Translation-dependent bioassay for amino acid quantification using auxotrophic microbes as biocatalysts of protein synthesis

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Abstract

Bioassay for amino acid quantification is an important technology for a variety of fields, which allows for easy, inexpensive, and high-throughput analyses. Here, we describe a novel translation-dependent bioassay for the quantification of amino acids. For this, the gene encoding firefly luciferase was introduced into Lactococcus lactis auxotrophic to Glu, His, Ile, Leu, Pro, Val, and Arg. After a preculture where luciferase expression was repressed, the cells were mixed with analytes, synthetic medium, and an inducer for luciferase expression. Luminescence response to the target amino acid appeared just after mixing, and linear standard curves for these amino acids were obtained during 15-60 min incubation periods. The rapid quantification of amino acids has neither been reported in previous works on bioassays nor is it theoretically feasible with conventional methods, which require incubation times of more than 4 h to allow for the growth of the microbe used. In contrast, our assay was shown to depend on protein translation, rather than on cell growth. Furthermore, replacement of the luciferase gene with that of the green fluorescent protein (GFP) or β-galactosidase allowed for fluorescent and colorimetric detection of the amino acids, respectively. Significantly, when a Gln-auxotrophic Escherichia coli mutant was created and transformed by a luciferase expression plasmid, a linear standard curve for Gln was observed in 15 min. These results demonstrate that this methodology can provide versatile bioassays by adopting various combinations of marker genes and host strains according to the analytes and experimental circumstances.

Key words

Bioassay, amino acid quantification, auxotroph, lactic acid bacteria

Introduction

Amino acid quantification is an important technology for a variety of fields and has applications in scientific research, healthcare (Hisamatsu et al. 2012; Miyagi et al. 2011; Palanza et al. 2016), and the animal and human food industries (Chalova et al. 2009; Mira de Orduna 2001). A variety of methods have been developed to meet these demands, such as instrumental methods (Kaspar et al. 2009) and enzymatic assays (Kameya and Asano 2014; Kameya et al. 2013; Liu et al. 2014; Matsui et al. 2015). Among these analytical methods, amino acid bioassays have been popular over the past 70 years because of their low cost, easy manipulation, coverage of wide range of amino acids, and applicability to high-throughput analysis (Cardinal and Hedrick 1948; Steele et al. 1949; Tamura et al. 1952). These assays utilize lactic acid bacteria, many of which are auxotrophic to specific amino acids (Christiansen et al. 2008), mixing them with an analyte and synthetic medium that contains all nutrients except the target amino acid. During a 24-72 h incubation period, growth of the auxotrophic bacterium depends on the amount of the target amino acid in the analyte. Thus, the concentration of a given amino acid in the analyte can be determined based on the resultant cell growth after the incubation by measuring the optical density (OD) of the culture. Thus, these conventional methods are referred to as “growth-dependent bioassays.”

One disadvantage of growth-dependent bioassays is the extended time necessary to complete the measurement, because the growth-dependent bioassay intrinsically requires a long incubation time to obtain a desired dynamic range. Specifically, in order to obtain x-fold increase in OD from a background control (reaction mixtures without the target amino acid), the incubation time must be longer than the time in which the cells can grow x-fold. For instance, in order to obtain signal-noise ratios of 10 and 100, the assays require an incubation time 3.3- and 10-fold times longer than the doubling time of the used bacterium, respectively. These durations prevent the rapid quantification of amino acids considering that the doubling time of most bacteria in synthetic medium is longer than 1 h (Paliy and Gunasekera 2007). This restriction cannot be solved by a superficial modification of the incubation and detection conditions as far as the bioassay depends on cell growth. Some recent studies have substituted auxotrophic Escherichia coli mutants for lactic acid bacteria (Li and Ricke 2003), since the former exhibits a lower doubling...
Bacterial strains

The basal synthetic medium contained 10 µg/mL guanine, 10 µg/mL adenine, 10 µg/mL uracil, 10 µg/mL xanthine, 1 µg/mL thiamine-HCl, 1 µg/mL riboflavin, 1 µg/mL pyridoxine, 1 µg/mL pyridoxal, 1 µg/mL calcium pantothenate, 1 µg/mL nicotinic acid, 0.2 µg/mL p-aminobenzoic acid, 0.01 µg/mL biotin, 0.01 µg/mL folic acid, 3 mg/mL NH₄Cl, 500 µg/mL KH₂PO₄, 500 µg/mL K₂HPO₄, 10 µg/mL NaCl, 20 µg/mL potassium acetate, 200 µg/mL MgSO₄·7H₂O, 10 µg/mL FeSO₄·7H₂O, 10 µg/mL MnCl₂·4H₂O, 200 µg/mL Ala, 100 µg/mL Cys, 500 µg/mL Glu, 400 µg/mL Asp, 200 µg/mL Phe, 100 µg/mL Gly, 100 µg/mL His, 200 µg/mL Ile, 200 µg/mL Lys, 200 µg/mL Leu, 200 µg/mL Met, 100 µg/mL Pro, 200 µg/mL Arg, 100 µg/mL Ser, 200 µg/mL Thr, 200 µg/mL Val, 100 µg/mL Trp, and 100 µg/mL Tyr (pH 6.8) as reported previously (Tamura et al. 1952). A series of deficient media was prepared by omitting one of the amino acids from the basal synthetic medium with the exception of Glu-deficient medium, which contained 200 µg/mL Asn instead of Glu and Asp.

For bioassays, L. lactis NZ9000 and NBRC 100933 were precultured at 30°C overnight without shaking in M17 medium (Merck, Whitehouse Station, NJ, USA) supplemented with 0.5% glucose (GM17 medium). Appropriate antibiotics (10 µg/mL chloramphenicol for cells carrying pNZ8148 and 10 µg/mL erythromycin for cells carrying pNZ9530) were added to GM17 medium if necessary. Cells grown to stationary phase were harvested and washed with 20 mM potassium phosphate buffer (pH 7.0) more than three times to remove nutrients in the preculture medium. The washed cells were resuspended in the deficient media and used for the following bioassays.

Materials and methods

Bacterial strains

Lactococcus lactis NZ9000 (Mierau and Kleerebezem 2005) and NBRC 100933 were purchased from MoBiTec (Göttingen, Germany) and the National Biological Resources Center (NBRC; Tokyo, Japan), respectively, and used as hosts for the cloning and expression of pNZ8140-derivative plasmids. E. coli BW25113 was obtained from Coli Genetic Stock Center (CGSC; New Haven, CT, USA). E. coli BW25113 and JM109 were used as hosts for the cloning and expression of firefly luciferase.

Media composition and culturing of L. lactis strains

The basal synthetic medium contained 10 µg/mL guanine, 10 µg/mL adenine, 10 µg/mL uracil, 10 µg/mL xanthine, 1 µg/mL thiamine-HCl, 1 µg/mL riboflavin, 1 µg/mL pyridoxine, 1 µg/mL pyridoxal, 1 µg/mL calcium pantothenate, 1 µg/mL nicotinic acid, 0.2 µg/mL p-aminobenzoic acid, 0.01 µg/mL biotin, 0.01 µg/mL folic acid, 3 mg/mL NH₄Cl, 500 µg/mL KH₂PO₄, 500 µg/mL K₂HPO₄, 10 µg/mL NaCl, 20 µg/mL potassium acetate, 200 µg/mL MgSO₄·7H₂O, 10 µg/mL FeSO₄·7H₂O, 10 µg/mL MnCl₂·4H₂O, 200 µg/mL Ala, 100 µg/mL Cys, 500 µg/mL Glu, 400 µg/mL Asp, 200 µg/mL Phe, 100 µg/mL Gly, 100 µg/mL His, 200 µg/mL Ile, 200 µg/mL Lys, 200 µg/mL Leu, 200 µg/mL Met, 100 µg/mL Pro, 200 µg/mL Arg, 100 µg/mL Ser, 200 µg/mL Thr, 200 µg/mL Val, 100 µg/mL Trp, and 100 µg/mL Tyr (pH 6.8) as reported previously (Tamura et al. 1952). A series of deficient media was prepared by omitting one of the amino acids from the basal synthetic medium with the exception of Glu-deficient medium, which contained 200 µg/mL Asn instead of Glu and Asp.

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Construction of luciferase, GFP, and β-galactosidase expression plasmids

The pNZ8148 (MoBiTec) backbone was used to construct the expression plasmids that can be introduced to L. lactis (Mierau and Kleerebezem 2005). A gene encoding firefly luciferase was excised from pGL3-Basic (Promega, WI, USA) by digestion with NcoI and XbaI and inserted into pNZ8148 to generate pNZ8148-luc. Two fragments of a variant GFP gene (AB124780.2) were amplified from pGreen (Miller and Lindow 1997) by PCR using two sets of primers, gfp-F1/gfp-R1 and gfp-F2/gfp-R2 (Table 1), and subsequently fused by the second PCR by using gfp-F1 and gfp-R2. The fused fragment was digested by NcoI

Table 1. Primer sequences used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Amplified gene or plasmid</th>
<th>Primer sequence (5’ to 3’)</th>
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</thead>
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<tr>
<td>gfp-F1</td>
<td>GFP</td>
<td>aacatggcagtaaaggagaagaacgccac</td>
</tr>
<tr>
<td>gfp-R1</td>
<td>GFP</td>
<td>aagttgctgcacgaagaacag</td>
</tr>
<tr>
<td>gfp-F2</td>
<td>GFP</td>
<td>cctgtccctggtgccac</td>
</tr>
<tr>
<td>gfp-R2</td>
<td>GFP</td>
<td>aaaaagctctattttgatagtctcatgctgcc</td>
</tr>
<tr>
<td>lacZ-F</td>
<td>β-Galactosidase</td>
<td>aaccatgagccagcttagatgacagcttcac</td>
</tr>
<tr>
<td>lacZ-R</td>
<td>β-Galactosidase</td>
<td>tataaggttattttgacacagcagacgttg</td>
</tr>
<tr>
<td>luc-F</td>
<td>Luciferase</td>
<td>aataactagtaggtgagcagcttagatgcttcag</td>
</tr>
<tr>
<td>luc-R</td>
<td>Luciferase</td>
<td>cgaaccetcatagagagatcttctcccg</td>
</tr>
<tr>
<td>pca-F</td>
<td>pCA24N</td>
<td>ttctctagttatctctttctcaagttcatgcttcag</td>
</tr>
<tr>
<td>pca-R</td>
<td>pCA24N</td>
<td>gcgttaagtttgacagcttcagacagcaagc</td>
</tr>
<tr>
<td>kan-F</td>
<td>Kanamycin resistance gene</td>
<td>gttacacaggacagactatccaaaatggtagtggctcg</td>
</tr>
<tr>
<td>kan-R</td>
<td>Kanamycin resistance gene</td>
<td>gcccagagacaggaagaaaatccatgcagcttgatcctcc</td>
</tr>
<tr>
<td>Δlac-F</td>
<td>pCA24N-luc</td>
<td>cctgaattgactcttcag</td>
</tr>
<tr>
<td>Δlac-R</td>
<td>pCA24N-luc</td>
<td>tgacgagcaacgcattatagtga</td>
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</table>
and HindIII, followed by insertion into pNZ8148 to generate pNZ8148-gfp. A gene encoding β-galactosidase was amplified by PCR from genomic DNA of E. coli W3110 (NC_007779.1) by using the primers lacZ-F and lacZ-R (Table 1). The amplified fragment was digested by NcoI and HindIII and inserted into pNZ8148 to generate pNZ8148-lacZ.

To construct a luciferase expression system in E. coli, the luciferase gene was amplified from pGL3 by PCR using the primers luc-F and luc-R (Table 1). A fragment of pCA24N vector, which contained a chloramphenicol resistance gene, a T5 promoter, a lac operator, and lacIq (Kitagawa et al. 2005), was amplified by PCR using pca-F and pca-R (Table 1). The two amplified fragments were fused using the In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan) to obtain a luciferase expression plasmid, designated pCA24N-luc. This plasmid harbors a lacIq gene to control expression of the luciferase gene, which was inserted downstream of the T5 promoter and lac operator.

To delete the lacIq gene, pCA24N-luc was amplified by PCR using the primers Δlac-F and Δlac-R (Table 1), and then subjected to blunt-end self-ligation. The resultant plasmid, pCA24N-lucΔlacIq, was used to evaluate the effects of lacIq repressor.

**Bioassay using the L. lactis strains**

The plasmids pNZ8148-luc, pNZ8148-gfp, and pNZ8148-lacZ were introduced to L. lactis NZ9000 and NBRC 100933. Due to the absence of nisRK in L. lactis NBRC 100933, which encodes a nisin sensor-regulator system, pNZ9530 (MoBiTec) was also introduced into L. lactis NBRC 100933 transformants.

The recombinants were precultured in GM17 medium supplemented with antibiotics, and the cells were harvested and washed as described above. No nisin was added to the preculture medium. The washed cells were suspended in synthetic medium deficient for the target amino acid, and 10 ng/mL nisin was added to induce the expression of the marker genes. Various amounts of the target amino acid were added to the suspension as an analyte. If necessary, 10 µg/mL ampicillin or erythromycin was added. This cell suspension is designated as the “bioassay mixture” henceforth. The volume of the bioassay mixtures was 200 µl, and the cell density in them was 2% that of the preculture. The bioassay mixtures were then incubated at 30°C for 15-60 min without shaking after addition of the target amino acid.

For the detection of in vivo luminescence, 0.5 mM luciferin was added to the bioassay mixtures prepared in a 96-well plate, and luminescence was monitored using an Infinite M200 (Tecan, Seestrasse, Switzerland) or Genios (Tecan) plate reader.

For the detection of in vitro luciferase activity, the cells were harvested from bioassay mixtures after a 1 h incubation and disrupted by a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). The cell debris were removed by centrifugation at 10,000 g for 5 min. The supernatants were mixed with 20 mM potassium phosphate buffer (pH 7.0), 5 mM MgSO4, 0.1 mM luciferin, and 0.1 mM ATP, and the luminescence was measured by an Infinite M200.

For the detection of GFP, the cells were harvested from bioassay mixtures after a 1 h incubation and suspended in 20 mM potassium phosphate buffer (pH 7.0). The fluorescence was measured using an Infinite M200 with excitation and emission wavelengths of 490 and 520 nm, respectively.

For the detection of β-galactosidase activity, the cells were harvested from bioassay mixtures after a 1 h incubation and suspended in 60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 50 mM 2-mercaptoethanol, 0.8% chloroform, 0.04% sodium dodecyl sulfate, and 4 mg/mL 2-nitrophenyl-β-D-galactopyranoside. Absorbance at 420 nm was then monitored with an Infinite M200.

**Preparation of frozen cells**

L. lactis NZ9000 (pNZ8148-luc) was precultured in GM17 medium with chloramphenicol, harvested, and washed as above. The cells derived from 2 ml of the preculture were suspended in 1 ml of 20 mM potassium phosphate buffer (pH 7.0) containing 10% glycerol solution, frozen by liquid nitrogen, and preserved at −80°C until use.

**Construction of Gln-auxotrophic E. coli**

A Gln-auxotrophic derivative of E. coli JM109 and BW25113 were constructed by disrupting glnA, which encodes glutamine synthetase (EC 6.3.1.2), a key enzyme for Gln biosynthesis in many organisms (Kameya et al. 2006). The FLP-FRT system (Datsenko and Wanner 2000) was used to disrupt the gene target without leaving the antibiotic selectable marker in the host cells, and the plasmids pKD46, pKD4, and pCP20 for this system were obtained from Coli Genetic Stock Center (CT, USA). The kanamycin resistance gene on pKD4 was amplified by PCR using the primers kan-F and kan-R (Table 1), and introduced into the strains harboring pKD46 to replace glnA on their genomic DNA. The kanamycin resistance gene was then eliminated by transformation with pCP20 expressing the FLP recombinase. The strains were cultivated in LB medium (0.5% yeast extract, 1% tryptone, and 1% NaCl) supplemented with appropriate antibiotics.

**Bioassay using the E. coli recombinants**

The glnA-deleted E. coli mutants harboring pCA24N-luc or pCA24N-lucΔlacIq were precultured in M9 medium (6 mg/mL Na2HPO4, 3 mg/mL KH2PO4, 2 mg/mL glucose, 10 µg/mL thiamine-HCl, 1 mM MgSO4, and 0.1 mM NaCl) supplemented with 1 mM Gln and 30 µg/mL chloramphenicol in the absence of isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells grown to stationary phase were harvested and
washed more than three times with M9 medium to minimize Gln contamination from the preculture medium. The cells derived from 100 µl of the preculture were then suspended in 200 µl of M9 medium supplemented with 0.5 mM luciferin, 0.5 mM IPTG, and various concentrations of Gln in a 96-well plate. Luminescence of the bioassay mixtures was monitored by an Infinite M200.

Results

Bioassay using a luciferase-expressing strain of *L. lactis* NZ9000

*L. lactis* is a well-studied lactic acid bacterium that is often used as a host for heterologous protein expression (Mierau and Kleerebezem 2005). The growth test of *L. lactis* NZ9000 in synthetic media revealed that this strain is auxotrophic to Glu, His, Ile, Leu, Val, and Arg.

A nisin-inducible luciferase expression plasmid was constructed and introduced into *L. lactis* NZ9000. Thus, luciferase was only expressed when nisin was added to culture medium (Mierau and Kleerebezem 2005). As expected, the obtained recombinant exhibited luminescence when cultivated in GM17 medium supplemented with nisin.

We then attempted to develop a bioassay for amino acids by inoculating this recombinant strain into synthetic media that contained various concentrations of amino acid. Surprisingly, *L. lactis* NZ9000 (pNZ8148-luc) exhibited a luminescence response more rapidly than expected. When recombinant cells precultured in the absence of nisin were mixed to synthetic medium containing nisin and various amount of Val, Val-dependent luminescence appeared immediately after the mixing (Fig. 1 a). In addition, it took

![Image](image1.png)

**Fig. 1.** *In vivo* luminescence response of *L. lactis* harboring pNZ8148-luc in the Val bioassay mixtures. (a) Time course of the luminescence of Val bioassay mixtures prepared using the *L. lactis* NZ9000 recombinant. The final concentrations of Val are indicated by the values on each curve. The reaction was started by the addition of Val to assay mixtures. Each plot represents the average of three samples. (b) Time course of the correlation coefficient between the Val concentration and the luminescence observed in the assay mixtures shown in panel a. (c) Relationship between the Val concentration and the luminescence intensity observed at a time point of 15 min of panel a. The highest luminescence in each panel was taken as 100%.

![Image](image2.png)

**Fig. 2.** Relationship between the concentrations of Arg, His, Ile, Leu, Glu, and Pro and *in vivo* luminescence intensities of *L. lactis* harboring pNZ8148-luc. The bioassay mixtures for each amino acid were prepared using the *L. lactis* NZ9000 (a-e) or NBRC 100933 (f). Each plot represents the average of three samples. The highest luminescence in each panel was taken as 100%.
less than 15 min to observe a linear relationship between the Val concentrations and luminescence intensities (Fig. 1b). This response speed is much faster than that of the conventional amino acid bioassays, which require significantly longer incubation periods. This strong, instantaneous response to Val was not observed when nisin was added to the pre-culture (not shown), suggesting that this bioassay requires strict repression of luciferase expression until preparation of the bioassay mixture. The linearity between Val levels and luminescence was sustained at least for an additional 45 min (Fig. 1b), yielding a reproducible linear standard curve for Val (Fig. 1c).

Similar results were obtained when bioassay mixtures for Glu, His, Ile, Leu, and Arg were prepared (Fig. 2a-e). These results indicate that the constructed bioassay allows for rapid and selective quantification of not only Val but also these amino acids. Meanwhile, these amino acids showed different concentration ranges where the standard curve is linear. For instance, linear standard curves for Arg and His were obtained up to 1 µM, while the linearity for Glu was sustained up to 400 µM (Fig. 2a, b, c). This difference was caused by the variation of sensitivity to each amino acid; i.e., sensitive response to low concentrations (< 1 µM) of Arg and His was observed while the same concentrations of Glu were too low to observe a significant luminescence response. When these amino acids in assay mixtures increased above the upper-limits, the luminescence intensity reached saturation at a constant level irrespective of amino acid.

When casamino acid solutions spiked with 0-800 nM Arg were analyzed by the Arg bioassay, a linear curve was obtained in response to the extrinsic Arg (Fig. S1). The slope of this curve indicated that almost all of the added Arg was recovered by the assay, estimating the recovery rate to be 98%. Chromatographic analysis (Kameya et al. 2013) estimated the Arg concentration in the same casamino acid solution to be 133 nM. This value is comparable to that estimated by the above bioassay (140 nM), demonstrating the successful measurement of an amino acid in crude samples by this bioassay.

Dependency on amino acid auxotrophy

*L. lactis* NZ9000 is auxotrophic to the six amino acids for which standard curves were obtained above. No significant response to the amino acids was observed when bioassay mixtures for other amino acids, such as Pro, were used with this strain due to the high increase in luminescence after the addition of nisin, even in the absence of the targeted amino acids (not shown).

Cultivation of related *L. lactis* strains in various synthetic media revealed that *L. lactis* NBRC 100933 is auxotrophic for Pro while *L. lactis* NZ9000 is not. Thus, the luciferase expression system was introduced into *L. lactis* NBRC 100933, and the recombinant was incubated in the bioassay mixture for Pro. The NBRC 100933 recombinant exhibited a luminescence response to Pro, yielding a reliable linear standard curve for this amino acid (Fig. 2f). These results indicate that the response to an amino acid is linked to the auxotrophy of the microbes utilized in our bioassay, similar to the methodology of conventional bioassays.

Assay dependence on cell growth or protein translation

Two antibiotics were used to investigate the effects of these compounds in the bioassay mixtures: erythromycin, which inhibits protein translation.
2003), and ampicillin, which halts cell growth and proliferation by inhibiting cell wall biosynthesis but not protein translation (Waxman and Strominger 1983). We confirmed that addition of either antibiotic (10 µg/mL) could completely inhibit the growth of *L. lactis* NZ9000 when added to the GM17 culture medium. When erythromycin was added to the Arg bioassay mixtures, the luminescence response to Arg disappeared (Fig. 3a). In contrast, addition of ampicillin did not significantly affect the luminescence response. These results indicate that the present bioassay requires protein translation as an essential biological activity, but does not depend on cell growth.

**Evaluation of the in vitro luminescence activity**

To obtain further evidence that the amount of the translated luciferase is a key determinant of the *in vivo* luminescence response, *in vitro* luciferase activity was measured in crude extracts of the cells incubated in the Arg bioassay mixtures. A significant correlation between *in vitro* luciferase activity and Arg concentrations was observed (Fig. 3b), as is the case with the *in vivo* assay. No significant difference was observed in the amount of total protein, clearly excluding the possibility that cell growth during the incubation served as a key factor for the *in vivo* luminescence response. These results demonstrate that the amount of the luciferase expression per cell increased in accordance with the level of the exogenous amino acid, which explains the rapidness of the *in vivo* luminescence response observed in the bioassay.

**Bioassay using GFP- or β-galactosidase-expressing strains of *L. lactis* NZ9000**

To verify whether the marker protein for this bioassay was limited to luciferase, a gene encoding GFP or β-galactosidase was introduced in *L. lactis* NZ9000 in place of the luciferase gene. GFP fluorescence or β-galactosidase activity was measured following 1 h incubation in the bioassay mixtures. Consequently, a positive correlation between the signal intensities and the amino acid concentrations was observed both for GFP- or β-galactosidase-expressing strains (Fig. 4). Therefore, this finding demonstrates that various proteins can be used as detection markers in this bioassay.

**Use of frozen cells**

To facilitate the preparation of the bioassay mixture, we tested the use of frozen cells of *L. lactis* NZ9000 (pNZ8148-luc). When cells stored at −80°C for three days were thawed and immediately used for the Arg bioassay, the cells yields a linear standard curve for Arg similar to the curve obtained with freshly harvested cells did (Fig. S2). No significant change was observed when the cells were stored at −80°C for one month (not shown). This result demonstrates that the translation-dependent bioassay allows using frozen cells, which saves labor and simplifies the preparation of cells for assays.

**Bioassay using Gln-auxotrophic strains of *E. coli***

To verify whether the bioassay can be used in other organisms, a Gln-auxotrophic *E. coli* JM109 mutant harboring a luciferase expression plasmid was generated. The strain was constructed by deleting *glnA* in strain JM109. JM109Δ*glnA* exhibited Gln auxotrophy when it was cultivated in M9 medium. Plasmid pCA24N-luc was designed to control luciferase expression under a T5 promoter and lac operator. The *E. coli* JM109Δ*glnA* (pCA24N-luc) was precultured in M9 medium supplemented with Gln and chloramphenicol.

A significant luminescence response depending on exogenous Gln was observed immediately after Gln bioassay mixtures were prepared and incubated with harvested cells (Fig. 5a). The luminescence intensities showed a linear relationship with Gln concentrations (0-50 nM) 15 min after the assay started (Fig. 5b).

Cultivation in LB medium often causes the unintended induction of the expression of genes under a *lac* operator due to residual lactose in medium (Studier 2014). When the recombinant was precultured in LB medium, rather than M9, no detectable luminescence response was observed in the bioassay mixtures due to the high background expression of luciferase that accumulated during the preculture period. This result is consistent with the above-mentioned idea that this assay requires strict repression of the detection marker until the cells are suspended in bioassay mixtures.

Similar results were observed when the host strain was replaced by *E. coli* BW25113 (Fig. S3a) that lacks the *lac* operator gene (Grenier et al. 2014). Interestingly, when the *lac* operator gene on pCA24N-luc was deleted from this recombinant, the luminescence response became undetectable (Fig. S3b), similar to that observed when cells were precultured in LB medium. This result shows that *E. coli* with *lac* operator expression is required for this assay in order to limit basal luciferase expression.

![Fig. 5. Luminescence response of *E. coli* JM109Δ*glnA* (pCN24N-luc) to exogenous Gln. (a) Time course of the luminescence of Gln bioassay mixtures. Duplicated assay mixtures were prepared for each condition, and no significant difference was observed between the duplicates (coefficient of variation <5%). Each plot represents the average of duplicate samples. (b) Relationship between Gln concentrations in bioassay mixtures and luminescence intensities observed at a time point of 15 min of panel a. The highest luminescence in each panel was taken as 100%.

Discussion

In this study, we developed a novel bioassay for amino acid quantification by analyzing the expression of a detectable marker protein in amino acid auxotrophs. This bioassay allows for rapid determination of amino acid concentrations within 60 min, a remarkably shorter time than necessary for any other conventional bioassays (Bertels et al. 2012; Cardinal and Hedrick 1948; Chalova et al. 2006; Erickson et al. 2000; Kim et al. 2010; Li and Ricke 2003; Palaniza et al. 2016; Steele et al. 1949; Tamura et al. 1952). The present results also demonstrate the high versatility of this bioassay in terms of both protein marker and the utilized microbe. Thus, users can select a desirable combination of marker proteins and host organisms according to their purpose, analytes, and experimental circumstances. An additional advantage of this bioassay over instrumental analyses is its suitability for high-throughput analyses of multiple samples at once, as emphasized by the previous works on conventional bioassays (Chalova et al. 2007). Our results also showed that frozen cells can be used without any issues, which improves the efficiency since it is not necessary to prepare new cells before every assay.

The present bioassay is seemingly similar to conventional growth-dependent bioassays in that microbes auxotrophic to a target amino acid are precultured and inoculated into synthetic culture medium. However, the rapid increase in signal intensity in this assay occurs over a significantly smaller period of time than that necessary for growth-dependent assays, which rely on the doubling time of the host microbe (Fig. 1a and Fig. 5a). Other results, such as the effects of antibiotics (Fig. 3a) and the negligible cell growth during the assay, clearly show that this bioassay is entirely independent of cell growth. Rather, we observed a positive correlation between the amino acid concentration and the amount of the marker protein translated during the assay (Fig. 3b). These facts indicate that the present bioassay can be called “translation-dependent bioassay.” This perspective is plausible considering that translation rates can be affected by the levels of amino acids available for protein synthesis. The translated protein can quickly accumulate in response to its induction. Thus, this new principle of bioassay allows rapid measurement that cannot be intrinsically achieved by growth-dependent assays.

The difference between growth- and translation-dependent bioassays is in the respective roles for amino acids in each bioassay: building blocks for cell structure or protein synthesis. One of the possible reasons why the use of protein synthesis has been overlooked for the purpose of bioassays seems to be the requirement of strict repression of the marker gene until cells were mixed with analytes. Our study demonstrates that the present Gln bioassay system with E. coli requires both the appropriate preculture condition and the presence of the lacF1 repressor.

Selectivity is a key important feature for these assay methods. It should be noted that our bioassay mixtures for L. lactis contained high concentrations of the 18 amino acids, except for the one targeted for quantification. The present results, in which significant response to the target amino acid was observed in the presence of the other amino acids, demonstrate that our bioassay using L. lactis is highly selective.

One interesting physiological observation in this study is the diversity in the dynamic range of each amino acid (Fig. 2A-E). The reason is unclear but it might be caused by differences between these amino acids with respect to their incorporation efficiency or in the affinity of aminoacyl-tRNA synthetases that transfer each amino acid onto tRNA for protein synthesis. Elucidation of this mechanism may allow for enhancement or adjustment of the assay and increase the sensitivity for each amino acid even further.

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Compliance with Ethical Standards

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Translation-dependent bioassay for amino acid quantification using auxotrophic microbes as biocatalysts of protein synthesis

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Supplemental material

**Fig. S1.** Spike and recovery test of Arg in casamino acid solution. Arg was added to the Arg bioassay mixtures at a final concentration of 0-800 nM in the presence (open squares) or absence (open circles) of 0.75 µg/ml casamino acid solution. The luminescence of the bioassay mixture containing 800 nM Arg and casamino acid solution was taken as 100%.

**Fig. S2.** Bioassay of Arg using cryopreserved *L. lactis* NZ9000 cells harboring pNZ8148-luc. The chart shows relationship between Arg concentrations in bioassay mixtures and in vivo luminescence intensities observed in Arg bioassay mixtures prepared by using the cells stored at −80°C. Each plot represents the average of three samples. The luminescence of the bioassay mixture containing 1000 nM Arg was taken as 100%.
**Fig. S3.** Time course of the luminescent response to Gln of *E. coli* BW25113 ΔglnA harboring pCA24N-luc (a) and pCA24N-lucΔlacI (b). The cells were precultured in M9 medium supplemented with 1 mM Gln and 30 µg/mL chloramphenicol. The bioassay mixtures contained 50 µM Gln (solid lines) or no Gln (dotted lines). The reaction was started by the addition of Gln to assay mixtures. The highest luminescence in each panel was taken as 100%. 