Topic Introduction

Expressing Cloned Genes for Protein Production, Purification, and Analysis

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Obtaining high quantities of a specific protein directly from native sources is often challenging, particularly when dealing with human proteins. To overcome this obstacle, many researchers take advantage of heterologous expression systems by cloning genes into artificial vectors designed to operate within easily cultured cells, such as *Escherichia coli*, *Pichia pastoris* (yeast), and several varieties of insect and mammalian cells. Heterologous expression systems also allow for easy modification of the protein to optimize expression, mutational analysis of specific sites within the protein and facilitate their purification with engineered affinity tags. Some degree of purification of the target protein is usually required for functional analysis. Purification to near homogeneity is essential for characterization of protein structure by X-ray crystallography or nuclear magnetic resonance (NMR) and characterization of the biochemical and biophysical properties of a protein, because contaminating proteins almost always adversely affect the results. Methods for producing and purifying proteins in several different expression platforms and using a variety of vectors are introduced here.

OVERVIEW OF EXPRESSION SYSTEMS

Escherichia coli is the first choice for expression of many heterologous proteins because of the vast fund of knowledge about its genetics, biochemistry, and molecular biology. Genetic manipulations are straightforward, cultures of *E. coli* are easily and inexpensively grown, and many foreign proteins are well tolerated and may be expressed at high levels. During the past 30 years, more than 30,000 recombinant proteins have been successfully expressed and purified using *E. coli* (Graslund et al. 2008). However, *E. coli* is not always the host of choice. For example, proteins whose full biological activity requires posttranslational modification (e.g., glycosylation or cleavage at specific sites) may best be expressed in a eukaryotic host, such as the yeast *Pichia pastoris* or the baculovirus-infected insect cell systems that have emerged since the late 1980s. Before embarking on an expression project, it is important to assess the final goal and to determine which host–vector system is most likely to be most appropriate for expressing the target protein.

Expression of proteins in *E. coli* is a relatively straightforward, cost-effective means for producing large quantities of simple proteins that do not require posttranslational modification. Eukaryotic proteins expressed in insect cells using baculovirus systems are likely to achieve important processing or modifications required for proper function. The baculovirus system is relatively approachable given many commercial options available to assist the user. Advantages of the *P. pastoris* expression system, in addition to eukaryotic processing, include the ease of generating expression vectors, the opportunity to coexpress partner proteins, and high biomass production. This system facilitates production of large quantities of native protein, enabling biochemical, physiological, or structural studies (Daly and Hearn

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2005). As such, the *Pichia* system represents a cost-effective, versatile alternative in the protein expression “toolbox” (Cregg et al. 2000). Protocol: Expression of Cloned Genes in *E. coli* Using IPTG-Inducible Promoters (Kielkopf et al. 2021a), Protocol: Subcellular Localization of Signal Peptide Fusion Proteins Expressed in *E. coli* (Kielkopf et al. 2021b), Protocol: Expression of Cloned Genes Using the Baculovirus Expression System (Kielkopf et al. 2020a), and Protocol: Expression of Cloned Genes in *Pichia pastoris* Using the Methanol-Inducible Promoter AOX1 (Kielkopf et al. 2021c) describe the methods for expression of proteins in *E. coli*, insect, or *Pichia* cells.

Fusions of the protein of interest with a carrier protein can increase solubility, change the localization, provide a means for affinity purification, or elicit a response in immunological assays. A variety of cleavable fusion tags may facilitate purification and detection. Procedures for cell lysis and purification of the common polyhistidine (6xHis) or glutathione S-transferase (GST)-fusion proteins are given in Protocol: Preparation of Cell Extracts for Purification of Soluble Proteins Expressed in *E. coli* (Kielkopf et al. 2021d), Protocol: Purification of Polyhistidine-Tagged Proteins by Immobilized Metal Affinity Chromatography (Kielkopf et al. 2020b), and Protocol: Purification of Fusion Proteins by Affinity Chromatography on Glutathione Resin (Kielkopf et al. 2020c). In some cases, proteins expressed in *E. coli* are found in insoluble inclusion bodies. Although baculovirus or *Pichia* expression systems (Protocol: Preparation of Cell Extracts for Purification of Proteins Expressed in *Pichia pastoris* [Kielkopf et al. 2021e]) provide viable alternatives for production of soluble, active protein, Protocol: Solubilization of Expressed Proteins from Inclusion Bodies (Kielkopf et al. 2021f) describes a procedure for isolating and recovering protein from inclusion bodies expressed in *E. coli*. Fundamental techniques for analyzing the quantity and purity of expressed proteins include SDS–polyacrylamide gel electrophoresis, immunoblotting (or western blots), and using the Bradford assay or A_{280} absorbance to determine protein concentration.

CHOOSING AN EXPRESSION SYSTEM

The attributes and drawbacks of several systems for recombinant protein expression are summarized in Table 1 and described below. Chief considerations when choosing an expression system include the size of the protein, the amount of protein required, the species from which the protein is derived, and any disulfide bonds or posttranslational modifications of the protein. If only small quantities of the target protein are needed—for example, when screening a series of site-directed mutants for enzymatic activity—there is little point in trying to optimize production. However, if purification of an active protein is necessary and/or if the protein is required in large quantities, it may be necessary to explore several different host–vector systems, expression conditions, and/or purification schemes before finding one that is workable on a large scale (Fig. 1).

E. coli

The *E. coli* system is usually the first choice for expression of small cytosolic proteins or domains. Protein production in *E. coli* is highly cost effective and convenient, given the simple media requirements and conditions for culture. Moreover, the speed of the *E. coli* system is exceeded only by cell-free expression (a few hours to produce protein), because the doubling times of *E. coli* (~20 min) are rapid compared with those of yeast (~2 h), insect, or mammalian cells (~20 h). In general, expression of recombinant proteins in *E. coli* involves (1) introducing a plasmid that encodes the desired protein into an expression host strain, (2) growing cells to exponential phase, and (3) inducing protein expression. Over the years, a wide array of *E. coli* strains, expression protocols, and vectors capable of producing very high levels of protein (>50% of total cell protein) have been developed (as described in Protocol: Expression of Cloned Genes in *E. coli* Using IPTG-Inducible Promoters [Kielkopf et al. 2021a] and Protocol: Subcellular Localization of Signal Peptide Fusion Proteins Expressed in *E. coli* [Kielkopf et al. 2021b]). However, for a variety of reasons, not all proteins can be successfully expressed in soluble, active form using the *E. coli* system. Some proteins may be toxic to *E. coli*

TABLE 1. Comparison of hosts for recombinant protein production

Attribute/host	<i>Escherichia coli</i>	Baculovirus-infected insect cells	<i>Pichia pastoris</i>	Mammalian cells	Cell-free translation
Yield	+ Often high yield	+ Often high yield	+ Easily scalable from small cultures to fermentation, high biomass production in fermenters	– Amount of expressed protein is typically small	– Typically lower yield than in vivo expression systems
Doubling time	+ Rapid doubling time (~20 min)	– Long doubling time (~20 h)	+ Reasonable doubling time (~2 h)	– Long doubling time (~20 h)	+ Rapid expression directly from plasmid or PCR product
Ease of use	+ Relatively straightforward culture and transformation procedures	+ Well-developed procedures	+ Commercially available host strains and reagents	– Often requires preparation of stable lines, which is very time-consuming. High-level expression requires extensive culture and selection of lines that give best yields.	+ “Open” system, can easily add components to enhance solubility or functionality
Cost	+ Commercially available host strains and reagents	– Time-consuming process for recombinant baculovirus production	+/- Growth conditions may require optimization.		+ Commercially available cell-free kits include <i>E. coli</i> , insect, yeast, and mammalian cell extract systems
Need for specialized equipment	+ Relatively low-cost media	– Experience with tissue culture advised	+ Relatively low-cost media	– ~15-fold higher cost than <i>E. coli</i>	– Large-scale expressions (several milligrams) can be very expensive.
Versatility of tags and host strains	+ Incubator and shaker	– Tissue culture hood, incubator, shaker	+ Incubator and shaker	– Tissue culture hood, incubator, spinner flasks, or roller bottle incubator	+ No specialized equipment needed
Quality of protein	+ Wide range of vectors with tags, signal peptides, and protease sites	+ Range of vectors with tags, signal peptides, and protease sites	– Few tags, host strains, and selection markers available	+ Native protein folding and posttranslational modifications	+ Can express a wide variety of tags
Posttranslational modifications	– Large proteins may fold improperly	+ Improved folding of large proteins	+ Improved folding of large proteins		– Few variations of host strains available
	– No eukaryotic posttranslational modifications	+ Eukaryotic protein processing with many posttranslational modifications	+ Eukaryotic protein processing with many posttranslational modifications including proteolytic processing, disulfide bridge formation, glycosylation, and phosphorylation	+ Recombinant proteins are most likely to be processed exactly as in normal tissues including mature N-linked and O-linked glycosylation, amidation, prolyl hydroxylation, myristoylation, palmitation, or sulfation.	+ Improved folding and posttranslational modifications
Toxic gene expression	– Certain proteins may be toxic to host cells.	– Proteins are core glycosylated (high-mannose type)	– Proteins are core glycosylated (high-mannose type).		+/- Modifications depend on the species of the cell-free system. Commercially available kits include <i>E. coli</i> , insect, yeast, and mammalian cell extract systems.
		– Usually a fraction of the expressed protein remains unmodified.	– Inability to perform certain complex posttranslational modifications		
Multigene expression	+ Plasmids with compatible origins of replication and/or dual expression cassettes are available.	+ Vectors with dual expression cassettes are available.	+ Many proteins have been successfully expressed that were toxic in <i>E. coli</i> .		+ Can express unstable and cytotoxic proteins
		– Multiple viruses may be used for coinfection; however, this does not ensure coexpression of different proteins in the same cell.	– Some secretory proteins may fold improperly and not traffic out of the endoplasmic reticulum.		
Membrane protein expression	+ High success for expression of prokaryotic membrane proteins, but may be problematic for eukaryotic membrane proteins	+ Often successful for expression of eukaryotic membrane proteins	+ Easy selection for multicopy integration using Zeocin		+ Multiple proteins can be expressed from different promoters in the same reaction by using multiple RNA polymerases.
			+ Multicopy vectors available for coexpression of partner proteins at defined stoichiometries		+ Allows greater flexibility in designing coexpression of multiple genes
					– Expression of membrane proteins is difficult.
					+ Can be expressed in the presence of detergents

FIGURE 1. Overall strategy for recombinant protein expression and purification.

when overexpressed (Saida 2007). Large proteins often aggregate; the fraction of proteins found in the soluble fraction of *E. coli* cell lysate decreases inversely with protein size (>100 residues) (Graslund et al. 2008). Furthermore, the *E. coli* host does not provide posttranslational modifications that may be important for the folding and/or activity of eukaryotic proteins. These large and/or eukaryotic proteins often collect in insoluble aggregates called “inclusion bodies” (Marston 1986). Although the recombinant protein may be purified from the inclusion bodies as described in Protocol: **Solubilization of Expressed Proteins from Inclusion Bodies** (Kielkopf et al. 2021f), the likelihood of refolding to produce soluble, active protein is low (Cabrita and Bottomley 2004; Graslund et al. 2008). Furthermore, the protein typically recovered after refolding is not always compatible with techniques that require large amounts of pure, active, and properly folded protein, such as crystallographic or nuclear magnetic resonance structure analysis. In some cases, altering the *E. coli* host strains, vectors, and growth conditions may improve protein recovery. In other cases, the alternative expression systems described below should be pursued to increase the chances of producing soluble, active protein.

Baculovirus-Infected Insect Cells

The general strategy of the baculovirus expression system is to infect insect cells with a virus that expresses a foreign protein at a very late stage of infection. For single-domain or prokaryotic proteins, the *E. coli* expression system is usually chosen over the baculovirus system because of the higher costs of insect cell media coupled with the time and expertise required to produce recombinant baculovirus. Nevertheless, the baculovirus expression system has several advantages for producing multidomain eukaryotic proteins, including the following.

1. The yields of protein per liter of insect cell culture can rival those produced by a comparable quantity of *E. coli* cells.
2. Because the protein of interest usually is expressed at a very late phase of virus infection, toxic genes can be expressed with few adverse effects on yield.
3. The relatively low temperature for insect cell growth (27°C–28°C) can facilitate folding of mutant or unstable proteins.
4. The foreign DNA sequences can be very large (>20 kb, corresponding to >7000 amino acids) because of the open-ended structure of the virus capsid.

5. Multiprotein complexes can be produced by simply coinfecting insect cells with more than one recombinant virus as well as by insertion of multiple expression cassettes into the viral genome.
6. Insect cells are capable of splicing introns from pre-mRNA transcripts, although cDNAs are recommended because alternative splicing patterns may cause complications.
7. Insect cells accomplish posttranslational modifications of eukaryotic proteins, with reported modifications, including phosphorylation (Miyamoto et al. 1985), acetylation (Kluwe et al. 1995), carboxymethylation (Rosenberg et al. 1998), prenylation (Khosravi-Far and Der 1995; Lindorfer et al. 1996), signal peptide cleavage (Smith et al. 1985), and proteolytic processing (Oker-Blom et al. 1989). Insect cells also perform *N*- and *O*-linked glycosylation of foreign proteins (Stiles and Wood 1983; Kuroda et al. 1990). However, the composition and length of the attached glycosyl groups usually do not correspond to those found in mammalian cells (Thomsen et al. 1990; Altmann et al. 1995).

The chief argument for choosing the baculovirus over the *E. coli* expression systems is the ability of insect cells to accomplish posttranslational modifications of eukaryotic proteins (see point 7, above). Unfortunately, the overexpressed protein often overwhelms the insect cell machinery so that only a fraction of it is modified (Hoss et al. 1990; Kuroda et al. 1991; Khosravi-Far and Der 1995; Hsu and Betenbaugh 1997). For example, only 10%–50% of ras-related proteins are prenylated when expressed using a baculovirus system (Lowe et al. 1991; Yang et al. 1992; Khosravi-Far and Der 1995). For applications that require homogeneous material, strategies must be developed to identify and separate the processed from the unprocessed forms of the recombinant protein. Alternatively, chaperones or enzymes may be expressed beside the protein of interest to increase processing of recombinant proteins (e.g., Hsu and Betenbaugh 1997; Ailor et al. 1999). This strategy also is used to introduce posttranslational modifications that insect cells lack. For example, the transgenic SfSWT-1 (marketed as the Mimic strain from Life Technologies) insect cell line stably expresses mammalian glycosyl transferases and produces complex sialylated glycoproteins when cultured with a source of sialic acid (such as fetal bovine serum) (Hollister et al. 2002). Despite these solutions, the ability to produce recombinant mammalian proteins with bona fide posttranslational modifications remains a strong rationale for choosing a mammalian over a baculovirus expression system.

As a system for producing eukaryotic proteins, *P. pastoris* has many advantages, such as proteolytic processing, protein folding, disulfide-bond formation, and glycosylation, while being as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae* (Cereghino and Cregg 1999). It is faster and less expensive than other eukaryotic expression systems and generally gives higher expression levels. Expression vectors are stably integrated into the genome via homologous recombination, in contrast to other expression systems in which the gene resides in episomal plasmids. Transformation is simple (requiring no viral stocks), and expression strains can be stored long term (>10 yr) without loss of viability. The low cost of culture media, the ease of scaling up from small cultures to fermentation cultures, and high biomass production in fermenters (up to 400 g/L) combine to make *Pichia* a very useful protein expression system. Several thousand heterologous proteins have now been expressed in *P. pastoris* (Cregg 2007), including many eukaryotic and integral membrane proteins that were nonfunctional when expressed in *E. coli* (Lueking et al. 2003; Parcej and Eckhardt-Strelau 2003; Long et al. 2005; Andre et al. 2006; Aller et al. 2009); see also Introduction: **Considerations for Membrane Protein Purification** (Kielkopf et al. 2021g).

Proteins expressed in *P. pastoris* are usually core glycosylated with short-chain *N*-glycans of the high-mannose type that may interfere with applications in which immunogenic reactions are undesirable (e.g., immunoglobulin G [IgG] production for medical applications) (Daly and Hearn 2005). Consequently, two groups have genetically engineered *P. pastoris* strains so that they mimic human glycosylation patterns with restricted or fully humanized, terminally sialylated *N*-glycans (Hamilton et al. 2006; Jacobs et al. 2009). Strains developed by the Contreras group (GlycoSwitch technology) are

Pichia pastoris

available to investigators and can be licensed through Research Corporation Technologies, Inc. Another potential drawback of *P. pastoris*, compared with mammalian systems, is the absence of certain complex posttranslational modifications, such as prolyl hydroxylation, amidation, and some types of phosphorylation, which may be important for proper folding and processing (Cereghino and Cregg 1999). Furthermore, some proteins may be toxic to *P. pastoris* when overexpressed and are consequently retained in the endoplasmic reticulum (ER), misfolded, or degraded by vacuolar and proteasomal degradation machineries (Sullivan et al. 2003; Schuck et al. 2009). Optimization of growth conditions or use of protease-deficient strains may rescue the protein in some cases. In other cases, insect cell or mammalian expression systems may be more suitable alternatives.

Other Expression Systems

Mammalian cells and cell-free expression systems derived from them are further alternatives to the *E. coli*, insect cell, and *P. pastoris* hosts are commonly used for protein expression. As summarized in Table 1, protein expression in mammalian cells offers the advantage of appropriate posttranslational modifications of mammalian proteins. However, drawbacks include (1) the time required for generating the desired amounts of the protein of interest and (2) the relatively high costs. Consequently, the cell-free expression systems have emerged recently as a popular alternative for rapid production of proteins on small or medium scales (up to 3 mg). As further described in Box 1, Cell-Free Systems for In Vitro Protein Expression, many cell-free expression kits are now commercially available and are well documented by manufacturer's protocols. The protocols cited in this introduction focus on use of the *E. coli*, insect cell, and *P. pastoris* systems for protein expression.

BOX 1. CELL-FREE SYSTEMS FOR IN VITRO PROTEIN EXPRESSION

The use of cell-free systems for the in vitro expression of target proteins is becoming increasingly popular and can provide a quick alternative to cell-based in vivo expression (for reviews, see Hoffmann et al. 2004; Murthy et al. 2004; Katzen et al. 2005; Endo and Sawasaki 2006; Falzon et al. 2006). Cell-free systems contain all of the necessary components, such as RNA polymerases, ribosomes, tRNAs, transcription factors, and regulatory proteins, required for protein synthesis. In vitro systems are typically derived from cell lysates but also can be reconstituted from purified components, although protein production is often less efficient in the latter case (Hillebrecht and Chong 2008).

Historically, cell-free protein expression systems were developed to study the mechanisms of protein biosynthesis, particularly the process of protein translation (Nirenberg and Matthaei 1961). In vitro protein synthesis has since evolved to become a tool used for the rapid production of proteins from either an mRNA or DNA template. DNA templates, derived from plasmids or polymerase chain reaction (PCR)-generated templates, typically contain (1) a bacteriophage promoter for transcription of the template DNA by a cognate, bacteriophage-encoded DNA-dependent RNA polymerase and (2) a Shine–Dalgarno sequence to increase the efficiency of translation. Mammalian expression systems usually require an internal ribosome entry site (IRES), Kozak sequence (Kozak 1987), followed by the target sequence, a signal for addition of poly(A), a transcriptional terminator, and a translation enhancer sequence (Hino et al. 2008). Transcription and translation of the target can be performed consecutively (uncoupled) or simultaneously (coupled), depending on the components of the in vitro reactions. Coupled reactions are preferred because they produce a higher yield in less time, but they require additional nucleoside triphosphates (NTPs) and a bacteriophage-encoded DNA-dependent RNA polymerase. The coupled reaction is initiated by combining an extract containing the transcription/translation machinery with the template nucleotide and amino acids. After a short incubation period (typically 30 min), the necessary cofactors and energy sources are added to produce the target protein. In uncoupled reactions, transcription and translation are performed in two separate stages, thus providing the opportunity to use mRNA as a starting template.

Advantages

In vitro cell-free expression can provide several benefits when compared with more traditional *E. coli* expression methods. One of the greatest advantages is being able to quickly produce the protein under

BOX 1. Continued

relatively native conditions. Using an expression system that more closely mimics the native expression conditions of the protein can allow for the proper posttranslational modifications and folding of the protein (see, e.g., Gibbs et al. 1985; Hillebrecht and Chong 2008). Working with a cell-free system also allows for the easy addition of components that may enhance protein solubility or functionality. The target protein can be produced at a much faster rate, often within a few hours (as short as 3 h in *E. coli* extracts), compared with traditional cell-based methods that are performed overnight for bacterial cultures or over the course of several weeks for proteins expressed in baculovirus vectors. Furthermore, expression of proteins in vitro helps to avoid protein aggregation into inclusion bodies and enables the synthesis of unstable, easily degraded, or cytotoxic proteins that cannot be produced in live cells. In vitro protein synthesis can be performed as a microliter-scale synthesis, which facilitates, for example, expression and screening of mutant forms of the target protein (Yokoyama 2003; Spirin 2004). Conversely, these reactions can be scaled up to express larger quantities of protein. However, large-scale production can often be more technically demanding and prohibitively expensive when compared with cell-based expression. Consequently, in vitro expression may not be a practical option, particularly considering how inexpensive and relatively effortless some in vivo expression methods can be.

Types of In Vitro Expression Systems

Cells of several species have been used to generate in vitro expression systems: *E. coli* (Zubay 1973), rabbit reticulocytes (Pelham and Jackson 1976), wheat germ (Roberts and Paterson 1973), insect (Glocker et al. 1993), and human (McDowell et al. 1972; Stueber et al. 1984; Mikami et al. 2008). Each species of cell-free expression system has its own advantages and is capable of adding different posttranslational modifications to the expressed protein (Endo and Sawasaki 2006; Falzon et al. 2006). Mammalian systems are capable of producing more complex modifications but are often expensive, more sensitive to additives, and generate lower yields. Prokaryotic systems, such as *E. coli*, produce much higher yields and are relatively tolerant of additives, but they cannot create the modifications found on some mammalian proteins nor can they provide environments conducive to correct folding of mammalian proteins.

Such issues create a possible trade-off between the quality and quantity of the protein. For example, cell-free systems from *E. coli* and wheat germ extracts are not able to glycosylate proteins but can produce high yields. Rabbit reticulocyte systems (with canine microsomal membranes added) can produce glycosylated proteins but with a lower overall protein yield. Because all of the posttranslational modifications and protein-folding differences between the above-mentioned expression systems are not well understood, it may be beneficial to test a few systems to determine which one will produce a functional gene product with the desired posttranslational modifications and yield. Several companies now provide in vitro expression systems derived from several species as kits that include the necessary transcription and translation components: Thermo Scientific Pierce, 1-Step Human Coupled IVT Kits (HeLa cell lysate; <https://www.thermofisher.com/us/en/home.html>); Life Technologies Invitrogen, Expressway Maxi Cell-Free *E. coli* Expression System or Ambion Retic Lysate IVT Kit (respectively, *E. coli* extract or rabbit reticulocyte lysate <http://www.thermofisher.com/us/en/home.html>); Promega, TNT Systems (wheat germ extract, insect cell lysate, or rabbit reticulocyte lysate; <https://www.promega.com/>); and QIAGEN, EasyXpress Protein Synthesis Systems (*E. coli* extract or insect cell lysate; <https://www.qiagen.com/us/>).

CHOOSING AN APPROPRIATE EXPRESSION VECTOR

Once a suitable host has been identified, a variety of DNA vectors are available with engineered cloning sites and noncoding sequences to drive protein expression. The promoters are commonly inducible to prevent expression of the target protein until adequate cell growth is achieved. Most vectors also provide the option of creating a fusion protein comprised of target sequences and any one of a number of tag proteins that may increase solubility and simplify subsequent purification (as described in the following section, Fusion Proteins). Common classes of expression vectors are arranged below according to the type of host system used for expression and are summarized in Table 2.

TABLE 2. Common vectors for protein expression

<i>E. coli</i> expression vectors ^a				
Vector series	Promoter	Fusion partners ^b	Protease sites ^c	Primary vendor
Vectors transcribed by <i>E. coli</i> RNA polymerase				
pGEX	<i>Tac</i>	GST; pGEX-2TK also carries protein kinase site for ³² P labeling.	Factor Xa, thrombin, or HRV 3C	GE Healthcare
pMAL	<i>Tac</i>	MBP; Selected vectors may carry the MBP signal peptide.	Enterokinase, Factor Xa, or Genenase I	New England Biolabs
pTrcHis	<i>Trc</i>	6xHis with c-myc or Xpress epitopes	Enterokinase	Life Technologies
pBAD	<i>pBAD</i>	6xHis; Selected vectors may carry hpTrxA; V5, Xpress, or c-myc epitopes; or gIII signal peptide	Enterokinase	Life Technologies
pQE	<i>T5-lac</i>	6xHis; Selected vectors may carry DHFR, Tag-100, or Strep(II)-tag fusions.	Selected vectors may carry TAGZyme or Factor Xa sites	QIAGEN
Vectors transcribed by T7 RNA polymerase				
pET	<i>T7-lac</i>	6xHis; Selected vectors may carry GST, Strep(II)-tag, hpTrxA, S-tag, CBP, NusA, Sumo, DsbA, DsbC; pelB signal peptide; HSV or T7-tag.	Enterokinase, Factor Xa, thrombin, HRV 3C, or TEV	Merck Biosciences; Gateway or TOPO cloning variants available from Life Technologies
pETduet	<i>T7-lac</i>	6xHis; S-tag	Enterokinase, thrombin	
pRSET	<i>T7</i>	6xHis with Xpress epitope; Selected vectors may carry a fluorescent protein or T7-tag.	Enterokinase	Life Technologies
pTriEx series	<i>T7-lac (p10, CMV/β-actin)</i>	6xHis; Selected vectors carry HSV-tag, S-tag, or Strep(II)-tag.	Thrombin, enterokinase, or HRV 3C	Merck Biosciences
pQE TriSystem	<i>T7-lac (p10, CMV/β-actin)</i>	None, 10xHis, or Strep(II)-tag	None or TAGZyme	QIAGEN
(continued)				

TABLE 2. Continued

Recombinant baculovirus DNAs and transfer vectors ^{d,e}		
Viral DNAs (Group A ^f)	Vendor	Features
BaculoGold	BD Biosciences Pharmingen	Invisible in the absence of recombination because of removal of an essential gene from the transfer region by restriction digestion. Nonrecombinant viruses produce blue plaques in the presence of X-Gal caused by a <i>lacZ</i> gene within the transfer region. The BaculoGold Bright variant expresses a GFP marker to monitor virus propagation.
<i>flashBac</i>	Oxford Expression Technologies	Invisible in the absence of recombination because of the absence of an essential gene from the transfer region. Circular viral DNA in which the <i>polyhedrin</i> locus has been replaced with sequences that allow propagation in <i>E. coli</i> . This ensures that nonrecombinant virus is absent from the progeny.
BacMagic	Merck Biosciences	Invisible in the absence of recombination because of absence of an essential gene from the transfer region. Circular viral DNA with <i>polyhedrin</i> locus replaced with a bacterial artificial chromosome for maintenance and propagation in <i>E. coli</i> . This ensures that nonrecombinant virus is absent from the progeny. BacMagic-2 lacks cysteine protease and chitinase genes; BacMagic-3 lacks these and additional genes that suppress production of recombinant protein yield.
BacVector Triple Cut series	Merck Biosciences	Invisible in the absence of recombination because of removal of an essential gene from the transfer region by restriction digestion. Nonrecombinant viruses produce blue plaques in the presence of X-Gal due to the <i>lacZ</i> gene located within the transfer region; recombinant plaques remain uncolored. BacVector-2000 lacks five nonessential genes that suppress the yield of recombinant protein; BacVector-3000 further lacks cysteine protease and chitinase genes.
ProEasy	AB Vector	The transfer region contains a gene that renders the virus inviable under standard growth conditions; recombination removes this gene so that only recombinant viruses are recovered.
ProGreen	AB Vector	Expresses a GFP marker to monitor virus propagation
ProFold series	AB Vector	Invisible in the absence of recombination because of removal of an essential gene from the transfer region by restriction digestion. Nonrecombinant viruses produce blue plaques in the presence of X-Gal due to the <i>lacZ</i> gene located within the transfer region. Expresses a GFP marker to monitor virus propagation
Bac-N-Blue	Life Technologies	ProFold-C1 and C2 coexpress Hsp40 and Hsc70 chaperones to facilitate target protein folding; ProFold-PDI coexpresses protein disulfide isomerase to facilitate folding of secreted proteins; ProFold-ERT also coexpresses calreticulin to facilitate folding of secreted glycoproteins. Linear, restriction-digested DNA that is inviable in the absence of recombination because of removal of an essential gene from the transfer region. When used with pBlueBac (discontinued) or pMelBac vectors, recombinant viruses produce blue plaques in the presence of X-Gal as a consequence of a <i>lacZ</i> gene donated by the transfer plasmid. Wild-type plaques remain uncolored. Bac-N-Blue is compatible with other transfer vectors but is not equipped for color selection.
BacPAK6	Clontech	Mimic host cells are not compatible because the <i>lacZ</i> recombinant trims N-glycans. Linear, restriction-digested DNA that is inviable in the absence of recombination because of removal of an essential gene from the transfer region. Nonrecombinant viruses produce blue plaques in the presence of X-Gal caused by a <i>lacZ</i> gene within the transfer region.

(continued)

TABLE 2. Continued

Transfer vectors (Group A ¹)	Promoter	Fusion partners ^b	Protease sites ^c	Primary vendor	Comments
pVL1392, pVL1393	<i>Polh</i>	—	—	BD Biosciences Pharmingen	Traditional transfer vector
pAChLT, pAcGHLT	<i>Polh</i>	6xHis; Protein kinase A site; pAcGHLT also carries GST.	Thrombin	BD Biosciences Pharmingen	—
pAcG1/2 T/3X	<i>Polh</i>	GST; pAcSecG2 T also carries signal peptide.	None, thrombin, or Factor Xa	BD Biosciences Pharmingen	—
pAcCp67	<i>Polh</i>	Signal peptide	—	BD Biosciences Pharmingen	—
pAcMP2, pAcMP3	<i>basic protein</i>	—	—	BD Biosciences Pharmingen	Mid-level expression during the late phase allows more complete processing. Recombines with <i>polyhedrin</i> locus despite the presence of alternate promoter
pAcAB3, pAcAB4	(<i>polh</i> , <i>p10</i> , <i>polh</i>) or (<i>polh</i> , <i>p10</i> , <i>polh</i> , <i>p10</i>)	—	—	BD Biosciences Pharmingen	Promoters/cloning sites for expression of three or four proteins, respectively pAcDB3 is a smaller version of pAcAB3.
pBAC -1, pBAC-2, pBAC-3	<i>Polh</i>	6xHis; pBAC-2 and pBAC-3 vectors also carry S-tag; pBAC-3 vectors also carry signal peptide.	Thrombin or enterokinase	Merck Biosciences	" <i>gus</i> " versions of the pBAC series transfer the <i>p6.9</i> promoter- β -glucuronidase gene as a positive control for recombinant virus production.
pBAC-5, pBAC-6	<i>gp64</i>	6xHis, S-tag; pBAC-6 vectors also carry signal peptide.	Thrombin or enterokinase	Merck Biosciences	Mid-level expression during the early and late phase for more complete processing
pBAC4x	<i>p10</i> , <i>polh</i> , <i>p10</i> , <i>polh</i>	6xHis	—	Merck Biosciences	Promoters/cloning sites for expression of four proteins
pIEX/Bac series	Tandem <i>hr5-ie1</i> and <i>p10</i> promoters	6xHis; Selected vectors carry GST or Strep(II)-tag.	Thrombin, enterokinase, or HRV 3C	Merck Biosciences	The recombinant gene is expressed throughout the baculovirus life cycle: The <i>hr5</i> enhancer and the <i>ie1</i> promoter use the insect cell RNA polymerase for intermediate early gene expression. The <i>p10</i> promoter drives very late gene expression. The <i>hr5-ie1</i> promoter also can be used for protein expression during transient transfection of insect cells.
pTriEx series	<i>p10</i> (T7-lac, CMV/ β -actin)	6xHis; Selected vectors carry HSV-tag, S-tag, or Strep(II)-tag.	Thrombin, enterokinase, or HRV 3C	Merck Biosciences	Contains sequences (T7-lac, CMV/ β -actin promoters) to produce protein in <i>E. coli</i> or mammalian cells as alternatives to baculovirus
pVL1393	<i>Polh</i>	—	—	AB Vector	Traditional transfer vector
pVL-GFP	<i>Polh</i>	GFP	—	AB Vector	For visualization of soluble protein expression
pAB series	<i>Polh</i>	MBP; 6xHis; 6xHis-MBP; GST; flag-6xHis; signal peptide	Thrombin (for 6xHis or MBP); HRV 3C (for GST)	AB Vector	Diverse options for expression and cleavage of fusion proteins
pQE-TriSystem series	<i>p10</i> (T7-lac, CMV/ β -actin)	None; 10xHis; or Strep(II)-tag	None or TAGZyme	QIAGEN	Contains sequences (T7-lac, CMV/ β -actin promoters) to produce protein in <i>E. coli</i> or mammalian cells as alternatives to baculovirus
pMelBac	<i>Polh</i>	Signal peptide	—	Life Technologies	This vector will not recombine with any viral DNA other than Bac-N-Blue.
pBacPak8, pBacPak9	<i>Polh</i>	—	—	Clontech	Traditional transfer vector

(continued)

TABLE 2. Continued

Viral DNAs (Group B ⁵)	Vendor	Features			
BaculoDirect	Life Technologies	No transfer vector is required per se; the gene of interest is transferred from a Gateway entry vector in vitro. Nonrecombinant viruses are inviable in the presence of ganciclovir because of a thymidine kinase gene that is replaced by the gene of interest. Nonrecombinant viruses produce blue plaques in the presence of X-Gal caused by a <i>lacZ</i> gene that is replaced by the gene of interest. BaculoDirect N-Term or C-Term DNAs encode amino- or carboxy-terminal V5-tag and 6xHis tags. The N-Term variant includes a Tev protease site. A "Secreted" variant also encodes a signal peptide.			
DH10Bac (Bac-to-Bac)	Life Technologies	BaculoDirect N-GST encodes amino-terminal GST fusion. A protease site needs to be added with the gene of interest. The <i>polh</i> promoter drives gene expression. The <i>E. coli</i> host strain, DH10Bac, contains a baculovirus shuttle vector (bacmid) and a helper plasmid that allows recombination with a pFastBac expression construct following transposition. The bacmid contains a kanamycin-resistance gene and mini- <i>att</i> Tn7 sites for transposition to insert the gene of interest from a pFastBac or pDest vector. The helper plasmid contains a tetracycline-resistance gene and expresses a Tn7 transposase.			
Transfer vectors (Group B ⁵) ^h	Promoter	Fusion partners ^b	Protease sites ^c	Primary vendor	Comments
pFastBac 1	<i>Polh</i>	—	—	Life Technologies	—
pFastBac HT	<i>Polh</i>	6xHis	TEV	Life Technologies	—
pFastBac dual	Separate <i>polh</i> and <i>p10</i> promoters	—	—	Life Technologies	For coexpression of two proteins from the same baculovirus
pFastBac/NT-TOPO or CT-TOPO	<i>Polh</i>	6xHis (amino- or carboxy-terminal)	TEV	Life Technologies	Two cloning sites, one each for the <i>polh</i> and <i>p10</i> promoters
pDEST8, 10, or 20	<i>Polh</i>	6xHis (pDEST10); GST (pDEST20)	TEV (pDEST10)	Life Technologies	TOPO cloning sites for gene insertion
<i>P. pastoris</i> ⁱ			TEV (pDEST10)	Life Technologies	For gene insertion using Gateway entry vectors such as pENTR
Vector system	Promoter	Fusion partners	Strains (genotype); phenotypes ^j	Comments	
pPIC-Z, pPIC-Zα series ^{k,l,m}	AOX1	c-myc epitope; 6xHis; pPIC-Zα vectors also carry signal peptide	X-33 (wild-type): Mut ⁺ KM71H (<i>aox1::ARG4; arg4</i>): Mut ^s SMD1168H (<i>pep4</i>): Mut ⁺ , <i>pep4</i> [−] <i>His</i> [−] variants: GS115 (<i>his4</i>): Mut ⁺ , His [−] KM71 (<i>his4, aox1::ARG4; arg4</i>): Mut ^s , His [−] SMD1168 (<i>his4, pep4</i>): Mut ⁺ , His [−] , <i>pep4</i> [−]	Methanol-inducible promoter Zeocin allows selection in <i>E. coli</i> and <i>P. pastoris</i> and facilitates screening for multicopy insertions; relatively small vector. <i>His</i> [−] variants allow for double plasmid cotransformation using different selection markers on each plasmid (e.g., <i>his4</i> of pPIC3.5 and Zeocin of pPIC-Z).	
pGAP-Z, pGAP-Zα ^m series	GAP	c-myc epitope; 6xHis; pGAP-Zα vectors also carry signal peptide.	—	Constitutive promoter	—
pFLDZ-Z, pFLD-Zα ^m series	FLD1	V5 epitope; 6xHis; pFLD-Zα vectors also carry signal peptide).	—	Zeocin selection Methanol- and methylamine-inducible promoter Zeocin selection	—
	AOX1			Original expression vectors based on <i>His4</i> selection	(continued)

TABLE 2. *Continued*

<i>P. pastoris</i> ⁱ				
Vector system	Promoter	Fusion partners	Strains (genotype): phenotypes ^j	Comments
pPIC9, pPIC3.5, pHIL-D2, pHIL-S1 ^k		pHIL-S1 vector also carries signal peptide.	GS115 (<i>his4</i>): Mut ⁺ or Mut ^S KM71 (<i>his4</i> , <i>aox1</i> :ARG4; <i>arg4</i>): Mut ^S SMD1168 (<i>his4</i> , <i>pep4</i>): Mut ⁺ or Mut ^S , <i>pep4</i> [−]	<i>His4</i> selection, and kanamycin selection for multicopy expression
pPIC9 K, ^l pPIC3.5 K, pAO815	AOX1	—	—	
pPink-LC, pPink-HC, pPink-α—HC ^k	AOX1	Signal peptide; pPink-α—HC vector also carries signal peptide.	PichiaPink Strain 1 (<i>ade2</i>): Mut ⁺ , "white" PichiaPink Strain 2 (<i>ade2</i> , <i>pep4</i>): Mut ⁺ , <i>pep4</i> [−] PichiaPink Strain 3 (<i>ade2</i> , <i>prb1</i>): Mut ⁺ , <i>prb1</i> [−] PichiaPink Strain 4 (<i>ade2</i> , <i>pep4</i> , <i>prb1</i>): Mut ⁺ , <i>pep4</i> [−] , <i>prb1</i> [−]	PichiaPink Expression Kit uses <i>ade2</i> complementation. Low-copy (LC) and high-copy (HC) vectors available Eight secretion signals for optimization of secreted proteins

^aUnless otherwise specified, all *E. coli* vectors are IPTG-inducible, have a pBR322-related replicon, and carry a *lac* repressor gene (*lac I* or *lac I*^q).

^b6xHis, GST, MBP, Strep(II)-tag, hpTrxA, S-tag, and CBP are carriers for the purification described in Table 3. NusA and Sumo may improve fusion protein solubility, DsbA or DsbC promote disulfide bond formation, and dihydrofolate reductase (DHFR) activity may distinguish soluble fusion proteins from inclusion bodies. The pelB and gene III signal peptides direct periplasmic localization of fusion proteins. The HSV, T7-tag, Tag100, V5, Xpress, and c-myc fusions provide epitopes for well-characterized, commercially available antibodies.

^cCommon site-specific proteases are described in Table 4.

^dAlthough the viral DNAs and transfer vectors of the same group are likely to be compatible, it is advisable to double-check that the homologous regions correspond.

^eMost transfer vectors carry the ampicillin-resistance gene and pUC replicon for high-copy-number replication in *E. coli*.

^fGroup A: Recombinant baculovirus generated by cotransfection of insect cells.

^gGroup B: Recombinant baculovirus generated before insect cell transfection.

^hTransfer vectors compatible with the Bac-to-Bac system carry a gentamicin gene within the transferred region. Most Gateway entry vectors are compatible with the BaculoDirect system, which does not have specific transfer vectors per se.

ⁱUnless otherwise described, *P. pastoris* vectors contain an ampicillin-resistance gene for propagation of the DNA in *E. coli*, a methanol-inducible AOX1 promoter, and a selection marker for *P. pastoris*.

^jThe phenotype for wild-type methanol utilization is termed Mut⁺, and methanol utilization slow is termed Mut^S; see text for details.

^kThe pPICZ vectors carry the *S. hindustanus ble*(*Sh ble*) gene for selection with the antibiotic Zeocin in both *E. coli* and yeast.

^lVector variants with different selection are available, for example, blasticidine or kanamycin (geneticine G114). Vectors with more selectable markers (*ADE1*, *ARG4*, *URA3*) can be requested from the Keck Graduate Institute <http://www.kgi.edu/faculty-and-research/jim-cregg>.

^mpPICZα, pGAPZα, pFLDZα, pHIL-S1, and pPINKα variants contain an amino-terminal pre-pro-α secretion signal (α-mating factor pre-pro peptide from *S. cerevisiae*) to direct secretion of recombinant proteins into the medium.

TABLE 3. Common fusion proteins used for recombinant protein purification

Fusion protein	Affinity ligand	Elution method	Number of residues	Reference(s)	Comments
Polyhistidine (6xHis)	Immobilized transition metal ions (e.g., Ni^{2+} or Co^{2+})	High concentrations of imidazole ($>100 \text{ mM}$ for Ni^{2+} -NTA)	6–10	Smith et al. 1988	Varying the type of resin and/or metal ion may improve the selectivity of protein separation (Gaberc-Porekar and Menart 2001). @Low pH and EDTA are alternative elution agents. @Denaturing conditions may be used during purification.
Glutathione-S-transferase (GST)	Immobilized glutathione	Reduced glutathione (10 mM)	220	Smith and Johnson 1988	GST and consequently uncleaved GST fusion proteins are usually dimeric (Armstrong 1997).
Maltose-binding protein (MBP)	Cross-linked amylose	Maltose (10 mM)	390	di Guan et al. 1988; Maina et al. 1988	
Strep(II)-tag	StrepTactin-resin	D-Desthiobiotin	8	Schmidt and Skerra 1993; Schmidt et al. 1996; Voss and Skerra 1997	StrepTactin is a streptavidin variant with increased affinity for Strep(II)-tag (Voss and Skerra 1997). @D-Desthiobiotin is a biotin derivative with weakened affinity to permit column regeneration (Hirsch et al. 2002).
His-patch thioredoxin (hpTrxA)	Immobilized transition metal ion (e.g., Ni^{2+} or Co^{2+})	Low concentrations of imidazole ($<100 \text{ mM}$ for Ni^{2+} -NTA)	109	LaVallie et al. 1993; Lu et al. 1996	Fusions with TrxA may in some cases be purified by taking advantage of improved thermostability compared with most <i>E. coli</i> proteins or by osmotic shock (despite its cytoplasmic location) (LaVallie et al. 2003).
S-tag	S-protein resin (Merck)	Denaturing conditions or protease cleavage	15	Kim and Raines 1993	S-tag/S-protein sequences are derived from subtilisin fragments of bovine pancreatic ribonuclease A (Richards and Vithayathil 1959). Consequently, reconstituted S-tag/S-protein complexes are active ribonucleases.
Calmodulin binding peptide (CBP)	Immobilized calmodulin	EGTA (2 mM)	26	Stofko-Hahn et al. 1992	Binding conditions include Ca^{2+} ions (2 mM).

Well-characterized antibodies are commercially available for all listed proteins. All fusion proteins, with the exception of S-tag, may be detected by absorbance at 280 nm.

TABLE 4. Common endopeptidases used for cleavage of recombinant fusion proteins

Protease	Recognition site ^a	Fusion tag	References	Comments
Enterokinase	DDDDK↓	—	Light et al. 1980; Dykes et al. 1988	Enterokinase, Factor Xa, and α -thrombin are inhibited by standard serine protease inhibitors such as phenylmethanesulfonyl fluoride (PMSF).
Factor Xa ^b	I-(E/D)-GR↓	—	Nagai and Thøgersen 1984; Jenny et al. 2003	
α -Thrombin ^c	LVPR↓GS	—	Gearing et al. 1989; Jenny et al. 2003	
HRV-3C ^d	LE-(V/A/T)-LFQ↓GP	GST or His tag	Cordingley et al. 1990; Walker et al. 1994	Serine protease, only slightly inhibited by PMSF and Zn ²⁺
Genenase I ^e	HY↓ or H↓Y	—	Carter and Wells 1987; Carter et al. 1989	Engineered subtilisin variant with increased specificity available from New England Biolabs Inhibited by standard serine protease inhibitors such as phenylmethanesulfonyl fluoride (PMSF)
Tobacco etch virus (TEV) protease	ENLYFQ↓(G/S)	His tag	Parks et al. 1994; Tropea et al. 2009	Cysteine protease sensitive to oxidation and inhibited by Zn ²⁺ (>5 mM) Autoproteolysis of wild-type TEV protease is reduced using a site-directed mutation (Kapust et al. 2001).

^aPositions where more than one type of amino acid is permitted are indicated in parentheses with a slash separating the interchangeable amino acids.

^bEnterokinase and Factor Xa will not efficiently cleave a site preceding a proline; Factor Xa also will not cleave a site preceding an arginine.

^cTwo types of optimum cleavage sites exist for α -thrombin: (i) p4-p3-P-(R/K)-p1'-p2', where p3 and p4 are hydrophobic amino acids; and (ii) p2-(R/K)-p1', where p2 or p1' is Gly (Chang 1985).

^dPreScission protease is the proprietary name of a GST-tagged HRV-3C protease marketed by GE Healthcare.

^eGenenase I has been shown to cleave following other histidines at rates that depend on the structural and sequence context (Carter et al. 1989). Rates of cleavage when the amino acid following the cleavage site (position p1') is Asp or Glu are slowest, and no cleavage is observed preceding Pro or Ile.

E. coli Expression Vectors Containing an Inducible Promoter

E. coli expression vectors typically include an origin of replication, a promoter followed by a cloning site, a transcription termination signal, antibiotic-resistance genes, optional fusion tags, and in some cases, coding sequences for repressor proteins. The replication origins of most *E. coli* expression vectors are based on replicons of the pBR322 family (colicin E1 or the closely related pMB1) and thus belong to the same incompatibility group. Specialized vectors for production of multicomponent complexes (such as the pET Duet system) have been developed to overcome this problem.

Many *E. coli* expression vectors make use of the *lac* operon to prevent induction of potentially toxic proteins until an appropriate cell density has been reached (Fig. 2). In general, the *lac* operator (*lac O*) is located downstream from the promoter of the target gene, so that binding of the *lac* repressor blocks transcription initiation until lactose or the isopropyl- β -D-thiogalactopyranoside (IPTG) analog is added. A *lac* repressor gene (*lac I*) (Farabaugh 1978) or the highly expressed *lac Iq* allele (Stark 1987; Amann et al. 1988) is constitutively expressed in *trans*. The strong promoters that drive target protein expression have been developed from either host promoters or bacteriophage sequences. These promoter options are discussed in more detail below. See also Protocol: Expression of Cloned Genes in *E. coli* Using IPTG-Inducible Promoters (Kielkopf et al. 2021a) and Protocol: Subcellular Localization of Signal Peptide Fusion Proteins Expressed in *E. coli* (Kielkopf et al. 2021b).

Host Promoters for Foreign Gene Expression in E. coli

***lac* Promoter.** Any general-purpose vector (pUC, pTZ, pSK, pBluescript, pGEM, etc.) designed for blue/white screening can be used to express a foreign protein, usually as a fusion protein with amino acids encoded by the amino terminus of the *lacZ* gene and/or the polylinker sequence. Although the *lac* promoter is not as strong as the *tac* or *trc* promoters (see below), the high copy number of most general-purpose vectors allows expression of foreign proteins at respectable levels. Maximum induction of the *lac* promoter requires the action of the cyclic adenosine monophosphate (cAMP) activator protein (CAP, the *crp* gene product), which is most active when cells are grown in medium lacking glucose (for review, see Reznikoff 1992). Thus, media with a minimal amount of glucose as a

FIGURE 2. Representative vectors for protein expression in *Escherichia coli*. (A) The *tac* promoter, found in the pGEX series of vectors, uses the host RNA polymerase. (B) The T7-*lac* promoter, found in the pET vectors, is transcribed by a T7 RNA polymerase (usually introduced in the host genome) and regulated by a *lac* operator.

carbon source (such as Luria broth) should be used for expression of genes cloned into vectors with the *lac* promoter.

***trp-lac* (*tac*) Promoter.** *tac* is a hybrid *trp-lac* promoter containing the –35 region of the *trp* promoter fused to the –10 region of the *lacUV5* promoter; it is regulated by the *lac* repressor and is independent of cAMP regulation mediated by the *crp* gene product (Amann et al. 1983; de Boer et al. 1983). A prevalent *tac* promoter series of GST-fusion plasmids (pGEX) (Smith and Johnson 1988) is available from GE Healthcare (Fig. 2A).

***trp-lac* (*trc*) Promoter.** *trc* is another version of the *lac* repressor-regulated hybrid *trp-lac* promoter containing the –35 region of the *trp* promoter fused to the –10 region of the *lacUV5* promoter (Amann and Brosius 1985). The only difference between the *trc* and *tac* promoters is the distance separating the –35 and –10 regions of the promoter. In the *trc* promoter, these two elements are separated by a consensus distance (17 bp), whereas in the *tac* promoter, they are separated by 16 bp. This difference has little or no effect on the expression levels of foreign proteins (Amann and Brosius 1985). The expression plasmid pTrcHIS, available from Life Technologies, carries the *trc* promoter.

The *l*-Arabinose Operon (*araBAD*) Promoter. The L-arabinose-inducible *bad* promoter is an alternative to the *lac* repressor-regulated vectors for protein production in *E. coli*. The pBAD series of vectors contains an *araC-P_{BAD}* fragment from the *ara* operon (Guzman et al. 1995), which encodes the regulatory AraC protein and a promoter that naturally controls the *araBAD* genes for arabinose catabolism (for review, see Schleif 2000). The *araBAD* promoter has valuable characteristics of both stimulating transcription in response to the L-arabinose-bound state of the AraC protein, as well as being repressed by the AraC protein in the absence of cofactor (Englesberg et al. 1969). Media that contain glucose as a carbon source should not be used to express recombinant genes under control of the *araBAD* promoter (Haggerty and Schleif 1975).

Bacteriophage Promoters for Foreign Gene Expression in *E. coli*

T7-lac Promoter. The pET series of vectors, originally developed by Tabor and Richardson (1985) and Studier and Moffatt (1986) and since expanded, allows regulated expression of foreign genes by bacteriophage T7 RNA polymerase (Fig. 2B). Depending on the specific pET vector, the multiple cloning sites allow a coding sequence to be inserted under control of the “natural” promoter for T7 RNA polymerase (the $\phi 10$ promoter) (Studier and Moffatt 1986), or more commonly under the control of the so-called T7-lac promoter, a derivative with the addition of a *lac* operator (*lac O*) for stringent regulation (Dubendorff and Studier 1991). For target protein expression, the host strain must contain an inducible copy of the T7 RNA polymerase gene. Usually, this is achieved using a DE3 lysogenic strain bearing the T7 RNA polymerase gene under the control of the *lacUV5* promoter. Because this promoter is subject to regulation by CAP as well as the *lac* repressor, media that contain a minimal amount of glucose as a carbon source (such as LB) should be used to increase target protein expression. However, glucose may be added to decrease leaky T7 RNA polymerase expression during cell growth. Coexpression of low levels of T7 lysozyme, a natural inhibitor of T7 RNA polymerase, serves as a complementary means for reducing basal activity of T7 promoters for stable gene expression (Studier 1991).

T5-lac Promoter. An alternative type of bacteriophage promoter efficiently competes with host RNAs to recruit the *E. coli* RNA polymerase and drive production up to 90% of the total host mRNAs. One common example is the T5 promoter (PN25) (Gentz and Bujard 1985) fused with the *E. coli lac* operator for transcriptional control (Bujard et al. 1987). The gene encoding the *lac* repressor is provided either on the vector or separately, for example, by a distinct plasmid such as pREP4 with the pQE series of vectors from QIAGEN.

Baculovirus Systems for Protein Expression in Insect Cells

The first continuous insect cell lines were established in the 1960s for the purposes of producing baculovirus as potential insecticides and studying insect cell metabolism (Gao 1958; Gao et al. 1959; Grace 1962). Subsequently in the 1980s, the first baculovirus vector system for expression of heterologous proteins using insect cell cultures was developed independently in the laboratories of Max D. Summers and Lois K. Miller (Fraser et al. 1983; Pennock et al. 1984). In subsequent decades, the popularity of the baculovirus expression system has accelerated because of advances in the methodology and, ultimately, commercial availability. For historical reasons, a limited number of species out of hundreds of known species of baculovirus and Lepidoptera cell lines are used for baculovirus expression systems (for review, see Lynn 2007). Most recombinant baculoviruses are derived from a single multiple nucleopolyhedrovirus, named AcMNPV after the *Autographa californica* (alfalfa looper) host in which it was originally identified. The common insect cell lines for protein expression include Sf-21 or Sf-9 from *S. frugiperda* (fall armyworm), Tn-368 or Tn-5 (High-Five) from *Trichoplusia ni* (cabbage looper), and transgenic variants. The expression levels of distinct recombinant proteins are likely to vary among these cell lines (Wickham et al. 1992; Davis et al. 1993). Below, we compare key differences among recipient viral DNAs and describe the general features of transfer vectors. In-depth descriptions of the baculovirus expression system are given in O'Reilly et al. (1994) and Murhammer (2007).

Three major steps are shared among baculovirus expression systems (Fig. 3A): (1) recombination between the double-stranded DNA virus and a transfer vector that contains the gene of interest, (2) transfection of insect cells to produce large quantities of recombinant baculovirus, and, finally, (3) infection of insect cells to express the desired protein. (See also Protocol: **Expression of Cloned Genes Using the Baculovirus Expression System** [Kielkopf et al. 2020a].) The large size of the baculovirus genome (134 kb) prevents the use of traditional ligation techniques to insert the target gene in the viral DNA. Instead, the gene of interest is subcloned into a transfer vector with features for propagation in *E. coli*. The transfer vector also contains a viral promoter, cloning site, transcription terminator, and selectable marker between two sequences with homology with viral DNA sites (Fig. 3B). This region of the plasmid is then transferred to the baculovirus genome by homologous

FIGURE 3. The baculovirus expression system. (A) Flow chart of the steps for baculovirus-mediated protein production in insect cells. (MOI) Multiplicity of infection. (B) Schematic diagram of the transfer vector containing the target gene (propagated in *E. coli*) recombining with the viral DNA.

recombination. In most currently used systems, recombination with the transfer vector replaces the polyhedron locus of the viral DNA (Smith et al. 1983b). Although polyhedron is a coat protein that is not essential for baculovirus replication in insect cell culture, its absence gives rise to a convenient plaque morphology that may be used by an experienced viewer to distinguish recombinant from wild-type baculovirus. The polyhedron region of commercial viral DNAs often is replaced with a *lacZ* gene for blue/white screening of recombinant virus. A traditional approach for generating recombinant baculovirus depends on homologous recombination within the insect cells following cotransfection of viral DNA and a transfer vector. However, cotransfection of the wild-type viral DNA with a transfer vector results in only ~0.1% recombinant baculovirus (Smith et al. 1983a). The desired progeny then must be identified by time-consuming techniques such as a viral plaque assay. To solve this problem, several improved strategies have been developed to generate and select the recombinant baculovirus, as summarized below and listed in Table 2.

Recombinant Baculovirus by Cotransfection of Insect Cells

Linear Viral DNAs. The fraction of recombinant virus produced by cotransfection may be increased to ~30% by introducing a unique Bsu36I restriction site and linearizing the viral DNA (Kitts et al. 1990). A second Bsu36I restriction site within an essential gene from the viral genome (ORF1629, a structural protein) further increases the recovery of recombinant baculoviruses to nearly 99% (Kitts and Possee 1993). Digestion of the two Bsu36I sites removes a portion of ORF1629 from the viral DNA, so that the recircularized baculovirus is no longer viable. Accordingly, the transfer plasmids used with this system must provide a complementary portion of ORF1629 in addition to the gene of interest. A *lacZ* gene located between the Bsu36I sites of the viral DNA marks nonrecombinant baculovirus with a blue color in the presence of X-Gal. Linear viral DNAs for this traditional method are available from several manufacturers, including BD Biosciences, Merck, Life Technologies, and Clontech.

Viral DNAs as Bacterial Artificial Chromosomes. Although digestion with Bsu36I significantly increases the fraction of recombinant baculovirus, contamination with undigested viral DNA is very difficult to avoid. This problem has been addressed by converting a viral genome that lacks ORF1629 to a low-copy, bacterial artificial chromosome (bacmid) that can be propagated in *E. coli* (Zhao et al. 2003). Cotransfection of transfer vectors with the bacmid versions of the viral DNAs that lack ORF1629 ensures that 100% of the recovered baculoviruses have recombined with the transfer

vector. The polyhedro (Oxford Expression Technologies) and BacMagic (Merck) viral DNAs offer this option for recombinant baculovirus production.

Recombinant Baculovirus before Insect Cell Transfection

Recombination of Linear Viral DNAs In Vitro. The BaculoDirect system from Life Technologies also makes use of viral DNA that has been linearized by several rounds of restriction digestion. However, *attR1* and *attR2* sites flanking the polyhedron locus have been engineered surrounding the polyhedron locus to enable in vitro recombination with Gateway entry vectors (Life Technologies). The products of the recombination reaction are then transfected into insect cells for baculovirus production. The viral DNA contains a thymidine kinase gene within the transfer region, so that only recombinant baculoviruses are able to propagate in media containing ganciclovir (Godeau et al. 1992). Because a transfection step is still required, the BaculoDirect procedure requires about the same length of time as cotransfections with viral bacmids, which do not require plaque purification. Furthermore, LR Clonase II mix (Life Technologies) must be purchased for the Gateway reaction. Nevertheless, an advantage of the BaculoDirect system over other strategies for generating recombinant baculovirus is the ability to use a single Gateway entry construct to generate expression constructs for both baculovirus and *E. coli* expression systems.

Recombination of Viral DNAs in Bacterial Hosts. The Bac-to-Bac Baculovirus Expression System (Life Technologies) is a popular strategy for generating recombinant viral DNA in an *E. coli* host using techniques that are familiar to most molecular biologists (Luckow et al. 1993). Recombination between a transfer vector and a viral bacmid DNA takes place in a bacterial host (DH10BacTM). The transfer plasmid and bacmid, respectively, contain mini-Tn7 and mini-*attTn7* sites that allow a Tn7 transposase provided by a helper plasmid in the DH10BacTM strain to transfer the gene of interest (Barry 1988). The transfer vector carries an antibiotic-resistance gene within the region to be transferred, and recombination disrupts a *lacZα* gene of the bacmid DNA. The *E. coli* colonies containing the recombinant bacmids gain an antibiotic resistance marker and are no longer colored in the presence of X-Gal. The high-molecular-weight DNA recovered from these amplified colonies is used to produce baculovirus by insect cell transfection. An advantage of the Bac-to-Bac System over other systems is the ability to select a single recombinant clone, which also can be sequenced before baculovirus production. All other systems use a mixture of recombinant DNAs for transfection, so that any mutant viruses may be amplified and compete with the viruses expressing the protein of interest.

Several transfer vectors and their salient features are listed in Table 2, with comprehensive information available from the manufacturers' websites. The transfer vectors share some general features with other *E. coli* vectors, including a selectable ampicillin-resistance gene, a pUC replicon for high-copy-number replication, and a cloning site. Like the vectors for protein expression in *E. coli*, the transfer vector may also carry coding sequences for protein purification, detection, or secretion in-frame with the recombinant gene product. Many of the baculovirus transfer vectors are equipped with 6xHis tags. Options are also available to monitor protein production. For example, fusions or coexpression with green fluorescent protein (GFP) variants allow the progress of protein expression to be easily monitored by fluorescence of the infected insect cells.

The polyhedron gene promoter is commonly used to drive expression of the foreign protein in insect cells (Smith et al. 1983b). Another option is to use a vector carrying the viral p10 gene (Zuidema et al. 1990). In both cases, a virally encoded RNA polymerase is used to drive transcription at a very late stage of baculovirus infection (~1–3 d postinfection) (Smith et al. 1983c), when the polyhedral viral coat normally would be assembled. The levels of expression are high, accounting for up to 50% of the total insect cell protein. One disadvantage of strong protein expression at a very late stage of infection is that levels of posttranslational modifications decline. A handful of transfer vectors with alternative promoters (e.g., basic protein or gp64) may be preferable for moderate expression of proteins with posttranslational modifications in the late stage of infection (~8–24 h postinfection) (Hill-Perkins and Possee 1990; Friesen 1997). Although the recombination sites of almost all commercially available

transfer vectors flank the polyhedron locus, given the variety of recombination sites, it is advisable to check with the manufacturer before mixing transfer and viral DNAs from different sources.

Vectors for Protein Expression in *P. Pastoris*

Most *P. pastoris* vectors rely on the strong, methanol-inducible *AOX1* promoter and *AOX1* transcription termination sequences to drive expression; the original vectors (Table 2) also contain 3' *AOX1* sequences. Because the *AOX1* promoter is tightly repressed by glucose and glycerol, these carbon source cultures can be grown to high density in media containing these compounds before shifting them to methanol-containing medium, which induces rapid, high-level expression (Daly and Hearn 2005). All *Pichia* vectors are shuttle vectors containing an origin of replication and antibiotic-resistance gene for plasmid maintenance in *E. coli*, plus an auxotrophic marker based on biosynthetic pathway genes (usually *His4*) for selection in yeast. For additional information, see Protocol: **Expression of Cloned Genes in *Pichia pastoris* Using the Methanol-Inducible Promoter *AOX1*** (Kielkopf et al. 2021c).

Examples of *Pichia* Vectors

pPIC-Zeocin Vectors. The newer-generation pPIC-Zeocin vectors (pPICZ and pPICZ α variants) (Table 2) combine an expanded multiple cloning site (MSC), a convenient carboxy-terminal myc epitope, and a 6xHis tag to facilitate detection and purification of the expressed proteins. These vectors also carry a *ble* gene for selection with the antibiotic Zeocin in both *E. coli* and yeast. Use of the *ble* gene has dramatically reduced the vector size (~3.6 kb) (see Fig. 1 in Protocol: **Expression of Cloned Genes in *Pichia pastoris* Using the Methanol-Inducible Promoter *AOX1*** [Kielkopf et al. 2021c]), facilitating cloning and downstream mutagenesis of the heterologous gene. The pPICZ α vector also contains the pre-pro- α secretion signal (α -mating factor pre-pro peptide from *S. cerevisiae*) to direct secretion of recombinant proteins into the medium. Promoters available for this vector include (1) pFLDZ, which can be induced by with methanol as well as methylamine, and (2) a constitutive promoter that may be the preferred option for expression using large-scale cultures.

Multicopy Integration Vectors. An advantage of the Zeocin vectors is that higher concentrations of the antibiotic can be used for direct selection of multicopy integrants among *Pichia* transformants (see Protocol: **Expression of Cloned Genes in *Pichia pastoris* Using the Methanol-Inducible Promoter *AOX1*** [Kielkopf et al. 2021c]) (Wang et al. 2006; Sunga et al. 2008). In vitro construction of tandem repeats of one or more genes, each containing its own *AOX1* promoter and *AOX1* transcription termination sequence, is another feature of the pPICZ vector series as well as the multicopy expression vector pAO815. This feature is useful for coexpression of partner proteins at defined stoichiometries (Cregg et al. 2000). However, large plasmids such as pAO815 are difficult to handle. In addition, they should not be linearized with enzymes that cut within the *AOX1* promoter because this significantly reduces their transformation efficiency.

***PichiaPink* Vectors.** The *PichiaPink* Vector Kit offered by Life Technologies takes advantage of *ADE2* complementation rather than antibiotic resistance. This *ADE2* gene is essential to de novo biosynthesis of purine nucleotides, and its deletion in the parental strains (*PichiaPink* strains 1–4) (Table 2) causes red-colored colonies because of the accumulation of purine precursors. This facilitates selection because the color of transformant colonies (more or less white) correlates indirectly with the expression level of the protein of interest. Another advantage is that the Life Technologies kit provides vectors equipped with *ADE2* promoters of different lengths, which influences the number of plasmid copies that integrate, and is useful for optimizing protein expression. A variety of secretion signal sequences in addition to the pre-pro- α -factor are offered for optimizing protein secretion into the medium.

Strains and Phenotypes Generated by Different Vectors. *Pichia pastoris* strains with a range of genotypes are available (Table 2). Strains X-33, GS115, SMD1168, and *PichiaPink* contain a functional copy of *AOX1*, the gene for the alcohol oxidase enzyme responsible for the majority of methanol utilization. These strains have a wild-type methanol utilization phenotype termed Mut⁺ (Daly and

Hearn 2005). Because the strains retain the Mut⁺ phenotype after genomic integration of the vector, they consume methanol rapidly, posing a potential fire hazard during large-scale fermentation. The “methanol utilization slow” or Mut^S strains such as KM71 contain a nonfunctional AOX1 gene and rely on methanol oxidation by the slower AOX2 enzyme, and thus grow much slower under methanol induction. Mut^S strains are sometimes better producers of heterologous protein than Mut⁺ strains. If necessary, their growth can be enhanced during induction by a mixed feed of methanol and an alternative carbon source such as sorbitol or mannitol, without repression of AOX1 (Inan and Meagher 2001). The Mut^S phenotype can also arise during transformation of strains GS115 or SMD1168 with the original expression vectors (pPIC9, pPIC3.5, pHIL-D2, pHIL-S1) when a double crossover occurs between the 5' AOX1 promoter and the 3' AOX1 sequence during transformation, which leads to replacement of the genomic AOX1 gene (Daly and Hearn 2005).

Strains such as GS115, KM71, and SMD1168 are defective in the histidinol dehydrogenase gene (*His4*), the auxotrophic selection marker provided by the original expression vectors. The protease-deficient strain SMD1168 is defective in the vacuolar peptidase A (*pep4*), a major aspartic protease in *Pichia* that also activates downstream proteases such as the acid/serine carboxypeptidase Y and the serine protease B1 (*prb1*). Variants SMD1165 and SMD1163, as well as the *Pichia*Pink strains 2, 3, and 4, are defective in *pep4* and/or *prb1*; these grow more slowly than wild-type strains but may be advantageous if proteolytic degradation of the heterologous protein by the vacuolar pathway (but not the ubiquitin/proteasomal pathway) is a problem.

Pichia Expression Kits

Pichia expression kits, including vectors and strains, are available through Life Technologies (<http://www.thermofisher.com/us/en/home.html>). Limitations of the commercial system include the lack of moderate-strength promoters and the existence of few selectable markers for *Pichia* transformation (Lin-Cereghino et al. 2008). Interestingly, AOX1 promoter analysis created a library of promoters based on AOX1 deletions/mutations for the fine-tuning of expression in *P. pastoris* (Hartner et al. 2008). Some vectors and strains with other selection markers (*ADE1*, *ARG4*, *URA3*) are available from James Cregg at the Keck Graduate Institute (<http://www.kgi.edu/faculty-and-research/jim-cregg>); detailed discussion of the vectors can be found in Lin-Cereghino and Lin-Cereghino (2007).

FUSION PROTEINS

Recombinant proteins and peptides often are expressed as fusion proteins, composed of carrier sequences linked to the target protein. Two or more open reading frames (ORFs) are joined together in the desired order to encode the fusion protein. Then, expression of the fused reading frames generates the hybrid fusion protein, in which the protein of interest is attached to the amino terminus or the carboxyl terminus of a carrier protein by a standard peptide bond (Itakura et al. 1977; for reviews, see Arnau et al. 2006; Esposito and Chatterjee 2006). Recovery of the target protein is facilitated by including a proteolytic cleavage site at the junction of the fused proteins.

Fusion proteins have a vast array of potential uses.

1. Attaching target proteins to a ligand-binding domain may provide a convenient method to “tag” and purify the target protein sequences.
2. Adding “carrier” sequences may protect the target protein from proteolysis.
3. Adding carrier sequences may improve the solubility of the target protein and may prevent the formation of insoluble inclusion bodies (see Optimization of Expression of Foreign Proteins for more information on dealing with insoluble proteins).
4. Adding well-characterized epitopes may allow the fusion protein to be detected by immunological methods.

5. Joining the target protein to translocation signals may allow the fusion protein to be directed to specific cellular compartments.

More than 20 different fusion systems have been designed or adapted for affinity purification, with several commonly used purification tags summarized in Table 3. Frequently, the attached target protein is a carrier with high affinity for a specific ligand, which enables almost any fusion protein to be purified by a single step of affinity chromatography. For example, the proteins GST and maltose-binding protein (MBP) are well-known tags for isolation of fusion proteins using chromatographic resins conjugated with their respective ligands, glutathione (Protocol: **Purification of Fusion Proteins by Affinity Chromatography on Glutathione Resin** [Kielkopf et al. 2020c]) or amylose. Furthermore, GST and MBP, as well as other carriers, often improve fusion protein solubility and accordingly may reduce inclusion body formation in *E. coli* expression systems (Esposito and Chatterjee 2006). Shorter peptides may be included in the fusion proteins, either as a handle for purification or as a convenient epitope for antibody-based methods. An artificial 6xHis sequence is a prevalent short carrier for purification of fusion proteins by affinity for columns carrying Ni^{2+} or Co^{2+} (Smith et al. 1988) as described in Protocol: **Purification of Polyhistidine-Tagged Proteins by Immobilized Metal Affinity Chromatography** (Kielkopf et al. 2020b). Short epitope tags are often used for antibody-based assays. The Flag tag is a well-known designer octapeptide that is both hydrophilic (to increase fusion protein solubility) and detectable with commercially available monoclonal antibodies (Hopp et al. 1988; for review, see Einhauer and Jungbauer 2001). Several different types of anti-Flag antibodies (called M1, M2, or M5) are marketed either for detection (e.g., using immunoblotting) or as immobilized forms for immunoprecipitation. The five carboxy-terminal amino acids of Flag constitute a recognition site for the protease enterokinase, which serves as a convenient means for removing the carrier peptide from the target protein, as further discussed below (Dykes et al. 1988; Hopp et al. 1988). However, the prohibitive costs of monoclonal antibodies render epitope tags impractical for large-scale protein purification.

Another major use of fusion proteins is to add signal peptides for protein translocation. Often, the oxidizing environment and chaperones of the periplasmic space of *E. coli* or endoplasmic reticulum (ER) of insect and yeast cells assist correct disulfide bond formation and folding. Furthermore, subcellular trafficking through the endoplasmic reticulum and Golgi network is required for *N*-linked glycosylation of proteins in eukaryotic expression systems. The natural signal peptide often appropriately directs secretion and processing of a recombinant protein expressed in a heterologous host (e.g., mammalian signals in insect cells) (Talmadge et al. 1980; Gray et al. 1985; Jarvis et al. 1993). However, the abilities of individual sequences to facilitate protein translocation and to be appropriately cleaved by the signal peptidase vary widely and may be less efficient in different expression systems (e.g., plant or bacterial signals may be inefficient in insect cells) (Iacono-Connors et al. 1990; Vernet et al. 1990). To avoid problems, a foreign protein's own signal peptide may be required with a well-characterized signal peptide from the expression vector. Popular examples of signal peptides used for recombinant protein localization include those derived from *Erwinia carotovora* pectate lyase B (pelB) (Keen and Tamaki 1986) or the *E. coli* outer membrane protein 3a (OmpA) (Ghrayeb et al. 1984) for *E. coli* hosts, the GP67 baculovirus glycoprotein (Murphy et al. 1993) or honeybee melittin (Tessier et al. 1991) in baculovirus systems, and the *S. cerevisiae* α -mating factor for expression in *Pichia* (Table 2) (Cregg 2007). Nevertheless, the efficiency of signal peptide translocation and cleavage depends on the amino-terminal structure and sequence of the fused target protein, so that further optimization still may be necessary.

Cleavage of Fusion Proteins

To obtain the polypeptide of interest in a native and biologically active form, it is often preferable to cleave the tag from the remainder of the fusion protein. Unlike signal peptides, which are normally cleaved by an endogenous signal peptidase following protein translocation, a variety of chemical and enzymatic methods have been developed to cleave the peptide bond between a carrier protein and the protein of interest. In practice, chemical cleavage is of limited utility because most target proteins

contain one or more potential cleavage sites and may be denatured under the harsh reaction conditions. Instead, efficient removal of a tag or carrier protein usually is achieved using site-specific proteases. Accordingly, the polycloning site of most commercially available vectors is located downstream from sequences encoding a carrier protein and a proteolytic cleavage site (Table 2). Several site-specific proteases commonly used to cleave fusion proteins are summarized in Table 4.

Usually several foreign amino acids remain attached to the amino terminus of the target protein after cleavage. A few proteases, such as enterokinase or Factor Xa, do not require substantial amino acids beyond the carboxyl terminus of the cleavage site. HRV-3C, TEV, and most other specific proteases leave 1–2 amino acids at the carboxyl terminus of the cleavage site, yet often are preferred because of their high sequence specificity and availability in recombinant, tagged form. Moreover, the restriction sites or recombination sites used for cloning into the vector frequently introduce additional amino acids into the target protein. In certain cases, the restriction sites can be carefully chosen to correspond to the sequences recognized by the protease. For example, the BamHI site encodes Gly-Ser and thus can be conveniently positioned at the carboxyl terminus of the thrombin and TEV cleavage sites. In practice, the presence of a few additional amino acids (usually at the amino terminus of the cleaved target protein) does not interfere to a significant extent with protein function. Nevertheless, it remains important to establish that the recombinant protein behaves in a similar manner to the protein composed solely of the native sequence.

In addition to the potential problems associated with introducing extra amino acids at the terminus of the target protein, three other issues are associated with removal of the fusion tag. First, the activity of a particular protease varies for each fusion protein. With many fusion proteins, the efficiency of the proteolytic cleavage reaction may be poor if the protease site is occluded in the context of the folded structure. In these cases, the protease may be induced to cleave once the fusion protein has been partially denatured or after a “spacer” has been inserted at the cost of artificially extending the amino terminus of the target protein. Second, either because the specificity of the protease is not absolute or because the enzyme used for cleavage is contaminated by other proteases, the protein of interest may be cleaved internally at sites that are related to the recognition site (Nagai and Thogersen 1984, 1987; Dykes et al. 1988; Lauritzen et al. 1991). Problems of this type can sometimes be solved by adjusting the digestion conditions (time, temperature, or protease-to-protein ratio). Otherwise, it may be necessary to choose a different protease for cleavage following appropriate adjustments to the expression construct. Third, the cleaved target protein must be isolated from the contaminating carrier protein and the protease. For this purpose, it is often convenient to choose a protease with a fusion tag that matches the carrier on the protein of interest. In this way, both protease and carrier protein can be removed by a single subtractive pass through the affinity resin. The 6xHis- and/or GST-tagged versions of HRV 3C or TEV proteases may be produced in *E. coli* or purchased from several sources. Although tagged versions of multichain, disulfide-linked proteases such as Factor Xa, thrombin, or enterokinase are more challenging to produce, traditional chromatography or immunological methods can be used to remove these proteases from the protein of interest.

OPTIMIZATION OF EXPRESSION OF FOREIGN PROTEINS

Optimization of Translation Efficiency: Initiation

Efficient initiation of translation requires a ribosome-binding site upstream of the initiation codon (Huttenhofer and Noller 1994). For example, during translation initiation in *E. coli*, the Shine–Dalgarno sequence, 5–9 nt in length, interacts with the 3′ end of 16S RNA (Shine and Dalgarno 1974; Steitz and Jakes 1975; Steitz 1979). The distance between the Shine–Dalgarno sequence and the initiating ATG codon affects translation efficiency. In vectors in which an ORF is inserted downstream from an initiating ATG, the distance between the Shine–Dalgarno sequence and the initiating codon will already have been optimized. However, if translation of the cloned ORF is initiated at its own

ATG, then the initiating codon should be positioned 5–7 nt downstream from the Shine–Dalgarno sequence (Ringquist et al. 1992).

The secondary structure of the translation initiation region also affects the efficiency of gene expression (de Smit and van Duin 1994a,b). In several cases, changing the sequences upstream and downstream from the Shine–Dalgarno sequence to reduce secondary structure has increased gene expression (e.g., see Chen et al. 1994). Similarly, translational coupling, which involves placing the coding region of the gene of interest downstream from a translated sequence, has led to high-level expression of several genes (e.g., see Makoff and Smallwood 1990; Rangwala et al. 1992). Most commercially available vectors contain appropriately spaced sequences for translation initiation. However, if the 5′ region of the gene of interest is predicted to contain significant secondary structure, it may be possible to increase expression by mutagenesis to decrease this secondary structure, or by fusion with a highly expressed carrier protein.

Codon Usage

The genetic code is redundant, using 61 codons to specify 20 amino acids. Only two amino acids (Met and Trp) are specified by a single codon, whereas the remaining 18 are each specified by multiple codons. The synonymous codons that specify a single amino acid are not used with equal frequency (Grantham et al. 1980a,b, 1981).

If the coding region contains a high level or a cluster of rare codons, removal of the rare codons by resynthesis of the gene or by mutagenesis can increase expression (Rangwala et al. 1992). In *E. coli*, particular problems have been observed when the target sequence contains the rare codons AGA and AGG because they can form fortuitous Shine–Dalgarno sequences within the coding region (Ivanov et al. 1992).

The DNA sequence encoding the amino terminus of the foreign protein can dramatically influence expression levels (Barnes et al. 1991; Bradshaw et al. 1998). For this reason, it is sometimes necessary to use the PCR or site-directed mutagenesis to change the DNA sequences of the first seven or eight codons of the expressed gene to those most frequently used in the particular expression system (Bennetzen and Hall 1982). Wherever possible, the (G+C) content of the 5′ end of the target gene should be reduced to <45% by these procedures.

Optimization of Growth Conditions

Irrespective of the expression system used, dramatic differences in expression levels are often realized in different media (Weickert et al. 1996). For example, a standard medium such as LB for *E. coli* can be used to establish the general parameters of expression, but optimum expression is usually achieved only after modification of the growth conditions, which may include using a minimal salts medium such as M9, defined salts medium, or rich media such as Terrific Broth, yeast tryptone (YT), or NZCYM. Similarly, minimal media such as minimal glycerol (MGY) and minimal medium (MM) are useful to establish expression of a heterologous protein in *P. pastoris*, whereas fine-tuning of pH (in buffered medium), temperature, and time almost always enhances yield and quality of the protein. For protein expression using baculovirus-infected insect cells, the time and multiplicity of infection are important parameters to optimize. For further information, see the Troubleshooting sections at the ends of the protocols on protein expression (Protocol: Expression of Cloned Genes in *E. coli* Using IPTG-Inducible Promoters [Kielkopf et al. 2021a], Protocol: Expression of Cloned Genes Using the Baculovirus Expression System [Kielkopf et al. 2020a], and Protocol: Expression of Cloned Genes in *Pichia pastoris* Using the Methanol-Inducible Promoter AOX1 [Kielkopf et al. 2021c]).

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