Measuring urea concentrations in water samples

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Feb 11, 2022

1

dx.doi.org/10.17504/protocols.io.b4wmqxc6

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Colorimetric assay for direct (vs. enzymatic) measurement of urea to a detection limit of 0.4 µM concentration. The reaction of urea with diacetylmonoxime (DAM) to form a colored product is enhanced by addition of thiosemicarbazide (TSC). The original direct method was adapted for use with a single mixed reagent and incubation at room temperature (vs. the “high temperature direct method” that incubates at 85ºC for 30 minutes). *NOTE: This protocol is written for measurement in 24-well plates*.

DOI

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Jacob Waldbauer, Amy Amy Zimmerman 2022. Measuring urea concentrations in water samples. **protocols.io** https://dx.doi.org/10.17504/protocols.io.b4wmqxc6

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Feb 10, 2022

Feb 11, 2022

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Spectrophotometer (or plate reader),
200 and 1000 µL pipettes,
Tube racks,
Vortexer;
200 and 1000 µL filter tips,
5 mL polypropylene tubes,
24-well microplate with lid, clear,
fresh Reagent A,
fresh COLDER reagent.

1 Making Standards.

1.1 Prepare 200 µM stock solution. Dilute 1:500 from 0.1 M solution => 20 µL + 9.980 mL nanopure water.

1.2 Dilute the stock solution to the following concentrations in nanopure water: 0, 0.5, 1.25, 2.5, 5, 7.5, 10 µM.

2 Making Reagents and Solutions

2.1 1. *Diacetylmonoxime (DAM) solution*: Dissolve 3.4 g in 100 mL nanopure or LC-MS water (34 g L^{-1} or 0.3363 M stock). Store solution at 4°C in dark. Stable for at least 1 month.
a. Also known as 2,3-butanedione monoxime.
b. Dissolve using rotisserie (hybridization oven) set to room temp (prop open door).

2.2 *Thiosemicarbazide (TSC) solution*: Dissolve 0.19 g in 20 mL nanopure water (9.5 g L^{-1} or 0.104235 M stock). Store solution at 4°C in dark. Stable for at least 1 month. a.Dissolve using rotisserie (hybridization oven) set to room temp (prop open door).

2.3 *Ferric chloride (hexahydrate) solution*: Dissolve 0.15 g ferric chloride ($FeCl_3$, $6H_2O$) in 10 mL nanopure water (15 g L^{-1} or 0.0554877 M stock). Store solution at 4°C in dark.

2.4 *Reagent A solution*: Mix 25 parts DAM with 1 part TSC. *Make fresh prior to*

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each analysis. (32.7 g L^{-1} or 0.3234 M DAM and 0.365 g L^{-1}or 0.004 M TSC)

2.5 *Reagent B solution*: Add 300 mL concentrated sulfuric acid (~98% or 18.4 M) to 535 mL nanopure water (final concentration H_2SO_4 = 35.2% or 6.6 M). Add 0.5 mL ferric chloride solution to diluted acid (8.977 mg L^{-1} or 0.03321 mM stock). Store solution at 4°C in dark. Stable for at least 1 month.

2.6 *Color developing reagent (COLDER)*: Mix 1 part of Reagent A with 3.2 parts of Reagent B. ***Use within 15 minutes***. a.Turbidity blank solution: Substitute nanopure water for Reagent A above for determination of optical turbidity blank.

3 Assay set-up.

3.1 Label four polypropylene tubes for each sample and all standards (7) (includes triplicate reactions and single turbidity blanks).NOTE: Assay requires >8 mL of each sample and standard (includes replication).

3.2 Calculate total volume of COLDER reagent needed to run triplicate reactions for each standard and sample: (7 standards + # samples + 1 extra) x 3 x 0.6 mL = total vol (mL).a.*If quantifying urea from 2 samples: 7+2+1 = 10 x3x0.6 = 18 mL reagent.*

3.3 Calculate total volume of turbidity blank solution needed to run duplicates for each standard and sample: (7 standards + # samples + 1 extra) x 2 x 0.6 mL = total vol (mL). a.*If quantifying urea from 2 samples: 7+2+1 = 10 x2x0.6 = 12 mL blank solution.*

3.4 Aliquot 2 mL of each sample and all standards into corresponding reaction tubes.

3.5 Prepare the volumes of COLDER reagent and turbidity blank solution needed by mixing 1 part of Reagent A (or nanopure water) with 3.2 parts of Reagent B as described below. ***Use reagent within 15 minutes***.
a.If need 18 mL, mix 4.5 mL of Reagent A with 14.4 mL of Reagent B (18.9 mL).
b.If need 12 mL, mix 3 mL of nanopure water with 6 mL of Reagent B (12.6 mL).

Add 0.6 mL of COLDER reagent (or turbidity blank) to each reaction tube.

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3.6 a. DAM: 1.7963 g L^{-1} or 0.01777 M final concentration
b.TSC: 0.02008 g L^{-1} or 0.0002203 M final concentration
c.$FeCl_3$: 0.001578 g L^{-1} or 0.00000584 M (5.84 µM)
d.H_2SO_4: 6.2% or 1.16 M final concentration

3.7 Mix (vortex) and incubate in the dark at room temperature (~22ºC) for 3 days.

3.8 After 72 hours, transfer/pour entire 2.6 mL volume of each reaction into corresponding wells of 24-well plates and measure absorbance on plate reader.

4 Reading plates.

4.1 Turn on Tecan Infinite 200 PRO plate reader 20-30 minutes prior to use.

4.2 Once warmed up, open the iControl software on MLCLab-PC.

4.3 Open file “Revilla_urea_24well”.

4.4 Load the plate—check whether the “plate with cover” box is checked (since using clear plates for this, can be read with lid on).

4.5 Read absorbance at 520±9 nm (25 flashes). Program automatically opens an Excel file that documents read parameters and data. ‘Save as’ before measuring 2nd plate.

5 Analyzing data.

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5.1 Subtract the average absorbance of sample turbidity blanks from the absorbance of the samples treated with COLDER reagent (= corrected sample absorbance).

5.2 Subtract the average absorbance of the nanopure water tubes (i.e., 0 µM urea) treated with turbidity blank solution from the absorbances of all the standards.

5.3 Plot corrected absorbance (y) vs. concentration (x) for all standards to establish a standard curve with linear regression.

5.4 Use the equation of the standard curve to calculate sample concentration from absorbance.

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