB respectively (figure 3, lane 3). A Sal I digest (figure 3, lane 2) generates three bands, as would be predicted: there are two Sal I sites from pVIB and one from pGreen. We named this new hybrid plasmid 'pTD' (from the Italian for 'Tutte Due', meaning 'Both'). Due to the large size and low yield of the pTD plasmid, it was necessary to combine and concentrate the DNA from several minipreps in order to have enough DNA to visualize the fragments on a gel. pTD transformants fluoresce much less brightly than pGreen, but are still distinctly green. The bioluminescence of pTD transformed cells is qualitatively as bright as that of pVIB transformants. We were able to use the pTD plasmid to transform competent DH5 alpha cells by the standard calcium chloride, heat-shock method.

We also realized that we should be able to easily deconstruct pVIB and obtain pBR322 intact. As pBR322 carries both ampicillin and tetracycline resistance genes, this would allow for direct selection on media containing both antibiotics. We simply did a ligation with a small amount of a Sal I digest of pVIB, transformed cells and selected on ampicillin + tetracycline media. Sure enough, we obtained a number of transformants that were resistant to both antibiotics and yielded a 4.5 kb fragment upon plasmid digestion with Hind III (figure 4, lane 3). Identically sized bands were also generated with EcoR I and with Sal I (data not shown). This is a simple but impressive demonstration of the power of direct selection. The students and I felt as if we had 'liberated' pBR322 after almost 30 years of being stuck together with 9kb of glow-in-the-dark genes.

Finally, a student suggested one more plasmid we ought to be able to make: since pBR322 has a single EcoR I site, as does pGreen, we ought to be able to ligate the two together, as we did with pGreen and pVIB. But since the EcoR I site in pBR322 is between the ampicillin and tetracycline resistance genes (7), we would be able to recover transformants by direct selection on amp + tet media, (as we did to recover pBR322 transformed cells). We combined equal amounts of EcoR I-cut pGreen and EcoR I-cut pBR322 and performed a standard ligation reaction, followed by transformation and selection on ampicillin + tetracycline agar plates. We recovered a number of transformants that were resistant to both antibiotics and were also fluorescently green but, like the pTD transformants, the fluorescence was diminished as compared with cells carrying the original pGreen plasmid. (Another 'why do you think that is?' question!). Restriction enzyme analysis of the new plasmid (coined pGRET, by the students) gave the predicted results: EcoR I generated two bands that migrate closely together on a gel - 4kb for pGreen and <4.5 kb for pBR322 (figure 4, lane 5). A Hind III digest generated a single band of about 8.5 kb (figure 4, lane 4). This confirmed our earlier finding that pGreen has no Hind III site. There should be a single Hind III site from the pBR322 plasmid and a Hind III digest should make a single cut in the new plasmid yielding a single band on a gel that should be the combined size of pGreen and pBR322.

Conclusion

Working with these plasmids has been and continues to be an instructive, rewarding, and challenging experience for biology majors. Starting with pGreen and pVIB we have generated three more plasmids we didn't have before: pBR322 (not new, but new to us-and of historical significance) and the two new plasmids we've named pTD and pGRET. The students gain familiarity and facility with basic techniques of molecular biology while generating restriction maps, characterizing transformant phenotypes, and making new plasmids. The transformants and plasmids are frozen away, quietly awaiting use in future classes.

I have expressly not included specific details on molecular protocols, as they are widely available and vary somewhat from lab to lab and kit to kit. There is no substitute for experience, for both teachers and students alike, when it comes to transformation, plasmid purification, restriction enzyme analyses, agarose gel electrophoresis, etc. 'Playing' with these (and other) plasmids is an excellent way to gain this experience. Please do not hesitate to contact me if you would like more specific details on the protocols used in our lab.

Finally, it should be noted that pGreen, as sold by Carolina, is patented and trademarked. There are other versions of plasmids carrying the green fluorescent protein (GFP) that are sometimes also called pGreen, they can be expected to behave differently than the 3927 bp Carolina product.

References

1) Anh-Hue T. Tu Transformation of Escherichia coli Made Competent by Calcium Chloride Protocol - http://www.microbelibrary.org/component/resource/laboratory-test/3152-transformation-of-escherichia-coli-made-competent-by-calcium-chloride-protocol

2) Carolina transformation manual - http://www.carolina.com/text/teacherresources/instructions/biotech/transformations_manual.pdf

3) Engebrecht, J., Nealson, K. and Silverman, M 1983 Bacterial Bioluminescence: Isolation and Genetic Analysis of Functions from *Vibrio fischeri*, Cell, Vol. 32, pages 773–781

4) Gilchrist, M.J., Greko, C., Wallinga, D.B., Beran, G.W., Riley, D.G. and Thorne, P.S. 2007 The Potential Role of Concentrated Animal Feeding Operations in Infectious Disease Epidemics and Antibiotic Resistance Environ Health Perspect. February; 115(2): 313–316

5) Molecular cloning manual online- http://www.scribd.com/doc/23261720/Molecular-Cloning-A-Laboratory-Manual-On-The-Web-Maniatis#outer_page_32

6) Online pGreen map from Carolina - http://www.carolina.com/product/pgreen+plasmid%2C+1+%26micro-g+%28200+%26micro-l-+0.005+%26micro-g-%26micro-l%29.do?keyword=pgreen+plasmid+map&sortby=bestMatches

7) Restriction map of plasmid pBR322 - http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/maps/pbr322_map.pdf

Figures and Figure legends

Figure 1 – pGreen. Lanes from L-R: EcoR I, Sal I, EcoR I + Sal I double digest, DNA marker. Note that that too much marker was used, generating large fuzzy bands, and making size comparisons more difficult).

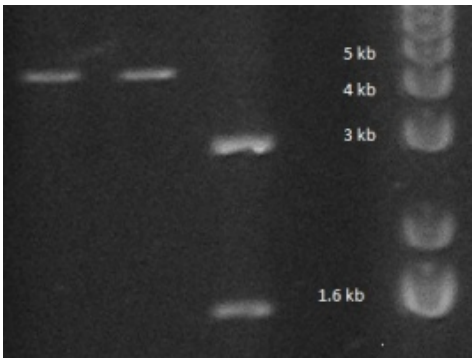

Figure 2 – pVIB. Lanes from L-R: Sal I, EcoR I, DNA marker

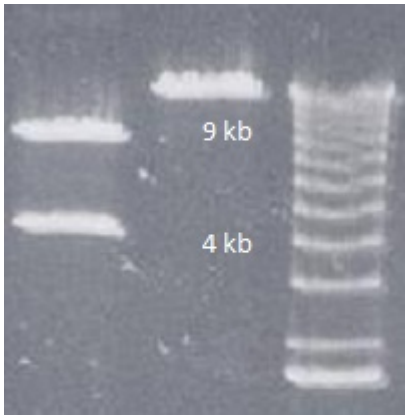

Figure 3 – pTD. Lanes from L-R: DNA marker, Sal I, EcoR I

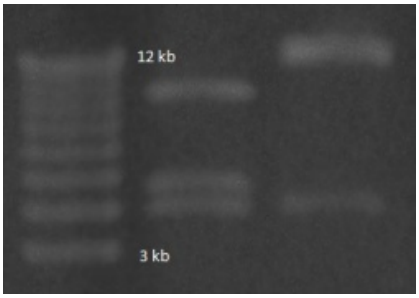

Figure 4 – Lanes from L-R: DNA marker, pGreen/Hind III, pBR322/Hind III, pGRET/Hind III, pGRET/Sal I, DNA marker