APPLICATION NOTE No. 339 I January 2015

Solving the Aggregation Problem of Human Embryonic Kidney 293 Cells Using the New Brunswick™ S41i CO_2 Incubator Shaker

Author
Stacey Willard and Ma Sha
Eppendorf, Inc., Enfield, CT, USA
Corresponding author: sha.m@eppendorf.com

Abstract

Human embryonic kidney 293 (HEK293) cells are among the most versatile hosts for recombinant protein expression. These cells are capable of expressing large membrane proteins such as G protein-coupled receptors (GPCRs) that are often not properly expressed by even the most popular biopharmaceutical production hosts such as Chinese hamster ovary (CHO) cells. Although an excellent host for protein expression, the issue of cell clumping in large-scale suspension culture of HEK293 cells has limited its use in bioprocess. Recently, many commercial entities have developed specialty media formulations and anti-clumping reagents to help combat this issue. Here, we show a successful example of the adaptation of a human recombinant protein-expressing HEK293 cell line from serum-supplemented attachment culture to serum-free single cell suspension culture using the New Brunswick S41i CO_2 incubator shaker. This adapted HEK293 cell line behaves like a typical aggregationless suspension cell line. We expect that such a cell line can be used in bioprocess applications using the typical batch or fed-batch methods established for conventional CHO cell culture in stirred-tank bioreactors.

Introduction

Within the biopharmaceuticals market, the most utilized model systems for protein production are, by far, mammalian cell lines. Although CHO cells make up the largest portion of these cell lines, some proteins still require a human intracellular environment for proper folding, post-translational modification including glycosylation, and function. For this reason, bioprocess applications involving HEK293 cells have become more relevant [1, 2]. This versatile human cell line benefits from a long history, extensive characterization, and successful protein expression in both transient and stable formats using plasmid and adenoviral vectors. In fact, in the case of the biopharmaceutical drug Xigris (activated Drotrecogin alfa), CHO cells were an inadequate host due to improper glycosylation which rendered the drug unsuitable for human injection. Used in the treatment of sepsis and marketed by Eli Lilly and Company, Xigris was the first biopharmaceutical generated in HEK293 cells to receive FDA approval [3 – 5].

Recently, a number of studies have begun to re-examine HEK293 cells as a platform for recombinant protein, vaccine, and biosimilar manufacturing [1, 2]. One such example involves the hemophilia treatment, recombinant coagulation factor VIII (rFVIII). Although classically produced in CHO or baby hamster kidney (BHK) cells, multiple recent reports have investigated the feasibility of changing the host cell line to HEK293 [6, 7]. In CHO, the expression levels of rFVIII are low, leading to higher production costs correlating with higher biomass requirements. In addition, possibly owing to the improper protein processing that can occur in non-human host cell lines, the protein is not efficiently secreted. Preliminary studies indicate that using HEK293 as a host increases manufacturing efficiency, reduces

costs, and eliminates the inclusion of immunogenic post-translational modifications [8].

Several problems have plagued large-scale HEK293 cell culture. Among them, one of the biggest hurdles has been that HEK293 cells tend to aggregate in suspension culture, especially at high cell density. Cell clumping in suspension culture is extremely detrimental to a growing population since it restricts the cells inside of the clump from access to sufficient oxygen and necessary nutrients, leading to increased cell death and toxin accumulation. To combat this well-known problem, many specialty media formulations and protocols are now commercially available and a number of anti-clumping reagents have been developed. Here, we aimed to evaluate the effectiveness of various anti-clumping methods using a commercially available human membrane protein-expressing HEK293 cell line. First, we demonstrate that the adaptation of protein-expressing HEK293 cells without cell clumping can be accomplished in a single step in the New Brunswick S41i CO_2 incubator shaker. Second, we show that the large 35 x 61 cm shaking platform of the New Brunswick S41i allows for the simultaneous evaluation of a large matrix of cell culture methods with multiple media formulations and varying doses of anti-clumping reagents.

Figure 1: The New Brunswick S41i CO_2 incubator shaker

Materials and Methods

Attachment cell culture

Untransfected HEK293 cells used for control experiments were obtained from the American Type Culture Collection (ATCC®, CRL-1573™). HEK293 cells expressing hemagglutinin-tagged human Toll-like receptor 4 (hTLR4-HA) were purchased from InvivoGen® (293/hTLR4-HA). 293/hTLR4-HA cells were created by stably transfecting HEK293 cells with a pUNO-hTLR4-HA plasmid. This plasmid encodes the hTLR4 gene fused at the 3'end to the influenza HA tag. The addition of the HA tag was shown to have no deleterious effect on the expression and function of the hTLR4 protein by InvivoGen [9]. Both cell lines, 293 and 293/hTLR4-HA, were initially cultivated in Dulbecco's modified Eagle medium (DMEM, Life Technologies®, 11960-044) supplemented with 4 mM L-glutamine (Life Technologies, 25030-149), 10 % Heat Inactivated Fetal Bovine Serum (HI-FBS, Life Technologies, 10438-026) and 1 X penicillin/streptomycin (Life Technologies, 15140-122). For the 293/hTLR4-HA cells, the medium was also supplemented with 10 µg/mL blasticidin (InvivoGen, ant-bl-1). All attachment cultures were grown in T-75 flasks and incubated at 37 ˚C with 5 % CO_2 on the static shelf of the New Brunswick S41i CO_2 incubator shaker. Standard cell culture techniques were used including passaging using trypsinization with 0.25 % Trypsin-EDTA (Life Technologies, 25200-056).

Suspension cell culture methodology

Adaptation to suspension culture was carried out using multiple methods (Table 1). Each method is comprised of a commercially-available base medium to which standard cell culture supplements such as HI-FBS and L-glutamine were added according to the manufacturer's adaptation protocol. In order to maintain plasmid stability and expression, blasticidin was added to each media formulation at an initial dose of 5 µg/mL. The manufacturer's protocol for inoculation and passaging was strictly followed. For example, the 293 SFM II medium protocol recommended a seeding density of 3 x 10^5 cells/mL, while the EX-CELL 293 medium recommended 6 x 10^5 cells/mL. These guidelines were followed in all cases. Cells were cultivated in single-use 125 mL vented-cap Erlenmeyer flasks (VWR®, 89095-262) with incubator shaker conditions set at 125 rpm, 8 % CO_2, and 37 ˚C. The cells were analyzed periodically (at least every 72 hours) by taking a 1 mL sample and evaluating three parameters: cell density, % viability, and cell clumping. Viable cell density and viability were determined using a Vi-CELL® automated cell counter (Beckman Coulter®) and cell clumping was evaluated by visual inspection under 10 X magnification using an EVOS® FL imaging system (Life

Technologies).

As shown in Figure 2, cell clumping was assigned a score from (–) indicating no clumping to (+++) representing large cell masses, based on the relative size of the cell clumps. Note that once a method was successful in producing a suspension culture that satisfied the three criteria (serum-free, no aggregation, and viable in the presence of blasticidin), no more modifications to the other methods were attempted. Therefore, full optimization of the media and conditions was not carried out and we do not know if other formulations would be successful with further experimentation. In addition, although blasticidin selection is required for this cell line, it may not be necessary for other protein-expressing HEK cell lines.

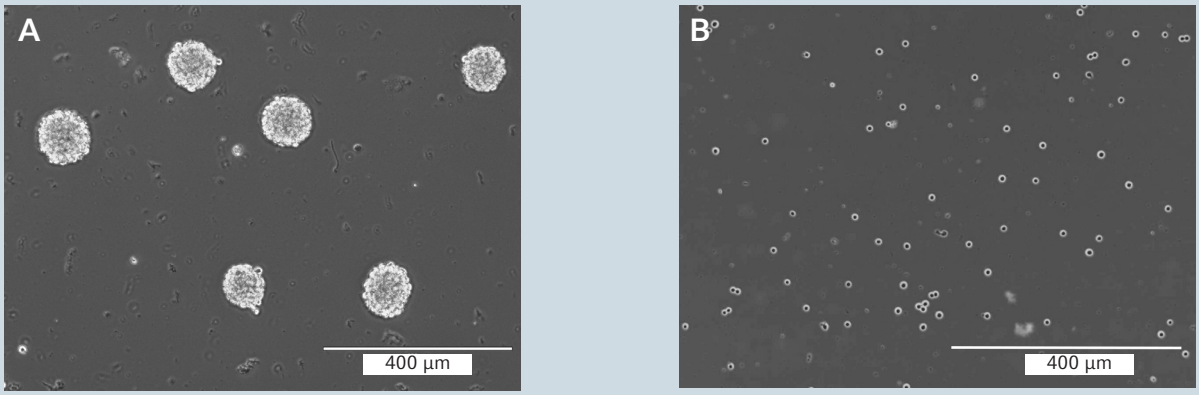

Figure 2: The evaluation of clumping in suspension 293/hTLR4-HA cells:
A: Cells formed large aggregates containing > 50 cells, this culture received an aggregation score of (+++).
B: Successful adaptation in which the culture showed no evidence of cell clumps greater than 2 – 4 cells; this culture was given a (–) for aggregation. Both images were taken at the same magnification (100 X), scale bars represent 400 µm in both panels.

Method	Base Medium	Medium order info.	Supplements	Modifications	Seeding Density (cells/mL)
1	DMEM	Life Technologies®/ 11960-044	• 0 – 10 % HI-FBS • 4 mM L-glutamine • 0 – 5 µg/mL blasticidin	• 0.1 % Pluronic® F-68 • 1 X Anti-clumping agent A • 0.5 mg/mL Bovine serum albumin (BSA)	3 x 10^5
2	CD 293	Life Technologies®/ 11913-019	• 4 mM L-glutamine • 5 µg/mL blasticidin	N/A	1 x 10^6
3	293 SFM II	Life Technologies®/ 11686-029	• 4 mM L-glutamine • 0 – 5 µg/mL blasticidin	• 0.5 mg/mL BSA • 0.1 % Pluronic F-68	1 x 10^6
4	EX-CELL® 293	Sigma-Aldrich®/ 14571C	• 4 mM L-glutamine • 0 – 5 µg/mL blasticidin	N/A	6 x 10^5
5	Pro293™ s-CDM™	Lonza®/ 12-765Q	• 5 – 0 % HI-FBS • 4 mM L-glutamine • 5 µg/mL blasticidin	• 1 X Anti-clumping agent A	5 x 10^5
6	PeproGro w *HEK293*	PeproTech®/ AF-CD-HEK293	• 5 µg/mL blasticidin	N/A	6 x 10^5

Table 1: Culture methods used to adapt adherent 293/hTLR4-HA cells to serum-free single cell suspension culture

Lysate preparation and western blotting

Protein lysates were created by harvesting the cells from confluent T-flasks or from suspension cultures at high density. Lysates were prepared from attachment 293 and 293/hTLR4-HA cells as well as from suspension-adapted 293/hTLR4-HA cells grown in EX-CELL 293 medium in the presence of 5 µg/mL blasticidin. The harvested cells were washed with Dulbecco's phosphate buffered saline (DPBS, Life Technologies, 14190-144) and resuspended in the lysis buffer formulation recommended by InvivoGen (Table 2). Before use, the lysis buffer was sterile-filtered and Halt™ protease inhibitor single-use cocktail (Thermo Fisher Scientific®, 78430) was added at a final concentration of 1 X. After incubation in lysis buffer on ice for 20 min, the lysate was cleared by centrifugation at maximum speed in an Eppendorf Centrifuge 5430 R with a fixed-angle rotor at 4 ˚C for 20 min. The cleared lysates were stored at -80 ˚C in a New Brunswick Premium U570 freezer to preserve protein integrity until western blotting. The protein concentration of each lysate was determined using the Pierce® BCA protein assay kit (Thermo Fisher Scientific, 23227).

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblotting were carried out using the following kits from Life Technologies. First, SDS-PAGE was performed using the Bolt™ mini gel system (B4477599) with the accompanying 4 – 12 % Bis-Tris plus gels (BG04125BOX), MOPS buffering system, and PVDF membranes (B0001 and LC2002, respectively). 20 µg of each sample was loaded and after transfer, the membranes were probed with a mouse anti-HA Tag antibody (InvivoGen, ab-hatag) at a 1:1000 dilution. Detection was performed using the WesternBreeze® chromogenic western blot immunodetection kit (Life Technologies, WB7103) with the included anti-mouse secondary antibody, according to the manufacturer's instructions.

Immunofluorescence

Attachment 293 and 293/hTLR4-HA cultures were subjected to immunostaining according to the protocol outlined previously [10]. A mouse Anti-HA Tag primary antibody was used at a 1:1000 dilution to detect expression of the hTLR4-HA protein combined with an Alexa Fluor® 594 goat anti-mouse secondary (Life Technologies, A-11005). Samples were counterstained with the nuclear dye, 4', 6-diamidino-2-phenylindole (DAPI), using ProLong® gold antifade mountant (Life Technologies, P-36931). Cells were imaged as described above.

Reagent	Company/Order no.	Final concentration
Tris HCl, pH 7.4	Sigma-Aldrich®/ T5941	10 mM
NaCl	Sigma-Aldrich®/ S1679	0.1 M
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich®/ E6760	1 mM
10 % Sodium dodecyl sulfate (SDS)	Life Technologies®/ 15553-027	0.1 %
Sodium deoxycholate	Sigma-Aldrich®/ 30970	0.5 %
Triton X-100	Sigma-Aldrich®/ T8787	1 %
Glycerol	Sigma-Aldrich®/ G2025	1 %

Table 2: Lysis buffer formulation as recommended by InvivoGen

Results and Discussion

Expression of hTLR4-HA in 293 cells

To confirm that 293/hTLR4-HA cells expressed the tagged hTLR4 receptor, attachment cells were stained with a mouse antibody raised against the HA tag and detected with a fluorescent anti-mouse secondary antibody. As Figure 3 illustrates, varying levels of expression of hTLR4 were detected in transfected cells, while no signal was found in untransfected cells. These data indicate that the transfected cells may represent a pool of transformants instead of a clonal population of cells. More uniform expression may be obtained if a clonal population was established.

Adaptation of 293/hTLR4-HA to serum-free single cell suspension culture

To adapt a human membrane protein-expressing 293 cell line to serum-free suspension culture without aggregation, 293/hTLR4-HA cells were subjected to multiple culture methods. Each culture was periodically analyzed for cell viability, density and clumping as described previously. As Table 3 indicates, varying levels of success were documented in each category using the tested media formulations; however, a successful adaptation was only achieved if the cells retained high viability and grew to high densities in the presence of blasticidin with no aggregation under serum-free conditions. If cell clumping was severe (+++), or viability was low, the culture was discontinued and adjustments to the method were made accordingly. For example, DMEM with 0 and 1 % HI-FBS resulted in large cell clumps of over 50 cells in suspension (Figure 2A). Therefore, after 48 h of culture, the formulation was adjusted to contain anti-clumping agents such as Pluronic F-68 and Anti clump A, and a new culture was established from attachment cells. As outlined in Table 3, DMEM was not able to support suspension cell growth without aggregation in this experiment. Another formulation, Pro293 s-CDM, was able to sustain the growth of 293/hTLR4-HA with serum supplementation, however, when serum weaning was complete, the cells did not survive for multiple passages (Figure 4A). Furthermore, it was clear that many cultures seemed to be extremely sensitive to blasticidin selection during the adaptation process as indicated by low viability in the days post-inoculation. Hence, some cultures were allowed to adapt to suspension culture before blasticidin was re-introduced. The most successful adaptation method using this strategy was Method 4 (EX-CELL 293) which, after blasticidin addition, resulted in virtually no cell clumps and reproducible high cell densities and viabilities without the presence of serum (Figure 4B). This method was deemed successful for the adaptation of this cell line to serum-free suspension culture without aggregation and optimization of the other methods was halted at this stage. Since no further changes to the other methods were attempted, it not clear whether or not other methods would have been found successful in future experiments.

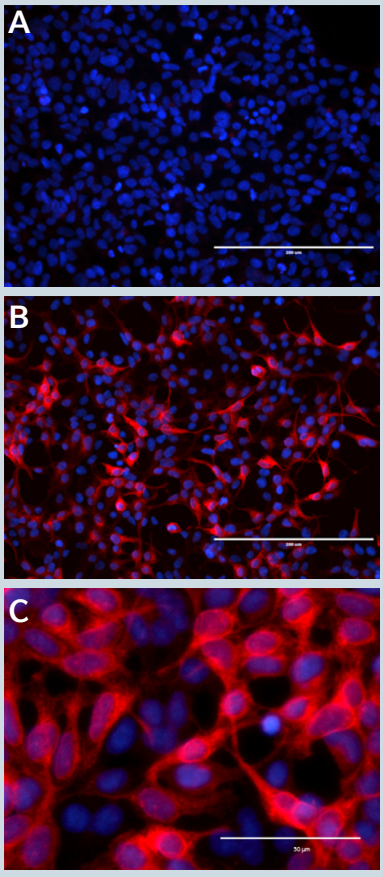

Figure 3: hTLR4-HA expression in untransfected (A) and transfected (B, C) 293 cells. In all panels, hTLR4-HA is detected in red and DAPI in blue.
A and B: Photographed at 200 X magnification, scale bar = 200 µm.
Panel C: Photographed at 400 X, scale bar = 50 µm.

Method	Base Medium	Formulation	Aggregation?	Viable with Blasticidin?	Serum-free?
1	DMEM	0 % HI-FBS	N/A	No	No
		1 % HI-FBS	+++	No	No
		1 % HI-FBS, Pluronic F-68	+++	No	No
		1 % HI-FBS, Anti-clumping agent A	++	No	No
2	CD 293	As recommended	+++	N/A	Yes
3	293 SFM II	No supplement	-	N/A	Yes
		5 µg/mL blasticidin	-	No	Yes
		1 % BSA, 5 µg/mL blasticidin	-	No	Yes
4	EX-CELL® 293	No supplement	-	N/A	Yes
		10 µg/mL blasticidin	-	No	Yes
		5 µg/mL blasticidin	-	Yes	Yes
5	Pro293™ s-CDM™	5 % HI-FBS, 5 µg/mL blasticidin	-	Yes	No
		2.5 % HI-FBS, 5 µg/mL blasticidin	-	Yes	No
		1 % HI-FBS, 5 µg/mL blasticidin	-	Yes	No
		5 µg/mL blasticidin	-	No	No
			-	No	Yes
6	PeproGro *HEK293*	As recommended	+++	N/A	Yes

Table 3: Result of suspension cell culture with the methods and formulations tested

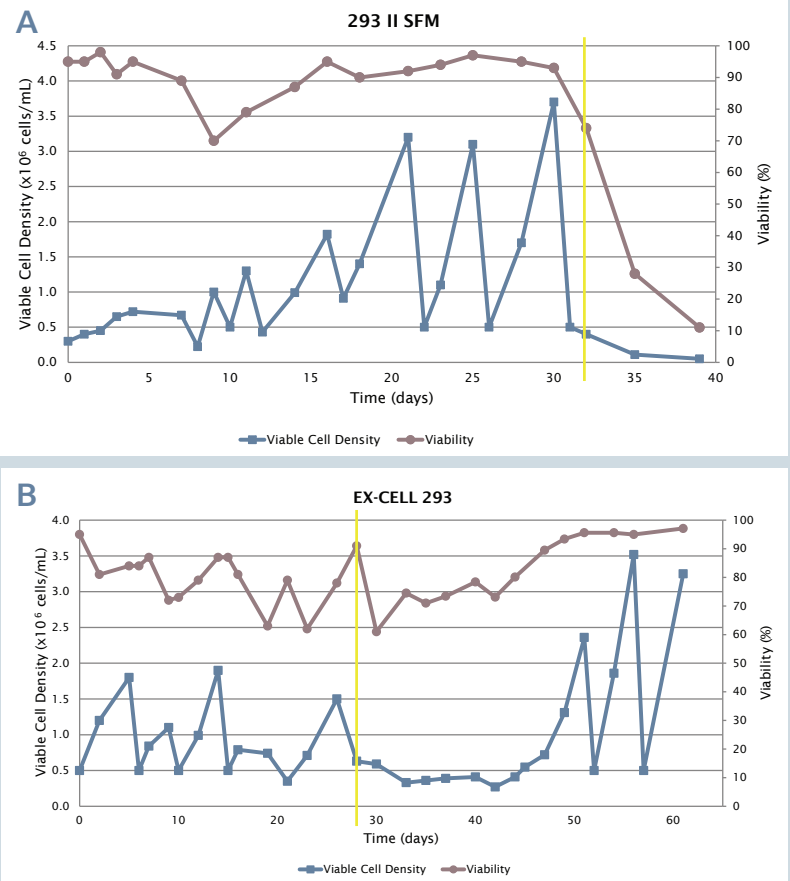

Figure 4: 293/hTLR4-HA cell adaptation to suspension culture.
A: Cells were able to adapt to suspension culture without serum, but died upon addition of blasticidin (yellow line). When blasticidin was present at the beginning of adaptation in this method, the cells died rapidly (data not shown).
B: Successful adaptation to single cell serum-free suspension culture. The yellow line denotes the addition of blasticidin. Note that after 45 days, the cells begin to grow reproducibly over multiple passages at high viability.

Protein expression after suspension adaptation
When acclimating a cell line to suspension culture in preparation for scale-up to a stirred-tank bioreactor, it is important to confirm that protein expression was not impacted by the adaptation process. Whole cell lysates from untransfected 293 and 293/hTLR4-HA were analyzed for hTLR4-HA expression by western blotting. As shown in Figure 5, no signal was detected in whole cell lysates from untransfected (293) early or late passage adherent cells. In contrast, early passage adherent 293/hTLR4-HA cells expressed a ~97 kDa HA-tagged protein. Suspension-adapted cells cultured in EX-CELL 293 media with 5 µg/mL blasticidin expressed an identical band of approximately the same intensity. This band closely matches the predicted size of the human TLR4 protein at 95 kDa with ~1 kDa added for the 9 amino acid HA tag. Although not quantitative, these data indicate that the expression of hTLR4-HA was not significantly impacted by the adaptation process.

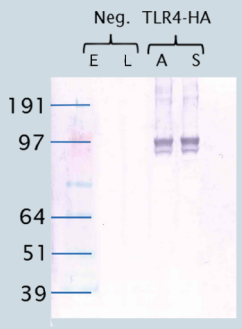

Figure 5: Post-adaptation hTLR4 expression confirmation by western blot. Untransfected (293) attachment cells of early (E; passage 3) and late (L; passage 21) passage and 293/hTLR4-HA attachment (A) and post-adaptation suspension (S) cell lysates were probed with anti-HA antibody and detected by the WesternBreeze chromogenic detection method. 20 µg of protein was loaded in each lane.

Conclusion

The number of FDA-approved biopharmaceuticals produced in HEK293 cells has been low, partially due to the well-known large-scale suspension culture aggregation issue in bioreactor conditions. In this work, we eliminated the clumping problem in our HEK293 cell line prior to the bioreactor production stage, leveraging commercially available serum-free adaptation methods. We have shown that the adjustment of a membrane protein-expressing HEK293 cell line to clump-free serum-free suspension culture can be accomplished by simultaneously testing multiple adaptation methods in the New Brunswick S41i CO_2 incubator shaker. This method cuts down on upstream process development time since it can support adherent and suspension cells simultaneously in the same chamber. Moreover, the large shaking platform can accommodate up to twenty-four 125 mL flasks. By eliminating aggregation before the bioreactor stage, we hope to address one of the major bottlenecks that has limited the bioprocess potential of this cell line.

Literature

[1] Liste-Calleja L, Lecina M, Cairo JJ. HEK293 cell culture media study towards bioprocess optimization: Animal derived component free and animal derived component containing platforms. *Journal of Bioscience and Bioengineering* 2014; 117(4):471-7.

[2] Dietmair S, Hodson MP, Quek LE, Timmins NE, Gray P, Nielsen LK. A multi-omics analysis of recombinant protein production in Hek293 cells. *PloS One* 2012; 7(8):e43394.

[3] Doig CJ, Zygun DA, Delaney A, Manns BJ. Drotrecogin alfa (activated; Xigris): An effective and cost-efficient treatment for severe sepsis. *Expert Review of Pharmacoeconomics & Outcomes Research* 2004; 4(1):15 – 26.

[4] Levi M, De Jonge E, van der Poll T. Recombinant human activated protein C (Xigris). *International Journal of Clinical Practice* 2002; 56(7):542 – 5.

[5] Larson AM. Xigris: Reducing mortality in adult patients with severe sepsis. *Urologic Nursing* 2002; 22(3):200-1.

[6] Fontes AM, Melo FU, Greene LJ, Faca VM, Lin Y, Gerson SL, Covas DT. Production of human factor VIII-FL in 293T cells using the bicistronic MGMT(P140K)-retroviral vector. *Genetics and Molecular Research* : GMR 2012; 11(1):775 – 89.

[7] Swiech K, Kamen A, Ansorge S, Durocher Y, Picanco-Castro V, Russo-Carbolante EM, Neto MS, Covas DT. Transient transfection of serum-free suspension HEK 293 cell culture for efficient production of human rFVIII. *BMC Biotechnology* 2011; 11:114.

[8] Valentino LA, Negrier C, Kohla G, Tiede A, Liesner R, Hart D, Knaub S. The first recombinant FVIII produced in human cells--An update on its clinical development programme. *Haemophilia* 2014; 20 Suppl 1:1 – 9.

[9] HEK-293 cells stably transfected with human HA-tagged TLR4a gene. InvivoGen, Inc. 2014 Version # 14G30-MT *www.invivogen.com*.

[10] Willard S, Philip L, Sha M. Hypoxic Cell Culture in the New Brunswick™ Galaxy® 170R Incubator: Normal Growth, Morphological Changes. 2013. *http://www.nbsc.com/files/AA331_Hypoxia_CO2_Incubators.pdf*

Ordering information

Description	Order no. International	Order no. North America
New Brunswick™ S41i CO_2 Incubator Shaker	S41I-230-0100	S41I-120-0100
Eppendorf Cell Culture Flask, T-75, Vented filter cap, Tissue culture treated	0030711122	0030711122
Eppendorf Centrifuge 5430 R	5427000410	022620623

Your local distributor: www.eppendorf.com/contact
Eppendorf AG · 22331 Hamburg · Germany
eppendorf@eppendorf.com · www.eppendorf.com

www.eppendorf.com