Fundamentals of Fermentation:
Techniques
For Benchtop Fermentors

Part ? *E. coli*

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The following document outlines step-by-step procedures for carrying out a benchtop fermentation, particularly when using an NBS fermentor. Provided in a question and answer format, this article covers such topics as what media formulation, tubing size, and concentration of various additives should be used, as well as the preparation, autoclaving and clean up procedures for the vessel and accessories. Unless otherwise indicated, our example refers to an *E. coli* fermentation in a benchtop BioFlo 3000® fermentor. However, in general, the information should be valid for any fermentation.

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Rev. 2

Question: What kind of MEDIA should be used, and does it differ from media used in shake flasks?

Answer: The media used in shake flasks does differ from the standard media used in a fermentation vessel. Shake flask media is generally of a much simpler composition. LB Broth (Difco catalog number 0446-17-3) and Tryptic Soy Broth (Difco catalog number 0370-07-5) are standard shake flask media.

An example of a more complex media used in a recombinant *E. coli* fermentation follows:

Chemical	**g/L**
KH_2PO_4	3.5
K_2HPO_4	5.0
$(NH_4)_2HPO_4$	3.5
$MgSO_4 \cdot 7H_2O$	0.5
Glucose	5.0 (for fed batch) 30.0 (for batch)
Yeast Extract	5.0
Trace Metals	1.0 ml/L
Antifoam	0.5 ml/L

Trace metals formulation:	
$FeCl_3$	1.6
$CoCl_2 \cdot 6H_2O$	0.2
$CuCl_2$	0.1
$ZnCl_2 \cdot 4H_2O$	0.2
$NaMoO_4$	0.2
H_3BO_4	0.05
Hcl	10 ml
H_2O	to 1000 ml

For fermentation, the glucose solution is usually sterilized in a separate flask. It is then added aseptically to the other (heat labile) components that can not be subjected to autoclaving, such as Ampicillin and the trace metal solution. These are prepared in advance by sterile filtration so that they are available as stock solutions. The magnesium sulfate is sometimes sterilized separately. Most materials are available from a variety of vendors. Note that Sigma and Difco are often the best sources for the more unusual biological and chemical materials. The exact formulations of the trace metals solution and the fermentation media for the fermentors will depend on the precise fermentation you wish to conduct. Various formulations can be found in the handbooks and literature.

Question: What kind of ANTIFOAM should be used, and in what concentration?

Answer: Antifoam such as Dow Corning's Antifoam 2210 or Compound A are usually suitable for use in fermentation. Calgene also produces antifoam -- M-10 is one example. The initial concentration of antifoam is usually 0.1-0.5 ml/L. When the foam probe is used the pumping of antifoam is controlled by the unit. In our labs we usually use Breox FMT 30 which is available from International Specialty Chemical Company Many other antifoams are available. Listings of other antifoaming agents can be found in the Sigma and Aldrich chemical catalogs. The pump should be set to add the minimum amount of antifoaming agent required to prevent foaming in your particular process. That amount varies depending on the amount of protein in the media, the amount of protein secreted by the microorganism, agitation speed, and other factors. Therefore, you will have to experiment to get the proper pump setting.

Question: What is the correct TUBING size for acid, base, antifoam and nutrient feed for a fed-batch run?

Answer: For vessels 5 liters or less, NBS part number TU202. This tubing will provide you with Marprene tubing with 1.6 mm inside dimension (ID). It has an OD of 4.8mm (3/16"NOM) and a wall thickness of 1.6mm.Larger tubing will be required for vessels over 5 liters. It may also be necessary to use a connecting fitting to allow two different tubing sizes to be used (in cases when the tubing size required for the pump and the size required for the direct connection to the vessel differ.) NBS recommends silicon tubing for use with the pumps heads (Watson-Marlow 101) which are built into the BioFlo fermentors. However, Marprene tubing may be used as well as long as the tubing size does not exceed 3/16" bore x 1/16" wall. Marprene tubing of this size and smaller can be used with the Watson-Marlow 101 pump heads under low pressure and with clockwise rotation. Take note that silicon tubing should not be used with hydrochloric acid (HCL), sulfuric acid ($H_2SO_{4)}$) or sodium hydroxide solutions since this material deteriorates rapidly when in contact with these solutions another reason for avoiding HCL is that HCL (and to a lesser extent H_2SO_4) causes corrosion of stainless steel. NaOH solutions equal to or less than 20% can be used in silicon tubing at temperatures less than 120 ?F without causing destruction of the tubing. Solutions of sulfuric acid less than 10% can cause moderate damage to silicon tubing.

Question: What concentration and type of ACID and BASE should be used?

Answer: The acid solution is 2 - 3N H_2SO_4. The base solution is either 5N NaOH or NH_4OH ~ 29% (which is the standard commercially available concentration.) Please be aware that these are fairly concentrated. The acid can effect the stainless steel parts of the fermentors vessel. To avoid damage to the entry ports it is a good idea to use a sterile disposable needle at the end of the addition tubing and add the acid (or base) through the disposable needle. The needle will corrode but it saves the

fermentor vessel. Insert the needle though a septum port so that the drip point is away from stainless steel components and fairly close to the liquid level. You may also use a more dilute solution of the acid or base. However be aware that this may cause the complication of adding a larger volume of liquid to the vessel. Also, it is not a good idea to add acid and base through a single double or triple port adapter. The combined effects of the two causes rapid corrosion of the adapter. The pump setting is usually 20.0 - 25.0 under acid or base mode.
For these concentrations of acid and base Marprene tubing should be used. To avoid damage to the stainless steel headplate, use a polymer side port or a septum port for introduction of these strong solutions into the vessel. If you are using silicon tubing reduce the concentration of H_2SO_4 to less than 8% (about 5%) and use a 20% solution of NaOH. When selecting an acid for use in fermentation, select the lowest concentration possible that allows for pH control.

Question: What is the proper concentration of GLUCOSE feed?

Answer: The glucose is 50% concentration. The feed rate is not usually a constant value as this will differ not only from run to run, but it will vary greatly over the course of a run depending upon the organism's growth. This operation can be controlled automatically by AFS-BioCommand, NBS' proprietary Windows-based software. (AFS is also available as a DOS-based system.) Glucose feeding can be set to respond to other sensor cues (such as D.O. level, the pH reading, the turbidity measurement, the glucose measurement, etc.). The pumping profile to be used must generally be determined through experimental experience.

Question: What are the recommended PROCESS CONTROL SETTINGS, (i.e. temp., pH, agitation speed, D.O. & gas sparge rate)?

Answer: For *E. coli,* temperature is usually set to 32^0 - 35^0C and pH is set at 7.0 - 7.2. For yeast the values are 30^0C and a pH value of 5.0. Agitation speed is usually set to a minimum of 200 - 300 rpm with a maximum value of 1000 rpm. Dissolved oxygen (or D.O.) level is usually 30%. The gas sparge rate is generally 0.5 to 1.0 VVM.

Question: Can you review the steps involved in SET UP through SHUT DOWN of a fermentation run?

Answer: To properly answer this, let's break the process down, as follows:

A. Vessel Preparation, prior to autoclaving

It is advisable to rinse the previously cleaned vessel prior to use. When doing this, remember that all clamps must be open and the valve for the sampling tube must be in

the open position. If the glass wool is going to be replaced for the run, then remove it and the rubber bulb of the sampler prior to rinsing. The protective cap for the motor drive adapter must also be in place. It will be necessary to hold the protective cap for the agitation shaft in place if you plan to invert the headplate while rinsing it. In this case it is usually advisable to also remove the clamps that hold the headplate onto the rest of the vessel, as failure to do so will result in their falling out during inversion. The pH and D.O. probes should not be in the headplate when it is rinsed. All gas filters must be removed prior to rinsing. The sparger must, in particular, be checked to ensure that it isn't clogged.

The headplate must be oriented in combination with the vessel and the internal baffle so as to allow for the exhaust condenser lines and the cooling jacket water lines to be connected. Also, the baffle must be positioned so that it does not interfere with the insertion of the pH and D.O. probes into their ports. Do ***not*** place the sample port to the rear of the vessel. The sampling port must be positioned so that ample room is available to take a sample. It is advantageous to have the addition ports for acid, base, etc., on the same side (or at least *not* the opposite side) as the pumps. The old grease (Dow Corning Silicone Grease) on the top of the glass cylinder should be wiped clean and then re-applied prior to placing the headplate on. The grease is applied by smearing a very thin layer around the top of the cylinder, usually with a fingertip. Care should be taken to ensure that no residual grease is left on your hands when you touch other parts of the vessel. When the headplate is attached, the headplate clamps must be tightened down properly.

All tubing connected to the headplate should be secured at the headplate connection point as well as to any addition bottles or connectors away from the headplate tie-gun is useful for this purpose. Note that both the air sparger and the exhaust line will have a terminal filter. (For the benchtop BioFlo 3000 vessel, the respective NBS part numbers are P0200-0491 for the small filter for the sparge line, and P0200-0490 for the large filter for the exhaust line.) All tubing connected to ports that have their terminus within the vessel below the liquid level (i.e. the harvest and sparge ports), must be clamped prior to autoclaving. The valve for the sampler must also be in the closed position. Other hoses, such as ones attached to base or addition ports should be clamped to facilitate sterile hook ups. We primarily use the following clamps: a Hosecock Clamp (Fisher catalog number 05-847) and a Side Tubing Clamp, Hoffman (Fisher catalog number 05-875B).

> Do not rely on polymer clamps to survive autoclaving. Polymer clamps often pop open in the autoclave. If you wish to use the newer polymer clamps during the running of your fermentation, then place the polymer clamps onto the tubing but leave them open. Use easily removable metal clamps to actually close the line during autoclaving. These may be removed after the vessel has been autoclaved. Be sure to use the polymer clamp to close off the tubing BEFORE you remove the metal clamp.
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> Clamps can be placed at any point of the tubing as long as they don't clamp down onto a port or connector, as that would interfere with proper sealing. The open end of the tubing should be covered with cotton, which is then covered with aluminum foil. The clamp on the tubing should go below this. The sparger filter

should also be covered, but not quite as tightly. The exhaust filter is not usually covered. All tubing should be inspected both prior to and after autoclaving to insure integrity. The above description holds true for any side harvest ports in use. Note that this type of port is often below the media fill line. It is also possible to use a hose that has been tied off and crimped at one end to provide a cap for the base port, addition port, as well as other ports. These caps must fit very securely over the port in order to avoid loss of sterility due to displacement while autoclaving.

All O-rings should be checked for damage prior to autoclaving. All fittings must be checked for tightness. A loose fitting is often an indication that the small O-ring in the fitting assembly requires replacement. All sealed side port plugs must be checked for tightness, and their O-rings must also be intact. The vessel must be checked to ensure that the bottom of the glass cylinder is properly secured to the vessel's base. The agitation shaft must have its protective cap on prior to autoclaving. It is advisable to check to ensure that the connectors from the unit to the vessel (cooling jacket water line and exhaust gas condenser) are compatible. This is a good time to check that the air and water lines to the unit are open and that (if required) an oxygen source is available and correctly connected.

The pH probe must be inspected prior to insertion to ensure that enough electrolyte is present and in good condition. The rubber stoppers must be checked to ensure that they are secure. The pH probe must be properly calibrated prior to insertion in the headplate. (Be sure to carefully follow the manufacturer's instructions for probe calibration which come with the probe and with the fermentor. See also Appendix at the end of this document for calibration information.) It is often necessary to coat the probe with a very thin layer of glycerin or deionized water in order to avoid jamming or breaking it during insertion. The pH probe must be inserted using carefully, using two hands, with one hand holding the base of the probe near the port opening. The insertion must never be forced. Never insert the pH probe until the headplate is properly secured.

(This is also true for the D.O. probe as well.) The D.O. probe must be checked to ensure that the required amount of electrolyte is present prior to insertion, and we usually exchange electrolyte for each new run. The D.O. probe's membrane must also be inspected prior to use. It is absolutely critical that both the pH and D.O. probes have their protective caps on prior to autoclaving, and these should, in fact, be on except when the probe is being hooked up to the unit. NEVER autoclave a pH probe or DO probe without the protective cap in place.

The glass wool for the sampler is prepared by rolling a small quantity up and inserting it into the small tube that attaches to the bulb. It may be necessary to trim any glass wool fibers that stick out. Note that it is possible and undesirable to pack glass wool too tightly. This can be checked for by using the bulb and a sampling tube to see if vacuum can be held and released properly, as when a sample is normally taken. A sample tube is attached prior to autoclaving. This tube should be 1/4 to 1/2 turn loose to avoid explosion or implosion. The glass wool is covered with a piece of foil.

B. Vessel Sterilization:

When autoclaving, the unit exhausts through the exhaust filter, so it is essential that the line be prevented from crimping and that the filter is good (unplugged). To insure that crimping does not occur, use a short piece of fairly rigid tubing. If rigid tubing is not available use a small splint to support the tubing. The vessel is normally sterilized for 45 minutes. Note that certain media formulations can not be sterilized for this length of time, as degradation will occur. (Check the media manufacturer's instructions.) The probes must never be autoclaved dry.

If it becomes necessary to sterilize the vessel without media, use a balanced salt (phosphate buffered saline) solution to cover the ends of the probes. Aseptically remove the PBS prior to filling the vessel with the desired media. NEVER PLACE PROBES IN DISTILLED OR DEIONIZED WATER. THIS WILL CAUSE YOUR PROBE TO LOSE ELECTROLYTE. The maximum fill is ~ 70% of the vessel's maximum volume. Autoclaving should be done in a unit with a liquid on a slow exhaust setting (see autoclave manufacturer's instructions for autoclaving liquids). Sterilization is at 121^0C. When sterilization is complete, it is important to check the exhaust line to ensure that it didn't crimp and the vessel's integrity must also be ascertained.

C. Post Sterilization Vessel Set Up:

The vessel must be gently handled when removed from the autoclave to prevent the media from boiling up. Confirm that any unprotected vented lines are clamped off upon removing the vessel from the autoclave. The vessel's integrity must be again ascertained. The vessel is then transported to the bench unit.

The vessel is placed on the unit using the "guide posts" on the console base. The orientation must allow for proper hook up to the cooling jacket water lines and the exhaust gas condenser lines. Note that this should be checked prior to autoclaving, as indicated above. Connect the water lines, ensuring that the water delivery and return lines are not inverted. Additionally, it is necessary to connect the outgoing lines prior to connecting the corresponding incoming lines. Insert the temperature probe into the thermowell. Check that the water lines to the unit are open. Set the temperature value to your desired PID value and set the control to "Prime". If using an external recirculating chiller, it must also be turned on at this time. The water level in the chiller should also be checked prior to use. After ~ 2 - 5 minutes, the unit can be switched from "Prime" to the desired PID temperature setting. This can be checked by making sure that water is truly leaving the unit by observing the water drained through the "Drain" or "Water out" port.

Remove the protective caps from the pH and D.O. probes and connect them to the unit. Be careful with the pH probe to avoid the temptation to twist the probe into its connection to the unit, as this can compromise sterility. The connection must be screwed onto the probe. The pH probe should also be checked to ensure that the secured rubber stoppers in it were not displaced. Note the time that the D.O. probe is connected, since the probe requires a minimum of 6 hours for polarization.

The protective cap on the agitation shaft should now be removed and the motor attached. Change the control panel on the unit to the Gasses screen and set the air from "Off" to "Manual". Return to the master screen and make sure that "air flow" is one of the four parameters displayed. Use the knob on the side of the machine to manually adjust the airflow to the desired setting. Connect the airline from the unit to the terminal filter of the sparger in as aseptic a manner as possible. Note that the filter will prevent external contamination but good technique never hurts. The clamp on the sparger line is then opened and the vessel can be visually observed to ensure that the air is flowing properly. Then set the agitation to the minimum desired value.

After set-up, the unit should be carefully observed to ensure that there are no problems, particularly as regarding water line leaks.

D. Vessel Operation:

The vessel must have any and all necessary addition bottles connected prior to use. If a bottle such as the glucose feed is not initially required, it can be hooked up later. The pH will probably need to be adjusted. This is done by setting the pH control to "PID". Note that due to a tendency for the unit to overshoot the target pH during this initial adjustment, it is desirable to set the initial pH PID a little conservatively. (Example: post sterilization pH reading is 6.8. Desired PID is 7.2. Set the unit to PID 7.0 when conducting the initial adjustment.) Note that the pH reading must be taken from a vessel that has already cooled down.

Additional media components that are not autoclaved can be added once the vessel has cooled sufficiently. The protocol for this is the same as for inoculation as described below.

Inoculation can be performed by-aseptically pouring liquids into the vessel through the inoculation port. However we normally use the harvest port to inoculate. A peristaltic pump or gravity is used to introduce the inoculum. The shake flask is connected to the port terminus using aseptic techniques, and then the clamps are opened to allow for addition. Once the material is all in (except for any residual inoculum which must be retained for testing) the clamps are secured and the shake flask is disconnected. At this point, the harvest port terminus must be covered up again with sterile cotton and foil. This must be performed in an aseptic manner.

To harvest from the vessel, a line can be attached to the harvest port and a peristaltic pump can be used to pump the culture broth out

E. Vessel Shut down and Cleaning:

When the fermentation run is complete, it is necessary to carefully shut the process down. First all operating parameters (agitation, temperature, D.O. level, pH, and gas feed) must be set from their current control modes (such as "PID", "D.O.", "Base") to the "Off" mode. Note that the manual air valve on the side of the unit should also be closed as well. Additionally, if a supplemental oxygen feed was used, it will be necessary to close the gas tank valve and the lines leading from it to the unit. If a recirculating chiller is in use, it should be shut off when the temperature control is shut off. Feed lines from any addition bottles which have been used should be clamped off prior to detaching them from the vessel, and should then be removed.

The next step is to disconnect the vessel from the unit. The temperature probe needs to be removed from the thermowell. Remove the motor and place the protective cap over the agitation shaft. Always disconnect the water lines by disconnecting the incoming lines prior to the outgoing lines. The air line must be disconnected from the sparger. Disconnect the pH and D.O. probes from the unit and replace their protective caps. The D.O. probe presents an easy removal as you simply unscrew the thread and gently pull it out. It should be immediately rinsed off and then wiped dry gently, always remembering to avoid touching the membrane at the tip. Some runs will result in an accumulation of biomaterial on the probe and it may be necessary to wipe the probe down more vigorously but in no case should the tip be touched. After cleaning the D.O. probe the tip can be visually inspected for damage. The probe should then be stored in a clean area in such a way as to protect the sensitive tip. Removing the pH probe is usually not as difficult a process as inserting it because the shaft will be wet and thus should be relatively easy to remove. However the danger of probe breakage is still very real and extreme care must be taken when removing it. It is still necessary to use two hands with one hand at the top of the port acting as a guide to ensure proper removal. A gentle pace for this is required. If at any point in this process the probe jams, it is essential to avoid trying to force it. It may be necessary to reinsert it part way and apply a lubricant such as glycerin to the shaft and port in order to effect the probes' removal. In extreme cases it may be necessary to remove the headplate with the probe still inside so that you can approach the problem from both ends. In this case, it is critical to remove the headplate very carefully. (We recommend that you have a spare probe available at all times, in case of breakage.) Once the pH probe has been removed, it should be immediately washed off with warm water. If biomaterial has accumulated on the probe, using a sponge (or an equivalent that will not scratch glass) and gentle pressure to clean the surface. The very tip of the probe should be handled with extreme care and a Kimwipe should be used to gently dry it off after washing. The probe should be stored with the tip immersed in either electrolyte or pH 7 buffer. This electrolyte/buffer can be reused, but it should always be inspected prior to each use for precipitation or contamination.

Now that the vessel is detached from the unit, it can be cleaned. To do this it will be necessary to remove any remaining cotton and foil covering the ports. The rubber bulb on the sampler should be removed and rinsed separately. The glass wool can be removed at this point as well. The sampling tube is detached and washed separately. The valve on the sampling port and all clamps on all tubing connected to ports will need to be open for proper washing. (Note that the media will need to be removed prior to

unclamping any tubing that is below the liquid level, as is the case with a side harvest line). The headplate should be detached by loosening and then removing the clamps that hold it to the rest of the vessel. Those clamps may require rinsing. The remaining culture broth should be sterilized or emptied into a bucket and disinfected by using bleach or other accepted disinfectant prior to disposal. Note that some media may be incompatible with this procedure, in which case the media can be placed into another container for sterilization prior to disposal. The headplate should be washed thoroughly with warm water and then de-ionized (DI) water. It may be necessary to scrub accumulations of biomaterial off. A pad that won't scratch the steel is required for this. The agitation shaft, the thermowell, harvest and sparger tubes and the short beveled tips of the interior portion of the base-type addition ports will often require special attention. All tubes and shafts must be cleaned. Note that there may be some residual base or acid left in those lines, so extreme caution and the use of chemically-resistant gloves is recommended for this procedure. It is often necessary to hand wipe surfaces with a paper towel in order to fully remove residual traces of small particulate debris.

The washing of the bottom portion of the vessel requires the same procedures as the headplate. Note that the sides of the vessel, particularly where the baffle was adjacent to it, and side ports (plugged or unplugged) may require special attention.

The vessel can now be cleaned by washing with detergent, or by using a cleaning solution. If the vessel is to be sterilized, all standard precautions must be taken. Note that for this purpose, the vessel does not need to be sealed except for those previously cited valves and tubing which run under the liquid level. It will be necessary to use water in the vessel. We recommend the use of DI water, and the fill should be at least as high as your standard level for a run. Unless you have already specifically wiped the residual grease off the top of the glass cylinder, there should be enough so that the headplate can be clamped to the lower portion of the vessel. Note that it is neither necessary nor desirable to fasten these clamps with the same force used for attaching the headplate prior to a run, as this could lead to vessel damage. Instead, the lightest possible pressure should be used. The advantage to sterilization is that not only are residual viable organisms killed, but residual debris will loosen and become removable by washing after the vessel has cooled. If a cleaning solution is required, we recommend a 10% dilution of Micro cleaning solution (International Products Corporation, catalog number 6732). Alternatively, if you are using the vessel for consecutive runs with the same media, a rinsing of it with warm tap water and the DI water may suffice. Note that if water will run over a vessel surface that has grease on it, the grease should be removed by wiping it off with a wet paper towel.

In cases where the vessel must be decontaminated prior to cleaning, add water so that the liquid level reaches the maximum working volume of the vessel. This will help prevent biological materials from adhering.

APPENDIX A.
BIOFLO 3000 Probes:

pH PROBE MAINTENANCE AND STORAGE

1. Check the level of the filling solution. It should be about 1 cm below the filling orifice. To add solution, *see pH Probe Preparation*.
2. Check for any trapped air bubbles in the electrode's tip to remove bubbles, hold electrode upright and shake electrode gently.
3. The probe should be stored standing upright. The electrode tip should be immersed in the solution of 3 molar KC1 or a buffer solution between pH 4 and pH 7. The two rubber "T" stoppers should be inserted. At no time should the electrode be allowed to rest on the tip.

pH PROBE CALIBRATION

✍ *NOTE:*

pH electrode is calibrated before autoclaving vessel.

1. Connect electrode to the pH connector using the appropriate cable.
2. Turn ON the main power switch.
3. Display calibration screen.

✍ *NOTE:*

The pH measuring system is calibrated using two external buffer solutions of known pH.

4. Immerse pH electrode into pH 7.0 buffer solution and allow a few minutes for the system to equilibrate.
5. Set the pH function to Zero.
6. Set the display to read 7.00.
7. Rinse the pH electrode with distilled water.
8. Immerse pH electrode into a second pH buffer solution which is several pH units above or below pH 7.00 (e.g. 4.00) and allow a few minutes for the system to equilibrate
9. Set the pH function to "Span".
10. Set the display to read the value of the second buffer solution.
11. Repeat steps 5 - 10 using the same buffer solutions.

✍ *NOTE:*

The pH calibration should be checked after autoclaving immediately prior to inoculation. This is performed by taking a sample from the vessel and comparing the value of pH displayed on the screen with that of an external pH meter. Any discrepancy should be adjusted with the function set to "Zero".

OPERATION OF DISSOLVED OXYGEN

1. Remove protective cap from electrode end. The membrane is delicate and care must be exercised to prevent accidental damage. Never rest probe on membrane.
2. To insure stable output, the probe should be subjected to two or three sterilization (autoclaving) cycles prior to use. The probe will be operable after the second cycle but will be more stable with additional sterilizations. The shorting plug should be installed on the probe during autoclaving or sterilization.
3. Install probe into vessel head plate assembly. If an Ingold probe is to be used, wrap the adapter threads with Teflon tape, screw into headplate and tighten with a wrench. Carefully insert the probe into the adapter. Finger tighten enough to compress the O-ring to insure a tight seal.

DISSOLVED OXYGEN ELECTRODE (D.O.) CALIBRATION

✍ *NOTE:*

D.O. Electrode is calibrated after autoclaving vessel.

✍ ***IMPORTANT NOTE:***

When the system is operated for the first time, or when the electrode has been disconnected from the voltage source (amplifier or polarized module) for longer than 5 to 10 minutes, the electrode must be connected to the operating O_2 amplifier for polarization purpose prior to calibration.

The electrode is polarized and ready for operation after six hours of polarization time.

1. There are two methods of obtaining zero on the BioFlo 3000. Use either Method 1 or Method 2.

   Method 1
   a. Remove the D.O. electrode cable from the D.O. electrode.
   b. Display calibration screen.
   c. Set the D.O. function to "Zero".
   d. Set the display to read zero by setting "Zero" to 0.
   e. Re-connect the D.O. electrode cable to the D.O. electrode.

   Method 2
   a. Connect the D.O. electrode cable to the D.O. electrode.
   b. Set agitation speed to 500 RPM.
   c. Sparge nitrogen* into the vessel via the filter on the head plate until the D.O. display is stable for approximately 10 minutes (this may take up to 30 minutes).
   d. Display calibration screen.
   e. Set the function to "Zero".
   f. Set the display to read zero by setting "Zero" to 0.

✍ ****NOTE:***

✍ *Connect Nitrogen to the N_2 gas inlet. On "2-Gas" screen, set mode to manual. Set N_2 to 100.*

2. Setting the "Span".

   a. Set the agitation rate of 500 RPM and agitation mode PID.
   b. Vigorously sparge air or oxygen into the vessel via the filter on the head plate until the display is stable for approximately 10 minutes (this may take up to 30 minutes).
   c. Set the function to "Span".
   d. Set the display to read 100 by setting "Span" to 10

APPENDIX B -- BIOFLO III PROBES

pH ELECTRODE PROBE CALIBRATION

pH probe is to be calibrated prior to autoclaving vessel.

1. Connect electrode to probe cable.
2. Turn the agitation switch OFF.
3. Turn ON power switch.

NOTE: *The pH measuring system is calibrated using two buffer solutions of a known pH.*

1. Set the selector switch to pH.
2. Set the mode switch to "ZERO".
3. Immerse the pH probe into an external pH 7.00 buffer solution.
4. Set the display to read the pH value of the buffer with INC/DEC switch.
5. Immerse the pH electrode in a second external buffer solution which is several
6. pH units above or below the pH selected in the previous step.
7. Set the mode switch to "SPAN".
8. Set the display to read the value of the second buffer solution with the INC/
9. DEC switch.

NOTE: *After autoclaving it is recommended that the calibration is checked by taking a sample from the vessel and measuring the pH with an external pH meter.*

BIOFLO III - DISSOLVED OXYGEN PROBE CALIBRATION

Probe is to be calibrated after autoclaving vessel.

1. There are two methods of obtaining zero on D.O.

METHOD 1 (Less Accurate)
Remove the D.O. probe cable from the D.O. probe (NOTE: Never disconnect D.O. probe more than 5-6 minutes). Set the selector switch to "DO".

Set the mode switch to "ZERO". With the INC/DEC switch adjust the display reading on the D.O. to read zero. Connect the D.O. probe cable to the probe.

METHOD 2

Connect the D.O. probe cable to the D.O. probe. Set the selector to D.O.

Set the agitation speed to 500 RPM.

Sparge Nitrogen into the vessel until the display is stable for approximately 5 minutes.

Set the mode switch to the "ZERO" position.

With the INC/DEC switch adjust the display for zero reading.

2. Set the "SPAN".

Set the selector to D.O.

Set the mode switch to "SPAN". Vigorously sparge air into the vessel. Set the agitation speed to 500 RPM.

After about 30 minutes observe the reading on the display. After the D.O. reading has stabilized adjust the D.O. display reading to 100 with the INC/DEC switch.