

Generation of monoclonal antibody-producing mammalian cell lines

Clinical effectiveness has driven the commercial success of monoclonal antibody (mAb) products. Mammalian cells are currently the preferred system for large-scale production as the mAbs produced are biochemically similar to human forms. The cell line generation process is tedious and time-consuming as clones with high productivity, stable long-term expression and good product quality are rare occurrences. Cell line generation efficiency and mAb quality can be improved through host cell engineering, vector optimization and high-throughput clone selection. Targeted integration into predetermined sites on the chromosome is a promising new area being explored. This review covers existing technology and recent progress made in improving various aspects of the stable cell line generation process for mAb production.

Early attempts at therapy with monoclonal antibodies (mAbs) were foiled by low protein amounts and highly immunogenic rodent sera cocktails [1]. These issues were addressed later by the development of hybridoma technology to generate larger amounts of product [2] and antibody humanization to reduce the immunogenic segments [3]. Fully human mAbs can now be generated with the recent inventions of phage display [4] and transgenic mice [5–7]. The improvement in efficacy and safety brought about by the aforementioned technologies has seen mAbs develop into the best-selling class of biologics.

The market for mAbs has seen 8.3% growth and US\$18.5 billion in sales for 2010 in the USA [8]. The highly specific targeting capability of mAbs is now used to treat various cancers, battle transplant rejections and fight autoimmune diseases. 28 mAb products have been approved for the market and over 350 are at various stages of clinical testing [9]. Five full immunoglobulin G (IgG) mAb products are currently listed as blockbusters (with over \$1 billion in annual sales each): Remicade®, Avastin®, Rituxan®, Humira® and Herceptin®. As the market continues

to mature, two new trends are also forming. Biotech companies are starting to target the smaller markets of orphan diseases as seen by the record number of biopharmaceuticals approved for such indications [10]. Biosimilars of existing blockbuster products are also being developed and gaining approval outside of the USA [10,11]. The increasing demand for existing mAbs and rapid innovation in mAb therapeutics has stimulated a parallel improvement in mammalian cell culture technologies used to produce a majority of the products. Faster and more efficient cell line development technologies for mAb production are now of utmost importance.

mAb production in mammalian cells can be performed either in transient or stable transfections. Transient transfections allow quick generation of small amounts of product for use during early stages of drug discovery [12]. There are several review articles available for information on large-scale transient transfections of mammalian cells [12–14]. Stably transfected cell lines are more widely used in large-scale industrial production. Cell lines used for manufacturing are from a single cell clone in order to obtain high amounts of consistent

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Key Terms

Biosimilars: Follow-on versions of an approved biopharmaceutical produced by a different company following expiry of the original patent.

Cell clone: Population of cells that are derived from the same ancestral cell.

product. The cell line development process (Figure 1) starts from transfection of a mammalian cell line with plasmid vectors carrying light chain (LC), heavy chain (HC), and a selection marker genes [15]. The plasmid vector comes in various designs, optimized for mAb production. Several cell types can be used, but mammalian

cells are the main workhorse for producing the safest and most effective mAb products. Plasmid delivery can be performed using calcium-phosphate precipitation,

electroporation, lipofection and polymer-mediated techniques [16]. After transfection, positive transfectants are selected for their drug resistance or growth advantage. If an amplifiable selection marker is used, gene amplification can be carried out to increase gene copies, leading to an increase in product expression. Single clones are then chosen for scale-up and characterization of product quality and long-term expression. The following sections will look at existing and upcoming materials and methods for generation of stably transfected mAb producing mammalian cell lines.

Mammalian cells

A survey of the mAbs currently approved in the USA or EU for the market shows a heavy reliance on mammalian cells for production. Of the 28 products, 12 are produced in Chinese hamster ovary (CHO) cells, 12 are produced in murine lymphoid cell lines NS0 or Sp2/0 and two are from hybridomas [9]. *Escherichia coli* microbial systems are only used for producing two antigen-binding fragment products. Mammalian cell types have become dominant for manufacturing due to their ability to produce large amounts of mAb with a consistent quality and to adapt well to culture in large-scale suspension bioreactors [15,17,18]. Another reason for the dominance of mammalian cells is their capability to perform the required protein folding, assembly and post-translational modifications such as glycosylation [19–21], so the mAb produced would be biochemically similar to human forms for increased product efficacy and safety [22].

» CHO

CHO cells were first isolated in 1958 and they quickly gained recognition for ease of culture and fast generation times [23]. Pathogenic human viruses such as HIV, influenza and polio do not replicate in CHO cells, greatly increasing the safety of the mAb produced and simplifying the downstream purification process [24]. The ease of genetic modification is another advantage of using CHO cells for mAb production [24]. CHO cells have a proven track record of producing safe, bio-compatible and bioactive mAbs, enabling products from these cells to gain regulatory approval more easily [25,26]. They will remain as the most widely used mammalian cells for therapeutic protein production in the near future, as evidenced by the continued usage of the cells for producing new mAb therapeutics [9,27]. The recently assembled genomic sequence of the ancestral CHO-K1 cell line will increase our understanding of these cells and further increase their popularity [28].

» Murine lymphoid cells

Murine lymphoid cells, NS0 and SP2/0 originate from differentiated B cells, which have the innate ability

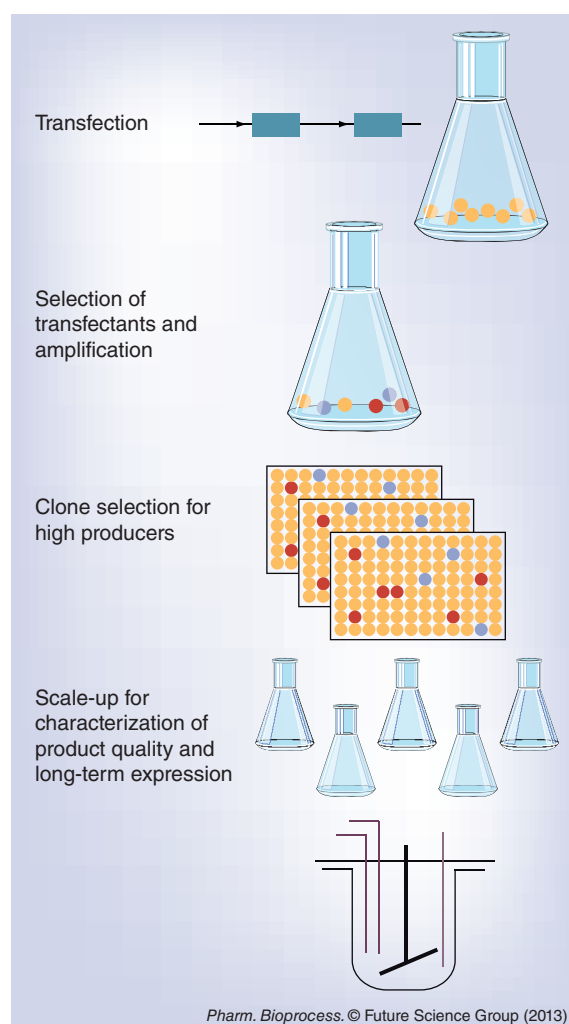


Figure 1. Cell line generation process. Mammalian cells are first transfected with plasmids carrying light chain, heavy chain and selection marker genes. Drug selection is then carried out to select for positive transfectants. An initial round of screening is carried out to identify high producers. Selected clones are scaled-up for characterization of product quality and long-term expression before production cell lines are selected for large-scale production.

to produce large amounts of immunoglobulin making them good candidates for manufacturing mAbs. Although there are currently a similar number of approved products from CHO cells and murine lymphoid cells, CHO-derived cells are becoming the preferred hosts. Four of the six products approved before the year 2000 were from NS0 or Sp2/0 cells, while two were from CHO. This changed in favor of CHO cells for products approved since 2010, where four were from CHO and only one from NS0. One reason for moving away from these cells is that glycoproteins from NS0 and Sp2/0 can have residues that are immunogenic and have a reduced *in vivo* half-life [29–31].

» Human cells

To ensure mAbs that are produced do not carry any antigenic carbohydrate groups, cells from a human source can be used for production [17]. Possible candidates include the human embryonic kidney-derived HEK293 cell line, immortalized human amniocytes from CEVEC and the human embryonic retinoblast derived Per.C6 cell line from Crucell [32]. HEK293 and amniocytes are reported to be better suited for transient protein production [12,33]. Per.C6 is currently the most promising candidate. This cell line can reach cell densities tenfold higher than CHO cells and has been reported to produce up to 8 g/l of protein in fed-batch reactors [32,34]. Several Per.C6-based products are currently undergoing clinical trials. Regulatory concerns exist regarding the use of human-based cell lines for production due to their lack of resistance against adventitious agents [32].

Host cell engineering

Mammalian cell culture performance can be improved by genetic engineering of either enzymatic or regulatory activities. Modifications to the cell phenotype can be achieved through traditional recombinant DNA techniques to overexpress target genes or to knock-down/knockout target genes by using more recent cell engineering techniques such as RNAi and zinc-finger nucleases (ZFN) [35–39].

» Apoptosis

Apoptosis is a form of programmed cell death that occurs during high stress conditions in dense and productive mAb-producing mammalian cell cultures [40]. Delaying the onset of apoptosis would benefit culture health and lifespan, making genes involved in the pathway interesting cell-engineering targets [41,42]. One approach is to overexpress anti-apoptotic genes, such as those in the Bcl-2 family, which have been shown to improve cell viability and increase mAb production [43–46]. Another approach involves downregulating pro-apoptosis genes such as Bax and Bak by RNAi [47] or

deleting the genes using ZFN [48]. Deleting the genes inhibited activation of downstream caspases in the presence of apoptotic stimuli, improving cell viability and increasing mAb expression by up to fivefold [48].

» mAb folding and secretion

mAb folding is an intricate process that is mediated by a series of chaperones and foldases [49,50], and is a possible bottleneck for mammalian cells producing high levels of the recombinant mAb [51]. Overexpression of protein disulphide isomerase, a foldase that catalyzes the formation of disulphide bonds, only saw moderate increases in mAb expression of CHO cells [52,53] or no effect [54]. BiP, a protein in the folding pathway that helps retain incompletely folded proteins by binding to exposed hydrophobic regions, caused a drop in mAb expression when overexpressed either alone or in tandem with protein disulphide isomerase [53]. The unfolded protein response is a cellular reaction to increased demand of the cells folding capacity by regulating the expression of a number of chaperones and foldases [55]. XBP-1 plays a major role in the unfolded protein response, but its overexpression generated no effects on mAb-expressing CHO cells [56]. The limited success of these attempts shows that engineering of the whole mAb folding pathway may be required for dramatic improvement of mAb expression.

» Glycosylation

mAb glycosylation is important for the product's pharmacokinetics, pharmacodistribution, stability, receptor binding and effector functions [57]. Despite its importance, there is no consensus for the 'correct' mAb glycosylation due to the inherent heterogeneity of the process and differences in activity [58]. It is still of great interest to both research and industry to generate mAbs with specific glycoforms to improve efficacy and safety. Although mAb glycosylation can vary through control of culture conditions [59,60], genetic approaches can be more efficient [61]. Many mAbs function through eliciting antibody-dependant cell cytotoxicity. Significant improvements were seen in antibody-dependant cell cytotoxicity activity for fucose-deficient IgG1 mAbs [62]. Knockout of the α -1,6 fucosyltransferase (*FUT8*) gene has been achieved by homologous recombination [63] and ZFN deletion [64]. Afucosylated Rituxan exhibited 100-fold improvements in antibody-dependant cell cytotoxicity activity [63]. It has also been demonstrated that normal mAb-producing cells can generate afucosylated product through siRNA knockdown of *FUT8* and GDP mannanose 4,6-dehydratase [65]. Fucose modification is currently the most successful method for improving mAb efficacy, and the first glycomodified afucosylated mAb produced from engineered CHO

cells was recently approved in Japan in March 2012 [66]. This approval will pave the way for more glycosylation-optimized biobetters produced from modified CHO cells.

» miRNA

Engineering of singular targets in complex mammalian pathways have yielded limited or mixed results [39]. miRNAs are non-coding dsRNA molecules able to globally modify gene expression levels to affect entire pathways [67]. Use of miRNA in CHO cells is a recent cell engineering technique first reported in 2007 [68]. The number of identified CHO miRNAs has increased exponentially since that report [69–71]. Although more studies still need to be conducted using mAb-producing cell lines to verify the usefulness of miRNAs, existing reports are promising. Overexpression of *cgr-miR-7* produced effects similar to growth arrest obtained by temperature-shifting and increased specific productivity [72]. Overexpression of miRNAs in the *cgr-miR-17-92* cluster enhanced cell growth [73].

Despite all the promise of cell engineering, none of the cell lines reported with improved growth or productivity have been used in industrial mAb production. With the approval of a glycomodified mAb from an engineered CHO cell line, and with over ten others under testing, we will likely see an increase in such products in the future [66]. Increased knowledge of the CHO genome [28,74,75], transcriptome [69,76] and proteome [77] would allow greater understanding of the complex interactions taking place for improved cell engineering approaches. Construction of dynamic computational models has worked well in the production of microbial cells and this extra information will eventually allow similar implementation in mammalian cells [39,78,79].

Vector design

» Signal peptide & codon optimization

Each IgG mAb molecule comprises two identical LCs and two identical HCs that are linked by disulphide bonds. Efficient expression of the LC and HC genes requires an appropriate signal peptide for transport of the LC and HC polypeptides to the endoplasmic reticulum for proper folding, assembly and post-translational modifications. Signal peptide derived from human albumin more than doubled the average specific productivity of a stable mAb-producing CHO cell mini pool to almost 40 pg/cell/day compared with using a native IgG LC signal peptide [80]. A signal peptide toolbox has also been generated to identify tailored signal peptides for each protein [81].

Codon optimization for gene expression is important due to differences in transfer RNA abundance in different cells and the effects on mRNA stability and second-

ary structures formed by the transcripts [82–84]. Early attempts at codon optimization for gene expression improved green fluorescence protein (GFP) expression in CHO cells by 42-fold using the empirical method of DNA shuffling [85]. More rational approaches are now available with increased understanding of mammalian cells, and a system known as codon adaptive indices is used to score how optimized a gene is [83]. A comprehensive assessment of codon usage was performed in a recent proteomic analysis of CHO cells, and preference for certain codons was observed to be especially prevalent for proline, threonine, aspartate and cysteine [77]. Applying optimization based on codon usage in CHO and human cells has seen improvements in mAb expression of 1.5–4-fold [84,86]. A combination of codon optimization and an optimized gene amplification protocol provides a way to obtain high-yield mAb producers with decreased effort [87]. An interesting application of understanding codon usage is to use it for generating mutant endogenous genes with ‘silent mutations’ for use in RNAi rescue experiments [88].

» Co-expression of LC & HC genes

Complex interactions occur between LC and HC during folding and assembly of an IgG mAb. The LC:HC peptide ratio plays an important role in the kinetics of mAb formation [89]. Excess LC is reported to be beneficial for higher mAb expression levels [89–92]. There are also reports that LC:HC ratios above 1.5 result in minimal product aggregation levels [92,93]. As LC:HC ratio could affect mAb assembly, it has been suggested that mAb glycosylation could also vary with ratio [91,92]. It is of interest to express these mAb subunits at optimal stoichiometric ratios for better mAb production.

LC and HC genes are traditionally introduced by co-transfecting on two separate vectors [94,95] or transfecting a single larger vector carrying all the required genes [96,97]. LC and HC peptide ratios can be varied under transient conditions when co-transfecting the genes on separate vectors by changing the relative amounts of each plasmid (Figure 2A) [91,98]. Controlling the ratio by this method in stable transfections is inefficient as the random integration process, gene copies and the site of integration cannot be controlled [99–101]. Single vectors provide better control of the ratio as all genes are integrated in the same site (Figure 2B). One possible issue that could arise with having multiple promoters in close proximity is the resulting transcriptional interference [102]. This interference suppresses gene expression to different degrees depending on the site of integration.

Single-promoter, single-vector systems for expressing LC and HC have a stricter control on the LC:HC ratio (Figure 2C). One such system uses internal ribosome entry site (IRES) elements to express LC, HC

and selection marker genes on one transcript [92,99]. An IRES element when placed between two genes mediates cap-independent translation of the second gene. This cap-independent translation of the second gene is less efficient than a typical cap-dependent translation, resulting in lower peptide levels for the second gene. Using such a design enables strict control of LC to HC peptide amounts. It has been shown that one IRES-mediated tricistronic vector with LC as the first cistron controlled LC:HC at a ratio of 4:1 in all clones [92]. This design was preferred as the excess LC aided in generating a greater proportion of high mAb-producing clones and the product had low aggregation and consistent glycosylation [92].

The multiple genes required can be expressed at equal amounts in a single open reading frame by using either the foot and mouth virus derived 2A self-processing sequence combined with a furine cleavage site (F2A) [103,104] or inteins [105]. 2A elements are only about 60–80 base pairs long, making it easy to incorporate them into vector designs. The 2A linked genes are expressed in one open reading frame and self-processing occurs to generate the two separate peptides [104,106–108]. Furin cleavage sequences are added to remove amino acid residues at the C-terminus of the protein upstream of 2A [104,109]. Productivity of clones from a F2A-based vector was comparable with clones generated using a reference vector using separate expression unit design [104]. Interestingly, Davies *et al.* recently reported that LC was still detected in excess in higher producing clones generated using the F2A system due to increased HC degradation [110]. One drawback of the F2A system is the possible formation of unwanted residues or fusion proteins [111,112]. Inteins have been tested for production of antibodies in HEK293 cells [105]. They are normally flanked by the two genes and translated together in a single polypeptide before splicing occurs to remove the intein to join the flanking proteins with a new bond. Mutations to the intein sequence inhibited the splicing while preserving the cleavage. When coupled with signal peptide optimization, functional mAbs could be produced using the constructs. More work is required to obtain higher mAb production levels using this system.

» Selection strategies

In order to efficiently select for stably transfected cells, selection marker genes that confer resistance to certain antibiotics or growth advantage in a nutrient-deficient condition are used. Antibiotics commonly used for selection include geneticin (also known as G418), hygromycin and puromycin and can act by disrupting protein synthesis in the cell. The cells are transfected with either neomycin phosphotransferase (NPT), hygromy-

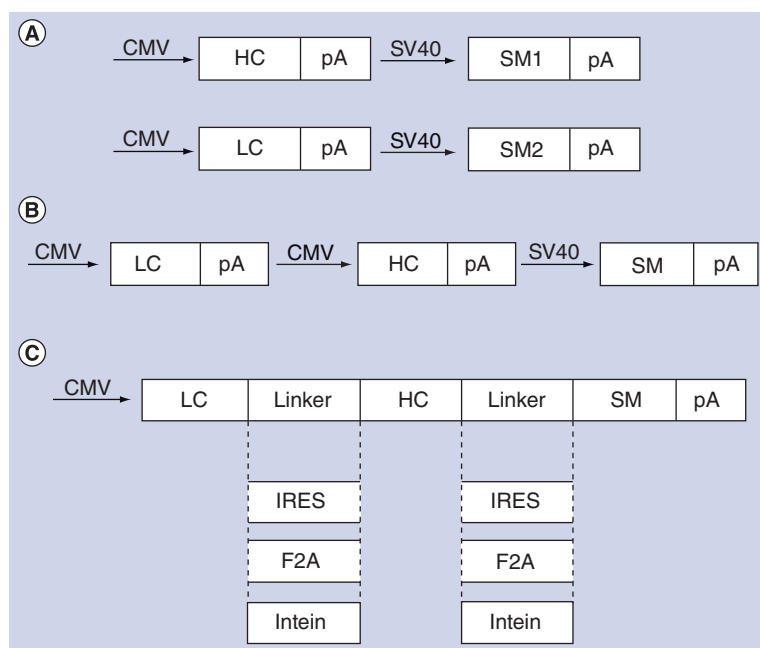


Figure 2. Different vector designs for expression of light chain and heavy chain for mAb production. (A) Co-transfection on two separate vectors. (B) Single vector with separate expression cassettes for each gene. (C) Single-vector, single-promoter vector with genes linked together on one expression unit. The linker element used can be an IRES element, a F2A sequence or intein sequences.

CMV: Human cytomegalovirus immediate-early gene promoter; F2A: Foot and mouth virus derived 2A self-processing peptide sequence with furin cleavage motif; HC: Heavy chain; IRES: Internal ribosome entry site; LC: Light chain; pA: Polyadenylation signal; SM: Selection marker; SV40: Simian virus 40 promoter.

cin phosphotransferase or puromycin acetyltransferase genes to gain resistance to the respective antibiotics. Benefits of antibiotics selection include the ease of use and no requirements for modified cell lines. One major drawback is that gene amplification cannot be conducted to increase expression levels.

The dihydrofolate reductase (DHFR) is an amplifiable selection marker typically used together with the DHFR-deficient DG44 or DXB11 CHO cells [113–116]. *DHFR*-knockout CHO-K1 mutants have also been generated using ZFN to overcome deficiencies such as poor growth and inefficient selection of current DG44 and DUXB11 cell lines [301]. DHFR is involved in the reduction of dihydrofolate to tetrahydrofolate, which is in turn needed for nucleic acid metabolism. First, selection is conducted in media devoid of hypoxanthine and thymidine. Amplification can be further carried out by adding a folic acid analog, methotrexate (MTX), which inhibits DHFR activity. In order to survive, cells will start to amplify the *DHFR* gene copy. The mAb genes located on the same transfected vector or in nearby sites are also amplified, increasing the gene copies and, thus,

Key Term

Gene silencing: Loss of expression of the transfected gene due to loss of gene copies or gene methylation inhibiting transcription.

expression levels after several stepwise increases in MTX levels [94–96,117].

The glutamine synthetase (GS) selection marker catalyzes formation of glutamine from glutamate and ammonia [97,118]. This allows successfully transfected cells to survive in media

lacking in glutamine. The GS selection system works well in NS0 cells that do not express their own GS. Using this system with mammalian cells with endogenous levels of GS requires the use of methionine sulphoximine, a GS inhibitor [118]. Similar to using MTX with DHFR, using methionine sulphoximine with GS forces cells to co-amplify the GS gene and the product gene [119]. There are concerns of instability when using the GS/methionine sulphoximine amplification in CHO K1 cell lines [120]. It has been reported that the GS system allows the generation of a high producing cell line without the need for gene amplification, and the timeline of cell line development is significantly reduced [97,118]. The effectiveness of the GS system in CHO cells was further improved when using the GS-knockout CHO cell lines. Usage of this mutant new cell line helped improve clone selection efficiency by sixfold, compared with using regular CHO cell lines [121].

Two novel selection methods have recently been reported that could be useful with further optimization. An amplifiable selection marker based on hypoxanthine phosphoribosyltransferase, which is required for purine synthesis, is currently under development [122]. A toxin/antitoxin method commonly used by bacterial cells to maintain plasmid stability has also been tested in mammalian cells [123].

Vector integration is a random event with no control over whether integration is into an active or inactive site. An active integration site leads to higher mAb expression levels. Enhancing selection stringency by reducing expression or activity of the selection marker can be used to identify integration into active sites. One way is using a weak promoter, such as the SV40 promoter, to drive expression of the selection marker gene [15]. Various forms of selection marker sequence manipulation or mutations can be carried out in tandem to further improve stringency. Using a non-traditional AUG start codon results in inefficient translation initiation and can further reduce expression [124,125]. Deoptimizing codon usage of the wild-type DHFR sequence to reduce translation efficiency improved the IgG expression level by threefold [126]. Reducing enzymatic activity of a NPT through amino acid mutations resulted in a 15-fold improvement of expression level as compared with wild-type NPT [127,128]. Protein and mRNA destabilizing elements can be used to reduce selection marker expression with-

out modifying the sequence [129]. This method could likely be applied with greater ease as no sequence modification is required.

When the selection marker gene is in an individual expression cassette, vector fragmentation can result in only the selection marker cassette being integrated intact [94,130,131]. Such clones would survive selection without expressing any mAb. Coupling of the selection marker to the product genes using IRES is a method used to minimize the number of these non-expressing clones [92,132–134]. Fragmented IRES-based vectors would have incomplete expression units and no transcription would occur. The reduced translation efficiency of IRES compared with typical cap-dependant expression also helps improve stringency. Increased stringency can be achieved by modifying the principal translation start site on IRES and further attenuating the translation [132]. Stringency of selection can be further improved by a combination of using IRES to couple product and selection gene and weaken the selection marker; for example, as design of IRES-mediated polycistronic vectors with activity-impaired NPT as selection marker genes [92,135].

» Chromatin-modifying DNA elements

The epigenetic status of the integration site can be altered by the DNA elements surrounding the site. Some of these elements have been isolated and have been tested in vector systems used for cell line generation, and have been shown to improve expression level and stability.

The ubiquitous chromatin opening element (UCOE) is an unmethylated CpG fragment isolated from a region around housekeeping genes that maintain the region in a transcriptionally active and open configuration [136,137]. UCOE from both human and mouse sources have been identified. The element is placed upstream of the promoter on the vector. Using UCOE increases the number of clones with higher expression levels [136]. Recombinant protein expression is also maintained for long periods as DNA silencing due to methylation is prevented [137]. The element is promoter-specific and some optimization of UCOE-promoter combinations is required [137].

Matrix attachment regions (MAR) create active chromatin loops within the chromosome and can regulate the epigenetic switching of the integration site to being transcriptionally active [138–140]. Transfecting it together with the plasmid vector helps maintain the integration site in an active configuration for a more homogeneous population with high and sustained product expression. Expression becomes gene copy number-dependant instead of site-dependant. mAb expression was most improved by either inserting one element upstream of

the promoter and co-transfecting another MAR-only plasmid or at both 5' and 3' ends of the expression cassette [139,141]. MAR from chicken lysozyme, the human genome and mouse genome have been identified and evaluated to have positive effects in CHO cells [139,140,142].

UCOE and MAR elements have been successfully used in gene expression platform technologies from Millipore and Selexis, respectively. Other DNA elements that could also prove to be useful with more evaluation include the expression augmenting sequence elements [143] and the stabilizing and antirepressor elements [144,145].

» DNA transposons

DNA transposons are repetitive genetic components found in eukaryotic cells that consist of a gene flanked by two inverted terminal repeats. A gene of interest flanked by the same inverted terminal repeats, together with a helper plasmid expressing the transposase, mediates transposition of the gene on the donor plasmid onto several possible sites in the chromosome [146,147]. This method is of interest to mAb cell line generation as most natural transposon sites are highly active. There is an increased level of transgene integration, and integration sites are less random than standard transfections [148]. Several DNA transposon systems have been tested to work well in mammalian cells and hold much promise in areas of cell culture [146,149]. Use of the technology to express a fusion protein in CHO cells saw an increased number of integrated gene copies compared with standard transfections [150]. Transposed clones had average specific mAb productivity of 26 pg/cell/day, more than threefold higher than regular transfected clones [150]. A 24-fold improvement in titer was observed in CHO pools transposed to express IgG mAb, with a significant increase in the proportion of clones having high productivities above 20 pg/cell/day [151].

» Targeted integration into 'hotspots'

A 'hotspot' commonly refers to a site in the genome that provides sustained high-level expression. Targeted integration into these hotspots is one attractive approach for cell line development. Transfected producer cells would have isogenic backgrounds, minimizing differences in genomic position effects and transgene copy numbers [152]. Productivity and growth behavior for these clones should be more similar than those generated using random integration, simplifying clone selection and large-scale culture process optimization. Epigenetic silencing effects can be circumvented to give stable production during long-term culture, a parameter as important as high productivity [153].

Cell line development using targeted integration has been demonstrated in a few studies based on site-specific recombinases, such as FLP, Cre and PhiC31 [152,154–162]. Identification of a hotspot in the genome by classical transfection of a screening vector, and subsequent screening of single copy integration, high expression level, and stability is first carried out. The screening vectors for hotspots usually contain an easily detectable marker gene, such as GFP, and a selection marker gene that may be mutated to enhance the stringency of selection for transcriptionally active sites [155,163]. Single copy integration is preferred to avoid repeat-induced **gene silencing** and for easier regulatory approval [164–167]. As hotspots are extremely rare in the genome, intensive screening of clones is required to identify an ideal master clone. This clone would then be reused to more effectively express any genes of interest using tag-and-target (**Figure 3A**) or tag-and-exchange strategies (**Figure 3B**) based on site-specific recombinase. The latter approach is more favorable as it avoids excision of the integrated product cassette and has a higher targeting efficiency by using two non-interacting recombination target sequences. A double-reciprocal recombination also enables removal of DNA between the two recom-

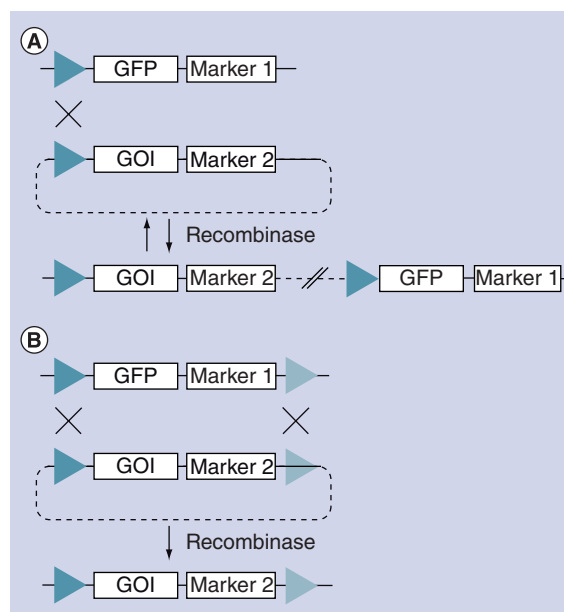


Figure 3. Site-specific recombinase-mediated strategies for cell line development. (A) Tag-and-target and (B) tag-and-exchange. Dark and light triangles represent wild type and mutated recombinase target sites, respectively, such as FRT for Flpe, LoxP for Cre, and attB for PhiC31. Markers 1 and 2 can be any antibiotic or enzyme-based selection marker.

GFP: Green fluorescent protein; GOI: Gene of interest.

Key Terms

Product quality: Monoclonal antibody attributes such as glycosylation, aggregation, charge variant and protein sequence that contribute to the efficacy and safety of the product.

Stable cell lines: Cell lines expressing a gene integrated into the host cell's chromosome as opposed to transient expression or the sustained recombinant protein expression over extended culturing of the transfected host.

ination sites in the screening vector, and avoids integration of extended plasmid backbone sequences of the targeting vector [154,166,167].

Cell line development using recombinase-mediated targeted integration has been shown to have advantages over random integration. More stable production and minimal clonal variations have been reported [154,155,159,160,168]. However, the mAb titer achieved is less than that achieved using random integration. More efficient screening strategies

to identify the rare, extremely transcriptionally active sites in the genome are required to increase the titer that can be achieved by targeted integration. Due to the low efficiency of targeting based on site-specific recombinase, drug selection and single cell cloning is still required to identify those correctly targeted clones [154,158]. As such, targeted integration has not yet significantly shortened the timeline of cell line development. Engineering recombinase for improved properties, such as enhancement of thermolability of Flp by cycling mutagenesis, is effective to improve targeting efficiency [169]. Other successful examples of enhancing targeting efficiency include addition of nuclear localization signal to PhiC31, application of strong promoters on Cre, optimization of Flp codons, optimization of the relative amount of transgene cassette and recombinase, and enhancing transfection efficiency of transgene cassette and recombinase [170–174].

Targeted integration can also be achieved by introducing a site-specific DNA double-strand break (DSB) on the chromosome that can stimulate homologous recombination efficiency by up to 1000-fold or more in mammalian cells [175–179]. DSB can be achieved using meganucleases, ZFN and transcription activator-like effector nucleases [171,180–182]. The DSB-mediated targeted integration efficiency is affected by the donor DNA structure, activity and sequence specificity of engineered endonuclease, and chromatin structure of the targeted locus [183,184]. Use of single-strand oligonucleotide donors [185] and *in vivo* cleavage of plasmid donors can improve targeting efficiency [186]. The sensitivity of endonuclease to methylation of targeted locus can be overcome by treatment of cells with a demethylation agent or design of an endonuclease that can avoid steric hindrance caused by cytosine methylation [187]. DSB-mediated targeted integration can reach a frequency of up to 15% in mammalian cell lines [183,188–191]. However, cell line development using DSB-mediated targeted integration has not been reported, probably due to intellectual property obstacles.

Clone selection

Random integration and amplification creates highly heterogeneous pools, making the selection of high mAb-producing clones an extremely time-consuming and tedious process. Most estimates place the number of clones required to ensure that sufficient high producers can be isolated to range between several hundred up to thousands [15,192]. Subsequent evaluation of long-term expression stability and **product quality** identify the few rare clones that satisfy the requirements of production cell lines. Recent developments in methods for selecting high-producing clones have also seen a heavier reliance on use of automation to save on labor and improve process consistency [193].

Flow cytometry applied to fluorescence-activated cell sorting (FACS) is one method of high-throughput selection for high mAb producers [194]. Millions of cells can be screened rapidly and specific subpopulations isolated from the highly heterogeneous pools. FACS can be applied to sorting of surface-labeled mAb-producing cell lines. The level of secreted proteins is proportional to the protein found on the cell surface. After three rounds of reiterative sorting, a 100-fold enrichment of MTX-amplified CHO cells was observed with specific mAb productivity reaching 42 pg/cell/day [195]. The cells can be chilled to below room temperature to slow down protein release from the membrane and endocytosis of the product-label complex to maximize the signal intensity [196]. Another way to use surface labeling for sorting is to co-express a surface protein not found in CHO cells, such as CD20 [197]. This allows surface labeling for cell sorting without the worry of signal loss. Surface labeling can also be conducted using a capture matrix composed of biotin, avidin and an antibody targeting the product. The matrix will cover the cell surface to trap the secreted mAb. The captured mAb can then be detected using another biotinylated secondary antibody for FACS sorting [198].

Linking the product gene to a fluorescent reporter by IRES is another way to allow use of FACS for sorting. Cells sorted for high fluorescent levels will be co-expressing high levels of the product gene [199]. High mAb producing cells can be sorted by dual fluorescent activated sorting where GFP and yellow fluorescent protein are linked to HC and LC expression, respectively [200]. Another alternative is to use two GFP fragments that reassemble to allow FACS of high producers [201].

Automated colony pickers are becoming an indispensable part of many cell line generation platforms. ClonePix™ from Genetix [202,203] and CellCelector from Aviso [204] are two systems that have been used for selecting recombinant protein-producing colonies. Cells expressing mAb are plated in semi-solid media containing a fluorescent detection antibody against the

mAb product. The viscous medium slows down dispersion of the secreted mAb, keeping it around the colony. This allows the detection antibody to label the product. Imaging software is then used to identify colonies with high productivities that fluoresce with a bright halo and are sufficiently isolated from low- or non-expressing cells for picking.

Production stability is a parameter as critical as productivity in mAb cell line development. The long-term stability required of a production clone was estimated to be around 60 generation to ensure sufficient time for expansion [118]. Studies have shown that intracolonial expression can be stochastic in nature, giving rise to heterogeneous clonal populations [205]. Identification of homogeneous clonal populations would identify those with expression at a steady state and more likely to be stable [205]. Screening can be performed using flow cytometry to check the population distribution [197]. Automated colony pickers can also be used by re-plating a clonally derived population back in the semi-solid media. **Stable cell lines** would have all subcolonies expressing mAb and the presence of non-expressing colonies would hint at unstable production. Methylation status of the promoter and gene copy number can also be used as early markers to identify unstable cell lines [206]. Other studies have also shown unstable cell lines to be more prone to apoptosis and relevant apoptosis-related markers such as caspase 3, Annexin V [207] and GADD153 [208] could also be used for early-stage screening.

Development of high-throughput analytical methods and frameworks would help alleviate some existing bottlenecks in the characterization process [209]. Early prediction of production level and product aggregation can be performed by selecting clones LC:HC mRNA ratios above 1.5 [93]. ER stress related protein GRP 78 was recently shown to be an indicator for high expressing clones and could be used to isolate high producing clones [210]. Miniaturized bioreactor systems such as ambr™ are also being used to mimic bioreactor conditions for medium-throughput characterization of clone performance under production conditions [302]. By using a combination of novel automated systems and recently identified early predictors for desirable cell phenotypes, significant effort can be conserved during the clone selection process.

Future perspective

The expanding use of mAb therapeutics and the development of the biosimilar market demands a parallel improvement in the production process. Initial issues of low production levels have been addressed with the past decade of research and development. Titrers have increased from milligrams per liter to easily reach grams

per liter in fed-batch cultures [15,211]. The timeline required for cell line development has also been reduced to a more manageable few months instead of over 1 year. These improvements in cell line generation have been achieved through innovations in cell line engineering, vector design and optimization, high-throughput automated clone selection devices, media design and process development [212]. Instead of looking for ways to reduce cost of goods through increasing titers, there is now a shift towards the shortening of development timelines and improving product quality and expression stability [213].

Technology advancement of available analytical tools such as chromatography and MS has seen a greater emphasis placed on the implementation of process analytical tools for analysis of products and control of processes. Critical process parameters can be tracked to identify quality attributes that will enhance the consistency and safety of mAb products [214]. Recent innovations in areas of genomics, transcriptomics, proteomics and metabolomics enable the detailed study of cellular processes. With this added knowledge, cell line development processes will move away from the empirical and towards more rational design, allowing quality-by-design concepts to be applied with greater accuracy and effectiveness [79]. Techniques such as hotspot targeting, cell and vector engineering and high-throughput automation will also see further technological advancements.

Along with new mAb therapeutics, the rise of follow-ons for existing blockbusters with expiring patents will increase over the next few years [215]. The need for robust platforms for biosimilar production is critical due to the number of competitors and the importance of maintaining similar product quality as the original innovator drugs [216]. Product characterization will need to be carried out with extra care as it is difficult to compare the quality and purity of biotherapeutics, unlike chemical generics [217]. Companies will increasingly rely on smaller scale disposable bioreactors to allow them to adopt flexible production lines for a wider array of products [213]. The approval of the first glyco-engineered mAb will also pave the way for biobetters of existing products and more novel products [66].

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Executive summary**Mammalian cells for monoclonal antibody production**

- » Existing cell types have been engineered to work better with selection markers and to produce products with high efficacy.
- » Novel candidates have been identified for possible use in the future.

Vector design

- » Signal peptide and codon optimization for improved expression levels.
- » Control of monoclonal antibody light chain and heavy chain subunits is important for efficient production.
- » Selection of positive transfectants can be improved using the appropriate selection marker and improved stringency.
- » Chromatin-modifying DNA elements can be incorporated into vectors to improve monoclonal antibody expression.

DNA transposon & 'hotspot'-targeted integration

- » Novel approaches are now available to control vector integration for improved cell line generation.

Clone selection

- » Heavier reliance on automated methods for high-throughput methods of picking clones.
- » Methods for early prediction of clone characteristics.

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