Codex® KRED Screening Kit

Frequently Asked Questions

WHAT IS THE CODEX® KRED SCREENING KIT AND WHY USE IT?

- The Codex® KRED Screening Kit is a collection of 24 ketoreductase (KRED) enzymes curated from more than 70 projects for their activity on a wide range of substrates and their high and diverse stereoselectivity. It is a feasibility tool that provides a high chance of finding active and selective enzymes for your transformation.
- Formatted as vials containing 250 mg lyophilized enzyme powder for each enzyme, the kit contains sufficient quantities for several screens and follow-on hit verification.
- Once a hit is identified, immediate follow-on quantities of 50–100 g of all kit enzymes are available as "made to stock" to enable rapid reaction optimization and scale-up.
- Enzymes in the Codex® KRED Screening Kit can be further engineered for greater activity, stability, and selectivity if such needs are identified after initial screening.

GENERAL INFORMATION

What is the purity of the enzymes in the Codex® KRED Screening Kit?

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

What is the shelf life of the Codex® KRED Screening Kit?

- We do not set expiration dates for our research enzymes. 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 in the Codex® KRED Screening Kit?

- The enzymes in the Codex® KRED Screening Kit generally have very high enantioselectivity; however, we do not assign a specific selectivity for each enzyme as it can vary with substrate. The enzymes were chosen to give broad selectivity (enantiomeric and diastereomeric) for a wide range of substrates.

PLANNING YOUR SCREENING EXPERIMENT

What can I use as a positive control substrate?

- Acetophenone and 4-phenylcyclohexanone are accepted by nearly all the ketoreductases in the kit and both will give high conversion when assayed according to the kit protocol. It is important to note that the activity ranking and selectivity towards these substrates may not necessarily be the same as it is towards your substrate.

What ranges of reaction conditions can be used with the Codex® KRED Screening Kit?

- Potential reaction conditions can include 1–200 g/L substrate, 1–10 g/L KRED, 0.1–1.0 g/L $NAD(P)^+$, 25–100 mM buffer (triethanolamine, phosphate or others) pH 6–9, and 10–50% isopropanol (IPA).

What cosolvents can be used with the Codex® KRED Screening Kit?

- The enzymes in the Codex® KRED Screening Kit will generally tolerate 10–50% IPA or 5–20% DMSO as a cosolvent. It is likely that many other organic cosolvents are also acceptable. For enzymes using IPA for cofactor recycling (see the Screening Protocol) at least 9% IPA is recommended in the reaction.

What is the pH range of the Codex® KRED Screening Kit?

- All the enzymes will operate between a pH of 5 and 8. Some variants are effective as low as pH 4.5, or as high as pH 11.

What is the temperature range of the Codex® KRED Screening Kit?

- Most enzymes in the kit retain activity up to 40 °C in aqueous buffer in the presence of IPA and cosolvent. Some variants will work at higher temperature. Cofactor stability can be an issue at temperatures above 60 °C.

COMMON SCREENING QUESTIONS

I made up my buffer, cofactor, or substrate solution last week. Can I still use it?

- We strongly recommend that you make up your solutions fresh on the day of the experiment as the substrate and cofactor may degrade over time and decrease the overall performance of the reaction. If reusing solutions is necessary, it is recommended they be stored cold between uses.

What can I do if the substrate is not fully soluble and the reaction mixture is cloudy?

- Once the substrate is added to the reaction, it does not need to remain fully soluble; a slurry or cloudy reaction is acceptable and is typically not detrimental. In the provided screening protocol, the substrate is first dissolved in DMSO before it is added to the reactions; most substrates are soluble in DMSO and the protocol can be followed. If your substrate is not soluble in DMSO it can be added from an aqueous stock solution or a DMSO-aqueous solution. The Codex® KRED Screening Kit has not been fully evaluated with other solvents, but they can be tried (such as methanol, acetonitrile, THF and others). The substrate can also be added as a solid, weighed out into each reaction vial or added from a slurry if it can be added uniformly to each reaction vial.

- For substrates with acidic or basic groups, these should first be neutralized before they are added to the reaction to prevent large pH changes.

What if low or no activity is found?

- Allow the reaction to run for a longer time, increase the temperature (e.g., 40 °C) and/or increase the enzyme concentration.

What if 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 lower enzyme loading or a higher substrate loading. For the latter, it is recommended to maintain DMSO concentration at <20% v/v.

OPTIMIZATION

What is the best way to optimize the reaction conditions once I find hits under screening conditions (e.g., temperature, pH, cosolvent, substrate to enzyme ratio)?

- Evaluate the performance of the reaction at different pH levels (pH 6–10 is recommended). Suggested buffers are Bis-Tris or sodium phosphate at pH 6–7, triethanolamine at pH 7–8.5, and sodium borate at pH 8.5–10. Other buffers can also be evaluated.

- Once an optimal pH is found, investigate the effect of temperature on the reaction performance (recommended temperature range is 30–60 °C).

- Evaluate the reaction at higher substrate loadings. Suggested conditions are 5 g/L KRED and substrate loadings ranging from 5 to ≥100 g/L.

- If high conversion is obtained at high substrate loadings (50–100 g/L), then investigate reducing the KRED concentration (recommended range is 1–5 g/L).

- Lowering the cofactor concentration can improve the process economics but may affect reaction performance if too low. Typical reactions use ≤0.5 wt. % relative to the substrate. In some cases, cofactor preference (NAD^+ vs. $NADP^+$) can be substrate dependent; it may be useful to evaluate both cofactors (separately) when optimizing the reaction conditions.

- Ensure that the IPA or glucose concentration is high enough that it is not limiting (especially important at high substrate loadings). Typically, 10% IPA or 1.5 molar equivalence of glucose should be sufficient. Depending on substrate and other factors, excess IPA can often be added to drive equilibrium to high conversion (see below). When using the glucose/GDH cofactor recycle system at high substrate loadings, the decrease in pH as the reaction proceeds due to the gluconic acid that is formed in the reaction can be detrimental. A pH stat is recommended in these cases to control the reaction pH. Alternatively, the phosphite/PDH cofactor recycle system can be used and the pH change for this system is minimal.

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 ketone reduction is an equilibrium reaction and may (depending on substrate) require a driving force to achieve high conversion. For reactions that use glucose and GDH or phosphite and PDH to recycle the cofactor, this is usually not a concern as these reactions are irreversible. When using IPA to recycle the cofactor, driving the equilibrium can be accomplished by using a high IPA 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 IPA; One way to adjust for this is to replenish the lost volume with 75% IPA in water. Alternatively, the nitrogen stream can be passed through an IPA/water solution at the same temperature and concentration as the reaction before passing it through the reaction.

- The reaction may also stall due to enzyme and/or cofactor inactivation under the given reaction conditions. This can be elucidated by adding fresh enzyme and/or cofactor 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 a KRED reaction is very similar to the procedure used for screening the KREDs. You will want to do at least some optimization first (see previous section), to determine optimal substrate and enzyme loading, cosolvent concentration, pH, temperature, and reaction time. Some important scale-up guidelines are listed below.

- Typical scale-up reaction conditions for **N-Protocol KREDs:**
    - Ketone substrate: 10–100 g/L
    - KRED: 1–10 g/L
    - GDH-105: 1 g/L
    - Glucose: 1–1.5 molar excess to ketone
    - NAD(P)$^+$: 0.5 g/L (refer to the Screening Protocol for correct cofactor to use: NAD$^+$ or NADP$^+$)
    - Magnesium sulfate: 2 mM
    - Buffer: 100 mM, pH 7 (triethanolamine, sodium or potassium phosphate)

  Note: At high substrate concentration there will be a decrease in pH as the reaction progresses due to the concomitant production of gluconic acid. The pH should be controlled at the starting pH using a pH stat. If a pH stat is not available, an inexpensive aquarium-type pH controller connected to a low flow rate peristaltic pump for base addition is usually sufficient for controlling pH. A 1–2 M solution of KOH or NaOH is recommended as the titrant for controlling the pH.

- Typical scale-up reaction conditions for **P-Protocol KREDs**:
    - Ketone substrate: 10–100 g/L
    - KRED: 1–10 g/L
    - IPA: 10–50%
    - NADP$^+$: 0.5 g/L
    - Magnesium sulfate: 2 mM
    - Buffer: 100 mM, pH 7 (triethanolamine, sodium or potassium phosphate)

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MISCELLANEOUS

What if I want to run the reaction in the oxidation direction rather than the reduction direction?

- For **N-Protocol KREDs**, we suggest you start with the following conditions:
  - Alcohol substrate: 5 g/L
  - $NAD(P)^+$: 1 g/L
  - Acetone or ethyl acetoacetate: ~5 molar equivalents to substrate (< 5% v/v)
  - KRED: 20 g/L
- For **P-Protocol KREDs**, please contact us.

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-alcohol stereocenter may improve the selectivity and rate. These conditions include increasing the reaction pH and/or increasing reaction temperature. Care should be taken that the conditions used are not detrimental to the enzyme, cofactor, substrate or product.

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

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