PROTOCOL FOR IN VITRO NDF DIGESTION EXPERIMENTS
Updated September 2013

I. Reference:

Goering, H.K., and P.J. Van Soest. 1970. Forage Fiber Analyses. Apparatus, Reagents, Procedures, and some applications. Agric. Handbook No. 379. ARS-USDA> Pages 12-15

II. Personal Protective Equipment:

A. Lab coat
B. Safety glasses/goggles
C. Latex gloves

III. Reagents:

A. pH 6.8 Buffer
   1. 7.5 g tryptone
   2. 1500 mL dH_2O
   3. 0.375 mL micromineral sol
   4. 750 mL buffer sol
   5. 750 mL macromineral sol
   6. 3000 mL total volume

B. pH 5.8 Buffer
   1. Same as pH 6.8 buffer with approx. 130mL 1M citric acid
      a. 1M citric acid: 105.5g citric acid in 500mL dH_2O (citric acid monohydrate, $C_6H_8O_2O$)

C. Micromineral Solution
   1. 100mL dH_2O
   2. 13.2g Calcium Dichloride $CaCl_2*2H_2O$
   3. 10.0g Manganese Chloride $MnCl_2*4H_2O$
   4. 1.0g $C_0Cl_2*6H_2O$
   5. 8.0g Ferric Chloride $FeCl_2*6H_2O$

D. Macromineral Solution
   1. 1L dH_2O
   2. 5.7g Sodium Phosphate Dibasic anhydrous Na_2HPO_4
   3. 6.2g Potssium Phosphate Monobasic anhydrous KH_2PO_4
   4. 0.6g Magnesium Sulfate $MgSO_4*7H_2O$

E. Rumen Buffer Solution
   1. 1L dH_2O
   2. 4g Ammonium bicarbonate (CH_5NNO_3)
   3. 35g Sodium bicarbonate ($CNaHO_3$)

Note: Start with ~ 100 mL 1M citric acid pH after bubbling CO_2 through the solution then add more citric acid as needed

IV. Preparation of substrate and tubes:

1. Determine number of 50 ml polypropylene tubes needed for in vitro run. Include blank tubes (no substrate added).

a. Typically, there will be blanks for every time point. The blanks are used to correct final NDF value for NDF contained in ruminal fluid inoculum.
b. Label appropriate number of tubes with marker near top of tube (remove after finished with tube using acetone).

2. Weight out 0.3000 ± 0.0040 g of substrate into each tube. Be sure to gravimetrically transfer sample from weight paper to bottom of tube.
   a. Static electricity can be a problem, so be careful. Make certain to have an accurately prepared spreadsheet so that you know what substrate is in each tube as you progress through the analyses.
   b. The standard particle size for in vitro fermentation is a 1-mm Wiley mill grind.
3. Randomly place tubes containing substrate into water bath with plexiglass holder for the tubes.
   a. If using dry sample add 1mL of ddH_2O to all tubes to reduce lose of sample. Treat all tubes the same.
   b. After inoculation double-check to ensure that all tubes are in proper order.
   c. It usually helps avoid confusion, if several people will be removing tubes, to label the rows of tubes as to treatment with colored tape and marker.

V. Procedure:

1. Calculate how much total buffer solution you will need for the entire run, at 30 ml per tube.
   a. For each buffer, make up approximately 300 ml more than needed so that the automatic dispenser will not draw air into the lines as you get to the last tubes to inoculate
2. Place buffer solutions in water bath, under CO_2, until they reach 39ºC and desired pH.
   a. Generally, it is best to place buffers in large graduated cylinders, use lead if less than 2L used in the cylinder donuts on the bottom to allow rapid warming and easy measuring of volumes when combining buffer and ruminal fluid
3. When buffers are at proper temperature and pH, collect inoculum from steer fed appropriate diet (in many cases alfalfa).
   a. Collect into a large graduated cylinder to ensure proper collection amount, strain through four layers of pre-folded cheesecloth, and immediately place into a pre-warmed thermos.
   b. Measure out enough fluid so that a 50% solution of ruminal fluid in buffer can be made up in the lab
4. Quickly bring ruminal fluid back to lab and combine with appropriate volume of buffer. **Refer to In-vitro Dry Matter Disappearance for a more detailed description of separatory methods.**
   a. Prior to mixing solutions, quickly measure pH of ruminal fluid and record.
   b. See attached chart for other quality control information to record for every in vitro run
5. Dispense 30 ml of buffer and RF mixture into each tube using Wheaton Unispense machine.
   a. Calibrate machine prior to inoculation.
   b. The total elapsed time from collection of RF to beginning of inoculation should not exceed 15 minutes, or 30 minutes to end of inoculation.
   c. Record starting time for tube removal

6. After inoculation, gently swirl all tubes and remove 0-h tubes and place in labeled rack and freeze immediately.
    a. When removing subsequent tubes, swirl all remaining tubes.
    b. Tubes should be swirled every 3 hours for the first 12 hours, and then every 6 hours through 24 hours

Record the following information:

Amount of citric acid added per 100 ml:_____.
Rumen fluid collected (time):_____.
Rumen fluid pH:__.
Buffer + RF temperature at inoculation:___.
Inoculation begin:__.
Inoculation end:__.
Tubes swirled:__.
Zero hour:__.

Note any problems during run: