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COMMUNICATION

# A High-throughput Screening Method for the Directed Evolution of Hydroxynitrile Lyase towards Cyanohydrin Synthesis

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Dedication ((optional))

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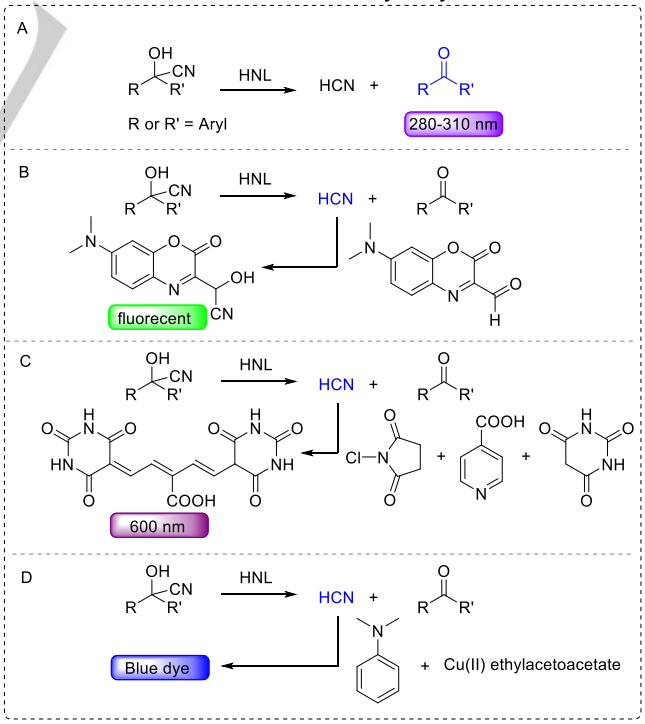
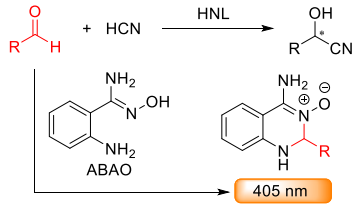
**Abstract:** Chiral cyanohydrins are useful intermediates in pharmaceutical and agricultural industries. In nature, hydroxynitrile lyases (HNLs) represent a kind of elegant tool for enantioselective hydrocyanation of carbonyl compounds. Currently available methods for demonstration of hydrocyanation reaction still stalled at precise but low throughput GC or HPLC analyses. Herein we report a chromogenic high-throughput screening (HTS) method feasible for cyanohydrin synthesis reaction. This method was highly anti-interference and sensitive, and could be used to directly profile the substrate scope of HNLs neither in cell-free extract or fermentation clear broth. This HTS method was also validated by generating of new variants of *Pc*HNL5 which presented higher catalytic efficiency and stronger acidic tolerance in variant libraries.

Hydroxynitrile lyases (HNLs; EC 4.1.2.10, 4.1.2.11, 4.1.2.46, and 4.1.2.47) catalyze the cleavage of cyanohydrins to form hydrogen cyanide and carbonyl compounds (mostly aldehydes).[1] The reverse hydrocyanation reaction has been successfully used for the synthesis of chiral cyanohydrins in pharmaceutical and agricultural industries.[2] As cyanohydrins easily degrade spontaneously in aqueous systems at pH >5, asymmetric biohydrocyanation is preferentially conducted under highly acidic conditions (pH <4) to prevent spontaneous racemization of the enzymatically formed product.[3] This presents a great challenge to both the specific activity and pH stability of HNLs, with very few enzymes tolerating such harsh conditions.[3a,4] Furthermore, to meet the demands of synthetic applications, protein engineering of HNLs is usually required to expand their substrate scope.

Directed evolution is a powerful tool for obtaining desired biocatalysts for target transformations.[5] However, this strategy relies on the mass screening of clones to obtain beneficial mutants, which depends on the availability of a reliable high-throughput screening (HTS) method. For HNLs, several HTS methods have been established for HNL screening or substrate profiling in the previous two decades. The most classic method depended on UV absorbance measurement of the enzymatic cleavage product (benzaldehyde) or its derivatives from corresponding cyanohydrins (**Scheme 1A**),[6] the beauty of this method relies on its very high sensitivity and easy to operate in laboratorial conditions. Aiming to develop a general method which are not strict to substrate. Methods for determination of the released HCN were subsequently developed, which involving using fluorescent probes (**Scheme 1B**),[7] König reaction assays (**Scheme 1C**),[8] and colony-based Feigl–Anger reaction assays (**Scheme 1D**)[9]. However, regarding fluorescent detection methods, strong background interference and reaction suppression are observed at acidic pH levels, which limited the substrate scope only to the mandelonitrile at pH 6.5. Although colony-based Feigl-Anger reaction allowed screening of massive random mutagenesis library of *E. coli* harboured recombinant *Gt*HNL toward 2-Cl-mandelonitrile dehydrocyanation, all above




**Scheme 1.** Examples of colorimetric assays reported based on cyanohydrin cleavage. (A) UV absorbance of aromatic aldehydes. (B) Coupling the fluorescent probe. (C) König reaction assays. (D) Feigl-Anger reaction assays.




**Scheme 2.** Quantification of residual aldehyde after cyanohydrin synthesis using chromogenic reagent ABAO.

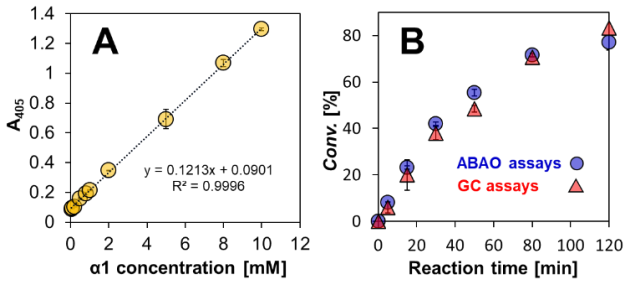



**Figure 1.** (A) Calibration curve of aldehyde **α1** concentration in citrate buffer (1 M, pH 2.5, 100 µL) by monitoring UV/Vis absorption at 405 nm after adding a stock solution of ABAO (50 mM) in citrate buffer (1 M, pH 2.5, 100 µL). Data shown are mean values of three replicates with standard deviations. (B) Biohydrocyanation progress curves of aldehyde **α1** (10 mM) with *Pc*HNL5 (supernatant of yeast culture broth), as monitored comparatively by ABAO and GC assays, respectively. See Supporting Information for more experimental details.

the HTS methods are based on the cleavage reaction of HNLs. For the requirements of practical synthesis, desired capability of HNLs is the reverse reaction, known as asymmetric hydrocyanation. According to the first rule of directed evolution, 'you get what you screen for', positive hits with higher activity toward the cleavage reaction do not always result in an improved ability in the reverse synthetic reaction.[6c] Therefore, the direct assay of hydrocyanation activity is still dependent on accurate but low-throughput high-performance liquid chromatography (HPLC) or gas chromatography (GC) analyses,[10] which are time consuming, labor-intensive, and unsuitable for HTS during the directed evolution of HNLs. Therefore, a general and rapid HNL assay method targeting cyanohydrin synthesis is still needed for the efficient evolution of HNLs. Herein, we report an HTS method for monitoring cyanohydrin synthesis using a modified 2-aminobenzamidoxime (ABAO) cycling assay (**Scheme 2**). This method allows direct chromogenic quantification of the remaining aromatic or aliphatic aldehyde after hydrocyanation.

In a recent study, Rudoff and coworkers[11] reported a fast cycling reaction of aldehydes using 2-amino-benzamidoxime derivatives in an activity assay of carboxylate reductases (CARs). As CARs only show activity under neutral conditions, a pH adjustment (to pH <4.5) was necessary after the enzymatic reaction to ensure iminium ion formation to facilitate cycling reactions when using self-synthesized 2-amino-5-methoxyl-benzamidoxime. We envisaged aldehyde quantification in the hydrocyanation reaction system using commercially available 2-amino-benzamidoxime (ABAO) as a general chromogenic reagent. Initially, the cycling reaction in the presence of ABAO (2 equiv.) was evaluated using aldehydes in an acidic monophase aqueous citrate buffer system (0.8 M, pH 4.5), which both suppressed background nonselective HCN addition to aldehydes and facilitated the cycling reaction. Herein, benzaldehyde (**α1**) was selected as a representative substrate because it is the scaffold found in most aldehydes accepted by most HNLs. However, the UV/Vis absorbance peak observed at 405 nm kept increasing even 10 min after ABAO was added, indicating that more ABAO might be needed. After further optimization 5 equiv. of ABAO was found to be sufficient to complete the cycling reaction within 2 min (**Figure S2**).

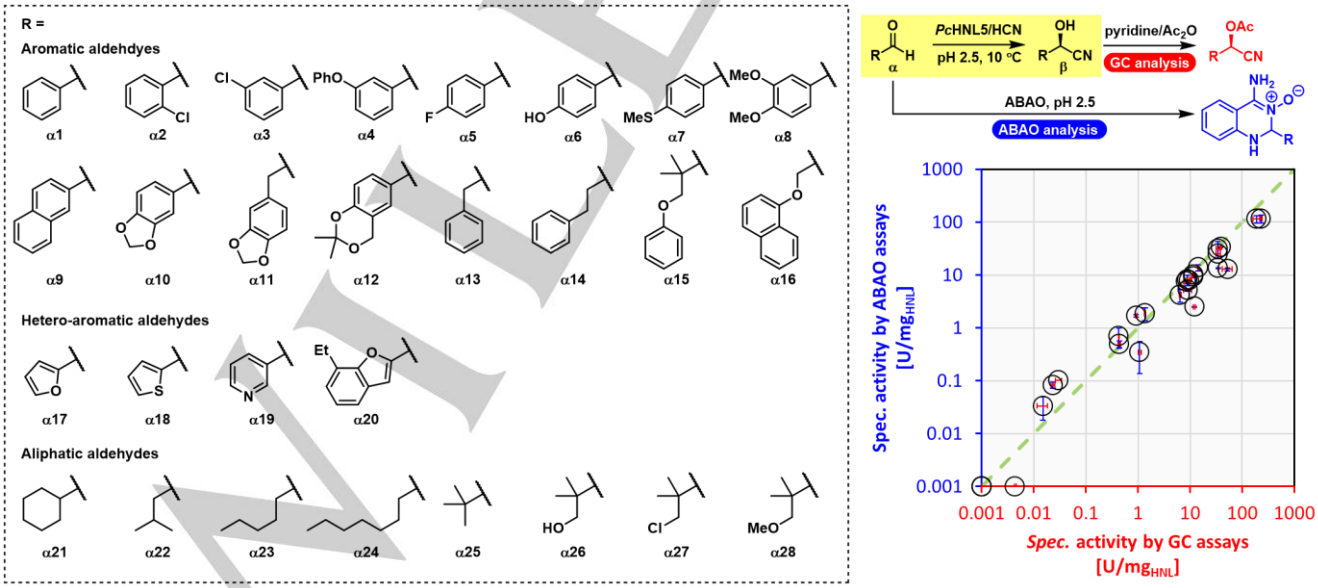



**Figure 2.** Correlation study of ABAO cycling assays with GC analysis results in profiling the substrate spectrum of wild-type *Pc*HNL5 (One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol cyanohydrin per minute under assay conditions). Specific activities toward various aldehydes were determined in 0.5-mL aqueous reaction systems. See Supporting Information for experimental detail and data.

As spontaneous nonselective HCN addition is highly pH-sensitive,[3a] cycling reactions at different pH values were next investigated. Surprisingly, lowering the aqueous phase pH led to a dramatic increase in the cycling reaction rate. At pH 2.5, the cycling reaction was finished almost immediately after ABAO was added to the system, which is ideal for hydrocyanation monitoring. Furthermore, the absorption intensity at 405 nm increased significantly with decreasing reaction pH (**Figure S3**). The assay also showed compatibility with most ions and organic cosolvents present in *E. coli* or yeast culture and the enzymatic reaction systems (**Figures S4**–**S6**). The only exception was some reductive sugars (such as glucose and lactose) that potentially interfere with the target aldehyde to be detected. However, these sugars could be replaced with other carbon sources, such as methanol and glycerol, in the cell culture process. Therefore, ABAO can be directly used to quench the reaction and start the chromogenic assay. To investigate the accuracy of our HTS assays, the measured level of coloration and resultant substrate conversion were correlated to the GC analysis results for the hydrocyanation of aldehyde **1** (10 mM) by *Pc*HNL5 (in the form of a supernatant of yeast culture broth)[12] in an aqueous system (**Figure 1**). The assay demonstrated a high compatibility and sensitivity, resulting in detectable UV/Vis absorption variance at a conversion difference of only 1%.

With optimized assay conditions in hand, the versatility of the ABAO assay methodology was investigated. The specific activities of wild-type *Pc*HNL5[12] toward a series of aldehydes (16 aromatic aldehydes (**α1**–**α16**), four heteroaromatic aldehydes (**α17**–**α20**), and eight aliphatic aldehydes (**α21**–**α24**)) were determined using the modified ABAO assay method. To confirm the reliability of this assay methodology, the specific activities of HNL for hydrocyanation were simultaneously determined by GC. Good correlation was observed toward almost all aldehydes tested (**Figure 2**), except substrate **α12**, owing to trace enzyme activity (4.4 mU/mg). However, ABAO showed relatively low reactivity toward ketones (**α29**–**α32**, **Figures S7**–**S10**) compared with aldehydes, indicating that the current HTS method was not suitable for ketone substrates.

The freedom of applying structurally diverse aldehydes prompted us to perform a substrate screening of several well-known HNLs to validate the current HTS methodology. We tested the enzymatic hydrocyanation reactions of reported (*S*)-*Me*HNL$_{H103L}$[13] and its variant H103L/W128A,[14] (*R*)-*Pc*HNL5[12] and its variant L331A,[15] and industrially applied (*R*)-*Pa*HNL5 variants N3I/I108M/A111G[6c] and V317A.[16] Four aldehydes, **α2**, **α4**, **α12**, and **α25**, were used as representative substrates (**Table 1**). The UV/Vis absorbance of the control reaction without any HNL preparation (*E. coli* cell lysate or yeast secretion supernatant) remained unchanged after adding ABAO. Hydrocyanation reactions catalyzed by the crude preparation of HNLs with relatively high acidities showed good correlation with the GC analytical results. Although both wild-type *Pc*HNL5 and *Pa*HNL5$_{V317A}$ showed quite poor activities toward bulky aliphatic aldehyde **α26**, a detectable difference was observed, with *Pa*HNL5$_{V317A}$ showing relatively high activity toward this substrate, in accordance with the reported results. Similar results were also obtained for the transformation of **α12** by wild-type *Pc*HNL5. However, this is worth to note enzymatic consumption of yeast endogenous proteins (e.g. aldehyde reductases or aldehyde oxidases) in the secretion led to spontaneous transformation of the aldehydes, but it didn't disturb for indentication of the beneficial variant. These results showed that the ABAO cycling method allowed quantitative determination of the hydrocyanation reaction at moderate conversion and qualitatitation of substrate conversion when HNLs had only very low activity.

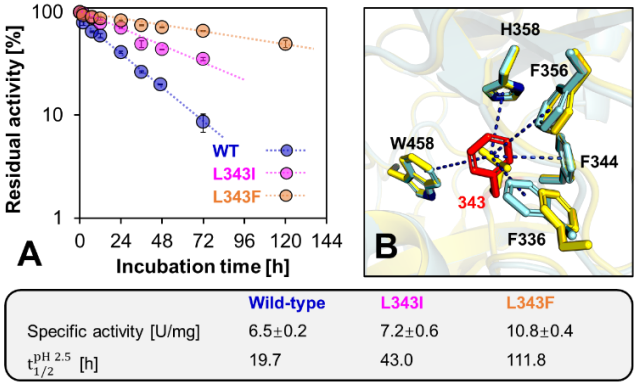
**Table 1.** Bioconversion of representative aldehydes by known HNLs or their variants, analyzed by ABAO or GC assay.[a]

| Substrate | HNLs | % Conv. by ABAO[b] *vs.* GC (listed in brackets)[c] |
|---|---|---|
| **α2** | *Pc*HNL5$_{WT}$ | 27.7 ± 0.1 (32.2 ± 0.1) |
| | *Pa*HNL5$_{N3I/I108M/A111G}$ | 73.8 ± 1.7 (75.4 ± 1.7) |
| **α4** | *Me*HNL$_{H103M}$ | 33.0 ± 4.0 (25.6 ± 0.7) |
| | *Me*HNL$_{H103M/W128A}$ | 94.7 ± 0.4 (76.9 ± 0.0) |
| **α12** | *Pc*HNL5$_{WT}$ | 8.5 ± 0.5 (0.39 ± 0.06) |
| | *Pc*HNL5$_{L331A}$ | 42.0 ± 0.1 (43.0 ± 2.8) |
| **α26** | *Pc*HNL5$_{WT}$ | 4.2 ± 0.4 (0.2 ± 0.1) |
| | *Pa*HNL5$_{V317A}$ | 7.9 ± 0.4 (1.1 ± 0.2) |

[a]Reactions performed on 0.5-mL scale by applying yeast fermentation supernatant or *E. coli* cell free extract with HNL activity (see Supporting Information for experimental details). [b]Conversions assayed using the ABAO cycling method. [c]Conversions assayed by chiral GC.

To demonstrate the practicability of this HTS methodology, a round of *Pc*HNL5 directed evolution toward aldehyde **α7**, whose corresponding (*R*)-cyanohydrin is a key intermediate in the synthesis of veterinary antibiotics, was attempted.[12] The beneficial sites were predicted in a substrate binding model according to a docking simulation. A total of 12 residues around the substrate binding pocket (**Figure S12**) were subjected to saturation mutagenesis, giving a library of ≥1200 yeast transformants. Based on the ABAO assay, positive hits with improved conversion were selected from the saturation mutagenesis library of site 343 (**Figure S13**). Rescreening of the beneficial clones by chiral GC led to the identification of two variants, L343I and L343F.

Variants L343I and L343F both showed significantly enhanced acid tolerance and higher specific activity than the wild-type (**Figure 3A**). In particular, L343F exhibited a half-life of 112 h at pH 2.5, showing stability much higher than that of the wild-type (19.7 h), and far beyond that of industrialized *Pa*HNL variants (about 9 h at pH 2.6).[17] Single-site mutagenesis in the active pocket leading to collaborative elevation of the acid tolerance and hydrocyanation activity of the HNL is surprising. To gain deeper insight into this phenomenon, the crystal structure of *Pc*HNL5$_{L343F}$ (PDB ID: 7CGS) was determined, indicating that five strong π–π stacking interactions were formed between the benzene ring of




| | Wild-type | L343I | L343F |
|---|---|---|---|
| Specific activity [U/mg] | 6.5±0.2 | 7.2±0.6 | 10.8±0.4 |
| $t_{1/2}^{pH\ 2.5}$ [h] | 19.7 | 43.0 | 111.8 |

**Figure 3.** (A) Plot of residual activities of *Pc*HNL5 mutants after agitation at 1000 rpm, pH 2.5, and 30 °C; (B) structural insight into strong π–π stacking interactions around the Phe343 side chain (red stick) by superposition of the crystal structures of wild-type *Pc*HNL5 (PDB ID: 6JBY, yellow) and PcHNL5 L343F (PDB ID: 7CGS, cyan).

residue Phe343 and the aromatic side chains of the surrounding residues, including Phe336, Phe344, Phe356, His358, and Trp458 (**Figure 3B**). As these residues are located at the flexible loops in the active pocket and the outer shell of *Pc*HNL5, these newly introduced hydrophobic interactions might dramatically enhance enzyme stability. Using aldehyde **α7** at a concentration of 1 M and a *Pc*HNL5 L343F (purified enzyme) dose of 1.0 mg per mmol substrate, the biohydrocyanation was complete within 12 h, affording 97% conversion and 99% *ee*. In contrast, the wild-type enzyme gave only 80% conversion and 98% *ee* (**Figure S14**). Approximately twice times of the wild-type enzyme compared with the variant was needed to obtain the same result.

In summary, we have developed a rapid and reliable colorimetric assay method that enables the high-throughput quantification of hydrocyanation reactions. We hope that this high-throughput assay methodology could provide opportunities for rapid evaluation of the hydrocyanation ability of HNLs toward tested aldehydes through directed evolution. Furthermore, as ABAO showed low reactivity toward ketones, the search for ketone-reactive chromogen compounds is now ongoing in our laboratory.

## Acknowledgements ((optional))

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**Keywords:** cyanohydrin • hydrocyanation • 2-amino-benzamidoxime • high-throughput screening • directed evolution

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## Entry for the Table of Contents

**Make biohydrocyanation measurable**: Hydroxynitrile lyases (HNLs) catalyze the enantioselective cleavage/formation of the cyanohydrins. To date, available methods for determination of hydrocyanation still stalls at HPLC or GC assays, which are not suitable for screening of massive of mutants for the protein engineering of this class of biocatalysts. We therefore demonstrate a chromogenic high-throughput screening method for cyanohydrin synthesis reaction, which was validated by either substrate profiling or directed evolution of HNLs.