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# Chapter 13

# Continuous High-Throughput Colorimetric Assays for α-Transaminases

**Egon Heuson, Jean-Louis Petit, Franck Charmantray, Véronique de Bérardinis, and Thierry Gefflaut**

## Abstract

Transaminases are efficient tools for the stereoselective conversion of prochiral ketones into valuable chiral amines. Notably, the diversity of naturally occurring α-transaminases offers access to a wide range of L- and D-α-amino acids. We describe here two continuous colorimetric assays for the quantification of transamination activities between a keto acid and a standard donor substrate (L- or D-Glutamic acid or cysteine sulfinic acid). These assays are helpful for kinetic studies as well as for high-throughput screening of enzyme collections.

**Key words** Transaminase, Amino acids, Screening assay, Cysteine sulfinic acid, High-throughput screening

## 1 Introduction

Transaminases (TA) offer an efficient access to chiral amines, which are found in numerous pharmaceuticals, and therefore have gained considerable attention in the past few years [1–3]. Notably, α-transaminases (α-TA) catalyze the conversion of α-keto acids into α-amino acids of both L- or D-series. Many L- or D-selective TA have already proven useful for the preparation of a range of rare or nonnatural amino acids with high stereoselectivity, which are key building blocks for biologically active peptides or pseudopeptides. With the aim of mining biodiversity to identify new useful biocatalysts within the α-TA family, we have developed two continuous colorimetric assays to monitor transamination reactions [4]. As shown in Fig. 1, these assays are based on the use of L- or D-cysteine sulfinic acid (CSA) as irreversible amino donor. In the direct assay, L- or D-CSA is used as unique amino donor substrate and leads upon transamination to the release of sulfur dioxide, readily converted into sulfite ions by hydration. Subsequently, the nucleophilic

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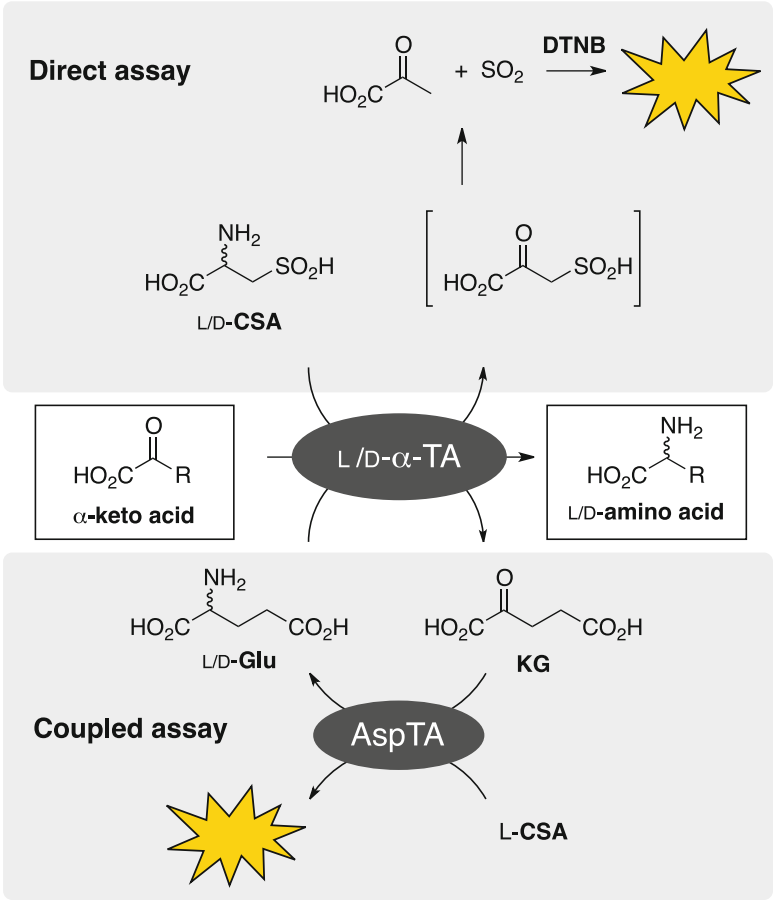


**Fig. 1** Principle of direct and coupled assays. In the direct assay, L-CSA or D-CSA must be accepted by the transaminase of interest. CSA conversion yields the instable beta keto sulfonic acid. $SO_2$ is then formed in a spontaneous decomposition and detected via a dye-forming reaction. Coupled assay: If the transaminase doesn't convert CSA, Asp-TA is employed as a shuttling enzyme that facilitates CSA conversion

sulfite specifically reacts with Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid), DTNB) to give a yellow thiolate anion ($\lambda = 412$ nm, $\varepsilon = 14{,}150\ M^{-1}\ cm^{-1}$) [5, 6]. Noteworthy, the use of L-or D-CSA offers the opportunity to identify TA with complementary stereoselectivities opening access to α-amino acids of the L- or D-series from a wide range of keto acids tested as acceptor substrates. In the coupled assay, L- or D-Glu is used as primary amino donor and gives upon transamination ketoglutaric acid (KG), which is converted back to L-Glu using aspartate transaminase (AspTA) as an auxiliary enzyme and L-CSA as secondary amino donor. The first enzymatic transamination reaction can therefore be monitored by colorimetric sulfite titration in the presence of Ellman's reagent DTNB, as described above for the direct assay. Considering that Glu is a preferred substrate for most α-TA,

this coupled assay thus allows for identifying a large variety of valuable TA.

With dynamic ranges of 0.05–80 mU/mL and 0.25–80 mU/mL for the direct and coupled assays respectively [4], these two new complementary assays can be used for high-throughput screening of large collections of TA, as well as for kinetic studies of a variety of enzymes accepting L/D-CSA or L/D-Glu as amino donor substrate. We describe here, as application examples, the determination of kinetic parameters of the two *E. coli* enzymes AspTA and branched chain transaminase (BCTA) as well as the screening protocol of an α-TA library to detect new enzymes for the stereoselective synthesis of amino acids from the corresponding keto acids. Using these new assays for screening a library of 232 α-TA from biodiversity, we could identify new valuable biocatalysts for the synthesis of L- and D-homophenylalanine [4].

## 2 Materials

### 2.1 Chemical Synthesis of L- and D-CSA

Use chemicals and solvents of reagent or analytical grade.

1. 95% Formic acid.
2. 37% Hydrochloric acid.
3. 30% Hydrogen peroxide.
4. 30% Ammonia.
5. L-/D-Cystine ($C_6H_{12}N_2O_4S$).
6. 1 M Formic acid.
7. 1 M Acetic acid (AcOH).
8. Sulfonic acid resin column: pour 250 mL Amberlite® IR120Na in a glass column (4 cm diameter, 30 cm length) equipped with a Teflon stopcock and a cotton piece to retain the resin. Wash the column with 2 M HCl (1 L) and then with water, until neutrality (typically 2 L).
9. Basic resin column ($AcO^-$ form): pour 200 mL Dowex® 1×8 (200–400 mesh, $Cl^-$ form) in a glass column (4 cm diameter, 30 cm length) equipped with a Teflon stopcock and a cotton piece to retain the resin. Wash the column with 1 M NaOH (1 L), with water until neutrality of the effluent (approx. 400 mL), with 1 M AcOH until acidity (approx. 400 mL) and finally with $H_2O$ until neutrality (approx. 400 mL).
10. Thin-layer chromatography (TLC): use silica gel 60F254 aluminum plates (Merck HX093822); elute with *n*-propanol–$H_2O$ (7:3); reveal by immersion in 2 g/L ninhydrine in EtOH, followed by heating at 200 °C with a heat-gun (Rf of CSA = 0.5).

### 2.2 Enzyme Production and Purification

Use chemicals and solvents of reagent or analytical grade (>98%). Prepare all solutions using deionized water. Store buffers at 4 °C unless otherwise specified.

1. AspTA and BCTA from *E. coli* are produced from transformed *E. coli* BL21(DE3) strains. The plasmid pET22b_AspTA contains the AspC gene (Uniprot Acc. No. P00509) coding for AspTA. Plasmid pET22b_BCTA contains the ilvE gene (Uniprot Acc. No. P0AB80) coding for *E. coli* BCTA. Both plasmids also contain a gene for ampicillin resistance for selection of transformed cells. A sequence coding for a His-tag was added to the AspC and IlvE genes, for enzyme purification by immobilized metal affinity chromatography (IMAC). Store stocks of transformed cells at −80 °C in LB medium (3 mL) supplemented with glycerol (10%).
2. Ampicillin stock solution: 200 mg/mL ampicillin in water.
3. IPTG solution: 50 mM isopropyl β-D-1-thiogalactopyranoside.
4. Luria-Bertani (LB)-ampicillin medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 100 mg/L ampicillin. In a 2 L beaker equipped with a stir bar, dissolve 50 g of solid LB mixture (Difco 244610) composed of tryptone (20 g), yeast extract (10 g) and NaCl (20 g) in 1.9 L water. Fill up to 2 L in a cylinder. Pour 200 mL of this solution in each of ten 0.5 L flasks equipped with a cotton stopper coated with an aluminum sheet and sterilize in an autoclave for 20 min at 121 °C. In each flask, add 0.1 mL ampicillin solution passed through 0.2 μM sterile syringe filters (Whatman FP30/0.2 CA-S).
5. 50 mM Phosphate buffer, pH 7.5: in a 0.5 L beaker, equipped with a stir bar, dissolve 6.80 g $KH_2PO_4$ in 900 mL water. Adjust pH with 5 M KOH (approx. 10 mL). Fill up to 1 L in a cylinder.
6. Lysis buffer: 50 mM potassium phosphate, 300 mM KCl, 10 mM imidazole, pH 8. In a 1 L beaker equipped with a stir bar, dissolve 6.80 g $KH_2PO_4$, 22.3 g KCl, and 0.68 g imidazole in 900 mL water. Adjust pH with 5 M KOH (approx. 10 mL). Fill up to 1 L in a cylinder.
7. Washing buffer: 50 mM potassium phosphate, 300 mM KCl, 20 mM imidazole, pH 8. Prepare as described for lysis buffer with 1.36 g imidazole.
8. Elution buffer: 50 mM potassium phosphate, 300 mM KCl, 250 mM imidazole, pH 8. Prepare as described for lysis buffer with 17.0 g imidazole.
9. Cleaning buffer: 50 mM potassium phosphate, 300 mM KCl, 500 mM imidazole, pH 9. Prepare as described for lysis buffer with 34.0 g imidazole.

10. Dialysis buffer: 50 mM potassium phosphate, 3 M $(NH_4)_2SO_4$, pH 7.5. In a 1 L beaker equipped with a stir bar, dissolve 6.80 g $KH_2PO_4$ and 396.4 g $(NH_4)_2SO_4$ in 600 mL water. Adjust pH with 5 M KOH (approx. 10 mL). Fill up to 1 L in a cylinder.
11. IMAC column: Ni-NTA Agarose (Qiagen). Prepare a 15 mL column and wash it with water (100 mL) and lysis buffer (100 mL). Between two purifications, use cleaning buffer (100 mL) and equilibrate with water (100 mL) and lysis buffer (100 mL). For storage, equilibrate with 20% EtOH (200 mL).
12. Dialysis membranes: 33 mm dialysis tubing cellulose membrane (Sigma-Aldrich).

### 2.3 Enzyme Assays, Kinetics, and Screening

Prepare all solutions using deionized water. Store buffers at 4 °C unless otherwise specified. Use chemicals and solvents of reagent or analytical grade (>98%).

1. Substrate and cofactor solutions: Prepare the various solutions listed below as follows: in a 50 mL beaker equipped with a stir bar, dissolve 0.34 g $KH_2PO_4$ and the appropriate amount of substrate (*see* below) in 40 mL water. Adjust pH to 7.5 with 1–5 M KOH (<5 mL) and bring to 50 mL in a cylinder. Concentrations and amounts of different substrates:

   KG solution, 20 mM: 0.15 g 2-ketoglutaric acid.

   MOPA solution, 20 mM: 0.15 g 4-methyl-2-oxopentanoic acid sodium salt.

   L-Glu solution, 200 mM: 1.48 g L-glutamic acid (*see* **Notes 1** and **2**).

   L-Asp solution, 200 mM: 1.33 g L-aspartic acid, (*see* **Note 1**).

   L-CSA solution, 200 mM: 1.53 g L-cysteine sulfinic acid (*see* **Notes 1** and **2**).

   Ammonium sulfate solution, 0.5 M: 3.30 g $(NH_4)_2SO_4$.

   DTNB solution, 10 mM: 0.198 g 5,5′-dithiobis(2-nitrobenzoic acid) previously dissolved in 5 mL EtOH.

   PLP stock solution, 10 mM: 0.133 g pyridoxal phosphate monohydrate.

   PLP solution, 0.5 mM: dilute 2.5 mL of 10 mM PLP-solution to 50 mL in a cylinder.

   NADH solution, 8.5 mM: 6 mg β-nicotinamide adenine dinucleotide reduced, disodium salt in 1 mL phosphate buffer just before use.

   Keto acid solution, 20 mM: For screening substrate specificity, prepare 20 mM solutions of various keto acids of your choice in phosphate buffer.

2. Glutamate dehydrogenase (GDH) from bovine liver (40 U/mg) and malate dehydrogenase (MDH) from bovine heart (3000 U/mg) are commercially available as suspensions in 3 M ammonium sulfate (Sigma-Aldrich). Prepare enzyme solutions just before use: in a 1.5 mL microcentrifuge tube, centrifuge the appropriate volume of suspension at 14,000 × *g* for 5 min. Discard the supernatant and gently dissolve the pellet in the appropriate volume of phosphate buffer, in order to reach the desired final concentration.
3. Bradford reagent (Bio-Rad).
4. BSA solutions, 0.01–0.1 mg/mL: prepare by serial dilution of bovine serum albumin (BSA) in phosphate buffer.
5. 96-Well microtiter plates, clear, flat bottom.
6. For screening tansaminase collections, enzyme solutions should be available as cell-free extracts, e.g., after cultivation and cell-disruption of recombinant *E. coli* clones in 96-well microtiter plates.

### 2.4 Equipment

1. Microplate reader.

## 3 Methods

### 3.1 Chemical Synthesis of L- or D-CSA

L- and D-CSA are prepared from commercially available L- and D-cystine following a modified procedure including oxidation of the disulfide bond of cystine with $H_2O_2$, followed by dismutation of the intermediate using ammonia [7]. A simple purification on an ion exchange column provides highly pure L- or D-CSA. Alternatively, L-CSA can be purchased from commercial sources.

1. In a 3-necked round bottom flask equipped with a stir bar and a thermometer, suspend cystine (20 g, 83.3 mmol) in 95% formic acid (417 mL).
2. Add to the suspension conc. HCl (15 mL, 174 mmol).
3. Add 30% $H_2O_2$ (20 mL, 200 mmol) dropwise, while keeping the temperature of the solution at 20 ± 3 °C using an ice bath and vigorous stirring. Stir then the mixture for 16 h at room temperature.
4. Concentrate the solution to dryness under reduced pressure (*see* **Note 3**) to obtain a yellow oil. Dissolve this oil in water (50 mL) and concentrate it again to dryness under reduced pressure. Repeat this step to completely remove formic acid by azeotropic distillation.
5. Dissolve the residue in water (200 mL) and adjust the pH value to 3.5 with 30% ammonia (approx. 5 mL). Cool the solution to 4 °C for 16 h. Filter the precipitate, and wash it three times with cold water (3 × 20 mL).

6. Suspend the precipitate in water (45 mL) and add 30% ammonia (45 mL). Stir the mixture for 2 h at room temperature.
7. Concentrate the mixture under reduced pressure to dryness. Dilute the residue in water (50 mL) and concentrate again to dryness under reduced pressure. Repeat this last step two times to completely remove ammonia. Suspend the residue in water (90 mL). Remove the precipitated cystine by filtration and wash it with cold water (10 mL). Concentrate the combined solution to approx. 20 mL under reduced pressure.
8. Pour the solution on the Amberlite® IR120 column ($H^+$ form, 250 mL). Elute with water and collect 25 mL fractions. Follow the product elution by TLC. Combine the CSA containing fractions and reduce the solution to approx. 20 mL under reduced pressure.
9. Pour the solution on the Dowex® 1X8 column ($AcO^-$ form, 200 mL). Wash successively with water (500 mL) and 1 M AcOH (500 mL), and elute with 1 M formic acid. Collect 25 mL fractions and follow CSA elution by TLC. Combine the CSA containing fractions and concentrate the solution under reduced pressure. Dilute the residue in $H_2O$ (20 mL) and concentrate again to dryness under reduced pressure. Repeat this operation two times to completely remove formic acid. Typically, CSA is isolated as a white solid in 75% yield (*see* **Note 4**).

### 3.2 Production of AspTA from Escherichia coli

We describe here the cell culture conditions and the purification of AspTA (*see* **Note 5**).

1. Preculture: in a sterilized 0.5 L flask, add a 3 mL stock sample of AspC recombinant cells to 100 mL of LB-ampicillin medium. Stir at 200 rpm for 24 h at 37 °C.
2. In each of five sterilized 0.5 L flasks containing 200 mL of LB-ampicillin medium, add 4 mL of preculture suspension and stir at 200 rpm at 37 °C until OD at $\lambda = 600$ nm reaches approx. 0.7.
3. To each flask, add 2 mL of a 50 mM IPTG solution and stir at 200 rpm for 24 h at 30 °C.
4. Centrifuge the culture broth at 8000 × *g* for 15 min, combine and resuspend the cell pellets in lysis buffer (20 mL) and centrifuge at 8000 × *g* for 15 min. Repeat this washing step with lysis buffer three times. Usually, approx. 3 g of wet cells are obtained per liter of culture medium.
5. Suspend the cells in 20 mL of lysis buffer and disrupt cell walls by sonication at 4 °C (*see* **Note 6**). Centrifuge at 25,000 × *g* for 30 min at 4 °C to remove cell debris.
6. Adjust the lysate volume to 28.5 mL and add 1.5 mL of 10 mM PLP.

7. Measure AspTA activity (*see* **Note 7**): In a 0.3 mL well, add 20 μL of the lysate diluted 100 times (*see* **Note 8**), 20 μL of 10 U/mL MDH, 20 μL of PLP solution, 20 μL of NADH solution, 20 μL of KG solution, 50 μL of phosphate buffer, and initiate the reaction by adding 50 μL of L-asp solution. Record the OD linear decay at 340 nm. The activity (U/mL = μmol/(min·mL) of AspTA in the supernatant is calculated from the slope ($min^{-1}$) as follows: Activity = ((slope/6220)/0.59)× $2{\cdot}10^{-4}\times50\times10^{6}$ (*see* **Note 9**). Usually a total activity of 25,000 U is found in the crude extract from 1 L culture.
8. Pour the cell lysate on a 15 mL IMAC column. Wash the column with the 150 mL of washing buffer and elute AspTA with 50 mL of elution buffer while collecting 5 mL fractions. Measure AspTA activity as described above and pool the AspTA-containing fractions. Usually the protein is obtained in fractions 3–5.
9. Fill a dialysis tubing with the AspTA solution, immerge in 200 mL dialysis buffer and stir slowly at 4 °C for 24 h. Refresh dialysis buffer (200 mL) two times after 8 and 16 h. After dialysis, remove the protein suspension from the tubing and store at 4 °C. Measure AspTA activity as described above. Usually around 10 mL of AspTA suspension is obtained with an activity of approx. 1500 U/mL. The suspension can be stored for months without loss of activity.

### 3.3 Production of BCTA from Escherichia coli

We describe here the recombinant production and purification of BCTA (*see* **Note 10**).

1. Prepare BCTA-containing cell lysate as described above for AspTA.
2. Measure BCTA activity as follows (*see* **Note 7**): In a 0.3 mL well, add 20 μL of cell lysate diluted ten times (*see* **Note 8**), 20 μL of 10 U/mL GluDH, 20 μL of PLP solution, 20 μL of NADH solution, 20 μL of ammonium sulfate solution, 20 μL of MOPA solution, 30 μL of phosphate buffer, and initiate the reaction with 50 μL of L-Glu solution. Record the OD linear decay. BCTA activity in the cell lysate is calculated as described above for AspTA. Usually a total activity of 2500 U is found in the crude extract from 1 L culture.
3. Purify BCTA by IMAC and dialyze as described for AspTA. Usually around 10 mL of BCTA suspension is obtained with an activity of approx. 100 U/mL. The suspension can be stored for months without loss of activity.

### 3.4 Enzyme Kinetics Using the Direct Assay

AspTA was chosen as a model of CSA-accepting enzyme to implement kinetic studies using the direct assay. All the measurements are done in triplicate for statistical reliability.

1. Measure the protein concentration of the purified AspTA suspension with the Bradford quantification method [8]. To get the calibration curve, add 20 μL of 0.01–0.1 mg/mL BSA solutions or phosphate buffer as a blank in different wells of a 96-well plate and add 180 μL of Bradford reagent. Incubate the plate at 30 °C for 15 min and measure OD at 595 nm. Plot OD as a function of BSA concentration. In a 1.5 mL microcentrifuge tube, centrifuge 20 μL of AspTA suspension in 3 M $(NH_4)_2SO_4$ at 14,000 × *g* for 5 min. Discard the supernatant and dissolve the pellet in phosphate buffer (1 mL). Dilute 100 μL of this solution to 1 mL. In a well, add the prepared AspTA solution (20 μL) and 180 μL of Bradford reagent (*see* **Note 11**). Incubate the mixture at 30 °C for 15 min and measure OD at 595 nm. Finally, determine AspTA concentration using the calibration curve obtained with BSA solutions.
2. Prepare a 0.1 U/mL AspTA solution: In a 1.5 mL microcentrifuge tube, centrifuge 10 μL of AspTA suspension produced in Subheading 3.2 at 14,000 × *g* during 5 min. Discard the supernatant and dissolve gently the pellet in 1 mL phosphate buffer. Dilute this solution to obtain a final activity of 0.1 U/mL (*see* **Note 12**).
3. Prepare by serial dilutions 16, 8, 4, 2, and 1 mM KG and 64, 32, 16, 8, 4, and 1 mM L-CSA solutions from 20 mM KG and 200 mM L-CSA solutions respectively.
4. Measure the activity of AspTA at various substrate concentrations (*see* **Note 7**): In a 0.3 mL well, introduce 0.1 U/mL AspTA (20 μL), 20 μL of PLP solution, 20 of μL DTNB solution, 20 μL of KG solution (various concentrations), 70 μL of phosphate buffer, and initiate the reaction with 50 μL of L-CSA solution (various concentrations) (*see* **Note 13**). Record the OD variation at 412 nm over 30 min and use the slope (in $min^{-1}$) to calculate the initial velocity of AspTA from the linear part of the curve as follows: $V_i$ (in mU) = $((slope/14150)/0.59)\times 2.10^{-4}$ (*see* **Note 9**).
5. Use a ping-pong bi-bi model to calculate the kinetic parameters of AspTA [9]. In our case, $K_M$ values of 10 ± 0.5 mM and 1 ± 0.1 mM were found for L-CSA and KG, respectively, and a $k_{cat}$ of 13,700 ± 700 $min^{-1}$ was determined from measured $V_{max}$ and protein concentration (*see* **Note 14**).

### 3.5 Enzyme Kinetics Using the Coupled Assay

BCTA is chosen as a model for enzymes accepting Glu as amino donor substrate and devoid of activity toward CSA. All the measurements are done in triplicate for statistical reliability.

1. Measure the protein concentration of the purified BCTA suspension as described above for AspTA (*see* **Note 15**).

2. Prepare as described above, a 10 U/mL AspTA solution and a 0.1 U/mL BCTA solution (*see* **Note 16**).
3. Prepare by serial dilutions 20, 15, 10, 8, 4, and 2 mM MOPA solutions from the 20 mM MOPA solution. Prepare 25 mM l-CSA by diluting 125 μL of 200 mM l-CSA to 1 mL.
4. Measure BCTA activity at various MOPA concentrations and 50 mM l-Glu (apparent $K_M$) (*see* **Note 7**): In a 0.3 mL well, add 20 μL of 0.1 U/mL BCTA, 20 μL of 10 U/mL AspTA, 20 μL of PLP solution, 20 μL of DTNB solution, 20 μL of MOPA solution (various concentrations), and 50 μL of 25 mM l-CSA, and initiate the reaction with 50 μL of l-Glu solution. Record the OD variation, calculate the initial velocities and determine the kinetic parameters as described above for AspTA. In our case an apparent $K_M$ value of $0.33 \pm 0.02$ mM for MOPA and a kcat value of $268 \pm 5$ $min^{-1}$ were found (*see* **Note 14**).

### 3.6 Screening of a TA Collection Using the Direct Assay

Both the direct and coupled assays are suitable for screening enzyme collections. In our study, we screened a library of putative transaminase genes from biodiversity, which were overexpressed in recombinant *E. coli* strains. 232 well-expressed TA were produced in 96-well microplates and screening with various keto acid substrates were performed on cell lysates after cell disruption by sonication [4].

1. Prepare the direct assay mixtures ($Mix_D$) for 100 assays: for each tested keto acid, mix 2 mL of L-CSA solution (*see* **Note 2**), 2 mL of DTNB solution, 100 μL of PLP stock solution, 2 mL of the desired keto acid solution, and 3.9 mL of phosphate buffer. The $Mix_D$ can be stored at 4 °C for 12 h.
2. Dilute the cell lysates in order to allow measurement of TA activities within the assay dynamic range (*see* **Note 17**).
3. In each 0.3 mL well of a microtiter plate, add 20 μL of the various diluted cell-lysates and 80 μL of phosphate buffer, and initiate the reaction by adding 100 μL of $Mix_D$.
4. Monitor OD variation at 412 nm over 30 min (*see* **Note 7**), and calculate initial velocity from the slope (in $min^{-1}$) of the linear part of the curve: $V_i$ (in mU) = (slope/14,150)/0.59)× $2.10^{-4}$.

### 3.7 Screening of a TA Collection Using the Coupled Assay

1. Prepare the coupled assay mixture ($Mix_C$) for 100 assays: for each tested keto acid, mix 2 mL of L-CSA solution, 2 mL of L-Glu solution (*see* **Note 2**), 2 mL of DTNB solution, 100 μL of PLP stock solution, 2 mL of keto acid solution and 1.9 mL of phosphate buffer. The $Mix_C$ can be stored at 4 °C for 12 h.

2. Dilute the cell lysates in order to allow measurement of TA activities within the assay dynamic range (*see* **Note 17**).
3. In each 0.3 mL well of a 96-well microtiter plate, add 20 μL of various diluted cell-lysates, 80 μL of 0.25 U/mL AspTA (*see* **Note 18**), and initiate the reaction by adding 100 μL of $Mix_C$.
4. Monitor OD variation and calculate initial velocities as described above for the direct assay.

## 4 Notes

1. As acidic amino acids (Glu, Asp, and CSA) are poorly soluble in water in their neutral form, a suspension is first obtained. Solubilization is observed during pH adjustment and is complete at pH 7.5.
2. This chapter describes screening protocols for L-specific transaminases. If you want to assay D-specific transaminases, D-CSA must be used in the direct assay instead of L-CSA. In the coupled assay, d-glutamate has to be employed instead of L-glutamate, but keep in mind that the aspartate transaminase (reporter enzyme) needs L-CSA in the coupled reaction!
3. The absence of residual peroxides should be checked by using test sticks before the concentration. In our case we used Quantofix® peroxide 25 and never evidenced any remaining peroxide in the solution.
4. The structure and purity of CSA can be checked by the following analyses: Melting point: 160 °C; $[\alpha]^{25}{}_D = +28°$ (c 1, 1 M HCl, L-enantiomer); $^1H$ and $^{13}C$ NMR (5 mg in 0.5 mL $D_2O$): 1H NMR (400 MHz) δ (ppm) 4.50 (1H, dd, J = 3.8, and 9.0 Hz), 2.99 (1H, dd, J = 3.8, and 14.2 Hz), 2.82 (1H, dd, J = 9.0, and 14.2 Hz); $^{13}C$ NMR (100 MHz, $D_2O$) δ (ppm) 179.6, 174.6, 138.0, 128.9, 128.3, 127.8, 59.9, 44.1, 40.3.
5. AspTA is also available from various organisms and commercial sources. Commercial enzyme preparations can also be used to implement the present colorimetric assays.
6. We used a Bandelin sonopuls sonicator, alternating 8 s on and 15 s off, for 60 min at 50% amplitude. The cell suspension is cooled by immersion in a water-ice bath during the sonication to avoid protein degradation.
7. Enzyme assays were automated on a Tecan Freedom EVO™ robotic platform. The workstation includes a multimode microplate reader (Safire™ II, Tecan) for UV/Vis absorbance measurement. All the experiments were performed in Greiner® 96-well plates incubated at 30 ± 2 °C and stirred 90 s before measurement.

8. If the measured activity is too high or too low, adjust the dilution of the supernatant to measure an enzyme activity of 1–4 mU/well.
9. The factor 0.59 is the calculated optical length of a well filled with 200 μL water.
10. As alternative to expression and protein purification, BCTA from various organisms and commercial sources can be used to implement the present colorimetric assay.
11. In our condition, the protein concentration of the purified AspTA suspension was about 16 mg/mL. The final protein concentration in the prepared solution (after centrifugation and 500-times dilution) was therefore around 0.03 mg/mL. This ensured that it was in the linear range of the calibration curve. If necessary, adjust the suspension dilution.
12. In our case, considering a starting activity of approx. 1500 U/mL for the AspTA suspension, 66 μL of AspTA solution were diluted to 1 mL to get a final mother solution of 0.1 U/mL.
13. All combinations of various KG and L-CSA concentrations are assayed.
14. Close values were measured using classical NADH assays based on reduction of pyruvate formed from CSA in the presence of lactate dehydrogenase, or on the reductive amination of KG formed from Glu in the presence of glutamate dehydrogenase.
15. In our condition, the protein concentration of the purified BCTA suspension was about 8 mg/mL. The final protein concentration in the prepared solution (after centrifugation and 200-times dilution) was then close to 0.04 mg/mL. If necessary, adjust the suspension dilution in order to be within the linear range of the calibration curve.
16. In our case, considering a starting activity of approx. 100 U/mL for the BCTA suspension, the pellet was dissolved in 0.5 mL before dilution of 50 μL of the solution to 1 mL.
17. In our case, lysates were diluted to get 0.1–0.5 mg/mL of protein.
18. The reference AspTA activity must be measured in the screening conditions (20 mM L-CSA, 2 mM KG, 1 mM DTNB, and 50 μM PLP) using the direct assay.

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