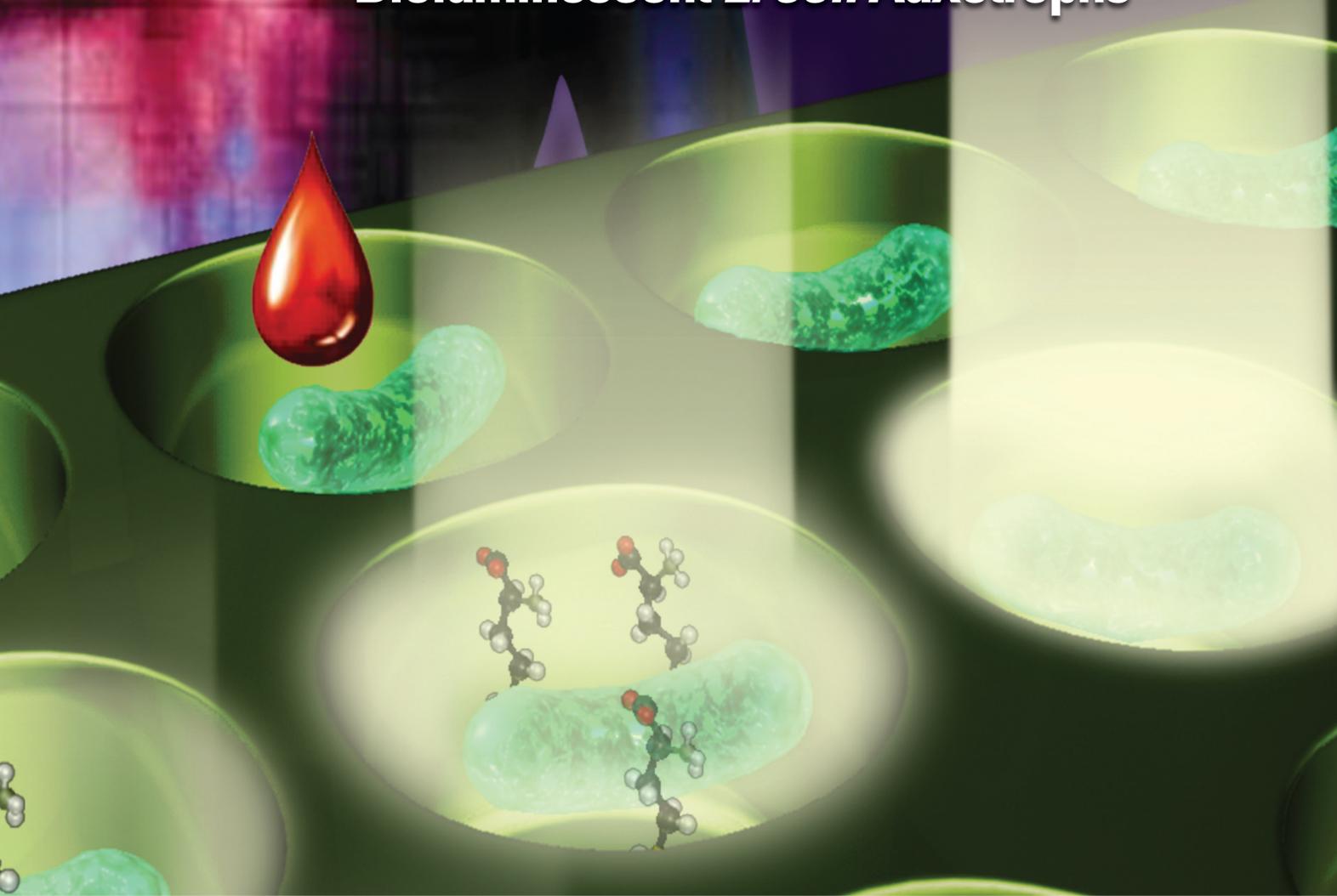


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Quantification of Homocysteine with Bioluminescent *E. coli* Auxotrophs



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Cell-Based Quantification of Homocysteine Utilizing Bioluminescent *Escherichia coli* Auxotrophs

Min-Ah Woo,[†] Moon Il Kim,[†] Byung Jo Yu,[‡] Daeyeon Cho,[§] Nag-Jong Kim,[†] June Hyoung Cho,[‡] Byung-Ok Choi,^{*,||} Ho Nam Chang,[†] and Hyun Gyu Park^{*,†}

[†]Department of Chemical and Biomolecular Engineering (BK21 Program), KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea

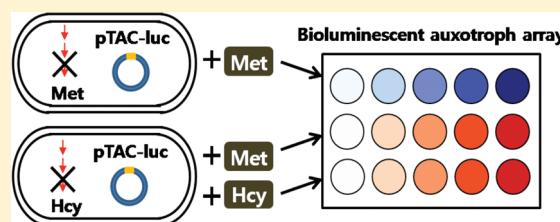
[‡]MD Science Inc., 258-1 Munji-dong, Yuseong-gu, Daejeon 305-701, Republic of Korea

[§]LabGenomics Co., Ltd., 1571-17 Seocho3-dong, Seocho-gu, Seoul 137-874, Republic of Korea

^{||}Department of Neurology, College of Medicine, Ewha Womans University, Mokdong Hospital, 911-1 Mokdong, Yangcheon-gu, Seoul 158-710, Republic of Korea

 Supporting Information

ABSTRACT: A cell-based quantitative assay system for Hcy has been developed by utilizing two *Escherichia coli* auxotrophs that grow in the presence of methionine (Met) and either homocysteine (Hcy) or Met, respectively. A bioluminescent reporter gene, which produces luminescence as cells grow, was inserted into the auxotrophs, so that cell growth can be readily determined. When the relative luminescence unit (RLU) values from the two auxotrophs immobilized within agarose gels arrayed on a well plate were measured, the amount of Hcy was quantitatively determined on the basis of differences between two RLU values corresponding to cell growth of two auxotrophs with excellent levels of precision and reproducibility. Finally, the diagnostic utility of this assay system was verified by its employment in reliably determining different stages of hyperhomocysteinemia in human plasma samples providing CVs of within and between assays that are less than 2.9% and 7.1%, respectively, and recovery rates of within and between assays that are in the range of 99.1–103.5% and 97.5–105.5%, respectively. In contrast to existing conventional methods, the new system developed in this effort is simple, rapid, and cost-effective. As a result, it has great potential to serve as a viable alternative for Hcy quantification in the diagnosis of hyperhomocysteinemia.



The critical role played by homocysteine (Hcy) in human blood was uncovered in the late 1960s, and since that time, many clinical studies have demonstrated that this amino acid is an important marker for cardiovascular disease.^{1–5} Recently, elevated Hcy levels, known as hyperhomocysteinemia, also have been shown to be related to Alzheimer's⁶ and Parkinson's disease,⁷ neural tube defects,⁸ pregnancy complications,⁹ and osteoporosis.¹⁰ Elevation of Hcy levels, which generally originate from genetic or metabolic disorders that cause an unbalance between the production and utilization of this amino acid in the human body,¹¹ has been demonstrated to directly trigger an increase in oxidative stress in the vasculature.^{12,13} However, the exact mechanism(s) for disease pathogenesis have not been fully characterized, and efforts to clearly establish the role of Hcy in such diseases are ongoing.

The main methods employed for Hcy quantification include chromatography and enzyme-based immunoassay. Chromatographic methods (eg, HPLC) are the most popular since they provide accurate results. However, these procedures are time-consuming since they require sample cleanup, isolation, and purification prior to instrumental analysis.¹⁴ More recently, immunological methods have been developed. One such method

involves fluorescence polarized immunoassay that typically relies on the enzymatic conversion of Hcy to S-adenosyl-L-homocysteine (SAH), which is measured using a competitive immunoassay. SAH generated from serum or plasma samples competes with fluorophore-tagged SAH in conjugating with a SAH antibody. After binding to the antibody, changes in fluorescence polarization of the fluorophore are detected giving a quantitative determination of Hcy.^{15,16} The immunological method for Hcy quantification is generally recognized to be more simple and faster than the chromatographic assay procedure and, consequently, it is currently used commercially. However, this method has several drawbacks that arise from the requirement for special instrumentation to introduce multiple reagents for the enzymatic reactions and to detect fluorescence polarization.

With the aim of overcoming the above-mentioned limitations of the conventional methods for Hcy quantification, recent studies have been carried out to develop more efficient methods based on new concepts. Representative of these efforts are

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investigations of enzymatic assays, which have been developed as highly selective and rapid methods that utilize recombinant homocysteine α,γ -lyase to convert Hcy to H_2S . This product subsequently reacts with *N,N*-dibutylphenylenediamine, leading to the formation of a fluorescent product.¹⁷ Modified chemical probes have also been employed to induce visible colorimetric changes or fluorescence turn-on through the reaction with Hcy and the other thiol-containing amino acid Cys.^{18–20} Other studies have focused on the structural and morphological changes of gold nanoparticles that take place in response to the presence of the thiol-containing amino acids Hcy and Cys.^{21–24} Although these recent explorations have resulted in substantial advancements in the determination of Hcy, the development of more advanced detection methods for this amino acid as part of procedures for the early detection and timely treatment and management of diseases associated with hyperhomocysteinemia are needed.

In a recent study, we devised a new cell-based strategy for Hcy quantification employing an *Escherichia coli* auxotroph, which generates luminescent signals that are proportional to cell growth. In the effort described below, we constructed two auxotrophs, designated as MetS and MetB, which grow only in the presence of methionine (Met) and either Met or Hcy, respectively. The Met concentration is first determined by the luminescence intensity produced by the MetS auxotroph, and the Hcy concentration is then determined on the basis of the Met concentration and the luminescence intensity produced by the MetB auxotroph which depends on both Met and Hcy. The analytical utility of this new cell-based strategy was successfully demonstrated in an array format by diagnosing hyperhomocysteinemia using clinical human blood samples with high selectivity, sensitivity, and precision.

■ EXPERIMENTAL SECTION

Construction of Two Bioluminescent *E. coli* Auxotrophs. The MetB auxotroph was created using the general method of chromosomal gene deletion.²⁵ A linear cassette, serving as a replacement for the chromosomal region of metB, consisted of a selectable chloramphenicol (*Cm*) resistance gene and two flanking homologous sequences. The assembly and amplification of the linear cassette was achieved using joint PCR of the resistance gene and two homologous fragments using a pair of primers (5'-GTG TAG GCT GGA GCT GCT TCG AAG TTC CTA-3' and 5'-CAT ATG AAT ATC CTC CTT AGT TCC TAT TCC GA-3') (Bioneer, Daejeon, Korea). *E. coli* W (ATCC1105)²⁶ was obtained from American Type Culture Collection (ATCC, MD, USA). A Red helper plasmid (pKD46), encoding Red recombinase, was first inserted into the *E. coli* by electroporation using a Gene pulser system (Bio-Rad, CA, USA). The cells were then grown in LB media containing ampicillin (50 $\mu\text{g}/\text{mL}$) at 30 °C to an OD₆₀₀ of 0.4, followed by additional incubation at 37 °C for 1 h after the addition of 1 M L-arabinose (Sigma-Aldrich, MO, USA). The prepared linear cassette was then inserted by electroporation into the *E. coli* carrying the ability for Red-mediated recombination. The metB gene-deleted *E. coli* (MetB auxotroph) was specifically selected in the *Cm*-containing (35 $\mu\text{g}/\text{mL}$) agar plate, and the pTAC-luc plasmid²⁷ was inserted into competent cells, which were prepared by washing the MetB auxotroph with ice-cold water three times. MetS auxotroph was obtained using the transposon mutagenesis method as described in a previous report.²⁷

Hcy Quantification on the Array of Two Bioluminescent *E. coli* Auxotrophs. MetB and MetS auxotrophs were cultivated in LB medium containing the respective antibiotics *Cm* and kanamycin at 37 °C for 9–12 h under shaking conditions. After the cells were washed with M9 media two times, the cell solution was mixed with 3% low melting agarose (Sigma-Aldrich, MO, USA) in a 1:1 volume ratio to make the final cell–agarose (1.5%) solution containing 2×10^6 cells per 100 μL and the 100 μL cell–agarose mixture which was poured into each well of a 96-well plate (Nunc, Roskilde, Denmark), followed by incubation at room temperature for 20 min to solidify the agarose gel. One hundred μL of the final assay solution consisting of 80 μL of M9 media with 1 nM cyanocobalamin (Sigma-Aldrich, MO, USA) and 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG; Sigma-Aldrich, MO, USA) and 20 μL of a test sample, such as an amino acid cocktail or human plasma, was next prepared and applied to the wells corresponding to MetB and MetS auxotrophs. All amino acids used to prepare the amino acid cocktail were purchased from Sigma-Aldrich (MO, USA), and the cocktail consisted of 3 μM aspartate, 20 μM methionine and tryptophan, 40 μM arginine, 45 μM cysteine, 50 μM glutamate and phenylalanine, 60 μM asparagines and tyrosine, 65 μM histidine and isoleucine, 100 μM leucine and serine, 120 μM threonine, 180 μM lysine, 200 μM proline, 220 μM glycine and valine, 300 μM alanine, and 500 μM glutamine. All blood samples were obtained from Labgenomics Clinical Laboratories (Seoul, Korea). After the blood samples were collected in the tube containing heparin, plasma was separated within 1 h by centrifugation at 6000 rpm at 4 °C for 30 min. The supernatant plasma was collected into sterile tubes and stored in aliquots at –70 °C until use.

After the assay solutions containing test samples were applied and incubated on the array at 37 °C for 4 h, luminescence was measured using a luminometer (Perkin-Elmer, MA, USA) and the scanned images were obtained by a cooled charge coupled device camera (Fujifilm, Japan) with a constant focal plane, magnification, and integration time.

Specificity, Linearity, and Precision Evaluations. To verify the specificity of the constructed MetB auxotroph toward either Met or Hcy, an array consisting of 22 wells of MetB cells was prepared in a 96-well plate. Each of 21 amino acids was dissolved in the assay solution to make 20 μM amino acid, which was then applied to the designated wells.

To test the linearity of MetS or MetB cell growth for Met, various concentrations of Met (0, 4, 8, 16, 32, 64, and 128 μM) in M9 media were added to each well of the MetS or MetB cell array. To evaluate the linearity of MetB cell growth for Hcy, various concentrations of Hcy (0, 4, 8, 16, 32, 64, 96, and 128 μM) in M9 media, 20 μM Met-fixed solution, or 20% amino acid cocktail were applied into each well of the MetB cell array.

The precision as well as reproducibility of the assay system was assessed by performing a recovery test using 2, 5, and 10 times diluted samples of original 50 μM Hcy and 20% amino acid cocktail in the assay solution. Another recovery test was also performed using 20% real plasma samples by determining the Hcy concentrations from five samples supplied with Hcy at concentrations of 10, 20, 30, 40, and 50 μM . The precision of the two recovery tests was evaluated using the coefficient of variation [$\text{CV} (\%) = \text{SD}/\text{mean} \times 100$] and recovery rate [recovery (%) = measured value/expected value $\times 100$]. The original Hcy concentrations of the samples used for the experiments described in Table 1B,C were determined using an enzymatic Hcy assay (Autolab Homocysteine, IVD Lab Co., Korea) followed by

Table 1. Precision Evaluation^a

| dilution | expected Hcy (μM) | (A) | | SD ^c | CV ^d (%) | Recovery ^e (%) | |
|---|--------------------------------|---|--------------------------------|---|---------------------|---------------------------|---------------------------|
| undiluted | 50 | measured Hcy ^b (μM) | | | | 100.0 | |
| 2 times | 25 | 24.94 | | 0.62 | 2.5 | 100.2 | |
| 5 times | 10 | 10.19 | | 0.16 | 1.6 | 98.1 | |
| 10 times | 5 | 4.92 | | 0.15 | 3.1 | 101.7 | |
| sample no. | original Hcy (μM) | added Hcy (μM) | expected Hcy (μM) | measured Hcy ^b (μM) | SD ^c | CV ^d (%) | recovery ^e (%) |
| 1 | 9.35 | 10 | 19.35 | 20.03 | 0.86 | 4.3 | 96.6 |
| 2 | 10.26 | 20 | 30.26 | 31.17 | 1.06 | 3.4 | 97.1 |
| 3 | 11.75 | 30 | 41.75 | 43.34 | 1.79 | 4.1 | 96.3 |
| 4 | 12.76 | 40 | 52.76 | 50.68 | 2.16 | 4.3 | 104.1 |
| 5 | 13.94 | 50 | 63.94 | 63.34 | 3.62 | 5.7 | 100.9 |
| (C) | | | | | | | |
| | sample 1 | sample 2 | | sample 3 | sample 4 | | |
| original Hcy (μM) | 7.97 | 9.20 | | 11.58 | 18.01 | | |
| added Hcy (μM) | 5 | 10 | | 40 | 90 | | |
| expected Hcy (μM) | 12.97 | 19.20 | | 51.58 | 108.01 | | |
| hyperhomocysteinemia | normal | moderate | | intermediate | severe | | |
| assay | sample 1 | sample 2 | | sample 3 | sample 4 | | |
| Within Assay | | | | | | | |
| measured ^b (μM) | 12.81 | 19.37 | | 50.28 | 104.32 | | |
| SD ^c | 0.37 | 0.26 | | 0.47 | 1.20 | | |
| CV ^d (%) | 2.9 | 1.4 | | 0.9 | 1.1 | | |
| recovery ^e (%) | 101.3 | 99.1 | | 102.6 | 103.5 | | |
| Between Assay | | | | | | | |
| measured (μM) | 12.70 | 19.46 | | 52.88 | 102.39 | | |
| SD | 0.90 | 0.46 | | 1.80 | 1.49 | | |
| CV (%) | 7.1 | 2.4 | | 3.4 | 1.5 | | |
| recovery (%) | 102.2 | 98.6 | | 97.5 | 105.5 | | |

^a (A) Recovery test for serially diluted samples from 50 μM Hcy in 20% amino acid cocktail solution. (B) Recovery test using human plasma samples containing unknown amounts of Met and Hcy supplied with different amounts of additional Hcy. (C) Within and between assay using four plasma samples prepared to represent a normal state and three different stages of hyperhomocysteinemia. ^b Mean of five measurements in (A) and (B) and three measurements in (C). ^c Standard deviation. ^d Coefficient of variation. ^e Measured value/expected value $\times 100$.

analysis using a chemistry analyzer (Hitachi 7180, Hitachi, Japan) according to the manufacturer's instructions and protocols.

Hcy Assay for Hyperhomocysteinemia Diagnosis. To represent both a normal state and three different stages of hyperhomocysteinemia, artificial samples containing four different levels of Hcy (10, 26, 70, and 120 μM) were prepared in M9 media, 20 μM Met-fixed solution, and 20% amino acid cocktail, and subjected to the Hcy assay. To assess the reliability and reproducibility of this method for hyperhomocysteinemia diagnosis, within and between assays were performed using four different plasma samples that contain Hcy at different concentrations (7.97, 9.20, 11.58, and 18.01 μM) and were additionally supplied with different amounts of Hcy (5, 10, 40, and 90 μM) to create hyperhomocysteinemia samples. The within-assay variation was determined from the results of three parallel experimental setups, and the between assay was performed by determining Hcy concentrations for each sample over 3 days.

RESULTS AND DISCUSSION

Hcy Quantification on the Array of Bioluminescent *E. coli* Auxotrophs. To develop the new cell-based Hcy assay system, two *E. coli* auxotrophs were constructed to quantify Met and Hcy in test samples. The MetS auxotroph was constructed using the transposon mutagenesis method involving repetitive selection of candidate mutants growing only in the presence of Met, as described in a previous report.²⁷ In contrast, the MetB auxotroph was constructed using chromosomal gene deletion of the metB gene, which is involved in the biosynthesis of Hcy as well as Met. Therefore, the metB gene-deleted cell (MetB auxotroph) was expected to grow in response to the presence of both Met and Hcy. The two auxotrophs were supplemented with the bioluminescence-generating plasmid pTAC-luc, constructed by inserting the luciferase gene (luc) derived from *Photinus pyralis* (firefly) into pET-pTAC containing an IPTG-inducible promoter.²⁷ As a result, the auxotrophs produced a bioluminescence signal during

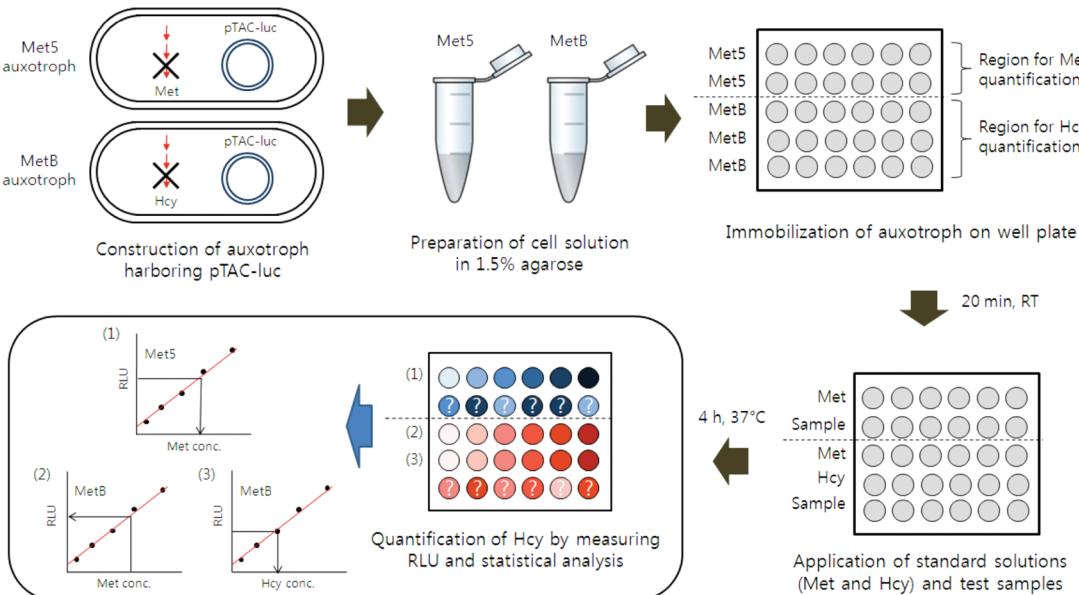


Figure 1. Overall scheme for Hcy quantification on arrays of bioluminescent *E. coli* auxotrophs. The array consists of two regions to quantify Met and Hcy. MetS and MetB cells in 1.5% agarose were immobilized in the regions for Met and Hcy quantification, respectively. Standard (Met or Hcy) and test (human plasma) samples were applied to the designated wells, and the luminescent signals were measured after 4 h incubation at 37 °C. From the curve (1) which indicates a correlation between MetS cell growth and Met, Met concentration in the test sample was determined and the RLU value corresponding to MetB cell growth induced by the determined Met was determined (2). The RLU value was then subtracted from the RLU value corresponding to MetB cell growth induced by both Met and Hcy. Finally, Hcy was quantified using the curve (3).

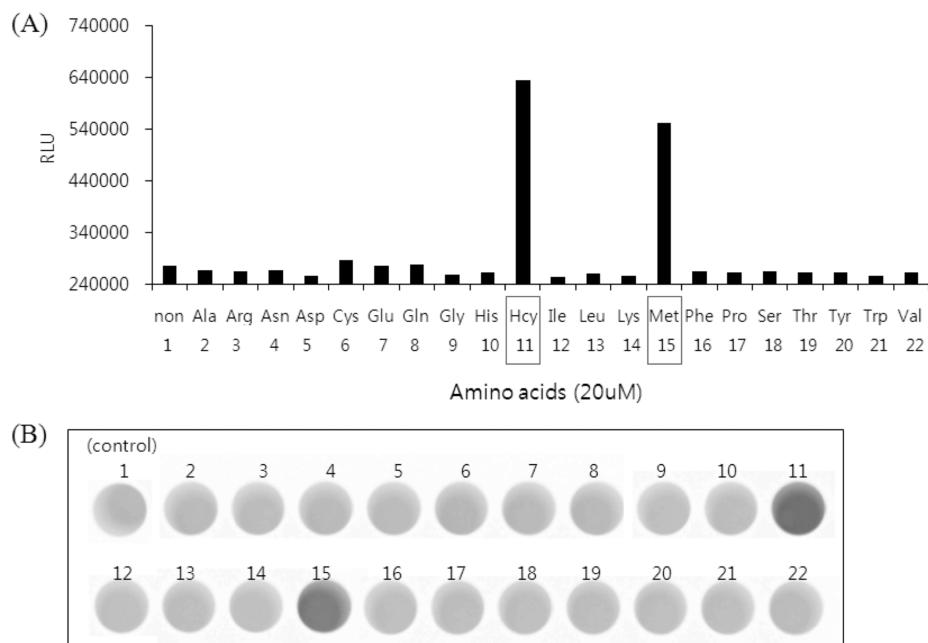


Figure 2. Specificity of MetB auxotroph toward Hcy and Met. (A) RLU values produced by MetB cell growth in the presence of each of 21 amino acids at 20 μ M. (B) Scanned image of the MetB cell array.

growth in response to the presence of Met and Hcy after IPTG induction without the requirement for any additional external reagents.

As depicted in Figure 1, the array system consists of two regions for Met and Hcy quantification in which wells were loaded with MetS and MetB auxotrophs in 1.5% (w/v) agarose, respectively. Met solutions with gradient concentrations were

then applied to both regions (usually the first rows of MetS and MetB regions) while Hcy solutions with gradient concentrations were applied to the second row of only the MetB region. Test samples were usually applied to the last rows of both the MetS and MetB regions. In response to applied Met and Hcy, the two auxotrophs grew on the array with simultaneous generation of luminescent signals, which were measured from (1), (2), and (3)

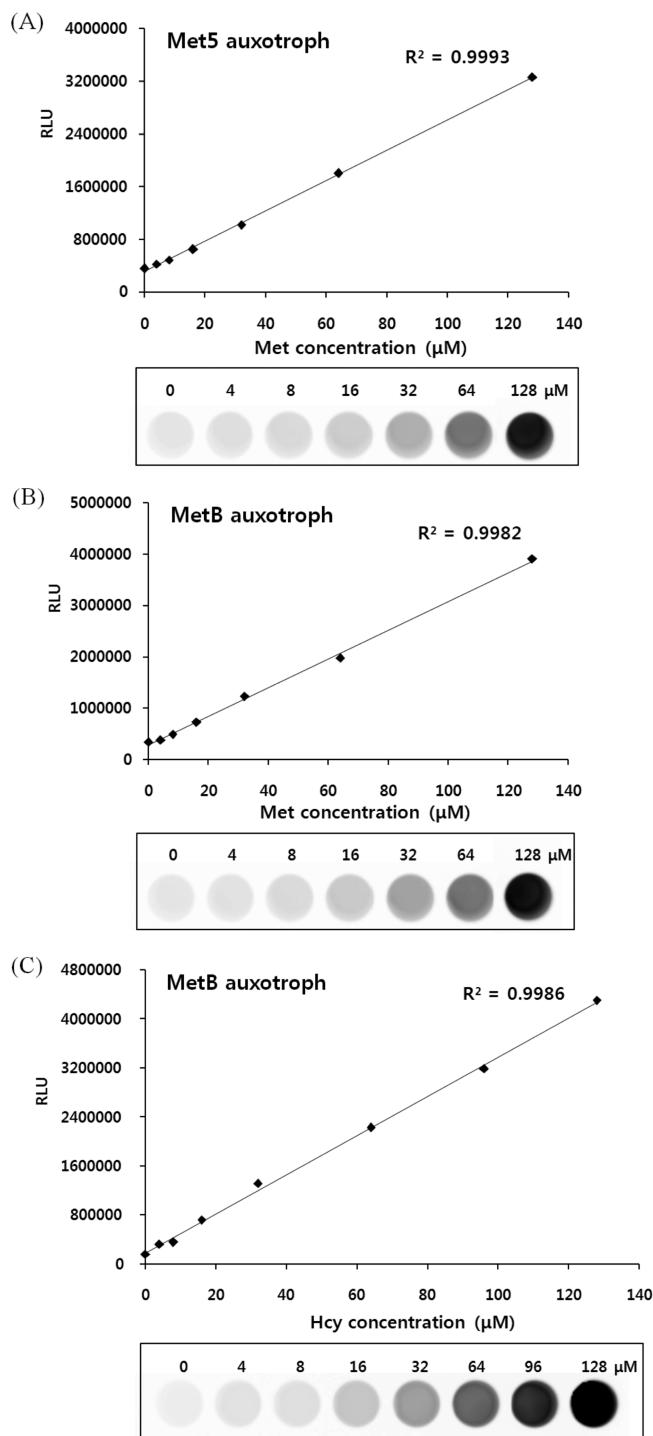


Figure 3. Linearity evaluation. A correlation between the luminescent signal produced by Met5 cell growth and Met (A), MetB cell growth and Met (B), and MetB cell growth and Hcy (C). The image below each graph shows luminescent signals produced by Met or Hcy at different concentrations.

regions and used to construct three standard curves for the quantification of Met and Hcy. On the basis of the relative luminescence unit (RLU) value obtained from a test sample in the Met quantification region, the Met concentration in the sample was first determined using the standard curve (1). The

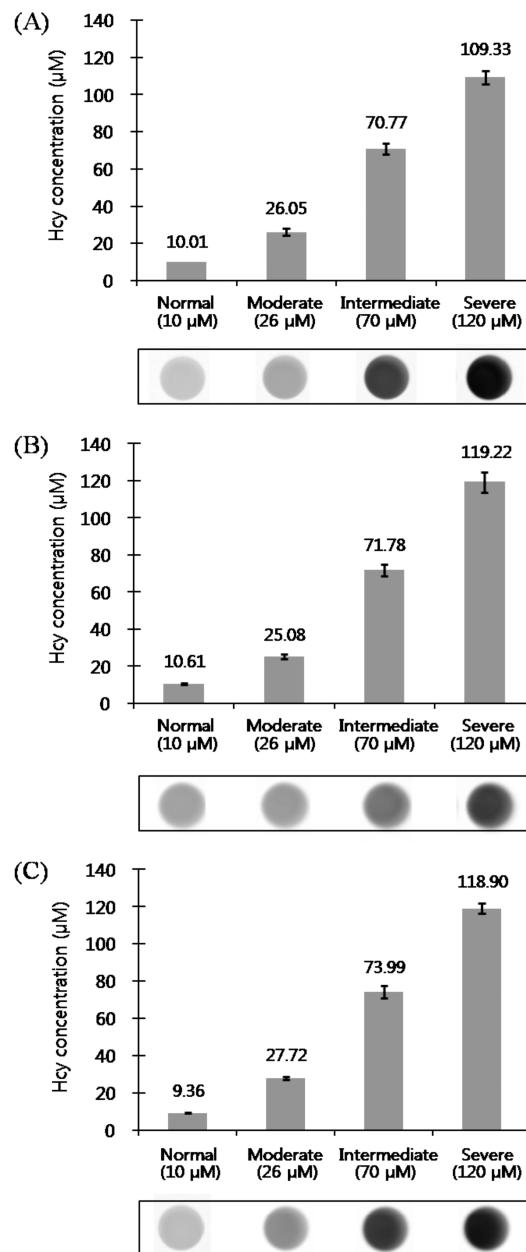


Figure 4. Quantification of four different levels of Hcy representing a normal state and three stages of hyperhomocysteinemia. Measurement of 10, 26, 70, and 120 μM of Hcy in M9 media (A), 20 μM -fixed Met solution (B), and 20% amino acid cocktail (C). The image below each graph shows luminescent signals produced by different concentrations of Hcy. All samples were analyzed with five replicates in a single run, and the results are averaged.

RLU value produced by MetB cell growth, induced by Met, was determined using the standard curve (2). The RLU value was then subtracted from the RLU value obtained from MetB cell growth that is induced by both Met and Hcy present in the sample. Since the difference between the RLU values is a result of MetB cell growth induced by only Hcy, the Hcy concentration corresponding to the subtracted RLU value can be determined using the standard curve (3).

Evaluations of Specificity, Linearity, and Precision. To evaluate the substrate specificity of the developed MetB auxotroph, MetB auxotroph was immobilized in 22 wells and 20 μM

each of 21 amino acid solutions were added to 21 wells, the last well being a control not containing any amino acids. Upon incubation of the array, luminescent signals were generated only from the two wells supplied with Met and Hcy (Figure 2). This observation revealed that the constructed MetB auxotroph exhibits an excellent specificity for both Hcy and Met. As demonstrated in our previous studies, the MetS auxotroph also displays highly specific growth only in the presence of Met.²⁷ Specifically, no luminescent signals are produced in the presence of Hcy in the concentration range probed (Supporting Information, Figure S-1). This result confirms that the MetS auxotroph can be used to determine Met concentrations in unknown samples without interference from other amino acids including Hcy.

The correlation between cell growth of the two auxotrophs and concentrations of corresponding amino acids, Met and Hcy, was evaluated next. MetS cell growth showed a linear correlation with Met concentration (Figure 3A), and MetB cell growth responds linearly with both the concentrations of Met and Hcy (Figure 3B,C). Moreover, the same linear correlation was found to exist between MetB cell growth and Hcy concentration even when other amino acids, including Met, were present in the sample (e.g., 20 μM Met-fixed solution and 20% amino acid cocktail mimicking real blood sample; see Supporting Information, Figure S-2). The combined results demonstrate that no interference takes place in the linear growth of MetB cells induced by Hcy when other amino acids are present in the sample.

The precision of the new method for Hcy quantification was assessed using analytical recovery tests with artificial and real samples. The recovery test was first performed by determining Hcy concentrations for serially diluted (2, 5, or 10 times) samples from 50 μM Hcy in 20% amino acid cocktail solution (Table 1A and Supporting Information, Figure S-3). As a result, the Hcy concentrations in the diluted samples were precisely determined and verified using CVs of five parallel data sets within less than 4% and with recovery rates from 98.1 to 101.7%. Another recovery test was performed by determining Hcy concentrations of real plasma samples, containing unknown amounts of Met and Hcy that are supplied with different amounts of additional Hcy (Table 1B). The original concentrations of Hcy were determined using the conventional enzymatic method. Met concentrations of the plasma samples were first determined on the basis of RLU values from the MetS cell array (Supporting Information, Figure S-4A), and the Hcy concentrations were determined by subtracting the RLU value corresponding to the determined Met concentration from the RLU value produced on the MetB cell array (Supporting Information, Figure S-4B). The results show that Hcy concentrations are nearly the same as those expected with CVs in the range of 4.3–5.7% and recovery rates from 96.6 to 104.1%.

Hcy Quantification to Diagnose Hyperhomocysteinemia. Hcy levels higher than 15 μM in blood are generally recognized to be indicators of hyperhomocysteinemia, and three different stages of hyperhomocysteinemia have been defined depending on the concentration of Hcy (moderate: $\leq 30 \mu\text{mol/L}$; intermediate: $31–100 \mu\text{mol/L}$; and severe: $> 100 \mu\text{mol/L}$).²⁸ The diagnostic capability of the Hcy assay method was demonstrated using four representative levels of Hcy, corresponding to the normal state and the three stages of hyperhomocysteinemia (normal: 10 μM ; moderate: 26 μM ; intermediate: 70 μM ; and severe: 120 μM). Sample preparation was accomplished by preparing various Hcy samples in M9 media, 20 μM Met-fixed solution, and 20% amino acid cocktail (Figure 4). Analysis of the

MetB cell array enabled quantification of Hcy in M9 media not containing Met (Figure 4A), but Hcy quantification in 20 μM Met-fixed solution and 20% amino acid cocktail requires simultaneous analysis of both the MetS and MetB cell arrays in order to evaluate the quantity of Met present in the samples that contributes to the RLU values produced by MetB cell growth. When this cell array strategy was employed, Hcy concentrations were determined with excellent CVs in a range of 3.7–5.2% (Figure 4B,C and Supporting Information, Table S-1A and 1B). The observations (Figure 4) show that the method serves as a precise Hcy assay for determining different levels of Hcy (Supporting Information, Table S-2).

The within- and between-assay precision of the method was evaluated using four plasma samples containing different concentrations of Hcy (Table 1C). The original concentrations of Hcy were determined using the conventional enzymatic method, and additional Hcy (5, 10, 40, or 90 μM) was added to each of the original samples to create artificial hyperhomocysteinemia conditions. The recovery rates of within and between assays of the samples were found to be in the respective ranges of 99.1–103.5% and 97.5–105.5%, verifying the excellent reliability of the method. The precision of the procedure, in comparison with currently used assays,^{29–31} was also found to be excellent as confirmed by the CVs of the within and between assays which were less than 2.9 and 7.1%, respectively.

CONCLUSIONS

A new cell-based assay, employing rapidly growing bioluminescent *E. coli* auxotrophs, was developed to determine Hcy concentrations in plasma samples. The assay, performed using an array format, displays excellent specificity for Hcy, high precision and reproducibility, and near perfect linearity, parameters that enable its use for the clinical diagnosis of hyperhomocysteinemia. On the basis of the results described above, we strongly believe that the new Hcy assay procedure will serve as a powerful alternative for high-throughput screening for cardiovascular disease as well as other diseases associated with hyperhomocysteinemia. Moreover, we envision that the strategy utilizing auxotrophic *E. coli* that specifically grows in the presence of a target metabolite and directly produces a luminescent signal will find general use in devising rapid, convenient, and economic diagnosis methods for various diseases.

ASSOCIATED CONTENT

S Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*H.G.P.: tel, +82-42-350-3932; fax, +82-42-350-3910; e-mail, hgpark@kaist.ac.kr. B.-O.C.: tel, +82-2-760-5257; fax, +82-2-760-5008; e-mail, bochoi@ewha.ac.kr.

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