



Short Communication

Generation of an expression library in the baculovirus expression vector system

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Abstract

The construction and screening of a small cDNA library consisting of 2×10^4 clones in the baculovirus expression vector system are described. This library consists of antibody heavy chain sequences isolated from the spleen of a mouse immunized with tetanus toxoid fragment C. A portion of this library was used to produce a pool of recombinant baculoviruses which were screened for production of antibody fragments reactive to tetanus toxoid without prior expression in *Escherichia coli*. The pool of 30 clones was found to contain at least 6 different populations of antibody indicating that diversity existed within the library. Positive clones were isolated from the baculovirus system and confirmed as being capable of producing a tetanus reactive antibody by expression as a β -lactamase fusion protein in *E. coli*. One of these clones was returned to the baculovirus system using a different transfer vector, and tetanus binding reconfirmed. The results presented here show that the concept of the construction and screening of a baculovirus expression library is feasible even with 'difficult' proteins, such as antibody heavy chain fragments, and that the baculovirus expression vector system has the potential to produce cDNA expression libraries which can be screened directly for the desired protein.

Keywords: Baculovirus; Antibody expression; Expression library; Eukaryotic expression

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The baculovirus expression vector system established by Smith et al. (1983) has been used widely to express many proteins (O'Reilly et al., 1992). Numerous reviews have been published on the use and advantages of this system (Bishop, 1992; Luckow, 1991; Maeda, 1989; Miller, 1988) and recent developments in virus vectors have improved the selection rates of recombinant virus to close to 100% (Kitts and Possee, 1993). Despite the advances in selection of recombinant viruses, the use of the baculovirus system, as for many other eukaryotic expression systems, relies upon first identifying a clone in *Escherichia coli* and then transferring the clone of interest to the expression system. The isolation of the clone of interest from complex samples such as tissues often involves large-scale screening strategies in prokaryotes employing plasmid- or bacteriophage-based systems, such as lambda and filamentous bacteriophages. Our aim was to illustrate that potential clones can be screened by expression in a eukaryotic expression vector system and to circumvent the usual route of screening for the desired clone in *E. coli* prior to transferring the clone to the baculovirus system. In this manuscript, we report our attempts to directly clone antibody heavy chain fragments from the spleen of an immunized mouse in a baculovirus plasmid transfer vector and demonstrate the feasibility of screening pools of recombinant transfer vectors by direct production of recombinant viruses and detection of expressed products.

Female Swiss–Webster mice were immunized by subcutaneous inoculation of 10 µg of tetanus toxin C-fragment (Boehringer–Mannheim, Indianapolis, IN) in Freund's complete adjuvant. Each animal was boosted 4 times at 7- to 10-day intervals with 10 µg of tetanus toxin C-fragment in Freund's incomplete adjuvant. The spleen from an immunized mouse was isolated and mRNA was extracted from the spleen cells using a Fast Track mRNA extraction kit (Invitrogen Corp., San Diego, CA). An aliquot of this mRNA was used for cDNA synthesis.

In order to amplify antibody sequences, a series of primers which were complementary to antibody sequences were synthesized. The C-terminal primer (Table 1) was designed to prime at the C-terminus of the C_{H1} domain of mouse IgG heavy chains based upon antibody sequences in Kabat et al. (1991). The primer contained stop codons in 3 different reading frames and restriction endonuclease sites for cloning purposes. The N-terminal primers (Table 1) contained homology to the N-terminal coding sequences of IgG antibodies as based upon the published sequences of antibodies (Kabat et al., 1991), and primers containing these homology sequences have been used previously to amplify antibody sequences for expression in *E. coli* (Ward et al., 1993). The N-terminal primers also restore the remainder of the heavy chain leader sequence lost by digestion of the baculovirus transfer vector with *Sph*I and *Bgl*II, thus producing a functional secretion signal precisely fused to the antibody sequence.

To synthesize first strand cDNA, the mRNA was denatured with 10 mM methyl mercury hydroxide for 10 min, and placed in a 20 µl reverse transcriptase reaction containing 0.5 µg of C-terminal primer (see Table 1), 10 mM DTT, 50 mM Tris-Cl pH 8.3, 8 mM MgCl₂, 70 mM KCl, 1 mM each dNTP, 5 U reverse transcriptase, and 1 mM vanadyl ribonucleoside complexes. The reaction was incubated at 42°C for 30 min and 1 µl used directly in a 100-µl PCR reaction containing 0.5 µg each primer (Table 1), 0.2 mM dNTPs, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-Cl pH 9.0, 0.1% Triton X-100, and 2 U of *Taq* DNA polymerase. The amplification procedure consisted of one cycle of

95°C/2 min, 50°C/2 min, 72°C/5 min, followed by 30 cycles of 95°C/30 s, 50°C/30 s, 72°C/1 min, and a final cycle identical to the initial cycle. The PCR products were analyzed by agarose gel electrophoresis.

A baculovirus transfer vector suitable for cloning antibody fragments unidirectionally and providing a heavy chain secretion signal was constructed. The plasmid pAcUW51 which contains a *pol* promoter and a *p10* promoter was altered to contain a modified version of a mouse heavy chain leader sequence (Sakano et al., 1990; Kabat et al., 1991) after the *p10* promoter (Fig. 1). Two oligonucleotides were synthesized and annealed to create an adapter which could be cloned into the *Bgl*II restriction endonuclease site immediately downstream of the *p10* promoter and regenerate the *Bgl*II site at the C-terminal end of the leader sequence but not at the N-terminal end. This leader sequence was modified to contain an *Sph*I restriction site near the C-terminus which caused a Ser to Ala change at codon position –5 (Table 1). The resulting plasmid was designated pAcLHS and is compatible with the PCR products generated using the primers described in Table 1.

To test the concept of producing a library and performing expression screening in the baculovirus system, an overall scheme was used, as shown in Fig. 1. Clones were generated in a polyhedrin negative transfer vector and used to generate recombinant viruses which were analyzed for tetanus binding activity. To confirm the presence of tetanus binding antibody chains, the potential clones were transferred to *E. coli* using a conserved *Bam*HI site found in most IgG heavy chains (Kabat et al., 1991), thus

Table 1
Nucleotide sequences of the heavy chain secretion signal, and PCR primers

<i>H-chain leader sequence in pAcLHS</i>	
Met →	<i>Sph</i> I <i>Bgl</i> II
ATG GCTGTCCTGGCATTACTCTTCTGCCTGGTAAACATTCCCA	GCA TGC ATCCTTTCC AGATCT
<i>N-Terminal Primers (5' to 3')</i>	
<i>Sph</i> I	<i>H-chain N-terminal homology</i>
TTA CTC GCT GCC GCA TGC ATC CTT TCC C _{GAG} GTG CAG CTG CAG CAG T/CCT GG	
TTA CTC GCT GCC GCA TGC ATC CTT TCC CAG GTA CAG CTG AAG GAG TCA GG	
TTA CTC GCT GCC GCA TGC ATC CTT TCC GAG GTG C _{AAG} CTG CTG GAG TCT GG	
<i>C-Terminal Primer (3' to 5')</i>	
<i>H-chain C_{H1} domain C-terminal homology</i>	<i>Bgl</i> II <i>Eco</i> RI
TGGTG/T CCA CCT GTT CTT T _T G _A AAC TGA CTC ACT TCTAGA CTT AAG CGG	

The sequence of the heavy chain leader sequence within the AcNPV transfer vectors pAcLHS and pAcLHSP is shown. The methionine start codon (ATG) is indicated as are the positions of the *Sph*I and *Bgl*II cloning sites. The N-terminal primers used for PCR amplification of IgG heavy chains are shown. The *Sph*I restriction site is indicated and the 3 amino acid residues removed from the leader sequence by digestion with *Sph*I and *Bgl*II, and replaced by the oligonucleotide primers, are indicated. The regions of the primers responsible for priming of the antibody sequence (*H-chain N-terminal homology*) are identical to the corresponding regions of primers reported previously (Ward et al., 1993) and have been shown to prime IgG1, IgG2a, and IgG2b antibodies. The C-terminal primer used for both priming of the heavy chain reverse transcriptase cDNA reaction and the amplification of the heavy chain sequences by PCR contains homology to the C-terminal region of the C_{H1} domain of IgG heavy chains and includes stop codons in all 3 reading frames plus a *Bgl*II restriction endonuclease site for cloning of the PCR products into the baculovirus transfer vectors.

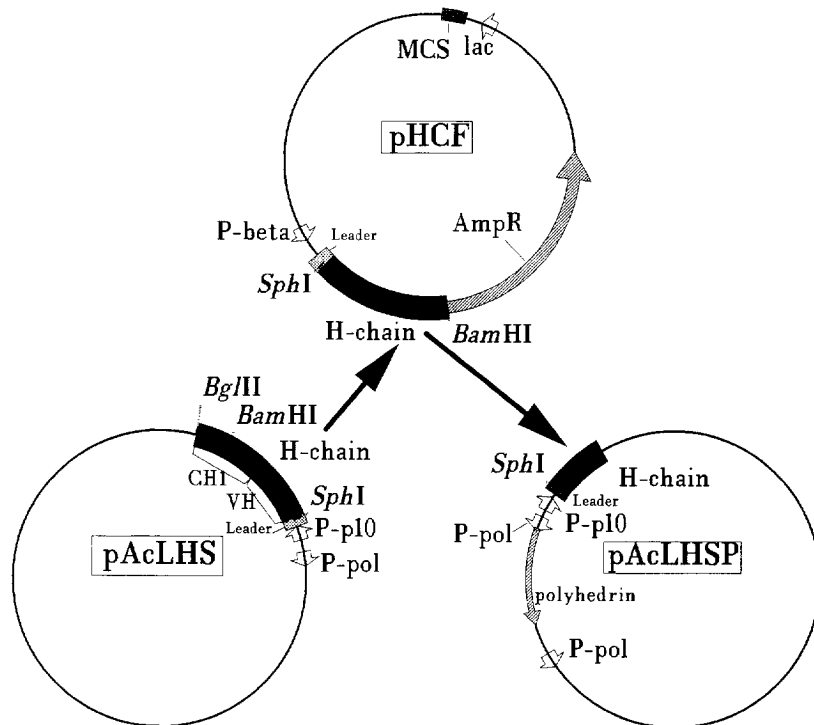


Fig. 1. Vectors and scheme for the construction and screening of mouse heavy chain fragment library. Heavy chain Fd fragments were cloned under the control of the baculovirus *p10* promoter at the *SphI* and *BglII* sites in the transfer vector pAcLHS, which had been engineered to contain a heavy chain leader sequence as described in the text. This vector was used to generate a heavy chain cDNA library from a mouse spleen and to subsequently make recombinant baculoviruses which were screened for production of antibody fragments reactive to tetanus toxoid. To 'rescue' the clones, the heavy chain encoding cDNA fragments were removed as *SphI*–*BamHI* fragments consisting of the V_H and part of the C_{H1} regions and transferred to the β -lactamase fusion vector pHCF. Successful cloning of an open reading frame in this vector regenerated ampicillin resistance by removal of the stop codon TGA. To confirm that a clone reactive in the baculovirus system had actually been rescued, the heavy chain fragment was reintroduced into the baculovirus system using the transfer vector pAcLHSP, which is a polyhedrin-positive derivative of pAcLHS.

confirming the presence of antibody sequences and allowing expression of a stable fusion construct in *E. coli*. To provide further evidence that the clones which expressed in *E. coli* could indeed express in the baculovirus system, the insert from an isolated clone was reintroduced into the baculovirus using a polyhedrin positive transfer vector (pAcLHSP).

The PCR products and the pAcLHS transfer vector were digested with *SphI* and *BglII*, and ligated using a TaKaRa ligation kit. *E. coli* strain XL1-blue was transformed by electroporation and screened for the presence of heavy chain clones by plasmid extraction and restriction endonuclease digestion analysis. Screening of 80 potential clones generated from ligation of the PCR products into the transfer vector pAcLHS by plasmid extraction and restriction endonuclease digestion indicated that 30 contained

heavy chain sequences of the correct size (37.5%). Based upon the fraction of the electroporated ligation which was plated, a total of 2×10^4 primary heavy chain containing clones were generated. By extrapolation of the fraction of the reverse transcription reaction and mRNA preparation used, a potential library approaching 10^6 clones could have been produced. To determine the diversity of the isolated clones, 26 of these clones were digested with *Pst*I restriction endonuclease and the restriction fragments compared to published antibody sequences (Kabat et al., 1991). At least 6 populations of clones were obtained. Comparison of the restriction fragments produced with calculated *Pst*I fragment sizes of various published antibody sequences (Kabat et al., 1991) suggested that IgG1a, IgG1b, IgG2a, IgG2b, and possibly IgG3 clones were present. While the clones cannot be definitively typed using a *Pst*I restriction endonuclease digestion, this result does show that the library was diverse.

To test the feasibility of screening potential clones in the baculovirus system a pool of 30 recombinant pAcLHS transfer vectors containing unknown heavy chain sequences were used to test co-transfection and screening procedures. *Spodoptera frugiperda* cells (Sf21) in ExCell 401 media (JRH Biosciences, Lenexa, KS) were cotransfected with linearized 'baculogold' AcNPV DNA (Pharmlingen, San Diego CA), DNA from the pool of transfer vector clones, and lipofectin (Gibco BRL) using standard procedures (O'Reilly et al., 1992). At 5 days post-cotransfection, the media were harvested and plaque assays performed (O'Reilly et al., 1992). From co-transfection of a pool of the 30 heavy chain clones, 48 individual viruses were plaque purified and amplified in individual wells of 24-well plates containing 5×10^4 cells per well. Assuming that the recombination efficiency is almost 100% when linearized, baculogold AcNPV DNA is used for co-transfection experiments, all of the transformants were presumed to be recombinants.

At 10 days postinfection an aliquot of media was harvested and tested for tetanus binding activity by ELISA. Goat anti-mouse (H + L) antibody (Boehringer–Mannheim, Indianapolis, IN) was diluted 1:2000 in 0.05 M carbonate/bicarbonate buffer pH 9.6 and used to coat individual wells of 96-well microtiter plates overnight at 4°C. The plates were washed with $0.1 \times$ PBS containing 0.05% Tween-20 (PBST) and 100 μ l of sample diluted 1:2 in PBS added to individual wells in triplicate. After 60 min at room temperature, the plates were washed with PBST and 100 μ l of tetanus-HRP tracer (Calbiochem, San Diego, CA) diluted in PBS was added to each well. After a further 60-min incubation at room temperature, the plates were washed and 100 μ l of substrate (100 μ g/ml TMB in sodium acetate/citrate buffer pH 5.5 containing 0.004% H_2O_2) added to each well. The reaction was stopped by the addition of 50 μ l of 4 N H_2SO_4 and the product measured at 450 nm in a V_{max} 96-well plate reader (Molecular Devices Corp, Menlo Park, CA). Analysis of media from cells infected with each individual purified virus is shown in Fig. 2A. Viral isolates 10, 25, 27, 39, and 45 provided evidence of antitetanus antibody expression. To ensure that the observed tetanus binding activity was to the sample and not to the microtiter plate, the media samples were serially diluted in PBS and assayed. The results showed that the binding activity could be progressively diluted, indicating that the observed signal was not due to non-specific binding of the tetanus-HRP tracer to the ELISA plate (data not shown).

To manipulate these or other clones further, it is important to be able to rescue the

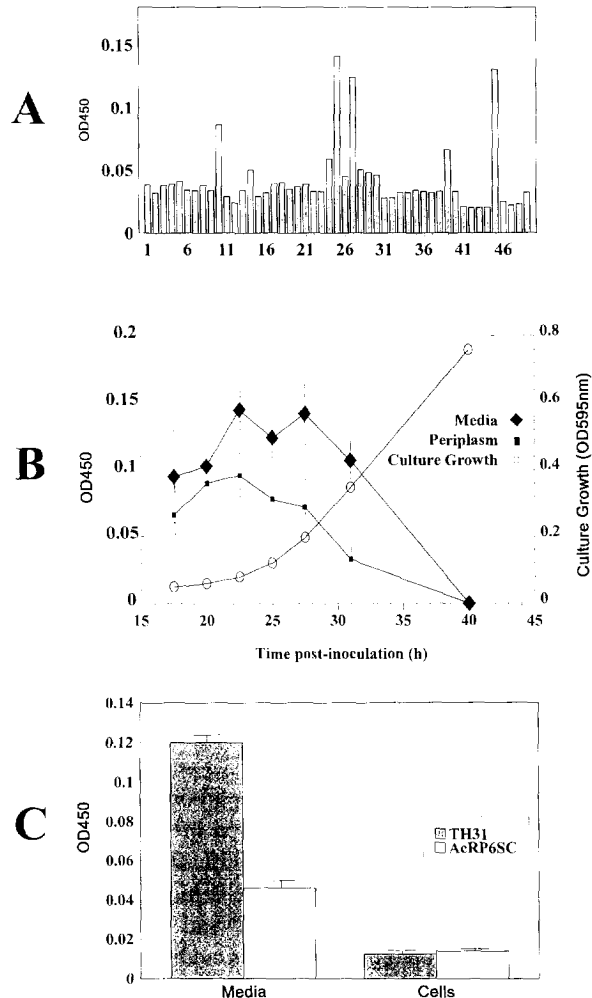


Fig. 2. Screening for tetanus binding activity. A: plaques of recombinant viruses were used to infect individual wells of *Spodoptera frugiperda* cells in 24-well tissue culture plates. The cultures were incubated for 10 days at 28°C before medium was harvested and assayed in triplicate by ELISA. The samples are 48 individual plaque picks (numbered 1–48) and the negative control (sample number 49) is non-recombinant AcNPV (AcRP6SC) culture medium. Binding of the tetanus–horse radish peroxidase probe was estimated at 450 nm in a Molecular Devices V_{max} plate reader. B: confirmation of heavy chain expression in *E. coli*. The β -lactamase fusion construct described in Fig. 1. was analyzed in a time course experiment to determine if tetanus-reactive antibody fragments were produced and exported to the periplasm or released to the culture media. The assays were performed in triplicate. The growth of the cultures was determined by the optical density of the culture at 595 nm. C: H-chain expression in pAcLHSP. The clone tested in Fig. 2B was isolated and transferred to the baculovirus system using a polyhedrin-positive transfer vector, pAcLHSP. Media and cell samples were produced and tested as described in the text. TH31 represents samples from baculovirus culture expressing a recombinant heavy chain fragment, and AcRP6SC represents samples from culture infected with control virus.

desired clone using an *E. coli* vector. In addition, we wanted to confirm the tetanus binding activity observed in the baculovirus. Because antibody fragments can be toxic to *E. coli* (Ward et al., 1993), and many PCR products derived from antibody sequences contain mis-sense mutations and deletions (unpublished observation), we constructed a pGEM 5Zf(–) based selection vector. The construction of this vector is described in Ward et al. (1995). Essentially, this vector has been modified at the junction of the β -lactamase leader sequence and mature coding sequence to introduce restriction endonuclease sites compatible with the heavy chain sequences cloned in this study. A stop codon was introduced to render the plasmid ampicillin-sensitive.

Because most IgG heavy chains contain a *Bam*HI restriction endonuclease site in the C_{HI} domain at a conserved Gly–Ser motif (Kabat et al., 1991) the heavy chain fragments from the pool of 30 baculovirus transfer vector clones were excised by digestion with *Bam*HI and *Sph*I. Insertion of the *Sph*I–*Bam*HI fragments into pHCF replaced the stop codon, and an in-frame fusion of the antibody open reading frame to the mature β -lactamase sequence regenerated ampicillin resistance (Fig. 1). This ensured that any clones containing frame shifts or mis-sense mutations as a consequence of the PCR reaction would not be viable. From the pool of 30 original clones, two ampicillin-resistant clones were obtained.

E. coli containing the heavy chain/ β -lactamase fusion plasmid were grown for 41 h at room temperature and aliquots harvested at different time points for determination of culture density and ELISA analysis of tetanus binding activity. Culture samples were centrifuged and the media supernatants harvested for analysis. The cell pellet was resuspended in the starting volume of fresh media and a periplasmic extraction performed by adding 1:10 volume of chloroform and mixing vigorously followed by a 15-min incubation at room temperature. The cells were pelleted in a microfuge for 2 min and the supernatant collected for analysis as the periplasmic fraction. The media and periplasmic samples were assayed directly by ELISA as described above. One of these (pHCF-TH2) showed tetanus binding activity as presented in Fig. 2B. Time course analysis confirms the difficulty of growing *E. coli* expressing individual antibody fragments, as reported previously (Ward et al., 1993). A duration of 24 h was required for the culture to reach even moderate growth, and log phase growth was never obtained. Despite the hybrid nature of the leader sequence, tetanus binding activity was found to be secreted to the periplasm and released into the medium indicating that this leader sequence is still functional. Another observation of Fig. 2B is that as the culture ages, the tetanus binding activity is lost, possibly due to protease degradation as the cells begin to lyse.

To confirm that the tetanus reactive chain isolated in *E. coli* was capable of binding tetanus toxoid when expressed in the baculovirus system, the clone was returned to the baculovirus system using a different version of the pAcLHS baculovirus transfer vector. This vector was constructed by removing the polyhedrin gene and promoter from the baculovirus transfer vector as a *Bgl*II–*Hind*III partial fragment into a pBluescript KS + vector which had been modified to contain a *Bgl*II restriction endonuclease site at the *lac* start codon. Two site-directed mutagenesis experiments were then performed to remove the *Kpn*I site (5'-AGTCGGTCCGATGT-3') and the *Bam*HI (5'-GAAAGG-GTCCTCAGC-3') site present in the polyhedrin gene. This caused a change in the

codon usage of polyhedrin without changing the amino acid sequence. An additional *Bam*HI restriction endonuclease site present in the multiple cloning site of this vector was removed by digestion with *Bam*HI, the 5'-overhangs filled in with T4 DNA polymerase, and the vector recircularized by T4 DNA ligase. This modified gene and promoter contains a *Xba*I site immediately upstream of the polyhedrin promoter and internal to the *Bgl*II cloning site, and a *Xba*I restriction endonuclease site present in the multiple cloning site of the vector. This *Xba*I fragment was removed and transferred to the *Xba*I restriction endonuclease site present between the *pol* and *p10* promoters of pAcLHS, to produce a polyhedrin-positive version of this vector which retained the heavy chain leader sequence. This vector was designated pAcLHSP (see Fig. 1).

The heavy chain clone present in the β -lactamase fusion vector was transferred as a *Sph*I–*Bam*HI fragment into the *Sph*I and *Bgl*II sites of pAcLHSP. This vector was used to produce recombinant baculoviruses as described above. To determine tetanus binding activity, infected cultures were harvested and cells removed by centrifugation. Media supernatant samples were collected, and the cell samples were resuspended in PBS to the starting volume and lysed by two freeze–thaw cycles followed by sonication for 2 min in an ice bath. Both fractions were assayed directly by ELISA as described above (Fig. 2C). The heavy chain fragment was found to be expressed and secreted to the medium.

In this study, it was successfully demonstrated that libraries can be constructed directly within the baculovirus expression system, and that foreign proteins can be expressed and screened using the standard baculovirus tools that are commercially available. As an example, antibody chains to tetanus toxoid were selected as a target because this antigen is highly immunogenic, produces high titers of circulating antibody, and reagents are readily available for analysis of the recombinant protein. A consequence of this research has been the construction of another member of the pAcUW series of vectors developed by Weyer et al. (1990) to include an antibody leader sequence containing a *Sph*I restriction endonuclease site internal to the end of the sequence and a *Bgl*II restriction endonuclease site at the end of the leader sequence which allows proteins to be fused to the secretion signal. The inclusion of a polyhedrin gene into the vector pAcLHS to produce pAcLHSP expands the usefulness of these vectors for development of orally infective baculoviruses and adds the convenient polyhedrin positive phenotype for determination and observation of baculovirus recombinants and their growth.

The ability to clone sequences directly within transfer vectors would be especially useful for proteins which are difficult to express in *E. coli* or remain inactive within the reducing environment of the bacterial cell. The ability to screen by expression in baculoviruses offers an alternative for these difficult proteins in the form of a eukaryotic expression system. One consideration when using the strategy employed here is that the cloned sequence cannot be manipulated within the baculovirus, hence standard experimental procedures such as site-directed mutagenesis, or sequencing cannot be performed readily. However, a number of strategies can be considered to transfer the clone of interest to a general plasmid vector. The approach was to use an *E. coli* fusion vector and screen the 30 transfer vector clones as a pool in order to isolate the desired clone. The decision to use an antibiotic fusion vector was based upon the unstable nature of

antibody fragments in *E. coli* and the observation that antibody clones derived from PCR amplification have a high frequency of mis-incorporations that cause frame shifts, stop codons, and deletions. The use of an antibiotic resistance fusion vector system was useful in overcoming these problems. Another strategy would be to perform individual co-transfection reactions on each member of a pool of transfer vectors once a positive pool had been determined. A single transfer vector containing the clone of interest could be manipulated readily, or the clone could be transferred to a more general purpose vector. Alternatively, if PCR derived mutations are not a problem, then the clone could be isolated directly by PCR of culture media containing plaque purified recombinant virus (O'Reilly et al., 1992) using the same primers as used to originally generate the clones. These PCR products could then be cloned into the desired vector.

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References

- Bishop, D.H.L. (1992) Baculovirus expression vectors. *Semin. Virol.* 3, 253–264.
- Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S. and Foeller, C. (1991) Sequences of proteins of immunological interest. US Department of Health and Human Services, and National Institutes of Health, Publication number 91-3242. Washington, 2nd edn.
- Kitts, P.A. and Possee, R.D. (1993) A method for producing baculovirus expression vectors at high frequency. *BioTechniques* 14, 810–817.
- Luckow, V.A. (1991) Cloning and expression of heterologous genes in insect cells with baculovirus vectors. In: A. Prokop, R.K. Bajpai and C.S. Hu (Eds.), *Recombinant DNA Technology and Applications*. McGraw-Hill, New York, pp. 97–152.
- Maeda, S. (1989) Expression of foreign genes in insects using baculovirus vectors. *Ann. Rev. Entomol.* 34, 351–372.
- Miller, L.K. (1988) Baculoviruses as gene expression vectors. *Ann. Rev. Microbiol.* 42, 177–199.
- O'Reilly, D.R., Miller, L.K. and Luckow, V.A. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*. W.H. Freeman, New York.
- Sakano, H., Maki, R., Kurosawa, Y., Roeder, W. and Tonegawa, S. (1990) Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy-chain genes. *Nature* 286, 676–683.
- Smith, G.E., Summers, M.D. and Fraser, M.J. (1983) Production of β -interferon in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol.* 3, 2156–2165.
- Ward, V.K., Schneider, P.G., Kreissig, S.B., Hammock, B.D. and Choudary, P.V. (1993) Cloning, sequencing and expression of the Fab fragment of a monoclonal antibody to the herbicide atrazine. *Protein Eng.* 6, 981–988.

- Ward, V.K., Hammock, B.D., Maeda, S. and Choudary, P.V. (1995) Development and application of recombinant antibodies to pesticide residue analysis. In: D. Kurtz, L. Stanker and J. Skerrit (Eds.), *Frontiers in agrochemical immunoanalysis*, AOAC International, Washington DC, in press.
- Weyer, U., Knight, S. and Possee, R.D. (1990) Analysis of very late gene expression by *Autographa californica* nuclear polyhedrosis virus and the further development of multiple expression vectors. *J. Gen. Virol.* 71, 1525–1534.