The Rotary Cell Culture System™

RCCS-1SC through 4SCQ

OPERATION MANUAL

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RCCS-4SCQ

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1.0 General Introduction to the Rotary Cell Culture System

Synthecon, Incorporated is the industry leader in the design and manufacture of the "Rotary Cell Culture system", (RCCS)—a unique line of bioreactors designed for the creation and propagation of three-dimensional tissue assemblies and fragile, hard-to-cultivate cells. The RCCS is a new, advanced technology for the growth of cells that has many advantages:

Fragile cell lines can be cultured that cannot grow in other systems

Cells can be grown with or without solid supports (scaffolding, microcarrier beads)

Versatility—over 50 different cell types have already been successfully cultivated

Standard culture medium formulations for cell growth are successfully used

Cells exhibit unique properties in the RCCS;
increased gene expression
enhanced production of bioproducts
spontaneous formation of 3D tissue assemblies
spontaneous differentiation of some cell types
propagation of mono- or co-cultures

The RCCS rotates the cell culture chamber horizontally to constantly suspend the inoculated cells in the culture medium. The RCCS provides an exceptional cell culture environment that enhances cell and tissue growth through:

minimal shear forces,
absence of air bubbles in the zero head space cell culture vessel,
high mass transfer of nutrients,
effective waste removal,
efficient oxygenation.

The RCCS-4SC is composed of the following parts shown in *Figure 1*.

Culture vessels- Autoclavable clear acrylic circular units with two sampling or injection Luer lock ports and a ½" drain/fill port. Vessels are available in 1, 2, 4 and 10 ml volume capacities.

Rotator Base- serves to support and rotate the culture vessel. This model has 4 rotation stations with a single speed control. It is constructed of a white Delrin/Acetyl plastic plate.

Power supply- control box containing the electronic motor speed controls. The front panel dial is used to adjust vessel rotation speed. A flat, multicolored, ribbon cable connects the rotator base to the power supply control box. CAUTION: THE CONTROL BOX SHOULD NEVER BE PLACED INSIDE AN INCUBATOR.

Figure 1: RCCS-4SC
Rotator Base, Vessels & Power Supply

2.0 READ BEFORE USING-LIMITED WARRANTY, LIMITED LIABILITY

SYNTHECON, INC. warrants that, for three hundred sixty-five days (365) days, under normal operating conditions and use, this equipment will be free from defects of materials and workmanship. SYNTHECON, Inc. will repair or replace defective parts at our option provided the customer returns the equipment to SYNTHECON, INC. immediately upon discovery of such a defect.

In no event shall SYNTHECON, INC. or its suppliers be liable for any indirect, special, or consequential damages, including but not limited to, loss of cells, medium, data, labor or equipment incurred by the purchaser or any third party arising from the use of, or inability to use this equipment. Alteration of the equipment voids the warranty on this equipment. In no

case shall SYNTHECON, INC. be responsible for any modifications or alterations to this equipment performed by anyone other than SYNTHECON, INC.

☑ The RCCS is currently intended for **RESEARCH USE ONLY**.

☑ ***Caution: performing any of the following can invalidate the warranty.***

- ☒ ***Do not*** attempt to *reuse* or resterilize disposable vessels.
- ☒ ***Avoid*** creating a vacuum within the vessel by forcefully pulling back on a syringe attached to a port ***without both ports being open and a compliant syringe containing culture medium on the second port. The plunger on the compliant syringe must be gently pulled while the plunger on the other, medium containing syringe is pushed.*** Failure to follow this procedure can break the oxygenation membrane and render the vessel unusable.
- ☒ Use of *corrosive chemicals* such as chromates will damage the parts and *abrasive cleaners* or strong organic compounds such as acetone will destroy the plastic and void all warranties.
- ☒ ***Do not*** autoclave the rotator base (see Figure 1).
- ☒ Storage of the rotator base in an incubator **while NOT in use** will corrode the motor eventually resulting in loss of function. ***Synthecon reserves the right to make discretionary determination of the cause of damage with returned rotators and deem whether the repair is covered under the limited warranty.***
- ☒ Placing the power supply/control box inside an incubator will result in loss of function. The flat ribbon power cable easily passes through the cell culture incubator door seal without compromising the interior incubator environment. The power supply can be conveniently located on top of or on either side of the incubator.

3.0 Getting Started- Unpacking and Inspection

The RCCS is carefully packaged for shipment to ensure the arrival of an intact, functional unit. Unfortunately, on rare occasions, the RCCS may incur some damage during handling by the freight carrier.

- ✦ Upon receipt, carefully unpack the system and visually inspect each component closely for visible or concealed damage.
- ✦ IF DAMAGE IS EVIDENT OR SUSPECTED, DO NOT ASSEMBLE OR OPERATE THE UNIT. Please call Synthecon, Inc. at (800) 853-0740 if you are in the USA. For assistance outside the USA, please call your closest distributor **listed in section 10.0 of this manual.**

4.0 Culture Vessel preparation required before culture initiation

1. Prepare the 10 or 50 ml vessel by rinsing with sterile culture medium (serum free is acceptable) or sterile phosphate buffered saline using the the fill port (see Figure 2). All procedures should be performed under sterile conditions in a laminar flow hood.

   Unscrew the one half-inch diameter fill port and place on a sterile alcohol prep pad. Fill the vessel by pipetting the medium through the open fill port until full. Replace the fill port cap. Wipe any medium that is spilled on any surface with alcohol preps and/or 70% ETOH.

   Allow the vessel to incubate for 5 minutes to overnight in the rinsing solution.

   Discard the solution used for rinsing before inoculating cells. Using the same procedure as above, open the fill port and use a sterile Pasteur pipette attached to a vacuum source to aspirate all of the medium. ***Take care not to puncture the membrane at the back of the vessel with the sharp end of the pipette.***

2. Clean any spilled media from the rotator base and/or culture vessel using 70% ETOH as this can encourage the growth of bacteria and fungi.

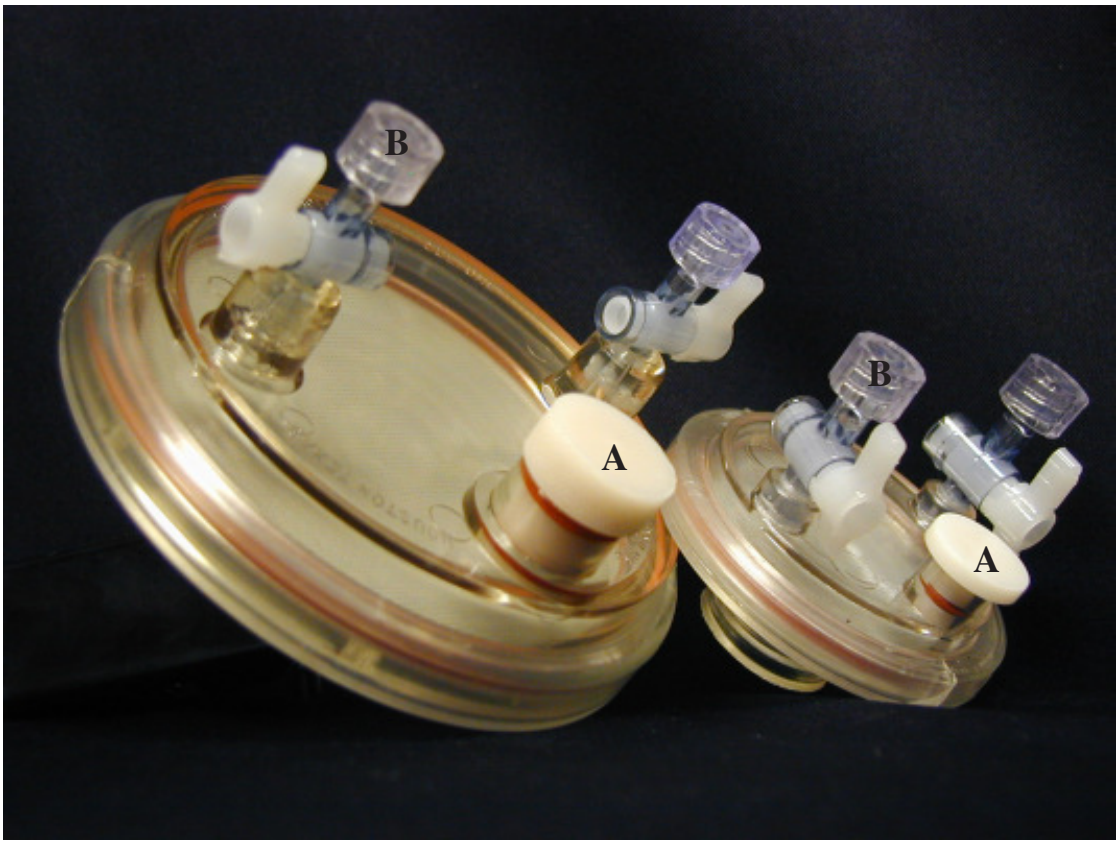


Figure 2: 50 & 10 ml Disposable Vessels
Fill Port - A; Syringe Ports - B

5.0 Cell culture Initiation

1. No special medium formulations are required for the growth of cells in the RCCS. Each cell type and application is unique. Cell culture medium formulations that you have previously used successfully with other cultivation methods (i.e., petri dishes, flasks, roller bottles, etc.) have generally been found to be appropriate for the RCCS. See the Bibliography (Section 11) and SYNTHECON website www.synthecon.com (bibliography periodically updated) for medium formulations successfully used in the past with the RCCS.

2. If microcarriers are to be used in growing the cells, these must be prepared and sterilized in advance according to the manufacturer's instructions. For the RCCS, a suggested starting concentration for microcarrier beads is 5 mg/ml of Cytodex beads.

3. Prepare single cell suspension according to the methods routinely used in your laboratory and specified for the cell lines being harvested. Obtain estimates of cell number using a Coulter counter or hemocytometer. **Culture vessels should be inoculated with a minimum of 200,000 cells/ml.** ***Please refer to Table 1 (below) and the bibliography included in this manual for example cell concentrations and additional information regarding cell concentrations and culture conditions that have been previously successfully used*.**

4. Once the desired cell concentration suspension has been prepared, it can be pipetted into the vessel through the fill port. Additional media is added until the vessel is nearly full and the fill port cap is replaced. Note that the lip of the fill port and fill port cap should be wiped with an alcohol swab to prevent contamination. Any solid support structures should also be introduced into the bioreactor at this time if being used.

   Microcarriers and small pieces of scaffolding material (1-3 mm) can be injected with the cell suspension using a syringe.

   Larger pieces of scaffolding material are best introduced through the fill/drain port.

5. After replacing the fill port cap there will be a bubble remaining which must be removed. This is done be placing a syringe on each syringe port, one of which has 2-3 ml of media in it. The ports are opened and the bubble is maneuvered underneath the port with the empty syringe attached. The bubble is pulled into the empty syringe while approximately the same volume of media is injected through the other syringe port. When all bubbles are removed, the valves are closed and any residual media is removed with a Pasteur pipet. The valve stems and covers are wiped with alcohol swabs and the covers replaced.

6. Attach the RCCS onto the rotator base by *slowly* turning in a clockwise direction. Then, place the RCCS and rotator base into the cell culture incubator. Alternatively, the RCCS can be attached to the rotator base while it is in the incubator. Turn on the power supply and make certain that the multicolored ribbon cable is attached to both the power supply and the rotator base.

NOTE: Make certain that the incubator is properly humidified (some incubators are designed with water trays in the bottom that must be filled). This will prevent excess medium evaporation from the vessels which can cause bubble formation.

Incubators should also be cleaned regularly according to manufacturer's instructions to reduce the risk of contamination.

7. Rotation Rate- initial settings

For anchorage dependent cells with microcarrier beads or scaffolding, begin rotation of the vessel at a speed of about 10-12 rotations per minute (RPM). Cells will attach to the microcarrier beads while the vessel is rotating. Attachment is usually complete within 24 hours after culture inoculation.

Sit and Spin Cycles—An alternative procedure that can enhance cell attachment in some cases is as follows. After placing the inoculated vessel on the rotator base in the incubator, allow it to rotate for 5 minutes to thoroughly mix the cells. Stop vessel rotation for 5 minutes. Rotate vessel for 5 minutes. Stop vessel rotation for 5 minutes. Repeat cycle one more time and then allow vessel to rotate continuously.

For cells not requiring solid support structures (microcarrier beads or scaffolding) such as tumor cell lines and lymphocytes, a beginning rotation rate of 8-10 RPM is recommended.

For minced particles of primary tissue, the rotation rate must be determined empirically due to size variations of the tissue pieces. Adjust the rate until the minced pieces remain suspended in the culture medium and do not hit the vessel wall.

8. Rotation rate- essential need for adjustment with culture time

The rotation speed must be adjusted with culture time as most types of cells form aggregates and the aggregates gradually enlarge. This results in an increased sedimentation rate of the aggregates within the culture vessel. Without adjustment, the cells will rapidly sediment toward the bottom and contact the walls of the vessel. This is detrimental to cell growth.

Adjust the rotation rate until the visible cell aggregates form a fluid orbit within the vessel, exhibit continual free fall, and do not contact the vessel wall. Rotation rate may need to be adjusted several times during the course of an experiment as cell cultures grow to form larger aggregates.

9. Monitoring Cell Cultures

It is recommended that that cultures be monitored daily to assess the condition of the cells and ensure the absence of contamination. Samples of cells can be removed using a

syringe and the syringe port in the culture vessel. This can be performed while the RCM is rotating in the incubator if desired or the RCM can be removed and taken to a laminar flow hood for this purpose.

During cell culture, multicellular tissue assemblies are often formed. As tissue assemblies increase in size, the rotation rate of the culture vessel should be gradually increased to keep them in suspension inside the culture vessel and avoid hitting the wall of the vessel.

6.0 *Replenishing Culture Medium*

As cell growth occurs with time, the culture media must be replenished. The time interval required before the medium needs to be replenished varies with the number of cells inoculated as well as cell type and *must be determined specifically for the cells and conditions you are using*. It is recommended that the pH, dissolved oxygen, and dissolved CO_2 be monitored using a blood gas analyzer if available.

1. Turn off the power to the rotator base. Remove the culture vessel from the rotating base by slowly turning in a counterclockwise direction. Restart rotation if using a multi-vessel rotator base.

2. Transport vessel to a sterile laminar flow hood. Remove the fill port and syringe port caps and place them on sterile alcohol pads.

3. Prop the vessel at approximately a 45 degree angel with the fill port at the highest point and allow the aggregates or microcarriers to settle into the opposite end of the vessel from the fill port. (A Styrofoam test tube rack can be used to prop the vessel) Open the fill port and remove as much media as possible with a Pasteur pipet attached to vacuum source without disturbing the cells. Approximately 2/3 of the media can be removed with this procedure. The vessel can then be placed flat and fresh media added. The fill port cap is replaced and the bubbles removed as in step 5 of **Cell Culture Initiation.** In some cases, such as single cell suspension cultures, cells will settle very slowly. In such a situation, it may be preferable to remove all the media and cells from the vessel, spin down the cells in a centrifuge, remove the spent media and resuspend the cells in fresh media and then reintroduce them into the vessel.

4. After completion of medium change, remove bubbles as detailed above and replace vessel on the rotator base in the incubator

Table 1-Selected list of type and number of cells successfully inoculated into rotary culture systems

Cell Type	Cells/ml Inoculated	Reference
Chondrocytes	5-6 X 10^5	Baker & Goodwin, 1997
Human intestine mesenchymal	2 X 10^5	Goodwin et al., 1993
Thyroid	4 X 10^5	Martin et al., 2000
Rat PC12	5.5 X 10^5	Lelkes et al., 1998
LNCaP human prostate	2 X 10^5	Zhau et al., 1997
LN1 mixed mullerian human ovarian cancer	2 X 10^5	Goodwin et al., 1992
Human cervical primary tumor	2 X 10^5	Chopra et al., 1997
16 different tumor cell lines	5-20 X 10^5	Ingram et al., 1997
MIP 101 human colon cancer	3 X 10^5	Francis et al., 1997
HepG2 human hepatoblastoma	1 X 10^6	Khaoustov et al., 1999
HT-29 colon adenocarcinoma	2 X 10^5	Goodwin et al., 1992

7.0 *Sampling Procedures*

It is desirable to periodically (daily is recommended) check the cell culture to monitor cell morphology, aggregation, ensure absence of contamination, glucose utilization, gases, etc.

1. **To take a cell and/or medium sample from the culture vessel, an empty syringe and media-filled syringe must be connected to the Luer lock ports.**

   This can be accomplished while the vessel is rotating to maintain a homogeneous sample.

   Remove the syringe port caps and place on a sterile alcohol swab.

   Wipe the ports with an alcohol swab, attach a sterile empty sampling syringe (1, 5, or 10 ml size) to one port and syringe with culture medium to the other port (10-20 ml size).

   Open both syringe port valves, turn on the power to allow the cells and/or tissue aggregates to become evenly distributed (2 min).

   Push medium into the vessel from the filled syringe into the vessel. A light pull on the sampling syringe will facilitate sample collection. Since the vessel is still rotating, this takes dexterity and may require some practice but provides a homogeneous, representative sample.
2. **Alternatively, a sample may be taken with the vessel removed from the rotator base.** With Luer lock syringes attached, slowly turn and rotate the vessel to evenly

distribute the cells, tissue aggregates, or explants within the vessel. Open both sample ports, and withdraw a sample as described above.

After removing the sample, close both syringe ports and remove the syringes. Place the syringe cap on the sampling syringe. Wipe the port with a sterile alcohol swab and replace the caps.

8.0 *Troubleshooting*

Problem	Possible cause/solution
Bubbles present in cell culture vessel	Incubator could be dry causing excessive evaporation Make certain that the trays in the bottom of the incubator are clean, filled with water and/or the humidifier system is functioning properly.
Culture medium leaking from vessel or observed on the rotator base	Check that valves are closed and fill port securely closedCheck that oxygenation membrane is not leaking in the back of the vessel
Vessel rotation is inconsistent	Check that the settings on the power supply are correct—make certain that the toggle switch is set for the correct rotation speed; i.e., high or low depending on the rotation rate.Check that the multicolored ribbon cable is fully inserted into both the connection to the rotator base and the power supply.
Vessel will not turn	Check that the power supply is plugged into a functional electrical outlet, the ribbon cable is securely connected between the power supply and the rotator base, and that the power switch is on

9.0 Example Cell Culture Protocols for first time users

It is often instructional for first time users of the system to become acquainted with the RCCS by first using the system with an established protocol before proceeding to actual experiments.

If microcarriers or scaffolding will be used, they should be prepared first for inoculation into the vessel *according to the manufacturer's instructions*. An example protocol for microcarrier beads is provided below.

Pharmacia Cytodex 3 Microcarrier Beads

The properties of these beads are as follows: a density of 1.04 g/ml, 175 μm size, a swelling factor of 15 ml/g dry weight, and 3 X 10^6 microcarriers/g dry weight.
Cytodex 3 consists of a layer of denatured collagen coupled to dextran beads. It is the microcarrier of choice for cells known to be difficult to grow in culture, for differentiated cell culture systems and cells with an epithelial-like morphology. It is commonly used as a general purpose microcarrier.

1. Weigh 1 gram of the dry microcarrier beads on a suitable balance and place into a 100 ml clean glass bottle. (Bottle should be pretreated with a siliconizing agent according to the manufacturer's instructions or excessive loss of beads will occur due to adherence to the glass).

2. Add 50-100 ml of 1X Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) to the bottle and incubate at room temperature for at least 3 hours to allow the beads to swell.

3. Remove supernatant by aspirating with a Pasteur pipette and vacuum source.

4. Wash microcarriers once with gentle agitation for a few minutes with PBS w/o Ca^{2+} and Mg^{2+}. Use 30-50 ml/g Cytodex of PBS.

5. Discard PBS and replace with fresh PBS w/o Ca^{2+} and Mg^{2+} adding 30-50 ml/g Cytodex of PBS.

6. Sterilize by autoclaving at 115°C, 15 min, 15 psi. Make certain that the cap of the bottle is loose before sterilization. **CAUTION: If microcarriers are autoclaved at higher temperatures and/or longer time intervals, beads may turn brown and performance affected.**

7. Prior to use, sterilized microcarriers are allowed to settle, the supernatant removed by aspiration as above and microcarriers rinsed in warm 37°C culture medium (serum is not required in culture medium).

8. Microcarriers are now ready to use for culturing and are extremely stable. Cytodex that has been hydrated and sterilized as above can be stored sterile in PBS for at least two years at 4°C.

Practice Protocol for Cell Culture using Cytodex 3 microcarrier beads

Perform procedures in a biological safety cabinet (sterile tissue culture hood).

1. A commonly available cell line to use for practice is baby hamster kidney (BHK-21). These can be obtained from the American Type Culture Collection (Rockville, MD) or colleagues. Alternatively, the same protocol can be used with MCF-7, a breast cancer cell line, also available from ATCC.

2. Before beginning, obtain a presterilized Synthecon cell culture vessel (10 or 50 ml size) and rinse with culture medium or phosphate buffered saline as described in Section 4, step 1 above.

3. Remove BHK-21 cells from culture flasks or dishes: Remove culture medium, add 0.25% trypsin/0.075% EDTA, incubate for 3-5 minutes. Check at minimum time to determine if cells are detaching from flask/dish by observation with an inverted microscope. ***Be careful not to over expose cells to trypsin/EDTA as this can result in loss of cell viability.***

4. After determining that the cells are detaching from the culture flask/dish by observation in an inverted microscope, add sterile, pre-warmed DMEM culture medium (containing 10% fetal bovine serum and penicillin/streptomycin or other antibiotic).

5. Remove the cells from the culture flask/dish by vigorously pipetting the medium (with the assistance of a Pipet Aid device) across the entire cell growth surface at least 5 times. This ensures that all cells are removed and facilitates creation of a single cell suspension. Inadequate pipetting of the cell suspension will result in a clumpy cell suspension which will not produce optimum results.

6. Place cell suspension in a sterile tube. Determine cell number using either a Coulter counter or hemocytometer.

7. Inoculate cells at a final concentration of 2-4 X 10^5/ml and microcarriers at 5 mg/ml (4,000 beads/mg) according to instructions in Section 5.

8. Place inoculated vessel carefully onto the rotator base by slowing turning in a clockwise direction. Place rotator base in culture incubator if not already in that location. Make certain that the ribbon cable is connected between the rotator base and power supply and that the power supply is plugged in. Begin the vessel rotation at a speed of 10-12 rpm.

9. The day after culture initiation, take a sample according to the procedures described in Section 7 above. If available, use a Beckman glucose analyzer and blood gas analyzer to assess glucose use, dO2, dCO2, and pH. Part of the sample should be placed in a small petri dish or on hemocytometer for observation. Cells should be visibly attached to the beads and some beads may be aggregated together in groups of two or three.

10. Day 3- Change medium according to procedures in Section 6 above and take another cell sample. Cell viability can be checked using trypan blue dye exclusion assay (Goodwin et al., 1992).

11. Day 4- A mixture of bead aggregates ranging from 2-16 should be seen.

Practice Protocol for Cell Culture without solid support

1. Before beginning, obtain a presterilized Synthecon cell culture vessel (10 or 50 ml size) and rinse with culture medium or phosphate buffered saline as described in Section 4, step 1 above.

2. Remove cells from culture flask/dish as described in the protocol for microcarrier beads above.

3. Determine the number of cells/ml using a Coulter counter or hemocytometer. Inoculate 8-1.0 X 10^6 cells/ml into the vessel.

4. Place inoculated vessel carefully onto the rotator base by turning in a clockwise direction. Place rotator base in culture incubator if not already in that location. Make certain that the ribbon cable is connected between the rotator base and power supply and that the power supply is plugged in. Begin the vessel rotation at a speed of 10-12 rpm.

5. The day after culture initiation, take a sample according to the procedures described in Section 7 above. If available, use a Beckman glucose analyzer and blood gas analyzer to assess glucose use, dO2, dCO2, and pH. Part of the sample should be placed in a small petri dish or on hemocytometer for observation. Cells should have aggregated and formed loose aggregates.

6. Change medium on days 2 and 4. Loose aggregates will be observed to condense and may form rounded structures.

NOTES AND CAUTIONS

Please read before using

- **Please complete and return the Limited Warranty Sheet on page 29 immediately. The Warranty will NOT be valid unless it is signed and returned to Synthecon.**

- ***Do not soak any part of the vessel in bleach, acidic or basic cleaning solutions.*** The plastic and rubber in the vessels can be impregnated with toxic chemicals from such solutions, rendering them toxic to cell growth. Soaking in chlorine bleach is guaranteed to cause extended toxicity problems.

- Corrosive chemicals such as chromates will damage the metal parts. Abrasive cleaners or strong organic cleaning compounds such as acetone will destroy the plastic and void all warranties.

- *The oxygenation membranes are particularly susceptible to damage.* The oxygenator membrane is a very delicate component consisting of silicone rubber, 0.005 inches thick, covering the polyester cloth backing. Care and attention should be given to the membrane during cleaning, sterilizing and removal of culture material. Use extreme caution when cleaning the oxygenator membrane. Using latex laboratory gloves, use the tip of the finger to gently cleanse with a mild laboratory detergent.

- The cleaning methods described in the instruction section are sufficient to clean other parts of the vessel. If necessary a soft brush can be used if applied gently and carefully. Harsh cleaning agents and stiff cleaning brushes will damage the plastic and rubber components.

- *Storage of the Rotator Base in an incubator while not in use will result in damage to the rotator components. Synthecon reserves the right to make discretionary determination of the cause of damage with returned rotators, and deem whether the repair is covered under the limited warranty.*

- Limited access to the RCCS™ cell culture equipment is desirable. Many people find it very difficult to refrain from tampering with new, different, rotating equipment. Well intentioned meddling has destroyed several RCCS™ cultures.

- The flat power cable passes easily through the cell culture incubator door seal without compromising the incubator environment. The power supply can be conveniently located on top of, or beside the incubator. ***The power supply must never be operated inside of the incubator.***

- This equipment is not a roller bottle and must not be operated as a roller bottle. Air bubbles in the chamber will cause turbulence and kill cells. Please follow the following instructions. Good sterile technique is probably the only directly applicable roller bottle protocol that can be transferred to the RCCS™.

- Pulling a strong vacuum on the vessel by forcefully pulling back on the syringes could burst the oxygenator membrane and require a replacement of the membrane.

- ***Do not over tighten bolts and fittings***. Excessive force will strip the threads in the plastic components.

- An autoclave cycle of 121°C for 20 minutes is recommended and has been proven to be sufficient. If concerns still remain following this procedure, do not raise the autoclave temperature, simply repeat the autoclaving procedure again. Excessively high autoclave temperatures will rapidly degrade plastic and rubber components and cause the sample port valves to seize.

- Residues in dirty autoclaves may impregnate the oxygenator membrane, plastic and rubber parts and cause the vessel to become toxic.

- We recommend that the vessel be filled with absolute ethyl alcohol and allowed to sit overnight before autoclaving. The alcohol is an excellent nontoxic decontaminant and it also will dissolve and remove any residual plasticizers. The vessel should be emptied and rinsed with culture grade water and emptied before autoclaving.

- At least one valve must be open during autoclaving to preclude buildup of internal pressure.

- The central core assembly screw of the Slow Turning Lateral Vessel should be backed off one turn prior to and during autoclaving.

- The peripheral screws on the High Aspect Ratio Vessel (HARV™) should be released one turn prior to autoclaving.

- The sample and syringe port fittings should not be loosened prior to autoclaving.

- After autoclaving, be sure to tighten those screws which have been loosened.

- The vessel should be autoclaved as an assembled unit. This eliminates the risk of contamination during assembly.

- Rinse the vessel with medium prior to inoculation. Most researchers prefer to rinse the vessel overnight.

- Since medium formulations vary with cell type and application, no attempt is made to prescribe a preferred medium. In general, those media formulations which produce good results with other cell culture equipment, will work very well in the RCCS™. Those media will provide a good starting point for the development of specialized formulations for specific applications. The publications in the reference section at the end of this manual also provide information on several media formulations that have been used successfully in the RCCS™ equipment.

- The use of a humidified tissue culture incubator reduces culture medium fluid loss through the silicone rubber oxygenation membrane. This is particularly important with the small 10ml HARV™.

- Only luer lock syringes should be used. Vibration and rotation forces cause non-locking syringes to detach. This exposes the syringe fitting openings, results in spilled droplets of medium and can cause contamination.

- Bubbles cause turbulence which kills cells. Although small bubbles will form, they should be removed as soon as they are observed (at least daily), by drawing back on the port syringe.

- For the best distribution of cells throughout the culture vessel, the rotator base should be level. This is more important with the Slow Turning Lateral Vessels (STLV™) than with the High Aspect Ratio Vessels (HARV™).

- Fifteen to twenty RPM is a good starting point for anchorage dependent cells on microcarrier beads. Rotation speed should be adjusted to compensate for changes in the sedimentation rate of the culture. Visible cell clumps should form a fluid orbit within the vessel and not contact the wall or central core of the vessel.

- Suspension cells such as lymphocytes can be easily suspended with a very slow rotation rate of ten revolutions per minute.

- If very small minced particles of primary tissue are used to start a culture, microcarrier beads may not be required.

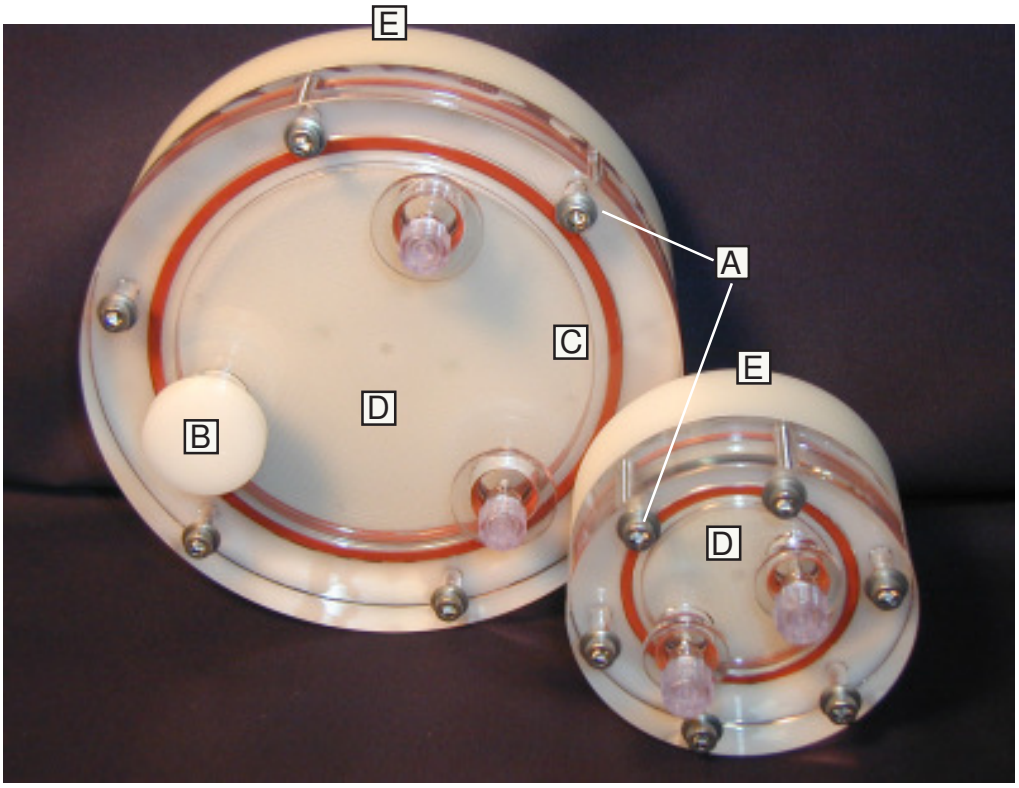
- Cells will readily attach to microcarriers while the vessel is rotating. It is not necessary or desirable to stop the vessel rotation during the attachment phase. Attachment of cells to microcarriers is normally complete within 24hrs. If rotation ceases, oxygenation stops and the cells will die.

- To obtain good representative samples, rotation should not be stopped during sampling. With a bit of practice, it is possible to manipulate the syringes and draw a sample while the vessel is rotating.

- Clean all media from the rotator base. Spilled media is corrosive and enables the growth of bacteria or fungus.

- The operation of electrical equipment inside an incubator causes additional heat. Your incubator should be calibrated according to the manufacturers instructions. Consult SYNTHECON™ in case of incubator overheating.

High Aspect Ratio Vessels



A. Peripheral Screws

B. 1/2" Fill Port (55ml only)

C. Syringe Ports

D. Front Plate

E. Back Plate

High Aspect Ratio Vessel Procedures

Microcarrier Preparation

If using microcarriers beads with anchorage dependent cells, prepare the microcarriers in accordance with the manufacturers instructions. One popular set-up in the RCCS™ is 5 mg microcarriers beads per ml media. It is estimated that 10 cells per bead are needed for efficient seeding of most cell types. (2.2×10^5 cell per ml). Cells will very readily attach to the microcarriers while the vessel is rotating. It is not necessary or desirable to stop the vessel rotation during the attachment phase. Attachment of cells to the microcarriers is normally complete within 24hr. Each cell and tissue type will have its own requirements, which must be individually assessed.

Vessel Preparation

- ***Note: Do not soak any part of the vessel in bleach, acidic or basic cleaning solutions. Vessels will absorb such solutions, rendering them toxic to cell growth. Abrasive cleaners or strong organic cleaning compounds such as acetone will destroy the plastic, void all warranties, and render the vessel useless. The cleaning methods described here are sufficient to clean the vessel properly.***

1. Using an Allen wrench, unscrew and remove the peripheral screws around the vessel. Gently separate the two halves to disassemble vessel.

- ***Note: it is not necessary or desirable to remove the silicone rubber oxygenator membrane from the back plate or the metal fittings and valves from the front plate.***

2. Place all pieces of vessel in 4-liter beaker filled with warm solution of mild detergent designed for tissue culture labware.

3. Scrub plastic parts with a soft bristle-brush as necessary to remove any residues.
4. Very gently clean oxygenator membrane with the tip of your finger using latex laboratory gloves.

- ***Note: Harsh scrubbing will damage membrane material. Do not use a brush to cleanse the membrane.***

5. Rinse vessel parts with continuous flow of ultra-pure water for 15-20 minutes.
6. Soak vessel parts in fresh ultra-pure water overnight.
7. Remove vessel parts from water and place on absorbent pads to dry.
8. Assemble vessel.
9. Fill unit with 70% ethanol and allow to soak for 24hrs.

- ***Note: The 10ml HARV™ does not have a ¼ inch port. With this vessel, all fluid transfer is conducted through the Luer-lock stopcock.***

10. Sterilize as described below.

Sterilization

1. Autoclave Method
   a. Empty vessel of 70% ethanol.
   b. Remove and dispose plastic valves and their caps. Remove fill port cap and autoclave seperate from vessel. Cover all ports with aluminum foil.
   c. Loosen peripheral screws (using Allen wrench) 1 turn.
   d. Wrap vessel and petri dish and autoclave for20min at 121°C. It is not necessary to slow-vent the autoclave.
   e. Remove from autoclave; cool to room temperature.

- ***Note: [1] These temperatures and times have been proven in actual use. Excessively high temperatures will rapidly degrade plastic and rubber components. [2] Toxic residues in dirty autoclaves may impregnate the plastic and rubber parts and render the vessel toxic and useless.***

   f. In a sterile environment, unwrap vessel, tighten peripheral screws, and install caps.

Cell Culture using High Aspect Ratio Vessel (HARV)

Vessel conditioning

1. If the peripheral screws have been loosened; in a sterile environment, gently tighten the screws with the Allen wrench.

- ***Note: Do not over tighten, as this will strip the threads in the plastic back plate.***

2. With a 10ml pipette, fill the 50ml vessel with medium of choice through the ¼ inch port.

2* With a 10ml syringe, fill the 10ml vessel through the syringe port.

3. Wipe port with sterile alcohol pad and attach sterile ¼ inch fill port cap if so equipped (50ml HARV™).
4. Close syringe port valves.

5. Fill a 10ml sterile syringe with growth medium. Wipe one syringe port with an alcohol pad and attach syringe.
6. Wipe the other syringe port with an alcohol pad and attach an empty 3 or 5ml sterile syringe.
7. Gently invert vessel and tap on sides to expel air bubbles from under the ports. Maneuver air bubbles under the empty syringe. With both valves open, gently press on the syringes to replace air bubbles with medium.
8. When all bubbles are removed, close the syringe valves and discard the small syringe. Wipe the port with an alcohol pad and replace the cap.
9. Leave the large medium-filled syringe on the unit with the valve open as the volume of the medium in the vessel will expand slightly as it warms to 37C.
10. Attach the vessel to the rotator base and place them in a humidified CO_2 incubator.
11. Attach the power cord to the rotator base and pass the flat cable through the incubator door seal. Attach the other end of the flat cable power cord to the power supply.

- ***Note: The power supply is always operated outside of the incubator.***

12. Insure that the rotator base is level.

13. Set the initial rotation speed to 15 to 20 rpm
14. Rotate the vessel overnight to check for leaks and sterility.

Experiment Start-up

1. Transfer the vessel to a sterile hood. Remove the end caps and place them on sterile alcohol pads or in sterile petri plates.
2. Aspirate medium from the 50ml HARV™ vessel through $^1/_4$ inch port with a sterile Pasteur pipette.

2* Aspirate the medium from the 10ml HARV™ vessel with a 10ml syringe through the luerlock port.

3. Fill the vessel to 50% of total volume with growth medium minus serum, allowing space to load cells and microcarrier beads, if used. (Serum addition at this time increases foaming and leads to difficulty in removing the air bubbles later).
4. Count cells to be used or mince primary tissue (ten 1mm pieces per 5ml of media).
5. Dilute the cells into separate container of medium to yield desired final concentration (2-3 x 10^5 per ml has been used by some authors).

6. Add appropriate amount of washed, prepared microcarrier beads (5mg/ml) to diluted cells.
7. With a 10ml pipette, load cell/bead/medium solution into the 50ml HARV™ through the ¼ inch port.

7* With a 10ml syringe, load cell/bead/medium solution into the 10ml HARV™ through the luer lock port.

8. Add the appropriate amount of serum and top off the vessel with medium.
9. Wipe the port with an alcohol pad, replace and tighten. Close the syringe port valves.
10. Fill a 10ml sterile syringe with growth medium. Wipe one syringe port with an alcohol pad and attach syringe.
11. Wipe the other syringe port with an alcohol pad and attach an empty 3 or 5ml sterile syringe. Open syringe valves.
12. Gently invert vessel and tap on sides to expel air bubbles from under the ports. Maneuver air bubbles under the empty syringe. With both valves open, gently press out the syringe to replace air bubbles with medium.
13. Discard the small syringe. Wipe the port with an alcohol pad and replace cap or new syringe.
14. Leave the large medium-filled syringe on the vessel with the valve open as the volume of the medium in the vessel may vary slightly with temperature.
15. Attach the vessel to the rotator base in a humidified CO_2 incubator. Check that the unit is level.
16. Turn power on and adjust to an initial rotation speed of 15 to 20 rpm for tissue particles or anchorage dependent cells on microcarriers or 10 rpm for suspension cell cultures.

- ***Note: Cells and cell aggregates should rotate with the vessel and not settle within the vessel nor should they collide with the edge of the vessel. When the speed is properly adjusted, the cells and cell aggregates will form a fluid orbit within the vessel. If suspension cells are being grown, slower speeds can be used and very little speed change will be required. If anchorage dependent cells are being cultured, the speed will need to be increased as the cell aggregate particles increase in size.***

Medium Renewal

1. Turn off power and immediately remove the vessel from the base and take it to a sterile environment (biological hood).
2. Let the cells or cell/bead aggregates settle to the bottom with the valves and ¼ port (50ml HARV™) rotated up.

- ***Note: Tissue particles and anchorage dependent cells on microcarrier beads settle quite rapidly. Most suspension cells settle very slowly.***

3. Open the valves.
4. Remove and discard any syringes that may be attached. Wipe ports with sterile alcohol pads.
5. Attach an empty 20ml syringe (10ml if using the 10ml HARV™)
6. Very carefully use the attached syringe to withdraw medium from the vessel. Usually ¼ to ½ of the conditioned medium is left in the vessel.
7. Use an appropriately sized syringe filled with warmed medium to very slowly inject fresh medium into the luer-lock port. (Do not disturb the cells or aggregate particles.)
8. Fill a 10ml (3 or 5 ml if using the 10ml HARV™) sterile syringe with growth medium. Wipe one syringe port with an alcohol pad and attach syringe.
9. Wipe the other syringe port with an alcohol pad and attach an empty 5 or 10ml sterile syringe.
10. Gently turn the vessel base down and tap on sides to expel air bubbles from under the ports. Maneuver air bubbles under the empty syringe. With both valves open, gently press on the syringes to replace air bubbles with medium.
11. When all bubbles are removed, close the syringe valves and discard the small syringe. Wipe the port with an alcohol pad and replace the cap or another small syringe for later sampling.
12. Leave the medium-filled syringe on the unit with the valve open as the volume of the medium in the vessel may vary slightly with temperature change.
13. Attach the vessel to the rotator base and replace them in the humidified CO_2 incubator.
14. Turn on the power and adjust speed as necessary.

- ***Note: As an alternate procedure with the 10ml HARV™, a 10ml syringe filled with medium can be attached to the luer-lock port to warm in place for the next medium change. An empty 10ml syringe is attached to the other port. At the next medium change, the warmed fresh medium in the syringe is very slowly and carefully injected into the vessel and spent medium is pushed into the other syringe. If the medium is injected too quickly, the cell culture that has settled to the bottom of the chamber will be disturbed and part of it will be transferred into the receiving syringe with the medium.***

Sampling Procedures

1. If a sampling syringe is not in place, stop the vessel rotation. Remove the syringe port cap and place it in a sterile petri dish. Attach a sterile empty 1, 3, or 5ml syringe to the valve. Both syringe port valves must be open.

2. Turn on the power to allow the cell/bead aggregates to be evenly distributed (2 Min).
3. Push medium into the vessel with the medium-containing 20ml (10ml if using the 10ml HARV™) syringe that is still attached from inoculation. It may also be necessary to pull on the smaller sampling syringe. This procedure provides a homogeneous representative sample, however, it may take some practice, as the vessel is still rotating.
4. When the desired sample has been drawn (usually 1-5 ml), turn off the power, close the valve in the sampling syringe port and remove the sampling syringe.
5. Attach another syringe and turn on the power, adjust speed if necessary.
6. If any bubbles are visible, turn off the power and utilize the bubble removal procedures provided in the prior sections.

- ***Note: Tissue particles to large to be drawn into a syringe can be removed with forceps through the fill port on the 50ml HARV™ (the 10ml HARV™ does not have a fill port) in some cases. Larger particles may require disassembly of the vessel.***

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