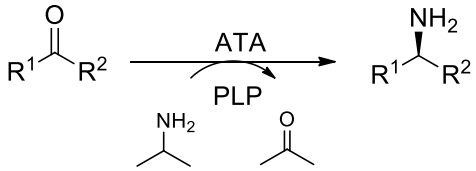
Codex® ATA Screening Kit

Screening Protocol

REACTION OF INTEREST



CODEX® ATA SCREENING KIT GENERAL INFORMATION

- The Codex® ATA Screening Kit contains 24 ω-transaminase enzymes (ATAs) that have been selected for their broad substrate range and diverse stereoselectivity for the synthesis of chiral amines from the corresponding ketone and amine donor. This kit is a useful tool to quickly determine the feasibility of using an ATA for an asymmetric transamination.

- The enzymes included in this kit were developed using Codexis' CodeEvolver® protein engineering technology and have been engineered for enhanced selectivity, activity, substrate range, and solvent and temperature stability.

- The 250 mg ATA Screening Kit contains sufficient enzyme to perform ~25 screens using the given protocol. Alternatively, fewer screens can be performed, and the remaining enzyme can be used for confirmation and optimization reactions.

- Recommended storage temperature for the enzyme powders is 4 °C when stored for up to 6 months and −20 °C when stored for longer periods.

SCREENING REAGENTS REQUIRED

- Codex® ATA enzyme powders, provided in the kit.
- Pyridoxal-5'-phosphate (PLP), provided in the kit.
- Triethanolamine, free base or HCl salt (Sigma catalog #90279 or #T1502, or similar), not provided in the kit.
- Isopropylamine, free base or HCl salt (Sigma #471291), not provided in the kit.
- Solvent, DMSO preferred, not provided in the kit.
- Substrate, approximately 150 mg, not provided in the kit.

SCREENING PROCEDURE

1. Weigh out approximately 10 mg of each ATA in the kit into separate labeled vials (vials should have at least 1.5 mL total volume). Plastic conical centrifuge tubes of 2 mL volume work well for this as the reaction can be extracted or quenched in the same vial.

2. For each full screen, add the following to a 50 mL vessel:

   a. 20 mL DI water
   b. 1.8 g (2.5 mL) isopropylamine (or 2.9 g isopropylamine-HCl)
   c. 0.5 g triethanolamine (or 0.6 g triethanolamine-HCl)
   d. 8 mg PLP

   Adjust the pH to 7.5 using HCl or NaOH and bring final volume to 30 mL. Final concentrations are 1 M isopropylamine, 0.1 M triethanolamine and 1 mM PLP. Prepare this solution fresh for each screen to avoid decomposition of the cofactor. The solution will be yellow.

3. Add 0.3 mmol of your ketone substrate to 3 mL of DMSO. Mix until dissolved.

4. Add 0.9 mL of the reaction solution from step **2** to the vials containing the ATA enzymes. Mix until enzyme is dissolved. Some cloudiness is acceptable.

5. Begin the reaction by adding 0.1 mL of the substrate-DSMO solution to each vial. It is acceptable if the substrate is not fully soluble under these conditions and typically doesn't hinder the reaction.

6. Mix the reactions at 30 °C. This is best done by placing the vials horizontally in a shaker, but any method that gives good mixing is acceptable.

7. After ~24 hours analyze your reaction by any preferred method to determine the conversion of ketone to amine. A general protocol follows.

CODEX® ATA SCREENING KIT WORK-UP AND ANALYSIS

1. Depending on the anticipated method of analysis, follow one of the protocols below:
   a. If using reversed-phase HPLC for analysis, the reaction can be quenched by adding 1 mL of acetonitrile and mixed well to dissolve any insoluble substrate and product.
   b. If using normal phase HPLC or GC for analysis, add 0.1 mL of 5 M NaOH (or similar base to increase reaction pH to >11) and extract into 0.9 mL of ethyl acetate or MTBE.

2. Centrifuge each mixture at ~4000 rpm for 2 min to separate the phases or to sediment any precipitated protein. If a centrifuge is not available, the quenched reaction can be filtered using a syringe filter or, for extractive workup, the phases can be allowed to separate unaided.

3. Transfer the aqueous acetonitrile or the organic phase from each quenched reaction vial to autosampler vials.

4. Analyze for conversion and selectivity by preferred method of analysis. Note: If analyzing by reversed phase HPLC, there will often be a large peak early in the chromatogram corresponding to the PLP cofactor.

WHAT TO DO IF…

- **…the substrate is very insoluble in water?**
  As long as the substrate is soluble in DMSO and can be added equally to each vial, partial insolubility in the reaction mixture is usually not an issue.

- **… low or no activity is found?**
  Allow the reaction to run for a longer time, increase the temperature (40 °C) and/or increase the enzyme concentration. Please see the ATA Screening Kit FAQs or contact Codexis to discuss further options of finding a suitable enzyme. You can also consider running a control reaction to verify your reaction solution. Using acetophenone and ATA-025, you can expect to see >30% conversion in 24 hours.

- **… there are too many hits and differences among them cannot be easily determined.**
  Stop the reaction at an earlier time or repeat the screen using a higher substrate loading. For the latter, maintain DMSO concentration at <20% v/v.

- **… a hit has been identified and the reaction needs to be optimized and scaled up?**
  Please see our ATA Screening FAQs for suggestions on optimizing the reaction conditions and scaling up your reaction.

- **… a different amine donor is desired?**
  Typical amine donors include alanine, ethylamine, 1- and 2-propylamine, 1- and 2-butylamine and others. The ketone/aldehyde form of these donors also act as good amine acceptors when performing chiral resolution.

- **… I have other questions?**
  See our ATA Screening Kit FAQs for answers to other questions you may have or feel free to contact us.

RECOMMENDED LITERATURE

- Savile, C. K.; Janey, J. M.; Mundorff, E. C.; Moore, J. C.; Tam, S.; Jarvis, W. R.; Colbeck, J. C.; Krebber, A.; Fleitz, F. J.; Brands, J.; Devine, P. N.; Huisman, G. W.; Hughes, G. J. Biocatalytic Asymmetric Synthesis of Chiral Amines from Ketones Applied to Sitagliptin Manufacture. *Science* **2010**, *329*, 305–309.

- Truppo, M. D.; Rozzell, J. D.; Turner, N. J. Efficient Production of Enantiomerically Pure Chiral Amines at Concentrations of 50 g/L Using Transaminases. *Org. Proc. Res. Dev.* **2010**, *14*, 234–237.

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Codex® ATA Screening Kit

Frequently Asked Questions

WHAT IS THE ATA SCREENING KIT AND WHY USE IT?

- The ATA Screening Kit is a collection of amine transaminases that will provide you with a high chance of finding a hit of meaningful activity and selectivity as the kit enzymes were selected based on the screening results of many projects.
- The enzymes in this kit are always in stock (>50 g) to allow for rapid delivery of follow-on quantities.
- The kit allows you to perform several screens and contains sufficient quantities of each enzyme for immediate scale up. It is typically suitable for determining feasibility and producing >1 g of product.
- Focused screening of just 24 enzymes leads to a shorter total analysis time.

GENERAL INFORMATION

What purity are your enzymes?

- We do not produce our enzymes to a specific purity specification. During production we do separate the enzyme from the majority of the cell debris and other fermentation components. Other compounds such as endogenous proteins and buffer salts may be present and often enhance stability.

What is the shelf life of your enzymes?

- We do not set expiration dates on research enzymes that have not yet been produced at large scale. From experience we have seen that if stored dry at –20 °C, most of the enzymes will remain stable for years.

What is the selectivity of the enzymes?

- The enzymes in the kit are highly selective and split about equally between (*R*) and (*S*) selectivity. We recommend that you screen all the enzymes rather than depend upon historical notation as the substrates can fit into the active site in unusual conformations. Note that the presence of non-carbon atoms in the substrate (such as halogens or sulfur) may give the opposite selectivity due to Cahn-Ingold-Prelog isomer naming conventions.

What are the Typical Parameters under which the enzymes work?

- The stability and activity of the ATAs are affected by several interdependent factors and can vary with different substrates and products. The table below gives typical performance parameters that might aid further optimization after screening.

Enzyme	Maximum [DMSO] (v/v)	Maximum Temperature	Optimal pH Range	Typical Selectivity
ATA-007	10%	30 °C	7–8.5	R
ATA-013	10%	35 °C	7–8.5	R
ATA-025	50%	50 °C	7–9	R
ATA-113	10%	30 °C	7–8.5	S
ATA-117*	10%	30 °C	7–8.5	R
ATA-200	10%	35 °C	7–8.5	S
ATA-217	20%	45 °C	7–9	S
ATA-234	25%	55 °C	7–9	S
ATA-237	20%	45 °C	7–9	S
ATA-238	25%	55 °C	7–9	S
ATA-251	20%	45 °C	7–9	S
ATA-254	20%	45 °C	7–9	S
ATA-256	20%	50 °C	7–9	S
ATA-260	20%	50 °C	7–9	S
ATA-301	40%	45 °C	7–10	R
ATA-303	40%	45 °C	7–10	R
ATA-412	50%	45 °C	7–10	R
ATA-415	20%	45 °C	7–10	R
ATA-P1-B04	10%	35 °C	7–8.5	S
ATA-P1-F03	10%	35 °C	7–8.5	S
ATA-P1-G05	10%	35 °C	7–8.5	S
ATA-P2-A01*	10%	30 °C	7–8.5	R
ATA-P2-A07*	10%	30 °C	7–8.5	R
ATA-P2-B01*	10%	30 °C	7–8.5	R
ATA-012^	10%	35 °C	7–8.5	R
ATA-024^	50%	50 °C	7–9	R
ATA-P1-A01^	10%	35 °C	7–8.5	S
ATA-P1-G06^	10%	35 °C	7–8.5	S

*Not sold in Japan, replacement enzymes are denoted with ^.

PLANNING YOUR SCREENING EXPERIMENT

What should I use as a control substrate?

- Acetophenone is a general substrate for most of the enzymes in this kit. If you would like to test your reaction system, expect ATA-025 to give >30% conversion with acetophenone when used as described in the screening protocol.

What are typical reaction conditions for the evolved enzymes?

- 1–200 g/L substrate, 100 mM triethanolamine hydrochloride, 1 mM PLP, 1 M isopropylamine (IPM-HCl), pH 7.5.

What buffers can be used with the enzymes?

- We suggest screening in triethanolamine hydrochloride buffer at pH 7.5. Once hit(s) are identified, the buffer type and pH can be optimized. Selectivity is typically not pH dependent, however activity may be. Borate buffer can be used to evaluate performance at high pH.

What solvents can be used with the enzymes?

- Over 50% of the kit enzymes are stable towards up to 20% v/v DMSO as co-solvent, see the **Typical Parameters** table for specific values. All the enzymes generally tolerate ~10% v/v ethanol, isopropanol, methanol, acetonitrile or THF.

What is the tolerance to pH?

- All enzymes operate between a pH of 7 and 8.5. A small subset is tolerant to pH 10. We do not recommend decreasing the pH below 7.0. See the **Typical Parameters** table for more details.

What is the tolerance to temperature?

- The variants have differing stability but range between 30 °C and 55 °C. See the **Typical Parameters** table for more details.

COMMON SCREENING QUESTIONS

I prepared my buffer/cofactor solution last week. Can I still use it?

We recommend that you make up your solutions fresh on the day of the experiment. The substrate and cofactor may degrade over time, decreasing the overall performance of the reaction. If the solution must be stored and reused, it should be kept cold and protected from light and, if possible, the performance checked against fresh solutions.

What can I do if substrate solubility in aqueous media is a challenge and the reaction mixture is cloudy?

- For many of the enzymes, 20% v/v DMSO can be used and the temperature can also be increased to 40–45 °C to increase solubility. See the **Typical Parameters** table for typical conditions tolerated for each enzyme.

- Increasing the pH may also help, but this is substrate dependent. Borate buffer can be used instead of triethanolamine at high pH. We do not recommend decreasing pH below 7.0.
- Cloudiness indicating that the substrate is not fully soluble in the reaction mixture is acceptable and typically does not hinder the reaction. It is best to add the substrate neat or from a DMSO (or similar solvent) solution to ensure it is added evenly to all reactions.

Is there an order in which reagents should be added?

- It is typically best to add the enzyme after all the other reagents and solvents have been added and the pH had been adjusted. If possible, first dissolve the enzyme in buffer before adding to the reaction mixture. Occasionally, changing the order of reagent addition can have a drastic effect on reaction performance (either beneficially or deleteriously).

OPTIMIZATION

What is the best way to optimize the reaction conditions (temperature, pH, co-solvent, isopropylamine concentration, substrate/enzyme ratio)?

- It is important to understand the inherent nature of the substrate and product since those are properties that the enzyme cannot affect (*e.g.* solubility, pH and temperature stability). In addition, you will want to avoid conditions that promote side reactions, create downstream issues or are otherwise incompatible with your substrate or product.
- Evaluate the performance of the reaction at different pH levels (pH 7.5, 9 and 10 are recommended using triethanolamine at pH 7.5 and sodium borate at pH 9 and 10).
- Once an optimal pH is found, investigate the effect of temperature on the reaction performance (recommended temperature range is 30–55 °C).
- Evaluate the reaction at higher substrate loadings. Suggested conditions are 5 g/L ATA and substrate loadings ranging from 5 to 100 g/L.
- Ensure that the isopropylamine (IPM) concentration is high enough that it is never limiting (especially important at high substrate loadings). Typically 1 M should be sufficient. Excess IPM is often required to drive equilibrium to high conversion (see below).
- If high conversion is obtained at high substrate loadings (50–100 g/L), then investigate reducing the ATA concentration (recommended range is 1–5 g/L).
- If the substrate is not soluble in the reaction mixture, substrate mass transfer limitation may occur. In this case, addition of a solubilizing co-solvent should be investigated. See the **Typical Parameters** table for solvent concentration tolerance (in addition to DMSO, other solvents such as short chain alcohols, DMF, THF and acetonitrile can also be investigated).
- See the **Typical Parameters** table for general tolerances of each ATA in the kit. It may be possible to exceed these tolerances depending on the particular reaction being performed.

What if the reaction appears to stall after a certain time or conversion?

- If the reaction seems to stall at a particular conversion, it is important to understand that a transamination is an equilibrium reaction, and often requires a driving force to achieve high conversion. This can be accomplished by using a high isopropylamine concentration (relative to the ketone substrate) and/or by removal of the acetone by-product. The latter can be achieved by applying a partial vacuum or by sweeping nitrogen across the surface of or through the reaction. This will result in some concurrent loss of isopropylamine and pH drop. The reaction pH should be monitored and if the pH decreases, it can be increased by drop wise addition of a 50% aqueous solution of IPM until the desired pH is obtained.
- The reaction may also stall due to enzyme inactivation under the given reaction conditions. This can be elucidated by adding fresh enzyme after the reaction has stalled and observing an increase in conversion.

SCALE-UP

What should I do once I find hits under screening conditions and want to scale up the reaction?

- Scaling up an ATA reaction is very similar to the procedure used for screening the ATAs. You will want to do at least some optimization first (see previous section) to determine optimal substrate and enzyme loading, co-solvent concentration, pH, temperature, and reaction time. Some general guidelines are listed below.

What conditions should I use to scale up an ATA reaction?

- Typical reaction conditions, which may change after optimization, are:
  - Ketone substrate: 10–100 g/L
  - ATA: 1–10 g/L
  - Isopropylamine: 1–2 M
  - PLP: 1 mM
  - Co-solvent: 0–20% DMSO, other solvents can also be evaluated
  - Buffer: 100 mM, pH 7–10 (triethanolamine, potassium phosphate, or sodium borate)

What is a typical reaction procedure for scaling up an ATA reaction?

1. Combine all reaction components except for the enzyme (retain some buffer for step **3**) and mix well until dissolved. If substrate will not fully dissolve, mix until well dispersed and any large masses of substrate have been broken up. If using a co-solvent such as DMSO it is often helpful to first dissolve the substrate in the co-solvent before adding it to the reaction buffer. If after mixing the substrate is not fully soluble, that is acceptable and typically does not hinder the reaction.

2. Check the pH of the substrate solution/mixture and adjust to the desired pH, if necessary.

3. Dissolve the ATA in the retained buffer. Enough buffer should be retained from step **1** such that the enzyme concentration in this step is <50 g/L.

4. Add the enzyme solution in step **3** to the substrate solution/mixture in step **1** and mix well. Check pH and adjust if necessary; avoid using concentrated acid or base for pH adjustment when enzyme is present. Typically 1 M acid or base (HCl or K/NaOH) concentration is sufficient. The pH adjustment should be done while the reaction is stirring.

5. Stir the reaction at the desired temperature. Ensure that the mixing is adequate, especially if the substrate is not fully soluble. However, vigorous mixing such that the reaction produces foam should be avoided.

6. The reaction time course can be monitored by taking in-process check (IPC) samples periodically (e.g. 2, 4, 6, 16, and 24 hrs.). Samples can be quenched with 1–2 volumes of acetonitrile or methanol or extracted with 1–2 volumes of ethyl acetate or MTBE prior to analysis (if extracting, IPC samples should be basified to pH >11 with K/NaOH prior to extraction). If the substrate has limited solubility in the reaction mixture, it can sometimes be difficult to get representative IPC samples and it is suggested multiple IPC samples be taken at each time point and averaged.

7. If the reaction has not reached high conversion (>80–90%) after 24 hrs., or if there is no difference in conversion between 16 and 24 hrs., additional ATA, isopropylamine, and/or PLP can be added. Generally adding 0.5–1 equivalent of what was initially used is usually sufficient to determine if this component is limiting the reaction. Samples can be checked after 4–6 hrs. to determine if this addition improved the reaction conversion.

MISCELLANEOUS

What do you suggest if I am trying to resolve a racemic amine and want to run the reaction in the deamination direction?

- For the deamination reaction, we suggest starting with the following reaction conditions.
  - 5 g/L amine substrate
  - 1 mM PLP
  - ~5 equivalents of acetone (<1% v/v)
  - 15 g/L enzyme loading

What do you suggest if I am trying to run a dynamic kinetic resolution?

- The basic protocol remains the same, but conditions that increase the racemization rate of the non-amine stereocenter may improve the selectivity and rate. These conditions include increasing the reaction pH, the substrate solubility (by increasing co-solvent concentration) and increasing reaction temperature. It is important that the conditions used are not detrimental to the substrate or product.

If I need help, is technical support available?

- Yes! Feel free to contact us at sales@codexis.com and we will be happy to assist you.