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**ANTIBODY ENGINEERING FOR EXPRESSION
IN INSECT CELLS AND LARVAE**

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RESEARCH AND TECHNOLOGY DIRECTORATE

August 2002

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13. ABSTRACT (Maximum 200 words) Antibodies are currently deployed as the recognition component of sensors that detect biological threat agents. Antibodies that detect simulants of bio-threat agents are also currently incorporated into detection platforms for testing and evaluating new devices and materials. Previously, we developed an anti-botulinum toxin antibody using a powerful genetic technology known as phage display, in which a very large library of immunoglobulin (antibody) genes are expressed on the surface of bacteriophage (bacterial virus) particles. However, antibodies expressed in bacteria lack molecular modifications made post-translationally by animal (eukaryotic) cells. It was therefore desirable to express Fab fragment antibody genes in insect cell lines and larvae. The use of insects as gene expression "bioreactors" poses far fewer ethical concerns than the use of mammalian systems. In this study, we improved an existing baculovirus expression vector by inserting the reporter gene DsRed, then modified and inserted the heavy and light chain genes encoding an anti-botulinum toxin-binding Fab antibody. The structures of all plasmids constructed were verified by restriction analysis and sequencing. Preliminary data demonstrate that the reporter gene DsRed is strongly expressed in larvae of <i>Trichoplusia ni</i> , suggesting that this system may be an economical manufacturing process for recombinant antibodies.				
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PREFACE

The work described in this report was supported by a grant from the Maryland Technology Development Corporation (Maryland TEDCO). The results fulfill ECBC's requirements as described in the Statement of Work in CRADA number 162, signed by ECBC and Chesapeake PERL, Inc., to develop a system for the expression of antibody genes in insect cells and larvae. The work was started in June 2001 and completed in May 2002.

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ANTIBODY ENGINEERING FOR EXPRESSION IN INSECT CELLS AND LARVAE

1. INTRODUCTION

Antibodies are the essential component in immunological sensors that detect biological warfare (BW) agents, conferring both sensitivity and selectivity for BW agents. The Army and the Joint Program Office for Bio-Defense (JPO-BD) purchase and field biosensor platforms (JBIDS, JPBDS, JBREWS, and PORTAL SHIELD systems) that incorporate antibodies produced in whole animals (polyclonal) and mammalian cell cultures (monoclonal) for BW agent detection.

Several groups have described bacteriophage-based systems for the selection and cloning of specific antibody gene sequences (Parmley and Smith, 1988; Hogrefe and Shopes, 1994). We have described previously the advantages of recombinant antibodies (O'Connell et al. 2001). They are genetically completely defined, in that their sequences are fully known; malleable, in that their sequences can be manipulated to alter their properties; and producible in a variety of organisms into which the antibody genes can be inserted. These systems include bacteria (e.g., *E. coli*). However, a drawback to the production of antibodies in bacteria is their lack of ability to properly modify the expressed antibodies with sugar molecules, a process called glycosylation. Glycosylation of antibodies is desirable when the antibodies are to be used as human therapeutics, as these sugar moieties reduce the antigenicity of the antibodies, and therefore reduce the body's tendency to recognize them as foreign. Glycosylation also affects how the immune system interacts with recombinant antibodies to facilitate binding to an antigen.

Recombinant antibodies can also be expressed in eukaryotic cells, such as yeast or cultured mammalian cells, or in whole transgenic higher organisms including mammals and plants. The time required to develop expression systems in transgenic animals or plants, however, is costly, making these approaches economically feasible only for very large scale expression.

The use of mammals in the production of antibodies and other proteins is becoming increasingly controversial for ethical reasons (National Research Council, 1999). **An attractive alternative to transgenic animal or plant expression for antibodies or indeed, any other proteins, is to express the desired genes in transformed insect larvae.** Insects are sufficiently molecularly sophisticated to glycosylate antibodies (Hsu et al. 1997; Davis and Wood 1995). Certain species, such as *Trichoplusia ni*, are also inexpensive to raise and can be grown in very large numbers in automated facilities. Most importantly, insect viruses (most harmless to humans) can be used to infect insects via ingestion and can be used as delivery vehicles for the genes of interest (Pham et al. 1999).

Upon eating a diet containing an engineered insect virus, the cells of a larva become infected and the desired gene is expressed at high levels along with the rest of the viral genome. The transfected insect cells produce the desired protein product in large quantities, and the product can be recovered by extraction from infected larvae. The process for gene expression in transformed insects is not foolproof, however, and not every larva expresses the desired gene at the same level. To reduce the amount of "background" insect protein in a preparation relative to the desired product, it is necessary to identify larvae that are the highest producers, and to extract the recombinant product from those larvae preferentially.

The purpose of this study was two-fold. The first objective was to modify an existing insect cell expression vector to contain a gene encoding DsRed, an auto-fluorescing protein derived from *Discosoma* coral. The DsRed gene was cloned into the vector in a manner permitting the fusion of the DsRed gene to any gene cloned between it and the promoter region. The result of such a cloning was the fusion of the desired gene with DsRed. Expression of the fused gene would give rise to a protein whose amino-terminal end is the desired product, and whose carboxy-terminal end is DsRed. Accumulation of the product would therefore be tied directly to the accumulation of DsRed, which can be monitored by illuminating the larvae with light in the visible portion of the EM spectrum (excitation =545nm, emission =620nm). In this way larvae producing a desired recombinant protein can be easily identified and selectively harvested. Such larvae could be selected either by hand, or by an automated larva "picker" guided by machine vision. A similar technology was described by Cha *et al.* (1999) in which green fluorescent protein (GFP) was used as the reporter.

The second objective was to demonstrate the effectiveness of the expression of a useful protein product using the DsRed fusion vector. This required the engineering of a set of antibody genes and their cloning into the DsRed fusion vector so that one of the antibody's light chain gene and the DsRed reporter protein would be expressed as a fusion protein. This fusion would allow the ready determination of the expression level of the antibody in a larva before beginning the process of antibody extraction and purification. The fusion protein was engineered in a way that allows the selective removal of the DsRed fluorescent marker after purification. To accomplish these goals, an existing gene set encoding the heavy and light chains of a recombinant anti-botulinum toxin Fab (Emanuel *et al.* 1996) was modified for expression in insect cells and cloned into both the original vector (without DsRed) and the DsRed-modified vector.

This report describes the cloning of the anti-botulinum Fab heavy and light chain genes (BotFab 5) into the insect baculovirus expression vector pAcAB3, the modification of pAcAB3 to contain the DsRed gene, and the cloning of the heavy and light chain genes into the resulting pAcAB3-DsRed vector.

2. METHODS

2.1 Bacteria, plasmids, and culture conditions. The host strain for all cloning in this study was *Escherichia coli* DH5 α (Hanahan, 1983), obtained from our in-house collection. Cultures of *E. coli* were grown at 37°C in LB broth (Sambrook et al., 1989) or on solid LB medium containing 1.5% agar. Carbenicillin was added to media for *E. coli* at 50 μ g/ml. Plasmid pAcAB3 was purchased from BD Pharmingen (San Diego, CA). Plasmid pDsRed was purchased from BD Biosciences (formerly Clontech; Palo Alto, CA).

2.2 Molecular biology. Plasmid DNA was prepared for use as PCR template and for sequencing from cells of *E. coli* using QIAGEN maxi-columns (QIAGEN Inc., Valencia, CA). Plasmids for cloning analysis were prepared from 2 ml cultures of *E. coli* with QIAGEN Spin Miniprep kits. PCR products were separated from unused and dimerized primers by gel electrophoresis (Sambrook et al., 1991), or purified with Qiaquick PCR Purification Kits. PCR products were extracted from agarose gel slices and purified using the Qiaquick Gel Extraction kit. All restriction enzymes were purchased from New England Biolabs (Beverly, MA) and used according to the manufacturer's instructions. PCR reactions contained 45 μ l PCR Supermix (Life Technologies, Gaithersburg, MD), 1 μ l (200 ng) of each primer, 1-3 μ l of template DNA, and molecular biology-grade H₂O was added to a total volume of 50 μ l.

DNA ligation reactions were set up as follows: 1 μ l of T4 DNA ligase (NEB), 2 μ l of T4 DNA ligase buffer, plus insert and vector DNA in varying amounts to bring the total volume of the reaction to 20 μ l. Ligations were performed in thin-walled PCR tubes in a Perkin Elmer 9600 temperature cycler set to maintain a temperature of 16°C. Products of ligation were transformed into electrocompetent cells of *E. coli* strain DH5 α and transformants were selected on solid LB medium containing carbenicillin.

Automated cycle sequencing reactions (fluorescent dye-terminator) were performed at the University of Maryland School of Medicine Biopolymer Laboratory, Baltimore, MD. DNA sequences were analyzed using LaserGene software (DNASTAR, Madison, WI).

2.3 PCR primer design. Primers were analyzed with Applera's Primer Express software, and secondary structure and dimer formation were checked with PrimerFinder software (<http://eatworms.swmed.edu/~tim/primerfinder/>). Primers were synthesized by The Midland Certified Reagent Co. (Midland, TX).

3. RESULTS

3.1 Amplification and cloning of DsRed. The gene encoding DsRed was amplified using primers 1 and 2 (Table 1). These primers were designed to amplify the DsRed open reading frame (ORF) and attach *Bgl*II and *B*lpl restriction sites on the 5' end and 3' end of the DsRed ORF, respectively. The forward PCR primer (primer 1, Table 1) was also designed to include *S*acI and *A*vrII restriction sites. These sites do not occur in plasmid pAcAB3, so their inclusion upstream of the DsRed ORF created two unique cloning sites into which genes of interest may be introduced to form translational fusions with DsRed. Plasmid pAcAB3 and the amplified DsRed fragment were then digested with *Bgl*II and *B*lpl, purified as described above, ligated as shown in Figure 1, and transformed into *E. coli*. DNA was isolated from transformants and cut with *Bgl*II and *B*lpl to verify the presence of DsRed insert DNA (Figure 2).

3.2 Bombyxin leader sequences. As constructed originally (Emanuel et al. 1996), the anti-botulinum antibody heavy and light chain genes were fused on the carboxyl-terminus to peptides derived from the *pe*lB gene of *Xanthomonas campestris*. The *pe*lB leader peptide targets the export of the heavy and light chain proteins from the cytoplasm of *E. coli* where they are translated from mRNA, to the periplasm where they fold and form the intact Fab antibody. The *pe*lB leader peptides, however, do not have this function in insect cells, and therefore it was necessary to replace them with sequences that encode a peptide that would direct their export from the cells. The protein bombyxin is exported avidly from insect cells, and the leader peptide from this protein was chosen as the replacement for *pe*lB. The replacement was performed as an integral part of PCR amplification of the heavy and light chain genes.

To perform the replacement, we synthesized PCR primers that had, at their 5' ends, the 3' portion of the bombyxin leader sequence, and the 5' end of the heavy (or light) chain at the 3' end (Figure 3, top PCR reactions). In Figures 3 and 4 the bombyxin leader-encoding sequence is diagrammed in red. PCR primers for the distal ends of the heavy and light chain were designed to include restriction sites to facilitate their cloning into pAcAB3. When amplified from the bacterial expression vector, the antibody genes were preceded by bombyxin-encoding sequences.

3.3 Antibody cloning strategies. Two strategies for antibody expression in insect larvae were employed. In one, we amplified the bombyxin leader-light chain gene fusion with primers that added *S*acI and *A*vrII restriction sites to allow the cloning of this gene into pAcAB3-DsRed, forming a bombyxin leader-light chain-DsRed fusion. The reverse PCR primers for this amplification (primers 4 and 6) also include a short sequence encoding an enterokinase cleavage site. This site is labile in the presence of enterokinase, a protease that will remove DsRed from the light chain after the fusion protein is produced and purified.

In the second strategy, we omitted the use of DsRed and cloned the bombyxin-light chain fusion directly into pAcAB3. This was done against the possibility that the fusion with DsRed might prevent proper assembly of heavy and light chains after expression.

In both approaches, we amplified the heavy chain gene with a bombxyin leader sequence built into the forward amplification primers, and left intact the existing 6 x histidine tag at the 3' end of the heavy chain gene. This bombxyin leader-heavy chain gene-his tag fusion was cloned into the unique *Xma*I site in both pAcAB3-DsRed (Figure 3) and pAcAB3 (Figure 4).

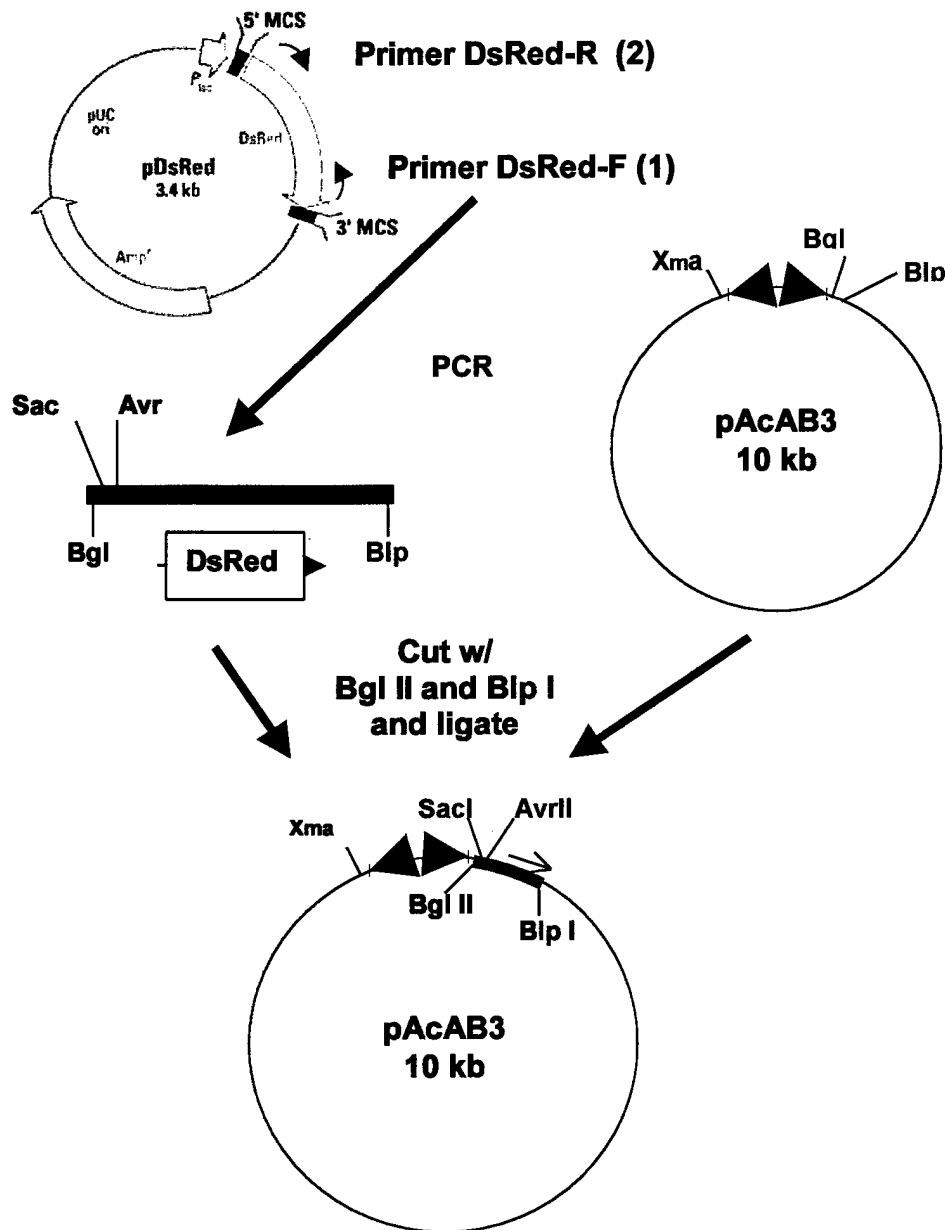


Figure 1. Cloning of DsRed into pAcAB3. Triangles in pAcAB3 represent the two divergent baculovirus p10 promoters that will drive gene expression in the insect host. The amplified DsRed gene, preceded by engineered unique *Sac*I and *Avr*II sites, was cloned downstream of one of these promoters. Numbers following each of the PCR primers (red arrows) for amplification of the DsRed gene correspond to the sequences listed in Table 1.

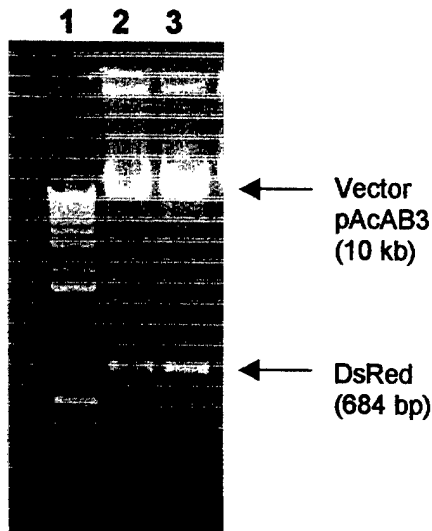


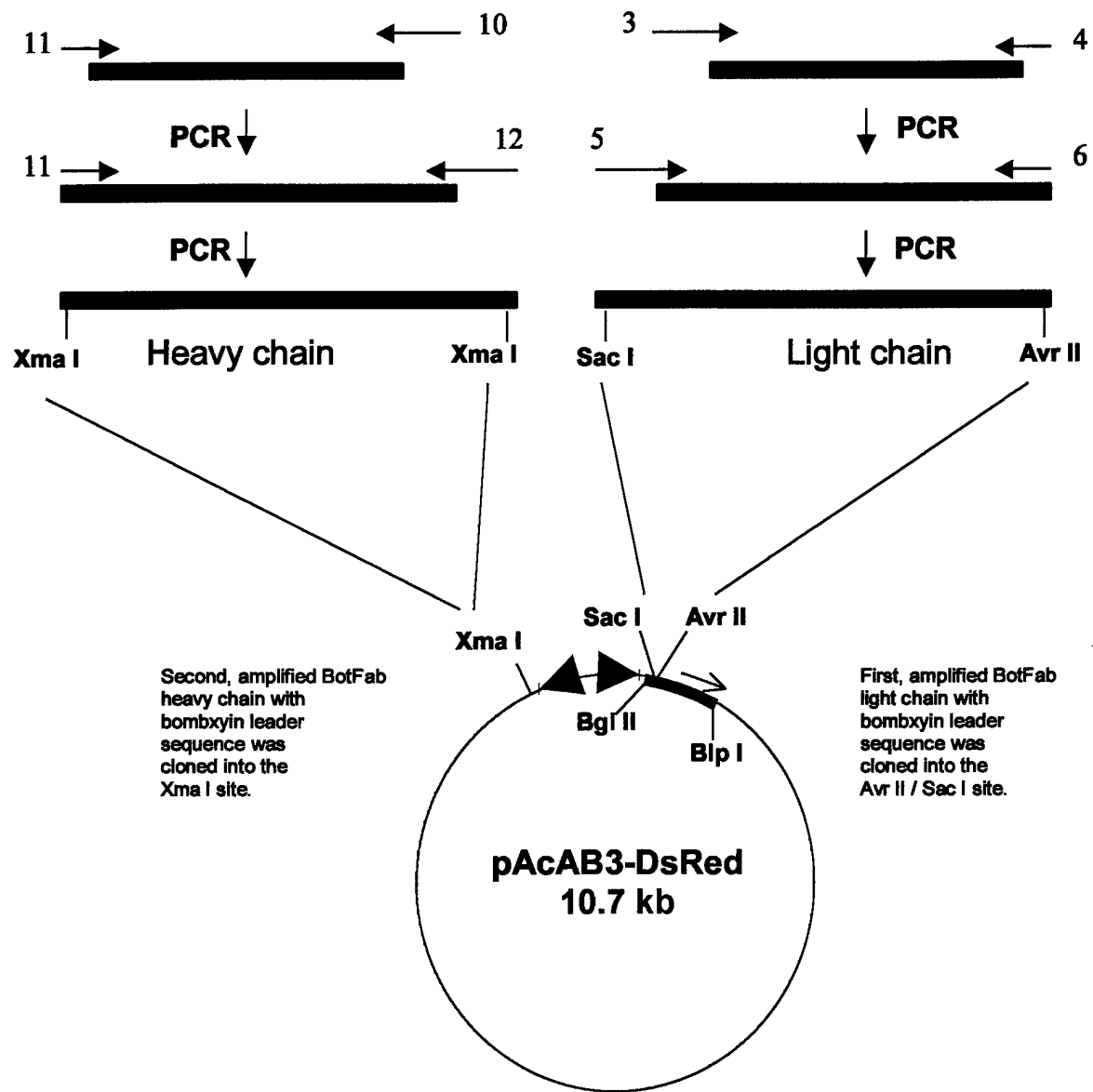
Figure 2. Verification of DsRed cloning. The plasmid resulting from the ligation of pAcAB3 and the amplified DsRed fragment was purified by miniprep, cut with *Bgl*I and *Bln*I, and analyzed by agarose gel electrophoresis. Two fragments of the expected sizes were generated, vector (10 kb) and insert (approximately 700 bp). Lane 1: 1 kilobase ladder size marker, lanes 2 and 3, digested pAcAB3-DsRed.

Table 1. Sequences of PCR primers used in this study.

Primer	Name	Direction	Sequence
1	DsRed	Forward	5' TCAGCAAGATCTACAGAGCTCACACCTAGGATGAGGTCTTCCAAGAAT 3'
2	DsRed	Reverse	5' CGTAGAGCTCAGCCTAAAGGAACAGATGGTGGCGTCC 3'
3	Light Chain 1	Forward	5' AATGTTGTCAACAGTAATGTGGGTGTCAACAGACATCCAGATGACCCAG 3'
4	Light Chain Reverse	Reverse	5' ACCTAGGCTTGTCTGTCGTCGTCACACTCATTCTGTTGAAGCTCTT 3'
5	Light Chain 2	Forward	5' GGAGCTCATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAA 3'
6	Light Chain 2	Reverse	5' ACCTAGGCTTGTCTGTCGTCGTCACACTCATTCTG 3'
7	Light Chain NO DsRed 2	Forward	5' TCAGCAAGATCTATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAAC 3'
8	Light Chain NO DsRed 1	Reverse	5' CGTAGAGCTCAGCTTAACACTCATTCTGTTGAAGCTCTTGACAATGGGT 3'
9	Light Chain No DsRed 2	Reverse	5' CGTAGAGCTCAGCTTAACACTCATTCTGTTGAAGCTCTTGAC 3'
10	Heavy Chain 1	Forward	5' ATTAATGTTGTCAACAGTAATGTGGGTGTCAACAGAGGTTGAGCTTCAAG 3'
11	Heavy Chain	Reverse	5' ACAGTCCCAGGTTAATGGTGATGGTGATGGTGCTACC 3'
12	Heavy Chain 2	Forward	5' TCAACGCCCGGATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAA 3'

Table 2. Characteristics of primers used in this study.

Primer	Name	Direction	Tm	%GC	Length
1	DsRed	Forward	79	44	48
2	DsRed	Reverse	79	57	37
3	Light Chain 1	Forward	82.5	44.9	49
4	Light Chain Reverse	Reverse	82.5	45	46
5	Light Chain 2	Forward	76.6	36.7	50
6	Light Chain 2	Reverse	78.2	54.3	35
7	Light Chain NO DsRed 2	Forward	76.6	36	50
8	Light Chain NO DsRed 1	Reverse	81.3	46.9	50
9	Light Chain No DsRed 2	Reverse	77.1	46.5	43
10	Heavy Chain 1	Forward	78	45	50
11	Heavy Chain	Reverse	79	54	39
12	Heavy Chain 2	Forward	81	45	49



Second, amplified BotFab heavy chain with bombxyin leader sequence was cloned into the Xma I site.

First, amplified BotFab light chain with bombxyin leader sequence was cloned into the Avr II / Sac I site.

Figure 3. PCR amplification strategy for heavy and light chains and cloning strategy for vector pAcAB3-DsRed. Heavy and light chain sequences were amplified from plasmid pHist5 (Emanuel et al. 1996) using forward primers beginning with bombxyin leader sequence (shown above in red) and ending with heavy or light chain sequence (top PCR reactions). Primer numbers correspond to those given in Tables 1 and 2. A second amplification of both light and heavy chain genes was needed to complete the addition of bombxyin sequence and add the 5' cloning sites to each gene (second PCR reaction). Light chain was cloned in first by necessity, as heavy chain is cut by SacI. Divergent baculovirus p10 promoters (large black triangles) drive expression of heavy and light chain genes in insect cells after transfection.

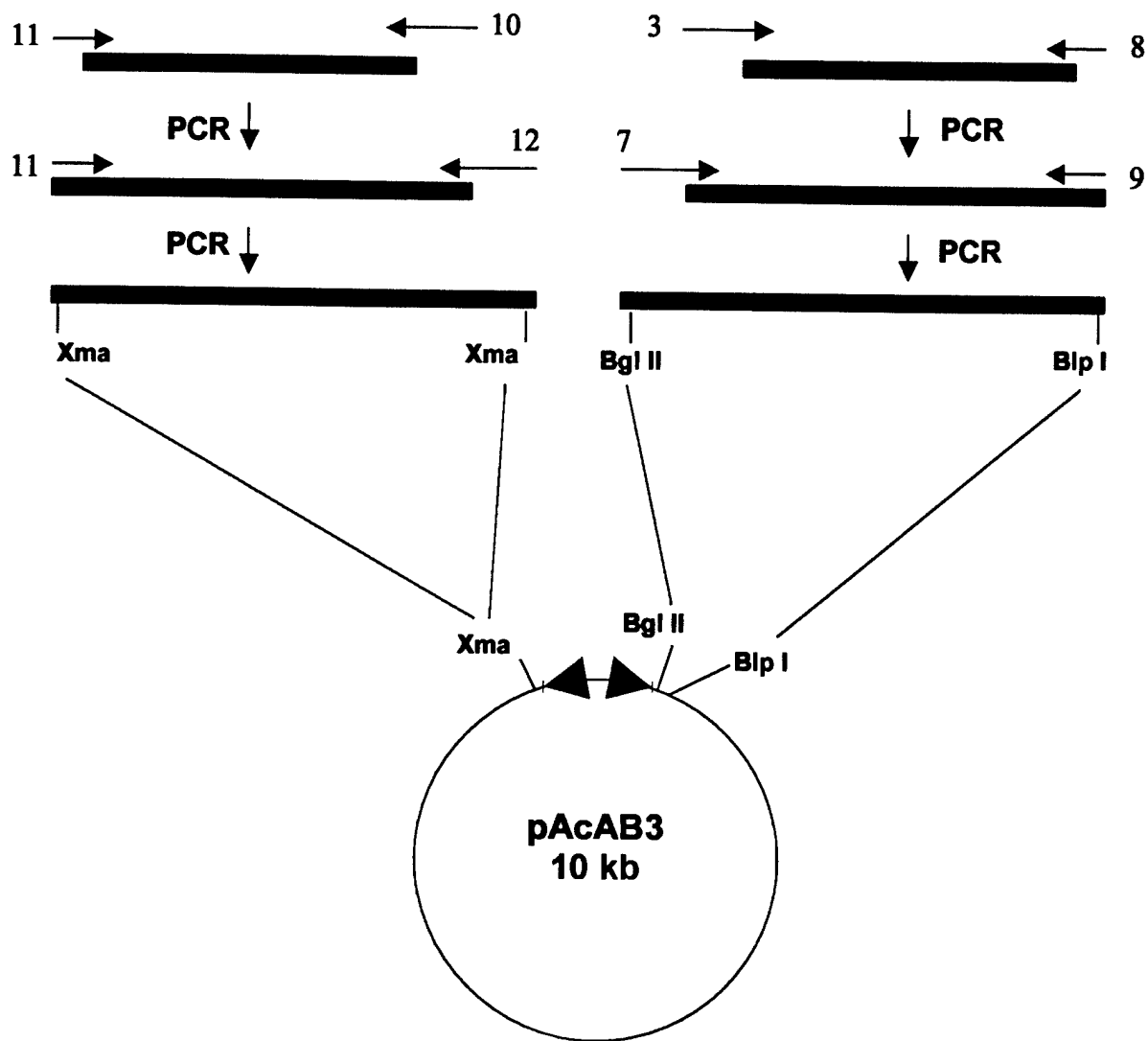


Figure 4. PCR amplification strategy for heavy and light chains and cloning strategy for vector pAcAB3. Heavy and light chain sequences were amplified from plasmid pHist5 using forward primers beginning with bombyxin leader sequence (shown above in red) and ending with heavy or light chain sequence (top PCR reactions). Primer numbers correspond to those given in Tables 1 and 2. A second amplification of both light and heavy chain genes was needed to complete the addition of bombyxin sequence and add the 5' cloning sites to each gene (second PCR reaction). Light chain was cloned directly into the *Bsp I* and *Bgl II* sites occurring in the vector as obtained. Light chain was cloned in first by necessity, as heavy chain is cut by *Sac I*. Divergent baculovirus p10 promoters (large black triangles) drive expression of heavy and light chain genes in insect cells after transfection.

3.4 Verification of cloning results. The insertion of light chain and heavy chain into each of the two vectors was confirmed by a set of restriction digests, with the desired result being predicted by analysis of the final sequences as assembled by computer. Figure 5 shows the *SacI* / *AvrII* digest of pAcAB3-DsRed containing the light chain gene. Additional digests were performed to verify that the light chain was inserted as a single copy (data not shown). Similar experiments were performed to verify the insertion of light chain into vector pAcAB3 (Figure 6).

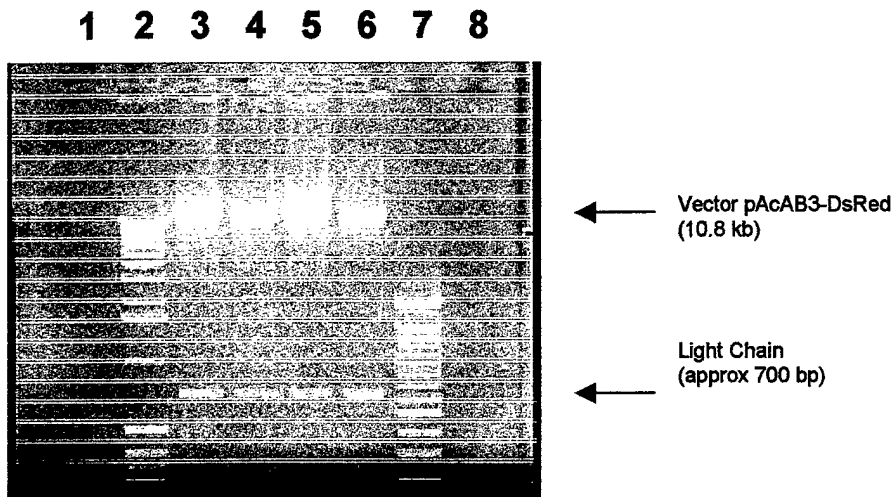


Figure 5. Verification of the cloning of light chain into vector pAcAB3-DsRed. Lanes 1 and 8, no sample. Lane 2, 1 kilobase ladder (size marker). Lanes 3-6, miniprep DNA from four of the colonies obtained after cloning, digested with *SacI* and *AvrII*, showing successful cloning attempts. Lane 7, 100 base-pair ladder.

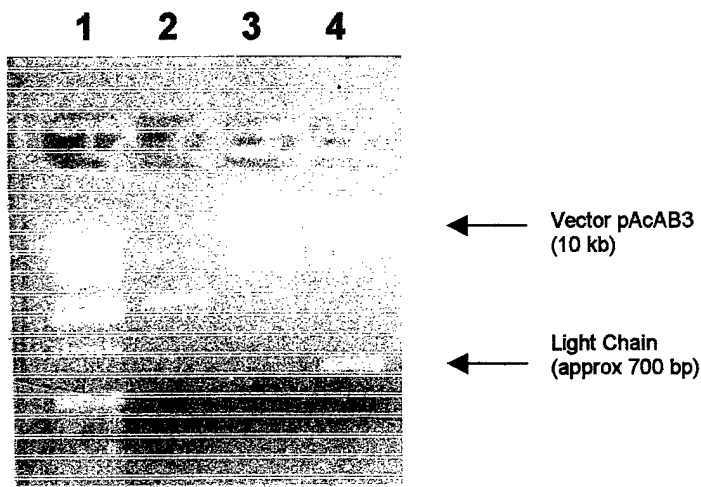


Figure 6. Verification of the cloning of light chain into vector pAcAB3. Lane 1, 1 kilobase ladder (size marker). Lanes 2-4, miniprep DNA from four of the colonies obtained after cloning, digested with *BspI* and *BglII*. Lanes 2 and 3 show DNA from colonies in which the vector DNA either shows deletions (lane 2) or does not contain the light chain (lane 3). Lane 4 shows a vector into which the light chain was successfully cloned.

3.5 Heavy chain amplification and cloning.

Heavy chain sequences were amplified with the primers shown in Tables 1 and 2, in the order shown in Figures 3 and 4, using pHist5 as template. The resulting heavy chain gene fused to the bombyxin leader peptide was digested with *Xma*I, and several attempts were made to clone this fragment into both pAcAB3 (light chain) and pAcAB3-DsRed (light chain). All of these attempts were unsuccessful, due to the possibility that *Xma*I might cut the PCR product poorly, resulting in low cloning efficiencies.

To circumvent this, we first cloned the final heavy chain PCR product into the unique *Xma*I site of cloning vector pUC18. This was done because the efficiency of cloning fragments into smaller vectors is much higher than when cloning into vectors 10 kb and larger. The resulting plasmid, pUC18-heavy chain, was then digested with *Xma*I, and the heavy chain separated from pUC18 by gel electrophoresis (Figure 7). This operation ensured that all heavy chain fragments recovered were in fact cleaved with *Xma*I at both ends before attempting to insert them into either of the larger vectors containing the light chain gene.

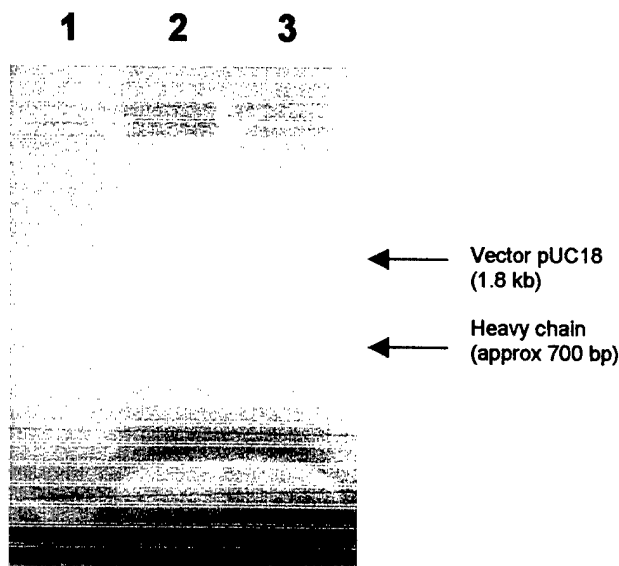


Figure 7. Cloning of heavy chain into, and digestion from, vector pUC18. Lane 1, 100 bp ladder. Lanes 2 and 3, *Xma*I digest of pUC18-heavy chain construct. Heavy chains were excised from the gel, purified, and ligated to *Xma*I-digested pAcAB3-DsRed (light chain) or pAcAB3 (light chain).

The heavy chain gene was cloned into each vector only after light chains were inserted. This was necessary because the heavy chain sequence contains restriction sites for *Bln*I (which would have interfered with light chain cloning if performed second in pAcAB3-DsRed) and *Sac*I (which would have interfered with light chain cloning if performed second in pAcAB3). The heavy chain could not be cloned “directionally”, as only one suitable unique restriction site in pAcAB3 is located downstream of the p10 promoter divergent from that driving light chain expression. Therefore, the single *Xma*I site had to be used, and the orientation and copy number of the heavy chain determined only after cloning (data not shown). Restriction digests that were instructive were: *Xma*I, *Bgl*II + *Spe*I, *Bam*HI, *Stu*I, and *Bln*I.

4. DISCUSSION

Currently the structures of all the plasmids constructed in this study are being verified by direct sequencing (U. of MD School of Medicine core facility). However, preliminary experiments have already been performed at Chesapeake PERL using the DsRed vector, to examine the expression of the reporter protein alone. Introduction of the DsRed construct into baculovirus and subsequent infection of *T. ni* larvae has demonstrated very striking results: larvae expressing quantities of DsRed that are so great that they turn bright pink, observable with the unaided eye (Figure 8). DsRed expression was also noted to be strong in cultured Sf9 cells (an insect cell line) (N. van Beek, unpublished results).

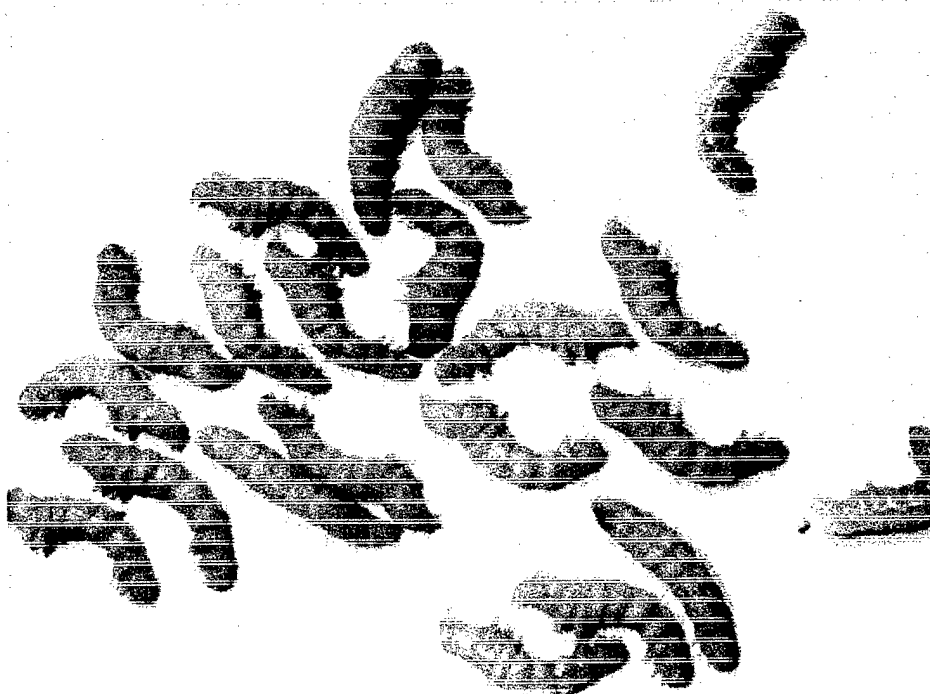


Figure 8. Larvae (pink, left) transfected with baculovirus containing the DsRed gene cloned in this study, driven by the p10 promoter. Note the color of the two green untransfected larvae (right). (Photo courtesy of Chesapeake PERL, Inc.)

The vector containing the antibody genes without DsRed has also been used by Chesapeake PERL staff to infect both cells and larvae. Preliminary results have shown that both the heavy and light chains of the antibody, cloned without the DsRed fusion, are also well-expressed (N. van Beek, unpublished results). Some larvae were estimated to produce as much as 1-3 mg antibody per larva. Samples of the antibody are currently being purified and will be tested with control lots of the Botfab to compare the affinities and specific activities.

The results obtained here are preliminary to the incorporation of the antibody gene-DsRed construct into baculoviruses and the introduction of the baculoviruses into *T. ni* larvae. This work will be a proof of concept experiment to demonstrate that the insect gene expression system. A complete demonstration of the system will include the expression of a desired recombinant protein in larvae fused to DsRed, the identification of high producers visually using DsRed as the marker, the purification of the recombinant protein, and determination that the recombinant protein has the desired activity. Features designed to test all these aspects of the system have been engineered into the vectors, as described in this report.

Variations of each feature of the system are possible, giving future investigators the ability to engineer around problems that may arise that are particular to individual products. For example, DsRed fusions to a recombinant protein might provide inconsistent red marking of larvae because the fusion partner perturbs the folding of DsRed. In such a case, DsRed could still be used as an indicator of the extent of viral infection in a larva by expressing it from another strong promoter in the same vector. For example, the vector used in this study, pAcAB3, has three suitable promoters, of which two were used. There are also several variations on the structure of protein fusions (such as the engineering of a GlyGlyGlyGlySer spacer arm) that can be incorporated into future vector designs. Similarly, if the assembly of a multi-polypeptide protein proves inefficient, the system could still be useful for the expression of single polypeptide proteins. This simplicity and adaptability are two of the most powerful benefits of the larval expression system.

Gene expression in insect cells should provide a useful means for the production of materials for human therapeutics. The manufacture of products for use in humans requires a strict adherence to cGMP (current Good Manufacturing Practices) to ensure the safety, quality and purity of product. The system described here should be amenable to cGMP production when insect cells are used as the production system. Cell cultures, just like larvae, can be monitored spectrophotometrically to determine the extent of protein expression, and the point at which protein expression is optimal can be numerically defined. Under cGMP, a similar quantification of product will likely need to be defined in a larva-based production regime. Also, since all facets of a manufacturing process must be controlled under cGMP, a production line relying on transfected larvae will need to obtain a supply of larvae or eggs that is similarly controlled and maintained.

Lastly, an economic analysis will need to be performed once data on product yield is obtained. Preliminary data indicate that, in the case of Botfab, approximately 300-1000 larvae may be required to produce one gram of antibody. Other proteins will need to be expressed, and the cost of raising, infecting, growing/harvesting larvae, and subsequent purification of product will have to be determined. Since several technological hurdles have already been overcome for the automation of a larval production line, it seems likely that only minor molecular modifications will be required to obtain an efficient production line. Market analysis, including the going rates for recombinant proteins made by other methods, and the overall demand for recombinant proteins, will ultimately determine the economic viability of this insect protein production system.

5. REFERENCES

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