Codex® KRED Screening Kit

Frequently Asked Questions

WHAT IS THE KRED SCREENING KIT AND WHY USE IT?

- The KRED screening kit is a collection of ketoreductases 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 more than 70 successful projects.
- The enzymes in this kit are always in stock (>50 g) so lead times for follow-up quantities are short.
- 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 our 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 KREDs generally have very high enantioselectivity; however, we do not assign a specific selectivity for each enzyme as it can be very substrate specific. The kit enzymes were chosen to give broad selectivity for as many substrates as possible. In our experience, there are enzymes in the kit that will produce both (*R*) and (*S*) products for nearly all substrates the enzymes are active towards. 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.

PLANNING YOUR SCREENING EXPERIMENT

What should I use as a control substrate?

- Acetophenone is a general substrate for nearly all the Ketoreductases in the kit. The following data is from a reaction of 5 g/L acetophenone, at 30 °C, with 5% THF as a co-solvent, after a 3-day reaction (many reached final conversion well before that). It is important to note that the activity ranking and selectivity towards acetophenone may not necessarily be the same as it is towards your substrate.

Enzyme	Conversion (%)	Stereoselectivity (% *R e.e.*)
KRED-P1-A04	99	100
KRED-P1-A12	n.d.	n.d.
KRED-P1-B02	97	9
KRED-P1-B05	75	−32
KRED-P1-B10	98	81
KRED-P1-B12	100	97
KRED-P1-C01	93	28
KRED-P1-H08	100	−83
KRED-P2-B02	99	−56
KRED-P2-C02	97	−60
KRED-P2-C11	99	61
KRED-P2-D03	72	36

Enzyme	Conversion (%)	Stereoselectivity (% *R e.e.*)
KRED-P2-D11	51	−61
KRED-P2-D12	67	52
KRED-P2-G03	100	100
KRED-P2-H07	100	100
KRED-P3-B03	19	−100
KRED-P3-G09	0	n.d.
KRED-P3-H12	4	−66
KRED-101	n.d.	n.d.
KRED-119	n.d.	n.d.
KRED-130	n.d.	n.d.
KRED-NADH-101	n.d.	n.d.
KRED-NADH-110	n.d.	n.d.

n.d.: Not determined.

What ranges of reaction conditions can be used with the evolved enzymes?

- Depending upon substrate and other factors, reaction conditions can include 1–200 g/L substrate, 1–10 g/L KRED, 0.1–0.5 g/L $NAD(P)^+$, 100 mM triethanolamine hydrochloride pH 7, 10–50% isopropanol (IPA). See the Screening Protocol for conditions for the natural (non-evolved) KREDs (last five in the list). The evolved enzymes can also be used with the glucose/GDH co-factor recycling system.

What buffers can be used with the enzymes?

- We suggest screening in triethanolamine hydrochloride buffer at pH 7. Once hit(s) are identified, the buffer type and pH can be optimized. Selectivity is typically not pH dependent, however activity may be. Other buffers we have been successful with include sodium or potassium phosphate, triethylamine hydrochloride, MES, and CAPS. Sodium borate buffer can be used to evaluate performance at high pH.

What solvents can be used with the enzymes?

- The KREDs in the screening kit will generally tolerate 10-50% IPA or 5% THF or DMSO as a co-solvent. It is likely that many other organic solvents are also acceptable. For most of the KREDs (see the Screening Protocol) at least 10% IPA is recommended in the reaction for cofactor recycling.

What is the tolerance to pH?

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

What is the tolerance to temperature?

- Most variants retain activity up to 40 °C in aqueous buffer in the presence of IPA and co-solvent. Some variants will work at higher temperature. Most of the KREDs in the screening kit will have reduced lifetimes above 60 °C.

COMMON SCREENING QUESTIONS

I made up my buffer/cofactor/substrate 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, 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?

- Reactions with KREDs that do **not** use GDH recycling (see the Screening Protocol) are typically performed with high IPA loadings to aid substrate solubility and many enzymes in the screening kit can tolerate up to 50–60% IPA. In addition, increasing the reaction temperature will improve substrate solubility. Many of the variants in the screening kit have been engineering for thermostability and can be evaluated at 35–40 °C.
- For reactions using GDH recycling, slightly higher concentration of DMSO (10-20%) or addition of ~ 10% PEG-200 as a co-solvent can be evaluated.
- 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 an IPA, DMSO or similar solvent solution to ensure it is added evenly to all reactions.

Is there an order for reagent addition?

- It is typically best to add the enzyme after all other reagents and solvents have been added and the pH has 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 once I find hits under screening conditions (temperature, pH, co-solvent, 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 6, 7, 8.5 and 10 are recommended) using Bis-Tris or sodium phosphate at pH 6, triethanolamine at pH 7 and 8.5, and sodium borate at pH 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, depending on substrate and other factors.
- 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.
- Ensure that the IPA or glucose concentration is high enough that it is never 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).

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 to recycle the cofactor, this is usually not an issue as this reaction is 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. The easiest 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, co-solvent concentration, pH, temperature and reaction time. Some important scale-up guidelines are listed below.

What conditions should I use to scale up a KRED reaction?

- Typical scale up reaction conditions, which depend on substrate and may change after optimization, for **N-Protocol KREDs** (KREDs 20–24):
  - 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 (25 mM) 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 (please contact us if more detailed information is needed). A 1–2 M solution of K/NaOH is recommended as the titrant for controlling the pH.

- Typical scale up reaction conditions, which depend on substrate and which may change after optimization, for **P-Protocol KREDs** (KREDs 1–19):
  - 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)

What is a typical reaction procedure for scaling up a KRED reaction?

1. Combine all reaction components except for the enzyme(s) and cofactor (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. For **P-Protocol KREDs** (KREDs 1-19) it is often helpful to first dissolve the substrate in the IPA before adding the substrate/IPA solution to the reaction buffer. If after mixing the substrate is not 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 enzyme(s) and cofactor in the retained buffer. Enough buffer should be retained such that the enzyme concentration in this step is <50 g/L.

4. Add the enzyme solution of 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 h). 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 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.

   If the reaction has not reached high conversion (>80–90%) after 24 h or if there is no difference in conversion between 16 and 24 h, additional KRED, GDH (for **N-Protocol KREDs**) and/or $NAD(P)^+$ 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 h to determine if this addition improved the reaction conversion.

MISCELLANEOUS

What if I want to run the oxidation reaction instead of the reduction?

- We suggest you start with the following conditions:
  - Alcohol substrate: 5 g/L
  - $NAD(P)^+$: 0.5 g/L
  - Acetone or ethyl acetoacetate: ~5 molar equivalents to substrate (< 5% v/v)
  - KRED: 15 g/L

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 stereo-center 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.