Whole-Genome Evolution Technology

Improving Protein and Antibody Yields in Scalable Mammalian-Cell–Based Manufacturing

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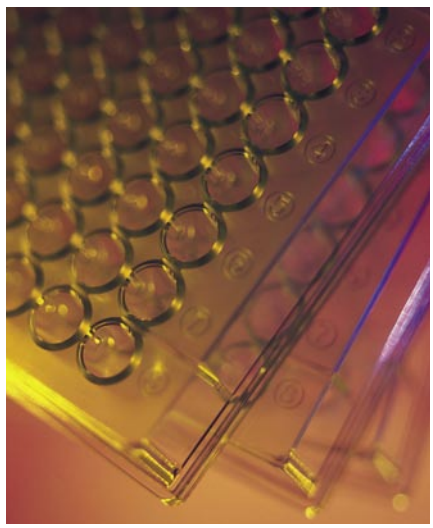
Over the past decade, therapeutic proteins and monoclonal antibodies (MAbs) have become one of the most successful classes of pharmaceutical agents because they can replace or block specific targets associated with disease (**1**). With the successful market performance of commercial products such as Remicade, Rituxan, Herceptin, Humira, and Avastin, MAbs have emerged as an important drug class, now representing about half of all new drug launches. Of all those launched to date, 40% are blockbuster drugs or have blockbuster revenue potential. Global sales of therapeutic MAbs exceeded $10 billion in 2004 and are projected to be in excess of $30 billion by 2008 (**2**). In the next five years, this should continue to be the fastest growing and most lucrative sector of the biotechnology and pharmaceutical industries — driven by technological evolution from chimeric (part human and part mouse) to humanized (CDR-grafted) to fully human antibodies.

Compared with traditional small molecules, MAb products offer greater specificity, less toxicity, and a more rapid development path to the clinic. Unlike small molecules, which exert their function on binding to a target, MAbs can make bigger therapeutic impact by using the immune system to elicit target-specific cytotoxic activity.



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Despite their huge success, efficient development of therapeutic MAbs remains quite challenging. Companies developing them must implement proper systems at early stages of development for successful GMP manufacturing, purification, and finishing processes. That ultimately affects time-to-market, efficacy, safety, and cost-of-goods. Also, MAb production remains challenging because of high manufacturing costs associated with the annual yields required to support dose volumes for therapy as well as high capital costs associated with adding production capacity. Manufacturing systems that generate large quantities of product as quickly as possible are needed to shorten development timelines and lower overall cost of goods by maintaining an abundance of production capacity within the marketplace.

Companies specializing in MAb manufacturing have developed several cellular systems that offer high MAb yields. These are based on bacterial, yeast, plant, and mammalian cells (**3**). Microbial platforms have provided some of the highest overall titer yields; however, the amount of active protein typically represents only a fraction of total protein generated because of improper folding or processing. No such systems have been successfully adopted to efficiently produce more complex proteins such as multichain macromolecules (including antibodies), which require additional processing (e.g., posttranslational modifications). In light of those microbial-based manufacturing limitations, mammalian cells remain one of the most reliable and widely used systems for large-scale GMP manufacturing of therapeutic antibodies and certain nonantibody proteins.

Currently 300 antibody development programs are in progress that will require significant manufacturing capacity for clinical materials and ultimately for commercial supply (**4**). Most of those programs use recombinant mammalian cell lines such as Chinese hamster ovary (CHO) and mouse myeloma (NS0, SP2) cells grown

in standard CGMP cell culture procedures using fed-batch or perfusion bioreactors (**5**). The burden on cell-based manufacturing capacity continues to grow because product demands increase as each program progresses through clinical trials and ultimately to the market. In light of the associated risk, alternative procedures may help ensure sufficient manufacturing capacity within the market and keep overall cost of goods similar to or lower than what is experienced in today's market.

A variety of approaches are being pursued to meet future needs for large-scale manufacturing: improvement of overall production and purification yields as well as use of alternative manufacturing sources such as transgenic strategies (**6, 7**). Even as alternative host systems are being validated for cost-effective scalability and regulatory compliance, the state-of-the-art remains mammalian cell culture. The ability to improve yields of antibody production in mammalian cells can be achieved by several methods:

- better bioreactor performance through culture conditions and/or media optimization
- improved vector expression through highly active promoters or amplified vector copy number
- optimized cell hosts with enhanced endogenous pathways that provide for better titer yields and cell growth at large scales.

Any of those improvements, or combinations thereof, can shorten the number of manufacturing runs required to produce annual product needs, thereby relieving overall capacity constraints in the marketplace. Here we focus on a process that improves cell host performance to enhance productivity for antibody production.

Host cells can be optimized by manipulation of endogenous pathways: e.g., mRNA transcription and maturation, protein synthesis and posttranslation modifications, protein secretion and cellular sublocalization, molecular trafficking between cytosol and organelles, and cell cycle and survival regulation. Subtle structural changes in proteins that are involved in regulation of one or more of those processes (Figure 1) can directly or indirectly affect the overall performance of a production cell line. A randomized, genome-wide mutagenic approach that can be screened for functional cellular phenotypes offers one approach to enhancing complex cellular processes regulating growth rate, survival at very high cell densities, and rates of protein synthesis and secretion. Unfortunately, the use of mutagens often causes genome-wide chromosomal instability, yielding unstable cell lines that are unsuitable for GMP manufacturing.

Previous studies have shown that inhibition of a postreplicative DNA repair mechanism called mismatch repair can induce genetic diversity within stable cells by increasing point mutations incorporated by DNA polymerase during replication (**8, 9**). This process has been successfully applied to several mammalian cell lines that produce recombinant therapeutic antibodies to derive evolved sibling (sib) cells with enhanced production titers (**10**). The process also can be applied to production cell lines for improving other cellular processes and growth properties that can improve manufacturing yields.

For example, cells can be genetically enhanced for faster growth and/or an ability to maintain productivity at high densities for longer culture runs by inducing structural changes in growth factors or biochemical receptors that sense accumulation of metabolic byproducts that perturb growth and survival. The model in Figure 2 simulates dramatic effects that enhanced growth by faster-growing (FGR) cells and/or those that grow at a high density (HD) can have on overall productivity for scalable manufacturing.

Figure 1: Pathways of antibody production in mammalian cells; many pathways exist to regulate protein production and cell growth, and alteration of one or more can be achieved through genetic manipulation to improve production and growth performance. Such pathways include (1) gene transcription; (2) mRNA stability and translocation; (3) protein synthesis; (4) protein posttranslation modification and trafficking; (5) folding within the endoplasmic reticulum; (6) protein maturation and glycosylation within the golgi; (7) secretory pathways; and (8) cell membrane expression. (GlcNAc = N-acetylglucosamine; P = phosphorylated residue)

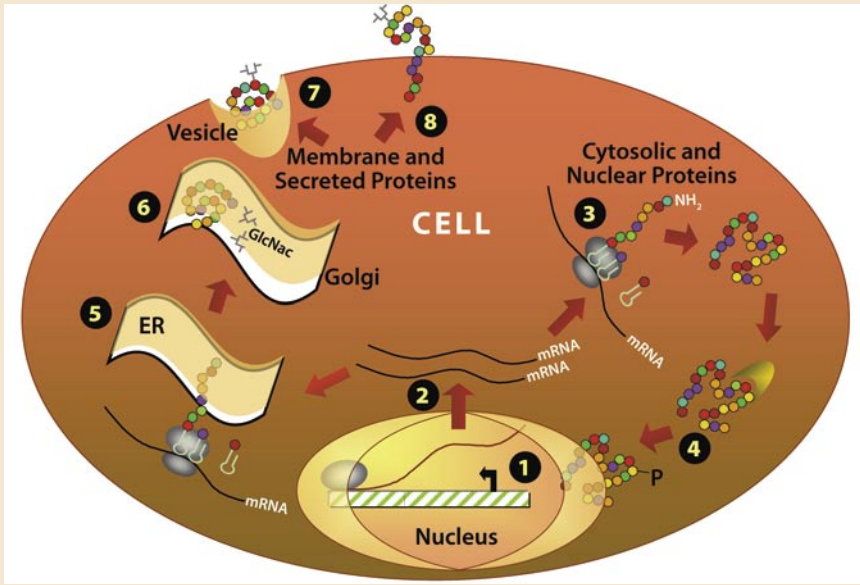


Figure 2: Production effects of cells with enhanced growth rates and stability at high cell density

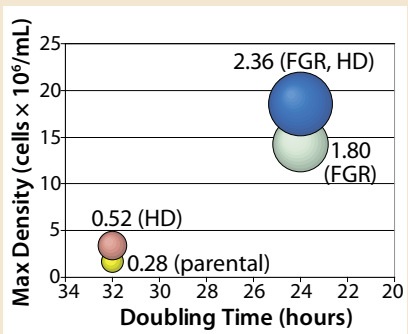
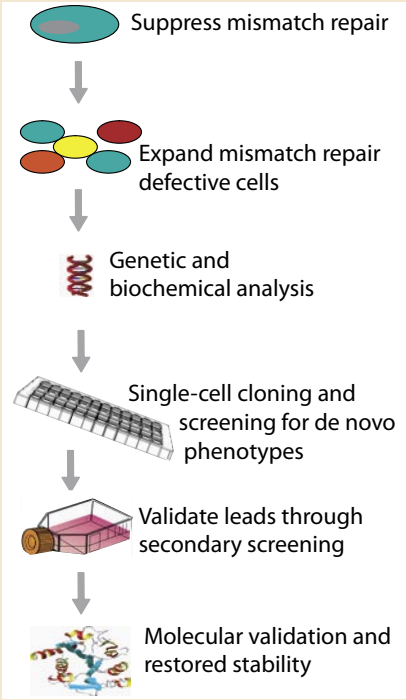


Figure 3: Whole genome evolution process flow chart



A 10-day culture was simulated for cells with enhanced growth rates or an ability to grow at high density for extended periods. The model assumes that as cell density increases, growth rate decreases; accumulation of antibody depends solely on cell number and pcd; and seeding at day 0 is 500,000 cells/mL. Doubling time of the parental line is 32 hours, maximal density of 1.7 × 106/mL is reached at day 5, and its specific productivity is 25 pg/cell/day (pcd). Under those conditions, a parental cell line would yield ~0.28 g/L upon completion of a run. As shown here, a cell line derived from the parental that reached a higher cell density (HD) of 3.4 × 106/mL at day 7 and would have a higher antibody yield — represented by the sphere's size and number expressing g/L. Another line with a faster doubling time (FGR) of 24 hours would produce an even higher yield than that achieved by cells capable of growing at higher density. A cell line exhibiting the combined improvement (HD and FGR) would perform best, reaching 2.36 g/L (compared with 0.28 g/L for the parenteral cell line).

Technologies that can improve those attributes within mammalian cell lines will address their ability to cost-effectively produce higher MAb yields fairly quickly. We have previously reported our use of a whole-genome evolution technology (WGET), termed *morphogenics* (www.morphotek.com) or *Revolution* technology (www.invitrogen.com), to evolve endogenous titer yields in CHO, NS0, and hybridoma cells (**9–11**). We recently expanded applications of the technology to generate mammalian cells with enhanced growth parameters for improving overall production at scale. Here we present the use of WGET to improve growth rates of recombinant mammalian cells with little affect on specific productivity.

Whole-Genome Evolution Technology

Whole-genome evolution technology harnesses the power of evolution for developing cells that yield proteins and antibodies with improved biological properties and/or enhanced titer yields for scalable manufacturing. A key distinction that separates this from other evolution-based technologies is the random, in vivo nature of the process. It uses the many genes and pathways that all cells innately possess, most of which go unexploited because of our incomplete understanding of cellular processes. So it generates unexpected mutants that are then identified by functional cell screens, leading to sib cells and gene products with desirable phenotypes. The process is time- and cost-efficient because it is applied in vivo to preexisting production strains for enhancement of whole-genome evolution, thus requiring no in vitro manipulation.

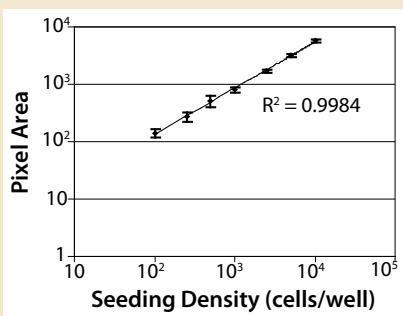
Reversible Inhibition of Mismatch Repair: DNA replication is a complex process that all cells undergo during proliferation to pass on their genetic information to "daughter" cells. As cells replicate their DNA, mutations occur within newly synthesized templates through a number of mechanisms, including polymerase infidelity. A series of postreplicative DNA repair processes — e.g., the mismatch repair (MMR) system (**12–14**) — have naturally evolved for organisms to retain their genotypic identity and are ubiquitously present in prokaryotic and eukaryotic cells.

MMR prevents accumulation of "naturally occurring" transition and transversion mutations through a secondary proofreading process that corrects discordant genetic information between parental and sibling DNA templates. WGET is based on reversible MMR inhibition. It is mediated by the activity of dominant negative proteins or chemical inhibitors blocking MMR within cells that yield therapeutic antibodies or proteins. Suppressing MMR allows inheritance of point mutations in the genomes of sib cells due to mutations that occur during DNA replication (**9, 12, 13**). This suppression allows naturally occurring mutations to be inherited at higher frequencies (up to 1000-fold enhancement) than are typically observed in MMR proficient cells. A genetically diverse population of sibs derived from WGET comprises a library of cells that can be screened through automated functional high-throughput screening (HTS) to identify subclones that secrete antibody with enhanced properties (e.g., affinity), that exhibit enhanced growth characteristics, or that produce antibodies at higher titers.

Process Outline

To enhance genetic evolution in mammalian cells, MMR is suppressed either by introducing an expression

Figure 4: Cell counting with microscopic image analysis; CHO-MAb cells were seeded in 96-well U-bottom plates at densities of 100–10,000 cells per well to determine linearity of imaging. Wells were imaged at 20× using the Meta imaging system. Colony size was determined by integrating the pixel area of each well covered by cells using the Metamorph software package. Data points are an average of 12 wells ± standard deviation.



vector containing an inhibitory protein or by incubating host cells in the presence of a chemical MMR inhibitor (**9, 10**). Both methods are effectively inhibit MMR and produce genetically evolved sib cells, and they have been used interchangeably.

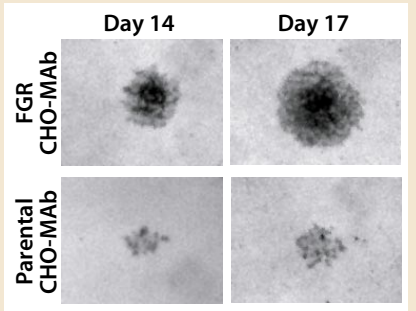
In either case, MMR-suppressed cells are passaged for about 30 generations, during which time genome-wide mutations accumulate in daughter cells. The genetically diverse pool of cells are then single-cell subcloned by limiting dilution into 96-well microplates, and clones are expanded for up to two weeks. At this stage, a variety of functional, automated HTS assays are used to identify subclones yielding proteins with enhanced pharmacologic activity or cells with enhanced titer yields and/or growth properties that may be suitable for improved, scalable manufacturing. MMR is then restored in desirable subclone(s) by removing the MMR inhibitor as previously described (**9, 10**).

At that point, MMR-proficient subclones exhibiting desired phenotypes are analyzed to confirm preservation and stability of the enhanced properties, restoration of wild-type MMR activity and a stabilized genome, and the integrity of protein structure and function. An attractive feature of WGET is that it can be directly applied to a manufacturing cell line for which there is a need to further optimize growth characteristics, titer yields, or both.

We applied WGET to improve growth rates of antibody-expressing mammalian cells to produce sufficient quantities for timely, scalable GMP manufacturing.

Enhancing Growth Rates: Generation of recombinant cells expressing antibodies at high titers has typically resulted in slower-growing hosts, making them impractical for scalable manufacturing (**15**). WGET can be applied to optimize growth parameters to yield faster-growing, high-titer cells of any species by virtue of the high degree of conservation and function of MMR in microbial, plant, and mammalian cell-based systems. As an example, we demonstrate the utility of the process in CHO cells producing a recombinant antibody.

Figure 5: Comparing colony size of CHO-MAb parental or WGET-derived clones subcloned into 96-well bar-coded plates and grown for 12 days in a CO_2 incubator at 37 °C; representative CHO-MAb colonies were imaged on days 14 and 17 using Metamorph imaging software to calculate pixel area of the colonies (growth ratio = day 17 area / day 14 area ÷ 3 days). Parental cells for the CHO-MAb line have a growth ratio of ~0.5. Cells exhibiting growth ratios >0.85 were expanded and analyzed in standardized growth assays. The inherent slower growth rate of parental cells typically results in smaller colonies at day 14 than clones that have evolved for faster growth, as expected.



The WGET process was applied as outlined in Figure 3. MMR was suppressed in parental cells using the chemical inhibitor morphocene. The MMR-suppressed cell line was propagated and subsequently subcloned to yield about 10,000 sibs, which were screened for a phenotype with enhanced cellular proliferation using a customized visualization platform and software that can monitor cell growth at low density.

Screening Cell Lines for Faster Growth: To screen for faster-growing (FGR) clones in WGET-derived cell pools, we developed a new image-based HTS method. This method eliminates the requirement of traditional time-consuming cell counting. Instead, it uses the Meta Imaging System from Molecular Devices Corporation (www.moleculardevices.com), which images cell colony size by a digital camera interfaced to an ORCA automated platform from Beckman Coulter, Inc. (www.beckmancoulter.com) that plates, feeds, and analyzes colony sizes of sib cell clones generated under sterile conditions. Using the Metamorph software package (Version 6.3r0 from Molecular Devices), the colony area image is quantified by pixels and exported to a spreadsheet that calculates ratios of cell colony size during a three-day growth period, then compares colony sizes at days 14 and 17. The imaging system has been refined to generate a linear correlation between image pixel area and cell number (Figure 4).

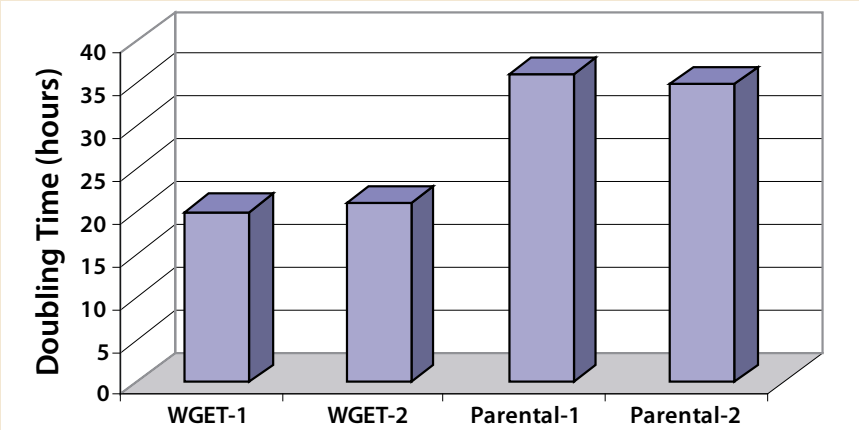
To identify FGR sibs in WGET-derived CHO-MAb cells, we seeded cells into 100–200 bar-coded, round-bottom, 96-well plates at 0.8 cells/well, using the Biomek FX robotic system from Beckman Coulter to ensure single-cell clones per well. The imaging method does not require sacrificing cells for quantification, so replica plates are not needed for this procedure.

Two weeks after the initial clonal seeding, we screened plates with the ORCA system for high-throughput analysis of clone growth as determined by microscopic imaging. Plates were housed in stacking incubators and returned to the incubator after image analysis with no disturbance of the colonies. We repeated that step three days later to determine the expansion of colonies, which we then used to determine cell growth ratios between the day 14 and 17 time points.

Cell growth is calculated as the ratio between colony sizes measured at day 17 and day 14 (dividing the colony area at day 17 by that at day 14 by three days). Figure 5 illustrates a typical analysis, in which parental or FGR CHO-MAb clones are imaged at day 14 and then again at day 17. As expected, the FGR cells show a larger ratio between day 17 and 14 than those derived from parental CHO-MAb. On average for this example cell line, parental clones show a growth ratio of ~0.5, as determined through a primary screening of ~20,000 independent parental CHO-MAb-derived subclones, whereas FGR clones exhibit a growth ratio of >0.85.

As a comparator standard, each plate contains clones derived from parental CHO-MAb cells. Figure 5 shows a typical profile of subclones derived from the parental line and

Figure 6: Representative growth data confirming faster-growing subclones; FGR and parental CHO-MAb cells were grown in seven-day shake-flask assays and analyzed for growth rates by cell counting at days 1, 4, and 7. Shown here are growth rates as a function of doubling time in hours for a subset of FGR (WGET-1 and WGET-2) and parental cell derived (Parental-1 and Parental-2) CHO-MAb subclones.



WGET-derived cells. In WGET derived CHO-MAb cells, ~5% of the wells screened exhibit a growth ratio of 0.8 or higher. Of those leads, 50% were confirmed to have improved growth rates, which is consistent with screens for other cell lines using the same assay.

To further validate increased growth rates, confirmed clones are grown in 3-mL static cultures over a 48-hour quantitative proliferation assay whereby cells are physically counted at days 0, 1, and 2 using the Cedex (Innovatis) automated cell counter. Figure 5 shows a representative result. The top-performing FGR clones were further expanded and evaluated in a 20-mL shake flask assay. Most clones that reach this level typically maintain their faster growth rate while retaining high antibody-specific productivity (Figure 6).

Structural analysis of antibodies derived from FGR subclones confirms that those produced retain similar genetic and biochemical properties to the parental antibodies. In addition, extended culturing of FGR cells demonstrated that the enhanced growth rate was stable and that overall titers during a three-month growth period remained constant in both parental CHO and FGR-derived cells (Figure 6).

Extensions of Technology

Generating FGR cells with high antibody titers is another application of WGET for development of cell lines suitable for scalable manufacturing. The ability to generate FGR cells with high specific productivities can lead to dramatic increases in overall production for antibody manufacturing with many cell types (Figure 2). The process also aids in development of isogenic cells that can be analyzed with a variety of genomic and proteomic tools to uncover genes and pathways involved in optimized cell growth or titer production.

By contrast with standard chemical mutagens, which induce aneuploidy as a result of chromosomal instability (**8**), the WGET process contributes to comparative genetic approaches because it allows subtle point mutations while leaving chromosomal stability and long-term viability intact. This feature eliminates the high mutation background and mutational "hotspots" seen in chemically mutagenized cells, which can show both genetically unstable genomes and recurring phenotype outcomes.

Not only does the WGET approach yield more robust outcomes, but it also makes differential gene/protein discovery easier by unequivocally identifying lead targets involved in pathways associated with enhanced growth and production. We successfully used WGET-derived MAb production cells to identify pathways in high-titer productivity by performing RNA microarray analysis of gene expression between sets of isogenic parental and high-titer WGET-derived sibs (**10**).

Identifying evolved pathways for high titers or faster growth can offer the opportunity to directly engineer high-performance cells for production of many products. This can be achieved by discovering modified pathways in WGET-derived cells with enhanced properties and recapitulating mutant phenotypes by cell engineering those pathways onto parental cell backbones. Extension of such accelerated growth rates into parental cell lines (either by WGET or directed pathway modifications resulting from it) can in turn accelerate speed to the clinic by reducing the time required to generate stable, high-titer production cell lines.

Although we have focused here on mammalian systems, similar approaches can be carried out to enhance growth and production properties in microbial production systems (such as yeast and *Escherichia coli*) as well as plant cells. In addition, other phenotypes could be generated within such alternative systems to enhance their use in antibody manufacturing, including proper glycosylation and folding of full-length antibodies to support industry needs for manufacturing of the numerous programs currently in development.

Whole-genome evolution technology can be considered a broad tool for supporting the scalable manufacturing needs of therapeutic antibodies in development. Its random nature and in vivo mode of action distinguish this process from other complementary technologies, providing alternative solutions to improve host cell performance. The speed with which a preexisting production strain can be optimized makes the WGET process suitable for satisfying current needs for rapid cell line optimization to produce FGR cells that exhibit high antibody titers at preclinical, clinical, or commercialization stages. Our application of WGET technology shown here yielded cell lines with faster growth properties and used an automated HTS platform to identify antibody production strains with enhanced growth properties.

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