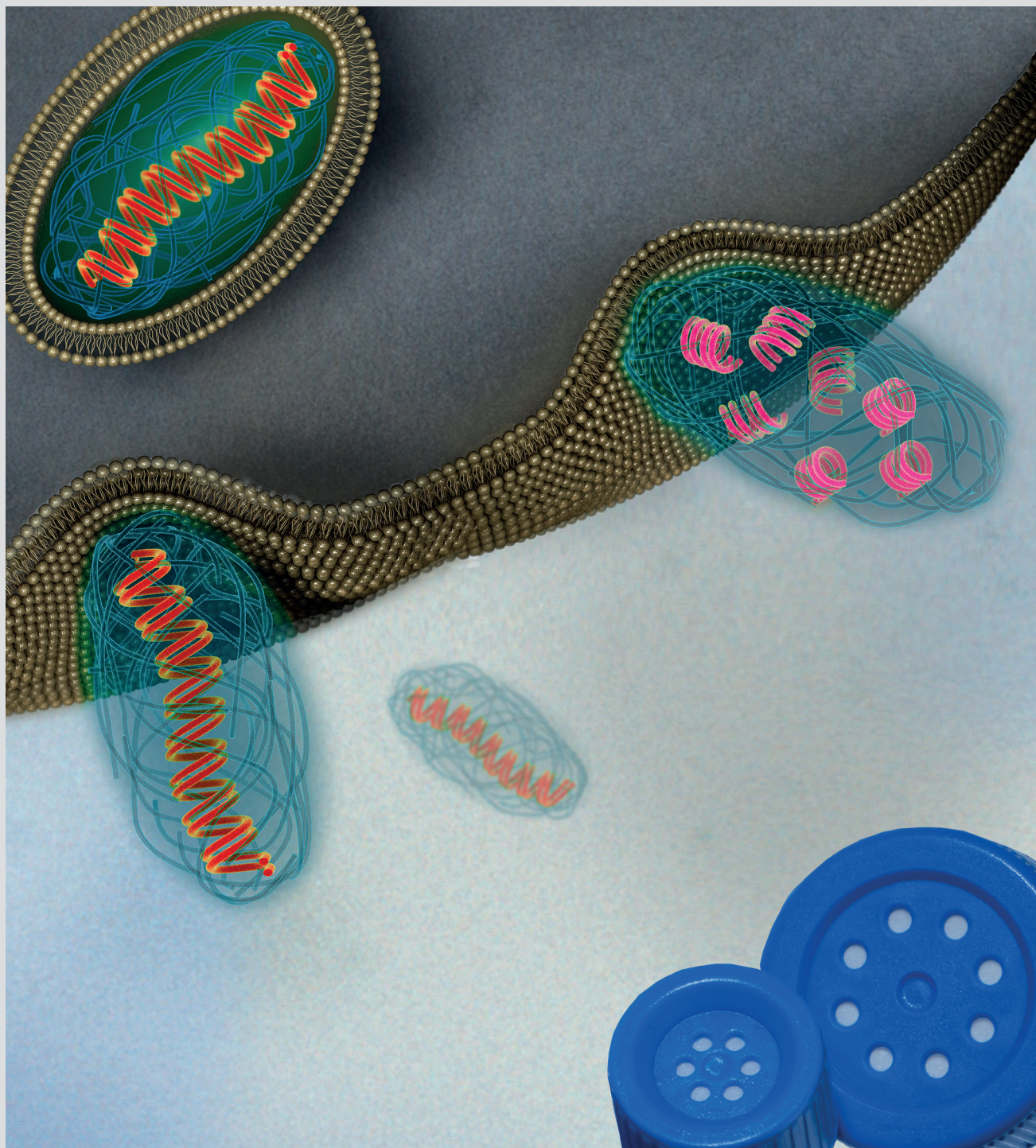


Application Note

Superior protein yields in suspension CHO cells
using FectoPRO™-mediated transient transfection
in CELLSTAR® CELLreactor™



1. Introduction

The introduction of nucleic acids into cells represents a key technology for research in life sciences (1). Transfection, which consists in introducing nucleic acids into eukaryotic cells, has enabled advances in many fields including cell and molecular biology, gene function, regulation studies as well as drug target identification and validation.

Transfection is a standard tool that has been widely used in bioproduction of recombinant proteins and antibodies to generate stable producer clones. Nowadays, transient gene expression (TGE) is also commonly used for medium scale production of biologics. This approach allows generation of sufficient protein amounts avoiding a major investment in production of stable cell lines prior to “proof of concept” studies or tools validation. Indeed, the speed and flexibility of TGE has enabled this technique to be widely adopted in bioproduction for early discovery, research applications and process developments (2).

Although widespread, transfection methods are evolving constantly in order to provide adapted solutions depending on the applications and the delivered biomolecules. To resolve the issue of low transfection efficiency of CHO cells, which can be a major bottleneck hampering protein production in TGE systems, Polyplus-transfection® has developed FectoPRO™, a specific transfection reagent for bioproduction applications. The FectoPRO™ kit, containing the transfection reagent and a booster, was designed after extensive screening of numerous chemical structures based on their transfection efficiency, protein production yield and cell viability. FectoPRO™ is suitable for transient transfection of suspension CHO and HEK-293 cells in various serum-free media and different culture vessels, using low DNA amount ($< 1 \mu\text{g}/\text{ml}$ of cell culture).

The most commonly used cells for production of recombinant proteins and antibodies are HEK-293 and CHO cells (3), usually grown in suspension in synthetic serum-free media in specific culture vessels, such as the CELLSTAR® CELLreactor™ from Greiner Bio-One. Culturing suspension cells in the CELLSTAR® CELLreactor™ has several advantages towards conventional systems such as shaker flasks. For screening purposes, only small volumes of cell cultures are needed, and the CELLSTAR® CELLreactor™ allows cells to grow in optimal conditions with as little as 3 mL of cell culture, reducing costs and handling. By using the tubes on an orbital shaker e.g. with a standard tube rack, a space-saving arrangement is achieved. In this way, cell growth can be monitored with a variety of culture conditions in a single run. If desired, centrifugation of cells without the need to transfer to another tube is achieved by the conical tube bottom.

Here we show that CHO cells grown in CELLSTAR® CELLreactor™ grow better and healthier than in other culture tubes. Moreover, CHO cells transiently transfected with FectoPRO™ produce higher protein yields in CELLSTAR® CELLreactor™. Taken together, our results show that the combination of CHO cells grown in CELLSTAR® CELLreactor™ and their FectoPRO™-mediated transient transfection result in amazing protein production yields.



The FectoPRO™ transfection reagent for bioproduction applications.



CELLSTAR® CELLreactor™ tubes are available as 15 ml and 50 ml tubes with filter cap.

2. Material and Methods

Mammalian cell culture

The commercially available FreeStyle™ CHO-S cells (Life Technologies, Carlsbad, CA) were grown in suspension culture in FreeStyle™ CHO Expression Medium (Life Technologies, Carlsbad, CA) supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin, 1% of Pluronic-F68, and 8 mM L-glutamine. Cell cultures were maintained between 0.25 and 3 x 10⁶ cells/ml in 125 ml Erlenmeyer flasks with vented cap, shaken at 130 rpm in an incubator at 37 °C with 8 % CO₂.

Plasmid

A pFUSE vector containing the gene coding the Fc fragment of the mouse immunoglobulin 3 was used for the transfection. Amplification and purification of the plasmid was performed by Tebu-bio (France).

Transfection

FectoPRO™ and FectoPRO™ Booster (Polyplus-transfection, Illkirch, FR) were used as transfection reagent. The day before transfection, cells were counted. 150 millions of cells were centrifuged. After elimination of the supernatant, the cells were resuspended in 150 ml of complete fresh FreeStyle™ Expression Medium. 16 CELLSTAR® CELLreactor™ tubes (Greiner Bio-One, Frickenhausen, DE), 16 tubes from Company A, and 16 tubes from company B were filled with each 3 ml of the 1 M/ml cell suspension. The tubes were then incubated overnight at 37 °C, 8 % CO₂ and shaken at 130 rpm. The day of transfection, cells were between 1.2 and 1.4 x 10⁶ cells/ml. 30 µg and 48 µg of plasmid DNA were diluted in 6 ml of cell culture medium without glutamine, antibiotics nor Pluronic-F68. Those diluted DNA preparations were then transferred in tubes containing respectively 60 µl and 72 µl of FectoPRO™. After 10 minutes at room temperature, 300 µl of the DNA/FectoPRO™ complexes were added directly to the 3 ml of cell culture. 0.75 µl of FectoPRO™ Booster was then added if necessary. Cell cultures were returned to the incubator at 37 °C, 8 % CO₂ and shaken at 130 rpm. Viable cell density and IgG3-Fc production were assessed every day during the 3 days following transfection.

Viable cell growth analysis

The TC-20™ Automated Cell Counter (Bio-Rad, Hercules, CA) was used to determine the viable cell density of the cultures. 10 µl of cell suspension were mixed with 30 µl of Trypan Blue (1:4 dilution) and then transferred into a counting slide chamber. 2 independent counting were done for each culture.

Capillary cytometry analysis

A Guava® flow cytometer (Merck-Millipore, Darmstadt, Germany) was used to determine the percentage of GFP-positive cells.

One day after transfection, 50 µl of cell suspension are mixed with 200 µl of PBS and transferred in a 96-well plate. The plate is then read with the flow cytometer and GuavaSoft 2.6 is used to determine the percentage of GFP-positive cells.

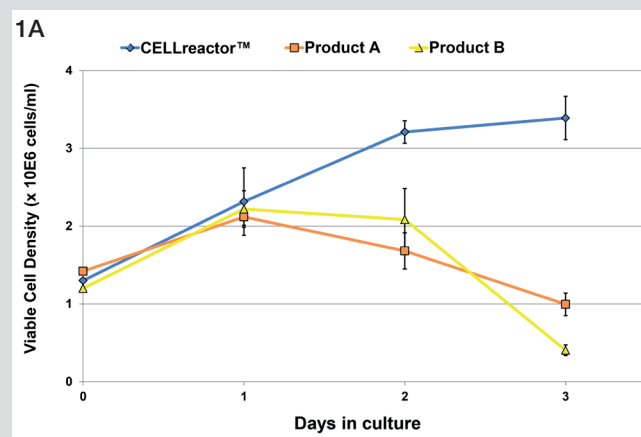
IgG3-Fc production analysis

For the IgG3-Fc production analysis, a whole 3 ml culture is centrifuged. The supernatant is filtered through a 0.22 µm filter and transferred into a glass HPLC vial. Using a Waters 2695 HPLC system, the samples are then injected in a POROS® G 20 µm Column (Life Technologies, Carlsbad, CA). The elution is done with a solution of NaCl 150 mM / HCl 12 mM and the purified protein is quantified using a spectrophotometry measurement at 220 nm. Using a standard curve and the measured area under the curve, the quantity of IgG3-Fc produced can be determined.

3. Results

CHO cell growth in CELLSTAR® CELLreactor™ and other tubes

Following the FectoPRO™ cell culture protocol, CHO-S cells were seeded at 1 x 10⁶ cells/ml the day before transfection in the different 50 ml culture tubes. The viable cell density (figure 1A) and viability (figure 1B) were monitored for the non-transfected cells during 3 days (figure 1). A similar cell growth and viability was determined for each transfection and culture condition after one day of cell culture. The cells cultured in the CELLSTAR® CELLreactor™ tubes continue to grow nicely at day 2 and day 3 until they reach almost 3.5 x 10⁶ viable cells per mL with a cell viability of 95-98 %. Comparatively, the cell cultured in the competitor tubes show a growth plateau at day 2 and then a decrease in the viable cell density at day 3. This growth arrest and decrease seem to be correlated with a lower viability. Those results show that the cells are healthier and grow better in the CELLSTAR® CELLreactor™ tubes than in the competitor tubes. The combination of high air permeability of the CELLreactor™ filter membrane and the large cap surface area that is available for aeration provides a key factor for successful cell growth.



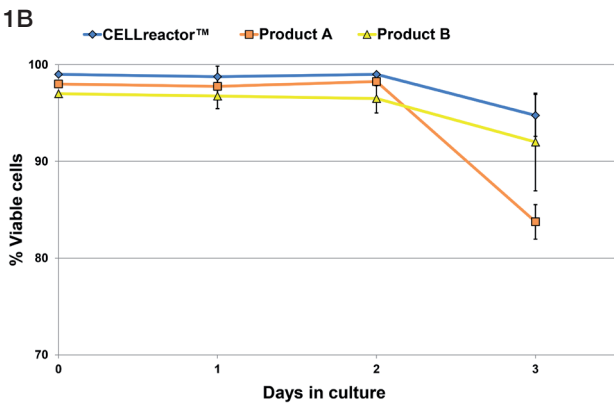


Figure 1: Growth and viability of non-transfected FreeStyle™ CHO-S cell are better when cultivated in CELLSTAR® CELLreactor™ tubes. The cells were seeded at 1×10^6 viable cells/ml in 3 ml at day 0 in CELLreactor™, Product A or Product B 50 ml tubes. The viable cell density (A) and the percentage of viable cells (B) were determined each day using Trypan Blue and an automated cell counter.

FectoPRO™ transfection efficiency in CHO cells

The cells cultivated in the different tubes were transfected with FectoPRO™ following the recommended protocol, with a plasmid coding for the Green Fluorescent Protein.

Transfection efficiency was determined as the percentage of GFP-positive cells 24 h after transfection using a capillary flow cytometer analysis (figure 2). Remarkably, transfection efficiency is significantly higher for the culture grown in the CELLSTAR® CELLreactor™ tubes than in other tubes. When no FectoPRO™ Booster is added to the cultures, a difference of transfection efficiency of almost 20 % between the cell cultures in product A (50.5 % GFP+) and the CELLreactor™ (69.3 % GFP+) is observed. This difference reaches 25% if the cell cultures in the CELLSTAR® CELLreactor™ tubes with the cell cultures in Product B (43.5 % GFP+) is compared. When adding the FectoPRO™ Booster following the transfection, the differences in transfection efficiency between the conditions tested decrease slightly as the Booster is a transfection inducer. Indeed, cultures grown in product A and product B tubes reach 71 % of fluorescent cells whereas the culture in the CELLreactor™ tubes reaches a transfection efficiency of 85 % GFP+, outperforming by a significant 15 % difference the other tubes. Here we show that cells are healthier and grow better in the CELLSTAR® CELLreactor™ tubes compared to 2 other tubes, leading to significantly better transfection efficiency.

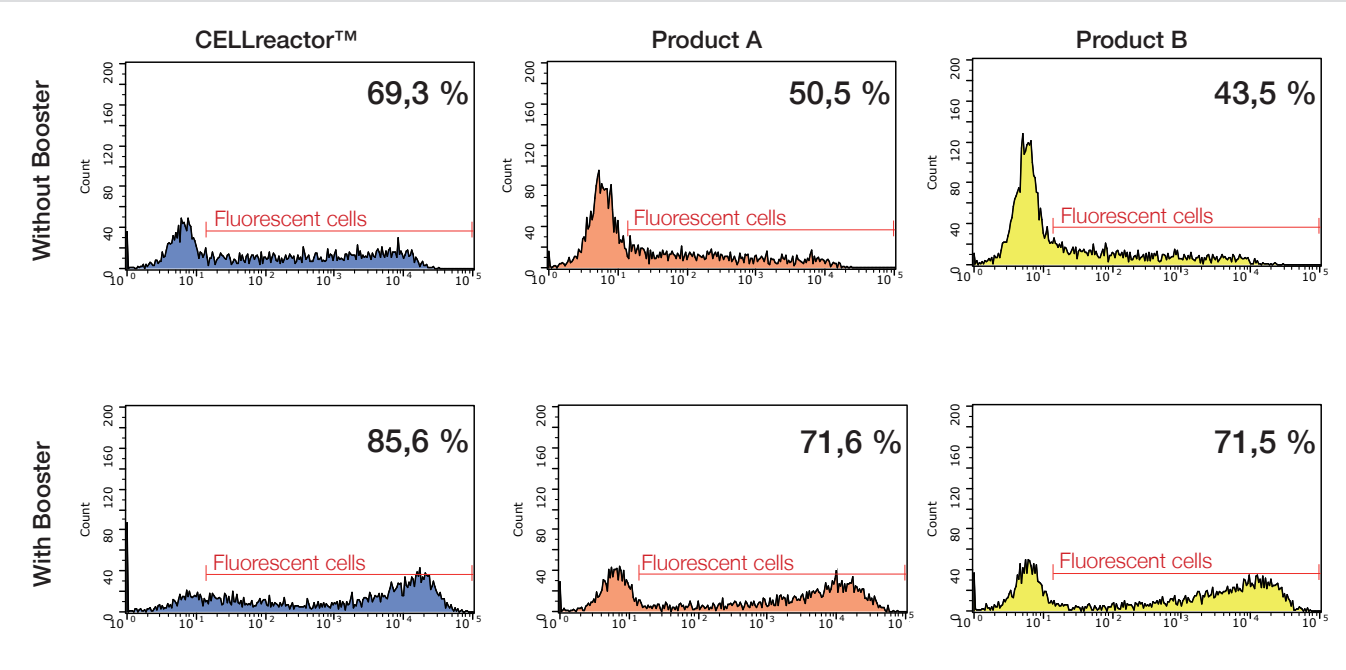


Figure 2: FectoPRO™-transfection efficiency is significantly higher with cells cultivated and transfected in CELLSTAR® CELLreactor™ tubes. The cells were transfected using FectoPRO™ reagent and a GFP-expressing plasmid either with Booster (0.5 µg of DNA/ml ratio 1:2) or without Booster (0.8 µg/ml ratio 1:1.5). Analysis of GFP-positive cells was done the day after transfection using capillary flow cytometry.

Protein production in CHO cells transfected with FectoPRO™

To investigate the impact of the variation of transfection efficiency observed in the different cell culture tubes on the production yield of a recombinant protein, we produced the secreted Fc fragment of the murine immunoglobulin G subclass III (IgG3-Fc) by transient gene expression in CHO cells. After cultivating and preparing the CHO cells in the different culture tubes, a plasmid coding for the IgG3-Fc was transfected according to the FectoPRO™ protocol. The concentration of secreted protein was then measured each day for 3 days (fig. 3). One day post-transfection, the IgG3-Fc concentration in the cell culture grown in Greiner tubes is already higher than in the culture using product A or product B tubes. This result is confirmed and even amplified at day 2 and 3, and is independent on the concentration of DNA transfected. Interestingly, we show that at day 1 and 2 post-transfection, the concentration of protein produced grows continuously in the 3 different tubes. However, between day 2 and 3, a drastic decrease of the IgG3-Fc concentration is observed in the culture in the Corning and TPP tubes. These results could be explained by the low viable cell density and low viability previously observed in those tubes. On the contrary, the CELLSTAR® CELLreactor™ tubes allow for an ideal cell growth and viability as well as for the highest transfection efficiency, both parameters which are converted into a continuous cell productivity of the IgG3-Fc protein up to day 3, resulting in an IgG3-Fc yield between 8 and 10 mg/L three days post-transfection and outperforming competitor tubes by a minimum of 30 % in terms of protein yields.

4. Conclusion

By carefully selecting culture and transfection conditions, cell culture and transfection efficiency can be significantly improved for bioproduction applications. Greiner Bio-One's CELLSTAR® CELLreactor™ has been shown to provide excellent cell viability for both untransfected and transfected cells. In combination with Polyplus' FectoPRO™ transfection kit, efficiencies of up to 85 % transfected CHO-S cells can be achieved, resulting in outstanding protein yield.

DISCLAIMER

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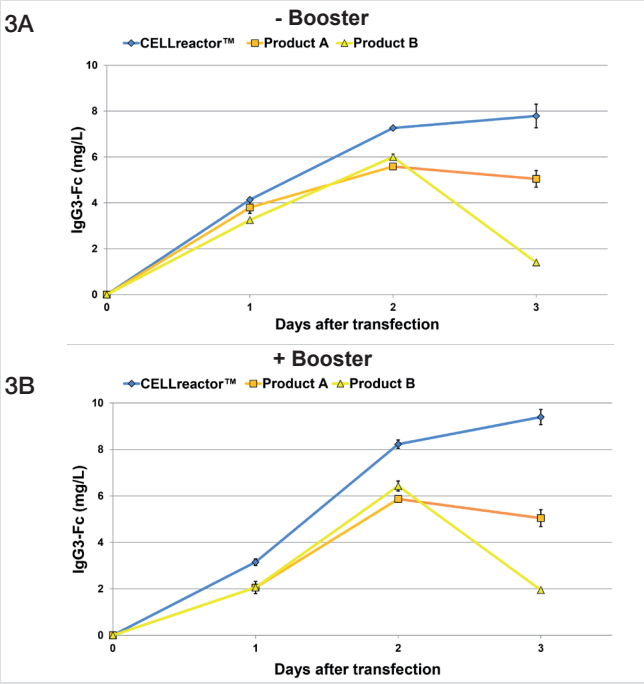


Figure 3: Protein production yields are higher and last longer with cells transfected and cultivated in CELLSTAR® CELLreactor™ tubes. The cells were transfected with FectoPRO™ reagent and a IgG3-Fc-expressing plasmid either without Booster (**A** - 0.8 µg/ml ratio 1:1.5) or with Booster (**B** - 0.5 µg of DNA/ml ratio 1:2). The secreted protein production analysis was performed using protein G affinity column (HPLC) each day after transfection.

Literature

- (1) Kim TK, Eberwine JH. Mammalian cell transfection: the present and the future. Anal Bioanal Chem. 397(8):3173-8 (2010)
- (2) Matasci, M., Hacker, D. L., Baldi, L. & Wurm, F. M. Recombinant therapeutic protein production in cultivated mammalian cells: current status and future prospects. Drug Discov. Today Technol. 5, e37–e42 (2008)
- (3) Wurm, F. M. Production of recombinant protein therapeutics in cultivated mammalian cells. Nat. Biotechnol. 22, 1393–1398 (2004)

Ordering Information

Cat.No.	Description	Quantity/Bag	Quantity/Case
GB0: 188 240	CELLSTAR® CELLreactor™ Filter Top Tube, PP, 15 ml, sterile	20	300
GB0: 188 299	Sample Pack CELLSTAR® CELLreactor™ Filter Top Tube, PP, 15 ml, sterile	2	n/a
GB0: 227 245	CELLSTAR® CELLreactor™ Filter Top Tube, PP, 50 ml, sterile	20	500
GB0: 227 299	Sample Pack CELLSTAR® CELLreactor™ Filter Top Tube, PP, 50 ml, sterile	2	n/a
Polyplus: 116-001	1 mL FectoPRO™ Reagent, 1 mL FectoPRO™ Booster	1	1
VWR: 10118-842	1 mL FectoPRO™ Reagent, 1 mL FectoPRO™ Booster	1	1
Polyplus: 116-010	10 mL FectoPRO™ Reagent, 10 mL FectoPRO™ Booster	1	1
VWR: 10118-444	10 mL FectoPRO™ Reagent, 10 mL FectoPRO™ Booster	1	1
Polyplus: 116-100	10x10 mL FectoPRO™ Reagent, 10x10 mL FectoPRO™ Booster	10	10
VWR: 10118-446	10x10 mL FectoPRO™ Reagent, 10x10 mL FectoPRO™ Booster	10	10

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