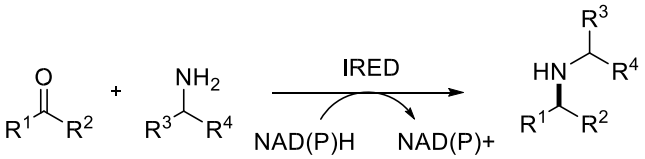
Codex® IRED Screening Panel

Screening Protocol

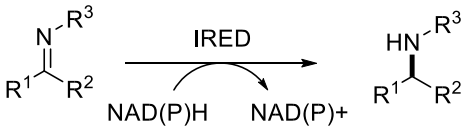
Reactions of Interest

Intermolecular Reductive Amination



or

Intramolecular Reductive Amination



Codex® Imine Reductase (Ired) Panel General Information

- The Codex® Imine Reductase (IRED) Screening Plate contains Imine Reductase enzymes (IREDs) that are active on a range of substrates with diverse stereoselectivity for the synthesis of secondary and tertiary chiral amines from the corresponding ketone and amine substrates and/or from pre-formed imines. This plate is a useful tool to quickly determine the feasibility of using an IRED for an asymmetrical reductive amination.

- Some of the enzymes included in this panel were developed using Codexis' CodeEvolver® protein engineering technology and have been engineered for enhanced selectivity, activity, substrate range, and solvent and temperature stability. The IREDs selected are a mixture of wild-type IREDs and evolved variants from four distinct lineages.

- The Codex® Imine Reductase (IRED) Screening Plate consists of one 96-well plate that contains 100 µL enzyme solution (lysate) in each well. There are 93 different IREDs in the plate with negative controls in wells E10 and E11 and a blank control (lysis buffer only) in well E12. This pre-arrayed 96-well panel presents an easy-to-use and convenient format for screening.

- Recommended storage temperature for the plates is-20 °C.

- Nicotinamide cofactor, NADPH or NADH, is used as the direct hydride source. To avoid using stoichiometric amounts of cofactor and to drive the equilibrium, the cofactor is recycled in situ. A second enzyme, glucose dehydrogenase (GDH) uses glucose as the reductant to convert the oxidized cofactor back to the reduced form. CDX-105, included in the panel, is capable of recycling both cofactors. We recommend that you add both cofactors to all wells; if you find a hit, we can recommend which cofactor to move forward with.

Screening Reagents Required

- Codex® Imine Reductase (IRED) screening plate, provided
- Glucose Dehydrogenase, CDX-105 enzyme powder, provided with the panel (13 mg)
- NAD+, provided with the panel (50 mg)
- NADP+, provided with the panel (50 mg)
- Glucose,(360 mg), to be provided by customer
- Potassium phosphate buffer pH 8.0, to be provided by customer
- Ketone substrate, to be provided by customer
- Amine substrate, to be provided by customer
- DMSO, to be provided by customer

Screening Conditions

Components	Final Concentration
1. Ketone	20 mM
2. Amine	40 mM
3. IRED Lysate	50% (v/v)
4. Glucose	40 mM
5. NAD^+	1 g/L
6. $NADP^+$	1 g/L
7. CDX-105	0.25 g/L
8. DMSO	5% (v/v)
9. Potassium phosphate buffer pH 8.0	95 mM

- *Note that higher temperature, pH, and/or DMSO levels may be used; however, not all the IREDs will be stable under these, potentially harsher, conditions. We don't recommend going above pH 10, 44 °C, and 15% DMSO, or below pH 7.0. Carbonate buffer can be used for screening at higher pHs. If you don't wish to use DMSO, some of the enzymes have been shown to be tolerant of up to 15% of diglyme, methanol, ethanol, and PEG200. If you find a specific hit, we may be able to provide more guidance as to the stability of the enzyme.*

Reactions of Interest

Screening Reagents Required

1. **GDH premix stock (5x final concentration):** Add the items listed in the table below to 10 mL of 100 mM potassium phosphate buffer, pH 8.0. Make premix stock fresh on the day of the experiment.

Components	Amount for 10 mL stock solution	Stock Concentration
Glucose	360 mg	36 g/L (200 mM)
NAD^+	50 mg	5 g/L
$NADP^+$	50 mg	5 g/L
CDX-105 (GDH)	12.5 mg	1.25 g/L

2. **Amine substrate stock (4x final concentration):** Prepare 10 mL of 160 mM amine substrate in 100 mM potassium phosphate buffer, adjust pH to 8.0 using HCl or KOH as needed.
3. **Ketone substrate stock (20x final concentration):** Prepare 2 mL of 400 mM ketone substrate in neat DMSO.

Reaction Set-Up

1. Thaw the Codex® Imine Reductase (IRED) Screening Plate at room temperature for approx. 30 min. Store plate at 4 °C until use; thaw plates only on the day of use.
2. Aliquot 40 µL of GDH premix stock, 50 µL of amine substrate stock, and 10 µL of ketone substrate stock to all wells of the plate. Note that the ketone substrate is added last to ensure that the enzyme does not temporarily experience high levels of DMSO; this is especially vital if you wish to screen with higher DMSO levels.
3. Quickly centrifuge the assay plate to remove residual droplets from the sides of the wells.
4. Seal the assay plate using a plate sealer (180 °C, 3 sec) or similar.
5. Allow the reaction to proceed overnight (16-22 hrs) while shaking at 30 °C.

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 200 µL acetonitrile and mixed well to dissolve any insoluble substrate and product. More acetonitrile can be added if one volume is not enough to solubilize the substrate(s) and product.
   b. If using normal phase HPLC or GC for analysis, add 50 µL of 5 M NaOH (or similar base to increase reaction pH to >11) and extract into 1 mL of ethyl acetate or MTBE.
2. Centrifuge the plate at ~ 4000 rpm for 10 min to separate the phases or to sediment any precipitated protein. If a centrifuge is not available an extractive workup can be done and the phases can be allowed to separate unaided.
3. Transfer the aqueous acetonitrile or the organic phase from each well to a shallow-well plate.
4. Analyze for conversion and selectivity by preferred method of analysis.

Useful Tips

- If the amine substrate is not soluble in buffer it can be added with the ketone in the DMSO stock solution; however, keep in mind that this may change the pH of the reaction.

- The following approaches are recommended, if low or now activity is found-
  - Allow the reaction to run for a longer time.
  - Consider running a control reaction to verify your reaction solution. You can use butylamine and 2methoxycyclohexanone as control substrates as this should yield reasonable amounts of product.
  - You can try coupling your amine with 2-methoxycyclohexanone and your ketone to butylamine or using a simpler surrogate substrate for one of the reaction components.
  - Consider using a much larger excess of the amine component (10 or 20 eq).
  - IRED starting activity on complex substrates is often quite low (this is an evolvable trait, please contact us for more information). If no activity is observed it is also possible that a more sensitive analytical method is needed such as mass spectroscopy.

- The best hits can be verified using lyophilized powder of the individual enzymes which can be custom made by Codexis.

- This IRED panel contains research enzymes to be used as starting points for evolution. By sharing the conversion and selectivity results from your screen with Codexis, we can provide suggestions on best approach for follow up development.

For further information or support, please contact us at sales@codexis.com.

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