Chapter 2

Process Optimization for Recombinant Protein Expression in Insect Cells

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Additional information is available at the end of the chapter

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Abstract

Insect cells can be used for the efficient production of heterologous proteins. The baculovirus expression vector system (BEVS) in *Spodoptera frugiperda* cells and the stable transformation of *Drosophila melanogaster* S2 cells are widely used for this purpose. Whereas BEVS is a transient expression system for rapid protein production, stable *D. melanogaster* cell lines are compatible with more complex processes modes. This chapter describes the setup of both systems, including steps for the generation of expression vectors and comprehensive optimization approaches. The genetic elements available in each system are described, as well as the use of different cloning and transfection methods and advanced process monitoring to achieve robust protein expression in larger-scale bioreactors.

Keywords: heterologous protein expression, BEVS, stably transformed *Drosophila melanogaster* S2 cells, recombinant protein expression, insect cell

1. Introduction

The cultivation of recombinant insect cell lines has been the subject of intense research since the 1980s and also allows the industrial production of recombinant proteins, vaccines and insecticides [1–4]. Since the first insect cell line was isolated in 1963, more than 500 different cell lines have become available [5, 6]. The most common expression systems are based on cell lines derived from *Spodoptera frugiperda* (Sf-9 and Sf-21), *Trichoplusia ni* (BTI-TN-5B1-4, marketed as High Five™) and *Drosophila melanogaster* (S2). The *S. frugiperda* and *T. ni* cell lines are used with the baculovirus expression vector system (BEVS) [7], which is the gold standard for protein production in insect cells. More recently, stably transformed *Drosophila* S2 cell lines have been used to express a wide variety of proteins [8]. In all cases, it is necessary to optimize protein

expression as well as the bioprocess conditions to achieve a robust and efficient upstream process. This includes the selection of suitable genetic elements, aspects of glyco-engineering, comprehensive screening for highly productive cell lines, appropriate aeration and mixing strategies and the selection of a robust process mode. Online analytical methods can be used to gain fundamental insights into the physiological state of the cells during the production process, with dielectric spectroscopy and online optical density measurements providing good results. In this chapter, we provide comprehensive, interdisciplinary guidance for the optimization of each process step and the upstream process as a whole.

2. Optimizing protein expression at the cellular level

2.1. Engineering the glycosylation profiles of insect cells

Many therapeutic proteins require a specific glycosylation pattern, so this aspect is a major issue when using insect cell lines for protein production. Generally, cell lines from *S. frugiperda, T. ni* and *D. melanogaster* can synthesize N-linked glycans. However, insect cells form shorter and less complex N-glycan structures than mammalian cells (**Figure 1**).

At least in some part, the alterations in the glycan patterns are of evolutionary origin. Studies revealed mutations in some enzymes of the apparatus for the protein glycosylation (e.g. transferases), occurring during the evolutionary split of vertebrates from invertebrates, 500 million years ago. This led to the future formation of distinct glycan patterns for the species [9].

In general, glyco-structures in insects thus formed in different patterns than in mammals. However, the complexity of the glycans is altered over the developmental stages in the insect [10], clearly hinting, that the glycans are involved in the development. The insect-derived cell lines do not express the respective enzymes in quantity and show therefore less complex glycosylation patterns [11].

The glycan structures of insect cells are mostly oligomannose or paucimannose forms with core fucose structures but no terminal sialic acid residues [12, 13]. In addition to human-like α1,6-linked fucose, insect-derived proteins may also carry a α1,3-linked fucose, which can induce immunogenic and allergic responses in humans [14]. About 70% of therapeutic proteins contain N-glycans and these structures can influence protein activity and tolerability, so the inability of insect cells to synthesize human-like glycosylation profiles is a disadvantage [15]. Several strategies have therefore been developed to address the issue of incomplete or incompatible N-glycosylation in insect cell expression systems.

The truncated paucimannose structures in *D. melanogaster* S2 cells were found to originate from an acetylglucosamidase (GlcNAcase) activity, which removes terminal N acetylglucosamine (GlcNAc) residues. The GlcNAcase was suppressed [16] and the simultaneous expression of a galactosyltransferase (GalT) resulted in more complex but still unsialylated and heterogeneous glycans [17].

The BVES can be improved by using genetically engineered host cells and/or baculoviruses (**Figure 1**). Cell lines have been modified to express recombinant glucosaminyltransferases,

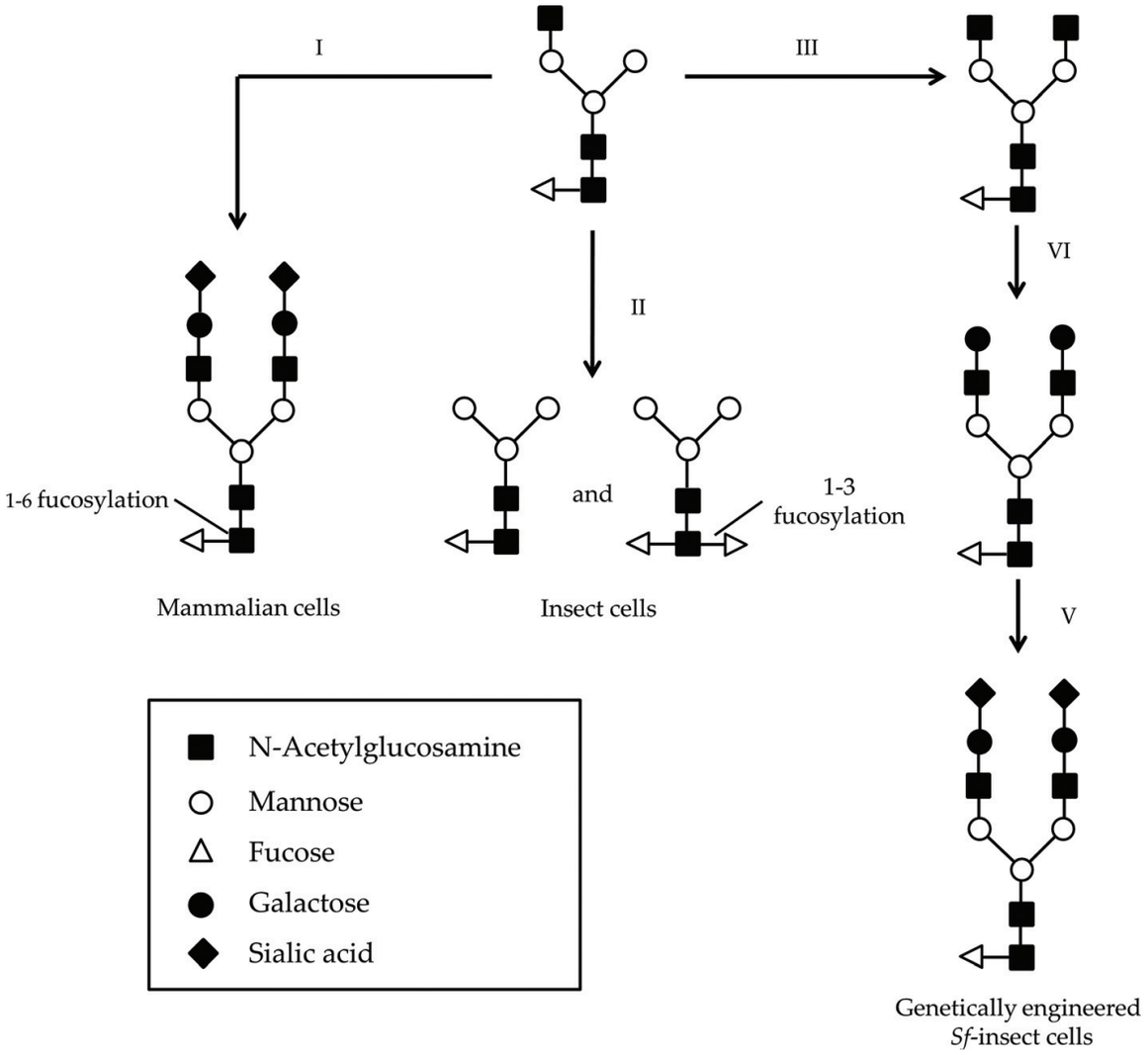

Figure 1. N-glycan profiles in different expression hosts. Mammalian and insect glycans share a common precursor. Mammalian cells (left branch) elaborate this precursor using N-acetylglucosaminyltransferase (I) and other enzymes. In the middle branch, the native paucimannose glycans of insect cells are synthesized by N-acetylglucosaminidase. Insect proteins typically contain both 1,3-linked and 1,6-linked core fucose. Genetically engineered insect cell lines (right branch) can express α-1,6-mannosylglycoprotein 2-β-N-acetylglucosaminyltransferase (III), β-galactosyltrasferase 1 (VI) and β-galactoside α-2,6-sialyltransferase. The symbols were used as previously defined [25]. The figure is based on earlier publications [13, 18].

galactosyltransferases and sialyltransferases. In the presence of the sialic acid precursor acetyl-D-mannosamine, human-like glycans can be synthesized in these cells [13, 18]. In order to reduce the metabolic burden, the cells can also be equipped with inducible promoters so that the mammalian glycosylation machinery can easily be switched on and off [19]. Initial attempts to produce mammalian glycans in the BEVS required the co-infection of insect cells with baculoviruses encoding the target protein and the enzymes required for glycosylation [20]. The yields of protein were low due to the relatively small statistical likelihood of co-infection, so alternative BEVSs have been developed such as the SweetBac® technique, in which the glycan-generating enzymes are carried on a separate 'glyco-module'. Although this simultaneous expression reduces the yield compared to traditional insect expression systems,

the previously established cell line *Tnao*38 can achieve results comparable to transient expression in mammalian cells [21, 22]. Additionally, the synthesis of core fucose can be prevented by using a baculovirus encoding an enzyme that diverts the precursor molecule into other pathways [23].

The ability of insect cells to synthesize O-linked glycan structures has not been explored in detail. Insect O-glycosylation profiles are less diverse than those produced by mammals, but the precise nature of these structures depends on the cell line and culture medium [24].

2.2. Transient expression using BEVS

2.2.1. General procedure for recombinant protein expression using BEVS

BEVS is based on the use of insect viruses known as baculoviruses (family baculoviridae) that are rod-shaped dsDNA viruses that infect lepidopteran species. The best characterized baculovirus is *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV). Its life cycle comprises two phases leading to different phenotypes [26]. Type 1 is known as a budded virus, which is enveloped with parts of the host cell membrane. After its release from the host cell between the early and late phases of the infection, the budded virus can spread and infect neighbouring cells. Type 2 is known as the occlusion-derived virus and is produced in the very late infection phase when the viral protein polyhedrin accumulates in the host cell and forms so-called occlusion bodies. Polyhedrin expression is controlled by the strongest viral promoter, the *polh* promoter. The promoters used for heterologous gene expression with BEVS are usually viral promoters, and these can be classed as early, late and very late promoters according to the timing of their activity post-infection. Early promoters are active directly after infection because they only require the host cell RNA polymerase. Early promoters drive the expression of genes encoding viral transcription factors and polymerases, which are in turn necessary for the expression of late genes. Very late genes, like polyhedrin, are even more active than the late genes, and are necessary for virus packaging. The *polh* promoter is the most widely used in BEVS because of its very high activity and due to the fact that occlusion body formation (and thus polyhedrin itself) is not necessary for baculovirus propagation in cell culture.

Recombinant protein expression using BEVS involves five different states, which are summarized in **Figure 2** and explained in more detail in the following sections. Many different products used in (veterinary) medicine have been produced using BEVS and selected examples are listed in **Table 1**.

2.2.1.1. Cloning the transfer vector

In the first step, the gene of interest (GOI) must be integrated into a transfer vector. This can be achieved using classic insertion-ligation technology, or state-of-the-art techniques such as golden gate cloning. Depending on which kit is used to generate the recombinant baculovirus, different transfer vectors are provided by the manufacturers. The most important differences among these vectors are the promoter system, the protein tag and the secretion signal. More information about the different kits and genetic elements can be found in Section 2.2.2.

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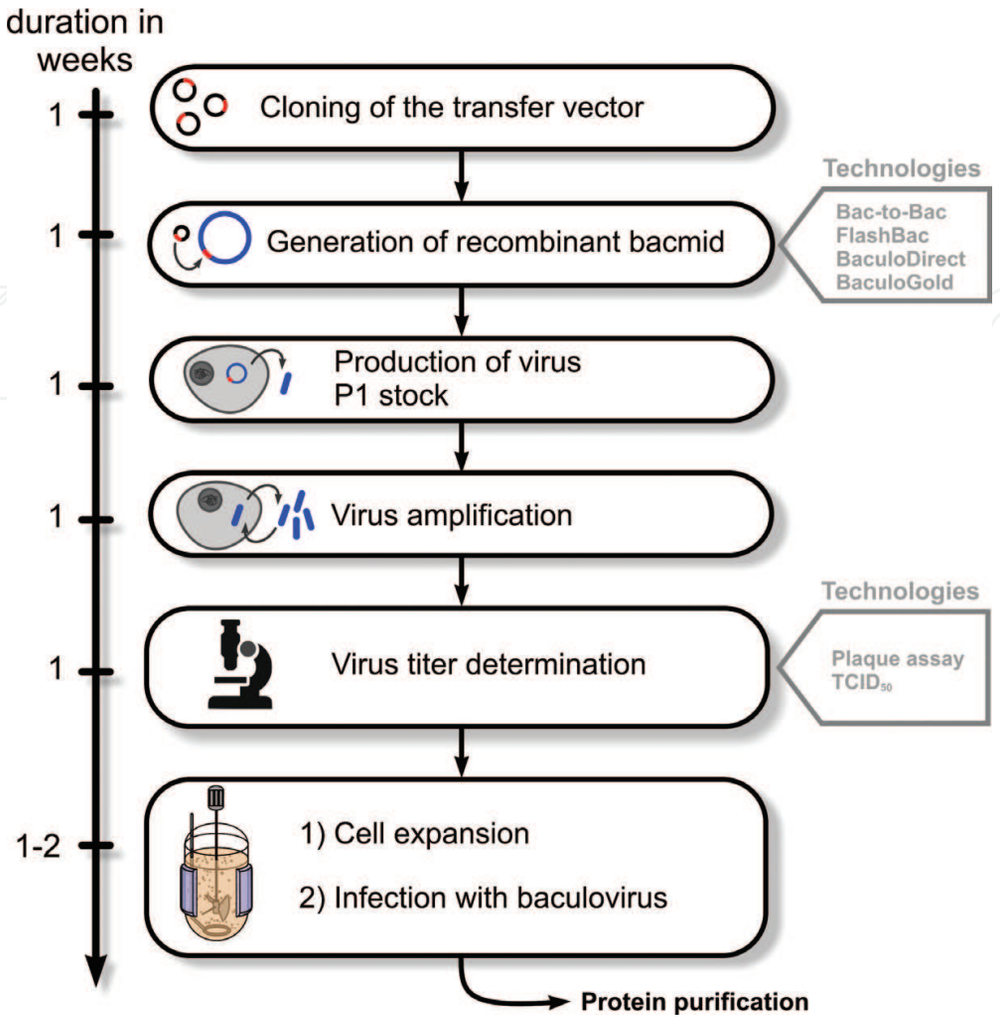

Figure 2. The process chain for recombinant protein production using BEVS.

Application	Product name	Company	Stage	References
For human use				
Cervical cancer	CERVARIX®	GSK	Approved	[27]
Prostate cancer	PROVENGE®	Dendreon	Approved	[28]
Influenza	FluBlok®	Protein Sciences	Approved	[29, 30]
Influenza	A/H5N1 Virus-like particle	Novavax	Phase I (NCT01596725)	[31]
For veterinary use				
Procrine circovirus 2 (PCV2)	Porcilis® PCV	Merck	Approved	[32]
PCV2	CircoFLEX®	Boehringer Ingelheim	Approved	[33]
Swine fever	Porcilis Pesti®	Merck	Approved	[34]

Table 1. Selected human and veterinary vaccines produced using BEVS.

2.2.1.2. Generation of a recombinant bacmid

When the transfer vector is ready, the desired parts need to be integrated into the baculovirus genome, resulting in a recombinant bacmid. Several commercial kits are available for this step, including the Bac-to-Bac® Baculovirus Expression System (Thermo Fisher Scientific), flashBAC (Oxford Expression Technologies), the BaculoDirect™ Baculovirus Expression System (Thermo Fisher Scientific) and BaculoGold™ (BD Biosciences).

2.2.1.3. Virus production

The recombinant bacmid DNA is then transfected into insect cells (Sf9, Sf21 or HighFive™) for virus production. The cells are usually transfected in a six-well plate (2 mL culture volume) to prepare the initial virus stock (P1 stock), which typically has a titre of 10^3–10^5 plaque forming units (pfu)/mL. Transfection can be achieved using the chemical and physical methods described in the following sections.

2.2.1.3.1. Calcium phosphate–DNA co-precipitation

One of the oldest transfection methods is calcium phosphate-DNA co-precipitation [35] which was adapted for insect cell lines in the 1980s [36]. Mixing calcium chloride with a phosphate-buffered DNA-containing solution results in the formation of a fine calcium phosphate/DNA co-precipitate that binds to the cell surface and penetrates the cells by endocytosis.

2.2.1.3.2. Lipid-mediated and polymer-mediated transfection

In the late 1980s, a transfection method was developed based on the synthetic positively charged lipid N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). The cationic head groups of DOTMA interact with the anionic phosphate backbone of DNA to form a complex can bind to the cell membrane and probably taken up by endocytosis [37]. Many different lipid formulations are available to achieve highly efficient transduction, including BaculoPORTER (Biocat), Cellfectin, Cellfectin II and Lipofectin (all Thermo Fisher Scientific).

Various non-lipid transfection reagents can also form complexes with DNA, including baculoFECTIN II and flashFECTIN (Oxford Expression Technologies), FuGENE 6 (Promega), GeneJuice® (Merck Millipore) and *Trans*IT®-Insect (Mirus Bioscience).

2.2.1.3.3. Electroporation

Electroporation is a convenient and efficient transfection method, but specialized equipment is required [38]. It is based on a short electrical pulse at an optimal voltage (specific for each cell line) to from transient pores in the plasma membrane. This in turn facilitates the intake of small molecules such as DNA, RNA or proteins [39].

2.2.1.4. Amplification

Following the generation of the P1 virus stock, the virus needs to be amplified to increase the titre and culture volume. The titre of the P2 stock is typically ≥10^8 pfu/mL. Insect cells are cultivated in shaker flasks with a culture volume of 50 mL. For industrial applications, virus

stocks with an even higher volume are necessary, so P3 stocks are generated using bioreactors. In the authors' laboratory, a low multiplicity of infection (MOI) of 0.01 pfu/mL and a cell density of 1×10^6 cells/mL are used for virus amplification.

2.2.1.5. Virus titre determination

The titre of infectious virus particles must be determined before the viruses can be used for protein expression. Two methods have been established for this purpose, namely the plaque assay and end-point dilution.

2.2.1.5.1. Plaque assay

The plaque assay determines the number of plaque-forming units, each of which is equivalent to a single infectious particle [40]. The infection of adherent insect cells with a highly diluted virus solution leads to plaque formation in the cell monolayer due to cell lysis caused by one infective virus particle. With an agarose overlay virus spreading is circumvented. Using neutral red, viable cells can be stained and white plaques appear when a cell has been lysed due to infection.

2.2.1.5.2. End-point dilution

End-point dilution is used to determine the infectious dose that leads to the infection of 50% of the cells, known as the 50% tissue culture infective dose ($TCID_{50}$) [40]. Cultured cells are infected with different virus concentrations and the number of infected and non-infected cultures is counted. For accurate titre determinations, 12 replicates of eight virus dilutions are analysed in the authors' laboratory. The readout can be conducted at 5 or 7 days post-infection. To facilitate evaluation, every baculovirus constructed and prepared in the authors' laboratory carries a green fluorescent protein (GFP) marker cassette driven by the OpIE1 promoter.

2.2.1.5.3. Comparison of plaque assay and end-point dilution

When choosing a titration method, different points need to be considered. Generally, the plaque assay is performed in six-well plates resulting in a high cell concentration needed to seed the cells confluent. Due to the plate format, only a few virus dilutions can be analysed and a low number of replicates are possible using one six-well plate resulting in the need of experienced experimenters knowing the suitable virus dilutions. In comparison to that, the end-point dilution method can be done in 96-well plates resulting in more virus dilutions and replicates, which can be analysed using one plate. Moreover, the cells can be seeded sub-confluent as they are not covered with agarose, which limits the cell growth. For the titre evaluation using the end-point dilution, it is important to remember that genomic integrations of marker proteins into the viral genome simplify the evaluation.

2.2.1.6. Protein expression

The protein expression step can be divided into two stages: cell expansion and infection. In the first step, the bioreactor is inoculated with a low cell concentration, and at the desired time of infection (TOI), the baculovirus stock is added to the cell suspension.

Industrial baculovirus-based processes use a volume of 10–100 m^3 [41]. Because of this size, the MOI strongly influences the virus volume required for one production cycle. Ideally the baculovirus stocks last for the whole process lifetime. A MOI > 1 statistically ensures the infection of all insect cells immediately after virus addition (see chapter 2.2.3). Using a lower MOI results in a two-stage infection process. In the first phase, only a proportion of cells are infected. These cells start to produce the recombinant protein and also produce new virus particles. In the second infection phase, these new virus particles infect further cells (**Figure 3**). A second effect of a low MOI is that the uninfected of the first infection phase continue to proliferate, resulting in a higher cell density at the start of the second infection phase.

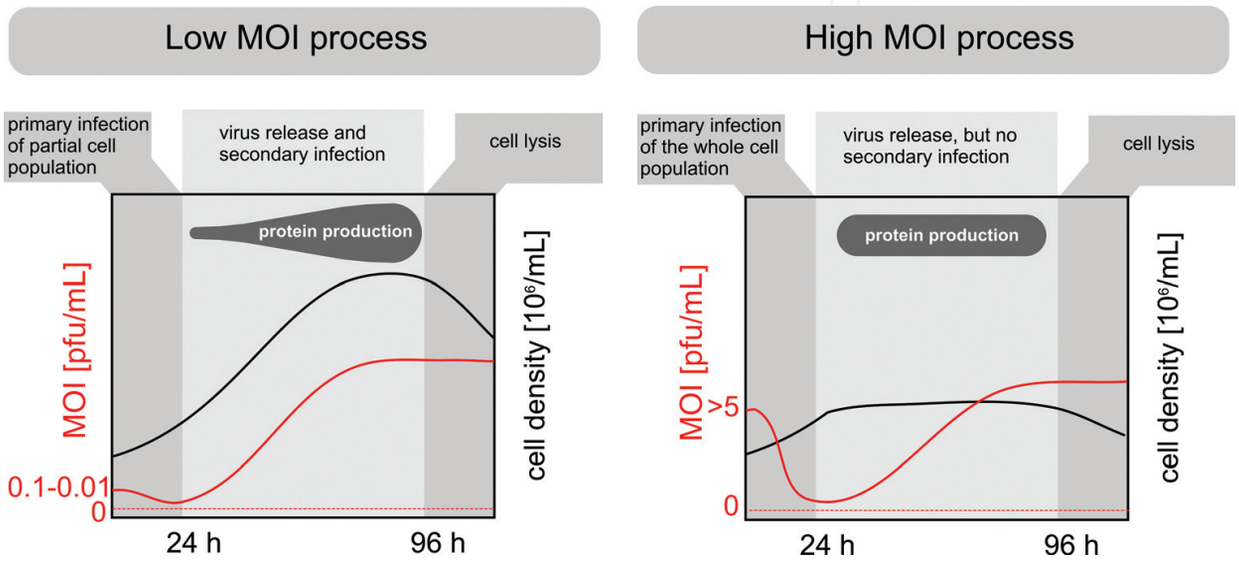

Figure 3. A comparison of BEVS processes with low and high MOI, respectively. The low MOI process is divided into two phases, whereas the high MOI process has only one phase.

2.2.1.7. Protein purification

The final step of the BEVS process is protein purification. Depending on the secretion signal (see Section 2.2.2.4), tag and characteristics such as protein size and pI, different purification methods can be used, many based on various forms of chromatography. Detailed coverage of this topic is beyond the scope of this chapter and the reader is referred to previous review articles [42, 43].

2.2.2. Historical overview and application of current BEVS kits

The BEVS patented by Max Summers and Gale Smith in 1983 (US Patent 4,745,051) used homologous recombination to integrate the GOI into a polyhedrin locus of the baculovirus genome. As stated above, polyhedrin is not essential for virus replication in cultured cells because its function is to form the occlusion bodies that protect the virus against UV light and high temperatures during the natural infection cycle. The original recombination-based method required two crossovers to integrate the GOI, which occurred at a low frequency

(~0.1%). This drawback was addressed by inserting three BsuI36 restriction sites and a *lac*Z cassette into the baculovirus genome [44]. Digestion with Bsu36I linearized the virus DNA (called BacPAK6) and co-transfection of the linearized virus DNA and transfer plasmid was followed by homologous recombination to restore the circularity of the virus DNA, leading to the replication of recombinant viruses. BacPAK6 was optimized by deleting the chitinase gene (*chi*A) that inhibits the secretory pathway in insects, resulting in higher protein concentrations when the product is membrane targeted or secreted. This kit is marketed by Oxford Expression Technologies as BacPAK6-Sec+.

To enable the replication of baculovirus DNA in bacteria, a bacterial artificial chromosome (BAC) was integrated into BacPAK6 to produce a bacmid vector. This was initially marketed by Oxford Expression Technologies as the flashBAC™ system (Patent no. EP1144666). After homologous recombination with the transfer plasmid, the BAC is replaced by the GOI, the essential ORF 1629 is restored and the GOI is expressed under the control of the *polh* promoter. Variants of the flashBAC system are available in the flashBAC GOLD, flashBAC ULTRA and flashBAC PRIME kits. If less protease activity is required, the flashBAC GOLD system is most suitable because the *v-cath* gene is deleted in addition to *chiA*. The flashBAC ULTRA system improves the protein yield and quality by deleting the p10, p26 and p74 genes in addition to *v-cath* and *chiA*. The deletion of p10 increases the *polh* promoter activity, the function of p26 is unknown but deletion does not inhibit viral replication and p74 is only required for virus attachment and fusion in midgut cells *in vivo* so its deletion improves biosafety [45]. In contrast to the kits described above, flashBAC PRIME does not carry any genetic modifications in the viral backbone resulting in cell lysis due to infection, facilitating the release and subsequent purification of more complex products such as virus-like particles (VLPs) or proteins formed in the cytoplasm or nucleus.

BD Biosciences markets linearized baculovirus DNA kits as BaculoGold™ and BaculoGold™ Bright. The manual that comes with each kit states that an essential gene is deleted (the gene is not specified), and BaculoGold™ Bright also carries a GFP marker gene allowing the detection of infected insect cells by fluorescence analysis. Another variant is the vEHuni baculovirus DNA, which features a *D. melanogaster* hsp70 promoter and a multiple cloning site with two Bsu36I sites integrated into the non-essential ecdysteroid UDP-glucosyltransferase (*egt*) gene, allowing the expression of diverse products and the production of baculovirus expression libraries [46]. Similarly, the vECuni baculovirus DNA carries a hybrid promoter consisting of $P_{capminXIV}$ and *polh* elements.

Thermo Fisher Scientific distributes the BaculoDirect™ kit that incorporates Gateway® cloning technology. Following the integration of the GOI into the Gateway® Entry Clone, *in vitro* LR recombination leads to the integration of the GOI into the BaculoDirect™ DNA, replacing the herpes simplex virus type 1 thymidine kinase negative selection marker. When insect cells are transfected with the recombinant vector and cultivated in the presence of ganciclovir, only recombinant baculoviruses are produced [47]. The BaculoDirect™ DNA can be combined with C-terminal or N-terminal V5 and His_6 tags. In the latter case, the tags are followed by a tobacco etch virus (TEV) protease cleavage site. BaculoDirect™ DNA is also available with an N-terminal glutathione-S-transferase (GST) fusion tag.

Recombinant baculoviruses can also be generated using transposon activity which is marketed as the Bac-to-Bac® system (Thermo Fisher Scientific). The baculovirus DNA in this kit also contains an integrated BAC, an antibiotic resistance gene, a *lac*Z cassette and an attachment site for the bacterial transposon Tn*7*. The cloning and amplification of recombinant viral DNA is therefore carried out in *Escherichia coli*. The corresponding transfer plasmid consists of the GOI flanked by two mini-transposon sites (Tn*7R* and Tn*7L*). The *E. coli* strain provided in the kit (DH10Bac) carries a helper plasmid providing all necessary transposon system elements. Following the transformation of the DH10Bac strain, clones carrying recombinant bacmid can be identified by blue/white screening and PCR analysis (The different kits discussed (see also **Table 2**) above are compatible…) [205].

The different kits discussed above are compatible with different transfer plasmids that are available from the same suppliers. Because most baculovirus DNA modifications are integrated at the *polh* locus by homologous recombination, common transfer plasmids facilitate gene expression under *polh* promoter control. Some transfer plasmids designed by BD Biosciences and Thermo Fisher Scientific also incorporate more than one promoter to allow multiple protein expression from the same recombinant virus. It is also possible to integrate the promoter into the transfer plasmid together with the GOI. Some of the commercially available transfer plasmids also include protein tags (Section 2.2.2.2) to facilitate protein purification and detection. If the tag is located upstream of the GOI, a protease cleavage site is integrated (Section 2.2.2.3). Secretion signals may also be integrated into the transfer plasmids (Section 2.2.2.4).

2.2.2.1. Promoters suitable for BEVS

The *Ac*MNPV-derived immediate early (IE-1) promoter is used in several commercially available kits. The activity of this promoter is rather low but protein expression starts immediately after infection, allowing more time for post-translational modifications that are necessary for the function of many complex proteins. This promoter can also be used for transient expression because it is active in the absence of other viral factors. To enhance recombinant protein expression, the IE-1 promoter can be combined with the homologous region 5 (*hr5*) enhancer [48].

The late protein p6.9, also known as basic protein, core protein or VP12 [40], is essential for the production of infectious baculoviruses because it mediates viral DNA condensation and packaging [49]. The expression of enhanced GFP under the control of the *p6.9* promoter could be detected as early as 6 hours post-infection, therefore also allowing more time for post-translational modifications than the very-late promoters.

The very-late *p10* and *polh* genes produce the very-late 10 kDa protein and polyhedrin, respectively. These are highly active promoters and are used in many BEVS kits, but they depend on proteins translated in earlier phases of the infection for their activity. The activity of the *polh* promoter can be increased even further if p10 expression is abolished [50].

2.2.2.2. Purification tags used in BEVS

Protein tags can be used to simplify the detection and purification of recombinant proteins produced using BEVS. Common tags include His_6 and GST. The His_6 tag comprises six

Kit name	Main component	Integration site	Special features	Manufacturer
BacPAK6	Linearized baculovirus DNA	*polh* locus	Bsu36I restriction sites within ORF 603, ORF 1629 (essential gene) and *lac*Z cassette	Oxford Expression Technologies
BacPAK6-Sec+	Linearized baculovirus DNA	*polh* locus	Deletion of chitinase gene (*chi*A) → expression of membrane targeted and secreted proteins	Oxford Expression Technologies
flashBAC™	Linearized baculovirus DNA	*polh* locus	Deletion of chitinase gene (*chi*A), integration of BAC	Oxford Expression Technologies
flashBAC GOLD™	Linearized baculovirus DNA	*polh* locus	Additional deletion of cathepsin protease (*v-cath*) → less protease activity	Oxford Expression Technologies
flashBAC ULTRA™	Linearized baculovirus DNA	*polh* locus	Deletion of *chi*A, *v-cath, p10, p26* and *p74* → improved yield and quality, expression difficult proteins	Oxford Expression Technologies
flashBAC PRIME™	Linearized baculovirus DNA	*polh* locus	No gene deletion in viral backbone	Oxford Expression Technologies
BaculoDirect	Linearized baculovirus DNA	*polh* locus	Negative selection of non-recombinant baculoviruses using thymidine kinase	Thermo Fisher Scientific
	BaculoDirect™ C term linear DNA	*polh* locus	C-terminal V5 and His_6 tags	Thermo Fisher Scientific
	BaculoDirect™ N term linear DNA	*polh* locus	Tobacco etch virus (TEV) cleavage site, N-terminal V5 and His_6 tags	Thermo Fisher Scientific
BaculoDirect™ GST Gateway	Linearized baculovirus DNA	*polh* locus	N-terminal GST tag	Thermo Fisher Scientific
BaculoGold™	Linearized baculovirus DNA	*polh* locus	Positive survival selection for recombinant baculovirus	BD Biosciences
	BD BaculoGold Bright linearized baculovirus DNA	*polh* locus		BD Biosciences
	AcRP23.lacZ linearized baculovirus DNA	*polh* locus	Intact *lac*Z gene after integration of GOI behind *polh* promoter	BD Biosciences
	AcUW1.lacZ linearized baculovirus DNA	*p10* locus	Intact *lac*Z gene after integration of GOI behind *p10* promoter	BD Biosciences
	vEHuni baculovirus DNA	*egt* locus	*hsp70* promoter	BD Biosciences
	vECuni Baculovirus DNA	*egt* locus	$P_{capminXIV}$ hybrid late/very late polyhedrin promoter	BD Biosciences
Bac-to-Bac®		*polh* locus	Site-specific transposition used to integrate genes from transfer vector into bacmid DNA using *E. coli* DH10Bac cells	Thermo Fisher Scientific

Abbreviations: *polh* = polyhedrin; *egt* = ecdysteroid UDP-glucosyltransferase.

Table 2. Commercial kits for the production of recombinant baculoviruses.

histidine residues and can be fused to either the N-terminus or C-terminus of proteins. This tag facilitates protein purification using nickel or cobalt ions or anti-histidine antibodies immobilized on a chromatography resin [51]. GST is a 26 kDa protein which is highly soluble and folds rapidly after translation. The tag is often used to increase protein solubility in prokaryotes. The affinity between this enzyme and its substrate means that immobilized glutathione can also be used for protein purification by affinity chromatography.

2.2.2.3. Common cleavage sites in BEVS

The purification tag is often removed from the recombinant protein after purification because its size and unique chemical properties can interfere with protein functions. For this purpose, a TEV protease cleavage site is often placed between the tag and the mature recombinant protein, allowing the tag to be released *in vitro* [52, 53]. Transfer vectors containing a TEV site are marketed by Thermo Fisher Scientific. Alternatively, transfer plasmids with thrombin cleavage sites are marketed by BD Biosciences and Oxford Expression Technologies.

2.2.2.4. Lead sequences to enhance protein secretion in BEVS

To improve the secretion of recombinant proteins produced in insect cells, secretion signals consisting of 15–30 amino acids can be fused to the N-terminus. Common secretion signals include those native to honeybee melittin (HBM) or the baculovirus envelope surface glycoprotein 67 (gp67). Transfer plasmids containing these secretion signals are available from BD Biosciences and LifeSensors. Further, signal peptides that enhance protein secretion have also been described [54].

2.2.3. Enhancing protein yields by optimizing the time and multiplicity of infection

The yields of recombinant proteins produced using BEVS can be enhanced by optimizing parameters such as the inoculum cell concentration, TOI, MOI and time of harvest. Both MOI and TOI are related to the cell concentration and therefore show significant correlation. The effect of low and high MOI on protein expression has been addressed in multiple experiments [55–59] and simulations [60–63]. The infection of a cell by a virus particle can be modelled using a Poisson distribution [61, 64]. The probability that a cell will absorb an infectious particle is therefore given in Equation 1, with n representing the number of absorbed baculovirus particles:

$$p\ (n, MOI) = \frac{MOI^n \cdot e^{-MOI}}{n!} \tag{1}$$

As shown in Equation 2, the proportion of uninfected cells can be estimated when $n = 0$. The proportion of cells infected with at least one virus particle can then be estimated by subtracting this value from 100% (Equation 3).

$$F_{uninfected\ n=0}\ (MOI) = e^{-MOI} \tag{2}$$

$$F_{infected\ with\ n\geq 1}\ (MOI) = 1 - e^{-MOI} \tag{3}$$

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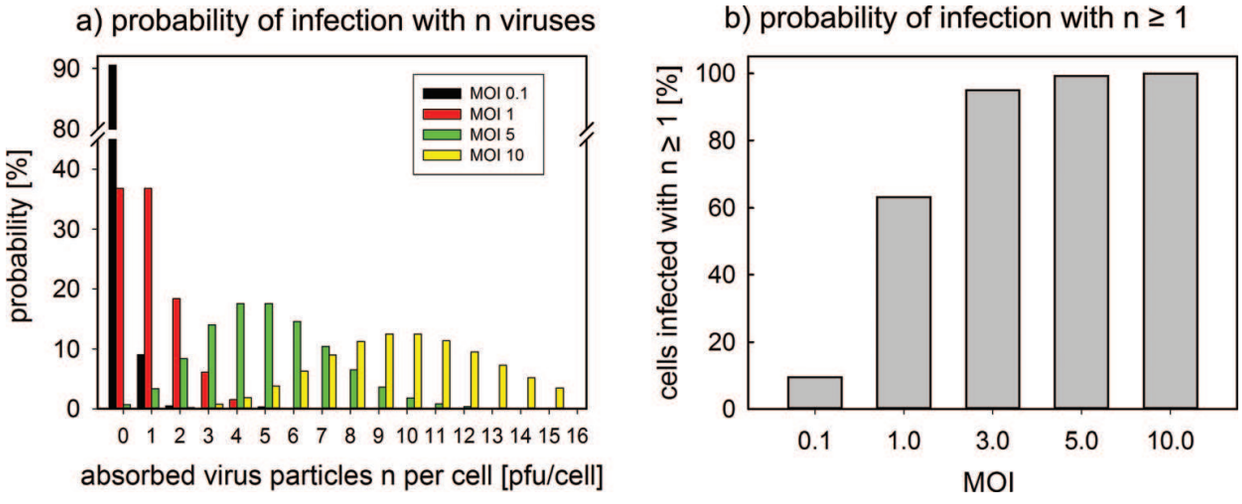

Figure 4. a) The impact of MOI on the number of absorbed virus particles per cell, according to the Poission model. b) The proportion of cells infected with at least one virus particle at different MOI values.

The simplified Poisson approach shown in **Figure 4** indicates that even with an MOI of 1, approximately 37% of the cells remain uninfected. To guarantee the simultaneous infection of all cells, the MOI should be 5 (99.326% infected cells) or 10 (99.995% infected cells).

As described in Section 2.2.1.6, the use of a low MOI for industrial processes has several benefits. A low MOI is easier to achieve and requires smaller volumes of virus stock, which is advantageous in large-scale cell cultures [56, 57]. Fewer virus amplification steps are required thus limiting the negative effects of passaging, such as the increasing proportion of defective viruses after each round of amplification [58]. Infection with a low MOI also results in the proliferation of cells not infected during primary infection, increasing the number of cells available for secondary infection and thus the number of cells producing the recombinant protein [58, 65]. On the other hand, the need for secondary infection also prolongs the process, but this drawback is outweighed by the advantages listed above. The final protein yield is not necessarily lower when starting with a low MOI compared to a high MOI [41, 62, 63, 66]. The early or mid-exponential phase is the optimal TOI when using a low MOI [56, 60, 61] because infection during the late exponential phase can lead to substrate limitation [67].

2.2.4. Virus purification and concentration methods

For laboratory-scale processes, centrifugation as a clarification step can produce virus stocks of sufficient purity and quantity. However, a virus concentration step is necessary for larger-scale processes, or for processes featuring a high MOI or high cell density. Very pure virus stocks are required for pharmaceutical applications such as the manufacture of vaccines [68] or the use of baculovirus vectors for in vivo gene therapy [69, 70], and it is particularly important to reduce host cell proteins and DNA to acceptable levels [71].

If the objective is to purify active virus particles with minimal loss, then the purification method must consider the stability of the virus. Baculovirus stability/activity has been tested against key parameters such as temperature, shear stress, ionic conductivity and pH. The virus is sensitive to high temperatures, i.e. it can be stored at 4°C for several months but higher

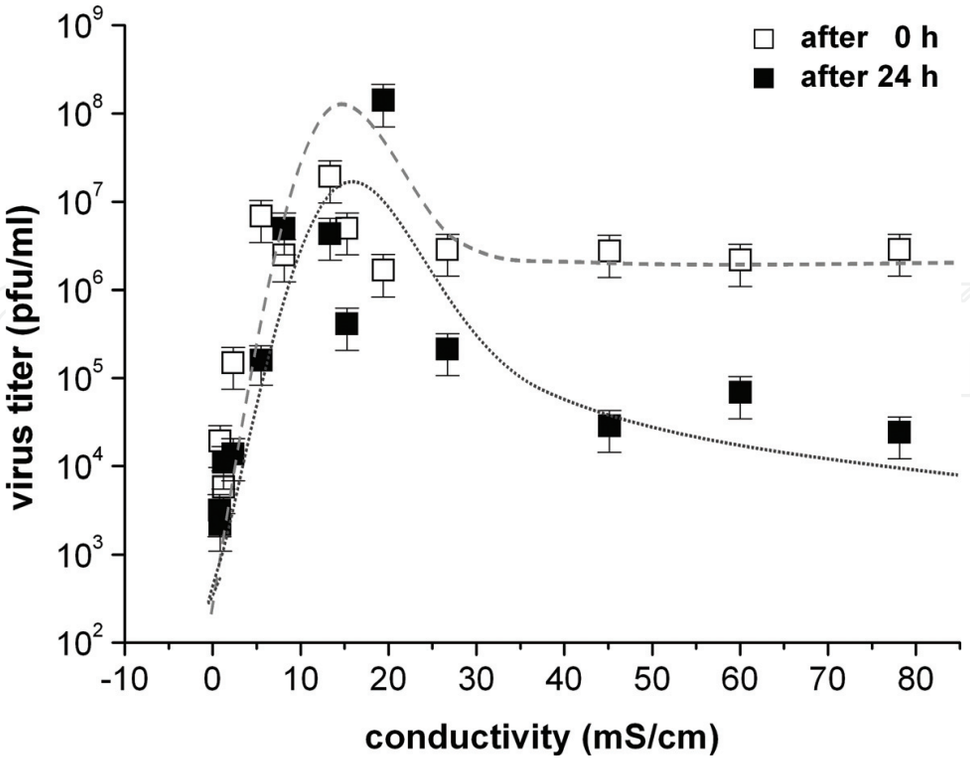

Figure 5. Infectivity of baculovirus particles under conditions of differing conductivity [73].

temperatures (especially above 40°C) induce a rapid decline in activity. The virus is insensitive to shear forces in a peristaltic pump and is stable over the pH range 6–8. The virus is also stable within the conductivity range 8–15 mS/cm (**Figure 5**). However, its activity decreases rapidly when the conductivity falls below 8 mS/cm and higher conductivities up to 80 mS/cm cause an immediate 10-fold loss of infectivity rising to more than 1000-fold if the conditions are prolonged, e.g. for 24 h [72, 73]. This suggests that the virus is sensitive to osmotic pressure but shows that high-conductivity environments up to 80 mS/cm can be tolerated briefly without total loss of activity.

The stability of the virus against shear forces allows it to be concentrated by tangential filtration. Polyethersulfone membranes with cut-offs in the range 100-1,000 kDa can be used to achieve a 20-fold concentration of virus particles at an average of 0.15 bar transmembrane pressure and 25 °C, but there is a considerable loss of flux due to fouling [74]. For medical applications, virus concentration alone does not meet the requirement to reduce the levels of host cell protein and DNA. These contaminants can be removed using ion exchange membranes, e.g. a polyethersulfone membrane with quaternary ammonium ligands achieved a three-fold concentration of virus particles while simultaneously reducing host cell DNA levels by nearly 90% and reducing host cell protein levels below the limit of detection [73]. **Figure 6** shows the virus and protein concentrations at each step of the purification process: adsorption of the viral particle to the column, washing and elution [73].

Another alternative method is monolithic anion exchange chromatography, which can achieve a 50-fold virus concentration while reducing the amount of host cell protein and host cell DNA

http://dx.doi.org/10.5772/67849

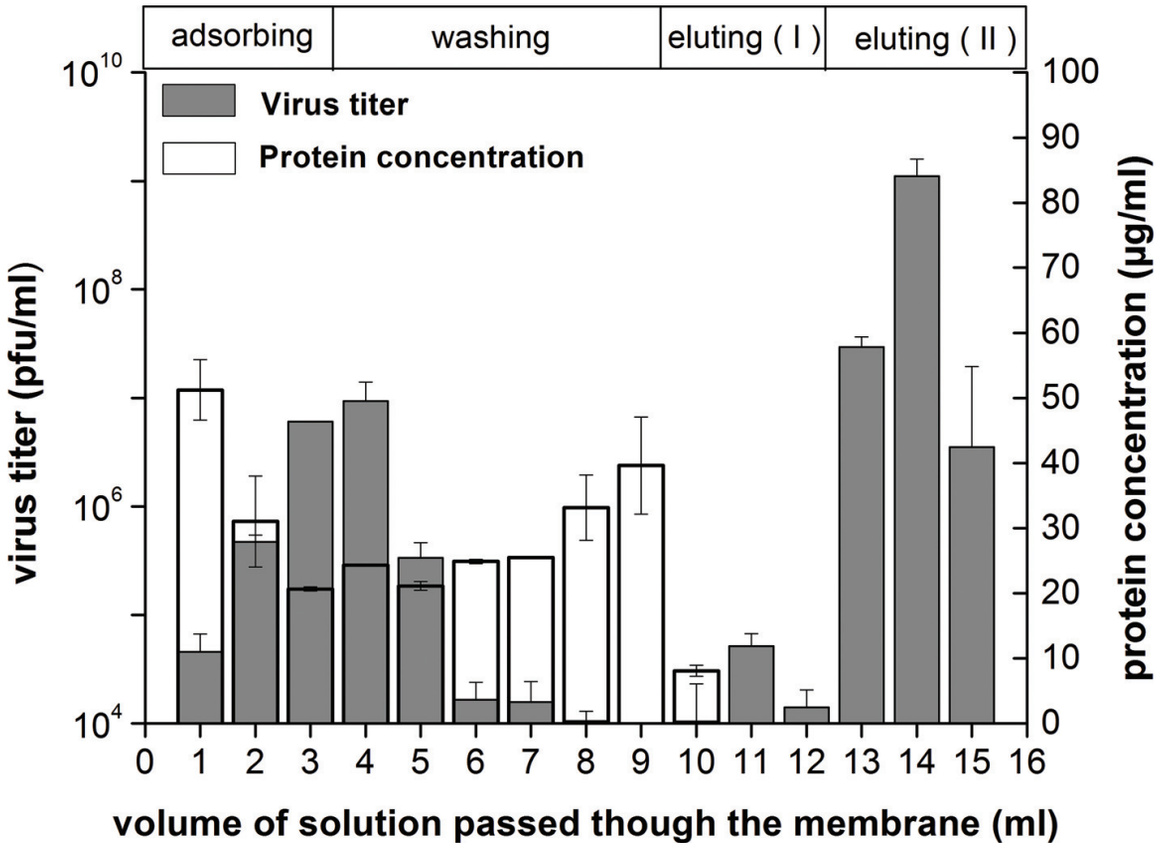

Figure 6. Steps in the purification of *Ac*MNPV using membrane chromatography, showing the virus titer and host cell protein concentration [73].

by 90 and 60%, respectively [75]. The most suitable method is therefore a matter of the required purity, process duration and scale.

2.3. Stable protein expression in *D. melanogaster* S2 cells

Although the BEVS platform is highly versatile and probably the most popular insect-based expression system, it is not the best choice for all products. Factors such as protein complexity, post-translational modifications and process mode must be considered during process development [70]. Recent studies show that recombinant *D. melanogaster* S2 cells (rS2 cells) can be used as an alternative and equally powerful expression platform [76, 77]. This system is based on an embryonic *D. melanogaster* cell line derived in 1972 [78]. Stable transformation of S2 cells with plasmid vectors facilitates the production of heterologous proteins. Since the 1980s, this system has been steadily refined and is now incorporated into commercial packages such as the DES® system (Thermo Fisher Scientific) and the ExpreS^2 platform (ExpreS^{2}ion Biotechnologies). The general procedure used to generate stable rS2 cell lines is the same for all packages and is summarized in **Figure 7**. A plasmid carrying the GOI is used to transfect S2 cells, and stable transformants that have integrated the expression construct are propagated under selection to yield a stable cell line. This line can be used for the isolation of a highly productive clone. Depending on the amount of protein required and the time available, several starting points can be used for protein production. For high-throughput screening or when small amounts of

protein are sufficient, transient expression may produce enough and the selection of cell lines is unnecessary [79]. If higher protein yields are required, stable cell lines can be established [80] and even single cell cloning may be necessary [81]. Although protein production in stable cell lines usually takes longer than the BEVS platform, the rS2 system retains some flexibility.

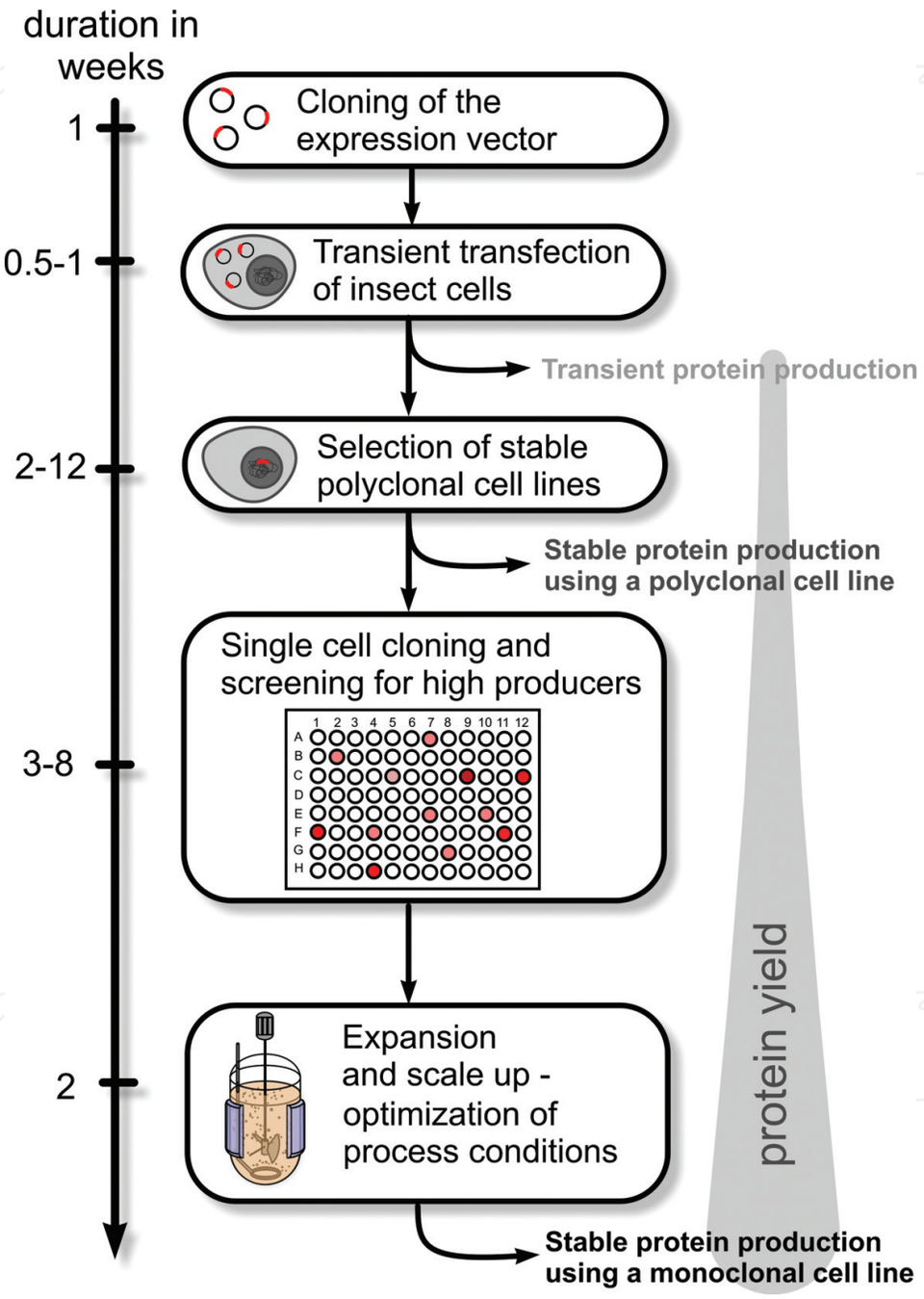

Figure 7. Overview of the general procedure to produce stably transformed *D. melanogaster* S2 cell lines for recombinant protein expression. Protein expression can be initiated at different points, starting with transient expression immediately after transfection followed by stable expression in a polyclonal cell line and finally the selection of a highly productive monoclonal cell line.

Stable rS2 cell lines are recommended for protein expression when the following aspects are important for the production process [76, 82]:

- Both, stable rS2 cells and BEVS share the advantage of minimal risk of contamination with human viruses because most human viruses cannot replicate in insect cells. This is particularly important for the production of pharmaceutical proteins intended for administration to humans.
- Stable rS2 cells are ideal when bacterial expression systems yield an inactive protein [83, 84] and where even BEVS is not efficient [84–86].
- Because of the non-lytic and stable nature of protein production, different bioprocessing modes such as batch or fed-batch cultures [87], chemostat cultures [88] and perfusion cultures [81] can be used. Perfusion mode in particularly achieves high protein yields [81][1].
- Stable rS2 cells can grow to considerably higher cell densities than other insect cell lines (20–70 $\times$ 10^6 cells/mL) and are robust concerning their hydrodynamic environment due to their small size (6–10 μm).
- No cell lysis occurs, so less host cell protein is released and the recombinant target protein is protected from proteolytic degradation. Therefore process-integrated product recovery is also conceivable.
- Stable rS2 cells also achieve high batch-to batch reproducibility between manufacturing runs, and generate a homogeneous glycan profile.

Several rS2-derived products have already entered clinical development, confirming that rS2 cells are not only used routinely in research but also for the commercial production of high value pharmaceutical proteins (**Table 3**).

2.3.1. Plasmids used to generate stable cell lines for recombinant protein expression

Stable rS2 cell lines are produced by transformation with suitable plasmid vectors carrying the GOI in an expression cassette and a selectable marker. Five general strategies have been developed, as summarized in **Figure 8**.

Classically, separate expression and selection cassettes with their own promoters can be combined in a single plasmid [97, 98] (**Figure 8b**). Alternatively, both features can be placed in one expression cassette, separated by an internal ribosome entry site (IRES) or a 2A-like sequence (T2A), resulting in bicistronic vectors with heterologous protein production and antibiotic

[1]Definitions: A **batch process** is cell growth and protein production in a fix amount of growth medium. The **fed-batch** operation augments a batch processes by continuous or intermittent addition of growth medium to prevent nutrient depletion and to increase cell density and productivity. **Chemostat** processes involve the continuous replacement of culture medium including a withdrawal of medium with cells and product. This mode of operation is more suitable for kinetic studies rather than protein production. **Perfusion** is the continuous replacement of culture medium with an additional cell retention system. This mode of operation prevents nutrient depletion and cell drainage while maintaining a constant reaction volume. It allows high cell densities and product titers.

Proteins in clinical development		
Recombinant placental malaria vaccine	Phase I	[76, 89, 90]
West Nile virus vaccine	Phase I	[76, 91]
HER-2 protein AutoVac™ (breast cancer)	Phase II	[76]
Proteins for research and process development		
HIV-1 VLP and soluble HIV gp120	VLP	[92]
Arabidopsis thaliana sterol glycosyltransferase	Enzyme	[93]
Psalmotoxin 1	Small peptide toxin	[83]
M2 muscarinic and glucagon receptor	G-protein-coupled receptor	[94]
Atlantic salmon serum C-type lectin	Lectin	[95]
Monoclonal antibody against H5N1 influenza hemagglutinin	Antibody	[81]
Enhanced green fluorescent protein (eGFP)	Fluorescent marker protein	[96]

Table 3. A selection of proteins produced in rS2 cells.

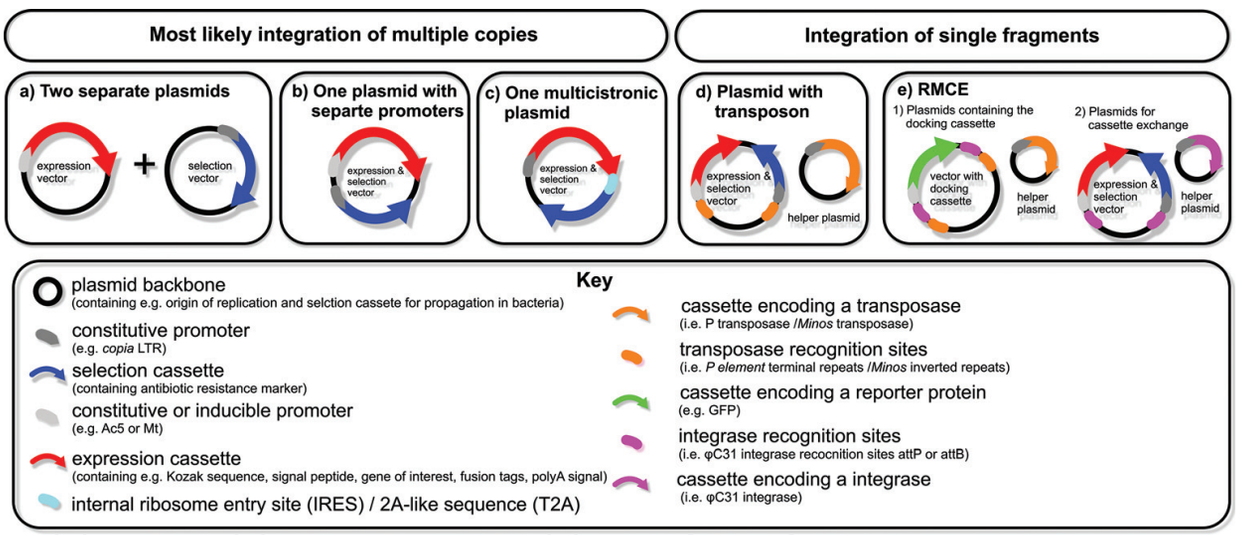

Figure 8. The different plasmid sets that can be used to generate stable rS2 cell lines.

resistance under the control of the same (constitutive) promoter [99, 100] (**Figure 8c**). However, the most common approach is the use of two separate plasmids, the first containing the expression cassette and the second containing the selectable marker (**Figure 8a**). This exploits the ability of dipteran cell lines to recombine different plasmids in long tandem arrays [82, 101]. The transfection or co-transfection of S2 cells with such plasmids followed by selection generally leads to the integration of multiple plasmid copies into the genome [102, 82]. The co-transfection of two plasmids using the calcium phosphate precipitation method (Section 2.2.1) can result in the integration of up to 4000 copies per S2 cell [82, 102]. The ratio of integrated plasmid sequences is similar to their proportions in the transfection mixture. When using two separate plasmids, it is therefore advisable to increase the initial ratio in favor of the expression plasmid. The commercial DES® system recommends a 1:19 ratio of selection plasmid to expression plasmid, but ratios ranging from 1:1 to 1:100 have been

successful [82, 103, 104]. The random integration of multiple plasmids generates heterogeneous cell populations and it is possible that rearrangements occur within the integrated array [102]. Nevertheless, this system can achieve high protein yields of 5–100 mg/L.

In contrast to the random transgene arrays described above, transposable elements can be used to insert a single copy of the GOI and selectable marker into the S2 cell genome (**Figure 8d**) [105, 106]. For this purpose, the expression construct and selectable marker are flanked by transposase recognition sites, i.e. *Minos* inverted repeats [107] or *P element* terminal repeats [108, 109]. Co transfection together with a helper plasmid encoding the corresponding transposase causes the GOI and marker to be inserted at a more or less random site. Transposition-mediated insertion events can occur more than once in the same genome, but the mutagenic nature of each insertion generally limits the number of integration events to between one and 10 copies [82]. This method is cumbersome because it is necessary to map the transposon insertion site and identify clones with single-copy insertions, and the low copy number limits the yield of recombinant protein. This method is more suitable when the goal is functional analysis rather than protein production, and can be very useful when combined with the technique of recombinase mediated cassette exchange (RMCE) as shown in **Figure 8e**. Two recent reports describe an RMCE system for *D. melanogaster* cell-lines based on integrase φC31 and its recognition sites *att*P and *att*B [108–110]. In this system, a single docking cassette flanked by the first recognition site (*att*P) is stably integrated into the genome by transposition. A second helper plasmid is then used to transiently express the integrase. The subsequent introduction of a plasmid with an expression cassette flanked by the corresponding recognition site (*att*B) promotes cassette exchange. Based on one parental cell line containing the docking cassette, different comparable clones can easily be generated, which is particular helpful in comparative studies (e.g. for promoter screening).

Although the classification of different plasmid types can simplify the principles underlying the generation of rS2 cells, it is not a fixed dogma. For example, the bicistronic system can be used to express two different proteins of interest, while co-transfection with a second plasmid provides the selection cassette [100]. Furthermore, co-transfection is not restricted to two plasmids. Indeed, up to four different proteins have been expressed simultaneously by co transfecting S2 cells with multiple vectors [111]. Finally, rS2 cells can be even combined with BEVS. Although baculoviruses cannot replicate in S2 cells, infection achieves successful protein production from the non-replicating vector [112–115]. Accordingly, rS2 cells appear to be a powerful and versatile tool for protein expression. Detailed protocols and more background information on the different techniques have been published [116–118, 82].

2.3.1.1. Genetic elements in the expression cassette

As described above, the expression plasmid contains various features required for protein production and the best combinations must be assessed for each process.

2.3.1.1.1. Promoter systems for rS2 cells

Several constitutive and inducible promoters have been used for the production of recombinant proteins in rS2 cells (**Table 4**). Strong constitutive promoters are generally favored for transient expression, because the protein must be expressed immediately after transfection.

Promoter	Comments	Example plasmids (source)	References
D. melanogaster actin 5C (*Ac5*)	Strong constitutive	pAc5.1/V5-His (Thermo Fisher Scientific) pUC-actGFP (DGRC 1219)	[119]
D. melanogaster metallothionein (*Mt*)	Strong inducible, induced by divalent metal ions such as Cu^{2+} and Cd^{2+}	pMT/Bip/V5-His (Thermo Fisher Scientific) pJACKS (-)	[120] [125]
D. melanogaster heat shock protein 70 (*hsp70*)	Strong inducible with some basal activity, induced by heat shock (i.e. 30 min at 37°C) and also by Cd^{2+}	pHSPCat1 (-) pHFHW (i.e. DGRC 1121)	[126]
D. melanogaster copia LTR	Strong constitutive, from *copia* retrotransposon; used in many selection cassettes	pCoBlast (Thermo Fisher Scientific) *copia*-CAT1 (-)	[127] [126]
D. melanogaster DS47	Moderate constitutive	pDS47/V5-His (Thermo Fisher Scientific)	[128]
*OpIE*1 or *OpIE*2	Constitutive, derived from *Orgyia pseudotsugata* multiple nucleopolyhedrovirus *Op*MNPV	PIZT/V5-His (Thermo Fisher Scientific)	[48, 129, 130]
D. melanogaster adh; α1-tubulin; PGK; CMV; EF1A; UBC; CAGG	Promoters with (lower) activity, not used routinely		[127, 131]
SV40 early; fibroin; herpes simplex virus thymidine kinase; Rous sarcoma virus LTR	These promoters are inactive in *D. melanogaster* cell lines		[106, 117, 127, 132]

Table 4. Promoter systems used in *D. melanogaster* S2 cells.

Inducible promoters can be more suitable in stable rS2 cell lines, particularly if the overexpressed protein is toxic to the host cell. Inducible promoters allow the decoupling of cell proliferation and protein expression, which can be appropriate for advanced process designs (i.e. two-step processes). The most widely used promoters in rS2 cells are the constitutive actin 5C (*Ac5*) promoter [119] and the copper-inducible metallothionein (Mt) promoter [120]. Other constitutive promoters, such as the *copia* long terminal repeat (LTR) promoter, can be used for the GOI but are usually paired with the resistance marker gene. The inducible *hsp70* promoter can also be used, but unlike the preferred Mt promoter it has a relatively high basal activity, and the heat shock required for induction can also induce endogenous heat-shock genes causing changes in gene expression and cell behavior that inhibit protein production [121]. Even so, the Mt promoter is usually induced with $CuSO_4$ or $CdCl_2$, both of which are cytotoxic at high concentrations [122] and Cd^{2+} also activates endogenous heat-shock promoters. Efficient protein production therefore requires a balance between promoter induction and toxicity, which

varies according to the exact experimental conditions between 200 and 1000 μM for $CuSO_4$ [95, 96, 123] and between 1 and 10 μM for $CdCl_2$ [95, 92, 85]. The addition of other divalent ions such as Zn^{2+} can improve protein production [85]. To rationalize all the different factors that affect protein expression (inducer concentration, time of induction, culture medium), a structured approach such as statistically designed experiments (DoE) may yield valuable information. As a future prospect, the doxycycline-inducible TRE promoter may also be useful because it can achieve good yields in *D. melanogaster* rS2 cells, although it has not yet been used widely [124].

2.3.1.1.2. Kozak sequence

The Kozak consensus sequence is required for the efficient initiation of translation. It is important to note that *D. melanogaster* has a different Kozak consensus sequence (cAAaATG) compared to vertebrates [133].

2.3.1.1.3. Signal peptides

Signal peptides are used to mediate protein trafficking, initiate proper folding or to ensure protein secretion to the supernatant. The signal peptides are cleaved off during or after translation. The most common signal peptide used in S2 cells is *D. melanogaster* BIP (homologous to the mammalian immunoglobulin heavy chain chaperone binding protein), which causes proteins to be secreted into the supernatant [134]. Signal peptides from human tissue plasminogen activator (tPa) [135] and from *Galleria mellonella* gloverin (GmGlv) also work in rS2 cells [136]. The proper folding of a dopamine receptor and its insertion into the cell membrane has been achieved using an influenza virus hemagglutinin signal sequence [125].

2.3.1.1.4. Fusions tags for protein detection and purification

As discussed above, fusion tags for protein detection and purification can be attached to either the C-terminus or N-terminus of a protein, with or without an additional protease cleavage site (e.g. enterokinase or thrombin). DES® plasmids usually contain a His_6 tag and a V5 epitope tag [93, 96, 100, 137]. The His_6 tag can be detected with an antibody, and purification can be achieved with the same antibody or by immobilized metal ion affinity chromatography (IMAC) [138, 136]. Other frequently used tags in the rS2 system include the BioEase™ tag (Thermo Fisher Scientific), the FLAG® and hemagglutinin epitope tags [139], the Myc tag [111)] and the S-tag™ [125].

2.3.1.1.5. Polyadenylation signals

As usual for eukaryotic organisms, mRNA must be polyadenylated in rS2 cells to maintain stability and support efficient protein synthesis [120, 132]. The late SV40 polyA signal (from simian virus 40) achieved the best performance, indicating that the polyadenylation mechanism is conserved between mammalian and insect cells. The early SV40 polyA signal and the polyA signals from *D. melanogaster* metallothionein (Mt) and alcohol dehydrogenase (adh) were also functional in rS2 cells.

2.3.1.2. Antibiotic selection markers

Several selection systems have been tested in rS2 cells as summarized in **Table 5**. Each system comprises a cytotoxic agent and a corresponding marker that confers resistance. The marker may encode an enzyme that catalyses the transformation of a selective agent into a harmless product, e.g. blasticidin and hygromycin resistance. Alternatively, the marker may encode a mutated enzyme that replaces an endogenous enzyme inhibited by the cytotoxic agent, e.g. α-amanitin and methotrexate resistance. Only cells that have integrated the selection cassette can survive and proliferate in the presence of the selective agent. The most appropriate selection system depends on time, cost and risk. For example, α-amanitin is fast and efficient but expensive and highly toxic to humans, making it less suitable for large-scale processes and pharmaceutical products. Blasticidin and puromycin are more expensive than hygromycin but also work faster. A high rate of spontaneous resistance has been reported for G418. It is always advisable to generate a kill curve in order to determine the 50% lethal dose (LD_{50}) as a starting point for the optimization of the selection protocol, because factors such as the presence of fetal

Selective agent (resistance marker)	Working concentration of the antibiotic	Plasmids (source)	References
Blasticidin S* (blasticidin-S deaminase - *bsd*)	25 μg/mL, range: 5–100 μg/mL	pCoBlast[a] (Thermo Fisher Scientific)	[140]
Hygromycin B* (hygromycin-B-phosphotransferase -*hph*)	300 μg/mL, range: 100–1000 μg/mL	pCoHygro[a] (Thermo Fisher Scientific) pUC-HygroMT[b] (DGRC 1059)	[103] [141]
Puromycin (puromycin N-acetyltransferase)	15–30 μg/mL, range: 2–30 μg/mL	pCoPuro[a] (RDB 08531) pMT-PURO[b] (RDB 08532)	[142] [97]
Methotrexate (resistant dihydrofolate reductase DHFR)	0.1 μg/ml, range 0.1–4 μg/mL	p8HCO[a] (DGRC 1003) pHGCO[a] (-)	[143] [144]
α-Amanitin (mutated RNA polymerase II)	5–10 μg/mL	pPC4[d] (DGRC 1217)	[145]
Geneticin (G418) (neo-aminoglycoside phosphotransferase type II)	1000 μg/ml	pUChsneo[a,d] (-) pAc5-STABLE1-Neo[c] (Addgene 32425)	[106] [99]
Zeocin™ (*Streptoalloteichus hindustanus ble*)	75 μg/mL	PIZT/V5-His[b] (Thermo Fisher Scientific)	[129]

Commonly used selective agents are indicated with an asterisk. Plasmids are available from the Drosophila Genomics Resource Center (DGRC), Riken Bioresource Center DNA Bank (RDB), Addgene and Thermo Fisher Scientific. Lower case letters indicate delivery methods: [a]co-transfection, [b]transfection with a single plasmid containing selection and expression cassettes, [c]transfection with a single bicistronic plasmid, [d]P-mediated transformation.

Table 5. Selection systems used for the development of rS2 cell.

bovine serum (FBS) and the overall medium composition can influence the potency of these selective agents.

2.3.1.3. Transfection methods for S2 cell lines

Several strategies for the transfection of insect cells were described in Section 2.2.1.3. All of these methods are appropriate for the production of stable rS2 cell lines, but it is important to consider that transfection causes stress to the cells and a recovery period may be necessary before selection commences.

2.3.1.3.1. Calcium phosphate–DNA co-precipitation

As described above for Sf cells, calcium phosphate-DNA co-precipitation was also one of the first methods used to generate rS2 cells [117] and was used extensively in the past [83]. However, the limitations of this technique include the need of a fixed amount of ~20 μg DNA per mL precipitate [38]. Calcium phosphate-DNA co-precipitation has therefore been largely replaced by more flexible techniques that achieve greater reproducibility [102].

2.3.1.3.2. Electroporation

Electroporation is a convenient method for the transfection of S2 cells [38] that can achieve efficiencies comparable to calcium phosphate–DNA co-precipitation [102]. This method also allows the uptake of DNA over a very large concentration range, making it useful for the transposon-mediated generation of cells with single-copy inserts [102].

2.3.1.3.3. Lipid-based systems

Several cationic lipid reagents achieve the efficient transfection of S2 cells, including the Thermo Fisher Scientific products Cellfectin [96, 146], Cellfectin II [147] and Lipofectin [148], as well as DOTAP-Liposomes [149] and DDAB [150].

2.3.1.3.4. Other transfection reagents

Specialized proprietary formulations for S2 cells have recently been introduced, including the *Trans*IT®-Insect transfection reagent (Mirus Bioscience), which is used routinely in the authors' laboratory. Other non-lipid transfection reagents have been used successfully with rS2 cells including FuGene 6 (Roche Molecular Biochemical) [38], Effectene (Qiagen) [99] JetPEI (PolyPlus-transfection) [151] and DEAE dextran [117].

2.3.1.4. Case study for the development of a polyclonal rS2 cell line

To illustrate the generation of a stable rS2 cell line, this section describes the expression of the reporter protein GFP and its time resolved detection during cell line establishment. *Trans*IT®-Insect-based transfection was carried out using a single plasmid with an expression cassette containing intracellular GFP under the control of the *Ac5* promoter and a selection cassette containing hygromycin phosphotransferase driven by the *copia* LTR promoter. After transfection, the cells were allowed to recover for 3 days before 300 μg/mL hygromycin was

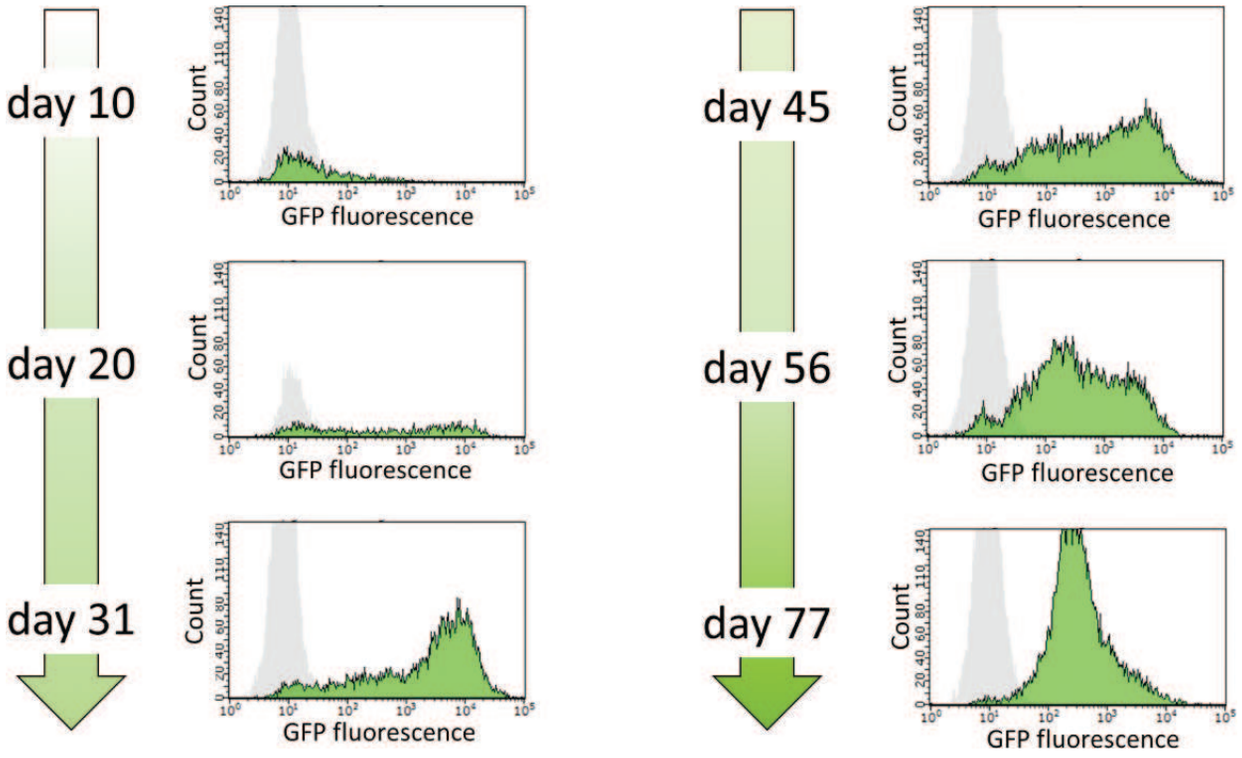

Figure 9. Time course showing the establishment of a stable rS2 cell line expressing GFP under the control of the constitutive *Ac5* promoter. GFP activity was recorded by flow cytometry. The transfected cell population maintained under selection pressure with 300 µg/mL hygromycin (green) was compared to a non-transfected control (gray).

added to the medium and renewed at each sub-culture interval. **Figure 9** shows the GFP expression profile monitored by flow cytometry. Within the first 10 days, most of the non-transfected cells died and only low levels of GFP were detected. After 20 days under selection, the cell population became increasingly GFP-positive, and a population producing high levels of GFP was established within 30 days. Further sub-culturing revealed the existence of different sub-populations with varying GFP expression profiles and different growth properties. By day 77, a population characterized by moderate levels of GFP became prevalent, suggesting that polyclonality is detrimental for long-term protein production. This can be overcome by single cell cloning, as discussed in the next section.

2.3.2. Single cell cloning for enhanced protein yields

Once a stable cell line is established, it can be used as a straightforward basis for subsequent up-scaling and protein expression even at the bioreactor scale. However, the expression profile within the polyclonal cell population is heterogeneous. Furthermore, high-copy-number transgenic loci confer an additional metabolic burden that may inhibit cell growth. Long-term subculturing therefore enriches subpopulations that have lost copies of the transgene, and protein expression declines [152] as illustrated with eGFP in **Figure 9**. As well as maintaining the cells under selection pressure, single cell cloning is necessary to minimize these effects and should commence before the highly-productive cells become overpopulated by their less-productive peers [118, 152]. However, the productive cells should only be chosen once they

have recovered from the stress of transfection because the success of a single cell cloning is highly dependent on clones that proliferate well.

Another important consideration is that rS2 cells grow very slowly at low densities and may arrest completely if seeded at less than 5×10^5 cells/mL [77, 116]. This reflects the demand for autocrine growth factors, which accumulate to sufficient levels only at high cell densities [77]. For example, the adenosine deaminase-related growth factor (ADGF) family is known to promote the growth of S2 cells [153, 154] but no studies have yet shown that ADGF alone can stimulate growth at very low cell densities. Augmenting the culture with conditioned medium or heat inactivated FBS can improve proliferation, but is also not sufficient to stimulate propagation of single cells. Feeder cells are therefore required to facilitate the proliferation of a single rS2 cell. There are two traditional cloning methods: cloning in soft agar and cloning by dilution. Both make use of non-transfected feeder cells, which are exposed to a γ or X ray source (e.g. 24 kR per 50 cm^2 T-flask) before co-culture [82]. Irradiated feeder cells do not divide, but remain able to secrete growth-promoting substances and provide an additional source of nutrients when they die [116]. Cloning in soft agar requires the mixing of concentrated feeder cells ($1–2 \times 10^6$ cells/mL) with much more dilute selected transformants (~25 cells/mL) in soft agar, which is then poured into a Petri dish. The cells grow in this semi-solid support and form colonies in the agar within 2 weeks. Cloning by dilution requires the mixing of feeder cells and transformants in such a way that approximately one clone per well can be seeded in a microtiter plate. The corresponding protocols have been described in detail [82, 38, 117, 118]. Both methods have been used successfully with rS2 cells for a long time, but their major drawback is the need for X-ray or γ sources which are not readily available routinely in cell culture laboratories. It is also necessary to ensure that the feeder cells are permanently unable to divide yet still survive at least 1 week post-irradiation to condition the medium during the early growth of the clones [117]. Robustness against ionizing radiation is cell line-dependent, so parameters such as radiation dose and distance from source must be empirically standardized to achieve the requirements described above. As an alternative to irradiation, feeder cells can be treated with mitomycin C to chemically block mitosis, but this method must fulfil the same requirements and empirical testing is still necessary [117].

A modified version of the limiting dilution protocol was recently reported that does not use radiation and therefore simplifies the cloning workflow [81, 95]. The method is based on the co-cultivation of single transformants with living, non-transfected feeder cells followed by antibiotic selection of the clones. Because the procedure is not yet well established, the steps in the protocol are summarized below:

- Seeding of approximately one transformant per well in 100 μL medium, containing 5×10^5 non-transfected, living feeder cells/mL.
- Co-cultivation for 1–3 days allows all cells to proliferate and ensures proper conditioning of the medium.
- Adding the antibiotic (e.g. 15–25 μg/mL blasticidin or 100–1000 μg/mL hygromycin) to initiate the selection phase. Feeder cells will slowly decay, while colonies from antibiotic resistant clones will expand during the next 2 weeks. If necessary, medium can be added to renew the antibiotic and avoid desiccation.

- Checking colony growth using a microscope: wells with multiple colonies should be discarded because they are not monoclonal (**Figure 10**).
- Picking colonies and successively expanding them in 48-, 24-, 12- and 6-well plates, followed by cultivation in T-flasks or shake flasks until cryopreservation in freezing medium containing 50% spent medium and 7.5–10% dimethylsulfoxide (DSMO).

For all methods, a negative control that only contains feeder cells is advisable. Feeder cells that have been irradiated, blocked with mitomycin C or treated with other antibiotics should show no evidence of proliferation after 2 weeks, otherwise the inactivation will not be successful and contamination of the single cell pool with less productive cells will remain possible (The method is based on the co-cultivation of single transformants with living, non-transfected feeder cells followed by antibiotic selection of the clones **(Figure 11)**).

To avoid extensive work during scale up, producer screening should be started soon after picking the single clones. The screening method depends on the expression strategy. If the recombinant protein is expressed constitutively then the cell lines can be screened directly,

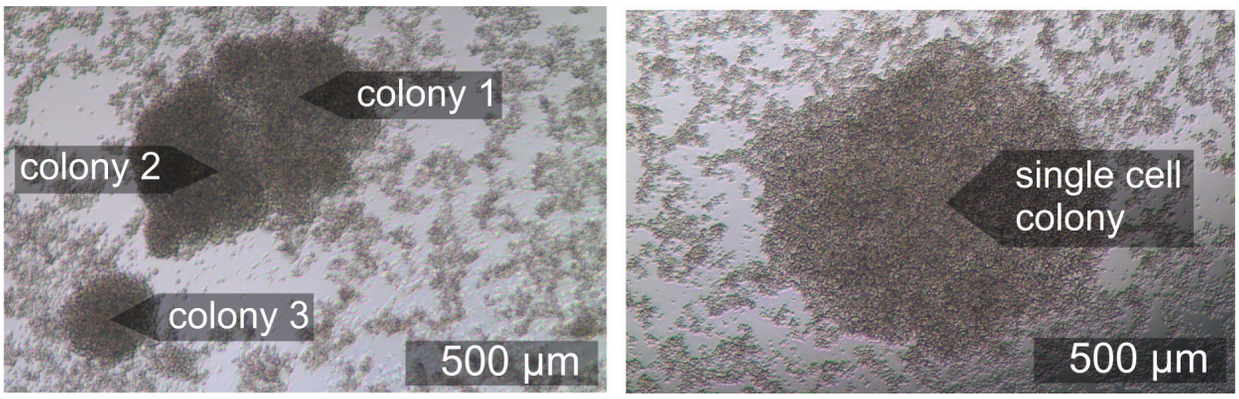

Figure 10. Phase contrast image of wells used for single cell cloning. Multiple colonies (left) and a single cell colony (right) on a decaying layer of feeder cells.

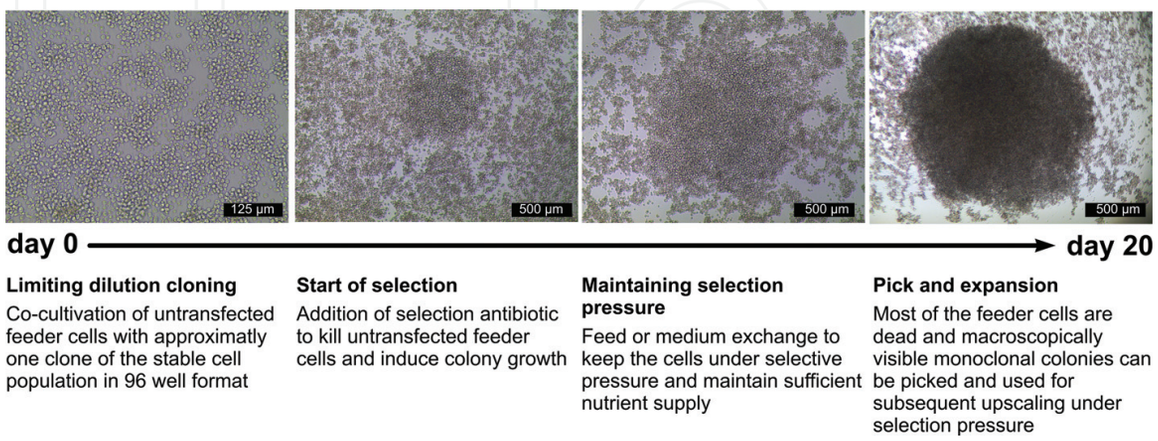

Figure 11. Time course for the establishment of a monoclonal population using the limiting dilution method and co-cultivation with non-transfected feeder cells under selection with 15 µg/mL blasticidin S.

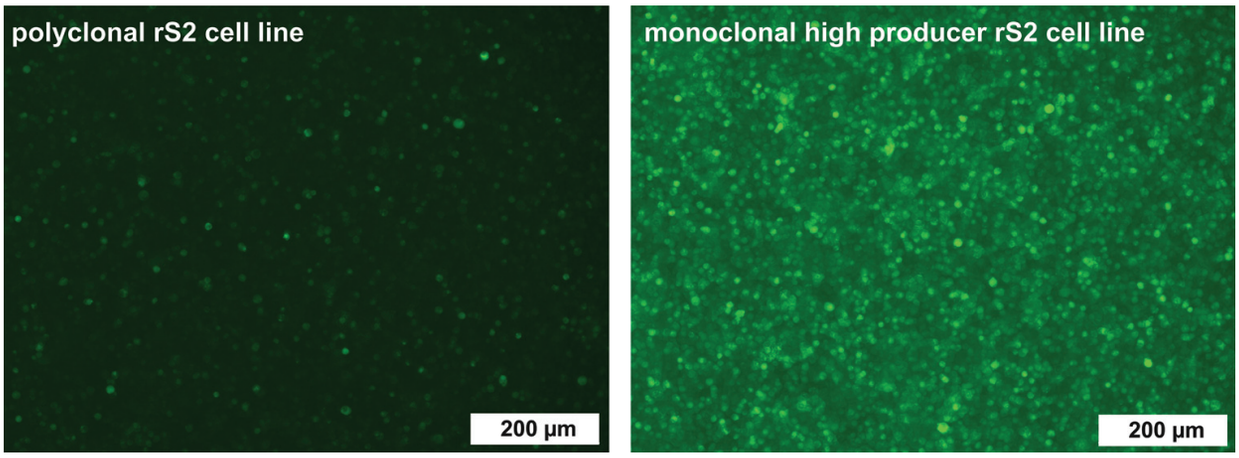

Figure 12. Fluorescence microscopy images of a parental polyclonal cell line (left) and a highly productive monoclonal rS2 cell line (right), expressing an eGFP fusion protein under the control of the Mt promoter (induced with 900 µM $CuSO_4$ at 1×10^6 cells/mL with viability > 97%). The images were captured using the same instrument settings 24 h post-induction.

whereas inducible cell lines must first be sub-cultured in a medium containing the inducer. The location of the target protein should also be considered, i.e. whether the protein accumulates in the cell or is secreted to the medium. Methods to screen for specific proteins include flow cytometry, fluorescence microscopy, western blot analysis, the enzyme linked immunosorbent assay (ELISA) and specific functional assays, e.g. enzyme assays. For example, **Figure 12** shows the comparison of an eGFP expressing parental polyclonal cell line and a highly productive monoclonal cell line by fluorescence microscopy. Note that the polyclonal cell line only contains a few highly productive cells, whereas the GFP fluorescence in the monoclonal cell line is much higher because all the cells produce large amount of the product. This indicates that a highly-productive cell line has been selected successfully. Although some authors report that single cell cloning does not always achieve enhanced protein production [116], others claim that the considerable effort is worthwhile [81, 95, 118, 155]. It is true that most clones do not show enhanced protein expression, but this is unsurprising because the frequency of highly-productive cells in the parental cell pool is usually low (**Figure 12**). Consequently, the likelihood of selecting a highly-productive cell reflects this initial ratio, and at least 500–1000 wells (i.e. 5–10 96-well plates) should be used for cloning and subsequent screening.

3. Scale up of insect cell cultivation processes

3.1. Assessment of the cost-effectiveness

In order to produce recombinant proteins cost effectively, a satisfactory expression level has to be achieved in one of several species available for recombinant protein expression. Suitable hosts include bacteria (*Escherichia coli*), yeasts (such as *Pichia pastoris*) and cell lines of mammalian or insect origin. These expression systems differ in terms of complexity, space-time-yield and the ability to support protein folding and posttranslational modification [156]. The system of choice depends on the properties of the protein that is to be produced and insect cell lines

are usually employed for the production of virus like particles and proteins that require folding and posttranslational modification [1, 156]. Generally the competitiveness of insect cell lines is demonstrated by the availability of different high value commercial products, such as the vaccines FluBlok®, CERVARIX® and PROVENGE® (se chapters 2.2.1 and 2.3), which are produced using baculovirus based systems. Furthermore a recent study showed that also stable rS2 cells can compete with established systems. Concretely rS2 cells were more suitable for the production of human coagulation factor IX than CHO cells [157] and also suitable to produce high titers of a monoclonal antibody [81].

3.2. General considerations on process design

Insect and mammalian cell lines both originate from tissues of multi-cellular organisms and therefore have comparable growth requirements, but insect cells offer some advantages in terms of process design [1]. They grow rapidly at lower temperatures (doubling time of approximately 24 h at 22–28°C) and tolerate higher levels of free amino acids and glucose without switching to overflow metabolism [158–160]. This allows the use of rich media, incorporating the nutrients for complete batch processes. Although first-generation media (e.g. Grace insect medium, TMH-FH and TC100) required complex additives such as FBS or insect hemolymph [160], optimized protein-free media are now available, including Sf-900™ II SFM (Thermo Fisher Scientific), ExCell® 420 (Sigma Aldrich) and Insect-XPress™ (Lonza). Defined media offer better lot-to-lot reproducibility, as well as simplified qualification and validation, and even contribute to higher protein titers. The optimal pH for insect cell lines is slightly acidic (6.2–6.9) and is usually maintained by a phosphate buffer. Therefore no CO_2 supply is required, unlike mammalian cell culture media which rely on the open bicarbonate buffer system. For industrial scale up, insect cells can be adapted to grow in suspension cultures, allowing the use of standard bioreactors resembling those typical for mammalian cell culture [158]. Beyond research, where more diverse culture devices are used [3, 161], stirred tank reactors (STRs) [87, 161] or wave bag reactors (e.g. GE Wave, AppliFlex or CultiBag) [81, 162, 163] are more suitable for the large-scale cultivation of insect cells. Both systems are scalable and are well established in the industry. Short reactor set-up times are important in particular for high-turnover baculovirus-based processes, which last 4–7 days. These processes are usually carried out in batch or fed-batch mode, because the virus-mediated lysis of the cells imposes a time limit on each production cycle. In contrast, continuous or perfusion mode is also compatible with stable rS2 cell lines. In each case, the main task of the bioreactor is to provide optimal growth and production conditions. The main challenge is therefore to ensure an adequate oxygen supply without generating destructive shear forces [164, 165].

3.3. Oxygen requirements of Sf and S2 cell lines

The essential nutrient oxygen must be delivered continuously because it is only sparingly soluble in cell culture medium. During scale up, it is not possible to achieve the high ratio of surface area to reaction volume, and in turn the high oxygen mass transfer through the headspace, which are characteristics of small-scale cell culture systems. It is therefore necessary to understand the oxygen requirements of the cultured cell line in detail in order to choose an appropriate oxygenation strategy. The cell-specific oxygen consumption rate $\dot{q}_{O_2}$ is a key

parameter for the physiological state of the cells, which provides useful information for scale up. It can be estimated by placing cells from a growing culture in a tightly sealed measurement chamber containing air-saturated medium. Given that oxygen transfer is negligible under these conditions, the saturation declines and a corresponding oxygen time course can be recorded (**Figure 13**). From the known cell concentration X and the slope of the curve $\frac{\Delta c_{O_2}}{\Delta t}$, the specific oxygen consumption rate $\dot{q}_{O_2}$ can be calculated according to Eq. (4). This method can also be adapted to determine $\dot{q}_{O_2}$ directly in a bioreactor. In order to do this, the oxygen supply must be closed and a sample must be withdrawn to determine the current cell concentration.

$$\dot{q}_{O_2} = -\frac{1}{X} \cdot \frac{\Delta c_{O_2}}{\Delta t} \tag{4}$$

Table 6 summarizes specific oxygen consumption rates for the cell lines discussed in this chapter. The data show that, $\dot{q}_{O_2}$ does not remain constant but is strongly dependent on the physiological state of the cells. For example, the infection of Sf cells with baculovirus increases the oxygen demand [166]. Furthermore, a wide range of $\dot{q}_{O_2}$ values has been reported for different stable rS2 cell lines, indicating that the expressed recombinant protein also affects

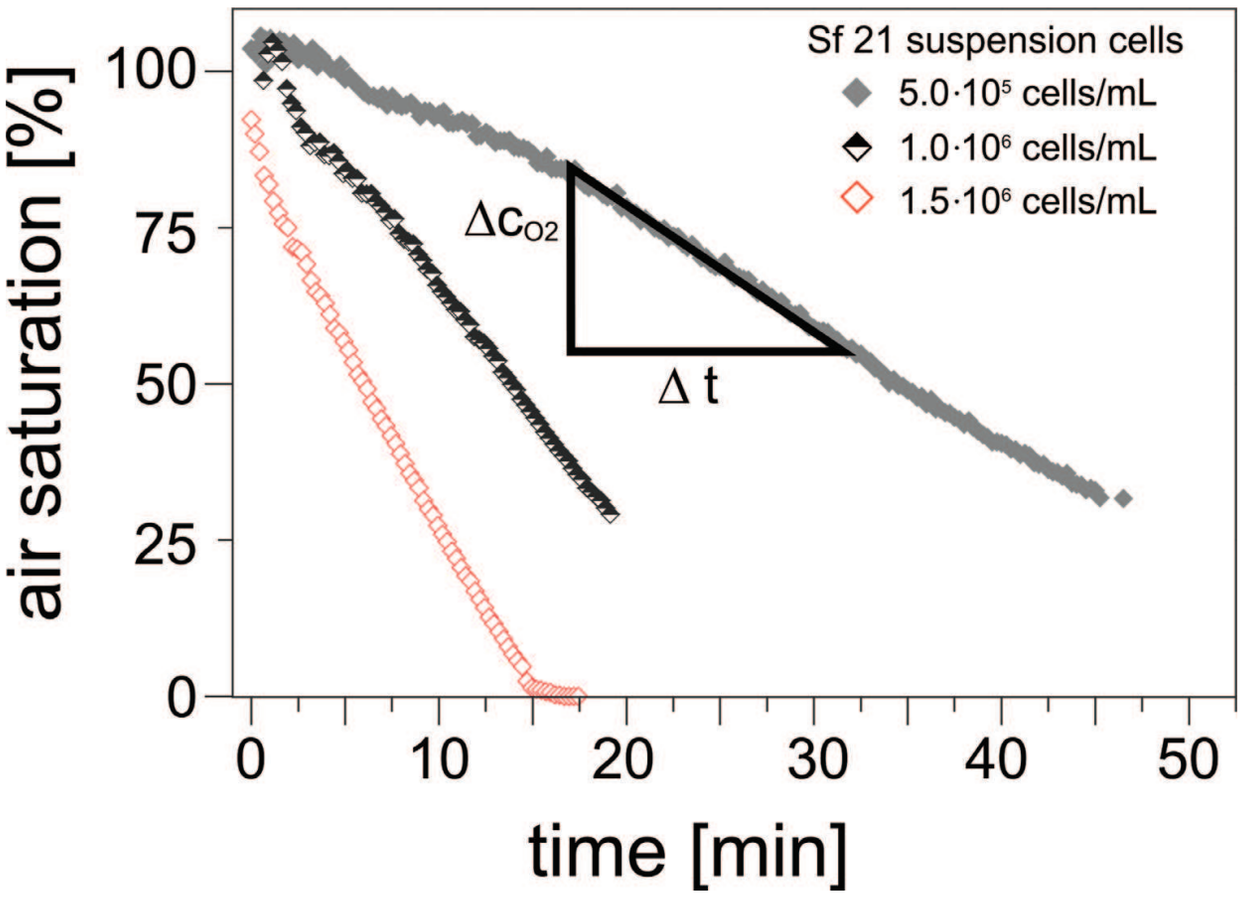

Figure 13. Time courses showing the declining oxygen saturation in a measurement chamber containing different concentrations of Sf 21 suspension cells in ExCell420 serum-free insect cell medium.

Cell line	$\dot{q}$ [10^{-12} mmol·Z^{-1}·min^{-1}]	Growth phase	Cultivation system	Infected	Medium	*FCS %*	References
Sf 9	2.58	exp.	Chamber	no	IPL-41	10	[167]
Sf 9	3.66	-	*STR*	yes	IPL-41	10	[168]
Sf 9	6.50	-	*STR*	yes	ICSF-WB	0	[168]
Sf 9	1.20–3.00	exp.	*STR*	no	Sf-900 II	0	[169]
Sf 9	1.50–2.70	-	*STR*	yes	Sf-900 II	0	[169]
Sf 9	5.50	exp.	*STR*	no	Excell 401	0	[170]
Sf 9	10.0	-	*STR*	yes	Excell 401	0	[170]
Sf 9	3.13–3.35	exp.	*Airlift*	no	IPL-41	10	[171]
Sf 9	1.33	stat.	*Airlift*	no	IPL-41	10	[171]
Sf 9	4.44	exp.	*STR*	no	Sf-900II		[172]
Sf 21	10.5	exp.	Chamber	no	Excell 420	0	*
Sf 21	2.33	exp.	Perfusion	no	IPL-41	5	[171]
S2 wt	1.5	exp.	STR	-	ExCell 420	0	*
S2 wt	0.45	exp.	*STR*	-	Sf-900 II	0	[172]
S2AcGPV	0.82	exp.	*STR*	-	Sf-900 II	0	[172]
S2AcGPV	0.6–1.2	exp.	*Spinner*	-	IPL 41		[173]
S2MtEGFP	0.8–1.5	exp.	*STR*	-	Sf-900 II	0	[172]

*Authors' own data. Abbreviations: exp. = exponential growth phase; stat. = stationary phase.

Table 6. Specific oxygen consumption rates for different insect cell lines and cultivation conditions.

the oxygen demand. In conclusion, $\dot{q}_{O_2}$ seems to be a function of cell status, overall medium composition and the reactor set up. Therefore, it is advisable to verify the values reported in the literature in each new experimental setting.

Knowing $\dot{q}_{O_2}$ and the current cell concentration X, the oxygen uptake rate (OUR) of the culture can be calculated according to Eq. (5):

$$\mathrm{OUR} = \dot{q}_{O_2} \cdot X \tag{5}$$

Similarly, the ability of a bioreactor to supply oxygen is characterized by its oxygen transfer rate (OTR), which is calculated according to Eq. (6):

$$\mathrm{OTR} = k_L a \cdot (c_{O_2}{}^{*} - c_{O_2}) \tag{6}$$

The volumetric mass-transfer coefficient $k_L a$ is a reactor-specific constant that describes the efficiency with which oxygen is transported to the medium under a given set of operating parameters [174]. The values $c_{O_2}{}^{*}$ and c_{O_2} are the maximum and actual oxygen concentration

in the medium, respectively. To ensure a sufficient oxygen supply, the OTR must be equivalent to the OUR at the maximum cell density X_{max} and the required k_La value of the cultivation system can thus be calculated according to Eq. (7):

$$k_La_{\text{ required}} = \frac{\dot{q}_{O_2} \cdot X_{\max}}{c_{O_2}{}^{*} - c_{O_2}{}^{\text{set}}} \tag{7}$$

Eq. (7) also reveals why oxygen supply is a major concern for both expression systems. Whereas Sf cells show high specific oxygen consumption rates, S2 cells consume less oxygen per cell, but grow to higher densities. Both situations lead to a considerable oxygen demand. As a rule of thumb, the oxygen saturation in the medium $c_{O_2}{}^{\text{set}}$ should not fall below 30% air saturation during cultivation [175, 158]. In order to avoid limitations at late process stages, the rate of oxygen transfer can be increased either by using highly efficient micro-porous spargers [165] or by using pure oxygen for aeration. Even with pure oxygen, sufficient gas flow must be maintained in order to strip out CO_2 that inhibits cell growth at higher concentrations. Increasing the stirrer speed and gas flow are a second option for STRs, but caution should be exercised because insect cells (especially infected and swollen Sf cells) are susceptible to shear damage. To encounter the danger of shear related cell damage, insect media usually contain up to 0.1% of the non-ionic block co-polymer Pluronic F68 as a shear force protecting agent [176]. Pluronic F68 adheres to the cell surface and thus stabilizes the membrane. A detailed discussion on the assessment of stirring and bubble related shear damage with its consequences for animal cell culture process design has been published [164].

3.4. Characterization of STR oxygenation capabilities for insect cell culture

The k_La value for each reactor setup must be determined and compared with the calculated value required to achieve sufficient oxygen transfer. The k_La can easily be determined using standard methods such as the dynamic method or the sulphate method [177]. In combination with a structured experimental design, appropriate settings for different cell lines are easy to find. One way to structure the experiments is to use the response surface method (RSM). This statistical method explores the relationship between a response variable (i.e. the k_La value) and different input variables (e.g. stirrer speed and aeration rate). Based on a set of designed experiments, the RSM approximates the coherences between the variables using a polynomial model [178]. The resulting model can then be used to predict the k_La value for each factor combination so that further experiments are not required. Once a suitable model is established and verified, optimal settings for new reactor set-ups can be deduced from a simple readout.

As an example, a respective model was determined for a water-filled 2-L bioreactor system (working volume 1000 mL) equipped with a drilled pipe ring sparger and a pitched blade impeller ($3 \times 45°$, $d/D = 0.57$). According to the typical cultivation conditions for insect cells, the temperature was set to 28°C. For RSM, the k_La values were determined by the dynamic method using a central composite design (CCD) for aeration rate $\dot{v}(0.01–0.09)$ and stirrer speed n (80–260). The resulting data were used to fit the significant terms of the general interaction model as shown in Eq. (8):

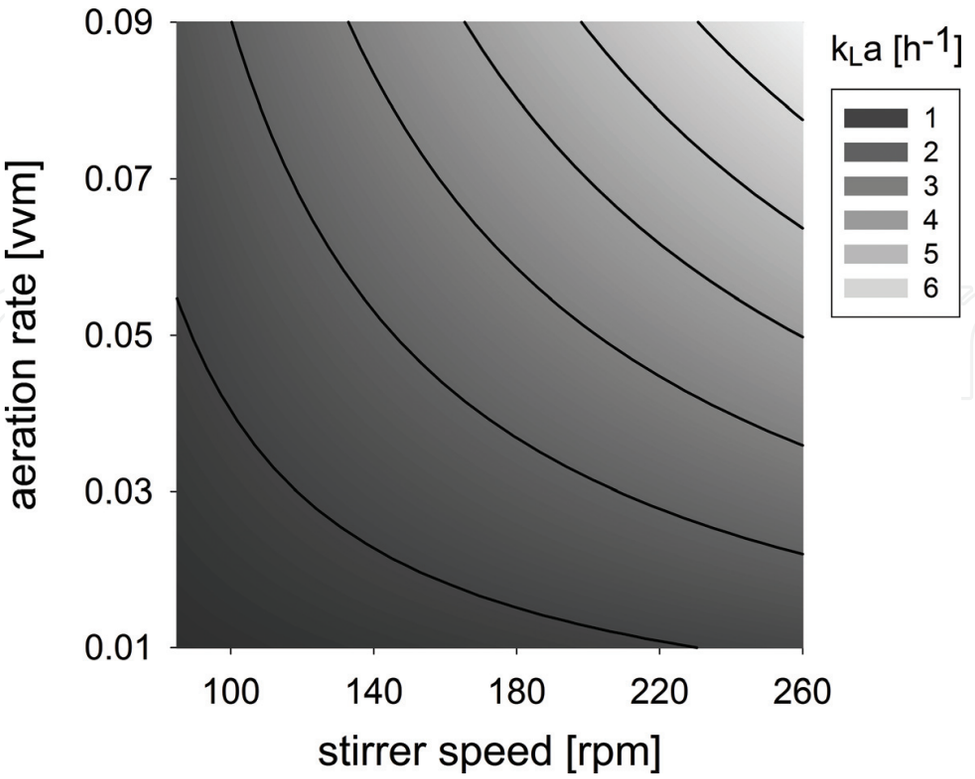

Figure 14. Relationship between k_La, aeration rate and stirrer speed for a 2-L autoclaveable bioreactor filled with water and operated under standard conditions for the cultivation of insect cells (28°C, 0.01–0.09 vvm, 80–260 rpm).

$$k_La = \beta_0 + \beta_1\dot{v} + \beta_2 n + \beta_{12}\dot{v}n \tag{8}$$

The results can be visualized as a contour plot (**Figure 14**) that describes the k_La value as a function of both input variables and thus provides a straightforward way to define the most suitable bioreactor settings. However, information about homogenization is also important, so it is advisable to use the same experimental design to establish models for mixing time. Combining both models provides a comprehensive overview of the potential operating window for each process. The workflow described in this chapter is not limited to STRs but can also be adapted for wave reactors easily, where k_La and mixing time are functions of the shaking frequency and angle.

4 Process monitoring

4.1. Process analytical technology as driving force for online monitoring

Complex recombinant proteins and vaccines are high-value products that are often intended for medical use. Such products must comply with high quality standards and recently the US Food and Drug Administration (FDA) released a guideline on process analytical technology (PAT), thus encouraging manufacturers to ensure product quality by improving their process understanding [179, 180]. This requires the measurement and control of critical process parameters, such as cell growth in the case of insect cells producing recombinant proteins. The

Method	Measured bioprocess variable	Direct or indirect biomass quantification	Representative commercial systems	References
Dielectric spectroscopy	Permittivity and conductivity	Direct viable cell density	Aber Futura Hamilton Incyte	[182]
Optical probing	Turbidity or backscattered light	Direct whole cell density and particles	Exner ExCell 230 Hamilton Dencyte Optek ASD Mettler InPro8100 Finesse TrueCell Cerexinc Wedgewood BT65	[183]
2D Fluorescence spectroscopy	Cellular fluorophores (NAD(P)H, flavins and aromatic amino acids)	Indirect from metabolic activity	Delta Light and Optics BioView	[184]
Biocalorimetry	Heat production from metabolic activity	Indirect from metabolic activity	Mettler Toledo eRC1	[185]
Off gas analysis	Respiratory activity (OUR and CER)	Indirect from respiratory activity	Blue Sens CellinOne	[186]
Soft sensors	Different process parameters	Indirect from correlation/models		[187]

Table 7. Summary of online biomass monitoring techniques.

dependency between cell growth and recombinant protein production exists because both processes require efficient protein synthesis machinery [181]. Optimal cell viability and comparable growth conditions during cultivation will therefore improve batch-to-batch reproducibility. **Table 7** lists online techniques that have been developed to estimate cell biomass, among which optical probing and dielectric spectroscopy have proven to be robust and easy to use. The following section therefore summarizes the principles of these two techniques and their application in insect cell cultures.

4.2. Dielectric spectroscopy to determine viable cell density

Dielectric or impedance spectroscopy is a noninvasive technique that is widely used to characterize materials in different research fields, including material testing, corrosion research and biological engineering [188, 189]. Historically, the application of dielectric spectroscopy for the characterization of cell suspension cultures dates back more than 150 years [190] and is now well established as a routine method [191, 192] even in industrial-scale processes [193]. Comprehensive reviews of the theory and application of this method have been published [194–199, 200].

The method involves exposing cell suspensions to an alternating current of low magnitude. This stimulus results in a phase-shifted, frequency-dependent voltage response, which in turn is recorded and used to calculate the dielectric properties of the material (**Figure 15a**). The measurement is not that simple and modern devices make use of special bridge circuits, network analysers and other advanced methods to calculate the real and imaginary part of

the complex impedance Z or admittance Y. Nevertheless, these measurements yield the two passive electrical properties of capacitance C and conductance G as function of the applied alternating current frequency. The capacitance C describes the ability of the material to store electrical charge, whereas the conductance G describes its ability to pass an electrical charge. Because these values depend on the electrode geometry, the relative permittivity ε and the conductivity κ are generally used to describe these electrical properties in an electrode-independent manner. The relationships are described in Eqs. (9) and (10), where C is the capacitance, ε is the relative permittivity, ε_0 is the permittivity of the vacuum (8.854 pF/m), G is the conductance, κ is the conductivity and z is the specific electrode constant (i.e. area/plate distance for a parallel plate capacitor):

$$C = \varepsilon \cdot \varepsilon_0 \cdot z \tag{9}$$

$$G = \kappa \cdot z \tag{10}$$

To simplify the explanation of the observed dielectric phenomena when ε is plotted against the applied frequency f, the cell suspension can be modelled as being composed of two parts [196]. The first part is the conducting aqueous cell culture medium that surrounds the second part (the cells), which are in turn composed of an insulating cell membrane and the conducting cytoplasm. Whereas the medium and cytoplasm are simple electrolytes with a certain resistance, the lipid cell membranes act as dielectric barriers and cells can therefore be regarded as small spherical capacitors. Electrically charged ions accumulate at the membranes of living cells in an alternating electric field, whereas leaking cells, cell debris, evolved gas bubbles, micro-carriers and other media components are essentially invisible to this method [195]. Only very high volume fractions of non-biomass materials close to the sensor may influence the measurement as the cells are replaced by non-reactive materials [198]. The build-up of electrical charge across the membranes is known as polarization and this occurs when the frequency of the excitation field is in the range 0.1–10 MHz (the radio frequency band). At low frequencies, the ions have sufficient time to reach the cell membrane, complete polarization takes place and a high permittivity signal is produced. At high frequencies, ions do not have enough time to move and they do not accumulate at the membranes before the electric field changes direction thus forcing them in the opposite direction. In this case, only a low permittivity is detected. This behaviour results in a sigmoid shaped $\varepsilon - log(f)$ relationship, the so called β-dispersion (**Figure 15b**). The plateau value of the permittivity $\varepsilon_{\max}$ depends on the quantity of polarizable cell membranes in the system. Hence, $\Delta\varepsilon$, the difference between $\varepsilon_{\max}$ and the residual permittivity ε_∞ (resulting from the medium), is a good measure of cell density. In 1957, Schwan derived Equation 11 predicts $\Delta\varepsilon$ for suspensions of ideal spherical cells [199]. The validity of the equation has been confirmed experimentally [189, 193].

$$\Delta\varepsilon = \frac{9 \cdot P \cdot r \cdot C_m}{4 \cdot \varepsilon_0} \text{ with } P = \frac{4 \cdot \pi \cdot r^3}{3} \cdot N \tag{11}$$

Here, P denotes the cell volume fraction (dimensionless), N denotes the cell density per unit volume, r *is* the cell radius and C_m is the capacitance per membrane area. It is clear that $\Delta\varepsilon$ is actually measuring the cell volume fraction instead of cell density, because it depends on the

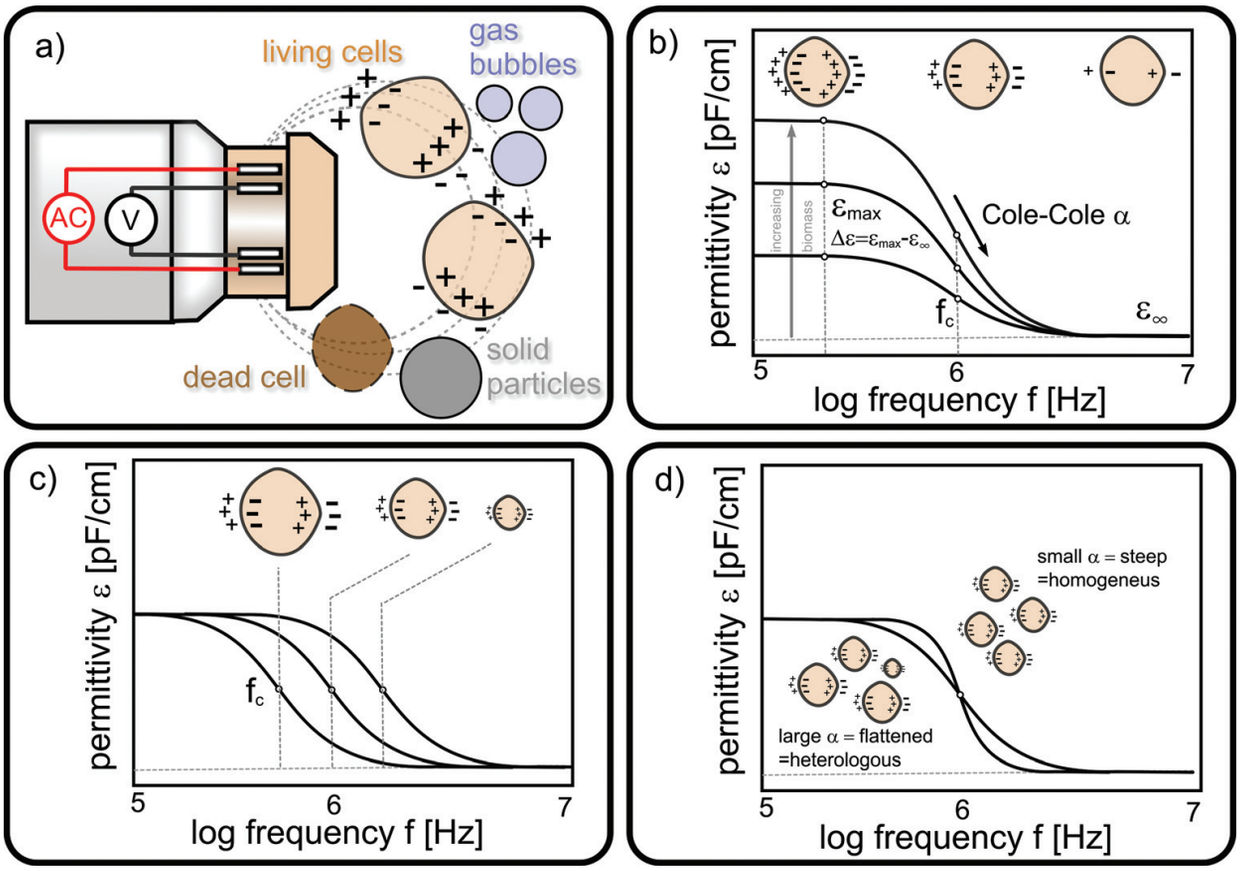

Figure 15. (a) Schematic illustration of a capacitive sensor: only cells with intact plasma membranes are polarized, whereas cell debris, gas bubbles and non-polarizable solid particles do not influence the measurement. (b) Schematic illustration of the β-dispersion spectrum of the observed permittivity: at low frequencies, the duration of the shift in the excitation field is sufficient to fully polarize the cells, whereas at high frequencies the rapid shift allows little polarization. Increasing the cell number increases the number of polarizable cell membranes and consequently leads to an overall increase in $\Delta\varepsilon$. (c) Theoretical comparison of three cell suspension cultures with equal biovolumes and conductivities, but composed of cells with different cell sizes. With decreasing cell size, the critical frequency f_c increases. (d) Theoretical comparison of two cell suspension cultures with equal biovolumes and average cell size, but different cell size distributions. A wider distribution leads to a flattened β-distribution. Panels (b) and (c) are modified from Ref. [202].

cell radius. However, if the radius does not change throughout cultivation, $\Delta\varepsilon$ is directly proportional to the viable cell density. Technically, the value of $\Delta\varepsilon$ can be determined either by scanning the whole frequency range or by measuring at two distinct frequencies. Recording a full β-dispersion spectrum is advantageous because it provides additional information. First, the so-called critical frequency f_c (Eq. (12)) represents the frequency at which one half of the potential polarization is achieved. It reflects changes in the cell radius as well as the conductivity of the cytoplasm σ_c and the surrounding medium σ_m (**Figure 15c**).

$$f_c = \frac{1}{2 \cdot \pi \cdot r \cdot C_m \cdot \left(\frac{1}{\sigma_c} + \frac{1}{2 \cdot \sigma_m}\right)} \tag{12}$$

Second, another variable known as the Cole-Cole α factor can be obtained by fitting the Cole-Cole model to the measured spectrum [197, 201]. The α factor has a value between 0 and 1 and

describes the steepness of the characteristic drop in β-dispersion. The α factor is assumed to reflect the homogeneity of the cell population [194, 201] as shown in **Figure 15d**.

Dielectric spectroscopy has proven to be a valuable tool for the online monitoring of insect cell cultures [195, 196, 203]. It is particularly useful for BEVS because the arrest of cell growth after infection and the swelling and lysis of infected cells can be monitored in real-time. In the context of insect cell cultures, dielectric spectroscopy was first used to monitor the growth of uninfected and infected Sf9 cells [204]. This study revealed a linear correlation between relative permittivity and viable cell density during the growth phase. However synchronous infection with a recombinant baculovirus encoding β-galactosidase at a MOI of 10 resulted in growth arrest, but permittivity increased further, indicating the successful detection of the infection-related cell swelling. During the late process stages, cell lysis associated with a decrease in cell viability and size was detected as a drop in the permittivity signal. In terms of process intensification by the optimization of feeding and infection strategies, the same authors reported the use of dielectric spectroscopy to monitor Sf9 and High Five^{TM} cells [205, 206]. In both studies, physiological parameters correlated with the impedance signal. Interestingly a peak in the CO_2 evolution rate (CER) observed at high MOI correlated with a temporary plateau in the permittivity signal. This was interpreted to represent the initial release of virus particles into the medium. In contrast, no permittivity plateau was detected for infections with a lower MOI (0.001). This behavior can be attributed to the fact that a lower MOI does not cause the simultaneous infection of all cells, hence there is no steep response in the cellular events caused by the infection, which are instead distributed throughout the cultivation process resulting in the delayed CER response (205). Dielectric spectroscopy has also been used for the optimization of a baculovirus-based production process for recombinant adeno-associated virus (rAAV) vectors at the 40-L scale [207]. The permittivity signal was used to pinpoint the optimal time of infection. An optimal time of harvest was also determined, because an increase in cell diameter was correlated to the yield of rAAV. Well-established off line methods (Vi-Cell® and CASY®) have been used to correlate the permittivity signal with the viable and total cell culture volume [208]. The authors observed an increase in the critical frequency f_c which coincided with the cell swelling after infection, but the influence of dynamic cell properties on f_c was not investigated.

Although there are only a few reports demonstrating the use of dielectric spectroscopy to monitor stable insect cell lines, this method is used routinely with a wide variety of mammalian cell lines [192, 195] and was proven to work with rS2 cells. In the production of an antimicrobial protein with rS cells under control of the Mt promoter, the dielectric spectroscopy was used to pinpoint the key transitional events of the process (induction and harvest) [209].

4.3. Optical density as a tool to determine absolute cell density

One of the most established methods for the online monitoring of cell suspension cultures is the measurement of optical density (OD) and its popularity is already reflected by the widespread availability of different commercial sensors (**Table 7**) [210]. Optical density probes exploit the ability of suspended particles (in this case cells) to scatter light in all directions. As shown in **Figure 16**, the scattering can be used in two main ways to derive information about cell density.

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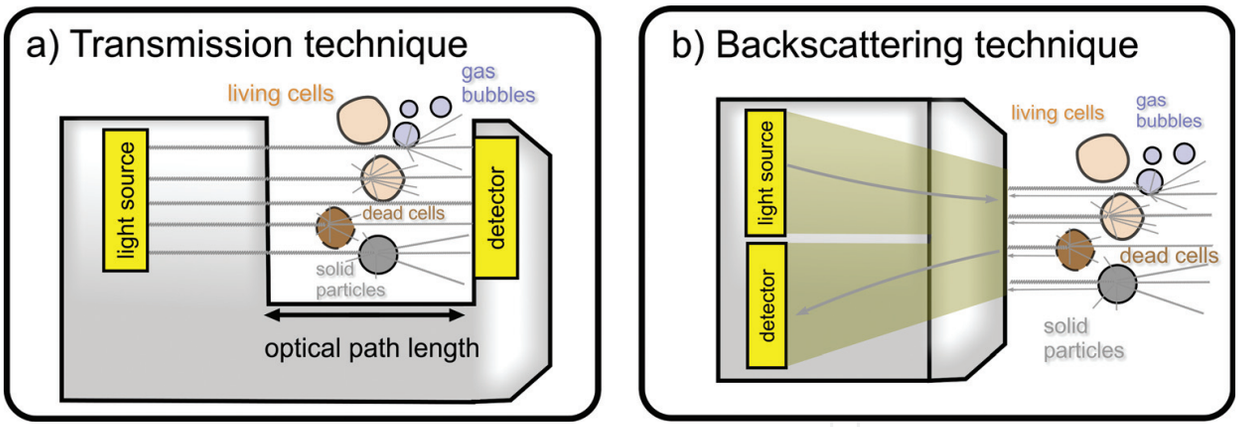

Figure 16. (a) Schematic illustration of an OD probe measuring light transmission. (b) Schematic illustration of an OD probe based on the measurement of 180° backscattered light.

Sensors of the first type (e.g. ExCell 230, Exner Process Equipment) measure the reduction in light intensity during its transmission through the medium. The availability of different optical path lengths for this type of sensor allows the analysis of samples containing a low density of particles. Sensors of the second type (e.g. InPro8100, Mettler Toledo) instead detect light that is backscattered at an angle of 180°. Both sensor types use near infrared (NIR) light in the range 780–1100 nm to avoid the signal being affected by changes in the medium colour [183].

In addition to their traditional use with microbial cells, online OD sensors have already been used to monitor mammalian and insect cells [175]. Accordingly, the density of uninfected, actively growing Sf9 cells has been shown to correlate well with the OD response, and the correlation persists until 24 h post-infection with a recombinant Baculovirus [202]. However, the OD signal becomes static or increases during cell lysis even though the viable cell number declines [202] This is because cell debris and dead cells contribute to light scattering to the same extent as viable cells, which is the major drawback of OD measurements: the method determines the overall abundance of light-scattering particles but cannot distinguish between cells and non-cellular particles nor between living and dead cells. Nevertheless, the method is highly robust for the quantification of viable cells and is therefore especially suitable for monitoring non-lytic expression systems such as rS2 cells [209]. Furthermore, some devices allow the simultaneous determination of dielectric properties and OD (e.g. the combined Hamilton Decyte and Incyte system). Combining both methods may improve process understanding because the ratio of OD and permittivity is an additional real-time parameter that can be used for process monitoring and control.

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