BUN (Serum or Plasma) by the QuantiChrom Urea Assay Kit (DIUR-100; BioAssay Systems, Hayward, CA)

10-28-20 mr/wk; rev 1-5-2021 wk

*Read package insert (**Addendum Figure)** before running this analysis. If the 2017 package insert has been updated, this procedure must be re-evaluated. wk*

Specimen: Serum or plasma (heparin, e.g., green top blood collection tube) separated from cells as soon as possible, non-hemolyzed. Store frozen (-20°C) until analysis.

Reagents:

Reagent A: 12 mL supplied. Store refrigerated at 4°C. Stable 12 months after receipt.

Reagent B: 12 mL supplied. Store refrigerated at 4°C. Stable 12 months after receipt.

50 mg/dL Urea Standard [i.e., 23.364 mg/dL Urea Nitrogen (BUN)]**:** Supplied ready to use. Store frozen at -20°C. Stable 12 months after receipt.

18 MOhm water (18MΩ H_2O): from water purification system in Lab 109/110.

Control: In-house pooled plasma or serum sample. Store frozen at -20°C. Thaw only the amount needed for the day; use a new aliquot each day. Control ranges (average ± 2SD) to be determined.

0.85% (w/v) Sodium chloride (for diluting high concentration samples): In a 500-mL volumetric flask, dissolve 4.25 g NaCl (MW = 58.44 g/mol) in ~400 mL 18MΩ H_2O. Mix. Bring up to 500 mL with 18MΩ H_2O. Mix. Store in labeled container refrigerated at 4°C; stable 6 months.

Procedure:

1. Check that the Synergy H1 Microplate Reader (Biotek, Winooski, VT) in Hultz 135 is available for use. Sign up to use it on the Departmental Microplate Calendar. Turn on the computer that controls the plate reader. The password is “Nutrition135.” Check that microcentrifuges in Lab 153 are also available.

2. Thaw serum or plasma SAMPLEs and CONTROL. Vortex gently to mix.

3. Remove **Reagent A, Reagent B,** and **50 mg/dL Urea Standard** from the refrigerator/freezer and allow to come to room temperature. *Note: Need 16 mL each of Reagent A and Reagent B for one, 96-well plate.* Before use, gently invert the reagent bottles a few times to thoroughly mix. *Do not shake*.

4. Prepare the Working STANDARDSs fresh, daily, in 1.5-mL microtubes following **Table 1** below.

Table 1. Working Urea Nitrogen standard set preparation using a 50-mg/dL Urea Standard for a total volume of 150 µL of each Working STANDARD. *Note: Working standards are expressed as Urea Nitrogen (BUN).*			
Standard number	**Amount of 50 mg/dL Urea Standard (µL)**	**18MΩ H_2O (µL)**	**Final *Urea Nitrogen* Working STANDARD concentration (mg/dL)**
BLANK	0	150	0
Std 1	15	135	2.336
Std 2	30	120	4.673
Std 3	60	90	9.346
Std 4	90	60	14.019
Std 5	120	30	18.692
Std 6	150	0	23.364

5. Plan the layout of a 96-well microtiter plate (96-well flat bottom, medium binding, polystyrene without lid; Greiner Bio-one 655101) according to the existing protocol. See **Figure 1** plate layout.

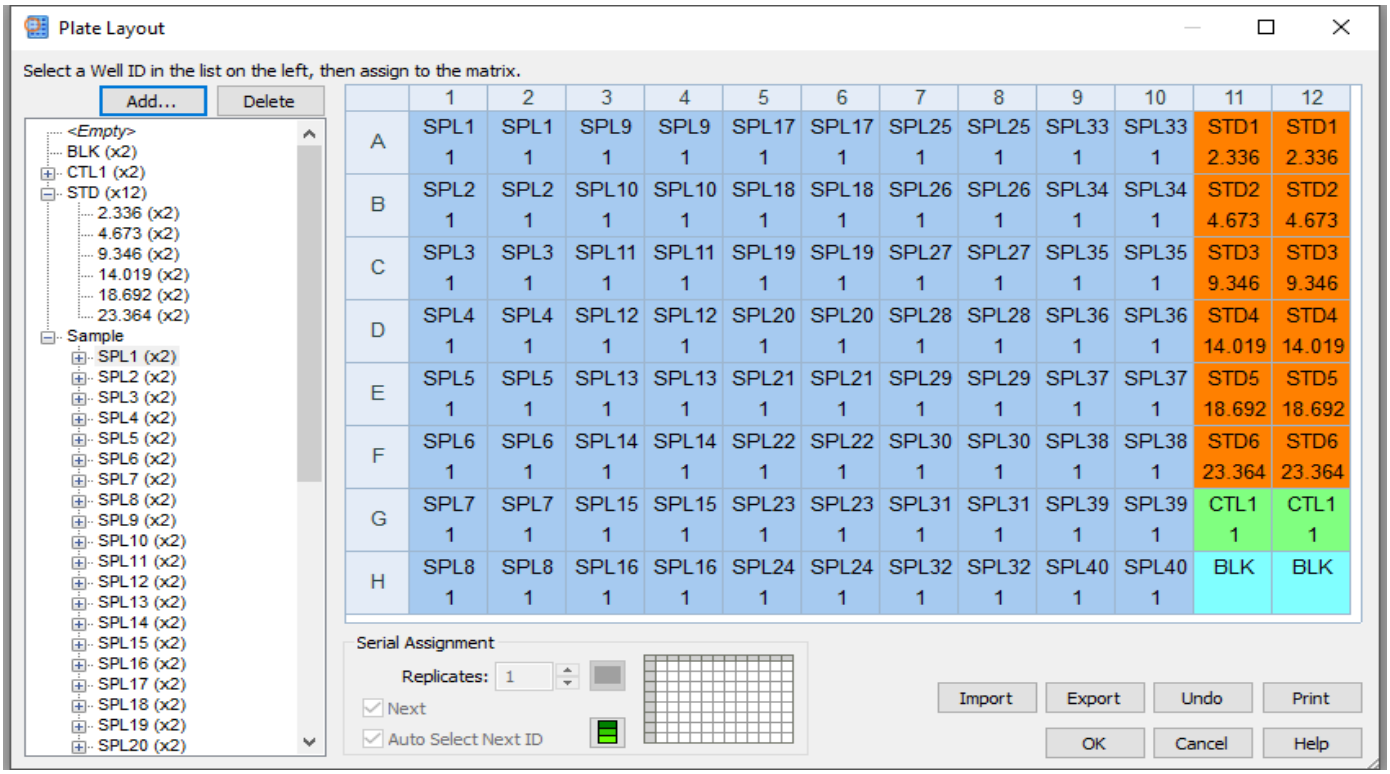


Figure 1. Urea nitrogen plate layout example. SAMPLES, STANDARDS, BLANKS, and CONTROL are run in duplicate. In this example, SAMPLES all have dilution factors of 1.

6. Turn on the Synergy microplate reader (right, bottom, front side). Open Gen5 2.04 program located on the desktop. Allow the instrument to do start up functions. Door opens when complete. Manually cl

Task Manager] menu

[Experiments] menu

[Create using existing protocol]. To find protocol go to [My Documents folder]; [Lab 135] folder; [BUN] folder; [Protocols] folder; filename BUN QuantiChrom Urea Assay Protocol Lab 135.prt.

Check the [Protocol] for correct [Procedure]. [Temp off; Shake: Linear 15 s; Read(A) 520].

Check [Plate Layout]; change accordingly. Check dilution factors. To change dilution factors, double click on samples (e.g., SPL 1) [Well ID] menu; [Type: Dilutions]. Enter dilution factor in SPL1:1. [OK].

7. Label 1.5-mL microcentrifuge tubes, in duplicate, to correspond to each SAMPLE, CONTROL, BLANK, and STANDARD to be run. For one, 96-well plate, a total of 192 microcentrifuge tubes will be needed.

8. Pipet 5 μL of each SAMPLE, CONTROL, BLANK, or prepared BUN Working STANDARD into respective 1.5-mL microcentrifuge tubes. *Change pipet tips between every sample, do not rinse the pipet tip in the sample, and pipet directly to the bottom of the tube for best results.*

9. Prepare **Working Reagent** immediately before use and only in the amount needed to run your plate:

In a labeled, 30-mL plastic bottle, combine equal parts of **Reagent A** and **Reagent B**: e.g., 16 mL of Reagent A and 16 mL of Reagent B for a total of 32 mL **Working Reagent** for a full 96-well microplate. Invert to mix. Use reagent within 20 minutes of mixing.

10. Using a Repeater pipet with a combi-tip attached, add 300 μL of **Working Reagent** into all samples already in respective microcentrifuge tubes. Close the microtubes, and vortex to mix. Incubate at room temperature (~ 22°C) for 20 minutes.

11. During the incubation, load the first batch of 66 tubes into microcentrifuges located in Lab 153 (two centrifuges, one with 36-tube capacity and one with 30-tube capacity). After the 20-minute delay, begin centrifuging at 15,000 x *g*, for 6 minutes and 22°C.

12. Once the first batch of tubes has finished spinning, remove from the microcentrifuge. Load the second batch and centrifuge as before. Continue in this manner until all tubes have been centrifuged.

13. During the centrifugation process, begin pipetting the first batch of samples into the 96-well plate. See plate layout example (**Figure 1)**. Using a new pipet tip for each sample tube, pipet 200 μL of each supernatant into its corresponding well on the 96-well microplate. *Note: Do NOT rinse pipet tips, it will disturb the precipitate.* Continue with the other samples as they come out of the microcentrifuge.

14. To load the plate into microplate reader, press the green triangular [Read] button on the top menu bar of the open Synergy Gen5 2.04 protocol file. The door to the microplate chamber should open. Place the microplate into the plate reader with well A1 on the upper left-hand side. Follow computer screen prompts. The instrument will perform the set protocol: shake for 15s, read at (A) 520 nm.

15. Results appear on the screen. The screen will prompt [SAVE]. Follow the guidelines below for editing/deleting points. Only edit results on [Plate 1] [Matrix] tab, [Data: Blank 520] dropdown menu.

a) On [Plate 1] [Matrix] tab, [Data: Blank 520] dropdown menu, check that the BLANK values are at or near zero and are consistent. [Mask] any BLANK that does not meet these criteria.

b) Check the standard curve (*Raw OD520 data has been transformed as follows: Blank Transformation and then linear regression calculated from Working BUN Standard concentration vs OD520*). [Plate 1] [Graphs] tab, [Results: StdCurve Fitting Results]. Is the R^2 between 0.995 and 1.00? Are the variables (slope and intercept) similar to previous BUN assays? If not, delete the bad points to get the best and most consistent standard curve. To do this, go to [Plate 1] [Matrix] tab, [Data: Blank 520] dropdown menu. Click on [Mask] button and then click on values to be masked [Apply changes] [Close] [Yes].

c) To check standard differences (assigned values vs values calculated and assigned based on the linear regression curve) go to [Plate 1] [Statistic] tab, [Data: Concentration] dropdown menu. Compare standard values in Concentration/Dil column with those in the Mean column. Are these values acceptable? If not, go back and [Mask] the appropriate standard values.

d) Is the CONTROL within control limits? Go to [Plate 1] [Statistics] tab, [Data:ConcentrationxDilution] dropdown menu.

e) Do the SAMPLE values fall within the standard curve values? Go to [Plate 1] [Statistics] tab, [Data:ConcentrationxDilution] dropdown menu. If not, repeat with different dilutions.

f) Are the SAMPLE values in normal range and/or what you expected?

g) To check CV, go to [Plate 1] [Statistics] tab, [Data: dilution factor calc] dropdown menu. Are the CV (%) of the SAMPLE ≤ 7%? If not, repeat the analysis on that sample.

16. [SAVE] the .exp file in the [Project] folder.

17. Export the results to Excel (Paper with blue arrow Icon on top menu bar). Add identifying info to the report (date, tech initials, experiment name, type of sample, and catalog and lot numbers of all reagents used), [SAVE] the .xlsx file in the [Project] folder, and PRINT. Fill out a *BUN QuantiChrom Urea Assay QC* sheet using the example below (**Figure 2**) for the project. Save QC files to the specific [Project] folder.

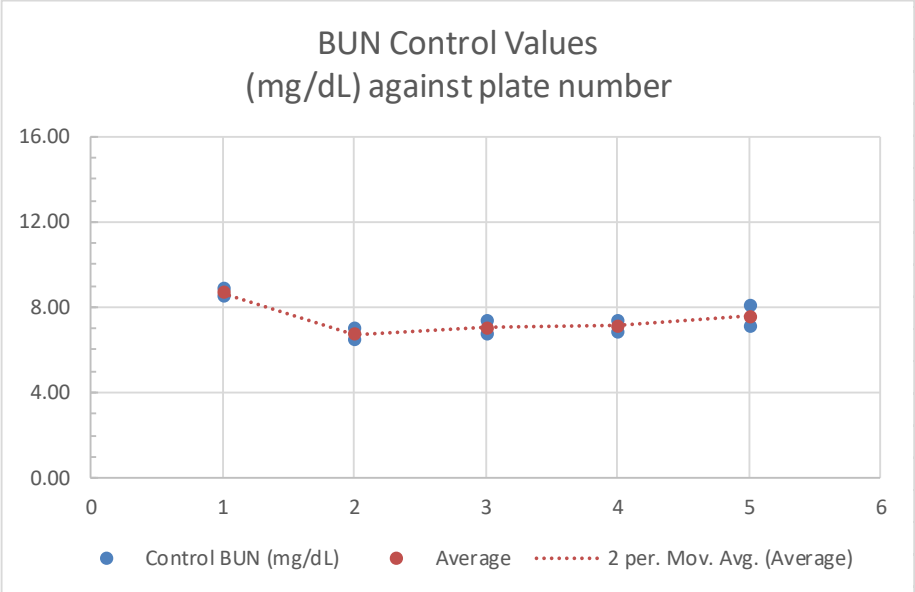
18. When finished using the microplate reader, close out of the program, and turn off the microplate master switch. Shut computer down at the end of the day, or when completed if you are the only user.

Repeat analysis on sample duplicates with CV greater than 7%.

Linearity: at least 23.364 mg/dL BUN.

To convert Urea Nitrogen (BUN) to Urea, multiply the results x by 2.14. (e.g. 23.364 mg/dL BUN * 2.14 = 50 mg/dL Urea).

Assay: BUN BioAssay Systems QuantiChrom Urea Assay Kit						Date/Tech: 11/16/20 Maddy Rehn		
Project: Carl Mature Bulls								
In-house pooled control made from ???								
Average	7.44							
1SD	0.79		In-house range:					
2SD	1.58		5.86	to	9.02	mg/dL		
3SD	2.37							
							Linear regression	
Date	Running Plate #	Control BUN (mg/dL)	Average	%CV			Slope A	Intercept B
11/2/2020	1	8.492	8.688	3.182			0.01150	-0.00108
	1	8.883						
11/3/2020	2	6.481	6.733	5.283			0.01150	0.00102
	2	6.984						
11/4/2020	3	6.753	7.055	6.044			0.01230	-0.00480
	3	7.356						
11/16/2020	4	6.868	7.139	5.368			0.01130	0.00529
	4	7.410						
11/16/2020	5	7.124	7.587	8.630			0.01130	0.00348
	5	8.050						
average			7.440			*average*	0.01158	0.00078
stdev			0.789			*stdev*	0.0004	0.0039
%CV			10.60	5.70		*%CV*	3.58	504.60
			%CV inter (between) assay	**%CV intra (within) assay**				



QuantiChrom™ Urea Assay Kit (DIUR-100)

Quantitative Colorimetric Urea Determination

DESCRIPTION

UREA is primarily produced in the liver and secreted by the kidneys. Urea is the major end product of protein catabolism in animals. It is the primary vehicle for removal of toxic ammonia from the body. Urea determination is very useful for the medical clinician to assess kidney function of patients. In general, increased urea levels are associated with nephritis, renal ischemia, urinary tract obstruction, and certain extrarenal diseases, e.g., congestive heart failure, liver diseases and diabetes. Decreased levels indicate acute hepatic insufficiency or may result from over-vigorous parenteral fluid therapy.

Simple, direct and automation-ready procedures for measuring urea concentration or blood urea nitrogen BUN in biological samples are becoming popular in Research and Drug Discovery. BioAssay Systems' urea assay kit is designed to measure urea directly in biological samples without any pretreatment. The improved Jung method utilizes a chromogenic reagent that forms a colored complex specifically with urea. The intensity of the color, measured at 520 nm, is directly proportional to the urea concentration in the sample. The optimized formulation substantially reduces interference by substances in the raw samples.

KEY FEATURES

Sensitive and accurate. Use 5 µL samples. Linear detection range 0.08 mg/dL (13 µM) to 100 mg/dL (17 mM) urea in 96-well plate assay.

Simple and high-throughput. The procedure involves addition of a single working reagent and incubation for 20 min. Can be readily automated as a high-throughput assay for thousands of samples per day.

Improved reagent stability and versatility. The optimized formulation has greatly enhanced reagent and signal stability. Cuvet or 96-well plate assay.

Low interference in biological samples. No pretreatments are needed. Assays can be directly performed on raw biological samples i.e., in the presence of lipid and protein

APPLICATIONS

Direct Assays: urea in serum, plasma, urine, milk, cell/tissue culture, bronchoalveolar lavage (BAL) etc.

Drug Discovery/Pharmacology: effects of drugs on urea metabolism.

Environment: urea determination in waste water, soil etc.

KIT CONTENTS (100 TESTS IN 96-WELL PLATES)

Reagent A: 12 mL **Standard:** 0.5 mL (50 mg/dL)

Reagent B: 12 mL

Storage conditions. The kit is shipped at room temperature. Store all components at 2-8°C. For long-term storage, keep standard at –20°C. Shelf life: 12 months after receipt.

Precautions: reagents are for research use only. Normal precautions for laboratory reagents should be exercised while using the reagents. Please refer to Material Safety Data Sheet for detailed information.

PROCEDURES

Reagent Preparation: Equilibrate reagents to room temperature. Prepare enough working reagent for all samples and standards by combining equal volumes of Reagent A and Reagent B shortly prior to assay. Use working reagent within 20 min after mixing.

Procedure for 96-well Plate

1. Serum and plasma samples can be assayed directly (n = 1). Urine samples should be diluted 50-fold in distilled water prior to assay (n = 50). Transfer 5 µL water (blank), 5 µL standard (50 mg/dL) and 5 µL samples in duplicate into wells of a clear bottom 96-well plate.

   For low urea samples (< 5 mg/dL), e.g. tissue/cell extract, BAL etc, transfer 50 µL water (blank), 50 µL ***5 mg urea/dL*** *(the 50 mg/dL standard diluted 10× in water) and 50 µL samples in duplicate into separate wells. For culture medium containing phenol red, transfer 50 µL medium (blank), 50 µL* ***5 mg urea/dL*** *(the 50 mg/dL standard diluted 10× in medium) and 50 µL samples in duplicate into separate wells.*

2. Add 200 µL working reagent and tap lightly to mix.
3. Incubate 20 min (*50 min for low urea samples*) at room temperature.
4. Read optical density at 520 nm. *For low urea samples, read OD at 430 nm.*

Procedure for Cuvettes

Prepare samples as described for 96-well plate assay. Transfer 20 µL water, standard (50 mg/dL) and samples to appropriately labeled tubes. *For low urea samples, use 5 mg/dL standard and 200 µL instead of 20 µL*. Add 1000 µL working reagent and tap lightly to mix. Incubate 20 min (*50 min*) and read OD_{520nm} (OD_{430nm}).

CALCULATION

Urea concentration (mg/dL) of the sample is calculated as

$$[\text{Urea}] = \frac{OD_{SAMPLE} - OD_{BLANK}}{OD_{STANDARD} - OD_{BLANK}} \times n \times [\text{STD}] \quad (mg/dL)$$

OD_{SAMPLE}, OD_{BLANK} and $OD_{STANDARD}$ are OD values of sample, blank and standard, respectively. n is the dilution factor. [STD] = 50 (*or 5 for low urea samples*), urea standard concentration (mg/dL).

Conversions: **BUN** (mg/dL) = **[Urea]** / 2.14.

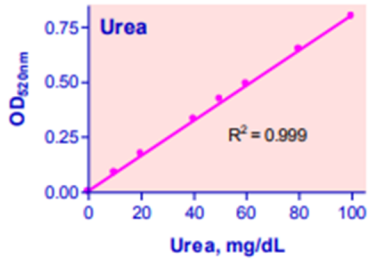
1 mg/dL urea equals 167 µM, 0.001% or 10 ppm.

MATERIALS REQUIRED, BUT NOT PROVIDED

Pipeting devices, centrifuge tubes, Clear flat-bottom 96-well plates (e.g. Corning Costar) or cuvettes, and plate reader or spectrophotometer.

EXAMPLES

Biological samples were assayed in duplicate using the 96-well protocol. The urea concentration (mg/dL) was 12.5 ± 0.9 for Commercial 2% reduced fat milk (Kirkland), 35.7 ± 0.1 for Invitrogen fetal bovine serum, 22.1 ± 0.9 for human serum, 22.3 ± 0.2 for human plasma, 31.8 ± 1.1 for rat serum, 42.6 ± 0.1 for rat plasma and 1501 ± 52 for a fresh human urine sample, 0.21 ± 0.03 in a human BAL sample, 0.15 to 2.7 mg/dL in cell culture.



Standard Curve in 96-well plate assay

PUBLICATIONS

1. Ji, H., Bachmanov, A.A. (2007). Differences in postingestive metabolism of glutamate and glycine between C57BL/6ByJ and 129P3/J mice. Physiol Genomics 31(3):475-82.
2. Snykers, S. et al (2007) Chromatin remodeling agent trichostatin A: a key-factor in the hepatic differentiation of human mesenchymal stem cells derived of adult bone marrow. BMC Dev Biol. 7:24.
3. Zeng, L. et al (2006). Multipotent adult progenitor cells from swine bone marrow. Stem Cells 24:2355–2366.