Published in final edited form as:

Methods Mol Biol. 2013 ; 978: 31–44. doi:10.1007/978-1-62703-293-3_3.

GFP Reporter Screens for the Engineering of Amino Acid Degrading Enzymes from Libraries Expressed in Bacteria

Olga Paley, Giulia Agnello, Jason Cantor, Tae Hyun Yoo, George Georgiou, and Everett Stone

Abstract

There is significant interest in engineering human amino acid degrading enzymes as non-immunogenic chemotherapeutic agents. We describe a high-throughput fluorescence activated cell sorting (FACS) assay for detecting the catalytic activity of amino acid degrading enzymes in bacteria, at the single cell level. This assay relies on coupling the synthesis of the GFP reporter to the catalytic activity of the desired amino acid degrading enzyme in an appropriate *E. coli* genetic background. The method described here allows facile screening of much larger libraries (10^6 – 10^7) than was previously possible. We demonstrate the application of this technique in the screening of libraries of bacterial and human asparaginases and also for the catalytic optimization of an engineered human methionine gamma lyase.

Keywords

Amino acid auxotrophs; GFP reporter; Enzyme engineering

1. Introduction

The screening of large libraries of enzyme variants for improved or altered catalytic function generally presents a daunting challenge. Unlike the screening of protein libraries for ligand binding, which can be accomplished using the same assay format regardless of the identity of the ligand, enzymes catalyze a plethora of reactions which in turn necessitates the use of specific individualized activity assays. Many, if not most, enzyme-catalyzed reactions are not easily amenable to high-throughput assays. This in turn limits the size of the sequence space that can be practically sampled in directed evolution experiments (1).

Many tumors are known to exhibit defects either in amino acid biosynthesis or alternatively to have a very high demand for certain amino acids relative to non-malignant tissues. Additionally tumor cells exhibit deregulation of stress responses that normally protect cells from the effects of nutrient starvation (2). The use of enzymes as a means for systemically depleting particular amino acids to restrict tumor growth with minimal toxicities has been under investigation for over 50 years and has resulted in significant clinical successes. Because of the paucity of human enzymes that display sufficient catalytic and pharmacological properties (e.g., serum stability and distribution, catalytic selectivity, ease of expression in heterologous hosts) until very recently amino acid depletion therapies in cancer have relied almost exclusively on the use of heterologous enzymes. However, heterologous enzymes are immunogenic leading to the elicitation of antibody responses that at best result in the neutralization of enzyme therapeutic function and at the worst, induce hypersensitivity and anaphylactic shock.

In recent years we have demonstrated the use of protein engineering strategies to develop human enzymes with the requisite properties for cancer therapy (3–6). Bacterial L-asparaginases so far constitute the only approved enzymes for amino acid depletion therapy and have been shown to dramatically enhance the response rate to combination chemotherapy in childhood acute lymphoblastic leukemia (7). However, immunogenicity of the bacterial enzymes is still an issue for a considerable fraction of patients that display hypersensitivity and for the treatment of patients that relapse, underscoring the need for a non-immunogenic human enzyme alternative. The enzymatic depletion of L-Methionine has been investigated for years but the bacterial enzyme is highly immunogenic in primates (8). As with L-asparaginase the engineering of a human L-methionine- γ -lyase (MGL) is of significant therapeutic interest, yet no human enzymes with these catalytic activities exist.

High-throughput screens for L-asparaginase and MGL activities can greatly aid the engineering of therapeutic enzymes by directed evolution of human enzymes that catalyze similar reactions. The development of such screens necessitates the engineering of unique *E. coli* strains that are auxotrophic for either L-aspartate (L-Asp) or α -ketobutyrate (a metabolite required for the synthesis of L-isoleucine (L-Ile)). These auxotrophies can then be rescued by the enzymatic hydrolysis of L-Asn to L-Asp or degradation of L-Met to produce α -ketobutyrate, respectively. In turn, these reactions can complement the auxotrophic strain enabling the synthesis of GFP. Thus a very active enzyme allows GFP to be translated at levels commensurate with nonselective conditions, whereas a less active or inactive enzyme results in reduced GFP expression due to the depleted amino acid pool in the auxotroph. Fluorescence activated cell sorting (FACS) can then be used to isolate individual clones based on their fluorescence profile from large libraries of enzyme variants expressed in *E. coli*. Employing several rounds of FACS sorting we can attain thousands of fold enrichment of clones with desired activity and then use a secondary assay method to analyze a handful of clones for the rank ordering of enzyme variants and assessment of kinetics. Figure 1 shows the general scheme and work flow of the method.

A high-throughput FACS screen for the activity of *E. coli* L-Asparaginase was reported recently (9). Here we demonstrate that this assay can be applied to the directed evolution of the human asparaginase-like protein 1 (hASRGL1), an enzyme that has β -aspartyl peptidase activity but also exhibits a low level of L-Asparaginase activity (10). The activity of hASRGL1 for L-Asn hydrolysis is about three orders of magnitude lower than the *E. coli* L-Asparaginase II enzyme approved for therapy. Briefly, *E. coli* BL21(DE3) [$\Delta aspC \Delta tyrB \Delta ansA \Delta ansB \Delta iaaA$] cells auxotrophic for L-Asn and stably transformed with a plasmid expressing GFP under control of an arabinose-inducible promoter are transformed with either parental hASRGL-1 or a mutagenized library of hAS-RGL-1 variants. Cells are grown and allowed to express hASRGL-1 or hASRGL-1 variants under nonselective conditions. The cells are then shifted to selective conditions concomitant with induction of GFP synthesis. Thus intracellular fluorescence is dependent on the availability of L-Asp, which in turn is proportional to the activity of the enzyme variant hydrolyzing L-Asn.

As a second example, we describe the application of our method for screening MGL activity. The non-mammalian enzyme MGL catalyses the α , γ -elimination of L-methionine to methanethiol, α -ketobutyrate, and ammonia. MGL from *P. putida* has shown efficacy in controlling tumor growth in a variety of xenograft models (11–18) but unfortunately has proven to be highly immunogenic in primates (8). Recently we employed rational design and scanning saturation mutagenesis to convert the structurally related human enzyme cystathionine- γ -lyase (CGL) into a human L-Methionine γ -lyase (MGL) that catalyzes the α , γ -elimination of L-Methionine to methanethiol, α -ketobutyrate, and ammonia but does not accept L-Met as a substrate. The assay available to screen this class of enzymes is a 96-well plate colorimetric assay for α -ketobutyrate production. *E. coli* produces α -ketobutyrate as an

intermediate metabolite in the de novo production of L-isoleucine (L-Ile) by action of threonine deaminase (*ilvA*). We thus deleted the *ilvA* gene to create an L-Ile auxotroph that could be rescued by α -ketobutyrate produced from L-Met degradation by MGL (Fig. 2a). Additionally, because CGL can also produce α -ketobutyrate from L-cystathionine and L-homocysteine which are metabolites in the *E. coli* pathway for L-Met production, we also deleted the *metA* gene (Fig. 2b).

2. Materials

2.1. Asparaginase Screen

Unless otherwise stated *E. coli* plates and cultures are incubated at 37°C.

1. *E. coli* strain BL21(DE3) $\Delta aspC \Delta tyrB \Delta ansA \Delta ansB \Delta iaaA$. This strain has deletions of the three native asparaginases and two aminotransferases that can produce L-Asp which for the sake of brevity we have named *E. coli D-aux* to reflect L-Asp auxotrophy (Fig. 3).
2. *Competent cells*: *E. coli D-aux* harboring pBAD-GFP prepared by an electrocompetent method (19).
3. Arabinose-inducible pBAD-GFP construct with a chloramphenicol marker as a reporter plasmid.
4. Isopropyl- β -D-1-thiogalactopyranoside (IPTG) inducible pET28a with a kanamycin marker as an expression plasmid for parental hASRGL-1 and libraries.
5. *Nonselective medium*: Medium supplemented with all amino acids; 1 $\times$ M9 media supplemented with 0.4% glucose, 3.5 μ g/mL thiamine, 1 mM MgSO₄, 0.1 mM CaCl₂, 160 μ g/mL of the amino acid L-Asp, 80 μ g/mL of the remaining 19 amino acids, 50 μ g/mL kanamycin, and 34 μ g/mL chloramphenicol. The nonselective medium can be supplemented with IPTG (see Note 1).
6. *Selective medium*: Medium deprived of L-Aspartate; 1 $\times$ M9 supplemented with 2% arabinose, 1% glycerol, 3.5 μ g/mL thiamine, 1 mM MgSO₄, 0.1 mM CaCl₂, no L-Asp, 5 μ g/mL L-Asn, 80 μ g/mL of the remaining 18 amino acids, 50 μ g/mL kanamycin, and 34 μ g/mL chloramphenicol.
7. *General medium*: 2 $\times$ YT supplemented with 50 μ g/mL kanamycin and 34 μ g/mL chloramphenicol.
8. *Plates*: 2 $\times$ YT agar supplemented with 50 μ g/mL kanamycin and 34 μ g/mL chloramphenicol.
9. Filter-sterilized 0.9% NaCl (4°C).
10. Filter-sterilized 1 $\times$ phosphate buffered saline at 4°C (PBS).
11. Filter-sterilized solution of 4 g/L L-Asp in water.
12. Filter-sterilized solution (1 M) of IPTG.
13. Culture tubes (5 mL).
14. Falcon tubes (15 mL).

¹The amount of enzyme required to rescue the auxotrophy depends on the catalytic activity and level of “leaky” expression of hASRGL1 and hASRGL1 libraries. In the case of the parental enzyme leaky expression is sufficient for the analyses. For a poorly expressed enzyme one can supplement with IPTG.

15. For flow cytometric analyses, a FACSAria instrument (BD Biosciences) with a 488-nm solid state laser for the excitation of GFP. A 530/30 band pass filter is used for the detection.
16. FACS tubes (BD Biosciences).

2.2. Methionine-γ-Lyase Screen

1. *E. coli* strain BL21(DE3) $\Delta ilvA\Delta metA$. For the sake of brevity this strain was named *E. coli MI-aux* to reflect its dual L-Met and L-Ile auxotrophies (Fig. 2).
2. *Competent cells*: *E. coli MI-aux* harboring pBAD-GFP prepared by an electrocompetent method (19).
3. Arabinose-inducible pBAD-GFP with a chloramphenicol acetyltransferase marker as a reporter plasmid.
4. IPTG-inducible pET28a with a kanamycin resistance marker as an expression plasmid for parental hCGL and pMGL.
5. *Nonselective medium*: Enzyme expression medium; M9 minimal medium supplemented with 0.4% glucose, 1 mM MgSO₄, 0.1 mM CaCl₂, 150 µg/mL of the amino acids L-Met and L-Ile, 75 µg/mL of the remaining amino acids excluding L-Leu and L-Val (see Note 6), 50 µg/mL kanamycin, and 34 µg/mL chloramphenicol.
6. *Selective medium*: GFP Expression Medium: M9 medium supplemented with 0.4% glucose, 1 mM MgSO₄, 0.1 mM CaCl₂, 150 µg/mL of L-Met, 75 µg/mL of the remaining amino acids (excluding L-Ile, L-Leu and L-Val), 50 µg/mL kanamycin, 34 µg/mL chloramphenicol, and 2% arabinose.
7. *General medium*: LB supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol.
8. *Plates*: 2xYT agar supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol.
9. Filter-sterilized 0.9% NaCl (4°C).
10. Filter-sterilized 1× PBS at 4°C.
11. Filter-sterilized solution of L-Ile (250 mM) in PBS.
12. Filter-sterilized solution (1 M) of IPTG.
13. Culture tubes (5 mL).
14. Falcon tubes (15 mL).
15. A FACSAria instrument (BD Biosciences) with a 488-nm solid state laser for the excitation of GFP. A 530/30 band pass filter was used for the detection.
16. FACS tubes (BD Biosciences).

⁶L-Val and L-Ile are excluded from the nonselective growth medium and selective growth medium because these branched-chain amino acids are known to inhibit the L-Ile biosynthesis pathway (21, 22). The addition of these two amino acids to the selective growth media without L-Ile in our experiments resulted in undetectable GFP expression in all variants tested.

3. Methods

3.1. Asparaginase Screen

Use your laboratories' favorite methods for library construction, competent cells, transformations, etc.

3.1.1. Cell Growth and GFP Expression—

1. Grow an overnight culture (5 mL) of *E. coli D-aux* cells co-transformed with pBAD-GFP and either a library or a single mutant in 2xYT (see Note 2).
2. Subculture the overnight culture into 10 mL nonselective medium supplemented with all the amino acids to a final OD₆₀₀ of 0.1. (To perform the FACS analysis of one sample (i.e., a round of a library or a single mutant) 10 mL of nonselective medium is sufficient).
3. Grow cells at 37°C with shaking at 250 rpm until OD₆₀₀ ~0.9–1.1.
4. Harvest cells by centrifugation of 1 mL aliquots of culture in 1.5 Eppendorf tubes at 13,000 × g for 5 min at 4°C. For each sample spin down at least 3 mL for subsequent positive control (step 8).
5. Re-suspend the pellets in cold 0.9% NaCl (by vortexing at low speed) and centrifuge at 13,000 × g for 5 min at 4°C. Use 1 mL of 0.9% NaCl per mL of culture harvested.
6. Repeat the wash with cold 0.9% NaCl (step 5) another three times (see Note 3).
7. Media shift: re-suspend cell pellets into selective media deprived of L-Aspartate. Use 1 mL of medium per tube.
8. Transfer culture into two separate 5 mL culture tubes (1 mL per tube): one sample tube and one positive control tube. In the positive control tube add L-aspartate to a final concentration of 160 µg/mL.
9. Shake the sample and positive control tubes at 250 rpm and 37°C for 2 h to induce the expression of GFP (see Note 4).
10. Harvest the sample and positive control cultures by centrifugation at 13,000 × g for 5 min at 4°C.
11. Re-suspend the sample and positive control pellets in cold PBS (by vortexing at low speed) (use 1 mL of PBS per tube) and centrifuge at 13,000 × g for 5 min at 4°C.
12. Repeat the wash with 1 mL cold PBS (step 11) another two times (see Note 5).
13. Re-suspend cell pellets in 1 mL cold PBS then dilute the positive control and the sample by 50–100-fold for flow cytometric analysis and cell sorting.

²We find it is easiest to prepare competent cells harboring the reporter plasmid pBAD-GFP and then transform with the expression plasmids of interest. For preparing highly competent *E. coli* cells we use an electrocompetent transformation method described previously (19).

³The multiple wash steps with 0.9% NaCl ensure that L-Asp is removed from the media.

⁴In the positive control the expression of GFP will be independent from the enzymatic activity, as the medium is nonselective and supplemented with L-Aspartate. In the sample the expression of GFP will be dependent on the hASRGL-1 asparaginase activity, as the medium is selective and deprived of L-Aspartate. A negative control can also be added using an inactive enzyme variant to assess background levels of GFP expression.

⁵The multiple wash steps with PBS ensure removal of the selective expression media for the subsequent cytometric analysis.

3.1.2. Cytometric Analysis FACS screening—The instructions reported here describe the *and Cell Sorting* procedure for a single sample (either a round of a library or a single mutant). For multiple samples adjust the volumes reported accordingly to the number of samples to analyze.

1. Adjust the throughput rate of cells to 4,000–5,000 events per second. Set a gate in the fluorescence channel to recover the 2% most highly fluorescent cells. Select the purity mode for the initial sort of each library, and then switch to the single cell mode and sort $\sim 10^7$ cells for each round.
2. Collect each round of sorting in 0.5 mL 2xYT medium and then spread onto 2xYT agar plates supplemented with 50 $\mu\text{g}/\text{mL}$ kanamycin and 34 $\mu\text{g}/\text{mL}$ chloramphenicol.
3. After overnight growth at 30°C, pool the clones and store them in 15% glycerol aliquots at –80°C.

3.1.3. Secondary Screening and Analysis—

1. Following the final round of sorting use the colorimetric asparaginase 96 wells plate assay using *L*-aspartic-acid- β -hydroxamate to isolate active hASRGL1 clones, as previously reported (9).
2. After rank ordering, select two or three clones for sequence verification.
3. Express, purify enzyme variants, and analyze rates of *L*-Asn hydrolysis by HPLC assay to determine steady-state kinetic parameters (10).
4. Performing this asparaginase FACS screening method on an error-prone library of hASRGL-1 variants resulted in a $\sim$ eight-fold increase in GFP signal after seven rounds of sorting (Fig. 4). A novel variant isolated from the screen had a corresponding twofold increase in k_{cat}/K_M ($\text{M}^{-1} \text{s}^{-1}$) for *L*-Asn hydrolysis relative to wt hASRGL-1.

3.2. Methionine- γ -Lyase Screen

Use your laboratories favorite methods for library construction, competent cells, transformations, etc.

3.2.1. Cell Growth and GFP Expression—

1. Grow an overnight culture (5 mL) of *E. coli* BL21(*MI-aux*) co-transformed with pBAD-GFP and plasmids either a library or a single mutant in 2xYT (see Note 2).
2. Inoculate 1 mL of *nonselective* media in a sterile culture tube with *E. coli* BL21(*MI-aux*) cells harboring plasmids of pBAD-GFP reporter and pET28a containing gene of interest to a final OD₆₀₀ of 0.1. The cell source for the inoculation can be previously frozen aliquots or overnight cultures of either a single mutant or a library of variants. Make sure to include tubes for a positive and negative control (1 mL each) for each round if planning to sort a library.
3. Grow cultures with shaking at 250 rpm at 37°C to an OD₆₀₀ of 0.3–0.4.
4. Add 1 M IPTG solution to a final concentration of 1 mM and shift the cultures to 25°C for 2 h with shaking at 250 rpm for enzyme expression (see Note 7).

⁷Some enzymes express better at lower temperatures thus this parameter can be adjusted on a case-by-case basis.

5. Harvest the cells by centrifugation ($4,000 \times g$, 3 min, 4°C). Wash the cells twice with cold 0.9% NaCl by re-suspending and centrifuging at $4,000 \times g$, for 3 min at 4°C (see Note 8). Use sterile 1.5 mL Eppendorf tubes to harvest the cells and at least 1 mL of the 0.9% NaCl solution for each wash.
6. Re-suspend the washed cells in 1 mL of GFP Expression Media (containing arabinose inducer for GFP expression). Split each 1 mL sample into two small culture tubes (500 μL per tube) and add 250 mM L-Ile solution to a final concentration of 1 mM in one of the two tubes. This will serve as a control for each sample (see Note 9).
7. Continue to grow at 25°C for an additional 2 h (shake at 250 rpm).
8. Harvest the cells by centrifugation ($4,000 \times g$, 3 min) and resuspend in PBS to a final OD_{600} ~0.05–0.1 for flow cytometric analysis and cell sorting.

3.2.2. Cytometric Analysis and Cell Sorting

1. Perform flow cytometric analysis with a FACS Aria (BD Biosciences) or an equivalent system. Adjust the throughput rate of cells to 4,000–5,000 events per second.
2. Perform all rounds of sorting in single cell mode, except for the initial sort, which should be in purity mode.
3. Set a gate in the fluorescence channel to recover the 4–5% most highly fluorescent cells. Additionally, set gates based on both the forward- and side-scatter channels to exclude sorting non-single cell events (see Note 10).
4. Sort tenfold the number of cells expected to cover the diversity for each round.
5. Collect the sorted cells in 0.5 mL of 2xYT medium and plate onto pre-warmed 2xYT medium supplemented with 50 $\mu\text{g}/\text{mL}$ kanamycin and 34 $\mu\text{g}/\text{mL}$ chloramphenicol (see Note 11).
6. Following overnight growth at 30°C , pool the clones and store aliquots in 15% glycerol at -80°C . At this step, you may also use the pool to inoculate the next round.

3.2.3. Enrichment—

1. Inoculate two culture tubes of *nonselective* medium with *E. coli* *Experiment with Controls* BL21(*MI-aux*) containing pBAD-GFP/pET28-pMGL for a positive control and pBAD-GFP/pET28-hCGL for a negative control. Follow the protocols from Subheading 3.2.1, steps 3–7, keeping the controls separate.
2. After GFP expression, measure the OD_{600} of the cultures and make several 100 μL aliquots of negative controls in 1.5 mL Eppendorf tubes.

⁸The multiple wash steps ensure that L-Ile is removed from the media.

⁹In the positive control for each sample the expression of GFP will be independent from the enzymatic activity, as the medium is nonselective and supplemented with L-Ile. In the sample the expression of GFP will be dependent on the L-Met degrading activity of the single mutant or of the library, as the medium is selective and deprived of L-Ile.

¹⁰During flow cytometric analysis the applied voltage may be adjusted to improve the signal separation of the controls. A higher voltage will result in higher signals for both the positive and negative samples, but will also improve the signal-to-noise ratio when the positive control signal is at the lower end of the optimal dynamic range for the instrument and the negative control is near 0 (FITC-A 0–10,000).

¹¹When plating cells collected from a round of sorting, optimal recovery is achieved if the agar plate is pre-warmed for 30 min to 1 h at 37°C .

3. Generate a pool that contains the negative control in a 10,000-fold excess by serial dilution of your positive control into your negative control aliquots.
4. Dilute this mixture in PBS to a final OD₆₀₀ ~0.05–0.1 for flow cytometric analysis and cell sorting.
5. Follow the remaining steps of the protocol described in Subheading 3.2.2.
6. Continue additional rounds of sorting until the fluorescence signal of your pool is either: (a) equivalent to that of the positive control alone or (b) no longer changing from one sort round to the next.
7. Sequence at least 20 random clones from the last round plate to verify identity of gene in the pET28a expression plasmid. Alternately, you can perform a *secondary* 96-well plate screen for MGL activity (20) to determine the makeup of the pooled sample.
8. Calculate the fold of enrichment achieved and note the number of sort rounds necessary to achieve the enrichment.
9. A successful Enrichment Test should yield results similar to Fig. 5. We achieved a 5,000-fold enrichment within three rounds (Additional rounds may be necessary under different conditions) (see Note 12).

References

1. Farinas ET. Fluorescence activated cell sorting for enzymatic activity. *Comb Chem High Throughput Screen.* 2006; 9:321–328. [PubMed: 16724923]
2. Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev.* 2004; 18:1926. [PubMed: 15314020]
3. Agrawal V, Woo JH, Mauldin JP, Jo C, Stone EM, Georgiou G, Frankel AE. Cytotoxicity of human recombinant arginase I (Co)-PEG5000 in the presence of supplemental l-citrulline is dependent on decreased argininosuccinate synthetase expression in human cells. *Anti-Cancer Drugs.* 2012; 23:51–64. [PubMed: 21955999]
4. Cantor JR, Panayiotou V, Agnello G, Georgiou G, Stone EM. Engineering reduced-immunogenicity enzymes for amino acid depletion therapy in cancer. *Methods Enzymol.* 2012; 502:291–319. [PubMed: 22208990]
5. Stone E, Chantranupong L, Gonzalez C, O’Neal J, Rani M, VanDenBerg C, Georgiou G. Strategies for optimizing the serum persistence of engineered human arginase I for cancer therapy. *J Controll Release.* 2011; 158:171–179.
6. Stone EM, Glazer ES, Chantranupong L, Cherukuri P, Breece RM, Tierney DL, Curley SA, Iverson BL, Georgiou G. Replacing Mn²⁺ with Co²⁺ in human arginase I enhances cytotoxicity toward l-arginine auxotrophic cancer cell lines. *ACS Chem Biol.* 2010; 5:333–342. [PubMed: 20050660]
7. Richards NG, Kilberg MS. Asparagine synthetase chemotherapy. *Annu Rev Biochem.* 2006; 75:629–654. [PubMed: 16756505]
8. Yang Z, Wang J, Lu Q, Xu J, Kobayashi Y, Takakura T, Takimoto A, Yoshioka T, Lian C, Chen C. PEGylation confers greatly extended half-life and attenuated immunogenicity to recombinant methioninase in primates, AACR. 2004:6673–6678.
9. Cantor JR, Yoo TH, Dixit A, Iverson BL, Forsthuber TG, Georgiou G. Therapeutic enzyme deimmunization by combinatorial T-cell epitope removal using neutral drift. *Proc Natl Acad Sci USA.* 2011; 108:1272–1277. [PubMed: 21209329]

¹²Various parameters may be adjusted to optimize the process. Optimization should maximize the fluorescence signal difference between the positive and negative control variants. The expression of enzyme can be tuned by changing the IPTG inducer concentration, altering the expression temperature and time, as well as by choosing a different OD₆₀₀ reading to initiate expression. GFP expression can be similarly controlled by altering the arabinose concentration in the selective media and varying expression time and temperature.

10. Cantor JR, Stone EM, Chantranupong L, Georgiou G. The human asparaginase-like protein 1 hASRGL1 is an Ntn hydrolase with beta-aspartyl peptidase activity. *Biochemistry*. 2009; 48:11026–11031. [PubMed: 19839645]
11. Yang Z, Wang J, Lu Q, Xu J, Kobayashi Y, Takakura T, Takimoto A, Yoshioka T, Lian C, Chen C, Zhang D, Zhang Y, Li S, Sun X, Tan Y, Yagi S, Frenkel EP, Hoffman RM. PEGylation confers greatly extended half-life and attenuated immunogenicity to recombinant methioninase in primates. *Cancer Res*. 2004; 64:6673–6678. [PubMed: 15374983]
12. Tan Y, Xu M, Tan X, Wang X, Saikawa Y, Nagahama T, Sun X, Lenz M, Hoffman RM. Overexpression and large-scale production of recombinant l-methionine- α -deamino-g-mercaptomethane-lyase for novel anticancer therapy. *Protein Expr Purif*. 1997; 9:233–245. [PubMed: 9056489]
13. Tan Y, Xu M, Guo H, Sun X, Kubota T, Hoffman RM. Anticancer efficacy of methioninase in vivo. *Anticancer Res*. 1996; 16:3931–3936. [PubMed: 9042315]
14. Lishko VK, Lishko OV, Hoffman RM. Depletion of serum methionine by methioninase in mice. *Anticancer Res*. 1993; 13:1465–1468. [PubMed: 8239522]
15. Tan Y, Zavala JSR, Han Q, Xu M, Sun X, Tan X, Magana R, Geller J, Hoffman RM. Recombinant methioninase infusion reduces the biochemical endpoint of serum methionine with minimal toxicity in high-stage cancer patients. *Anticancer Res*. 1997; 17:3857–3860. [PubMed: 9427792]
16. Hu J, Cheung NK. Methionine depletion with recombinant methioninase: in vitro and in vivo efficacy against neuroblastoma and its synergism with chemotherapeutic drugs. *Int J Cancer*. 2009; 124:1700–1706. [PubMed: 19089915]
17. Yang Z, Wang J, Yoshioka T, Li B, Lu Q, Li S, Sun X, Tan Y, Yagi S, Frenkel EP, Hoffman RM. Pharmacokinetics, methionine depletion, and antigenicity of recombinant methioninase in primates. *Clin Cancer Res*. 2004; 10:2131–2138. [PubMed: 15041734]
18. Yoshioka T, Wada T, Uchida N, Maki H, Yoshida H, Ide N, Kasai H, Hojo K, Shono K, Maekawa R, Yagi S, Hoffman RM, Sugita K. Anticancer efficacy in vivo and in vitro, synergy with 5-fluorouracil, and safety of recombinant methioninase. *Cancer Res*. 1998; 58:2583–2587. [PubMed: 9635582]
19. Varadarajan N, Cantor JR, Georgiou G, Iverson BL. Construction and flow cytometric screening of targeted enzyme libraries. *Nat Protocols*. 2009; 4:893–901.
20. Takakura T, Mitsushima K, Yagi S, Inagaki K, Tanaka H, Esaki N, Soda K, Takimoto A. Assay method for antitumor l-methionine-lyase: comprehensive kinetic analysis of the complex reaction with l-methionine. *Anal Biochem*. 2004; 327:233–240. [PubMed: 15051540]
21. Leavitt RI, Umbarger H. Isoleucine and valine metabolism in *Escherichia coli* XI. K-12: Valine inhibition of the growth of *Escherichia coli* strain. *J Bacteriol*. 1962; 83:624–630. [PubMed: 14463257]
22. Gollop N, Chipman DM, Barak Z. Inhibition of acetohydroxy acid synthase by leucine. *Biochimica et Biophysica Acta: Protein Struct Mol Enzymol*. 1983; 748:34–39.

Fig. 1.

(1) Engineer *E. coli* strain that can no longer make a metabolic intermediate needed for growth. (2) Create mutagenized libraries of enzymes that have the potential to make the missing metabolic intermediate. (3) Co-transform auxotrophic strain with library and GFP plasmids that have mutually exclusive promoters. (4) Culture under nonselective conditions and induce expression of enzyme plasmids. (5) Shift culture to selective conditions and induce expression of GFP. If an enzyme variant can rescue the auxotrophy, then the cell will express greater levels of GFP. (6) Use rounds of FACS sorting to select cells with high GFP

fluorescence. (7) Use secondary screen/assay to rank order clones and if necessary repeat mutagenesis on variants with increased activity.

Fig. 2.

Summary of genes deleted in *E. coli* to create L-aspartic acid auxotroph strain that can be rescued by recombinant asparaginase activity.

Fig. 3. Flow cytometric analysis of *E. coli* *D-aux* strain expressing an error-prone library of hASRGL-1 variants. Representative fluorescence histograms are shown from round 1 (R1) of sorting to round 7 (R7), along with a positive control containing L-Asp (see Note 4). Mean of the fluorescence histograms is reported in FACS diagram.

Fig. 4. (a) Summary of the pathway and gene deletions in *E. coli* needed to create an α -ketobutyrate auxotrophic strain that can be rescued by recombinant methionine- γ -lyase activity. (b) Summary of the pathway and gene deletions needed to create an L-methionine auxotroph to prevent parental cystathionine- γ -lyase activity from rescuing α -ketobutyrate auxotrophy.

Fig. 5. Histogram showing enrichment of positive enzyme control out of a mixed pool of 10,000:1 hCGL to pMGL over the course of four rounds as well as the signals of a positive enzyme control alone (pMGL-Ile). (*Inset*) A histogram of a positive and negative control experiment using hCGL in the presence or absence of L-Ile (+/- Ile).