DOI: 10.46765/2675-374X.2022v3n1p156

ADVANCED CELL THERAPY PRODUCT RELEASE CONTAINING CAR-T CELLS

Juliana Aparecida Preto de Godoy[1], Lucila Nassif Kerbauy[1], Raquel de Melo Alves Paiva[1], Andrea Tiemi Kondo[1], Oswaldo Keith Okamoto[2], José Mauro Kutner[1]

1 Department of Hemotherapy and Cell Therapy, Hospital Israelita Albert Einstein, São Paulo, SP, Brazil.
2 Human Genome and Stem Cell Research Center, Department of Genetics and Evolutionary Biology, Biosciences Institute, University of São Paulo (USP), Sao Paulo, Brazil.

Correspondence to: ju.pgodoy@gmail.com

ABSTRACT

Immunotherapy consisting of genetic modification of T cells such as CAR-T cells need to address several tests and exams attesting the quality of the advanced therapy product. These tests are based on the Resolution of the Collegiate Board (RDC – Resolução da Diretoria Colegiada - Anvisa) number 508 of May 27, 2021 that guide the necessary release tests of advanced cells/gene therapy for use in humans. These tests include the total count number of the cells, identity of cells populations, cell viability, purity test, aseptic tests, cytogenetics, potency test and nucleid acid detection for some virus in case of allogenic use and for gene therapy product, tests carrying out identity, integrity, purity and potency of the vector used for the genetic modification. The final release of the advanced cell/gene therapy product should attest the safety and characteristics of the cells.

Keywords: Immunotherapy, Adoptive.

AIMS

Describe the release tests required after the manufacturing of CAR-T cells in accordance with current regulations.

INTRODUCTION

Manufacturing of customized gene or cell therapy products such as CAR-T cells is complex and depends on release tests andexams that can attest to a consistent quality standard for each product. The quality of CAR-T cell products is subject to donor variation, but also includes the manufacturing environment, as well as the quality and availability of materials and reagents. Quality must be carefully monitored and integrated into the manufacturing process.

Risk-based approach should guide the advanced cell therapy product manufacturing process so that quality assurance is achieved[1] (EMA/CAT/CPWP/686637/2011). Unintentional variability in cell cultures such as differences in starting material, vector conditions and concentration, transduction efficiency, multiplicity of infection (MOI) can result in quantitative and/or qualitative differences in product quality.

According to Resolution of the Collegiate Board number 508 of May 27, 2021[2], the release of advanced cells/gene therapy for use in humans must follow the Good Practices in Human Cells for therapeutic, as summarized in the table below:

TABLE 1 - Release Tests on a Sample of the Advanced Cell/Gene Therapy Final Product

Release Tests on a Sample of the Advanced Cell/Gene Therapy Final Product
a. total count of relevant cells
b. identity testing or appropriate phenotyping for the product and quantification of cell populations present
c. cell viability
d. purity test
e. microbiological tests
f. cytogenetics
g. potency test
h. nucleic acid detection of CMV, HIV-1 and HIV-2, HTLV-I and HTLV-II, EBV, HBV, HCV and B19 viruses in case of allogeneic use
i. Gene Therapy: carrying out tests of identity, integrity, purity and potency, related to the stem cell line and vector.

ADVANCED THERAPY PRODUCT RELEASE TESTS

According to the Good Manufacturing Practices (GMP) regulations, quality is incorporated into the design of the manufacturing process[3]. A carefully list of release tests is required to provide adequate evidence of identity, safety, purity, potency and cytogenetics. The identity of CAR-T cell products is commonly characterized by surface expression of CAR but it also could be assessed by qPCR[4,5]. Safety is related specially to the absence of any possible contamination such as endotoxin, mycoplasma, microorganisms and, also to the lack of lentiviruses replicating[4,5]. Purity is specified by number of viable T cells and, also' CD3+ and CAR-T+ cells; regarding purity, a panel could be done in flow cytometry to identify the populations within the final cell therapy product. Impurities may be present in CAR-T cell product and it could be evaluated by observing under the microscopy for residual possible magnetic beads; CD19+ B cells could be determined by flow cytometry and expressed by % of unwanted cells. Potency of CAR-T cells could be determined by in vitro cytotoxicity assay or cytokines secretion, such as interferon-γ, when CAR-T cells are cultured with cells expressing the target such as CD194,[5]. Table 2 summarizes examples of release assays for CAR-T cells using different genetic modifications.

TABLE 2 - Quality Controlo f CAR-T cells product[4,5].

Advanced Therapy Product Release Tests			
Safety	Genetic modification by retroviral or lentiviral vector	Genetic modification by transposon	Genetic modification by electroporation (mRNA)
	Gram stain/microbiologicals	Gram stain/microbiologicals	Gram stain/microbiologicals
	Mycoplasma by qPCR	Mycoplasma by qPCR	Mycoplasma by qPCR
	Endotoxin quantification	Endotoxin quantification	Endotoxin quantification
	Determination of VSV-G DNA by qPCR		
Purity	% viable T cells	% viable T cells	% viable T cells
	% CD3+ T cells	% CD3+ T cells	% CD3+ T cells
	% CAR-T cells	% CAR-T cells	% CAR-T cells
Identity	% CAR-T cells – by PCR or flow cytometry		
cytogenetics	T lymphocyte karyotype		
Potency	in vitro cytotoxicity assays or interferon-γ release in response to cells expressing the target molecule		
Quantity	Number of viable cells and calculation of the dose		

SAFETY

- Gram staining/microbiological assays

Microbiological tests are performed at determined key points in the manufacturing process; the method and timing of testing will provide assurance of sterility of the advanced therapy product. If the final product is cryopreserved, the tests must be performed before freezing process so that all results will be released at the time of infusion in the patient; however, if there is any type of manipulation of this product after thawing before infusion such as washing, there will be a need to repeat the microbiological tests or to perform a rapid test before infusion and follow-up of the standard microbiological culture.

If the product must be administered right after manufacturing (fresh infusion) before the results of microbiological tests are ready, there is a need for validation of an additional test that guarantees the sterility of the product. Some regulatory agencies, such as the Food and Drug Administration (FDA), recommend certain tests:

- Microbiological test on a sample 48 to 72 hours before the end of the manufacturing process;
- A rapid microbial detection test such as the Gram stain;
- 21 CFR 610.12 compliant sterility test (sterility) in the final product formulation6.

In this way, the advanced therapy product would be released based on the results described above; the culture of the final product must be continued until 14 days of incubation even after the product has been administered to the patient, and a final result, without growth of microorganisms, will confirm that the aseptic technique was maintained. If there is a positive result, an investigation should be carried out to determine the cause of the sterility failure.

The principal investigator should evaluate the patient for any signs of infection that may be related to the product. If the patient has any serious adverse reaction, which may be due to the failure of sterility of the advanced therapy product, a report must be sent to the health surveillance.

According to Brazilian Health Surveillance (Anvisa), The Cell Processing Center must have mechanisms to identify, investigate and execute corrective and preventive actions related to Technical Complaints and Adverse Events.

MYCOPLASMA

Mycoplasma contamination can happen from 2 main sources: the serum of animal or human origin and the facilities where the cells are cultivated in an open system, whose contamination can come from the operator of the process[7]. Performing the mycoplasma test on the final product is essential to detect possible contamination.

The PCR test for the detection of mycoplasma species is the most recommended, but rapid detection assays can be performed since providing that they demonstrate adequate sensitivity and specificity.

- Endotoxin

The endotoxin test detects a lipopolysaccharide in the cell membrane of gram-negative bacteria; endotoxin is released in the environment after bacterial cells death and it can cause troubling effects such as fever, septic shock and, even death[8]. According to the Brazilian Pharmacopoeia section 5.5.2.2, bacterial endotoxin test is used to detect or quantify endotoxins from gram negative bacteria[9]. The aqueous extract of circulating amebocytes from Limulus polyphemus or Tachypleus tridentatus is used.

There are two techniques with different sensitivity for this test:

- Gel Coagulation Method: based on clot or gel formation (semi-quantitative method);
- Photometric Methods: that is a quantitative method and could be divided in 2 types - Turbidimetric Method or Chromogenic Method.

The validation of the chosen method should be performed and some parameters such as maximum valid dilution as well as potential inhibitors factors must be carried out.

- Number of copies of the transgene/lentivirus replication

Replication Competent Virus (RCV) testing is performed to confirm the absence of RCV (using validated and sensitive assays) in the starting material after use of the viral vector and to exclude RCV formation during the manufacturing of genetically modified cells. In this case, a risk assessment must be submitted to address the potential generation of RCVs during the manufacturing process. Whenever possible, retention samples should be stored for future analysis.

FDA has guidelines recommendations regarding the replication competent virus (Testing of Retrovi-

ral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up); according to these guidelines, the possible pathogenicity of the retroviruses must be evaluated to monitor replicating particles in the final product10. When manufacturing cells to be transduced using retroviruses, agency recommends the test to be done in the cells including the ones cultivated for 4 days or less.

In ex vivo genetic modification of cells, the RCV in the vector should be at minimum level, however, the manufacturing procedure can provide a favorable environment for RCV amplification so the test should be performed for each lot of cells regardless the lenght if time the cells were cultivated. This chapter also presents some assays for RCV analysis.

Regarding patient monitoring, FDA recommends that samples from the patient should be analyzed at some specific time-points: pre-treatment, testing after 3, 6 and 12-months after the gene therapy, and yearly for up to 15 years.

- Purity

Purity of advanced cell therapy is related to cell type and transduction efficiency; purity must be defined considering the nature and use of its production consistency, the production method and also the degree of production process. Purity criteria must be determined and be within specified limits.

When a viral vector is used for transduction, the level of replicating particles in the final product must be determined and kept below a justified threshold. When using transposon vectors, it must be shown that the final cell population is free of transposase activity.

In the case of genome editing, the persistence of gene editing tools in cells must be assessed; ideally, they are no longer present when the cells are released for clinical use.

- Identity

Flow cytometry is one of the most used techniques to identify cells and protein biomarkers. Detection is performed on cell samples from an incident laser beam that allows measurement of the scattering and fluorescence of the reflected laser. In this way, it is possible to obtain fast and accurate information with the identification of numerous intrinsic and extrinsic characteristics contained in the cells, accurately recognize the size and granularity by reading the intensity of the fluorescence reflected in cells previously stained with fluorescent antibodies.

In this context, flow cytometry fits as a technique used for the purpose of releasing advanced therapy product from CAR-T cells. On the day of product release, two immunophenotyping panels are required for accurate detection and quantification of cells present in the final product. Panel A has markers that allow the evaluation of all immune components present in the final composition of the product, while panel B has markers for the quantification of positive CAR cells (Table 3), allowing a reliable calculation of the dose to be infused in the patient.

TABLE 3 - Immunophenotypic profile of CAR T cells

Panel A	**Panel B**
Immune Composition Profile	**Transduction Profile**
CD45+	CD45+
CD4+	CD4+
CD8+	CD8+
CD3+	CD3+
CD56/CD16	CAR+
CD19	7-AAD
7-AAD	

- Cytogenetics

According to RDC No. 508/2021, performing cytogenetics is mandatory in case of extensive manipulation and applies to the release of the CAR-T cells product. In this context, the G-band karyotype is performed to detect clonal and non-clonal changes in each sample[2]. Ten metaphases are analyzed and, in case of alteration, a total of 20 metaphases must be evaluated. If confirmation of the change occurs, a FISH analysis (fluorescence in situ hybridization) is requested for safer detection of any possible change.

- Potency

Potency of genetically modified cells must be assessed to determine the functionality of the cells; this test should provide quantitative information about the function of the cells and the transgene product. Whenever possible, a reference lot of cells with assigned potency should be established and used to calibrate tests[11].

Potency testing should not be limited to cell functionality but also include other relevant tests such as cell viability. Potency test for products containing genetically modified T cells against tumor cells is preferably based on the cytotoxic potential of the T cells. Tests on the potential of CAR-T cells can be performed with the analysis of the release of cytokines and/or cytotoxic molecules or the expression of T cells activation markers, providing the data of tumor cells death[12].

- Advanced Therapy Product Release Template

General Information	
Grant:	Patient ID:
Patient weight: kg	Dose:
Cultivation data	
Culture Start date: / / /	Duration of cultivation: days
Transduction Percentage: %	Cell Count Starting Material:
Cell Count Final Product:	CART Cell Count:
Cell Viability:	
Results Exams Release	
Karyotype	Normal ( ) Alteration ( ) Type de alteration:
Immunophenotype	CD3+ ( ) CD4+ ( ) CD8+ ( ) CD45+ ( )
Microbiological analysis	Negative ( ) Positive ( )
Mycoplasma Analysis	Negative ( ) Positive ( )
Endotoxin Analysis	Negative ( ) Positive ( ) _____EU/mL
Potency Test	% Citotoxicity _____
Product release	
Product: Released ( ) Conditionally Released ( ) Blocked ( )	
checked by: _____ Date ____/____/____	
Released by: _____ Date ____/____/____	

- Frequency of training or competence assessment

Initial training for all employees involved in the process of releasing the product containing CAR-T cells. Annual retraining required.

- Critical Points and Risks

Critical Points:

- Refinement of acceptability criteria for effective detection of positive or negative from advanced therapy product release tests;
- Preventive maintenance of equipment used in the release of advanced therapy products;
- Validation and qualification of reagents used in advanced therapy product release assays.

Risks:

- Associated with the type of vector used;
- False positive or false negative results.

REFERENCES

1. European Medicines Agency. Guideline on the risk-based approach according to annex I, part IV of Directive 2001/83/EC applied to Advanced therapy medicinal products [Internet]. [s.l.]; 2013 [cited 2021 Dec 13]. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-risk-based-approach-according-annex-i-part-iv-directive-2001/83/ec-applied-advanced-therapy-medicinal-products_en.pdf

2. ANVISA. Resolução RDC 508/2021 – dispõe sobre as Boas Práticas em Células Humanas para Uso Terapêutico e pesquisa clínica, e dá outras providências [Internet]. Brasília; 2021 [cited 2021 Dec 13]. Available from: https://www.in.gov.br/en/web/dou/-/resolucao-rdc-n-508-de-27-de-maio-de-2021-323013606

3. Rathore AS, Winkle H. Quality by design for biopharmaceuticals. Nat Biotechnol. 2009;27(1):26-34.

4. Tyagarajan S, Spencer T, Smith J. Optimizing CAR-T Cell Manufacturing Processes during Pivotal Clinical Trials. Mol Ther Methods Clin Dev. 2020;16:136-44.

5. Wang X, Rivière I. Clinical manufacturing of CAR T cells: foundation of a promising therapy. Mol Ther Oncolytics. 2016;3:16015.

6. FDA. § 610.12 Sterility [Internet]. Silver Spring; 2021 [cited 2021 Dec 13]. Available from: https://www.govinfo.gov/content/pkg/CFR-2011-title21-vol7/pdf/CFR-2011-title21-vol7-sec610-12.pdf

7. Nikfarjam L, Farzaneh P. Prevention and detection of Mycoplasma contamination in cell culture. Cell J. 2012;13(4):203-12.

8. Su W, Ding X. Methods of Endotoxin Detection. J Lab Autom. 2015;20(4):354-64.

9. ANVISA. Farmacopeia Brasileira: Volume 1 [Internet]. Brasília; 2010 [cited 2021 Dec 13]. Available from: https://www.gov.br/agricultura/pt-br/assuntos/inspecao/produtos-vegetal/legislacao-1/biblioteca-de-normas-vinhos-e-bebidas/farmacopeia_volume-1_2010.pdf

10. FDA. Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up: Guidance for Industry [Internet]. Silver Spring; 2020 [cited 2021 Dec 13]. Available from: https://www.fda.gov/media/113790/download

11. Levine BL, Miskin J, Wonnacott K, Keir C. Global Manufacturing of CAR T Cell Therapy. Mol Ther Methods Clin Dev. 2017;4:92-101.

12. Kiesgen S, Messinger JC, Chintala NK, Tano Z, Adusumilli PS. Comparative analysis of assays to measure CAR T-cell-mediated cytotoxicity. Nat Protoc. 2021;16(3):1331-42.