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Cell Banking of HEK293T cell line for clinical-grade lentiviral particles manufacturing

Unai Perpiñá[1,2,3,4], Cristina Herranz[1,2,3,4], Raquel Martín-Ibáñez[1,2,3,4,5], Anna Boronat[4,6], Felipe Chiappe[1,2,3,4], Verónica Monforte[1,2,3,4], Gemma Orpella-Aceret[1,2,3,4], Ester González[1,2,3,4], Myriam Olivé[1,2,3,4], María Castella[4,7,8], Guillermo Suñé[4,7], Álvaro Urbano-Ispizua[4,7,9,10], Julio Delgado[4,7,9,11], Manel Juan[4,6,9,12] and Josep M. Canals[1,2,3,4*]

Abstract

Background: Cell banks are widely used to preserve cell properties as well as to record and control the use of cell lines in biomedical research. The generation of cell banks for the manufacturing of Advanced Therapy Medicinal Products, such as cell and gene therapy products, must comply with current Good Manufacturing Practice regulations. The quality of the cell lines used as starting materials in viral-vector manufacturing processes must be also assessed.

Methods: Three batches of a Master Cell Bank and a Working Cell Bank of the HEK293T cell line were manufactured under current Good Manufacturing Practices regulations. Quality control tests were performed according to product specifications. Process validation includes the training of manufacturing personnel by performing simulation tests, and the continuous measurement of environmental parameters such as air particles and microorganisms. Cell number and viability of cryopreserved cells were periodically measured in order to define the stability of these cellular products.

Results: All batches of HEK293T Master and Working Cell Banks met the acceptance criteria of their specifications showing the robustness and homogeneity of the processes. In addition, both Master and Working Cell Banks maintained the defined cell viability and concentration over a 37 month-period after cryopreservation.

Conclusions: Manufacturing cell banks under Good Manufacturing Practice regulations for their use as raw materials or final cellular products is feasible. HEK293T cell banks were used to manufacture clinical-grade lentiviral particles for Chimeric Antigen Receptor T-cell based clinical trials.

Keywords: ATMP, Good Manufacturing Practices, Cell therapy, Gene therapy, HEK293T, CAR-T, lentivirus

* Correspondence: jmcanals@ub.edu
[1]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Laboratory of Stem Cells and Regenerative Medicine, Institute of Neurosciences, University of Barcelona, C/ Casanova 143, 08036 Barcelona, Spain
[2]Production and Validation Center of Advanced Therapies (Creatio), Faculty of Medicine and Health Sciences, University of Barcelona, Barcelona, Spain
Full list of author information is available at the end of the article

Background

Advanced Therapy Medicinal Products (ATMPs) such as cell therapy, gene therapy and tissue engineering [1, 2] have emerged as promising candidates for the treatment of currently incurable diseases. This is the case of Alofisel®, a cell therapy product based on adipose-derived mesenchymal stem cells, used to treat perianal fistulas in Crohn's disease, [3] and Holoclar®, a tissue engineered product used to repair damaged corneal tissues, based on fibrin membranes coated with a population of corneal cells, including limbal stem cells [4].

In recent years, the number clinical trials based on *ex vivo* and *in vivo* gene therapies has increased due to the great potential of ATMPs for monogenic disorders such as cystic fibrosis, adenosine deaminase deficiency (ADA-SCID) or hemophilia B (factor IX deficiency) among others [5]. European Medicines Agency (EMA) and Food and Drug Administration (FDA) regulatory bodies recently approved several gene-derived ATMPs. This is the case of Zolgensma®, a treatment for spinal muscular atrophy (SMA) based on the *in vivo* administration of adeno-associated viruses (AAVs), which deliver a functional version of the survival motor neuron 1 (SMN1) gene; [6, 7] and Zynteglo®, an autologous *ex vivo* gene therapy for beta thalassemia that uses lentiviruses to insert a functional hemoglobin subunit beta (HBB) gene in the hematopoietic stem cells of the patient [8].

Chimeric Antigen Receptor (CAR) T-cell therapy might be considered the most revolutionary *ex vivo* gene therapy in oncology because it allows targeting specific surface antigens in a wide range of tumor cell populations. Anti-CD19 CAR T-cells for relapsed/refractory B-ALL patients, the first cancer gene therapy approved on August 2017 by the FDA, is being used worldwide because of its excellent clinical outcomes [9–11]. Anti-CD30 CAR T-cells and Anti-BCMA CAR T-cells are also promising therapies based on the CAR T-cell technology [12, 13].

Most of the research and development on ATMPs during the early clinical stages (Phase I/II trials) is carried out in academic institutions, rather than in the pharmaceutical industry [14]. Thus, academic institutions should get familiar with the regulations controlling ATMP manufacturing to transition from basic research to clinical research. Good Manufacturing Practices (GMP) are mandatory for all manufacturing sites dedicated to the ATMP production, including academic institutions. Given the nature and complexity of these products, it is of crucial importance that the development of new ATMPs provides safe, effective and high-quality products, thus guaranteeing the robustness and homogeneity of the manufacturing process [15]. Therefore, manufacturing procedures should be validated according to GMP and, for this reason, a careful assessment of the whole process - from raw materials to final products - and of the starting materials' product specifications is required before manufacturing begins. Here we present the GMP-compliant generation of a Master Cell Bank (MCB) and Working Cell Bank (WCB) of HEK293T cells at Creatio, the production and validation center of advanced therapies at the University of Barcelona. HEK293T adherent cells are widely used and are a consolidated model of viral-vector production because of their high transfectability. MCB and WCB were used as packaging cells to obtain GMP-grade lentiviruses for clinical trials using CAR T-cell therapies: phase I clinical trial CART19-BE-01 (EudraCT Number: 2016-002972-29) and phase II clinical trial CART19-BE-02 (EudraCT Number: 2019-003038-17), based on anti-CD19 CAR T-cell therapy; and phase I clinical trial (EudraCT Number: 2019-001472-11), based on anti-BCMA CAR T-Cell therapy.

This GMP-grade cell banking approach may provide guidance to academic centers willing to generate GMP-grade lentiviruses, and at the same time represents a reference to generate different viral vectors.

Methods

Quality System

Creatio Quality System operates under the current GMP regulations and is authorized by the Spanish Agency of Medicines and Medical Devices (PE010-1570, AEMPS, Spain). A specific organizational chart is employed at Creatio. Functions and responsibilities of each staff member of the facility are clearly defined and documented. Also, a specific document management system is implemented at Creatio and includes: risk assessment approaches, change control documentation, incidents and nonconformities records, calibration, validation and qualification annual strategy, audit programs, personnel training programs, preventive maintenance systems, reagents and starting materials record and traceability, quality control management, manufacturing management, batch certification and release system.

Facility and equipment

Creatio is an academic facility of the University of Barcelona dedicated to the validation and manufacturing of ATMPs for their use in phase I/II clinical trials. The facility has 4 B Grade cleanrooms in which all procedures can performed in a Grade A laminar flow cabinet. MCB and WCB of the HEK293T cell line (CRL-11,268; ATCC, Mansassa, VA, USA; obtained under a License Agreement between The Rockefeller University and Hospital Clínic de Barcelona; October 17, 2016) were handled in the cleanroom dedicated to cell therapy. The access to the cleanroom consists of an entrance to the pre-dressing room (Grade D), a pre-dressing room (Grade C), a dressing room (Grade B), and a distributor (Grade

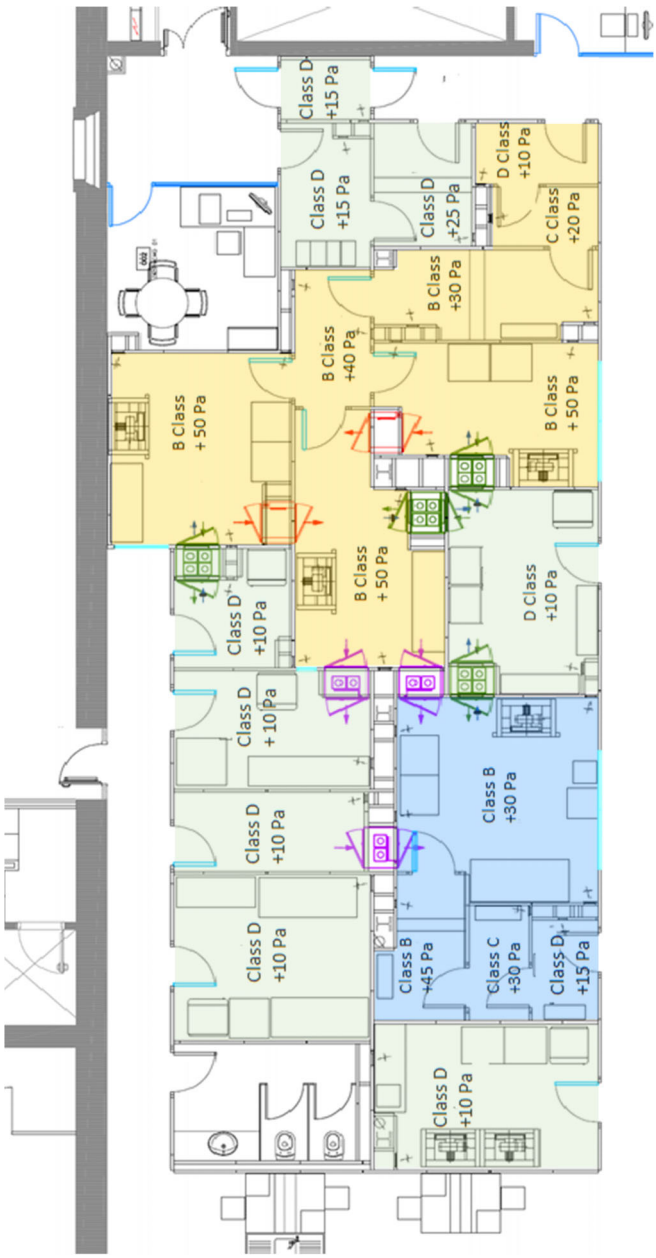


Fig. 1 Layout of Creatio GMP facility. The facility is controlled by three independent Heating Ventilation and Air Conditioning (HVAC) systems (orange, blue and green). In white, non-classified areas. In orange, HVAC-1. In blue, HVAC-2. In green, HVAC-3. Rooms belonging to HVAC-3 have a pressure of 10 Pa and are dedicated to storage and product conditioning. Cell therapy products are manufactured in HVAC-1. Cleanrooms (Grade B) belonging to HVAC-1 have a pressure of 50 Pa, adjacent rooms have a differential pressure of 10–15 Pa. HVAC-2 is dedicated to gene therapy products. Access to gene therapy cleanrooms (Grade B) is granted through dressing rooms which have a differential pressure of 10–15 Pa. In this case, Grade B cleanroom is under pressured (30 Pa) compared to the adjacent dressing room (40 Pa) as a physical containment barrier to avoid viral spreading

B) (Fig. 1). Adjacent rooms are at different pressures (Δ10–15 Pa between rooms) reaching 50 Pa in the Grade B cleanroom. Creatio facility has two storage rooms (Grade D) and a Grade D product conditioning room. Pressure, temperature, and humidity are continuously monitored and regulated by a specific control software (Controlli Delta Spain, Barcelona, Spain). The facility is validated once a year according to specific procedures of validation management. Equipment is validated and calibrated according to a pre-determined annual planning. Parameters of incubators, refrigerators, freezers, ultra-freezers, nitrogen tanks, and cell

incubators CO_2 levels are continuously monitored by a specific control software (Sirius, Nirco, Rubí, Spain).

HEK293T source and cell line specifications

The HEK293T cell line, which expresses the Simian Virus 40 (SV40) large antigen T, was generated at the Rockefeller University from the HEK293 cell line. According to the certificate of analysis, the product obtained from the ATCC had a passage number of 17. In addition, the certificate of analysis of the HEK293T cell line reports the results of microbiological quality control tests, Short Tandem Repeats (STRs) analysis, and PCR-based assays for human pathogenic viruses: HIV, HepB, HPV, EBV, CMV. This information was documented and used as a starting point of our process, and no further manipulations were performed between cell line purchase and its use for cell banking.

Cell line identification

The following STR profile of the HEK293T cell line was analyzed: Amelogenin: X,CSF1PO: 11, 12; D13S317: 12, 14; D16S539: 9, 13; D5S818: 8, 9; D7S820: 11; THO1: 7, 9.3; TPOX: 11; vWA: 16, 18, 19.

HEK293T cell culture

HEK293T cells were incubated for 2 min at 37 °C in a thermoblock and quickly transferred to a tube containing DMEM (Thermo Fisher Scientific, Walthman MA, USA) supplemented with 10% of Fetal Bovine Serum (FBS) (Thermo Fisher Scientific). Subsequently, cells were centrifuged (1250 rpm, 5 minutes), supernatant was removed, and cells were resuspended in fresh cell culture medium for Trypan blue-based cell counting. Cells were seeded in a sterile and pyrogen-free flask at a density of 1×10^6 cells x 25 cm^2 and incubated at 37 °C/ 5% CO_2 for 3–4 days until cells reached 80% confluence. For cell passaging, spent culture medium was removed, and cells washed with Phosphate-Buffered Saline (PBS) (Thermo Fisher Scientific). TrypLE™ (Thermo Fisher Scientific) was added and incubated for 1 min to detach cells from the flask. Once detached, cells were transferred to a tube containing fresh medium and centrifuged (1250 rpm, 5 min). Cells were counted, centrifuged again (1250 rpm, 5 min) and seeded at a density of $1{\cdot}10^6$ cells·25 cm^2 in as many flasks of 25 or 75 cm^2 as necessary. Cell passages were made to reach more than $200{\cdot}10^6$ cells for the MCB and $1000{\cdot}10^6$ for the WCB.

HEK293T cryopreservation

Once the required cell number was reached, cells were centrifuged (1250 rpm, 5 min) and the pellet was resuspended in cryopreservation medium to achieve a density of $10{\cdot}10^6$ cells/ml for MCB and $20{\cdot}10^6$ cells/ml for WCB. Cryopreservation medium was composed of 95% of cell culture medium (10% FBS-supplemented DMEM) and 5% of DMSO (Scharlab, Setmenat, Spain). Cryotubes were properly labelled and filled with 1 ml of the required concentration. Cryotubes were transported from the cleanroom to the cryopreservation area and temperature was monitored during transport. Cryopreservation was carried out in a controlled-rate freezer (Criomed™, Thermo Fisher Scientific) following a freezing program that lowers the temperature 1 °C/minute. The resulting cryotubes were then transferred to a gas phase nitrogen tank at -180 °C ± 20° C.

Personnel training and validations

Creatio adopts an ongoing personnel training program. Manufacturing staff is also assessed and validated once every 6 months with simulation tests of ongoing production processes. Personnel involved in the production of MCB and WCB undertook three consecutive simulation tests before starting the manufacturing process. Tryptic Soy Broth medium (TSB; Becton Dickinson, Madrid, Spain) was used to carry out simulation tests. Simulation tests were designed considering all steps of the process and emphasizing the worst-case scenarios, and were carried out with the same materials and equipment used to manufacture the MCB and WCB. During simulation tests, the presence of airborne microorganisms and particles was continuously monitored according to GMP standards. To verify the maintenance of the aseptic conditions during the simulation process, sampling points were set at the beginning and at the end of the process (negative control and simulation of the final product, respectively).

Environmental monitoring

A laser particle counter (CLiMET, Redlands, CA, USA) was used to monitor airborne particles in the cabinet (Grade A) during the manufacturing process and during simulation tests. According to the current GMP regulations, the maximum permitted airborne particle concentrations in Grade A areas are as follows: particle size equal or greater than 0.5 μm, 3520 particles per m^3; particle size equal or greater than 5 μm, 20 particles per m^3 [15].

Settle plates (Becton Dickinson) were used to monitor the presence of airborne microorganism (bacteria and fungi) in the cabinet (Grade A) during the manufacturing process as well as during the simulation tests. In addition, glove prints (finger dabs) of Grade A personnel were sampled for microbiological analysis (Becton Dickinson) at the end of each working day. In accordance with current GMP regulations, acceptance criteria of less than 1 colony forming units (> 1 CFU/gloves and > 1 CFU/plates) were established [15].

Animal origin-derived products and adventitious viruses risk assessment

In accordance with GMP regulations, the use of animal products in the manufacturing process should be avoided. Furthermore, the use of animal-derived substances must be controlled to avoid the transmission of pathogens to the final product. In this sense, the only animal-derived product used in this work was the Fetal Bovine Serum (FBS) (Thermo Fisher Scientific) of Australian origin, whose certificate of analysis indicates the absence of bluetongue virus, bovine adenovirus, bovine parvovirus, bovine respiratory syncytial virus, bovine viral diarrhea virus, hemadsorbing agents, rabies virus, reovirus, and spongiform encephalopathy virus. Given that FBS is the only animal-derived reagent used in the cell bank production process, it can be guaranteed that the manufacturing process is not introducing animal viruses into the final product. However, for greater safety, an adventitious virus test is carried out on the final product, as described below.

MCB and WCB process validation

One cryovial containing HEK293T cells was thawed and split into three HEK293T populations which were independently expanded in order to obtain three cell stocks for the MCB. Each HEK293T batch was expanded to reach a concentration of $200{\cdot}10^6$ cells. Every batch was composed of at least 20 cryotubes totaling ≥ 60 cryotubes for MCB. Quality control tests were carried out for every MCB batch. Three consecutive and independent batches of WCB were prepared from the MCB. HEK293T cells from the MCB were thawed and expanded to reach a concentration of $1000{\cdot}10^6$ cells. Every batch of the WCB was composed of at least 50 cryotubes totaling ≥ 150 cryotubes for WCB. Quality control tests were run for every WCB batch.

Sterility test and growth promotion test

Cell cultures and TSB media were subjected to a growth promotion test according to Chap. 2.6.1 of the European Pharmacopoeia to ensure that growth media met the proper sterility requirements.

Sterility tests on TSB, MCB, and WCB samples were performed by using the direct inoculation method described in Chap. 2.6.1 and 2.2.27 of the European Pharmacopoeia [16].

Mycoplasma test

Venor®GeM qOneStep kit (Minerva Biolabs®, Berlin, Germany) was used for the detection and identification of Mycoplasma in TSB, MCB, and WCB samples. The method is based on the amplification of the Mycoplasma 16S rRNA coding region in agreement with the method described in Chap. 2.6.7 of the European Pharmacopoeia [16].

Karyotype test

Karyotype was determined by G-band staining. HEK293T were seeded and incubated until 80% confluence was observed. 12 μl of KaryoMAX™ Colcemid™ (Thermo Fisher Scientific) was diluted in 6 ml of DMEM. Subsequently, 6 ml of KaryoMAX™ Colcemid™ working solution was added to the cell culture and incubated at 37 °C/5% CO_2 for an hour. Cell culture medium was removed, and cells were washed with 3 ml of PBS (Thermo Fisher Scientific). After removing PBS, 1 ml of TrypLE™ (Thermo Fisher Scientific) was added and cells were incubated for 1 minute at 37 °C. Then, 5 ml of culture medium was added, and cells were collected by centrifugation for 5 minutes at 1250 rpm. After removing the supernatant, the pellet was washed with PBS and centrifuged again for 5 minutes at 1250 rpm. 10 ml of pre-warmed (37 °C) 0.075M KaryoMAx™ KCl solution was gently added to the cells and, after 10 minutes of incubation at 37 °C, cells were fixed with Carnoy fixative solution.

Adventitious viruses test

Adventitious viruses testing is based on the analysis of cytopathic effects in host cells. HEK293T cell samples from MCB and WCB were disrupted by shaking with 0.2- and 3-mm stainless steel beads. Cell homogenate was centrifuged (1250 rpm, 5 min, 4 °C) and 0.9 ml of supernatant was added to MR5, VERO and RD cell cultures (Vircell, Granada, Spain) and incubated at 35–37 °C. Cell cultures were analyzed 14 days after inoculation. Positive and negative controls were included in the analysis. Real Time (RT)-PCR for *Enterovirus* identification was performed on samples with no cytopathic effects.

In-Process Controls (IPCs): cell morphology, cell confluence and viability

Cell morphology, cell confluence and cell viability were used as IPCs during the entire process of MCB and WCB generation. Morphological inspections of HEK293T cells and cell confluence were carried out visually under a microscope. Trypan blue exclusion test was carried out at every cell passage step to test cell viability. Samples with a viability < 80% were not used in the following production steps.

MCB/WCB stability after cryopreservation: viability and cell number

Trypan blue exclusion test was carried out on each batch of MCB and WCB to test cell viability after cryopreservation. To ensure the optimal cryopreservation conditions, the acceptance criteria of the viability test after thawing were set equal or greater than 50%.

A stability program has been carried out over a period of 37 months after WCB generation to determine the optimal storage conditions of cell banks. Trypan blue exclusion

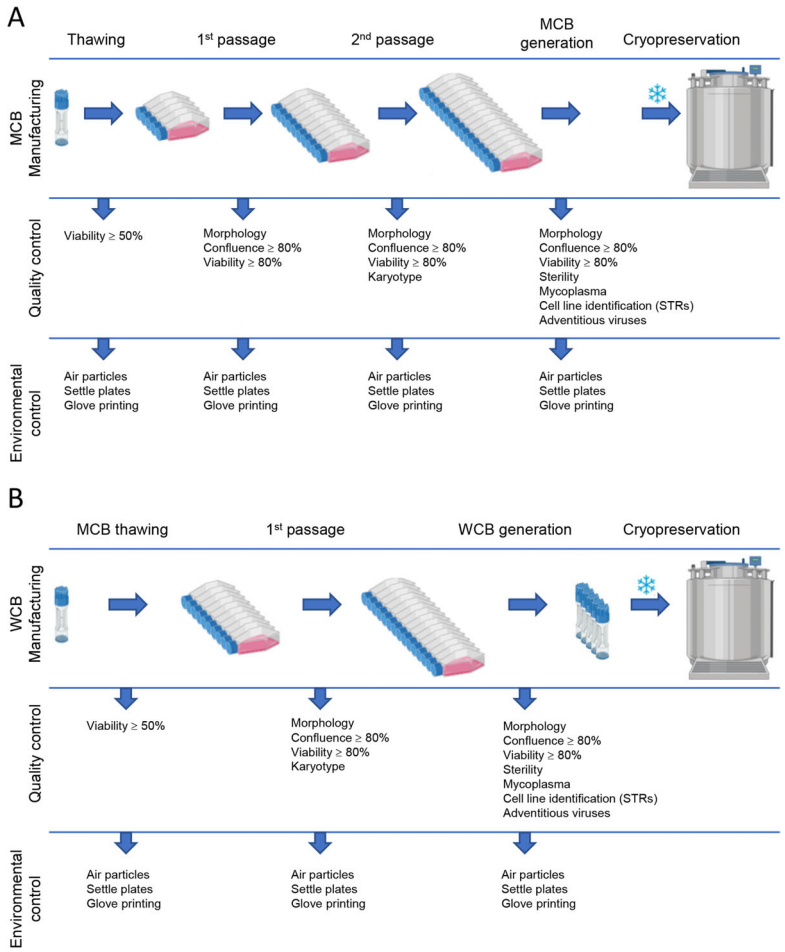


Fig. 2 a MCB workflow. MCB was composed of 3 independent batches containing 20 or more cryotubes. The HEK293T cell line cryovial was thawed and expanded until reaching $200{\cdot}10^6$ cells. Two cell passages were needed to obtain the final number of $200{\cdot}10^6$ cells. After the second passage, cells were transferred in cryotubes at a concentration of $10{\cdot}10^6$ cells per tube and frozen using a ramp program that lowers the temperature 1 °C/minute. MCB cryotubes were transferred to a gas phase nitrogen tank at -180 °C ± 20° C. WCB was composed of 3 independent batches containing 50 or more cryotubes. Environmental controls, IPCs and quality control test results are shown in the image. **b** WCB workflow. MCB HEK293T were thawed and expanded to reach $1000{\cdot}10^6$ cells. Only one cell passage was needed to obtain $1000{\cdot}10^6$ cells. Cells were transferred into cryotubes at a concentration of $20{\cdot}10^6$ cells per tube and frozen using a ramp program that lowers the temperature 1 °C/minute. WCB cryotubes were transferred to a gas phase nitrogen tank at -180 °C ± 20° (C) Environmental controls, IPCs and quality control test results are represented in the image

assay was used to determine cell viability and cell number of the WCB during the ongoing stability program.

Results

MCB process validation

MCB validation was performed as shown in Fig. 2a. Environmental controls and IPCs were carried out during the entire procedure of MCB generation (Table 1). No incidents were detected and, concerning MCB specifications, all batches met defined acceptance criteria (Table 2). Sterility tests indicated that all batches were negative for bacterial and fungal contaminations. All batches tested negative for Mycoplasma. No cytopathic effects were detected in any of the three batches indicating the absence of viruses in the final product. Morphological analysis by visual inspection showed adherent cells with the presence of thin extensions. Complex karyotypes were detected in all batches, in agreement with previous studies [17, 18]. Results showed a hypertriploid cell line with the following alterations: der (1) t (1;5) (p10;q10); der (1)t (1;15) (q42;q13)x2; dup (13)(q14q34); der (22) t (17;22) (q21;q13). These alterations were observed in the three MCB batches. Cell viability after thawing was over 90%, in line with the specifications. In general terms, the MCB met all defined acceptance criteria, thereby demonstrating that the process is robust and homogenous.

Table 1 Environmental parameters of MCB process validation. Table summarizing the environmental parameters obtained during the production of MCB batches. Airborne particles concentration in the sterile cabinet (Grade A) was measured at each step of the procedure. Particles equal or greater than 0.5 μm met defined acceptance criteria. Particles equal or greater than 5 μm met defined acceptance criteria. Glove prints of personnel operating in the cabinet (Grade A) were sampled at each step of the procedure. No microorganisms were detected in agreement with the acceptance criteria. The presence of airborne microorganisms in the cabinet (Grade A) was monitored at each step of the procedure by using settle plates. No microorganisms were detected on the plates, meeting defined acceptance criteria

Environmental Parameters	Culture Day	Batch code: MCB-HE-01	Batch code: MCB-HE-02	Batch code: MCB-HE-03
≥ 0.5 μm particles/m^3	HEK293T Thawing	Pass	Pass	Pass
	1st passage	Pass	Pass	Pass
	2nd passage	Pass	Pass	Pass
	MCB Generation	Pass	Pass	Pass
≥ 5 μm particles/m^3	HEK293T Thawing	Pass	Pass	Pass
	1st passage	Pass	Pass	Pass
	2nd passage	Pass	Pass	Pass
	MCB generation	Pass	Pass	Pass
Glove Print CFU	HEK293T Thawing	0	0	0
	1st passage	0	0	0
	2nd passage	0	0	0
	MCB generation	0	0	0
Settle plates CFU	HEK293T Thawing	0	0	0
	1st passage	0	0	0
	2nd passage	0	0	0
	MCB generation	0	0	0

WCB process validation

WCB production process validation was performed as shown in Fig. 2b. Environmental controls and IPCs were carried out during the entire process of WCB preparation (Table 3). No incidents were detected, and all batches met defined acceptance criteria. Regarding WCB specifications, all batches met the acceptance criteria (Table 4). Sterility tests indicated that all batches were negative for bacterial and fungal contaminations. All batches tested negative for Mycoplasma. Morphological analysis by visual inspection showed adherent cells with the presence of thin extensions. Complex karyotypes

Table 2 MCB specifications. Table summarizing results collected during MCB batch analyses. All MCB batches met defined acceptance criteria

Parameter	Method	Acceptance Criteria	MCB-HE-01	MCB-HE-02	MCB-HE-03
Morphology	**Visual Inspection**	**Adherent cells with thin extensions**	**Adherent cells with thin extensions**	**Adherent cells with thin extensions**	**Adherent cells with thin extensions**
Identification	PCR	Presence of: D5S818: 8/9 D13S317: 12/14 D7S820: 11 D16S539: 9/13 vWA: 16/18/19 TH01: 7/9/3 Amelogenin: X TPOX:11 CSF1PO: 11/12	8/9 12/14 11 9/13 16/18/19 7/9.3 X 11 11/12	8/9 12/14 11 9/13 16/18/19 7/9.3 X 11 11/12	8/9 12/14 11 9/13 16/18/19 7/9.3 X 11 11/12
Sterility	Eu. Ph 2.6.1	Sterile	Sterile	Sterile	Sterile
Mycoplasma	Eu. Ph 2.6.7	Absent	Absent	Absent	Absent
Karyotype	G-Band Staining	Informative	Complex	Complex	Complex
Adventitious viruses	Internal method	Absence of cytopathic effect	Absence	Absence	Absence
Viability after thawing	Trypan Blue Assay	≥ 50.0%	91.9%	96.9%	93.8%

Table 3 Environmental parameters of WCB validation. Table summarizing the environmental parameters collected during the analyses of WCB batches. The concentration of airborne particles in cabinet (Grade A) was measured at each step of the procedure. Particles equal or greater than 0.5 μm met defined acceptance criteria. Particles equal or greater than 5 μm met defined acceptance criteria. Glove prints of personnel operating in the cabinet (Grade A) were sampled at each step of the procedure. No microorganisms were detected, in line with the acceptance criteria. The presence of airborne microorganisms in the cabinet (Grade A) was monitored at each step of the procedure by using settle plates. No microorganisms were detected meeting defined acceptance criteria

Environmental Parameters	Culture Day	Batch code: MCB-HE-01	Batch code: MCB-HE-02	Batch code: MCB-HE-03
≥ 0.5 μm particles/m^3	MCB Thawing	Pass	Pass	Pass
	1st passage	Pass	Pass	Pass
	WCB Generation	Pass	Pass	Pass
≥ 5 μm particles/m^3	MCB Thawing	Pass	Pass	Pass
	1st passage	Pass	Pass	Pass
	WCB generation	Pass	Pass	Pass
Glove Print CFU	MCB Thawing	0	0	0
	1st passage	0	0	0
	WCB generation	0	0	0
Settle plates CFU	HEK293T Thawing	0	0	0
	1st passage	0	0	0
	WCB generation	0	0	0

were detected in all batches, in agreement with previous studies [17, 18]. Cell viability after thawing was over 90%, in line with the specifications. In general terms, WCB met defined acceptance criteria and the manufacturing process was robust and homogenous.

Simulation tests validation

Before starting the validation process, operating personnel was involved in the validation of aseptic processing conditions by reproducing all steps of MCB and WCB production with TSB medium. All samples from simulation batches remained sterile throughout the process (Table 5). During the simulation process, glove print and settle plate analyses demonstrated the absence of viable particles meeting the acceptance criteria for Grade A working areas (Table 5). These results indicate that the manufacturing staff was properly trained to carry out aseptic productions and were not source of contamination.

Table 4 WCB specifications. Table summarizing results obtained during the analyses of WCB batches. All WCB batches met the acceptance criteria

Parameter	Method	Acceptance Criteria	WCB-HE-01	WCB-HE-02	WCB-HE-03
Morphology	**Visual Inspection**	**Adherent cells with thin extensions**	**Adherent cells with thin extensions**	**Adherent cells with thin extensions**	**Adherent cells with thin extensions**
Identification	PCR	Presence of: D5S818: 8/9 D13S317: 12/14 D7S820: 11 D16S539: 9/13 vWA: 16/18/19 TH01: 7/9/3 Amelogenin: X TPOX:11 CSF1PO: 11/12	8/9 12/14 11 9/13 16/18/19 7/9.3 X 11 11/12	8/9 12/14 11 9/13 16/18/19 7/9.3 X 11 11/12	8/9 12/14 11 9/13 16/18/19 7/9.3 X 11 11/12
Sterility	Eu. Ph 2.6.1	Sterile	Sterile	Sterile	Sterile
Mycoplasma	Eu. Ph 2.6.7	Absent	Absent	Absent	Absent
Karyotype	G-Band Staining	Informative	Complex	Complex	Complex
Adventitious viruses	Internal method	Absence of cytopathic effect	Absence	Absence	Absence
Viability after thawing	Trypan Blue Assay	≥ 50.0%	97%	94.4%	96.4%

Table 5 Simulation tests results. Table summarizing results obtained with the three simulation test batches. Particles equal or greater than 0.5 μm and particles equal or greater than 5 μm in the cabinet (Grade A) met defined acceptance criteria. Gloves of the operating personnel and airborne microorganisms in cabinet (A grade) were monitored at each step of the simulation test. No microorganisms were detected in agreement with the acceptance criteria. Samples of the TSB, used throughout the simulation, were subjected to sterility tests

Parameter	Culture Day	Batch code: MF-HE-01	Batch code: MF-HE-02	Batch code: MF-HE-03
≥ 0.5 μm particles/m^3	1st entrance	Pass	Pass	Pass
	2nd entrance	Pass	Pass	Pass
	3rd entrance	Pass	Pass	Pass
	4th entrance	Pass	Pass	Pass
≥ 5 μm particles/m^3	1st entrance	Pass	Pass	Pass
	2nd entrance	Pass	Pass	Pass
	3rd entrance	Pass	Pass	Pass
	4th entrance	Pass	Pass	Pass
Glove Print CFU	1st entrance	0	0	0
	2nd entrance	0	0	0
	3rd entrance	0	0	0
	4th entrance	0	0	0
Settle plates CFU	1st entrance	0	0	0
	2nd entrance	0	0	0
	3rd entrance	0	0	0
	4th entrance	0	0	0
TSB sterility test	TSB used during the entire procedure	Sterile	Sterile	Sterile

HEK293T cell bank stability over time

Thawing tests of MCB and WCB batches showed an average cell viability of 94.2% and 95.3% respectively, indicating that the cryopreservation procedure did not affect cell viability. WCB cryovials were regularly thawed over a period of 37 months after cryopreservation to test cell viability. Over time, cell viability was always > 50%, meeting specifications (Fig. 3a and b), and cell number was > 10 million cells per batch (Fig. 3c and d). Altogether, these results confirmed the stability of the cell line after cryopreservation.

Discussion

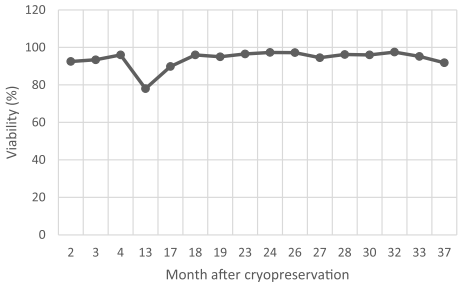
In the present work we demonstrated the feasibility, but also the potential issues, of generating MCB and WCB under current GMP regulations [15] in academic institutions. According to current regulations, it is crucial to first implement a risk-based assessment to evaluate every stage of the manufacturing process (cell culture conditions, cell passages, quality controls and IPCs requirements) as well as to evaluate the need of scaling up the procedure to reach a required cell number per batch. The main objective of the risk-based assessment is to detect those steps in the manufacturing procedure that may require mitigation actions to obtain homogeneous and robust products. In this regard, it is important to involve in these evaluations all key workers in the manufacturing process (i.e. quality control personnel, manufacturing personnel, quality assurance personnel and qualified persons). The present work shows the approach established at Creatio to validate the production of GMP-compliant MCB and WCB of the HEK293T cell line which are currently being used as packaging cells for GMP-grade lentivirus manufacturing in several CAR T-cell clinical trials [11, 19].

When a basic research protocol is applied to clinical studies, the quality of the reagents and starting materials has to be changed. Frequently, new ATMPs developed in academic institutions with research-grade reagents and non-tested starting materials do not achieve the minimum quality standards required to start the manufacturing step. The HEK293T are widely used in research as packaging cells for different viral-vector production systems such as adeno-associated viruses (AAVs), retroviruses, adenoviruses (AD) or lentiviruses [20]. For this reason, before using any packaging cell line such as HEK293T, it is mandatory to analyze its quality parameters and attributes. In this regard, although according to Chap. 8 of current GMP for ATMP manufacturing the generation of cell banks is not mandatory, [15] it is highly recommended when they are used as starting materials in manufacturing processes,

A

Month after cryopreservation	Cell Viability (%)
2	92.5
3	93.4
4	96
13	78
17	89.8
18	96
19	95
23	96.5
24	97.3
26	97.2
27	94.5
28	96.2
30	96
32	97.5
33	95.2
37	91.8

B



C

Month after cryopreservation	Cell number/cryotube
2	1.75E+07
3	1.40E+07
4	1.45E+07
13	1.10E+07
17	1.88E+07
18	1.88E+07
19	1.90E+07
23	1.40E+07
24	1.85E+07
26	1.83E+07
27	2.63E+07
28	1.90E+07
30	1.80E+07
32	1.98E+07
33	1.56+E07
37	1.38E+07

D

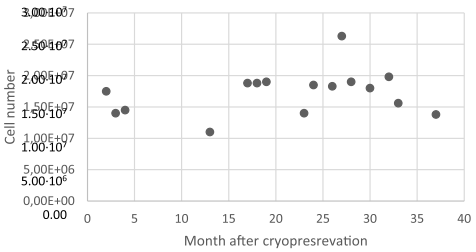


Fig. 3 Stability program of the WCB. Table (**a**) and graph (**b**) showing cell viability after thawing of WCB over a period of 37 months after cryopreservation. Table (**c**) and graph (**d**) showing cell number after thawing of WCB over a period of 37 months after cryopreservation

since this guarantees the product robustness and homogeneity.

We analyzed the sterility of three independent batches from MCB and WCB. The results showed that all the tested batches of HEK293T MCB and WCB were sterile. Due to the nature of ATMPs, which are considered as live products, sterility must be kept throughout the manufacturing process. Accordingly, cleanrooms must be validated to guarantee the adequate environment in terms of pressure, temperature and humidity conditions as well as of viable and non-viable particles contents. In addition, a personnel training program, which includes the simulation of the aseptic process, should be established in every pharmaceutical quality system because workers participating in the manufacturing process are considered the main source of contamination [15]. Creatio's personnel involved in MCB and WCB generation was successfully trained and validated to carry out the processes under current GMP regulations. Importantly, a cleaning validation of the Creatio facility was previously performed to guarantee and keep record of the cleaning procedures, considered as a critical step. Another critical aspect is the presence of Mycoplasma in the raw materials used for ATMPs manufacturing. For this reason, Mycoplasma test is a mandatory quality control test. According to the current GMP regulation for ATMPs manufacturing, [15] the presence of Mycoplasma in raw materials must be evaluated before starting the manufacturing process under a risk-based approach. The original HEK293T stock and the three independent batches of MCB and WCB were free of Mycoplasma. In terms of safety, MCB and WCB must be also free of adventitious viruses. No cytopathic effects were detected in any batch analyzed, so we can guarantee that raw materials such as FBS or HEK293T cells used in the manufacturing process were not a source of adventitious viruses.

Even though the HEK293T cell line will not be used as a final product, it is necessary to characterize the identity of this line to ensure its biological properties such as

growth rates and packaging functions. In this sense, the presence of specific STRs in MCB and WCB cells coincides with those reported in the certificate of analysis of the original HEK293T line. Thus, no alterations of the STRs Amelogenin: X; CSF1PO: 11, 12; D13S317: 12, 14; D16S539: 9, 13; D5S818: 8, 9; D7S820: 11; THO1: 7, 9.3; TPOX: 11; vWA: 16, 18, 19 were detected after the passages performed during the cell banking processes. Moreover, we also analyzed the karyotype to detect any alterations caused by cell manipulation and cell amplification. The HEK293T line has a complex karyotype which remains constant between batches. HEK293T MCB and WCB showed a hypertriploid karyotype with numerous aberrant chromosomic alterations such as duplication and chromosomic derivation, as described elsewhere [17, 18]. These alterations are mainly due to the genetic instability of this cell line. For this reason, karyotype testing results are only indicative and do not need to meet specific acceptance criteria. Nonetheless, we consider mandatory reporting and documenting karyotype information in order to correlate possible alterations in viral vectors packaging yield with chromosomic alterations.

Other crucial aspects of cell banking are cryopreservation and storage conditions [21, 22]. The cryopreservation of HEK293T MCB and WCB was obtained using a freezing ramp of 1 °C/min. Cell viability after thawing was over 90% indicating that cryopreservation procedure did not affect the quality of the product. However, different freezing ramps and cryopreservation media should be evaluated when the banking of new cell lines is planned [23]. Creatio's cryopreservation tanks use a gas phase liquid nitrogen system that reduces cross-contaminations and ensure a more homogeneous temperature than those using liquid phase nitrogen systems. By using this system, cryopreserved HEK293T cells displayed a long-term stability > 37 months. Taken together these data show that cell banking of HEK293T offers an adequate approach to generate homogeneous cellular products and GMP-grade MCB and WCB banks with a long-lasting stability which can help researchers to design ATMPs manufacturing protocols.

The present approach indicates the minimal requirements academic institutions should meet when using non-GMP cell lines as starting material in a manufacturing process. Although the present work focuses on the banking of an adherent cell line, it would be equally possible to establish MCB/WCB of suspension cells following the same strategy [24–26]. The development of new technologies for cell therapy products involving adherent or suspension cell culture is currently underway. Bioreactors are emerging as closed systems which reduce reagent consumption and handling time [27, 28]. In this sense, the present procedure can be applied to generate cell banks in bioreactor systems. MCBs and WCBs have been successfully used in different ATMP manufacturing processes including, among others, mesenchymal stem cells, [29, 30] fibroblasts, [31] induced pluripotent stem cells, or embryonic stem cells [32, 33] which have been used as a final product ready for administration. We would like to highlight the importance of GMP-compliant cell banking for ATMP manufacturing and translational research.

Conclusions

The manufacturing of a vast majority of ATMPs requires the use of specific cell lines to generate cell or gene therapy delivery products such as lentivirus- or adenovirus-based vectors. In this sense, cell banking is a powerful tool to generate and preserve high-quality cell lines. Most of the advancements on ATMPs are usually generated in academic contexts, therefore it is crucial for researchers to be aware of the regulations applying to ATMP manufacturing processes. However, academia seems unaware about the most common regulatory frameworks governing ATMPs manufacturing in pharmaceutical industry. This work presents a GMP-compliant approach for the cell banking of HEK293T cells in an academic context. Our work may help academic scientists understand the rationale behind manufacturing process design and quality control testing for the generation of cell banks, an approach that can be adapted to other manufacturing processes.

Abbreviations

AAVs: Adeno-Associated Viruses.; ADA-SCID: Adenosine Deaminase Deficiency.; ALL: Acute Lymphoblastic Leukemia.; ATMPs: Advanced Therapy Medicinal Products.; BCMA: B Cell Maturation Antigen.; CAR: Chimeric Antigen Receptor.; CD: Cluster of Differentiation.; DMSO: Dimethyl Sulfoxide.; EMA: European Medicines Agency; FBS: Fetal Bovine Serum; FDA: Food and Drug Administration; GMP: Good Manufacturing Practices; HBB: Hemoglobin Subunit Beta; HEK: Human Embryonic Kidney; IPCs: In-Process Controls; MCB: Master Cell Bank; PBS: Phosphate-Buffered Saline; PCR: Polymerase Chain Reaction; rRNA: Ribosomal Ribonucleic Acid; SMA: Spinal Muscular Atrophy; SMN1: Survival Motor Neuron 1; STRs: Short Tandem Repeats; TSB: Tryptic Soy Broth; WCB: Working Cell Bank

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Declarations of interest

None.

Authors' contributions

UP and RM designed the experiments. CH and VM carried out the validation of manufacturing process. GO, EG, MO carried out Quality Control tests. MC, GS, AB carried out the stability tests. FC and UP reviewed validations and GMP documentation. AU, JD, MJ critically read the manuscript. JMC coordinated and supervised the study. The author(s) read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate
Not Applicable.

Consent for publication
Not Applicable.

Competing interests
Authors declare no competing interests.

Author details
[1]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Laboratory of Stem Cells and Regenerative Medicine, Institute of Neurosciences, University of Barcelona, C/ Casanova 143, 08036 Barcelona, Spain. [2]Production and Validation Center of Advanced Therapies (Creatio), Faculty of Medicine and Health Sciences, University of Barcelona, Barcelona, Spain. [3]Networked Biomedical Research Centre for Neurodegenerative Disorders (CIBERNED), Barcelona, Spain. [4]August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Barcelona, Spain. [5]Present Address: Cedars-Sinai Biomanufacturing Center , Regenerative Medicine Institute , Los Angeles, USA. [6]Department of Immunology, Centro de Diagnóstico Biomédico, Hospital Clínic de Barcelona, Barcelona, Spain. [7]Department of Hematology, Institut Clínic de Malalties Hematològiques i Oncològiques, Hospital Clínic de Barcelona, Barcelona, Spain. [8]Present Address: Immunotherapy Unit. Banc de Sang i de Teixits , Barcelona, Spain. [9]Faculty of Medicine and Health Science, University of Barcelona, Barcelona, Spain. [10]Josep Carreras Leukemia Research Institute, Barcelona, Spain. [11]Centro de Investigación Biomedica en Red de Cancer (CIBERONC), Barcelona, Spain. [12]Institut de Recerca Pediàtrica Hospital Sant Joan de Déu, Universidad de Barcelona, Esplugues de Llobregat, Spain.

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References
1. Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use. Official Journal of the European Communities, ISSN 0378-6978, L 311, Volume 44, 28 November 2001.
2. Comission E. Regulation. EC N 1394/2007 of the European Parliament and of The Council of 13 Nivember 2007 on advanced therapies medicinal products 2007.
3. Avivar-Valderas A, Martín-Martín C, Ramírez C, Del Río B, Menta R, Mancheño-Corvo P, et al. Dissecting allo-sensitization after local administration of human allogeneic adipose mesenchymal stem cells in perianal fistulas of Crohn's disease patients. Front Immunol 2019;10. https://doi.org/10.3389/fimmu.2019.01244.
4. Pellegrini G, Ardigò D, Milazzo G, Iotti G, Guatelli P, Pelosi D, et al. Navigating Market Authorization: The Path Holoclar Took to Become the First Stem Cell Product Approved in the European Union. Stem Cells Transl Med. 2018;7:146–54. https://doi.org/10.1002/sctm.17-0003.
5. Ginn SL, Amaya AK, Alexander IE, Edelstein M, Abedi MR. Gene therapy clinical trials worldwide to 2017: An update. J Gene Med 2018;20. https://doi.org/10.1002/jgm.3015.
6. What Is ZOLGENSMA® (onasemnogene abeparvovec-xioi) n.d. https://www.zolgensma.com/what-is-zolgensma (Accessed 4 May 2020).
7. Al-Zaidy SA, Mendell JR. From Clinical Trials to Clinical Practice: Practical Considerations for Gene Replacement Therapy in SMA Type 1. Pediatr Neurol. 2019;100:3–11. https://doi.org/10.1016/j.pediatrneurol.2019.06.007.
8. Schuessler-Lenz M, Enzmann H, Vamvakas S. Regulators' Advice Can Make a Difference: European Medicines Agency Approval of Zynteglo for Beta Thalassemia. Clin Pharmacol Ther. 2020;107:492–4. https://doi.org/10.1002/cpt.1639.
9. Novartis N, Portfolio G, Data F. Novartis Receives First Ever FDA Approval for a CAR-T Cell Therapy, Kymriah (TM)(CTL019), for Children and Young Adults with B-cell ALL That Is Eefractory 2017.
10. Porter DL, Hwang WT, Frey NV, Lacey SF, Shaw PA, Loren AW, et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. Sci Transl Med 2015;7. https://doi.org/10.1126/scitranslmed.aac5415.
11. Castella M, Boronat A, Martín-Ibáñez R, Rodríguez V, Suñé G, Caballero M, et al. Development of a Novel Anti-CD19 Chimeric Antigen Receptor: A Paradigm for an Affordable CAR T Cell Production at Academic Institutions. Mol Ther - Methods Clin Dev 2019. https://doi.org/10.1016/j.omtm.2018.11.010.
12. Brudno JN, Maric I, Hartman SD, Rose JJ, Wang M, Lam N, et al. T cells genetically modified to express an anti–B-Cell maturation antigen chimeric antigen receptor cause remissions of poor-prognosis relapsed multiple myeloma. J Clin Oncol. 2018;36:2267–80. https://doi.org/10.1200/JCO.2018.77.8084.
13. Ramos CA, Ballard B, Zhang H, Dakhova O, Gee AP, Mei Z, et al. Clinical and immunological responses after CD30-specific chimeric antigen receptor-redirected lymphocytes. J Clin Invest. 2017;127:3462–71. https://doi.org/10.1172/JCI94306.
14. Meij P, Canals J, Lowery M, Scott M. Advanced Therapy Medicinal Products. LERU publications, Briefing paper No. 3, September 2019.
15. The Rules Governing Medicinal Products in the European Union. Good Manufacturing Practice guidelines, 2015, Volume 4, EudraLex, European Commission.
16. European Pharmacopoeia. (Ph. Eur.) 10th Edition | EDQM - European Directorate for the Quality of Medicines n.d. https://www.edqm.eu/en/european_pharmacopoeia_10th_edition (Accessed 4 May 2020).
17. Stepanenko AA, Dmitrenko VV. HEK293 in cell biology and cancer research: Phenotype, karyotype, tumorigenicity, and stress-induced genome-phenotype evolution. Gene. 2015;569:182–90. https://doi.org/10.1016/j.gene.2015.05.065.
18. Lin YC, Boone M, Meuris L, Lemmens I, Van Roy N, Soete A, et al. Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. Nat Commun 2014;5. https://doi.org/10.1038/ncomms5767.
19. Castella M, Caballero-Baños M, Ortiz-Maldonado V, González-Navarro EA, Suñé G, Antoñana-Vidósola A, et al. Point-Of-Care CAR T-Cell Production (ARI-0001) Using a Closed Semi-automatic Bioreactor: Experience From an Academic Phase I Clinical Trial. Front Immunol. 2020;11:482. https://doi.org/10.3389/FIMMU.2020.00482.
20. Van Der Loo JCM, Wright JF. Progress and challenges in viral vector manufacturing 2015. https://doi.org/10.1093/hmg/ddv451.
21. Li Y, Ma T. Bioprocessing of cryopreservation for large-scale banking of human pluripotent stem cells. Biores Open Access. 2012;1:205–14. https://doi.org/10.1089/biores.2012.0224.
22. Shafa M, Walsh T, Panchalingam KM, Richardson T, Menendez L, Tian X, et al. Long-term stability and differentiation potential of cryopreserved cGMP-compliant human induced pluripotent stem cells. Int J Mol Sci 2020;21. https://doi.org/10.3390/ijms21010108.
23. Martn-Ibez R, Hovatta O, M. J. Cryopreservation of Human Pluripotent Stem Cells: Are We Going in the Right Direction? Curr. Front. Cryobiol., InTech; 2012. https://doi.org/10.5772/34853.
24. Olmer R, Lange A, Selzer S, Kasper C, Haverich A, Martin U, et al. Suspension culture of human pluripotent stem cells in controlled, stirred bioreactors. Tissue Eng - Part C Methods. 2012;18:772–84. https://doi.org/10.1089/ten.tec.2011.0717.
25. Zweigerdt R, Olmer R, Singh H, Haverich A, Martin U. Scalable expansion of human pluripotent stem cells in suspension culture. Nat Protoc. 2011;6:689–700. https://doi.org/10.1038/nprot.2011.318.
26. Kwok CK, Ueda Y, Kadari A, Günther K, Ergün S, Heron A, et al. Scalable stirred suspension culture for the generation of billions of human induced pluripotent stem cells using single-use bioreactors. J Tissue Eng Regen Med. 2018;12:e1076–87. https://doi.org/10.1002/term.2435.
27. Valkama AJ, Leinonen HM, Lipponen EM, Turkki V, Malinen J, Heikura T, et al. Optimization of lentiviral vector production for scale-up in fixed-bed bioreactor. Gene Ther. 2018;25:39–46. https://doi.org/10.1038/gt.2017.91.
28. Leinonen HM, Lipponen EM, Valkama AJ, Hynynen H, Oruetxebarria I, Turkki V, et al. Preclinical Proof-of-Concept, Analytical Development, and Commercial Scale Production of Lentiviral Vector in Adherent Cells. Mol Ther - Methods Clin Dev. 2019;15:63–71. https://doi.org/10.1016/j.omtm.2019.08.006.

29. Lechanteur C, Briquet A, Giet O, Delloye O, Baudoux E, Beguin Y. Clinical-scale expansion of mesenchymal stromal cells: A large banking experience. J Transl Med. 2016;14:145. https://doi.org/10.1186/s12967-016-0892-y.
30. Cooper K, Viswanathan C. Establishment of a Mesenchymal Stem Cell Bank. Stem Cells Int 2011;2011. https://doi.org/10.4061/2011/905621.
31. Prathalingam N, Ferguson L, Young L, Lietz G, Oldershaw R, Healy L, et al. Production and validation of a good manufacturing practice grade human fibroblast line for supporting human embryonic stem cell derivation and culture. Stem Cell Res Ther 2012;3. https://doi.org/10.1186/scrt103.
32. Devito L, Petrova A, Miere C, Codognotto S, Blakely N, Lovatt A, et al. Cost-Effective Master Cell Bank Validation of Multiple Clinical-Grade Human Pluripotent Stem Cell Lines From a Single Donor. Stem Cells Transl Med. 2014;3:1116–24. https://doi.org/10.5966/sctm.2014-0015.
33. Shafa M, Yang F, Fellner T, Rao MS, Baghbaderani BA. Human-induced pluripotent stem cells manufactured using a current good manufacturing practice-compliant process differentiate into clinically relevant cells from three germ layers. Front Med. 2018;5:69. https://doi.org/10.3389/fmed.2018.00069.

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