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CTS™ Rotea™ Counterflow Centrifugation System Process Design

USER GUIDE

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Revision A.0

For Research Use or Manufacturing of Cell, Gene, or Tissue- Based Products.

ThermoFisher SCIENTIFIC

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For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

The information in this guide is subject to change without notice.

Revision history: Pub. No. MAN0018754

Revision	Date	Description
A.0	20 October 2020	New document. User guide for process design workflow.

Contents

1 Product information

IMPORTANT! Before using this product, read and understand the information in the “Safety” appendix in this document.

Product description

The Gibco™ CTS™ Rotea™ Counterflow Centrifugation System is an automated, closed-flow benchtop instrument that can perform a wide variety of cell processing steps across different cell types.

This *CTS™ Rotea™ Counterflow Centrifugation System Process Design User Guide* provides the user with an overview of the Rotea Protocol Builder application and describes some of the commonly used features and strategies to assist the user with the development of protocols.

Note: The user is responsible for the development, optimization, qualification, storage and management of protocols used on the CTS™ Rotea™ Counterflow Centrifugation System.

System overview

CTS™ Rotea™ Counterflow Centrifugation Instrument

The CTS™ Rotea™ Counterflow Centrifugation Instrument is an automated benchtop instrument that incorporates several important features that enable the user to develop and optimize protocols while ensuring the integrity of the process.

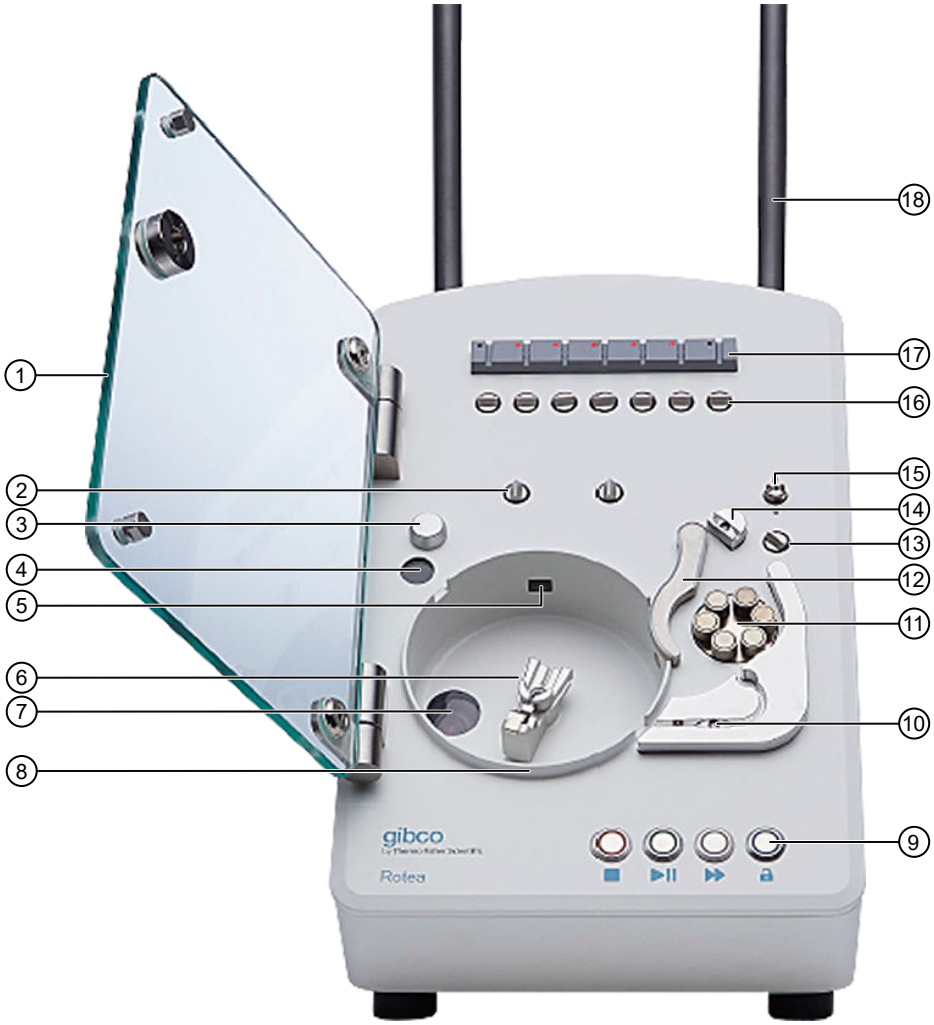


Figure 1 Instrument – Front View

1. Instrument Door
2. Pinch Valves (J & K)
3. Kit Location Button
4. 2D Barcode Reader
5. CFC Chamber Detector
6. CFC Chamber Carrier
7. Camera
8. Moisture Sensor
9. Pushbutton Controls
10. Optical Density Sensor & Pressure Sensor (P1)
11. Peristaltic Pump
12. Pump Clamp Arm
13. Pinch Valve (H)
14. Kit Location Button & Pressure Sensor (P2)
15. Door Latch
16. Pinch Valves (A, B, C, D, E, F, & G)
17. Bubble Sensors
18. Hanger Posts

CTS™ Rotea™ Single-Use Kit

The CTS™ Rotea™ Single-Use Kit can be flexibly configured by the user to perform a range of cell processing applications such as separation, isolation, buffer exchange, wash and concentrate. The Single-Use Kit comprises eight sterile weldable tubes to which the user connects input and output bags/vessels to suit their specific process. The Single-Use Kit also includes a 2D Barcode for kit identification and labelling to aid kit assembly.

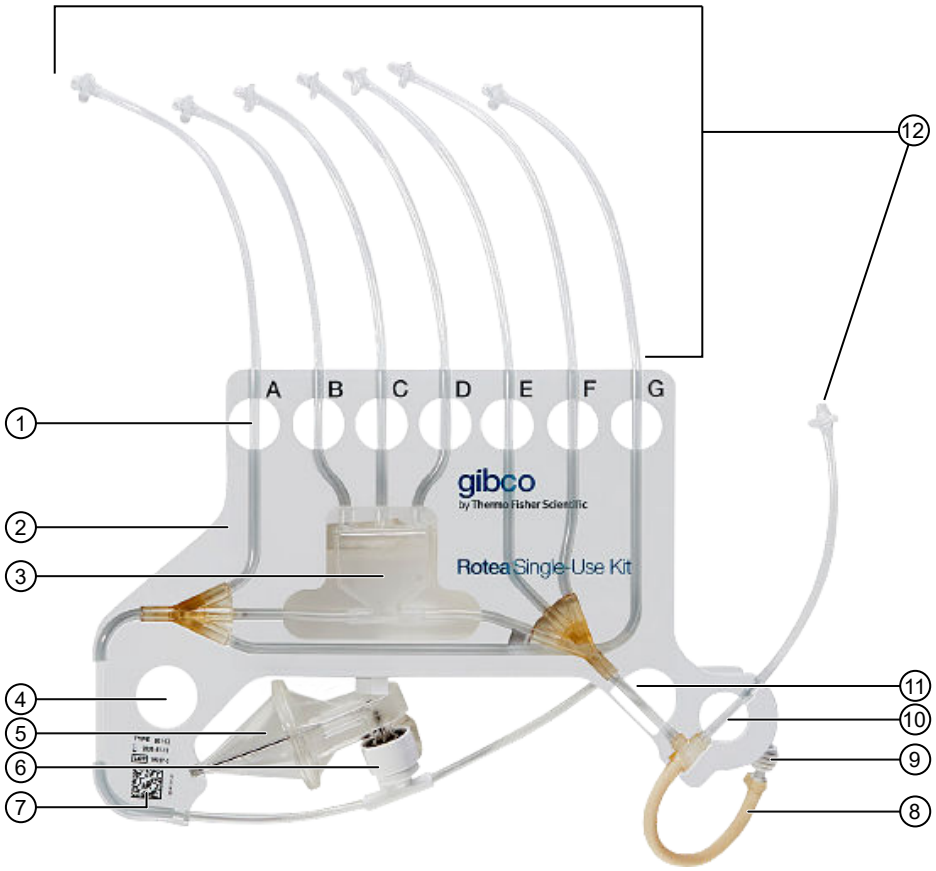
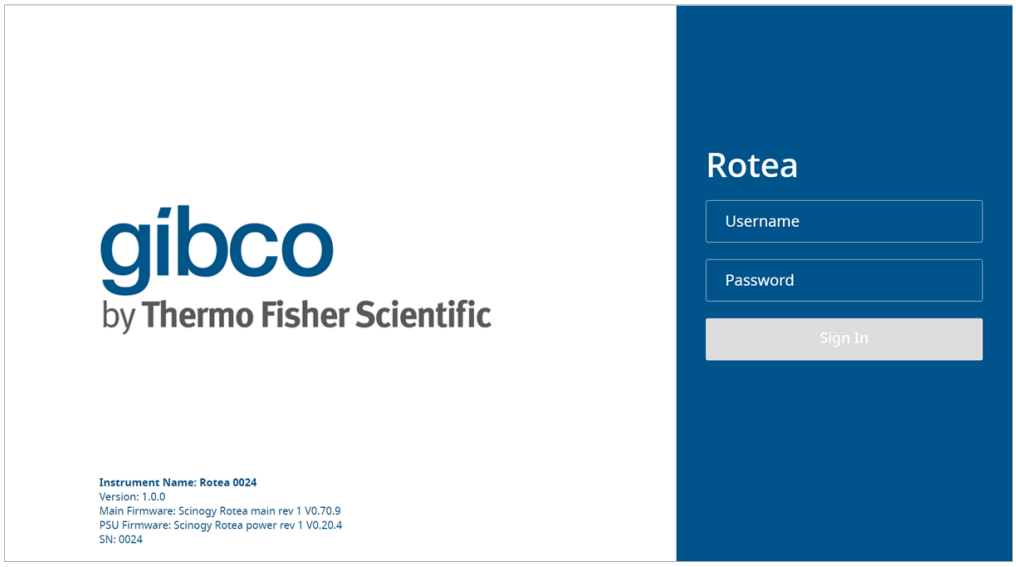


Figure 2 CTS™ Rotea™ Single-Use Kit

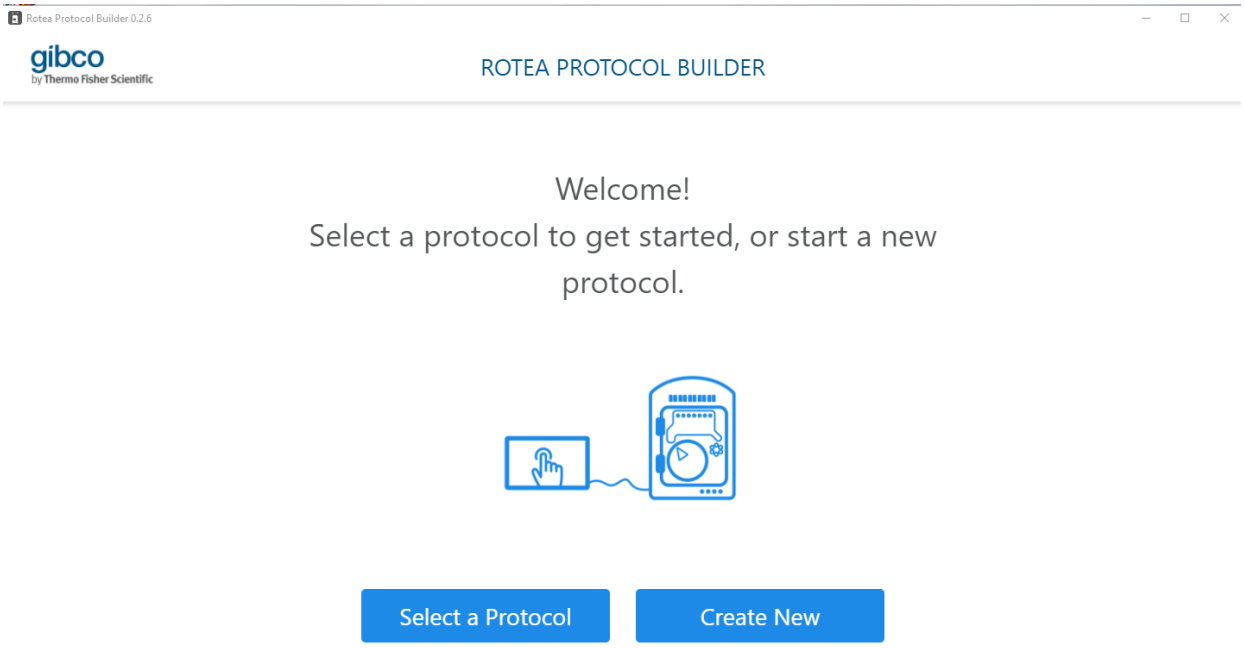
1. Valve Hole & Tube ID
2. Carrier Frame
3. Bubble Trap
4. Location Hole
5. CFC Chamber
6. Rotary Coupling
7. 2D Barcode
8. Pump Tubing
9. Tube Retainer
10. Valve H Hole
11. Location Hole
12. Kit Tubing (Input and Output)

CTS™ Rotea™ Graphical User Interface (GUI)



The CTS™ Rotea™ Graphical User Interface (GUI) enables users to download and run protocols on the instrument. The Rotea™ GUI also includes the ability to make in-process adjustments to the G force and flow rate and has an integrated high-resolution color camera to visualize the behavior of cells in the CFC Chamber in real-time. This provides the user with an interactive environment for protocol development and optimization.

Rotea Protocol Builder

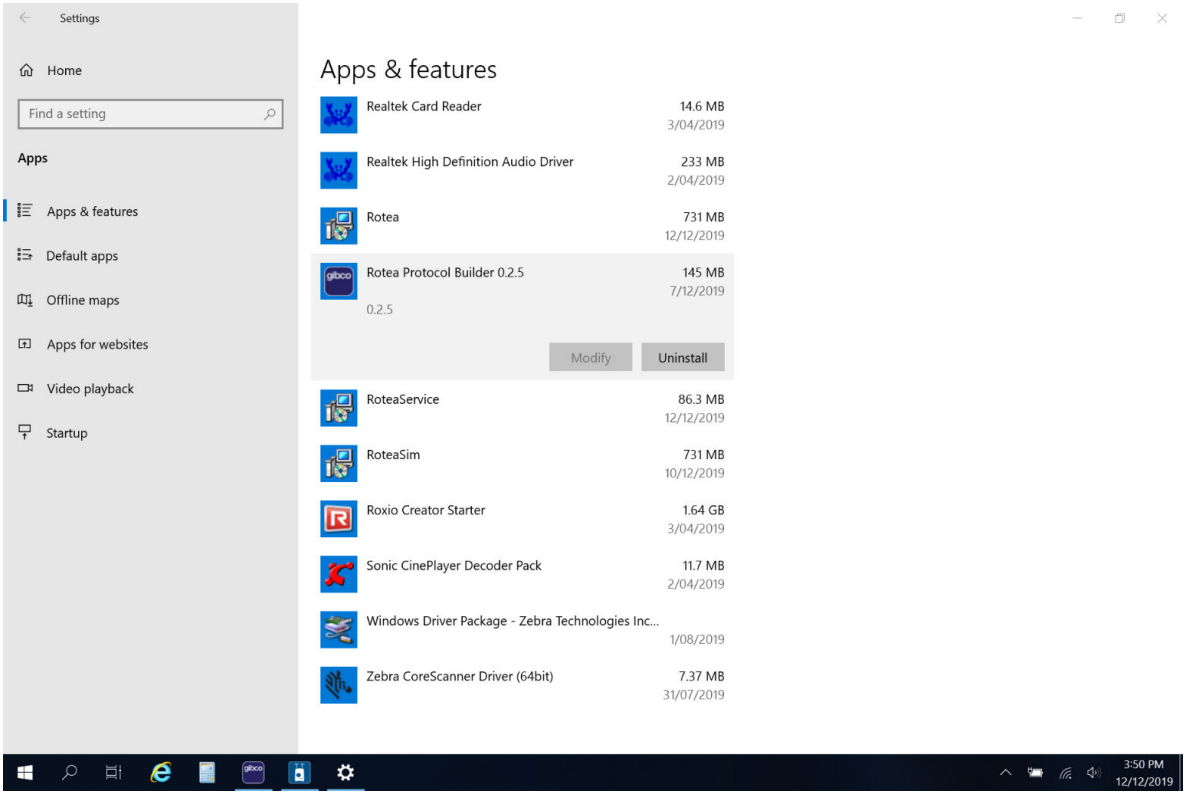


The Rotea Protocol Builder is a Windows-based application that enables users to download, create, modify and save protocols.

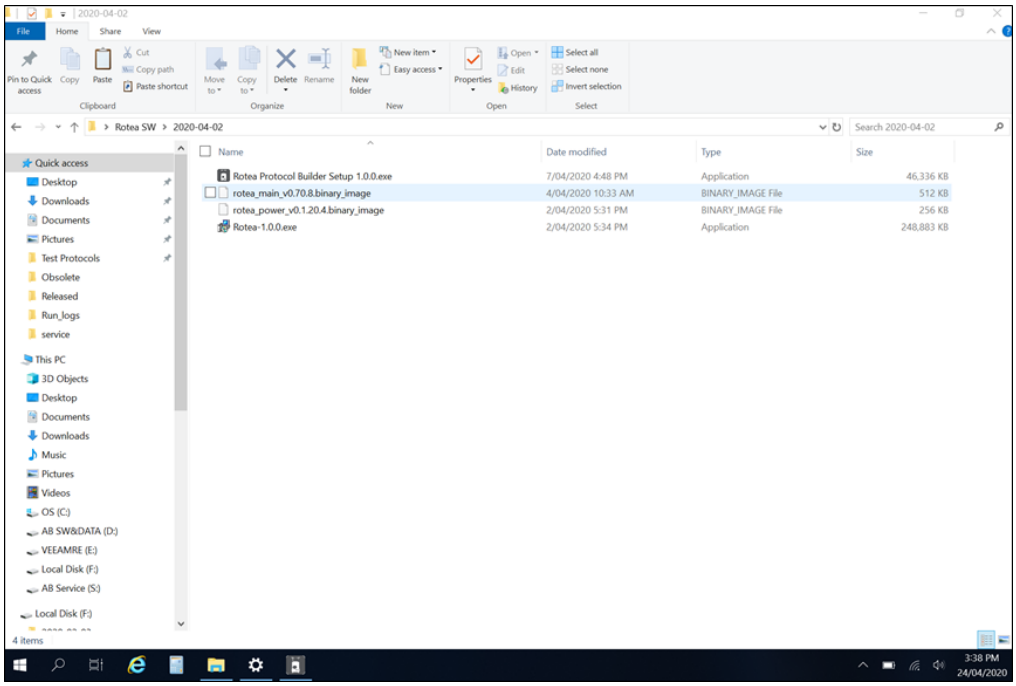
2 Software installation

Software installation

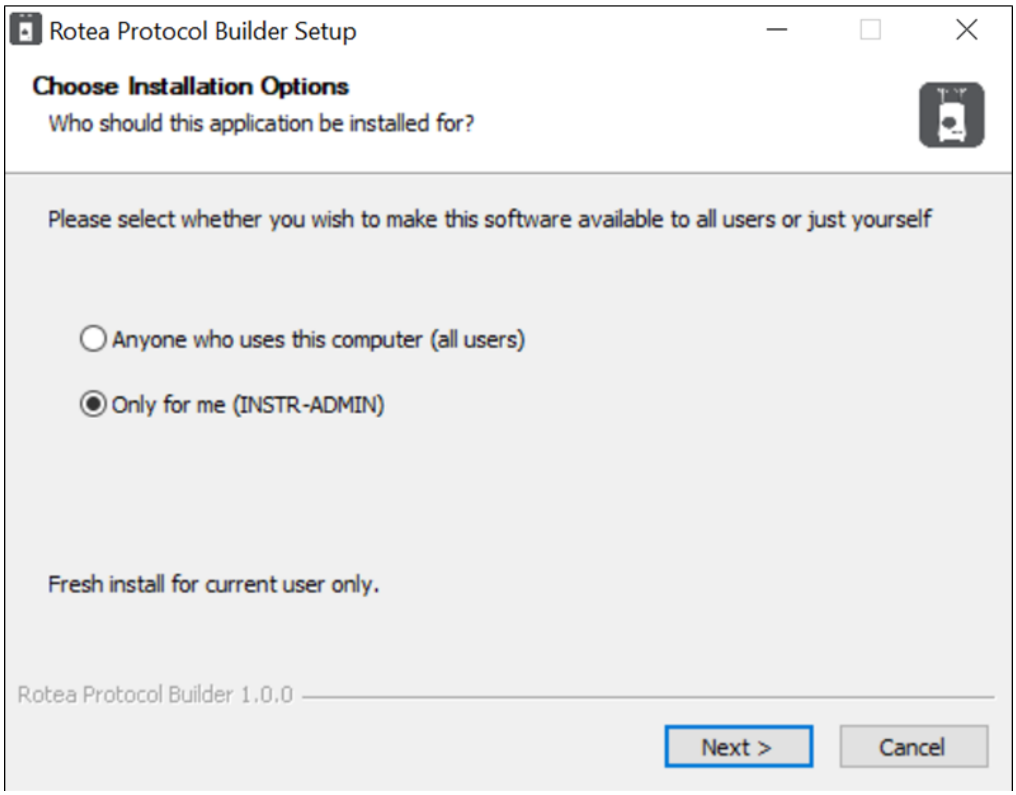
1. Prior to installing a new release of the Rotea Protocol Builder application, first un-install any previous versions.



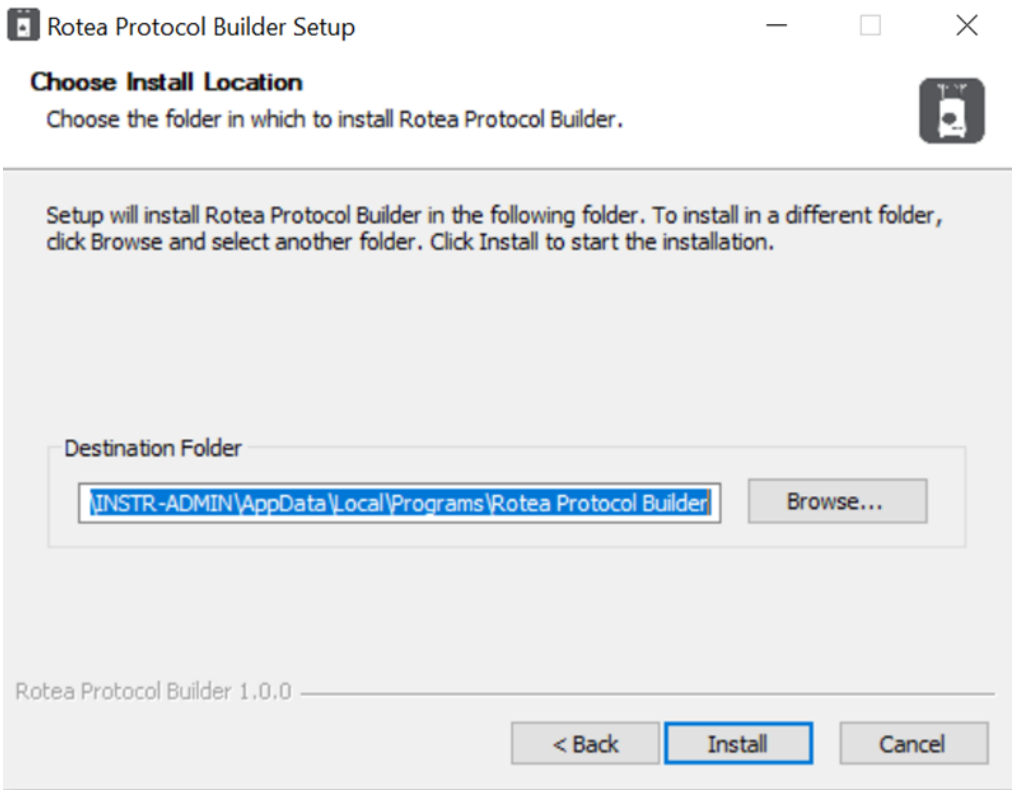
2. Locate the latest release of the **Rotea Protocol Builder Setup** on your PC or accessible directory (in this example a USB Drive). Double click it to begin installation.



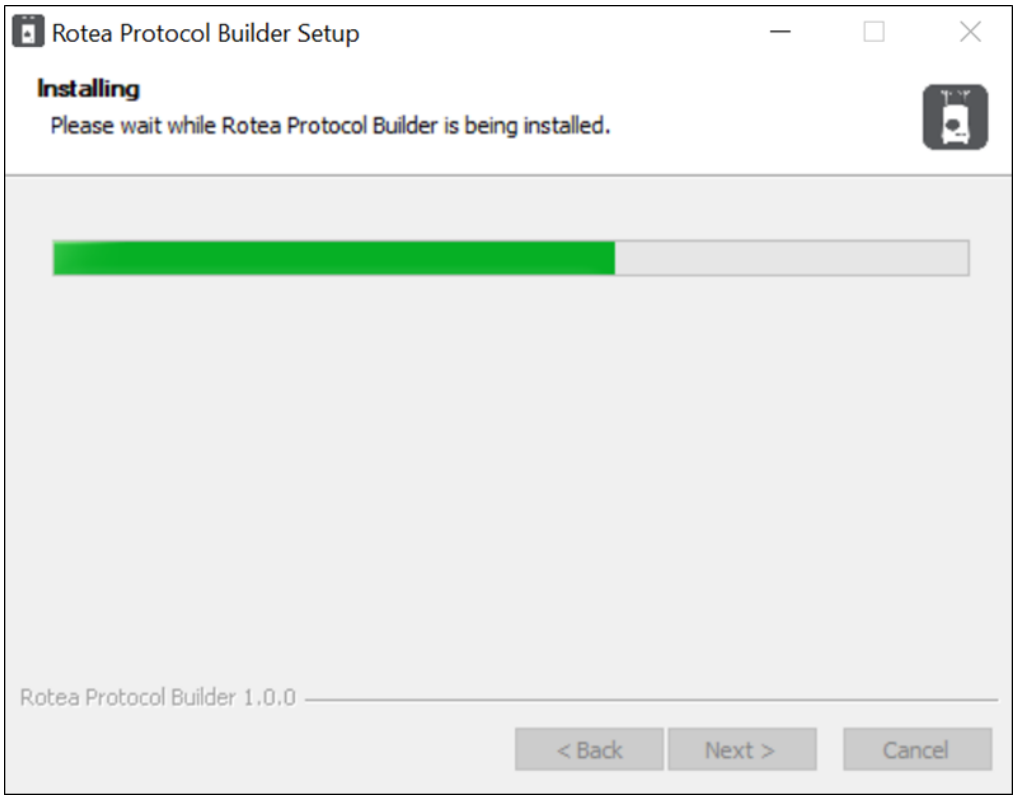
3. Select whether the software will be installed for the current user or all users on the device, and click **Next**.



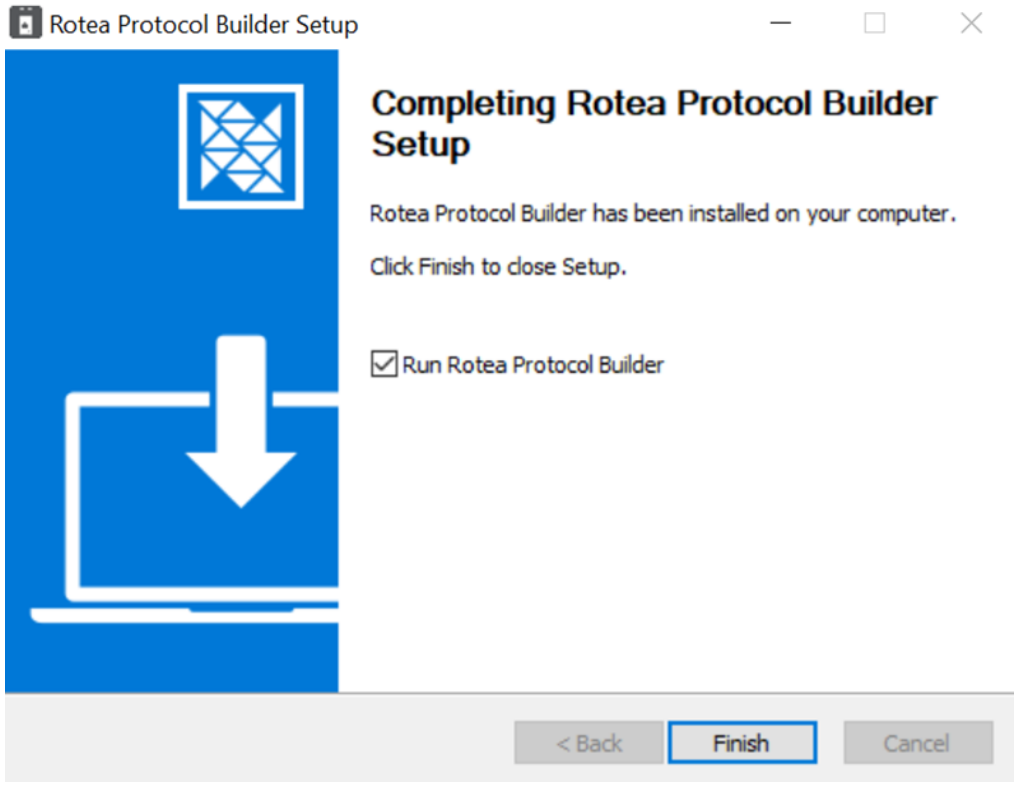
4. Choose where you want the software to be installed. By default, a folder called **Rotea Protocol Builder** is created in **Program Files** on your `C:\ drive`. Then click **Install**.



5. Wait for the installation to complete.

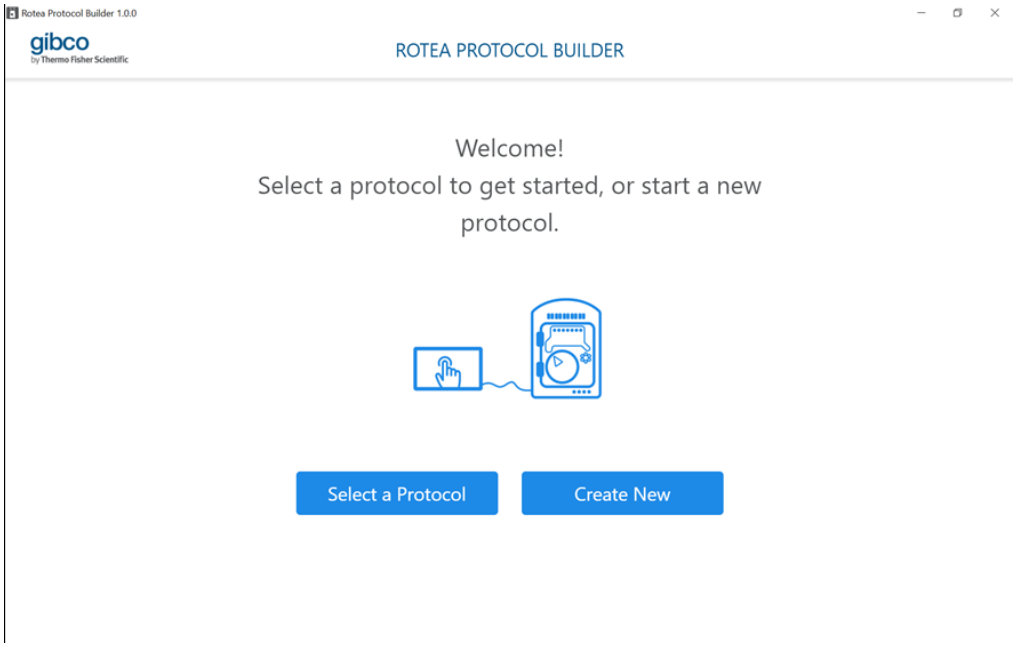


6. Choose whether you want **Rotea Protocol Builder** to open once installation has finished, and click **Finish** to close the installer.



3 Rotea Protocol Builder Application

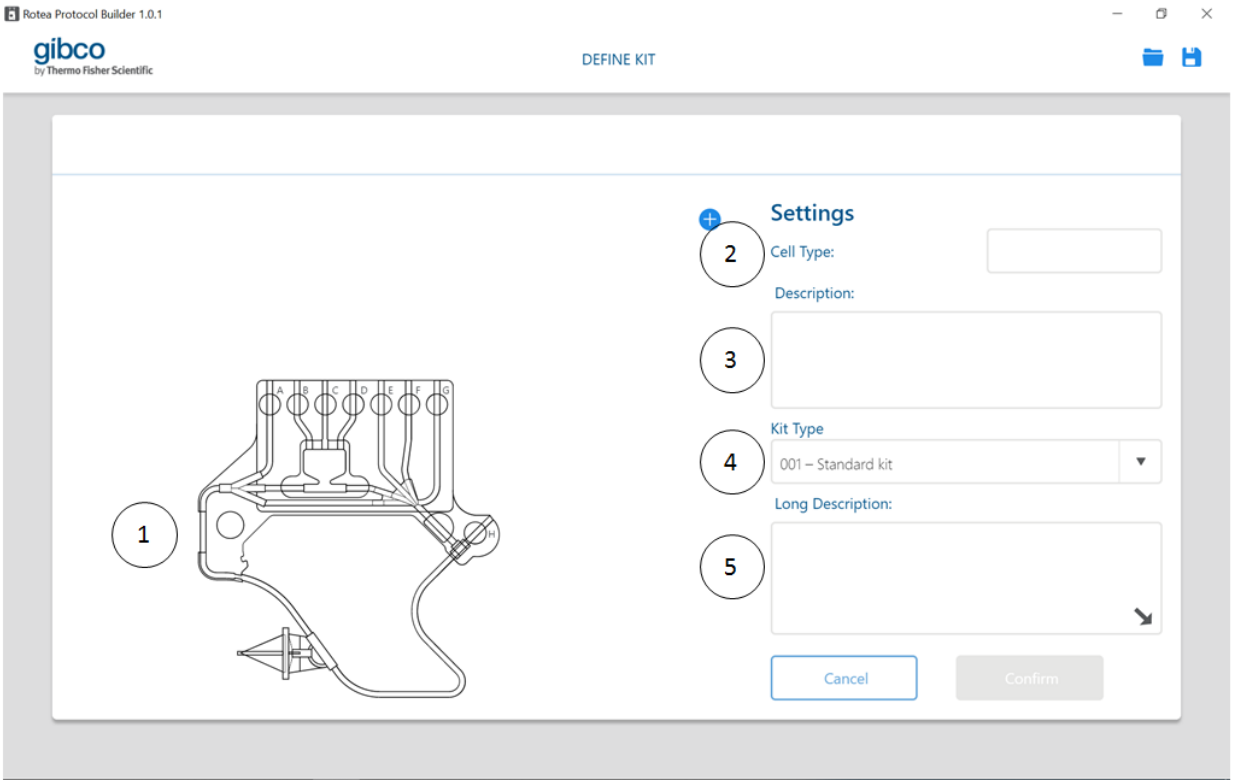
Create or open a protocol



This is the home screen of the **Rotea Protocol Builder**. Select **Create New** to create a new protocol, or **Select a Protocol** to open an existing protocol from an accessible local or external folder.

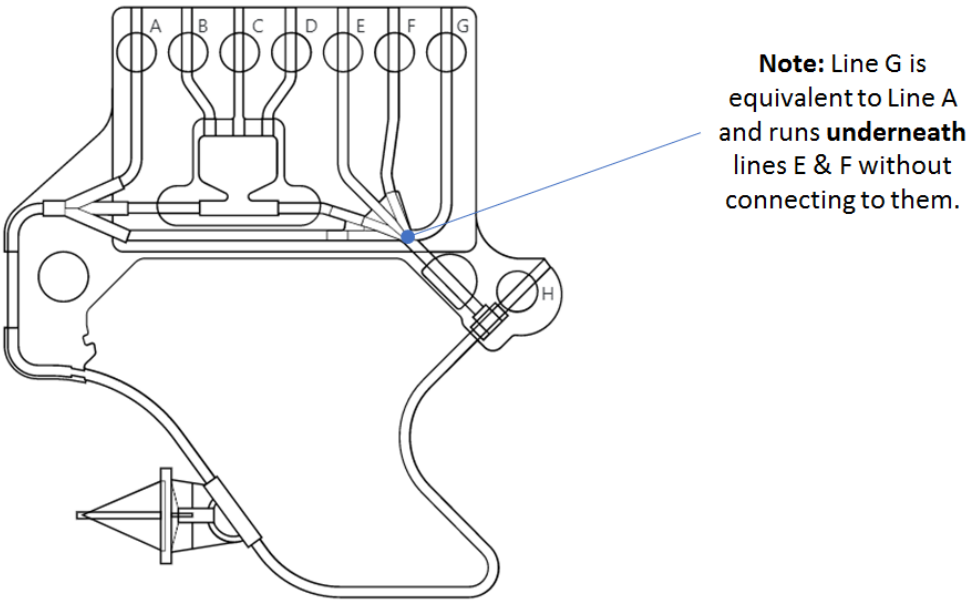
Define Kit

Select **Create New**, to go to the **Define Kit** screen.



① CTS™ Rotea™ Single-Use Kit diagram
② **Cell Type**
③ **Description**
④ **Kit Type**
⑤ **Long Description**

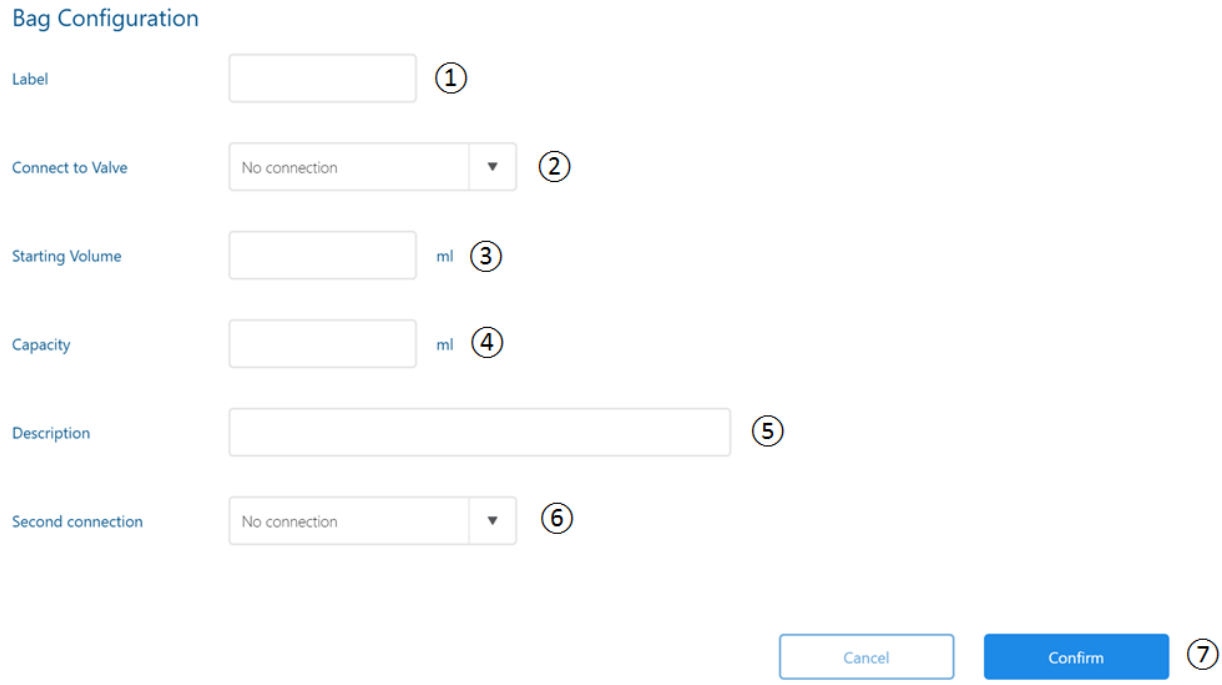
CTS™ Rotea™ Single-Use Kit diagram



On the left of the **Define Kit** screen is a diagram of a CTS™ Rotea™ Single-Use Kit, with no bags attached. The Single-Use Kit configuration is defined by the user by adding bags to the various lines required to perform the protocol.

Bag Configuration

To create a new bag, select the ⊕ to open the **Bag Configuration** window.



① REQUIRED: Enter a label for the bag here (maximum 20 characters). The name can be anything you want but should be a logical reference for the operator of the instrument. This label will appear in multiple places throughout both the Rotea Protocol Builder Application and the Rotea™ GUI as the primary way of identifying the function of the bag. It is very important that the name given is clear and provides the user with an adequate understanding of the bags function and/or contents.

② REQUIRED: Select which valve the bag will be connected to for the protocol from the dropdown menu.

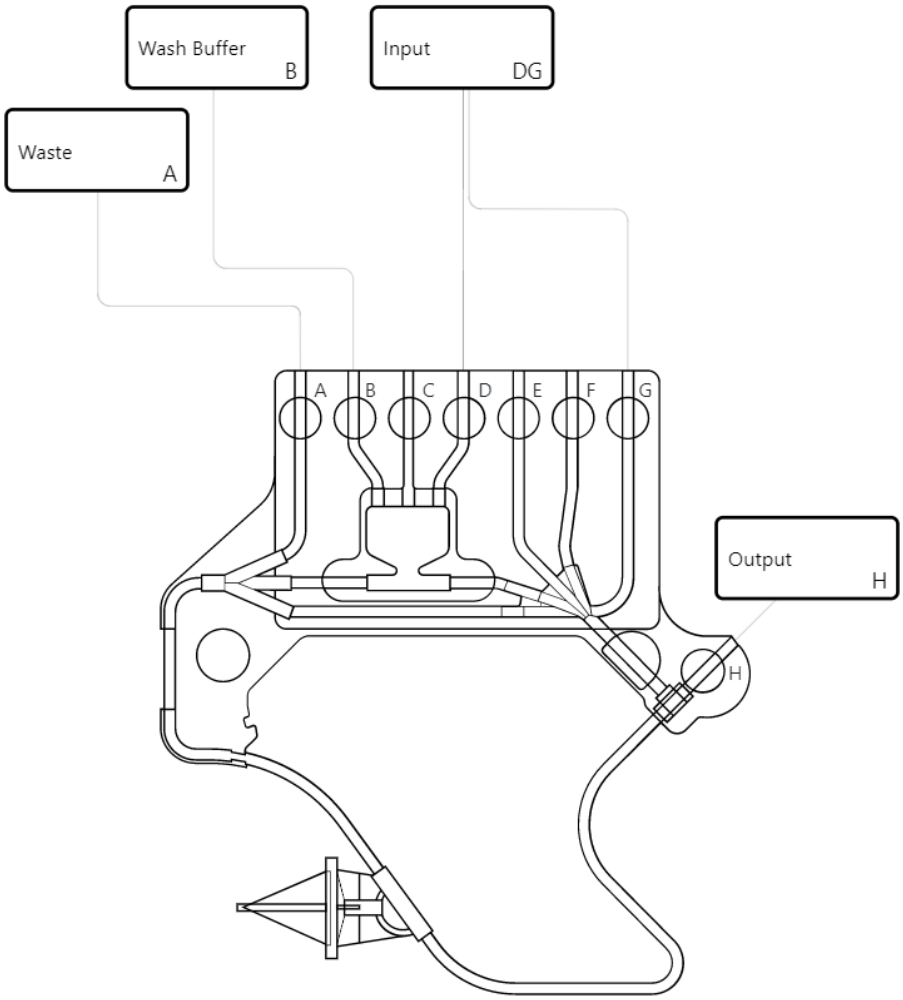
③ OPTIONAL: Set the volume of fluid that should be in the bag at the beginning of the protocol. This is used as a reference so that the user can ensure there is enough fluid to run the protocol.

④ OPTIONAL: Enter the maximum volume the bag can contain, for user reference.

⑤ OPTIONAL: In the **Description** box you can enter a more detailed description for the bag (maximum 50 characters). This is for reference only and can be anything you wish.

⑥ If the bag has a second connection, you can select one from the dropdown menu. Only valid options will be presented.

⑦ Click **Confirm** to save the bag, or **Cancel** to return to the **Define Kit** screen.



An example of what the CTS™ Rotea™ Single-Use Kit diagram might look like after bags have been defined.

Note: Not all tubes need to be attached to a bag and only those being used will be displayed on the kit diagram.

Cell Type

You have the option to enter the cell type your protocol will be used for here. This is for reference only.

Description

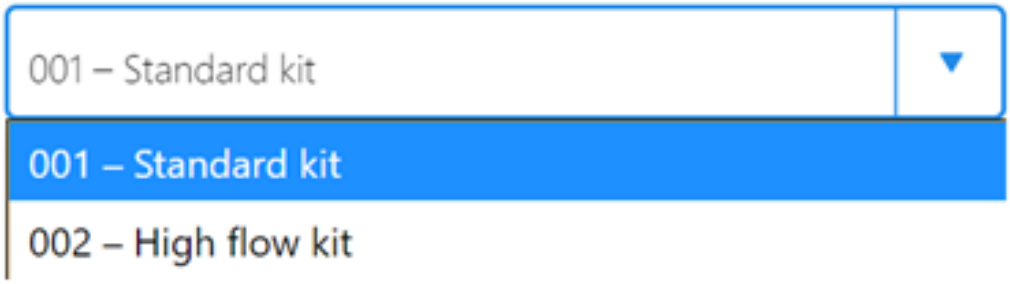
REQUIRED: Enter a description for your protocol here. This is used for reference only, it provides users with a summary of the protocol. Include any additional instructions in the long description.

Kit Type

There are two kit types currently available, defined by the maximum flow rate that is possible using the Single-Use Kit:

- 001 – **Standard kit**: Suitable for flow rates 4 to 110 mL/min
- 002 – **High flow kit**: Suitable for flow rates 6.5 to 180 mL/min. The Protocol Builder application allows 6.5 mL/min to 165 mL/min for the High Flow Kit.

In the **Kit Type** box, select the required kit type from the dropdown menu:



Ensure that the kit type selected matches one the user has available for running protocols.

Note: A 2D Barcode on the underside of the kit frame is read by a 2D Barcode Reader in the instrument on loading of the Single-Use Kit. The instrument will automatically verify that the Single-Use Kit loaded is the correct kit type for the protocol to be run. Pump calibration settings will also be automatically adjusted based on the kit type selected.

Once you have set at least one bag, filled in a description, chosen your kit type and filled in any of the optional entries, select **Confirm** from the **Define Kit** screen to go to **Step** view.

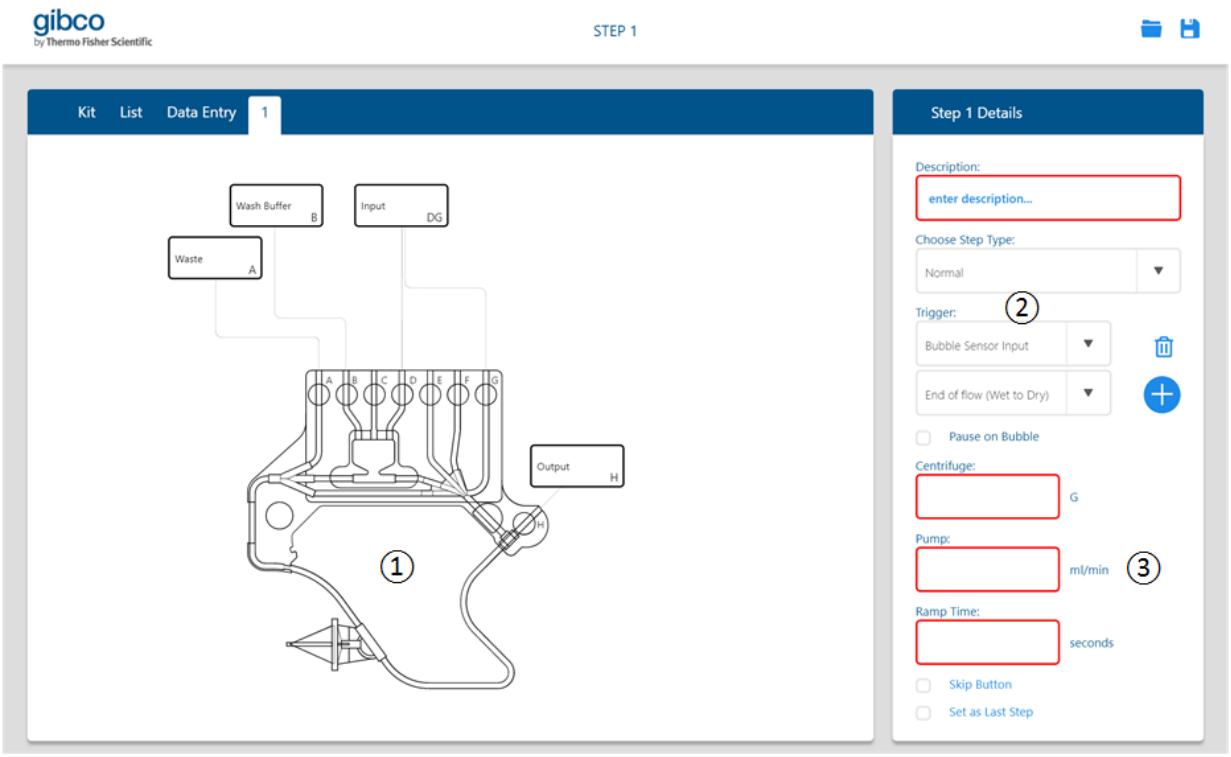
Long Description

Long Description include instructions on how to run the protocol, including descriptions of any data entry. You can enter any information required by the user in this section.

Step view

Protocols comprise individual steps. Each step is automatically assigned a sequential number as it is created.

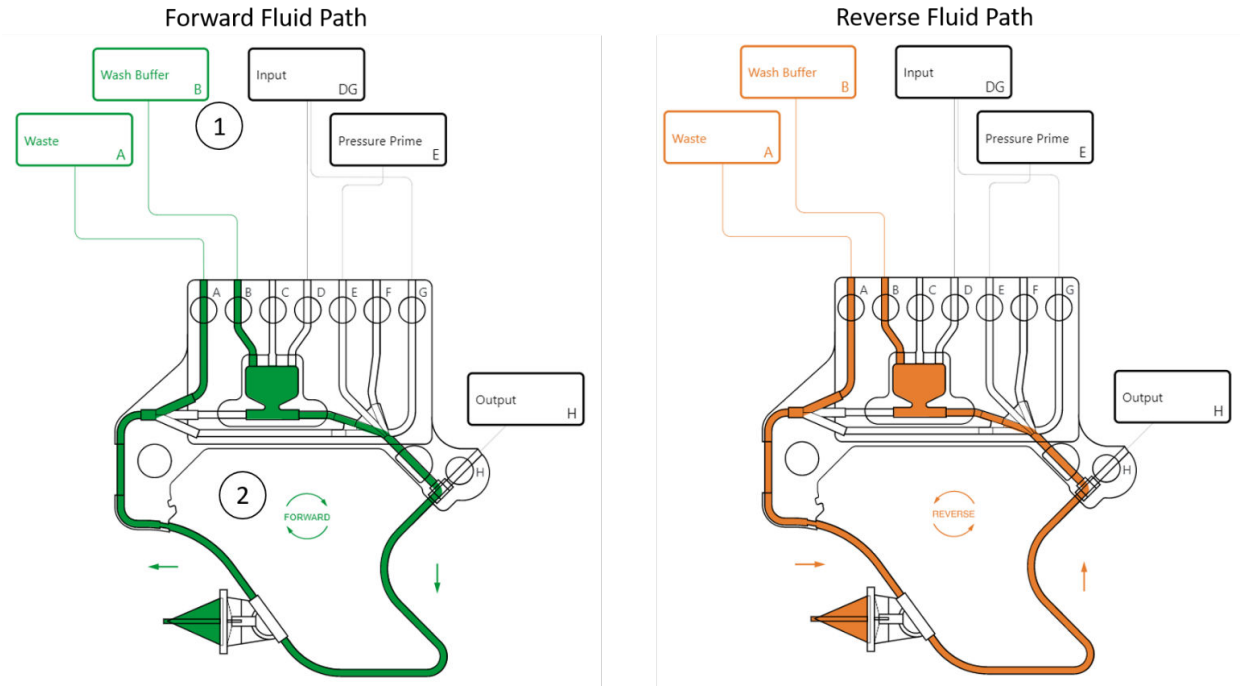
Step numbers are displayed across the top banner and can be selected by clicking on the required number. Where the number of steps exceeds the banner width, “<<, <, > and >>” will be added to the banner to enable navigation.



Step view showing the first step in a new protocol

① Fluid path and direction of flow
② **Choose Step Type** and **Triggers** that determine when the step is complete
③ Operating parameters for the **Centrifuge** (G), **Pump** (mL/min) and **Ramp Time** (seconds)

Define fluid path and direction of flow

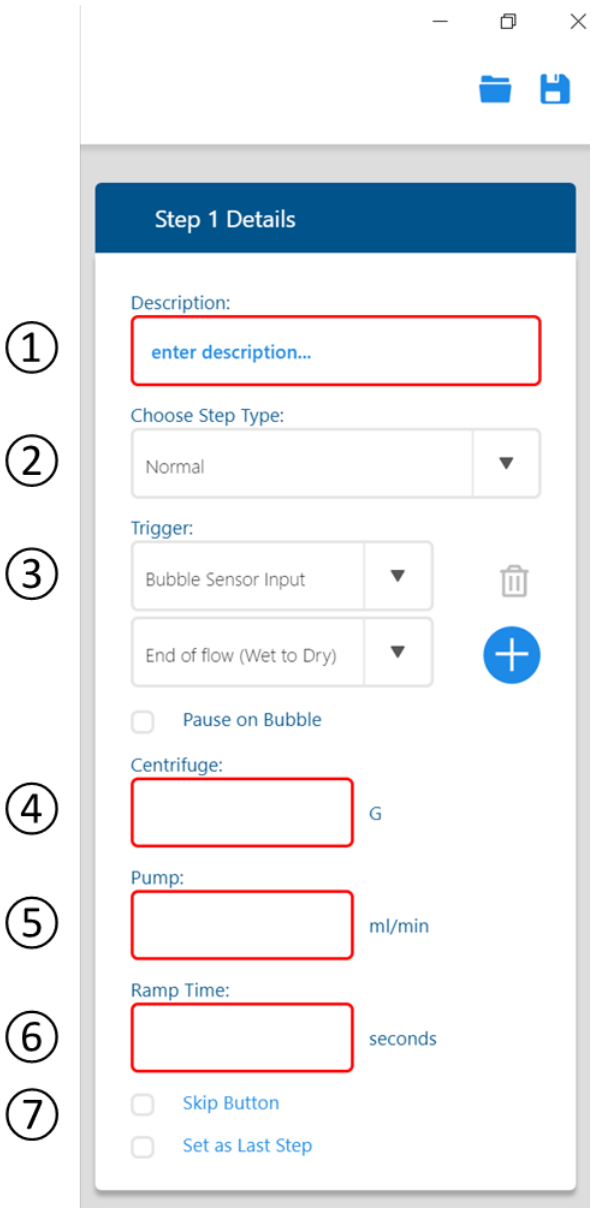


Define the fluid path used in each step using the Single-Use Kit diagram.

① Click on the bags that you want the fluid to flow between. This will highlight a valid fluid path between the selected bags in green. If one or more of the bags has a second connection, multiple valid paths between the bags will be available to select from; click the bags to cycle through your options.

② Change the direction of the pump by clicking on FORWARD or REVERSE. The flow direction is indicated by the arrows and the colour of the fluid path: green is forward, while orange is reverse.

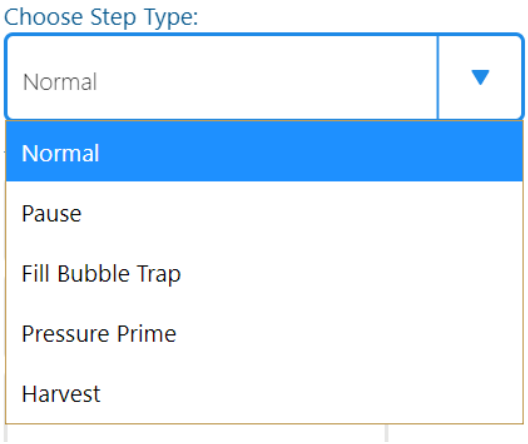
Step Details



Step Details window

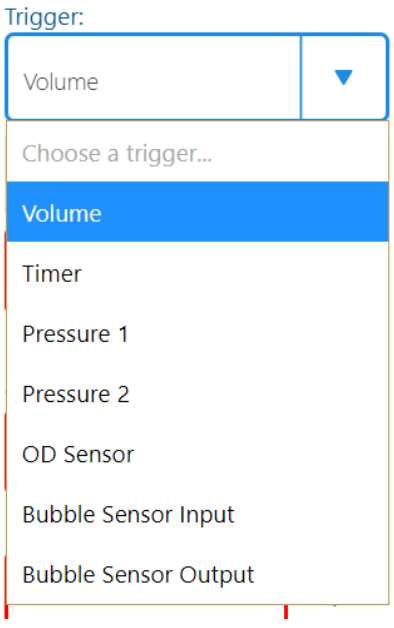
① **Description**: Enter a description for your step. Keep your descriptions clear and concise to enable the user to quickly comprehend what each step does. This description will also be displayed in the **List** view.

② **Choose Step Type**: Choose the step type from the options in the dropdown menu.

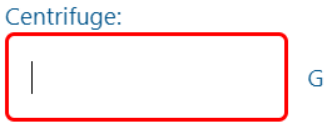


③ **Trigger**: Define the triggers that will be used to advance to the next step.

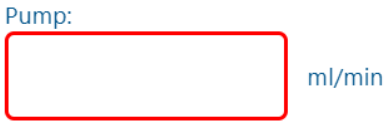
Note: Multiple triggers can be selected for each step. The first trigger reached will cause the protocol to advance.



④ **Centrifuge**: Enter the desired G force to be generated by the centrifuge.



⑤ **Pump**: Enter the pump speed in mL/min



⑥ **Ramp Time**: When the ramp time is set to zero, default speed ramps will be applied to the change in G force and pump speed between steps. When there is a significant difference in speeds between steps, it may be desirable to ramp speeds more gradually to ensure a smooth transition. Inserting a ramp time will cause both the G force and pump speed to change to their new speed settings over the specified time.

Note: Ramp time must be less than or equal to the step time.

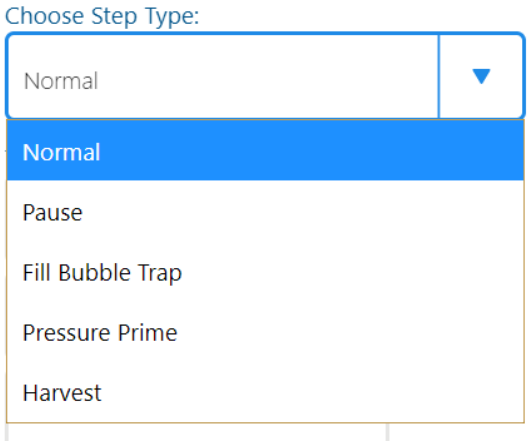
⑦
- Click the **Skip Button** check box if you wish to enable the **ADVANCE** button on the instrument so that the user can manually skip to the next step during operation of the protocol.

Note: The default for the **Skip Button** is off. The **ADVANCE** function on the instrument is very useful during process development, however, use with caution on processes where manual intervention needs to be restricted e.g. qualified processes. The skip button can also be used as a step trigger.

- Click the **Set as Last Step** checkbox to have the protocol end after the step has run.

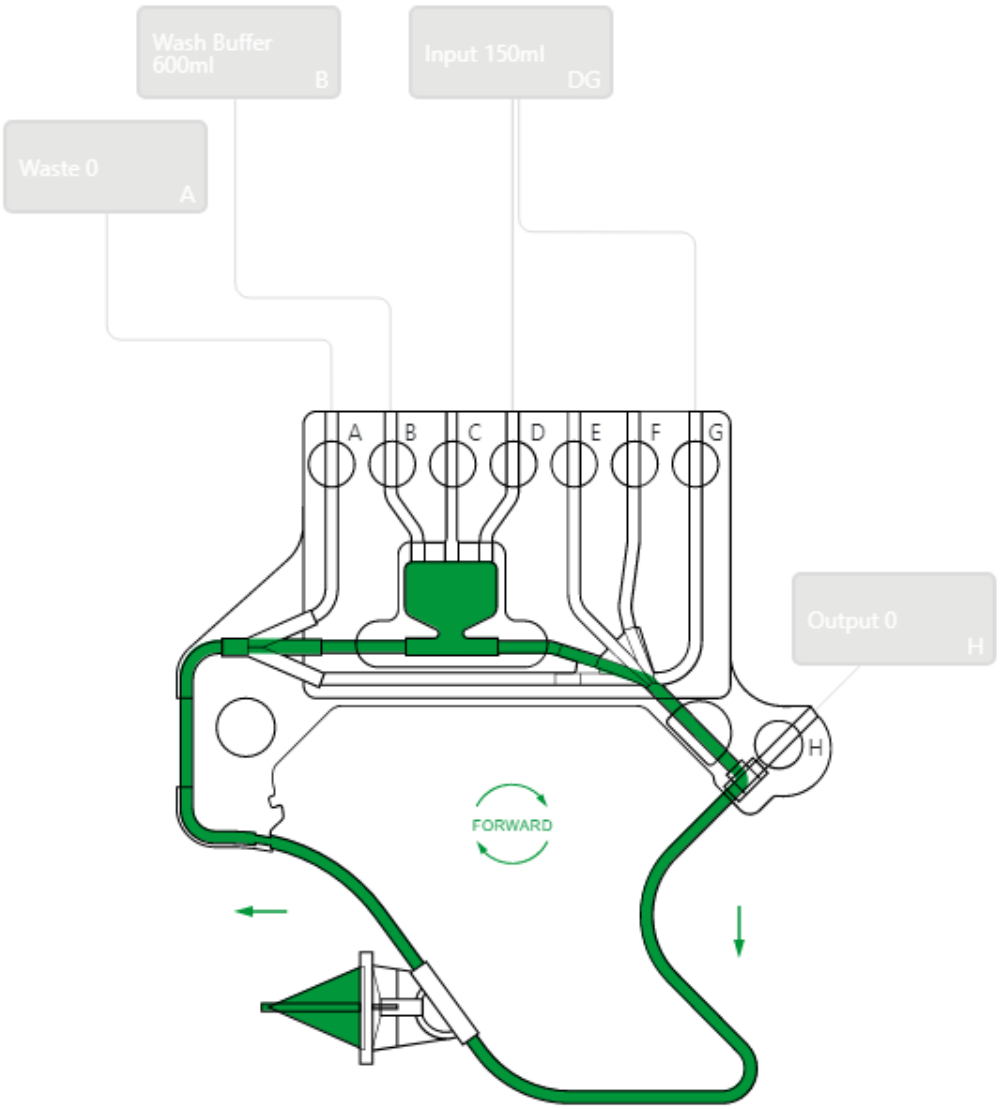
Step Type

There is a selection of standard step types available, each with a different function.

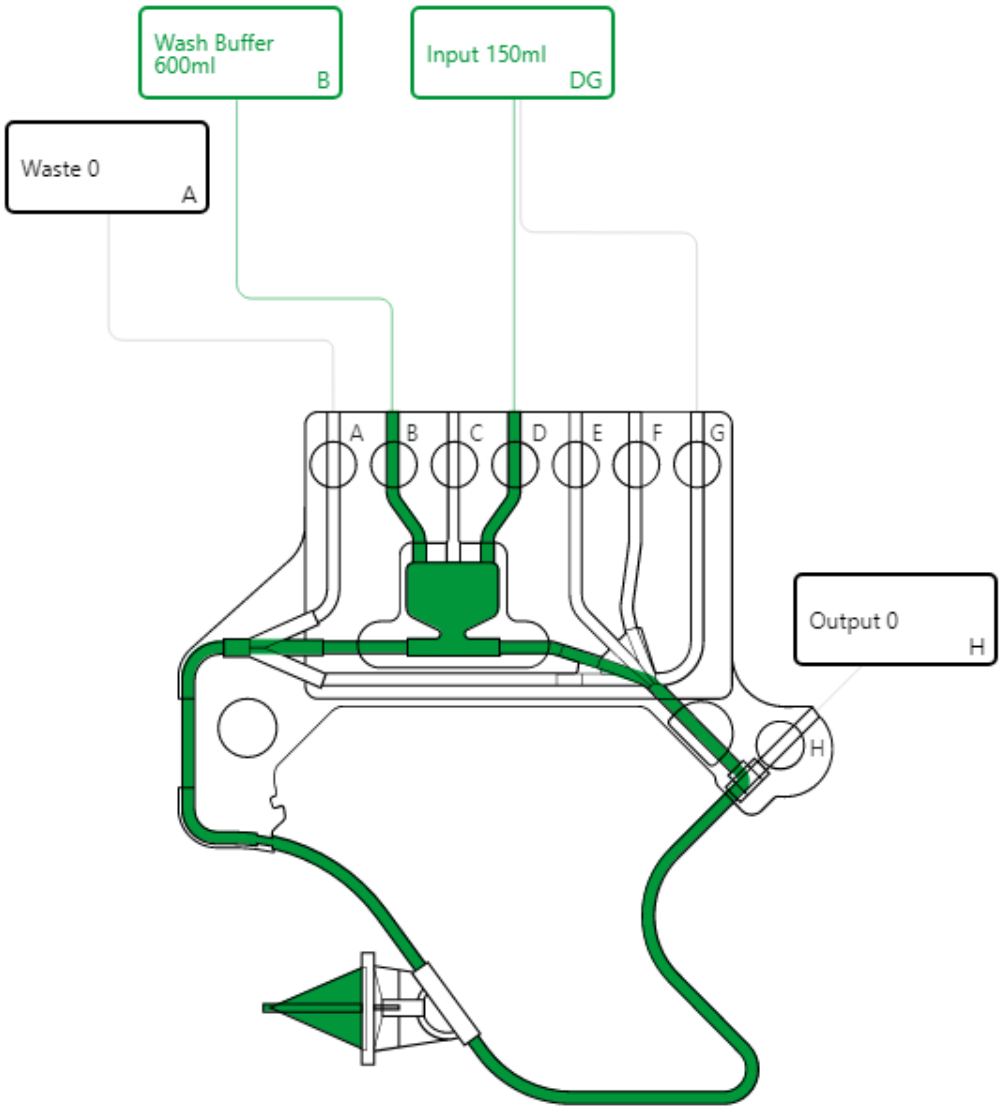


Note: Some step types may be greyed out (unavailable for selection) dependent upon fluid path settings and bag layout.

1. **Normal**– all functions in **Step** view are available to the user to define the step.
2. **Pause**– creates a closed loop fluid path within the kit that is isolated from the bags. The flow direction can be changed by clicking on the **Forward/Reverse** button.

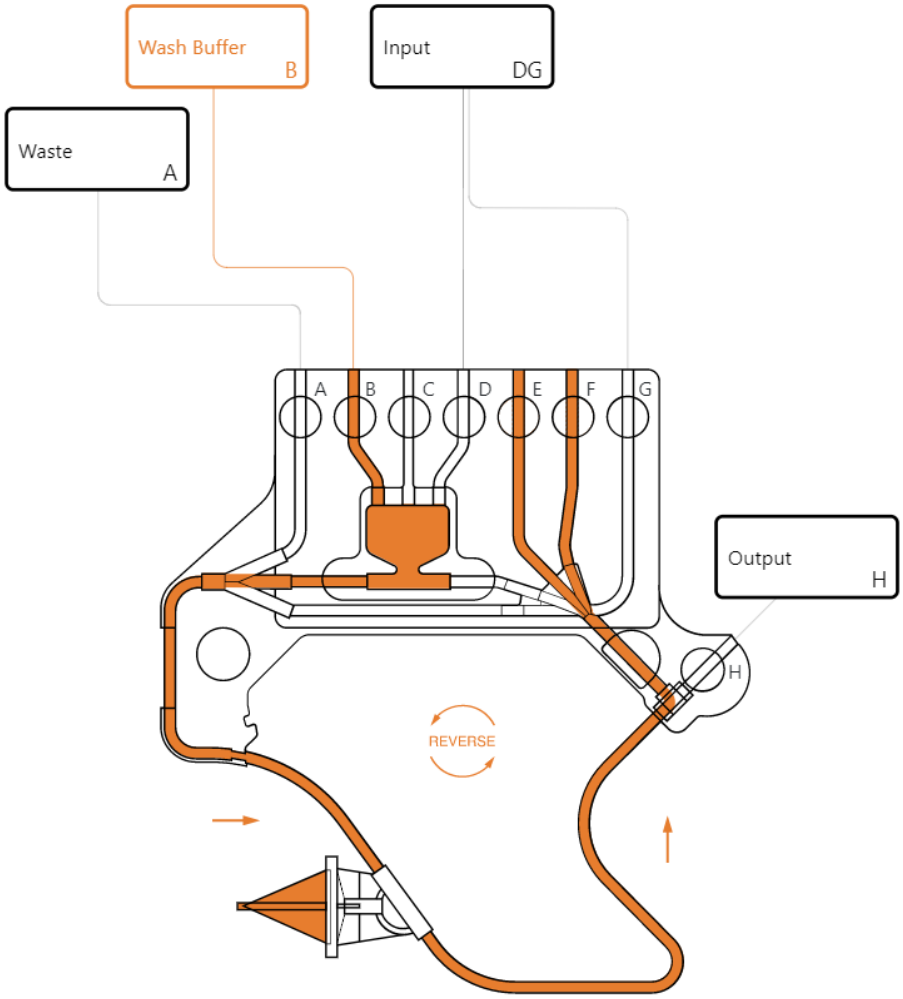

Wash Buffer 600ml
B
Input 150ml
DG
Waste 0
A
A
B
C
D
E
F
G
Output 0
H
H
FORWARD

3. **Fill Bubble Trap**– enables the Bubble Trap to be re-primed during a protocol without disrupting the fluidized bed. Only bags connected to lines B, C and D are available for selection for this step type.

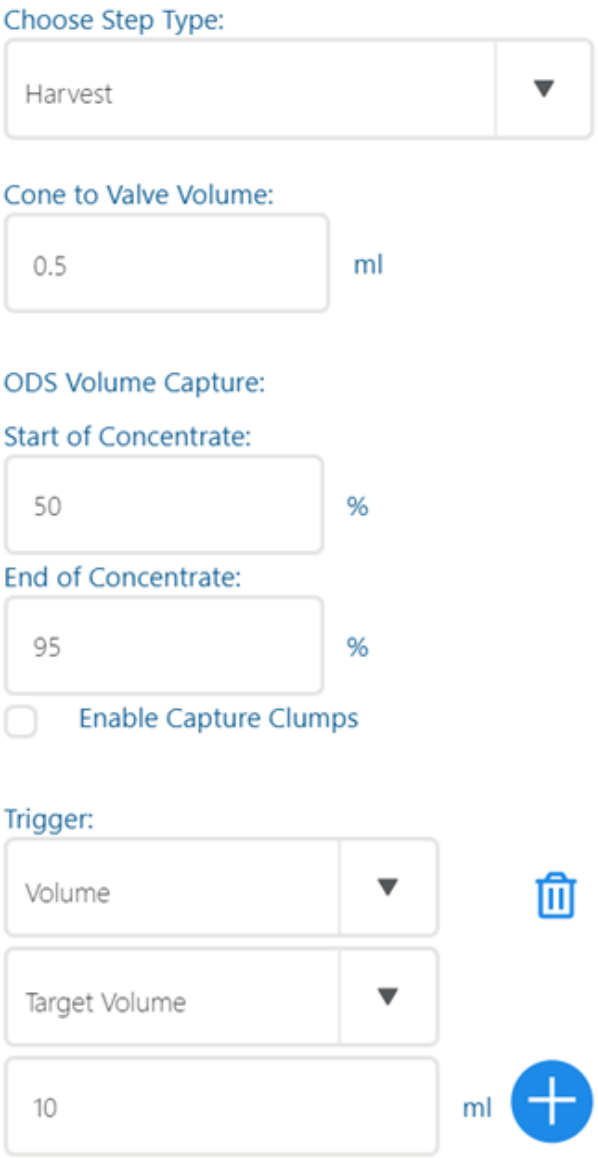


4. **Pressure Prime** – primes lines E and/or F when they are not connected to a bag to minimize losses to these lines during a **Harvest** step.

Note: Only valid bags may be selected while using this step type, and only unconnected lines will be pressure primed.

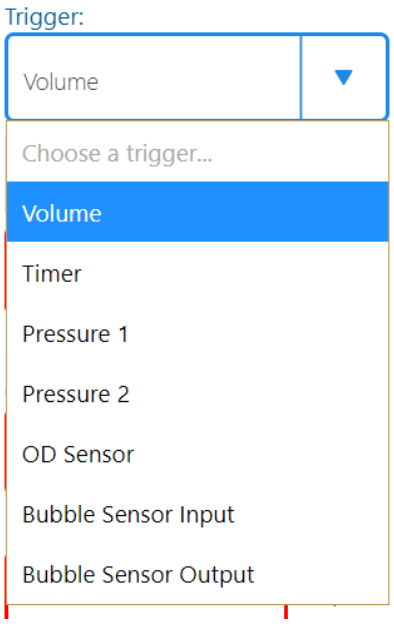


5. **Harvest**– is used to deliver a high concentration of cells to valves E, F or H with less carryover of the buffer. During harvest the valve for the destination bag is kept closed until the volume entered in **Cone to Valve Volume** has been diverted past the valve. The valve for the destination bag is then opened, dispensing the concentrate. The default **Cone to Valve Volume** is set at 0.5 mL and can be optimized to suit the different valves. During a **Harvest** step the OD Sensor will always record the optical density and calculate the concentrate volume. This is stored as **OD Sensor Volume** in **Volume Register** and can be used as a trigger.



Trigger

The Rotea™ software uses triggers to automate the progression from one step to the next in a protocol. Each step must have at least one valid trigger. A trigger is a condition that, when met, causes the protocol to advance to the next step.



To create triggers:

1. Select a **Trigger** type from the dropdown menu.

   Note: The **Skip Button** box at the bottom of the **Step** view is also a valid trigger as it enables the user to manually advance to the next step.

   Each trigger type has different parameters. These are addressed in the following subsections.

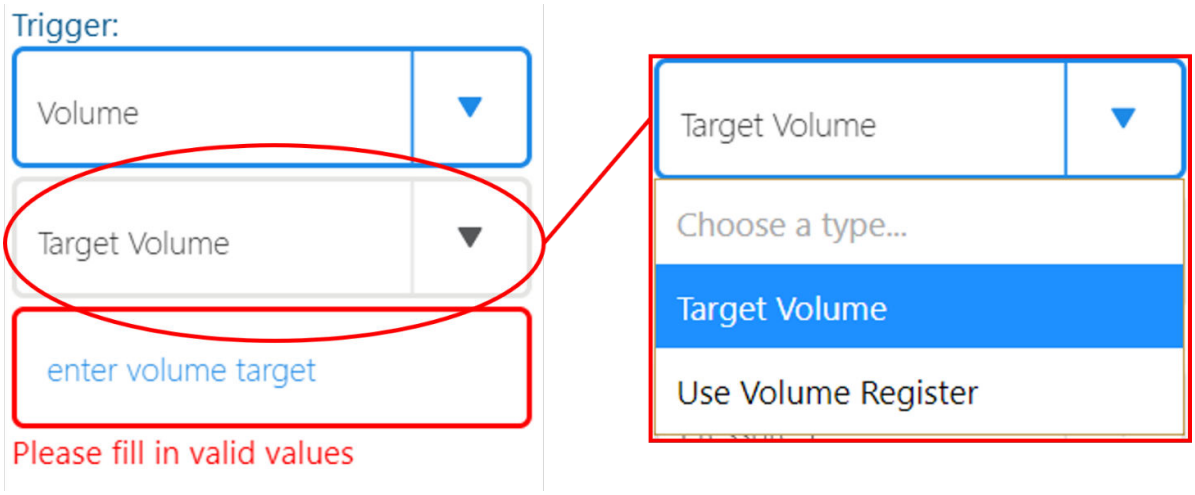
2. Add triggers by clicking ⊕ or delete by clicking the 🗑 adjacent to the trigger.

3. If you want the instrument to go into a pause state instead of automatically progressing to the next step, click **Pause on Trigger**. **Pause on Trigger** is only available on **Pressure 1**, **Pressure 2**, **Bubble Sensor Input** and **Bubble Sensor Output** step types. When this option is used the instrument will require a manual advance using the **Skip Button** before the next step will begin.

Volume

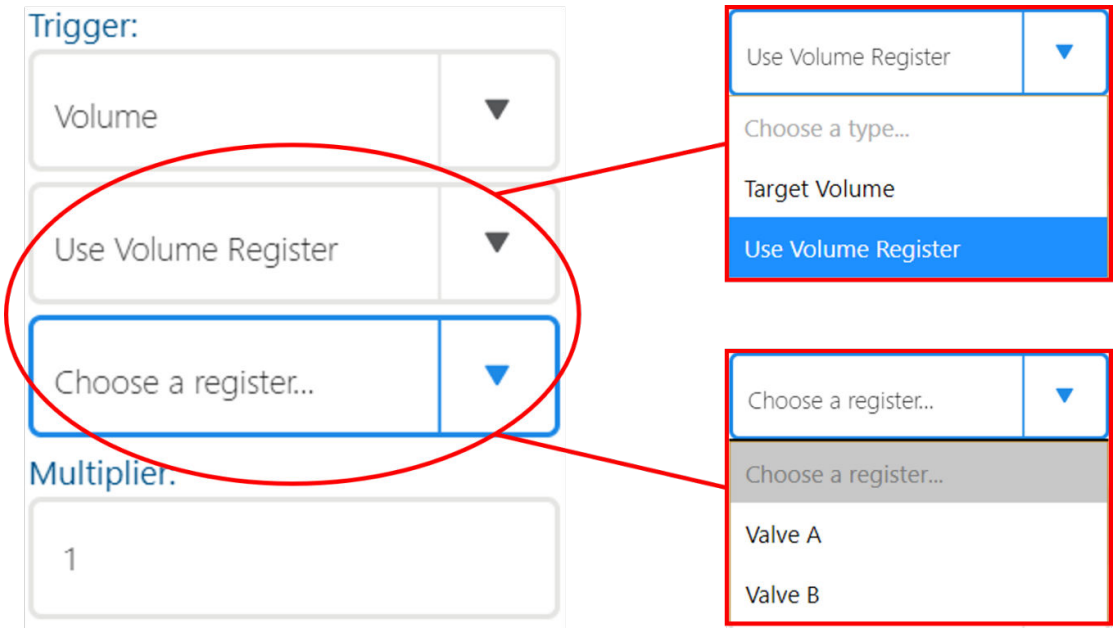
A **Volume trigger** means that the step will continue until a defined volume of fluid travels through the pump. There are two options for setting this volume.

Target Volume



Target Volume will cause the step to continue until the defined volume of fluid passes through the pump.

Volume Register



Selecting **Use Volume Register** allows you to select any valid variable from a dropdown menu including:

- Variables defined in the **Data Entry** screen
- Valves that are being used within the protocol.
- OD Sensor volume if a harvest step type is being used

> **Note:** The volume used for a valve trigger is the absolute value of the volume that has passed through the valve over the course of the protocol. Flow into a bag is tracked as a positive and flow out of a bag is tracked as negative.

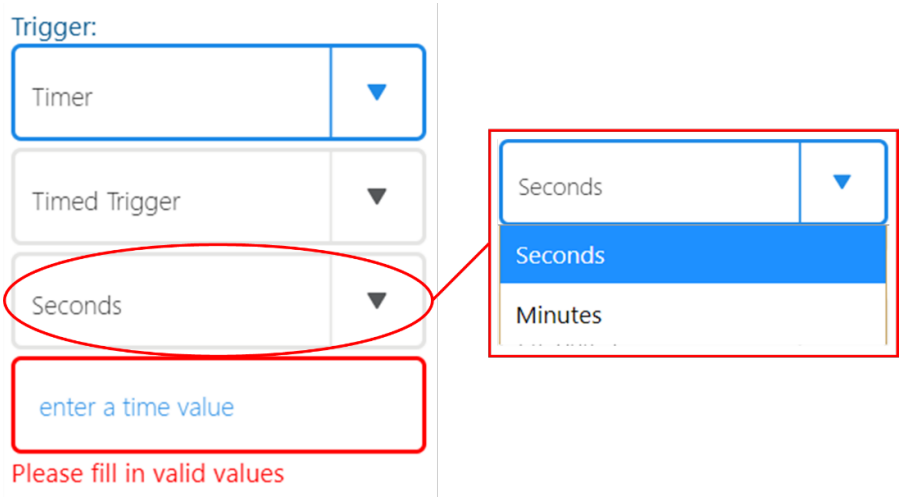
You can also apply a multiplier to the volume trigger.

A volume register trigger will advance to the next step when the volume pumped during the step equals the volume in the **Volume Register** at the beginning of the step multiplied by the multiplier.

Note: All types of volume triggers are measured from the pump, not the bag. Setting a volume of 30 mL from B to A does not guarantee that 30 mL will end up in A, just that 30 mL will travel through the pump.

Timer

Timer trigger causes the step to continue for the specified length of time, then advance to the next step automatically.



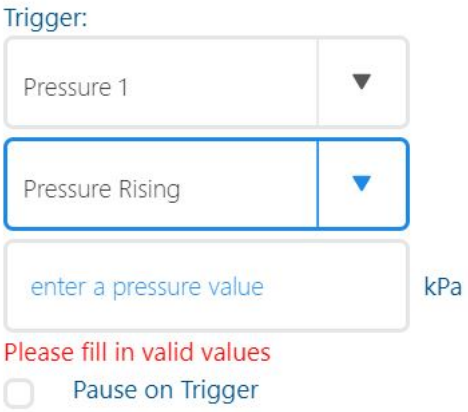
You can choose seconds or minutes for the timer.

Note: The Rotea™ GUI displays the step time remaining in seconds for each step where it can be calculated.

Pressure 1 and Pressure 2

There are two pressure sensors on the Rotea™ instrument, **Pressure 1** and **Pressure 2**. A **Pressure 1** or **Pressure 2 Trigger** means that the step will continue until the specified pressure is reached in the selected pressure sensor. Pump speed must be set to 10 mL/min or lower during pressure sensor triggers.

Note: The instrument has an over-pressure limit of 120 kPa. It is recommended that a pressure value of <50 kPa be used for pressure triggers.

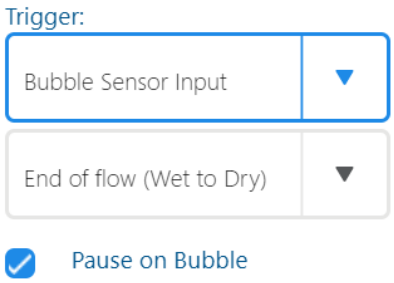


Pressure Rising will be automatically selected, causing the step to end when the pressure rises to the set value. This is the only option.

You can click the **Pause on Trigger** checkbox at the bottom of the above figure to have the step go into pause mode when the trigger conditions are satisfied rather than automatically advancing to the next step, requiring a manual use of the **Skip** button to progress.

Bubble Sensor Input and Bubble Sensor Output

Bubble Sensor are present on lines B, C, D, E & F to detect whether fluid or air is present in that section of the tube.

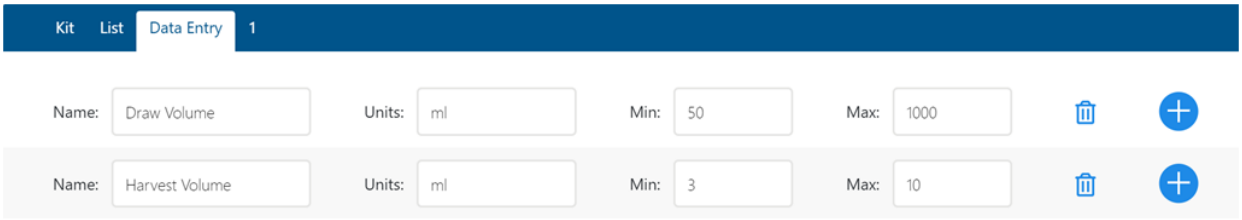


A **Bubble Sensor Input** trigger detects change using the bubble sensor on the source bag of the fluid path, while a **Bubble Sensor Output** trigger detects change using the bubble sensor on the destination bag of the fluid path.

Use the dropdown menu to select whether you want the trigger condition to be met when the sensor first detects air (**Wet to Dry**), or when it first detects fluid (**Dry to Wet**).

Select the **Pause on Bubble** check box to put the instrument into a paused state when the trigger occurs rather than advancing to the next step, requiring a manual use of the skip button to advance.

Data Entry



The **Data Entry** screen enables the creation of variables for use with the **Volume Register** function and as a **Repeat Count**, **Process Counter** or **Timer Loop** condition. To define a variable:

1. Click the + to add a new variable.
2. Enter a name for the variable. This name will appear in **Step** view when selecting a **Volume Register** trigger, or in the dropdown when setting up a **Loop** condition.

   Note: A **Data Entry** window will also be displayed by the Rotea™ GUI immediately after initiating the protocol. The user will be prompted to enter a value for each **Data Entry** variable so the name should be concise and easily understood.
3. Enter what unit the variable is measured in. If using the **Data Entry** in conjunction with **Volume Register**, the unit entered must be able to be converted to **mL** with a simple multiplier. If using this **Data Entry** for a **Loop** condition, ensure that the units entered match those used in the **Loop** condition (i.e. number of repeats for **Repeat Counter** and **Process Counter**, **mL** for **Valve Volume**, or **seconds** for **Timer**).
4. Enter a minimum and maximum value for entering the variable value into the Rotea™ GUI. These are safeguards to prevent the user from entering a value that would be too high or too low for the process to run correctly.

Click the ⊕ to add another variable. Click 🗑 to delete the adjacent variable.

Note: Users are prevented from deleting a **Data Entry** variable that is currently being used within the protocol and a warning will be displayed highlighting all steps that use the variable.

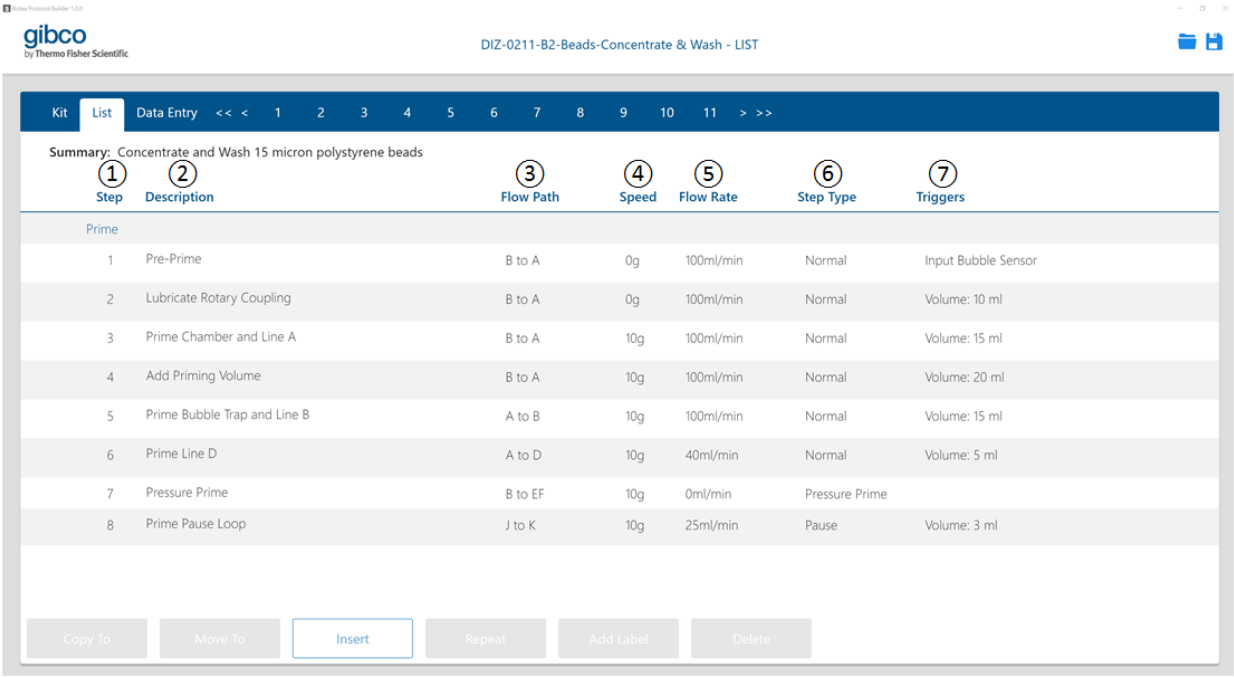
List view

The **List** view summarizes the steps of the protocol in table form and provides a convenient way to review and edit a protocol. It is also the view used to create loops within a protocol.

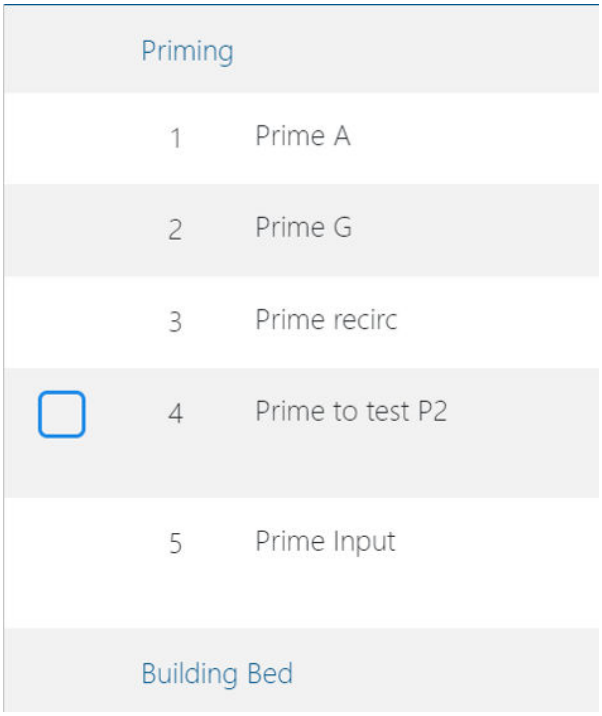
Note: The **List** view is also reproduced by the Rotea™ GUI to enable the user to see the entire protocol and importantly, is the screen from which the user would recover from an abnormal situation using either:

- **Start the Protocol from this step**
- **Run the selected step only**

Step summary

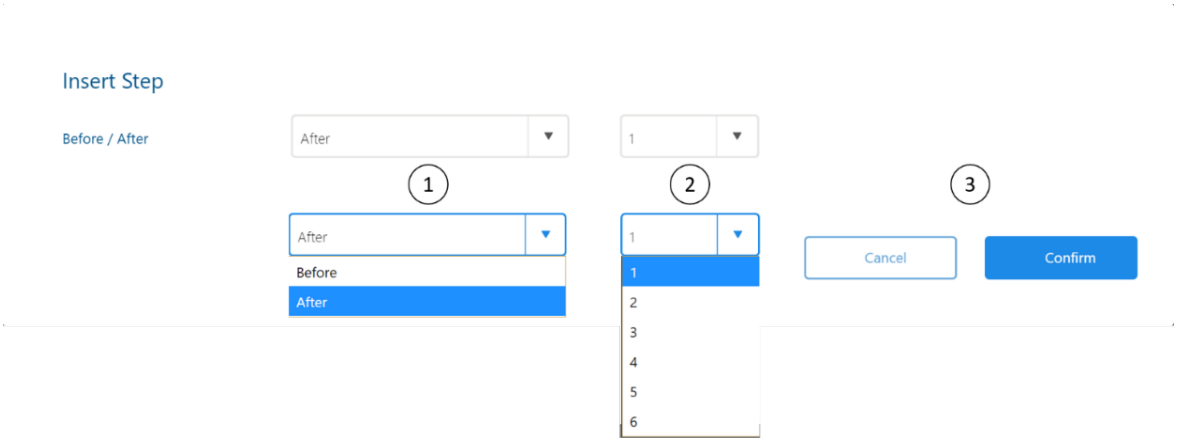


① **Step**: Step number
② **Description**
③ **Flow Path**: Flow path in a simple text format, indicating the start point and end point.
④ **Speed**: Centrifuge speed in terms of G force
⑤ **Flow Rate**: Pump flow rate in mL/min
⑥ **Step Type**
⑦ **Triggers**: All set triggers, along with the values set as their conditions



The blue checkbox on the left can be clicked to select the step. Multiple steps can be selected at a time.

Edit using List view



① Select whether you want the step to be inserted before or after the selected step.
② Select the step number that you want the new step to be inserted before or after.
③ Click **Confirm** to add your new step.

When a single step is selected you have several options:

① Duplicate the step at the designated location. The **Copy To** window is identical to the **Insert Step** window.
② Move the step to the designated location. The **Move To** window is identical to the **Insert Step** window.
③ Add a label to your list view, which appears above the selected step. This can be used to divide your protocol into sections, for ease of use.
④ Delete the selected step.

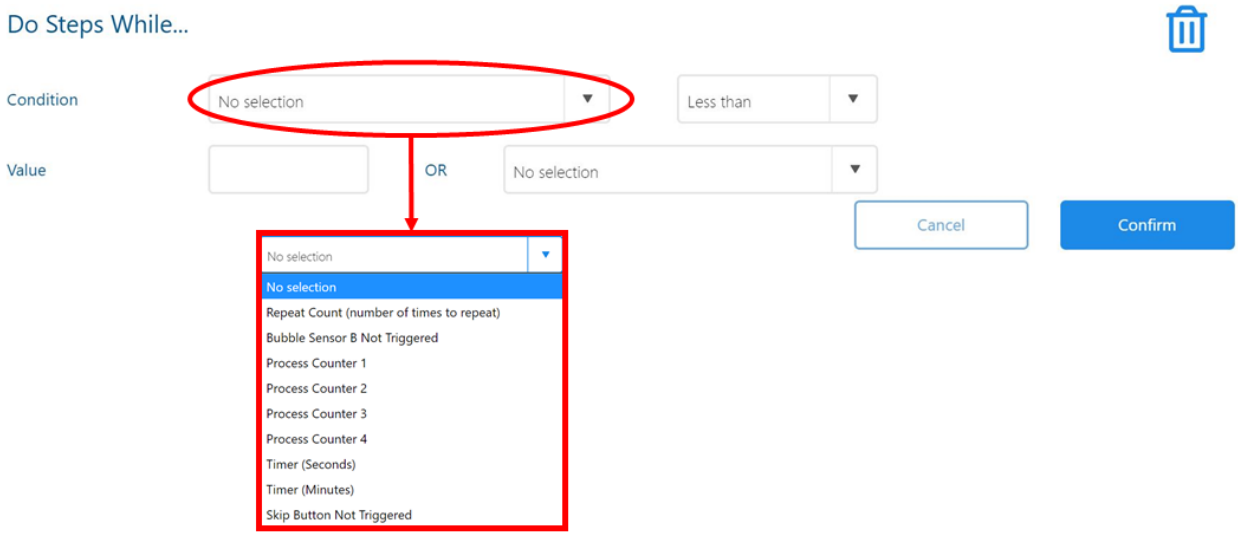
When two or more steps are selected you are left with the option to delete or move all currently selected steps.

Note: If the selected steps are not all adjacent, they will be grouped together after the move.

Loops

Select any two steps in **List** view to bring up the option to create a loop of them and all steps between them.

Click **Repeat** to bring up the **Loop** creation parameters:



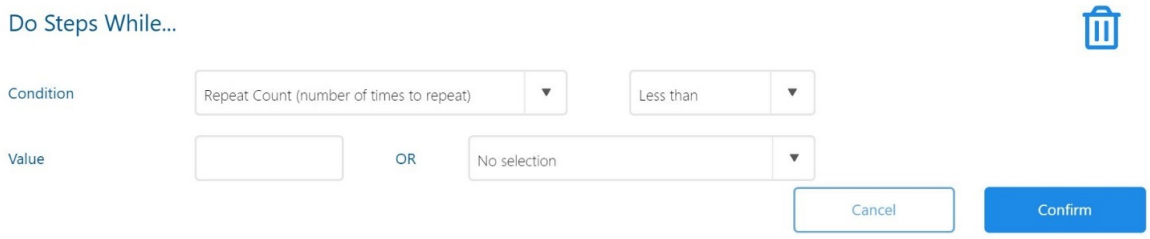
A loop is a selection of steps within a protocol that repeat. Each loop has a **Do Steps While** condition that the software checks prior to performing the loop. If the condition is true, the loop runs. If it is false, the protocol skips the loop and advances to the first step directly after the loop.

For example, a user wants the loop to continue until the Bubble Sensor on line B is triggered. The **Bubble Sensor B Not Triggered** condition is selected from the dropdown, making the condition **Do Steps While Bubble Sensor B Not Triggered**.

When the protocol reaches the loop for the first time, the software checks whether Bubble Sensor B has been triggered. Since it has not, the condition is true, and the loop runs.

Each time the loop reaches the end, it jumps back to the start and checks the condition again. If Bubble Sensor B is triggered at any point during a loop, the next time the loop condition is checked, the condition will be false, and the protocol will not perform the loop.

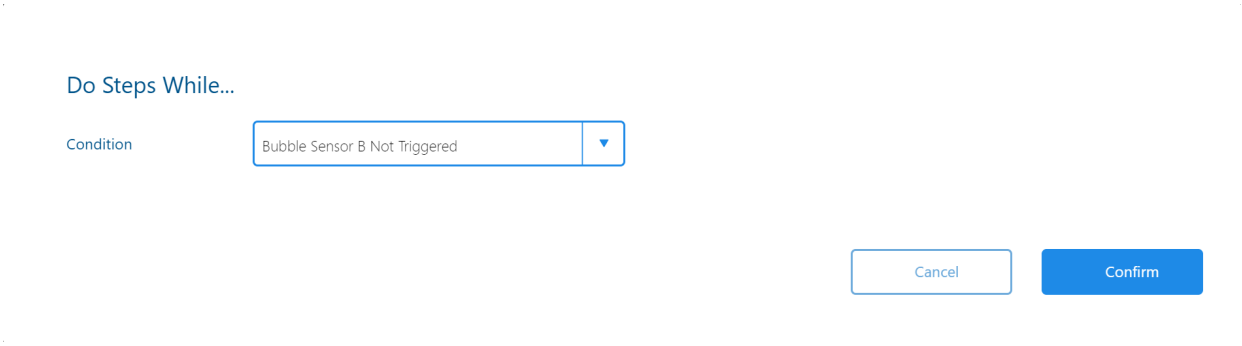
Repeat Count



A **Repeat Count** condition causes the loop to repeat until it has repeated the number of times entered in the value box.

Note: Once the loop condition is met, the **Repeat Count** resets to zero.

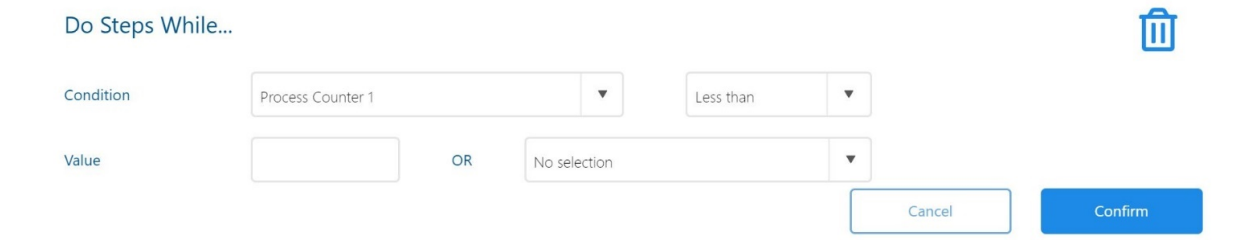
Bubble Sensor Not Triggered



If any of the steps within a loop uses Bubble Sensors B, C, D, E or F, a **Bubble Sensor Not Triggered** condition causes the loop to continue until the Bubble Sensor is triggered (**Dry to Wet** or **Wet to Dry**).

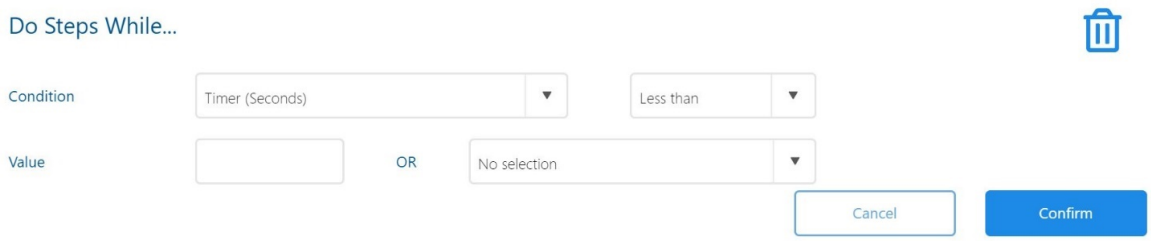
Note: Multiple options will appear in the list if more than one Bubble Sensor is available for use with this condition. Only one option can be selected.

Process Counter



For loops within loops where the internal loop can be performed multiple times, **Process Counters** can be used to count how many times a loop has occurred during the entire protocol. Unlike **Repeat Count**, the **Process Counter** for the loop does not reset to zero after each loop sequence is completed.

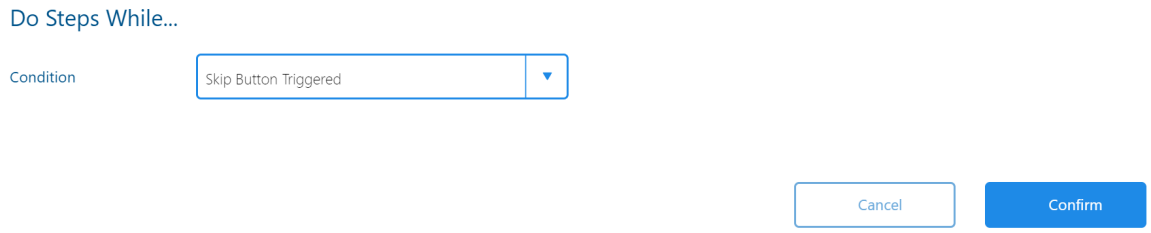
Timer (Seconds) and Timer (Minutes)



A **Timer (Seconds)** or **Timer (Minutes)** condition causes the loop to continue until the entered length of time has passed.

Note: The condition is checked at the start of each loop. If the timer value is set to 30 seconds, and it reaches that time in the middle of the loop, it will finish the current loop before continuing out of it.

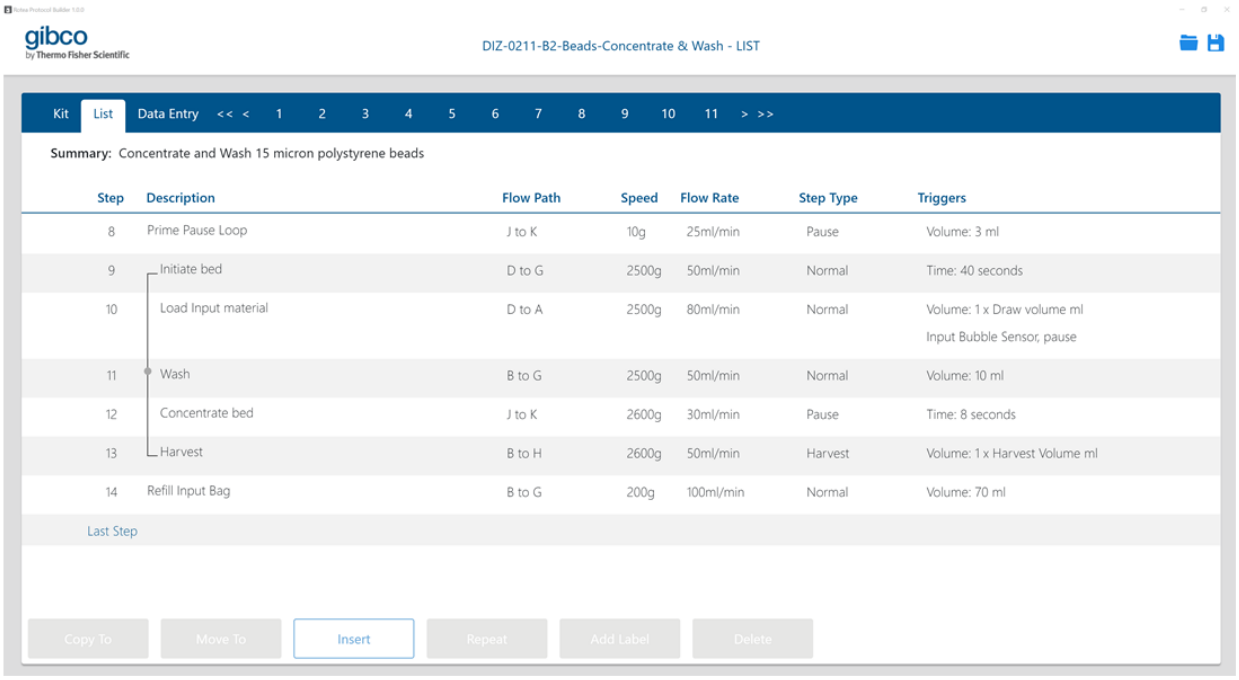
Skip Button Triggered



A **Skip Button Triggered** condition will cause the loop to continue until the **Advance** button on the instrument is manually pressed by the user.

Editing loops

When a loop has been created, it will appear in **List** view as a bracket from the first to last step in the loop. Clicking the small circle in the centre of the loop bracket allows you to edit the loop condition.



4 Protocol Building Blocks

Priming

Overview

The first steps in any protocol are used to prime the system. Priming has two main functions:

- lubricate the Rotary Coupling
- replace air in the Single-Use Kit with fluid

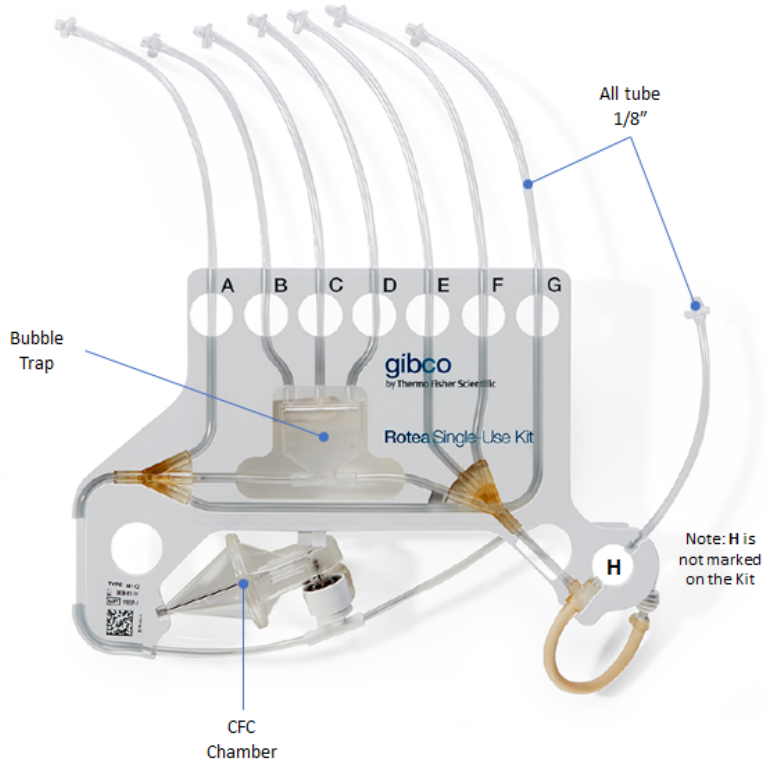
It is essential to prime any line through which fluid will enter the system. Any further priming of lines is dependent upon the specific protocol.

WARNING! Insufficient wetting of the Rotary Coupling can lead to premature failure of the Single-Use Kit. Whilst recommended priming steps and settings are described in this section of the user guide, adequate wetting is ultimately dependent on the media used, the pump flow rate, centrifuge G force settings and the protocol itself.

Bubbles

It is important to remove all air from the system. If bubbles enter the process line feeding the centrifuge at operating speed, they accumulate within the Rotary Coupling causing the pressure to rapidly increase. To protect the integrity of the Single-Use Kit, the instrument will detect this over pressure condition through the Pressure Sensor and trigger an automatic stop.

Priming volumes



Description	Prime Volume (ml)
CFC Chamber	13.2
Bubble Trap	5.0
1/8" Tube (100mm)	0.7
CFC Chamber to H	1.6
CFC Chamber to Bubble Trap	2.4
CFC Chamber to E	2.6
CFC Chamber to F	2.6
CFC Chamber to A	1.7
Bubble Trap to B, C & D	0.3

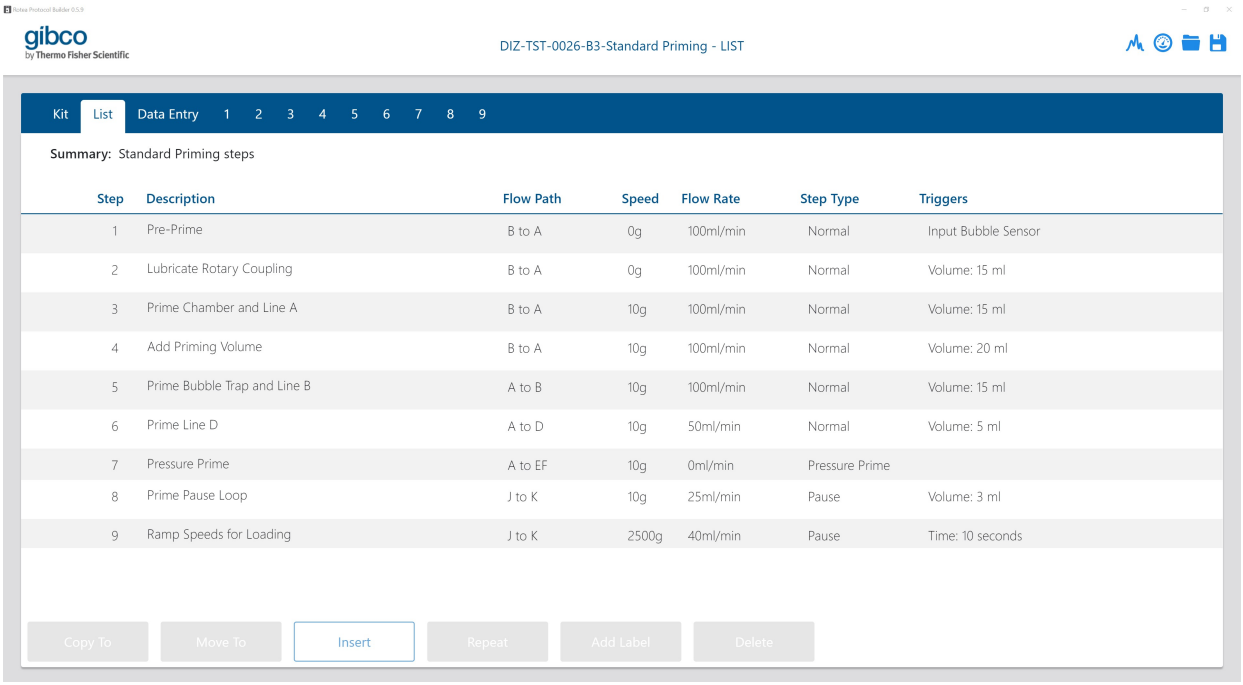
Description	Harvest Offset (ml)
Valve H	1.0
Valve E	1.2
Valve F	1.2

When priming it is important that enough volume is pumped through the system to remove all air from the CFC Chamber, Bubble Trap and fluid lines. The table shows the approximate volumes of the Single-Use Kit components to assist with calculating the correct priming volumes.

Note: Always include additional volume in each priming step to ensure adequate priming.

Basic priming steps

A typical priming sequence consists of 9 steps, as shown in this example:



Note: The priming sequence will always be protocol dependent. It is the users responsibility to determine the priming steps that are appropriate to their specific application, the basic steps and settings shown below are recommended.

Pre-Prime

The **Pre-Prime** step is used to ensure fluid has entered the Single-Use Kit regardless of the length of tube that connects to the bag containing the priming fluid. To achieve this, the **Bubble Sensor Input** trigger is set **Dry to Wet** and hence will detect the arrival of fluid at the Bubble Sensor. The centrifuge should be at 0 G (not yet spinning), and the pump should be at a high flow rate (100 mL).

A typical option for the **Pre-Prime** step is from your wash buffer to your waste bag – B to A in this example.

Lubricate Rotary Coupling

After the Bubble Sensor goes off and advances to the next step, the pump delivers 15 mL of priming fluid through the system before starting centrifuge rotation. The centrifuge should be set to 0 G and with a high pump flow rate e.g. 100 mL/min.

The fluid path is the same as the **Pre-Prime** step – in this example, B to A.

Prime Chamber and Line A

Start centrifuge rotation slow (10 G), continuing to pump the liquid through the CFC Chamber. Centrifuge spinning will drive any air to the inside of the chamber, allowing it to be pushed out by priming fluid entering the chamber. A volume trigger of 15 mL is usually enough.

Add Priming Volume

Calculate the amount of volume needed in bag A to complete priming and pump slightly more than that volume from B to A. The same fluid path, pump speed, and centrifuge speed as the previous step is maintained. To calculate this volume simply add together the priming volume required in the subsequent priming steps.

Reverse flow and fill Bubble Trap

Reverse the pump to fill the Bubble Trap and prime the input line from bag B with priming fluid, typically 10–15 mL.

Prime remaining input lines

Any other lines through which product is drawn into the system must be primed, e.g. lines C, D, E and F. Smaller volumes can usually be used now that the Bubble Trap and CFC Chamber have been filled. Each line requires its own step. A to D is shown here as an example.

Note: It is particularly important to prime lines E and F when being used as inputs as they are not protected by the Bubble Trap.

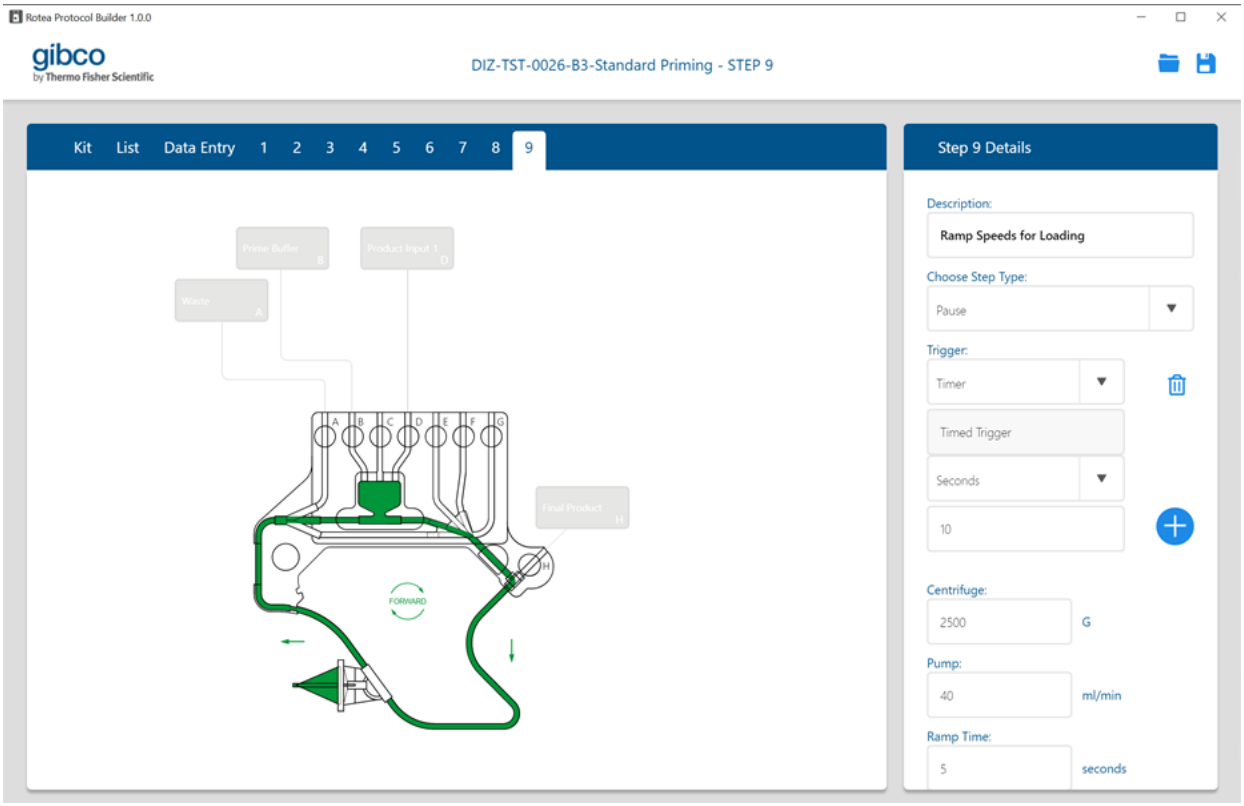
Pressure Prime

If lines E or F are not connected to a bag, they should be primed using a **Pressure Prime** step type. This improves the performance and reproducibility of the Harvest step. In this example, Bag B is selected as the origin bag and the **Pressure Prime** step type automatically primes lines not being used within the Single-Use Kit. The centrifuge is ideally set at the same G as the prior step with all other settings being default settings.

Prime Pause Loop

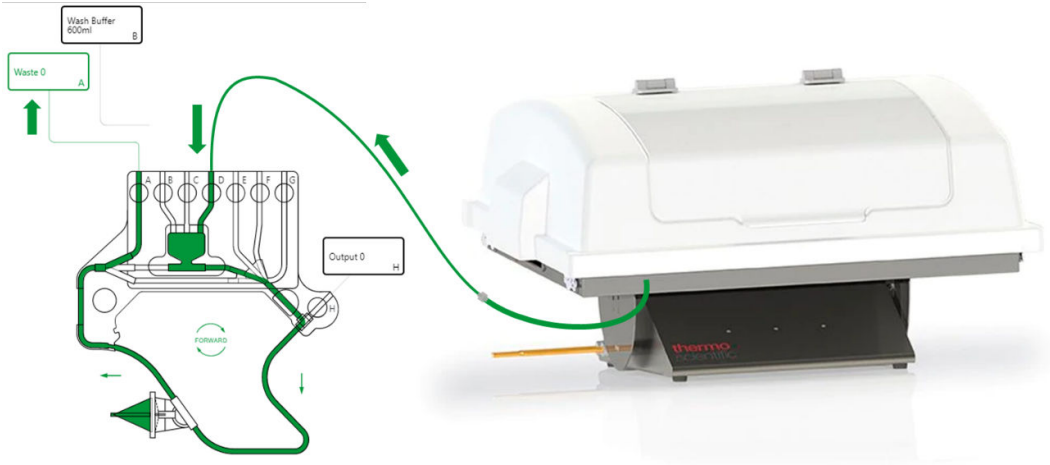
The recirculation loop is primed using a brief **Pause** step type running in the forward direction. Set a small volume trigger or short timer trigger on a **Pause** step type with the centrifuge set to 10 G and a low pump flow rate e.g. 25 mL/min. This will help to ensure that bubbles in the J–K line don't skip across the bottom of the Bubble Trap and remain trapped in the recirculation loop.

Ramp to next step



The next step after priming will often require a significantly higher centrifuge speed and a change in the pump flow rate. It is preferable to insert a pause step with speed ramp immediately after priming to enable the user to check that priming has been successfully achieved, provide an opportunity for residual air within the CFC Chamber to be released and stabilize the system at the new speeds in a controlled manner. In the example above we have created a 10 second pause step with a 5 second ramp time.

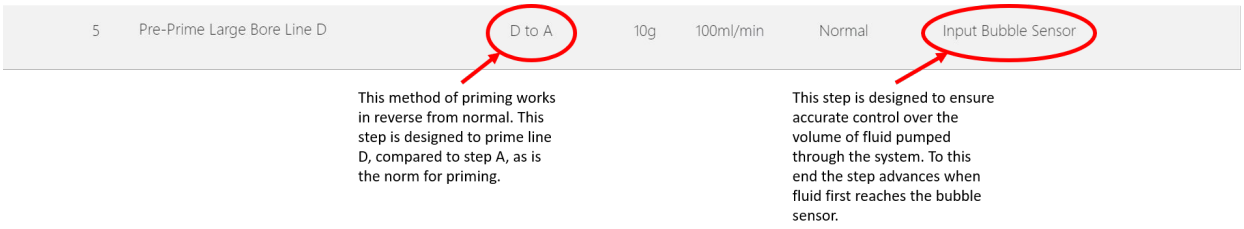
Prime remote sources



When drawing cell product from a bioreactor or large vessel the supply line can often contain too much air volume for the bubble trap to accept before fluid arrives. A lot of bioreactors also use large bore tubing (i.e. >1/8”) that can allow fluid to flow past bubbles.

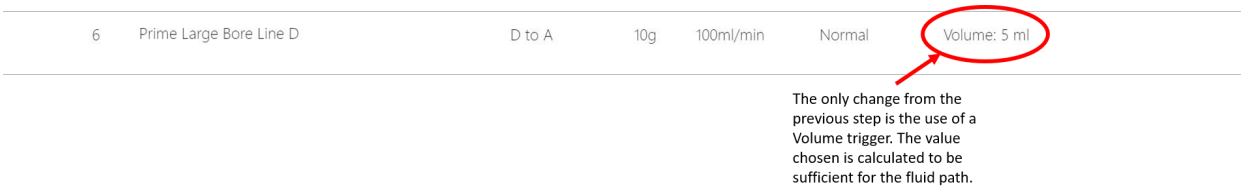
Priming bags that use large bore tubing requires a two-step priming process.

Pre-Prime Large Bore Line



The goal is to draw the air into the system and pull it into the Bubble Trap – or through it to another bag. Set a fluid path from the input source using the large bore line to your waste bag (in this example D to A). Set a Bubble Sensor (Dry to Wet) trigger, and check that **Pause on Bubble** is not selected. This is to ensure control over the volume you pump in the next step. The flow rate does not affect this step, so a faster flow rate e.g. 50–100 mL/min, can be used. The centrifuge should be set to a low G force, to avoid issues if the bubbles travel through the centrifuge.

Prime Large Bore Line



Draw the air brought into the Bubble Sensor down into the Bubble Trap or out into another bag. All settings should remain the same as the previous step, except using a **Volume** trigger instead of a **Bubble Sensor Input** trigger. The trigger volume should be calculated using the **Priming Volumes** table to ensure any air not caught in the bubble trap travels all the way into the waste bag. In this example (D to A) we use a volume of 5 mL.

Manage air in the system

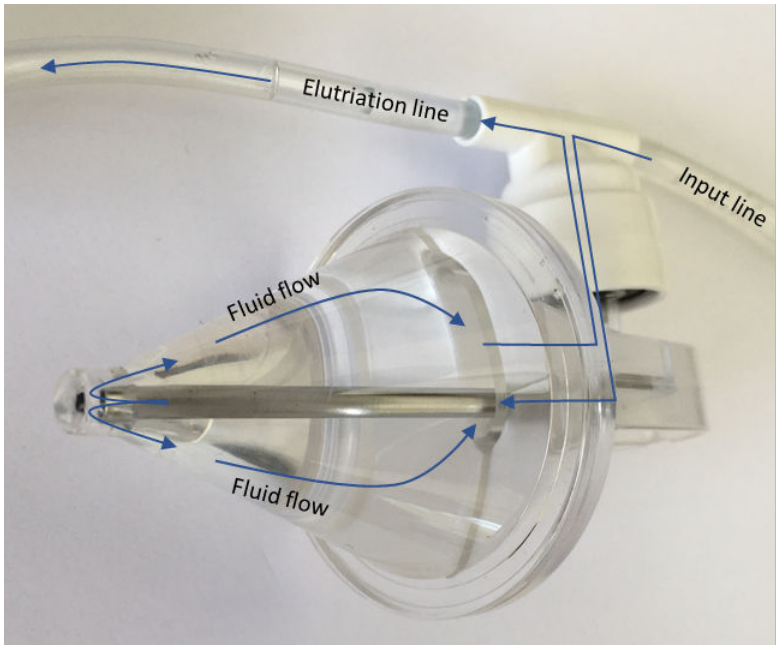
When first priming it is possible to shift air around the Single-Use Kit with the peristaltic pump. If, for example, the output bag is destined for cryo-storage it is usually preferred that no air is in the bag once filled. In this situation use the method shown above.

Establish a fluidized bed

Overview

A stable fluidized bed is the central concept that counterflow centrifugation and Rotea™ instrument is built around. The most essential part of creating a good protocol using the Rotea™ instrument is understanding how to establish a good fluidized bed.

Example of fluid path through the CFC Chamber:



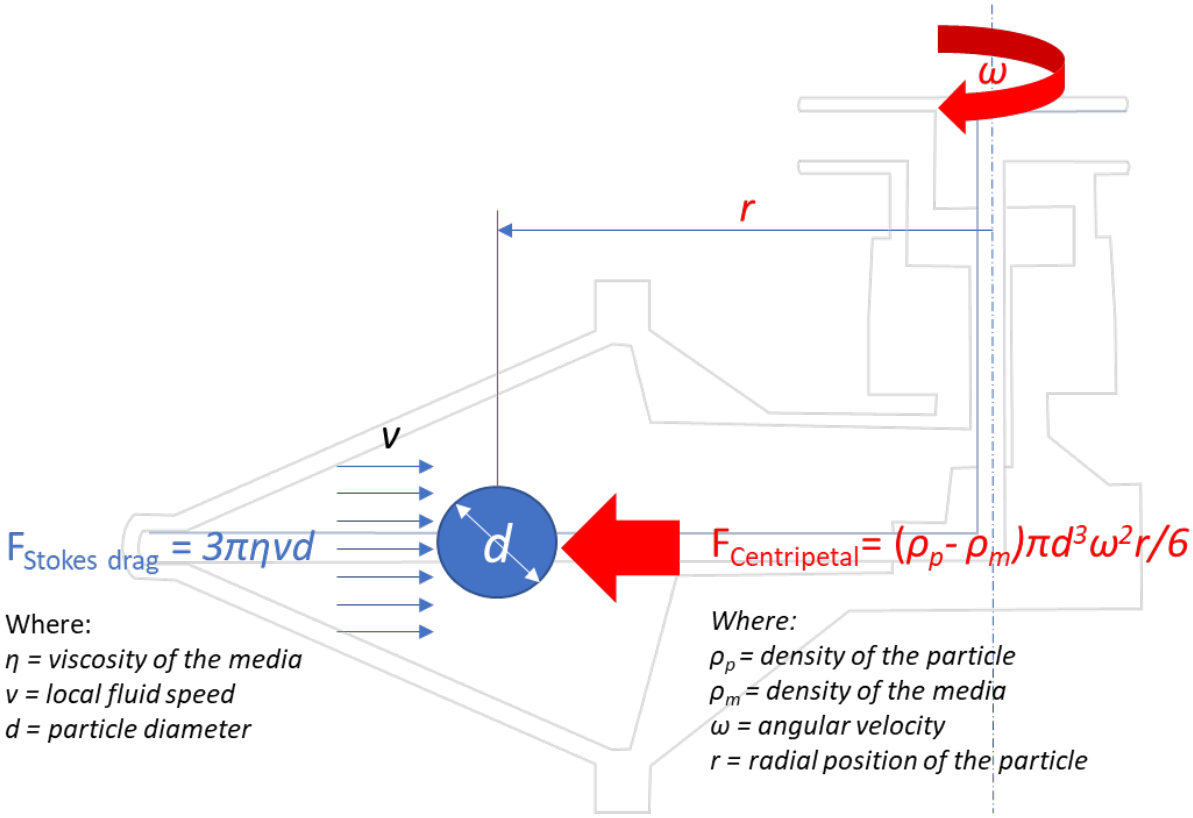
Media containing both large and small cells enters the Rotary Coupling via the input line and flows into the CFC Chamber, exiting at the tip of the stainless-steel Dip Tube.

The media immediately changes direction and flows back towards the axis of rotation.

As the media passes through the CFC Chamber, larger cells are loaded to the fluidized bed whilst smaller cells are elutriated and exit the CFC Chamber via the output.

The seven parameters for establishing a fluidized bed

The theoretical model and key process parameters that define the behaviour of cells in the CTS™ Rotea™ Counterflow Centrifuge is described in the formula below (see Chapter 5, "Rotea™ Process Model" for further details).



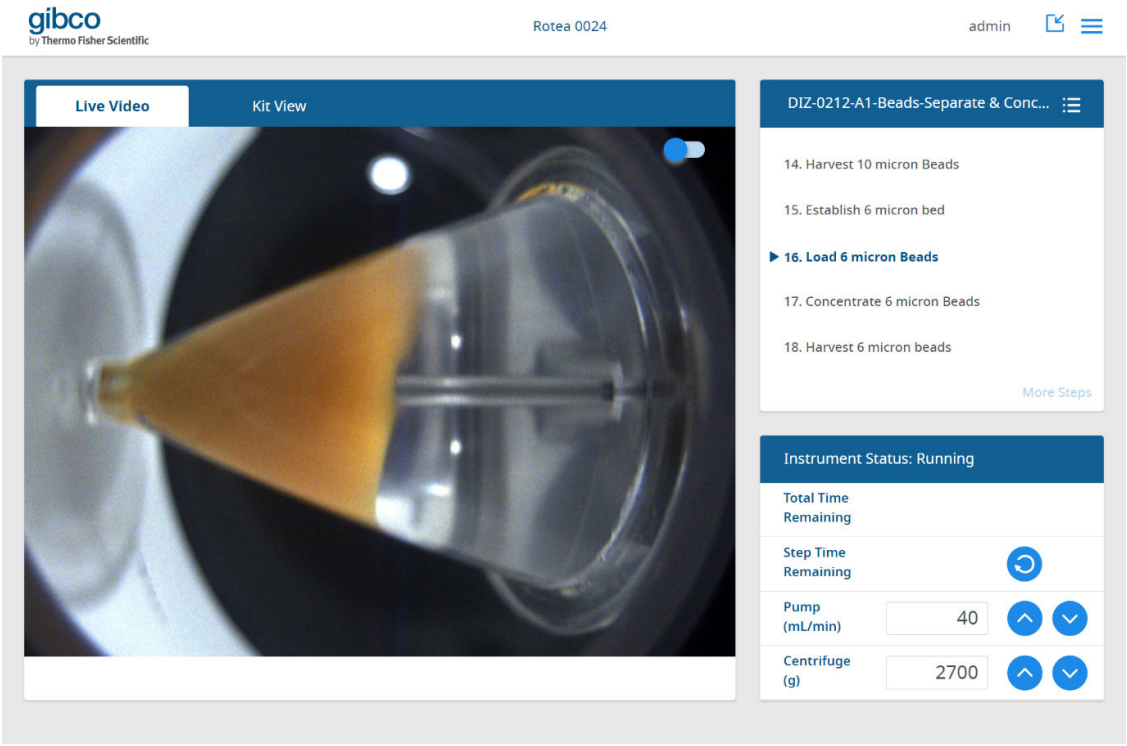
$$F_{Net} = F_{Centripetal} - F_{Stokes\ drag}$$

Seven parameters affect formation of a fluidized bed to varying degrees and need to be considered when developing a protocol.

1. Cell diameter and cell density are fixed for the cell type.
2. G force and flow rate are readily adjustable settings in the CTS™ Rotea™ Counterflow Centrifugation System.
3. Density and viscosity of media can sometimes be optimized to suit the protocol.
4. Cell concentration is not considered in the formula but has a significant influence on the ability of the bed to form.
5. The conical shape of the chamber has been specifically designed such that the increasing cross-sectional area of the chamber varies the flow rate of the media as it passes through the chamber. This counteracts the change in centripetal force due to the radial position of the particle.
6. The Rotea™ Process Model uses this formula to predict the behavior of cells under different conditions.

Optimize G force and flow rate

The two parameters most easily controlled during process development are the G force and the flow rate. Entering the cell type and media used into the Rotea™ Process Model application enables you to predict the settings for both parameters.



Further optimisation of the settings can be performed based on observation of the fluidized bed using the integrated camera and in-process sampling as adjustments are made to G force and flow rate.

Cell size (*d*)

- **Small cells**: Small cells take longer to settle with less opportunities for inter-cellular collisions within the fixed CFC Chamber volume. Higher G forces, lower flow rates and a longer time are usually required to establish a bed.
- **Large cells**: Large cells settle faster and the probability of inter-cellular collisions is higher. Establishing a bed with large cells can occur quite quickly and less cells are required.
- **Mixed cells**: A mixed cell population comprising both small and large cells will tend to form quickly, with the fluidized bed of large cells creating a stable environment for the small cells. The challenge with mixed populations is finding settings that retain small cells without pelletizing the large cells.

Media density (ρ)

It is important that there is enough difference in density between the cells (ρ_p) and the media (ρ_m).

Note: If the media is denser than the cells, then the cells cannot settle and a bed will not form.

Even very subtle changes in media density can affect the stability of the bed and the settings for loading and separating cells. The higher the density of the media, the higher the G force or lower the flow rate needed to form a bed.

Cell concentration

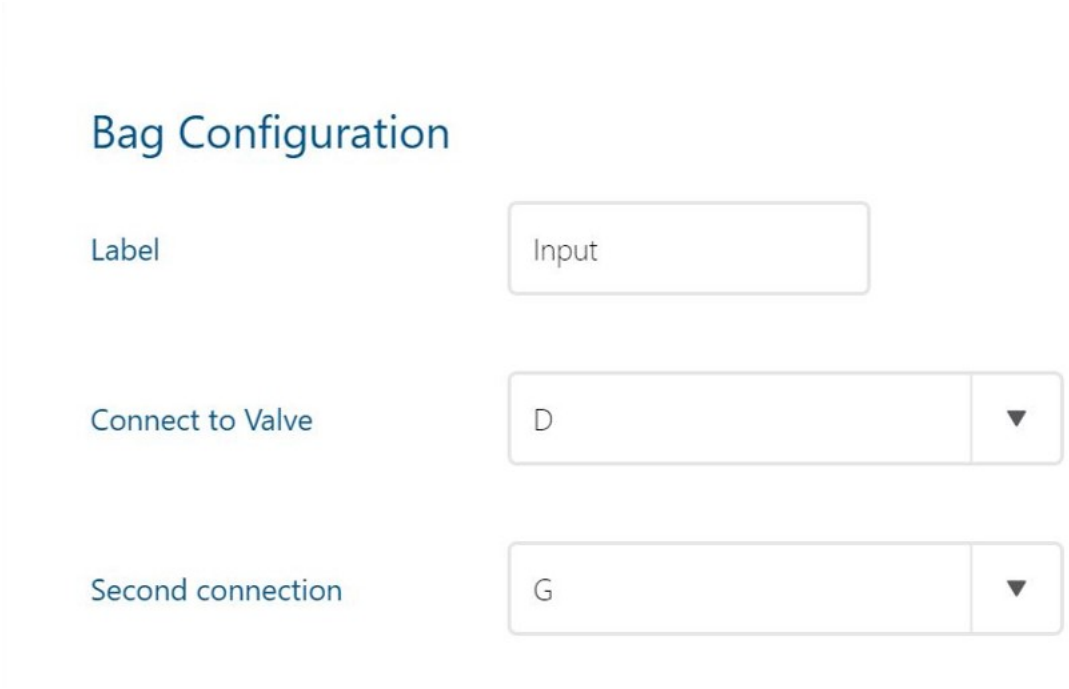
The number of cells required for a fluidized bed to form is strongly influenced by the cell diameter (*d*) and the density differential ($\rho_p - \rho_m$) but as a guide:

- MSC's: >20 × 10^6 cells
- PBMC's: >30 × 10^6 cells
- When the cell concentration in the input material is low, it will take longer to reach the cell concentration in the CFC Chamber and hence for the fluidized bed to form.
- While the Rotea™ instrument is capable of processing comparatively small numbers of cells, small beds are much more sensitive to changes in conditions. Where possible, it is best to work with larger numbers of cells

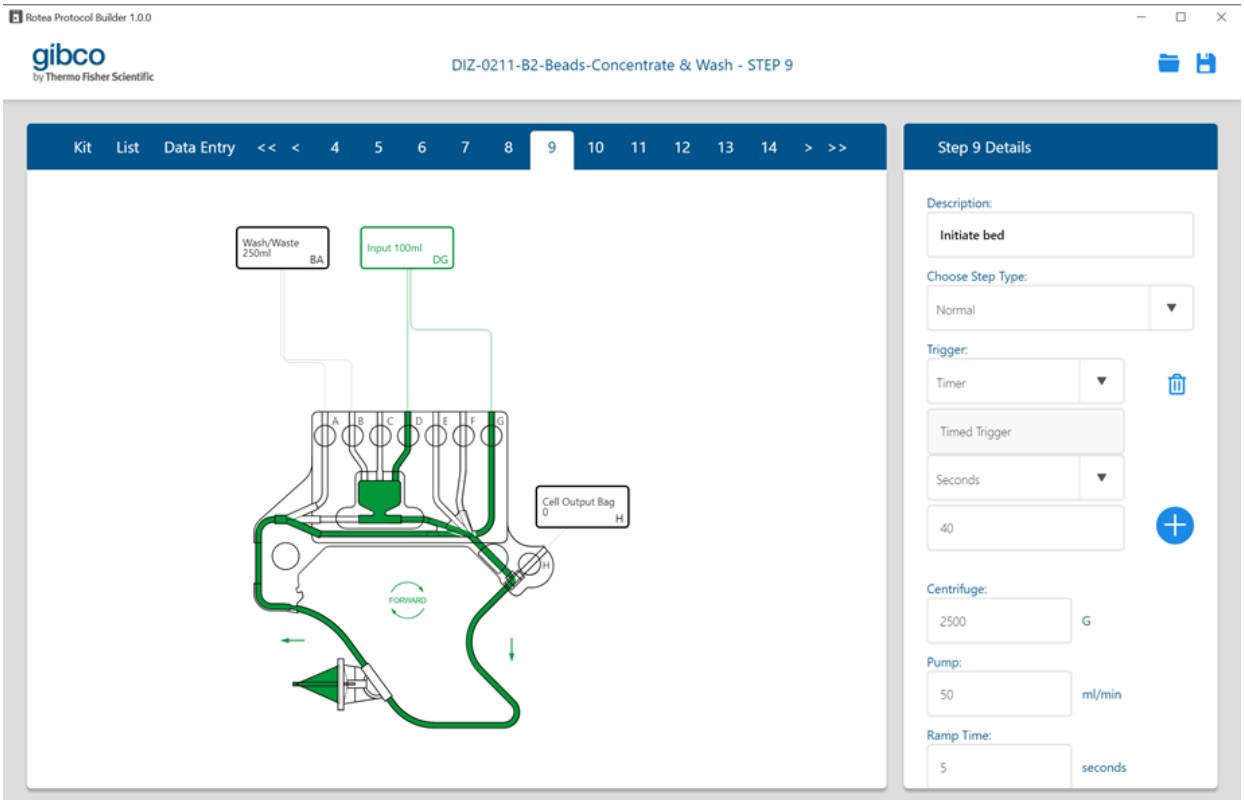
Establish a bed in recirculation

Under most situations it is preferred to establish a bed in such a way that no cells are lost to the waste bag during the formation of the bed. This technique is particularly important when the cells are small, the input concentration low or the media dense.

A dual-connection input bag contains the cells in media. At the define kit screen, define a bag with two connections. The Rotea Protocol Builder will give you the available second connection options.

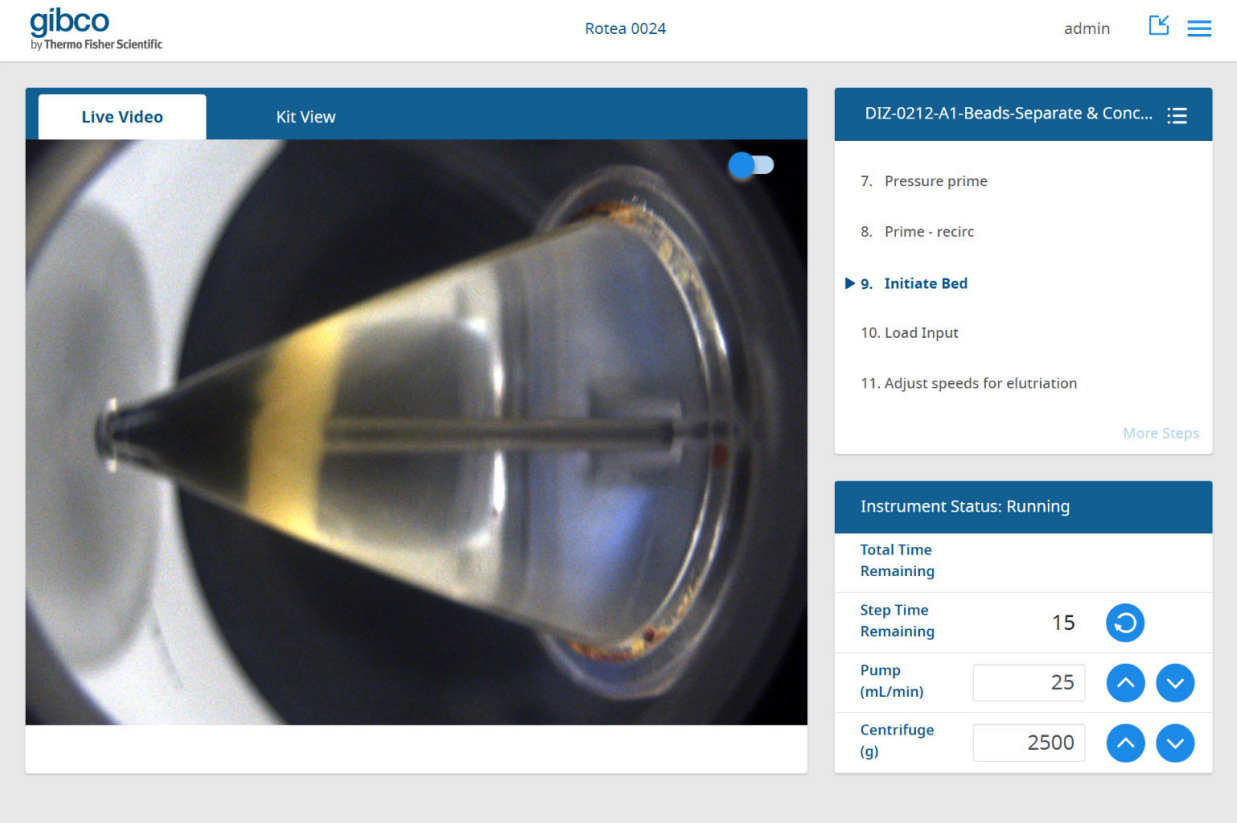


In this example, D and G are the two connections. This will change depending on the protocol. When establishing a bed, flow direction must be forward. Set your fluid path to go into and out of the same bag, with a forward flow direction.



Once the fluidized bed forms we advance to the cell loading step where cells continue to be collected in the chamber whilst the output is now directed to the waste bag.

If the bed has not established within the defined **Step** time, press the reset button to extend the **Step Time Remaining**.



Establish a bed without recirculation

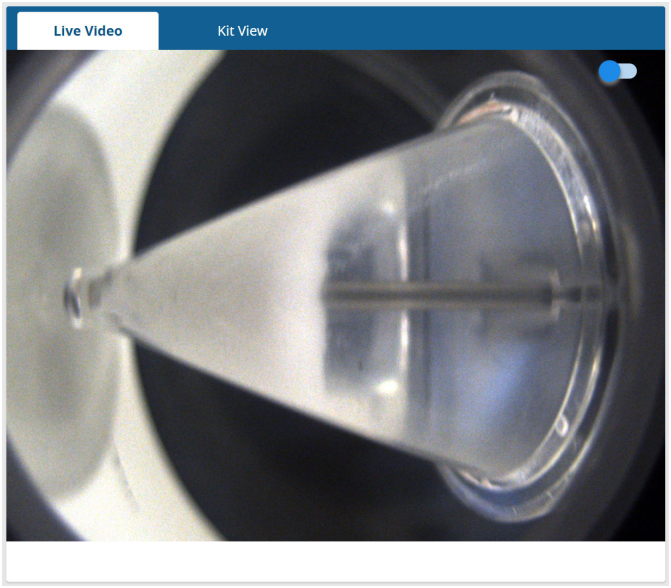
When processing large cells or aggregates that settle quickly, or when the input material has a very high concentration of cells, such as a leukopak, it is possible to establish a bed as part of the loading step rather than using recirculation.

Using a higher G force or lower flow rate to minimize losses whilst the bed is being established is often desirable, because any cells lost during establishment of the bed are being sent to waste.

What a good bed looks like

In order to optimize a process using counter flow centrifugation, it is essential to understand what a good bed looks like. In general, the factors to look for are:

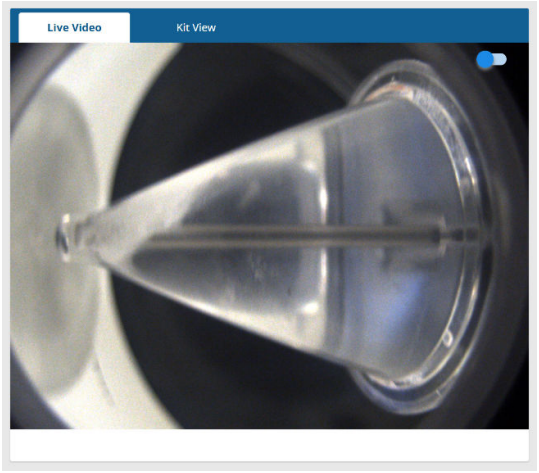
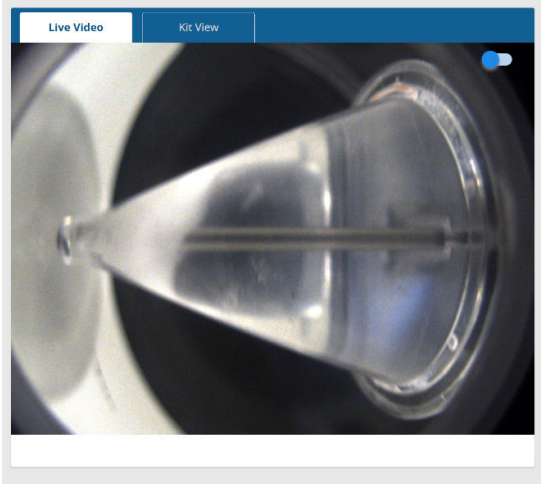
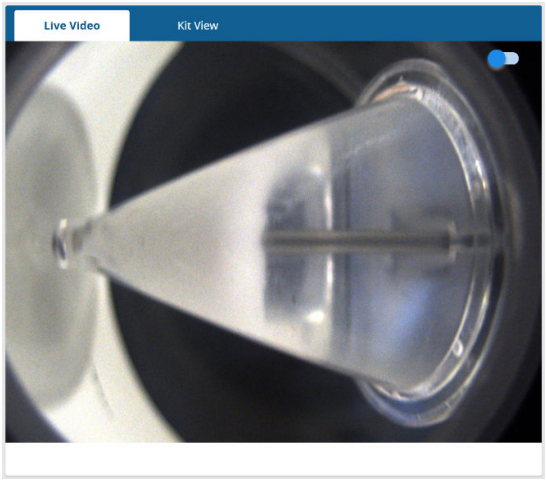
- The top surface of the bed is mostly flat with minimal turbulence
- Cells are not flowing up the top edge of the cone and ultimately exiting the CFC Chamber
- The stainless-steel Dip Tube is visible at the tip of the CFC Chamber and between the surface of the bed and the CFC Chamber exit port.



What a good bed looks like will vary depending on the cell type, the size of the bed, the media density and the process you are wanting to perform. The following images show the typical stages in bed formation.

Note: Depending on the media, the clarity of the liquid above the fluidized bed is often a good indicator that cells are not leaving the CFC Chamber. We would typically expect the exit port on the right to be clearly visible.

Fluidized bed formation

Image	Description
	In the early stages of establishing a bed, cells can be seen entering the chamber through the tip, and flowing up the top edge of the chamber, a consequence of Coriolis flow.
	As more cells enter the chamber the bed begins to stabilize. The cells are still affected by Coriolis flow at this point, but the angle of the bed is getting closer to vertical.
	The number of cells in the chamber has increased and we can see that the bed is nearly vertical with minimal turbulence.

Establish a bed: Troubleshooting

Observation	Possible cause	Recommended action
Small number of small cells	When the number of cells being processed is small e.g. <20 to 30 × 10^6 cells, a stable fluidized bed may not form and in some instances may not be visible at all, despite using recirculation.	**Increase the G force and/or reduce the flow rate**: Increasing the G force and/or reducing the flow rate, even for only a few seconds, can be enough to cause the bed to form. This suggests that a low-density bed exists in the chamber away from the tip that isn't sufficiently concentrated to be visible. Increasing the G force and/or reducing the flow rate biases the cells to the tip of the CFC Chamber where they are more concentrated enabling the bed to form and become visible.
		Increase the flow rate: Provided you are establishing the bed in recirculation, increasing the flow rate reduces the time it takes to accumulate enough cells in the CFC Chamber for a bed to form without losing cells to waste. **Note:** if the flow rate is too high, the cells will be continuously elutriated and will not accumulate in the CFC Chamber.
		Support particles: Adding support particles to the input material can encourage a bed to form comprising the target cells and the support particles. These particles might be other cells such as a lymphocytes and monocytes in PBMC, RBC's in a leukopak or beads. The important thing to remember with this strategy is that what you add most likely will have to be removed.
Large cells and aggregates	Rapidly settling cells and aggregates can accumulate in the Bubble Trap and may be difficult to recover.	It is recommended to load these types of cells through lines E or F. Care must be used to avoid bubbles in the process line. It is not recommended to use line H as an input, as it does not have a Bubble Sensor.
		Adding protein such as HSA to the priming and wash buffer can also often change the behavior of these cells.

Observation	Possible cause	Recommended action
Clumps	Clumps will behave like very large particles in the CFC Chamber and hence will be concentrated at the tip.	It is recommended that clumpy material be drawn from a bag lying on its side or a vessel where the lowest point is adjusted mid process so rapidly settled clumps are not the first material to be loaded. Mixing of the input bag during loading can also help to prevent settling of clumps. The Rotea™ instrument can be used to separate good cells from these large clumps by elutriating the good cells to an intermediate bag, removing the clumps to a waste bag, such as line E or F, then re-loading the clump free cells for wash and recovery. This process should be performed at relatively high flow rates to minimize the potential for blockage of the tip during recovery of the clumps. **Note:** • Clumps often reflect DNA being released from the disruption of cells damaged by upstream processes such as thawing. The clumps may not always be evident prior to processing. Keeping the centrifuge speed as low as practical is recommended for such processes. • If clumps in the input material settle near to the Rotea™ input line, they will be drawn into in the CFC Chamber at the very beginning of bed formation and accumulate at the tip of the CFC Chamber. The accumulated clumps can then disrupt the fluid flow, making it difficult for the cell bed to form. • Accumulated clumps have the potential to cause blockages of the Dip Tube. While they can sometimes be cleared by cycling back and forth in pause or recirculation at high flow rates, some clumps, e.g. DNA, can be difficult to remove. This can be especially challenging when processing large batches containing clumps in multiple bites. • The clumps will usually be recovered along with the rest of the cells in the fluidized bed and may be an undesirable impurity.

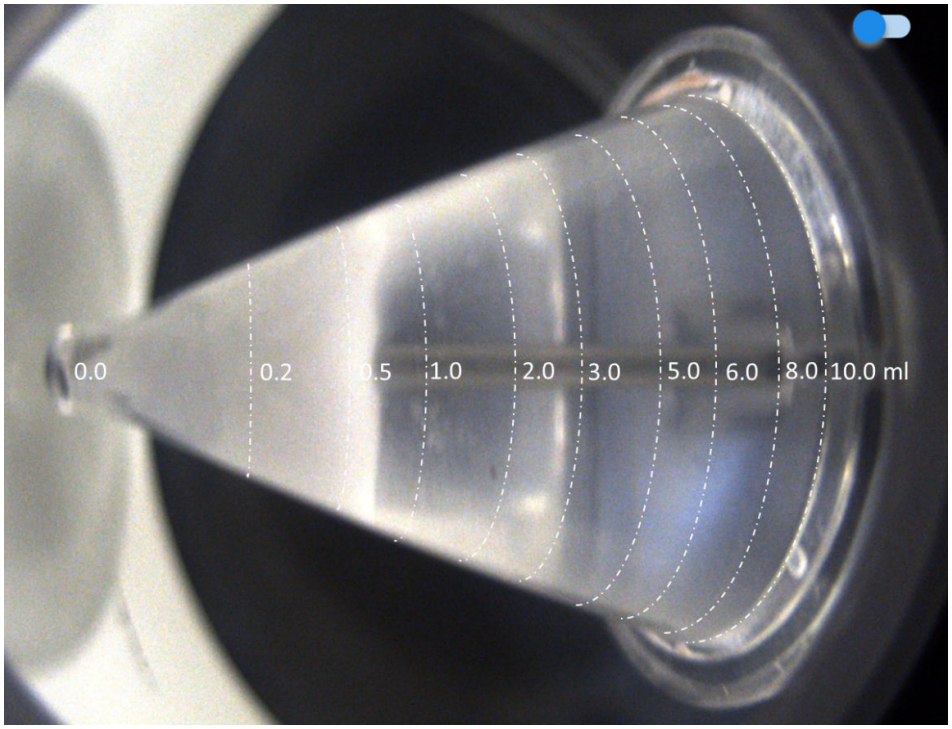
Cell loading

Overview

Cell loading is the process of capturing target cells in the fluidized bed as the input material passes through the CFC Chamber.

Cells loaded into the CFC Chamber

The CFC Chamber has a fluidized bed volume of 10 mL. Due to its conical shape, a small volume of cells represents a reasonable size bed as can be shown in the following illustration:



The number of cells that can be processed in the CFC Chamber at a time is dependent on the following:

- Size and concentration of the cells
- Density of the media
- G force and flow rate settings that will deliver acceptable cell recovery and viability

There are two practical limitations to the number of cells in the bed that can be determined through experimentation:

- When the top surface of the bed extends past the CFC Chamber rim closest to the axis of rotation (ie >10 mL), cells will be washed out of the CFC Chamber
- When the fluidized bed is too concentrated to recover

Note:

- Up to 5 billion T-cells have been loaded in the CFC Chamber using high G and a very low flow rate but 2 – 3 billion T-cells is more common.
- Cells <4 µm, e.g. platelets, cannot be concentrated in the CFC Chamber and hence do not need to be considered when calculating the volume of input material that can be loaded.
- Elutriation requires a lower G force and higher flow rate than loading, reducing the concentration and hence number of cells in the fluidized bed.
- When loading cells prior to an elutriation step, be sure to allow enough room in the CFC Chamber to retain the larger cells as the fluidized bed expands.

Bed disruption during loading

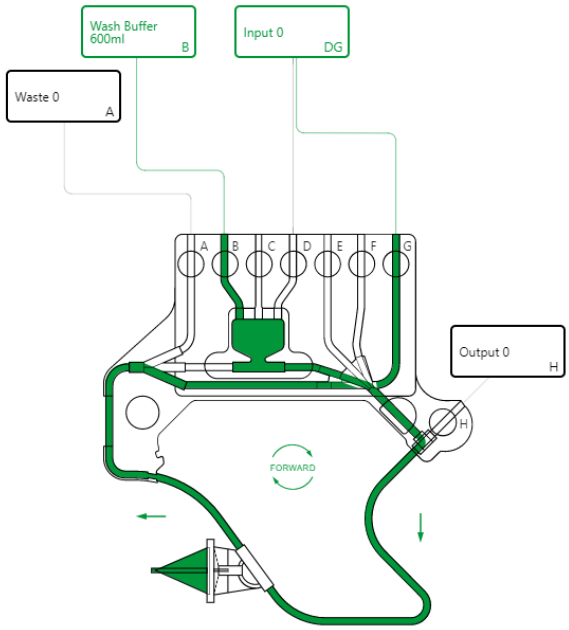
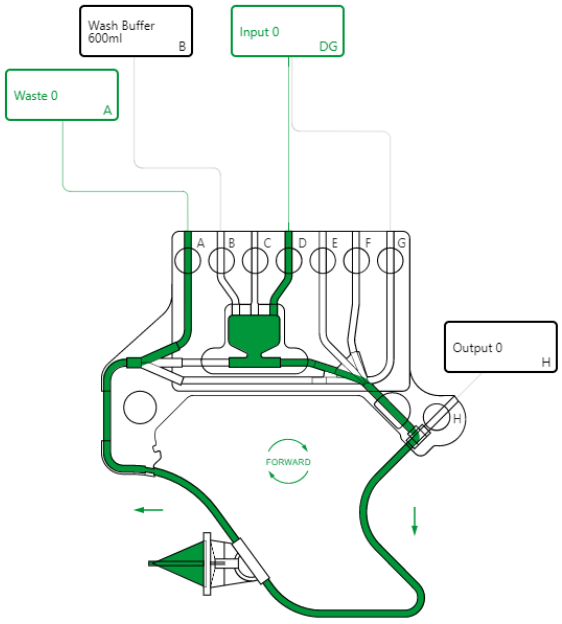
In certain conditions, low density immiscible media may accumulate as a separate layer of fluid in the input bag. When this fluid enters the CFC Chamber, it is highly buoyant and can disrupt the established fluidized bed. It is important to keep materials of this nature mixed during loading.

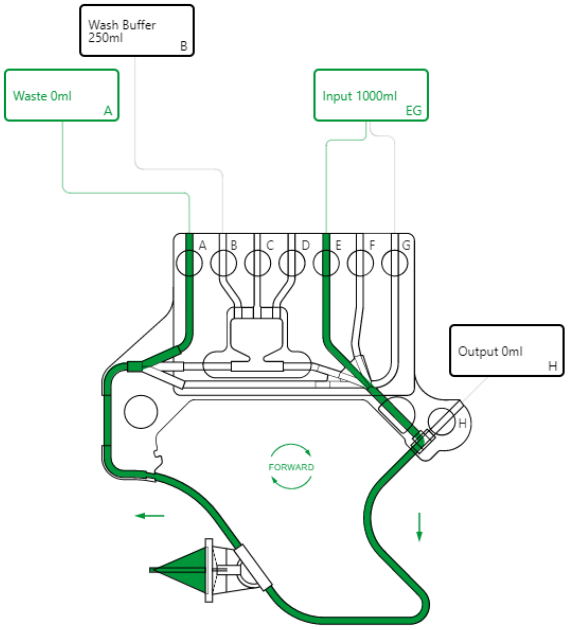
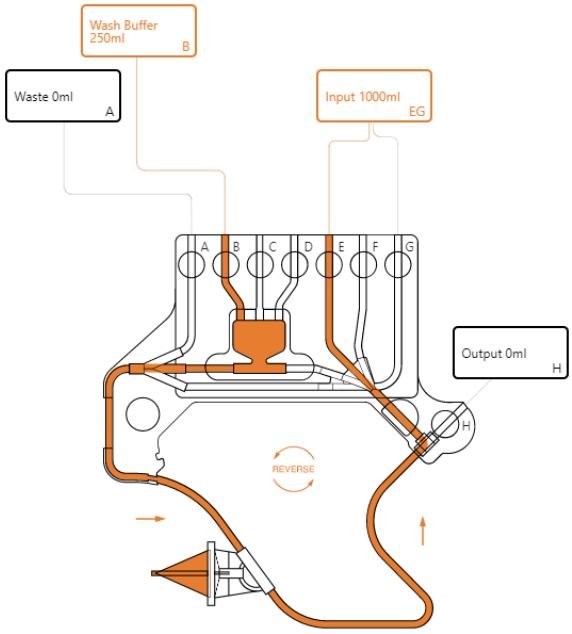
Cell separation during loading

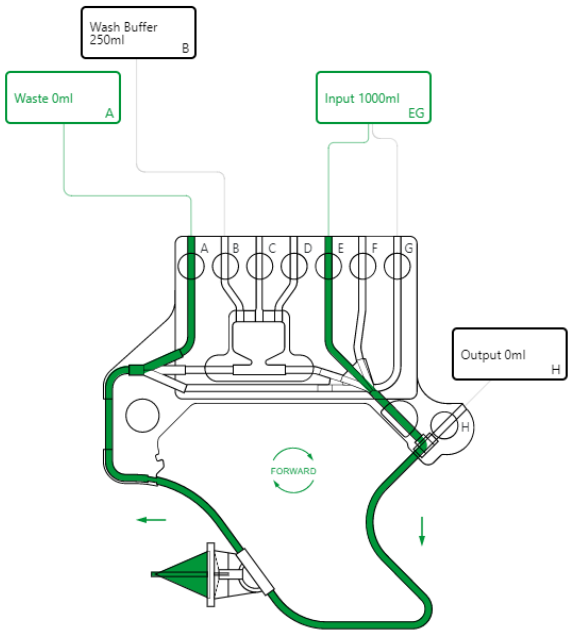
While it is common practice to be conservative during cell loading to avoid losses of target cells, the loading step can be an efficient way to remove debris and lighter cell materials. For example, this strategy can be used during leukopak processing where debris and platelets are washed out as the PBMC is loaded.

Re-use input bags

Rinsing and re-priming input bags can enable re-use of the bag for subsequent steps in a protocol.

Option 1: Two-port bag used for recirculating bed formation.	
	With the cells loaded in the fluidized bed, wash buffer is delivered through the bed and into the input bag through line G. This fluid is then used to “rinse” the input bag. **Note:** This is also a good wash strategy if there is potential for the wash step to disrupt the bed due to differential media densities.
	The loading conditions are re-established, and the rinse fluid is drawn into the fluidized bed until the bubble sensor triggers. **Note:** It may be necessary to re-prime the input line (D in this case) prior to loading the rinse fluid.

Option 2: Large batches where multiple process "bites" (processing media in small portions at a time) are needed.	
	Use one of lines E, F or H as the product input line to take a bite from the input bag and build the fluidized bed.
	After recovering the concentrate from the previous bite, rinse media connected to lines B, C or D is directed to the input bag, re-priming the input line for the next bite.

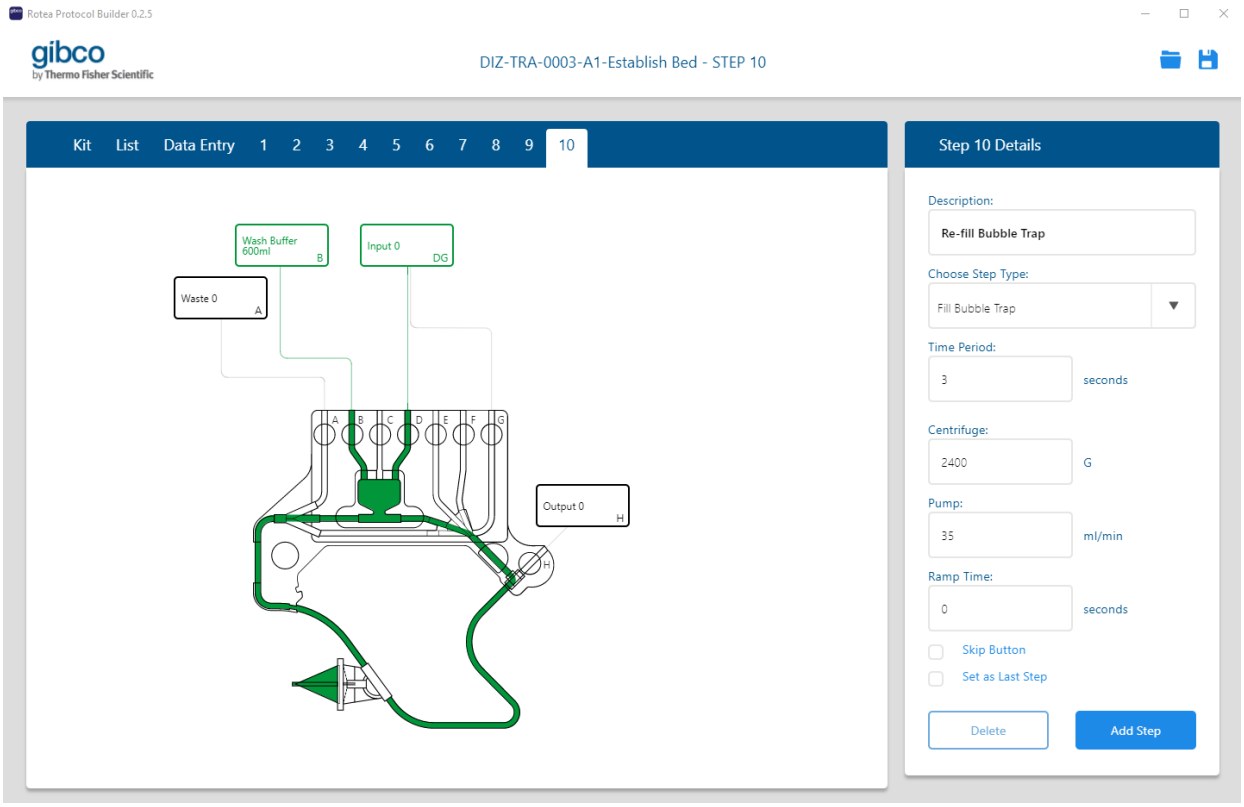
	The loading conditions are established and processing of the next bite commenced.

Refill the Bubble Trap

Loading fluid through an input line that has been previously emptied using a Bubble Sensor trigger and not re-primed, will result in air being drawn into the system. A common example of this is multi-bite operations where an input bag is repeatedly used.

In such instances, the input line should ideally be connected to the Bubble Trap i.e. lines B, C or D, to prevent the air from entering the CFC Chamber.

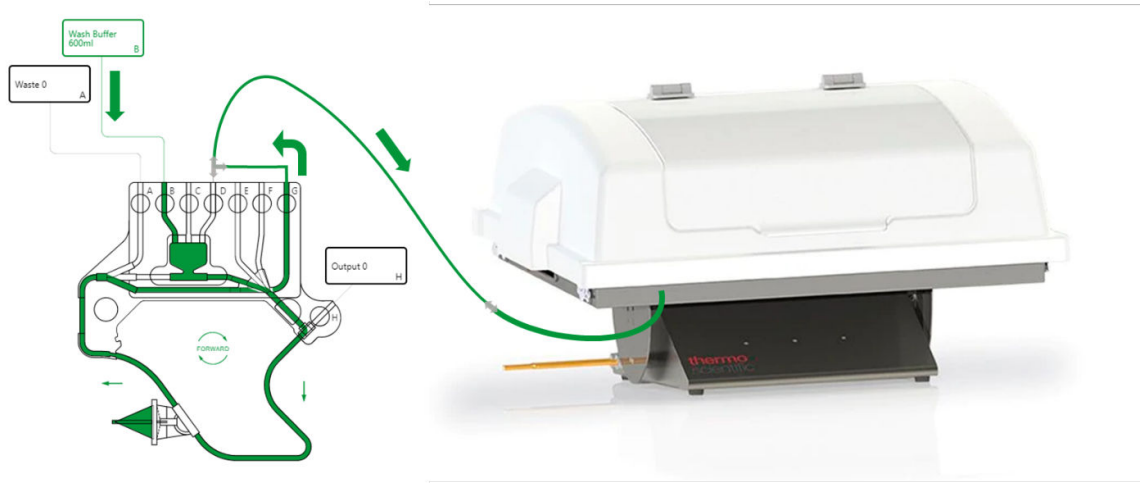
Even so, drawing in air will eventually deplete the Bubble Trap and it will need to be re-primed before further use.



The **Fill Bubble Trap** step type enables the Bubble Trap to be refilled whilst cells are held in the fluidized bed. With the system in pause (the fluid flowing around the recirculation loop), the media supply and product input lines are opened for a short period of time, for example 3 seconds, allowing liquid to flow from whichever bag has the highest liquid level to the other, refilling theBubble Trap in the process.

Note: Only valid bag options are available for selection when using the **Fill Bubble Trap** step type.

Connect to bioreactors



The Rotea™ instrument can be directly connected to many bioreactors provided there is a suitable fluid connection and that priming is adequate (see “Prime remote sources” on page 52).

If the bioreactor is a rigid vessel, it must be vented.

CAUTION! The user is responsible for ensuring that the risk of excessive pressure within the total system is assessed and appropriate steps taken to prevent over-pressure conditions from eventuating.

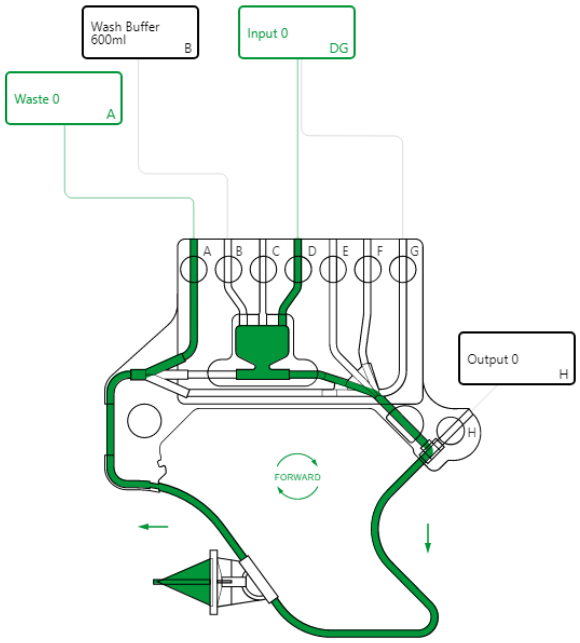
The tubing connecting the bioreactor chamber to Rotea™ instrument should ideally not be more than 1/8” ID, (3.2 mm). If the tubing is >1/8” then bubbles can be difficult to drive out of the tubing. If bubbles/air remain in the tubing, air will be drawn into the Rotea™ instrument when it starts the loading process.

If the input line Bubble Sensor triggers during loading from a bioreactor, the entire input line may be empty. This can represent a significant volume that requires re-filling before loading from the input line can continue. To address this, a separate step to re-prime the input line can be added, either as a standard step within the protocol or as a pre-prepared recovery step. One strategy is to connect line G as close as possible to the input line. This enables wash buffer to be pumped through the CFC Chamber and out through G to the bioreactor without disrupting the fluidized bed.

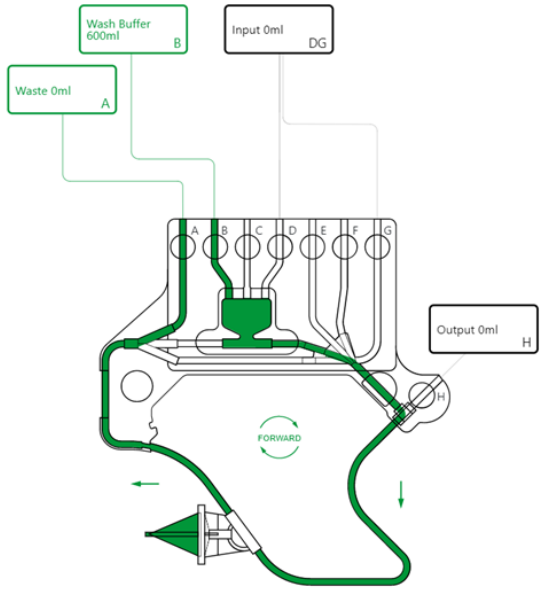
Wash cells and buffer exchange

Overview

Counterflow centrifugation depends on the formation of a fluidized bed where cells are surrounded by, and in intimate contact with, the media flowing through the bed. This means that cells in the fluidized bed are rapidly exposed to new media resulting in very fast and efficient buffer exchange using a small volume of wash media. The basic cell washing steps using counterflow centrifugation are as follows:

Image	Details
	Create a fluidized bed of cells in the original media.

(continued)

Image	Details
	Pump new wash media through the fluidized bed displacing the original media. **Note:** The amount of original media remaining (residual) is dependent on the volume of new wash media that passes through the bed but 3 to 5 CFC Chamber volume changes (e.g. 30 to 50 mL) is often adequate.

Media selection

Depending on the cell type, sensitivity to processing and the properties of the wash media, the rapid exposure to the new media in counter flow centrifugation can contribute to higher than expected cell losses. Washing with the same media, culture media (e.g. CTS™AIM-V™ Medium) or PBS with 1–2% HSA, can significantly improve cell recovery.

Note: Forming a fluidized bed in dense media requires higher G force and/or lower flow rates compared to lighter media. The bed will also take longer to form, especially if the cells are small.

Wash to similar density media

If the wash media has very similar density to the original media, washing can often be conducted at the loading speed or slightly higher G force (or lower flow rate) with minimal disruption of the fluidized bed.

Wash to more dense media

If the wash media is substantially more dense or higher viscosity than the original media, cells can be slow to settle. There is also a risk that the denser wash media will not mix with the original media as it tries to pass through the fluidized bed, instead pushing the bed of cells out of the CFC Chamber. Sometimes this issue can be addressed by washing at high G and very low flow rates.

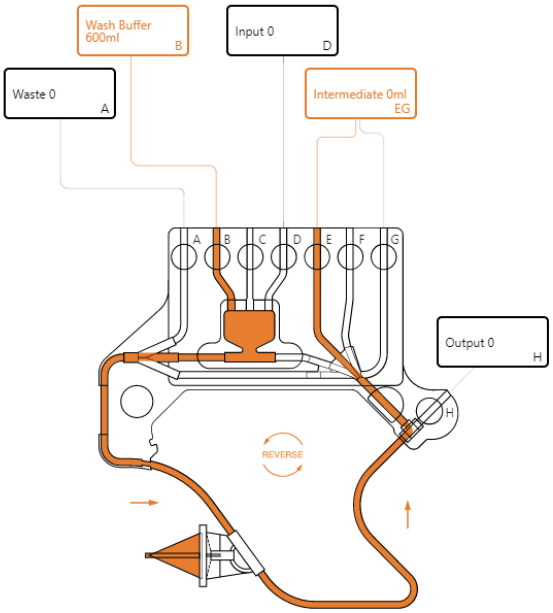
Wash to lower density media

Transferring to a lower density media such as PBS can disrupt the fluidized bed due to the dramatically increased buoyancy effects caused by the spinning CFC Chamber. When the lower density media enters the tip of the CFC Chamber, cells can be entrained by the rapidly rising lighter media and washed out of the chamber. This can often be clearly seen in the live video and can be quantified by sampling the media exiting the chamber through line A or G. Sometimes simply increasing the G force and/or reducing the flow rate will be enough to address this issue.

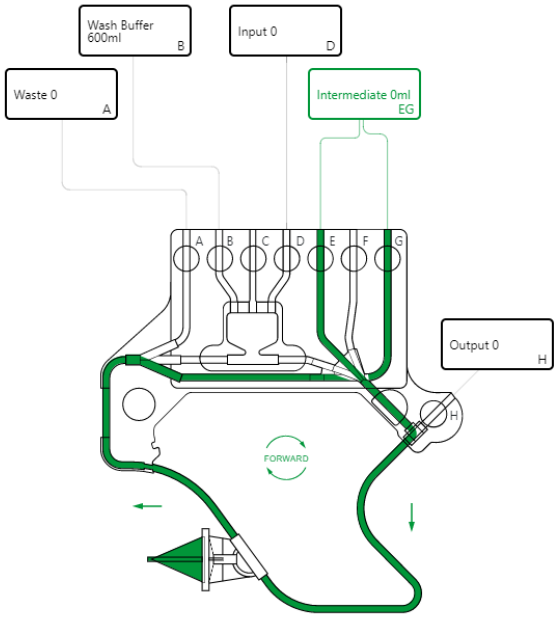
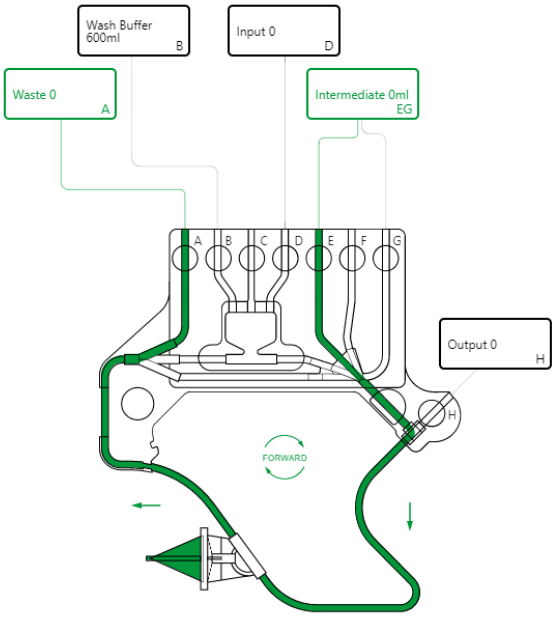
Note: Residual low-density wash media in the Pause loop and/or Bubble Trap can potentially disrupt the fluidized bed as it enters the CFC Chamber. Allow time for the bed to re-settle when moving to a Pause step.

Defensive washing strategies

The following defensive washing strategies can be applied when the properties of the new media are different to the original media. They are also particularly helpful during process development when the properties of the media and cells are not well defined.

Image	Details
Recover as concentrate to intermediate bag and dilute to new media	
	After loading the cells into the CFC Chamber, recover the cells as concentrate to an intermediate bag connected to line E or F. Transfer additional new media to the intermediate bag, re-priming the line and diluting the concentrate such that its density is similar to the new media.

(continued)

Image	Details
	The intermediate bag is then mixed, and the cell bed re-established using recirculation.
	Re-load the diluted cells into the CFC Chamber.

(continued)

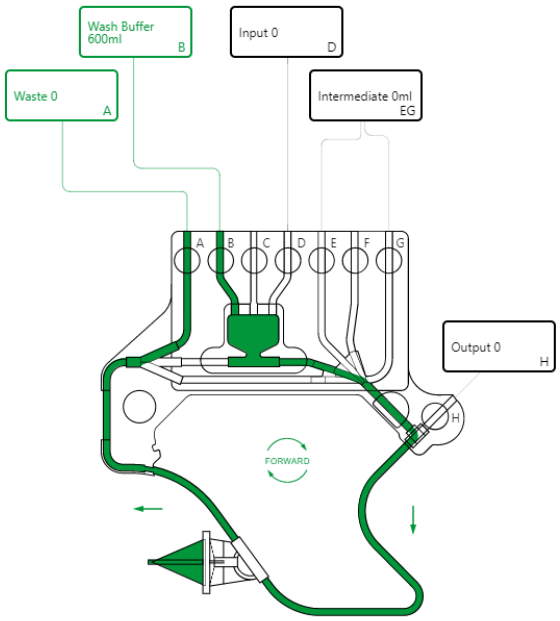
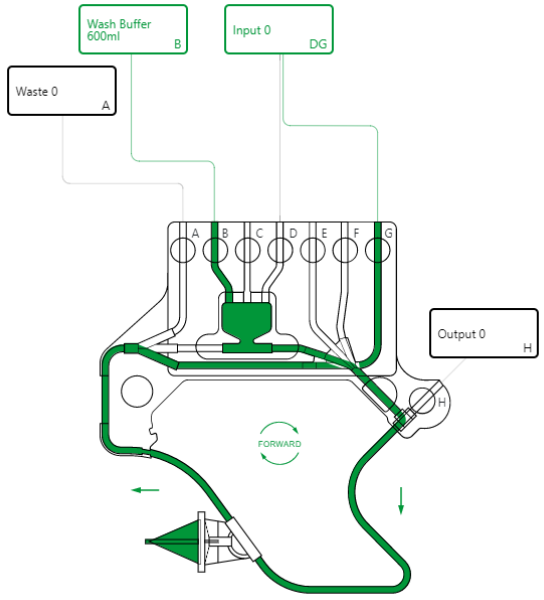
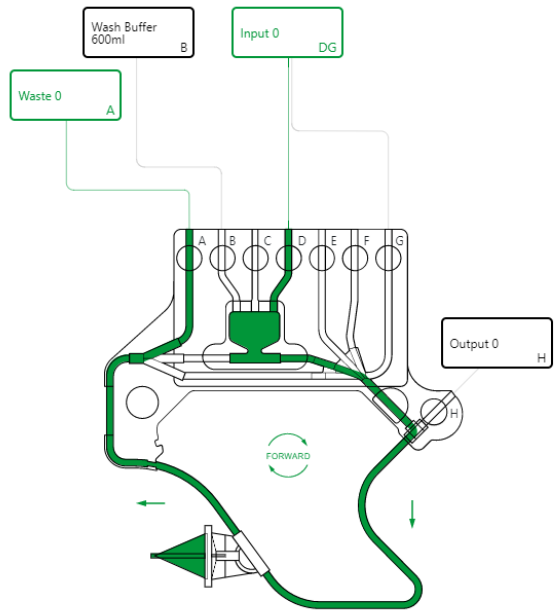
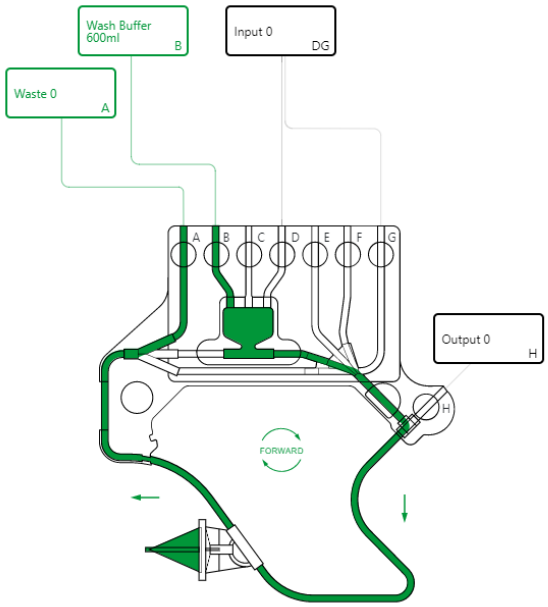
Image	Details
	Perform a final wash of the cells using the new media to remove residual original media.

Image	Details
Wash to intermediate bag and recover	
	Wash the cells in the fluidized bed, directing the output to a dual port intermediate bag connected to line G. Additional wash volume is then pumped to the bag, diluting the contents. • Since we will be re-processing the wash output, this step can be performed at low G and high flow rate to intentionally disrupt the bed and mix the diluted cells. • This wash step can also be performed at normal wash settings to separate the wash waste from normal waste during process development as a means of identifying the source of any yield losses.

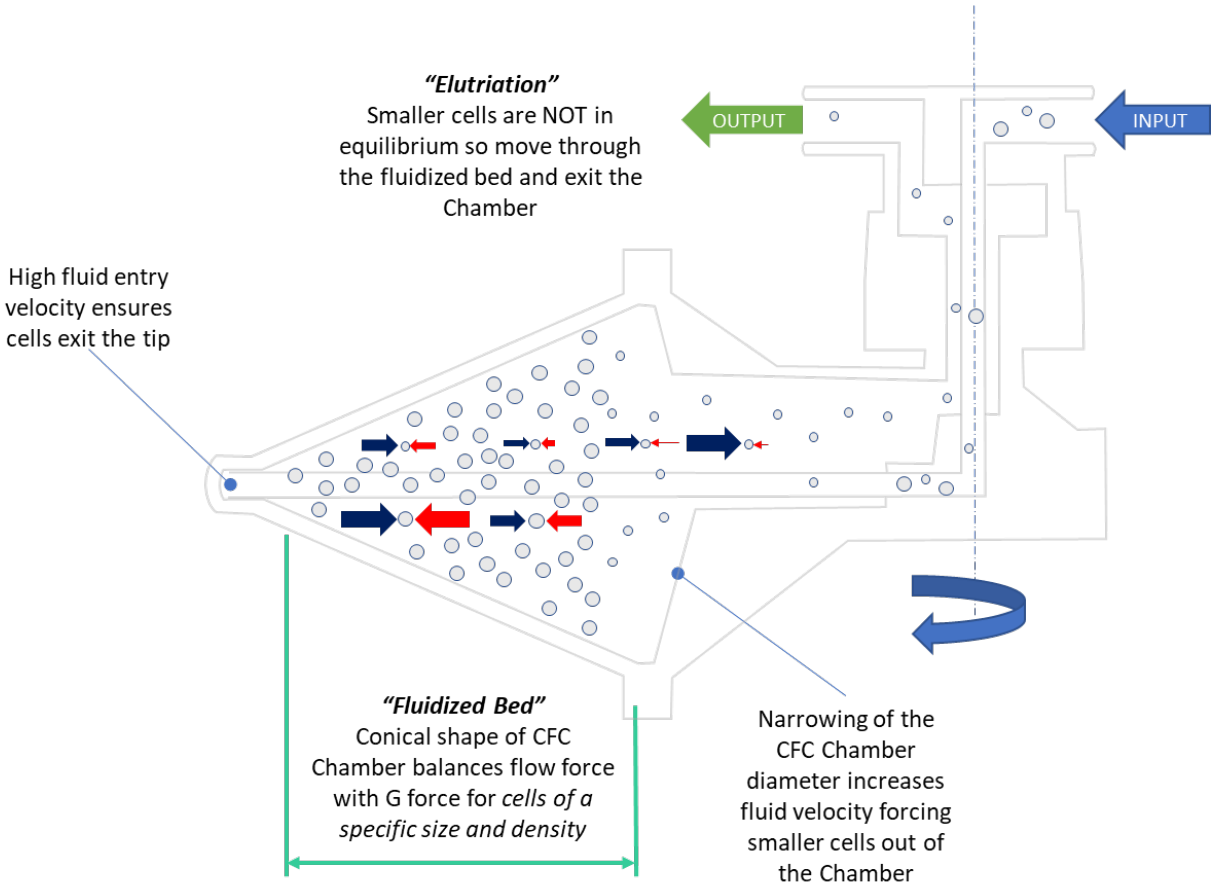
(continued)

Image	Details
	Re-establish the fluidized bed using recirculation and then load the cells, directing the output to the waste bag. **Note:** It may be necessary to re-prime the input line prior to this step.
	A final wash of the cells using the new media can now be completed with low risk of cell loss.

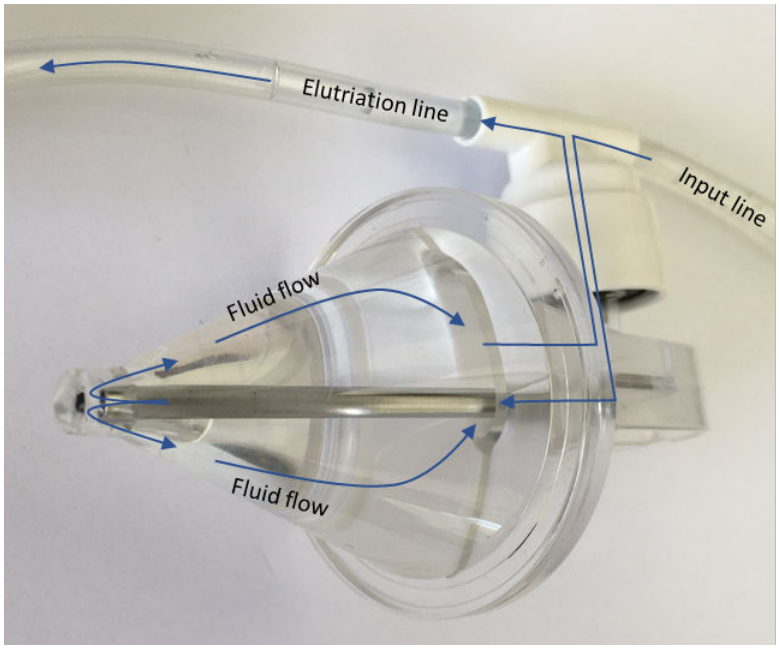
Elutriation

Overview

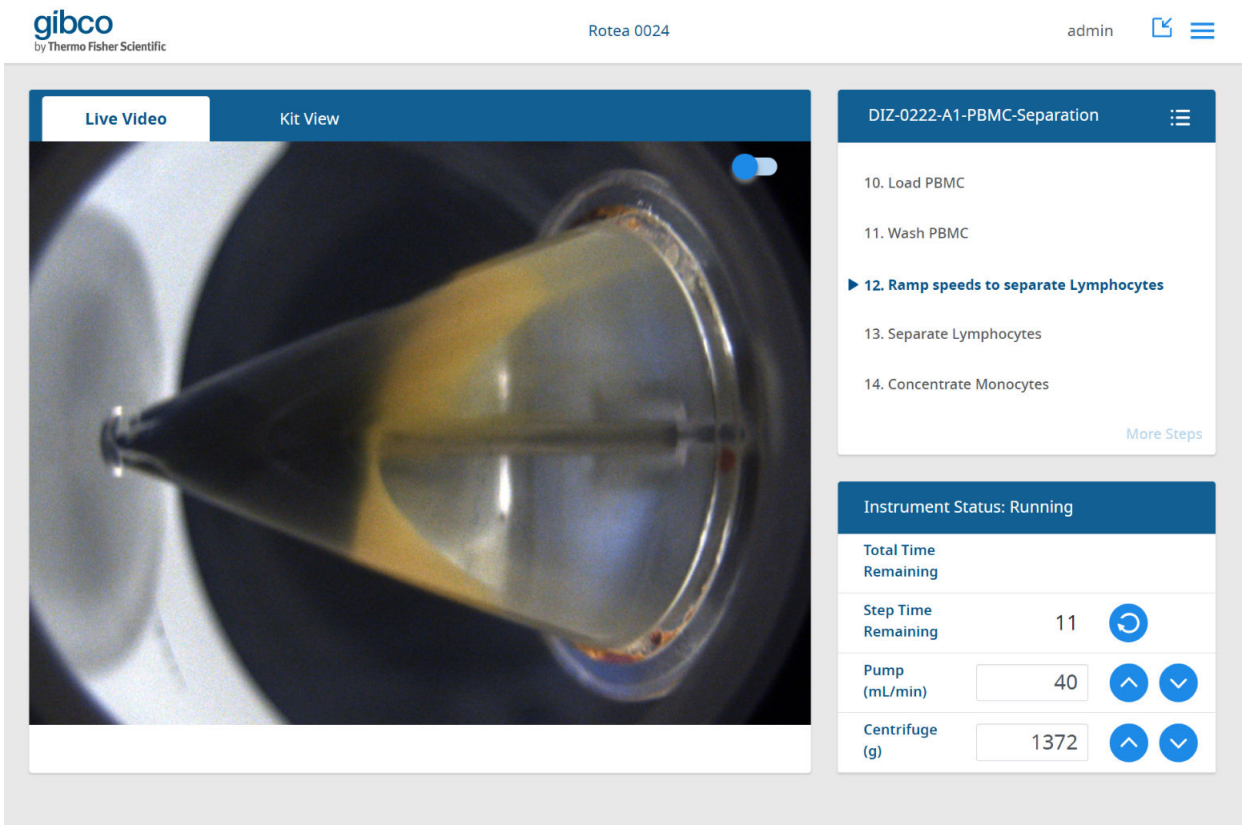
Elutriation is a process unique to counter flow centrifugation that enables particles of different size and/or density to be separated from each other.



For a specific set of processing conditions (G force and flow rate), a larger cell can be in a stable state in the fluidized bed whilst a smaller cell is not stable and will be washed (elutriated) from the bed.



Cells that reach the rear section of the CFC Chamber will accelerate as the diameter reduces and exit via a hole in the back of the CFC Chamber.



6 µm yellow beads being separated from a bed of 10 µm blue beads

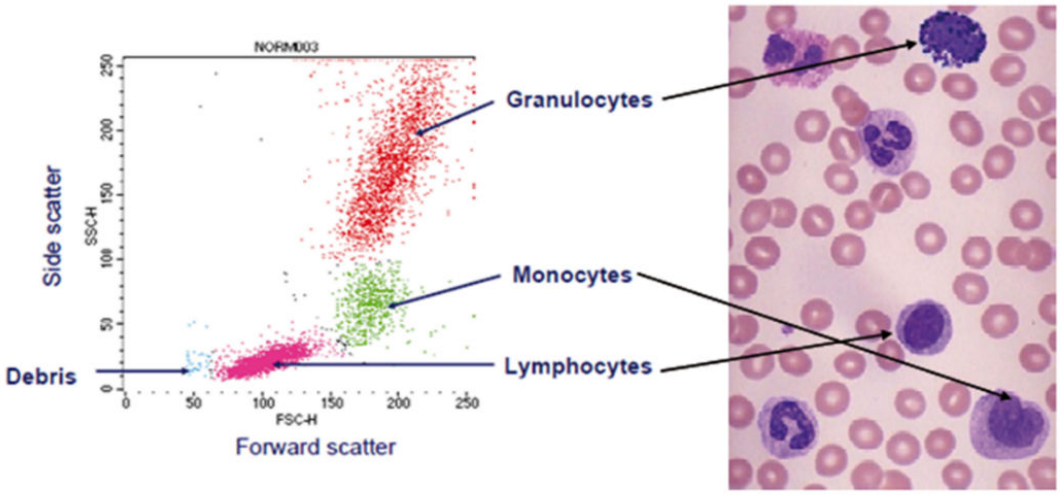
Improve cell viability

Viability is defined by *Number Live Cells* / *Total Number Cells*. Dead cells are smaller and have a different density to that of live cells. The viability of a cell population can therefore be increased by elutriating dead cells to waste while retaining viable cells in the fluidized bed. Common applications include processing thawed Input material or following transfection or electroporation.

Note: The settings that will elutriate dead cells but retain live cells can be a delicate balance and may require a compromise between recovery and viability.

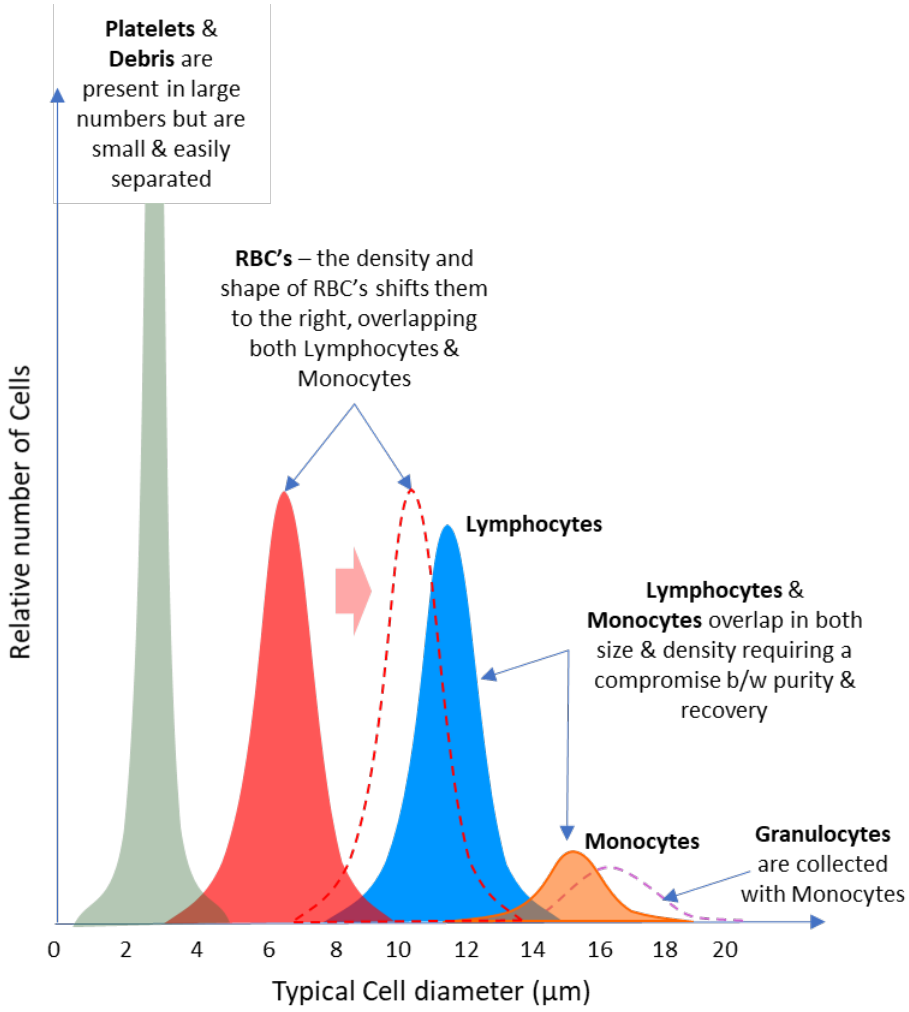
Leukopak processing

A leukapheresis contains cells of different shapes, sizes, densities and quantities that can be separated using the Rotea™ instrument.



Composition for a leukapheresis expressed in terms of the cell diameter and relative number of cells.

Typical leukaphoresis composition



The distribution of cells based on diameter in a leukapheresis shows quite discrete cell populations or "fractions". Several important factors need to be considered when developing a protocol to separate these fractions using elutriation:

- Removal of platelets and debris is readily achieved as part of the PBMC loading step since they are significantly smaller and will be washed out of the fluidized bed
- The number of cells in each fraction can vary by a significant amount, e.g. some leukapheresis products have very low monocyte numbers that can make it difficult to establish a monocyte bed. Red blood cells (RBCs) also vary depending on the donor patient, the process used and the skill of the leukapheresis operator
- The average size of a lymphocyte is smaller than a monocyte. However, both fractions have a size distribution, with large lymphocytes overlapping with small monocytes. This overlap in cell diameter results in a compromise needing to be made between purity and recovery. If you want high recovery of lymphocytes you will also recover small monocytes. Conversely, to achieve high purity lymphocytes, you will need to use processing parameters that will not collect the smaller monocytes which also means you won't collect the larger lymphocytes

- RBC's are typically smaller than PBMC's, but they are denser and non-spherical. In the Rotea™ instrument RBC's behave in a similar way to lymphocytes and small monocytes when processed in culture media or wash buffer. This means that using elutriation alone, the majority of RBC's will be in the lymphocyte fraction.

  Note: Lysing the RBC's enables them to be elutriated from the PBMC's. The density of the buffer can also be manipulated, e.g. Ficoll to deplete RBC's from PBMC based on the density differential

- Granulocytes typically end up with the monocyte fraction due to their size and density

Lentivirus clarification

Lentivirus is very small relative to the HEK producer cells so can be elutriated leaving the concentrated HEK cells in the CFC Chamber. In this instance, the product of interest is being elutriated from the input material leaving the concentrated waste behind.

Separate cells from microcarriers

Microcarriers are very large e.g. 200 µm relative to the adherent cells they are producing. Once separated from the microcarriers, the cells can be elutriated from the bed leaving the concentrated microcarriers behind.

- The microcarriers will settle very rapidly in the CFC Chamber. To avoid pelletizing them in the tip, it will be necessary to process at very low G and high flow rates.
- Despite the significant size difference between microcarriers and cells, it is difficult to guarantee that there won't be any microcarriers in the output due to the potential catch points and dead zones within the fluid path. Avoiding the Bubble Trap during processing can reduce the number of trap points for microcarriers but requires careful attention to priming.
- The large diameter, quantity of microcarriers and batch volume typically being processed means that the CFC Chamber is likely to require emptying on a frequent basis to complete a batch
- Since separation of adherent cells requires the addition of enzymes, the Rotea™ instrument can also be used to wash away the enzyme

Elutriation techniques

The Rotea™ instrument provides the tools to develop and optimize elutriation processes and settings.

Lines A and G of the Single-Use Kit direct elutriated material (media and cells) to either a waste bag or intermediate bag for subsequent processing.

Note: If line E or F is being used as the input, it is also possible to direct elutriated material through the Bubble Trap from the left and out via lines B, C or D.

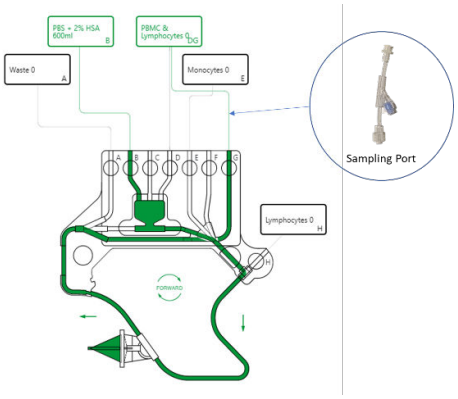
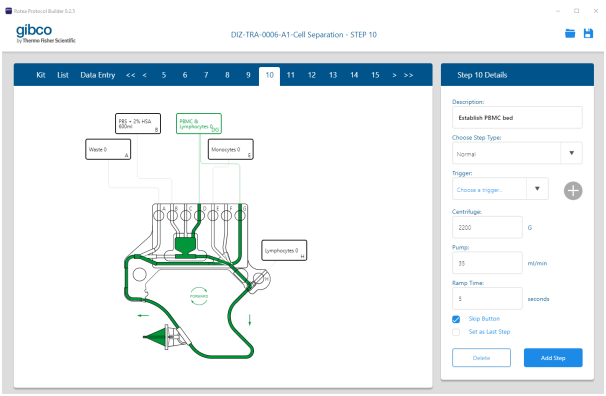
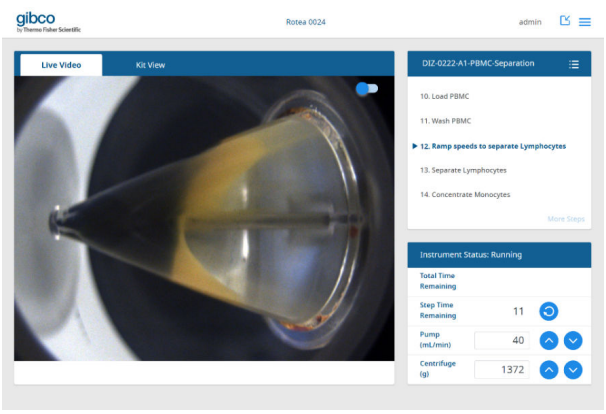
When developing an elutriation method, the challenge is to understand:

- Which cells am I trying to retain in the fluidized bed and which cells am I trying to elutriate?
- At what “speed” do the cells I want to elutriate start to leave the CFC Chamber?
- How long do I need to elutriate at a given “speed” to achieve an acceptable level of purity?

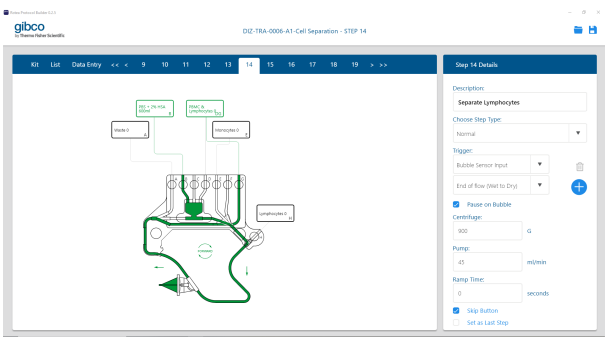
Note: Speed represents a combination of flow rate and G force where the ratio of G force : Flow Rate is roughly constant for the same fluidized bed conditions. For example, 1,000 G : 50 mL/min is equivalent to 2,000 G : 100 mL/min.

The following section describes a method for determining suitable elutriation speeds for processing PBMCs but the same method could equally apply to other applications.

Elutriation process development

Image	Details
	Set up a 2-port input bag between lines G and D. Insert a sampling port in line G.
	Establish a bed of PBMC's using recirculation in the planned media with **Skip Button** as the only trigger.
	Manually adjust the pump flow rate or centrifuge speed using the Rotea™ GUI, allowing a few seconds for the system to reach a steady state condition. Collect a sample of the elutriation output from the sampling port in line G using a syringe. Repeat at different settings to determine the first break point i.e. when the smaller lymphocytes are being elutriated. Continue to increase the flow rate or reduce the G force until the larger monocyte cells are detected, defining the second break point.

(continued)

Image	Details
	To determine the elutriation volume required to achieve the required purity: • Load the PBMC into the bed and elutriate at the speed for lymphocyte separation. • Take samples from the sampling port every 50 mL or so to determine when the lymphocytes stop emerging.

Note: The elutriation break points can be quite a sharp step change, with subtle changes in speed having a significant impact on the purity and rate of elutriation. Determining and optimizing these break points may take several iterations.

Integrate elutriation and cell concentration functions

The Rotea™ instrument enables cells that have been separated through elutriation to then be concentrated as part of an automated process.

In some cases, the target cells are retained in the fluidized bed and can simply be recovered as concentrate.

When the target cells are being elutriated from the chamber, they can be directed to a dual port intermediate bag through line G. This leaves the concentrated non-target cells in the chamber which can be recovered to ports E, F or H.

The elutriated target cell product can now be loaded into the CFC Chamber through port D, washed if required, and delivered as concentrate to port E, F or H.

Concentrate Recovery

Overview

The primary objective of **Concentrate Recovery** is to recover all cells contained within the fluidized bed. Additional objectives might include:

- Minimize carryover of wash buffer
- Maximize cell concentration
- Achieve a target cell concentration
- Achieve a target volume
- Dilute with media in preparation for the subsequent process

Concentrate recovery is achieved by reversing the pump with the CFC Chamber still spinning.

The fluidized bed emerges from the CFC Chamber as a concentrated “slug” of cells in the media used for the prior step e.g. wash buffer, EP buffer or culture media.

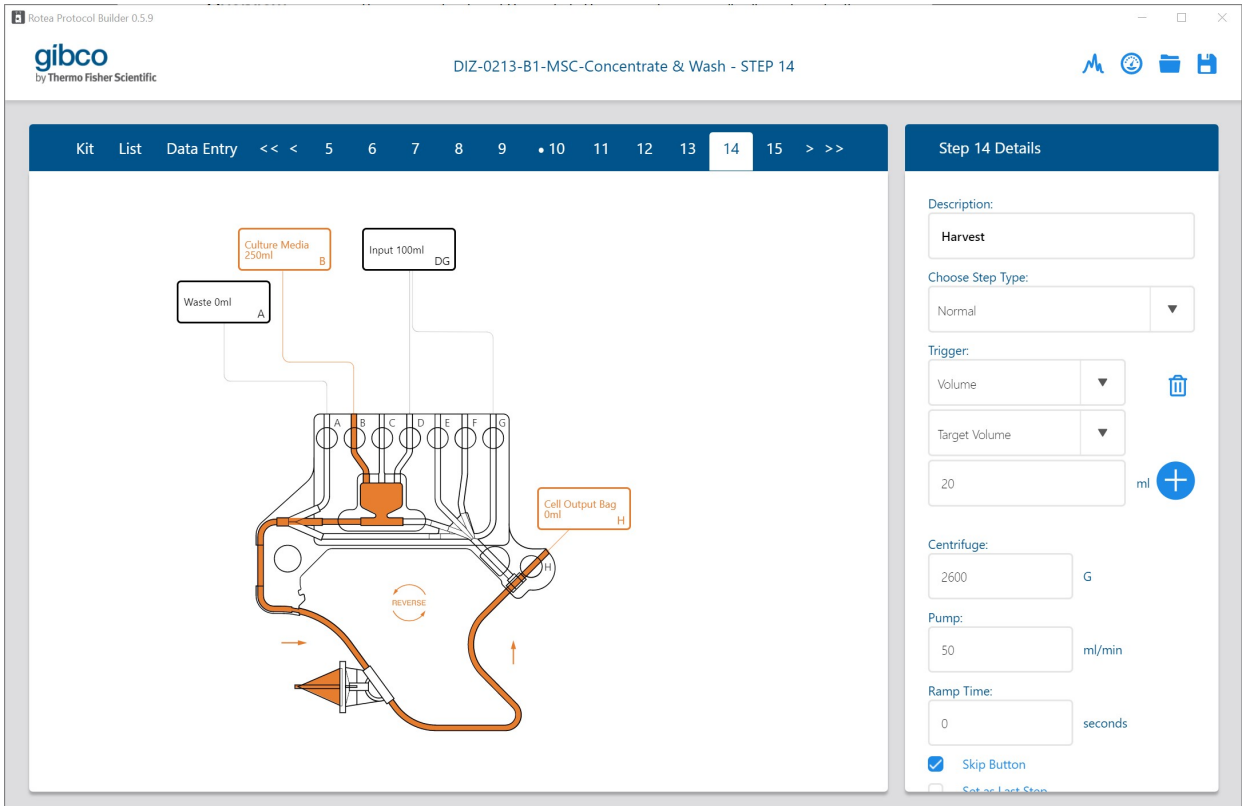
Lines E, F and H are specifically concentrate output lines since cells can be delivered with minimum dead volume or disruption of the concentrate.

Note: The Rotea™ system has been designed to optimize cell recovery with high cell density in a small controlled volume.

The following sections describe the tools available for programming and optimizing the concentrate recovery sequence.

Normal step

The simplest method for **Concentrate Recovery** is to program a **Normal** step where the pump is reversed, and set to deliver a **Target Volume** to an output bag using Wash Buffer as the source fluid.



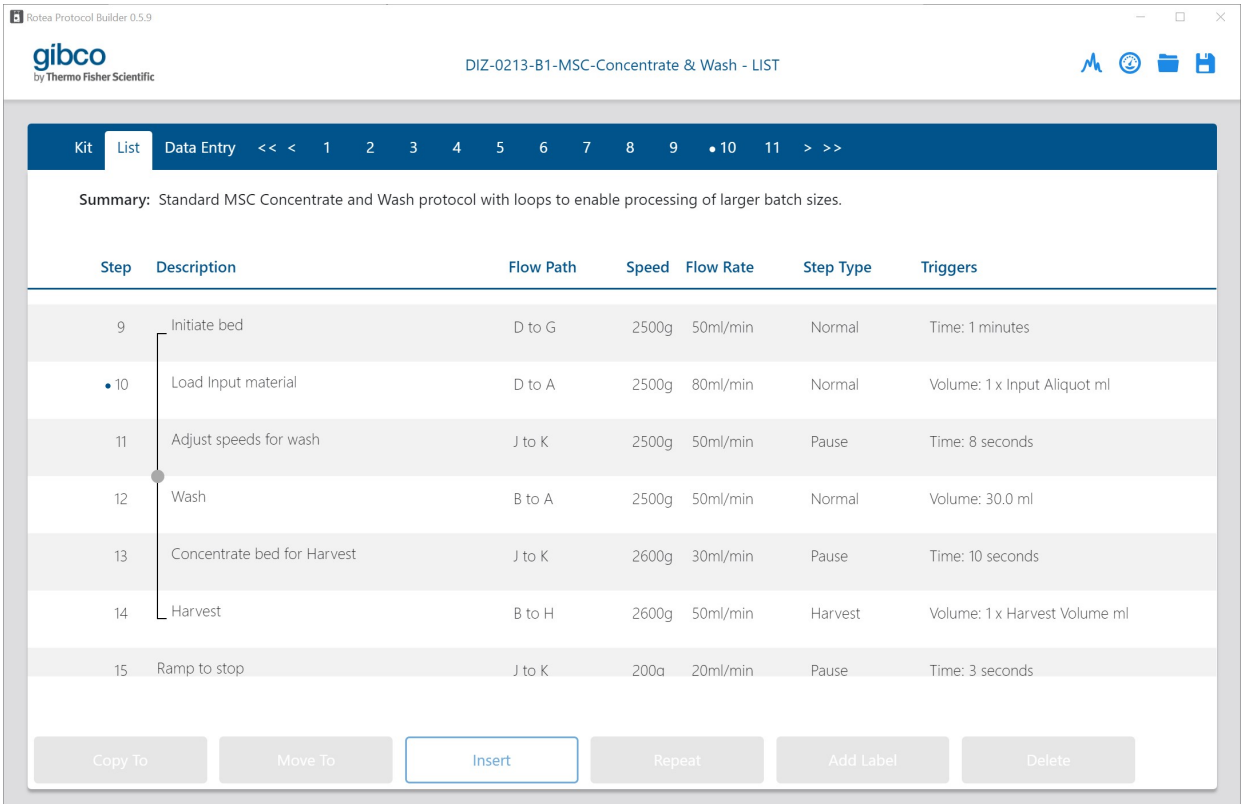
Speed settings

Different circumstances and cell types require process specific settings but a good starting point in process development is to simply reverse the pump flow with the G force constant and the flow rate equal to or slightly greater than in the prior step.

Pause step

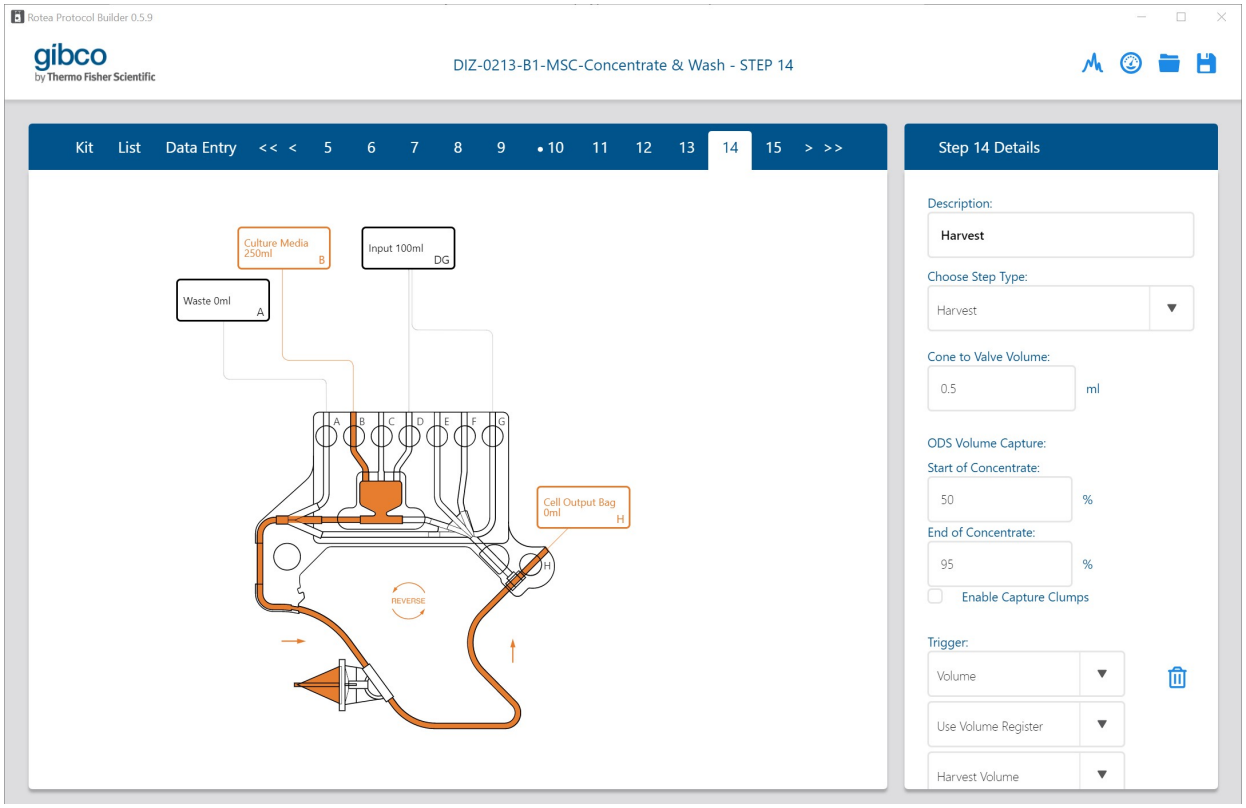
A recommended strategy is to include a brief (5 to 10 second) **Pause** step prior to **Concentrate Recovery** to adjust the speeds and hence concentration of the fluidized bed. This provides several benefits:

- The **Pause** step actuates valves that may not have been recently used but will be important during **Concentrate Recovery**
- It is a convenient step from which to restart a protocol after an interruption or stoppage
- Concentrate recovery typically happens directly after washing where it may have been necessary to concentrate the bed to minimize disturbance of the fluidized bed. It can be advantageous to slightly expand the bed and flush compacted cells from the tip of the cone prior to **Concentrate Recovery**
- Adding a **Speed Ramp** to the **Pause** step provides a smooth speed transition to expand or concentrate the bed without loss of cells.
- If you are using a **Harvest** step the **Pause** loop is the ideal destination for fluid prior to the valve changeover.



Harvest step

The **Harvest** step enables a high product concentration to be delivered by minimizing dead volume in the fluid path between the tip of the CFC Chamber and the output valve. It can only be selected as a step type if one of the output valves E, F or H is active for the step and the pump is set in the reverse flow direction, eg:



The **Harvest** step holds off the opening of the output valve until the **Cone to Valve Volume** has been pumped from the tip of the cone.

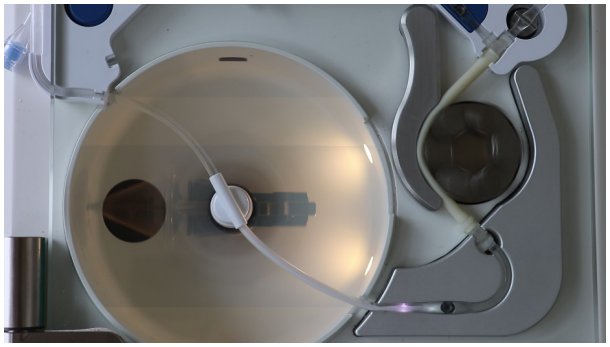
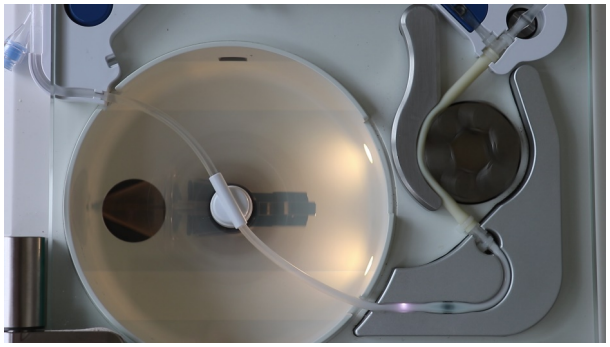
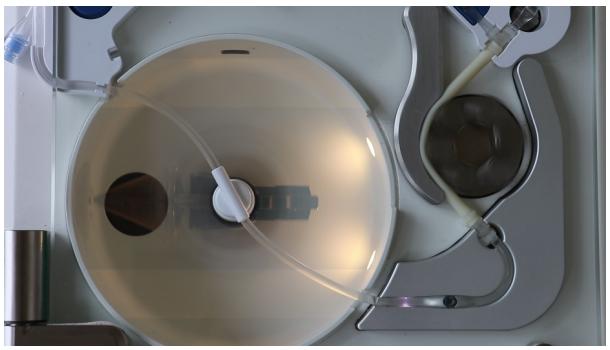
Since the **Harvest** step delays changeover of the valves, the step before **Harvest** needs to have a legitimate flow path that can be sustained during the initial harvest period, such as a **Pause** step.

The **Cone to Valve Volume** relates to the volume in the rotary coupling, pump tube and kit tubing between the tip of the cone and the output valve. The default **Cone to Valve Volume** is 0.5 mL and can be adjusted to further optimize the **Harvest** step.

Once the **Cone to Valve Volume** has been drawn out of the CFC Chamber, the programmed valve settings for the step are enforced and the triggers enabled.

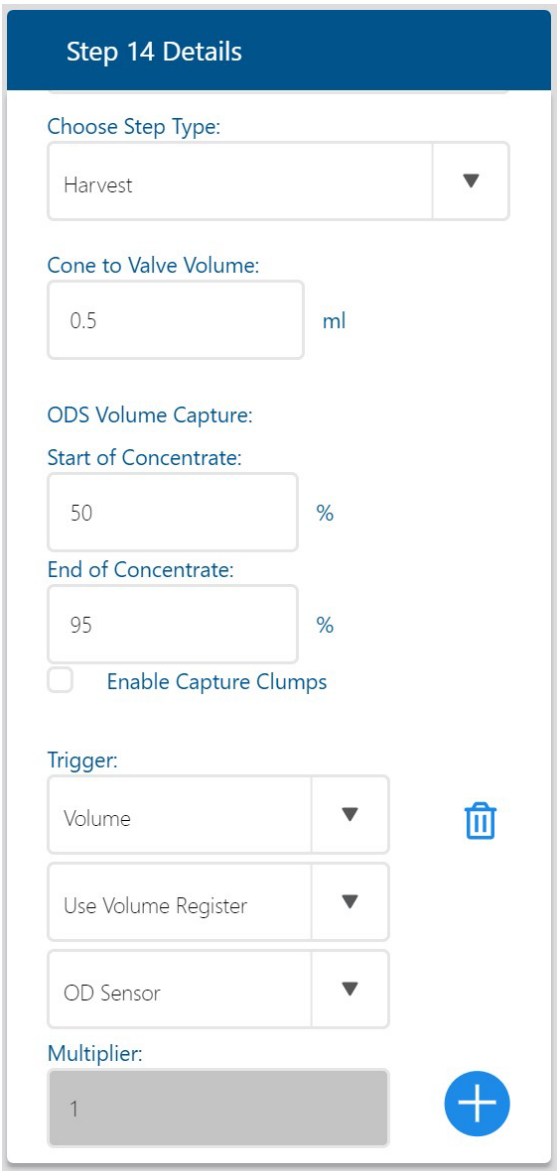
From a practical perspective:

- If you set a volume trigger of 4 mL without a **Harvest** step, a volume of 4 mL will be delivered to the output bag, but this will include the **Cone to Valve Volume** which does not have cells from the fluidized bed in it.
- If you enable the **Harvest** step and place a volume trigger of 4 mL on that step, then 4 mL of concentrate will be delivered to the output bag.

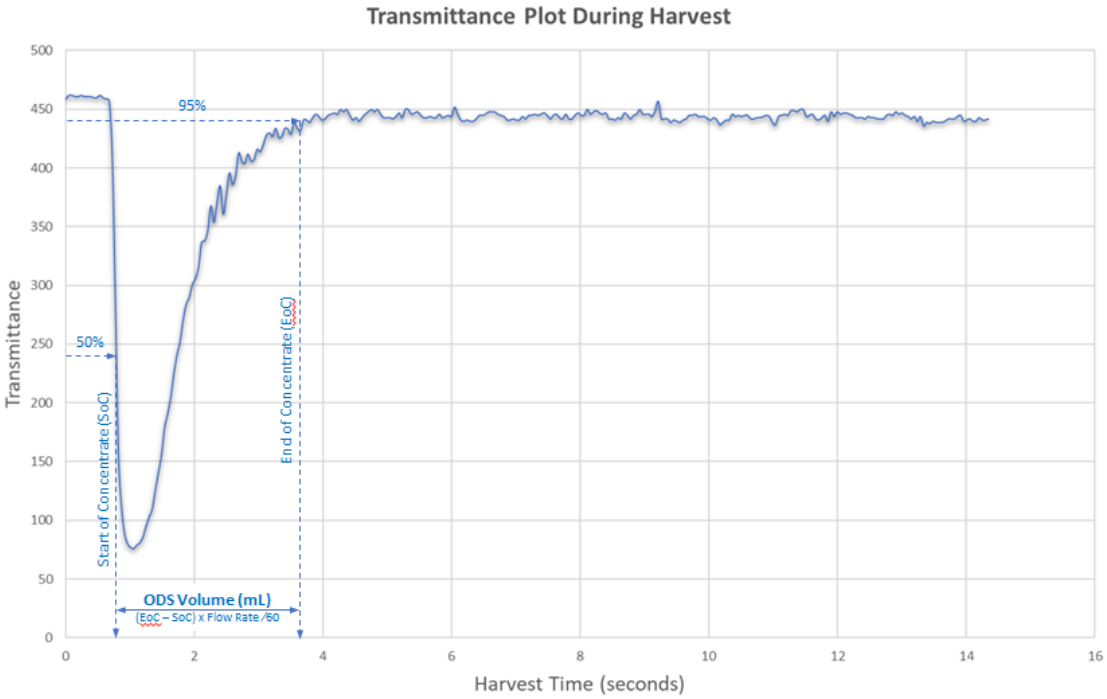
Image	Details
	The pump direction has reversed and the **Harvest** step is in progress. The concentrated cells have already reached the ODS and the **Cone to Valve Volume** is being diverted past Valve H, exiting the T-Fitting to the left.
	The pump has finished diverting the **Cone to Valve Volume** and **Valve H** has just opened. The concentrate is now being delivered into the output bag.
	
	The **Harvest** step is complete and valve H has closed.

Harvest using OD Sensor

The Rotea™ instrument comes with an Optical Density (OD) Sensor included. The OD Sensor measures the transmittance of the light from an LED through the tubing and the process fluid in order to determine when concentrate passes it.



The OD Sensor reads the transmittance of the media in the tube at the start of each step and uses this as its new baseline value (similar to zeroing on a set of scales).



When product is being recovered from the chamber, a high density “slug” of cells is drawn out by the pump. The OD Sensor detects this as a sudden drop in transmittance.

As the last of the concentrate is drawn from the chamber, the transmittance increases back to the value determined at the start of the step.

The OD Sensor is used to calculate the volume of the concentrate and stores this as a volume in the **Volume Register** for use as a **Volume Trigger**. When using it, set a percent of light transmittance for the trigger to read as the start of concentrate, and a percent of light transmittance for the end of concentrate. The step will advance when the OD Sensor detects the **End of Concentrate** % value entered.

When using the **OD Sensor Volume** within a **Harvest** step, the volume Multiplier will be set at 1.0 meaning that the **Harvest** step will deliver the calculated OD Sensor volume.

To dilute the concentrate to a target ratio, a **Normal Step** can be added immediately after the **Harvest Step** using a volume trigger and the OD Sensor volume from the **Volume Register** with a multiplier that provides the appropriate dilution.

Multi-bite processing

Overview

The CFC Chamber in the Single-Use Kit has a working volume of 10 mL which is often sufficient to collect and concentrate all of the cells in a batch.

The size of the CFC Chamber enables Rotea™ instrument to:

- Operate at high G's (this results in a relatively high throughput rate and the ability to concentrate small cells)
- Process as few as 50 × 10^6 cells which is ideal for autologous samples and process development

Where the number of cells in a batch exceeds the capacity of the CFC Chamber, the Rotea™ instrument can be programmed to automatically load and empty the CFC Chamber multiple times ("bites") using Loops.

Determine the number of cells per bite

Cell loading

While 5 billion T-cells have been successfully loaded and recovered in a single CFC Chamber load, this can only be achieved by loading very slowly and at high G which isn't always practical. Using more "typical" loading conditions for 10 to 20 µm cells, the CFC Chamber can hold 2 to 3 billion cells before overwhelming the chamber.

This is a good starting point for process development but ultimately it is dependent on what you are processing and the settings that you choose to run at.

Note: All cells being loaded into the CFC Chamber need to be included in the cell number estimate, for example, if you are processing PBMC or leukopak, then you need to include the number of RBC's. In the case of buffy coat, the number of RBC's typically far exceeds the number of PBMC's making processing of buffy coat difficult.

Elutriation

Elutriating cells from the fluidized bed requires relatively high pump speeds and low G force that expand the bed. If there are too many cells in the bed prior to the elutriation step, the bed can expand beyond the 10 mL working volume and cells you want to retain will be washed out. Process development runs are recommended to find the right balance between elutriation speed and the size of bed that can be sustained at that speed.

Note: One common strategy is to slowly increase the elutriation flow rate as the volume of the fluidized bed reduces through elutriation of cells.

The other side to elutriation is when there are too few cells in the bed for the elutriation settings. Elutriation settings are bed size dependent. If, when using a multi-bite process, the final bite is a small number of cells, then the subsequent elutriation step may not perform as expected.

Best practice for multi-bite processing is to set a bite volume so the last bite before the bubble sensor trigger will be 70% to 90% of the normal cells per bite. This can be manipulated by adjusting the volume per bite or the volume of a dilution step.

Maximize throughput

Some processes eg post expansion, require a significant volume of input material to be processed within a limited time frame. In counter flow centrifugation, processing at high G force also means running at a higher flow rate to sustain the fluidized bed. The Rotea™ instrument also has a high flow Single-Use Kit option for cell and media combinations that allow it.

The optimum flow rate for a given process will be a balance of throughput and yield determined from process development runs.

Having determined the optimum process flow rate, the number of cells that can be retained within the fluidized bed can be estimated through experimentation and from this, the volume per bite that can be loaded into the CFC Chamber.

With smaller cells e.g. 6 to 8 µm T-cells, a significant time per bite can be consumed in establishing the bed. One approach is to not recover the full cell population after processing each bite. Establishment of the subsequent bed is then commenced using the residual cells from the prior bite to accelerate bed formation.

- The loading speed for a stable fluidized bed is affected by the size of the bed, with higher flow rates possible as the bed increases in size. Slowly increasing the flow rate as the fluidized bed builds can reduce the total processing time.
- The **Concentrate Recovery** step is very fast compared to loading. It may be better to do more bites at higher flow rates with less cells per bite than to load at a very low flow rate and create a highly concentrated bed of cells.

Process integrity

Overview

The CTS™ Rotea™ Counterflow Centrifugation System includes several features that minimize the risk of process errors and provide a means of recovery should something go wrong. Following are just some of the process integrity features and strategies that users can incorporate into protocols.

CTS™ Rotea™ Single-Use Kit

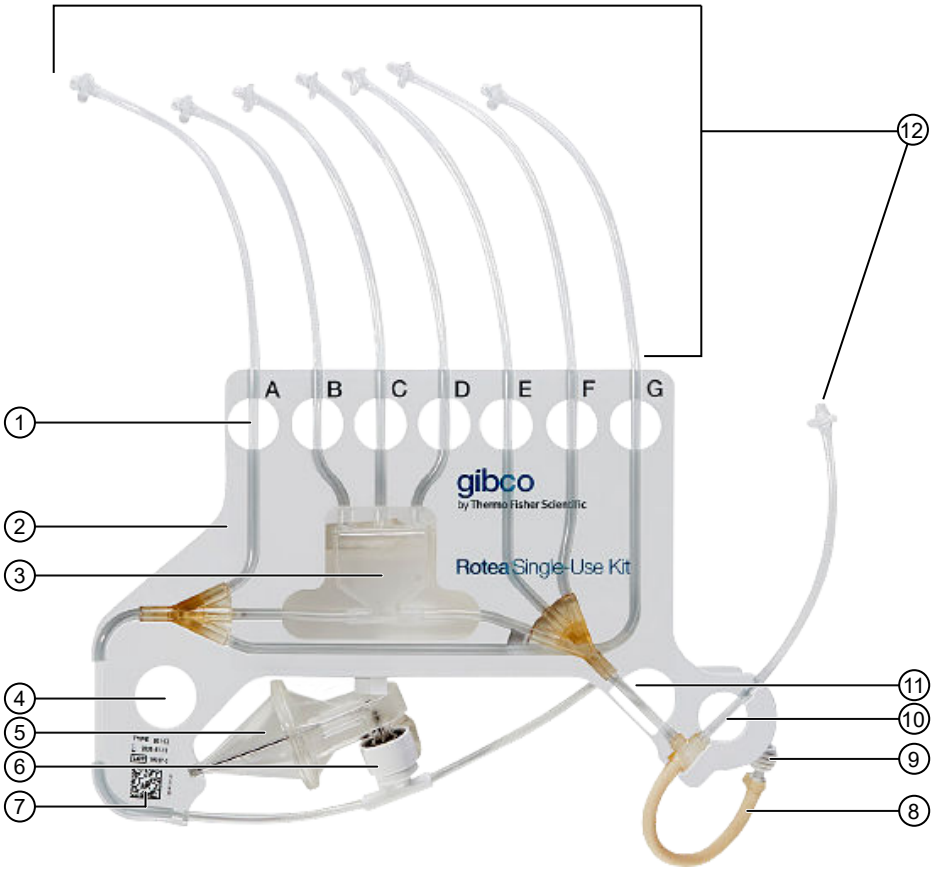


Figure 3 CTS™ Rotea™ Single-Use Kit

1. Valve Hole & Tube ID
2. Carrier Frame
3. Bubble Trap
4. Location Hole
5. CFC Chamber
6. Rotary Coupling
7. 2D Barcode
8. Pump Tubing
9. Tube Retainer
10. Valve H Hole
11. Location Hole
12. Kit Tubing (Input and Output)

Kit definition

The Single-Use Kit label includes the Valve ID for lines A to G (Note: Line H is not labelled). It is recommended that reagent packs and other materials that will be attached to a Single-Use Kit, include matching identification labels. Adding Bag volumes and descriptions when defining the Single-Use Kit can also aid in kit preparation.

2D Barcode

The 2D Barcode defines the Single-Use Kit type. The 2D Barcode will be automatically checked upon loading of the Single-Use Kit to the instrument to confirm that it matches the kit defined in the protocol.

Check manual clamps

A **Pause** step with the **Skip** button selected as the only Triggercan be added at the beginning of every protocol to remind the operator to confirm that the manual clamps are open.

Verify reagent connected and not clamped

Create a pre-priming step with a Bubble Sensor Trigger set **Dry to Wet** and **Skip** button not selected. Set centrifuge = 0 speed and pump at 50 mL/minute. The system will draw down on the reagent bag until the Bubble Sensor indicates fluid is available to proceed.

Check for closed clamps

The Pressure Trigger provides a means to check each line before the protocol commits to processing any product.

Add a Pressure Trigger - Pressure Rising to all priming steps as follows:

- **Pressure 2** when priming lines B,C,D, E,F or H from bag A or G
- **Pressure 1** when priming lines A or G from lines B, C, D, E, F or H
- Set the pressure value at 40 kPa.

Include a **Pause on Trigger** for the step with **Skip** button not selected. If the Pressure Trigger goes off before the volume is delivered, the instrument will go into pause and cannot proceed until the clamp is released and the target volume has been delivered.

5 Rotea™ Process Model

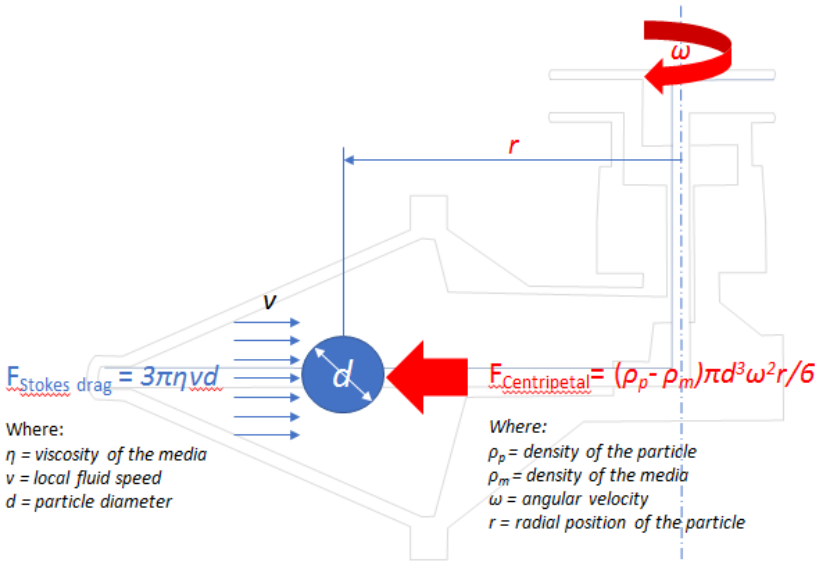
Counter flow centrifugation forces

Counter flow centrifugation is based on the interaction of two opposing forces on a particle of a specific diameter (d) and distance from the axis of rotation (r):

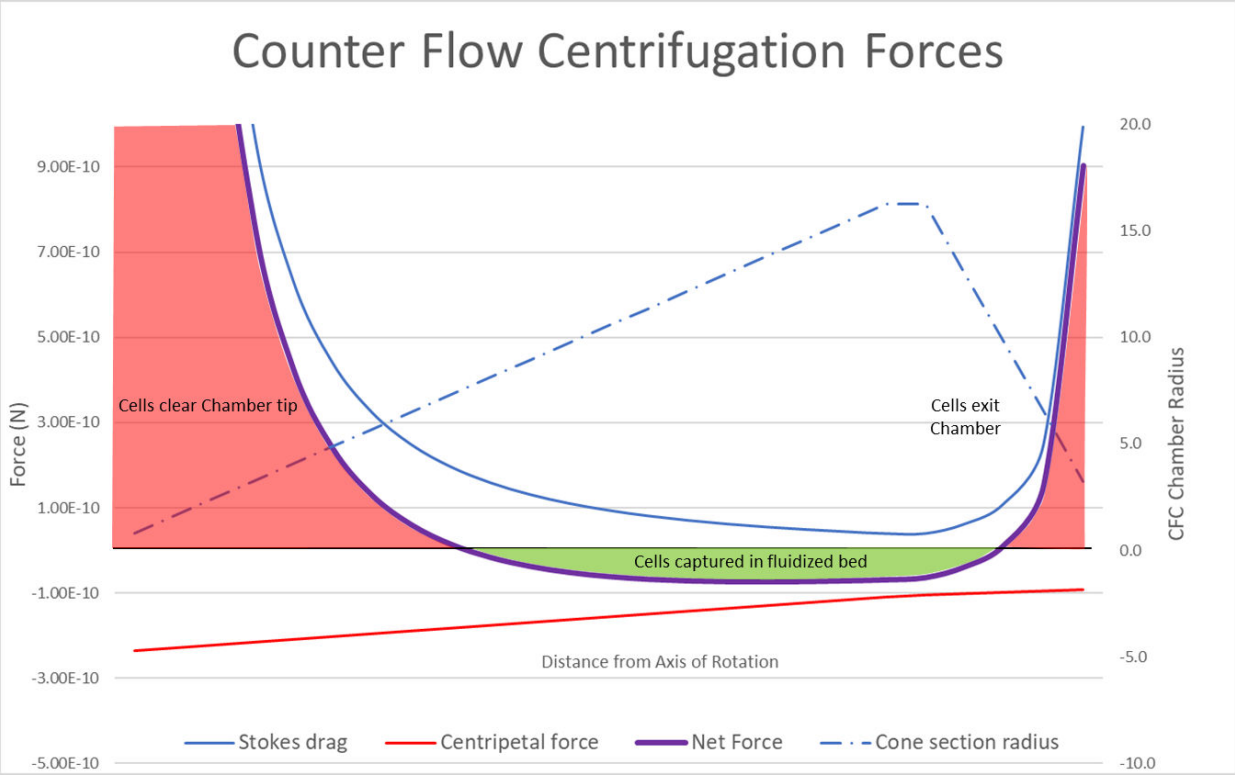
- $F_{Centripetal}$ – Acceleration force due to rotation of the CFC Chamber
- $F_{Stokes\ drag}$ – Stokes drag force due to fluid flow, applicable when small particles and low fluid velocity are involved

The Rotea™ Process Model calculates the Net Force (F_{Net})**net Force (F_{Net})** on the particle in the CFC Chamber due to these two opposing forces, as illustrated:

Stokes Law
When small particle size and low velocity are involved, the Reynolds number is small and the flow is laminar. Under this condition, inertial forces are negligible compared to viscous forces, Newton's Law is no longer valid and ***Stokes Law*** *shall be used.*



Note: The conical shape of the CFC Chamber provides an approximate match of the local fluid speed to the local acceleration with changing radius from the axis of rotation.



The graph shows the forces acting on a particle at different radial positions.

Note: The axis of rotation is to the right of the graph and the tip of the cone is at a radius of 67 mm.

The primary vertical axis is the estimated force acting on the particle in Newton. The secondary vertical axis is the radius of the CFC Chamber.

The Centripetal force is the mass x acceleration force acting on the buoyancy mass of the particle in the fluid. If the fluid is denser than the particle, the particle will float. Cells are normally denser than the media, so they tend to settle.

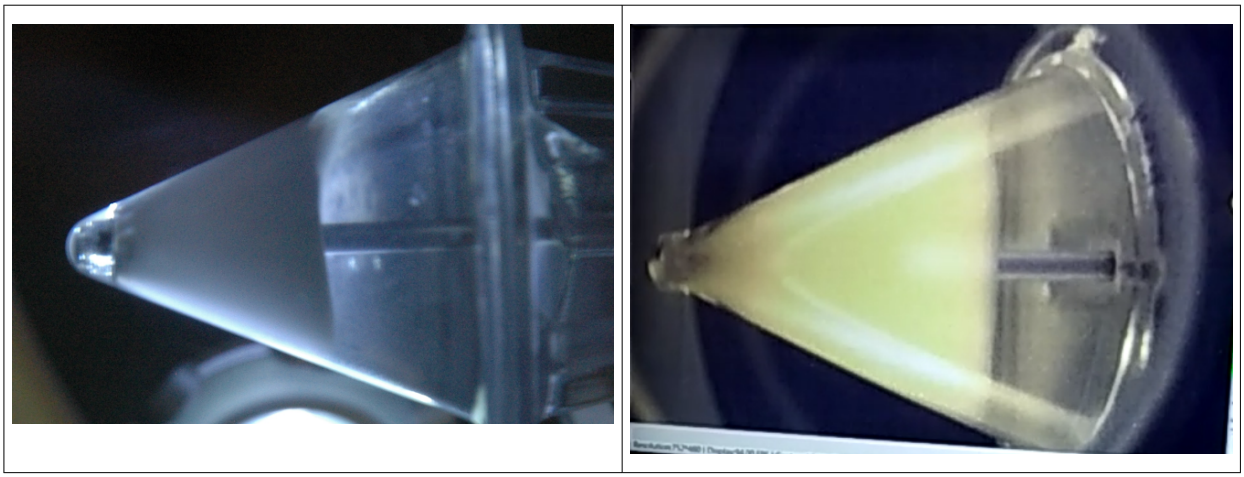
How quickly the cells settle is influenced by the interaction with the fluid e.g. a high viscosity fluid slows settling. The cells will settle at a speed where the settling velocity creates a drag force that matches the acceleration force. The drag force arises from fluid pressing against the particle. This is the Stokes drag force.

The shape of the particle affects this interaction, for simplicity the Rotea™ Process Model assumes the cells are spherical. The blue Stokes drag line shows how the force on the particle changes with the position in the chamber. The fluid flow starts at the tip of the chamber on the left and moves towards the axis of rotation to the right.

The tip of the chamber has a small cross-sectional area so the local fluid velocity and Stokes drag is high.

The cross-sectional area of the CFC Chamber increases as the fluid moves back towards the axis of rotation, reducing the local fluid velocity. The back section of the CFC Chamber beyond the fluidized bed zone, reduces in diameter as it approaches the outlet port, increasing the fluid velocity.

The net force on the particle is plotted in purple. Where the net force is >0, the particles are driven inwards towards the axis of rotation by the Stokes drag rather than settling under centrifugal acceleration. Where the net force is below the line, the particles will settle towards the tip of the chamber.



Examples of fluidized beds highlight predicted behavior. The left image is of beads. The very clear zone in the tip clearly reflects the expected clear zone highlighted by the red zone in the chart. The image on the right is CHO cells with a well-formed bed.

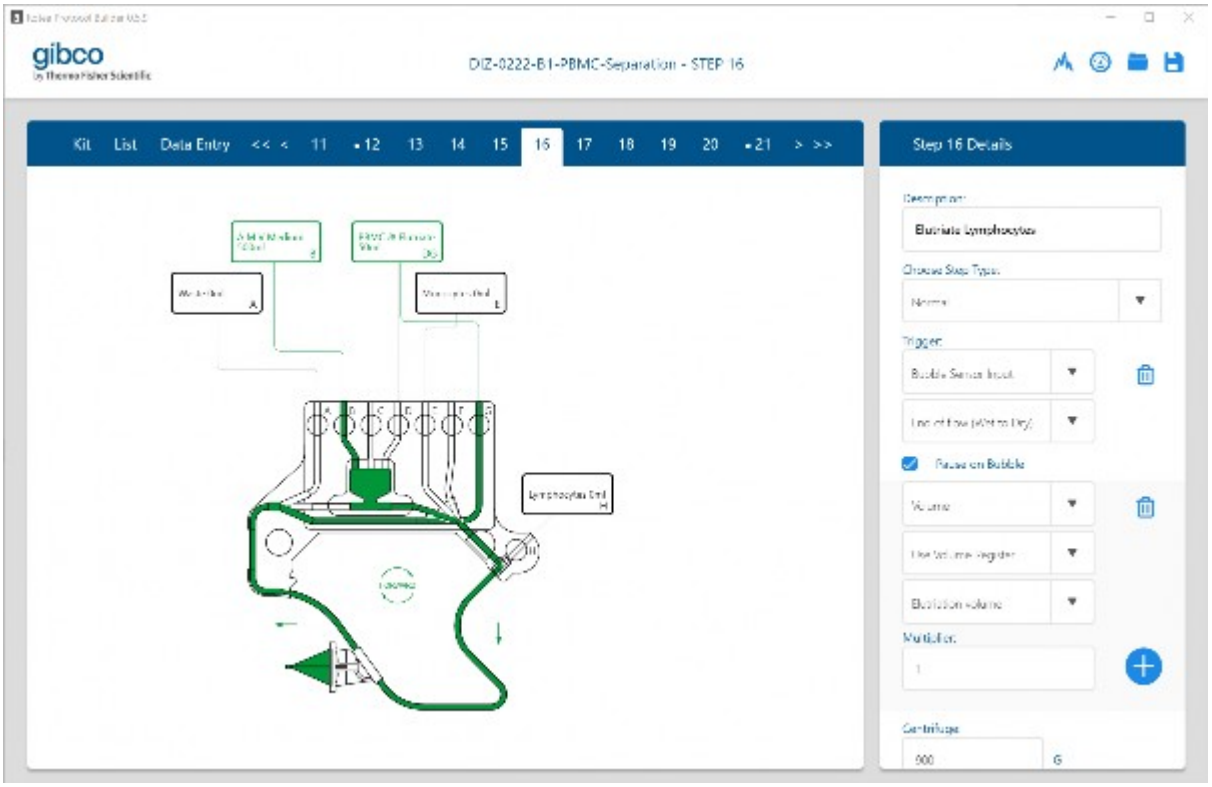
Note: The tip of the cone is not clear in this case suggesting the flow rate could be higher. Alternatively, there may be larger cells in the population that are settling at the tip while the smaller cells are in the fluidized bed.

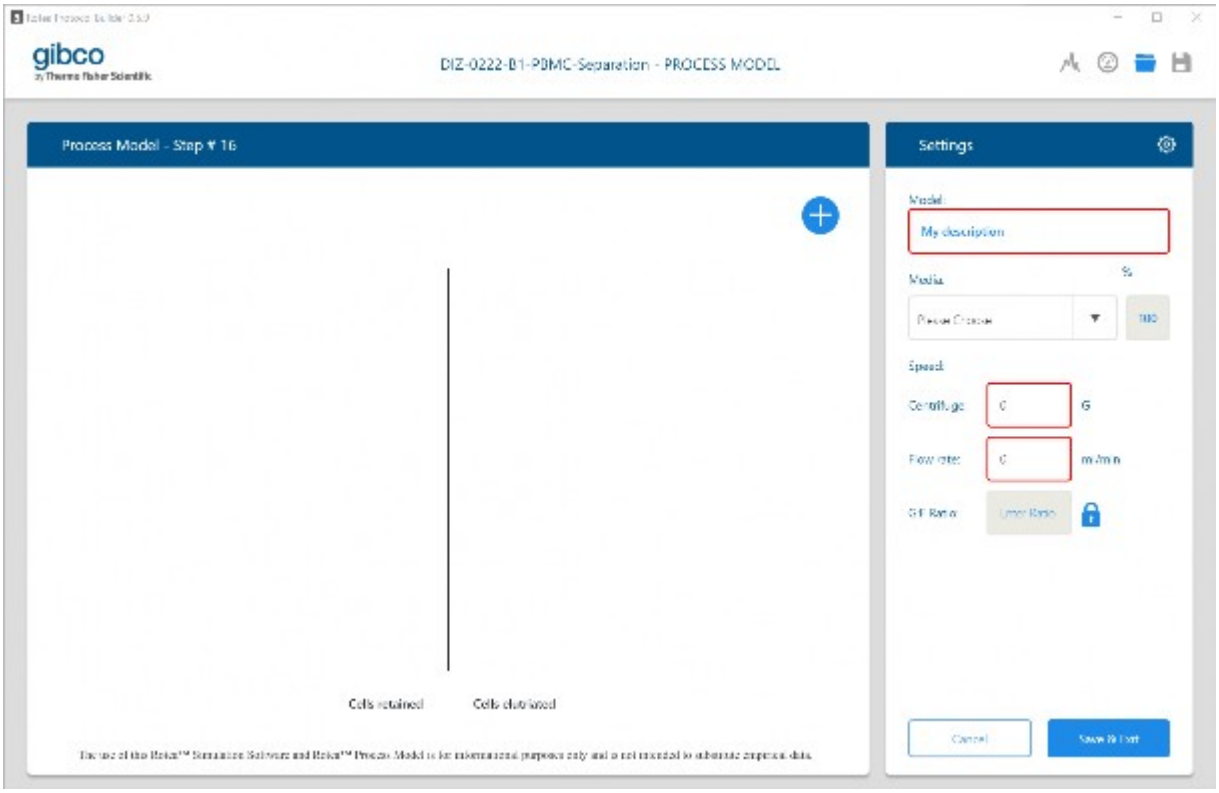
Predict process settings

The Rotea™ Process Model is based on the theoretical model, see “Counter flow centrifugation forces” on page 96, including several assumptions.

In the absence of proven settings from a prior protocol, the Rotea™ Process Model allows the user to estimate the flow rate and g force required to either concentrate or elutriate different cell types in various media.

To open the Rotea™ Process Model, select the protocol step number that you wish to model e.g. **Step 16** and click on in the top right corner of the **Process Model** home screen.





The step number for the process model will be displayed in the top left corner of the **Process Model** screen.

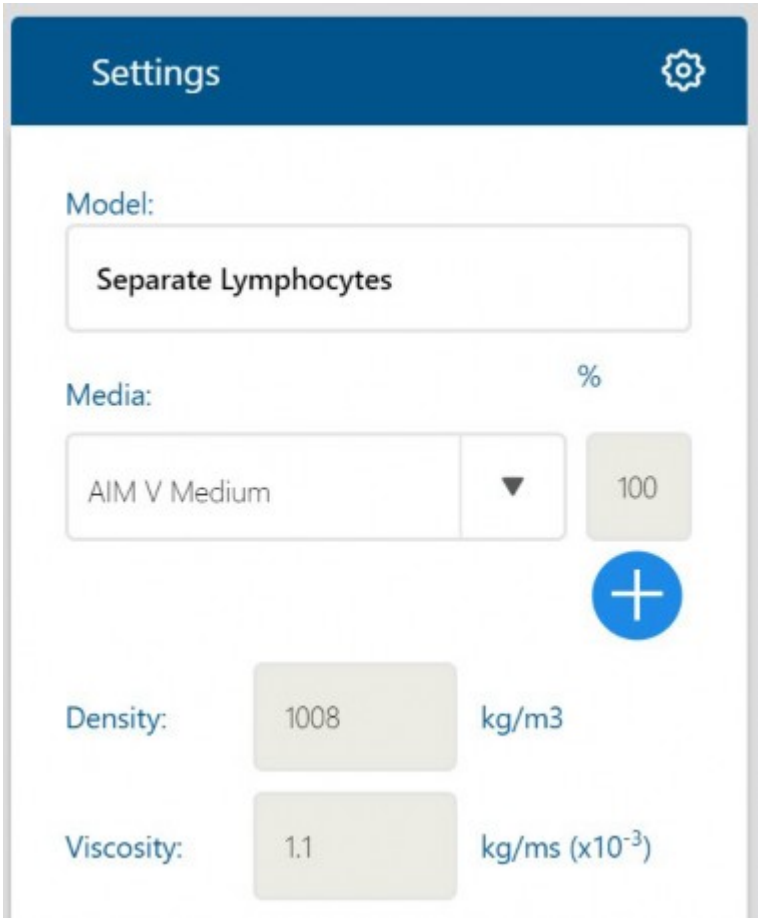
The process settings for the **Step** are displayed in the **Settings** panel on the right of the screen.

Insert a brief description for the **Model** in the **My description** field.

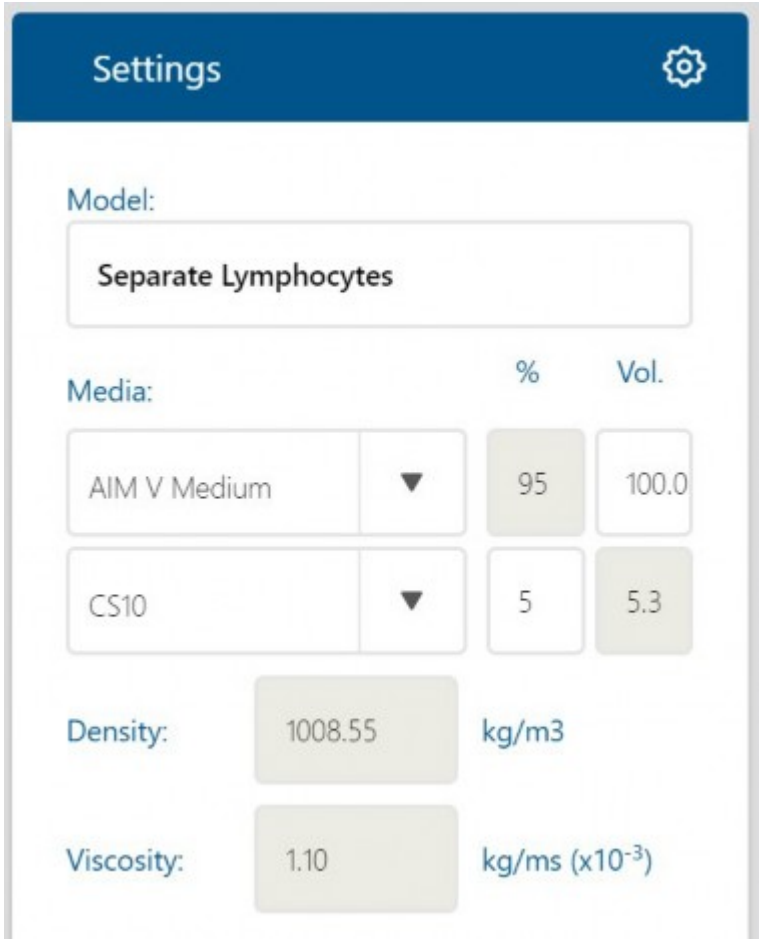
The Rotea™ Process Model includes several standard media and cell types with pre-defined density, viscosity and diameter ranges that can be used.

Select and format media

1. Click ▼ in the **Media** box in the **Settings** panel and select the preferred meda from the dropdown list. This will display the density (kg/m^3) and viscosity (kg/ ms×10^{-3}) of the selected media.



If processing a blended media, click on ⊕ to add a second media.



2. Insert the volume (mL) of the first media and the % V/V for the second media.

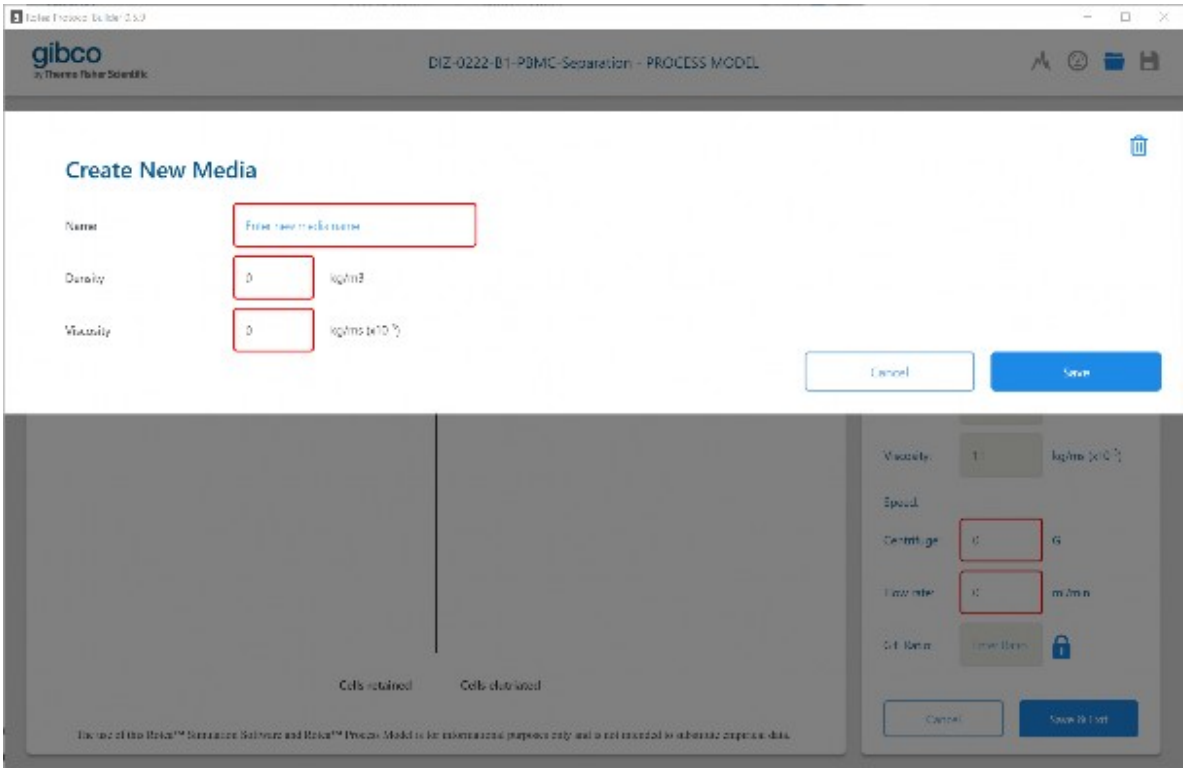
   The density and viscosity of the resultant media is automatically re-calculated based on the %V/V. The volume of the second media is automatically calculated and displayed in the **Vol** field for the second media.

Note: To delete the second media, click on the ▼ of the second media and select **Please Choose** from the dropdown list.

Create custom media

Within the **Media** drop-down list is the option to select from custom media previously created or create a new custom media.

1. Click on **Create new** in the drop-down list to bring up the **Create New Media** screen.

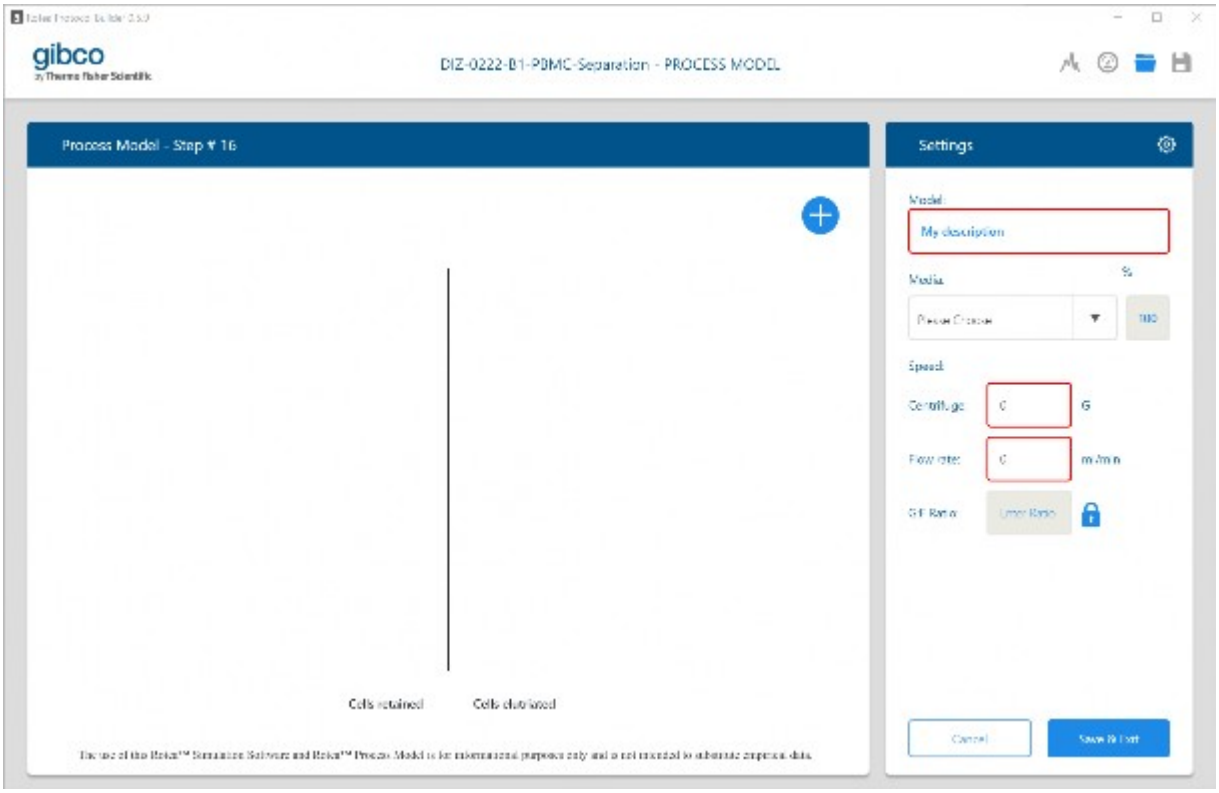


2. Input the following parameters:
   - New media name
   - Density (kg/m^3)
   - Viscosity (kg/ms ×10^{-3})

3. Click **Save** to add the new custom media to the drop-down list or **Cancel** to exit.

Model particle behavior

The panel on the left of the **Process Model** will display the outputs of the process model.

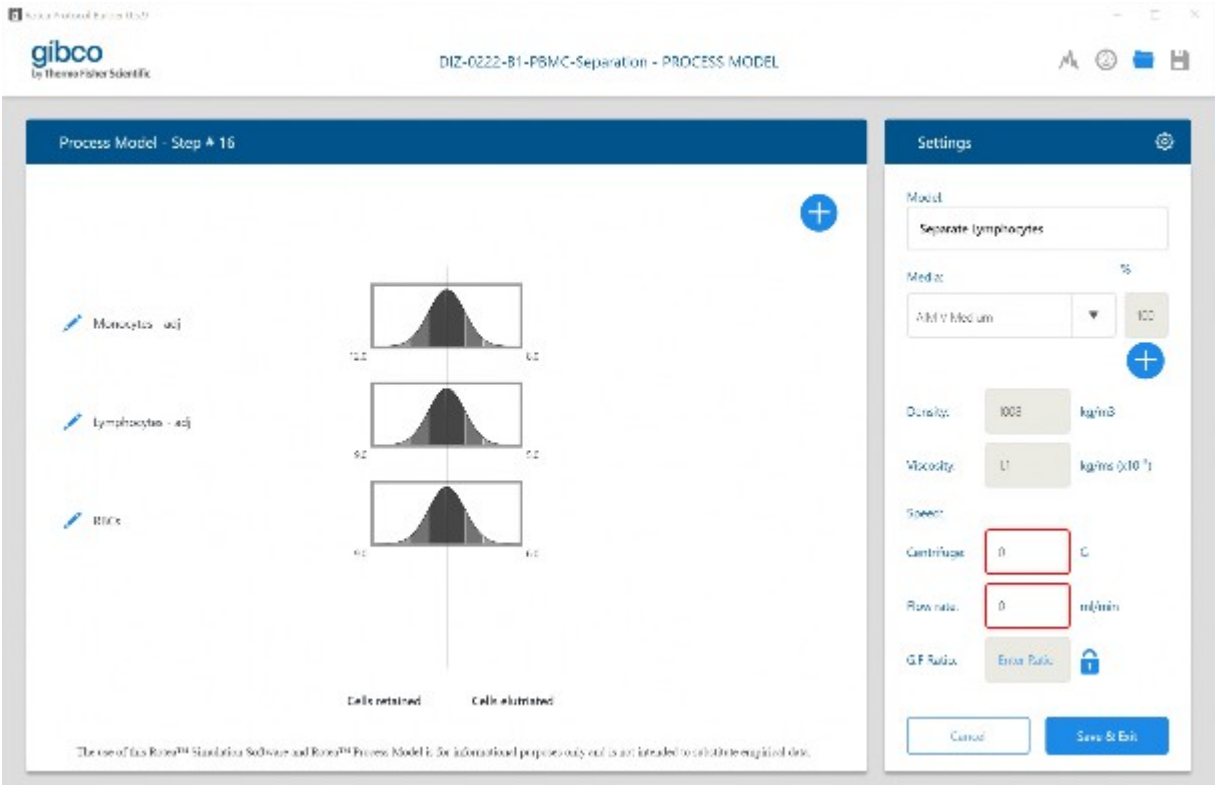


The vertical line in the middle of the panel represents the zero-force line i.e. where the centripetal and Stokes drag force on the particle are in equilibrium. This means that:

- Particles to the left of the zero line will be retained in the CFC Chamber – **Cells retained**.
- Particles to the right of the zero line will be elutriated – **Cells elutriated**.

Click on ⊕ to add a cell/particle type from the drop-down list.

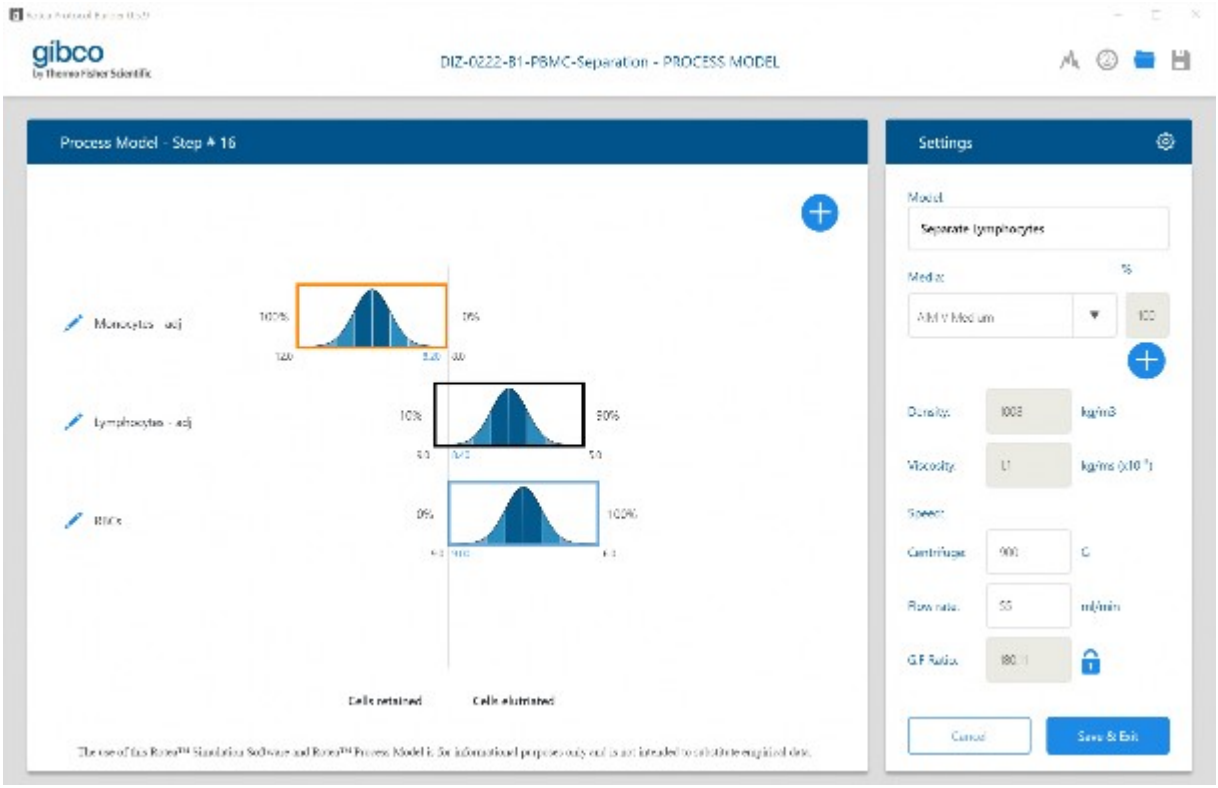
The behaviour of up to 4 cells/particle types can be modelled for any given step. In the example below we have selected two custom cell types (lymphocytes- adj, monocytes-adj) and RBC's from the **Standard Particles** dropdown list.



The Rotea™ Process Model assumes that the cell diameter is a normal distribution between the maximum and minimum cell diameter defined for each cell type.

Prior to inputting values for the centrifuge speed and flow rate, the normal distribution curve for the particles will be shaded grey and positioned centrally about the zero line.

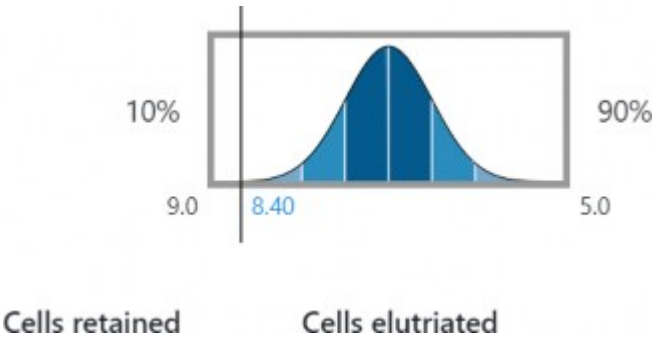
Adjust the centrifuge speed (G) and flow rate (mL/min) to model the behaviour of the cells within the CFC Chamber under the different processing conditions until you achieve the desired outcome:



The position of the normal distribution curves shows the population of each cell type relative to the zero line.

In the above example, the monocytes will be retained in the CFC Chamber and most of the lymphocytes will be elutriated along with the RBC's.

The % at each end of the normal distribution curves indicates the predicted percentage of cells that will be retained (e.g. 10%) and elutriated (e.g. 90%) at the current settings.



The figures at the bottom of each graph reading from left to right are:

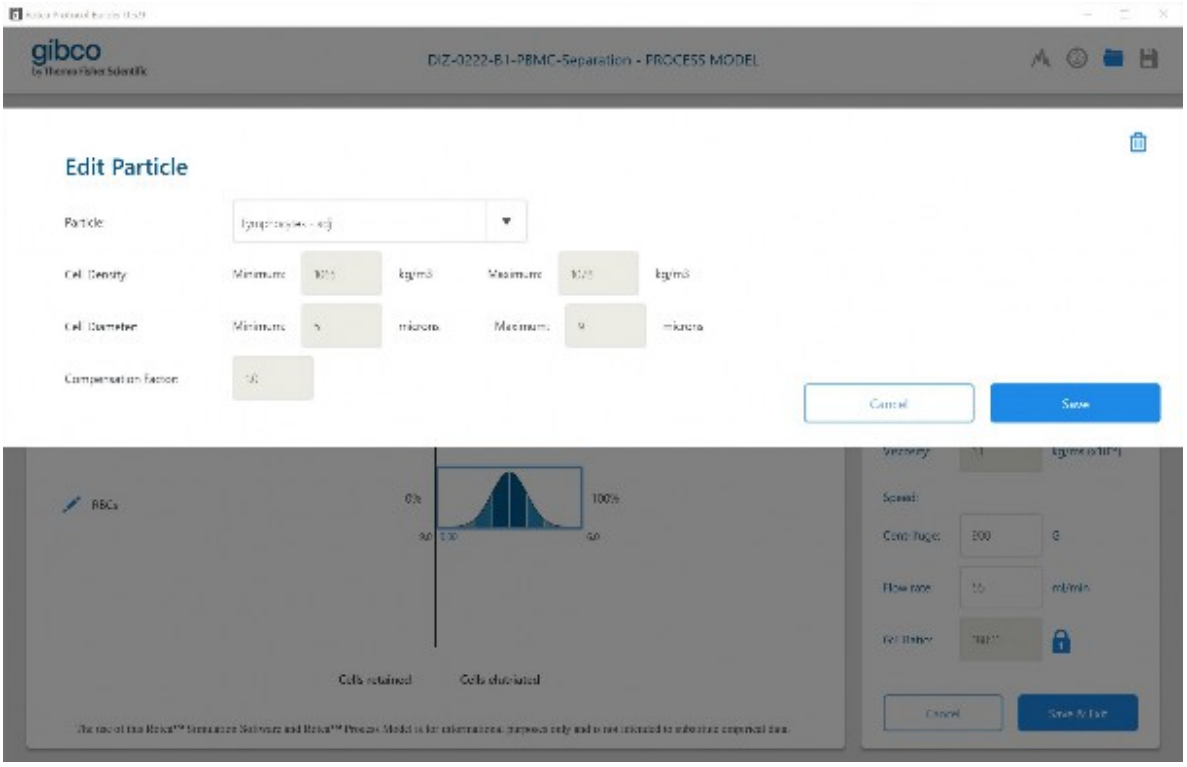
- maximum cell diameter (e.g. 9.0 micron)
- theoretical cell diameter at the zero line (e.g. 8.4 micron)
- minimum cell diameter (e.g. 5.0 micron)

The formula used in the model predicts that the Flow rate to G Force ratio (G:F) will be a constant for a specific particle and media combination e.g. 1000 × G at 50 mL/min will produce the same result as 2000 × G at 100 mL/min.

Once the appropriate G:F ratio has been defined, it can be locked by clicking on the . Adjusting either the flow rate or the G force will now automatically adjust the other parameter to retain the G:F ratio.

Change and delete particles from a model

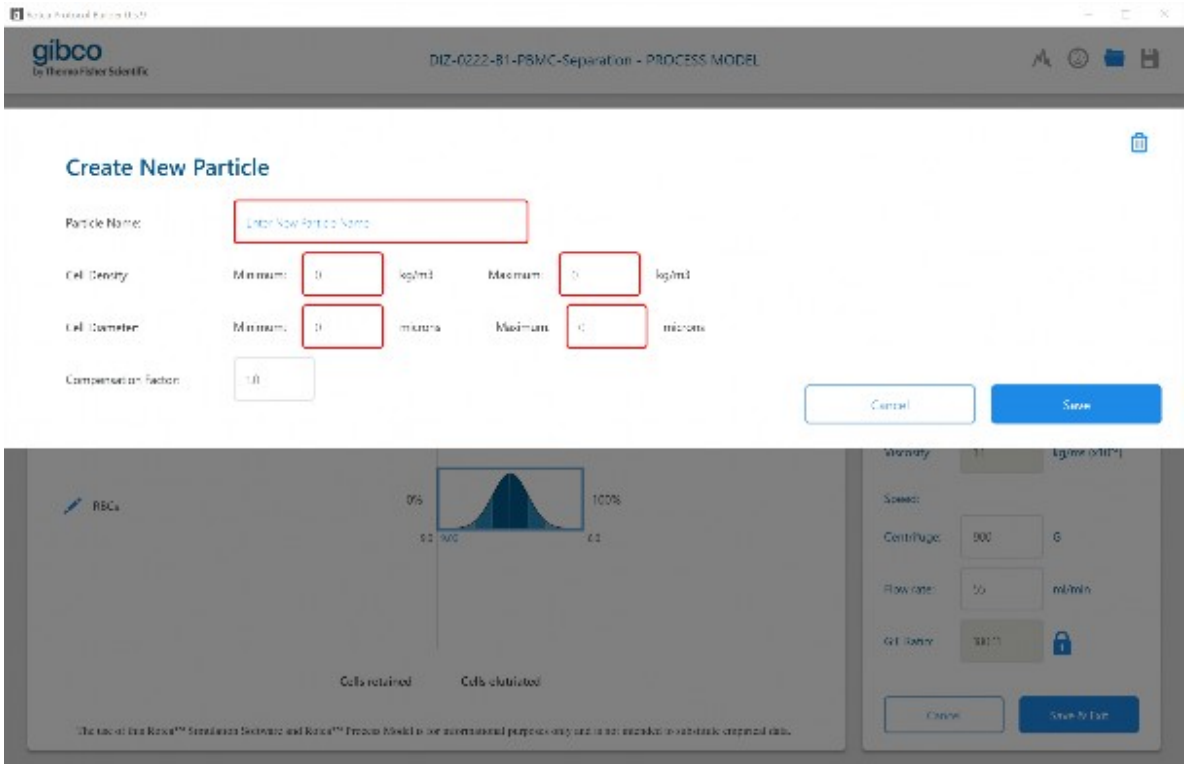
1. Click on the to show the parameters for the particle.
2. Click on the to select a different particle or the to delete the particle from the model.



Create custom particles

Within the **Particle** drop-down list is the option to select from **Custom Particles** previously created or create a new particle.

1. Click on the **Create new** text in the drop-down list to bring up the **Create New Particle** screen.



2. Input the following parameters:
   - **New Particle Name**
   - **Cell Density** - minimum and maximum (kg/m^3)
   - **Cell Diameter** - minimum and maximum (microns)
   - **Compensation Factor**

   Note: The Compensation Factor will be applied to the cell diameter in the model and has a default value 1.0 with a range between 0.1 and 10.

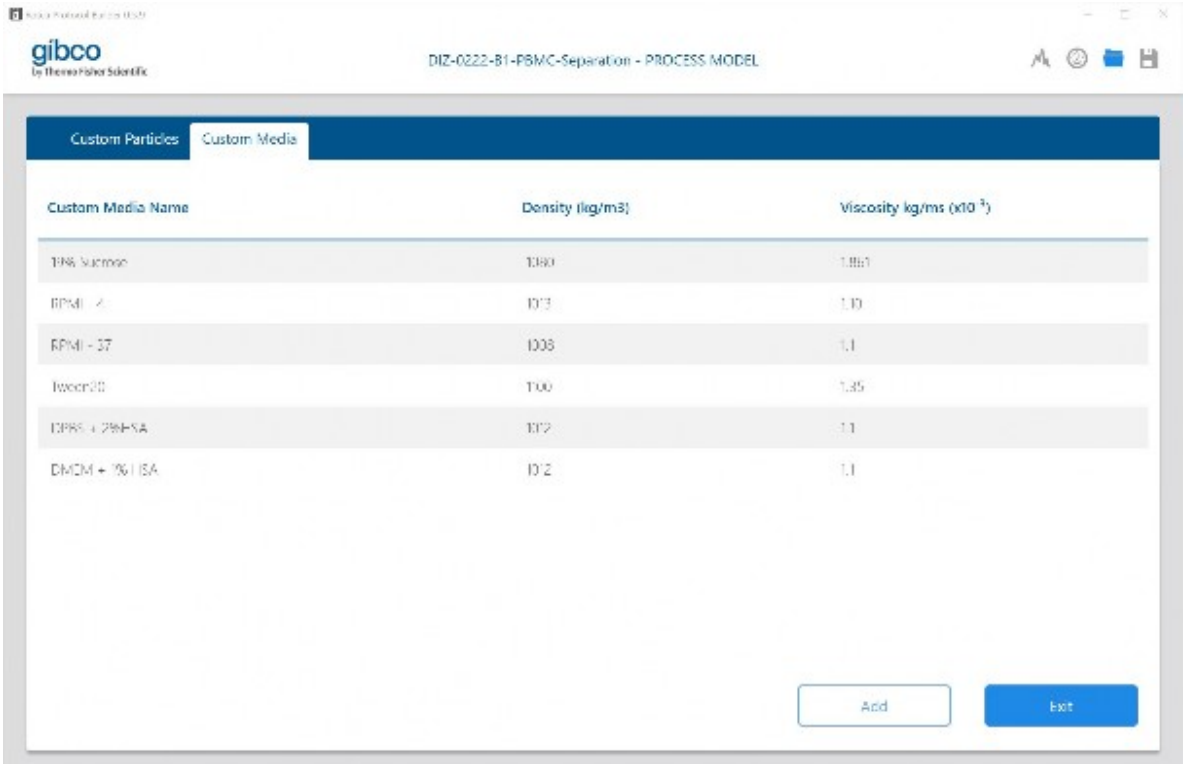
3. Click on **Save** to add the new custom particle to the dropdown list or **Cancel** to exit.

Edit custom particles and custom media

When custom particles and media are created, they are stored locally on the specific computer used to create them. When a protocol is saved, any custom particles or media used in a process model will be

saved with the protocol and visible when opened using any computer. However, these custom particles and media will not be automatically added to the dropdown list on the new host computer.

1. To edit custom particles and media, click on the settings icon in the top right corner of the **Process Model** to display the table of **Custom Particles** and **Custom Media**.



2. Select the item you wish to edit, update the parameters and click **Save**.

   You can also add **Custom Particles** and **Custom Media** from this screen by clicking on the **Add** button.

6 Rotea™ Simulation software

The use of the Rotea™ Simulation software is for informational purposes only and is not intended to subsitute empirical data.

Rotea™ Simulation software

The purpose of the Rotea™ Simulation software is to enable the user to:

1. Simulate and visualize the total protocol including data entry variables to check that it functions as intended.
2. Check that all fluid lines being used have been primed.
3. Determine the starting volume required in each bag to complete the protocol.
4. Check that each bag has sufficient capacity to hold the maximum volume that it will need to hold at any point during the run.
5. Estimate the run time for each step and the overall protocol.
6. Check which triggers will be activated when the protocol runs.

The Rotea™ Simulation software is access by clicking on the icon in the top right corner of the **Protocol Builder** home screen.

The Rotea™ Simulation software runs the protocol using all input variables and step settings as defined within the protocol including Bag Volume, Bag Capacity, Flow Rate, G force and Triggers.

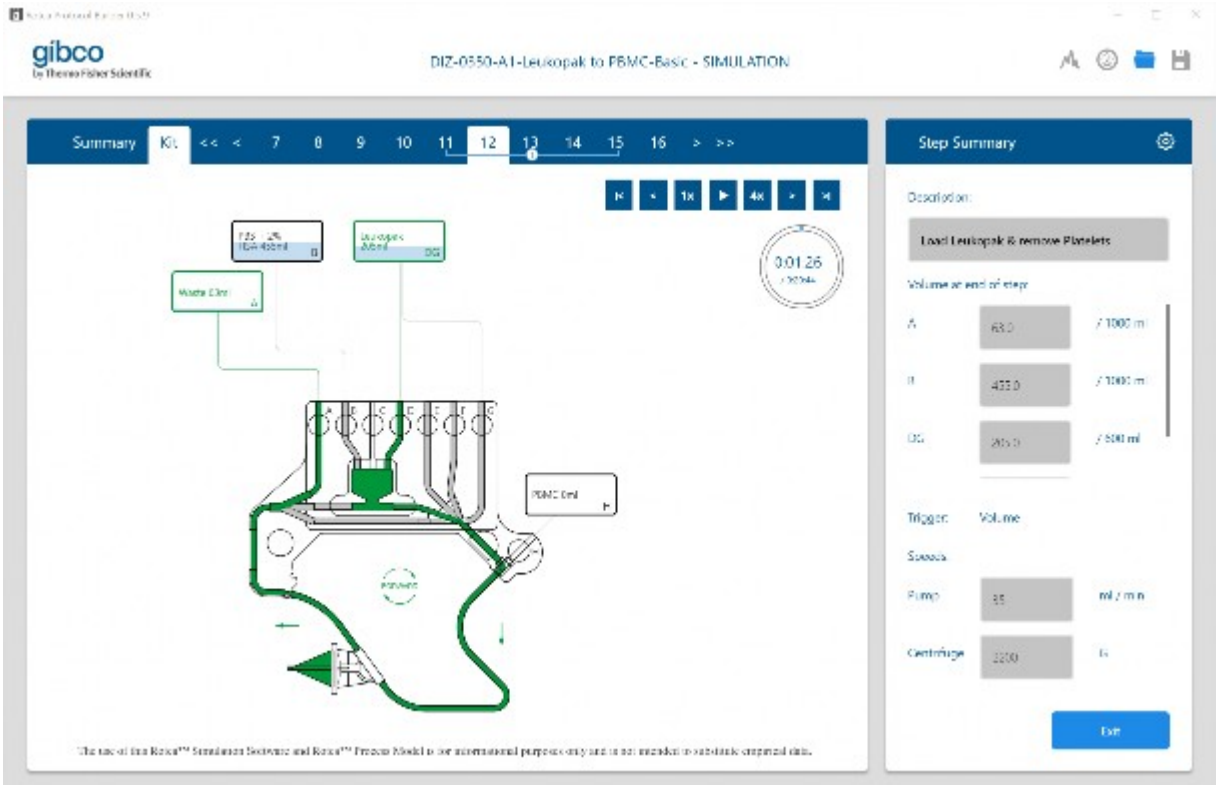
After opening the Rotea™ Simulation software, the user will be prompted for any data entry values defined in the protocol.

Input the data and click **Confirm**.

Note:

- Only valid data entry values can be input.
- Values that are input after opening the Rotea™ Simulation software are not updated in the protocol.

The **Kit View** tab for the Rotea™ Simulation software:



The protocol steps are listed across the top ribbon as per the **Protocol Builder**. Click on one of the step numbers to take you to the step selected and show you the information specific to that step.

The example shows the details for step 12, **Load Leukopak & remove Platelets**. The volumes displayed in the volume boxes in the **Step Summary** tab, are the volumes in each bag at the completion of the step. The maximum capacity for each bag is shown to the right of the box. If the volume in the bag at the end of the step exceeds the capacity, it will be highlighted in red.

Note: A scroll bar is provided at the right of the bags to display additional bags in use.

Step Summary also shows the following for each step:

- **Trigger** that will cause the step to end
- **Pump** flow rate (mL/min)
- **Centrifuge** speed (G)

Loops within a protocol are also illustrated in the ribbon at the top including which loop number is currently being performed. In the example shown we are on step 12 of the first loop comprising steps 11 to 15.

Below the step ribbon are several control buttons:

Hit the ▶ to run the simulator in real time. The 1x, 4x and 8x (not currently visible) enable the user to speed up or slow down the simulation. You can also pause, advance, rewind and skip to the beginning or end of the protocol by clicking on the appropriate button.

Directly below the control buttons, a clock shows the cumulative time (top number) and total time for the protocol in hrs:mins:secs. The colored ring will also be highlighted as the simulation runs showing the proportion of the total time elapsed.

With the simulator paused, the top number in the clock will be the total time to complete the selected step, in this case 1 minute and 26 seconds and the total protocol time is 20 minutes and 44 seconds.

The **Kit View** tab also shows the fluid path, direction of flow and the volume in each bag at the completion of the step.

The liquid level in the bags adjusts in real time as the simulation runs.

Any fluid line that has been primed will be shaded grey.

Note: The Rotea™ Simulation software compensates for the volume of the CFC Chamber and the Bubble Trap during priming using approximate volumes for these components and their associated fluid lines. Fluid lines are only shaded grey when fully primed.

Hitting the exit button will exit the Rotea™ Simulation software and take you back to the main **Protocol Builder** tab.

The Rotea™ Simulation software also includes a Summary View that tabulates the **Estimated Bag Volume at End of Step** for all active bags. In the example, bags A, B, DG and H are being used in the protocol hence bags C, E & F are not displayed.

At the bottom of the table the following volumes are also displayed:

- **Maximum Volume Achieved**
- **Maximum Capacity** – as defined in **Kit** view in the **Protocol Builder**

In the example above, the **Maximum Volume Achieved** for bag H is 60.0 mL whereas the bag capacity is only 50 mL, then the step line will show red.

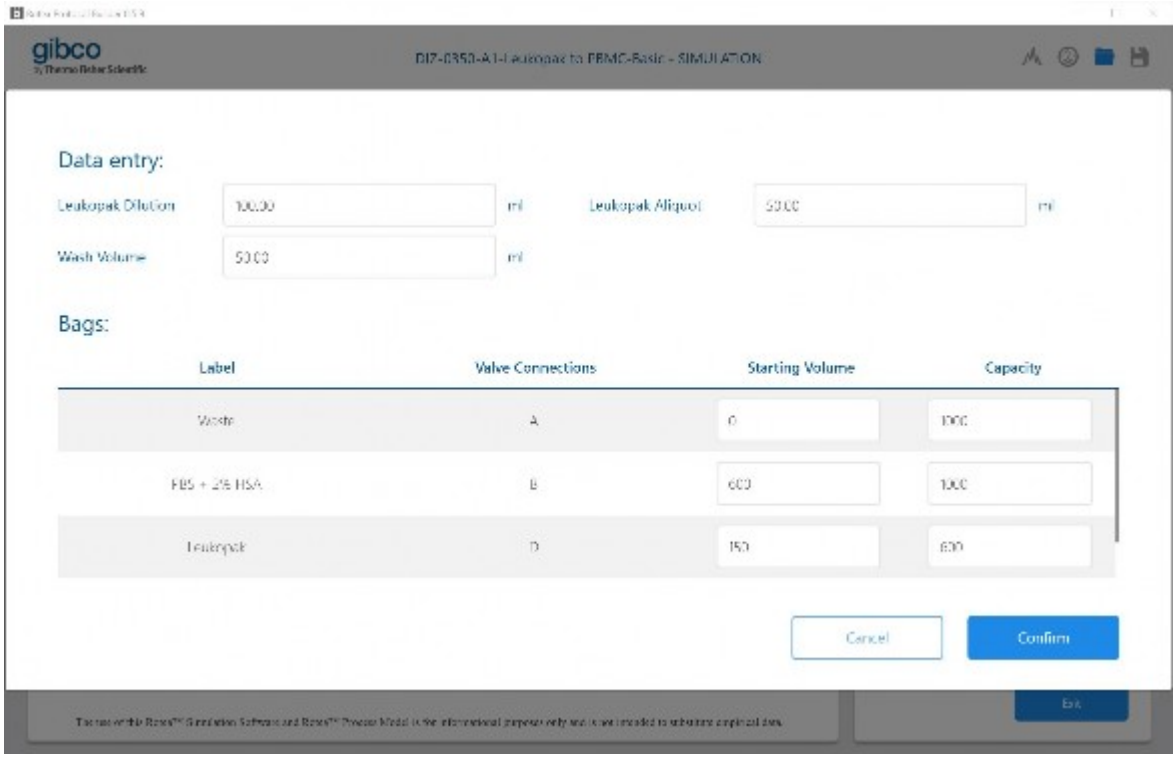
The Rotea™ Simulation software runs until each step reaches its designated trigger. If, for example, a volume trigger and Bubble Sensor trigger have been selected to detect an empty bag, the Rotea™ Simulation software will continue to run until the first trigger is reached.

If a Bubble Sensor trigger has not been included, the Rotea™ Simulation software will run until the specified volume is delivered. If the volume of media in the input bag is insufficient to complete the step, the input bag volume will turn negative.

The protocol will need to be modified to prevent this from occurring by either adding a Bubble Sensor trigger, increasing the **Bag Input Volume** or reducing one or more of the **Volume** triggers within the protocol.

To enable different scenarios to be quickly simulated, a separate input screen is provided that can be accessed by clicking on the ⚙ in the **Setting** box. The input screen contains the **Data Entry** values defined within the protocol and the **Starting Volume** and **Capacity** for all bags used.

Note: These volumes are editable for running the simulation but will not be automatically updated in the protocol.



7 Design new protocols

Plan the kit configuration

Consider what fluids you want from where and layout a Single-Use Kit identifying the reagents and any recirculation bag links using the **Define Kit** screen in the **Protocol Builder**.

Review the layout thinking through challenges like rinsing bags, intermediate bags and concentrate waste disposal.

Plan the protocol

Write a first draft of the protocol incorporating recommended priming steps and the various step types available.

Use the Rotea™ Simulation software to:

1. Estimate the total run time for the protocol and the time to complete each step.
2. Estimate the bag volume at the end of each step.
3. Compare the bag volume with the bag capacity and highlight when the capacity is exceeded.
4. View each step in the protocol to see the bag volumes (numerical value and graphical representation), fluid path, flow rate, G force, the trigger that would be activated to move to the next step and the time to complete the step.
5. Play the Rotea™ Simulation software in real time, 4x or 8x speed, pause and skip.
6. Simulate the impact of changes to input volumes, bag capacities and data entry values without changing the original protocol.

Where possible for early process development work with new cells and media, the output line for steps processing cells, e.g. loading, washing and harvesting, should be directed to separate bags. The combined total of cells in the output bags should be equal to what was put in. If the combined total is not equal, or the amount of cells in any bag is not what is expected, having separate bags will enable you to pinpoint in which steps cells were lost.

It can often be helpful to separate the protocol into sections, e.g. a sequence of priming steps followed by a block of processing steps that are performed multiple times until a large batch of cells is processed. Where loops are ultimately required, initially create and test the protocol on a smaller batch without loops. Add the loops once you are happy with the protocol settings.

Prepare for the first trial run

Complete the protocol definition including checking of speeds and triggers from similar processes and cells. When these are not available, use the Rotea™ Process Model to better understand the potential behaviour of particles within the fluidized bed for any Step within the protocol including:

1. Estimate the percentage of cells retained or elutriated based on the input parameters, assuming a normal distribution of particle size.
2. Estimate the theoretical cell size at the zero line (the transition point between cells being retained or elutriated when the opposing forces on the cell are balanced).

If a scoping run is planned, use a recirculation step through an intermediate bag to enable in-process samples to be drawn at different speeds.

Water runs

Set up a test Single-Use Kit and run the protocol with water to verify that steps, speeds and triggers behave as you expected.

Use this opportunity to refine the settings, practice setting up the Single-Use Kit and practice with any user interactions you have included.

First run with cells

For initial runs use small quantities of cells, e.g. 50 to 100 million cells, to verify loading speed and wash behavior.

Carefully measure the cell count and viability of both the input and outputs. The focus should be on viable cells only.

Analyze the outcome

Confirm the output cell count and viability.

The CTS™ Rotea™ Counterflow Centrifugation System is a closed system. If recovery is <95% then a mass balance should be conducted:

- Mix and sample from every bag with fluid in it.
- Rinse the original cell supply bag to determine how many cells remain.
- Drain the kit and count cells.
- If cells are found in places other than the output, then refine the protocol to minimize the lost cells.

Refine the protocol to achieve target cell outcomes

Progressively increase the input volume to be representative of your final process and refine the protocol to optimize the outcome.

Add process integrity features

Add process integrity features to the protocol e.g. test that manual valves have not been left clamped as part of the priming cycle.

A Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the "Documentation and Support" section in this document.

Symbols on this instrument

Symbols may be found on the instrument to warn against potential hazards or convey important safety information. In this document, the hazard symbol is used along with one of the following user attention words.

- **CAUTION!**—Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.
- **WARNING!**—Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.
- **DANGER!**—Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury.

Standard safety symbols

Symbol and description
CAUTION! Risk of danger. Consult the manual for further safety information.
CAUTION! Risk of electrical shock.
CAUTION! Potential biohazard.

Control and connection symbols

Symbols and descriptions	
	On (Power)
	Off (Power)
	Earth (ground) terminal
	Protective conductor terminal (main ground)
	Direct current
	Alternating current
	Both direct and alternating current

Conformity symbols

Conformity mark	Description
	Indicates conformity with safety requirements for Canada and U.S.A.
	Indicates conformity with China RoHS requirements.

(continued)

Conformity mark	Description
CE	Indicates conformity with European Union requirements.
	Indicates conformity with Australian standards for electromagnetic compatibility.
	Indicates conformity with the WEEE Directive 2012/19/EU. **CAUTION!** To minimize negative environmental impact from disposal of electronic waste, do not dispose of electronic waste in unsorted municipal waste. Follow local municipal waste ordinances for proper disposal provision and contact customer service for information about responsible disposal options.

Safety information for instruments not manufactured by Thermo Fisher Scientific

Some of the accessories provided as part of the instrument system are not designed or built by Thermo Fisher Scientific. Consult the manufacturer's documentation for the information needed for the safe use of these products.

Instrument safety

General

CAUTION! Do not remove instrument protective covers. If you remove the protective instrument panels or disable interlock devices, you may be exposed to serious hazards including, but not limited to, severe electrical shock or crushing.

CAUTION! Solvents and Pressurized fluids. Wear eye protection when working with any pressurized fluids. Use caution when working with any polymeric tubing that is under pressure:

- Extinguish any nearby flames if you use flammable solvents.
- Do not use polymeric tubing that has been severely stressed or kinked.
- Do not use polymeric tubing with tetrahydrofuran or nitric and sulfuric acids.
- Be aware that methylene chloride and dimethyl sulfoxide cause polymeric tubing to swell and greatly reduce the rupture pressure of the tubing.
- Be aware that high solvent flow rates (~40 mL/min) may cause a static charge to build up on the surface of the tubing and electrical sparks may result.

CAUTION! Do not lean on the operating instrument. Do not stay within 11.8" (300 mm) of the instrument longer than necessary for operational reasons. Do not deposit any potentially hazardous materials within 11.8" (300 mm) of the instrument.

Physical injury

CAUTION! Moving and Lifting Injury. The instrument is to be moved and positioned only by the personnel or vendor specified in the applicable site preparation guide. Improper lifting can cause painful and permanent back injury.

Things to consider before lifting or moving the instrument or accessories:

- Depending on the weight, moving or lifting may require two or more persons.
- If you decide to lift or move the instrument after it has been installed, do not attempt to do so without the assistance of others, the use of appropriate moving equipment, and proper lifting techniques.
- Ensure you have a secure, comfortable grip on the instrument or accessory.
- Make sure that the path from where the object is to where it is being moved is clear of obstructions.
- Do not lift an object and twist your torso at the same time. Keep your spine in a good neutral position while lifting with your legs.
- Participants should coordinate lift and move intentions with each other before lifting and carrying.
- For smaller packages, rather than lifting the object from the packing box, carefully tilt the box on its side and hold it stationary while someone else slides the contents out of the box.

Electrical safety

WARNING! Fuse Installation. Before installing the instrument, verify that the fuses are properly installed and the fuse voltage matches the supply voltage. Replace fuses only with the type and rating specified for the unit. Improper fuses can damage the instrument wiring system and cause a fire.

WARNING! Ensure appropriate electrical supply. For safe operation of the instrument:

- Plug the system into a properly grounded receptacle with adequate current capacity.
- Ensure the electrical supply is of suitable voltage.
- Never operate the instrument with the ground disconnected. Grounding continuity is required for safe operation of the instrument.

WARNING! Instrument protective bonding. For safe operation of the instrument regularly check grounding continuity to the instrument chassis, front plate and bag hangers.

WARNING! Power Supply Line Cords. Use properly configured and approved line cords for the power supply in your facility.

WARNING! Disconnecting Power. To fully disconnect power either detach or unplug the power cord, positioning the instrument such that the power cord is accessible.

Cleaning and decontamination

CAUTION! Cleaning and Decontamination. Use only the cleaning and decontamination methods that are specified in the manufacturer user documentation. It is the responsibility of the operator (or other responsible person) to ensure that the following requirements are met:

- No decontamination or cleaning agents are used that can react with parts of the equipment or with material that is contained in the equipment. Use of such agents could cause a HAZARD condition.
- The instrument is properly decontaminated a) if hazardous material is spilled onto or into the equipment, and/or b) before the instrument is serviced at your facility or is sent for repair, maintenance, trade-in, disposal, or termination of a loan. Request decontamination forms from customer service.
- Before using any cleaning or decontamination methods (except methods that are recommended by the manufacturer), confirm with the manufacturer that the proposed method will not damage the equipment.

Safety and electromagnetic compatibility (EMC) standards

The instrument design and manufacture complies with the following standards and requirements for safety and electromagnetic compatibility.

Safety standards

Reference	Description
EU Directive 2014/35/EU	European Union “Low Voltage Directive”
IEC 61010-1 EN 61010-1 UL 61010-1 CAN/CSA C22.2 No. 61010-1	*Safety requirements for electrical equipment for measurement, control, and laboratory use – Part 1: General requirements*
IEC 61010-2-020 EN 61010-2-020	*Safety requirements for electrical equipment for measurement, control and laboratory use – Part 2-020: Particular requirements for laboratory centrifuges*
IEC 61010-2-081 EN 61010-2-081	*Safety requirements for electrical equipment for measurement, control and laboratory use – Part 2-081: Particular requirements for automatic and semi-automatic laboratory equipment for analysis and other purposes*

EMC standards

Reference	Description
EU Directive 2014/30/EU	European Union "EMC Directive"
EN 61326-1 IEC 61326-1	*Electrical Equipment for Measurement, Control and Laboratory Use – EMC Requirements – Part 1: General Requirements*
AS/NZS CISPR 11	*Limits and Methods of Measurement of Electromagnetic Disturbance Characteristics of Industrial, Scientific, and Medical (ISM) Radiofrequency Equipment*
ICES-001, Issue 4	*Industrial, Scientific and Medical (ISM) Radio Frequency Generators*
FCC Part 15 Subpart B (47 CFR)	*U.S. Standard Radio Frequency Devices* This equipment has been tested and found to comply with the limits for a Class A digital device, pursuant to part 15 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference when the equipment is operated in a commercial environment. This equipment generates, uses, and can radiate radio frequency energy and, if not installed and used in accordance with the instruction manual, may cause harmful interference to radio communications. Operation of this equipment in a residential area is likely to cause harmful interference in which case the user will be required to correct the interference at his own expense.

Environmental design standards

Reference	Description
Directive 2012/19/EU	European Union "WEEE Directive"—Waste electrical and electronic equipment
Directive 2011/65/EU	European Union "RoHS Directive"—Restriction of hazardous substances in electrical and electronic equipment
SJ/T 11364-2014	"China RoHS" Standard—Marking for the Restricted Use of Hazardous Substances in Electronic and Electrical Products For instrument specific certificates, visit our customer resource page at **www.thermofisher.com/us/en/home/technical-resources/rohs-certificates.html**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

B

Documentation and support

Customer and technical support

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- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.