Chemistry
A European
Journal

Chemistry Europe
European Chemical Societies Publishing

Accepted Article

Title: Enzymatic Cleavage of Double-Stranded DNA-Encoded Libraries (DELs) to Single-Stranded DELs with Compounds at the 3' End: Its Application in Photo-crosslinking Selection

Authors: Shin Itoh, Masatoshi Niwa, Jun Hayashida, Munefumi Tokugawa, Takeshi Nanya, Masako Tanabe, Naoko Honda, Takehiko Inohana, Hajime Fukano, Yukihiro Shigeta, and Takeshi Kuboyama

To be cited as: *Chem. Eur. J.* **2024**, e202403233

Link to VoR: https://doi.org/10.1002/chem.202403233

WILEY VCH

10.1002/chem.202403233

WILEY VCH

RESEARCH ARTICLE

Enzymatic Cleavage of Double-Stranded DNA-Encoded Libraries (DELs) to Single-Stranded DELs with Compounds at the 3' End: Its Application in Photo-crosslinking Selection

Masatoshi Niwa,[a] Jun Hayashida,[b] Munefumi Tokugawa,[a] Takeshi Nanya,[b] Masako Tanabe,[b] Naoko Honda,[a] Takehiko Inohana,[a] Hajime Fukano,[d], [e] Yukihiro Shigeta,[c] Takeshi Kuboyama,[c] and Shin Itoh*[a]

[a] M. Niwa, Dr. M. Tokugawa, N. Honda, T. Inohana, Dr. S. Itoh
Chemical Research Laboratories
Nissan Chemical Corporation
10-1 Tsuboi-Nishi 2-chome, Funabashi, Chiba, Japan
E-mail: itoushi@nissanchem.co.jp

[b] J. Hayashida, T. Nanya, M. Tanabe
Biological Research Laboratories
Nissan Chemical Corporation
1470, Shiraoka, Shiraoka, Saitama, Japan

[c] Y. Shigeta, Dr. T. Kuboyama
Head Office
Nissan Chemical Corporation
5-1, Nihonbashi 2-chome, Chuo-ku, Tokyo, Japan
E-mail: shigetay@nissanchem.co.jp

[d] H. Fukano
Biological Research Department
Daiichi Sankyo RD Novare Co., Ltd.
1-16-13, Kitakasai, Edogawa-ku Tokyo, Japan

[e] Present institution: Daiichi Sankyo Co., Ltd.

Abstract: DNA-encoded library (DEL) technology is a crucial tool in pharmaceutical research, rapidly identifying compounds that bind to a target of interest from an extensive pool of compounds. In this study, we propose a new method for generating single-stranded DELs (ssDELs) with compounds at the 3' end. The introduction of uniquely designed hairpin-shaped headpieces containing deoxyuridine (NC-HP) and the use of a cleavage enzyme facilitate the conversion from double-stranded DELs (dsDELs) to such ssDELs. Moreover, Klenow fill-in provides the dsDELs with photo-crosslinkers covalently linked to the coding region, which exhibit durability even under stringent washing conditions and enable photo-crosslinking with a high signal-to-noise ratio, as also confirmed in cell-based photo-crosslinking selections.

Introduction

DNA-encoded libraries (DELs) are extensive pools of compounds (ranging from 10^5 to 10^{10}) widely used by pharmaceutical companies and academic institutions for identifying hit compounds.[1] Each compound is tagged with a DNA sequence that records its synthetic history and functions like a barcode. When very small quantities of compounds that bind to a specific target molecule are extracted from the DEL, the DNA sequence is read to determine the identity of the compounds with the desired biological activity quickly. These compounds and DNA sequences are bound via the headpiece (HP). Currently, the HP developed by Praecis/GSK, which covalently links two DNA strands to form hairpin-shaped double-stranded DNA (dsDNA), is widely used as a starting material for DEL synthesis.[2] One reason is that short dsDNA of about 9–13-mers with 2-mer sticky ends enables the encoding process through enzymatic ligation, and another is that the robust double-stranded structure prevents damage to the nucleobase parts under reaction conditions during library synthesis.

On the other hand, single-stranded DELs (ssDELs) have been gaining attention for their application in DEL selections.[3] Although ssDELs are less stable in chemical reactions than double-stranded DELs (dsDELs) and, thus, limit compound diversity, they can form double strands with complementary DNA strands having various functional groups. Utilizing these functional groups, various selection methods, including crosslinking, have been developed. However, these methods cannot be directly applied to conventional hairpin-shaped dsDELs. Recently, two approaches have been reported to solve this trade-off by converting dsDELs to ssDELs.[4] One method involves degrading one strand of the hairpin-shaped DELs with lambda exonuclease (λ exo) (Scheme 1(a), top). The other method utilizes an HP with a 3-cyanovinylcarbazole nucleoside on one strand. This technique forms covalent dsDELs by photo-crosslinking with thymine on the opposite strand, followed by degradation of one strand by λ exo and further cleavage by UV irradiation to give ssDNA (Scheme 1(a), bottom). Considering the polarity of the DNA strand and compounds, both methods provide

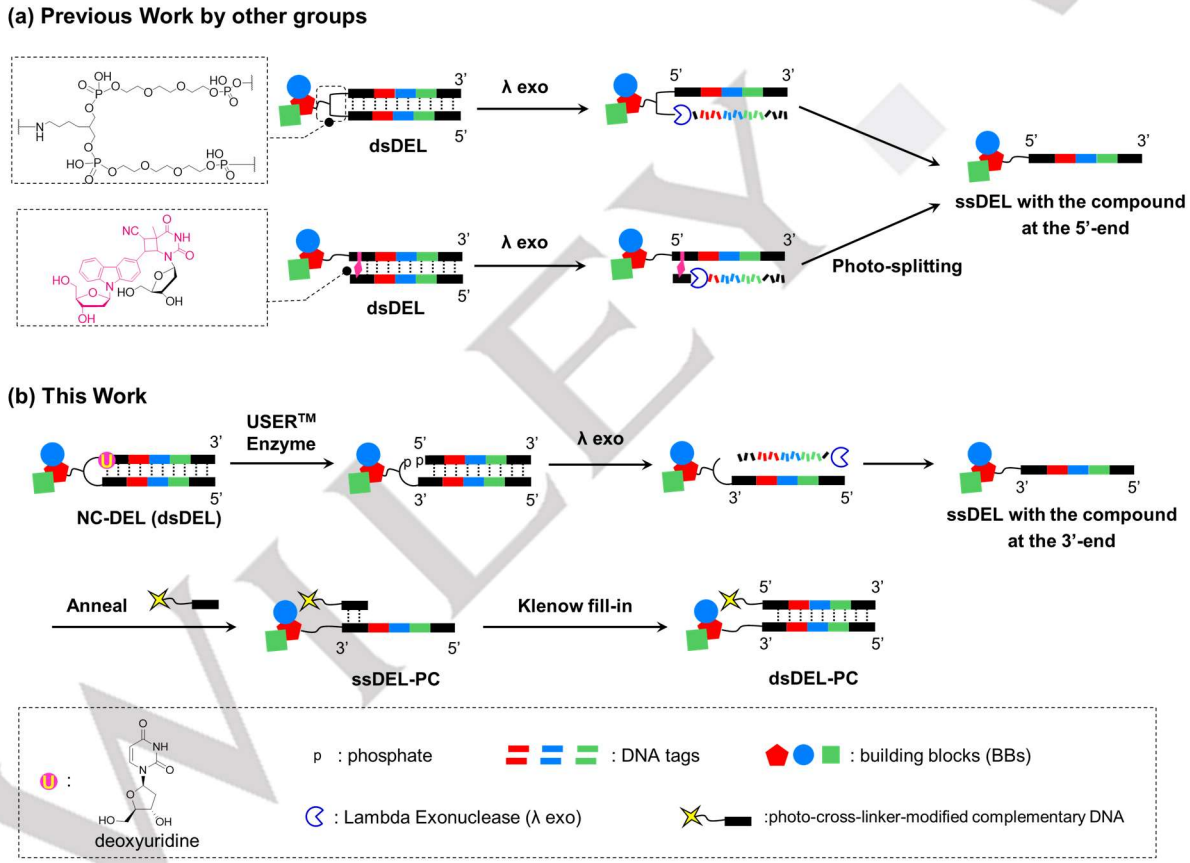
10.1002/chem.202403233

RESEARCH ARTICLE

ssDELs with compounds at the 5' end because of the degradation direction (5' → 3') of λ exo. Although the use of Exonuclease III, a 3' → 5' exonuclease, has been reported, [4a] it fails to produce ssDELs with compounds at the 3' end because it digests both DNA strands of hairpin-shaped dsDELs. To date, no method for inducing ssDELs with compounds at the 3' end has been reported.[4a] In this study, we propose cleavable hairpin-shaped DELs (NC-DELs) as a novel method for generating ssDELs with compounds at the 3' end.

Briefly, we developed a new HP containing deoxyuridine as a cleavable site (NC-HP). Deoxyuridine has similar chemical stability to natural DNA and is expected to allow cleavage by enzymes without the risk of compound damage. dsDELs constructed using NC-HP produced ssDELs with compounds at the 3' end by cleaving the hairpin part with Uracil-Specific Excision Reagent (USER™) enzyme and then digesting one strand with λ exo. USER™ is an endonuclease cocktail that removes uracil, including uracil DNA glycosylase (UDG) and endonuclease VIII; it removes uracil in double-stranded DNA to generate a one-base gap and cleave the DNA strand. In the USER™ process, UDG first removes a uracil base to produce an abasic site, after which the endonuclease decomposes a phosphodiester bond to liberate a baseless deoxyribose and a one-base gap. The ssDELs with compounds at the 3' end are considered more useful in DEL selection than those at the 5' end. This is because one can anneal a crosslinker (such as a photo-crosslinker (PC-linker) and other reactive groups) with short DNA complementary to the constant region (ssDEL-PC; Scheme 1(b)) and then introduce the coding region by Klenow fill-in (dsDEL-PC; Scheme 1(b)), thus covalently linking the DEL coding information and the crosslinker.

Further, we demonstrate that ssDEL with compounds at the 3' end converted from NC-DEL can be successfully changed into ssDEL-PC and then dsDEL-PC. This dsDEL-PC was applied to photo-crosslinking selection and yielded affinity ligands with a high signal-to-noise (S/N) ratio. In addition, this methodology also enabled DEL selection on membrane proteins in living cells. Our approach opens possibilities for the utilization of selection methods that require ssDELs and expands their utility in the drug discovery process.



Scheme 1. (a) (top) Synthesis of the ssDEL with the compound at the 5′-end from dsDEL by λ exo. (bottom) Synthesis of the ssDEL with the compound at the 5′-end from dsDEL with a reversible covalent headpiece. (b) Synthesis of ssDEL-PC with the compound at the 3′-end from NC-DEL (dsDEL) and its conversion to dsDEL with a photo-crosslinker (dsDEL-PC) utilizing Klenow fill-in.

10.1002/chem.202403233
WILEY VCH

RESEARCH ARTICLE

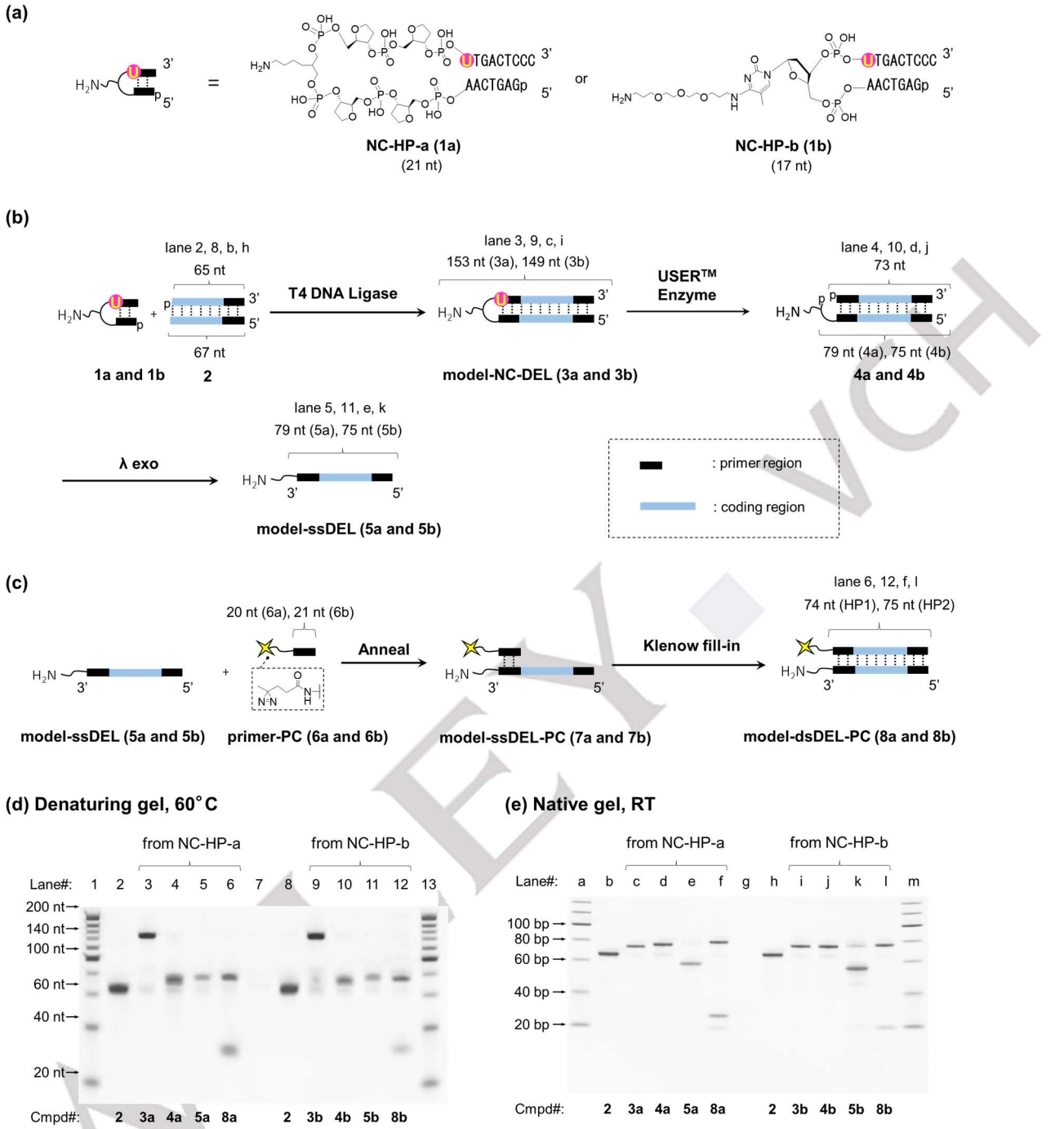


Figure 1. (a) Structures of NC-HP-a (**1a**) and NC-HP-b (**1b**) with deoxyuridine on the 3' side of the amino linker moiety. (b) Synthesis of model-ssDEL from NC-HP-a (**1a**) and NC-HP-b (**1b**). In short, **1a** and **1b** were tagged by T4 DNA ligase to model-NC-DELs (**3a** and **3b**) (lanes 3, 9, c, and i). Model-NC-DELs (**3a** and **3b**) (lane 3, 9, c, and i) were cleaved by the USER™ enzyme to compounds (**4a** and **4b**) (lanes 4, 10, d, and j). Compounds (**4a** and **4b**) (lanes 4, 10, d, and j) were digested by λ exo to model ssDEL (**5a** and **5b**) (lanes 5, 11, e, and k). (c) Synthesis of model dsDEL-PC from model-ssDEL. Crosslinker-modified complementary DNA was installed to the model-ssDEL (**5a** and **5b**) (lanes 5, 11, e, and k) by annealing to model-ssDEL-PC (**7a** and **7b**) and the coding region was transferred by Klenow fill-in to model-dsDEL-PC (**8a** and **8b**) (lanes 6, 12, f, and l). (d) Denaturing gel electrophoresis at 60 °C of the library construction steps. Lane 1 and 13: DNA ladder, 7: blank. (e) Native gel electrophoresis at RT of the library construction steps. Lane a and m: DNA ladder, g: blank.

10.1002/chem.202403233

Results and Discussion

We designed two types of NC-HPs having a hairpin structure containing deoxyuridine (**1a** and **1b** in Figure 1(a)). Both NC-HPs have deoxyuridine on the 3' side of the amino linker moiety. Using these HPs as starting materials, we investigated the feasibility of USER™ enzyme cleavage and subsequent dsDNA to ssDNA conversion strategy (Figure 1(b)). First, we treated two model-NC-DELs (prepared from NC-HPs **1a** and **1b** and a model DNA tag 2) with the USER™ enzyme and confirmed cleavage of almost all substrates by denaturing gel electrophoresis (Figure 1(d), lanes 4 and 10). Pleasingly, the USER™ enzyme worked successfully even when deoxyuridine was adjacent to nonnatural linkers (**3a**) or present in heavily distorted hairpin sections (**3b**).[5] The upper strand of the cleaved product (**4a** and **4b**) has a phosphate group at the 5' end, a substrate structure readily recognized by λ exo. Therefore, digestion with λ exo degraded only the upper strand as expected (Figure 1(e), lanes e and k), as shown in the non-denaturing gel electrophoresis diagram in Figure 1(e), achieving an inductive strategy to ssDELs with compounds at the 3' end (**5a** and **5b**). These series of products were also identified by liquid chromatography–mass spectrometry (LC-MS) (see Supporting Information). We further attempted to install a photo-crosslinking group into this model-ssDEL (**5a** and **5b** in Figure 1(c)) by annealing the primer region of the model-ssDEL to a DNA strand with a diazirine group as a photo-crosslinking group at the 5' end (**6a** and **6b**). Then, Klenow fill-in transferred the coding region of model ssDEL-PC (**7a** and **7b**) and produced model-dsDEL-PC (**8a** and **8b**), which was confirmed in lanes f and l of Figure 1(e). NC-HP-a and NC-HP-b exhibited similar degradation efficiencies, although NC-HP-a seemed to be slightly more efficient. A series of model experiments proved that the dsDEL-PC structure can be easily constructed by our method.

In DEL selection, improving the S/N ratio is a critical challenge. To achieve this, research on photo-crosslinking selection has been vigorously studied.[6] Generally, stringent washing conditions can improve the S/N ratio; however, we expected the previously reported ssDEL-PCs with compounds at the 5' end to be incompatible with stringent washing conditions because the DNA duplex structure would not tolerate them and the DEL's coding region would be lost from the target protein (Figure 2(a))[4a] [6a,c]. On the other hand, we hypothesized that our approach using dsDEL-PC would allow for the use of stringent washing conditions because the strand with the crosslinker also contains a coding region (Figure 2(b)).[4a,6a,6c] We also expected this would lead to further improvements in the S/N ratio.Therefore, we conducted model selection experiments using carbonic anhydrase IX (CAIX) as the target protein, following the method previously reported by Neri *et al*.[6d]. As shown in Figure 3(a), single-stranded DNAs (ssDNAs) conjugated with known ligands of varying binding affinities (4-sulfamoyl benzoic acid (SABA); **9a**, 4-chloro-3-sulfamoyl benzoic acid (Cl-SABA); **9b**, 3-sulfamoyl benzoic acid (m-SABA); **9c**, and amino as a negative control; **9d**), were synthesized through amide formation reaction between these ligands and **5b** (a model-ssDEL with an amino group at the 3' end). They were annealed with a complementary strand containing a photo-crosslinker to give ssDNA-PC-CAIX (**10a**-**d**). Klenow fill-in yielded dsDNAs conjugated with ligands and photo-crosslinker (dsDNA-PC-CAIXs; **11a**-**d**). The dissociation constant (K_d) values listed under each ligand structure are based on Neri's report and increase in order SABA < Cl-SABA < m-SABA.[6d] Note that all these samples have the same DNA sequence, which includes a 75-mer DNA sequence that can be quantified by quantitative polymerase chain reaction (qPCR) (see Supporting Information). The model selection experiment was conducted as follows. First, CAIX with a His tag and 50 fmol (3×10^{10} molecules) of the sample were incubated in solution.

Next, 365-nm UV irradiation was applied (denoted UV(+)) for 20 min (control experiments without UV irradiation were conducted simultaneously, denoted UV(-)). Subsequently, stringent washing conditions were applied by heating at 90 °C. It was expected that most of the DNA double strands would dissociate on the 90 °C treatment. Finally, the amount of target DNA in the solution eluted from the beads was measured by qPCR. The results are shown in the bar graph in Figure 3(b). After UV irradiation, dsDNA-PC-CAIX (**11a**-**d**) showed significantly higher DNA recovery amounts for all ligands compared to ssDNA-PC-CAIX (**10a**-**d**) (ssDNA UV(+) vs. dsDNA UV(+)). Although the recovery amount of the negative control (amino; **11d** vs. **10d**) also increased slightly, the increase in the recovery of the conjugates of SABA (**11a** vs. **10a**), Cl-SABA (**11b** vs. **10b**), or m-SABA (**11c** vs. **10c**) exceeded it, showing a 6- to 200-fold improvement. In addition, although ssDNA-PC-CAIX gave an insufficient S/N ratio, it was challenging to detect the weakest ligand, m-SABA (**10c** in ssDNA UV(+)), as a hit. However, detection was possible when utilizing dsDNA-PC-CAIX (**11c** in dsDNA UV(+)). These results reflect the significance of the direct binding between the PC-linker and the coding region, as indicated by our hypothesis in Figure 2. In the absence of UV irradiation (ssDNA UV(-) and dsDNA UV(-)), the recovery was much lower, possibly because most ligands were intolerable under stringent washing conditions. From these model experiments, we concluded that the dsDEL-PC structure could improve the S/N ratio for photo-crosslinking selection, effectively identifying low-affinity ligands and reducing false-positive hits. Finally, we conducted cell-based photo-crosslinking selection experiments using the DELs synthesized for screening (NC-DEL-001). Generally, data obtained from cell-based DEL selections tend to have more noise compared to selections using purified proteins, making data analysis more complex and the identification of hit compounds more challenging.[7] Therefore, we considered it appropriate to use cell-based DEL selection for proving the utility of our ssDEL-to-dsDEL conversion strategy.

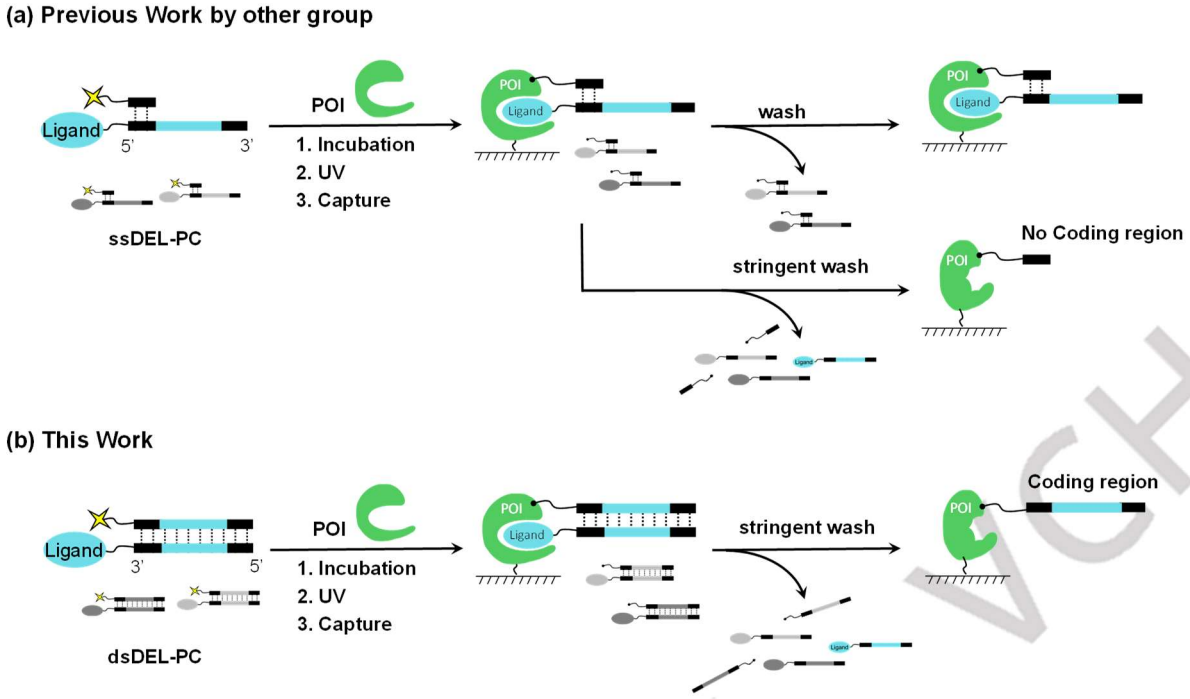


Figure 2. (a) Photo-crosslinking selection with ssDEL-PC (crosslinker does not contain a coding region). The coding information is lost under stringent washing conditions. (b) Photo-crosslinking selection with dsDEL-PC (crosslinker contains a coding region). Even under stringent washing conditions, the coding information remains associated with the POI (protein of interest) via a covalent bond.

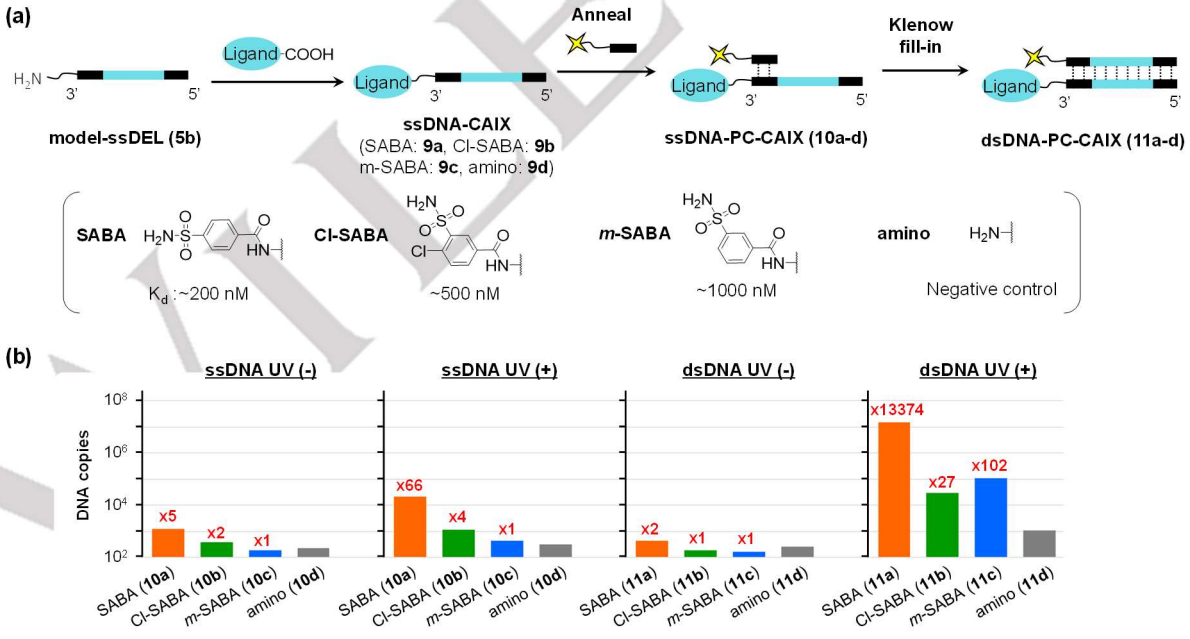


Figure 3. (a) Synthesis of ssDNA or dsDNA containing a photo-crosslinker and various CAIX ligands. (b) The results of the model selection experiment targeting CAIX with ssDNA-PC-CAIX (**10a-d**) and dsDNA-PC-CAIX (**11a-d**). ssDNA UV(-) and ssDNA UV(+) show the results of ssDNA-PC-CAIX without or with UV irradiation. dsDNA UV(-) and dsDNA UV(+) show the results of dsDNA-PC-CAIX without or with UV irradiation. The red numerals above each bar in these graphs represent the fold change of "SABA," "Cl-SABA," and "m-SABA" relative to "amino" (the negative control) within each graph.

10.1002/chem.202403233

RESEARCH ARTICLE

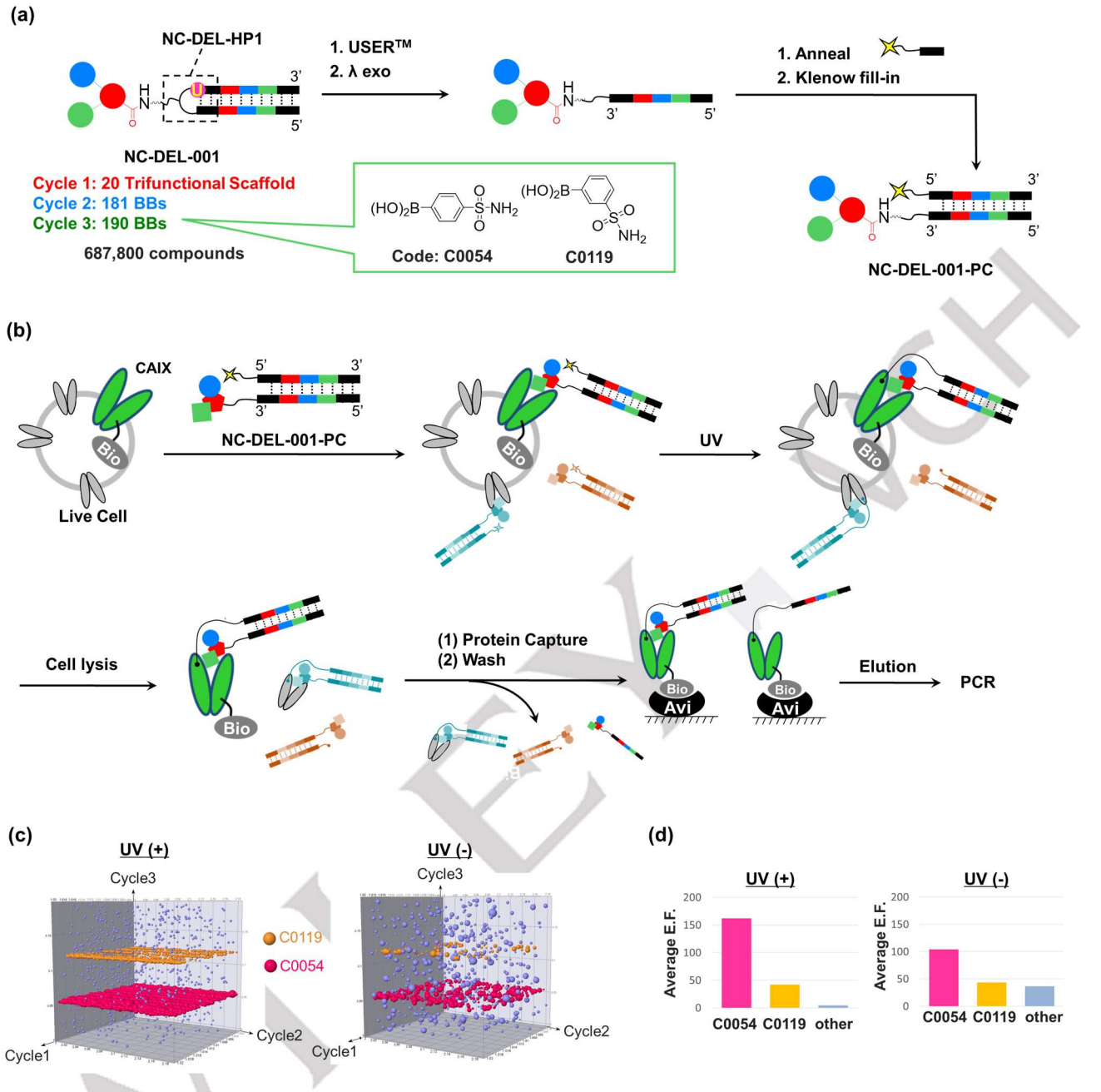


Figure 4. (a) Synthetic scheme of NC-DEL-001-PC (containing 20 × 181 × 190 = 687,800 compounds). (b) Cell-based photo-crosslinking selection experiment targeting CAIX with NC-DEL-001-PC. (c) 3D plots of DEL selection results with (left) or without (right) UV irradiation condition. UV irradiation improved the S/N ratio, making the hit structures (C0119 and C0054) clearly visible. (d) Average enrichment factor of each building block used in library construction (C0054, C0119, and other sequences) with (left) or without (right) UV irradiation.

The synthesis of DEL (NC-DEL-001) was conducted as shown in Figure 4(a): Using NC-HP-a (**1a**) as the starting material based on the results shown in Figure 1 and employing the split-and-pool method widely used in industry.[8] We synthesized NC-DEL-001, which contains 687,800 compounds, by repeating the tagging with dsDNA and the introduction of building blocks (BBs) over three cycles. In cycle 1, we introduced 20 trifunctional BBs (structures undisclosed) that were uniquely designed and synthesized to yield NC-HPs. In cycles 2 and 3, we introduced 181 and 190 BBs, respectively, to the compounds constructed in the previous cycles. Cycle 3 includes two types of sulfonamide reagents (sequence codes: C0054 and C0119), which are useful as positive controls in selections targeting CAIX. For the introduction of the photo-crosslinking group, we conducted the method established in the previous validations. We converted the NC-DEL-001 to the NC-DEL-001-PC by introducing a photo-crosslinking group (a diazirine group). High conversion efficiency was confirmed by gel electrophoresis (see Supporting

Information). The cell-based photo-crosslinking selection was conducted as shown in Figure 4(b). NC-DEL-001-PC was incubated with living cells (T-Rex-293) expressing biotinylated CAIX. After UV irradiation at 365 nm for 20 min, the cells were lysed, and the biotinylated proteins were captured using streptavidin beads and washed five times under protein-denaturing conditions (sodium dodecyl sulfate (SDS), room temperature). Subsequently, the eluted library was PCR amplified and sequenced using next-generation sequencing (NGS). The 3D plot in Figure 4(c), derived from NGS analysis, investigates the enrichment factor (EF) for each sequence, where the axes correspond to each BB used in library construction, and the dot size is proportional to the EF. Under UV irradiation conditions (UV(+)), numerous dots representing sulfonamides C0054 (magenta) and C0119 (orange), which are expected to have high binding affinity with CAIX, are visible, forming a surface. According to structure–activity relationship reported by Schreiber *et al*., C0054 derivatives have higher affinity than those of C0119.[9] This is reflected in the bar graph in Figure 4(d), which shows the average EF. On the other hand, sequences other than C0054 and C0119 were almost undetectable, indicating a clear difference in EF. The conditions without UV irradiation (UV(-)) were also examined, but the difference in EF between C0054, C0119, and other sequences was not clear. Particularly for C0119, there is hardly any difference in the average EF compared to other sequences, making it difficult to identify as a hit. In addition, in cells not expressing biotinylated CAIX, no distinctive sequences were detected, regardless of UV irradiation (see Supporting Information). In summary, the cell-based photo-crosslinking selection with the dsDEL-PC structure has been demonstrated to be a high S/N ratio evaluation system, as we expected.

Conclusion

In this study, we developed a new approach for converting dsDELs to ssDELs using cleavable hairpin-shaped DELs with deoxyuridine (NC-DELs). The USER™ enzyme cleaved deoxyuridine with high selectivity without damaging the chemical moiety or DNA. This method makes the dsDEL-PC structure readily available, and because the coding region is present on the side of the PC-linker, it allows for photo-crosslinking selection with a high S/N ratio without loss of coding under stringent washing conditions. Cell-based photo-crosslinking selection experiments using the synthesized DEL (NC-DEL-001) demonstrated a sufficient S/N ratio. Further, this method, which allows for the addition of new functional groups to the coding region after the construction of the DEL, can be adapted for various DEL selections beyond photo-crosslinking selection,[10] potentially achieving higher S/N ratios. Currently, our research group is synthesizing multiple DELs, including millamolecules (macrocyclic peptides), using NC-DEL technology, conducting selections against various targets, and advancing the applications for drug discovery.

Supporting Information

The authors have cited additional references within the Supporting Information. [11]

Acknowledgements

We thank Dr. Masahiro Kamaura, Shigetada Sasako and Junji Kamon (Nissan Chemical Corporation), Dr. Michiko Murakoshi (Daiichi Sankyo RD Novare Co., Ltd. when this study was conducted and is currently employed by Daiichi Sankyo Co., Ltd.) for helpful discussions on DNA-encoded Libraries.

Conflict of Interest

M. Niwa, J. Hayashida, M. Tokugawa, T. Nanya, M. Tanabe, N. Honda, T. Inohana, Y. Shigeta, T. Kuboyama, and S. Itoh are (or were) employees of Nissan Chemical Corporation H. Fukano was an employee of Daiichi Sankyo RD Novare Co., Ltd. when this study was conducted and is currently employed by Daiichi Sankyo Co., Ltd. The authors have a pending patent application related to the findings presented in this paper.

Keywords: DNA-encoded libraries • USER™ enzyme • dsDELs to ssDELs • photoaffinity labeling • drug discovery

[1] (a) A. A. Peterson, D. R. Liu, *Nat. Rev. Drug Discov.* **2023**, *22*, 699–722; (b) L. Plais, J. Scheuermann, *RSC Chem. Biol.* **2022**, *3*, 7–17; (c) A. Gironda-Martínez, E. J. Donckele, F. Samain, D. Neri, *ACS Pharmacol. Transl. Sci.* **2021**, *4*, 1265–1279; (d) D. Guan, J. Liu, F. Chen, J. Li, X. Wang, W. Lu, Y. Suo, F. Tang, L. Lan, X. Lu, W. Huang, *J. Med. Chem.* **2024**, *67*, 3778–3794; (e) J. W. Mason, Y. T. Chow, L. Hudson, A. Tutter, G. Michaud, M. V. Westphal, W. Shu, X. Ma, Z. Y. Tan, C. W. Coley, P. A. Clemons, S. Bonazzi, F. Berst, K. Briner, S. Liu, F. J. Zécri, S. L. Schreiber, *Nat. Chem. Biol.* **2024**, *20*, 170–179.

[2] M. A. Clark, R. A. Acharya, C. C. Arico-Muendel, S. L. Belyanskaya, D. R. Benjamin, N. R. Carlson, P. A. Centrella, C. H. Chiu, S. P. Creaser, J. W. Cuozzo, C. P. Davie, Y. Ding, G. J. Franklin, K. D. Franzen, M. L. Gefter, S. P. Hale, N. J. V. Hansen, D. I. Israel, J. Jiang, M. J. Kavarana, M. S. Kelley, C. S. Kollmann, F. Li, K. Lind, S. Mataruse, P. F. Medeiros, J. A. Messer, P. Myers, H. O'Keefe, M. C. Oliff, C. E. Rise, A. L. Satz, S. R. Skinner, J. L. Svendsen, L. Tang, K. Van Vloten, R. W. Wagner, G. Yao, B. Zhao, B. A. Morgan, *Nat. Chem. Biol.* **2009**, *5*, 647–654.

[3] (a) Y. Zhou, W. Shen, Y. Gao, J. Peng, Q. Li, X. Wei, S. Liu, F. S. Lam, J. Mayol-Llinàs, G. Zhao, G. Li, Y. Li, H. Sun, Y. Cao, X. Li, *Nat. Chem.* **2024**, *16*, 543–555; (b) B. Shi, Y. Deng, X. Li, *ACS Comb. Sci.* **2019**, *21*, 345–349; (c) A. I. Chan, L. M. McGregor, T. Jain, D. R. Liu, *J. Am. Chem. Soc.* **2017**, *139*, 10192–10195; (d) B. Shi, Y. Deng, P. Zhao, X. Li, *Bioconjug. Chem.* **2017**, *28*, 2293–2301; (e) K. E. Denton, C. J. Krusemark, *Medchemcomm* **2016**, *7*, 2020–2027; (f) L. M. McGregor, T. Jain, D. R. Liu, *J. Am. Chem. Soc.* **2014**, *136*, 3264–3270; (g) P. Zhao, Z. Chen, Y. Li, D. Sun, Y. Gao, Y. Huang, X. Li, *Angew. Chemie - Int. Ed.* **2014**, *53*, 10056–10059.

[4] (a) Y. Gui, C. S. Wong, G. Zhao, C. Xie, R. Hou, Y. Li, G. Li, X. Li, *ACS Omega* **2022**, *7*, 11491–11500; (b) G. Zhao, S. Zhong, G. Zhang, Y. Li, Y. Li, *Angew. Chemie - Int. Ed.* **2022**, *61*, DOI 10.1002/anie.202115157.

(c) G. Zhao, X. Fan, Y. Li, G. Zhang, Y. Li, *ChemBioChem* **2022**, *23*, e202200025.

[5] (a) M. Ghosh, N. V. Kumar, U. Varshney, K. V. R. Chary, *Nucleic Acids Res.* **2000**, *28*, 1906–1912; (b) M. Ghosh, N. V. Kumar, U. Varshney, K. V. R. Chary, *Nucleic Acids Res.* **1999**, *27*, 3938–3944; (c) N. V. Kumar, U. Varshney, *Nucleic Acids Res.* **1997**, *25*, 2336–2343.

[6] (a) B. Cai, A. El Daibani, Y. Bai, T. Che, C. J. Krusemark, *JACS Au* **2023**, *3*, 1076–1088; (b) H. Wei, T. Zhang, Y. Li, G. Zhang, Y. Li, *Chem. - An Asian J.* **2023**, *18*, DOI 10.1002/asia.202300652; (c) Y. Onda, G. Bassi, A. Elsayed, F. Ulrich, S. Oehler, L. Plais, J. Scheuermann, D. Neri, *Chem. - A Eur. J.* **2021**, *27*, 7160–7167; (d) A. Sannino, A. Gironda-Martínez, émile M. D. Gorre, L. Prati, J. Piazzi, J. Scheuermann, D. Neri, E. J. Donckele, F. Samain, *ACS Comb. Sci.* **2020**, *22*, 204–212; (e) Y. Zhou, J. Peng, W. Shen, X. Li, *Biochem. Biophys. Res. Commun.* **2020**, *533*, 215–222.

[7] R. Hou, C. Xie, Y. Gui, G. Li, X. Li, *ACS Omega* **2023**, *8*, 19057–19071.

[8] (a) P. R. Fitzgerald, B. M. Paegel, *Chem. Rev.* **2021**, *121*, 7155–7177; (b) E. Lenci, L. Baldini, A. Trabocchi, *Bioorganic Med. Chem.* **2021**, *41*, 116218.

[9] C. J. Gerry, M. J. Wawer, P. A. Clemons, S. L. Schreiber, *J. Am. Chem. Soc.* **2019**, *141*, 10225–10235.

[10] B. Cai, A. B. Mhetre, C. J. Krusemark, *Chem. Sci.* **2023**, *14*, 245–250.

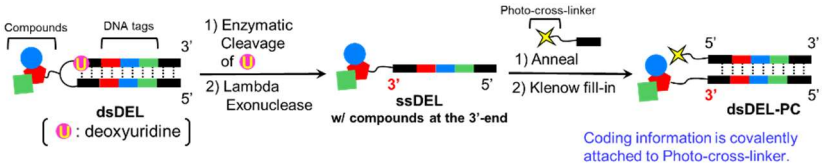
[11] G. Bassi, N. Favalli, M. Vuk, M. Catalano, A. Martinelli, A Trenner, A Porro, S. Yang. C. L. Tham, M. Morglu, W. W. Yue, S. J. Conwey, P. K. Vogt, A. A. Sartori, J. Scheuermann, D. Neri, *Adv. Sci.* **2020**, *7*, 2001970.

10.1002/chem.202403233

WILEY VCH

RESEARCH ARTICLE

Entry for the Table of Contents



In this study, we developed a novel method for converting double-stranded DNA-encoded libraries (dsDELs) to single-stranded DELs (ssDELs) with compounds at the 3' end using cleavable-deoxyuridine. Moreover, such ssDELs are converted to dsDELs with the coding information covalently attached to photo-crosslinkers (dsDELs-PC). This approach enhances photo-crosslinking selection efficiency, maintaining high signal-to-noise (S/N) ratios under stringent conditions.