Q-Box NF1LP Nitrogen Fixation Package

Manufactured and Distributed by Qubit Systems Inc., Kingston ON, Canada
613-384-1977, info@qubitsystems.com
www.qubitsystems.com
Dec 2020

Table of Contents:

Overview of Q-Box NF1LP Nitrogen Fixation Package:

The Q-Box NF1LP Nitrogen Fixation Package provides the only safe and inexpensive means to study Nitrogen Fixation in legume plants that evolve H_2 gas as a by-product of the nitrogenase enzyme activity. Traditionally, "N_2 Fixation" has been measured by the acetylene reduction assay, which requires the use of a gas chromatograph to measure the reduction of acetylene to ethylene by N_2 fixing organisms. Gas chromatographs are expensive and the 10% acetylene required for the assay is explosive. In addition, it has been shown that the acetylene reduction assay inhibits the nitrogenase reaction! The use of Q-Box NF1LP Nitrogen Fixation package avoids these complications and provides an accurate and inexpensive way of measuring nitrogen fixation as a H_2 evolution rate in real time.

The Q-Box NF1LP Nitrogen Fixation Package can only be used with legume symbioses that lack the enzyme Uptake Hydrogenase (HUP). This enzyme oxidizes H_2, and prevents its release from nodules where the N_2 fixing bacteria reside. The Q-Box NF1LP package includes soybean seeds (*Glycine max*) and a peat-based inoculant of *Bradyrhizobium japonicum* strain 532c (Becker Underwood Inc.) which, when used according to the plant cultivation section in this chapter, will produce H_2-evolving legumes within a 28 day period. Other legumes may be used with the N_2 Fixation package, but they should be inoculated with the correct (*Brady*-) *Rhizobium* strain lacking the HUP enzyme, so that H_2 gas is evolved from the nodules during the process of N_2 fixation.

The Q-Box NF1LP Nitrogen Fixation Package provides all the equipment required to set up an open flow gas exchange system. Gas is supplied to the pot with the plant from a gas bag via a Q-G268 Flow Monitor and Pump. Gas is sub-sampled at a lower flow rate from the growth pot with the plant via a Q-G267 Flow Monitor and Pump and delivered to the Q-S121 H_2 Analyzer. From the H_2 Analyzer, gas enters the Q-S102 O_2 Analyzer and then vents to the atmosphere. The analog signals from the Flow Monitors, the H_2 and O_2 analyzers plus a temperature probe (S132) that can be inserted in the plant pot, are converted to digital signals via three integrated interfaces (9 available channels). Data are displayed, recorded and manipulated on a PC or Macintosh computer using Logger Pro software. Changes in the concentrations of H_2 and O_2 plus temperature of the growth medium can be monitored in real time.

The Q-Box NF1LP Package also contains 4 gas exchange pots for the cultivation of the plants. The plants must be cultivated in the correct planting medium to allow gases to flow from the nodulated roots to the H_2 Analyzer. We recommend silica sand as an ideal medium, since it has the correct porosity, and contains no nitrogenous matter that may inhibit N_2 fixation. Silica sand is otherwise known as industrial quartz (Unimin Corp., phone 203-966-8880). Size 3.0 (sandblasting equivalent) mesh should be used, the effective grain size of the sand being 0.65 mm. **Soil should not be used**, as this may contain micro-organisms that possess the HUP enzyme and re-oxidize the H_2 released from the nodules. Also, most soils are too dense to allow proper movement of gases from the nodules to the analyzer.

Included in the Q-Box NF1LP package is a flow through chamber for measurements of detached nodules. The package can also be used for measurements of H_2 production or consumption in the range of 0-100ppm of any soil or bacterial samples. Contact Qubit for other flow through chambers of various sizes and for various applications.

It is recommended to read the section "General Introduction to Nitrogen Fixation" of this manual (page 22) before using the Q-Box NF1LP.

Components of Q-Box NF1LP:

Q-G268 Flow Monitor and Pump (0-2L/min)

Q-G267 Flow Monitor and Pump (0-1L/min)

Q-S121 H_2 Analyzer (range: 0-100ppm)

Q-S102 O_2 Analyzer compensated in the software for pressure changes (Range: 0-25% and 0-100%)

Drying column for Q-S121 and S102 with Drierite (blue) (2x) (Q11784)

G113 Flow-through Chamber (1.6 cm ID x 10 cm L)

S132 Stainless Steel Temperature Probe

C301 Event Switch (for marking in the software change from air to Ar:O2 gas)

G122 Large Gas Bag (30 Litres x 2)

G132 Plant Growth and Gas Exchange Pots with connectors, stopper, lid and sealant (4x)

B201 Soybean Seeds and *Bradyrhizobium japonicum* innoculant

Integrated Data Acquisition Interface C610 LabQuest Mini x3

C901 Logger Pro Data Acquisition Software

C404 Customized Setup Software

Q-Box Accessory Kit (includes tubing, filters, luer connectors and T-pieces, wool, wrench for needle valve adjustment, qubitac sealant, screwdriver for calibration of analyzers, 2 x 3-way valves for gas bypass of the pot during reference measurement)

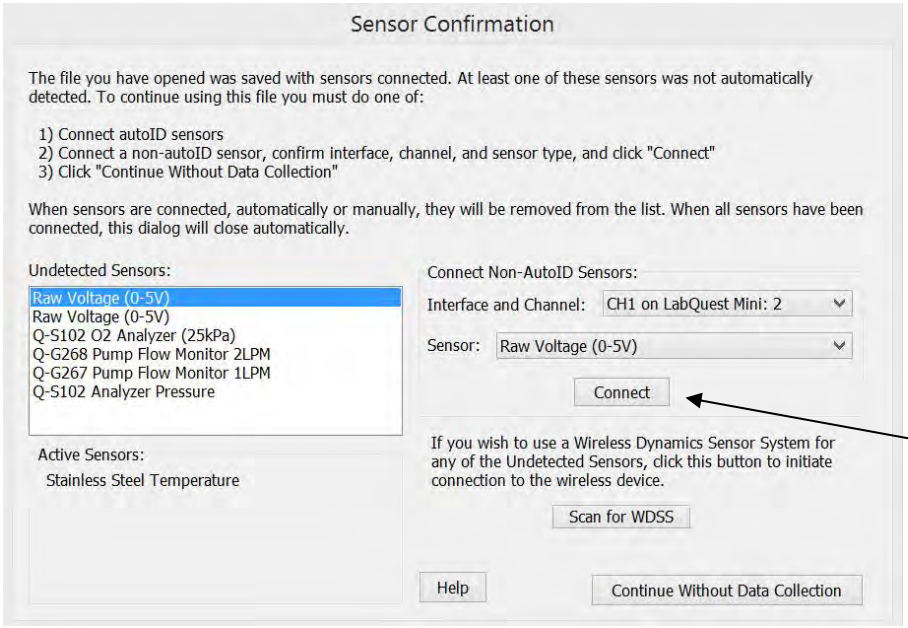
Manual

Individual power supplies for all the sensors (for use in stand-alone mode)

Rugged weather proof case

Quick Start Up Steps:

1. Load Logger Pro 3 software onto the computer (follow instructions on page 26).
2. Load C404 customized software (follow instructions from page 28)
3. Plug the Q-Box into a power supply
4. Turn on the Q-G268, Q-G267, Q-S121 and Q-S102
5. Allow the Q-S121 H_2 analyzer to warm up for at least 1hr with air flowing past it to obtain stable and accurate readings.
6. Connect three USB cables from the Q-Box frame to the computer directly (A USB hub may be required if 3 USB ports are not available on the computer) (may hear 3 audible sounds as the three interfaces are recognized and drivers are loaded). **Connect USB1 first and then USB2 followed by USB3.**
7. Open the "Q-Box NF1LP Setup" file to start Logger Pro software. The following sensor confirmation window will be visible.



8. Assign the undetected sensors to appropriate channels on the 2 interfaces by selecting the sensor and clicking "connect" button as follows:
    a. Ch. 1 LabQuest Mini 1 = Raw Voltage (Q-S121 H_2 Analyzer)
    b. Ch. 2 LabQuest Mini 1 = Raw Voltage (0-5V) (C301 Event switch)
    c. Ch. 1 LabQuest Mini 2 = Q-S102 O_2 Sensor (O2)
    d. Ch. 2 LabQuest Mini 2 = Q-G268 Pump Flow Monitor (V)
    e. Ch. 3 LabQuest Mini 2 = Q-G267 Pump Flow Monitor (V)
    f. Ch. 1 LabQuest Mini 3 = Q-S102 Analyzer Pressure

    Note Ch. 3 LabQuest Mini 1 automatically recognizes the S132 Stainless Steel Temperature Sensor and places it in active sensors list.

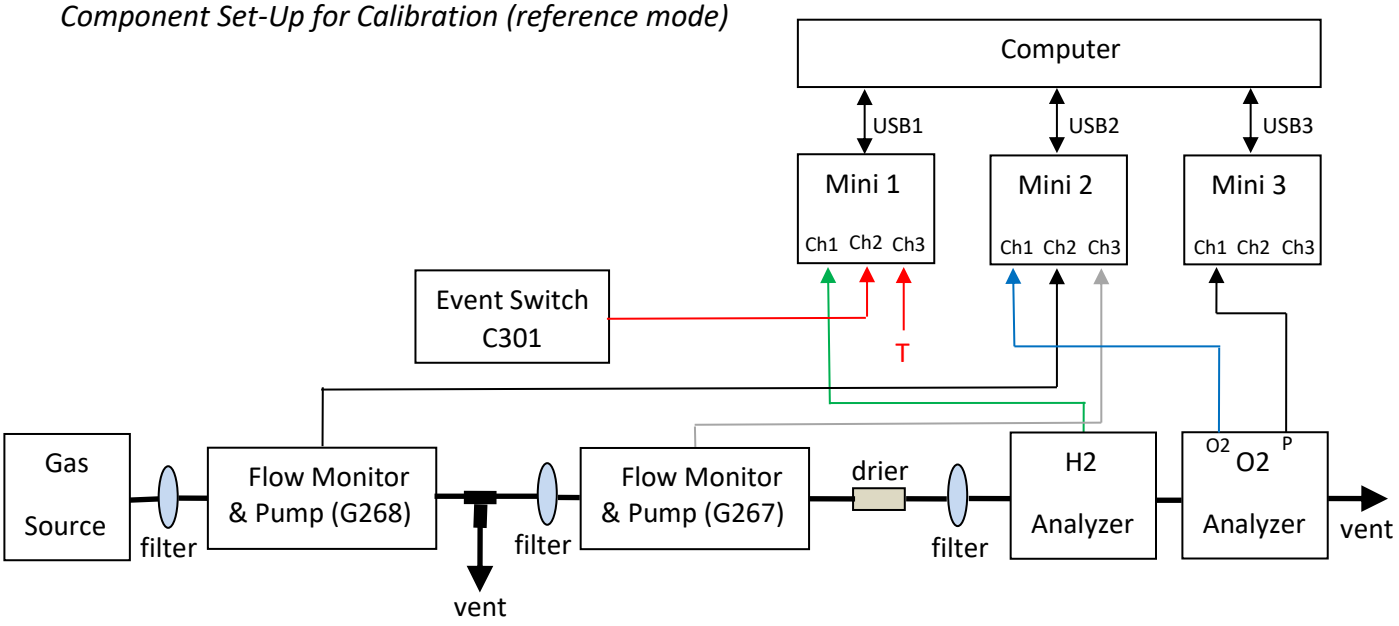
The list of the sensors and their connections is included in a window on Page 1 of the Q-Box NF1LP Setup file for easy reference and is shown below.

Sensor Connections

Mini 1:	Ch1 - Voltage (Q-S121 H2) Ch2 - Voltage (C301; Event Switch) Ch3 - S-132 (T)
Mini 2:	Ch1 - Q-S102 (O2) Ch2 - Q-G268 (Flow Monitor & Pump) Ch3 - Q-G267 (Flow Monitor & Pump)
Mini 3:	Ch1 - Q-S102P (Pres)

9. Check and set up the plumbing of the system by gently removing the tray with all the sensors from the Q-Box and placing it outside of the box with the back of the sensors facing up. The system should be configured for calibration before it is used in experiments.

Component Set-Up for Calibration (reference mode)



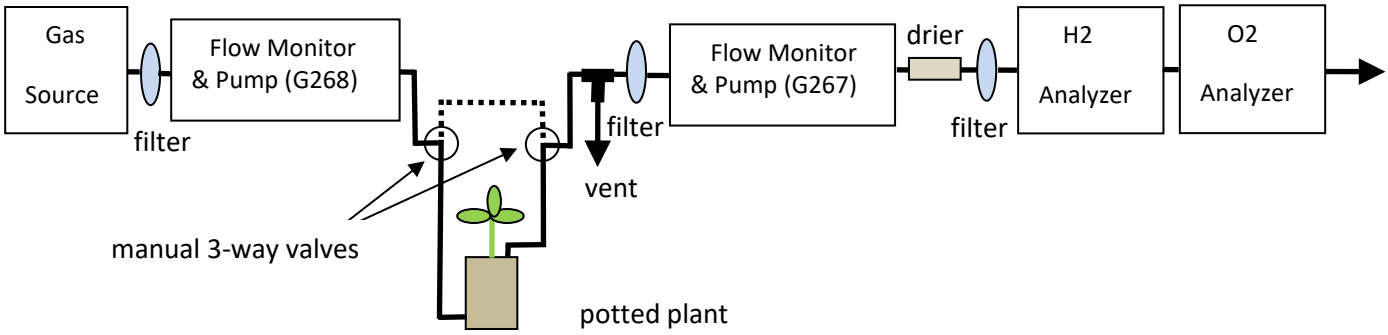
a. The gas supply tubing from the gas source bag or other constant source of H_2 is attached to "PUMP IN" port on the Q-G268 Flow Monitor and Pump via a blue particle filter (25µm).
b. Short tubing from "PUMP OUT" port of the Q-G268 goes to "FLOW IN" port of the Q-G268.
c. Tubing from "FLOW OUT" port of the Q-G268 initially (reference mode) should go to "PUMP IN" port of the Q-G267 Flow Monitor and Pump via a T-Piece. The T-piece allows the extra reference gas to vent while Q-G267 Flow Monitor and Pump subsample this gas **at a lower flow rate (suggest 100ml/min) than that of Q-G268** for supply to the gas analyzers.

Back of Flow Monitor and Pump

d. Short tubing from "PUMP OUT" port of Q-G267 is connected to "FLOW IN" port of the Q-G267 and the tubing from "FLOW OUT" port is connected via a drierite column and a blue filter to the "GAS IN" port of the Q-S121 H_2 Analyzer.
e. (Note that only one drying column needs to be used when QS121 and QS102 are used in series. Gas should be well dried before entering the H2 analyzer. A second drying column is provided for stand-alone use of the Q-S102 or for quick change when first one is spent)
f. From "GAS OUT" port of the Q-S121 H_2 Analyzer, short tubing attaches to "GAS IN" port of the Q-S102 O_2 Analyzer.
g. Gas vents from the O_2 analyzer via the "GAS OUT" port.
h. Three particulate filters (blue) should be placed in the gas line, one before each "PUMP IN" port of Q-G268 and Q-G267 and one before the inlet into the H_2 analyzer to prevent particulate matter from entering the system or the analyzers.

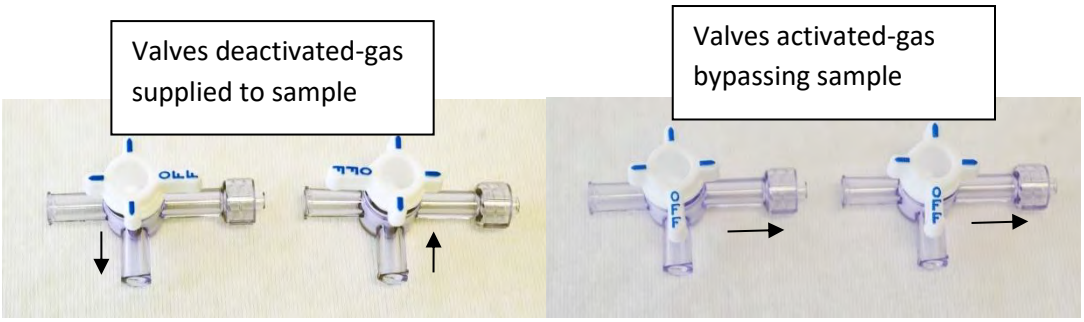
10. After initial calibration and a check of the system, configure the Q-Box for measurements of Hydrogen evolution from the plant pot in an open-flow system:

Component Set-Up for Sample Measurements in Open-Flow System

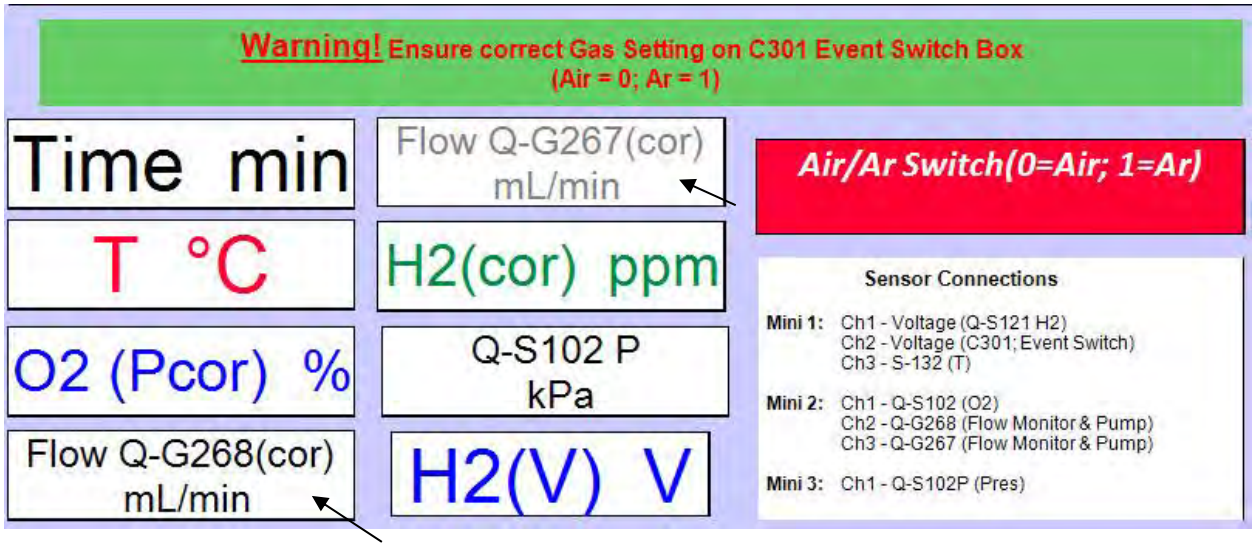


a. Maintain all connections as in the reference mode except the tubing from "FLOW OUT" port of the Q-G268 should be connected to the gas inlet at the bottom of the sealed plant pot (See instructions on how to seal the plant pot for gas exchange measurements on page 16).
b. Attach the tubing from the lid of the pot (gas outlet of the pot) to the "PUMP IN" port of the Q-G267 via a T-Piece. The blue filter should be placed right in front of the "PUMP IN" port of the Q-G267 to prevent any sand particles from the pot entering the system. The T-piece will allow the excess gas from the pot to vent to the atmosphere while the gas pump of the Q-G267 sub-samples the gas and delivers it to the Flow Monitor of the Q-G267 and the analyzers.
c. The rest of the connections remain as described for the reference mode above.
d. Note that the blue particulate filters should be checked regularly and cleaned or replaced as they may get clogged over time.

Note: The manual 3-way valves are installed as in the above diagram to direct the flow of gas either through the pot to obtain a measurement, or to bypass the pot to obtain reference levels measurements.



11. Set the flow rate through the system. Open the "Q-Box NF1LP Setup" file so the Q-G268 and Q-G267 meters are active and visible (see image below).



Please ensure that the event switch Is set in the correct position (Air at the start of the experiment) as the Flow rate displayed in the meters is corrected for the background gas. Set the flow of gas to the plant pot with the Pump of the Q-G268. This flow rate depends on the H_2 evolution rates of the plant and initially may be set around 200ml/min. Once the H_2 evolution rate is detected, this flow may be adjusted accordingly to either increase or decrease the H_2 signal. When the flow through the pot is set, then the flow rate of the sub-sampling pump of the Q-G267 is set and it should be less than the flow through the pot (i.e. if the flow through the pot is 200ml/min, the flow to the analyzers could be 100ml/min). The excess gas will vent through the T-piece placed before the Q-G267. The flow rates of gas entering the Q-S121 and Q-S102 analyzers should be at least 5-10ml/min and should not exceed 650ml/min and it should be maintained constant through the experiment.

The flow rate of the pumps in both Q-G268 and Q-G267 is set using the two needle valves on the back of the instrument. It is recommended to use the valve across the "IN" ports first to bring the flow half way to the desired value (as displayed in Logger Pro) and use needle valve across the "OUT" ports to adjust the flow down to the final rate. Turning the valves counter clockwise

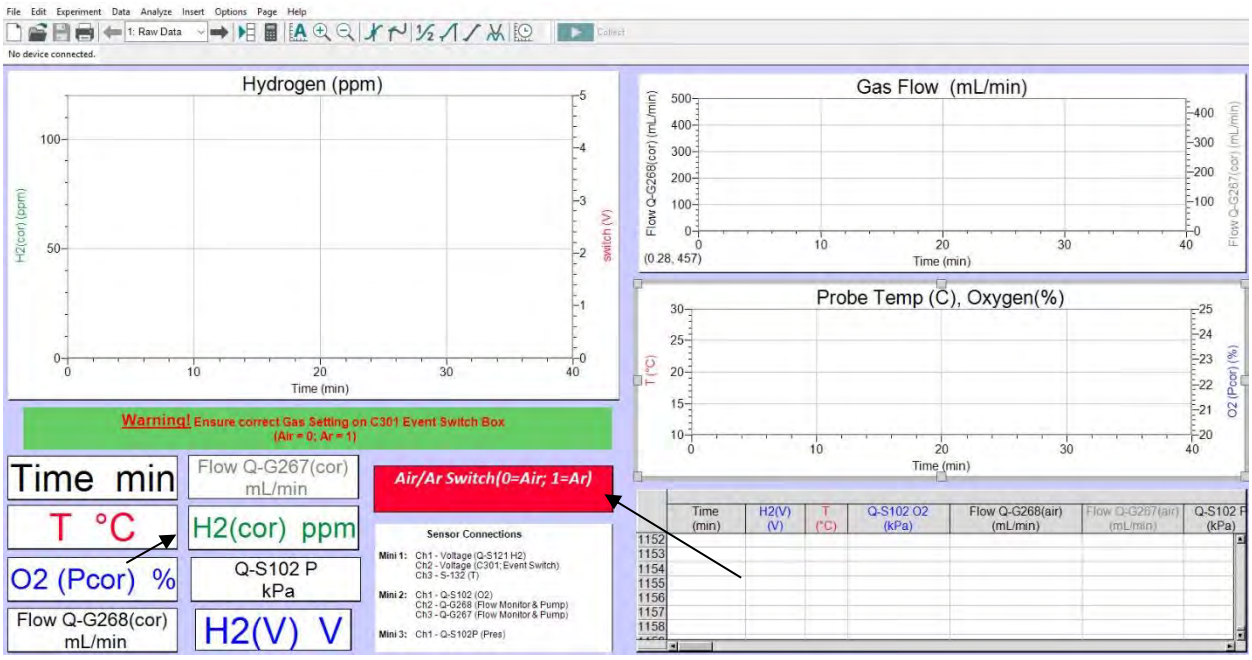
increases the flow and clockwise reduces it. Once the flow is adjusted to the desired rate, use the small wrench provided in the accessory pack to lock the valves in place to avoid accidental changes in the flow rate during experiments. Note that flow rate is recorded in software, so any changes will be recorded. The tray holding all the sensors can then be placed back in the Q-Box.

12. The Q-G268 and Q-G267 Flow Monitors plus S132 Temperature Sensor are factory calibrated and no additional calibrations are required. The Q-S121 H_2 Analyzer and Q-S102 O_2 Analyzer have also been factory calibrated but should be checked at the start of each day of experiments and recalibrated or adjusted if necessary. The Q-S102 Oxygen Analyzer requires only 2 point calibration as its output is linear. The first point should be zero (i.e. N_2) and the second point should be a standard O_2 concentration within the range at which the analyzer will be used. The Q-S121 H_2 Analyzer has a non-linear output and the factory calibration employs a 5^{th} level polynomial equation that has been incorporated into the software. The response of the Q-S121 H_2 analyzer is affected by the background gas, oxygen concentration and to a lesser degree, the flow rate past the analyzer. The Q-Box NF1LP Package has been designed for use at Air levels of oxygen concentration (21%). The software corrects the response of the analyzer for changes in the background gas from air to Ar:O_2 mixture (at 21% O_2) **if the event switch C301 is correctly set** (see instructions below on page 11). For optimal results the calibration check of the H_2 analyzer should be done daily in air or a N_2:O_2 79:21 mixture. Also small adjustments of the "H_2 zero" and "H_2 Span" should be done as part of the daily calibration check that involves 2 points, zero and a known H_2 standard within the range of 0-100ppm. If the H_2 Analyzer is to be used at O_2 concentrations different from 21% a custom calibration of the analyzer needs to be performed at each of the O_2 concentrations and the polynomial equation for each of the O_2 concentrations needs to be incorporated into the software. This is beyond the scope of this package. Contact Qubit Systems for information on the components required for custom calibration at different O_2 levels.

13. **Calibration check of the Q-S121 H_2 Analyzer** (two point calibration):

a. The Q-S121 H_2 analyzer should be plugged in and warmed up with air flowing past the sensor for at least an hour before use. The calibration check should be done in Air and at the flow rate past the H_2 analyzer as set with the Q-G267 that will be used in the experiment. The response of the H_2 Analyzer is optimized by a brief initial exposure to high H_2 concentration (0.5 to 1% H_2 is ideal to prime the sensor but if only calibration gas such as 100ppm is available it will be sufficient). It is therefore recommended to flush the H_2 sensor with high H_2 concentration for a couple of minutes before proceeding with the calibration.

b. Ensure that the Logger Pro software is running during the calibration check. Open the "Q-Box NF1LP Setup" file and after sensor selection as described above on pg. 5and proceed with calibration.



c. Once the sensor has been warmed up and primed, supply H_2 free –air to the analyzer by attaching the bag with zero H_2 gas to the "PUMP IN" port of the Q-G268. Note that room air levels of H2 gas are not zero. Observe in the Logger Pro software on Page 1 of the "Q-Box NF1LP Setup" file the H2 ppm signal (shown in the meter, left hand bottom corner). H2 ppm signal is the signal corrected for the background gas (either air, which is 79% N_2 and 21%O_2, or Ar:O_2 (79:21) mixture) as long as the C301 Event switch is in correct position. **Note that the C301 switch has to be set manually by the user according to what background gas is being used**. When the switch is in Air or Ar position the software will apply the correct calibration to H2 signal and correct H2 levels will be displayed in the "H2 (cor) ppm" meter or in the Hydrogen (ppm) graph. When Air is selected, the "Air/Ar

Switch" in software (left hand bottom corner) will display 0. When Ar is selected 1 will be displayed. (See page 31 for more details). If the H2 ppm reading is 0.0 (+/-0.1) no adjustments are needed to the H_2 Analyzer at this point.

d. If the H2 ppm reading in the software is different from 0.0 use the "**H_2 Zero**" potentiometer on the front of the Q-S121 H_2 Analyzer to bring the reading to 0.0. Turning the potentiometer clockwise with the small green screwdriver provided will increase the signal and counter clockwise it will decrease the signal.

e. Once the Zero H_2 has been established (about 5 min), proceed to the second point in the calibration check. Attach a gas bag with a known H_2 concentration, for example 100ppm (and the same background gas) in the expected range to be measured to the "PUMP IN" port of the Q-G268.

f. Wait until the H2 ppm reading in the software becomes stable (about 5 min). If the reading in the software matches the H_2 concentration of the standard gas, no further action is required. If the reading in the software is different, use the "**H2 Span**" potentiometer control on the front of the instrument to adjust the reading in the software to that of the standard gas. Use the green screwdriver provided to adjust the "H2 Span" potentiometer clockwise to increase the signal and counter clockwise to decrease the signal.

g. If adjustment to the "H2 Span" was made, it is advisable to go back to the zero H_2 check and make sure that the zero reading on the analyzer has not shifted.

h. The H_2 Analyzer is now ready for measurements. It is advisable to keep the H_2 analyzer powered continuously if used regularly so it stays warmed up and maintains its calibration.

Please note that if the H2 analyzer will be used at other O2 concentrations than 21% then a full calibration of the analyzer needs to be done at that O2 concentration. A gas mixing system will be required for such a calibration or precise calibrated gas pumps like wosthoff pumps will be required to provide a whole range of H2 concentrations at that O2 level.

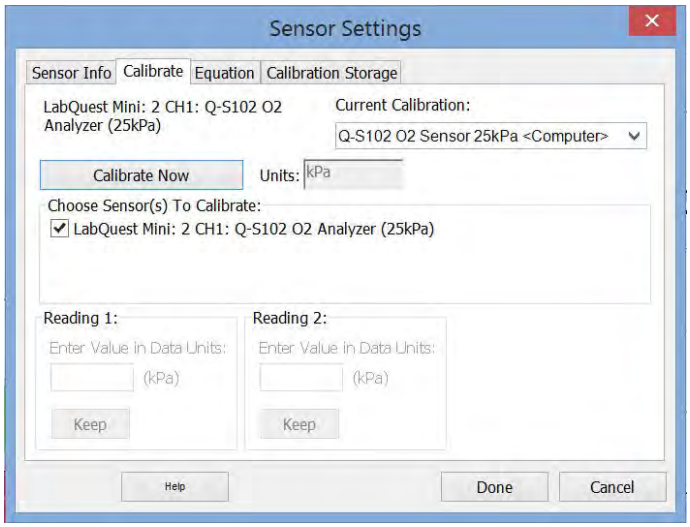
14. **Calibration and check of the Q-S102 O_2 analyzer** (2 point calibration):

Note the Q-S102 analyzer measures partial pressure (kPa) of O_2 in a gas, not concentration (%) of O_2 in the gas. Only at a pressure of exactly 100kPa does the O_2 partial pressure correspond to % O_2. The partial pressure of O_2 will follow atmospheric pressure changes whereas the concentration is constant and independent of pressure variation. The O_2 analyzer also measures total gas pressure in the system. Correction of the O_2 signal for pressure changes is done in software according to changes in total gas pressure to produce **O_2(Pcor)** reading in % units. These corrected readings (see calculation below) are displayed in the software in a meter and in the graph.

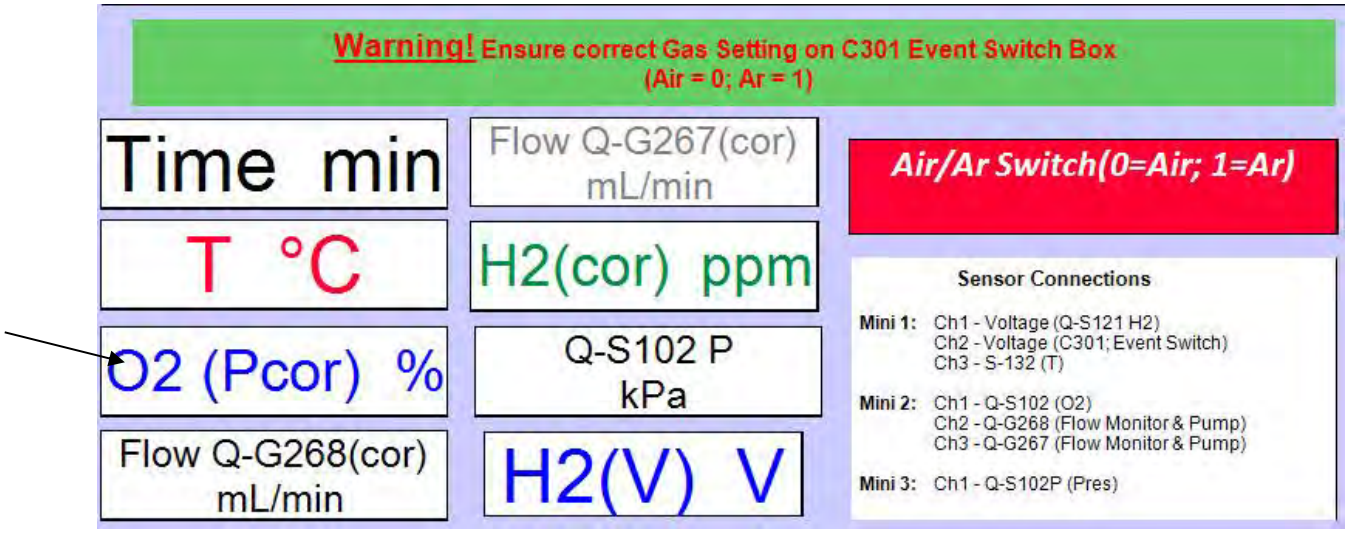
$$O2\ (Pcor) = ((Q\text{-}S102\ O2\ )/(Patm))*100$$

Q-S102 O2 is the recorded O_2 partial pressure, and *Patm* is the (total) gas pressure (i.e. atmospheric pressure) as measured by the pressure sensor of the Q-S102 O2 analyzer.

15. To check and adjust the calibration of the Q-S102 follow these steps:
    a. Set the Q-S102 O_2 analyzer in the 25% range (the default range for measurements with this package).
    b. In the software select from top menu ***Experiment>Calibrate>LabQuest Mini 2 Ch1>Q-S102 O2 Analyzer (25KPa)***



c. Supply O_2 free – gas (i.e. N_2 gas) to the analyzer by attaching the O_2-free gas source (gas bag) to the "PUMP IN" port of the Q-G268. As the O_2-free gas enters the Q-S102 O_2 Analyzer, the reading in the software on Page 1 - **O2 (Pcor)** will decrease toward zero (the first point of the calibration)



d. When the reading in the software, O2 (Pcor), stabilizes, use the small green screwdriver provided to adjust the "O_2 Zero" potentiometer on the Q-S102 O_2 analyzer to **set O2 (Pcor) in the software to read 0.0 %**.

e. Once the zero O_2 is established, proceed to the second point in calibration. Attach the gas bag with known O_2 concentration or a dry room air source (contains 20.95% O_2) to the "PUMP IN" port of the Q-G268.

f. When the **O2 (Pcor)** reading in software stabilizes, adjust the "O_2 Span" control on the Q-S102 O2 analyzer until the reading of **O2 (Pcor)** in the software corresponds to the O_2 concentration supplied to the analyzer.

g. If a significant adjustment had to be made to "O_2 Span", it is advisable to go back to the zero check and make sure that the zero reading on the analyzer has not shifted. It is not necessary to do the zero check while in the calibration mode of the software.

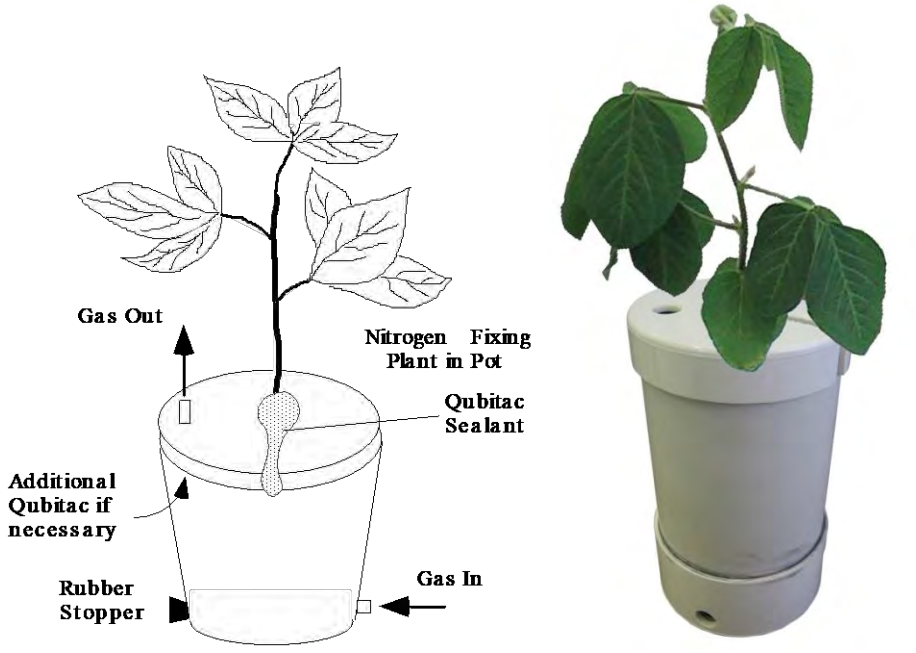
16. The Q-Box NF1LP system is now ready for use with the plant or a flow through chamber containing detached nodules or other H_2 evolving samples. See instructions below on preparing the plant pot for root gas exchange measurements.

17. For detached nodule gas exchange measurements, assemble the flow through chamber by placing the temperature probe in the opening of one of the end caps as shown. If the temperature probe requires additional sealing around the opening, use the blue Qubitac sealant provided in the Q-Box accessory kit.

18. Place the nodules in the chamber and attach the chamber to the system in place of the plant pot as shown in item #10 above for an Open-Flow system. Placing the nodules on a moist tissue paper will prevent them from drying out.

19. **Preparing the Plant For the Gas Exchange Measurements**:

The pots supplied with the Q-Box NF1LP Nitrogen Fixation Package are ideal for the growth of soybeans and other legumes (beans, peas, lupins etc.) for use in gas exchange experiments. Additional pots may be purchased from Qubit Systems. Plants need to be grown in an inert medium such as silica sand for best results in gas exchange experiments. ***See the included instructions for cultivation of plants for gas exchange.*** It is important that the root system is sealed well in the pot for gas exchange measurements.



The base of the pot contains two inlet holes. One hole is used to attach the tubing from the Q-G268 Flow Monitor and Pump (via the use of a hollowed stopper fitting). The drainage hole on the other side of the pot should be plugged with a rubber stopper. The lid of the pot has a slot to accommodate the stem of the plant and the temperature probe (S132). The outlet hole in the lid attaches, via tubing and the T-Piece assembly to the "PUMP IN" port of the Q-G267 Flow Monitor and Pump. Before placing the lid over the pot, ensure that the edge of the lid is lined with a thin layer of the blue Qubitac sealant, so when it is pressed against the edge of the pot a gas tight seal will be formed.

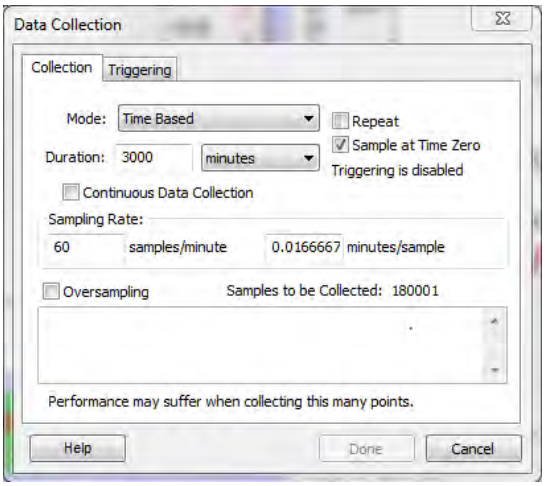
The lid snaps down to seal around the perimeter of the pot while the stem of the plant and the temperature probe are positioned in the slit of the lid. The slit in the lid must then be sealed from the outside with the blue Qubitac sealant to render it gas-tight. Seal the slit by placing a long piece of Qubitac around the plant stem and over the perimeter of the slit and press the Quibitac together and gently down against the lid to seal the plant in the pot.

Ensure that the Qubitac is tight around the stem so no gas leaks are formed. Once the gas is being supplied to the pot through the bottom gas inlet, check that gas is coming out of the pot through the gas outlet in the lid. When the T-piece is attached to the gas outlet from the lid and the "PUMP IN" port on the Q-G267, check that excess gas is venting through the T-piece outlet.

IT IS EXTREMELY IMPORTANT TO PROPERLY SEAL THE POT TO PREVENT LEAKS

Note that the blue Qubitac sealant can be reused many times after some cleaning and stretching to remove sand particles from previous use.

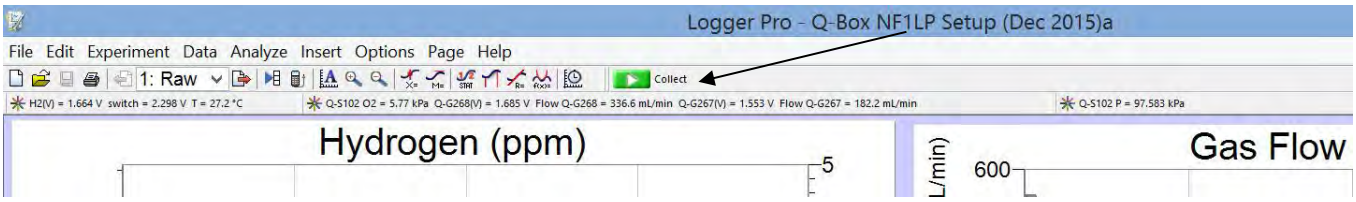
20. Before starting to collect data with Logger Pro software, select ***Experiment > Data Collection*** in the main menu or click on the clock icon. The following dialog box will appear:



Input experiment length and data sampling rate as required. The default rate is 60 samples per minute. Click "Done".

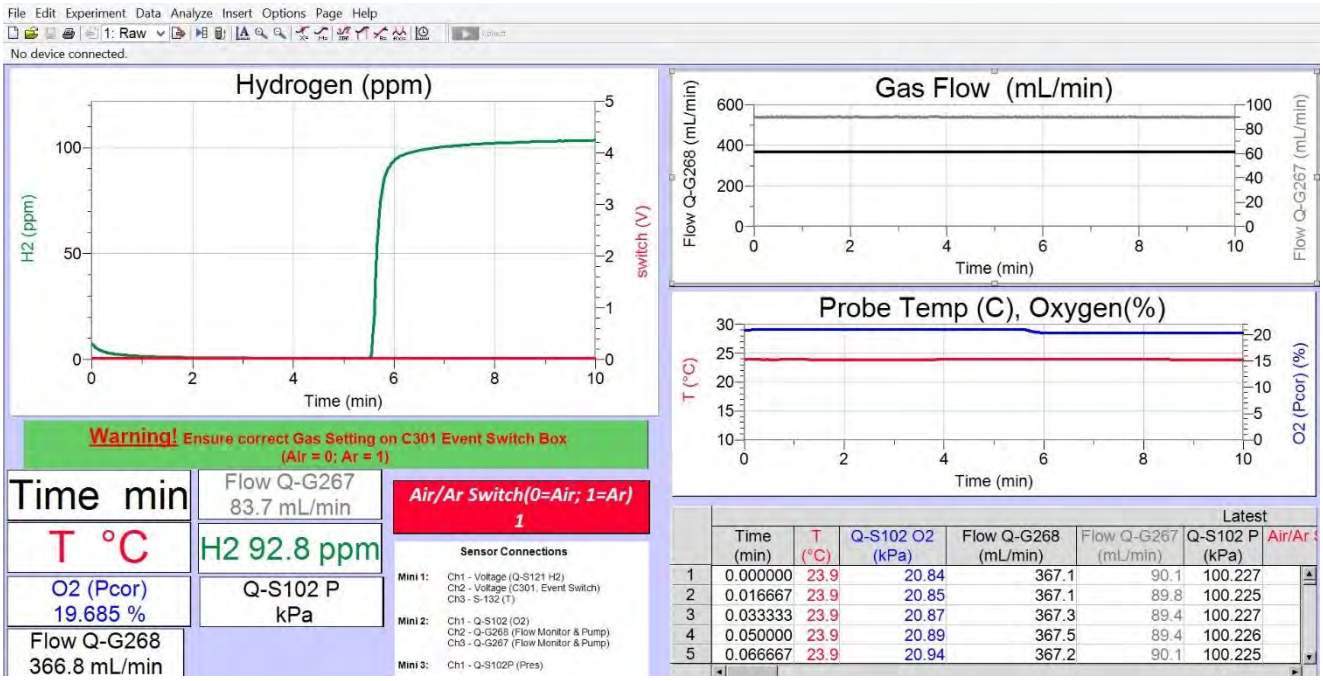
Select: ***File> Save As*** to save the experiment settings under a new File Name so that the original set up file (Q-Box NF1LP Setup) is not over- written.

Start data collection by clicking the green button "Collect"

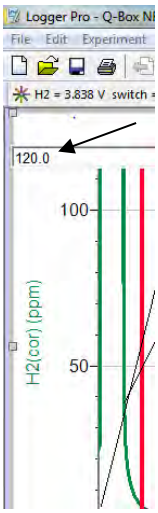


21. Stop data collection by clicking the red button “Stop” (“collect” button turns into “stop” button during data collection),

22. Logger Pro software displays data on Page 1 “Raw Data” as they are collected in graphs, meters and spreadsheet columns. Sample data are shown below



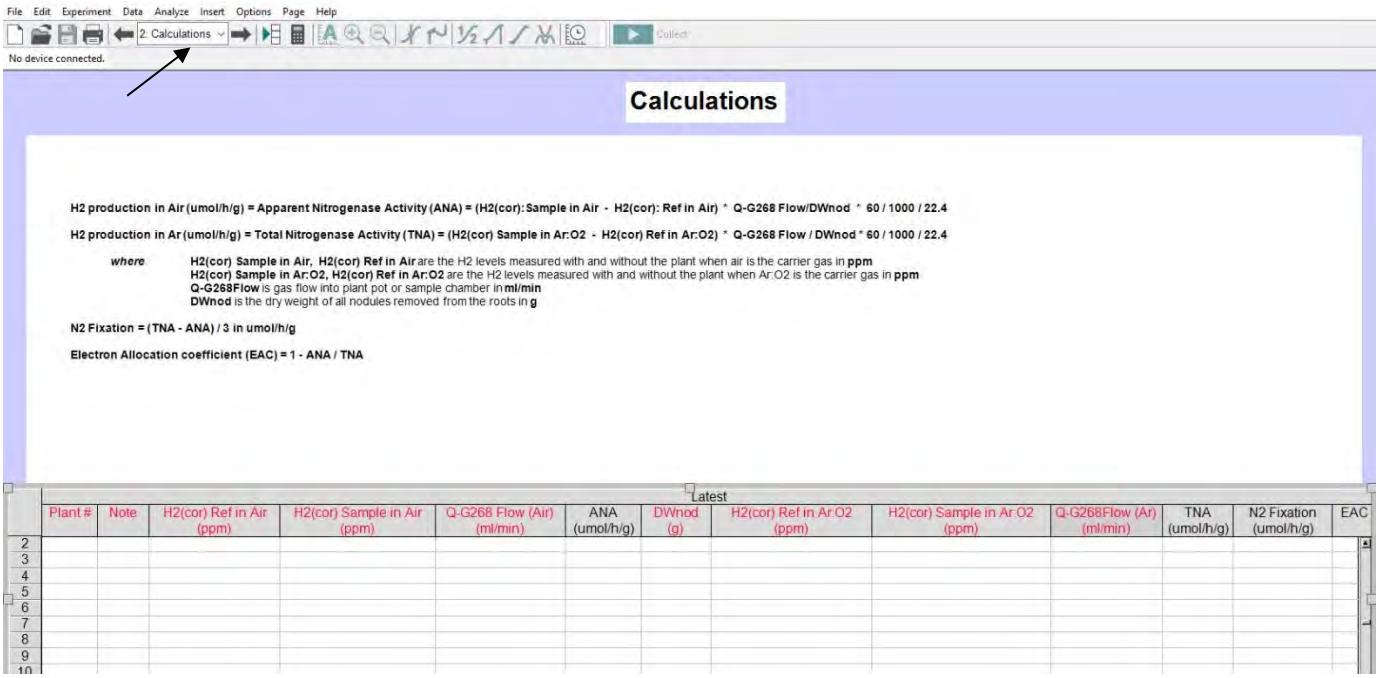
23. During the experiment, the x and y axis ranges can be adjusted by clicking on the lowest or highest number and typing in the desired new value.



24. Upon completion of the experiment, the data can be analyzed directly in Logger Pro using the various analysis tools in the ***Analyze*** menu, or using icons selected in the top menu bar. Further calculations can be done directly in Logger Pro on Page 2 called "Calculations". The data can also be directly exported as a text file (***File > Export As > CSV***), which can then be opened in Excel for further analysis and calculations.

Calculations of Nitrogenase Activity and Nitrogen Fixation rates

To fully understand the calculations, read page 22, "General Introduction to Nitrogen Fixation".
Upon completion of an experiment, the raw data can be analyzed on Page 2 of the Q-Box NF1LP Setup file – "Calculations". On this page data can be entered in the columns highlighted in red, and calculations are done automatically in the black columns.

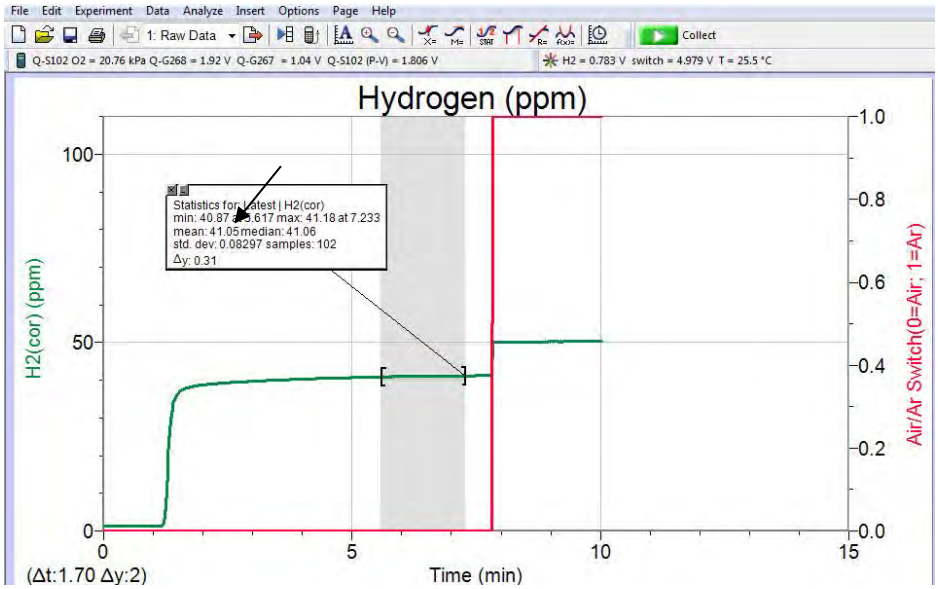


H2(cor) Ref in Air is the value of corrected H_2 signal in ppm measured in Air during a reference run (no pot attached to the gas exchange system or 3-way valves set to bypass the pot). **H2(cor) Sample in Air** is the corrected H_2 signal in ppm with the plant attached to the system. The difference between these two values equals the H_2 evolved by the root system of the plant in Air. **Q-G268 Flow (Air)** is the flow rate through the pot in ml/min during ANA measurement (in air) and **Q-G268 Flow (Ar)** is the flow rate through the pot in ml/min during TNA measurement (in Ar:O2). Please note that there is a small change in the flow rate when background gas is changed from Air to Ar:O2. The software corrects for this change and displayed corrected flow rates for the both Q-G268 and Q-G267 Flow monitors when the even switch is depressed correctly. **DWnod** is the dry weight of the nodules (detached after the completion of the experiment and oven dried). The software calculates the value of **ANA** or the Apparent Nitrogenase Activity in µmol/hr/gDWnod. Values of **H2(cor) Sample in Ar:O2** and **H2(cor) Ref in Ar:O2** are the corrected H_2 signal in ppm during the exposure of the root system to $Ar:O_2$ and the reference reading in $Ar:O_2$ without the plant in line (or pot bypassed by 3-way valves), respectively. These values are used in calculation of Total Nitrogenase Activity (**TNA**) by the software. From ANA and TNA, values the software calculates the rate of Nitrogen Fixation and the Electron Allocation Coefficient (**EAC**) according to the following equations:

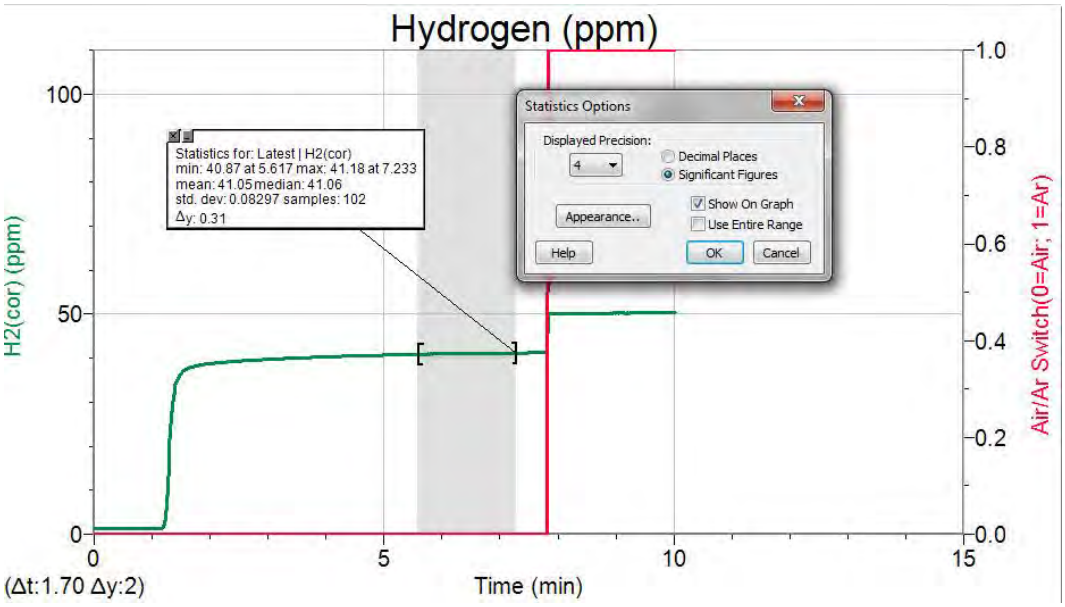
$$\mathbf{N_2\ Fixation\ Rate} = \frac{(\mathbf{TNA - ANA})}{\mathbf{3}}$$

$$\mathbf{EAC} = \mathbf{1} - \left(\frac{\mathbf{ANA}}{\mathbf{TNA}}\right)$$

To obtain the raw data for the calculations of ANA and TNA, numerous data analysis tools of Logger Pro can be used. These are displayed in the top menu as individual icons. Data can be selected on each graph by dragging the mouse over the portion of interest on that graph. When the data have been selected, the appropriate analysis tool can be chosen i.e. Statistics function to obtain mean or average data over a period of stable readings (see example below).



The statistics will then be shown in a separate window for the selected data on each of the graphs. The display of the values in the analysis window can be adjusted in the options window by right clicking on the statistics window and selecting the number of decimal spaces to be displayed.



The mean values for each of the parameters can then be copied from the stats windows into the calculation page in the appropriate red columns. **This has to be done manually by the user**. Once the red columns with the input parameters are filled, the calculation columns (black) will fill automatically with the calculated values.

General Introduction to Nitrogen Fixation

Biological N_2 fixation is a process of fundamental importance to life on earth. Annually, 200 million tons of nitrogen is added to the soil by nitrogen fixing organisms - 4 times the amount provided by inorganic fertilizers. Most of the nitrogen entering the environment by fixation is derived from bacteria that form symbiotic associations with certain plants. It has been known for centuries that these plants (especially legumes such as clover and alfalfa) improve the yield of other crops when they are grown with them in rotation.

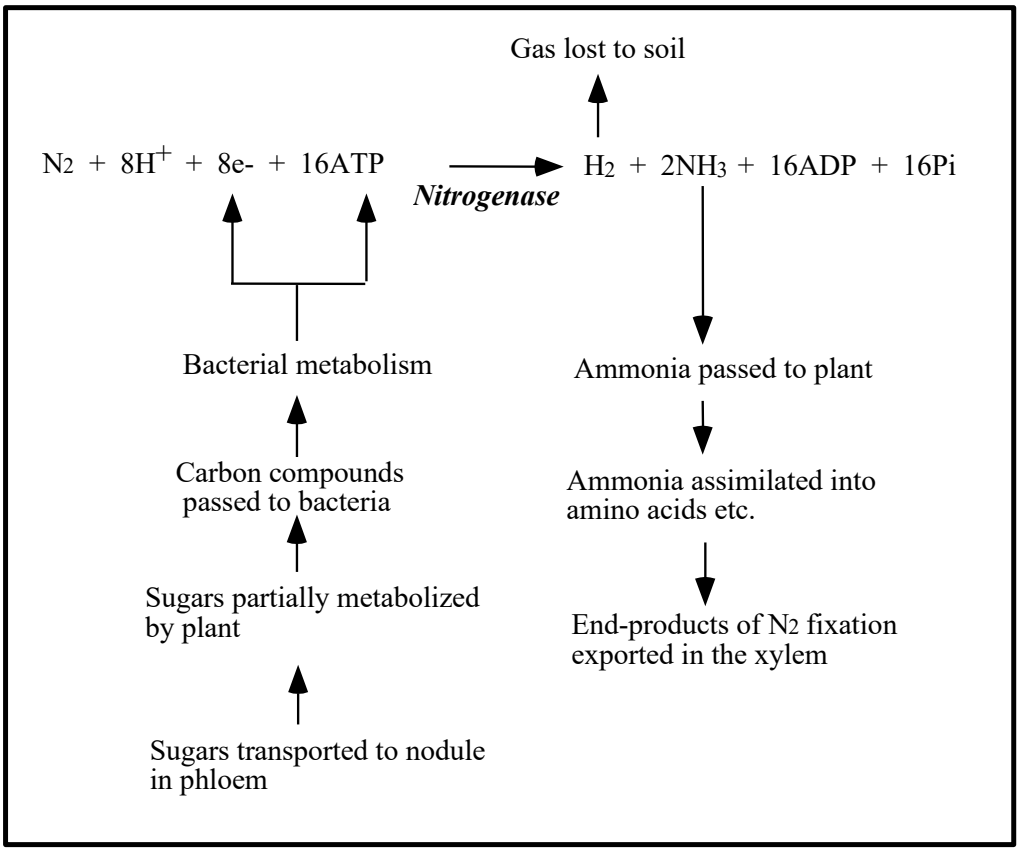
Biological N_2 fixation in leguminous plants requires the development of a symbiotic relationship between soil bacteria and the plant root. The most common endosymbionts found in the roots of crop legumes are bacteria of the genera *Rhizobium* and *Bradyrhizobium*. An extremely complex recognition process has evolved between the plant and bacteria, so that the interactions between the plant and the bacteria are species specific. The Q-Box NF1LP Nitrogen Fixation Package includes soybean seeds and *Bradyrhizobium japonicum* inoculants for studies of N_2 fixation and nitrogenase activity in this Soybean x *Bradyrhizobium* symbiosis.

As in all legume symbioses, *B. japonicum* inhabits swollen outgrowths of the soybean root called nodules. The root nodule can be subdivided into specific zones. The outer periderm consists of loosely packed cells and lenticels through which gases diffuse into the outer cortical layer. These gases include the N_2 required for N_2 fixation, as well as O_2 required for plant and bacterial respiration. The cells of the outer cortex are also loosely packed, and contain large air spaces, which offer little resistance to the diffusion of gases. However, in the inner cortex, the cells are smaller and much closer together, and here the gases may have to diffuse through the contents of the cortical cells to reach the central zone of the nodule, since open intercellular spaces are infrequent. This part of the nodule is thought to act as a barrier to gas diffusion. The central zone of the nodule contains plant cells that are infected with thousands of bacteria. In their symbiotic form, these bacteria are called bacteroids, and a typical soybean root may provide a home for 2,000,000,000 of these bacteroids.

The bacteroids are responsible for reducing the N_2 gas that diffuses into the central zone to ammonia, which can then be assimilated by the plant cells. The diagram below shows the exchange of materials that must occur between the plant and the bacteroids to allow for the fixation of N_2 and the assimilation of ammonia. A carbon source from the plant is supplied via the phloem to root nodules housing the N_2 fixing bacteria. The carbon source is partially metabolized by the plant and the resulting carbon compounds are imported into the bacteria. The bacteria contain the enzyme nitrogenase, which is responsible for catalyzing the reduction of N_2 gas to ammonia.

The carbon compounds entering the bacteria are metabolized to produce the ATP and reductant required in the nitrogenase reaction. The ammonia produced in the reaction is transported to the plant where it

is assimilated into organic nitrogenous compounds. These are exported in the xylem to the rest of the plant where they are used in the synthesis of amino acids, nucleic acids and other nitrogen-containing compounds.



Measurement of Nitrogenase Activity

Nitrogenase is a promiscuous enzyme that is capable of reducing a wide range of substrates. The traditional method of assaying nitrogenase activity is by the acetylene reduction assay, in which nodulated roots are supplied with 10% acetylene. This gas is reduced by nitrogenase to ethylene, and the amount of ethylene produced per unit of time is measured by gas chromatography. This assay is dangerous to perform, since 10% acetylene is explosive, and it uses a non-physiological substrate for the nitrogenase reaction. A better assay is to measure the H_2 that is produced as an obligate by-product of the N_2 fixation reaction:

$$\mathbf{N_2 + 8H^+ + 8e^- + 16ATP \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16\,(ADP + Pi)}$$

Many *Rhizobium* and *Bradyrhizobium* species possess an enzyme called uptake hydrogenase (HUP), which re-oxidizes the H_2 produced in the nitrogenase reaction. However, a great many other *Rhizobium* and

Bradyrhizobium species lack this enzyme, and the H_2 produced within the nodule diffuses out into the soil. The Q-Box NF1LP package includes the inoculum (black powder) with a bacterial species (*B. japonicum*) that lacks HUP and therefore evolves H_2. The rate of H_2 evolution from the nodules provides a measurement of nitrogenase activity. The great advantage of measuring nitrogenase activity by H_2 evolution, is that measurements can be made with minimal disturbance of the plant tissue.

Measurement of H_2 production from nodules provides only a measurement of ***Apparent Nitrogenase Activity*** **(ANA)**. This is because of the 8 electrons passing through nitrogenase in the reaction shown above, a minimum of 2 of these are used to produce H_2, while the others are used in the reduction of N_2 to ammonia (NH_3). H_2 production rate provides only a measurement of that proportion of the enzyme activity that is being used to reduce protons, and while this must represent at least 25% of the total enzyme activity, the allocation of electrons between N_2 and H^+ reduction is variable. To measure ***Total Nitrogenase Activity*** **(TNA)**, it is necessary to replace the N_2 in the atmosphere surrounding the nodule with an inert gas such as Ar. When this is done, all the electrons that were previously allocated to N_2 reduction are used for H^+ reduction and the rate of H_2 evolution from the nodule increases rapidly. The maximum rate of H_2 evolution from the nodule after a switch from a N_2:O_2 atmosphere to an Ar:O_2 atmosphere represents TNA. To determine the rate of N_2 fixation that was occurring under initial conditions the following equation is used:

$$\mathbf{N_2\ Fixation\ Rate} = \frac{(\mathbf{TNA - ANA})}{\mathbf{3}}$$

A denominator of 3 is used because reduction of N_2 to NH_3 requires 3 electron pairs, whereas reduction of H^+ to H_2 requires only 1 electron pair.

As stated above, in air at least 25% of total electron flux through nitrogenase is used for H^+ reduction but this value is not constant, and often more than 25% of electron flux through nitrogenase is "wasted" in H_2 production. To determine the relative allocation of electrons between H^+ and N_2, the ***Electron Allocation Coefficient*** of nitrogenase (EAC) may be calculated:

$$\mathbf{EAC} = \mathbf{1} - \left(\frac{\mathbf{ANA}}{\mathbf{TNA}}\right)$$

EAC is an important parameter, because higher values of EAC indicate that a greater proportion of nitrogenase activity is being used for the production of nitrogenous products for export to the plant.

The Regulation of Nitrogenase Activity

The nitrogenase reaction requires a great deal of energy, consuming at least 16 ATP and 4 pairs of electrons for every molecule of N_2 reduced to ammonia. This energy is derived from carbohydrates provided by the plant, and nitrogenase activity therefore represents a drain on the plant's resources. To ensure that nitrogenase functions with benefit, rather than detriment to the plant, regulatory

mechanisms have evolved to reduce, or to halt, nitrogenase activity when the plant is supplied with an adequate source of combined nitrogen. Since carbohydrate supply to the nodule is essential to sustain nitrogenase activity, it is no surprise that any factor, which limits phloem sap supply to the nodule, also causes nitrogenase inhibition. However, the mechanisms by which nitrogenase is inhibited by phloem sap deprivation, or by combined nitrogen, are complex and require an understanding of the regulation of respiratory processes in the nodule as outlined below.

Nitrogenase Activity and Oxygen

The nitrogenase enzyme is peculiar in that to function, it requires ATP derived from oxidative phosphorylation, yet at the same time it is extremely O_2-labile. Therefore, the nodule must have mechanisms for delivering a high flux of O_2 to support respiration in the infected cells, while maintaining the free O_2 concentration in these cells at an extremely low level. It has been estimated that the O_2 concentration in the infected cells of the nodule may be as low as 30 nM, compared to an O_2 concentration of 250 000 nM in the cells of the nodule outer cortex. This low O_2 concentration in the infected cells is maintained by a barrier to O_2 diffusion in the nodule inner cortex, which severely limits the diffusion rate of O_2 to the central zone. Also, the high rate of bacteroid respiration ensures that the O_2 diffusing into the central zone is consumed swiftly and does not accumulate. In fact, the O_2 concentration in the infected cells is so low that O_2 cannot diffuse fast enough by itself to support the demands of bacteroid respiration. To alleviate this limitation, the infected cells contain a red pigmented protein called leghemoglobin, which acts as an O_2 carrier, and facilitates the diffusion of O_2 to the bacteroids. In both its structure and its function, leghemoglobin is very similar to myoglobin in human blood, and it is an example of how plants and animals have evolved similar mechanisms to cope with similar problems. The leghemoglobin in nodules is easily visible, and its red colour clearly delineates the central zone of the nodule from other nodule tissues.

Regulation of the O_2 Diffusion Barrier

It has been shown that the barrier to O_2 diffusion in the legume nodule does not have a fixed resistance, but can vary the degree to which it limits O_2 diffusion into the central zone. If the nodulated root is placed in a low O_2 environment, the diffusion barrier relaxes its resistance to maintain an adequate flux of O_2 to the infected cells. Conversely, when the nodulated root is placed in a high O_2 environment, the diffusion barrier increases its resistance to prevent too much O_2 entering the infected cells and causing nitrogenase inhibition. Although low O_2 environments are common in nature (such as in waterlogged soils), environments containing supra-ambient levels of O_2 are extremely rare. However, the ability of the nodule to respond to high levels of O_2 in the infected cells is very important, since any process which limits the respiration rate of these cells will reduce the rate at which O_2 is consumed and cause O_2 to accumulate. Therefore, under these circumstances the nodule increases its resistance to O_2 diffusion to reduce the flux of O_2 to the central zone. It is this increase in diffusion barrier resistance that is mostly responsible for the inhibition of nitrogenase activity that occurs when nodules are supplied with combined nitrogen, or are deprived of phloem sap.

When combined nitrogen is supplied to the nodulated root, continued nitrogenase activity would represent a waste of the plant's carbohydrate resources. Therefore, a signal is given to the nodule to increase its resistance to O_2 diffusion. This causes a reduction in the rate of infected cell respiration, and insufficient ATP and reductant are generated for the nitrogenase reaction to continue at a high rate. When the supply of phloem sap to the nodule is prevented (e.g. by severing the plant shoot (de-topping), or removing its leaves (defoliation), or by picking the nodules off the plant), the reduced amount of carbohydrate entering the nodule could eventually lead to a reduction in infected cell respiration and a consequent increase in infected cell O_2 concentration. Therefore, a signal is given to the nodule to increase its resistance to O_2 diffusion. This has the combined effect of limiting O_2 entry into the central zone (thereby protecting nitrogenase from inactivation) and of reducing respiration rate (thereby conserving carbohydrates that may eventually become scarce). The O_2 supply to the nodule plays a critical role in the regulation of nitrogenase activity.

Installing and Running Logger Pro 3

PC Users:

(1) To start, a complete copy of Logger Pro 3 must be installed on the computer. Before starting the installation, make sure that all USB cables are disconnected from the computer. Failure to do so may cause an error in the installation of the USB drivers.

(2) Run the installation and do not change the default destination directory. Logger Pro 3 will be installed in C:/Program Files/Vernier Software/Logger Pro 3.

(3) The setup process will automatically load the USB drivers for connecting the LabQuest mini or other interfaces to the computer.

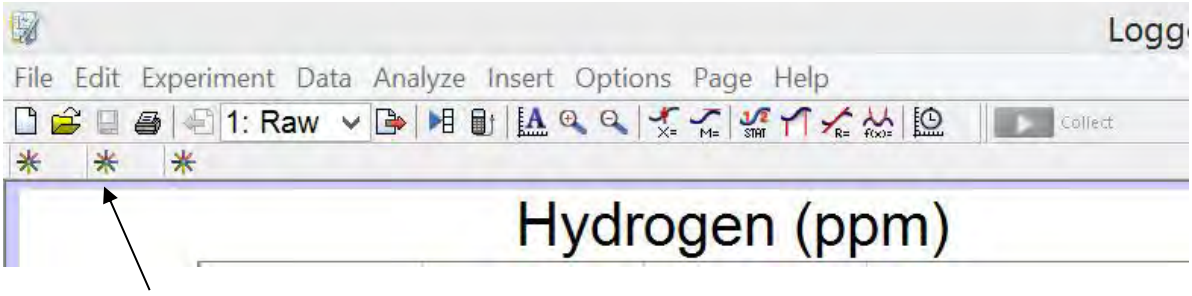
(4) If QuickTime 6 (or greater) is not installed on the computer, it is advised that it be installed when prompted. QuickTime will allow the use of picture and movie features of Logger Pro 3.

(5) You will be prompted to connect the LabQuest mini interfaces to the computer via the USB connection. The LabQuest minis will be connected to the computer by connecting the USB1, USB2 and USB3 cables **(in that order)** from the Q-Box frame into the computer (via the USB hub if necessary).

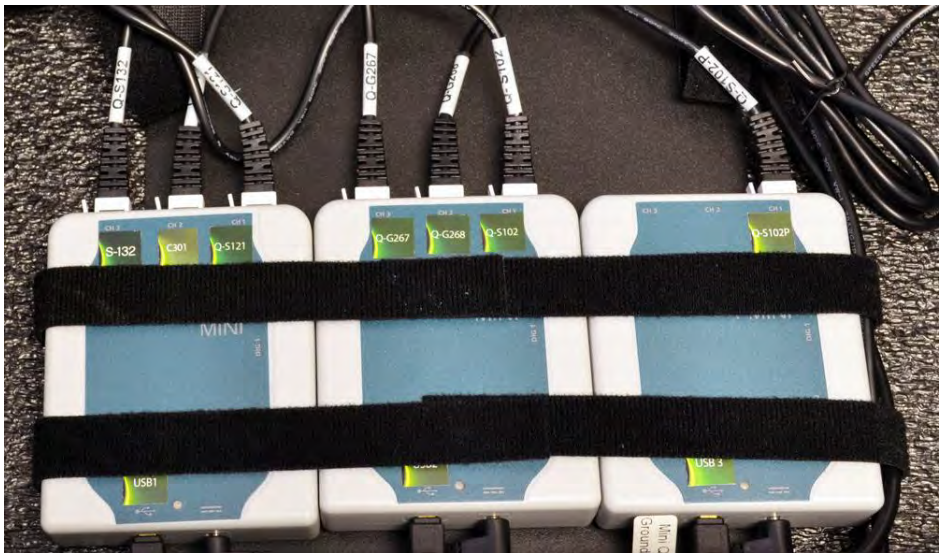
(6) Click 'Finish' to complete the installation process.

(7) Proceed to C404 installation (below) before opening the Logger Pro software with the "Q-Box NF1LP Setup" file.

(8) Double click the "Q-Box NF1LP Setup" file (create a shortcut on the desktop once the file has been moved from the C404 disk) to start Logger Pro and data collection. If Logger Pro detects the 3 interfaces, the Logger Pro screen will appear with three LabQuest Mini stars in the top left side



(9) If Logger Pro cannot detect the Interfaces a message will appear “no device connected” or only one icon will be visible. Check first that the interfaces are connected to the computer via the three USB cables and connections 1, 2 and 3 on the frame of the Q-Box or via the USB Hub if necessary. The LED power lights on the minis should be green. No LED light indicates no power is being supplied to the mini. A red LED indicates that power is on but there is no communication between the mini interfaces and the computer. In this case, exit the “Q-Box NF1LP Setup” file and unplug the USB cables from the computer. Plug in the USB cables 1, 2 and 3 back into the computer (via USB hub if necessary) in that order. Allow the computer to apply the drivers for the interfaces and re-open the “Q-Box NF1LP Setup” file again. Alternatively, one can open the “Q-Box NF1LP Setup” file first, and then plug the 3 USB cables in the correct order one at a time and wit for the 3 stars to appear in the software as each interface is recognized. Then proceed to connection of the sensors as described above.



Macintosh Users:

(1) To start, a complete copy of Logger Pro 3 must be installed on the computer (running at least OS 9.2). Run the “Complete Installation” and make sure that all TI GRAPH_LINK and USB cables are disconnected. The most recent version of Logger Pro (3) is included with this package. Following instructions are the same as for PC users.

C404 Custom Setup Files Installation:

Qubit Systems' C404 Custom Setup Files CD contains Qubit Experiment files designed by Qubit Systems for the package purchased, in this case "Q-Box NF1LP Setup". The Experiment files contain the setup (i.e. graphs, table, calculations etc) for the various experiments, as well as the calibration constants for the Qubit sensors. The C404 disk also contains a manual for this package and the LabQuest Mini data interface. All Files from this disk files can be copied to user specified location on the computer and the experimental file of interest "Q-Box-NF1LP Setup" should be placed in an accessible location or have a shortcut created on the desktop to the file. We highly recommend for the user to make a copy of the original file and keep it in a safe place on the computer in case the original is accidentally altered.

Using the Q-S121 H_2 Analyzer

The H_2 sensor of the Q-S121 H_2 Analyzer is a semi-conductor device incorporating a heated alumina ceramic tube. O_2 binds to the surface of the semi-conductor, and H_2 that comes into contact with it combusts, causing the resistance of the semi conductor to change depending on the concentration of both the O_2 and H_2 present. The output of the H_2 sensor is, therefore, dependent on both the H_2 and O_2 concentrations of the gas that flows through it. The response to H_2 concentration is non-linear, but the Logger Pro "Q-Box NF1LP Setup" file provided with the Q-Box NF1LP N_2 Fixation Package incorporates a 5^{th} order polynomial function so that voltage readings from the sensor at O_2 concentrations of 21% can be converted to H_2 concentrations in ppm. This polynomial function differs depending on the nature of the balance gas (N_2 or Ar). Hence the user needs to manually **indicate which background gas is being used (Air or Ar:O_2 by depressing the C301 event switch** in a correct position. The output of the H_2 Sensor is also affected by the O_2 concentration; however the **Q-Box NF1LP Package has been designed for use at O_2 concentrations close to Air levels of 21%.** If the H_2 Analyzer is to be used at O_2 concentrations different from 21% a custom calibration of the analyzer needs to be performed at each of the O_2 concentrations and the polynomial equation for each of the O_2 concentrations needs to be incorporated into the software. This is beyond the scope of this package. Contact Qubit Systems for information on the components required for custom calibration at different O_2 levels.

The H_2 Sensor of the Q-S121 H_2 analyzer requires heating to operate, therefore, it functions optimally when it is activated for several hours before use. A **warm up time of at least one hour with air flow past the analyzer** is required and if the H_2 Analyzer is to be used regularly over a period of time it is recommended to leave it powered up continuously. The **response time of the H_2 Analyzer is greatly enhanced if the sensor is exposed to a high concentration of H_2** (0.5 to 1% is ideal, but calibration gas of 100ppm will also work well). A brief exposure (2-5min) before daily use is recommended. This can be done by filling a syringe with H_2 gas of an appropriate concentration and injecting the gas into the inlet port of the sensor.

The **H2 sensor is also greatly affected by moisture** so the gas supplied to the sensor needs to be well dried. It is important to check the drying column before using the system and possibly using two in a series to ensure that gas entering the analyzer is dry

Using the Drying Column

The Q-S121 and Q-S102 are designed to analyze dry gas samples. Moisture in the gas can significantly affect the response of the H2 sensor so it is important to dry all gases delivered to the H2 analyzer (both from the nodulated pot and from a gas bag filled with calibration gas from a tank. A desiccant column is provided which is filled with DRIERITE to dry the gas before analysis. The column is supplied ready for use. The column should be mounted **vertically** on the back of the analyzer with "wet" gas entering at the bottom and dry gas exiting from the top.

DRIERITE contains an indicator that is blue when the column is functional and pink when the DRIERITE is spent. When spent, the DRIERITE should be replaced or reconditioned. To recondition, remove the DRIERITE from the column and place it in a drying oven at 210^{o}C for 1 hour, or until the pink coloration disappears. The replacement DRIERITE is #8 mesh, order #23005 from drierite.com.

Magnesium Perchlorate is an alternative drying agent which is more effective than Drierite. However, magnesium perchlorate will turn into liquid with time so it must be changed frequently. It is recommended that a trap (2 PTFE filters are included in the Q-Box accessory kit for this application) is installed downstream if using magnesium perchlorate. **Qubit Systems is not responsible for any damage caused to any components of the Q-Box due to magnesium perchlorate entering any part of the system.**

Using the Q-S102 O_2 Analyzer

The Q-S102 O_2 Analyzer contains an O_2 sensor which is a galvanic cell (a lead-oxygen battery) consisting of a lead anode, an O_2 cathode, and an acid electrolyte. It also incorporates an O_2-permeable Teflon FEP membrane with a gold electrode bonded to its surface. Oxygen diffusing through this membrane is reduced electrochemically at the gold electrode. A resistor and a thermistor (for temperature compensation during diffusion across the Teflon membrane) are connected between the anode and the cathode. The output of the instrument is proportional to the current flowing through the resistor and thermistor. This is, in turn, proportional to the partial pressure of O_2 in contact with the Teflon FEP membrane. The signal from the oxygen sensor is transmitted to the computer via the LabQuest mini interface (i.e. Q-S102 O2) and it is also displayed on the analyzer in kPa. The Q-S102 O_2 Analyzer has two analog signal outputs, one for O_2 and one for total gas pressure at the O_2 sensor. The pressure readings from Q-S102 is recorded in software and is used in correcting the O_2 signal in KPa to O_2 in % (**O2 Pcor**).

Because of changes in total gas pressure at the O_2 sensor, the O2 (Pcor) reading in the software (corrected for pressure changes) may not be the same as the Q-S102 O2 reading. Only at a gas pressure of 100KPa will both the Q-S102 O2 (kPa)and the O2 (Pcor) (%) be the same.

The galvanic cell is housed in a brass cylinder for greater temperature stability and more stable O_2 signal. Pressure (Q-S102 P) is measured at the O_2 cell and can be recorded in the software. However in the NF1LP package, this pressure value is not recorded, rather a user parameter value of atmospheric pressure, Patm, is used to convert the partial pressure of O_2 (**Q-S102 O2 in kPa**) to a concentration **O2 (Pcor) in %** which is independent of pressure.

$$O2\ (Pcor)=((Q\text{-}S102\ O2)/(Patm))*100$$

The pressure corrected concentration is displayed in a meter and a graph on Page 1 of the "Q-Box NF1LP Setup" file.

The maximum flow rate through the Q-S102 O_2 analyzer should not exceed 650 ml/min. The minimum flow rate through the O_2 analyzer should not drop below 5mL/min to avoid local depletion of O_2 at the membrane of the sensor. The analyzer should not be exposed to pressures that are +/- 5 PSIG or the damage to the sensor can result. The **expected life of the O_2 Analyzer's galvanic cell is 3 – 5 years**. If it is impossible to adjust the O_2 analyzer's signal amplitude by adjusting the gain, a new sensor must be installed. Replacement of the sensor is simple. Contact Qubit Systems to make arrangements to replace the galvanic cell of the analyzer.

The Q-S102 is supplied with its own drying column filled with Drierite. When used in a series with the Q-S121 H_2 Analyzer, only one drying column is necessary (in front of the Q-S121). When using the Q-S102 in a stand-alone mode, gas should be dried before entering the O_2 Analyzer. Place the drying column in the bracket mounted on the back of the analyzer and connect it to the "in" port via the blue filter (25μm). Check the filter frequently to ensure that it is not plugged. Plugged filters will result in reduced gas flow to the analyzer and should be replaced.

Troubleshooting the Q-S102 O_2 Analyzer

If the O_2 **signal can no longer be adjusted** with the "O_2 Span" and the Analyzer has been used for 3-5 years the galvanic cell should be replaced. Contact Qubit Systems for instructions on ordering the new cell and the replacement procedure.

If **significant periodic jumps** (0.5-1%) in the O_2 readings are observed, this may indicate pressure damage to the Teflon membrane inside the sensor cell. If the signal shows unexpected drift, check for other damage to the membrane by removing the Q-S102 O_2 Analyzer from the Q-Box tray (loosen screws on the back of the tray that hold the analyzer in place). While monitoring the O_2 readings, tilt the analyzer 90 degrees. If there is a large change in the signal following the tilt, the Teflon membrane is likely damaged. If no large change in signal is observed, then the drift may be associated with atmospheric pressure changes. In case of any damage to the membrane, the galvanic cell should be replaced. Contact Qubit Systems for replacement instructions. In case of drift in the signal due to atmospheric pressure changes, base line (reference) measurements should be obtained more frequently.

If **small pulses in the O_2 readings** are observed when running a stable gas, this may indicate pressure changes in the system which may occur as a result of unstable gas delivery. Check the system for any obstructions or restriction in the gas lines first and eliminate these. Second check the O_2 analyzer by unplugging the gas supply line from it and monitor the O_2 readings. The reading should become stable shortly after detachment of the gas supply line. A stable reading when no gas is running past the analyzer indicates that the gas delivery to the analyzer is the problem and should be further investigated.

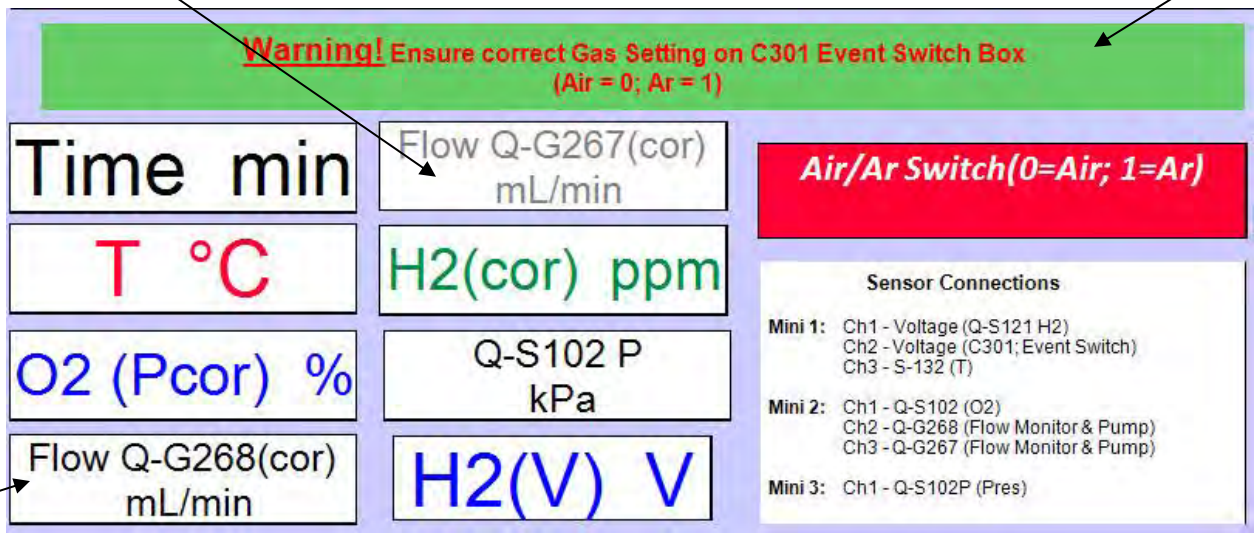
Using C301 Event Switch (Air/Ar)

The C301 event switch is connected to channel 2 of LabQuest Mini 1 and when the switch is in Air position zero voltage signal is recorded in Logger pro. When background gas running through the system is changed to Ar:O_2 mixture, the switch **<u>needs to be manually depressed in the Argon position</u>** with the red LED light on. The signal recorded by the software and displayed in the Air/Ar switch meter is 1.

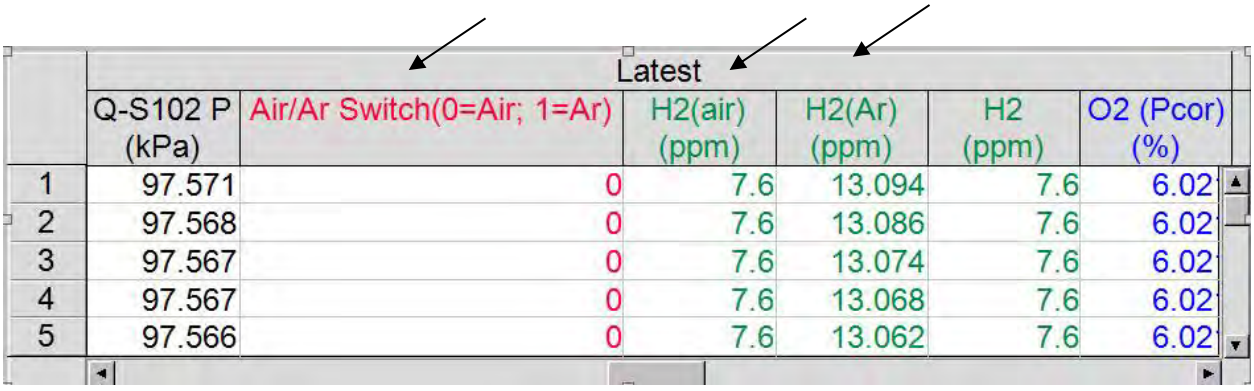
This change in event switch position is necessary for the software to correct the H_2 signal for the background gas used since the output of the H_2 analyzer is affected by the background gas.

Also the flow rate of the Q-G268 and Q-G267 Flow Monitors is affected by the change from Air to Ar:O_2 mixture and this event switch is used to correct the gas flow rate through the pot and through the system**. The meters and the graphs on page 1 of the software display corrected flow rates.** The corrections for both flow monitors have been calculated using mass flow controllers. The values for both the Q-G268 and Q-G267 should drop while the argon mixture is passing through them. The values shown are true representations of the flow rate that account for the difference in mass between argon and air.

Logger Pro uses different coefficients in the 5th order polynomial which converts H_2 voltage to H_2 concentration (ppm) for the different background gases. **If the user forgets to change the position of the event switch when the background gas is changed, the H2(cor) data will not be displayed correctly.** Hence the Warning message in the software is shown to remind the user to make this change.



In case the change of event switch position is forgotten, the correct data can still be obtained from the calculation columns of the raw data table in columns labeled H2(air), H2(Ar) as shown below:



	Latest					
	Q-S102 P (kPa)	Air/Ar Switch(0=Air; 1=Ar)	H2(air) (ppm)	H2(Ar) (ppm)	H2 (ppm)	O2 (Pcor) (%)
1	97.571	0	7.6	13.094	7.6	6.02
2	97.568	0	7.6	13.086	7.6	6.02
3	97.567	0	7.6	13.074	7.6	6.02
4	97.567	0	7.6	13.068	7.6	6.02
5	97.566	0	7.6	13.062	7.6	6.02

The data in the H2(Air) column is H_2 data calculated for Air as background gas and H2(Ar) column has H_2 data calculated for Ar:O2 mixture as the background gas. When the data is exported ***File>Export as>CSV*** and then opened in a spreadsheet program such as Excel, these columns will be accessible.

Using the S132 Temperature Probe

The temperature sensor is a thermistor mounted on the end of a stainless steel probe. It is nonlinear with an operational range from –40°C to +125°C. The calibration for the temperature sensor is fixed in the Logger Pro software. The temperature sensor should not require calibration, however this is possible in the Logger Pro software using the menu commands **Experiment>Calibrate**. The sensor is recognized automatically and calibration is loaded when the "Q-Box NF1LP setup" file is opened. The sensor is mounted on a stainless steel support, which fits through the temperature sensor ports in the flow through chamber, or it can be placed in the pot for measurements of root system temperature. The temperature sensor port in the flow through chamber must be plugged with a solid white rubber stopper when the sensor is not in use.

Using the G122 Gas Bags

The 30 L gas bags are made from a gas-impermeable nylon-polyethylene laminate and are heat-sealed. Tygon tubing is attached to each bag by a luer-lock fitting. The fitting on the other end of the tubing attaches directly to the fittings "PUMP IN" port of the Q-G268 Flow Monitor and Pump via the blue filter. These bags can be filled with air or another gas mixture as required by the experiments. Bags should not be over-inflated as this can cause weakening of the seams and eventual leakage. After use, the bags should be fully deflated, preferably by attachment to a vacuum-line.

Troubleshooting Q-G268 and Q-G267 Flow Monitor and Pump

The Q-G268 and Q-G267 Flow Monitor and Pump have been factory calibrated and should not require further calibration. However, if with time the flow values appear to be erroneous, it may be that the "Flow Zero" has drifted. To reset zero, adjust "Flow Zero" with no gas running through the system (disconnect the "pump gas out" from "flow gas in") until a zero flow value is displayed in Logger Pro. **Be sure not to accidently adjust the "Flow Span".** If "Flow Span" needs adjusting, a calibrated flow standard (mass flow controller) must be used to calibrate the "Flow Span". Otherwise the flow monitor may be returned to Qubit for recalibration. The speed of the pump should be adjusted using the two needle valves on the back, however the speed potentiometer adjustment on the front of the instrument is also available if the max flow rate with the needle valves fully open is not being reached.

Cultivation of Plants

The Nitrogen Fixation package includes seeds (Soybean: *Glycine max*, cultivar Maple Arrow), and inoculum (*Bradyrhizobium japonicum*), which, if treated properly, will yield H_2-evolving legume symbioses that are suitable for use in experiments 28 days after planting. Grow the plants as follows:

(1) Surface sterilize seeds in 5% bleach solution for 2 min and then rinse in running water for 10 min.
(2) Sterilize pots in 10% bleach solution and rinse thoroughly.
(3) Place 1/4"- grade gravel in the base of the pots sufficient to cover the drainage hole, and then fill the pots with a porous inorganic growth medium. Grade 16 industrial quartz (silica sand) is ideal. Unimin Corp. (203-966-8880) is a U.S. supplier of industrial quartz but local suppliers are available. Note that soil cannot be used as it contains microorganisms that oxidize some, or all, of the H_2 released by the nodulated roots. Also, growth material with a small pore size will create too much backpressure to allow gas flow through the pots during experiments.
(4) Water the quartz and make 2 holes, 1/2" deep, near the centre of the pots.
(5) Place one seed in each hole together with a very small amount of peat inoculums (pinch). Cover the hole and place the pot in a growth cabinet or greenhouse.
(6) Soybeans will grow well in the greenhouse during the summer months, or in a growth cabinet with a 16 h photoperiod at a constant 20°C. Use as high a light intensity as available in the growth chamber to promote growth.
(7) Flush the pots **twice daily** with a nitrogen-free nutrient solution. It is essential that the plants are not exposed to even moderately high levels of nitrogen, since this will inhibit the nitrogenase that is present and prevent further expression of the nitrogenase enzyme. An ideal nutrient solution for legumes is composed of the following components per litre of solution:

(a)	257 µmol	KH_2PO_4
(b)	57 µmol	K_2HPO_4
(c)	502 µmol	K_2SO_4
(d)	243 µmol	$MgSO_4 * 7H_2O$
(e)	246 µmol	$MgCl_2 * 6H_2O$
(f)	748 µmol	$CaCl_2 * 2H_2O$
(g)	10 µmol	$MnSO_4 * H_2O$
(h)	1.0 µmol	$CuSO_4 * 5H_2O$
(i)	1.0 µmol	$ZnSO_4 * 7H_2O$
(j)	31 µmol	H_3BO_3
(k)	0.5 µmol	$Na_2MoO_4 * 2H_2O$
(l)	0.2 µmol	$CoSO_4 * 6.5H_2O$
(m)	38 µmol	Fe from Fe- Sequestrine

Adjust final solution to pH 6.8 with acid or base as required.

Each of these nutrient components may be made up as 2000-fold concentrated stock solutions (see following page for details) and may be stored in the following combinations:

(a) plus (b), (c) alone, (d) plus (e), (f) alone, (g) to (l) combined, (m) alone

Any other combinations of stock solutions may cause precipitation of solutes, and nutrient deficiencies. During the first 10 days after planting, it is beneficial to supplement the nutrient solution with KNO_3 to a final concentration of 0.5 mM. This will stimulate nodulation and enhance N_2 fixation capacity. However, this treatment should be stopped when the plants begin to fix N_2.

(8) When the seedlings germinate, remove one seedling from each pot and dispose of it. Leave the seedling that is most centrally located, in the pot.

(9) Plants are ideal for experiments after 28 days, although older plants will produce greater amounts of H_2.

(10) After harvesting the plants, sterilize the pots in 10% bleach solution, and store for re-use.

Stock Nutrient Solutions for Legumes

The following solutions represent 2000-fold concentrations of the working solutions:

1	**0.514 M**	**KH_2PO_4**	**(69.90 g/L)**
2	**0.114 M**	**K_2HPO_4**	**(19.84 g/L)**
3	**1.004 M**	**K_2SO_4**	**(174.70 g/L)**
4	**0.486 M**	**$MgSO_4 * 7H_2O$**	**(119.70 g/L)**
5	**0.492 M**	**$MgCl_2 * 6H_20$**	**(100.00 g/L)**
6	**1.496 M**	**$CaCl_2 * H_2O$**	**(219.80 g/L)**
7	**0.020 M**	**$MnSO_4 * H_2O$**	**(3.38 g/L)**
8	**0.002 M**	**$CuSO_4 * 5H_2O$**	**(0.50 g/L)**
9	**0.002 M**	**$ZnSO_4 * 7H_2O$**	**(0.55 g/L)**
10	**0.062 M**	**H_3BO_3**	**(3.83 g/L)**
11	**0.001 M**	**$Na_2MoO_4 * 2H_2O$**	**(0.24 g/L)**
12	**0.0004 M**	**$CoSO_4 * 6.5H_2O$**	**(0.11 g/L)**
13	**0.076 M**	**Fe from Fe Sequestrine**	**See packet for Fe content**

Add 0.5 mL of each per litre of the working strength solution. Adjust final pH to 6.8 acid or base as needed.

Solutions may be stored in the following combinations:

(1) plus (2), (3) alone, (4) plus (5), (6) alone, (7) to (12) combined, (13) alone

Specifications of Q-S102 O_2 Analyzer:

- Operating principle - Acid Electrolyte, Teflon Diffusion Membrane
- Detection Range - 0-25% and 0-100 %O2 (Linear)
- Resolution - ±50 ppm
- Accuracy ± 0.21% of Full Scale
- Response Time (90%) - 12 Seconds
- Life Expectancy of sensor - 3-5 years
- Sensor easily replaceable
- Influence by Other Gases - Ammonia and Ozone
- min flow - 5 mL/min
- max flow - 650 mL/min
- Built in total gas pressure reading at the sensor (for pressure correction in the software)
- Pressure Range - 0.5 atm to 1.5 atm
- Pressure Effect - Output voltage changes proportionally
- Shock Resistant - to 2.7 G
- Avoid strong vibration
- Operating Temperature - 5 to 40^{o}C (Effective range)
- Passive temperature control of the O2 sensor
- Weight – 1.35 kg
- Dimensions (cm) – (H x W x D: 5.5 to 9.5 x 9.5 x 17)
- Output - 0 to 5 volt
- Power Supply - 12 Volts
- warranty: 1 year

Specifications of Q-S121 H2 Sensor

- Operating Principle: Tin-dioxide semi-conductor sensing element
- Range: 0 to 100 ppm H2 (higher range in customized units)
- Output: 0 to 5 V analog to digital interface
- Output sensitive to O2 concentration and background gas
- Calibration at 21% O2 in N2 and Ar is provided with the H2 Analyzer
- Sensitive to organic solvents and CO
- Response time (90%) – 20 sec
- Resolution – 0.001ppm
- Long life expectancy of the sensor
- Dimensions (cm) – (H x W x D: 5.5 to 9.5 x 9.5 x 17)
- Weight – 0.85 kg
- Operating Temperature - 5 to 40^{o}C
- Power supply – 12 Volts
- warranty: 1 year

Qubit Systems Warranty Information

QUBIT warrants all its instruments to be free from defects in materials or workmanship for a period of **one year** from the date of invoice/shipment from QUBIT.

If at any time within this warranty period the instrument does not function as warranted, return it and QUBIT will repair or replace it at no charge. The customer is responsible for shipping and insurance charges (for the full product value) to QUBIT. QUBIT is responsible for shipping and insurance on return of the instrument to the customer.

No warranty will apply to any instrument that has been (i) modified, altered, or repaired by persons unauthorized by QUBIT; (ii) subjected to misuse, negligence, or accident; (iii) connected, installed, adjusted, or used otherwise than in accordance with the instructions supplied by QUBIT.

The warranty is return-to-base only, and does not include on-site repair charges such as labour, travel, or other expenses associated with the repair or installation of replacement parts at the customer's site.

QUBIT repairs or replaces the faulty instruments as quickly as possible; maximum time is one month.

QUBIT will keep spare parts or their adequate substitutes for a period of at least five years.

Returned instruments must be packaged sufficiently so as not to assume any transit damage. If damage is caused due to insufficient packaging, the instrument will be treated as an out-of-warranty repair and charged as such.

QUBIT also offers out-of-warranty repairs. These are usually returned to the customer on a cash-on-delivery basis.

Wear & Tear Items are excluded from this warranty. The term Wear & Tear denotes the damage that naturally and inevitably occurs as a result of normal use or aging even when an item is used competently and with care and proper maintenance.

Return Procedure

Before returning any instrument to QUBIT:

Consult the operating manual or contact Qubit to ensure that the instrument(s) is in fact faulty and has not just been set up improperly.

Contact QUBIT before sending anything back. We will issue an RMA number and provide shipping instructions. QUBIT will refuse any goods that are returned without an RMA number or which are sent in a manner outside of QUBIT'S stipulations.

If you have encountered a program failure, we would need a printed copy of any faults you have seen, including how to reproduce them. Include these in the return package along with your mailing address.

Include a copy of the Invoice on which the product was shipped to you.

All returns must be shipped prepaid. Unpaid packages will not be accepted.

In case of questions contact QUBIT by

E-mail: info@qubitsystems.com,

by phone: (01)-613 384 1977,

or by fax: (01)-613 384- 9118.