G-Rex bioreactors incubated in Heracell Vios CR CO_2 Incubators help prevent cross contamination and bacterial contamination in cell and gene modified cell therapy production

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Abstract

Background and Aim: For Cell and Gene Modified Cell Therapy (CGT) manufacturing, a robust contamination control strategy (CCS) is necessary to ensure compliance with current Good Manufacturing Practices (cGMP). A successful CCS helps ensure safety, purity, efficacy, and quality of a CGT product. CGT manufacturers should implement Closed Systems wherever possible and employ equipment that maintains a stable, clean environment with advanced monitoring. We show that Closed System G-Rex® bioreactors combined with Thermo Scientific™ Heracell™ Vios™ CR carbon dioxide (CO_2) incubators enable simplified parallel processing capabilities and significantly reduced contamination risks in a highly efficient production.

Methods, results and conclusion: Culturing T cells or NK cells in a Closed System G-Rex® bioreactor (Wilson Wolf Manufacturing, LLC) reduces the risk of contamination. The G-Rex approach eliminates most manual handling, a leading source of contamination. G-Rex bioreactors include weldable tubing for simple Closed System sterile connections and do not require interventions for feeding. Importantly, Closed system (CS) G-Rex bioreactors feature a validated sterile fluid path that reliably maintains integrity throughout manufacturing as shown by Microbial ingress testing based on ASTM E-3251. The bioreactors withstand full immersion in a challenge media solution containing bacteria for at least fourteen days. G-Rex bioreactors also passed Viral Penetration testing based on ASTM Method F1671, demonstrating suitability for use in CAR-T and other CGT applications requiring use of virus.

Heracell Vios CR CO_2 Incubators provide recovery of all parameters in 10 minutes or less, and uniformity of +/- 0.3° C per DIN 12880. These parameters are maintained even when culturing ten G-Rex 500M-CS bioreactors simultaneously. Thermo Scientific™ CultiMaxx™ shelving maximizes production capacity per footprint. Incubating multiple G-Rex bioreactors in a single chamber means the incubator must offer proven contamination control features. An on-demand cycle delivers 12-log sterility assurance level (SAL). In-chamber HEPA filtration generates air cleanliness in the chamber equal to ISO Class 5. Data show the Heracell Vios CR CO_2 incubators are certified for use in ISO Class 5, GMP Grade A/B cleanrooms.

Together, G-Rex bioreactors incubated in Heracell Vios CR CO_2 incubators offer a high yield, parallel processing method with reduced contamination risk in a highly efficient footprint enabling robust CCS in a cGMP environment.

Materials and methods

G-Rex microbial ingress test: Tests were performed by a third-party test lab, Wuxi AppTec (Atlanta GA USA). Briefly, a $1x10^8$ challenge solution was prepared using *Brevundimonas diminuta*. The test sample G-Rex bioreactors were aseptically filled with Nutrient Broth Media through each of the tubing lines, including the sample port, the reduction lines, and the harvest lines to sufficiently wet all internal surfaces. 5,000 mL of Nutrient Broth Media was introduced into each test sample G-Rex bioreactor. 12 Liters of challenge solution were filled into a lined container (Figure 1A). The G-Rex bioreactors were weighted to ensure full immersion in the challenge solution during the entire 14-day incubation test period (Figure 1B). The vent filter was unclamped and in an upright position to ensure that it did not make contact with the challenge solution.

To confirm no growth, the test bioreactors were carefully removed from the challenge solution and placed inside a new sterile bag. The bioreactors were then gently swirled to mix the contents, and a 1.0 mL aliquot of Nutrient Broth was removed and added to a 10 cm Petri dish in triplicate per bioreactor and incubated 2-3 days then counted.

G-Rex membrane viral penetration test: Viral penetration testing was performed by a third-party test lab, Nelson Laboratories (Salt Lake City UT, USA) according to their internal protocols. Tests were performed on the G-Rex membrane material according to ASTM F1671 [1]. Briefly, a challenge suspension was prepared using bacteriophage Phi-X174 maintained at a concentration of at least 1.0×10^9/mL. A total of 32 test articles (G-Rex membrane material) were prepared by loading each test article membrane into a test apparatus as shown in Figure 2.

The bolts around the test frame were torqued to create a perimeter seal. Each test reservoir containing the filter sample was then filled with 60 mL of PhiX174 bacteriophage challenge suspension and pressured to 2.0 psig (~103 mm Hg) for 1 minute. Air pressure was then vented, and the test articles were allowed to sit for 54 minutes with no applied pressure while the surface of the membranes was observed for liquid penetration. As confirmation, an assay titer was performed for each test article in addition to positive and negative controls. For this test, the observed side of the membranes was rinsed with a sterile medium and assayed for the presence of Phi-X174 bacteriophage.

CO_2 Incubator temperature uniformity mapping: Validated PT100 temperature probes were placed in 27 locations in the incubator chamber, with 9 equidistant probes on each of three shelves, according to DIN 12880 [2]. The temperature was set to 37°C , the humidity reservoir was filled to the maximum and the humidity was set to maximum. The incubator operated at these conditions undisturbed for 12 hours before commencing the test measurement. Each measurement had a 10 second duration and the test continued for 22 hours, in an ambient temperature of 22.8 °C. Uniformity equals the difference between the highest and lowest recorded temperatures.

CO_2 Incubator recovery tests: For temperature, the tests were performed similarly to the uniformity mapping, except the incubator "Low humidity" setting was used and the temperature probe for the incubator was placed in the center of the center shelf. 10 G-Rex 500M-CS bioreactors were each filled with 5 L of water pre-heated to 37 °C. Both the inner and outer doors of the incubator were opened for 60 seconds, then closed. Recovery is defined as returning to 98% of the set value.

For CO_2 gas recovery testing, the incubator was set to the most commonly used concentration of 5%, temperature was set to 37 °C, and the "Low Humidity" setting was used. CO2 was measured using a GMM221 infrared sensor (Vaisala, Finland) used without the protective cover and placed in the center of the middle shelf between the 4 G-Rex bioreactors (10 total in the incubator chamber). Recovery is defined as returning to 98% of the set value.

For humidity, the "Low Humidity" setting was used. Humidity was measured using a FHAD 462 relative humidity (RH) sensor (AHLBORN, Germany) positioned in the center of the middle shelf between the 4 G-Rex bioreactors (10 total in the incubator chamber). Recovery is defined as returning to 98% of the set value.

HEPA filtration tests: Tests were performed in a room at 22 °C, 50% RH. Ambient particles were counted, an average of 4,269,400m3 was recorded (ISO Class 8). The incubator chamber and glass door were wiped with 70% ethanol to remove any surface residual particles. A calibratied particle counter, ACS Plus 328 (KM Optoelectronik GmbH, Germany) was used with an airflow setting of 1.0 cubic foot per minute (cfm). Particles were generated to boost to ISO Class 8-9 levels using an aerosol generator, CFG290 LMT (Topaz Gmbh, Germany). The sample tubing for the particle counter was located in the center of the empty chamber and the return air located in the top left rear of the chamber. The return air velocity was set to 2.1 m/sec. The test sample tubing and the return air tubing were run through the access port in theleft upper rear wall. The space around the tubing was sealed, and the incubator water drain was sealed inside the chamber to ensure no passive air. A new HEPA filter was installed in the incubator chamber according to the user manual. Samples were collected for 30 seconds and purge time was 2 seconds between samples. Particles of 0.5 µm and larger were counted. The particle counter was operated in "Automatic" mode for a minimun of 10 minutes.

Cleanroom compatibility tests: Tests were performed by an industry specialist, TÜV SÜD (Munich, Germany). Briefly, working in an ISO Class 4 cleanroom, the entire incubator was manually wiped and analyzed for surface particle shedding. The entire incubator exterior was then sampled using a particle counter to determine the areas of highest emission. This area was then sampled for 100 minutes with a sample taken every minute during the approximately 12-hour sterilization cycle at 180 °C. Particles of 0.5 and larger were counted.

Results Summary

Closed-System G-Rex Bioreactors prevent bacterial contamination and viral penetration

G-Rex Bioreactor validations enable fully closed system manufacturing
G-Rex Closed System Bioreactor validations include Shipping Simulation (ASTM D 4169), Environmental Conditioning (ISTA Procedure 3A), Accelerated and Real-Time Shelf-Life Studies (3 yr. per Q10 Theory principles and ASTMF 1980-16), and Sterilization Validation (Sterile Fluid Path) per Method VDmax25 ANSI/AAMI/ISO 1137 10^6 SAL Dose Substantiation and Max Dose validations. This validation testing supports the shelf life and sterile fluid path claims listed on G-Rex Certificates of Compliance. G-Rex is manufactured by Wilson Wolf Manufacturing LLC, in accordance with cGMP, and is 100% leak tested prior to release. To further substantiate G-Rex Closed System and sterile fluid path integrity claims during use, third-party microbial ingress testing and viral penetration testing were performed.

Incubating multiple G-Rex bioreactors simultaneously in Heracell Vios CR CO_2 incubators represents minimal risk due to in-chamber protections

Heracell Vios CR CO_2 incubators offer proven contamination control
Based on a history of incubation innovation, Heracell Vios CR CO_2 incubators are the first-to-market certified cleanroom compatible CO_2 incubators. [3] In this way, the Heracell Vios CR models protect the cleanroom environment as an extension of the proven protection for cells incubated in the incubator chamber. This in-chamber protection includes a HEPA filtration system to capture airborne viable and non-viable particles, a 180°C 12-log sterilization cycle which has been proven effective by a third-party test lab. [4] Humidity is provided by a covered, protected water reservoir which is easily drained and fully opened for easy cleaning and disinfection. All interior surfaces are electropolished to reduce microscopic structures where microorganisms could attach and to provide enhanced chemical resistance. The exterior casing is sealed, brushed stainless steel with ingress protection (IP) 54 rated electronics. A unique Active Particle Control system captures particles that would otherwise be emitted to the cleanroom.

Results Summary

Microbial ingress testing verifies sterile fluid path integrity after rigorous microbial challenge
For microbial ingress testing, a very small bacterium, *Brevundimonas diminuta*, is considered an ideal challenge. *B. diminuta* is a highly motile, gram-negative bacterium. Due to its small size (0.3 – 0.6 µm), it is the preferred indicator organism for testing filter integrity and pore size.

As described in the Methods, three test G-Rex bioreactors, three negative control bioreactors, and three positive control bioreactors were prepared. All bioreactors were immersed in the challenge solution and placed in an incubator for 14 days. After incubation, the bioreactors were examined for the presence or absence of bacterial growth. All three test bioreactors showed no bacterial growth after 14 days of incubation under test conditions.

After the completion of the 14-day test, no growth was observed in the nutrient broth tested from the test bioreactors (Table 1), confirming the results of the microbial ingress testing, and verifying sterile fluid path integrity despite full immersion in the bacterial challenge solution.

Similar microbial ingress testing has been performed according to the protocol described above for all available sterile Closed System G-Rex models resulting in no growth in any of the test bioreactors. These results confirm that the G-Rex bioreactors are a closed system.

Figure 1A. Loading the bioreactors: 12 Liters of challenge solution were filled into the lined container and the bioreactor completely immersed except the vent filter.

Figure 1B. During the test: The bioreactors were weighted to ensure full immersion for the complete 14-day test.

Table 1. Microbial ingress test show no growth: None of the tested bioreactors showed any growth, indicating that they passed the microbial ingress test challenge and demonstrating that the G-Rex bioreactors operate as a closed system.

	Replicate 1	Replicate 2	Replicate 3
Test Bioreactor — Pre-Subculture	no growth	no growth	no growth
Test Bioreactor — Post Subculture	no growth	no growth	no growth
Positive Control	growth	growth	growth
Negative Control	no growth	no growth	no growth
Growth Promotion	growth	growth	growth

Membrane viral penetration testing confirms non-porous membrane structure and no viral penetration
G-Rex bioreactors include a highly gas-permeable membrane comprised of thin silicone rubber. According to Fick's law of diffusion [5], gas molecules diffuse through the non-porous membrane's molecular structure into the liquid medium inside the bioreactor. The oxygen consumption rate of cells at the bottom of a static flask easily exceeds the diffusion rate of oxygen through the overlying culture medium.[5] Importantly, gas diffusion through the membrane surface at the bottom of the bioreactor negates reliance on oxygen diffusion at the gas-liquid interface above the medium inside the bioreactor for sufficient oxygen delivery to cells. Unconstrained by height, enough media can be present in the device at onset of culture to completely eliminate the need for medium exchanges.

The membrane material at the bottom of the bioreactor was tested for viral penetration according to ASTM F1671 (Figure 2). This test method is intended to evaluate blood-borne pathogens of major concern, including hepatitis B virus, hepatitis C virus, and human immunodeficiency virus. The Phi-X174 bacteriophage has the following attributes: it is a non-enveloped 15-27 nm virus with an icosahedral or nearly spherical morphology, excellent environmental stability, a limit of detection which approaches a single virus particle, grows rapidly, and can be cultivated to reach high titers.

Figure 2. Membrane viral penetration test set-up: The test membrane was loaded into the test apparatus, then the bolts tightened to seal the perimeter. The test reservoir was filled with the bacteriophage challenge suspension and pressurized. Following the venting, the membrane surface was visually inspected for liquid penetration. Then each test reservoir was tested for the bacteriophage.

Table 2. Viral Penetration Tests show no penetration. No bacteriophage was found in any of the 32 test samples, providing further evidence that the G-Rex bioreactors are a closed system.

Test Article Number	Pre-Challenge Concentration (PFU/mL)	Post-Challenge Concentration (PFU/mL)	Assay Titer (PFU/mL)	Visual Penetration	Test Result
1-32	$2.5x10^8$	$3.0x10^8$	<1[a]	None Seen	PASS
Negative Control	$2.5x10^8$	$3.0x10^8$	<1[a]	None Seen	Acceptable
Positive Control	$2.5x10^8$	$3.0x10^8$	TNTC1[b]	Yes	Acceptable

[a]A value of <1 plaque forming unit (PFU)/mL is reported for assay plates showing no plaques.
[b]TNTC = PFUs were too numerous to count.

As shown in Table 2, all 32 test articles passed viral penetration testing, no liquid penetration was observed, and no (<1) plaque-forming units (PFU/mL) were reported for each test sample matching the negative control assays and confirming no viral penetration of the membrane.

These test results support closed-system G-Rex bioreactors in cell and gene-modified cell therapy manufacturing processes in low-cost and lower-grade cleanrooms. Additionally, because G-Rex bioreactors are structured with sufficient wall heights to contain medium at unconventional and uniform volumes above the gas-permeable cell growth surface, immune cells can expand from a minimum cell density per square centimeter of gas-permeable cell growth surface area to a maximum cell density per square centimeter of gas permeable cell growth surface area without medium change. An optimal ratio of 10 mL of medium per square centimeter of cell growth surface area eliminates all interventions during expansion, including medium exchanges or cytokine spikes, further reducing contamination risks and simplifying cell manufacturing processes [6].

CultiMaxx shelving increases G-Rex capacity without compromising incubator performance

CultiMaxx Shelving is specifically designed to increase the incubator chamber capacity from 4 to 10 G-Rex 500M-CS units. Like the rest of the chamber, these shelves are electropolished to increase cleanability and reduce areas where microorganisms could attach. Due to the modified shelving configuration, we wanted to determine effects on environmental uniformity throughout the incubator chamber, because reactive T cells and NK cells are demonstrably affected by culturing conditions. [7]

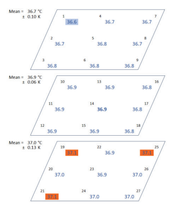


Figure 3. Temperature uniformity with CultiMaxx shelving for G-Rex units remains within specifications of +/- 0.3 °C. For the G-Rex specialized CultiMaxx shelving, Heracell Vios incubator shelves have been extended and the lowest shelf sits lower in the incubator chamber compared to the standard shelving. Conceivably these changes could negatively affect the uniformity of culturing conditions. When testing the new shelving in an empty Heracell VIOS incubator at 27 points according to DIN 12880, results show that the uniformity specification of +/- 0.3°C is maintained, similar to the standard shelving (results not shown). The temperature uniformity is shown to be +/- 0.25 °C and the temperature fluctuation was 0.22 to 0.26 °C

Conditions recover quickly following a long door opening, even with 10 G-Rex bioreactors in the chamber simultaneously. Especially for primary patient immune cells, it is important that culturd cells spend their maximum time at their ideal conditions [8]. Because incubator recovery to set conditions following a door opening could be affected by size, type, and placement of large culture vessels, we tested recovery of each parameter following a sixty second door opening with ten filled G-Rex 500M-CS in place in the incubator chamber. Results show that all parameters recover quickly. Recovery is similar to the 10-minute or less performance specification for the standard Heracell Vios incubator (see figures 4, 5 and 6), which is based on a 30-second door opening. We have defined recovery as 98% of the set value.

Figure 4. Temperature recovers in 5.3 minutes from a 60-second door opening. With the Heracell Vios CO2 incubator and CultiMaxx shelving filled with ten G-Rex 500M-CS units, each containing 5 L of water, both incubator doors were opened for sixty seconds. The temperature recovered to the set conditions of 37 °C in 5.3 minutes.

Figure 5. CO_2 gas recovers in 5 minutes from a 60-second door opening. With the Heracell Vios CR CO2 incubator and CultiMaxx shelving filled with ten G-Rex 500M-CS units, each containing 5 L of water, both incubator doors were opened for sixty seconds. Carbon dioxide recovered to the set 5% CO_2 in 5.0 minutes.

Figure 6. Humidity recovers in 15 minutes from a 60-second door opening. With the Heracell Vios CR CO_2 incubator and CultiMaxx shelving filled with ten G-Rex 500M-CS units, each containing 5 L of water, both incubator doors were opened for sixty seconds. Relative humidity recovery following a sixty second door opening was 15 minutes, longer than our standard specification of 10-minute recovery from a 30-second door opening. Here,in 10-minutes, humidity recovery has reached 95% of the "Low Humidity" parameter of 90%. The slower recovery here is is due to the "Low Humidity" setting being switched on, rather than the standard high humidity with faster recovery. A shorter door opening or the standard high humidity setting would speed humidity recovery.

Results Summary

Vios CR Incubator protects cells with proven contamination control technologies

Many CO_2 incubators today offer features to help limit contamination inside. However, there is a wide range of efficacy of these technologies. For cell therapy manufacturing, proven technologies should be employed.

HEPA filtration
It is a common misconception that HEPA filtration only captures particles 0.3 µm or larger. This stems from the Most Penetrating Particle Size (MPPS) classification. Different physical processes are involved including impaction, interception and diffusion [9] such that for an H13 HEPA filter, the MPPS is 99.97% efficient at particles of 0.3 µm but as shown in Figure 7, smaller and larger particles are captured with higher efficiency approaching 100%.

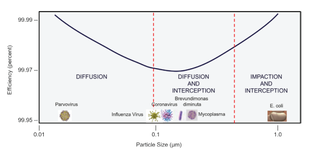
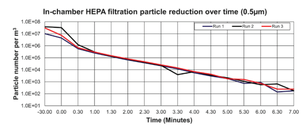


Figure 7. In-chamber HEPA filtration captures all particles regardless of size. In an H13 HEPA filtration system, all particles are captured regardless of size. The MPPS is 99.97% at 0.3 µm, meaning smaller particles including the smallest viruses and mycoplasma bacteria are also captured with even greater efficiency.

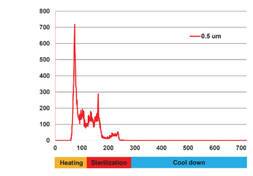
A HEPA filtration system is driven by a circulating fan. Thermo Scientific™ THRIVE ™ Active Airflow system works with the H13 HEPA filter to clean the air over time, as a dilution, where the entire chamber air volume is passed through the HEPA filter every sixty seconds. We wanted to ensure that the Heracell Vios 250i CR CO_2 incubator chamber reaches ISO Class 5 cleanroom conditions in 5 minutes after a 30 second door opening. Normal indoor room air is ISO Class 8-9, ISO Class 8 = 3.5 x 106 particles of 0.5 µm or larger per cubic meter of air. A Grade B cleanroom is equal to ISO Class 5 when at rest, ISO Class 7 in operation. These tests were conducted in an ISO Class 7 room at 23 °C. Particles were injected in the chamber to equal approximately ISO Class 8-9. Samples were taken according to ISO 14644-1. [10,11] The results show that conditions inside the chamber reach ISO Class 5 conditions in about 5 minutes and continue to get cleaner over time. This system helps to protect cultures from any microorganisms entering the incubator when the doors are opened.

Figure 8. In-chamber HEPA filtration provides ISO Class 5 conditions in 5-minutes after a door opening. Representative test results showing that in the Heracell Vios 250i CO_2 incubator, the in-chamber HEPA filtration combined with the THRIVE Active Airflow generates ISO Class 5 conditions in five minutes following a thirty second incubator door opening (both doors) and the air continues to get cleaner over time, reaching less than ISO Class 4 conditions in seven minutes.



Cleanroom compatibility certification
An estimated 70% of particulates in a cleanroom come from the staff, and an estimated 15% comes from the process equipment.[12] As that process equipment is heated, more particles are shed into the air. For this reason, a CO2 incubator with a high temperature sterilization cycle represents a greater risk and should be certified for use in a cleanroom. Heracell Vios CR CO2 incubators include an exhaust filtration system that protects the cleanroom even during sterilization. They are certified compatible with EU Grade A/B, ISO Class 5 cleanrooms by an industry specialist. This is discussed further elsewhere. [12] As shown in Figure 9, the particles sized 0.5 µm or larger given off during the 12-hour (720 minutes) sterilization cycle fall below ISO Class 5, which has a limit of 3520 particles/m3 0.5 µm or larger. During normal operation at 37°C, the incubator is also certified for use in ISO Class 5 conditions.

Figure 9. The unique Active Particle Control filtration system limits particle emissions in a cleanroom even during sterilization. Representative test results show particle emission 0.5 µm or larger released from the Heracell Vios CR CO2 incubator during the Steri-Run 180 °C sterilization cycle. Results show that at all times during the 12-hour sterilization cycle, the device is certified for use in an ISO Class 5 environment. Tests were repeated three times by an independent industry specialist, TÜV SÜD (Munich, Germany).

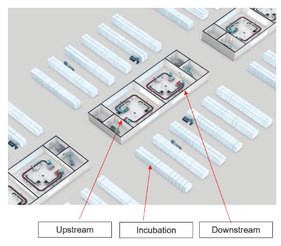


Closed system G-Rex bioreactors in combination with Heracell Vios CR CO_2 Incubators enable simplified and efficient cGMP cell therapy manufacturing

Figure 10. Stacked Heracell Vios CR Incubators allow the production of 400 billion cells in about 3 square feet. The results of this testing tangibly demonstrate the possibility of high throughput parallel processing of cell and gene-modified cell therapies. The modular approach is easily automated and scaled in a highly efficient facility layout. Without intervention, G-Rex bioreactors regularly achieve 40 million cells per square centimeter of gas-permeable cell growth surface area [13]. Each G-Rex 500M-CS can produce 20 billion cells, and each Heracell Vios CR CO2 Incubator can hold 10 G-Rex 500M-CS. Thus, when the incubators are stacked, up to 400 billion cells can be produced in just over 3 square feet of floor space. This predictability enables repeatable, robust, and low-risk cell production for large-scale allogeneic and/or autologous processes.

Figure 11. Novel facility layout enabled by combined technologies. A scalable, low-cost, and high-throughput manufacturing facility layout is now possible. Production set-up can occur in a small cleanroom, which houses upstream cellular processing equipment and reagents for high throughput closed system cell production set-up (i.e., apheresis wash, media fill, activation, etc.) and closed system inoculation of G-Rex bioreactors, followed by transfer of G-Rex bioreactors to a separate incubation room, which houses numerous incubators for parallel expansion processes with no risk of bacterial or cross-contamination.

Upon completion of the expansion phase, closed system G-Rex bioreactors can be removed from the incubators and moved to a cleanroom dedicated to downstream processing. The downstream cleanroom houses downstream cell processing equipment and reagents for cell harvest, final formulation, and fill processes. The resulting facility will produce significantly more doses in a smaller space than conventional facility designs.



Conclusions

Concise statement of the findings, indicating future research directions.

- G-Rex bioreactors are shown to operate as a closed system using multiple tests including microbial ingress testing and viral penetration testing.
- Heracell Vios CR CO2 Incubators are shown to retain their specified uniformity and recovery specifications even when filled with 10 G-Rex bioreactors,and protect cells with ISO Class 5 conditions inside the chamber, and are certified for use in ISO Class 5, EU GMP Grade A/B cleanrooms environments.
- Heracell Vios CR CO2 incubators can hold up to 10 G-Rex 500M-CS bioreactors, producing up to 400 billion cells in a small footprint. Closed System G-Rex in combination with Heracell Vios CR incubators enable novel facility design for high throughput and easily automated cell therapy manufacturing.

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Contact us: info@scaleready.com

ScaleReady is a Joint Venture formed by Bio-Techne, Fresenius Kabi, and Wilson Wolf. Combining selected offerings from the three partners, the ScaleReady manufacturing platform combines tools and technologies for cell culture, cell activation and expansion, gene editing, and cell processing.

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