COUNTING RUMINAL PROTOZOA
Updated September 2013

I. References:

Boyne, A. W., J. M. Eadie and K. Raitt. 1957. J Gen Microbiol. 17:414.
Dehority, B. A. 1984. Appl Environ Microbiol. 48:182.

II. Reagents:

A. Phosphate buffer:

1. Sodium Chloride (NaCl) 0.50%
2. Anhydrous sodium acetate ($C_2NaO_2H_3$) 0.13%
3. Potassium phosphate monobasic (KH_2PO_4) 0.03%
4. Potassium phosphate dibasic (K_2HPO_4) 0.10%
5. Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$) 0.01%
    a. Adjust pH between 6.9 and 7.2

B. 10% formalin solution

C. Glycerol-Buffer-methyl green mixture:

1. Phosphate buffer 70%
2. Glycerol 30%
    a. Place on a stir plate and mix. While mixing, add methyl green dye powder sparingly until desired color is reached.
    b. If glycerol solution is too light expect poor staining.

III. Ruminal Sampling:

A. Ruminal protozoa can be counted per gram of ruminal contents or per ml of ruminal fluid.

1. For counts per gram of ruminal fluid.
    a. Ruminal contents are hand mixed as thoroughly as possible and a 50 ml plastic beaker is used to collect the sample.
    b. If hand mixing is not possible, composite samples of contents from various locations (reticulum, cranial sac, dorsal sac, and ventral sac).
2. For counts per ml of ruminal fluid.
    a. Use the suction strainer device to sample ruminal fluid in a 50 or 100 ml plastic tube.

IV. Dilution of Ruminal Contents:

A. **For counts per gram of ruminal fluid**.

1. Pipette approximately 10 ml of ruminal contents with a wide mouth pipette into a tared beaker and note the weight of the sample.
2. To the beaker, pipette 10% formalin solution with a pasteur pipette to equal the weight of ruminal contents (1:1 dilution).
3. A 2.0 ml aliquot of the well mixed, formalinized sample is pipetted with a wide orifice pipette into a test tube containing 18 ml glycerol-buffer-methyl green mixture (1:20 final dilution).
4. The mixture is allowed to stand for at least 4 h before counting, overnight results in better staining (After protozoa are fixed, stain within one week).
5. Make preparations in duplicate.

B. **For counts per ml of ruminal fluid**.

1. Add 5 ml of ruminal fluid to 5 ml of formalin solution (dilution 1:1) and mix.
2. Then place a 2.0 ml aliquot of the well mixed, formalinized sample with a wide orifice pipette into a test tube containing 18 ml glycerol-buffer-methyl green mixture (1:20 final dilution).
3. The mixture is allowed to stand for at least 4 h before counting, overnight results in better staining (After protozoa are fixed, stain within one week).
4. Make tubes in duplicated.

V. Counting the protozoa:

A. Mix the tube well immediately before counting.
B. Fill the counting chamber (Sedgewick-Rafter Cell) and place a coverslip on the chamber carefully, without causing formation of air bubbles.
C. The slide is set for 10 minutes to allow settling of protozoa.
D. If dilution is too concentrated, further dilution with phosphate buffer prior to counting works well (7-8 protozoa per field is desirable).
E. Use the eyepiece to count the number of organisms.
F. Count at least 25 microscopic fields using a 10X objective.
   1. It is better to count 50 fields through all the chambers.

VI. Constants and Calculations (for 50 fields):

A. **Constants**:

1. Eye piece standard square units (one field): $0.469225\ mm^2$
2. Depth of Sedgewick-Rafter cell: 1.00 mm
3. Standard volumetric units: $0.469225\ mm^3$
4. Volume of 50 field: $23.461250\ mm^3$
5. Organisms per count ($1000\ mm^3 / 23.46125\ mm^3$): 46.6235

VII. Calculations:

Number of organisms in 50 fields x dilution x organisms per count = Organisms/ml or g

Example: 184 (50 fields) x 20 x 46.6235 = 156,854/ml or g

Note: For identification of species use an atlas of microbiology. Practice the procedure before you experiment. Be consistent on time of sampling relative to feeding time.