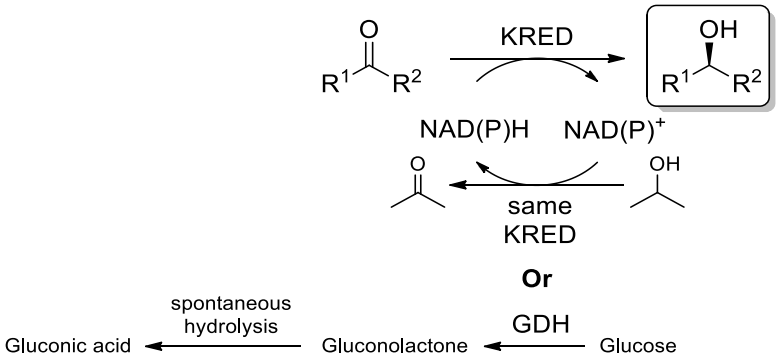
Codex® KRED Screening Kit

Screening Protocol

REACTION OF INTEREST



CODEX® KRED SCREENING KIT GENERAL INFORMATION

The Codex® KRED Screening Kit contains 24 ketoreductases (KREDs) that have been selected for their broad substrate range, high and diverse enantioselectivity, and high stability cumulated from more than 70 screening projects. This kit is a useful tool to quickly determine the feasibility of using a KRED for an asymmetric ketone reduction.

Codexis' KREDs have been engineered for enhanced selectivity, activity, substrate range, and solvent and temperature stability. Most of them are tolerant to high concentrations of isopropanol (IPA) and can use IPA as a substrate. This is advantageous as using IPA as a co-solvent will assist in dissolving compounds with poor water solubility and the IPA can act as a substrate for the recycling of NAD(P)H cofactor as shown in the scheme above. The reaction equilibrium is driven to product by using high concentrations of IPA relative to the substrate and/or stripping off the acetone product.

The five natural enzymes (KREDs 20–24 in the table on the next page: KRED-101, KRED-119, KRED-130, KRED-NADH-101 and KRED-NADH-110) work poorly with IPA and therefore use glucose and glucose dehydrogenase (GDH) instead of IPA for cofactor recycling.

KREDs 23 (KRED-NADH-101) and 24 (KRED-NADH-110) use NADH as a cofactor instead of NADPH.
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.

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

CODEX® KRED SCREENING KIT CONTENTS

Item	Enzyme	Amount	Screening Procedure	Cofactor Required	Cofactor Recycling System
1	KRED-P1-A04	250 mg	P	NADPH	Isopropanol
2	KRED-P1-A12	250 mg	P	NADPH	Isopropanol
3	KRED-P1-B02	250 mg	P	NADPH	Isopropanol
4	KRED-P1-B05	250 mg	P	NADPH	Isopropanol
5	KRED-P1-B10	250 mg	P	NADPH	Isopropanol
6	KRED-P1-B12	250 mg	P	NADPH	Isopropanol
7	KRED-P1-C01	250 mg	P	NADPH	Isopropanol
8	KRED-P1-H08	250 mg	P	NADPH	Isopropanol
9	KRED-P2-B02	250 mg	P	NADPH	Isopropanol
10	KRED-P2-C02	250 mg	P	NADPH	Isopropanol
11	KRED-P2-C11	250 mg	P	NADPH	Isopropanol
12	KRED-P2-D03	250 mg	P	NADPH	Isopropanol
13	KRED-P2-D11	250 mg	P	NADPH	Isopropanol
14	KRED-P2-D12	250 mg	P	NADPH	Isopropanol
15	KRED-P2-G03	250 mg	P	NADPH	Isopropanol
16	KRED-P2-H07	250 mg	P	NADPH	Isopropanol
17	KRED-P3-B03	250 mg	P	NADPH	Isopropanol
18	KRED-P3-G09	250 mg	P	NADPH	Isopropanol
19	KRED-P3-H12	250 mg	P	NADPH	Isopropanol
20	KRED-101	250 mg	N	NADPH	GDH/glucose
21	KRED-119	250 mg	N	NADPH	GDH/glucose
22	KRED-130	250 mg	N	NADPH	GDH/glucose
23	KRED-NADH-101	250 mg	N	NADH	GDH/glucose
24	KRED-NADH-110	250 mg	N	NADH	GDH/glucose
25	KRED Recycling mix N	10 g	N	N/A	GDH/glucose
26	KRED Recycling mix P	20 g	P	N/A	Isopropanol

The reconstituted KRED Recycle Mix N contains 263 mM sodium phosphate, 1.7 mM magnesium sulfate, 1.1 mM $NADP^+$, 1.1 mM NAD^+, 80 mM D-glucose, 4.3 U/mL glucose dehydrogenase, pH 7.0.
The reconstituted KRED Recycle Mix P contains 128 mM sodium phosphate, 1.7 mM magnesium sulfate, 1.1 mM $NADP^+$, pH 7.0.

CODEX® KRED SCREENING KIT—SCREENING PROCEDURE P (KREDs 1–19)

1. Weigh out approximately 10 mg of each KRED (1–19) in the kit into separate labelled 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, reconstitute the **KRED Recycle Mix P** by adding 33 mL deionized water to 0.6 g dry **KRED Recycle Mix P**. Prepare this solution fresh for each screen to avoid decomposition of the cofactor.
3. Add ~1 mmol of your ketone substrate to 4 mL isopropanol (IPA). Mix to dissolve.
4. To begin the reaction, add 0.9 mL of reconstituted **KRED Recycle Mix P** (**2.**) to each vial containing KRED. Mix well until the enzyme is dissolved and then add 0.1 mL of the IPA-substrate solution (**3.**) to each vial.
5. ALTERNATIVE METHOD IF KETONE IS AQUEOUS SOLUBLE: Add the IPA-substrate solution (**3.**) to the Recycle Mix solution (**2.**), mix well until dissolved. Add 1 mL of this solution to each vial containing KRED.
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. Assay the reaction mixture after ~24 hours by any preferred method to monitor the conversion of ketone to alcohol. A general work-up protocol is given below.

CODEX® KRED SCREENING KIT—SCREENING PROCEDURE N (KREDs 20–24)

1. Weigh out approximately 10 mg of each KRED (20–24) in the kit into separate labelled 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, reconstitute the **KRED Recycle Mix N** by adding 6 mL DI water to 0.3 g dry **KRED Recycle Mix N**. Prepare this solution fresh for each screen to avoid decomposition of the cofactor.
3. Add ~0.3 mmol of your ketone substrate to the reconstituted **KRED Recycle Mix N**. If the ketone is very insoluble in water, see the “WHAT TO DO IF” section.
4. To begin the reaction, add 1 mL of reconstituted **KRED Recycle Mix N** containing your ketone substrate to the vials containing the KRED enzymes.
5. Mix the reactions at 30 °C. Assay the reaction mixture after ~24 hours by any preferred method to monitor the conversion of ketone to alcohol. A general work-up protocol is given below.

CODEX® KRED SCREENING KIT WORK-UP AND ANALYSIS

1. Depending on the anticipated method of analysis, for normal phase HPLC or GC, add 1 mL ethyl acetate (preferred), propyl acetate or methyl *t*-butyl ether (preferred) to each reaction. For reversed phase HPLC analysis, quench the reaction by addition of 1 mL acetonitrile. Mix well to ensure substrate and product have been fully solubilized or extracted.
2. Centrifuge each mixture at ~4000 rpm for 2 min to separate the phases and 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 organic phase or the aqueous acetonitrile 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 NADP(H) cofactor.

WHAT TO DO IF…

- **… the substrate is very insoluble in water?**

  This is typically not an issue unless the substrate solubility is extremely low (essentially undetectable); a cloudy reaction mixture is acceptable. For KREDs 20–24, you can also try using a co-solvent such as IPA (preferred), DMSO (preferred), methanol, THF, 2-Me-THF or toluene but we recommend that the solvent does not exceed 5% of the total volume of the reaction solution for screening purposes. For KREDs 1–19 you can try increasing the IPA concentration to 20% or 30%. It is important that the substrate is added equally to each enzyme. If this cannot be done from an aqueous or IPA stock solution, it can be added neat to each reaction vial.

- **… 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 KRED Screening Kit FAQs or contact Codexis to discuss further options of finding a suitable enzyme.

- **… a hit has been identified and the reaction needs to be optimized and scaled up?**

  Please see our KRED Screening FAQs for suggestions on optimizing the reaction conditions and scaling up your reaction.

- **… 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.

- **… I have other questions?**

  See our KRED Screening FAQs for answers to other questions you may have or feel free to contact us at sales@codexis.com.

RECOMMENDED LITERATURE

- Pollard, D.; Truppo, M.; Pollard, J.; Chen, C-y.; Moore, J. Effective synthesis of (S)-3,5-bistrifluoromethylphenyl ethanol by asymmetric enzymatic reduction. *Tetrahedron: Asymmetry* **2006**, *17*, 554–559.
- Kosjek, B.; Tellers, D. M.; Biba, M.; Farr, R.; Moore, J. C. Biocatalytic and chemocatalytic approaches to the highly stereoselective 1,2-reduction of an α,β-unsaturated ketone. *Tetrahedron: Asymmetry* **2006**, *17*, 2798–2803.
- Wei, L.; Makowski, T.; Martinez, C.; Ghosh, A. Efficient synthesis of (R)-3-amino-1,1,1-trifluoropropan-2-ol via a Dakin-West reaction followed by enantioselective reduction. *Tetrahedron: Asymmetry* **2008**, *19*, 2648–2651.
- Liang, J.; Mundorff, E.; Voldari, R.; Jenne, S.; Gilson, L.; Conway, A.; Krebber, A.; Wong, J.; Huisman, G.; Truesdell, S.; Lalonde J. Highly enantioselective reduction of a small heterocyclic ketone: biocatalytic reduction of tetrahydrothiophene-3-one to the corresponding (R)-alcohol. *Org. Proc. Res. & Dev.* **2010**, *14*, 188–192.
- Gooding, O. W.; Voldari, R.; Bautista, A.; Hopkins, T.; Huisman, G.; Jenne, S.; Ma, S.; Mundorff, E. C.; Savile, M. M. Development of a practical biocatalytic process for (R)-2-methylpentanol. *Org. Proc. Res. & Dev.* **2010**, *14*, 119–126.
- Liang, J.; Lalonde, J.; Borup, B.; Mitchell, V.; Mundorff, E.; Trinh, N.; Kochrekar, D. A.; Cherat, R. N.; Pai, G. G. Development of a biocatalytic process as an alternative to the (-)-DIP-Cl-mediated asymmetric reduction of a key intermediate of Montelukast. *Org. Proc. Res. & Dev.* **2010**, *14*, 193–198.

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