

A live bacteria enzyme assay for identification of human disease mutations and drug screening

Corresponding Author: Prof Bernhard Palsson

Version 0:

Decision Letter:

Dear Dr Palsson,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "A live bacteria enzyme assay facilitates rapid identification of mutations associated with human genetic diseases and drug screening". The manuscript has been seen by three experts, whose reports you will find at the end of this message.

You will see that the reviewers appreciate the work. However, they articulate concerns about the translational utility of the approach, and provide useful suggestions for improvement. We hope that with substantial further effort you can address the criticisms, increase the strength of the evidence, and convince the reviewers of the merits of the study. In particular, we would expect that a revised version of the manuscript provides:

- * Robust validation of the assay.
- * Evidence of the assay's utility in the identification of less-characterized gene variants.
- * Evidence of the assay's utility in a translationally relevant setting.

When you are ready to resubmit your manuscript, please [upload](#) the revised files, a point-by-point rebuttal to the comments from all reviewers, the [reporting summary](https://www.nature.com/authors/policies/ReportingSummary.pdf), and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

- * Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).
- * If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).
- * If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).
- * Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.
- * The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).
- * Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We expect that you will be able to resubmit the manuscript within 16 weeks of receiving this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*.

We hope that you will find the referee reports helpful when revising the work. Please do not hesitate to contact me should

you have any questions.

Best wishes,

Pep

Pep Pàmies

Chief Editor, Nature Biomedical Engineering

Reviewer #1 (Report for the authors (Required)):

Summary: This original research article presents a simple and rapid method for investigating human enzymopathy through the growth rate measurement of *E. coli*, expressing human mutant enzymes. The replacement of *E. coli* genes with their human orthologs is performed according to a previously published method by the same research group (Sandberg, et al., Nat Ecol, 2020). By knocking out the bacterial phosphoglucose isomerase (*pgi*) and glucose-6-phosphate dehydrogenase (*zwf*) genes, glycolytic flux is forced through the recombinant human enzymes glucose-6-phosphate isomerase (GPI) or glucose-6-phosphate dehydrogenase (G6PD). Growth rate is then shown to correlate well with previously reported values of enzymatic activities for different mutations. The method is sufficiently sensitive to detect dose-dependent and mutation-dependent pharmacodynamics of selected known small-molecule activator and drug inhibitors. While the presented method is simple and elegant the manuscript in its current scope should not be considered for publication in Nature Biomedical Engineering due to the following major concerns:

- 1) The translational potential of the method is not sufficiently supported, as expected by the journal's standards. This may include, but is not limited to, screening genetic variants within a real clinical patient pool, and verifying inhibitor/activator pharmacodynamics via hemolysis assays, high-throughput cellular assays, or animal in vivo models.
- 2) The authors lightly dismiss some major analytical challenges they indicate, including differences in membrane transport between bacterial and mammalian cells, the toxicity of some studied compounds to *E. coli*, and discrepancies between the presented method and cell-based assays.
- 3) The presented work lacks the use of tools or principles from physical sciences, engineering or mathematics as required by the journal's scope. For example, how could the proposed method be incorporated within a medical device? Could it be coupled with an algorithm to diagnose mutations or prescribe a drug?

Minor concerns

-
- 1) Growth rates reported in this work are very low, specifically for the humanized *E. coli* for G6PD. This issue should be addressed and explained.

Stylistic recommendations:

-
- 1) The work could be presented more concisely
 - a. Line 91 – describes a previous study – could be shortened to one sentence
 - b. Line 103 – this paragraph does not contribute to the understanding of the results
 - c. Line 122-130 – should be included in the introduction
 - d. Line 170 – this paragraph does not contribute to the understanding of the results
 - 2) Results could be presented more concisely with better format consistency:
 - a. Figure 2a,c could be combined in Figure 1 and presented in the same format. Growth curves in Figure 2b could be in Extended data
 - b. Growth curves in Figure 4 could be in Extended data, and Figure 3 could be combined into Figure 4 and presented in the same format as all other dose-response plots (Extended data Figure 3)

Reviewer #2 (Report for the authors (Required)):

Identification of genetic variations associated with human diseases is crucial for advancing our understanding and treatment of these conditions. Genome-wide association studies (GWAS) are an effective method for uncovering these variations. However, inferring the functional impact of these variations remains a challenge, often necessitating specialized assays and time-consuming analytical methods and instrumentation.

The authors propose an innovative strategy, termed LEICA, which leverages humanized *E. coli* to study human genetic enzymopathies and to screen candidate drugs targeting metabolic pathways. Specifically, they focus on GPI and G6PD, which are implicated in common hereditary enzymopathies. By substituting selected *E. coli* metabolic enzymes with human

orthologs and their variants, they demonstrate that the growth rate of *E. coli* reflects the in vivo activity of heterologously expressed human enzymes. This method enables high-throughput screening of drug effects on human enzymes and bridges the gap between in vitro assays and cell-based assays. The use of live bacterial cells for enzyme activity assessment offers a rapid and accurate means of identifying the impact of genetic mutations, providing valuable insights into the causality of genetic disorders.

The authors also suggest that LEICA holds potential for personalized medicine, offering a cost-effective platform for identifying drug sensitivities and developing targeted therapies. The growth rate of *E. coli*, which is dependent on glycolytic flux or rate-limiting steps, serves as a proxy for the activity of human enzymes expressed in the bacteria. *E. coli* strains carrying different enzyme mutants displayed distinct growth rates, reflecting variations in enzyme activity induced by mutations. This approach eliminates the need for laborious protein purification steps typical of traditional in vitro assays.

Several concerns need to be addressed:

Assessment of Catalytic Activity Relevance: On line 206, the authors state, "Human genetic disorders result from a complex interplay of biological factors, including variations in catalytic activity, expression levels, and zygosity, posing challenges in identifying causality. LEICA insulates enzyme activity from such biological factors by mimicking the homozygous state and providing stable expression levels, offering a rapid means of assessing only catalytic activity as a variable." While this statement is well-founded, I question the overall value of developing a technology like LEICA that correlates catalytic activity with cell growth. Given the complex interplay of factors in human genetic disorders, LEICA's application may be limited to well-characterized variants. If so, what is the utility of LEICA in studying and diagnosing less-characterized variants? Could it risk complicating interpretations rather than simplifying traditional assays? I suggest that the authors include genetic variants with limited background information for this study.

Validation of Variants: Although the authors demonstrate that GPI and G6PD variant activities correlate strongly with *E. coli* growth rates, the sample size ($n = 6$) is too small to robustly validate the hypothesis or generalize the findings. A larger number of GPI and G6PD variants should be tested to strengthen the correlation and provide more comprehensive data.

Screening of Small Molecules: While LEICA appears promising for screening small molecules like AG1, there is skepticism about its effectiveness for screening inhibitors. Given that LEICA might also select compounds with antibacterial properties or effects on primary metabolism, it may result in many false positives when screening for G6PD inhibitors. To validate LEICA's utility for drug screening, it is recommended that the authors conduct screens using e.g., commercially available compound libraries to evaluate its efficacy in identifying both activators and inhibitors.

Application to Personalized Medicine: This manuscript focuses on validating LEICA with previously known data. However, it does not demonstrate how LEICA could provide new insights or expand our understanding. I question whether LEICA genuinely contributes valuable information for personalized medicine.

Overall, while LEICA is an intriguing approach with potential applications, the study would benefit from addressing these concerns to enhance its robustness and applicability.

Reviewer #3 (Report for the authors (Required)):

Choe and Palsson report an approach for high-throughput screening of candidate drugs in a humanized *E. coli* platform (LEICA) in an effort to develop a precision medicine approach to human genetic disorders. The relationship between a detected genetic variant and its potential functional significance is a major issue in the practice of medicine. By replacing *E. coli* glycolytic enzymes with their human equivalents, the growth rate of *E. coli* was found to correlate highly with previously reported activities of recombinant enzymes. By incorporating genes coding for human glucose-6-phosphate isomerase (G6PD) and GPI into the LEICA system, human enzyme activities can be measured in an intracellular environment that provides the physiological milieu for proper enzyme function. This system offers a relatively straightforward and rapid way to measure human enzyme activity compared to current specialized assays of enzyme activities. Such a screening technique could be adapted to test small molecules for their potential to act as therapies for specific genetic enzymopathies.

Focusing on six representative mutations (2 benign, 4 pathogenic) in the gene coding for GPI, the authors showed that strains carrying different GPI variants showed different growth rates compared to the wild-type humanized *E. coli* for GPI. Strains harboring pathogenic variants showed significantly lower growth rates; benign variants caused weakly impaired or no change in growth rate compared to the wild type control. In short, LEICA can be used to estimate activities of a human enzyme.

Studies focusing on human glucose 6-phosphate dehydrogenase (G6PD), showed that this enzyme is also functional in the *E. coli* system. Similarly to the GPI studies, differential growth, related to the pathogenic severity of the underlying genetic alteration, was seen in the LEICA system when different G6PD gene variants were studied.

The LEICA system also exhibited potential as a drug-screening platform, as the use of a small molecule activator of G6PD activity (AG1) resulted in growth rates of humanized strains for various G6PD enzymes that correlated to known activation profiles reported in other assay systems. In addition, seven other compounds with document effects on G6PD activity were studied. LEICA generally generated results similar to cell-based recombinant assays versus in vitro assays and was considered to be a suitable platform for high-throughput screening. This system also has potential as a precision medicine

tool, as the studied G6PD genetic variants showed different responses to each tested compound.

The manuscript is clearly written and background related to the studied enzymes is provided to assist with understanding these studies in context.

The LEICA system appears to be robust in interrogating enzymes related to glycolysis. Given the interest in finding novel therapies for inborn errors of metabolism, such as PKU, MSUD, urea cycle disorders, organic acidemias, fatty acid oxidation disorders, etc. this platform would appear to have multiple potential applications. Do the authors have thoughts related to how this E. coli system might perform while evaluating other enzymes related to human disease in different biochemical pathways, e.g., those involving small molecules, such as amino acid or fatty acid metabolism, or organelle disorders, such as those involving lysosomes, peroxisomes, or mitochondria?

Minor comment:

Gene names should be italicized.

Version 1:

Decision Letter:

Dear Bernhard,

Thank you for your revised manuscript, "A live bacteria enzyme assay facilitates rapid identification of mutations associated with human genetic diseases and drug screening". Having consulted with the original reviewers (whose comments you will find at the end of this message), I am pleased to write that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*.

We will be performing detailed checks on your manuscript, and in due course will send you a checklist detailing our editorial and formatting requirements. You will need to follow these instructions before you upload the final manuscript files.

Please do not hesitate to contact me if you have any questions.

Best wishes,

Pep

Pep Pàmies

Chief Editor, *Nature Biomedical Engineering*

Reviewer #1 (Report for the authors (Required)):

Summary: The impact of the work has been significantly enhanced through the extensive inclusion of the following findings:

- Identification of seven potential lead compounds for G6PD inhibition within a 160-member drug library, with four of the hits being novel discoveries.

- Analysis of four less-characterized G6PD variants with no previously reported biochemical properties.
- Demonstration of a linear correlation between the growth rates of humanized E. coli expressing G6PD and catalytic constants (k_{cat}) for 13 additional well-characterized variants.
- Expansion of LEICA beyond glycolysis to screen the activity of human argininosuccinate lyase (ASL).

The revised manuscript now more effectively highlights the translational potential of this straightforward and cost-effective method for screening genetic variants within a real clinical patient pool.

The analytical limitations of the method are now more thoroughly addressed, with a detailed comparison to other gold-standard methods. The authors have clarified the specific application of LEICA for investigating enzyme activity independently of other pathogenic factors, emphasizing both its advantages and drawbacks.

All inquiries were carefully addressed and clarified, and all stylistic suggestions were implemented.

Optional comment: Regarding the drug library screening, did you encounter any false-negative results? Specifically, were there compounds known to inhibit G6PD but not identified as such by LEICA?

Reviewer #2 (Report for the authors (Required)):

All of my concerns have been thoroughly addressed, and I now recommend acceptance.

Reviewer #3 (Report for the authors (Required)):

Since the initial submission, the authors have performed additional experiments and have made significant additions to the manuscript. In addition to expanding the work related to glycolytic enzymes, argininosuccinate lyase (ASL), an essential urea cycle enzyme, was also studied in the LEICA system. Interestingly, humanized *E. coli* expressing pathogenic ASL variants showed markedly reduced growth rates compared to wild-type cells. I appreciate this addition, as the LEICA method has now shown applicability to metabolic pathways related to amino acid metabolism. As there are numerous biochemical genetic disorders related to impaired metabolism of amino acids (and of course other substrates), I look forward to following the results of this screening assay as it relates to an expanding number of conditions.

Version 2:

Decision Letter:

Dear Prof Palsson,

I am happy to inform you that your manuscript, "A live bacteria enzyme assay for identification of human disease mutations and drug screening", has now been accepted for publication in *Nature Biomedical Engineering*.

Over the next few weeks, the figures will be checked for production quality, the text edited to ensure that it conforms to house style, and the manuscript typeset.

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Thank you for having submitted this work to *Nature Biomedical Engineering*.

Best wishes,

Barbara Cheifet

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A live bacteria enzyme assay facilitates rapid identification of mutations associated with human genetic diseases and drug screening

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Keywords: Live *Escherichia coli* assay (LEICA), humanized *E. coli*, *in vivo* screening system

ABSTRACT

Advances in high-throughput sequencing have enabled the identification of genetic variations associated with human disease. However, deciphering the functional significance of these variations remains **challenging**. Here, we propose an alternative approach **that utilizes** humanized *Escherichia coli* to study human genetic enzymopathies and to screen candidate drug effects on metabolic targets. By replacing selected *E. coli* metabolic enzymes with their human orthologs and their sequence variants, we demonstrate that the growth rate of *E. coli* reflects the *in vivo* activity of heterologously expressed human enzymes. This approach accurately reflected enzyme activities of known sequence variants, thus enabling **rapid** screening of **causal sequence variations associated with human diseases**. This approach bridges the gap between *in vitro* assays and cell-based assays. Our findings suggest that the proposed approach using a humanized *E. coli* strain holds promise for **drug discovery**, offering a **high-throughput** and cost-effective platform for identifying **new compounds targeting human enzymes**. Continued research and innovation in this field have the potential to impact the development and practice of precision medicine.

INTRODUCTION

The human genome sequence varies between individuals. Everyone typically harbors between 250 to 300 loss-of-function sequence variations relative to the reference human genome¹. These sequence variations often underlie inherited disorders, making the identification of causal mutations an **essential** pursuit in human biology and personalized medicine. This imperative is underscored by recent strides in *in vivo* genome editing, which offer promising avenues for treating conditions like eye and liver disease^{2,3}.

Traditionally, the identification of genotypes associated with genetic disease has relied on direct patient analysis and subsequent documentation as case reports. Once genetic variations linked to specific disorders are mapped, they are studied through various methodologies. One common approach involves *in vitro* studies utilizing materials obtained from patients or recombinant proteins to characterize mutant proteins. However, this approach is constrained by sample availability and necessitates labor-intensive purification processes. Moreover, it often requires specialized assay systems tailored to individual proteins, alongside time-consuming analytical methods and instrumentation. **Additionally**, dilute *in vitro* conditions do not replicate crowded and dense gel-like physiological conditions, necessitating an *in vivo* system for an accurate biochemical assay⁴.

Alternatively, large-scale computational surveys, such as genome-wide association studies based on population sequencing datasets⁵, have proven effective in identifying potential associations between thousands of genetic changes to pathological conditions⁶. However, such associations do not establish causality, particularly given the vast number of uncharacterized genetic variations and confounding factors of zygosity and genetic linkage^{5,7}. **Furthermore, variations that induce severe pathogenicity from early onset (such as in newborns and infants) are rare in the human population, making it challenging to obtain samples or detect such variations in population databases. As a result, pathogenic mutations remain practically undetectable, while detected mutations are more likely to have only a minor impact on enzyme activity.**

In prior work, we demonstrated the replacement of *Escherichia coli* glycolytic genes with **their** human orthologs⁸. Despite the evolutionary distance between bacteria and humans, they share significant metabolic similarities, such as a universal glycolytic framework across phyla, where glucose is metabolized through identical chemical reactions, albeit with different enzymes. **Thus**, *E. coli* lacking key native enzymes **for glucose metabolism could utilize glucose with the** introduction of corresponding human enzymes without alterations to their coding sequence.

Thus, we proposed an alternative approach to investigate the activities of human mutant enzymes by developing a live *Escherichia coli* assay (LEICA). Specifically, we focused on glucose-6-phosphate isomerase (GPI) and glucose-6-phosphate dehydrogenase (G6PD), which are associated with the most common human hereditary enzymopathies^{9,10}.

As the growth rate of *E. coli* is contingent upon glycolytic flux¹¹, it serves as a surrogate measure for the activity of heterologously expressed human enzymes. Harnessing the ease of genetic manipulation in *E. coli*, we replicated human mutations in these enzymes. *E. coli* strains carrying different mutants exhibited distinct growth rates, reflecting variations in enzyme activity induced by mutations. Notably, the growth rates demonstrated a high linear correlation with enzyme activities previously determined through *in vitro* assays with recombinant proteins. This live bacterial cell assay provides an accurate and rapid means of screening for enzyme activity change resulting from human mutations, offering insights into the causality of genetic disorders attributable to genetic variations. We also expanded LEICA to screen argininosuccinate lyase (ASL), a key urea cycle enzyme whose deficiency results in argininosuccinic aciduria¹². Complementation of arginine auxotrophy in *E. coli* lacking ASL with human equivalents demonstrates LEICA's broader applicability across diverse enzymes.

LEICA revealed other potential uses. Through experimentation with small molecules using LEICA, we found that chemical compounds targeting human G6PD could either enhance or inhibit growth when administered directly to cells in culture. We confirmed inhibitory effects of known G6PD inhibitors, highlighting the drug screening capability of LEICA on human drug targets. Screening a library of 160 human metabolism modulators revealed seven lead compounds, including the rediscovery of three with known G6PD inhibitory effects or antimalarial activity. Lastly, we observed enhanced growth of *E. coli* carrying the G6PD Canton mutant when treated with the recently discovered G6PD agonist AG1¹³. LEICA thus serves not only as a drug screening platform but also holds promise for use in developing personalized medicine.

RESULTS

Growth of *Escherichia coli* carrying human glucose-6-phosphate isomerases (GPI) reflects *in vivo* enzyme activity: In a previous study, we demonstrated the successful replacement of *E. coli* GPI with its human ortholog⁸. During adaptive laboratory evolution to optimize growth, we observed that achieving optimal GPI function in live bacteria did not necessitate mutations in the protein sequence. Instead, enhanced gene expression, facilitated by two point mutations in the promoter, proved to be the key factor for growth improvement.

Furthermore, various *in vitro* studies use recombinant human enzymes expressed from *E. coli* lysates^{14,15}, indicating *E. coli* as a potential host for functional expression of human enzymes.

In both humans and *E. coli*, glucose is mainly utilized by glycolysis. Thus, we hypothesized that replacing a glycolytic enzyme in *E. coli* with its human counterpart would reflect human enzyme activity (Fig. 1a). However, glucose can also be utilized via the oxidative pentose phosphate pathway (oxPPP), also known as hexose monophosphate (HMP) shunt. Therefore, to specifically assess human GPI activity in *E. coli*, it is essential to disengage the HMP shunt. This was achieved by deleting the gene for G6PD (*zwf*) that enables an alternate glucose utilization. We thus knocked out *zwf* from the *E. coli* strain 20.71—an evolved *E. coli* K-12 MG1655 strain¹⁶ carrying in-frame *pgi* swap to human GPI and promoter mutations (Fig. 1b). In the resulting strain—a humanized *E. coli* for GPI—growth in a glucose-containing medium is solely dependent on GPI activity (Extended Data Fig. 1).

We sought to examine the effects of genetic variations in GPI found in the human population that are associated with hemolytic anemia. To explore the effects of both benign and pathogenic mutations, we selected six representative mutations (Supplementary Table 1), some of which are supported by direct biochemical evidence¹⁴. Two of the six mutations are known to be benign, with population frequencies ranging from 1 in 49 to 1 in 4,400 individuals (Genome Aggregation Database; gnomAD¹⁷), while four pathogenic variations were found at frequencies of 1 in 57,000 to 1 in 390,000 individuals (gnomAD), with some reported from patients exhibiting hemolysis^{18–20}, indicating their disease association.

To evaluate the functionality of a live bacterial assay, we replaced wild-type (WT) human GPI in the humanized *E. coli* for GPI with the six mutant GPIs to investigate activity differences induced by the mutations. Strains expressing different GPI variants showed growth rate changes ranging from -13.1 to +2.2% compared to the WT control (Fig. 1c). Growth rates of strains carrying pathogenic mutations were significantly lower than those with WT GPI, while two benign mutants displayed either a mild decrease in growth (-5.6%) or growth indistinguishable from the WT control.

Comparison of biochemically determined properties of recombinant mutants¹⁴, Fukuoka, Iwate, and Matsumoto, with the live bacterial assay revealed a high linear correlation between enzyme activity and the growth rate. This result indicates a direct impact of GPI activity on the growth rate of humanized *E. coli* strains (Fig. 1d). This demonstrates that a live *Escherichia coli* assay (LEICA) is capable of inferring activities of human enzymes. LEICA gives an impetus for rapidly screening genetic variants causing enzyme deficiency. Compared to

conventional biochemical assays using hemolysate from isolated red blood cells (RBCs) or recombinant enzymes that require specialized assay systems and laborious purification steps²¹, LEICA calls for only a single gene replacement (and variants therein) with a straightforward growth measurement, as all the necessary substrates and cofactors are present in live bacteria.

Diagnosing human glucose-6-phosphate dehydrogenase (G6PD) deficiency using LEICA: Having demonstrated the concept of LEICA analyzing human enzyme activity, we sought to explore another human enzyme, G6PD. G6PD deficiency is the most common cause of RBC enzymopathy, affecting an estimated 300-400 million people worldwide²². G6PD plays a critical metabolic role in RBCs as the HMP shunt is the main source of NADPH required to maintain redox homeostasis²³. Hereditary G6PD deficiency is caused by sequence variations in *G6PD*, which is highly polymorphic with over 230 sequence variants identified^{6,10}, due to its association with malaria resistance²⁴. Despite the growing number of variants identified through populational sequencing, clinical or molecular characterizations remain limited⁶.

To assess *G6PD* variations using LEICA, we first examined whether human G6PD could functionally express in *E. coli* (Fig. 1e). Since the *E. coli* 20.71 Δ *pgi* Δ *zwf* double knockout strain is unable to utilize glucose as a carbon source (Extended Data Fig. 1), growth in a glucose medium would be directly coupled with activity of the heterologously expressed human G6PD, creating a humanized *E. coli* for G6PD. The double knockout strain expressing human G6PD exhibited comparable growth to the *E. coli* Δ *pgi* single knockout strain, whose glycolytic flux is supported by its endogenous G6PD (*Zwf*) (Extended Data Fig. 2). As demonstrated with the *GPI* gene replacement, human G6PD proved functional in *E. coli*.

Next, we selected 13 human *G6PD* sequence variations associated with varying severities of G6PD deficiency for LEICA application (Supplementary Table 2)⁶. Strains expressing G6PD variants showed markedly different growth profiles to those carrying the WT enzyme (Extended Data Fig. 3a, b). Specifically, the humanized *E. coli* carrying G6PD-Volendam, a class I G6PD variant associated with chronic hemolysis, grew at half the rate of the WT-expressing strain (Fig. 1f). Growth of the relatively mild deficiency variant, G6PD-Mahidol (class III) was also apparent (84% of WT, *p*-value of 1.412×10^{-4} ; Welch's *t*-test), indicating high dynamic range of the growth assay. Moreover, growth rates of humanized *E. coli* carrying different G6PD variants correlated linearly with biochemical properties of the corresponding recombinant enzymes (Pearson's R^2 of 0.84, Fig. 1f)^{25–33}. This result further illustrates LEICA's ability to rapidly screen human genetic variants.

Given the linear correlation observed, we sought to test less-characterized variants with no reported biochemical properties using LEICA. We constructed humanized *E. coli* strains carrying two predicted benign variants, one pathogenic variant, and one variant with uncertain clinical significance (Supplementary Table 2). LEICA detected marginal growth rate reductions in the two predicted benign variants (Fig. 1g and Extended Data Fig. 3b), indicating a mild decrease in enzyme activity. However, the association of these variants with disease should be considered carefully, as LEICA primarily screens for enzyme activity. If these mutations are revealed to be clinically associated with diseases, it may imply that pathogenicity arises from factors other than enzyme activity decrease, such as enzyme stability or expression level. Additionally, a variant with insufficient evidence to determine its role in disease (N426K) displayed similar results to the benign variants, suggesting N426K induces no significant enzyme deficiency.

In contrast, the G6PD-Sumare variant exhibited a notable reduction in growth (86% of WT, p -value of 6.736×10^{-5} , Welch's t -test), comparable to that of class III variants (A⁺, Mahidol, and Murcia) associated with mild anemia³⁴. Although the biochemical properties have never been studied, the results from LEICA, along with reduced blood G6PD activity in patients carrying this variation, indicate a possible association of the G6PD-Sumare variant with disease³⁵.

Incorporating G6PD and GPI into LEICA enables accurate measurement of catalytic activities of human enzymes in an intracellular environment. Unlike an *in vitro* assay, the bacterial host provides the necessary substrates and physiological conditions for enzyme activity. This approach allows for an *in vivo* enzyme assay by simple growth measurement, independent of specialized chemical and reporter systems. Human genetic disorders result from a complex interplay of biological factors, including variations in catalytic activity, expression levels, and zygosity, posing challenges in identifying causality. LEICA insulates enzyme activity from such biological factors by mimicking the homozygous state and providing stable expression levels, offering a rapid means of assessing only catalytic activity as the primary variable.

Small-molecules activate or inhibit glucose 6-phosphate dehydrogenase in humanized *E. coli*: Human G6PD is functionally active only in a dimer or tetramer, and some mutations have been shown to hamper oligomerization³⁶. A recently discovered small-molecule activator of G6PD, named AG1, promotes and stabilizes dimer formation^{13,15}. Thus, it activates G6PD mutants that have a reduced oligomerization state, e.g., G6PD-Canton^{13,15}. According to the previous report, AG1 improved activity of the G6PD-Canton variant up to 1.7-fold with half maximal effective concentration (EC₅₀) of 3.4 μ M¹⁵. It also increased basal activity of WT G6PD

by approximately 20%¹⁵. Thus, we examined the effect of AG1 using LEICA to determine if it could be used to screen for the effect of small molecules.

The effect of AG1 exposure on growth of *E. coli* carrying its endogenous *G6PD* (*E. coli* 20.71 Δ *pgi*) was examined. AG1 treatment showed no effect on growth, thus ruling out its effect on endogenous *E. coli* metabolism (Fig. 2). When humanized strains for G6PDs were examined, AG1 improved the growth rates of humanized *E. coli* expressing WT G6PD and Canton variant up to 12.8% and 20.1% at 0.3 μ M, respectively (Fig. 2a); a result that is consistent with the previous study (Supplementary Note 1)¹⁵. Activated G6PD-Canton supported growth of humanized *E. coli* better than untreated WT and there was no significant difference in the activities when maximally activated (*p*-value of 0.055; Welch's *t*-test, Fig. 2a). It is worth noting that the effective concentrations of AG1 needed in LEICA experiments were much lower than those of *in vitro* and cell-based screens¹⁵. The discrepancy of effective concentrations might have originated from different concentrations of reaction constituents (i.e., substrates, cofactors, and enzymes) in the *in vitro* reconstituted system and bioavailability of small molecules across bacterial and mammalian cell membranes. Nonetheless, LEICA for G6PD successfully reflected the known activation of human G6PD by small molecule activator AG1 without any specialized reporter-based assay system.

Given that LEICA can measure the effect of AG1 on human G6PD, we aimed to evaluate its ability to screen various compounds that target G6PD. As G6PD deficient patients face a high risk of hemolysis when exposed to certain drugs, specific foods, or infections³⁷, it is important to evaluate the impact of drugs on G6PD when devising treatment strategies for G6PD-deficient individuals. Additionally, drugs that act on G6PD are active areas of clinical research, as it is linked to antimalarials, immune response, and tumors^{38–41}.

Seven compounds with known effects on G6PD were chosen for screening (Supplementary Note 2). These include: (1) the local anesthetic proparacaine (PPC), (2) the non-selective inhibitor of NADP-dependent enzymes 6-aminonicotinamide (6AN), (3) the energetic compound, radiosensitizer RRx-001, (4) the α_2 adrenergic agonist brimonidine (BMN), (5) the steroid hormone dehydroepiandrosterone (DHEA), (6) the small molecule G6PD inhibitor G6PDi-1, and (7) the pyrazolonic analgesic metamizol (MMZ). Previous reports indicate that PPC has no significant effect on G6PD⁴², while G6PDi-1⁴¹, DHEA⁴³, BMN⁴², and MMZ⁴⁴ exhibit inhibitory effects. RRx-001 and 6AN are non-selective inhibitors^{45,46}, either generating reactive radicals or non-selectively inhibiting NADP-dependent enzymes. Each compound was supplemented to culture media at a concentration of 100 μ M and 10 μ g/ml tetracycline (Tc) was used as a positive control for bacterial growth inhibition.

LEICA treated with PPC exhibited no difference in growth profile compared to the untreated control (Fig. 2b and Extended Data Fig. 4), aligning with previous observations⁴². Treatment with Tc, RRx-001, and 6AN completely halted the growth of humanized *E. coli* for G6PD in LEICA (Fig. 2b). However, the growth of *E. coli* Δ pgi was also suppressed by RRx-001 and 6AN (Extended Data Fig. 5), indicating their toxicity to *E. coli* and prompting us to exclude them as hits.

The growth of humanized *E. coli* for G6PD (WT) was significantly inhibited by G6PDi-1, DHEA, and BMN (Fig. 2b,c and Extended Data Fig. 4), with no effect observed on *E. coli* Δ pgi (Extended Data Fig. 5), underscoring the inhibitory effect of these compounds on human G6PD, consistent with the previous reports^{41–43}. However, MMZ showed no discernible effect. This is likely attributable to the high inhibitory concentration of MMZ (IC₅₀ of 17 mM) required to inhibit G6PD activity⁴⁴, in contrast to G6PDi-1 and BMN, whose IC₅₀s are in the sub-millimolar range (0.07–30 μ M).

Two compounds—G6PDi-1 and BMN—which exhibited inhibitory effects in the primary screen, underwent further examination in assays with multiple doses to validate the screening results. We also included DHEA that has been reported to have varying G6PD inhibitory effect depending on the assay format. LEICA was performed with each compound at concentrations ranging from 5 to 300 μ M, revealing a dose-dependent response with IC₅₀ of 111, 138, and 1309 μ M, respectively (Fig. 2c-e). These IC₅₀ values differ from those obtained in *in vitro* assays where the IC₅₀ of G6PDi-1, DHEA, and BMN were reported as 0.07, 9, and 30 μ M, respectively^{41,42}. However, IC₅₀ values measured from LEICA are more closely aligned with results from cell-based assays, where the IC₅₀ value for G6PDi-1 ranged from 13 to 31 μ M depending on the cell type. Interestingly, DHEA exhibited an inhibitory effect in LEICA, contrary to the lack of inhibitory effect observed in cell-based assays⁴¹. Previous reports indicate the antiproliferative effects of DHEA on various cells may stem from indirect effects that are not fully understood⁴⁷. Thus, the behavior of LEICA in this context resembles that of a recombinant assay. Despite these discrepancies, LEICA effectively screened compounds with comparable IC₅₀ values, making it a suitable format for high-throughput screening applications.

Drug library screening identifies potential lead compounds for G6PD: Having demonstrated the sensitivity of LEICA with known compounds that modulate G6PD activity, we screened a drug compound library to identify new lead compounds with potential effects on G6PD. The screening involved two stages: (1) a single-dose growth assay using LEICA,

and (2) validation of primary hits through dose-response assays (Fig. 3a). Of the 160 human metabolism modulators screened, 7 compounds (primary hits) were identified that inhibited the growth of *E. coli* expressing human G6PD, while showing no effect on the strain with endogenous *E. coli* G6PD (Fig. 3b).

The screening successfully re-discovered G6PDi-1 as a primary hit, confirming the assay's robustness. Two other primary hits, methotrexate and gossypol, have also been previously linked to G6PD. Methotrexate, a dihydrofolate reductase inhibitor, has reported G6PD inhibitory effects (IC₅₀ of 114 µM)⁴⁸ and antimalarial activity⁴⁹. Gossypol, known for its antimalarial properties, inhibits oxidoreductases, including G6PD in *Trypanosoma cruzi*⁵⁰. Given that G6PDi-1 was originally discovered as an inhibitor of *T. cruzi* G6PD⁵¹, it logically follows that gossypol, with its reported effects on *T. cruzi*, might also inhibit human G6PD. These three previously known compounds detected in the screen validate the sensitivity and reliability of the assay.

The four remaining compounds have no previously reported association with G6PD activity; however, three completely inhibited the growth of *E. coli* expressing human G6PD. Further characterization using dose-response assays revealed reliable dose-dependent effects from all the primary hits (Fig 3c). Among the four newly identified compounds, three displayed higher potency with IC₅₀s ranging from 15.6 to 109 µM, compared to G6PDi-1, methotrexate, and gossypol (IC₅₀s of 111-148 µM). This result suggests that these newly identified compounds are candidate leads for novel antimalarial drugs as potent G6PD inhibitors. The drug screening demonstrates LEICA's potential as a versatile high-throughput screening tool capable of identifying compounds that influence human enzymes within a bacterial context.

Expanding LEICA's application for enzymes other than glycolysis: To broaden LEICA's applicability to enzymes outside glycolysis, we focused on argininosuccinate lyase (ASL), a critical enzyme in the urea cycle (Fig. 4a)⁵². Although *E. coli* does not have a urea cycle, it contains an ASL enzyme encoded by the *argH* gene, which is involved in arginine biosynthesis. Since knockout of *argH* results in arginine auxotrophy, it creates a suitable platform for assaying human ASL activity by growing cells in arginine-free conditions (Fig. 4b). We found that human ASL could complement the arginine auxotrophy in an *E. coli argH* knockout strain, indicating successful functional expression of human ASL in *E. coli* (Extended Data Fig. 6).

We then examined various ASL sequence variants that are known to cause argininosuccinic aciduria (ASA), with residual enzyme activity ranging from 0 to 6.09% (Supplementary Table

3) ^{12,52}. The humanized *E. coli* for ASL whose arginine biosynthesis is supported by different pathogenic ASL variants exhibited significant growth reductions (ranging from 16 to 100%) compared to cells expressing WT human ASL (Fig. 4c and Extended Data Fig. 7). Specifically, the growth rates of humanized *E. coli* aligned with the residual activities of recombinant ASL variants: D87G, which results in a complete loss of activity, could not complement the arginine auxotrophy, while the least severe R12Q variant supported growth to 84% of WT levels.

The ASL example demonstrates LEICA's versatility in screening enzymes from diverse metabolic pathways. Its expandability suggests LEICA could be used as a platform to study a wide range of human metabolic enzymes, further broadening the potential applications in identifying pathogenic sequence variations and therapeutic compounds for metabolic disorders.

DISCUSSION

High-throughput sequencing has revolutionized our understanding of the human genome, providing vast amounts of data on genetic variations. The mere knowledge of genetic sequences is insufficient, however; the key challenge lies in deciphering the functional significance of these variations, particularly those associated with genetic disorders. Genome-wide association studies (GWAS) have identified mutations linked to various disorders, but understanding the causality of these mutations remains a significant hurdle.

To address this challenge, we developed a live bacteria assay, named LEICA, to measure activities of heterologously expressed human enzymes. Unlike traditional *in vitro* assays and cell-based assays, this system leverages the metabolic capabilities of bacterial cells to rapidly characterize genetic variations associated with enzyme deficiencies. By coupling bacterial growth with enzyme activity, this approach provides a simpler and more cost-effective alternative to existing screening methods as it highly correlates with the activities reported from previous studies. A notable feature of LEICA is its ability to screen for pathogenic variations through the coupling of bacterial growth with enzyme activity. This alternative approach offers distinct advantages over traditional screening methods, by rapidly providing insights into the functional consequences of genetic variations.

Beyond its utility in studying genetic variations, LEICA proves effective for screening drug effects on human enzymes. By directly assessing the impact of various compounds on enzyme activity in an intracellular environment, it bridges the gap between *in vitro* assays and

cell-based assays. While offering advantages such as simplicity, speed, and cost-effectiveness, it also presents some limitations, such as its inability to screen certain compounds with antimicrobial properties. However, incorporating a pairwise comparison of the humanized *E. coli* with the strain carrying the endogenous bacterial gene effectively prevents false-positive calls, as demonstrated with 6AN treatment (Fig. 2b, 3b, and Extended Data Fig. 5). Also, we acknowledge that bacterial cells may have different permeability to chemical compounds compared to human cells, which could affect the assay's representation of drug efficacy in human cells. However, *E. coli* cells are non-selectively permeable to small molecules (<600 Da)⁵³, and LEICA's demonstrated robustness in identifying seven effective compounds from the drug library supports its reliability as a screening tool. Nonetheless, its ability to operate under intracellular conditions makes it a valuable approach for compound screening. Looking ahead, LEICA holds significant promise for high-throughput screening and personalized drug testing (Supplementary Note 3).

In conclusion, LEICA represents an alternative approach for studying genetic enzymopathies, screening drug effects, and advancing precision medical research. By leveraging the metabolic machinery of bacterial cells, LEICA offers insights into the functional consequences of genetic variations and facilitates the rapid screening of drug libraries. Moving forward, continued research and innovation in this field holds the potential to impact the identification of lead compounds for human targets that can be manifested in *E. coli*.

ONLINE METHODS

Bacterial strains and culture conditions

E. coli strain 20.71, which is an adaptively evolved strain with an in-frame substitution of its endogenous phosphoglucose isomerase with human homolog, was constructed in a previous study⁸. The *E. coli* 20.71 Δ *pgi* strain was constructed by substituting the human *GPI* with the *sacB*-cat dual-selection cassette using lambda recombination⁵⁴. The *E. coli* 20.71 Δ *pgi* Δ *zwf* double knockout strain was constructed by substituting the *zwf* gene with a kanamycin resistance cassette on the *E. coli* 20.71 Δ *pgi* background using lambda recombination. Humanized *E. coli* carrying either the wildtype (WT) GPI or GPI variants were constructed by introducing human WT GPI or variant genes into the double knockout strain using lambda recombination. Human GPI variants were constructed by assembling split human GPI fragments (upstream and downstream of the variation) amplified by primers containing genetic variations. Full GPI constructs were assembled using Overlap Extension PCR (OE PCR). Briefly, 5 ng each of the fragments were first ligated by 15 cycles of the following PCR reaction: 98°C for 30 s, 68°C for 30 s, and 72°C for 90 s. Ligated products were amplified in the same tube by adding two outermost primers (HsaGPI_F and HsaGPI_R) with 20 cycles of PCR reaction: 98°C for 30 s, 70°C for 30 s, and 72°C for 90 s. The *E. coli* Δ *argH* strain was constructed by substituting the *argH* of *E. coli* strain K-12 substrain MG1655 with the *npt-II* kanamycin resistance cassette (amplified from pKD13) using lambda recombination. The kanamycin cassette was subsequently removed by flippase-mediated recombination with a helper plasmid pCP20⁵⁴. Q5 High-Fidelity DNA Polymerase (NEB) was used to perform PCR as instructed by the manufacturer. Primer sequences are summarized in [Supplementary Table 4](#). Successful recombinants were screened by *sacB* counterselection on salt-free LB-agar plate (1% tryptone, 0.5% yeast extract, and 1.5% agar) containing 10% sucrose. Insertion and mutations of the human GPI gene were confirmed by Sanger sequencing. Cells were propagated in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl). For growth assays, M9 glucose medium (4 g/l glucose, 47.75 mM Na₂HPO₄, 22.04 mM KH₂PO₄, 8.56 mM NaCl, 18.70 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, and trace elements) was used. Trace elements were prepared in 2000× concentrated solution (100 mM FeCl₃, 9.54 mM ZnCl₂, 8.41 mM CoCl₂, 8.27 mM Na₂MoO₄, 0.75 mM CaCl₂, 0.91 mM CuCl₂, and 0.5 mM H₃BO₃ in 3.7% (w/w) hydrochloric acid solution). Kanamycin (50 µg/ml) was added when screening humanized *E. coli* for GPI. To screen humanized *E. coli* for G6PD, 50 µg/ml kanamycin, 100 µg/ml carbenicillin, and 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) were added to media. To screen humanized *E. coli* for ASL, 100 µg/ml carbenicillin and 0.01 mM IPTG were added to media. Growth profiles of *E. coli* strains were monitored using a Tecan Infinite 200 Pro microplate reader (Tecan). Cells were incubated in 96-well microplates at 37°C with a culture

volume of 100 μ l. Microplates were sealed with Breathe-Easy gas-permeable sealing membranes (Diversified Biotech). Optical density (A600nm) was monitored every 15-60 min.

Cloning G6PD variants and construction of humanized G6PD *E. coli* model

The human *G6PD* gene was chemically synthesized (IDT, sequence available in [Supplementary Note 4](#)) and cloned into the pTrcHis2A plasmid (Invitrogen) using Gibson assembly (NEBuilder HiFi DNA Assembly Kit, NEB). Briefly, 5 fmol pTrcHis2A plasmid backbone (linearized by PCR) and 20 fmol G6PD gene fragment were mixed in 6 μ l reaction followed by incubation at 50°C for 15 min. Different G6PD variants were constructed by assembling split human G6PD fragments (upstream and downstream of the variation) amplified by primers containing genetic variations. Full G6PD constructs were assembled by OE PCR as follows: 5 ng each of the fragments were ligated by 15 cycles of the following PCR reaction: 98°C for 30 s, 68°C for 30 s, and 72°C for 90 s. Ligated products were amplified in the same tube by adding two outermost primers (HsaG6PD_F and HsaG6PD_R) with 20 cycles of PCR reaction: 98°C for 30 s, 67°C for 30 s, and 72°C for 90 s. The assembled constructs were cloned into pTrcHis2A using the aforementioned method. Then, plasmid carrying each *G6PD* variant was introduced into the *E. coli* 20.71 Δ *pgi* Δ *zwf* double knockout strain to construct humanized G6PD *E. coli* model. Glycolytic flux was solely supported by the HMP shunt in *E. coli* 20.71 Δ *pgi* strain or 20.71 Δ *pgi* Δ *zwf* having human *G6PD*, which induces a slow growth rate, as reported elsewhere⁵⁵. Thus, we monitored the growth of these strains for up to 60 hrs. Primer sequences are summarized in [Supplementary Table 4](#).

Measuring effects of small molecules using LEICA

The G6PD activator AG1, RRx-001, brimonidine (BMN), 6-aminonicotinamide (6AN), proparacaine hydrochloride (PPC) were purchased from MedChemExpress (USA). Tetracycline hydrochloride, metamizole (dipyrone) monohydrate (MMZ), and G6PD Δ -1, were purchased from Sigma-Aldrich (USA). Dehydroepiandrosterone (DHEA) was bought from ApexBio (USA). RRx-001 was prepared as a 100 mM dimethyl sulfoxide (DMSO) solution. AG1, G6PD Δ -1, and DHEA were prepared as 30 mM DMSO solutions. BMN, PPC, MMZ, and 6AN were prepared as 10 mM aqueous solutions. Tetracycline was prepared as a 10 mg/ml aqueous solution. Compounds were treated at appropriate concentrations with a final DMSO concentration of 1%. DMSO was treated (1%) as an untreated vehicle control.

Drug library screening

Cellular Metabolism Screening Library (Cayman Chemical, Cat #33705, Batch #0609421) was used for drug library screening. 1.2 μ l of 10 mM compound solutions (DMSO) were dispensed to 96-well microplates. *E. coli* 20.71 Δ *pgi* and *E. coli* 20.71 Δ *pgi* Δ *zwf* carrying pTrc_G6PD-

WT was incubated in 3 ml LB medium containing 50 µg/ml kanamycin, 25 µg/ml chloramphenicol, and 1 mM IPTG. 100 µg/ml carbenicillin was added to cells carrying plasmid. 1 ml of overnight cultures were washed with 1 ml of M9 glucose medium to remove excess nutrients of LB. The washed resuspensions were inoculated into 3 ml fresh M9 glucose medium (with initial OD of 0.03) containing 50 µg/ml kanamycin, 25 µg/ml chloramphenicol, and 1 mM IPTG. 100 µg/ml carbenicillin was added to cells carrying plasmid. Then cultures were incubated at 37°C for 3 hrs and 120 µl of cultures were transferred to 96-well microplates having compounds resulting in 100 µM final concentration. Each plate comprises three replicated cultures of 15 compounds and vehicle control (1% DMSO) for both *E. coli* 20.71 Δpgi and *E. coli* 20.71 $\Delta pgi \Delta zwf$ carrying pTrc_G6PD-WT. Cell growth was monitored every 60 min in a BioTek LogPhase 600 microplate reader (Agilent; set at 37°C with 800 rpm shaking).

Cloning ASL variants and construction of humanized ASL *E. coli* model

The human ASL ORF clone was obtained from GenScript (Clone ID OHu22033, Acc. NM_001024943.2) and cloned into the pTrcHis2A plasmid using Gibson assembly. Briefly, 5 fmol pTrcHis2A plasmid backbone (linearized by PCR) and 20 fmol ASL gene fragments were mixed in 6 µl reaction followed by incubation at 50°C for 15 min. Different ASL variants were constructed by assembling split human ASL fragments (upstream and downstream of the variation) amplified by primers containing genetic variations. Full ASL constructs were assembled by OE PCR as follows: 5 ng each of the fragments were ligated by 15 cycles of the following PCR reaction: 98°C for 30 s, 68°C for 30 s, and 72°C for 90 s. Ligated products were amplified in the same tube by adding two outermost primers (HsaASL_F and HsaASL_R) with 20 cycles of PCR reaction: 98°C for 30 s, 67°C for 30 s, and 72°C for 90 s. The assembled constructs were cloned into pTrcHis2A using the aforementioned method. Primer sequences are summarized in [Supplementary Table 4](#).

Statistical analysis

To compare the difference of means, a two-sided Welch's *t*-test was used with Bonferroni correction for multiple hypothesis testing. Dose-response curve was fitted to the Hill curve to estimate EC₅₀ or IC₅₀. For drug library screening, 15 compounds were first compared with untreated control using a two-sided Welch's *t*-test corrected for multiple hypothesis testing using Bonferroni method. Compounds that induced a significant growth difference (*p*-value < 0.001) in *E. coli* 20.71 $\Delta pgi \Delta zwf$ carrying pTrc_G6PD-WT strain, while not inducing a significant difference (*p*-value ≥ 0.001) for *E. coli* 20.71 Δpgi were selected as primary hits.

DATA AVAILABILITY

The main data supporting the results in this study are available within the paper and its Supplementary Information. Source data are provided with this paper. All compounds in the drug library (n=160) are anonymized using proxy identifiers. For transparency, the actual chemical names of all compounds have been provided to the journal's editorial team in a separate document. Researchers interested in reproducing or building on this work may contact the corresponding author to request access to the compound names and structures, which will be made available under a confidentiality agreement to support ongoing research and development efforts. Full disclosure of the identity of the candidate lead compounds is deferred to a later date.

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AUTHOR CONTRIBUTIONS

B. O. P. conceived and supervised the study. D. C. and B. O. P. designed the experiments. D. C. performed the experiments. D. C. and B. O. P. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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FIGURES LEGENDS

Figure 1. A bacterial system represents activities of human enzymes by growth rate. **a**,

Schematic representation of the bacterial system used for the screening of activities of genetic variants of human metabolic enzymes. **b**, Growth of the *E. coli* phosphoglucose isomerase (*pgi*) and glucose-6-phosphate dehydrogenase (*zwf*) double-knockout strain is dependent on human glucose-6-phosphate isomerase (GPI) activity enabling a live *Escherichia coli* assay (LEICA). Growth rate of the *E. coli* 20.71 $\Delta pgi \Delta zwf$ strain carrying human GPI (humanized *E. coli* for GPI) reflects activity of the human enzyme. EMPP: the Embden-Meyerhof-Parnas pathway. HMP shunt: hexose monophosphate shunt. G6P: glucose-6-phosphate. 6PG: 6-phosphogluconate. F6P: fructose-6-phosphate. FBP: fructose-1,6-bisphosphate. TCA cycle: tricarboxylic acid cycle. **c**, Activities of human GPI variants were represented by growth rates of the humanized *E. coli* for GPI. Data are presented as mean values $\pm$ SD. Error bars indicate SD of ten replicated cultures. Numbers are relative differences of growth rates compared to the wild-type (WT) enzyme; in percentage. ns, no significant difference. **p*-value = 0.022. ***p*-value < 0.005 (two-sided Welch's *t*-test with Bonferroni correction). Circles indicate ten independent cultures. **d**, Growth rates of the *E. coli* with human gene swap shows high linear correlation with previously reported activities of recombinant enzymes (Pearson's R^2 of 0.96; dashed line)¹⁴. Data are presented as mean values $\pm$ SD. Error bars indicate SD of biological replicates (*n*=10). Specific activities of recombinant enzymes were pulled from a previous report¹⁴. **e**, Growth of the double knockout strain expressing human G6PD (*E. coli* $\Delta pgi \Delta zwf$ + pTrc_human G6PD; humanized *E. coli* for G6PD) is governed by activity of the human G6PD. **f**, Growth rates of humanized *E. coli* for G6PD and catalytic constants (k_{cat}) of recombinant enzymes^{25–30} had high correlation (Pearson's R^2 of 0.84; dashed linear regression line). Data are presented as mean values $\pm$ SD. Error bars indicate SD of five replicated cultures. WT: wild-type. For G6PD-Volendam, the specific activity was used³¹, because k_{cat} is not available. **g**, Activities of a few less-characterized human G6PD variants were represented by growth rates of the humanized *E. coli*. Data are presented as mean values $\pm$ SD. Error bars indicate SD of five replicated cultures. Circles indicate five independent data points.

Figure 2. Effect of small molecules on glucose 6-phosphate dehydrogenase. **a**, Effect of

a G6PD activator AG1 on growth rate of humanized *E. coli*. Half-maximal effective concentration (EC_{50}) is calculated from the dose-response curve (dashed lines). Circles are individual data points (*n*=10). **b**, Growth rates of humanized *E. coli* in LEICA with different compounds. Vehicle: untreated control (1% DMSO). Tc: tetracycline. PPC: proparacaine. 6AN: 6-aminonicotinamide. RRx-001: bromonitroimidazole. BMN: brimonidine. DHEA:

dehydroepiandrosterone. G6PDi-1: inhibitor of G6PD 1. MMZ: metamizol. All compounds were treated with the final concentration of 100 μ M, except for tetracycline (10 μ g/ml). ND: no observable growth was detected. Data are presented as mean values $\pm$ SD. Error bars show SD of three replicates. Dots are individual data points. **ns: no significant difference. * p -value = 0.025 (BMN) and 0.0135 (G6PDi-1)** (two-sided Welch's t -test with Bonferroni correction, compared to the untreated control). **c-e**, Dose-response validation of inhibitory effect of three primary hits G6PDi-1 (**c**), DHEA (**d**), and brimonidine (**e**). Half-maximal inhibitory concentration (IC_{50}) was calculated from a Hill curve (four parameters; dashed line) fitted to the dose response curve. Dots are individual data points ($n=5$). * p -value < 0.05. ** p -value < 0.005. *** p -value < 0.001 (two-sided Welch's t -test with Bonferroni correction, compared to the untreated control).

Figure 3. Drug library screening using LEICA. a, Various compounds are supplemented to LEICA and growth is monitored using a plate reader. Change in G6PD activity in response to chemical compounds is reported as the growth difference of the humanized *E. coli* at a fixed concentration compared to untreated control (primary hits). Primary hits are further validated by dose-response assay, resulting in discovery of lead compounds. **b**, Growth rates of *E. coli* expressing human G6PD compared to that of *E. coli* expressing endogenous G6PD. Seven compounds induced significant (p -value < 0.001; two-sided Welch's t -test, compared to the untreated control) growth difference, while inducing no significant difference on *E. coli* expressing endogenous G6PD. All compounds were treated with the final concentration of 100 μ M. Data are presented as mean values $\pm$ SD. Error bars show SD of three replicates. **c**, Effect of primary hit compounds at different concentrations on humanized *E. coli* for G6PD WT. Half-maximal effective concentration (IC_{50}) is calculated from the dose-response curve (dashed lines). Circles are individual data points ($n=5$).

Figure 4. Expanding LEICA for screening human argininosuccinate lyase (ASL). a, Schematic representation of the human urea cycle. BC: bicarbonate. CP: carbamoyl phosphate. CIT: citrulline. ASA: argininosuccinate. ORN: ornithine. Fum: fumarate. **b**, Arginine auxotrophy induced by *argH* knockout is complemented by human argininosuccinate lyase (ASL). **c**, Growth rates of wild-type (WT) *E. coli*, *argH* knockout strain, and *argH* knockout strain expressing WT and variants of human ASL. *in vitro* activities of the recombinant enzymes^{12,52} are indicated above. ND: no observable growth was detected. Data are presented as mean values $\pm$ SD. Error bars show SD of five replicates. Dots are individual

742 data points. *** p -value < 0.001 (two-sided Welch's t -test with Bonferroni correction, compared
743 to the WT).
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EXTENDED DATA FIGURES

Extended Data Figure 1. Growth profiles of *E. coli* carrying human glucose-6-phosphate isomerase (GPI) or its mutants in M9 glucose medium. WT: wild-type human GPI. Data are presented as mean values $\pm$ SD. Error bars indicate SD of ten replicated cultures.

Extended Data Figure 2. Growth of *E. coli* lacking phosphoglucose isomerase (*pgi*) or glucose-6-phosphate dehydrogenase (*zwf*) in M9 glucose medium. **a**, Growth profiles of *E. coli* Δpgi and $\Delta pgi \Delta zwf$ strains in M9 glucose medium. Human glucose-6-phosphate dehydrogenase (G6PD) rescued glucose utilization of $\Delta pgi \Delta zwf$ knockout strain. Data are presented as mean values $\pm$ SD. Error bars show SD of five replicates. **b**, Growth rates of Δpgi and $\Delta pgi \Delta zwf$ strains. *E. coli* utilizing glucose with its endogenous G6PD (*Zwf*; *E. coli* Δpgi) or human G6PD exhibited no significant difference in growth rate. Data are presented as mean values $\pm$ SD. Error bars show SD of five replicates. ND, no growth was detected. ns, no significant statistical difference (p -value = 0.346, two-tailed Welch's t -test). Dots represent individual data points.

Extended Data Figure 3. LEICA monitors activities of G6PD variants as growth rates are contingent on G6PD activity. **a**, Growth profiles of humanized *E. coli* for G6PD expressing well-characterized variants. **b**, Growth profiles of humanized *E. coli* for G6PD expressing or less-characterized variants. Data are presented as mean values $\pm$ SD. Error bars show SD of five replicates.

Extended Data Figure 4. Effect of various compounds on humanized *E. coli* for G6PD. Growth profiles of humanized *E. coli* for G6PD (*E. coli* 20.71 $\Delta pgi \Delta zwf$ + *HsaG6PD*) strain under different drug treatments. veh: untreated control (1% DMSO). Tc: tetracycline. PPC: proparacaine. 6AN: 6-aminonicotinamide. RRx-001: bromonitroizidine. BMN: brimonidine. DHEA: dehydroepiandrosterone. G6PD Δ 1: inhibitor of G6PD 1. MMZ: metamizol. All compounds were treated with the final concentration of 100 μ M, except for tetracycline (10 μ g/ml). Data are presented as mean values $\pm$ SD. Error bars show SD of three replicates.

Extended Data Figure 5. Effect of various compounds on *E. coli*. **a**, Growth profiles of *E. coli* *pgi* knockout strain under different drug treatments. veh: untreated control (1% DMSO). Tc: tetracycline. PPC: proparacaine. 6AN: 6-aminonicotinamide. RRx-001: bromonitroizidine. BMN: brimonidine. DHEA: dehydroepiandrosterone. G6PD Δ 1: inhibitor of G6PD 1. MMZ: metamizol. All compounds were treated with the final concentration of 100 μ M, except for tetracycline (10 μ g/ml). Data are presented as mean values $\pm$ SD. Error bars show SD of three replicates. **b**, Growth rates of the strain treated with the treatments. Data are presented

as mean values $\pm$ SD. Error bars show SD of three replicates. Dots represent individual data points. No significant difference in growth rate was observed (two-tailed Welch's *t*-test with Bonferroni correction).

Extended Data Figure 6. Growth profiles of wild-type (WT) *E. coli*, *argH* knockout strain, and *argH* knockout strain expressing WT and variants of human ASL. Data are presented as mean values $\pm$ SD. Error bars show SD of five replicates.

Point by point response to reviewers

Reviewer #1

Summary: The impact of the work has been significantly enhanced through the extensive inclusion of the following findings:

- Identification of seven potential lead compounds for G6PD inhibition within a 160-member drug library, with four of the hits being novel discoveries.
- Analysis of four less-characterized G6PD variants with no previously reported biochemical properties.
- Demonstration of a linear correlation between the growth rates of humanized *E. coli* expressing G6PD and catalytic constants (k_{cat}) for 13 additional well-characterized variants.
- Expansion of LEICA beyond glycolysis to screen the activity of human argininosuccinate lyase (ASL).

The revised manuscript now more effectively highlights the translational potential of this straightforward and cost-effective method for screening genetic variants within a real clinical patient pool.

The analytical limitations of the method are now more thoroughly addressed, with a detailed comparison to other gold-standard methods. The authors have clarified the specific application of LEICA for investigating enzyme activity independently of other pathogenic factors, emphasizing both its advantages and drawbacks.

All inquiries were carefully addressed and clarified, and all stylistic suggestions were implemented.

Optional comment: Regarding the drug library screening, did you encounter any false-negative results? Specifically, were there compounds known to inhibit G6PD but not identified as such by LEICA?

Response: We appreciate the reviewer's efforts and constructive suggestions in the review process, which helped improve the manuscript.

Regarding the question on false-negatives: Among the 160 compounds tested, we did encounter any compounds previously reported to inhibit human G6PD that failed to be identified by LEICA.

Reviewer #2

All of my concerns have been thoroughly addressed, and I now recommend acceptance.

Response: We sincerely appreciate the reviewer's feedback throughout the review process.

We are glad that our revisions have addressed your concerns, and we thank you for your recommendation for acceptance.

Reviewer #3

Since the initial submission, the authors have performed additional experiments and have made significant additions to the manuscript. In addition to expanding the work related to glycolytic enzymes, argininosuccinate lyase (ASL), an essential urea cycle enzyme, was also studied in the LEICA system. Interestingly, humanized *E. coli* expressing pathogenic ASL variants showed markedly reduced growth

rates compared to wild-type cells. I appreciate this addition, as the LEICA method has now shown applicability to metabolic pathways related to amino acid metabolism. As there are numerous biochemical genetic disorders related to impaired to the metabolism of amino acids (and of course other substrates), I look forward to following the results of this screening assay as it relates to an expanding number of conditions.

Response: Thank you for your encouraging and insightful feedback. We are glad that the additional experiments strengthened the manuscript. Your recognition of LEICA's potential to be applied to a wider range of metabolic pathways and genetic disorders is greatly appreciated. We share your enthusiasm for expanding this platform and look forward to future developments in this area.