

# ***In Vitro* Gas Production Procedure**

(Updated April 2016)

## **1) Procedure:**

- ✓ This method allows estimation of the digestibility and metabolizable energy (ME) content of ruminant feedstuffs
  - The amount of gas released during incubation of feedstuffs is closely related to digestibility and therefore the ruminant ME value for (Menke and Steingass, 1988).
- ✓ Have an inter-run standard sample, with a known gas production history, in each run (e.g., an alfalfa hay) to standardize gas readings between runs.
  - If gas values for standards within a run diverge from the historical average an adjustment should be made, unless divergence is extreme in which case the run should be dropped.
  - Average gas values for the standard sample at specific time points can be used to adjust gas values among runs.
  - Once the quantity of the standard becomes low, a new standard should be created and run with the old standard.
- ✓ Using blanks
  - Blank syringes containing only rumen fluid/buffer solution – no feed – as many as deemed necessary, usually 2 to 4.
  - Mean gas production value of all blank syringes are the blank value.
  - Blanks correct for gas production from fermentation of residual particles in rumen fluid.

## **Tips:**

### **❖ Test samples:**

- Run the alfalfa standard and compare with known previous values.
- Unusual values indicate a problem in technique and/or chemicals mixed.
- Gas values should be adjusted using the average of all previous standard values (i.e., inter-run standard) to standardize across runs.

### **❖ Repeatability:**

- However samples are treated, keep it the same among all samples.

### **❖ Speed:**

- When filling more than 20 syringes, get another person to help to speed up the process, otherwise later reading will become difficult and prolonged.
- One person should fill and mix syringes while the other person places syringes in water and records values.

## 2) Preparation:

- ◇ Collect all required equipment/instruments
- ◇ Find/purchase all required chemicals
- ◇ Grind samples
- ◇ Create and print sample list with corresponding syringe number & datasheet
- ◇ Set up lab bench
- ◇ Prepare Solutions (*that can be kept*)
- ◇ Have spreadsheet ready for gas results
  - Usually add DM and OM analysis for samples

## 3) Apparatus:

- ◇ Lab coat
- ◇ Gloves – medium/large
- ◇ Pens
- ◇ Paper towel
- ◇ Water Bath (large) and plastic cover with syringe holes (1)
- ◇ Thermostat with circulation pump – 39°C (2)
- ◇ Metallic water bath (small, 10L)
- ◇ Re-pipette dispenser (no bottle) – set to 30 ml
- ◇ Magnetic agitator bar – small (for buffer mineral medium – not too large, do not create a vortex)
- ◇ Magnetic agitator large enough to hold water bath
- ◇ Timer & battery
  - Time settings (15 min)
- ◇ Glass syringes (with piston) – 100 ml, graduated cylinder
  - Elastic tubes pushed onto inlet of syringe
  - Plastic clip attached to elastic tube
  - Extra clips in cup
  - ✓ **Check that elastic tube is not blocked**
- ◇ Trolley with high sides to transport syringes
- ◇ Weighing spoon
- ◇ Vaseline
- ◇ Sharpie to mark syringes
- ◇ Scale (micro, measure in milligrams)

- ◇ Weighing boat with removable stem
  - Long enough to put material at the bottom of syringe without leaving sample on the sides
- ◇ Thermometer - glass
- ◇ Funnel to filter rumen fluid
- ◇ Thermo flask to collect rumen fluid in
- ◇ Cheesecloth
- ◇ 3 x Strong alligator clips
- ◇ Graduated glass cylinder (500 ml)
- ◇ Pipette filters
- ◇ Pipette tips
  - 10 x 5 ml
  - 1 x 1 ml
- ◇ Pipettes
  - 1 x 5 ml – 1N NaOH etc.
  - 1 x 1 ml – Trace mineral, Resazurin
- ◇ Glass Jar
  - 2 x 5 L – to keep Rumen fluid & Buffer mineral medium warm
- ◇ Glass volumetric flasks
  - 2 x 100 ml – 1N NaOH, Resazurin
  - 1x 50 ml – 0.04N NaOH
  - 1 x 25 ml – 0.04N NaOH extra
- ◇ Conical volumetric flasks
  - 2 x 4 L – Main element, Buffer solution
  - 1 x 250 ml – Trace element
  - 1 x 125 ml – Reduction solution
- ◇ Glass flasks
  - 1 x 500 ml – Rumen fluid drip cup
  - Multiple small for each of solutions



#### 4) Chemicals:

- ✓ Calculations:
  - ❖ Max 60 syringes/run for this protocol
    - 2 blank + 3 inter-run sample = 5
- ◇ Sodium hydrogen carbonate (**NaHCO<sub>3</sub>**)
- ◇ Ammonium hydrogen carbonate (**NH<sub>4</sub>HCO<sub>3</sub>**)

- ◇ Disodium hydrogen phosphate (**Na<sub>2</sub>HPO<sub>4</sub>**)
- ◇ Potassium dihydrogen phosphate (**KH<sub>2</sub>PO<sub>4</sub>**)
- ◇ Magnesium sulphate (**MgSO<sub>4</sub>.7H<sub>2</sub>O**)
- ◇ Calcium chloride (**CaCl<sub>2</sub>.2H<sub>2</sub>O**)
- ◇ Manganese chloride (**MnCl<sub>2</sub>.4H<sub>2</sub>O**)
- ◇ Cobalt chloride (**CoCl<sub>2</sub>.6H<sub>2</sub>O**)
- ◇ Iron chloride (**FeCl<sub>3</sub>.6H<sub>2</sub>O**)
- ◇ Sodium hydroxide (**NaOH**)
- ◇ Sodium sulphide (**Na<sub>2</sub>S.7H<sub>2</sub>O**)
- ◇ Resazurin
- ◇ **CO<sub>2</sub>** – check pressure gauge to ensure enough remains in bottle

➤ *If H<sub>2</sub>O content of chemicals differ from what is required, recalculate molecular weight and proportion of molecule that comes from the mineral, adjust required amount of mineral for the adjusted molecular weight (e.g., Calculate molecular weight of **Na<sub>2</sub>S.7H<sub>2</sub>O** and the % of **Na<sub>2</sub>S** in it. Multiply % of **Na<sub>2</sub>S** with grams added to solution = weight of **Na<sub>2</sub>S** required. Adjust that weight for the % of **Na<sub>2</sub>S** in molecular weight of **Na<sub>2</sub>S.9H<sub>2</sub>O** to add up to the same amount of grams in the solution.*

### **Solutions:** *Make extra if can be kept*

#### 1) Main Element - *(can be kept)*

- 5.7 g **Na<sub>2</sub>HPO<sub>4</sub>**
- 6.2 g **KH<sub>2</sub>PO<sub>4</sub>**
- 0.6 g **MgSO<sub>4</sub>.7H<sub>2</sub>O**
- Fill to 1 L

#### 2) Trace Element - *(can be kept)*

- 13.2 g **CaCl<sub>2</sub>.2H<sub>2</sub>O**
- 10 g **MnCl<sub>2</sub>.4H<sub>2</sub>O**
- 1 g **CoCl<sub>2</sub>.6H<sub>2</sub>O**
- 8 g **FeCl<sub>3</sub>.6H<sub>2</sub>O**
- Fill to 100 mL

#### 3) Buffer - *(can be kept)*

- 35 g **NaHCO<sub>3</sub>**

- 4 g **NH<sub>4</sub>HCO<sub>3</sub>**
  - Fill to 1 L
- 4) Resazurin (0.1%) - (*can be kept*)
- 100 mg **resazurin**
  - Fill to 100 mL
- 5) 1N NaOH - (*can be kept*)
- 4 g **NaOH** granules
  - Fill to 100mL
    - NaOH molecular weight = 40, thus 40 g in 1 L water = 1 N solution
- 6) 0.04N NaOH - (*make fresh daily*)
- 2 ml **1N NaOH** - Fill to 50 ml
  - 1 ml **1N NaOH** - Fill to 25 ml (*make extra 25 ml if > 50 syringes*)
- 7) Reduction solution - (*make fresh daily*) (*make extra 25 ml if > 50 syringes*)
- With **Na<sub>2</sub>S.7H<sub>2</sub>O**, add 285 mg to 50 ml **0.04N NaOH**
  - With **Na<sub>2</sub>S.9H<sub>2</sub>O**, add 335.3 mg to 50 ml **0.04N NaOH**

## 5) Set up for analysis (day before):

- 1) Check water level of large water bath – refill with warm water if needed
- 2) Switch on thermostat heater
  - Often takes ~3h to warm up to 39°C
- 3) Set up required # of syringes needed
  - Push elastic tubes onto syringe inlet
    - Make sure tube is not blocked/congested with old sample
    - Assure that the clip works and tube does not break off
  - Attach plastic clip to end of elastic tube – close the clip
- 4) Mark sample number on syringe as well as piston (top)
  - Lay out syringe on trolley – angled with inlet pointing downward
  - Rub pistons with Vaseline & lay out on trolley
    - This makes pistons gas- and watertight and prevents piston from getting stuck
    - Do NOT go further than red line – tip should be clean
- 5) Write sample info on Sample List

6) Filling syringes with standard & sample:

- Material should be milled to pass a 1mm screen
  - ✓ *Small sample size necessitates fine material. However, this can cause a faster fermentation rate compared to coarsely ground samples*
- Take trolley with all weighing equipment to scale:
  - Empty tray with paper towel
  - Weighing boat with removable stem
  - Weighing spoon
  - Standard and other samples
  - Sample list and writing pen
- Tare weighing boat on scale
- Weigh out samples and standard(s) into a weighing boat
  - 200 mg (easily degradable material) = 0.2 g
  - 300 mg (poorly degradable material) = 0.3 g
  - ✓ *Keep sample sizes similar since increased substrate can affect gas production linearly above 200 mg DM in the incubated sample*
- Attach weighing boat to removable stem
- Tip syringe on side with clip closed
  - Keep clip opening facing upward to unclip it easily
- Carefully place the sample all the way down at the very bottom of the syringe, below the inlet
  - Do NOT let the sample cover the syringe inlet

7) Place syringes back on tray, angled so that samples doesn't move out

8) Insert piston into syringe:

- Hold syringe securely with one hand, open clip with thumb and insert piston
  - While pushing air out - take care not to push the sample out
- Not closer than 20 ml
- Close plastic clip
- Place syringes back onto tray, angled so that samples doesn't move

9) Set up equipment for small water bath

- Small water bath (10L) filled with water – big enough to fit 2 x 5L glass jars
- Thermostat with circulation pump – set to 39°C
- Magnetic stirrer – under bowl

- Magnetic bar in glass flask (Buffer mineral medium)
- Repipette dispenser (set to 30 ml) – attached to insert into 5L glass jar
- CO<sub>2</sub> bottle
  - Elastic tube from CO<sub>2</sub> bottle to 5 L glass jar with hard tip to bubble into rumen fluid
  - Check pressure – enough available?

10) Check number of final syringes and adjust amount of chemicals and solutions required to fill all syringes

- Add extra 300 ml to allow easy filling of syringes

## 6) Method:

08:00 – **Set up in the lab**      - *Time = 45 min until rumen fluid is added*

- ◇ Switch on small water bath (39°C)
  - Refill with warm water if needed
- ◇ Check temperature of large water bath
- ◇ Prepare rumen fluid collection equipment – place in bucket
- ◇ Fill Flasks (1L) with hot water (large enough for 600 ml - 50 syringes)
  - Place in bucket with other rumen fluid equipment
- ◇ Prepare fresh **0.04 N NaOH**
- ◇ Prepare fresh **REDUCTION SOLUTION**
  - Takes a few minutes to dissolve
- ◇ Prepare **BUFFER MINERAL MEDIUM** in Glass jar/Woulff flask (*1500 ml for 50 syringes*) in the following order:
  1. 474 ml **DI H<sub>2</sub>O**
  2. 0.12 ml **Trace** element solution
  3. 237 ml **Buffer** solution
    - This will increase the pH to ~ 8.8
  4. 237 ml **Main** element
    - Place jar in water bath and turn on stirrer. pH declines to ~8.2
  5. 1.22 ml **Resazurin** solution
- ◇ Warm Buffer mineral medium for rumen fluid to 39°C
  - Mix with magnetic agitator

- Check temp of mineral medium
  - Takes ~30 min to warm up to 39°C
- Insert elastic tube from CO<sub>2</sub> bottle into Buffer mineral medium
  - Tip must be inside the liquid
- Close jar with foil

08:45 - **Collect rumen fluid:** - Time = 45 min

- ✓ *Always advisable to collect a rumen fluid mixture from 2 donor animals to assure higher constancy of fluid incubated*
- ✓ *It makes no difference if sheep instead of cattle are used as donors, as long as blanks and standards are used to correct values*
- ✓ *Recommended feeding of donor animals: 50% hay & 50% concentrate. Two meals a day, cover maintenance requirements (affects quality and stability of rumen fluid)*
  - *It is not meaningful in all cases to feed donor animals feedstuffs to be tested in vitro*
  - *More important is to ensure minimum rumen fluid activity*
    - *Rumen fluid collection longer than 16 h after last feeding will have low fluid activity*
- ✓ *Collect rumen fluid before morning feeding. If fed continuously, this it is not as important*
- ✓ *If donor animals are fed no concentrates, only straw, gas production can be reduced by 25%*
  - *Blank value indicates microbial activity in the fluid*
  - *Minimum rumen fluid activity is indicated by a blank value of >7 ml/24 h. (12-16 ml)*
  - *If blank value is lower, feed more hay to donor animals to increase microbial activity*

◇ Equipment required (from Lab) – place in bucket:

- Gloves (short and long)
- Thermo Flasks (2x if more than 50 syringes) – place in bucket
  - Fill with hot water prior to rumen fluid collection to warm up
    - Rumen fluid should be kept at 39°C – a drop in temperature can effect measurement values due to delayed fermentation
    - For a longer journey, fill flask with CO<sub>2</sub> prior to fluid collection and fill the flask to the brim during collection to limit oxygen exposure
      - ✓ When CO<sub>2</sub> bubbles over top of flask (it can be smelled) it is full
- Boots & Plastic cover booties
- Rumen fluid collection kit, store in large bucket
  - Large syringe, Plastic sample tube with holes to attach to syringe, PVC perforated strainer pole
  - Flasks (filled with hot water)



◇ Procedure:

1. Fill bucket with warm water (not hot)
2. Identify the cannulated cows (preferably 2 donors)
3. Open cannula
  - Place hand in middle of cannula and push cap into the hole (make a note of which side faces inwards), turn cap sideways and pull out
  - Place it in the bucket with warm water
4. Carefully insert the strainer pole into the rumen, all the way to the bottom to ensure that a sufficient amount of rumen fluid flows into pole
5. Insert the plastic sample tube into the strainer pole. Attach the syringe to the outside end of the tube
6. Slowly draw rumen fluid into syringe
  - Once all the air is removed from the tube and the rumen fluid flows freely, remove syringe and let the fluid flow into the flask
  - If it doesn't flow, fluid in the syringe can slowly be ejected into the flask
  - Avoid large amounts of air coming in contact with fluid – don't bubble
7. Fill the flask completely and close it immediately (let rumen fluid pour over the side when closing).
8. Insert cannula cap again
  - Ensure it is inserted correctly – run fingers around on the inside to ensure cap is completely inserted
9. Repeat with 2<sup>nd</sup> animal

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10:00 – **Prepare Buffer medium and rumen fluid** - Time = 45 min

- ◇ Turn CO<sub>2</sub> on LOW and flush through the buffer medium (tip submerged in fluid)
- *This makes the medium anaerobic and lowers pH from 8.2 to ~6.9*
- ◇ Pour **Reducing agent** (all 50 ml) into Buffer mineral medium under continuous CO<sub>2</sub> flushing
- ✓ *This lowers the redox potential and generates an anaerobic environment*
  - Reducing agent = reddish color
  - Medium should become **colorless** before rumen fluid is added
    - If it doesn't change color, something is wrong (do not add rumen fluid)
- ◇ Set Repipette to 30 ml
- ✓ *Use of a semi-automatic pipette speeds up filling process*

- ◇ In the meantime, Set up to strain rumen fluid:
  - 3 layers of cheesecloth
    - Attach to sides of funnel with strong alligator clips
  - Large funnel (that will fit into glass jar)
  - Place glass jar in small water bath @ 39 °C to keep warm
    - Use weight to keep jar submerged in water
- ◇ Filter rumen fluid through cheese cloth into heated glass jar
  - Lift cheese cloth to allow air to enter bottle otherwise it goes to slow
- ◇ Wait for medium indicator to become colorless before rumen fluid is added
- ◇ Transfer **474 ml** rumen fluid into Buffer medium (Rumen fluid:Buffer = 1:2)
  - Use graduated glass cylinder
  - Cover flask with foil
  - Set timer = 15 min, while CO<sub>2</sub> is flushing
  - pH ~ 6.6
- ◇ After 15 min – remove CO<sub>2</sub> nozzle from fluid but keep flushing (Keep foil cap on)
  - Prepare repipette to fill syringes - remove all air bubbles
- ◇ Put foil cap back on to keep anaerobic, **keep stirring slowly** (do not vortex)
- ◇ **Fill syringes with buffer medium:** -Time = 30 min
  - ✓ *If possible, syringes should be pre-heated in a 39°C water bath, however, a short drop in temperature has less effect on gas production than over-heating, which can selectively damage microbial activity*
    - Note start time
    - Make sure there are NO air bubbles in pipette spigot
    - Carefully attach elastic tube of syringe onto pipette spigot
    - Open plastic clip - Pipette 30 ml of solution into syringe
      - No need to hold on to plunger, it will not come out
    - Close plastic clip and gently pull from the spigot
    - Put hand ON PISTON and pull while opening clip – suck back the fluid that is in the tube
    - Expel air up to 3 ml space
    - Close clip - mix feed with solution (do not turn syringe upside down)
    - Open clip while holding the piston
    - Slowly expel all air from syringe – stop before solution goes into inlet

- Don't spend too much time getting bubbles out as temp is falling
  - ✓ *It is difficult to remove air with roughage samples. Gas bubbles of about 1 ml can be tolerated without affecting microbes*
- Close the plastic clip on the elastic
- ◇ Invert syringe and record initial reading (Time = 0)
  - Reading at red line on plunger
- ◇ Place syringe into water bath at 39°C
- ◇ Note down end time when all syringes are in water bath

### **Reading gas values:**

- ◇ Record reading at specific time intervals
  - e.g., 4, 6, 24, 30, 48, 72, 96 h
  - Do readings midway between the start and end filling times to allow sufficient production time for last syringes filled
- ◇ Note start time before any readings are made, note end time when complete
- ◇ Lift syringe up using the piston
  - BUT, take hold of the syringe as soon as possible to reduce pulling force on the piston
  - If the piston pulled out slightly, wait for it to settle back down before reading is made
- ◇ Holding syringe upright with the red ring on the piston at eye level, note down reading on left of syringe (graduated from 0 to 100)
  - Reading notes in ¼ points (0, 0.25, 0.50, 0.75)
  - Ensure that current reading is higher than previous – otherwise there is a problem
  - Carefully swirl syringe to mix all sample floating on top, into the rumen fluid.
  - Place syringe back into the water bath
  - Note end time after all readings were made
- ◇ Evacuation of gas
  - In some cases gas production in the allotted time exceeds the 100 ml volume available in the syringes. In this case, gas must be evacuated during the procedure:
    - Read gas production at current time as described above
    - Carefully tilt the syringe sideways until the opening is no longer covered by rumen fluid
    - Remove any water that may have collected in the plastic tube at the tip of the syringe opening
    - Open the clip while holding the piston
    - First pull the piston out to suck in any rumen fluid and sample that may have collected in the syringe opening

- Push the piston inward to expel gas
  - Expel gas until the red marker on the piston is exactly at 40 ml of gas
  - Two options exist – either expel a constant amount of gas from all syringes, or expel gas from syringes to the same amount for all samples
- Close the clip, Return syringe to the water bath
- Note quantitative extraction of gas on datasheet
  - Values should be added to the final readings for all times after this point
  - If one syringe needs evacuation, it is recommended that the same is done for all syringes, and to limit this action to one time for all samples

## 7) Calculations

### 1. Correct for 0 h gas:

- Deduct 0 h gas from recorded gas volume
- This corrects for fluid volume inserted into syringe at start
  - ✓  $\text{Gas volume @ Time X} - \text{Gas @ Time 0}$

### 2. Correct for Blanks:

- Deduct mean blank value from recorded gas volume
- This corrects for gas production from residual feed present in rumen fluid at start
  - ✓ 
$$\frac{\text{Gas volume @ Time X}}{\text{Average Gas volume for all Blanks @ Time X}}$$

### 3. Correct for weight:

- Correct gas production back to ml gas/gram of material (as is)
- This corrects for deviations between sample weights inserted into syringes
  - ✓ 
$$\frac{\text{Gas volume @ Time X}}{\text{Sample weight (mg) / 1000}}$$

### 4. Correct for DM %:

- Correct gas production to ml gas/gram of dry material
- This corrects for differences in material DM, which affects total gas production
  - ✓ 
$$\frac{\text{Gas volume @ Time X}}{\text{Air DM \% * Lab DM \%}}$$

### 5. INTER-RUN Correction:

- Correct for Inter-run standard (with known history of gas production)
- This corrects for differences in rumen fluid activity between days, animals as well as differences in atmospheric pressure
  - ✓ 
$$\frac{\text{Gas volume @ Time X}}{\text{Historical STD average (\%DM) / Average STD ml/g DM of current run}}$$

### 6. Correct for OM%:

- Correct gas production to ml gas/gram of organic material
  - ✓ 
$$\frac{\text{Gas volume @ Time X}}{\text{Organic Matter \%}}$$

## 8) Summary:

- ✓ This method allows estimation of the digestibility and ME content of ruminant feedstuffs
  - The amount of gas released during incubation of feedstuffs is closely related to digestibility and therefore the ruminant ME value.
- ✓ The function of the incubation medium is to create and maintain an environment suitable for fermentation.
- ✓ This is achieved by supplementing nutrients and a buffering system to maintain medium pH so that fermentation is not compromised over time, and to provide enough volume (low DM content) to prevent fermentation end-products from inhibiting substrate degradation.
- ✓ Functions of components:
  - Buffer medium
    - Phosphate and Bicarbonate buffers (synthetic saliva) -  $\text{PO}_4$  &  $\text{HCO}_3$ 
      - Maintain incubation medium pH above ~5.5 by neutralizing acidic fermentation end-products (*i.e.*, acetic, butyric, propionic or lactic acids = VFA)
      - Creates osmotic pressure similar to rumen fluid – high osmotic pressure prevents end-products from becoming inhibitory
    - Inorganic salts
      - Provides nutrients required by microbes (trace elements *e.g.*, Ca, Co, Mg, Mn, Fe) to ensure adequacy of fiber digestion of poor quality substrates.
    - Nitrogen
      - Ammonium carbonate supply additional N for bacterial growth as rumen fluid alone can supply insufficient N.
      - Without adequate N, gas production will plateau early (~ 8 h) due to limited microbial activity supported by N in rumen fluid, especially in dry cows fed poor nutrient quality hay-based diets.
  - Saturation with  $\text{CO}_2$ 
    - Displaces dissolved oxygen to create an anaerobic environment
    - Balancing the bicarbonate buffering system which must be saturated by  $\text{CO}_2$
    - Gassing also brings pH in the range for rumen bacteria (from >8 to ~6.5)
    - Carboxylation reactions in AA synthesis
  - Reduction solution
    - Sodium sulphide reduces Buffer medium (lowers redox potential) to create an anaerobic environment by lowering medium affinity for oxygen.

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[Swanepoel/Robinson of UC Davis (April 2016)]