Rotofor® System

Instruction Manual

BIO-RAD

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Note

To insure best performance from the Rotofor cell, become fully acquainted with these operating instructions before using the cell to separate samples. Bio-Rad recommends that you first read these instructions carefully. Then assemble and disassemble the cell completely.

Bio-Rad also recommends that all Rotofor cell components and accessories be cleaned with a suitable laboratory cleaner (such as Bio-Rad Cleaning Concentrate, catalog number 161-0722) and rinsed thoroughly with distilled water before use.

Model_____

Catalog No._____

Date of Delivery_____

Warranty Period_____

Serial No._____

Invoice No._____

Purchase Order No._____

Warranty

Bio-Rad Laboratories warrants the Rotofor cell against defects in materials and workmanship for 1 year. If any defects occur in the instrument during this warranty period, Bio-Rad Laboratories will repair or replace the defective parts free. The following defects, however, are specifically excluded:

1. Defects caused by improper operation.
2. Repair or modification done by anyone other than Bio-Rad Laboratories or an authorized agent.
3. Use of fittings or other parts supplied by anyone other than Bio-Rad Laboratories.
4. Damage caused by accident or misuse.
5. Damage caused by disaster.
6. Corrosion due to use of improper solvent or sample.

For any inquiry or request for repair service, contact Bio-Rad Laboratories. Be prepared to provide the model and serial number of your instrument.

Section 1
General Information

1.1 Introduction

Bio-Rad's unique Rotofor System fractionates complex protein samples in free solution using preparative isoelectric focusing. The Rotofor system is designed for the initial clean up of crude samples and for use in purification schemes for the elimination of specific contaminants from proteins of interest that might be difficult to remove by other means.

The Rotofor cell provides up to 500-fold purification for a particular molecule in less than 4 hours. Because electro-focusing is carried out in free solution, fractions from an initial run can be easily collected, pooled and refractionated, resulting in up to 1000-fold enrichment for a particular molecule. Purification using isoelectric focusing is especially advantageous when protein activity needs to be maintained. Bioactivity is maintained because the proteins remain in solution in their native conformation.

The Rotofor cell incorporates a cylindrical focusing chamber with an internal ceramic cooling finger. Rotation at 1 rpm around the focusing axis stabilizes against convective and gravitational disturbances. Nineteen parallel, monofilament polyester screens divide the focusing chamber into 20 compartments, each holding one fraction. After focusing, the solution in each compartment is rapidly collected without mixing using the harvesting apparatus supplied with the unit.

The Rotofor system is designed to accommodate a range of sample volumes using interchangeable focusing chambers. The Mini Rotofor chamber is used for sample volumes of 18 milliliters containing micrograms to milligrams of total protein. The large Rotofor chamber is used for samples of 35 to 60 milliliters containing milligrams to grams of total protein.

The Rotofor cell is used to purify a wide range of proteins. These include monoclonal antibodies, cell surface receptor proteins, integral membrane proteins, cytosolic and secreted enzymes, chemotactic factors, and recombinant proteins. It has been used to separate isoenzymes, lipoproteins, and apolipoproteins.

Should a final purification step be required, we recommend the Model 491 Prep Cell. The Prep Cell is a continuous elution gel electrophoresis device that uses SDS-PAGE or Native-PAGE to completely purify individual proteins of interest. For examples of published Rotofor cell applications, please refer to the Rotofor Technical Folder (request Bulletin 1555A).

*Patent No. 4,588,492

1.2 Specifications

Construction

Focusing chambers	Acrylic
Vent buttons	Porous polytetrafluoroethylene (PTFE) membrane in molded plastic
Gaskets	Silicone rubber
O-Rings	Fluorocarbon elastomer
Cooling finger	Ceramic
Housing	Polycarbonate and acrylic
Harvest box and lid	Polycarbonate and acrylic
Tubing	Polyvinyl
Needle array	Stainless steel and acrylic
Electrodes	Platinum, 0.010 inch diameter
Membrane Core	Molded polyethylene with polyester membranes
Chemical compatibility	The Rotofor cell components are not compatible with chlorinated hydrocarbons (*e.g.* chloroform), aromatic hydrocarbons (*e.g.* toluene, benzene), or acetone. Use of organic solvents voids all warranties.
Shipping weight	9 kg
Overall size	45.7 cm (L) x 16.5 cm (W) x 22.8 cm (H)
Cell voltage limit	3000 VDC
Cell power limit	15 W
Cooling	The Rotofor cell must be run with cooling or excessive heating may occur, damaging the unit. A refrigerated circulating water bath is recommended to keep the coolant temperature at 4 °C.
Maximum coolant flow rate	12 L/minute
Minimum coolant flow rate	50 ml/minute
Sample volume	18–58 ml
Electrical connection	3 wire cord
Input power Requirements	120 V Model: 100-120 VAC, 50/60 Hz, 12W 240 V Model: 220-240 VAC, 50/60 Hz, 12W
Fuses	250 mA Type T (1 required, 1 spare)

1.3 Isoelectric Focusing

Isoelectric focusing (IEF) is a gentle, non-denaturing technique; antibodies, antigens, and enzymes usually retain their biological activities. IEF is also a high resolution technique capable of resolving proteins that differ in pI by fractions of a pH unit. IEF in the Rotofor has the added advantage that the proteins can be easily recovered once they are focused.

Separation of proteins by isoelectric focusing is based on the fact that all proteins have a pH-dependent net charge. The net charge is determined both by the amino acid sequence of the protein and the pH of the environment. When a protein is electrophoresed through an established pH gradient, it will migrate until it reaches the pH where the net charge on the protein is zero; at that point it will stop migrating and is said to be focused at its isoelectric point or pI.

Ampholytes which are small, charged buffer molecules are used to establish the pH gradients increasing in pH from anode to cathode. When voltage is applied to a system of ampholytes and proteins, all the components migrate to their respective pIs. Ampholytes rapidly establish the pH gradient and maintain it for long periods allowing the slower moving proteins to focus.

A protein with a net positive charge, for example, in a particular region of the pH gradient will tend to migrate toward the cathode while concurrently giving up protons. At some point, the net charge on the molecule will be zero and the protein will cease to migrate. If the protein diffuses into a region of net charge, the resultant electrical force on it will drive it back to its pI, so that the molecule becomes focused at that point.

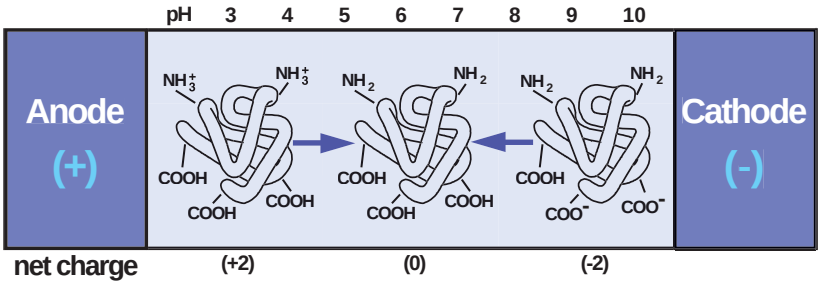

Fig. 1.1. Acidic Protein "Focusing" in a pH gradient.

1.4 Safety

This instrument is intended for laboratory use only.

This product conforms to the “Class A” standard for electromagnetic emissions intended for laboratory equipment applications. It is possible that emissions from this product may interfere with some sensitive appliances when placed nearby or in the same circuit as those applicances. The user should be aware of this potential and take appropriate measures to avoid interference.

Power to the Rotofor preparative IEF cell is to be supplied by an external DC voltage power supply. This power supply must employ a safety isolation transformer to isolate the DC voltage output with respect to ground. All of Bio-Rad’s power supplies meet this important safety requirement. Regardless of which power supply is used, the maximum specified operating parameters for the cell are:

3000 VDC	maximum voltage limit
15 Watts	maximum power limit
50 °C	maximum ambient temperature limit

Current to the cell, provided by the external power supply, enters the unit through the lid assembly, providing a safety interlock. Current to the cell is broken when the lid is removed. Do not attempt to circumvent this safety interlock, and always turn the power supply off before removing the lid, or when working with the cell in any way.

Important: This Bio-Rad instrument is designed and certified to meet IEC1010-1* safety standards. Certified products are safe to use when operated in accordance with the instruction manual. This instrument should not be modified or altered in any way. Alteration of this instrument will:

- Void the manufacturer’s warranty
- Void the IEC1010-1 safety certification
- Create a potential safety hazard

Bio-Rad is not responsible for any injury or damage caused by the use of this instrument for purposes other than for which it is intended or by modifications of the instrument not performed by Bio-Rad or any authorized agent.

*IEC1010-1 is an internationally accepted electrical safety standard for laboratory instruments.

Section 2
Description of Major Components

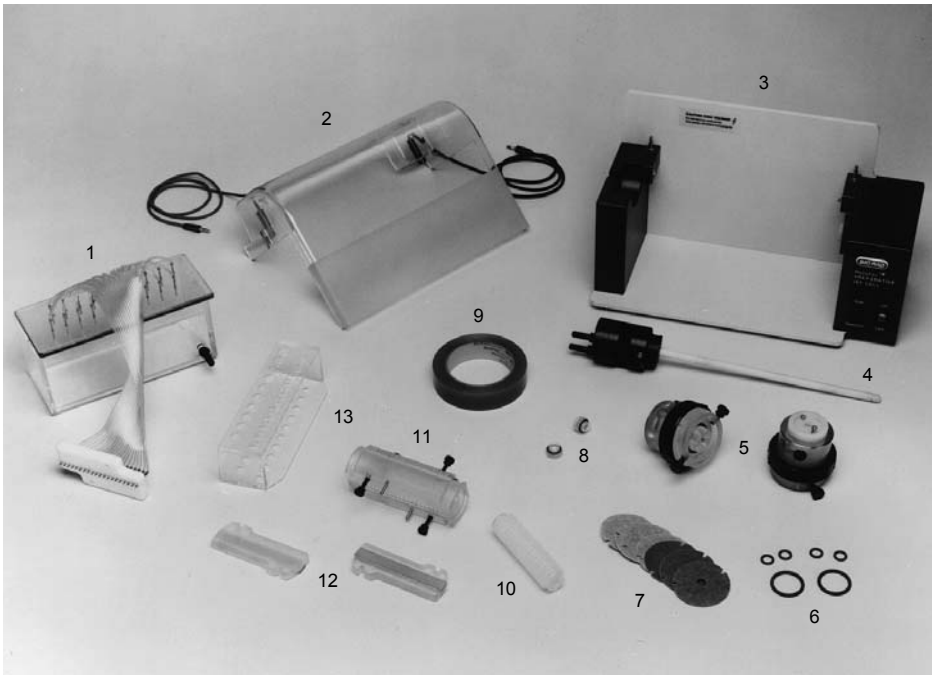

Fig. 2.1. Rotofor components. Harvesting apparatus (1), safety cover (2), housing (3), cooling finger (4), electrode assemblies (5), O-rings (6), ion exchange membranes (7), vent buttons (8), sealing tape (9), membrane core (10), focusing chamber (11), cell covers (12), test tube rack (13).

Focusing chambers - Two focusing chambers are available with the Rotofor cell. The Mini focusing chamber holds 18 ml of sample and should be used for fractionating micrograms to milligrams of total protein. The Mini chamber is also ideal for refractionation. The standard chamber holds from 35 to 60 ml of sample and is used to fractionate milligrams to 3 grams of total protein. The focusing chambers are machined acrylic cylinders 120 mm long. Twenty evenly-spaced ports are bored in opposite sides for sample filling and collection.

Membrane core - The membrane core divides the focusing chamber into 20 compartments. The core assembly is a stack of 19 membrane units made from monofilament polyester screens of 10 µm nominal pore size. This assembly is inserted in the focusing chamber to stabilize the zones of focused proteins.

Electrode assemblies - There are two electrode assemblies. The assemblies hold the cathode and anode electrolyte solutions and provide electrical contact between the focusing chamber and the power supply. They are not interchangeable; alignment pins prevent improper assembly. Ion exchange membranes, inserted in the assemblies, isolate the electrolytes from the sample in the focusing chamber while allowing establishment of an electrical field across the chamber. A plastic gear mounted on the cathode assembly engages the drive motor to rotate the focusing chamber.

Ion exchange membranes - Ion exchange membranes are used in the electrode assemblies to separate the electrolytes from the sample while allowing current

flow. The anion-exchange membrane is notched to fit only the cathode assembly (black button) and the cation exchange membrane will fit only the anode assembly (red button).

Before the initial use, the membranes must be equilibrated overnight in the appropriate electrolyte. Once wetted they cannot be allowed to dry. If they dry out, membranes should be discarded. Membranes generally last 4–5 runs.

Anion Exchange Membranes are equilibrated in 0.1 M NaOH.

Cation Exchange Membranes are equilibrated in 0.1 M H_3PO_4.

Gaskets - Four grey colored silicone rubber gaskets are provided to seal the ion exchange membranes within the electrode assemblies. These will fit either electrode assembly.

Vent buttons - Both electrode assemblies have filling ports. Vent caps containing integral, gas-permeable, PTFE membranes provide pressure relief form the gases which build up in the electrolyte chambers during the run. The vent buttons will fit either electrode assembly.

Housing - The stand supports the assembled focusing chamber during the run and houses the rotation motor. Focusing power is transmitted to the focusing chamber through brass contacts that are spring-loaded to maintain constant electrical contact between the focusing chamber and the housing. The assembled focusing chamber fits on the stand, with the anode (red) compartment to the left. If assembled correctly, the cathode electrode assembly will engage with the gear on the housing. If any connections are loose, the unit will not fit. Electrical contact to the case is through jacks on the safety cover. The safety cover must be in place for safe operation of the Rotofor cell.

Harvesting apparatus - A test tube rack which holds 20 test tubes (12 x 75 mm culture tubes) is enclosed in the harvesting box. This box has a fitting for connection to a vacuum source. House vacuum is usually sufficient for harvesting. Stainless steel tubes on the lid of the box are connected to an array of needles by flexible tubing. Individual fractions are collected through the tubing into the test tubes.

Cooling finger - The ceramic cooling finger extends through the focusing chamber and the electrode assemblies. The cooling finger is in contact with the sample and provides efficient heat dissipation up to 20 W.

Section 3
Setting Up For A Run

Assemble the anode and cathode electrolyte chambers first. Alignment pins prevent misassembly of the two electrodes. The anion-exchange membrane is notched to fit only the cathode compartment (black button) and the cation exchange membrane will fit only the anode assembly (red button). The four silicone rubber gaskets can be used in either electrode assembly. The procedure is identical for assembly of both the mini focusing chamber and standard focusing chamber.

3.1 Equilibration of the Ion Exchange Membranes

Ion exchange membranes are used in the Rotofor cell to separate the sample from the electrolyte while allowing current flow. The ion exchange membranes used in the Rotofor cell are of two types: cation exchanger and anion exchanger. The cation exchanger is negatively charged and repels negatively charged ions, preventing them from contaminating the anolyte. The anion exchanger works in the opposite way; it is positively charged and repels positive ions.

Using the ion exchange membranes gives a concentration gradient of the corresponding ions at the respective ends of the sample chamber. The highest concentration of negative ions will be next to the cation exchanger and the highest concentration of positive ions will be next to the anion exchanger.

Prior to assembly, the ion exchange membranes must be equilibrated overnight in the appropriate electrolyte solution. Ion exchange membranes are used for 4–5 runs prior to replacement.

Anion Exchange Membranes: These membranes are lighter in color than the cation exchange membranes when dry. The color of the two membranes is similar when wet. These membranes are equilibrated in 0.1 M NaOH. They are stored in distilled water or electrolyte between runs.

Cation Exchange Membranes: These membranes are darker colored than the anion exchange membranes when dry. These membranes are equilibrated in 0.1 M H_3PO_4. They are stored in distilled water or electrolyte between runs.

Note: The membranes can be stored indefinitely when dry. After rehydration, they must be kept moist. If the membranes dry out, they should be discarded.

3.2 Assemble the Electrodes

1. Examine the inner portion of an electrode assembly. For the Standard Rotofor there should be a small O-ring in the central hole on the flat side, and a large O-ring seated in the large groove around the central shaft on the other side. For the mini chamber, the outer portion contains only one large O-ring. Place a gasket over the alignment pins and seat it on the flat surface of the inner assembly. The three oblong holes in the ion-exchange gaskets should align with the six holes of the electrolyte chamber. When properly aligned, the gasket should not obstruct the six holes in any way.

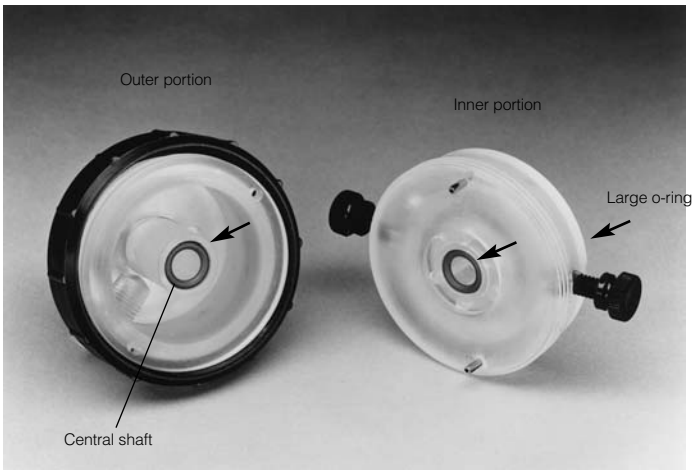

Fig. 3.1. Outer and inner portions of the electrode assemblies. Arrows indicate O-rings. Electrolyte buffer should just cover the central shaft when completely assembled. For the mini focusing chambers, the six holes in the inner portion of each electrode assembly are much smaller in diameter than six holes in the inner portion of the electrode assemblies used with the larger focusing chamber. In addition the six holes for the mini chamber are drilled at a distinct angle to the central axis of the assembly. These parts are not interchangeable!

2. Place the proper ion exchange membrane on the gasket by aligning the notches in the membrane around the pins, and complete a “sandwich” with a second gasket on top of the membrane. The cathode holds one anion exchange membrane and the anode holds one cation exchange membrane.

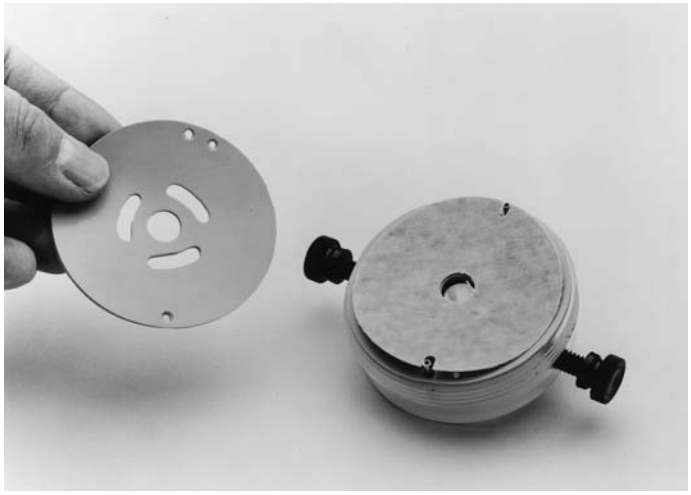

Fig. 3.2. Ion exchange membrane and gasket sandwich on inner portion of electrode assembly.

3. Make sure that there is a small O-ring inset in the central shaft of the large, outer portion of the electrode assembly and fasten the halves together with the captive, threaded sleeve.
4. Repeat the assembly process for the second electrode.
5. Fill the electrode chambers with electrolytes immediately after assembly to prevent the membranes from drying. Filling is most easily accomplished with the assembled focusing chamber mounted on its stand. The anode (+) electrode assembly (red button), containing the cation exchange membrane, is filled with acidic electrolyte, usually 0.1 M H_3PO_4. The cathode (–) electrode assembly (black button), containing the anion exchange membrane, is filled with basic electrolyte, usually 0.1 M NaOH. To fill the compartments, remove the vent buttons, add 25–30 ml of the appropriate electrolyte to each chamber, so that the chambers are about 65% full, and replace the buttons. The electrolyte should just barely cover the central shaft of the chamber. Excessive electrolyte does not provide sufficient air space to allow gases to escape. Pressure may build up inside the electrode assembly and cause leaking from the vent buttons or ion exchange membranes.

   The vent buttons are interchangeable and can be used with either electrode assembly. The life of these buttons is usually 4–5 runs. After 4–5 runs, electrolyte may begin to leak from the vent buttons during the run. If a vent button is inadvertently perforated or, if during focusing an inordinate amount of electrolyte leaks from the filling port, stop the run and replace the vent cap.

   When the cell is used for the first time, the electrode assemblies will contain fresh electrolyte. If the cell has been run previously, the distilled water or electrolyte solutions must be left in the electrode assemblies between runs to maintain hydration of the ion exchange membranes. Use fresh electrolytes for each run. If the membranes are allowed to dry, they must be replaced. Empty the electrode assemblies and fill with fresh electrolyte solution before each focusing run.

3.3 Assemble the Focusing Chamber

1. Slide the assembled anode electrode assembly over the ceramic cooling finger so that the two protruding screw heads fit into the holes in the black plastic base of the cooling finger support assembly.

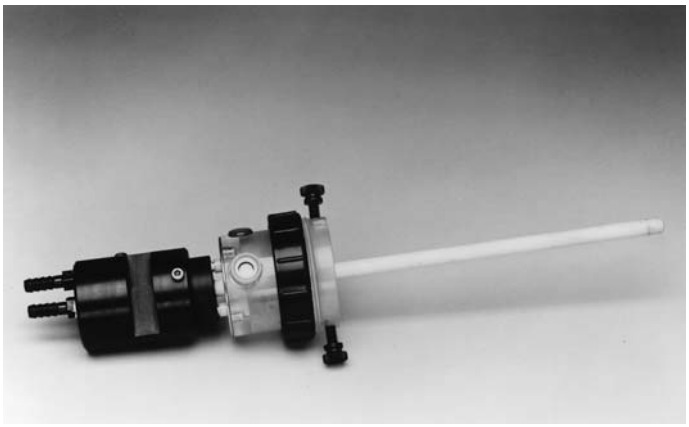

Fig. 3.3. Anode electrode assembled on the cooling finger.

2. Slide the membrane core onto the ceramic cooling finger, making sure the core abuts the acrylic ridge on the anode chamber.
3. Slide the focusing chamber over the membrane core, inserting the metal pin into the small hole in the anode chamber. Position the focusing chamber so that each membrane screen lies between two adjacent ports. These ports must not be blocked by the membrane screens at either side, load or harvest. If the ports are blocked, remove the focusing chamber, and slide it once more over the membrane core. Tighten the black, nylon retaining screws. Check again to make sure the membrane screens do not block the ports of the chamber.

Fig. 3.4. Slide the focusing chamber over the membrane core.

4. Slide the assembled cathode compartment over the cooling finger, aligning the metal pin and hole in the cathode chamber, and tighten the nylon retaining screws.

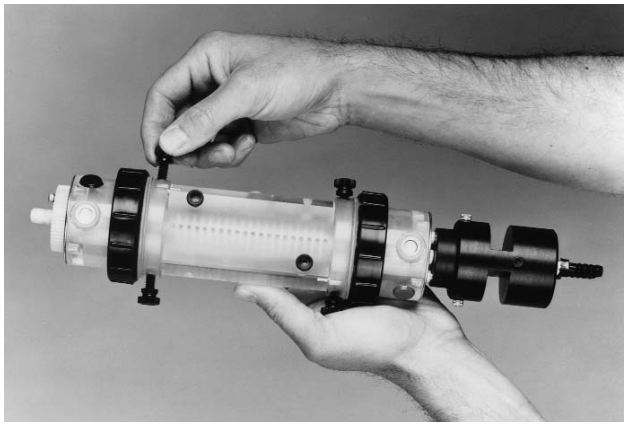

Fig. 3.5. Assembled focusing chamber.

5. Mount the assembled focusing chamber in the stand. The gear on the cathode electrode assembly should be fully engaged with the gear on the stand. If the focusing chamber does not slide in easily, remove it to check that all parts are properly assembled.
6. Attach the power cord to the back of the unit and connect it to an electrical outlet.

3.4 Prepare the Focusing Chamber

With the cell mounted on the stand, rotate the focusing chamber so the 20 collection ports, identified by the two metal alignment pins, are facing up. Cover the ports with a piece of the sealing tape provided with the cell. Reinforce the taped ports with one of the two acrylic cell-cover blocks, and finger tighten the screws. We recommend pre-running the cell with pure water for the first use or after cleaning the components in the focusing chamber with NaOH. Pre-running the cell with water for 5 minutes at 5 watts constant power will remove residual ionic contaminants from the membrane core and ion exchange membranes before addition of the sample.

3.5 Load the Sample

Rotate the cell so the filling ports face up. This is easily accomplished by flipping the toggle switches to **ON** and **HARVEST**. In the harvest mode the focusing chamber will automatically stop with the filling ports facing up and the collection ports facing down. Fill the cell with sample through the ports using a 50 ml syringe with a 1-1/2 inch 19-gauge needle. Typically, every other port is filled, and the sample spreads into the adjoining compartments. For the large focusing chamber, the minimum sample volume must be sufficient to cover the cooling finger. For the mini focusing chamber load the maximum sample volume of 18 ml.

3.6 Seal the Loading Ports

A. Mini Rotofor chamber: Place the grey rectangular, silicone gasket in the slot containing the loading ports then place the second cell cover block over the gasket (tape is unnecessary), and the Rotofor cell is ready for operation.

B. Standard Rotofor chamber: Seal the filling ports with only the second cell cover block (tape is unnecessary), and the Rotofor cell is ready for operation.

3.7 Remove Air Bubbles

During filling, air bubbles can become trapped in the 6 ports of the electrolyte chamber. This is especially true with the mini focusing chamber. If the bubbles are not removed, they will produce occasional fluctuations in the voltage and currents due to the discontinuity they create in the electrical field. Some power supplies, such as the Bio-Rad Power Pac 3000, have safety sensors that may trip and shut off the voltage in response to the resistance change that occurs when a bubble rotates into the electrical circuit. Thus, bubbles must be eliminated prior to commencing electrophoresis. Remove the assembled, loaded cell from the stand, turn it vertically and tap the electrode chamber to dislodge the bubbles. Then turn the cell 180° and tap the other chamber. If any air bubbles remain in the 6 ports between the sample and the ion exchange membranes, repeat this process. When all the bubbles are eliminated from the electrode ports, return the cell to the stand and start the fractionation.

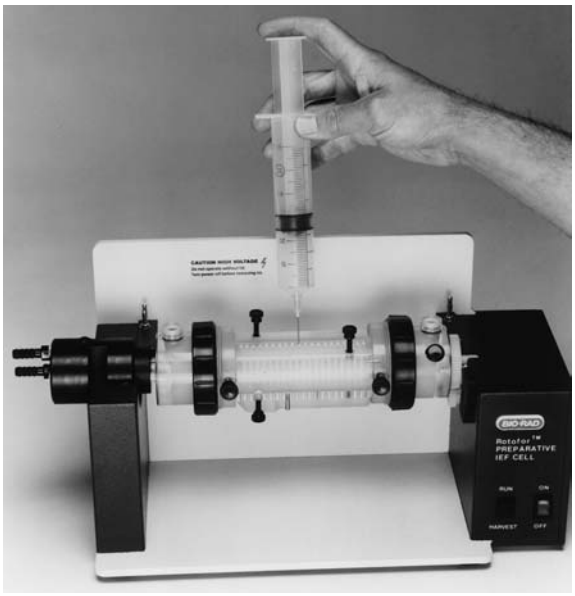

Fig. 3.6. Loading the sample.

Section 4
Running Conditions

4.1 Starting the Fractionation

Excessive heating may denature proteins and damage the Rotofor cell. Connect the ports of the cooling finger to a source of recirculating coolant and begin coolant flow. The ports are interchangeable, so either one may be connected to the coolant inlet. It is usually sufficient to set the chiller at 4°C. For more critical temperature control, the chiller can be adjusted accordingly. At 12 W constant power (normal operating mode) the coolant temperature should be set at 10°C less than the temperature desired for the sample. In other words, if the coolant is -6°C than the sample temperature will be maintained at about 4°C. Attach the cover of the unit, mating its jacks to the plugs on the base. Allow the system to come to thermal equilibrium at the cooling temperature before beginning the run, approximately 10–15 minutes.

4.2 Power Supply

1. Never operate the Rotofor cell with the cover removed. When focusing power is applied to the jacks without the cover in place, several high voltage elements become exposed. To avoid personal injury due to accidental contact with these elements, always operate the cell with the cover in place.
2. Attach the high voltage leads to the power supply, and the Rotofor cell is ready for use. To begin rotation, flip the toggle switches to **ON** and **RUN**.
3. Power supply:

   Standard Rotofor chamber - Set the supply to 15 W constant power and begin the run.

   Mini Rotofor chamber- Set the supply to 12 W constant power and begin the run.

   The starting voltage and current will vary depending on the salt concentration of the sample. For example, if the salt concentration of the sample is 10 mM, the starting voltage will be 300–500 V, and the current will be 24–40 mA. The maximum power that can be dissipated is about 15 W for an initial fractionation when the Rotofor cell is operated at 4°C. If more than 15 W is applied to the cell, overheating can damage the cell. The applied power is too high if the current increases or remains constant, rather than decreases, during a run. If a constant power supply is not available, check the graph in Figure 4.1 to determine the optimum starting voltage and increase the voltage manually in increments over time. The voltage should be increased as the run progresses to keep the power at a constant 12 W.
4. A typical run is completed in 3–5 hours. To monitor the progress of a run under conditions of constant power, observe the voltage increase over time. The run is complete when the voltage stabilizes. At that point, allow the run to continue for 30 minutes before harvesting. The total run length should not exceed 6 hours. Longer run times do not tighten the focusing and may begin to break down the gradient.

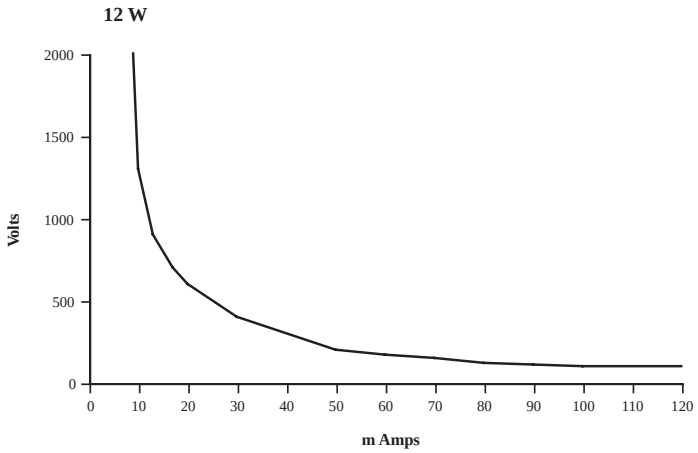

Fig. 4.1. The maximum power that should be applied to the Rotofor cell is 12-15 W. The graph shows the voltage and current readouts for setting a constant voltage power supply. If the current reading is too high at the set voltage (in the danger zone), reduce the voltage until a safe power level is obtained. **Watts = voltage x current.**

4.3 Fraction Collection

1. Load the test tube rack with twenty 12 x 75 mm culture tubes and place it inside the harvest box. Place the lid on the box, making certain that each stainless steel collecting tube is inside a test tube. Connect a vacuum source to the vacuum port on the box and turn on the vacuum to hold the lid in place. A vacuum pump or house vacuum of 10–50 mm Hg is recommended.
2. When focusing is completed, move the black toggle switch to the HARVEST position. This stops the cell rotation with the cell properly aligned for sample collection, i.e., with the alignment pins and taped collection ports on the bottom of the focusing chamber. All manipulations which follow the end of rotation should proceed as quickly as possible to minimize mixing.
3. Turn the power supply off, disconnect the power supply, remove the cover, and move the Rotofor cell and the harvesting box next to one another. Remove both the upper and lower focusing chamber cell cover blocks. Mount the needle array on the two alignment pins on the bottom of the chamber. Grasp the needle array with the fingers of both hands while placing the thumbs on the top of the focusing chamber. Take care not to block any of the uppermost ports. Quickly push the needles firmly and uniformly all the way through the sealing tape into the chamber. This will cause all 20 fractions to be simultaneously aspirated from the cell and delivered to the collection tubes.

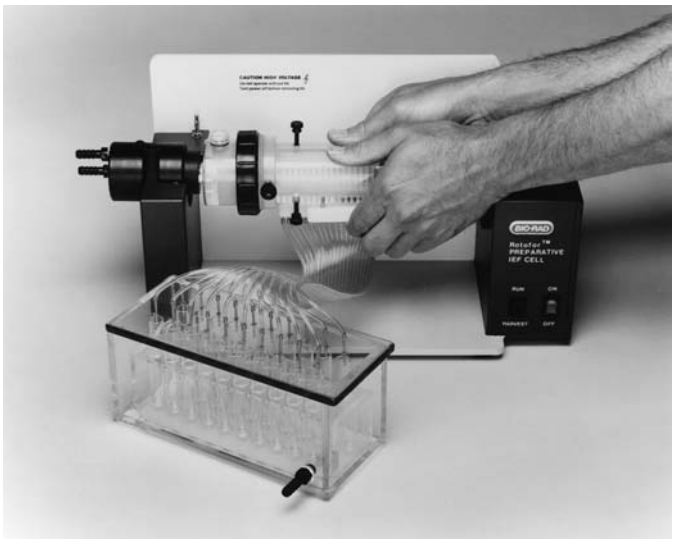

Fig. 4.2. Harvesting samples after focusing is complete. Make sure thumbs do not cover the uppermost ports.

4. Turn off the vacuum source and remove the test tube rack. Note that all the odd numbered fractions are in one row and the even numbered fractions are in the other row of the rack.

4.4 Refractionation

The fractions containing the protein of interest may also contain other, contaminating proteins after the initial fractionation. Refractionation of Rotofor fractions is one way to increase sample purification. Because of its lower volume requirement the Mini Rotofor chamber is ideal for refractionating pooled fractions.

After screening the samples collected from the first fractionation, pool the fractions containing the protein of interest. These pooled samples (typically 3–5 fractions) can be reapplied either to the Rotofor or Mini Rotofor for refractionation. Rotofor fractions obtained from an initial run contain ampholytes whose range spans the pI of the protein of interest. It is best to add no additional ampholytes to the sample to be refractionated.

Because ampholytes and salts are not added prior to the refractionation, higher voltages can be obtained because of the low ionic strength of the sample. High voltages lead to better resolution during focusing. Upon refractionation, the ampholyte range is much narrower and more specific to the protein of interest. The pooled fractions contain a small part of the initial pH range which spans the pI of the protein of interest. This is spread across the length of the chamber during refractionation, providing a shallow pH gradient, and thereby increasing the likelihood of obtaining one or more fractions of pure protein.

4.5 Final Purification

The Rotofor is designed to quickly separate proteins of interest from other proteins in a sample. Bio-Rad's Model 491 Prep Cell can purify individual proteins from Rotofor fractions by continuous-elution electrophoresis. Conventional gel electrophoresis buffer systems and media are used with the Model 491 Prep Cell. Using SDS-PAGE or native-PAGE, the Prep Cell can isolate specific components from complex mixtures containing micrograms to 200 milligrams of total sample. Up to 5 milligrams per band can be resolved. Using SDS-PAGE the cell isolates molecules that differ in molecular weight by 2%. Using non-denaturing PAGE the cell can isolate molecules that differ in charge by 0.1 pH units. Electrophoretic purification can also be effective in removing ampholytes from samples. See Section 12.

Section 5
Disassembly and Cleaning

1. Rinse the needle array and its associated tubing with water as soon as possible after use. Do not use the vacuum box to pull water through the needle array. This may damage the box. Rinse the box with water.
2. Take the focusing chamber from the stand. Loosen the nylon screws and remove the cathode chamber.
3. Leave the cathode and anode chambers intact. The ion exchange membranes must be stored wet. Remove the electrolyte and fill the electrode chambers with distilled water. If properly stored, the membranes will not decrease in performance between runs. Before starting a new run, the electrolytes must be replaced with fresh solutions.
4. Loosen the nylon screws on the anode chamber and remove the focusing chamber and membrane core. Rinse all chamber components with water and air dry. Do not expose the focusing chamber to concentrated acid, base, or alcohol. The membrane core requires additional care, especially if there has been protein precipitation during the run. A spatula can be used to loosen and remove caked precipitates. Soak the membrane screens in saline and then in detergent or 0.1 M NaOH to remove traces of protein. An ultrasonic cleaner will facilitate the cleaning process. Finally, rinse the screens with water. For complete removal or residual NaOH or other cleaning compounds, assemble the Rotofor cell with the membrane core, add distilled water, and apply 5W power until the current stabilizes. Then discard the solution and add the sample. Cleaning with strong oxidizing agents, such as hypochlorite, or organic solvents should be avoided, as they will damage the membrane core. If properly cleaned, the membrane core can be immediately reused.

Section 6
Sample Preparation

6.1 Salt Concentration

1. Samples should be desalted (e.g., by dialysis or Bio-Gel® P-6 chromatography) prior to ampholyte addition to insure that the nominal pH range of the ampholyte will extend over the full length of the focusing chamber and that the maximum voltage can be applied. It is best to limit salt concentrations in the samples to about 10 mM for optimum fractionation. However, the maximum salt capacity will vary with the application, therefore optimum running conditions should be determined empirically. During focusing, all salts migrate to the compartments next to the anode and cathode, effectively desalting the sample.
2. The sample should not be in a buffer greater than 10 mM concentration. Buffers add to the conductivity of a sample and decrease resolution. Also, buffering solutions may flatten the pH gradient in the region of the pK_a of the buffer.

6.2 Clarification

Turbid sample solutions should be clarified by filtration or centrifugation to remove extraneous cellular debris that might clog the membrane core.

6.3 Solubility

If the solubility of proteins presents a problem, adjusting the sample to 3–5 M urea is recommended. Higher urea concentrations, up to 8M urea, can be used. Be sure to deionize the urea using AG® 501-X8 mixed bed ion exchange resin (catalog number 143-7424). Addition of non-ionic detergents, such as: CHAPS, CHAPSO, octylglucoside, digitonin, or Triton X-114 is also valuable in maintaining the solubility of focused proteins. The concentration of detergents used is usually between 0.1% and 1%. Alternatively, solubility can sometimes be maintained by increasing the Bio-Lyte ampholyte concentration in the sample. Check the solubility of the protein by diluting it in detergent or urea and running it on an analytical IEF gel. If the protein does not show signs of precipitation in the IEF gel, it should not precipitate in the Rotofor cell.

Section 7
Optimizing Fractionation

7.1 Ampholyte Choice

1. Bio-Lyte® ampholytes are complex mixtures of synthetic buffering electrolytes with closely spaced pI's and high conductivity. Bio-Lytes are supplied at concentrations of 40% (w/v), except in the pH ranges 3-5 and 8-10, which are at 20%. The final concentration of Bio-Lytes used in the Rotofor system depends on the protein concentration in a given sample:

Protein per milliliter	**Bio-Lyte Ampholytes**
>2 mg	2%
1 mg	1.5%
0.5 mg	1%
0.25 mg	0.5%

2. Up to 8% (w/v) ampholyte concentrations have been used for various applications. Ampholytes at 1% permit higher applied voltages and are recommended if refractionation is not required. 2% ampholytes will provide greater buffering and are necessary when refractionation is performed. If protein precipitation occurs during the run because of the desalting effect of focusing, sample solubility may be maintained with higher ampholyte concentrations. Use the following formula to determine the appropriate volume (V_1) of a 40% Bio-Lyte ampholyte solution to give a desired final concentration in your Rotofor sample.

   For the equation: $\mathbf{(C_1)(V_1)=(C_2)(V_2)}$, solve for $\mathbf{V_1}$.

   where: C_1 = Starting concentration of Bio-Lyte (40%)
   V_1 = Unknown volume of 40% Bio-Lyte to give desired final concentration
   C_2 = Final or desired concentration of Bio-Lyte
   V_2 = Final volume of the sample to be applied to the Rotofor (35–58 ml) or Mini Rotofor (18 ml)

3. The pI of the protein of interest can be determined by running the sample on a flatbed slab IEF cell (such as the Model 111 mini IEF cell) using the broad pH range of 3-10. IEF markers (catalog number 161-0310) run in the same gel allow the pI of the protein of interest to be estimated. Alternatively, the pI can be estimated by running the sample in the Rotofor cell using a broad range 3–10 ampholyte. The pI of the protein of interest will correspond to pH of the Rotofor fraction where the protein of interest focuses.
4. A narrow pH range of ampholytes spanning the pI of the protein of interest should be used for the initial fractionation. Narrow range fractionation first separates the protein of interest from the bulk of its contaminants. The pI of the protein of interest should fall in the middle of the ampholyte range.

5. An example of the importance of using the proper ampholytes in a fractionation is demonstrated by a purification of Japanese water moccasin snake venom. The protein of interest has a pI of 6.1 as determined by IEF gels. Bio-Lyte ampholytes pH 6-8 were used for the initial fractionation. The fractions were analyzed and the ones containing the specific protein were pooled. After refractionation, the fractions were again analyzed on IEF slab gels and multiple bands were observed in all fractions. When the same snake venom sample was initially fractionated in 5-7 Bio-Lyte ampholytes the results were dramatically different. After refractionation, the protein of interest was almost completely free of contaminating proteins. The conditions for both experiments were identical except for the initial Bio-Lyte range; however, much greater purity was obtained from the experiment using the 5-7 Bio-Lyte ampholytes.

7.2 Sample Capacity

Choosing between the Standard Rotofor Chamber and the Mini Rotofor Chamber is a matter of sample size. The Standard Rotofor Chamber is designed to optimally fractionate milligrams to grams of total protein. The Mini Rotofor chamber is designed to fractionate micrograms to milligrams of total protein. The smaller volume of the Mini Rotofor decreases sample volume and is best suited for use with samples of low protein concentration. Protein concentrations should be adjusted for desired yield or to provide convenient assays of focused material, assuming each component will focus in 1–3 channels, approximately equal to 3 ml/fraction in the Rotofor and 800 µl/fraction in the Mini Rotofor.

For example, if Rotofor fractions are analyzed by SDS-PAGE on a Mini-PROTEAN® II cell (catalog number 165-2940) and silver stained, the sample should contain a minimum of 50.0 µg per component. More sensitive assays, such as activity assays, decrease the necessary protein load.

The maximum protein load varies with the solubility of each sample and must be determined empirically. However, preparative fractionation of 51 ml of lyophilized cell culture supernatant containing 2.4 g of protein has been successfully performed using the Rotofor Cell.

7.3 Power Conditions

We recommend running the Rotofor cell at constant power. During the initial fractionation the voltage values will vary between samples depending on the relative concentration of proteins and salts. The Mini Rotofor should be run using 12W constant power and standard Rotofor should be run using 15W constant power.

When voltage is applied to a system of ampholytes and proteins, all the components migrate to their respective pIs. In electrofocusing, the higher the voltage the better the resolution. The limiting factor in achieving high resolution is how efficiently electrically generated heat can be dissipated.

In a constant power mode, voltage gradually increases as the components focus. The progress of the run is easily monitored by observing the voltage increase over time. When the sample is focused, voltage levels off at a maximum. Runs typically last 2-4 hours at 12 watts constant power and can require up to 3000 volts.

7.4 Cooling

Sample temperature affects activity and resolution. Many proteins, especially enzymes, are temperature labile. The water recirculation chiller should be set about 10 °C cooler than the temperature required to maintain stability of your protein. The

heat generated during IEF keeps the temperature inside the focusing chamber approximately 10 °C higher than that of the circulating coolant. Temperature settings for chillers are generally between - 10°C and 4°C.

Diffusion rates of proteins are proportional to their temperature in solution. Because proteins at steady state diffuse in and out of their focused zones it is advisable to run the Rotofor cell at the lowest possible temperature to offset this effect.

7.5 Electrolytes

The recommended electrolytes for the anode and cathode are 0.1 M H_3PO_4 and 0.1 M NaOH, respectively. Because there can be a slight amount of electrolyte exchanged through the ion exchange membranes during the focusing run, the first one or two channels may be very acidic (<pH 3) and the last one or two channels may be very basic (>pH 10). The result will be a concentration of the effective pH gradient in the middle channels. This will have minimal affect on the final results of the experiment. Alternative electrolytes, e.g., amino acids, acetic acid, etc., may be used and perform as well as H_3PO_4 and NaOH. These include:

pH range of Bio-Lyte	Anode Electrolyte	Cathode Electrolyte
3-5	0.5 M acetic acid	0.25 M HEPES
4-6	0.5 M acetic acid	0.5 M ethanolamine
5-7	0.1 M glutamic acid	0.5 M ethanolamine
6-8	0.1 M glutamic acid	0.1 M NaOH
7-9	0.25 M MES	0.1 M NaOH
8-10	0.25 M MES	0.1 M NaOH

7.6 Pre-running the Cell

The unit should be cleaned with distilled water prior to loading the sample. Simply fill the focusing chamber with 55 ml of distilled water and run at standard power for 5 minutes. Drain the unit using the harvesting apparatus. This will insure that extraneous ions have been removed from both the cell and the surface of the ion exchange membranes.

7.7 Prefocusing

Loading the sample into the Rotofor cell is usually accomplished by injecting a homogeneous solution of the prepared sample containing ampholytes, the protein of interest, and any required solubility agents into the focusing chamber. However, some proteins are especially sensitive to rapid pH shifts or to extremes of pH and may precipitate or become denatured. To avoid exposing your protein to these potentially damaging conditions during initial focusing, “prefocus” the focusing media (i.e. Bio-Lyte ampholytes and solubility additives), without protein for about an hour. This will establish the pH gradient. Then inject your protein sample into the sample chamber at or near the point in the pH gradient that corresponds either to the pH of the protein sample solution or the pI of your protein of interest. To avoid disrupting the pH gradient during injection of the sample, this technique requires that the volume of the solution containing the protein sample be as small as possible. Prefocusing decreases exposure of proteins to rapid pH shifts and pH extremes, minimizes the amount of time the protein spends in the Rotofor cell, and may reduce run times by up to 50%.

7.8 Refractionation

Better separation may be achieved by refractionating the sample. The mini Rotofor is ideal for refractionation because samples are minimally diluted in its chamber. After analyzing the fractions from the initial separation, the fractions containing the protein of interest should be diluted in distilled water and reloaded in the standard Rotofor cell or the Mini Rotofor cell. Upon refractionation the ampholyte concentration should be at least 0.5%. We recommend that no less than 4–5 fractions be pooled and reapplied for a second Rotofor run. If urea or non-ionic detergents are needed to maintain protein solubility add the same concentration as used in the first fractionation. Do not add additional ampholytes or salts at this stage.

1. Dilute pooled fractions appropriately, *e.g.*, with water, up to 8 M urea, or a solution containing non-ionic detergent for solubility, to a final volume of 55–60 ml in the standard Rotofor or 18 ml for the Mini Rotofor. The customized ampholyte blend obtained will span the pI of the protein of interest. Do not add additional ampholyte to the refractionation mix; the amount present in the pooled samples is suitable for focusing and provides a narrow range pH gradient to increase separation of the protein of interest.
2. Load the diluted sample and re-run. Since the ionic strength of the sample will be lower upon refractionation, higher voltages, yielding better separations can be achieved. Refractionations of low ionic strength solutions have been carried out at 2,000–3000 volts. Do not exceed the power limit of the cell. Focusing is usually complete in 3–5 hours. The upper limit for voltage is dependent on how well heat can be dissipated. Set the coolant temperature between -5°C and -10°C for high voltage separations.

Section 8
Analysis of Results

8.1 Fraction Analysis

After harvesting, it is important to analyze the fractions to determine which contain the protein of interest. There are many different ways of doing this, and the best method is dependent on the protein being analyzed.

SDS-PAGE analysis or an IEF gel, usually pH 3-10, will give an accurate representation of the fractionation. Other methods for assaying which channels contain the protein of interest are dependent on the particular protein and include activity assays and antibody tests. Analytical gels should be silver stained for high sensitivity detection of contaminants.

8.2 Separation of Ampholytes From Proteins

Many applications can tolerate the presence of ampholytes in protein solutions. However, ampholytes can interfere with some assays such as amino acid analysis. Several methods for separating ampholytes from focused proteins are listed below.

1. Preparative Electrophoresis - Rotofor fractions containing the protein of interest and any remaining contaminating proteins can be pooled and applied to a preparative continuous-elution electrophoresis cell such as Bio-Rad's Model 491 Prep Cell. Using the Model 491 Prep Cell as second and a final purification step, samples (Rotofor fractions) are electrophoresed through a polyacrylamide gel. In this way, the contaminating proteins and the ampholytes are effectively separated from the protein of interest.

2. Dialysis - Probably the simplest method for ampholyte removal is dialysis. Adjust the pooled sample to 1 M NaCl. This will effectively strip electrostatically bound ampholytes from proteins by ion exchange. Then dialyze into the buffer appropriate for further uses.
3. Ammonium sulfate precipitation of proteins may also be effective in removing ampholytes from samples.
4. Any number of chromatographic techniques, such as gel filtration, ion exchange, hydroxylapatite, affinity chromatography, or use of AG 501-X8 resin, can be used to separate proteins from ampholytes.

Section 9
Troubleshooting Guide

This guide is designed to answer common Rotofor cell questions. For further information, please contact your local Bio-Rad representative. In the U.S., our Technical Service department is available Monday to Friday, from 7:00 am to 5:00 P.M. Pacific Time to answer all of your technical inquiries involving Bio-Rad equipment and reagents. You can reach us by dialing 1-(800)-4BIORAD.

9.1 Solubility and Precipitation of Proteins

1. By definition, a protein at its isoelectric point (pI) has no net charge. Because little charge repulsion exists between focused molecules, hydrophobic interactions between proteins become predominant causing proteins to aggregate. Maintaining the solubility of proteins in this case requires overcoming protein-protein interactions. Several agents promote protein solubility. Detergents provide a hydrophobic environment for proteins to mask interprotein interactions. Disulfide bridges also may form between proteins leading to aggregation. This effect may be overcome by the addition of reducing agents to the focusing media. Because the solubility of proteins varies greatly, there is no one answer to the problem of insolubility. Generally, the easiest method of getting proteins to remain in solution is to add nonionic detergents, zwitterionic detergents, and/or chaotrophic agents to the sample mixture.

   In addition, glycerol from 5-25% (v/v) in the sample is highly effective for maintaining the solubility and stability of proteins. Glycerol stabilizes water structure and the hydration shell around proteins.

Table 9.1. Recommended Solubilizing Agents for the Rotofor System

Non-Ionic Detergents	Zwitterionic Detergents	Reducing Agents	Chaotropic Agents
0.1-3.0% Digitonin	0.1-3.0% CHAPS	DTE 5-20 mM	1.0-8.0 M Urea
0.1-3.0% Octylglucoside	0.1-3.0% CHAPSO	DTT 5-20 mM	0.1-2.0% Glycine
0.1-3.0% Triton X-114		BME 1-5 mM	0.1-2.0% Proline

2. When the solubility of a protein depends on maintaining high ionic strength during focusing, increasing the concentration of Bio-Lyte ampholytes up to 5–8% in your sample will help keep proteins in solution.

3. Decreasing the protein load will also help keep the protein in solution. The largest amount of protein concentration in solution that has been successfully fractionated in the Rotofor cell is 4 grams. The lower limit for protein loading depends on the sensitivity of your detection system.

9.2 Factors Affecting the pH Gradient

Non-linear pH gradients are rarely observed when sample is prepared properly and the Rotofor cell and its parts are carefully maintained. A non-linear pH gradient may be caused by one or more of the following:

1. **Electrolyte leakage.** Excessive leakage of electrolyte across the ion exchange membranes into the focusing chamber will decrease the number of fractions on the linear portion of the pH gradient and reduce the effective voltage across the sample. To determine if this is occurring, check the pH of the fractions. Alternatively, fill the Rotofor focusing chamber with distilled, deionized water and run the Rotofor at 12 W constant power. If the amperage does not decrease to < 6 mA and the voltage does not increase to near 2,000 V within 5–10 minutes, the chances are good that you have electrolyte leaking into your sample. Some common causes are:

   A) Expired **Vent Buttons**. Vent buttons lose their capacity to vent the gases produced during electrolysis over time and when there is too much electrolyte in the chamber. The pressure that results within the electrolyte chambers forces electrolytes into the focusing chamber. Replace the vent buttons (catalog number 170-2957) every 4 to 5 runs.

   B) Worn **O-rings and/or electrode Gaskets.** The Rotofor repair kit contains replacement parts for these items (catalog number 170-2953). Lubricating the O-rings with a small amount of silicone O-ring grease or Cello-Seal™ will extend their useful lifetime (catalog number 170-2954).

   C) Cracked, dehydrated, or worn out **Ion-exchange Membranes** (catalog number 170-2956). These last 4 to 5 runs.

2. **Uneven harvesting.** Variations in the volumes of harvested fractions may affect the linearity of the collected pH gradient. Be sure to remove both harvesting and loading port covers before piercing the sealing tape with the harvesting block needles. Also make sure that the harvesting tubes are clean and clear of blockages by soaking in Bio-Rad cleaning concentrate (catalog number 161-0722) or dilute 0.05 M NaOH and rinsing well with DDI H_2O after each run. Dry the tubes by aspirating each individual tube with a vacuum line. Be careful not to block the loading port holes with your fingers during harvesting.

   The compartments of the focusing chamber contain unequal volumes at the end of the run. As proteins become focused the osmotic pressure in each Rotofor compartment may vary. If the focusing chamber is not completely full, this may cause unequal distribution of fluids in the 20 compartments. This effect will vary as a function of protein load and concentration of solubilizing additives. Reproducibility of results, especially where isolation of a protein in a particular fraction number is expected, will depend on the constancy of these factors. To alleviate the osmotic effect, the Rotofor cell should be run with the focusing chamber completely filled.

3. **Premature harvest.** Too short a run will result in a partially-formed pH gradient and poorly focused proteins. The Rotofor cell is normally run for 3 to 6 hours. To assure complete focusing, continue the run for 1/2 hour after the voltage stabilizes, then stop the electrophoresis and harvest the focused protein.

4. **High salt sample.** The salt (or buffer) concentration in the sample may be too high. This will decrease the effective voltage across the sample and may reduce the number of fractions on the linear portion of the pH gradient. Resolution is dependent on both high voltage and maximizing the number of fractions on the linear gradient. If a particularly high ion concentration is necessary to preserve the stability and/or activity of your protein, Bio-Lyte ampholytes (which are ionic molecules) may be substituted for salts. For example, preparation of the enzyme aldose reductase (pI ~ 5.0) from porcine lens for purification using the Rotofor cell required that the protein sample be at low ionic strength (< 1.0 mM buffer) to maximize voltage and resolution[10]. Since aldose reductase is unstable under these conditions, the following procedure was used to avoid exposure to low ionic strength:

   1.0 ml of 5% Bio-Lyte ampholytes, previously fractionated using the Rotofor cell at 4.5 to 5.5 pH range, were added to 1.0 ml of 5.0 mg / ml protein solution in a 10.0 mM phosphate buffer. This solution was exhaustively dialyzed against 25.0 ml of the same 5% Bio-Lyte solution, thereby making the final phosphate concentration less than 1 mM. The salt concentration in the sample was reduced to a reasonable level while the ionic strength required to maintain the stability of the enzyme was retained.

   If the pH gradient plateaus or dips near the middle, this may be due to the presence of excess buffer in the protein sample solution. The pH of the gradient will be buffered at the pK of this buffer, creating a dip or plateau in the gradient in this region. The symptom may be many fractions with the same pH. Reduce buffer salts to < 10 mM.

5. High sample temperature. At 12 W, the temperature inside the chamber is generally 10 degrees higher than the temperature of the circulating coolant. The cooler the run, the more stable the proteins will be. 4 °C is the optimum sample temperature.

6. Non-reproducible pH gradients. Use sufficient concentration of ampholytes. Batches and brands of ampholytes may also vary. Do not run the Rotofor cell more than 1–2 hours after voltage stabilization. Reduce salt to below 10 mM. Always run the sample at or below 4°C. Check the integrity of the Bio-Lyte ampholytes. Ampholytes should be stored at 4°C in the dark. Guaranteed shelf life of opened Bio-Lyte ampholytes is 1 year.

9.3 Recovery of Biological Activity

1. **pH.** Some proteins are especially sensitive to rapid pH shifts and to extremes of pH that exist at the extreme ends of the Rotofor focusing chambers. To avoid exposing your protein to these potentially damaging pH extremes during initial focusing, “prefocus” the focusing media (i.e. Bio-Lyte ampholytes, additives, water, etc.), without protein, for about an hour. This will establish the pH gradient. Then inject your protein sample into the sample chamber at or near the point in the pH gradient that corresponds either to the pH of the protein sample solution or to the pI of your protein of interest. Addition of your protein sample solution in as small a volume as possible decreases exposure to rapid pH shifts and pH extremes, minimizes the amount of time the protein spends in the Rotofor cell and maintains native tertiary structure.

2. **Temperature.** Many proteins, especially enzymes, are temperature labile. Make sure that the water recirculation chiller is set 10°C cooler than the temperature required to maintain stability of your protein. The heat generated during IEF keeps the temperature inside the focusing chamber approximately 10°C higher than that of the circulating coolant. Temperature settings for chillers are generally between - 10°C and 4°C.

   Diffusion rates of proteins are directly proportional to their temperature. Because proteins at steady state diffuse in and out of their focused zones it is advisable to run the Rotofor cell at the lowest possible temperature to offset this effect.

3. **Ampholytes.** Ampholytes may form weak electrostatic complexes with proteins. They can be removed by bringing pooled fraction(s) to 1.0 M NaCl and dialyzing against appropriate buffer or water. The salt effectively exchanges for the ampholytes on the protein. This may be followed by dialyzing against appropriate assay buffer. Other methods for ampholyte removal include electrophoresis; ammonium sulfate precipitation; and gel filtration, ion-exchange, and hydroxylapatite chromatography. Be sure to measure the pH of the fractions before manipulating them to remove ampholytes.

4. **Urea.** Urea in the focusing media at 3 M generally alleviates precipitation. Without the use of urea, loss of activity due to precipitation may be excessive. Urea at higher concentrations (4-8 M) is often used. Following focusing, dialysis will remove urea from the solution.

5. **Detergents.** Both the concentration and the type of detergent used play an important role in recovery of activity. Use the least amount of compatible detergent required to maintain the solubility of your protein. Also try other non-ionic or zwitterionic detergents. Removal of detergent from Rotofor fractions may be necessary for full recovery of activity.

6. **Precipitation.** Protein-protein interactions may result in activity loss. Decreasing the protein load, and addition of detergents, glycerol, reducing agents and/or chaotropic agents keep proteins from forming complexes during focusing.

7. **Proteins are not always active at their pI.** Adjust the pH of the solution for assay.

8. **Some proteins require the presence of a particular ionic species for activity** (i.e. mono- or divalent cations like Na^+ or Mg_2^+). Replace the ions, if necessary, for assay.

9.4 Maximizing Resolution

1. Diffuse or multiband protein IEF patterns can arise from molecular interactions and conformational changes as well as from inherent isoelectric microheterogeneity. Ampholytes can reversibly bind to proteins, proteins can undergo sequential pH dependent conformational changes, and proteins can interact with one another. These types of reactions can artifactually alter the pH profiles of proteins. On the other hand, many proteins are inherently heterogeneous, consisting of isoelectric isomers. To distinguish between artifactual and inherent heterogeneity, it may be necessary to run an analytical IEF gel in the presence of all constituents to be used during focusing in the Rotofor cell (i.e., detergents, urea, glycerol, etc.) in the same proportions to be used in the Rotofor cell. Single focused bands should be cut out and rerun. If this single band splits into many bands, artifact formation is indicated. In this case the Rotofor “prefocusing” protocol is recommended.

2. Clarify all sample solutions before focusing. Membrane cores are composed of polyester membranes with a pore diameter of approximately 10 µm. The membrane core can become clogged with insolubles in sample solutions. Starting solutions can be clarified by centrifugation. To clean the membrane core, soak it in detergent, dilute NaOH, or sonicate. Rinse the core well in distilled water after cleaning. For complete removal of residual base, assemble the Rotofor cell with the membrane core and prerun the cell with H_2O until the voltage stabilizes. Then discard the water and add sample. Generally the membrane cores will last at least 20 runs if they are well cared for.

3. Protein samples that contain a charged detergent, like SDS, may experience a shift in apparent pI and migrate to one or another end of the cell as a result of acquired net charge. Use only non-ionic or zwitterionic detergents for this reason.

   Some proteins are inherently associated with phospholipids, heme groups, or other charged groups that affect the electrophoretic migration of proteins in a pH gradient. A means must often be found to neutralize the effects that these charged groups have on proteins during focusing. For example, the non-ionic detergent, digitonin, has been found to be effective at disassociating negatively charged phospholipid from integral membrane proteins. Digitonin provides a suitable hydrophobic environment for maintaining the stability and biological activity of these proteins during focusing in the Rotofor cell.

9.5 Power Conditions

Voltage is the driving force behind isoelectric focusing. Maximizing the voltage is the best way to increase resolution. The cooling finger is capable of dissipating up to 15 W of power generated in the large focusing chamber. The main factor limiting voltage is efficiency of heat dissipation. These are common problems related to the application of power.

1. **Voltage fluctuations are** caused by air bubbles trapped between the sample and the ion-exchange membranes. Remove the assembled Rotofor core, hold it vertically and tap on it to dislodge the bubbles. Turn the cell 180° and repeat the bubble removal process. The minimum running volume of sample solution should not be less than 35 ml for the standard focusing chamber and 18 ml for the mini chamber.

2. **Voltage decreasing at the beginning of the run is normal.** At the beginning of a run mobile charge carriers migrate through the chamber creating a relatively high initial current. Eventually, desalting ceases and the pH gradient forms. As the run proceeds, the resistance of the focusing medium increases and voltage climbs. Do not set a limit on the voltage below 2,000 volts. When the voltage finally plateaus, steady state has been achieved. Let the run continue for an additional 15-30 minutes, then harvest.

3. **Arcing between the anode and/or the cathode contact plate(s) and the contact assembly(s)** may occur for either of two reasons: 1) the solid brass points of the contact assembly(s) are worn down and electricity is jumping across the gap, or 2) there is a leak in the coolant from the cooling finger housing and the coolant is making the electrical connection. Either replace the contact assemblies left side (catalog number 100-3780) or right side (100-3790) or repair the leaking cooling finger with new O-rings from the Cooling Finger Repair Kit (catalog number 170-2954).

9.6 Uneven Harvesting

1. **Use a stronger vacuum.** Use vacuum that pulls >5 inches mercury.
2. **Remove both harvesting and loading port covers.** Before puncturing the sealing tape to aspirate the fractions, remove both upper and lower covers. Do not cover loading port holes with your fingers or gloves.
3. **Run the Rotofor cell completely full.** Volumes will vary in compartments as the result of protein concentrations in each.
4. **Check the needles in the harvesting block, the tubing and the harvest block lid.** They should not be loose, kinked, plugged, or unequal in height.
5. **Check the plastic harvest tubing.** If necessary, soak the tubes in dilute NaOH (0.1 M) to remove residual matter then rinse the tubes with water.

   Caution: DO NOT insert all of the needles into a water bath at the same time with the vacuum on, as the harvest box may implode! Dry the tubes one at a time.

9.7 Mechanical Problems

1. **The cell doesn't rotate.** Make sure that the core is assembled tightly, and that the gear teeth are meshing well. With each hand, grab the big black rings that hold the halves of the electrolyte chambers together and tighten them simultaneously for better leverage. (Also make sure that the switch is set to "run" and not "harvest"). Refer to the manual for proper assembly.
2. **The unit leaks.** Leaks from different places indicate different problems:

   A) **Vent buttons** may become wet under normal operation, but this is usually attributable to condensation during the run, not to leaking. If they are old (>4–5 runs), or the electrolyte chamber is more than 2/3 full, they may actually leak. Replace the vent buttons if they are old. Make sure electrolyte solutions are 0.1 Molar.

   B) **Check the ion exchange gaskets** between the two halves of the electrolyte chamber to make sure that there is a good seal between them. They should not be wrinkled, pinched, or out of alignment.

   C) **Check the O-rings** that seal the chamber from the cooling finger. If one or more of them is twisted, cracked, or missing, the unit may leak. To keep the O-rings from twisting as you put the unit together, lubricate them with a small amount of silicone grease, or Cello-Seal.

   D) The cell must be seated firmly in the electrodes with the screws tightened.
3. **The focusing chamber is too long for the base.** Push the assembled components all completely together on the cooling finger. To properly seat the pieces together may require some force.

Section 10
Maintenance Guide

10.1 Vent Buttons

The vent buttons should last four or five runs. They should be inspected before use for tears in the fabric. If there are any tears, or if leaking occurs during the run, replace the vent buttons (catalog number 170-2957). Leaking may be due to overfilling the electrode assemblies. Make sure the assemblies are not filled more than 65% full. Air space is needed above the electrolyte to allow gas to escape through the buttons. If the air space is insufficient, pressure may build up and cause leaking.

10.2 O-rings

The O-rings in the electrode assemblies should be inspected after the first 20–30 runs. If there are any signs of wear, replace them using the O-rings supplied in the Repair Kit (catalog number 170-2953). If the O-rings are unworn after the first 20 runs, check them every five runs after that until they need to be replaced.

10.3 Cooling Finger O-rings

Inspect the cooling finger O-rings every 200 runs or every year, which ever comes first. If there are signs of wear, replace them using the O-rings supplied in the Cooling Finger O-ring Kit (catalog number 170-2954).

10.4 Membrane Core

The membrane core does not have a replacement schedule. It should be inspected after each run. If it becomes deformed (through overheating or mechanical stress), it should be replaced.

Section 11
Rotofor References

Methods

Whether alone or in combination with other techniques- polyacrylamide gel electrophoresis (PAGE), electroelution, or chromatography, for example- the Rotofor system integrates into any purification scheme. The following articles illustrate the use of the Rotofor in a variety of different protein purification and enrichment methods.

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Rapid Commun Mass Spectrom. 15(6): 428-32.

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Preparative 2-D Applications

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Section 12
Rotofor Application Notes

Bulletin #	Title
2-D Applications	
2859	Combination of 2-D Gel and Liquid-Phase Electrophoretic Separations as Proteomic Tools in Neuroscience
1773	Preparative 2-D Electrophoresis System Purifies Recombinant Nuclear Proteins from Whole Bacterial Lysates
1776	A Rapid Method for the Purification of Analytical Grade Proteins from Plant Using Preparative SDS-PAGE and Preparative Isoelectric Focusing
2043	Purification of Proteins *from Mycobacterium tuberculosis* by Simultaneous Electro-Elution of the Mini Whole Gel Eluter
1744	Preparative 2-D Purifies Proteins for Sequencing or Antibody Production
1953	Preparative SDS Gel Electrophoresis of Hydrophobic Cell Wall Proteins from *Candida albicans*
RP0014	Isoelectric Focusing Nonporous RP HPLC: A Two-Dimensional Liquid Phase Separation Method for Mapping of Cellular Proteins with Identification Using MALDI-TOF Mass Spectrometry
RP0015	Identification of Protein Vaccine Candidates from *Helicobacter pylori* Using a Preparative Two-Dimensional Electrophoretic Procedure and Mass Spectrometry
Native Proteins	
1508	Isolation of a Toxic Phospholipase D from *Corynebacterium pseudotuberculosis*
1520	Isolation of an *Escherichia coli* Heat Stable Enterotoxin (STb)-Alkaline Phosphatase Fusion Protein by Preparative Isoelectric Focusing
1899	Isolation of Multiple Lipoprotein (a) Charge Forms in Human Plasma by Liquid Phase Isoelectric Focusing
1521	Isolation of Recombinant HIV1 Protease Expressed in *E. coli* and *S. cerevisiae*
1516	Separation of Secreted immunosuppressive Proteins of the Fish Pathogen, *Renibacterium Salmoninarum*, from Culture Medium and Infected Fish Tissues
1519	Isolation and Purification of a Turkey Seminal Plasma Protease
1539	Separation of Aldose – Reductase Isoelectric Forms Using the Rotofor Cell

Bulletin #	Title
Detergents or Denarurants used	
1518	Purification of Bacterial Eukaryotic Fusion Proteins Using the Rotofor Cell
1517	Rotofor Fractionation of Intestinal Brush Border Membrane Proteins
1514	Isolation of a Membrane Bound Immunoregulatory Molecule from Metastatic Lymphoma Cells
1515	Preparation of Spinach Cold Acclimation Proteins for Gas Phase Sequencing, Oligonucleotide Derivation and Monoclonal Antibody Production
1475	Isolation of Monoclonal Antibodies to Phencyclidine from Ascite Fluid

Section 13
Application for Preparative Two Dimensional Electrophoresis System

Preparative electrofocusing in the Rotofor Cell is typically used for the initial fractionation of proteins from crude mixtures. Following primary purification in the Rotofor Cell, a final purification step may be needed to isolate a specific component. Bio-Rad's Model 491 Prep Cell is a continuous elution polyacrylamide gel electrophoresis device that is designed to be used as a secondary, and final purification step following the Rotofor Cell. The following example illustrates the usefulness of Bio-Rads unique "preparative two-dimensional electrophoresis system".

13.1 Introduction

We report here a new preparative two-dimensional (2-D) electrophoresis system for purification of proteins. The system is based on the same principles of isoelectric focusing and gel electrophoresis as analytical two-dimensional electrophoresis. The procedure, which combines the Rotofor® preparative isoelectric focusing (IEF) cell and the new Model 491 Prep Cell for preparative gel electrophoresis (PAGE), is applicable to a wide range of biological samples. This preparative 2-D system is designed to purify individual proteins from crude, complex mixtures for detailed compositional analysis and antibody production and is especially advantageous for isolating proteins present in low concentrations in the specimen.

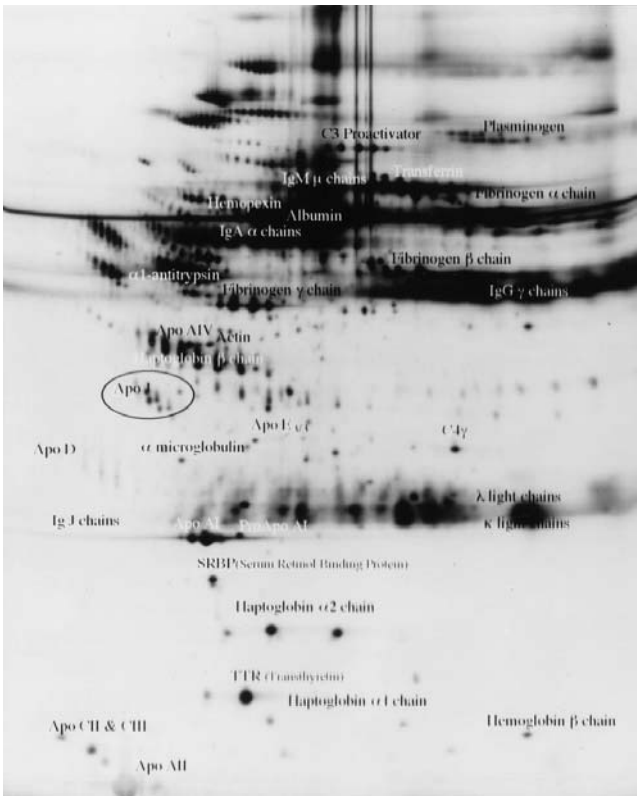

Fig. 1. Analytical 2-D gel map of whole human plasma. This silver stained second-dimension gel demonstrates the complexity of the starting sample. The positions of the glycosylation-induced isoforms of Apo J, and the uncharacterized 49 kd protein of interest, purified by preparative 2-D electrophoresis, are indicated.

Analytical two-dimensional (2-D) gel electrophoresis is now a routine procedure for reproducible separation of proteins in complex biological samples[1,2,3]. Over 3000 tissue proteins and more than 1000 plasma proteins can be resolved by this method. However, analytical 2-D electrophoresis procedures are incapable of supplying sufficient amounts of low abundance proteins for further characterization. It has been necessary to recover proteins from several gels for sequence analysis[4], assay, or antibody production[5].

In preparative 2-D electrophoresis the first step fractionates proteins into defined pH ranges by liquid-phase isoelectric focusing in the Rotofor cell. The Rotofor is capable of 500-fold, or more, purifications of proteins from complex mixtures. Proteins are concentrated in discrete liquid fractions at their respective isoelectric points. In the second purification step, preparative polyacrylamide gel electrophoresis (PAGE) in the Model 491 Prep Cell, individual proteins are isolated on the basis of their size differences.

Samples such as plasma pose a particular problem for electrophoretic techniques due to the presence of high concentrations of albumin immunoglobulins, which together make up more than 65% of the total plasma protein. The high protein load severely limits the volume of plasma that can be purified by conventional electrophoretic means. The preparative-2D method circumvents this problem. The method is illustrated with purification of a 70 kd dimeric (34 and 36 kd) apolipoprotein (Apo J) and a 49 kd uncharacterized protein which in previous blotting experiments appeared to have a blocked amino-terminus. Both have glycosylated isoforms with pIs ranging from 4.9 tom 5.3. Apo J which is in the 0.05 mg/ml concentration range represents less than 0.15% of total plasma protein. The low plasma concentration of Apo J and the 49kd uncharacterized protein is evident from analytical 2-D PAGE of whole plasma (Figure 1) where they are barely visible with silver staining.

13.2 Methods

Analytical 2-D PAGE

5 microliters of whole human plasma were diluted with 10 microliters of dithioerythritol (DTE, 1% w/v), containing SDS (10% w/v). After a 5 minute incubation at 95 ÞC the sample was diluted to 500 microliters with DTE (1% w/v), CHAPS (4% v/v), urea (9M) and ampholytes (pH range 3-11, 5% v/v). Aliquots of 30 microliters (containing 18 micrograms of protein) were used for analysis on 2-D gels. See Figure 1. Protein containing fractions obtained from the Rotofor cell were similarly treated.

Sample preparation for preparative 2-D electrophoresis

Whole plasma (20.0 ml) was first dialyzed (2 hours, Mr cut off 10,000) against distilled water. Following dialysis, urea (21 g, final concentration 7M), CHAPS (1.0 g, final concentration 2% w/v) and DTE (0.232 g, final concentration 30 mM), were added. After stirring for 15 minutes, carrier ampholytes [Bio-Rad Bio-Lytes®; pH range 3-10 (2.5 ml) and pH range 5-7 (0.5 ml)] were added and the volume was brought to 50 ml with distilled water.

Preparative Isoelectric Focusing

The sample (50 ml) containing 1.2 grams of total protein, was loaded into the Rotofor cell for initial fractionation in a wide-range pH gradient (pH 3-10). Constant power (10 W) was applied for 5 hours with the system cooled to 4 ÞC. Runs were terminated when the voltage had stabilized (1500 V) for about 30 minutes. 20 Rotofor fractions were collected. Selected fractions were analyzed by 2D-PAGE. Rotofor fraction 5 (pH 4.3) was substantially free of the bulk plasma proteins, albumin and immunoglobulins, and highly enriched for Apo J and the unknown protein. This step provided approximately 500-fold purification of the protein of interest (Figure 2a).

Refractionation

Rotofor fractions 4,5 and 6 were collected, pooled, and refractionated in the Rotofor cell without additional ampholytes. The total protein load was 50 milligrams. Upon refractionation, an overall 1000-fold purification of Apo J and the unknown protein was obtained in Rotofor fraction 11. See Figure 2b.

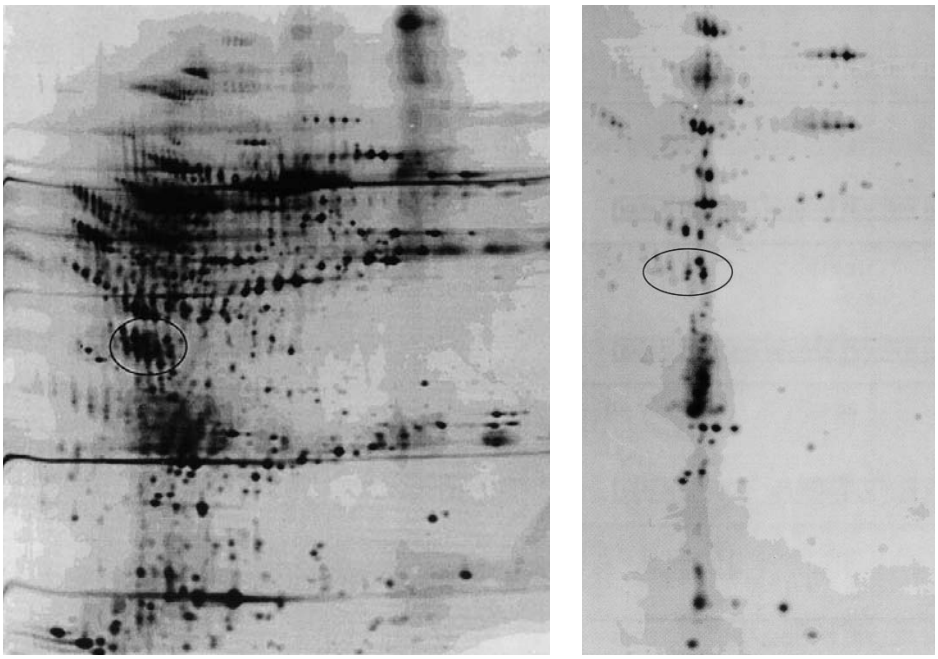

Fig. 2. Analysis of Rotofor fractions by 2-D gel electrophoresis. 2a) Initial Rotofor fractionation provided enrichment for the proteins of interest in Rotofor fraction 5, shown here. 2b) Refractionation of Rotofor fraction 5 shown here resulted in 1000-fold purification of Apo J and the unknown 49 kd protein by comparison to the starting sample in Figure 1. Analytical 2-D gels were silver stained.

Preparative SDS-PAGE

For preparative gel electrophoresis, the discontinuous buffer system of Laemmli was used[5]. The total acrylamide concentration (%T) of the separating gel was optimized at 12%.

The sample (Rotofor fraction 11) contained approximately 2.5 mg of total protein dissolved in 2.0 ml of sample buffer (see Table 1). After a 5 minute incubation at 95°C, the sample was loaded onto the Prep cell and the gel run for 16 hours. Running buffer was pumped through the elution chamber at a rate of 0.5 ml per minute.

Table 1. Model 491 Prep cell running conditions

Resolving gel:	12% acrylamide/ 2.6% C (PDA crosslinker)
Resolving gel length:	8 cm in 37 mm gel tube
Resolving gel buffer:	Tris-HCl (0.375 M) pH 8.8
Stacking gel:	4% T/2.6% C (PDA crosslinker)
Stacking gel buffer:	Tris-HCl (125 mM) pH 6.5
Running buffer:	Tris-Glycine-SDS (25 mM-192mM-0.1%)

Elution buffer:	Tris-Glycine-SDS (25 mM-192mM-0.1%)
Sample buffer A:	10% SDS + 2.32% DTE
Sample buffer B:	1% g DTE + 4% CHAPS + 9 M urea + 5% ampholytes pH 9-11
Sample:	Rotofor Fraction 11 was dialyzed and freeze dried then dissolved in 100 microliters of sample buffer A. Then 1900 microliters of sample buffer B was added.
Elution rate:	0.5 ml/min
Power:	start: 50 mA 177 V 8 W finish: 50 mA 301 V 12 W

Fraction Collection and Analysis

The elution chamber outlet of the Model 491 Prep cell was connected to a fraction collector (Bio-Rad Econo System) and 80 5-ml fractions were collected. Fraction number one was the first fraction containing visible amounts of the bromophenol blue marker dye. In order to locate the fractions containing Apo J and the 49 kd uncharacterized protein, 30 microliters from every fifth fraction were analyzed by SDS-PAGE. (Figure 3a). Once the elution positions of the Apo J and 49kd protein were determined, 30 microliters of every fraction near the peak of the eluted proteins of interest were analyzed by SDS-PAGE. (Figure 3b).

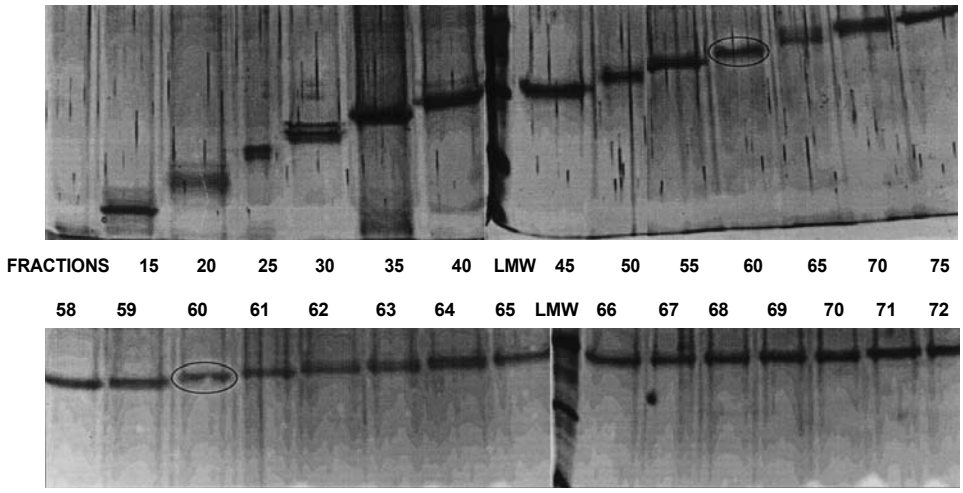

Fig. 3. Analysis of protein fractions eluted from the Prep cell. 3a) Aliquots from every fifth Prep cell fraction were analyzed on silver stained. SDS-PAGE gels. The elution position of Apo J was fraction 60. and the 49kd protein eluted in fraction 70. (3b). Every Prep cell fraction near the peak of eluted Apo J and the 49kd protein was then analyzed. The fractions containing Apo J were 60, 61 and 62. and the ones containing the 49 kd protein were 71, 72, and 73.

Antibody Production

Rotofor fraction 11 containing Apo J was assayed for protein[6] and frozen at -20°C until used. Polyclonal antibodies were raised in rabbits against proteins purified as described above using conventional immunization procedures.[7] Polyclonal antibodies were specific for Apo J and did not cross-react with other plasma proteins. Using preparative 2-D electrophoresis (combined Rotofor and

Prep cell) we were able to obtain a pure preparation of Apo J, free of cross-contamination, despite the presence of a series of presumably glycosylation-induced isomers. Figure 1.

Sequence Analysis

Prep cell fractions 71, 72 and 73 containing the 49 kd unknown protein, were pooled and concentrated to 500 microliters by freeze drying. The final concentrations of components in the 500 microliter sample was: Tris (200 mM) - glycine (1.6 M) - SDS (0.8%). The sample was then reduced with DTT (2.0 µM, 2 hours at 37 ÞC) and carboxymethylated with iodoacetic acid (ICH_2COOH, 5 µM, pH 8) for 30 minutes in the dark. Following dialysis against water for 48 hours, the sample was again freeze dried, and the SDS extracted.[8] The protein was then digested with TPCK-trypsin in 4 molar urea, pH 8.0. Prior to sequencing, peptides were separated with a Microbore C8 HPLC column (1x100mm) with a 0.1% TFA/Acetonitrile system.[9] Sequence analysis was done on an ABI 473 A sequenator. We have found no sequences similar to those of the 49 kd protein in data base searches.

13.3 Results

This report describes a rapid electrophoretic procedure for purification of proteins from crude extracts in concentrations where comprehensive sequence analysis and antibody production are feasible. Apo J and the 49kd uncharacterized protein were obtained in a highly purified state. The preparative 2-D procedure typically yields from 20-40 micrograms of the proteins.

The advantages of the primary fractionation step (liquid phase IEF) cannot be over-emphasized, notably with respect to the fractionation of plasma proteins. Here, high plasma concentrations of certain proteins, such as albumen, alpha-1-antitrypsin, immunoglubulins or transferrin limit the volume of plasma that can be processed. Pre-fractionation of plasma with the Rotofor confines these proteins to their respective pl ranges. It is then possible to undertake a sequential, detailed analysis of the different Rotofor fractions. Each fraction represents a defined, restricted pl interval, containing an adequate quantity of protein for the preparative PAGE purification step.

In conclusion, this procedure can be considered a viable means of obtaining highly purified preparations of plasma proteins, even those present in low concentrations. Yields are such that comprehensive sequence data can be generated on amino-terminally blocked proteins and antibody production is feasible. The procedure offers great potential as a firstline protein purification procedure, whether applied to plasma or other biological samples.

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Section 14
Product Information

Catalog Number	Product Description
Rotofor Cell and Mini Rotofor Cell[1]	
170-2986	**Rotofor Purification System**, 100/120 V, includes 60 ml focusing chamber, 18 ml focusing chamber, and starter kit
170-2987	**Rotofor Purification System**, 200/220 V, includes 60 ml focusing
170-2914	**Rotofor Purification System with PowerPac 3000 Power Supply**, 100/120V
170-2906	**Rotofor Purification System with PowerPac 3000 Power Supply**, 220/240V
170-2950	**Standard Rotofor Cell**, 100/120V, includes 60 ml focusing chamber and starter kit
170-2951	**Standard Rotofor Cell**, 220/240V, includes 60 ml focusing chamber and starter kit
170-2988	**Mini Rotofor Cell**, 100/120V, includes 18 ml focusing chamber and starter kit
170-2989	**Mini Rotofor Cell**, 220/240V, includes 18 ml focusing chamber and starter kit
170-2910	**Rotofor Starter Kit**, includes 10 ml Bio-Lyte ampholytes (pH range 3-10), 60 ml syringe, colored protein sample, 2 vent buttons, one each of the ion exchange membranes, hydrated
170-2919	**Colored Protein Sample**, 1 ml (included in Rotofor Starter Kit)
Rotofor Adaptor Kits	
170-2990	**Adaptor Kit,** Rotofor cell to mini Rotofor cell, includes mini focusing chamber and mini membrane core, 18 ml
170-2959	**Adaptor Kit**, mini Rotofor cell to Rotofor cell, includes standard focusing chamber and membrane core, 60 ml

[1] The Rotofor Cell comes with all necessary parts for initial set up and operation. A repair kit, extra membrane cores, ion exchange membranes and vent buttons are recommended as spare parts.

Catalog Number	Product Description
Replacement Accessories	
170-2991	**Mini Membrane Core**, for 18 ml focusing chamber, 2
170-2952	**Membrane Core**, for 60 ml focusing chamber, 2
170-2953	**Repair Kit**, includes O-ring kit, 4 ion exchange gaskets, 4 port cover screws, 4 electrolyte chamber screws, 2 port gaskets
170-2954	**Cooling Finger O-ring Kit**, with 4 O-rings
170-2956	**Ion Exchange Membranes**, 5 pair
170-2957	**Vent Buttons**, 8
170-2958	**Cooling Finger**
170-2960	**Sealing Tape**
170-2961	**Test Tube Rack**
170-2963	**Harvest Box**
170-2964	**Harvest Tubing**
170-2965	**Harvest Box Lid**
170-2966	**Harvesting Needle Array**
170-2967	**Anode Electrolyte Chamber**, universal
170-2968	**Cathode Electrolyte Chamber**, universal
100-3780	**Contact Assembly**, left side
100-3790	**Contact Assembly**, right side
800-2056	**Inner Anode Membrane Holder Assembly**, Rotofor
800-2057	**Inner Cathode Membrane Holder Assembly**, Rotofor
800-2074	**Inner Membrane Holder Assembly**, mini Rotofor
920-2093	**Recess Gasket for Mini Rotofor**

Catalog Number	Product Description
Solubilizing Agents	
161-0730	**Urea**, 250 g
161-0731	**Urea**, 1 kg
161-0460	**CHAPS**, 1 g
161-0465	**CHAPSO**, 1 g
161-0717	**Glycine**, 250 g
161-0718	**Glycine** 1 kg
Auxiliary Instruments	
170-2926	**Model 491 Prep Cell**, 100/120 V, includes buffer recirculation pump and reagent starter kit with protein standard
170-2927	**Model 491 Prep Cell**, 220/240 V
165-5056	**PowerPac 3000 Power Supply**, 110/120 V
165-5057	**PowerPac 3000 Power Supply**, 220/240 V
Bio-Lyte Ampholytes	
163-1112	**Bio-Lyte 3/10 Ampholyte**, 40%, 10 ml
163-1132	**Bio-Lyte 3/5 Ampholyte**, 20%, 10 ml
163-1142	**Bio-Lyte 4/6 Ampholyte**, 40%, 10 ml
163-1152	**Bio-Lyte 5/7 Ampholyte**, 40%, 10 ml
163-1192	**Bio-Lyte 5/8 Ampholyte**, 40%, 10 ml
163-1162	**Bio-Lyte 6/8 Ampholyte**, 40%, 10 ml
163-1172	**Bio-Lyte 7/9 Ampholyte,** 40%, 10 ml
163-1182	**Bio-Lyte 8/10 Ampholyte**, 20%, 10 ml
163-1113	**Bio-Lyte 3/10 Ampholyte**, 40%, 25 ml
163-1153	**Bio-Lyte 5/7 Ampholyte**, 40%, 25 ml
163-1193	**Bio-Lyte 5/8 Ampholyte**, 40%, 25 ml
163-1163	**Bio-Lyte 6/8 Ampholyte**, 40%, 25 ml

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