

# Protein Bioproduction without Antibiotics

## Tool Specifically for Use in *E. coli* Gets Around Problems with Antibiotic-Resistance Genes

Cédric Szpirer, Ph.D., and  
Philippe Gabant, Ph.D.

Since the beginning of DNA engineering, plasmid instability has been a significant concern, especially in recombinant protein production. Typically, protein-production processes in prokaryotes require the use of bacterial plasmids as vectors to carry the gene of interest encoding the protein to be overexpressed.

It is well known that the growth of plasmid-bearing cells is significantly reduced relative to plasmid-free hosts simply because the protein production (corresponding to the gene of interest overexpression) and the vector represent a burden on host metabolism.

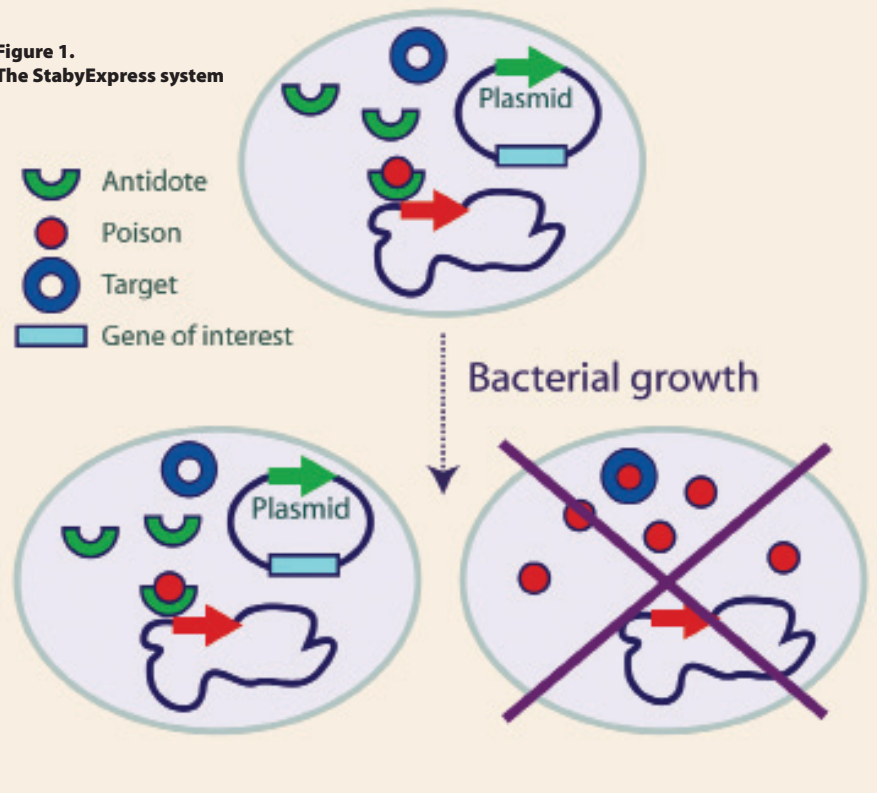
Antibiotic-resistant genes are the selectable markers typically used in fermentation processes to avoid plasmid-free cells overgrowing the culture. Antibiotic and antibiotic markers represent a problem from a

regulatory standpoint, and today, only the Kanamycin resistance gene is still tolerated by regulatory authorities. The Kanamycin resistance gene, however, represents a metabolic burden that limits the production yield of the bioproduct of interest.

The only way to avoid antibiotic-resis-

tance spread in the environment and transfer to pathogenic strains is the complete absence of antibiotic-resistance genes in recombinant constructions. Thus, it is expected that, in the near future, there may be zero tolerance toward antibiotic-based selection and production systems at least in the biopharma-

**Figure 1.**  
The StabyExpress system



**Figure 2.**  
Researchers can obtain access to StabyExpress technology by purchasing research kits that contain strains and vectors to produce their protein of interest. The purchase of the kits includes a license for research use of the technology.



### Fermentor Production Yield

|   | Induction at Early Stage of Growth   |                        | Induction at Advanced Stage of Growth |                        |
|---|--------------------------------------|------------------------|---------------------------------------|------------------------|
|   | System Based on Kanamycin Resistance | Antibiotic-free System | System Based on Kanamycin Resistance  | Antibiotic-free System |
| Cell Dry Weight (g/L)                   | 28                                   | 23                     | 22                                    | 24                     |
| Plasmid Retention (%)                   | 5                                    | 100                    | 90                                    | 98                     |
| Product Yield (mg/L)                    | 36                                   | 350                    | 280                                   | 603                    |
| Specific Productivity (mg product/gCDW) | 1                                    | 15                     | 13                                    | 25                     |

Two conditions were analyzed: induction at  $A_{600}=25$  (advanced stage of growth), close to standard production, and induction at  $A_{600}=1$  (early stage of growth) to increase the number of cell divisions between induction and end of fermentation and introduce an extended high level of stress over the cells. In normal conditions the plasmid maintenance is increased and productivity doubles. For extended stress conditions, the differences between antibiotic-free and standard are dramatic, both in terms of plasmid maintenance and protein productivity.

ceutical field.

Several alternatives to antibiotics have been proposed but none of them has been widely adopted. This can be explained by the complexity of the proposed systems, which requires complex or specific media, or non-conventional strains.

An alternative to antibiotic markers that is less energy consuming for the host metabolism is required for more efficient recombinant bioproduction processes.

With these challenges in mind, **Delphi Genetics** ([www.delphigenetics.com](http://www.delphigenetics.com)) developed a stabilization system called StabyExpress™ based on the use of natural bacterial antidote/poison genes and more precisely the *ccd* operon. This operon was isolated from the F plasmid naturally present in *E. coli* and encodes two genes (*ccdA* and *ccdB*) that are very well characterized.

The *ccdB* poison gene codes for a small stable protein, whereas the *ccdA* antidote gene codes for a small unstable protein that neutralizes the poison protein both transcriptionally and via protein-protein interactions. These genes were chosen because they are not toxic to humans.

StabyExpress technology takes full advantage of the natural properties of these genetic elements. Indeed, the functions of these genes have been polished by evolution in order to interact very efficiently with *E. coli* physiology so as to trigger bacterial death in very defined conditions.

The observed efficiency of the StabyExpress technology comes from this natural evolution. The *ccd* operon belongs to the poison/antidote bacterial gene family. These loci share common features and are widespread in plasmids and bacterial chromosomes. They represent a natural toolbox for applications in bacterial species presenting an industrial interest.

#### **Bacterial Poison/Antidote as an Alternative to Antibiotic Markers**

In the StabyExpress system, the *ccdB* poison gene has been introduced into the chromosome of *E. coli*, and the vector contains a copy of the *ccdA* gene encoding the antidote (*Figure 1*). When the plasmid is lost, the production of the bacterial toxin is induced, causing bacterial death. Consequently, only bacteria-harboring plasmids are growing and can use nutritional resources. To limit the burden of energy for the host and selection of mutants, the *ccdA* antidote production was reduced to a maximum (the gene is cloned under a weak constitutive promoter and for all intents and purposes the protein is not detectable).

Moreover, we used a transcriptional negative feedback loop to avoid *ccdB* expression when *ccdA* is present. Since *ccdB* poison protein is specifically targeting the bacterial

DNA gyrase, it is thus not toxic for eukaryotic and human cells.

Staby technology was combined with a T7 expression system and introduced in research kits. Different laboratories (academic and industrial) have used these kits to produce their protein of interest in *E. coli*. The system is easy to use and is compatible with any growth medium. It is also possible to integrate StabyExpress technology into any vector: the GetStaby kit contains a *ccdA* cassette designed for easy combination with any protein-expression system in *E. coli*.

In 2009, Delphi Genetics signed a non-exclusive commercial agreement for StabyExpress with **Sanofi-Pasteur**, which subsequently showed that the technology allows recombinant protein production at industrial scale without the use of antibiotic markers (and antibiotics).

Moreover, Sanofi-Pasteur showed that StabyExpress technology improves production yield in fermentors. As shown in the *Table*, when standard fermentation conditions are applied, the plasmid maintenance is at least equivalent or superior when StabyExpress selection is compared to standard vector (98% versus 90%). More interesting, in these conditions, the amount of expressed antigen increases twofold with StabyExpress (603 mg/L versus 280 mg/L).

In high-stress conditions, the plasmid loss is extremely rapid with standard vectors as compared to StabyExpress (5% versus 100%), indicating stringent selection of the StabyExpress system.

In that case, the amount of expressed antigen increases tenfold (36 mg/L versus 350

mg/L). In order to evaluate the potential for scaleup, the fermentation process previously established for standard conditions was transposed at a 30 L scale. Values obtained for both optical densities at different times and plasmid maintenance are almost similar (twofold and tenfold increase).

Delphi Genetics provides StabyExpress licensee strains that are compliant with cGMP standards in terms of culture quality (animal and GMO free). In addition, strains containing StabyExpress technology have been completely sequenced. This allows industrial researchers to develop an antibiotic-free production process starting from completely genetically characterized material.

#### **Conclusion**

Improving yield and quality of recombinant protein production remains a challenge. Necessary improvements include the re-engineering of genetic elements (host and vectors) used in these processes. By using the natural properties of bacterial poison and antidote genes, Delphi Genetics has designed a powerful alternative to antibiotic markers for expression-vector selection in *E. coli*.

StabyExpress can be integrated into any *E. coli* genetic background making it a potential universal alternative to antibiotics/antibiotic markers in this host. In light of new regulatory guidelines limiting the use of antibiotics, this development is significant. **GEN**

*Cédric Szpirer, Ph.D., is co-founder and CEO, and Philippe Gabant, Ph.D. (pgabant@delphigenetics.com), is co-founder and head of business development at Delphi Genetics. Web: [www.delphigenetics.com](http://www.delphigenetics.com).*