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Bacterial Whole-Cell Biosensor for Glutamine with Applications for Quantifying and Visualizing Glutamine in Plants

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A whole-cell biosensor for glutamine (GlnLux) was constructed by transforming an *Escherichia coli* glutamine (Gln) auxotroph with a constitutive *lux* reporter gene. Measurements of Gln in plant extracts using GlnLux correlated with quantification using high-performance liquid chromatography (Spearman's $r = 0.95$). GlnLux permitted charge-coupled-device (CCD) imaging of Gln from whole plant organs.

Microbial whole-cell biosensors have been used to quantify analytes from biological extracts into which they are coinoculated (6, 8, 21). Such biosensors have also been employed for *in vivo* imaging of metabolites following their leakage from plant organs onto agar embedded with biosensor bacteria expressing a visible reporter gene (12, 14, 19, 22).

Glutamine (Gln) is a critical intermediate in nitrogen metabolism. In plants, soil nitrogen is assimilated into Gln, which then serves as a nitrogen donor for other biochemical reactions (18). Gln is also a major long-distance transport form of nitrogen (e.g., root to shoot) (20). Today, free Gln is primarily quantified using tissue extracts by high-performance liquid chromatography (HPLC) (1, 17), not *in vivo* imaging.

Here we constructed a microbial *lux* biosensor for Gln. *Escherichia coli* Gln-auxotrophic strain JW3841-1 (*E. coli* Genetic Stock Center CGSC 10775, Yale University) was previously generated by disrupting *glnA* with a kanamycin cassette (*glnA*732Δ::Kan) (2). The auxotroph failed to grow in M9 minimal medium except when it was supplemented with Gln. We transformed JW3841-1 with pT7-*lux* (Amp^r) (16), containing a *luxCDABE* operon from *Vibrio fischeri* driven by a constitutive T7 promoter from *Xenorhabdus luminescens*, creating strain GlnLux.

Gln standards were used to optimize GlnLux for luminometer assays. In the optimized protocol, GlnLux was grown (optical density at 595 nm [OD_{595}] of 0.3, LB, 37°C, 250 rpm) and then pelleted (centrifugation at 700 × *g*, 25°C, 10 min). To deplete endogenous free Gln, the pellet was washed 3 times (in M9 medium lacking Gln), diluted (1/10, $OD_{595} = 0.025$, M9 medium), and then incubated (37°C, 250 rpm, 14 h) (9). Diluting GlnLux cultures in this way resulted in a subsequent reduction of background noise and an increased assay sensitivity (see Fig. S1 in the supplemental material). The culture was then incubated with Gln standards (96-well opaque white plates; Fisher CS003912): GlnLux (10 μl) and M9 medium containing Gln standards (90 μl) were added into each well, covered (sterile breathable film; Sigma A9224), and then incubated (37°C without shaking). Increasing the incubation time of Gln with GlnLux cells shifted the linear range of the assay higher (see Fig. S2 and S3 in the supplemental material). For lux quantification, plates were reread hourly (1-s reads; MicroLumat Plus LB96V; Berthold Technologies, Germany). All media were supplemented with 50 μg/ml kanamycin and 100 μg/ml carbenicillin.

The GlnLux assay using Gln standards achieved a sensitivity of 0.0001 μg/ml (0.68 nM) (see Fig. S2 in the supplemental material), which is similar to that of the most sensitive HPLC-tandem mass spectrometry (HPLC-MS/MS) assay (7, 11). The linear range of the assay extended across 3 orders of magnitude (0.0001 to 0.1 μg/ml Gln) (see Fig. S2 in the supplemental material). GlnLux did not significantly react to other nitrogenous compounds at concentrations expected in plant extracts following a 1,000-fold dilution, the dilution needed to shift extracted Gln into the linear assay range. There was no significant cross-reactivity from NH_4Cl (≤90 μM, already present in M9 medium), KNO_3 (≤200 μM), $NaNO_2$ (≤0.3 μM), or a 19-amino-acid cocktail (3 to 110 μM). However, higher nitrate or amino acid concentrations could potentially activate GlnLux (data not shown).

The accuracy of the GlnLux luminometer assay for quantifying Gln in plant extracts was determined by comparing the results with those from HPLC (1) (see the supplemental material for the HPLC methodology and plant treatments). For the GlnLux assay, maize/corn (*Zea mays* L.) leaves were ground (1 to 10 mg) with sand in liquid nitrogen, treated (9 μl double-distilled water [ddH_2O] with 1% [vol/vol] protease inhibitor cocktail [PIC; Sigma P9599, pH 7.0] per mg), vortexed, and centrifuged (20 min, 4°C, 10,000 × *g*). The supernatant was transferred to a fresh tube on ice, diluted 1,000-fold (ddH_2O, pH 7.0), and used immediately. Using 96-well plates, each well was prepared (80 μl 1.25× M9 medium, 10 μl GlnLux as noted above, 10 μl plant extract). The PIC inhibited suspected proteases in plant extracts (data not shown). An excellent correlation was found between measurements using the GlnLux assay and those using HPLC (Spearman's $r = 0.95$, $P < 0.0001$, degrees of freedom [df] = 39) (Fig. 1). Remarkably, GlnLux leaf measurements could distinguish between plants fertilized with and without ammonium/nitrate (Fig. 1) using only the equivalent of 1 μg of tissue per well.

For imaging of Gln in whole plant organs, tissues were freeze-thawed to cause Gln leakage, placed on agar preembedded with

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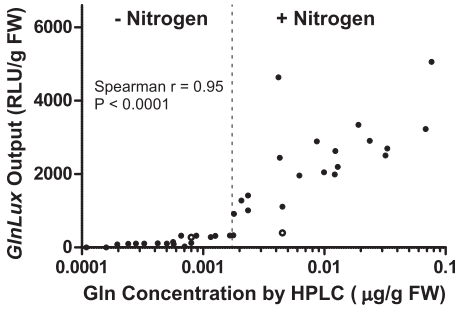

FIG 1 Validation of the GlnLux luminometer assay as a method to quantify free Gln in plant tissue extracts by comparing results to those of a standard HPLC assay. Leaf extracts of maize seedlings previously treated with fertilizer solution with or without nitrogen were quantified using HPLC and independently tested using the GlnLux luminometer assay, with the latter using 1/1,000 dilutions of maize extract, corresponding to 1 μg tissue (fresh weight [FW]). Each value is from an extract from one leaf of one seedling. The coefficient of correlation (Spearman's *r*) between the GlnLux and HPLC assays is indicated. HPLC and GlnLux readings segregated based on the prior nitrogen fertilizer treatment (vertical dotted line), with the exception of two outliers (open circles). The 0-μg/ml Gln standard reading was subtracted from all lux values, which were read in randomized replicates of 3 to 4 in an endpoint assay set to the "integrate" function. RLU, relative light units.

GlnLux (GlnLux agar), and then imaged using a photon capture camera (22). Briefly, a GlnLux starter culture (LB with 50 μg/ml kanamycin, 100 μg/ml carbenicillin, 0.2 mM Gln, 4.0 mM glucose; 16 h, 37°C, 250 rpm) was pelleted (centrifugation at 1,100 × *g*, 25°C, 10 to 20 min), washed 3 times (0.01 M potassium phosphate buffer, pH 7.0), and resuspended (M9 medium with an OD_{595} of 1.0). To prepare GlnLux agar, concentrated M9 medium (with Bacto agar, 10 g/liter) was cooled (42°C), supplemented with Casamino Acids solution (5 g/liter of the final solution), mixed with GlnLux (10% [vol/vol], OD_{595} = 1.0), and then poured. Flash-frozen tissues were thawed (room temperature, 30 s), pressed onto GlnLux agar, inverted, incubated (37°C, 1 to 6 h), and imaged hourly (ChemiProHT, Roper) (4) with a charge-coupled-device (CCD) chip precooled to −80°C (5) (200- to 600-s exposures). GlnLux agar permitted imaging of Gln at a sensitivity threshold of 0.01 to 0.1 mM (data not shown).

For plant imaging, maize seedlings were fed with nitrate once or with only water (14 h prior to freeze-thawing). GlnLux agar under nitrate-treated roots emitted a noticeably higher signal than the same agar under water-treated roots (Fig. 2A and B). Leaves were also imaged after their roots were exposed to fertilizer with or without ammonium/nitrate (five times, ~50 to 10 h prior to freeze-thawing). The signal from GlnLux agar under leaves from nitrogen-treated seedlings was dramatically higher than that of the same agar under leaves from nitrogen-deficient seedlings (Fig. 2A to H). Thus, GlnLux agar was successful in visualizing root nitrogen uptake, assimilation into Gln, and long-distance transport to leaves (18).

Apart from uptake, free Gln can accumulate at the base of a senescing leaf from scavenged proteins at the tip; scavenged Gln is exported through vascular cells to growing organs (10). In previous studies using maize leaf 2, Gln levels were highest at the leaf base, decreasing by 50% in the middle and by >90% near the tip

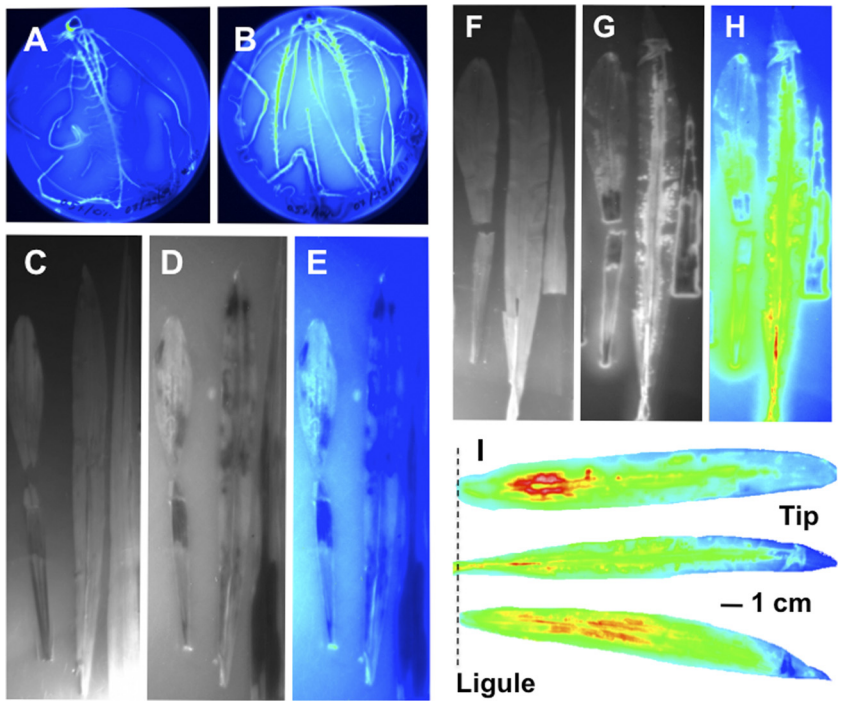

FIG 2 Luminescence *in vivo* imaging of Gln in plant organs using GlnLux agar. (A to H) Two-week-old maize seedlings either were treated with nitrogen fertilizer or were not, and harvested plant organs were freeze-thawed to cause Gln leakage and then placed on GlnLux agar; the opposite agar surface was then imaged using a photon capture CCD camera. Representative pictures of a root system not previously exposed to nitrate (A), a root system previously exposed to nitrate (B), leaves from unfertilized plants (C to E), leaves from ammonium nitrate-fertilized plants (F to H), and leaf number 2 from different ammonium nitrate-fertilized plants showing consistent spatial distribution of Gln (I) are shown. (C to H) The leaf tip numbers in each panel from left to right are 1, 2, and 4. The light images (C and F), white lux images (D and G), and false-colored lux images (A, B, E, H, and I) are shown.

(3), consistent with peak glutamine synthetase expression at the leaf base (13, 15). Using GlnLux agar, *lux* expression in fertilized leaf 2 was consistently highest at the base-to-middle zones along the midvein vasculature and lowest at the tip (Fig. 2I). GlnLux agar could thus resolve *in vivo* spatial variation for Gln. Nevertheless, imaging using GlnLux agar is only semiquantitative. Future applications may include high-throughput screening for nitrogen uptake/metabolism mutants. GlnLux is complementary to a recent plant Forster resonance energy transfer (FRET)-based Gln biosensor providing subcellular resolution (23).

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SUPPLEMENTAL MATERIALS AND METHODS

Leaf sampling - Plant growth conditions and treatments (Fig. 1, Fig. 2C-I). *Zea mays* L. (hybrid CG102 X CG108) seed was used for all experiments. Seeds were germinated on wet Kimwipes for 3 d then transplanted into individual flat inserts (6 x 8 x 5 cm) containing a clay substrate (Turface MVP, Profile Products, USA) and watered with ddH_2O only. Growth chamber (Model PGR15, Conviron) conditions were set to 28ºC/20ºC (day/night) with a 16 h photoperiod with cool white Supersaver CW/VHO/SS (Sylvania) and enhanced spectrum fluorescent bulbs [(Gro Lite WS GL/WS/VHO (Gro Lite Industrial) and VitaLux Ultra-High Output (Duro-Test)] at 120-150 µmol m^{-2} s^{-1} at pot level. At the 13th day after transplanting, plants were watered with a modified Hoagland's nutrient solution that either contained all nutrients required for growth including nitrogen (+N solution containing 20 mM total N), or all nutrients excluding nitrogen (-N). Both nutrient solutions were at pH 5.7 and contained: 0.1 mM K_2SO_4 (Fisher P-304), 1 mM KCl (Sigma P9333), 2 mM KH_2PO_4 (Sigma P0662), 1 mM $MgSO_4 \cdot 7H_2O$ (Sigma 230391) and 0.03 g/liter Plant-Prod Chelated Micronutrient Mix (Plant Products). The +N nutrient solution also contained 6 mM $Ca(NO_3)_2 \cdot 4H_2O$ (Sigma 237124) and 4 mM NH_4NO_3 (Sigma A3795), whereas the –N nutrient solution contained 6 mM $CaCl_2 \cdot 2H_2O$ (Sigma C-79). Plants were each watered with a single 20 ml dose of nutrient solution at 40 h before sampling and another 25 ml does at 25 h before sampling. At 17, 10 and 1 h before sampling the excess nutrient solution was poured from each pot and re-watered with 15 ml of fresh nutrient solution per plant. There were 4-5 pots/treatment and the positions of the

pots were randomized within a treatment block and changed every other day to minimize positional effects in the growth chamber.

Root sampling - Plant growth conditions and treatments (Fig. 2A,B). For root imaging, plants were germinated and grown in vertical germination bags (16 x 16 cm) to allow roots to grow flat for easier lux imaging. The seedlings were treated with ddH_2O only for 14 d using the above growth chamber conditions. At 14 h before sampling, the plants were watered with a single dose of 10 mM NO_3 or water.

Plant sampling for *GlnLux* versus HPLC comparisons (Fig. 1). At the 15th day after transplanting, leaf tissue was collected at various time points from + N and – N groups, frozenZ in liquid nitrogen and stored at -80˚C. After grinding, each homogenized sample was divided into two, for parallel HPLC and biosensor analysis.

HPLC measurements of plant Gln (Fig. 1). Fifteen to two hundred milligrams of weighed frozen homogenized samples were re-ground using a chilled mortar and pestle with silica sand and 0.6-2.0 ml of sulphosalicylic acid (30 mg/ml) (2). The homogenate was centrifuged for 20 min at 16,000 x *g*, the supernatant was collected and the pH adjusted to 7.0 using 4 N NaOH. The sample was stored at -20ºC for up to 24 h. For HPLC analysis, the amino acid samples were separated by reverse-phase HPLC following automatic derivatization with o-phthalaldehyde

(1) using an Agilent Model 1100 series HPLC [Agilent Technologies Inc. (Mississauga, Canada) equipped with a degasser, binary pump, autosampler, temperature-controlled column holder, and fluorescence detector].

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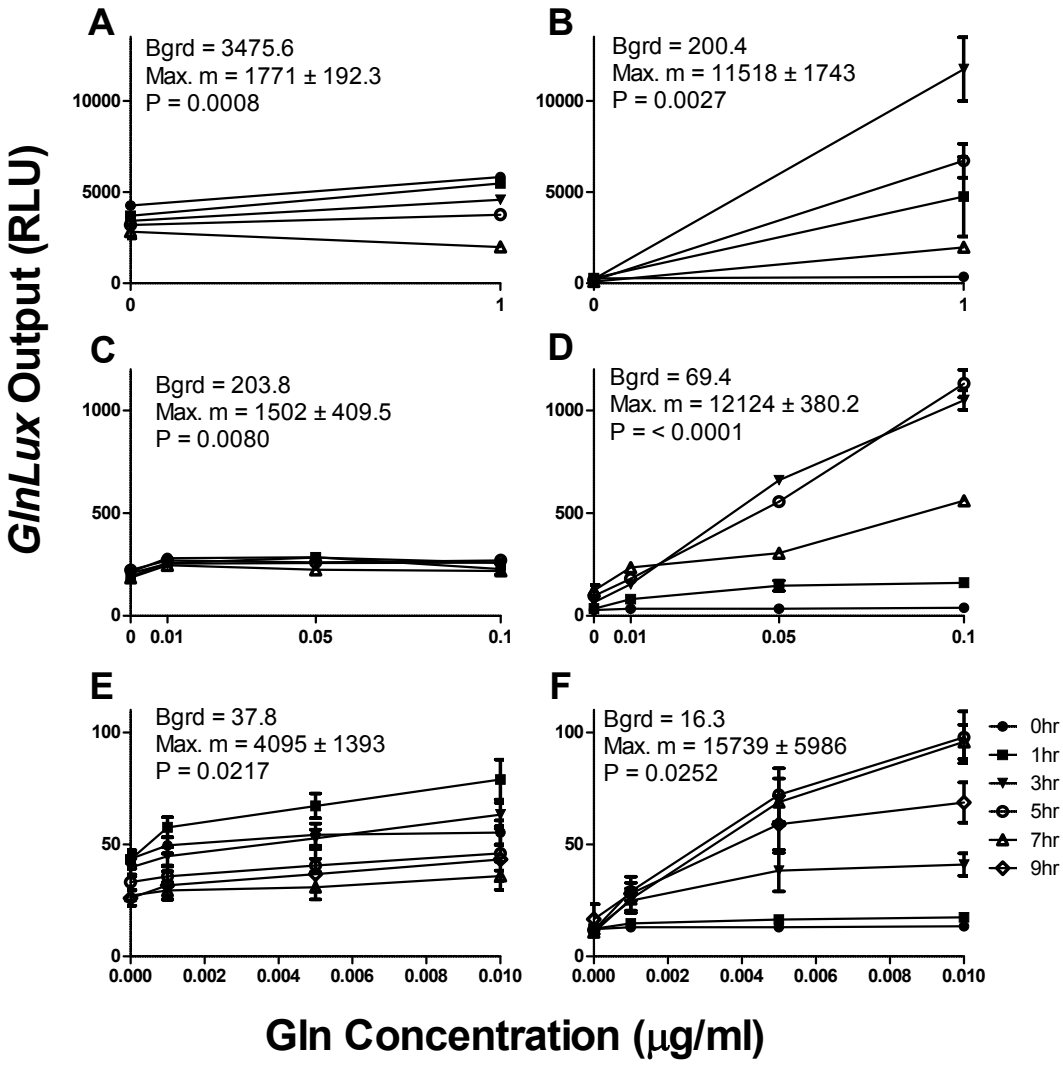
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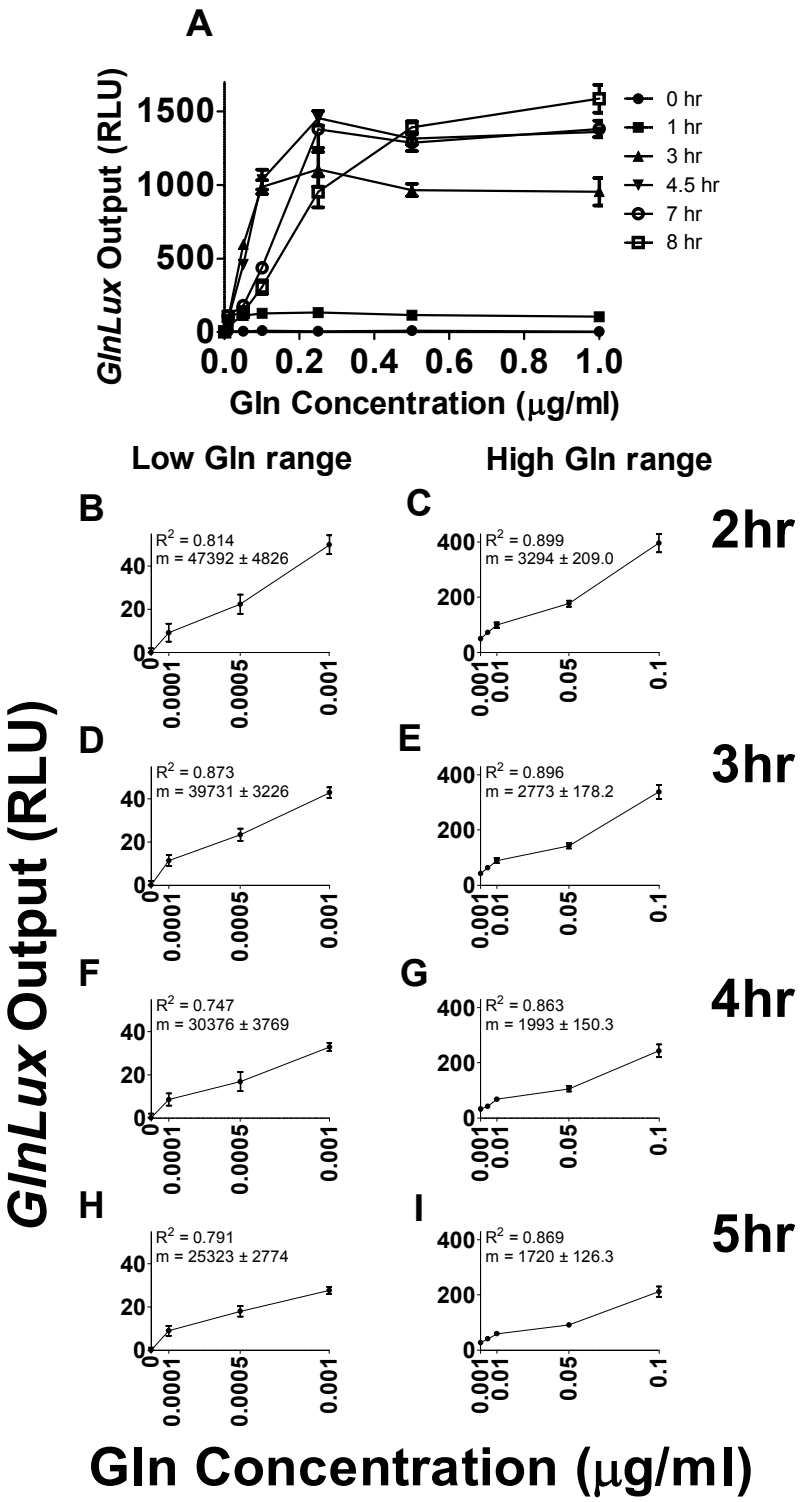
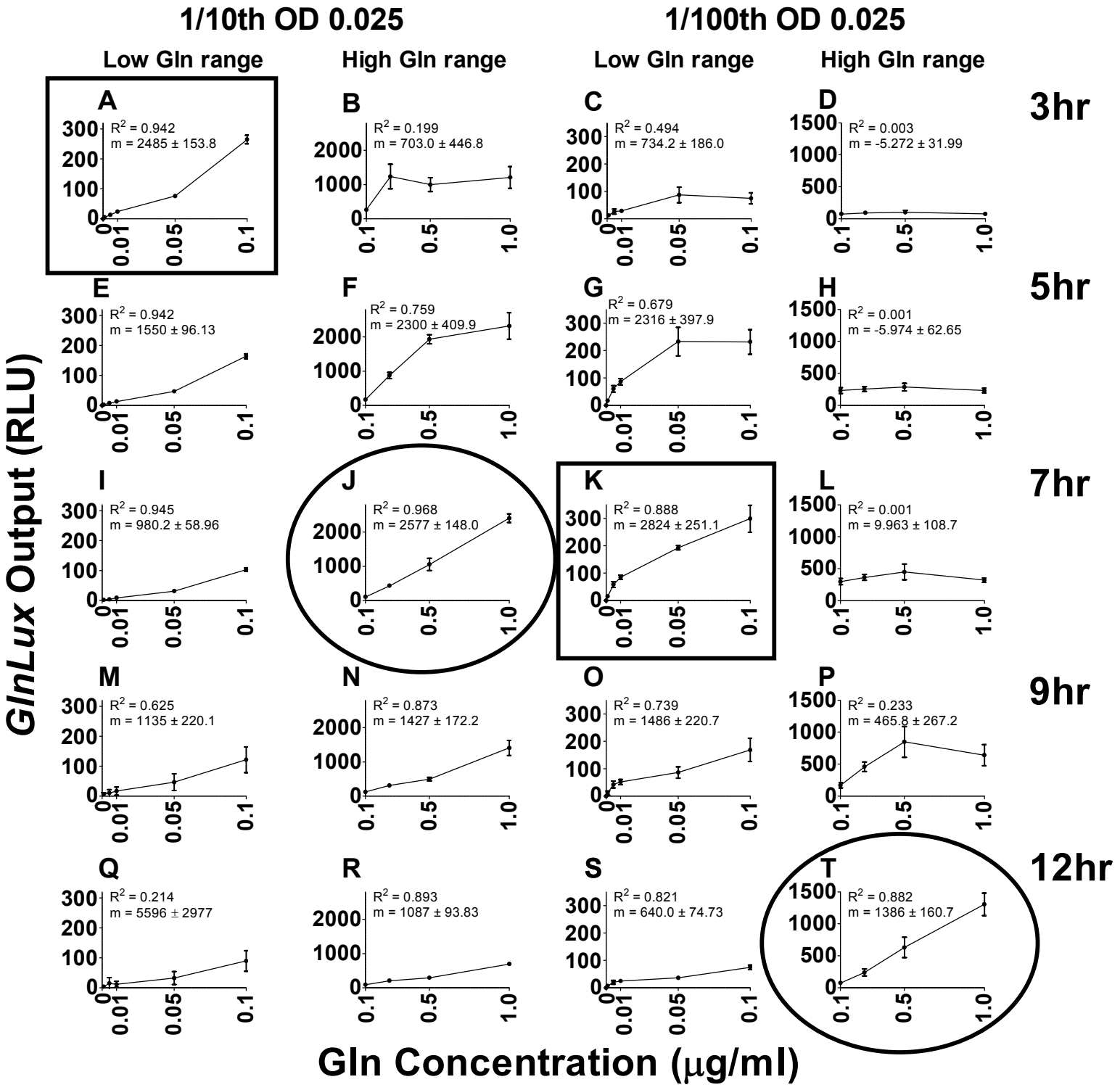
B
Bgrd = 200.4
Max. m = 11518 ± 1743
P = 0.0027
C
Bgrd = 203.8
Max. m = 1502 ± 409.5
P = 0.0080
D
Bgrd = 69.4
Max. m = 12124 ± 380.2
P = < 0.0001
E
Bgrd = 37.8
Max. m = 4095 ± 1393
P = 0.0217
F
Bgrd = 16.3
Max. m = 15739 ± 5986
P = 0.0252
0hr
1hr
3hr
5hr
7hr
9hr
GlnLux Output (RLU)
Gln Concentration (μg/ml)

Suppl 1


A
GlnLux Output (RLU)
1500
1000
500
0
0.0 0.2 0.4 0.6 0.8 1.0
Gln Concentration (μg/ml)
0 hr
1 hr
3 hr
4.5 hr
7 hr
8 hr
Low Gln range
High Gln range
B
C
D
E
F
G
H
I
2hr
3hr
4hr
5hr
R2 = 0.814
m = 47392 ± 4826
R2 = 0.899
m = 3294 ± 209.0
R2 = 0.873
m = 39731 ± 3226
R2 = 0.896
m = 2773 ± 178.2
R2 = 0.747
m = 30376 ± 3769
R2 = 0.863
m = 1993 ± 150.3
R2 = 0.791
m = 25323 ± 2774
R2 = 0.869
m = 1720 ± 126.3
GlnLux Output (RLU)
Gln Concentration (μg/ml)

Suppl 2


1/10th OD 0.025
1/100th OD 0.025
Low Gln range
High Gln range
Low Gln range
High Gln range
3hr
5hr
7hr
9hr
12hr
A
R² = 0.942
m = 2485 ± 153.8
B
R² = 0.199
m = 703.0 ± 446.8
C
R² = 0.494
m = 734.2 ± 186.0
D
R² = 0.003
m = -5.272 ± 31.99
E
R² = 0.942
m = 1550 ± 96.13
F
R² = 0.759
m = 2300 ± 409.9
G
R² = 0.679
m = 2316 ± 397.9
H
R² = 0.001
m = -5.974 ± 62.65
I
R² = 0.945
m = 980.2 ± 58.96
J
R² = 0.968
m = 2577 ± 148.0
K
R² = 0.888
m = 2824 ± 251.1
L
R² = 0.001
m = 9.963 ± 108.7
M
R² = 0.625
m = 1135 ± 220.1
N
R² = 0.873
m = 1427 ± 172.2
O
R² = 0.739
m = 1486 ± 220.7
P
R² = 0.233
m = 465.8 ± 267.2
Q
R² = 0.214
m = 5596 ± 2977
R
R² = 0.893
m = 1087 ± 93.83
S
R² = 0.821
m = 640.0 ± 74.73
T
R² = 0.882
m = 1386 ± 160.7
GlnLux Output (RLU)
Gln Concentration (μg/ml)

Suppl Fig 3