STANDARD IN SITU PROCEDURE FOR FORAGES
Updated September 2013

I. References:

Broderick, G.A. 1994. Quantifying forage protein quality. In: G.C. Fahey, Jr. (Ed.) Forage Quality, Evaluation, and Utilization. American Society of Agronomy, Inc., Crop Science Society of America, Inc., and Soil Science Society of America, Inc., Madison, WI.

Klopfenstein, T.J., R.A. Mass, K.W. Creighton, and H.H. Patterson. 2001. Estimating forage protein degradation in the rumen. J. Anim. Sci. 79 (E. Suppl.): E208-217.

Mass, R.A., G.P. Lardy, R.J. Grant, and T.J. Klopfenstein. 1999. In situ neutral detergent insoluble nitrogen as a method for measuring forage protein degradability. J. Anim. Sci. 77:1565-1571.

NRC. 2001. Nutrient Requirements of Dairy Cattle (7th Ed.). pp 59-62. National Academy Press, Washington. DC.

Vanzant, E.S., R.C. Cochran, and E.C. Titgemeyer. 1998. Standardization of in situ techniques for ruminant feedstuff evaluation. J. Anim. Sci. 76: 2717-2729.

II. Supplies:

A. Ankom rumen in situ bags
   a. 5 cm X 10 cm (#R510), 50 Φm pore size (small bags)
   b. 10 cm X 20 cm (#R1020), 50 Φm pore size (large bags)

B. Ankom heat sealer - #1915

III. Personal Protective Equipment:

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

IV. Sample Preparation:

A. Forage Protein Analysis
   1. Freeze dry sample
   2. Grind to pass through a 2-mm screen

V. Procedure:

A. Animal(s)
   1. Animal(s) should be on desired diet for at least three days before starting any incubations. If the previous diet is not similar to the desired diet, then allow seven days for adaptation.
   2. Feed at desired intake, noting intake as a percentage of body weight (~1.8% of BW).

B. Sample Labeling
   1. Label in situ bag with simple number or code
   2. Use a black Sharpie (permanent marker)
   3. Label lower portion of the bag

C. Initial Weights
   1. Obtain weight of empty labeled bag
   2. Obtain weight of samples (as-is material)
      Forages: 1.25g (small bags)
      5.00g (large bags)

D. Bag Sealing-Seal bags completely with Ankom heat sealer

1. Place upper portion of bag on sealer strip leaving only 1-2 cm space
2. Hold down arm of heat sealer firmly so that the red light illuminates
3. Once red light goes off, wait 3-6 seconds before releasing arm
4. Gently remove bag and repeat to **add a second seal** below the first

E. Mesh Bag
1. 32 X 42 cm polyester with a nylon zipper
2. Weight bags down with two heavy bolt nuts
3. Tie off corner of bag to contain nuts with a cable tie
4. Place in-situ bags in mesh bag so that they are not stacked
5. Close zipper to ensure that it does not come open

F. Pre-incubation
1. Soak mesh bag(s) containing in-stiu bags in warm water (39°C) for 20 minutes prior to incubation
2. Allow excess water to drip before placing in the rumen

G. Incubation
1. Number of bags
   a. Up to 50 small in-situ bags may be incubated in mesh bags
   b. Up to 20 large in-situ bags may be incubated in mesh bags
   c. Up to 6 mesh bags may be incubated in the ventral sac of the rumen of a large (>500 kg) fistulated bovine
2. Time Points
   a. 0 hour, 75% TMRT, 96 hour
   b. 0 hour material is not incubated but will be rinsed later in Neutral Detergent Fiber Solution and analyzed for N to determine the potentially available NDIN (original pool)
   c. Total Mean Retention Time (TMRT) is estimated from IVDMD analysis using the following equation:
      kp = [0.07 * IVDMD (%)] - 0.20
      MRT = 1/kp
      TMRT = MRT + 10 hours (lag time)
   d. Incubate 96 hour samples first, followed by 75% TMRT. This will allow all bags to be removed from the rumen at the same time (Complete Exchange Method).

H. Bag Rinsing
1. Remove mesh bag(s) simultaneously from rumen and take in-situ bags out of mesh bag(s).
   All nylon bags can be rinsed at the same time using a washing.
2. Rinse bags in washing machine with 0.375 L of 39°C water/in-situ bag.
3. Rinse bags five times (a rinse consists of a one minute agitation and a two minute spin).

I. NDIN
1. To remove microbial contamination from bag residue, reflux in-situ bags in Neutral Detergent Fiber Solution (NDFS).
   a. In an in-situ Fiber Analyzer, place in-situ bags (3 bags per tray) in rack (24 bags will fit in the rack at a time) and pour 1600 ml of NDFS in reservoir.
   b. Turn heat (97°C) and agitator on and bulk reflux bags for 75 minutes.
   c. Drain NDFS and fill reservoir with 1600 ml boiling (100 °C) distilled water–heat may be turned off at this point. Agitate bags in the boiling distilled water for 5 minutes. Drain water and repeat twice more.
   d. Using distilled water, individually rinse each in-situ bag. Rinse material to the

bottom of the bag, roll the bag top down to the residue exposing the label, and gently squeeze excess water from each bag.

f. 0 hour bags (not incubated) should also be bulk refluxed in NDFS, rinsed, rolled, and dried like other samples.

J. Drying

1. Dry the washed bags in a 60°C forced-air oven overnight (12-**48** hours).
2. Place bags in a desiccator and retrieve a dry weight on the samples.
3. After drying allow the bags to air equilibrate to room conditions for at least three hours before weighing.
4. Once the bags are equilibrated take get the air equilibrated wt of the samples as well.

K. Analysis Nitrogen

a. Determine the N concentration (%) of a representative sub-sample for each residue (~.25 g for combustion N analysis). This value is the Neutral Detergent Insoluble Nitrogen (NDIN) remaining at each in situ time point.

b. Analyze original sample for DM and CP.

c. Express NDIN on a DM basis.

VI. Calculations:

A. UIP (% DM) = $\frac{\text{(Residue N * Residue weight) * 6.25}}{\text{(Original Sample DM in)}}$

B. Rate of Degradation (k_d)

1. Subtract the 96 hour NDIN (indigestible fraction; fraction C) from all 0 hour 75% TMRT NDIN values to get the potentially digestible NDIN at each time point.
2. Divide the remaining NDIN (96 hour corrected) by the potentially digestible NDIN at 0 hour (96 hour corrected) to get the potentially digestible fraction of NDIN remaining (Fraction B).
3. Multiply by 100 to transform the percent (as a decimal) to percent as a number. **Note**: At 0 hour, 100% of the potentially digestible NDIN remains.
4. Take the natural logarithm (LN) of the percent (as a number) of potentially digestible NDIN remaining and subtract from 4.6 (LN of 100 %).

0 to 75% TMRT rate (% kd) =
[(4.6 - LN 75% TMRT potentially digestible NDIN remaining)/75% TMRT hours] * 100

C. UIP using Equations:

The potential UIP pool left after a lag in passage (X hours) is determined by multiplying the percent of the original pool remaining after the lag by the original pool concentration. The percent of the original pool remaining after the X hr lag is calculated by subtracting the rate of digestion (k_d) from 1 and raising that quantity to the X (hr of lag) power.

1. Percent remaining of original pool = $(1-k_d)^X$, where k_d is the rate of digestion and X is the number of hours in the lag of passage.
2. B = original (0 hr) pool
3. UIP (% DM) = $[B(1-k_d)^X][(k_p/(k_p + k_d)] + C$

Notes: Fraction B must be on a DM basis:
UIP (% CP) = UIP (% DM) / CP (% DM)