Long Lab, Stanford University
http://cmgm.stanford.edu/biology/long/

ACETYLENE REDUCTION ASSAYS
SHIMADZU GAS CHROMATOGRAPH GC-8A1F

Set up plants for assays:

Set up one plant per BNM slant tube. Plants are often assayed at 3 weeks for nitrogenase assay. Earlier timepoints can also be taken if, for example, delayed nitrogen fixation is a possible phenotype. Plants assayed early (e.g. 8 days) or late (e.g. 7 weeks) show a lot of plant to plant variability.
Alternatively, if your plants haven been growing on plates, take a plant and lay it on a 5 x 0.5" strip of filter paper, and slide it into a tube containing 0.5 ml of 1/2X BNM. The liquid will soak the filter paper and keeps the plant from drying out.
If you want to assay individual nodules, cut off the nodule and treat as above. When you run the GC you may need to increase the sensitivity (see below) in order to measure the ethylene

Addition of acetylene to start the assay:

1) Cap tubes with red septum stoppers. The stoppers go in easier if you wet them with some water.
2) Partially fill a Tedlar 1 liter gas bag (Supelco #2-4633, phone 1-814-359-3441) with some acetylene.
The gas bags are "disposable", but since they cost $10 each I reuse them until the septum is destroyed. I label used bags with tape so that different gases aren't mixed.
The acetylene is normal grade (i.e. it isn't necessary to buy high quality). We order a B size tank. The tank is the standard tank that welders use for acetylene torches.
3) Use a 1 ml syringe and a 25 gauge needle to remove 1 ml of gas from the bag and inject into each tube. Note time.
4) Inject an empty tube with 1 ml acetylene to serve as a control.
5) Place tubes in growth chamber for a few hours or overnight.
Incubating overnight can result in lower calculated levels of nitrogenase activity, perhaps because the level drops over time. Therefore, shorted incubation times are probably best and all plants should be incubated for about the same length of time.

Gas chromatography:

1) Detailed instructions for starting the machine are on p. 9 of the Gas Chromatograph manual. Settings are:
Inj/Det 120° C
Column 80° C
For lighting the FID: Carrier gas (nitrogen) 70-80 kPa
Hydrogen 100 kPa
Air 10 kPa
There is a loud pop when the flame is ignited. It can take several tries to get the flame to stay lit. Check ignition by holding aluminum foil next to the hole. Condensation on the surface indicates ignition and is very obvious. After the flame is lit, however, it may take a minute or so until the condensation can be seen.
Once the FID is lit, change to running settings: Carrier gas (nitrogen) 200 kPa
Hydrogen 50 kPa
Air 50 kPa
I've increased the carrier gas to 250 kPa and still been able to resolve the peaks.

2) Set range to 102. The attenuation knob on the front of the GC is not hooked up to the Chromatopac integrator so the setting does not matter (it would be used to attenuate the signal if it went directly to a plotter.).
3) Instructions for zeroing the baseline, setting the parameters, and automatically setting the slope are on p. 4-1 of the Chromatopac manual.
The parameters that I change from the file's default are: WIDTH (0)=1
SPEED=30
METHOD=2021
FORMAT=2040
Perform S.TEST as described to automatically determine the appropriate slope (where the beginning and end of peaks will be defined). The slope will be about 400.
The parameters can be printed by pressing <LLIST> <PARA> <return>.

Parameters, methods, and formats are described in section 7, 9, and 10 of the Chromatopac manual.
4) Set the date and time as described p. 5-1, section 5.2, of the Chromatopac manual so that the date and time of each analysis will be printed.
5) Running a sample.
Use a 1 ml syringe and a 25 gauge needle to inject gas into the injection port. Push the needle down into the injection port keeping a finger on the plunger to prevent the backpressure from pushing up the plunger. Push down plunger. Pull needle and syringe out. All should be done quickly to load the gas tightly.
Press <START/STOP1> to start recording and analyzing the peaks. Ethylene will peak at about 0.7-0.8 minutes. Acetylene will peak at about 1.2 minutes.
Once the end of the final peak is reached (the display will no longer say "peak"), press <START/STOP1> again to stop the run and have the baseline and analysis printed out.
I reuse the syringe and needle pumping air in and out 4-6 times between samples. A little residual acetylene is detectable (~0.05% of the original quantity) but no ethylene is detectable.
6) Create a standard curve for ethylene to allow quantitation.
Use the needle attachment on the can of 1% ethylene (4 liter can of mix 855, Scott Specialty Gases, phone 1-510-659-0162) and pump some gas into a Tedlar bag.
Using a fresh needle and syringe to avoid contamination from previous samples, take 1 ml of 1% ethylene and inject into a capped tube. Final concentration is 0.0352%.
An empty capped tube contains 27.4 ml of air.
Take 1 ml of 1% ethylene and inject into a capped tube. Take 1 ml from that tube and inject into a capped tube to give a final concentration of 0.00124%.
Note that it is most accurate to set up these tubes starting each time from the bag because otherwise the tube of the first dilution would have less gas in it after making the serial dilutions.
Repeat doing three serial dilutions to give a final concentration of 4.37 x 10-5%.
This sample is the most difficult to make accurately because it's at such low concentration. For example, any leftover ethylene in the syringe can change the amount significantly so it's especially important to use fresh needles and syringes. I often just skip this dilution since I've checked that the machine is linear throughout this whole range.
Inject 1 ml of each sample (including straight 1%) and run it on the GC to determine the area of each peak. See below for analyzing the data using the standard curve.
6) Run a positive control for ethylene and acetylene by taking the empty tube with 1 ml of acetylene from the previous day and injecting 1 ml of 1% ethylene. Inject 1 ml of the mix on the GC.
The Chromatopac can read signals from -5 mV to 1 V. At the 102 range (which is very sensitive for the ethylene), the acetylene sometimes will go just off scale and the peak will be marked with an "E" in the analysis report. This doesn't matter in that we are only interested in quantifying the amount of ethylene. It is useful to keep the acetylene on scale, however, so that the concentration percentages reported are accurate and to make sure that the right amount of acetylene was added to the tube and that the sample was injected properly. The problem can be solved by either injecting less acetylene in the tube in the first place or sampling less than 1 ml (e.g. 0.8 ml).
With no attenuation set on the Chromatopac, the acetylene peak on the chromatogram will be off the paper, but as long as the signal is not higher than 1 V the area listed in the analysis will be accurate. I don't set the attenuation higher, because then the ethylene peaks get really small. The data can be reanalyzed and printed with attenuation if desired.
At the 102 range, conversion of 0.002 % acetylene to ethylene is detectable (determined empirically).
7) Run samples.
8) Directions to shut down the machine are located on p. 10 of the Gas Chromatograph manual.

Keeping the plants:
If you want to keep the plants, uncap the tubes and put a clear cap back on. The acetylene and ethylene will be gone in around a day. I've been leaving the tubes in the hood overnight rather than the growth chamber since acetylene isn't good to breath. To remove the gases more quickly, use a sterile Pasteur pipet and an automatic pipettor to blow air into the very bottom of the tube for 10-12 seconds.
The plants can be assayed again. However, ethylene is a plant hormone (and high levels of acetylene act like ethylene), and the plants do not look as healthy as those that have not seen the gases. In addition, M. truncatula seems to be more sensitive to the acetylene and ethylene than alfalfa. Ideally, therefore, it's

probably not advisable to reassay the plants. Alternatively, it might help to do the assay for only two hours and then immediately flush the tubes as described above.

Analyzing the data:

The Chromatopac can be programmed to quantitate the injected samples. However, since further calculations are required to convert to nmol/plant/hour, I simply enter the area of each the ethylene peak into an Excel spreadsheet and do all the quantitation in Excel.

1) All gas calculations are done at normal temperature and pressure (NTP) (pressure = 760 mm Hg or 1 atm; temperature = 273° K). Convert 1 ml of ethylene to ml of ethylene at NTP using the gas law relationship:

P1V1/T1 = P2V2/T2

Where P1 = 760 mm Hg, V1 = unknown, T1 = 273° K

Where P2 = 760 mm Hg, V2 = 1 ml, T2 = (273° + 23°) K

->1 ml of ethylene at room temperature and 1 atm (760 mm Hg) = 0.922 ml at NTP

2) Convert ml of ethylene to nmoles:

1 mole of C2H4 at NTP occupies 22.4 liters.

--> 0.922 ml of ethylene at NTP = 4.12 x 10-5 mol

--> 1 ml of 1% ethylene injected into the GC = 412 nmol

3) Plot standard curve as a log plot with area on the x-axis and nmoles on the y-axis:

% ethylene	**nmoles in 1 ml injected**
1	4.12
0.0352	14.5
0.00124	0.511
4.37 x 10^-5	0.018

Use a power equation to define the line.

4) Calculate acetylene reduction as nmol of ethylene produced per plant per hour. *I have an Excel spreadsheet that will do all the calculations. I find it most convenient to have the spreadsheet open as I run the GC and transfer the data as it comes off the machine.*

Convert peak areas of samples to nmoles injected.

Multiply by 21.5 to determine the nmoles present in the tube (a 1-3 week old stoppered tube with agar and a plant contains 20.5 ml of air plus 1 ml of the injected acetylene). Also correct at this point if less than 1 ml was injected.

At 7 weeks a stoppered tube with agar and a plant contains 22.9 ml of air plus 1 ml of injected acetylene.

Calculate the number of hours for the reaction.

Calculate nmol/plant/hour.