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Validation of the media fill method for Cytokine-Induced killer cells manufacturing process

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Abstract

Background Media fills are used to demonstrate the adequacy of the aseptic conditions of a pharmaceutical production process, according to good manufacturing practice (GMP), using a growth medium in place of the product solution. For advanced therapy medicinal product (ATMP), where the final product consists of viable cells, the media fill is still a challenge, but represents the starting point for process validation.

Methods The aim of this paper is to describe the media fill test procedure in the context of ATMP manufacturing, in particular of Cytokine-Induced Killer (CIK) cell expansion process under GMP conditions, including quality control tests and environmental monitoring. The media fill test has been designed to cover all the critical steps of the process, including worst cases and deviations.

Results From July 2019 to August 2022, we performed 16 media fill tests. During these years, the media fill protocol has been gradually improved and the worst cases were designed to be closer to reality and occurring cases. Although some deviations occurred, all the media fills performed were compliant.

Conclusions A good media fill design combined with a robust environmental monitoring program provides a high degree of assurance of the microbial safety of ATMPs.

Keywords Validation, Good manufacturing process, ATMP

Background

The aseptic process simulation or Media Fill (MF) test consists of a simulation of the sterile product manufacturing by using a growth medium such as tryptic soy broth (TSB) in place of the product solution, able to highlight microbial contamination after an appropriate incubation time [1, 2]. It is considered one of the most effective ways to validate a pharmaceutical production process, according to good manufacturing practice (GMP), used both to highlight contamination in the final cell product and to qualify operators [3, 4].

Media fill test can be performed as prospective validation, including the process validation, the qualification of

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facility, utilities and operators, and shall be re-validated regularly.

The process simulation test allows a perfect simulation of the regular aseptic manufacturing process including all steps and worst cases, according to GMP regulation [4].

TSB is a perfect medium supporting the growth of a wide variety of microorganisms, especially common aerobic and facultative anaerobic bacteria [5]. During aseptic preparation, the overall microbial contamination risk results from various risk factors including the environmental conditions, type and complexity of the preparation process, and the skills of the operators [6].

Media fills are used to demonstrate the adequacy of the aseptic conditions and procedures in use, in order to detect a contamination rate of 0.1% (1:1000) at a 95% confidence level, that means that at least 3000 units must be filled [7]. However, media fills routinely conducted in a standard Facility do not come anywhere near this level, typically numbering one media fill per person once or twice a year. In a small advanced therapy medicinal product (ATMP) Facility, only zero is acceptable, consistently achievable when human operators are involved. Any rate of contamination should be rigorously investigated [8]. This is also compliant to Pharmacopeia for batches < 3000 units [7].

Therefore, media fill tests should simulate the aseptic conditions and procedures as close as possible and should also represent worse conditions and the highest risk level to be expected during normal cell production workflow preparation [8].

A prospective simulation test should be performed before starting the real manufacturing process using three consecutive tests (recommended to be performed in different days), and is required for new products, processes and equipment.

The media fill must be performed as a "start-up" or "on going" test as needed [9]. A start up media fill consists in three runs that should be conducted on three separate days. It must be conducted before qualifying a new production process, whenever a significant change in the aseptic process is introduced (e.g., changes in personnel, method, or equipment) and whenever there is evidence of a failure to maintain product sterility. Media fills performed to validate an aseptic process should be done by an operator who previously has been trained and qualified in aseptic GMP techniques (dressing, handling sterile materials, sanitization practices). The "on-going" simulation has to be performed at least one time per year. It is necessary to verify and monitor the maintenance of both the aseptic condition of the production process and the operators' qualification [10].

Media fill test is routinely used in the pharmaceutical industry, while in the ATMP contest is still a challenge. In ATMP production, the final product consists of viable cells on which it is not possible to proceed with final sterilization or filtration [9, 11]. Moreover, the expansion process may require long permanence of the cells in culture, with aseptic techniques often required throughout manufacture, and a complete quality control at batch release may not be available [12].

The purpose of this paper is to explain the media fill procedure stepwise in the context of ATMP manufacturing, in particular of Cytokine-Induced Killer (CIK) cell expansion process under GMP conditions.

CIK cells are a very promising cell population, which raise growing interest in the field of cellular antitumor therapy mainly due to their easy expansion method and their cytotoxic antitumor activity [13]. These NK-like T cells, characterized by a very high cytolytic potential, displayed potent cytotoxic activity against haematological and solid tumors in autologous and allogeneic settings in the presence of very limited toxicity [14–16].

CIK cells are expanded under GMP conditions by cultivating peripheral blood mononuclear cells in standard conditions in presence of Interpheron-gamma, OKT-3 and Interleukin-2 for 3 weeks of expansion, reaching the requested number of cells to be cryopreserved and used in clinical trial [17, 18].

The media fill test has been designed to cover all the steps of the process from the raw material acceptance to the freezing procedure of the final product, including worst cases and deviations.

Methods

Media fill design

The media fill process has been carefully designed to ensure that the simulation is representative of all the aseptic manipulations performed during production, through a careful risk analysis of the process. Regarding the frequency, in case of initial validation, 3 runs were performed by each qualified operator (QO); in case of maintenance of validation, one run for each operator was required every year.

The simulation runs have been carried out in the same locations where the production usually occurs (laboratory named L2, L3, and L4), using the same instruments, and employing the broadest range of possible manipulations that could take place during production.

We designed the media fill protocol with defined and logical sequences of process steps. A system of checkpoints was used to guide the operator through the process, including all the phases related to the reagents preparation, cell manipulation, instruments use and environmental microbiological controls. The process followed all the steps of CIK production process, as illustrated in Fig. 1.

In particular, the following steps were simulated:

1) Preparatory Phase: flow of qualified materials to the Cell Factory (CF); initialization and sanitization of all the equipment according to validated internal standard operating procedures (SOPs); control and preparation of materials and reagents necessary for simulation; login to the Facility database.
2) Day 0: cell separation and culture seeding;
3) Day 1: cytokines addition;
4) Day 2 to 21: CIK observation, evaluation of cellular count and viability, expansion and/or feeding;
5) Day 21 (± 3): quality control (QC) sampling and CIK freezing.

The environmental microbiological controls were performed during all the steps of the media fill process and they were the same carried out during the routine aseptic production process.

The worst-case situations were also defined taking into account the potentially worsening events that could occur or that occurred during the regular production process.

Materials and instruments

The flow of qualified material used during MF was previously validated. In general, the more critical material (i.e. tubes, pipettes, bags etc.) was triple wrapped. However, all the other supplies were carefully sanitized according to internal SOPs and working instructions (WI) and were included in a previous flow material validation.

1. Reagents

TSB from 3 different suppliers (Biotec srl, Grosseto Italy; Merck Life Science GmbH, Eppelheim Germany; Agricons Ricerche, Piazzola sul Brenta PD Italy) replaced all the media and supplements used during the normal CIK production.

The volume of TSB must normally correspond to that used during the production process in relation to the size of the container (flasks, bags, tubes) and must take into account the average daily production of each process and any risk situations that could arise during processing. Based on the validated CIK expansion protocol, we calculated the total TSB volume for each run, which was higher in the worst-case simulations.

2. Consumables

50 ml tubes (Greiner Bio-One, Cassina de Pecchi, Italy) were used to simulate density gradient separation, cellular washes and final cellular product pool.

Pipettes of different size (Greiner Bio-One, Cassina de Pecchi, Italy; Corning Incorporated Life Science, Kennebunk ME, USA) and tips (Gilson, Villiers-le-bel, France) were used to simulate product transfers, addition of culture medium/cytokines and for QC sampling.

Different size of flasks were used: T75 flasks (Greiner Bio-One, Cassina de Pecchi, Italy) were used for the first culture steps (up to day+13), when the cell culture volume was moderate. From day 13 to day 21, T150 flasks (Corning Incorporated – Life Science, Durham NC, USA) replaced T75 ones to handle a larger volume of culture medium.

Safe2 SH-50B ethylene vinyl acetate (EVA) Bags (Paolo Gobbi Frattini, Tovo S. Agata, SO, Italy) were used to simulate cell preparation for freezing step and for sterility/fertility analysis. 600 mL bags (Fresenius Kabi, Bad Homburg, Germany) were used for the simulation of the pre-freezing and freezing solution preparation.

2.5 ml syringes (Farmac-Zabban, S.p.A., Calderara di Reno, BO, Italy), 30 and 60 ml syringes (Bewnefis s.r.l. Via Gualco 14 16,455 Genova) were used for bag transfers and addition of solutions.

Triptic Soy Agar (TSA) plates 90 mm and 50 mm (Liofilchem s.r.l., Roseto degli Abruzzi, TE, Italy) were used for the microbiological environmental active, passive and surface controls, performed during all the steps of MF.

3. Sanitizers

Cleaning and disinfection of environments, materials and equipment took place according to internal validated SOPs and WIs. The validated sanitizers used were the following: Isopropanol 70% Spray, Sterile Klercide 70/30 IsoPropyl Alcohol (IPA), Klerwipe Sporicidal Low Residue Peroxide Blended, Klerwipe Klercide Amine Blended and Sterile Klerwipe Quat/Biguanide Blended (Ecolab - Life Sciences, Neath, UK). These were alternated to avoid resistance, according to the cleaning validation previously performed.

4. Equipment

The instruments used during the media fill procedure were the laminar flow cabinet, the centrifuge, the micropipettes and the automatic pipettes. For the final step, a welder and vacuum were also used for the sealing of the bags.

Surface Air System (SAS) samplers were used for active microbiological environmental assay.

All equipment was qualified and maintained according to an annual established program.

5. Environmental control

Air particle was monitored during processing using the integrated system for continuous air quality monitoring Edo 2000 (Airnova S.r.l., Limena PD). This system monitors grade B environments and grade A biohazard cabinet, in compliance with the limits recommended by Annex 1.

Pressure, temperature and humidity were monitored using Desigo Insight software (Siemens Italia), according to technical procedures.

Operators

All the QOs, including Production and QC operators have been previously qualified to perform all the operations simulated in media fill. Staff qualification included theoretical and practical training in aseptic manipulations, instruments use, correct entrance and dressing, material entrance and sanitization, and negative results in environmental controls performed on dressing and gloves.

Three or four QO were involved during the media fill process: 2 Production QOs executed the processing steps, 1 CQ QO performed the environmental microbiological controls during the process. In the worst-case simulation, an extra Production QO was present, as a supporter, in the initial step (day 0) and in the freezing step (day 21), considered the most critical ones of the entire production process.

Documentation

Before starting the MF, the Validation Protocol was drafted, including written specifications on the process along with the procedure, test method, equipment handling, specifications, acceptance criteria, data reporting and approval. This document was created to help the QOs to follow the process and contained the following parts: objective and scope, glossary, responsibilities, personnel involved, reference material, reference documents, documentation, methods, acceptance criteria, deviations, attachments. The annexed Validation Report included the raw data, a conclusive summary on their compliance and the final approval, according to the validation protocol and acceptance criteria.

In addition, we collected all the data in the electronic batch record (BR) Biomanagement (SOL, SOL SpA, Via Borgazzi, 27–20,900 Monza Italy, www.solgroup.com), according to internal SOPs and WIs.

Media fill execution

We defined the possible worst situations in order to simulate them in media fill.

In particular, the worst-case (WC) conditions we challenged were:

1) Container removal or replacement due to accidental brake or culturing medium leak;
2) Maximum number of flasks that can be contained in the cabinet, due to a large culture volume: this situation causes a great encumbrance in the cabinet and increases the risk of contamination.
3) Staff break: in this case, the QO has to stop the production procedure, leave the CF, undress correctly and restart the process after a new dressing step.
4) Maximum/minimum number of QOs employed presence of an extra operator that could affect environmental conditions.
5) Accidental braking of gloves or the suspicion thereof, which requires their change during the procedure.
6) Use of flasks with different size and dimension.
7) Accidental leak from flask or EVA bag: this situation leads to the necessity of an extraordinary cleaning of the cabinet surface and the temporary interruption of the step.

Not all the seven WCs were simulated in all the MFs. More precisely, only those situations that are most likely to occur (staff break, different sizes of the expansion flasks) were simulated. Other less frequent worsening situations (flask breakage, temporary operation interruption) were simulated if they actually occurred in the previous year. Therefore, each year is representative, in terms of worst case, of the real situation of the Facility.

The media fill execution followed the steps of the CIK production process previously described (Fig. 1). For the whole process, the gestures of each step were simulated, but the times were shortened. All the steps were performed, except those that do not directly affect the sterility of the product (for example the preparation of Burker chamber in the QC step), as shown Fig. 2.

For each run the filling of the bags representing the final preparations with a maximum capacity of 30 mL have been simulated, which constitute the maximum number of final containers set up in routine and intended for cryopreservation before infusion to patients, according to the approved trial (Protocol C.A.S.T. CIK Cells Advanced Sarcoma Trial, Eudra CT 2017-002257-11).

The process has been recorded in the electronic batch record (BR) Biomanagement.

Following the CIK production process, we simulated:

1. Preparation of the reagent. The TBS distributed in the 50 ml tubes simulated the reagent used for gradient separation of PBMCs. The culture medium was simulated by TSB.
2. Preparation of starting material (peripheral blood). A 600 ml bag filled with 300 ml of TSB was representative of the starting material.
3. Centrifugation. All centrifugation steps, both for the gradient separation phase and for washes or pooling, were simulated by reducing the centrifugation time to 1 min. In order not to damage the TSB, the centrifuges worked at the minimum speed allowed (200 rpm).
4. Seeding, feeding and expansion. TSB was taken from the original glass bottle. When the maximum capacity of the flasks was reached (80 ml in T75, 180 ml in T150), we increased the flasks number,

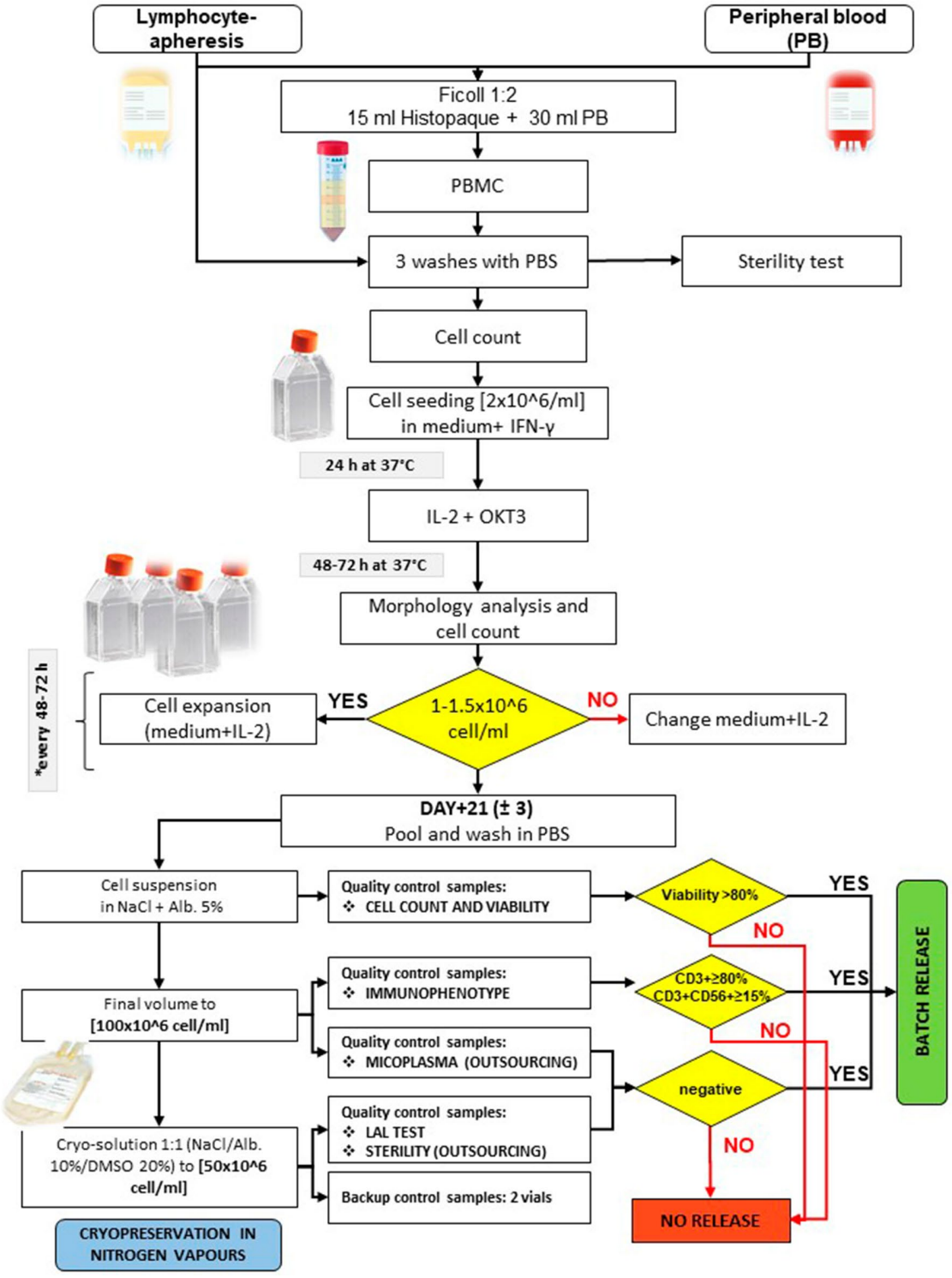

Fig. 1 CIK production process flow chart. Graphical illustration of the CIKs expansion method, including production and quality control steps

up to 35 flasks, which was the maximum capacity of incubator. The flasks were maintained on the bench, not in the incubator, in order not to damage the TSB under the temperature of 37 °C.

5. Cytokine addition to each flask with micropipettes and tips.
6. Pool flask creation. At the beginning of each expansion step, it is necessary to perform a count to establish the cellular concentration. The pool flask is

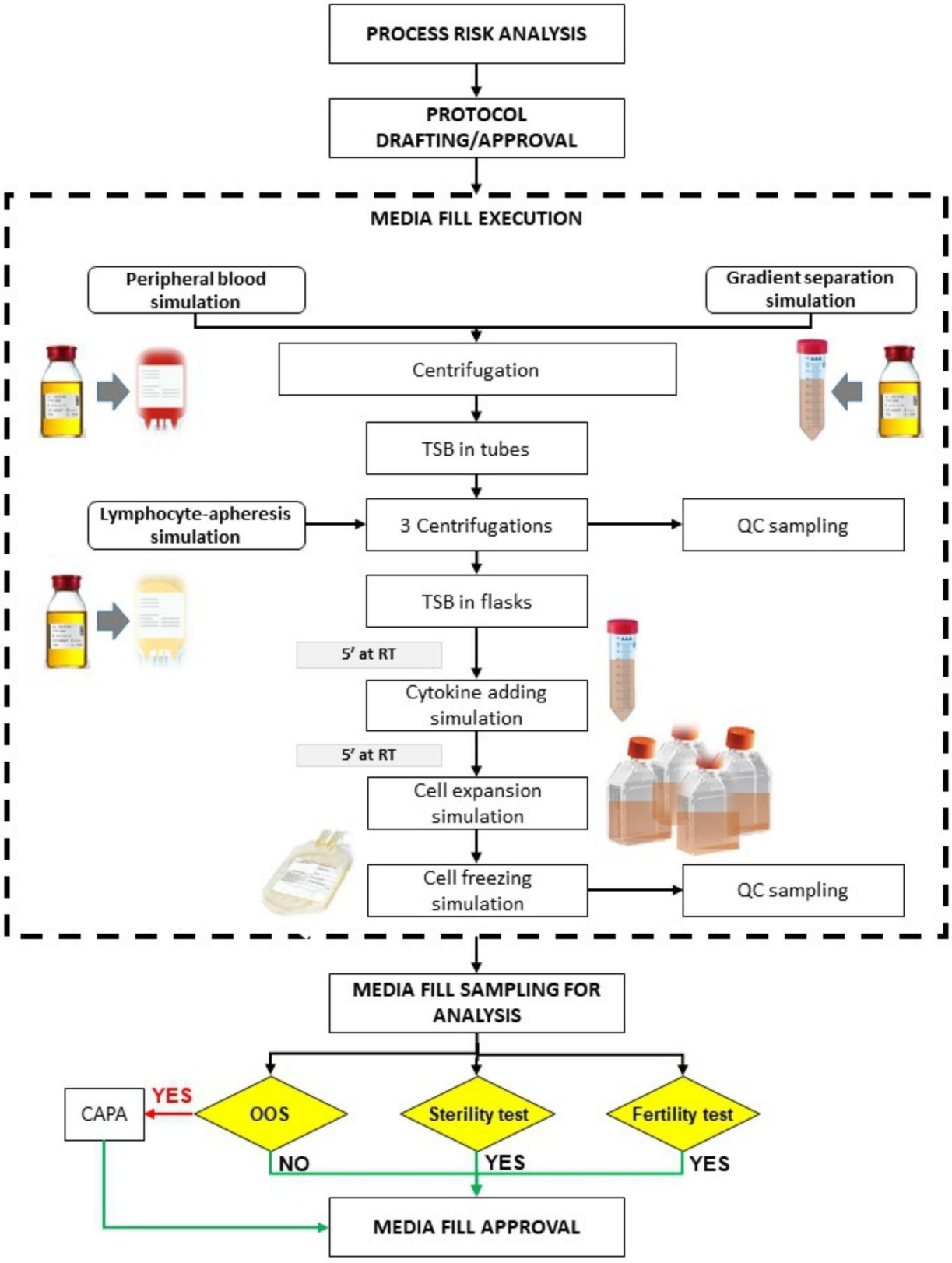

Fig. 2 Media fill process flow chart. Graphical illustration showing how the CIK production process steps have been shortened in the MF

representative of the entire cellular culture and we simulated it taking the same quantity of TSB from each flask. From the pool flask, a TSB sample was taken to simulate cellular count and viability.
7. QC sampling. We simulated this phase by collecting the adequate TSB quantity, representative of the requested QC test, from the flask or bag.
8. Bag filling and freezing. The TSB representing the final cell product was transferred into a 600 ml bag after the simulation of pooling and washing operations. Then we simulated the preparation of pre-freezing (TSB pre-freezing) and freezing (TSB freezing) solutions by transferring the TSB with syringes in the 600 ml bags. The TSB pre freezing solution was added to the final product bag to simulate the adjustment of the correct concentration. We added the TSB freezing solution just before splitting the bag in the cryopreservation EVA bags, filling them to the maximum capacity, by transferring TSB with syringes from the single bag.
9. Sampling for media fill tests analysis. We identified three representative checkpoints of the (a) initial phase (day 0), (b) day 4 (± 2) and (c) day 21 (± 2) freezing step respectively. We took the same quantity of TSB from each flask in order to obtain the representative sample of both phases (a) and (b) which were used for the sterility and fertility analyses while for step (c) we analyzed all the bags.

Environmental monitoring

The microbiological environmental controls were performed during all the steps of the media fill and they were the same carried out during the routine production process.

The microbiological sampling points were defined by a previous risk analysis. The monitoring procedures consisted in air active or passive sampling, surface sampling and QO monitoring through glove prints and they are crucial to identify, define and treat possible out of specifications (OOS) that may occur during critical steps.

At the end of each production step, the QC operator performed patches on Production operators' gloves and on the central flow cabinet surface while at the end of each work session more patches were added on lateral and frontal flow cabinet walls.

Active and passive sampling were also performed during the entire production process.

Air particle and environmental conditions were monitored continuously during processing.

Media fill analysis: sterility and fertility test

At the end of all runs, the units have been inspected to verify the absence of breakages or defects, and turned upside down and shaken to allow all internal surfaces to be exposed to the culture medium. All the units underwent sterility test by incubation for a period of 14 days (7 days at 22.5 ° C and 7 days at 32.5 °C). The first 7 days of incubation could be delayed for 1–2 days due to the transport of the units to the microbiology laboratory. At the end of the sterility test, 6 bags of each run, whose sterility has been demonstrated, are used for the TSB medium fertility test. Briefly, known microorganisms (Staphilococcus aureus (ATCC-6538), Bacillus subtilis (ATCC-6633), Pseudomonas aeruginosa (ATCC-9027), Candida albicans (ATCC-10,231), Aspergillus brasiliensis (ATCC-16,404) have been inoculated in the 5 designed bags. The sixth was used as negative control. An incubation period of 5 days was followed. The incubation temperatures were the same used for the sterility test. The media fill was considered compliant if at the end of the incubation time all the samples had maintained their characteristics of clearness and the fertility test was positive. If the TSB medium sterility and/or fertility was not demonstrated, the media fill must be invalidated and repeated [10]. The sterility and fertility tests were performed in outsourcing (Itelpharma, Via Antonio Labriola 70,037 Ruvo di Puglia (BA) Italy, and SDS S.r.l. Via Galvani, 9/G 31,027 Spresiano (TV), Italy).

Media fill result analysis and acceptance criteria

The optimal result of a media fill is to obtain zero contaminated units. According to Pharmacopeia for batches < 3000 units if no one units are contaminated, the run is compliant. On the contrary, the presence of even just one contaminated unit determined the non-compliance of the media fill and the possible detection of a problem within the production process (equipment, environmental conditions), or the operating methods, of the operators involved.

In case of non-compliant results, the corrective actions performed were different in case of start-up validation (three runs) or ongoing validation (one run) as showed in Fig. 3.

All the results have been resumed in the final report, which included also a conclusive judgment on compliance with the acceptance criteria established in the validation protocol.

Results

From 2017, we perform annual validation through Media fill analysis. Here, we reported the results of the last four years. From July 2019 to August 2022, we performed 16 Media fill tests. During these years, the media fill protocol has been gradually improved and the worst-cases were designed to be closer to reality and occurring cases. The characteristics and the results of the runs are illustrated in Table 1.

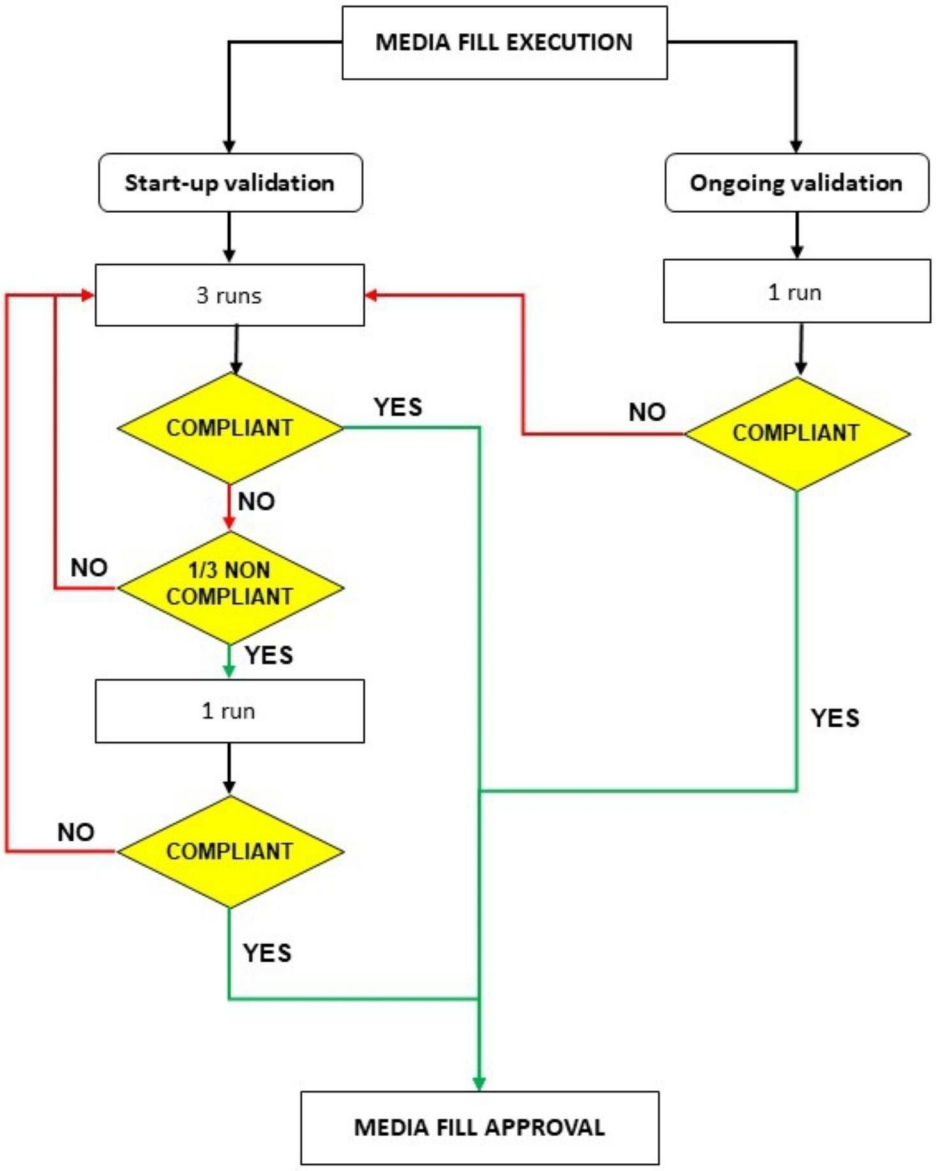

Fig. 3 MF approval process. Graphical illustration of decision-making steps for start-up and ongoing MF validation

In 2019 QO1 and QO2 were qualified operators needing to follow a maintenance protocol (1 run), while QO3 was a new operator needing to implement validation protocol with 3 runs. QO3 performed two small MF runs alone (MF3 and MF4) and the third worst case run in tandem with another already qualified operator (MF2). On these bases, a total number of four MF was sufficient to guarantee and check the OQ qualification level in 2019.

In 2020, QO4 was a qualified operator previously checked (data not shown). Only the new QO5 needed the entire cycle of qualification performing 3 runs (MF5, MF7 and MF8).

In 2021, no new operators were introduced, while in 2022 the QO6 completed his validation protocol, as previously defined.

Al the MFs have been performed in the three rooms of the CF over these years. During 2021, for technical reasons, the room called L2 was not used. Therefore, to guarantee the regular functioning of the Cell Factory, room L4 was also introduced and qualified for the process.

Based on sterility test on all the final samples, all the media fill performed were compliant, as requested by Pharmacopeia for small batches (<3000 units).

Air particle has been monitored thanks to the EDO2000 system, that monitors real-time alert and alarm thresholds and keeps under control the environment conditions

Table 1 Characteristics and the results of the runs performed

	Protocol	ID	Date	Production QO	L	TSB total volume (ml)	Sterility (N° of Units)	Fertility (N° of Units)	WC	OOS/DEV	Compliance
2019											
MF1	MAN	19095	08/07/2019	QO1/ QO2	L3	12,000	10 d0 tubes, 10 d4 tubes, 6 bags	5 bags	YES	1	YES
MF2	MAN/ VAL	19094	08/07/2019	QO1/ QO3	L3	12,000	10 d0 tubes, 10 d4 tubes, 6 bags	5 bags	YES	3	YES
MF3	VAL	19100	05/08/2019	QO3	L2	6,000	4 d0 tubes, 4 d4 tubes, 6 bags	5 bags		NO	YES
MF4	VAL	19101	06/08/2019	QO3	L2	6,000	4 d0 tubes, 4 d4 tubes, 6 bags	5 bags		1	YES
2020											
MF5	MAN/ VAL	20127	21/07/2020	QO1/ QO5	L2	11,000	10 d0 tubes, 10 d4 tubes, 6 bags	5 bags	YES	4	YES
MF6	MAN	20133	27/07/2020	QO4/ QO3	L2	11,520	10 d0 tubes, 10 d4 tubes, 6 bags	5 bags	YES	4	YES
MF7	VAL	20156	08/09/2020	QO5	L3	6,000	4 d0 tubes, 4 d4 tubes, 6 bags	5 bags		NO	YES
MF8	VAL	20176	12/10/2020	QO5	L3	6,000	10 d0 tubes, 10 d4 tubes, 6 bags	5 bags		NO	YES
2021											
MF9	MAN	21115	21/06/2021	QO4/QO5	L3	12,000	1* d0 tubes, 1*d4 tubes, 12 bags	12 bags	YES	NO	YES
MF10	MAN	21133	26/07/2021	QO3/QO4	L4	12,000	1* d0 tubes, 1* d4 tubes, 12 bags	12 bags	YES	NO	YES
MF11	MAN	21143	24/08/2021	QO5/QO3	L4	12,000	1* d0 tube, 1* d4 tube, 12 bags	12 bags	YES	6	YES
2022											
MF12	MAN	22099	05/07/2022	QO4/QO3	L4	12,000	1* d0 tubes, 1* d4 tubes, 12 bags	12 bags	YES	NO	YES
MF13	MAN	22106	26/07/2022	QO5/QO4	L3	12,000	1* d0 tubes, 1* d4 tubes, 12 bags	12 bags	YES	NO	YES
MF14	VAL	22146	27/09/2022	QO6	L3	6400	1* d0 tubes, 1* d4 tubes, 6 bags	6 bags		NO	YES
MF15	VAL	22174	07/11/2022	QO6	L3	6,400	1* d0 tubes, 1* d4 tubes, 6 bags	6 bags		NO	YES
MF16	VAL	22179	14/11/2022	QO4/QO5/ QO6*	L3	12,000	1* d0 tubes, 1* d4 tubes, 12 bags	12 bags	YES	NO	YES

DEV, deviation; d0, day 0 (±2); d4, day 4 (±2); ID, identification number; L, laboratory; MAN, maintenance; MF, media fill; OOS, out of specification; QO, qualified operator; VAL, validation; WC, worst case

*Pool of total tubes, *QO5 and QO6 alternated alongside QO6

during processing, thus having the chance to make any corrective measures in case of OOS or deviations. The microbiological monitoring was also performed during the simulation procedures.

As reported in Table 1, some OOS/deviations were found. Any deviation encountered during MF execution has been investigated, with an accurate root cause analysis; the most frequent OOS/deviations were related to microbial contamination. In particular, in 2019 the microbial contamination happened in 2 WC MFs (MF1 and MF2) and in 1 standard MF (MF4). In 2020, the OOS/Deviations occurred only in 2 WC MFs (MF5 and MF6). In 2021, we performed three WC MFs, and the deviations occurred only in the MF11. In 2022 we did three WF MF (MF12, 13, 16) and 2 standard MF (MF14, MF15). only microbiological OOS/deviations were recorded and occurred in MF14.

The highest number of microbial OOSs/deviations mainly concerned the operators (26/27 positive contact plate in 2019, 1/4 positive contact plate in 2020, 19/23 in 2021 and 6/9 in 2022), (Fig. 4, panel A), with a median number of colony forming units (CFUs) of 4.85 in 2019, 0.75 in 2020, 16.21 in 2021 and 0.89 in 2022 respectively. On the contrary, in 2020, most of the microbial OOSs concerned the grade A (3/4 positive contact plate with a median number of CFUs 0.75), (Fig. 4, panel B). The Fig. 4, panel C shows the percentage distribution of the microbial OOSs during the years.

The most frequent contaminants were environmental microorganisms, in particular Bacilli and fungi. However, these results were homogenous and compliant to the routine trend of the Facility and contamination rates found in our routine monitoring program during years (data not shown).

Furthermore, most of the OOS results were consistent with the microbial standard environment situation of our Facility, and the MFs results were always compliant.

Discussion

The media fill test is a fundamental step that allows validating the production process, qualifying the operators and keeping under control new critical events that may occurred during the routine process. Personnel are the largest risk factor in aseptic manufacturing processes. All operators shall be trained and qualified on manufacturing using aseptic procedures.

All the steps of the CIK manufacturing process have been simulated, challenging the most critical situations, in at least one WC MF for each OQ. The goal of a media fill is to demonstrate that a QO can follow the routine aseptic production process maintaining the sterility of

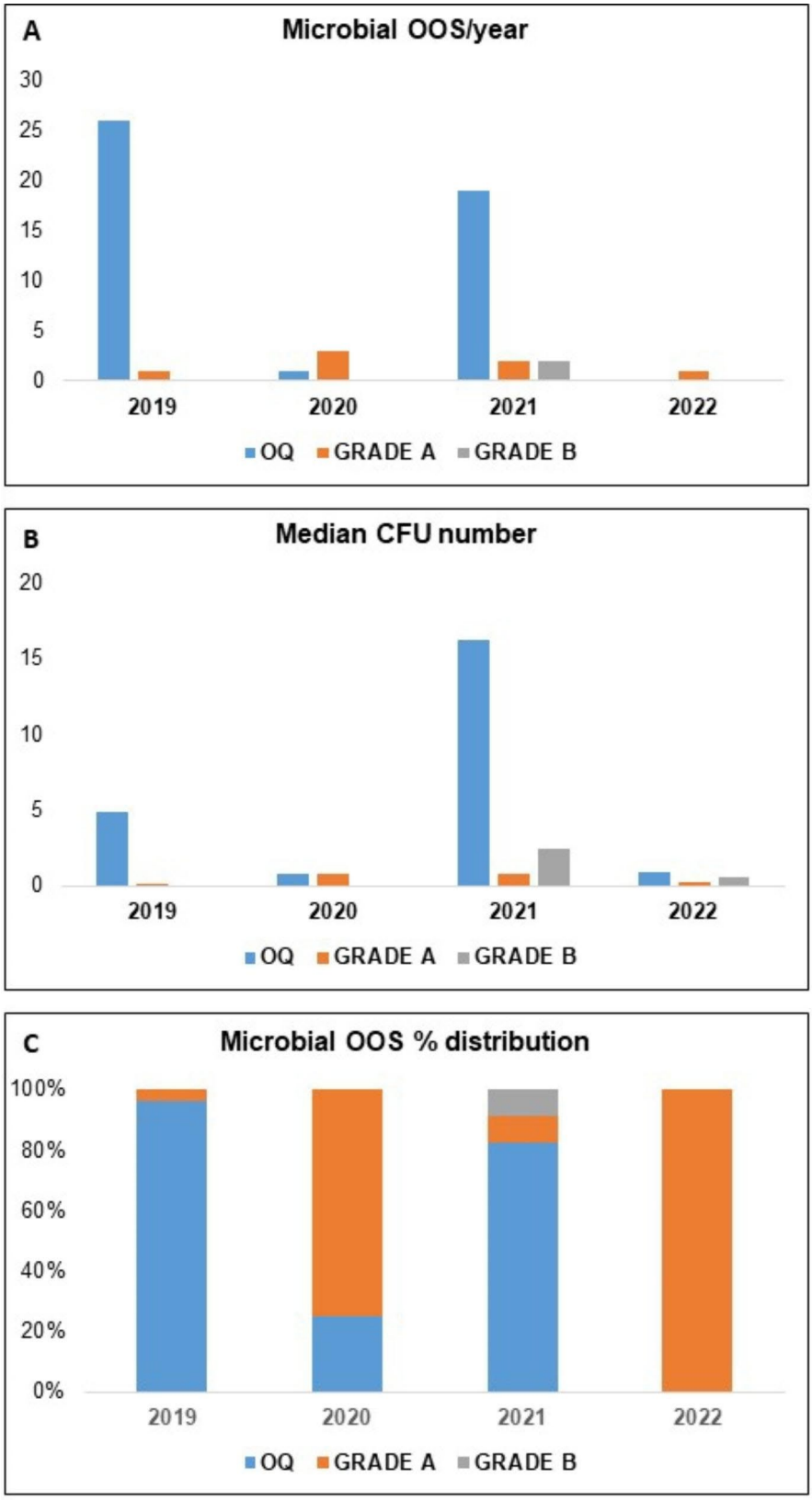

Fig. 4 OOS distribution during years. A: number microbial OOS/year on QO gloves, grade B surfaces, grade A surfaces. B: median number of CFUs in samples from QO gloves, grade B surfaces, grade A surfaces. C: percentage distribution of the microbial OOSs during the years

the final batch, without resulting in contamination. Using rigorous and worst-case media-fill tests can help in this.

In aseptic manufacturing, environmental microbiological monitoring plays a pivotal role, and documents the state of quality control of the Facility rooms.

Thanks to microbiological monitoring, in fact, it is possible to identify new critical steps and highlight incorrect operators' behaviour that can preclude the sterility and the safety of the process and of the cell therapy product consequently.

Some deviations occurred during MF processes. Environmental microbiological OOS/deviations occurred during the MF remained consistent with what befallen during the routine, and they never precluded the sterility of media fills.

All OOS/deviations and deviations were treated by a careful cause analysis. In particular, the transfer of the flasks from the cabinet (grade A) to the bench (grade B) and vice versa could be the main cause of the OOSs found. The flasks cannot be sanitized in all their parts, to avoid damaging the TSB or medium inside.

According to GMP Annex 1, the QO CFU gloves fingers print number must be <1 in grade A. For those working in grade B the limit is <5. Our results are important, because the MFs' compliance demonstrates that the passage to and from the two classified environments does not affect the process and the cellular product safety. On the other hand, corrective and preventive action (CAPA) plan is necessary to minimize and control the risk of contamination of the cellular product. For instance, we implemented an increase number of random microbiological samples during the process, and the presence of a QO as a support of the production only during the most critical phases of the process.

Another critical step encountered during MF was the manipulation of TSB bottles, as the spillage of the broth from the cup can be difficult. We processed three different type of bottles with different type of caps. In some cases, the cleaning and disinfection of cap surface by wiping was less effective. The screw cap that bottles of culture media are normally equipped with is the best choice and can prevent contamination. However, spillage of broth during the filling process can yield a sticky film, which is difficult to remove. Therefore, the bioburden is higher and cleaning and disinfection of the broth vial surfaces can be less effective. For this reason, the CFU increased number on the fingertips of the operators can be higher than during the routine activity [5]. In general, this evidence could be important to guide Cell Factory operator in the choice of TSB medium in order to avoid the risk to invalidate the MF itself. In this very delicate and critical process, even the smallest details could make the difference.

From our cause investigation, the number of microbial OOSs occurred especially in summer, with hot temperature and humidity and frequent HVAC (heating, ventilation and air conditioning) malfunctions. Therefore, the decision to carry out MFs at this period of the year allows us to simulate the most critical environmental situation. In this way, process control checkpoints can be further strengthened even under extreme conditions.

Moreover, important evidence raised from our data analysis: during the last three years, the QOs improved the worst-case management. In fact, in 2019 the microbiological OOS occurred in 2 WC MFs and in 1 standard MF. In 2020, the OOS occurred in 2 WC MFs, and in 2021 only in 1 WC MF, when HVAC system suffered some malfunctions due to extreme temperature conditions. In 2022, the OOS was only microbiological and occurred in MF14, the first of the three validation run for the new QO. No other OOSs were recorded during this year. All the data together demonstrated the important evidence that, even under some stressful conditions, the QOs have improved their skills over the years.

In 2021 no new QOs were introduced, therefore this event cannot be related to operator turnover. However, the production activity in Cell Factory increased in 2021, and then decreased again in 2022. As the most frequent OOS occurred in summer, we hypothesized a correlation with environmental conditions. The summer are always the most critical period, especially in terms of environmental contamination of fungal species. However, in 2022, as in previous years, the preventive and corrective measures were effective in containing contaminations and did not affect the quality and safety of the processes. This aspect reinforces the concept that a strong risk assessment and the implementation of corrective and preventive actions could play a pivotal role in the maintenance under control all the processes.

The MFs helped us to validate the CIK manufacturing process. In recent years, we have carried out 7 validation runs and 8 drug runs, which were all compliant for sterility. This demonstrates that, even in the presence of microbial OOSs, the final drug is not compromised.

Conclusions

The aim of this paper is to describe the media fill procedure stepwise in the context of ATMP, in particular of CIK cell expansion process under GMP conditions, defining the correct number of checkpoints in the process. In general, the experience led us to adopt more and more effective corrective and preventive actions. As a direct consequence, the number of checkpoints, already emerged during the previous risk assessment phase, increased as well as the safety and quality of the production process.

The MFs and the analysis of the microbiological OOSs are important instruments to evaluate the CAPA effectiveness and to guide QOs in the risk management. The implemented CAPA included, first, the microbial identification, in order to choose the most suitable sensitization strategies (extraordinary cleaning of surfaces and rooms) for the eradication of contaminants, in particular Bacilli and fungi. Another important focus was the retraining of the operators on dressing and sanitization of disposables and materials, and the revision of SOP/WI. The reduction of contamination and the compliance of the MFs demonstrated the effectiveness of CAPA plan.

During the CIK production process, some DEV/OOS happened. They concerned microbiological surfaces and QO glove print contaminations, but the process compliance and the batch release were always achieved thanks to simulation of similar DEV/OOS during the media fill test.

A hospital Facility can often encounter significant obstacles in developing ATMPs, because of lack of financial support and logistical or engineering difficulties. With this MF planning and design we were able to guarantee the qualification of the process and of the QOs, taking in account the Facility sustainability. The MF design, moreover, is the result of an accurate risk analysis, involving multiple processes, from the flow of operators and materials to the environmental monitoring performed every year, and allowed us to focus on the most challenging steps to guarantee the quality and safety of the process itself.

A good MF design combined with a robust environmental monitoring program provides a high degree of assurance of the microbial safety of ATMP.

Abbreviations

ATMP	Advance therapy medicinal product
BR	Batch record
CAPA	Corrective actions and preventive actions
CF	Cell Factory
CFUs	Colony forming units
CIK	Cytokine-Induced Killer
DEV	Deviation
EVA	Ethylene vinyl acetate
GMP	Good manufacturing practice
HVAC	Heating, ventilation and air conditioning
IPA	IsoPropyl Alcohol
L	Laboratory
MF	Media Fill
OOS	Out of specifications
QC	Quality control
QO	Qualified operator
SAS	Surface Air System
SOPs	Standard operating procedures
TSA	Triptic soy Agar
TSB	Tryptic soy broth
WIs	Working instructions

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Authors' contributions
IF: Study design, data analysis and interpretation, manuscript writing; SC: data analysis and interpretation, manuscript writing; AA, AM, FS, LG: production process; DR, ML, GP: quality control process; KM: critical reading of the paper; FF: financial support, critical reading of the paper. All authors read and approved the final manuscript.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflicts of interest
The authors declare that they have no competing interests.

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Not applicable.

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Not applicable.

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