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Enzymatic Spectrophotometry Assay to Measure D-, L-, and Total Lactate in Canine Feces

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1 Works for me dx.doi.org/10.17504/protocols.io.hrqb55w

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EXTERNAL LINK

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Blake AB, Guard BC, Honneffer JB, Lidbury JA, Steiner JM, Suchodolski JS (2019) Altered microbiota, fecal lactate, and fecal bile acids in dogs with gastrointestinal disease. PLoS ONE 14(10): e0224454. doi: 10.1371/journal.pone.0224454

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Triethanolamine	T58300	Sigma Aldrich
Trichloroacetic acid solution	T0699	Sigma Aldrich
D-/L-Lactic acid kit	11112821035	

Aliquot feces

1 Aliquot 120-130 mg feces into 1.5 mL microcentrifuge tube. Store at -80°C until deproteinization.

Begin deproteinization. Add TEA buffer

2 Make 0.1 M triethanolamine buffer (pH 9.15) using scale for accuracy (to the 0.01 mg). Then add 750 µL buffer to each aliquot. Vortex and place in refrigerator (-4°C) for three hours, vortexing every hour to ensure thorough mixing.

Triethanolamine
by Sigma Aldrich
Catalog #: T58300

Centrifuge #1

3 Centrifuge samples at 13,000 x g for 5 minutes at 4°C. Carefully pipette 495 µL of supernatant into a new 1.5 mL microcentrifuge tube. Store supernates at -80°C overnight to provide ample time for next processing steps.

From empirical experience, the extra freeze-thaw cycles favorably increased protein pellet size while not affecting lactate concentration.

Add TCA

4 Thaw samples at room temperature for 20-40 minutes. Once thawed, add 10 µL of 6 M trichloroacetic acid to each sample. Vortex for 10 seconds and place immediately in ice bath for 20 minutes.

Performed this step and following steps (up to dilution with TEA) under a chemical fume hood with proper personal protective equipment.
http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=T0699&brand=SIGMA&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsigma%2Ft0699%3Flang%3Den

Trichloroacetic acid solution
by Sigma Aldrich
Catalog #: T0699

Centrifuge #2

5 Vortex samples again upon removing from ice bath, and centrifuge at 4,500 x g for 20 minutes at 4°C. After centrifugation, a protein pellet was noted in the bottom of each tube. Next, remove 400 µL of supernatant and place in a clean 2 mL microcentrifuge tube.

Dilute samples

6 Add 1600 µL 0.1 M triethanolamine buffer (pH 9.15) to each sample to achieve a neutral or alkaline pH (between 7 and 10). These deproteinized fecal extracts were either used immediately for lactate analysis or stored in -80°C for later use.

Make standards

7 Make standard dilutions with ultra-pure water and D- or L-lactate provided in enzymatic kit (D-/L-Lactate Enzymatic Kit, R-Biopharm Inc.)

Standard	Dilution	Concentration (g/L)
1	1:4	0.05175
2	1:10	0.0207
3	1:20	0.01035
4	1:40	0.005175
5	1:80	0.0025875
Stock concentration = 0.207 g/L		

D-/L-Lactic acid kit
Catalog #: 11112821035

Make Master Mix

8 Make master mix (MM) solution on an as needed basis depending on the number of samples. In a 15 or 50 mL tube, mix together (100 x n) µL solution 1, (20 x n) µL solution 2, and (2 x n) µL solution 3, with n equal to the total number of blanks, standards, and unknown samples.

Solution Number	Description*
1	approx. 30 ml solution, consisting of: glycylglycine buffer, pH approx. 10.0; L-glutamic acid, approx. 440 mg
2	approx. 210 mg NAD, lyophilizate, reconstituted in 6 ml redist. Water
3	approx. 0.7 ml glutamate-pyruvate transaminase suspension, approx. 1100 U
4	approx. 0.7 ml D-lactate dehydrogenase solution, approx. 3800 U
5	approx. 0.7 ml L-lactate dehydrogenase solution, approx. 3800 U
*Descriptions obtained from manufacturer protocol	

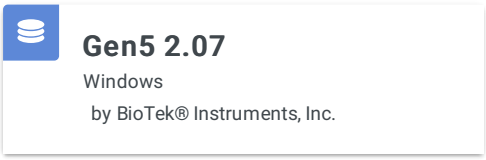
Plate your samples and incubate

9 Pipette blanks, standards, and samples in duplicate onto 96-well plate and place on plate shaker for one minute. Shake at low enough speed that a plate cover is not needed. Then incubate the plate in plate incubator at 25°C for 15 minutes.

Well type	Volumes
blanks	122 µL MM, 100 µL water
standards (D- and L-lactate)	122 µL MM, 100 µL standards 1-5
unknown samples	122 µL MM, 100 µL fecal extract
MM = master mix	

Plate reader and settings

10 Use path length adjustment settings in plate reader software to account for using a protocol originally designed for use with 1 cm diameter cuvettes. Set standards curve to quadratic and adjust lactate concentrations by subtracting the absorbance difference of the blank.



A1 and D-LDH

11 After incubation, take the first absorbance reading (A1) at 340 nm wavelength. Then immediately add 2 µL D-LDH to each well, shake plate for one minute, and then incubate at 25°C for 30 minutes.

A2 and L-LDH

12 Take second absorbance reading (A2) at 340 nm wavelength. Add 2 µL L-LDH to each well, shake for one minute, and incubate at 25°C for 30 minutes.

A3

13 Take third absorbance reading at 340 nm wavelength.

Data processing

14 Calculate standard curve and lactate concentrations using software above. Then, if D- or L-lactate concentrations are below their respective lower limits of quantification, adjust concentrations to their respective cutoff values. Ours were 0.002 g/L for D-lactate and 0.0007 g/L for L-lactate. Also adjust final lactate concentrations based on starting weight of feces, dilution factor, and dry matter content.