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Transient gene expression (TGE) from mammalian cells is an increasingly important tool for the rapid

production of recombinant proteins for research applications in biochemistry, structural biology, and bio-

medicine. Here we review methods for the transfection of human embryo kidney (HEK-293) and Chinese

hamster ovary (CHO) cells in suspension culture using the cationic polymer polyethylenimine (PEI) for

Polyethyleneimine-based transient gene expression processes for suspension-adapted HEK-293E and CHO-DG44 cells

ABSTRACT

gene delivery.

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Review

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Introduction

There is a growing interest in the rapid production of recombinant proteins in cultured animal cells for applications in medicine and fundamental research [1–6]. Currently, the two major approaches to rapid protein production are non-viral transient gene expression (TGE)¹ using mammalian cells [7–11] and infection of insect cells with a baculovirus expression vector [12,13]. Cost-effective protein production is achievable by both methods when performed with suspension-adapted cells cultivated in simple and scalable systems.

One of the major differences between the two expression systems is the manner in which the gene of interest (GOI) is delivered to the production host. Construction of the recombinant baculovirus begins with the cloning of the GOI into a transfer vector that allows recombination-mediated transfer of the GOI into the baculovirus genome while it is maintained as an episomal DNA element (bacmid) in Escherichia coli [12,13]. After recovery of the recombinant bacmid DNA from E. coli, it is transfected into insect cells, resulting in a productive viral infection. The baculovirus stock is then used for the large-scale infection of insect cells to produce the recombinant protein of interest. Unfortunately, the baculovirus infection of insect cells is cytolytic, limiting the production phase to a relatively short period of 2-5 days. In contrast, TGE requires the cloning of the GOI into a mammalian expression vector. After amplification of the plasmid in E. coli, it can to be transfected into cells. A protein production phase of up to 2 weeks is possible depending on the protein and the culture conditions. In optimized cultures, volumetric yields up to 1 g/L have been reached [14]. A major drawback of this method, however, is the amount of plasmid DNA, typically 1 mg or more, required per liter of transfection. By comparison, the generation of the recombinant baculovirus vector is time-consuming, but its continued propagation is relatively simple and inexpensive.

Both mammalian and insect cells support correct protein folding, multi-protein complex formation, and post-translational modifications. However, there is a major difference between the two animal cell hosts with regard to *N*-linked glycosylation. Insect cells mainly synthesize oligomannosidic and paucimannosidic glycans with low levels of galactose and sialic acid [13,15]. In contrast, mammalian cells synthesize complex glycans containing mannose, *N*-acetylglucosamine, galactose, and sialic acid [16,17]. For applications in structural biology, it is often beneficial to produce glycoproteins with homogenous oligomannosidic glycans that can be efficiently removed by glycosidases [18–20]. For therapeutic proteins, on the other hand, it is preferable to have complex glycans to enhance both the half-life and functionality of the protein *in vivo* [21–23].

This article focuses on TGE methods with the two major mammalian production hosts, human embryo kidney 293 (HEK-293) and Chinese hamster ovary (CHO) cells, using polyethyleneimine (PEI) for DNA delivery. A non-exhaustive list of examples of proteins which have been produced in these cells is provided in Table 1. Since 2004, over 20 structures have been resolved using proteins produced transiently in HEK-293 cells. In addition, the method has been successfully applied to the production of virus vectors for the purpose of gene delivery (Table 1). Despite these successes, there may be perceptions that TGE is unaffordable for many academic labs and that the cultivation of mammalian cells in suspension is technically difficult. Fortunately, the production yields from transiently transfected mammalian cells have improved considerably in the last decade, and innovative cost-effective, non-instrumented cultivation systems for suspension-adapted mammalian cells have been developed [24–26]. These technical improvements have dramatically reduced protein production costs.

Our objective is to present a practical overview of the key components, methods, and limitations of PEI-based TGE production processes in HEK-293 and CHO cells based on our extensive experience in the Protein Expression Core Facility and the Laboratory of Cellular Biotechnology at the École Polytechnique Fédérale de Lausanne. The point is to provide access to the technology so that new users may better understand the critical steps in order to develop protocols to suit their own needs. We are not providing a comprehensive review of transient transfection methods with animal cells. Other recent reviews can be consulted for a broader perspective of TGE [2,10,11]. In addition, we and others have published step-bystep protocols on TGE using mammalian cells as host [27–33].

Cells

To achieve an economic transient production process, two properties of the host cell are essential. They must be able to grow to a high density (>5 × 10⁶ cells/mL) in single-cell suspension culture, and they must be efficiently transfected (DNA uptake in more than 50% of cells) with a low-cost DNA delivery vehicle. The ability to grow in the absence of serum is also highly desirable if the protein product is secreted. Although the TGE methods described here can be adapted to many other mammalian cell lines, most of them do not meet these criteria. Consequently, most efforts to develop TGE systems have focused on HEK-293 and CHO cells as hosts.

HEK-293 cells

HEK-293 cells (American Type Culture Collection, Molsheim, France) were generated from embryonic human kidney tissue by stable transfection with sheared human adenovirus DNA, resulting in cells overexpressing the adenovirus E1A and E1B genes [34]. The cells were initially used to propagate adenovirus mutants deficient in these genes. Due to their ease of cultivation and transfection, they eventually gained popularity as a TGE host. Subsequently, HEK-293T (American Type Culture Collection) and HEK-293E cells, resulting from stable transfection of the parental line with the simian virus 40 (SV40) large T antigen (LT) gene and the Epstein-Barr Virus nuclear antigen 1 (EBNA1) gene, respectively, were generated [35,36]. Both EBNA1 and SV40 LT function in viral DNA synthesis by binding to the cognate viral origin of DNA replication (ori) to recruit the cellular DNA replication machinery. These two cell lines were developed with the expectation that they would support the episomal replication and maintenance of a transfected plasmid DNA bearing the appropriate viral ori [37]. For the TGE system described here, HEK-293E cells were used. Interestingly, the highest protein yields in these cells were achieved under conditions of growth arrest following transfection with plasmids that did not bear the EBV ori [14]. However, there may be other TGE conditions in which episomal replication of the plasmid has a positive effect on recombinant protein yield [38-40].

More recently, HEK-293 subclones, adapted to suspension growth in commercial serum-free media, were made available. HEK-293F[™] and Expi293F[™] cells (Life Technologies Europe, Zug, Switzerland) were selected for high-density suspension growth in FreeStyle293[™] and Expi293[™] media (Life Technologies), respectively. A suspension-adapted cell line lacking

¹ Abbreviations used: TGE, transient gene expression; CHO, Chinese hamster ovary; PEI, polymer polyethylenimine; GOI, gene of interest; HEK-293, human embryo kidney 293; EBNA1, Epstein-Barr virus nuclear antigen 1; DHFR, dihydrofolate reductase; HT, hypoxanthine and thymidine; Py, polyomavirus; OSRs, orbitally shaken bioreactors; k_La, oxygen mass transfer coefficient; DO, dissolved oxygen; hCMV, human cytomegalovirus; mIE, major immediate early; mCMV, mouse CMV; UTR, untranslated region; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; CaPi, calcium phosphate; PDI, polydispersity index.

Table 1

Examples of proteins and virus vectors produced by PEI-mediated transient transfection of HEK-293 and CHO cells.

A function studies version version <thversion< th=""> version <thversion< th=""></thversion<></thversion<>	Protein	Host	Affinity tag	Secreted	Refs.
Applications receiper (Sk2p) 293E Fear Strephis No (98) Transforming yound heard & receiper (Gr-RBU (DP)) 293E None Ves (100) Scrinder (Groben) (GC4) 293E None Ves (100) Resident (Groben) (GC4) 293E None Ves (100) Resident (GC4) 293E His Ves (106) Resident (GS1) 293E His Ves (106) Feadermal growth factor 293E His Ves (107) Feadermal growth factor 293E None Ves (107) Feadermal growth factor 1001 293E None Ves (107) Feadermal growth factor 1001 293E None Ves (110) Resider (GR) 293E Red <t< td=""><td>A. Function studies</td><td></td><td></td><td></td><td></td></t<>	A. Function studies				
Transforming growth factor (TCF-RBII (MPD) 233E His Vec [9] Screeted clusterin (SCU) 233E None Yes [10] Screeted clusterin (SCU) 233E His Ves [10] Rein factor 233E His Ves [10] Rein factor 233E Honolay Yes [10] Rein factor 233E Honolay Yes [10] Rein factor 233E Honolay Yes [10] Rein factor Yes [10] [20] [10] Insulin full growth factor [10] [23] [23] [10] [10] Rein factor [10] [23] [23] [10] [10] [10] Rein factor [23] [23] [10] [23] [10] [10] Rein factor [23] [23] [10] [10] [10] Rein factor [10] [23] [11] [11] [11] Rein factor [23	Alpha-factor receptor (Ste2p)	293E	Fc or Strep/his	No	[98]
Vacuar enderficial growth factor (VGG) 292E None Yes [10] Sitt honology proten 2 (SIL) 291E His Yes [10] Sitt honology proten 2 (SIL) 291E His Yes [10] Sitt honology proten 2 (SIL) 291E His Yes [10] Existin (STM) 293E His Yes [10] Existin (STM) 293E His Yes [10] Existin (STM) 293F Sift None Yes [10] Factor VII 293F Sift None Yes [10] Lexistin (STM) 293F Sift None Yes [10] Lexistin (STM) 293F Sift None Yes [11] Lexistin (STM) 293F Sift None Yes [11] Lexistin (STM) 293F Sift None Yes [11] Lexistin (STM) 293F Sift None Yes [12] Lexistin (STM) 293F Sift None Yes [13] <	Transforming growth factor & receptor (TGF-RRII) (FD) and TGF- RRIII (MPD)	293E	His	Yes	[99]
scretced dustrin (stdl) 292E His Yes [10] Reistin kondowy ducital 2(SI2) 293E His Yes [10] Reistin (STN) 293E His Yes [10] Epidermal growth factor 233E PC or His Yes [10] Epidermal growth factor 233E PC or His Yes [10] Epidermal growth factor 233E PC or His Yes [10] Leinner (Hig protein 7 (IGFBY7) 233E None Yes [10] Registron Symptal Wins Kison protein (ESV F) 239E None Yes [11] Registron Symptal Wins Kison protein (ESV F) 239E FL/G/His None Yes [82] CF complex (TCL, STN and TEN1) 239E FL/G/His None Yes [80] CF complex (TCL, STN and TEN1) 239E FL/G His None Yes [80] CF complex (TCL, STN and TEN1) 239E FL/G No [13] HV grid 0 triner 239E FC Yes [80]<	Vacular endothelial growth factor (VEGF)	293E	None	Yes	[100]
Shi homology proten 2 (SH2) 297 116 Yes 102, (19) Shi homology proten 2 (SH2) 297 116 Yes 105 Chineric heavy chain antibody (GH2h) 293 293 Cor bins Yes 105 Insulin-like growth factor funding protein 7 (IGFBP7) 293 293 Cor bins Yes 103 Caternical pericean fragment (LG3) 293 Cor bins Yes 107 Responde 293 Cor bins Yes 103 Responde 293 Cor bins Yes 112 Responde 293 None Yes 112 Responde 293 None Yes 112 Deckopf (Dk4) 293 None Yes 112 Cor transition protein (SV-F) 293 None Yes 113 Calkopf (Dk4) 293 Cor Yes 104 104 Corrent perice (Cr1, STM and TEAL) 293 Corrent Parkow 103 104 Corrent perice (Cr1, STM and TEAL) 293	Secreted clusterin (sCIII)	293E	His	Yes	[101]
instruction page its Yes [104] Epidermal growth factor 293E PC or His Yes [106] Epidermal growth factor 293E PC or His Yes [106] Eactor VII 293F His Yes [107] Eactor VII 293F His Yes [107] Eactor VII 293F None Yes [107] Respiratory Structure (IG1) 293F None Yes [107] Respiratory syncytial vins factor vactor (IG1) 293E None Yes [101] Respiratory syncytial vins factor vactor (IG1) 293E None Yes [101] Dickopfin (Dact) 293E None Yes [101] Dickopfin (Dact) 293E PC Yes [101]	Slit homology protein 2 (Slit2)	293E	His	Yes	[102 103]
Thimmer, beawy chain antibody (HCAb) 393 None Yes 105 Insulin-like growth factor funding protein 7 (IGEBP7) 233 Bit or Yill Yes 104 Extor VII 2335-SF6 None Yes 104 Insulin-like growth factor funding protein 7 (IGEBP7) 2335 His Yes 114 C-terminal preferant factor 2335 Cillo None Yes 114 C-terminal preferant factor 2335 None Yes 114 Regularity synthylicity livins factor protein (RV-F) 2335 None Yes 112 Dickkopf (Dkk1) 2335 Gril and 2337 None Yes 113 IIVI 23140 trime 2335 Gril and 2337 None Yes 103 IIVI 23140 trime 2335 Gril and 2337 None Yes 113 IIVI 23140 trime 2335 Fic Yes 103 IIVI 23140 trime 2335 Fic Yes 103 IIVI 23140 trime 2335 Fic Yes	Resistin (RSTN)	293E	His	Ves	[104]
Defension 299E For THis Yes 106 Insidin-Kierg work factor building protein 7 (IG/RP7) 293E His Yes 103 Factor VIII 233S-3F6 None Yes 103 Human [GC1 antibidy 233E His Yes 103 Magodi 233E None Yes 110 Leucine-rich gliona inactioned 1 (G1) 233E None Yes 61 Disk dopt 233E None Yes 61 CST complex (TC1, SNN and TNN) 233E RAC(N No 113 Pointerase processivity factor (POT1-TPP1) 233E Fic Yes 60 Vill Acid Stal antigen 233E Fic Yes 60 114 Calsynamin 1 (Calst (PD1) 233E Fic Yes 60 114 Calsynamin (SerV) 233E Fic Yes 60 114 Calsynamin (SerV) 233E Fis Yes 116 Calsynamin (SerV) 233E Fis Yes	Chimeric heavy chain antibody (cHCAb)	293E	None	Ves	[105]
institution B& growth Darron building protein 7 (IGTBP7) 299T 118 Yes 1091 Pactor VII 2935-2765 Nonce Yes 1081 C-terminal perferan fragment (IG3) 2932. C100 None Yes 1109 Nago66 2937. C100 None Yes 1109 Respiratory synoptial virus fusion protein (RSV-F) 293E None Yes 1111 Deckopti (DX1) 293E None Yes 1131 Deckopti (DX1) 293E None Yes 1131 Deckopti (DX1) 293E Fc Yes 1131 Deckopti (DX1) 293E Fa Yes 1131 Deckopti (DX1) 293E Fc Yes 1131 HV Jg 40 frimer 293E Fc Yes 1161 C101 (E01) 293E Fc Yes 1161 C116 (D1) 293E His Yes 1161 C116 (D2) 293E His Yes 1171 C116 (D2) <td>Endermal growth factor</td> <td>203E 203F</td> <td>Fc or His</td> <td>Ves</td> <td>[106]</td>	Endermal growth factor	203E 203F	Fc or His	Ves	[106]
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Turnin (sc) antibody 293 C. (10) None Yes [14,79] Nago66 293 C. (10) None Yes [110] Leucine-rich glioma inactivated 1 (LG1) 293 E None Yes [110] Leucine-rich glioma inactivated 1 (LG1) 293 E None Yes [111] Expiratory synchial virus fasion protein (RSV-F) 293 E F. C Yes [111] Dickkopf (Dk1) 293 E F. C Yes [113] Telomerase processivity factor (PT1-TPP1) 293 E F. C Yes [114] Adaymetin 1 (Cals) (ED) 293 E His Yes [114] Galymetin 1 (Cals) (ED) 293 E F. C Yes [116] CD1 (ED) 293 E His Yes [117] Single chain antibody fargenet (scFv) 293 E His Yes [117] CD1 (ED) 293 E His Yes [117] CD1 (ED) 293 E His Yes [117] CD1 (ED) 293 E His	C-terminal perfector fragment (LC3)	29331-510 203F	Hic	Vec	[100]
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Deckerp Participant 2337 His Yes [112] CSC complex (CCL, SN1 and PATTN1) 2395 FLAG(His No [113] Felometare processivity factor (POTI-TPP1) 2395 GTT and 2937 None Yes [114] Calsynthemin 1 (Cals1) (ED) 2395 GTT and 2937 None Yes [80] Prion protein (PT) 2395 Fc Yes [80] [80] [80] VIA class I landing 2395 Fc Yes [80] [80] [80] [80] [80] VIA class I landing 2395 Fc Yes [117] [80] [80] [80] [80] [80] [80] [80] [80] [80] [80] [80] [81] [81] [80] [80] [81]	Ricylic pantidas	203E	Fc	Vec	[30]
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Langman (Lang) 295 Fr. Yes [60] Hick class I angles 2931 Fr. Yes [80] Prior protein (PP) 2931 Fr. Yes [15] Single chain antibody fragment (sCPv) 2932 His Yes [16] CD1 (ED) 2932 His Yes [17] CD1 (ED) 2935 His Yes [18] Sonic fordgenos (Shi ED) 2935 His Yes [18] Sonic fordgenos (Shi ED) 2931 Halo Yes [12] Coactivator-associated arginine methyl transferase 1 (CARM1) 2931 Halo Yes [12] Coactivator-associated arginine methyl transferase 1 (CARM1) 2931 Halo Yes [12] Coactivator-associated arginine methyl transferase 1 (CARM1) 2931 Halo Yes [12] Leukernia inhibitory factor (LFE) CHO Fc Yes [12] Pupel Bitecoprop protein protein protein protein weak for the protein (PEP) 2931 His Yes [12]	Calsuntenin 1 (Cals1) (ED)	203F	Hic	Vec	[90]
Index transform 252 Fc Ves [80] HCMV ULS (D) 293E Fc Ves [115] Single chain antibody fragment (scFv) 293E His Ves [116] CD1 (D) 293E His Ves [117] CD1-antiHER2 scFv fusion 293E His Ves [117] Erythrocyte membrane protein 1 (PEMP1 var2CSA ED) 293E His Ves [118] Sonic hedgehog (Shh ED) 293E None Ves [121] Coccitivator -associated arginie methyl transferase 1 (CARM1) 293T Halo Ves [121] Coccitivator -associated arginie methyl transferase 1 (CARM1) 293T His Ves [121] It like receptor 2 (TLR-2) (EC) 293T His Ves [123] Luckmain hinbitory factor (TNF RED) CHO Fc Ves [126] Rattrace statis CHO Fc Ves [126] MCH-9 SASK coronavirus 293T His None [123] Parant B2 (phB2) (ED) </td <td>HIA class II antigen</td> <td>203E</td> <td>Fc</td> <td>Vec</td> <td>[80]</td>	HIA class II antigen	203E	Fc	Vec	[80]
Ham Noter (H) 295 Fc	Drion protein (DrD)	203E	Fc	Vec	[80]
Item Data His Visit 112 CD1 (D) 293E His Visit 1117 CD1 (D) 293E His Visit 1117 CD1 (D) 293E His Visit 1117 Erythrocyte membrane protein 1 (PEMP1 var2CSA ED) 293E His Visit 1117 Erythrocyte membrane protein 1 (PEMP1 var2CSA ED) 293T and 293S GnTi His Visit 1120 Coactivator associated arginine methyl transferase 1 (CARM1) 293T Malo Visit 1121 Toll like receptor 2 (TIR-2) (EC) 293E His Visit 1121 Leukemai inhibitory factor (UF) CHO Fc Visit 1121 Tumor necrosis factor receptor (TIR ED) CHO Fc Visit 1121 B Structure studies Type IIB receptor protein tyrosine phosphatase (RPTPµ and RPTPor) 293T His None 1221 Ephitin B2 (EphB2) (ED) 293T His None 1281 1281 Media avirus glycoprotein (HV-C) (ED) 293T His </td <td>HCMV III 18 (FD)</td> <td>203E</td> <td>Fc</td> <td>Vec</td> <td>[115]</td>	HCMV III 18 (FD)	203E	Fc	Vec	[115]
Jung Attion antonody inspiration (Set Y) 252 His Yes 1171 CD1 (ED) 293E His Yes 1171 CD1-antHER2 serv fusion 293E His Yes 1171 CD1-antHER2 serv fusion 293F His Yes 1181 Sonic hedgehog (Sh ED) 293T and 293S GnTi His Yes 1120 Coactivator-associated arginine methyl transferase 1 (CARM1) 293T Halo Yes 121 Toll like receptor 2 (TR-2) (EC) 293E His Yes 122 Leukemia inhibitory factor (LIF) CHO Fc Yes 189 Tumor necrosis factor receptor (TNFR ED) CHO Fc Yes 123-1251 Human (gG1 antibody 293T His Yes 123-1251 Nipah virus glycoprotein (Niv-G) (ED) <td>Single chain antibody fragment (scEy)</td> <td>203E</td> <td>Hic</td> <td>Vec</td> <td>[115]</td>	Single chain antibody fragment (scEy)	203E	Hic	Vec	[115]
CD (LD) CD (LD) <t< td=""><td>CD1 (FD)</td><td>203E</td><td>Hic</td><td>Vec</td><td>[117]</td></t<>	CD1 (FD)	203E	Hic	Vec	[117]
Description Description Description Description Description Description Sonic hedgehog (Sh ED) 2931 and 293S GnTi- His Yes [120] Coactivator-associated arginine methyl transferase 1 (CARM1) 2931 Halo Yes [121] Coactivator-associated arginine methyl transferase 1 (CARM1) 2931 Halo Yes [122] Leukemia inhibitory factor (LIF) CHO Fc Yes [89] Tumor necrosif factor receptor (TNFR ED) CHO Fc Yes [123–125] B. Structure studies T Type IB receptor protein tyrosine phosphatase (RPTPµ and RPTPor) 293T His Yes [128] Nipah virus glycoprotein (NV-G) (ED) 293T His Yes [129] Hedra virus glycoprotein (NV-G) (ED) 293T His Yes [129] Hedra virus glycoprotein (NV-G) (ED) 293T His Yes [129] Hedra virus glycoprotein (NV-G) (ED) 293T His Yes [129] Hedra virus glycoprotein (NV-G) (ED) 293T His <t< td=""><td>CD1-antiHFR2 scFv fusion</td><td>293E 293F</td><td>His</td><td>Ves</td><td>[117]</td></t<>	CD1-antiHFR2 scFv fusion	293E 293F	His	Ves	[117]
Laymond intervent (Linear Force (Linear Force) 2021 His Yes [13] Erythropoietin (EPO) 2935 None Yes [12] Coattuvator-associated arginine methyl transferase 1 (CARM1) 2937 Halo Yes [12] Toll like receptor 2 (TLR-2) (EC) 2938 His Yes [12] Coattuvator-associated arginine methyl transferase 1 (CARM1) 2937 Halo Yes [12] Toll like receptor 2 (TLR-2) (EC) CHO Fc Yes [9] Leukemia inhibitory factor (LIF) CHO Fc Yes [123-125] Tumor necrosis factor receptor (TNFR ED) 2937 None Yes [123-125] Human IgC1 antibody 2937 None Yes [12] Patricure studies Yes [12] Patricure studies [12] His Yes [12] Patricure studies [12] Human IgC1 antibody 2937 His Yes [12] Hedgehog interacting protein (NN-G) (ED) 2935 GnTT His Yes<	Erythrocyte membrane protein 1 (PfFMP1 var2CSA FD)	293E	His	Ves	[117]
John Rogeng (JML D/) 251 and 555 GML Ind Yes [12] Erythropoietin (EPO) 293E None Yes [12] Coactivator-associated arginine methyl transferase 1 (CARM1) 293T Halo Yes [12] Leukemia inhibitory factor (UF) CHO Fc Yes [9] B. Structure studies Tumor necrosis factor receptor (TNFR ED) 293T His Yes [12] Phuman (SC Inbibody 293T None Yes [12] Phuman (SC Inbibody 293T His No [12] Phuman (SC Inbibody 293T His No [12] Phyne B2 (PBA2) (ED) 293T His No [12] Hedgehog increatcing protein (NW-G) (ED) 293T His Yes [12] Hedgehog increatcing protein (Hu) (ED) 293T His Yes [12] Inderacting protein (Hu) (ED) 293T His Yes [12] Inotrophic glutamate receptor 2 (ED) 293S GnT- His Yes [13]	Sonic hedgebog (Shh ED)	200E 2035 CnTi ⁻	His	Ves	[110]
Lay Imojochi, 1907 2.3.2. Hole Test Test Contrivator-associated agnine methyl transferase 1 (CARM1) 2931 Haio Yes [121] Toll like receptor 2 (TIR-2) (EC) 2932 His Yes [89] Tumor necrosis factor receptor (TIRR ED) CHO Fc Yes [89] B. Structure studies Type IIB receptor protein tyrosine phosphatase (RPTPµ and RPTPor) 2937 His Yes [122]-125] Human IgC1 antibody 2937 His Yes [123] ORF-98 SARS coronavirus 2937 His Yes [123] Hedgehog interacting protein (NV-C) (ED) 2937 His Yes [129] Hedgehog interacting protein (HeV-G) (ED) 2937 His Yes [129] Hedgehog interacting protein (HeV-G) (ED) 2937 His Yes [129] Interacting protein (HeV-G) (ED) 2935 GnT- His Yes [12] Interacting protein (HeV-G) (ED) 2935 SGnT- His Yes [130] Intera	Fruthropoietin (FDO)	203F	None	Vec	[120]
Continuou-basicitation aginine function function of the	Coactivator-associated argining methyl transferase 1 (CARM1)	2031	Halo	Vec	[120]
John Reference (LF) ESSE His Fee Yes [89] Leukemä inibilistyr factor (LF) CHO Fc Yes [91] <i>B. Structure studies</i> Tumor necrosis factor receptor (TNFR ED) CHO Fc Yes [12] <i>B. Structure studies</i> Type IIB receptor protein tyrosine phosphatase (RPTPµ and RPTPor) 293T His Yes [12] Fphrin B2 (EphB2) (ED) 293T His None Yes [12] Ephrin B2 (EphB2) (ED) 293T His Yes [12] Inotrophic glutamate receptor 2 (ED) 293T His Yes [12] Hendra virus glycoprotein (NiV-C) (ED) 293T His Yes [12] Hendra virus glycoprotein (HeV-G) (ED) 293T His Yes [13] Iontorophic glutamate receptor 2 (ED) 293T His Yes [12] Ephrin receptor A2 (EphA2) (ED) 293T His Yes [13] Ephrin receptor A2 (EphA2) (ED) 293S GnT- His Yes [13] Ephrin A5 lig	Toll like recentor 2 (TLR-2) (EC)	293F	His	Ves	[122]
Link minimum precode (LTM) Link Link <thlink< th=""> Link Link <thl< td=""><td>Leukemia inhibitory factor (LE)</td><td>CHO</td><td>Fc</td><td>Ves</td><td>[89]</td></thl<></thlink<>	Leukemia inhibitory factor (LE)	CHO	Fc	Ves	[89]
B. Structure studies Fig. 1 Fig. 1 Fig. 1 Fig. 1 Type IB receptor protein tyrosine phosphatase (RPTPµ and RPTPor) 293T His Yes [123-125] Human IgG1 antibody 293T His None Yes [127] Ephtrin B2 (EphB2) (ED) 293T His Yes [129] Heddra virus glycoprotein (NV-G) (ED) 293T His Yes [129] Heddra virus glycoprotein (NV-G) (ED) 293T His Yes [129] Hedgehog interacting protein (HeV-G) (ED) 293T His Yes [129] Hedgehog interacting protein (Hib) (ED) 293T His Yes [128] Ephtrin receptor A2 (EphA2) (ED) 293T His Yes [128] Ephtrin receptor A2 (EphA2) (ED) 293T His Yes [131] WIF domain of Wnt inhibitory factor 1 (WIF-1(WD)) (ED) 293S GnTT His Yes [132] Netrin G igands (NGL1 and NGL2) 293S GnTT His Yes [134] ULT creceptor related protein 6 (LRF0) (ED) 293S GnTT His Yes [134] Plexin-81 (ED	Tumor necrosis factor receptor (TNFR ED)	СНО	Fc	Yes	[91]
b. Structure studies Type IB receptor protein tyrosine phosphatase (RPTPµ and RPTPσ) 293T None Yes [126] Human IgC1 antibody 293T None Yes [127] Bergen SARS coronavirus 293T His Noe [127] Ephrin B2 (EphB2) (ED) 293T His Yes [129] Hedra virus glycoprotein (HeV-G) (ED) 293T His Yes [129] Hedra virus glycoprotein (HeV-G) (ED) 293T His Yes [129] Hedgehog interacting protein (Heiv) (ED) 293T His Yes [128] Ephrin receptor A2 (EphA4) (ED) 293T His Yes [128] Ephrin receptor A2 (EphA4) (ED) 293T His Yes [128] Ephrin receptor A2 (EphA4) (ED) 293S GnTT His Yes [131] WIF domain of Wnt inhibitory factor 1 (WIF-I(WD)) (ED) 293S GnTT His Yes [43] LDL receptor related protein 6 (LRP6) (ED) 293S GnTT His Yes [132] IgG Fc 293S GnT					
Type its receptor protein tyrosine prospiratase (kFr1P4 and kFr1P6) 2931 Fis Yes [126] ORF-98 SARS coronavirus 2937 His None [126] ORF-98 SARS coronavirus 2937 His No [127] Ephrin B2 (EphB2) (ED) 2937 His Yes [128] Nighah virus glycoprotein (NV-G) (ED) 2937 His Yes [129] Hedgeng interacting protein (Hip) (ED) 2937 His Yes [129] Hedgeng interacting protein (Hip) (ED) 2937 His Yes [128] Ephrin receptor A (EphA4) (ED) 2937 His Yes [128] Ephrin receptor A2 (EphA2) (ED) 2937 His Yes [131] WF domain of Wn tinhibitory factor 1 (WIF-1(WD)) (ED) 2935 GnTT His Yes [132] Netrins G1 igands (NGL1 and NGL2) 2935 GnTT His Yes [132] LDL receptor related protein 6 (LRP6) (ED) 2935 GnTT His Yes [134] Plexin-B1 (ED) 2935 GnTT His Yes	B. Structure studies	2027	11 ¹ -	V	[100, 105]
Human igU antibody 2931 None Yes [126] CRF-9B SARS coronavirus 293T His No [127] Ephrin B2 (EphB2) (ED) 293T His Yes [128] Nipah virus glycoprotein (NV-C) (ED) 293T His Yes [129] Hedgabog interacting protein (HeV-C) (ED) 293T His Yes [128] Ionotrophic glutamate receptor 2 (ED) 293S GnTi- His Yes [128] Ephrin receptor A4 (EphA4) (ED) 293T His Yes [128] Ephrin receptor A2 (EphA2) (ED) 293S GnTi- His Yes [128] Ephrin A5 ligand 293S GnTi- His Yes [131] WF domain of Wnt inhibitory factor 1 (WF-1(WD)) (ED) 293S GnTi- His Yes [132] Netrins G1 and C2 (ED) 293S GnTi- His Yes [132] LDL receptor related protein 6 (LRPG) (ED) 293S GnTi- His Yes [131] IgG Fc 293S GnTi- His Yes [132] IgG Fc 293S GnTi- His Yes [134] <tr< td=""><td>Type IIB receptor protein tyrosine phosphatase (RPTPμ and RPTPσ)</td><td>2931</td><td>HIS</td><td>Yes</td><td>[123-125]</td></tr<>	Type IIB receptor protein tyrosine phosphatase (RPTP μ and RPTP σ)	2931	HIS	Yes	[123-125]
DM-99 SARS Containing 2931 His No [127] Biphrin B2 (EphR2) (ED) 293T His Yes [128] Nipah virus glycoprotein (NiV-G) (ED) 293T His Yes [129] Hedgehog interacting protein (Hilp) (ED) 293T His Yes [130] Ionotrophic glutamate receptor 2 (ED) 293T His Yes [42] Ephrin receptor A4 (EphA4) (ED) 293T His Yes [128] Ephrin receptor A2 (EphA2) (ED) 293T His Yes [128] Ephrin receptor A2 (EphA2) (ED) 293T His Yes [128] Ephrin receptor A2 (EphA2) (ED) 293T His Yes [131] WIF domain of Wnt inhibitory factor 1 (WIF-1(WD)) (ED) 293S GnTT His Yes [132] Netrin G Ligands (NGL1 and NGL2) 293S GnTT His Yes [132] DL receptor related protein 6 (LRP6) (ED) 293S GnTT His Yes [134] Plexin-A2 (ED) 293S GnTT His Yes [134] Semaphorin 6A (ED) 293S GnTT His Yes	OPE OP SAPS coronavirus	2931	None	No	[120]
Eprim D2 (Eprinz) (ED) 2931 Fis Fes [129] Mipah virus glycoprotein (NiV-G) (ED) 293T His Yes [129] Hendra virus glycoprotein (NiV-G) (ED) 293T His Yes [129] Hedgehog interacting protein (Hhip) (ED) 293T His Yes [130] Ionotrophic glutamate receptor 2 (ED) 293S GnTI ⁻ His Yes [128] Ephrin receptor A4 (EphA4) (ED) 293T His Yes [128] Ephrin receptor A2 (EphA2) (ED) 293T His Yes [128] Ephrin S1 igand 293S GnTI ⁻ His Yes [131] WIF domain of Wnt inhibitory factor 1 (WIF-1(WD)) (ED) 293S GnTI ⁻ His Yes [43] DL receptor related protein 6 (LRP6) (ED) 293S GnTI ⁻ His Yes [131] LD receptor related protein 6 (LRP6) (ED) 293S GnTI ⁻ His Yes [132] ID receptor related protein 6 (LRP6) (ED) 293S GnTI ⁻ His Yes [133] IgG Fc 293S GnTI ⁻ His Yes [134] Plexin-A1 (ED) 293T	CRF-9D SARS COLONAVILUS	2931		NO	[127]
And an Virus gytophotent (NEV-C) (ED) 2931 His Tes [129] Hendra virus gytoprotent (NEV-C) (ED) 293T His Yes [130] Hendra virus gytoprotent (NHV) (ED) 293T His Yes [42] Ionotrophic glutamate receptor 2 (ED) 293S GnTr His Yes [42] Ephrin receptor A4 (EphA4) (ED) 293T His Yes [128] Ephrin receptor A2 (EphA2) (ED) 293T His Yes [131] WIF domain of Wnt inhibitory factor 1 (WIF-1(WD)) (ED) 293S GnTr His Yes [132] Netrins G1 and G2 (ED) 293S GnTr His Yes [132] Netrin G ligands (NGL1 and NGL2) 293S GnTr His Yes [132] IDL receptor related protein 6 (LRP6) (ED) 293S GnTr His Yes [132] IgG Fc 293S GnTr His Yes [134] Plexin-81 (ED) 293S GnTr His Yes [134] Semaphorin 6A (ED) 293S GnTr His Yes [134] Semaphorin 3A (ED) 293S GnTr His Yes [1	Nipob virus glucoprotoin (NiV C) (ED)	2551	Lic	Voc	[120]
Initial wins given of the (IPCO) (ED) 2931 His Fes [129] Hedgehog interacting protein (Hih) (ED) 2933 GnTT His Yes [120] Ephrin receptor A4 (EphA4) (ED) 2933 GnTT His Yes [128] Ephrin receptor A2 (EphA2) (ED) 2933 GnTT His Yes [131] WIF domain of Wnt inhibitory factor 1 (WIF-1(WD)) (ED) 2935 GnTT His Yes [132] Netrins G lagads (NGL1 and NGL2) 2935 GnTT His Yes [132] Decreptor related protein 6 (LRP6) (ED) 2935 GnTT His Yes [132] LDL receptor related protein 6 (LRP6) (ED) 2935 GnTT His Yes [132] LDL receptor related protein 6 (LRP6) (ED) 2935 GnTT His Yes [133] Plexin-B1 (ED) 2935 GnTT His Yes [134] Semaphorin 6A (ED) 2935 GnTT His Yes [134] Neurophilin 1 (Nrp1) 2935 GnTT His Yes [135] Semaphorin 3A (ED)	Hendra virus glycoprotein (HeV-C) (ED)	2951 203T	Hic	Ves	[129]
Integrating internation protein (Intrip) (ED) 293 Find	Hedgebog interacting protein (Hbin) (FD)	2037	Hic	Vec	[120]
bit of the equivalation (Epo) (ED) 2937 His Pts [42] Ephrin receptor A4 (EphA4) (ED) 2937 His Yes [128] Ephrin receptor A2 (EphA2) (ED) 2937 His Yes [131] Ephrin receptor A2 (EphA2) (ED) 2937 His Yes [131] Ephrin receptor A2 (EphA2) (ED) 2935 GnT1 ⁻ His Yes [132] Netrins G1 and G2 (ED) 2935 GnT1 ⁻ His Yes [43] Netrin G ligands (NGL1 and NGL2) 2935 GnT1 ⁻ His Yes [112] LDL receptor related protein 6 (LRP6) (ED) 2935 GnT1 ⁻ His Yes [112] Chaperone mesoderm development (Mesd) 2935 GnT1 ⁻ His Yes [134] Plexin-B1 (ED) 2935 GnT1 ⁻ His Yes [134] Semaphorin 6A (ED) 2935 GnT1 ⁻ His Yes [134] Neurophilin 1 (Nrp1) 2935 GnT1 ⁻ His Yes [134] Semaphorin 3A (ED) 2935 GnT1 ⁻ His Yes [135] Human telomerase 2935 GnT1 ⁻ His Yes [135	Ionotrophic dutamate recentor 2 (FD)	2035 CpTI-	Hic	Vec	[130]
Ephrin receptor A2 (EphA2) (ED) 2931 His Yes [128] Ephrin receptor A2 (EphA2) (ED) 2935 GnTI ⁻ His Yes [131] WIF domain of Wnt inhibitory factor 1 (WIF-1(WD)) (ED) 2935 GnTI ⁻ His Yes [43] Netrins G1 and G2 (ED) 2935 GnTI ⁻ His Yes [43] IDL receptor related protein 6 (LRP6) (ED) 2935 GnTI ⁻ His Yes [43] IDL receptor related protein 6 (LRP6) (ED) 2935 GnTI ⁻ His Yes [112] Chaperone mesoderm development (Mesd) 2935 GnTI ⁻ His Yes [134] IgG Fc 2935 GnTI ⁻ His Yes [134] Plexin-81 (ED) 2935 GnTI ⁻ His Yes [134] Semaphorin 6A (ED) 2935 GnTI ⁻ His Yes [134] Semaphorin 3A (ED) 2935 GnTI ⁻ His Yes [134] Neurophilin 1 (Nrp1) 2935 GnTI ⁻ His Yes [135] Human telomerase 2935 GnTI ⁻ His Yes [136] HUY p140 trimer 2935 GnTI ⁻ His Yes	Endrin recentor AA (End AA) (ED)	2955 6111	Hic	Vec	[128]
Image: Control (Coping (CD)) 2931 Image: Coping (Coping (CD)) 1129 Ephrin AC (Ephre) (ED) 2935 GnTT His Yes [131] WIF domain of Wnt inhibitory factor 1 (WIF-1(WD)) (ED) 2935 GnTT His Yes [43] Netrins G1 and G2 (ED) 2935 GnTT His Yes [43] Netrin G ligands (NGL1 and NGL2) 2935 GnTT His Yes [41] LDL receptor related protein 6 (LRP6) (ED) 2935 GnTT His Yes [112] Chaperone mesoderm development (Mesd) 2935 GnTT His Yes [133] IgG Fc 2935 GnTT None Yes [134] Plexin-81 (ED) 2935 GnTT His Yes [134] Semaphorin 6A (ED) 2935 GnTT His Yes [134] Semaphorin 3A (ED) 2935 GnTT His Yes [135] Human telomerase 2935 GnTT His Yes [135] Human telomerase 2935 GnTT His Yes [135] Human telomerase 2935 GnTT His Yes [136] HIV1 gp140	Ephrin receptor A2 (EphA2) (ED)	2037	Hic	Vec	[120]
Diffinit Dright 2535 GnT1 Ins Its It	Ephrin A5 ligand	2035 CpTI ⁻	Hic	Vec	[120]
With domains of with unsufficient (With Fight) (ED)2935 GnT1HisFits[12]Netrins G1 and G2 (ED)2935 GnT1-HisYes[43]Netrin G ligands (NGL1 and NGL2)2935 GnT1-HisYes[112]LDL receptor related protein 6 (LRP6) (ED)2935 GnT1-HisYes[112]Chaperone mesoderm development (Mesd)2935 GnT1-FLAGYes[112]IgG Fc2935 GnT1-NoneYes[134]Plexin-B1 (ED)2935 GnT1-HisYes[134]Semaphorin 6A (ED)2935 GnT1-HisYes[134]Semaphorin 6A (ED)2935 GnT1-HisYes[134]Neurophilin 1 (Nrp1)2935 GnT1-HisYes[135]Semaphorin 3A (ED)2935 GnT1-HisYes[135]Human telomerase2935 GnT1-HisYes[135]Huvin pi 140 trimer2937NoneYes[137]Repulsive guidance molecule B (RGMB) (ED)2937HisYes[138]C. Virus vector2935F-3F6 and 293None[139]Lentivirus (LV) vector293 and 293ENone[140,141]	WIE domain of What inhibitory factor 1 (WIE-1(WD)) (FD)	2935 GITI 2035 CnTI-	Hic	Vec	[132]
Netrin G Ligands (NCL1 and NGL2) 2935 GnT1 ⁻ His Yes [43] LDL receptor related protein 6 (LRP6) (ED) 2935 GnT1 ⁻ His Yes [112] Chaperone mesoderm development (Mesd) 2935 GnT1 ⁻ His Yes [112] IgG Fc 2935 GnT1 ⁻ FLAG Yes [133] Plexin-B1 (ED) 2935 GnT1 ⁻ His Yes [134] Plexin-A2 (ED) 2935 GnT1 ⁻ His Yes [134] Semaphorin 6A (ED) 2935 GnT1 ⁻ His Yes [134] Neurophilin 1 (Nrp1) 2935 GnT1 ⁻ His Yes [134] Neurophilin 1 (Nrp1) 2935 GnT1 ⁻ His Yes [134] Neurophilin 1 (Nrp1) 2935 GnT1 ⁻ His Yes [135] Semaphorin 3A (ED) 2935 GnT1 ⁻ His Yes [135] HUN 1g140 trimer 2937 None Yes [136] Huwan telomerase 2937 None Yes [137] Repulsive guidance molecule B (RGMB) (ED) 2937 His Yes [138] Neogenin (Neo1)	Netrins C1 and C2 (FD)	2935 GHT	His	Ves	[43]
Internet of lights (Ref) and Ref2)2935 GnT1HisFitsFitsFitsLDL receptor related protein 6 (LRP6) (ED)2935 GnT1HisYes[112]IgG Fc2935 GnT1NoneYes[133]Plexin-B1 (ED)293THisYes[134]Plexin-A2 (ED)2935 GnT1HisYes[134]Semaphorin 6A (ED)2935 GnT1HisYes[134]Neurophilin 1 (Nrp1)2935 GnT1HisYes[135]Semaphorin 3A (ED)2935 GnT1HisYes[135]Human telomerase293EFLAGNo[136]HIV1 gp140 trimer293TNoneYes[137]Repulsive guidance molecule B (RGMB) (ED)293THisYes[138]Neogenin (Neo1)293THisYes[138]C. Virus vector293THisYes[139]Lentivirus (LV) vector293 and 293ENone[140,141]	Netrin Cligands (NCL1 and NCL2)	2035 CnTI-	Hic	Vec	[43]
Diama and a problem of clark of (EM of (ED)) 2935 GmTi and a maximum of this and a maximum of the formation of th	IDL recentor related protein 6 (IRP6) (ED)	2935 GITI 2035 CnTI-	Hic	Vec	[112]
IgG Fc 2935 GnTr None Yes [13] Plexin-B1 (ED) 2937 His Yes [134] Plexin-A2 (ED) 2935 GnTr His Yes [134] Semaphorin 6A (ED) 2935 GnTr His Yes [134] Neurophilin 1 (Nrp1) 2935 GnTr His Yes [134] Semaphorin 3A (ED) 2935 GnTr His Yes [135] Semaphorin 3A (ED) 2935 GnTr His Yes [135] Human telomerase 2935 GnTr His Yes [135] Huwan telomerase 2935 GnTr His Yes [136] HUY1 gp140 trimer 2937 None Yes [137] Repulsive guidance molecule B (RGMB) (ED) 2937 His Yes [138] Neogenin (Neo1) 2937 His Yes [138] C. Virus vector 2935 Gad 293 None [139] Adeno-associated virus (AAV) vector 293 and 293E None [139]	Chaperone mesoderm development (Mesd)	2935 GITI 2035 CnTI-	FLAC	Vec	[112]
Igo It 2935 GHT Note 163 [134] Plexin-B1 (ED) 2935 GnTT His Yes [134] Semaphorin 6A (ED) 2935 GnTT His Yes [134] Neurophilin 1 (Nrp1) 2935 GnTT His Yes [134] Neurophilin 1 (Nrp1) 2935 GnTT His Yes [135] Semaphorin 3A (ED) 2935 GnTT His Yes [135] Human telomerase 2935 GnTT His Yes [136] HIV1 gp140 trimer 2937 None [137] Repulsive guidance molecule B (RGMB) (ED) 2937 His Yes [138] Neogenin (Neo1) 2937 His Yes [138] C. Virus vector 2935F-3F6 and 293 None [139] Adeno-associated virus (AAV) vector 293 and 293E None [139]		2935 GITI 2035 CnTI-	None	Vec	[12]
Plexin-A2 (ED) 2931 Fils Fils Fils Fils Plexin-A2 (ED) 2935 GnTI ⁻ His Yes [134] Semaphorin 6A (ED) 2935 GnTI ⁻ His Yes [134] Neurophilin 1 (Nrp1) 2935 GnTI ⁻ His Yes [134] Semaphorin 3A (ED) 2935 GnTI ⁻ His Yes [135] Human telomerase 2935 GnTI ⁻ His Yes [136] HIV1 gp140 trimer 2937 None Yes [137] Repulsive guidance molecule B (RGMB) (ED) 293T His Yes [138] Neogenin (Neo1) 293T His Yes [138] C. Virus vector 293SF-3F6 and 293 None [139] Adeno-associated virus (AAV) vector 293 and 293E None [139]	Devin_B1 (FD)	2955 6111	Hic	Vec	[134]
Initial Product (D) 2935 GmT Initial Product (D) Initial Product (D) Semaphorin 6A (ED) 2935 GmT His Yes [134] Neurophilin 1 (Nrp1) 2935 GmT His Yes [135] Semaphorin 3A (ED) 2935 GmT His Yes [135] Human telomerase 2935 GmT His Yes [136] HIV1 gp140 trimer 2937 None Yes [137] Repulsive guidance molecule B (RGMB) (ED) 2937 His Yes [138] Neogenin (Neo1) 2937 His Yes [138] C. Virus vector 2935F-3F6 and 293 None [139] Adeno-associated virus (AAV) vector 293 and 293E None [140,141]	$P[exin_{A2}(ED)]$	2035 CpTI ⁻	Hic	Vec	[134]
Schuppenin (LD) 2935 GmT His Fes [135] Neurophilin 1 (Nrp1) 2935 GmT His Yes [135] Semaphorin 3A (ED) 2935 GmT His Yes [135] Human telomerase 2932 FLAG No [136] HIV1 gp140 trimer 293T None Yes [137] Repulsive guidance molecule B (RGMB) (ED) 293T His Yes [138] Neogenin (Neo1) 293T His Yes [138] C. Virus vector 293SF-3F6 and 293 None [139] Adeno-associated virus (AAV) vector 293 and 293E None [139]	Semanhorin 6A (FD)	2935 GHT	His	Ves	[134]
Returbining (Rupp)2935 GnTHisFes[135]Semaphorin 3A (ED)2935 GnTHisYes[136]Human telomerase293EFLAGNo[136]HIV1 gp140 trimer293TNoneYes[137]Repulsive guidance molecule B (RGMB) (ED)293THisYes[138]Neogenin (Neo1)293THisYes[138]C. Virus vector293F-3F6 and 293None[139]Adeno-associated virus (AAV) vector293 and 293ENone[140,141]	Neurophilin 1 (Nrp1)	2935 GITI 2035 CnTI-	Hic	Vec	[134]
Human telomerase2935 cmHisHCS[13]Huvan telomerase293EFLAGNo[136]HIV1 gp140 trimer293TNoneYes[137]Repulsive guidance molecule B (RGMB) (ED)293THisYes[138]Neogenin (Neo1)293THisYes[138]C. Virus vector293SF-3F6 and 293None[139]Adeno-associated virus (AAV) vector293 and 293ENone[140,141]	Semanhorin 34 (FD)	2935 GITI 2035 CnTI-	Hic	Vec	[135]
Human contrast255LFLAGNO[136]HIV1 gp140 trimer293TNoneYes[137]Repulsive guidance molecule B (RGMB) (ED)293THisYes[138]Neogenin (Neo1)293THisYes[138]C. Virus vector293SF-3F6 and 293None[139]Adeno-associated virus (AAV) vector293 and 293ENone[140,141]	Human telomerase	2935 GITT 293F	FLAC	No	[136]
Invergence under2551None[157]Repulsive guidance molecule B (RGMB) (ED)293THisYes[138]Neogenin (Neo1)293THisYes[138]C. Virus vector293SF-3F6 and 293None[139]Adeno-associated virus (AAV) vector293 and 293ENone[140,141]	HIV1 gn140 trimer	293L 293T	None	Ves	[130]
Repairing guarance inforcation b (ROWB) (ED)2551FitsFets[138]Neogenin (Neo1)293THisYes[138]C. Virus vector293SF-3F6 and 293None[139]Adeno-associated virus (AAV) vector293 and 293ENone[140,141]	Repulsive guidance molecule B (RCMB) (FD)	293T	His	Ves	[137]
C. Virus vector293SF-3F6 and 293None[139]Lentivirus (LV) vector293 and 293ENone[139]Adeno-associated virus (AAV) vector293 and 293ENone[140,141]	Neogenin (Neo1)	2931 293T	His	Vec	[138]
C. virus vector293SF-3F6 and 293None[139]Lentivirus (LV) vector293 and 293ENone[140,141]		2331	1115	103	[130]
Lentivirus (LV) vector293SF-3F6 and 293None[139]Adeno-associated virus (AAV) vector293 and 293ENone[140,141]	C. Virus vector		Nama		[120]
Adeno-associated virus (AAV) vector 293 and 293E None [140,141]	Lentivirus (LV) vector	2935F-3F6 and 293	None		[139]
	Aueno-associated virus (AAV) vector	293 and 293E	None		[140,141]

Abbreviations: ED, ectodomain; MPD, membrane-proximal ligand-binding domain; Strep, streptavidin tag; His, 6×-histdine tag.

N-acetylglucosaminyltransferase-I activity (HEK-293S GnTI⁻) was generated by chemical mutagenesis. These cells produce glycoproteins with immature *N*-linked glycans having a high-mannose (Man) content [18], and they have served as a host for both stable and transient recombinant protein production [20,41–45].

CHO cells

In the 1950s the original CHO cell lines were recovered as spontaneously immortalized cells from primary Chinese hamster ovarian cultures [46]. CHO-DG44 cells were derived from one of these original lines by two rounds of gamma irradiation and then screened for the absence of dihydrofolate reductase (DHFR) activity [47]. These auxotrophic cells must be grown in the presence of hypoxanthine and thymidine (HT) whose synthesis requires the DHFR gene [47]. These cells were used as the host for the TGE method described here because they are efficiently transfected, grow to a high density in suspension culture, and are widely used in the biopharmaceutical industry to generate stable cell lines for the production of therapeutic proteins [1].

Two other lines, CHO-K1 and CHO-S, were separately derived from the original immortalized CHO cell lines with the latter being adapted to suspension culture [48,49]. Both have been transiently transfected with PEI [50]; Rajendra, unpublished data]. Unfortunately, a commercially available cell line adapted for growth in suspension culture using FreeStyle™ CHO medium (Life Technologies) is also designated CHO-S, but it may not be the same as the CHO-S line developed in the 1960s. CHO-T cells were generated by stable transfection of CHO-K1 cells with the Polyomavirus (Py) LT gene for the purpose of supporting the replication of plasmids carrying the Py ori [51]. However, they have only been transfected in suspension culture with liposomes [52]. Lastly, CHO mutants deficient in lectin binding have been isolated [53,54]. These cell lines synthesize glycoproteins with immature N-linked glycans, but to our knowledge they have not been used for largescale TGE.

Other host cells

Three human cell lines have recently emerged as potential TGE hosts, but the results are limited so far. CEVEC's amniocyte production cell line (CAP-T[®]) (Köln, Germany) expressing the SV40 LT can be cultivated in suspension and efficiently transfected with PEI [55]. HKB-11 cells, a hybrid between HEK-293 and Burkitt's lymphoma-derived 2B8 cells, and human Per.C6[®] cells (Crucell, Leiden, The Netherlands) have only been transiently transfected in suspension with liposomes [56–59].

Cell cultivation

Media

Suspension cultivation of HEK-293E and CHO-DG44 cells is usually performed in serum-free media that support densities of 5×10^{6} cells/mL or more. We routinely cultivate HEK-293E cells in EX-CELL[®]293 (SAFC Biosciences, St. Louis, Missouri) and CHO-DG44 cells in ProCHO5[™] (Lonza, Verviers, Belgium). However, many other serum-free media are available for high-density cell cultivation. As expected, the formulations of these high-performance media are not made available by the providers, but they often contain soy-derived peptones and one or more recombinant growth factors. Due to the lack of information on the formulation, choosing and trouble-shooting media are difficult since the various steps of the TGE process - cell growth, transfection, production, and purification - have different requirements, and unknown medium components may interfere with any one of them. Most, if not all, of the commercial media contain polymers such as Pluronic® F-68 (SAFC Biosciences) to reduce shear forces generated by the mixing of cells in suspension. To our knowledge, this polymer does not interfere with any step in the methods described here.

Cell cultivation system

One of the key factors required for the development of a costeffective TGE method with mammalian cells is an efficient system that provides the physico-chemical environment for high-density cell cultivation. The major consumable cost in TGE processes is that of the medium. Therefore, maximizing medium utilization by growing cells to a high density is the best approach to making protein production more economical. This is not only important for the protein production phase but also for the provisioning of cells prior to transfection. Having a cultivation system that supports high-density cell growth is therefore necessary to minimize the overall costs of protein production.

Several options are available for suspension cell cultivation including orbitally shaken containers, stirred-tank bioreactors (STRs), spinner flasks, and WAVE bioreactors (GE Healthcare Europe, Glattbrugg, Switzerland) [1,60-62]. To select among these options, one must take into account the cost and engineering parameters of the equipment. Spinner flasks are inexpensive and available in nominal volumes of 100 mL-36 L. However, due to poor mixing and gas transfer, they do not adequately support high-density cell cultivation in the absence of active aeration. The WAVE bioreactor relies on disposable bags with nominal volumes of 500 mL-500 L [63-66]. The single-use bags are a major operating expense, and cultures in WAVE bags require active aeration for optimal cell growth. The other shortcoming of the WAVE bioreactor is that the simultaneous operation of multiple cultures requires multiple agitation/heating units, increasing the space and equipment needs. Finally, STRs are difficult to maintain, setup and operate. They are also impractical for the performance of simultaneous cultures and for small-scale operations. Their cost may also be prohibitive for many potential users.

We use orbitally shaken bioreactors (OSRs) for cell cultivation and TGE due to the simplicity of operation from small to large volumetric scales and for their proven superior engineering characteristics for efficient mixing and gas transfer [26,67–70]. Orbitally shaken containers include the disposable TubeSpin[®] bioreactor 50 and 600 tubes (TPP, Trasadingen, Switzerland) with nominal volumes of 50 and 600 mL, respectively, cylindrical glass bottles with nominal volumes of 100 mL–5 L, square-shaped glass bottles with nominal volumes of 100 mL–1 L, and shake flasks with nominal volumes of 250 mL–5 L. We perform most of our operations in TubeSpin[®] bioreactors and in glass bottles [24,26,68]. Small-scale operations at volumes of 2–10 mL and medium-scale operations of 100–500 mL are well-suited to the TubeSpin[®] bioreactor 50 and TubeSpin[®] bioreactor 600, respectively (Fig. 1) [24,71]. These disposable containers are shaped like conical centrifuge tubes,



Fig. 1. Image of TubeSpin[®] bioreactor 50 and 600. The two disposable containers are shown in their appropriate racks fixed on the shaking platform of a incubator shaker.



Fig. 2. Images of typical glass containers used for suspension cell cultivation in OSRs. (A) Square-shaped glass bottle of 1-L nominal volume attached to the shaker platform with double-sided tape. (B) Cylindrical glass bottle of 5-L nominal volume attached to the shaker platform with vertical supports.

but they are equipped with a ventilated cap having a membrane filter as a sterility barrier. Cultures in these tubes are maintained in an incubator shaker with 5% CO_2 and 85% humidity in racks attached to the shaker platform (Fig. 1). It is important that the tubes fit tightly in the rack to prevent their rotation during agitation. The tubes double as centrifuge tubes to facilitate cell handling. Alternatively, it is possible to perform small-scale transfections in multi-well plates, but this cultivation system is limited by high evaporation rates [72,73].

When using cylindrical or square-shaped glass bottles, the working volume is 30–40% of the bottle's nominal volume [26]. The cultures are maintained on the shaker platform with double-coated removable foam tape (3M, Rüschlikon, Switzerland) (Fig. 2A) or with supports fastened to the platform in the case of 5-L bottles (Fig. 2B). It is best to maintain the cultures in an incubator with 5% CO₂. Suitable incubator shakers with CO₂ control are commercially available from several manufacturers. It is possible to substitute shake flasks for glass bottles, but they have a larger footprint than glass bottles of the same nominal volume.

In OSRs, gas exchange takes place at the liquid surface rather than by the sparging of gas into the liquid as in STRs. As a consequence gas transfer into and out of the culture is dependent on rapid mixing to create a high renewal rate at the liquid surface. At shaking frequencies suitable for cell culture, all the OSRs mentioned above have mixing times (the time needed to mix to homogeneity) under 20 s [74]. Importantly, efficient mixing can be achieved with a low power input, resulting in less shear stress on cells, by an order of magnitude, than observed in STRs [25,70,75–77].

An oxygen mass transfer coefficient (k_La) of 7 h⁻¹ is needed to avoid oxygen limitations in high-density cultures of mammalian cells in OSRs [78]. The k_La values for the OSRs described here are above this minimum level at shaking frequencies suitable for mammalian cell cultivation [69,78]. The efficient mixing and gas exchange in OSRs also means that the CO₂ produced by cells is effectively removed from the culture so that the dissolved CO₂ concentration is maintained by the CO₂ level in the incubator, keeping the pH in a suitable range (6.6–7.1) for cell cultivation [75,78]. Therefore, after appropriate optimization of the cultivation parameters, it is not necessary to continuously monitor and control the dissolved oxygen (DO) concentration and pH during bioprocesses performed in OSRs [75].

Routine maintenance of cells

Both HEK-293E and CHO-DG44 cells are subcultivated twice per week in EX-CELL[®]293 and ProCHO5TM media, respectively, at a cell density of 0.3×10^6 cells/mL. The cells are maintained as a 10-mL culture in a TubeSpin[®] bioreactor 50 or as a 100-mL culture in a 250-mL glass bottle with agitation at 180 or 120 rpm, respectively. All shaking speeds described here are based on a shaking diameter of 5 cm. For shakers with a smaller shaking diameter, the shaking speed must be higher.

We do not normally keep the cells in culture longer than 20 passages. For long-term storage, CHO-DG44 and HEK-293E cells are frozen in liquid nitrogen at a density of 15×10^6 cells/mL in aliquots of 1 mL or more, in the cultivation medium containing 10% DMSO (Sigma–Aldrich, Buchs, Switzerland).

Expression vector

Although many strong, constitutive promoters/enhancers are available for heterologous gene expression in mammalian cells, the human cytomegalovirus (hCMV) major immediate early (mIE) promoter/enhancer is usually the best choice for TGE in both CHO-DG44 and HEK-293E cells, and most commercially available expression vectors carry it. The mIE promoter/enhancer of mouse CMV (mCMV) and the human elongation factor 1-alpha (hEF-1 α) promoter/enhances serve as adequate substitutes. We have found that the minimal expression vector can be constructed with the hCMV mIE promoter/enhancer, a polyadenlyation site, and either a splice site in the 5' untranslated region (UTR) or the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) in the 3' UTR [14]. Currently, we use an expression vector (pXLG^{HEK}) that has the bovine growth hormone polyadenlyation site and a chimeric β -globin-IgG splice site [14,79].

Modifications of the minimal expression vector are possible, depending on the needs of the user. For example, if the vector is frequently used for the production of secreted or membrane-associated protein, then it may be convenient to include the coding sequence for a signal peptide in the expression cassette. If this is necessary, the kappa light chain signal peptide (M E T D T L L L W V L L L W V P G S T G D) serves as a good choice. Otherwise, it is possible to include the native signal sequence of the protein of interest. An expression vector that includes the coding sequence of an affinity tag may also be desirable.

For transient antibody production, it may be advantageous to clone the genes into separate vectors in order to vary the ratio of the light and heavy chain genes to achieve the highest yield possible [80,81]. We have not observed higher antibody yields by expressing the light and heavy chain genes from a bicistronic vector with an internal ribosome binding site [82] or as a polyprotein with the heavy and light chains separated by the picornavirus 2A self-processing peptide [83].

Polyethyleneimine

Although several non-viral gene delivery methods are available for the transfection of mammalian cells, few of these are both scaleable and cost-effective. Among the scalable methods, the least expensive reagents are calcium phosphate (CaPi) and cationic polymers [11]. The former, however, requires the presence of serum in the transfection medium [84]. Since serum-free conditions are often preferred for the production phase, it is necessary to perform a medium exchange following gene delivery with CaPi [85]. Moreover, the formation of the CaPi-DNA complex is time-dependent, increasing the technical difficulty of its use at large scale. PEI-mediated gene delivery, on the other hand, does not require serum in the medium [86], and time-dependent pre-complex formation between PEI and DNA is not required [29,87]. Cationic liposomes are arguably the most efficient DNA delivery vehicles [2], but their high cost is a limitation for their use at large scale [10,11].

Linear 25 kDa PEI (Polysciences, Eppelheim, Germany) is dissolved in water to generate a stock solution at a concentration of 1 mg/mL and pH 7.0. Dissolving PEI requires acidification of the solution to pH 3 by addition of 1 N HCl. When all the PEI is in solution, the pH is increased to 7.0 by addition of 1 N NaOH. The solution is filter sterilized and stored at -20 °C. Linear 25 kDa PEI in this form usually has a number-average molecular weight (M_n) of 6-7 kg/mol with the range being 2-26 kg/mol [88]. Its polydispersity index (PDI) is about 1.9, an important property for efficient DNA delivery in HEK-293E cells [88]. In the synthesis of PEI from 2ethyloxazoline, there is an acidification step to remove *N*-propionyl groups from the amine. However, this reaction may not be complete, resulting in batch-to-batch variation in the percentage of protonatable amine groups [88]. The presence of *N*-propionyl groups reduces both the charge density of PEI and the strength of its interaction with DNA. Nevertheless, we observed that the transfection efficiency of various batches of PEI with 4–15% *N*-propionyl groups did not vary [88].

Until recently, it was thought that pre-formation of PEI-DNA complexes (polyplexes) prior to their addition to the culture was critical for efficient transfection. However, it has been observed that the direct addition to the culture of DNA followed by PEI is possible when transfecting at a low or high cell density [29,87]. We have employed this approach with both HEK-293E and CHO-DG44 cells [30,89–91].

HEK-293E					
Vessel volume [L]	1	0.25	0.25	5	
Working volume [L]	0.4	0.05	0.05	1	
Shaking speed [rpm]	110	120	120	120	
Cell density [cells/mL]	4 x 10 ⁶	20 x 10 6	20 x 10 6	1 x 10 ⁶	
Medium	EX-CELL ®293	RPMI + 0.1% F68	RPMI + 0.1% F68	Production	
Temperature [°C]	37	37	37	37	

СНО			
Vessel volume [L]	5	5	5
Working volume [L]	1.5	1	1
Shaking speed [rpm]	120	120	120
Cell density [cells/mL]	4 x 10 ⁶	5 x 10 ⁶	5 x 10 ⁶
Medium	ProCHO5 ™	ProCHO5 ™	ProCHO5 ™
Temperature [°C]	37	31	31

Fig. 3. Schematic diagrams of a one-liter TGE process in HEK-293E (top) and CHO cells (bottom). Production media for HEK-293E cells include EX-CELL[®]293, Pro293s[®], FreeStyle[™]293, and FreeStyle[™]F17. For cell scale-up, the approximate cell density one day after the inoculation is given. The clock indicates time-dependent steps. *Abbreviations*: F68, Pluronic[®]-F68.

Transfection processes

HEK-293E cells

HEK-293E cells, grown in EX-CELL® 293 medium, are passaged to fresh medium on the day before transfection at a density of $1.5-2.5 \times 10^6$ cells/mL. The next day, the appropriate volume of culture is centrifuged, and the cell pellet is resuspended at 20×10^6 cells/mL in RPMI 1640 containing 0.1% Pluronic F-68 (transfection medium). As an example, for a 1-L (final volume) transfection, 1×10^9 cells are resuspended in 50 mL of transfection medium in a 250-mL glass bottle (Fig. 3). The DNA is then added at $1.5 \,\mu$ g/million cells (1.5 mg total), and the culture is mixed by hand. Immediately, 3.0 mL of a 1 mg/mL PEI stock solution is added [87,90,91]. The culture is again swirled and then transferred to an incubator shaker with 5% CO₂ for 60–90 min at 37 °C with agitation at 120 rpm. The transfection can also be performed in a TubeSpin[®] bioreactor 600 with agitation at 180 rpm. At the end of the transfection phase, the culture is transferred to a 5-L glass bottle containing 950 mL of pre-warmed production medium (see below) to give a density of 1×10^6 cells/mL. The transfection can be performed at any volumetric scale with proportional adjustments of the cell number and the DNA and PEI amounts.

Valproic acid (VPA: SAFC Biosciences), a histone deacetylase inhibitor, can have a positive effect on the production of recombinant proteins in HEK-293E cells, but this is not universal [92,93]. The effect of VPA on yield must be determined for each protein, and the optimal amount of VPA must be optimized for each protein and each HEK-293 cell line. The stock solution is prepared by dissolving VPA in water at 0.5 M followed by filter sterilization and storage at -20 °C in 50-mL aliquots. In general, the maximum level of protein accumulation is observed on day 2–3 post-transfection for transmembrane and intracellular proteins, and on day 3-7 post-transfection for secreted proteins. In the presence of VPA, cells double once post-transfection and then cease dividing [92]. Due to this effect, it is possible to dilute the transfected culture to a density of $2-4 \times 10^6$ cells/mL, instead of 1×10^6 cells/mL. This allows for a greater accumulation of biomass in the culture, but it does not change the amounts of DNA and PEI added, since they are based on the total cell number rather than the final culture volume.

We use different media for production, depending on the protein. For all intracellular and transmembrane proteins we use EX-CELL[®] 293 or FreeStyle[™] F17 (Life Technologies Europe). This is also the case for secreted proteins that are affinity-purified with protein A, protein G, or an anti-FLAG antibody. For secreted proteins that have a histidine-tag, the production medium is either FreeStyle293[™] or Pro293[™] (Lonza) since both EX-CELL[®] 293 and FreeStyle[™] F17 contain a component(s) that interferes with nickel-affinity chromatography.

To estimate the transfection efficiency (defined as the percentage of transfected cells), we replace 2% of the plasmid carrying the GOI with one for the expression of the enhanced green fluorescent protein (eGFP) gene. At day 2 post-transfection, an aliquot of the culture is visually inspected by fluorescence and light microscopy to estimate the transfection efficiency [94]. Alternatively, an aliquot of the culture is centrifuged and resuspended in PBS, and the percentage of eGFP-positive cells is determined by flow cytometry. We expect 80–90% eGFP-positive cells by day 2 post-transfection.

CHO-DG44 cells

As with HEK-293E cells, a medium exchange must be performed the day before transfection for CHO-DG44 cells grown in ProCHO5TM medium. This culture is started at a density of $1.5-2.5 \times 10^6$ cells/mL. The next day the appropriate volume of

culture is centrifuged, and the cell pellet is resuspended at a density of 5×10^6 cells/mL in ProCHO5TM medium pre-warmed to 31 °C. DNA and PEI are added sequentially at 0.6 µg/million cells and 3.0 µg/million cells, respectively. After each addition the culture is mixed by swirling. For a 1-L (final volume) transfection, 5×10^9 cells are resuspended in 1 L of medium in a 5-L cylindrical glass bottle (Fig. 3). Immediately, 3 mg of DNA and 15 mg of PEI (1 mg/mL stock solution) are added sequentially with brief mixing after each addition [89]. The culture is then transferred to an incubator shaker at 31 °C with 5% CO₂ and agitated at 120 rpm with the bottle cap slightly open [79,89,95]. Under these conditions, the cells go through one doubling by day 1 post-transfection and then stop dividing [79,89]. The cells can be maintained at 31 °C for up to 10 days before the cell viability falls below 50% [79,89,95]. We have not experienced problems with the affinity purification of secreted proteins in ProCHO5[™] medium, and so this medium can be used for all steps of the TGE process. With CHO-DG44 cells the transfection efficiency, as determined by co-transfection with an eGFP expression vector as described for HEK-293E cells, is usually 50-60%. The maximum accumulation of transmembrane and intracellular proteins is usually observed at 2-4 days post-transfection, while for secreted proteins the maximum level can be observed at 4-10 days post-transfection.

Limitations of TGE

For TGE processes using CHO-DG44 and HEK-293E cells, we have observed yields up to 300 mg/L and 1 g/L, respectively, for a recombinant IgG antibody [14,89]. For membrane and intracellular proteins, the highest yields have been about 30 mg/L [96]. Note that these levels will not be achieved with every protein. For any given protein, the yield is usually higher from HEK-293E cells than from CHO-DG44 cells, but there are exceptions to this rule. Therefore, we routinely check the expression of each protein in both hosts.

Even though the TGE yields are satisfactory for most proteins and for most research applications, some aspects of the TGE processes described here are not ideal if a large amount of protein is needed. One of the main limitations of these methods is the need to passage the cells the day before transfection to achieve the optimal transfection efficiency and protein yield. This constitutes an important cost item, and it makes large-scale TGE (>10 L) very challenging due to the need to centrifuge large culture volumes.

We have investigated the molecular limitations of the TGE processes described here using a monoclonal antibody as the model protein. We did not observe a limitation to plasmid DNA uptake in either host. Instead, the steady-state level of transgene mRNA, as measured by quantitative PCR, did reach a plateau as the amount of plasmid DNA uptake increased [93]. A transcriptional limitation for transiently transfected HEK-293E has been independently reported [97]. For both CHO-DG44 and HEK-293E cells, most of the intracellular plasmid DNA remains intact, at least up to day 3 post-transfection [93]. However, the majority of this DNA is present as linear or nicked circular DNA rather than supercoiled DNA, as judged by southern blot analysis of total cellular DNA [93]. Unfortunately, it is not known if all three forms of plasmid DNA are competent for transcription in mammalian cells. From these results, it appears that further improvements in TGE yields will need to overcome the limitation in transgene transcription.

As stated before, there are problems with regard to some of the commercial media used for TGE. For example, EX-CELL[®] 293 inhibits efficient transfection with PEI, and both EX-CELL[®] 293 and Free-Style[™] F17 inhibit the affinity purification of secreted His-tagged proteins. We have also observed lot-to-lot variation in the quality of commercial media, leading to large variability in cell viability,

maximum cell density, transfection efficiency, protein yield, and protein recovery by ion exchange chromatography. Therefore, it is advisable to test every lot of medium before purchase. Unfortunately, this may not be practical for users who only purchase a few liters at a time.

Conclusion

As summarized in Table 1 over 50 proteins have been overexpressed by PEI-mediated transfection of HEK-293 and CHO cells since 2004. This list includes over 20 proteins whose structure has been resolved. In addition, adeno-associated virus and lentivirus vectors have been transiently produced in HEK-293 cells by the methods described here. Undoubtedly, the number of proteins produced by TGE will continue to grow as more researchers see the benefits of this approach. It is for this reason that we have outlined a detailed guideline for developing an in-house cost-effective method for recombinant protein expression by TGE using suspension-adapted HEK-293E and CHO-DG44 cells grown in OSRs. Importantly, the methods described here have been optimized for our own cells and the corresponding medium formulation for their expansion. Variations from these "optimal" conditions are expected for CHO and HEK-293 cells maintained in other laboratories under different culture conditions.

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