

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

Alamar Biosciences, Inc.
Petitioner

v.

Olink Proteomics AB
Patent Owner

U.S. Patent No. 7,883,848

**DECLARATION OF MARIA P. GARCIA
UNDER 37 C.F.R. § 1.68**

I, Maria P. Garcia, hereby declare as follows:

1. I am making this declaration on behalf of the Petitioner for the Petition for *Inter Partes* Review of U.S. Patent No. 7,883,848. Unless otherwise stated, the facts stated in this declaration are based on my personal knowledge.

2. I am the Intellectual Property Research Librarian at Jones Day, 555 South Flower Street, Fiftieth Floor, Los Angeles, California 90071-2300. I received a Bachelor of Science in Chemical Engineering from the University of Southern California in May 1989. I worked in the Circulation and Acquisition Department at the Education and Information Science Library at the University of Southern California from December 1983 to February 1989. I formerly worked at Lyon & Lyon, where I was responsible for all aspects of patent and trademark research.

3. I am familiar with library procedures for receiving, cataloging, and indexing periodicals in libraries. I am also familiar with library procedures for entering and indexing print and electronic sources in publicly available databases such as MEDLINE, PubMed, the Internet Archive Wayback Machine, and University of Wisconsin Catalog, among others.

Kanan

4. **Exhibit 1010** is Kanan et al., “Reaction discovery enabled by DNA-templated synthesis and *in vitro* selection,” *Nature* 2004 Sept.; 431:545–549 (hereinafter “Kanan”), published on September 30, 2004.

5. Appendix A to this declaration is a copy of the MEDLINE database record for Kanan. Appendix A indicates that Kanan was published in *Nature* Vol. 431, No. 7008, pp. 545–549. Appendix A indicates the Kanan article was entered into the MEDLINE database on October 14, 2004. The general public would have been able to search the database and locate the Kanan article on or shortly after the entry date.

6. Appendix B to this declaration is a copy of the cover of Vol. 431, No. 7008 of *Nature* and the Kanan article that spans pages 545–549 of that volume from the University of Wisconsin – Madison. I have compared the copy of the Kanan article from the University of Wisconsin library with Exhibit 1010 and attest that Exhibit 1010 is a true and correct copy of the Kanan article published on September 30, 2004. The table of contents of the volume bears a library stamp from the University of Wisconsin library indicating a date of October 11, 2004. According to library procedure, Vol. 431, No. 7008 of *Nature*, including the Kanan article therein, would have been shelved, locatable by search, and made available to library patrons or visitors (*i.e.*, the general public) for review and/or photocopying within a week of its stamped date.

Kanan Supplementary Information

7. **Exhibit 1013** is Kanan et al., “Reaction discovery enabled by DNA-templated synthesis and *in vitro* selection, Supplementary Information,” 431 *Nature* 2004 (hereinafter “Kanan Supplementary Information”).

8. Kanan Supplementary Information is available online at *Nature*'s website at the current URL <https://www.nature.com/articles/nature02920>. *Nature*'s website indicates Kanan and its Supplementary Information was published on September 30, 2004. I contacted *Nature* and confirmed Kanan's Supplementary Information was published on September 30, 2004. Appendix C is a true and correct copy of the e-mail I received from *Nature* confirming the public availability date. Accordingly, Kanan Supplementary Information was publicly available online at *Nature*'s website as of September 30, 2004.

9. In addition, I also reviewed the Internet Archive's Wayback Machine to determine when it first archived the Kanan Supplementary Information. The Internet Archive is a website that provides access to a digital library of Internet sites and other cultural artifacts in digital form. Like a paper library, it provides free access to researchers, historians, scholars, and the general public. The Internet Archive's Wayback Machine makes it possible to browse billions of pages stored in the Internet Archive's web archive. Visitors to the Wayback Machine can search archives by URL (i.e., a website address). If archived records for a URL are available, the visitor will be presented with a display of available dates. The visitor may select one of those dates, and begin browsing an archived version of the Web. Links on archived files in the Wayback Machine point to other archived files (whether HTML pages or other file types), if any are found for the URL indicated by a given link. For instance, the

Wayback Machine is designed such that when a visitor clicks on a hyperlink on an archived page that points to another URL, the visitor will be served the archived file found for the hyperlink's URL with the closest available date to the initial file containing the hyperlink. The archived data made viewable and browsable by the Wayback Machine is obtained by use of web archiving software that automatically stores copies of files available via the Internet, each file preserved as it existed at a particular point in time.

10. The Internet Archive assigns a URL on its site to the archived files in the format `http://web.archive.org/web/[Year in yyyy][month in mm][day in dd][Time code in hh:mm:ss]/[Archived URL]` (an "extended URL"). For example, the extended URL `http://web.archive.org/web/19970126045828/http://www.archive.org/` would be the URL for the record of the Internet Archive home page HTML file (`http://www.archive.org/`) archived on January 26, 1997 at 4:58 a.m. and 28 seconds (1997/01/26 at 04:58:28).

11. A web browser may be set such that a printout from it will display the URL of a web page in the printout's footer. The date indicated by an extended URL applies to a preserved instance of a file for a given URL, but not necessarily to any other files linked therein. Thus, in the case of a page constituted by a primary HTML file and other separate files (e.g., files with images, audio, multimedia, design elements, or other embedded content) linked within that primary HTML file, the primary HTML file and

the other files will each have their own respective extended URLs and may not have been archived on the same dates.

12. In 2004, the general public would have been able to navigate to Kanan's Supplementary Information from *Nature's* homepage (www.nature.com/nature) by navigating to the September 30, 2004 issue, Volume 431.

13. The Internet Archive extended URL for the contents of *Nature's* September 30, 2004 issue is <https://web.archive.org/web/20041017141016/http://www.nature.com/cgi-taf/DynaPage.taf?file=/nature/journal/v431/n7008/index.html>, which shows that the link was archived on October 17, 2004 at 14:10:16 (Appendix D).

14. On that page, the user could then click on the link to Kanan Supplementary Information, titled "Supplementary Information," as shown in Appendix D at 5 (highlighted). This link takes the user to a webpage with a direct link to the Supplementary Information, again titled "Supplementary Information," as shown in Appendix E. The extended URL for the direct link to Kanan's Supplementary Information is <https://web.archive.org/web/20041028131204/http://www.nature.com/nature/journal/v431/n7008/supinfo/nature02920.html>, which shows that the link was archived on October 28, 2004 at 13:12:04 (Appendix E).

15. The Internet Archive extended URL for Kanan's Supplementary Information is <https://web.archive.org/web/20041028221019/http://www.nature.com/nature/journal/v431/n7008/extref/nature02920-s1.pdf>. (Appendix F). Because the archived PDF

document does not print a URL in the footer, I am also attaching as Appendix G a printout from a web browser reflecting the first page of Kanan's Supplementary Information, which shows the extended URL. The extended URL shows that Kanan's Supplementary Information (Appendix G) was archived on October 28, 2004 at 22:10:19.

16. I have compared the copy of the Kanan Supplementary Information from the Wayback Machine captured on October 28, 2004 (Appendix F) with Exhibit 1013 and attest that Exhibit 1013 is a true and correct copy of the Kanan Supplementary Information captured on October 28, 2004.

Famulok

17. **Exhibit 1012** is Famulok, "Bringing picomolar protein detection into proximity," *Nature Biotechnology* 2002 May; 20:448–449 (hereinafter "Famulok").

18. Appendix H to this declaration is a copy of the MEDLINE database record for Famulok. Appendix H indicates that Famulok was published in *Nature Biotechnology* Vol. 20, No. 5, pp. 448–449. Appendix H indicates the Famulok article was entered into the MEDLINE database on September 5, 2002. The general public would have been able to search the database and locate the Famulok article on or shortly after the entry date.

19. Appendix I to this declaration is a copy of the cover of Vol. 20, No. 5 of *Nature Biotechnology* and the Famulok article that spans pages 448–449 of that volume from

the University of Wisconsin – Madison. I have compared the copy of the Famulok article from the University of Wisconsin library with Exhibit 1012 and attest that Exhibit 1012 is a true and correct copy of the Famulok article available on May 1, 2002. The table of contents of the volume bears a library stamp from the University of Wisconsin library indicating a date of May 8, 2002. According to library procedure, Vol. 20, No. 5 of *Nature Biotechnology*, including the Famulok article therein, would have been shelved, locatable by search, and made available to library patrons or visitors (*i.e.*, the general public) for review and/or photocopying within a week of its stamped date.

Declaration

I declare that all statements made herein on my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Dated: August 22, 2024

By: Maria Garcia
Maria P. Garcia

APPENDIX A
TO
DECLARATION OF
MARIA P. GARCIA

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AN 2004488734 MEDLINE [Full-text](#)
DN PubMed ID: 15457254
TI [Reaction discovery enabled](#) by DNA-templated synthesis and in vitro
selection.
AU [Kanan Matthew W](#)
CS Department of Chemistry and Chemical Biology, Harvard University, 12
Oxford Street, Cambridge, Massachusetts 02138, USA.
AU Rozenman Mary M; Sakurai Kaori; Snyder Thomas M; Liu David R
SO Nature, (2004 Sep 30) Vol. 431, No. 7008, pp. 545-9.
Journal code: 0410462. E-ISSN: 1476-4687. L-ISSN: 0028-0836.
Report No.: PMC-PMC2814052; MID-NIHMS171113.
DOI <http://dx.doi.org/10.1038/nature02920>
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APPENDIX B
TO
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September 2004

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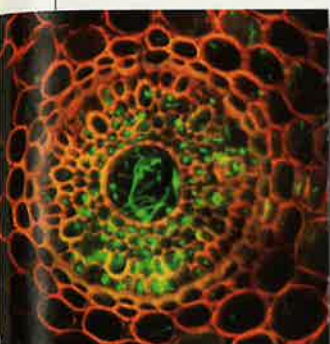


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Published online 29 August 2004, doi:10.1038/nature02899

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Paul Smaglik

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Alamar v. Olink

Reaction discovery enabled by DNA-templated synthesis and *in vitro* selection

Matthew W. Kanan, Mary M. Rozenman, Kaori Sakurai,
Thomas M. Snyder & David R. Liu

Department of Chemistry and Chemical Biology, Harvard University,
12 Oxford Street, Cambridge, Massachusetts 02138, USA

Current approaches to reaction discovery focus on one particular transformation. Typically, researchers choose substrates based on their predicted ability to serve as precursors for the target structure, then evaluate reaction conditions^{1–6} for their ability to effect product formation. This approach is ideal for addressing specific reactivity problems, but its focused nature might leave many areas of chemical reactivity unexplored. Here we report a reaction discovery approach that uses DNA-templated organic synthesis^{7–10} and *in vitro* selection to simultaneously evaluate many combinations of different substrates for bond-forming reactions in a single solution. Watson–Crick base pairing controls the effective molarities of substrates tethered to DNA strands; bond-forming substrate combinations are then revealed using *in vitro* selection for bond formation, PCR amplification and DNA microarray analysis. Using this approach, we discovered an efficient and mild carbon–carbon bond-forming reaction that generates an enone from an alkyne and alkene using an inorganic palladium catalyst. Although this approach is restricted to conditions and catalysts that are at least partially compatible with DNA, we expect that its versatility and efficiency will enable the discovery of additional reactions between a wide range of substrates.

A reaction discovery system capable of simultaneously evaluating many combinations of substrates for bond-forming reactivity in a single solution must meet several requirements. First, the system must organize complex substrate mixtures into discrete pairs that can react (or not react) without affecting the reactivity of the other substrate pairs. Second, the system requires a general method for separating reactive substrate pairs from unreactive pairs. Last, the reactive substrate pairs must be identified efficiently.

Recent developments in DNA-templated organic synthesis^{7–10} indicate that DNA annealing can organize many substrates in a single solution into DNA sequence-programmed pairs. To this end, we prepared two pools of DNA-linked substrates, with n substrates in pool A and m substrates in pool B. Each substrate in pool A is covalently linked to the 5' end of a set of DNA oligonucleotides containing one 'coding region' (uniquely identifying that substrate) and one of m different 'annealing regions' (Fig. 1a). Each of the m substrates in pool B is attached to the 3' end of an oligonucleotide containing a coding region that uniquely identifies the substrate and complements one of the m annealing regions in pool A.

When pools A and B are combined in a single aqueous solution at nanomolar concentrations, Watson–Crick base pairing organizes the mixture into $n \times m$ discrete pairs of substrates attached to complementary sequences. Only substrates linked to complementary oligonucleotides experience effective molarities in the millimolar range⁸; substrates linked to non-complementary oligonucleotides experience nanomolar solution concentrations and hence do not react with each other at a significant rate. This effective molarity-based design enables reactions that might otherwise be suppressed by the preferential dimerization of one or both substrates. The possibility of interference by the structure of DNA during reactions between substrates is minimized by using long and flexible substrate–DNA linkers¹¹.

The separation of reactive pairs of substrates is based on selection concepts used in the directed evolution of catalytic RNA and DNA^{12,13} (Fig. 1b). We covalently linked each pool B substrate to its corresponding oligonucleotide by a linker containing a biotin group and a disulphide bond (Fig. 1b and Supplementary Information). After incubation under a set of chosen reaction conditions, followed by cleavage of the disulphide bonds, only pool A sequences encoding bond formation between a pool A and pool B substrate remain covalently linked to biotin. Streptavidin affinity selection of the resulting solution separates biotinylated from non-biotinylated sequences. In contrast to many existing reaction discovery screens^{1,5} focused on a particular reaction type, our selection-based approach does not depend on any specific substrate or product property but instead can identify all substrate pairs capable of forming a covalent bond under the reaction conditions.

Reactive substrate pairs are identified by amplification of the selected sequences by polymerase chain reaction (PCR) followed by DNA microarray analysis (see below). Because PCR amplification is extremely sensitive, femtomole quantities of substrates are sufficient for the entire reaction discovery process.

We prepared pool A and pool B containing 12 substrates each (Fig. 2a), representing 144 heterocoupling combinations (see Supplementary Methods and Supplementary Fig. S1 for details). We also prepared DNA-linked substrates to enable the detection of homocoupling of any of the 24 different substrates, bringing the total number of unique substrate combinations to 168 (Fig. 2a). The 24 substrates in Fig. 2a were chosen to represent simple functional groups commonly encountered in organic molecules. New reactions forming bonds between simple functionalities are of special interest because they might provide more accessible alternatives to coupling reactions that require more complex substrates. Although our approach requires the preparation of DNA-linked substrates, a single nanomole-scale preparation of each pool provided sufficient material for more than 1,000 reaction discovery experiments that could collectively evaluate more than 168,000 combinations of substrates and reaction conditions.

To test the ability of our system to detect a single reactive combination of substrates out of 168 possibilities, we combined pool A and pool B in the presence of Cu(I), conditions known to promote a cycloaddition between a terminal alkyne (A5 in Fig. 2a) and an azide (B9 in Fig. 2a)¹⁴. Pool A members (2 pmol total) were combined with 2 pmol total of pool B members (12 fmol of material encoding each possible substrate combination) in the presence of 500 μ M Cu(I) in a total volume of 12 μ l. After 10 min at 25 °C, the salts were removed and disulphide linkages were cleaved with 0.1 M tris-carboxyethylphosphine hydrochloride. A control experiment lacking Cu(I) but identical in all other respects was also performed.

Sequences encoding bond-forming substrate pairs were captured with streptavidin-linked magnetic particles and amplified by PCR with a DNA primer labelled with the cyanine fluorophore Cy3. For comparison, an aliquot of the pool A sequences before selection was amplified by PCR with a Cy5-labelled primer. The Cy3-labelled and Cy5-labelled PCR products were combined and hybridized to a DNA microarray containing all 168 possible reaction-encoding sequences. The ratios of Cy3 (green) to Cy5 (red) fluorescence for all array locations were calculated and ordered by rank, and spots with green/red fluorescence ratios significantly higher than the majority of spots (in the experiments below, ratios above 1.5) were considered to be positive. A pre-quantified internal standard (bottom right corner of the array images in Fig. 2b) corresponding to a moderate level of reactivity was used as a positive control and as a reference for comparing different arrays.

For the experiment performed in the presence of Cu(I), the array spot corresponding to the combination of the alkyne (A5) and the azide (B9) was the sole spot that had a significant green/red fluorescence ratio (A5 + B9 = 8.5, standard = 2.8; see Fig. 2b and Supplementary Data). Alamar v. Olink

in the absence of any added metal yielded no green array spots other than the standard (Fig. 2b). These results show that the above method can detect a single bond-forming substrate combination from a complex mixture of 168 combinations, and confirm the high chemoselectivity characteristic of the Sharpless-modified Huisgen cycloaddition reaction¹⁵.

Cu(I) damages DNA by promoting radical-mediated processes¹⁶. Indeed, we observed 48% degradation of a DNA oligonucleotide exposed for 10 min to the Cu(I)-containing conditions (Supplementary Data). The successful detection of the alkyne-azide cycloaddition under conditions that degrade DNA shows that reaction conditions do not need to be fully DNA compatible, because the extreme sensitivity of PCR amplification¹⁷ enables bond formation to be revealed even if a significant fraction of the total DNA in an experiment is destroyed.

To validate this reaction discovery system further, we performed a selection for bond formation after exposure to organic reagents (1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride and *N*-hydroxysuccinimide at pH 6.0) known to promote the coupling of amines with carboxylic acids¹⁸. Microarray analysis revealed one positive; this spot indeed corresponded to the combination of the carboxylic acid from pool A (A10) and the amine from pool B (B12) ($A_{10} + B_{12} = 6.9$, standard = 15.6; see Fig. 2b and Supplementary Data).

After successfully 'rediscovering' known bond-forming reactions, we examined the reactivity of a simple Pd(II) salt. Inorganic Pd salts and complexes containing Pd are known to mediate diverse coupling reactions¹⁹, indicating that Pd can activate a variety of organic

functional groups. Selection for bond formation in the presence of 500 μ M Na_2PdCl_4 for 1 h at 37 °C resulted in five strong positives (A_7+B_3 , A_5+B_3 , A_4+B_8 , A_5+B_5 and A_5 homocoupling) with significant green/red fluorescence ratios (3.6 to 2.6; standard = 2.9). In addition, we observed five weaker positives (A_9+B_3 , A_8+B_3 , A_8+B_8 , A_5+B_8 and A_5+B_9) with lower, but possibly significant, green/red fluorescence ratios ranging from 1.9 to 1.6 (Fig. 2b and Supplementary Data). One positive representing the combination of aryl iodide (A_7) and acrylamide (B_3) is consistent with the well-known Heck reaction^{20,21} (assuming the formation of some Pd(0) under the reaction conditions). Other positives could be rationalized with mechanistic steps preceded in known Pd-mediated chemistries. For example, the spot indicating bond formation between an olefin (A_4) and an aryl boronic acid (B_8) is consistent with transmetalation of Pd(II) by the aryl boronic acid²² followed by the insertion of an olefin into the Pd-aryl bond and subsequent β -hydride elimination²³.

To determine whether these array results reflect genuine bond-forming events, we examined the putative reactions corresponding to the above ten spots in separate DNA-templated reactions. Denaturing polyacrylamide gel electrophoresis (PAGE) analysis indicated that all five strong positives and three of the five weak positives corresponded to authentic DNA-templated reactions, whereas two weak positives (A_9+B_3 and A_5+B_9) showed little or no product formation (Fig. 3 and Supplementary Fig. S2). We observed no product formation in control reactions in which Na_2PdCl_4 was omitted, or in which either of the reactive substrates was replaced with an unreactive alkane group. These findings are

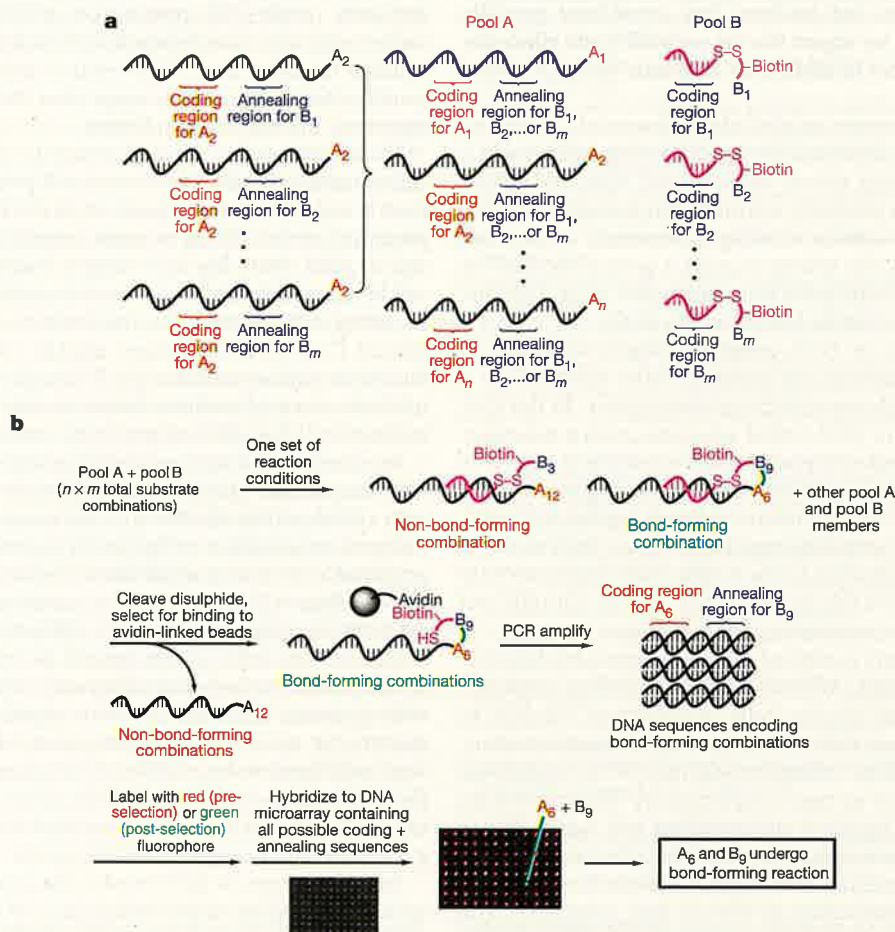


Figure 1 Key elements of a new approach to reaction discovery. **a**, Two pools of DNA-linked organic functional groups that associate each of $n \times m$ substrate

combinations with a unique DNA sequence. **b**, A general one-pot selection and analysis method for the detection of bond-forming reactions between DNA-linked substrates.

consistent with the detection of Pd-dependent reactions that couple substrate groups and do not couple the functionality present in DNA.

We further characterized the PAGE-validated Pd-mediated DNA-templated reactions using matrix assisted laser desorption ionization–time-of-flight (MALDI–TOF) mass spectrometry (Fig. 3). The observed product masses are consistent with covalent bond formation. We suggest possible structures consistent with the observed masses for each of the reaction products but note that other products or mixtures of products are also consistent with the data. Together with the PAGE analysis, these results show that this reaction discovery system reliably and efficiently reveals bond-forming events even under conditions that cause multiple combinations of substrates to react.

Increasing the stringency of the reaction conditions by decreasing the temperature to 25 °C and quenching the reaction after 20 min

decreased the number of strong positives to four (A5+B5, A5+B3, A4+B8 and A5 homocoupling; green/red fluorescence ratios = 3.7 to 2.7; standard = 3.5) and the number of weak positives to two (A5+B8 and A5+B9; fluorescence ratios = 1.9 and 1.7; see Fig. 2b and Supplementary Data). Consistent with these results, the five authentic bond-forming reactions among this set (all four strong positives and weak positive A5+B8) showed substantial product formation by PAGE analysis when performed under the 25 °C conditions, whereas the three additional reactions listed in Fig. 3 but not appearing as positives in the 25 °C experiment (A8+B8, A8+B3 and A7+B3) showed significantly weaker product formation (Fig. 3). Varying the stringency of the reaction conditions before selection can therefore efficiently distinguish substrate combinations on the basis of their level of reactivity.

The above results suggested that the Pd(II)-mediated carbon–carbon bond formation between a simple terminal alkyne and

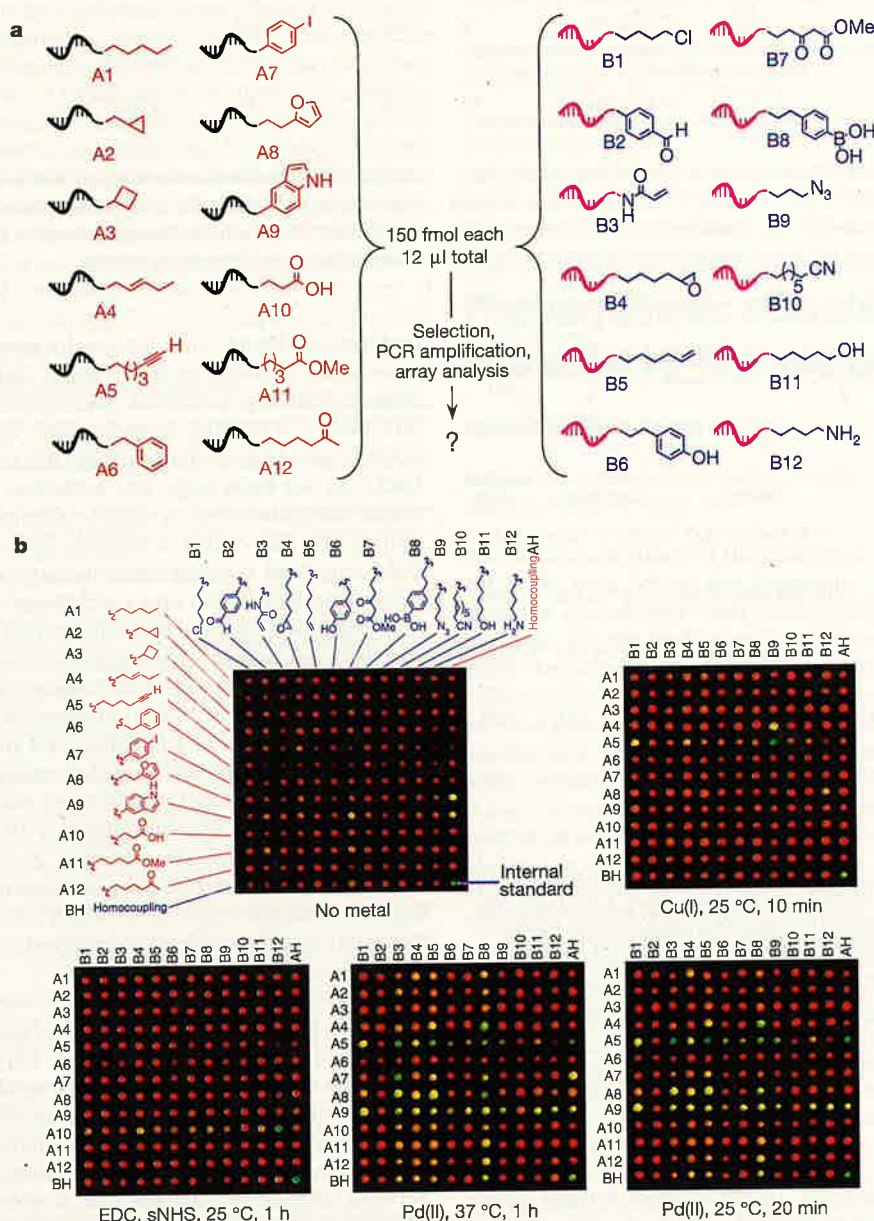


Figure 2 Results from reaction discovery selections and analysis. **a**, Pool A and pool B substrates used in this work. **b**, Qualitative results of reaction discovery selections after exposure to the reaction conditions listed below each array image. Spots that are significantly green suggest bond formation between the corresponding substrates

(quantitative fluorescence ratios in Fig. 3 and the Supplementary Data are used for actual interpretations). The 840 reaction possibilities in these five experiments were evaluated by one researcher in two days. See Supplementary Methods for detailed reaction conditions.

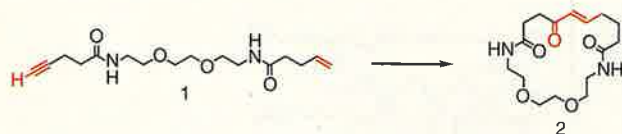
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Substrates		Green/red fluorescence ratios		DNA-templated yields (%)		Product consistent with observed mass
		37°C	25°C	37°C	25°C	
A5	B5	2.7	3.7	35	31	
A5	B3	3.5	3.1	28	20	
A5	B8	1.6	1.9	36	34	
A5	Homocoupling	2.6	2.7	45	42	
A4	B8	3.0	2.8	57	39	
A8	B8	1.8	<1.2	30	10	
A8	B3	1.8	<1.2	19	<10	
A7	B3	3.6	<1.2	39	14	

Figure 3 Characterization in a DNA-templated format of array positives resulting from exposure to 500 μ M Na_2PdCl_4 at 37 °C for 1 h, or at 25 °C for 20 min. Putative reactions were screened by PAGE and MALDI-TOF mass spectrometric analysis (Supplementary Information). Template architectures and reaction conditions were chosen to match those

used in the selection rather than to maximize product yields. Product structures other than those proposed are possible. The green/red fluorescence ratio for the internal standard at 37 °C and 25 °C is 2.9 and 3.5, respectively. For A8 + B3, reactivity was not sufficient to obtain reliable product mass characterization.



Entry	Metal(s)	Solvent	Conditions	Isolated yield
a	1 equiv. Na_2PdCl_4	1 M NaCl in H_2O	25 °C, 15 h	86%
b	5 mol% Na_2PdCl_4 1 equiv. CuCl_2	100 mM NaCl in H_2O	25 °C, 2 h	90%
c	5 mol% Na_2PdCl_4 1 equiv. CuCl_2	9:1 THF: H_2O	25 °C, 4 h	91%
d	15 mol% Na_2PdCl_4 1 atm O_2	9:1 THF: H_2O	25 °C, 14 h	73%
e	1 equiv. CuCl_2	100 mM NaCl in H_2O	25 °C, 4 h	0%
f	1 equiv. CuCl	100 mM NaCl in H_2O	25 °C, 4 h	0%

Figure 4 Characterization of a new alkyne-alkene macrocyclization reaction in a non-DNA-templated format. The macrocyclic enone product (**2**) was characterized by ^1H -NMR, ^{13}C -NMR, COSY, UV-visible spectrometry and high-resolution mass electrospray (Supplementary Information). We speculate that product formation proceeds through the following sequence: soft deprotonation of the alkyne to form a $\text{Pd}(\text{II})$ -alkynyl intermediate; insertion of the alkene into the Pd -alkyne bond; β -hydride elimination to form a conjugated enyne; $\text{Pd}(\text{II})$ -catalysed hydration of the alkyne to form an enol π -allyl Pd complex; enol tautomerization and π -allyl Pd protonation to generate the *trans*-enone.

terminal alkene (A5+B5) proceeds efficiently to generate a possible enone product (Fig. 2b and Fig. 3), prompting a detailed investigation of this reaction. We synthesized a small-molecule substrate (**1**) to study a non-DNA-templated, intramolecular version of this alkyne-alkene coupling reaction on a multi-milligram scale (Fig. 4). The addition of **1** to one equivalent of $\text{Pd}(\text{II})$ in 1 M aqueous NaCl over 15 h followed by purification by reverse-phase high-

performance liquid chromatography provided the 20-membered macrocyclic *trans*-enone **2** as a single olefin stereoisomer in 86% isolated yield (Fig. 4, entry a). The structure of **2** was confirmed by ^1H -NMR, ^{13}C -NMR, homonuclear correlated spectroscopy (COSY) and high-resolution mass spectrometry (Supplementary Data). To our knowledge, this is the first example of macrocyclic enone formation from a simple alkyne-alkene precursor. The finding also shows that a reaction discovered by a selection for DNA-templated covalent bond formation can operate in a non-DNA-templated format on a much larger (10^9 -fold) scale.

Because the formation of an enone from an alkyne and an alkene represents an oxidative coupling, we proposed that the reaction could be performed with catalytic quantities of Pd by introducing an oxidant such as CuCl_2 + air to reoxidize $\text{Pd}(0)$ to $\text{Pd}(\text{II})$ (ref. 24). Indeed, the addition of **1** to 5 mol% Pd and 1 equivalent of CuCl_2 over 2 h in water at 25 °C provided enone **2** as the sole observed product in 90% isolated yield (Fig. 4, entry b). Significantly, this reaction maintains its high efficiency in solvent containing 9:1 tetrahydrofuran:water (Fig. 4, entry c). Product was formed in similar yields, although at a slower rate, using 1 atm O_2 instead of CuCl_2 to reoxidize $\text{Pd}(0)$ (Fig. 4, entry d). Control reactions with CuCl_2 or CuCl alone yielded no observed product formation (Fig. 4, entries e and f).

The discovery of this alkyne-alkene coupling reaction suggests the value of searching a large number of substrate combinations for unexpected reactions. While aqueous $\text{Pd}(\text{II})$ has been known for more than 40 years to oxidize alkenes rapidly to ketones^{24,25}, which are unreactive towards alkynes under these conditions (Fig. 2b and Supplementary Data below Supplementary Fig. S4), our approach to reaction discovery revealed that carbon-carbon bond formation between alkynes and alkenes can outcompete alkene oxidation. Although other enone-forming coupling reactions such as the Horner-Wadsworth-Emmons reaction²⁶ or aldol condensation are known, the mild reaction conditions, simple hydrocarbon starting materials and high efficiency of the transformation discovered here might render it an attractive alternative for addressing

some macrocyclization problems in organic synthesis. In addition, the compatibility of this reaction with both organic and aqueous solvents might facilitate the synthesis of highly functionalized macrocyclic enones, of which there are numerous known biologically active examples²⁷.

Six of the eight observed heterocoupling reactions in Fig. 3 involve a substrate that couples with itself under the reaction conditions (reactions involving either the alkyne or the aryl boronic acid; see Supplementary Data below Supplementary Fig. S4). In a DNA-templated format, heterocoupling without competitive homocoupling is possible because a substrate experiences its sequence-programmed heterocoupling partner at a much higher effective concentration than the concentration of another identical substrate molecule.

Biological macromolecules have previously been used to address specific problems in chemical reactivity^{28–30}. In contrast, our approach uses the ability of nucleic acids to direct effective molarities and undergo *in vitro* selection and amplification to reveal bond-forming reactivity in a general manner. Once DNA-linked substrate pools are prepared, a single researcher can evaluate thousands of combinations of substrates and reaction conditions in a two-day experiment. Our approach requires water-soluble catalysts and aqueous reaction conditions that are at least partially compatible with DNA; however, as illustrated by the alkyne–alkene coupling reaction, some of the discovered reactions will proceed in a non-DNA-templated format in organic solvents. We expect that using this approach in a broad examination of reaction conditions including transition-metal complexes, Lewis acids, mild oxidants or reductants, and organic reagents will lead to the discovery of additional bond-forming reactions between simple and relatively unreactive functional groups. □

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Photosynthetic microbial mats in the 3,416-Myr-old ocean

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Recent re-evaluations of the geological record of the earliest life on Earth have led to the suggestion that some of the oldest putative microfossils¹ and carbonaceous matter were formed through abiotic hydrothermal processes^{2,3}. Similarly, many early Archaean (more than 3,400-Myr-old) cherts have been reinterpreted as hydrothermal deposits rather than products of normal marine sedimentary processes^{4,5}. Here we present the results of a field, petrographic and geochemical study testing these hypotheses for the 3,416-Myr-old Buck Reef Chert, South Africa. From sedimentary structures and distributions of sand and mud, we infer that deposition occurred in normal open shallow to deep marine environments. The siderite enrichment that we observe in deep-water sediments is consistent with a stratified early ocean^{6,7}. We show that most carbonaceous matter was formed by photosynthetic mats within the euphotic zone and distributed as detrital matter by waves and currents to surrounding environments. We find no evidence that hydrothermal processes had any direct role in the deposition of either the carbonaceous matter or the enclosing sediments. Instead, we conclude that photosynthetic organisms had evolved and were living in a stratified ocean supersaturated in dissolved silica^{8,9} 3,416 Myr ago.

The Buck Reef Chert (BRC) is a 250–400-m-thick unit of carbonaceous and ferruginous chert that regionally marks the base of the Kromberg Formation in the Barberton greenstone belt, South Africa¹⁰ (Fig. 1). Along the west limb of the Onverwacht anticline, it includes three main facies reflecting deposition under progressively deeper-water conditions: first, a basal evaporite facies 5–40 m thick that interfingers with a siliceous sandstone

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APPENDIX E
TO
DECLARATION OF
MARIA P. GARCIA

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Kanan, M. W. *et al.* Reaction discovery enabled by DNA-templated synthesis and *in vitro* selection
Nature **431**, 545-549 (2004) *Letters to Nature*

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APPENDIX F
TO
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“Reaction Discovery Enabled by DNA-Templated Synthesis and In Vitro Selection”

Supplementary Information Index

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Supplementary Methods

General Experimental Methods

DNA oligonucleotides were synthesized using standard automated solid-phase phosphoramidite coupling methods on a PerSeptive Biosystems Expedite 8909 DNA synthesizer. All reagents and phosphoramidites for DNA synthesis were purchased from Glen Research. Oligonucleotides were purified by reverse-phase High Pressure Liquid Chromatography (HPLC) using a C18 stationary phase and an acetonitrile/100 mM triethyl ammonium acetate (TEAA) gradient and quantitated using UV spectroscopy. Labeled oligonucleotides were purified by HPLC and characterized by MALDI-TOF mass spectroscopy using a hydroxypicolinic acid/ammonium citrate matrix. Observed masses were within 0.075% of expected masses. All H₂O used in the manipulations below was obtained from a Milli-Q purification system.

For purification and characterization of small-molecule products arising from non-DNA-templated reactions, analytical HPLC was performed on an Agilent 1100 Series instrument equipped with a Varian Pursuit C18 reverse-phase semi-preparative column. Preparative HPLC was performed on a Varian ProStar instrument equipped with a Varian Pursuit C18 1.0 gram-scale preparative reverse-phase column. Proton magnetic resonance (¹H-NMR), carbon nuclear magnetic resonance (¹³C-NMR), and COSY spectra were obtained using Varian Mercury 400 (400 MHz) and Varian INOVA500 (500 MHz) NMR spectrophotometers at 25°C. Small-molecule products were further analyzed by electrospray mass spectroscopy.

Preparation of Pool A DNA-linked Substrates

Pool A Oligonucleotide Sequence Design

Complete pool A templates contain (from 5' to 3'): a 15-base 5' PCR primer-binding site, a 15-base annealing region complementary to a pool B sequence, an 8-base constant region, a 12-base coding region used to identify the attached substrate, and a 15-base 3' PCR primer binding site (Fig. S1). Each pool A small-molecule substrate is attached to a pool A oligonucleotide through an amide linkage at the 5' end of the oligonucleotide.

Preparation of Component Oligonucleotides for the Modular Assembly of Pool A

Pool A DNA-linked substrates were assembled in a convergent, modular manner using four types of oligonucleotides: “coding”, “annealing”, “splint”, and “substrate-linked” (Fig. S1). The description and preparation of each type are detailed below.

Coding oligonucleotides are 35 bases long and consist of (5' to 3'): a 15-base PCR primer-binding site, a 12-base coding region, an 8-base constant region, and a 3'-modified group (arising from the C7 amino CPG). This 3' modification ensured that only the annealing oligonucleotides (which have natural 3'-OH groups,) were substrates for primer extension during pool A assembly (Fig. S1).

Annealing oligonucleotides are 28 bases long and contain (5' to 3'): a 5'-phosphate installed with Chemical Phosphorylating Reagent II, a 5-base portion of the 5'-PCR primer binding site, a unique 15-base annealing region, and an 8-base constant region complementary to the 8-base constant region present in the coding oligonucleotide. The 5'-phosphate group enabled these sequences to undergo a ligation reaction with the 3'-OH of the “substrate-labeled” oligonucleotides during pool A assembly (see below).

Splint oligonucleotides are 25-base sequences consisting of (5' to 3'): 15 bases complementary to an annealing region, 5 bases complementary to the 5' end of the annealing oligonucleotides, and 5 bases complementary to the 3' end of the substrate-linked oligonucleotides.

Substrate-linked oligonucleotides are 10-base sequences containing (5' to 3'): a 5' amino group (installed using the Amino-Modifier 5 phosphoramidite), and 10 bases representing the 5'-end of the 5'

PCR primer binding site. This oligonucleotide was labeled with 12 different carboxylic acids (or activated carboxylic acid derivatives) in separate reaction vessels. For a typical labeling reaction, a carboxylic acid was pre-activated as an NHS ester by combining 0.9 M solutions of the carboxylic acid, N-hydroxysuccinimide (NHS), and 1,3-dicyclohexyl carbodiimide (DCC) in a 1:1:1 ratio and letting the reaction proceed at 25° C for 40 min. The DCU precipitate in these reactions was pelleted by microcentrifugation and the supernatant was used directly in a labeling reaction. For those substrates that were commercially available as activated carboxylic acid derivatives, a 10 mg/mL stock solution in DMF was prepared and added directly to the labeling reaction. A typical labeling reaction consisted of 10 nmol of amino-terminated oligonucleotide in 100 μ L of 0.2 M phosphate buffer at pH 8.0, to which 45 μ L of the NHS ester solution was added. After 2 h at 25° C, labeling reactions were passed through a Nap-5 sephadex gel-filtration column (Amersham Biosciences) and the labeled oligonucleotide was purified by reverse-phase HPLC. See table below for MALDI characterization of each substrate-linked oligonucleotide used in the assembly of the pool A members.

The carboxylic acids (or carboxylic acid derivatives) used for the preparation of pool A were all obtained from commercial sources as follows: hexanoic acid (A1, Aldrich), cyclopropylacetic acid (A2, Oakwood Products), cyclobutanecarboxylic acid (A3, Aldrich), *trans*-3-hexenoic acid (A4, Aldrich), 6-heptynoic acid (A5, Aldrich), phenylacetic acid (A6, Aldrich), 4-iodobenzoic acid (A7, Aldrich), 3-(2-furyl)propanoic acid (A8, Acros), indole-5-carboxylic acid (A9, Aldrich), succinic anhydride (A10, Aldrich), methyl *N*-succinimidyl adipate (A11, Pierce), 6-oxoheptanoic acid (A12, Aldrich).

Modular Assembly of Pool A Members

The complete pool A DNA-linked substrates were assembled from the above components by primer extension and ligation (Fig. S1). A one-pot primer extension appended the coding sequence for a pool A member to 13 different annealing oligonucleotides and a subsequent ligation assisted by the splint oligonucleotides linked the pool A substrate encoded by the coding sequence to each of the 13 primer extension products. Primer extensions were performed on a 500 pmol scale (5 μ M coding sequence and 5 μ M total annealing sequences) in 100 μ L at 25° C for 3 h using Klenow *exo*⁻ (New England Biolabs). In addition to the coding and annealing sequences, 1 nmol of the splint oligonucleotides necessary for the ligation step was added to the primer extension step to minimize the formation of internal secondary structure within the annealing sequences.

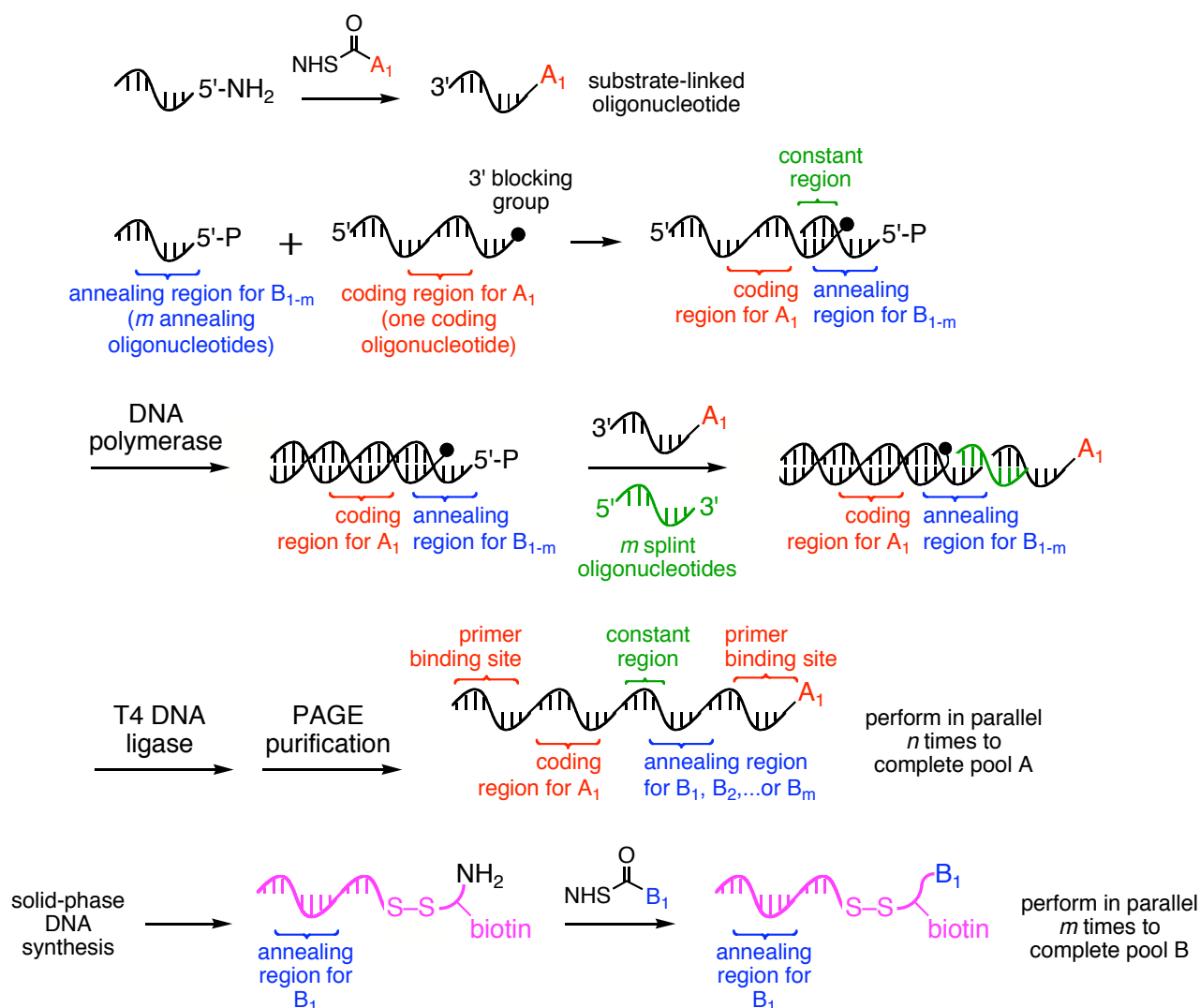


Fig. S1. Assembly of pool A and pool B.

Ligations were performed directly following buffer exchange of the primer extension reactions. One nmol of the appropriate 5'-pool A substrate-linked oligonucleotide (two equiv. with respect to the primer extension products, one equiv. with respect to the splint oligonucleotides) was added to the buffer-exchanged reaction and the ligation was performed overnight at 16° C using T4 DNA ligase. Ligation reactions were precipitated with ethanol and full-length single-stranded pool A DNA-linked substrates were purified by denaturing PAGE. Typically, a 500 pmol-scale assembly of a pool A DNA-linked substrate yielded ≥ 100 pmol of purified full-length templates. The mass spectroscopic analysis of the substrate-linked oligonucleotides used in the assembly of pool A is shown below in Table S1.

substrate	expected mass of DNA-linked substrate	observed mass
hexanoic acid (A1)	3281.7	3281 $\pm$ 3
cyclopropylacetic acid (A2)	3265.6	3267 $\pm$ 3
cyclobutane carboxylic acid	3265.6	3267 $\pm$ 3

(A3)		
3-hexenoic acid (A4)	3279.6	3280 $\pm$ 3
6-heptynoic acid (A5)	3291.6	3292 $\pm$ 3
phenylacetic acid (A6)	3301.6	3304 $\pm$ 3
4-iodobenzoic acid (A7)	3413.5	3414 $\pm$ 3
3-(2-furyl)propanoic acid (A8)	3305.6	3306 $\pm$ 3
indole-5-carboxylic acid (A9)	3326.6	3327 $\pm$ 3
succinic acid (A10)	3283.6	3385 $\pm$ 3
adipic acid monomethyl ester (A11)	3325.6	3326 $\pm$ 3
6-oxoheptanoic acid (A12)	3309.6	3310 $\pm$ 3

Table S1. MALDI-TOF characterization of pool A substrate-linked oligonucleotides.

Preparation of Pool A DNA-linked Substrates for the Detection of Pool B Homocoupling

In addition to the 12 pool A members described above (each containing 13 different sequences), 12 individual pool A sequences were prepared, each linked to a different pool B substrate. These DNA-linked substrates were designed to enable detection of homocoupling of any of the pool B substrates. Each of these DNA-linked substrates was prepared in a manner analogous to the preparation of pool A members described above except that only one annealing oligonucleotide and only one splint oligonucleotide were used in the primer-extension and ligation steps. Additionally, all of these DNA-linked substrates were prepared using the same coding oligonucleotide (rather than coding for a substrate, this coding sequence encoded for homocoupling). Mass spectroscopic characterization of the substrate-linked oligonucleotides is provided in Table S2.

substrate	expected mass of DNA-linked substrate	observed mass
5-chlorovaleric acid (B1)	3301.6	3303 $\pm$ 3
<i>p</i> -formylbenzoic acid (B2)	3315.6	3317 $\pm$ 3
acrylic acid (B3)	3237.6	3240 $\pm$ 3
5-oxiranyl pentanoic acid (B4)	3309.6	3313 $\pm$ 3
6-heptenoic acid (B5)	3293.7	3293 $\pm$ 3
3-(4-hydroxy-phenyl)propionic acid (B6)	3331.6	3332 $\pm$ 3
5-methoxycarbonyl-4-oxopentanoic acid (B7)	3339.6	3342 $\pm$ 3
4-(2-carboxyethyl)benzeneboronic acid (B8)	3515.8 (citric acid adduct)	3516 $\pm$ 3
4-azidobutyric acid (B9)	3294.6	3296 $\pm$ 3
8-cyano-octanoic acid (B10)	3334.7	3336 $\pm$ 3
6-hydroxycaproic acid (B11)	3297.6	3298 $\pm$ 3
unlabeled amine (no carboxylic acid) (B12)	3183.6	3185 $\pm$ 3

Table S2. MALDI-TOF characterization of pool A substrate-linked oligonucleotides for the detection of pool B homocoupling.

Preparation of Pool B

Pool B DNA-linked substrates are 16 bases in length and contain (5' to 3'): a 15-base annealing region complementary to one of the annealing regions in pool A, a single C, a disulfide linker, a biotin

group, and an amide-linked substrate (Fig. S1). The modified groups were introduced using the 3' PT Amino-Modifier C6 CPG, the biotin phosphoramidite, and the thiol modifier C6 S-S. The 3' amine-terminated oligonucleotides were labeled with the appropriate activated carboxylic acid using the protocol described in the preparation of pool A. The purified labeled oligonucleotides were characterized by mass spectroscopy as is shown below. In all, 24 DNA-linked substrates were prepared and characterized in this manner comprising 12 DNA-linked pool B substrates and 12 additional DNA-linked substrates for detection of homocoupling of any of the pool A substrates.

The following carboxylic acids or carboxylic acid derivatives used in the preparation of pool B were available from commercial sources: 5-chlorovaleric acid (Aldrich, B1), *p*-formylbenzoic acid N-hydroxysuccinimide ester (Aldrich, B2), acrylic acid (Aldrich, B3), 6-heptenoic acid (Aldrich, B5), 3-(4-hydroxy-phenyl)propionic acid N-hydroxysuccinimide ester (Aldrich, B6), 4-(2-carboxyethyl)benzeneboronic acid (Lancaster, B8), and 6-hydroxycaproic acid (Aldrich, B11). 5-methoxycarbonyl-4-oxopentanoic acid (B7) was prepared according to a literature procedure (Oku, A., Numata, M. *J. Org. Chem.* **65** 1899-1906 (2000)). 4-azidobutyric acid (B9) was prepared according to a literature procedure (Kusumoto, S., Sakurai, K., Shiba, T. *Bull. Chem. Soc. Jpn.* **59**, 1296-1298 (1986)). 5-oxiranyl pentanoic acid (B4) was prepared from 6-heptenoic acid using the epoxidation procedure described in Corey, P. F., Ward, F. E. *J. Org. Chem.* **51**, 1925-1926 (1986). ¹H NMR (CDCl₃): δ = 1.50-1.61 ppm (m, 4H), 1.67-1.74 (m, 2H), 2.38 (t, 2H), 2.48 (dd 1H), 2.76 (t, 1H), 2.92 (m, 1H). HRMS: calculated = 162.1130 (NH₄⁺); observed = 162.1125. ¹³C NMR (CDCl₃): δ = 24.63, 25.66, 32.28, 34.06, 47.29, 52.34, 179.66.

Preparation of 8-Cyano-octanoic Acid (Substrate B10):

The preparation of the cyano-carboxylic acid representing substrate B10 was adapted from a procedure for the preparation of 4-(2-cyanoethyl)benzoic acid (Delia, T. J., Baumann, M., Bunker, A. *Heterocycles*, **35**, 1397-1410 (1993)). To 501 mg (2.25 mmol) of 8-bromooctanoic acid in 7.0 mL of methanol was added 520 mg NaCN (10.6 mmol) in 2.0 mL H₂O. The solution was heated at reflux for 2.5 h. After cooling to 25 °C, the solution was acidified to pH ~1.5 with concentrated HCl (*caution*: HCN gas evolves with the addition of HCl) and diluted with 40 mL EtOAc. The resulting suspension was washed twice with 15 mL H₂O. The organic layer was dried by filtration through MgSO₄, and the solvent was removed *in vacuo* to afford 282 mg (74%) of a light brown oil. The ¹H NMR spectrum agreed with previously reported data in Cotarca, L., Delogu, P., Maggioni, P., Nardelli, A., Bianchini, R., Sguassero, S. *Synthesis* **1997**, 328-332.

Mass spectroscopic characterization of pool B members are shown in Tables S3 and S4.

substrate	expected mass of DNA-linked substrate	observed mass
5-chlorovaleric acid (B1)	5922.3	5922 ± 5
4-formylbenzoic acid (B2)	5896.2	5895 ± 5
acrylic acid (B3)	5778.2	5778 ± 5
5-oxiranyl pentanoic acid (B4)	5952.3	5952 ± 5
6-heptenoic acid (B5)	5874.3	5874 ± 5
3-(4-hydroxy-phenyl)propionic acid (B6)	5823.2	5823 ± 5
5-methoxycarbonyl-4-oxopentanoic acid (B7)	5960.3	5960 ± 5
4-(2-carboxyethyl)benzeneboronic acid (B8)	6145.3 (citric acid adduct)	6145 ± 5
4-azidobutyric acid (B9)	5755.2	5754 ± 5

8-cyano-octanoic acid (B10)	5906.3	5907 $\pm$ 5
6-hydroxycaproic acid (B11)	5869.3	5869 $\pm$ 5
unlabeled amine (no RCOOH) (B12)	5775.3	5775 $\pm$ 5

Table S3. MALDI-TOF characterization of pool B members.

Hexanoic acid (A1)	5835.3	5835 $\pm$ 5
Cyclopropylacetic acid (A2)	5855.3	5854 $\pm$ 5
Cyclobutanecarboxylic acid (A3)	5917.3	5917 $\pm$ 5
3-hexenoic acid (A4)	5891.3	5891 $\pm$ 5
6-heptynoic acid (A5)	5845.2	5843 $\pm$ 5
Phenylacetic acid (A6)	5873.3	5873 $\pm$ 5
4-iodobenzoic acid (A7)	6003.2	6003 $\pm$ 5
3-(2-furyl)propionic acid (A8)	5877.3	5877 $\pm$ 5
Indole-5-carboxylic acid (A9)	5876.3	5875 $\pm$ 5
Succinic acid (A10)	5815.2	5814 $\pm$ 5
Adipic acid monomethylester (A11)	5906.3	5906 $\pm$ 5
6-oxoheptanoic acid (A12)	5819.3	5818 $\pm$ 5

Table S4. MALDI-TOF characterization of pool B members for the detection of pool A homocoupling**Preparation of DNA Microarrays**

Commercial 5'-amino-modified oligonucleotides (Illumina) were printed on aldehyde-silane glass slides (Schott-Nexterion) using a robotic microarrayer (GeneMachines). Stock solutions for printing were 20 μ M in 3X SSC buffer. Fifteen copies of the microarray were printed on each slide, in 5 columns of 3 copies each. This enabled each analyte sample to be hybridized to three different microarrays per experiment. In all cases, the three replicate microarrays per experiment gave virtually indistinguishable signals. Printed oligonucleotides were immobilized and unreacted aldehydes on the slides were blocked according to the manufacturer's protocol with one 15 s concentrated NH_4OH wash followed by a 1 min H_2O wash added to the end of the blocking procedure. After blocking, slides were stored in a dessicator covered with aluminum foil and used within two weeks.

Each printed oligonucleotide representing a unique combination of substrates was a 35-mer consisting of (5' to 3') one of the 15-base annealing regions, an 8-base constant region, and one of the 12-base coding regions from the pool A sequences. The 169th oligonucleotide used to detect the internal standard (see Selections for Bond Formation) shared only the constant region with any of the other 168 sequences and was printed in the lower right corner of each array.

Selections for Bond-Formation***Internal Standard Oligonucleotide:***

To compare fluorescence intensity ratios between arrays, a biotinylated oligonucleotide was included in each selection at an amount equal to 1/10th of the amount of an individual pool A sequence encoding a unique substrate combination. This oligonucleotide served as an internal standard representing ~10% DNA-templated reactivity in a selection. The reference oligonucleotide was synthesized with a 5' biotin group using the 5' Biotin Phosphoramidite and purified by Oligonucleotide Purification Cartridge (OPC, Applied Biosystems, Inc.)

Universal Clamp Oligonucleotide

To minimize secondary structure in the pool A oligonucleotides, a “universal clamp” oligonucleotide was included in each selection for bond formation. This oligonucleotide consisted of (5' to 3') 15 bases complementary to the 3' primer binding site of the pool A sequences and 8 bases complementary to the constant region of the pool A sequences.

Selection Components

A typical selection for bond-formation used five components: pool A, pool B, a “universal clamp”, the biotinylated internal standard, and the metal complex or reagents being tested. For selections, stock solutions were prepared for each pool. The stock solution for pool A was 1 μ M in total DNA and contained equal amounts of each of the 168 different oligonucleotides (encoding the 144 heterocoupling and 24 homocoupling substrate combinations) in addition to 1/10 of an equivalent of the biotinylated internal standard oligonucleotide. The stock solution for pool B was 2 μ M in total DNA and contained equal amounts of each of the 12 pool B oligonucleotides and 1/12 of one equivalent of each of the “self-A” homocoupling-encoding oligonucleotides.

Reaction Discovery Experiments and Selection Procedure

Each reaction discovery experiment was performed in 12 μ L total solution containing 12 fmol of material representing each unique substrate combination. Two pmol of total pool A material, 2 pmol of total pool B material, 8 pmol of the universal clamp, and 1.2 fmol of the biotinylated internal standard were combined in buffer and allowed to hybridize at 25 °C for 30 min. Reactions were initiated by adding 1 μ L of a solution containing the metal or non-DNA-linked reagents. The detailed conditions for each experiment shown in Fig. 2b are as follows:

- No metal: 50 mM MOPS, 0.5 M NaCl, pH 7.0, 37 °C, 1h.
- Cu(I): 500 μ M CuSO₄, 500 μ M sodium ascorbate, 50 mM MOPS, 0.1 M Na₂SO₄, pH 7.0, 25 °C, 10 min.
- EDC + sNHS: 20 mM EDC, 15 mM sNHS, 50 mM MES, 0.5 M NaCl, pH 6.0, 25°C, 1h.
- Pd(II), 37 °C: 500 μ M Na₂PdCl₄, 50 mM MOPS, 0.5 M NaCl, pH 7.0, 1h.
- Pd(II), 25 °C: 500 μ M Na₂PdCl₄, 50 mM MOPS, 0.5 M NaCl, pH 7.0, 20 min.

After the reaction times indicated above, the solution was diluted with 20 μ L of H₂O and passed through a gel filtration microcentrifuge column (Princeton Separations) to remove small-molecule reactants. To the eluant was added 150 μ L of a 0.1 M aqueous solution of *tris*(2-carboxyethyl)phosphine hydrochloride in 1.0 M sodium phosphate, pH 8.0 to effect disulfide cleavage. After 1 h at 25 °C, this solution was added to a 20 pmol binding-capacity aliquot of streptavidin-linked magnetic particles (Roche Biosciences) suspended in 300 μ L of 0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5 (“loading buffer”). The supernatant was removed after 15 min. The particles were rinsed once with 200 μ L H₂O, and once with 200 μ L 1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5 (“washing buffer”). The particles were then resuspended in 20 μ L of 95% formamide-5% 10 mM EDTA and incubated at 65 °C for 10 min. Fifteen μ L of this eluant was diluted with 60 μ L H₂O and added to a fresh aliquot of streptavidin particles suspended in 350 μ L of loading buffer. After 15 min, the supernatant was removed and the particles were rinsed with H₂O and washing buffer as above. The particles were resuspended in 15 μ L of 95% formamide-5% 10 mM EDTA and incubated at 90 °C for 10 min. Ten μ L of this eluant was diluted with 35 μ L H₂O and passed through a gel filtration spin-column (Princeton Separations). This solution was used directly in PCR reactions for microarray analysis.

Microarray Analysis of Selections

Preparation of PCR Primers

Primer 1 was synthesized using standard automated methods and purified using an OPC column to avoid trace contaminants from HPLC purification that could be amplified in a PCR reaction. Primer 2 was synthesized with the C6 Amino-dT phosphoramidite in place of the dT monomer and also purified by OPC. (See the DNA Oligonucleotide Sequences section for the primer sequences). A solution of 0.5 μ mol primer 2 in 200 μ L of 0.1 M sodium carbonate pH 9.3 was added to a Cy-3 Mono-Reactive Dye Pack (Amersham Biosciences) and the solution was agitated in the dark at 25 °C for 1h before being passed through a gel filtration spin-column and a Nap-5 gel filtration column. The Nap-5 eluant was quantitated by UV and stored at –80 °C until it was used in a PCR reaction. A 0.5 μ mol aliquot of primer 2 was labeled with Cy-5 using an identical procedure with the Cy-5 Mono-Reactive Dye Pack.

Blocking Oligonucleotides

Twenty-three-base oligonucleotides complementary to each annealing region + constant region and 20-base oligonucleotides complementary to each constant region + coding region present on the array were purchased from Illumina. These oligonucleotides were included in the hybridization solution to maximize annealing fidelity.

PCR Amplification of Pre- and Post-Selection Sequences and Microarray Analysis

Ten μ L aliquots of selected sequences (see Selections for Bond Formation) were added to a 250 μ L PCR reaction containing 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 40 nM primer 1, and 400 nM primer 2 labeled with Cy-3. The sequences were amplified with 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 20 s. Forty fmol of an equimolar mixture of the 169 pool A sequences (including the internal standard sequence) was amplified by PCR under identical conditions using primer 2 labeled with Cy-5. The PCR reactions were extracted with phenol-chloroform and precipitated with EtOH. A pre-selection pellet (Cy-5-labeled) and post-selection pellet (Cy-3-labeled) were resuspended together in 24 μ L of 20 mM HEPES pH 7.5, 1X SSC containing 10 μ g BSA and 10 μ M blocking oligonucleotides. The solution was filtered using a 0.45 μ M microcentrifuge filter (Millipore). Six μ L of formamide and 0.45 μ L of 10% SDS were added to the filtrate and the solution was heated to 100 °C for 2 min. After cooling to room temperature, this solution was hybridized at 65 °C for 12 h to the DNA microarray described above. After hybridization, the slides were washed with 0.025% SDS, 0.6X SSC (5 min) and 0.05X SSC (5min) and scanned immediately using an Axon GenePix 4000B scanner (Axon Instruments).

DNA Oligonucleotide Sequences and Linker Structures

Pool A oligonucleotide sequence:

5'-CGTTGATATCCGCAGXXXXXXXXXXXXXXXXX CACACACC
 YYYYYYYYYYYYGCCAGCTGCTAGCTT-3'

where XXXXXXXXXXXXXXXX (the annealing region) is one of the following:

- | | |
|--------------------|---------------------|
| 1) AACTTCCTCTCGGGA | 8) CTTAGTTTCGCGCAC |
| 2) ACGCGATGTTTCGAC | 9) GAGGGTGATGCATGT |
| 3) AGCGTTATGGTCCGA | 10) GCTGGACTAGCTACA |
| 4) ACATGAGCCCCACTA | 11) TAGACACGCATGTGC |
| 5) CCACTGTTACTAGGG | 12) TCGGACTTTGATGGC |
| 6) CGTGCTTGAGGAGAA | 13) ACCGAAGGGCAATAC |
| 7) AGGCCTCTTTAGACC | 14) GTCAGCCCTTGGTAT |

15) CACCTACCGGTAATC
16) CAGTCCGGTACCTAA
17) CCTGAGAAAGAACCG
18) TTGATGACCAGGCCA
19) TGCGCAACTGGTCTT

20) TGGCAAATCCAGCGT
21) GACCTTGGCGTTTAG
22) TCCAGGGGATGCATA
23) GCACGTACATTGCTG
24) GGTGTATACGTGGCT

and **YYYYYYYYYYYY** (the coding region) is one of the following:

1) AAGCTGATCACG (**A1**)
2) TAGTGCCGTTGA (**A2**)
3) ACCATATCCCCT (**A3**)
4) ACGTATAGCGGT (**A4**)
5) AGAAAGTGTGCG (**A5**)
6) ATAGTTGGCTCC (**A6**)
7) ATCTGGAACCTC (**A7**)

8) ATTCAACCGTCG (**A8**)
9) CACCGAAGTAAC (**A9**)
10) TTGGAGCCTGAT (**A10**)
11) CCCTCCTTATCA (**A11**)
12) TGTCGGGACAAT (**A12**)
13) TGCTAATGACGC (SELF-B)

Annealing and coding sequences were generated by computer algorithms to minimize undesired reactivity arising from the mismatched hybridization of a pool B member with a non-complementary pool A member. The overall GC/AT ratio of these sequences was kept constant to minimize differences in melting temperatures between different annealing and coding sequences.

Internal standard oligonucleotide sequence:

5'CGTTGATATCCGCAGGTGCATTAGCACGCACACACACCTCTAACACAGCCGCCAGCTGC
TAGCTT-3'

Pool B oligonucleotide sequences are as follows:

1) TCCCGAGAGGAAGTTC (**B1**)
2) GTCGAAACATCGCGTC (**B2**)
3) TCGGACCATAACGCTC (**B3**)
4) TAGTGGGGCTCATGTC (**B4**)
5) CCCTAGTAACAGTGGC (**B5**)
6) TTCTCCTCAAGCACGC (**B6**)
7) GGTCTAAAGAGGCCTC (**B7**)
8) GTGCGCGAACTAAGC (**B8**)
9) ACATGCATCACCTCC (**B9**)
10) TGTAGCTAGTCCAGC (**B10**)
11) GCACATGCGTGTCTAC (**B11**)
12) GCCATCAAAGTCCGAC (**B12**)

13) GTATTGCCCTTCGGTC (SELF A1)
14) ATACCAAGGGCTGACC (SELF A2)
15) GATTACCGGTAGGTGC (SELF A3)
16) TTAGGTACCGGACTGC (SELF A4)
17) CGGTTCTTTCTCAGGC (SELF A5)
18) TGGCCTGGTCATCAAC (SELF A6)
19) AAGACCAGTTGCGCAC (SELF A7)
20) ACGCTGGATTTGCCAC (SELF A8)
21) CTAAACGCCAAGGTCC (SELF A9)
22) TATGCATCCCCTGGAC (SELF A10)
23) CAGCAATGTACGTGCC (SELF A11)
24) AGCCACGTATACACCC (SELF A12)

Sequences used in DNA-templated characterization of array positives:

30-base oligonucleotide: 5'- CGTTGATATCCGCAGACGCGATGTTTCGAC

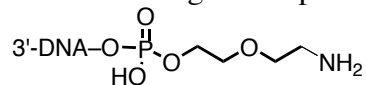
16-base oligonucleotide: 5'- GTCGAAACATCGCGTC

Sequences used in MALDI-TOF analysis of DNA-templated reactions:

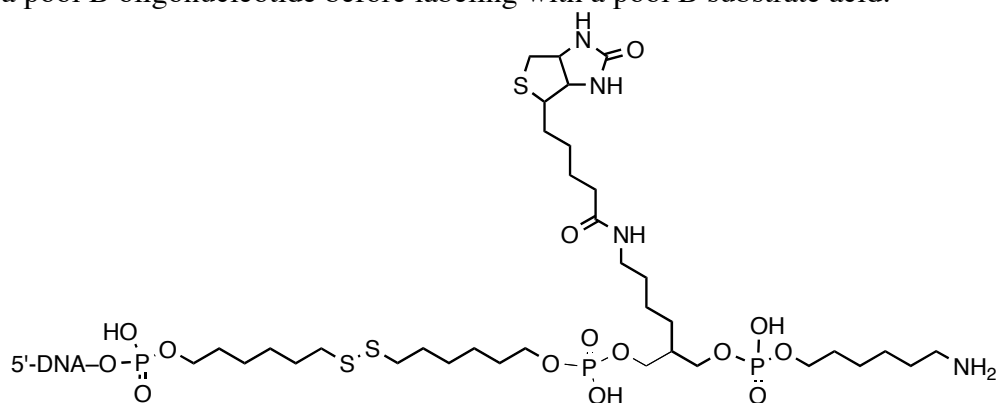
20-base oligonucleotide: 5'- CATTACCATGTACATACCAG

15-base oligonucleotide: 5'- CTGGTATGTACATGG

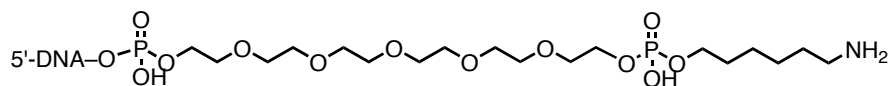
Structure of a pool A oligonucleotide before labeling with a pool A substrate acid:



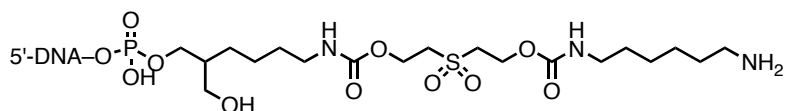
Structure of a pool B oligonucleotide before labeling with a pool B substrate acid:



Structure of an unlabeled 16-base oligonucleotide used in the DNA-templated characterization of array positives:



Structure of an unlabeled 15-base oligonucleotide with a cleavable sulfone linker used in the MALDI-TOF characterization of DNA-templated reactions:



Supplementary Data

Quantitative Microarray Data

The ratio of median green:red fluorescence intensities of the spots on the arrays were calculated using the GenePix Pro 5.0 software. The values for the spots with the highest green:red ratios of median fluorescence in each of the arrays in Fig. 2B are as follows:

- Cu(I): A5+B9 = 8.5; standard = 2.8
- EDC + sNHS: standard = 15.6; A10+B12 = 6.9
- Pd(II), 37 °C: A7+B3 = 3.6; A5+B3 = 3.5; A4+B8 = 3.0; standard = 2.9; A5+B5 = 2.7; A5+B13 = 2.6; A9+B3 = 1.9; A8+B8 = 1.8; A8+B3 = 1.8; A5+B8 = 1.6; A5+B9 = 1.6
- Pd(II), 25 °C: A5+B5 = 3.7; standard = 3.5; A5+B3 = 3.1; A4+B8 = 2.8; A5+B13 = 2.7; A5+B8 = 1.9; A5+B9 = 1.7

Characterization of Putative Reactions in a DNA-Templated Format

Reactions corresponding to array positives from the Pd(II) selections were performed in a DNA-templated format on a 25 pmol scale and analyzed by denaturing PAGE. Two oligonucleotides were used for these experiments: a 30-base oligonucleotide labeled with a substrate at the 5' end and a 16-base oligonucleotide (complementary to a region of the 30-base oligonucleotide) labeled with a substrate at the 3' end. The 30-base oligonucleotide was synthesized with a 5' amine using the 5'-Amino-Modifier 5 phosphoramidite. The 16-base oligonucleotide was synthesized with a 3' amine using the 3'-PT Amino-Modifier C6 CPG and the Spacer 18 phosphoramidite (see Supplementary Methods for the complete structure). The Spacer 18 phosphoramidite enabled the number of bonds between the substrate and the 3' base of the 16-base oligonucleotide to match the number of bonds between the substrate and the 3' base in a pool B DNA-linked substrate. Both oligonucleotides were labeled with appropriate substrates as described in the preparation of the substrate-linked oligonucleotides in pool A. Labeled oligonucleotides were characterized by MALDI-TOF mass spectroscopy (Table S5).

substrate	expected mass of labeled 30-base oligonucleotide	observed mass
3-hexenoic acid (A4)	9456.6	9457 ± 9
6-heptynoic acid (A5)	9468.6	9468 ± 9
4-iodobenzoic acid (A7)	9590.5	9591 ± 9
3-(2-furyl)propionic acid (A8)	9482.6	9481 ± 9
indole-5-carboxylic acid (A9)	9503.6	9502 ± 9
hexanoic acid (negative control)	9458.7	9457 ± 9

substrate	expected mass of labeled 16-base oligonucleotide	observed mass
acrylic acid (B3)	5441.05	5441 ± 5
6-heptenoic acid (B5)	5497.12	5497 ± 5
4-(2-carboxyethyl)benzeneboronic acid (B8)	5719.11 (citric acid adduct)	5717 ± 5
6-heptynoic acid (A5)	5495.10	5494 ± 5
hexanoic acid (negative control)	5485.10	5484 ± 5

Table S5. MALDI-TOF characterization of DNA-linked substrates for evaluating array positives in a DNA-templated format.

To perform a DNA-templated reaction, 25 pmol of substrate-linked 30-mer was combined with 37.5 pmol of substrate-linked 16-mer in 360 μL of 0.1 M MOPS, 1 M NaCl, pH 7.0 and allowed to hybridize for at least 20 min. Reactions were initiated by adding 40 μL of a 5 mM solution of Na_2PdCl_4 in H_2O and allowed to react for either 20 min at 25 $^\circ\text{C}$ or 1 h at 37 $^\circ\text{C}$ before the Pd was quenched with 4 μL of 2 M β -mercaptoethanol and the reactions were EtOH precipitated. Ten pmol aliquots of the reactions were electrophoresed on denaturing polyacrylamide gels and the efficiencies of the reactions were determined by quantitation of ethidium bromide staining. Control reactions in which Pd was omitted showed no product formation. Control reactions with Pd in which one of the substrates was replaced with an alkane also showed no product formation, although occasionally we observed faint diffuse higher molecular weight bands that we attribute to Pd-complexed species. In the case of the reaction discovery selections, cleavage of the disulfide linker prevents Pd complexation from generating false positives. Reaction yields were quantitated by UV visualization of stained gels and densitometry of product and starting material bands using a Stratagene Eagle Eye II densitometer. Yield calculations assumed that species in denaturing gels stain with comparable intensity per nucleotide. The DNA-templated yields of the nine positives arising from the 37 $^\circ\text{C}$ Pd(II) array shown in Fig. 2b are shown in Fig. S2.

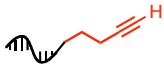
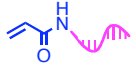
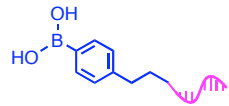
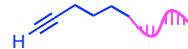
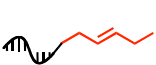
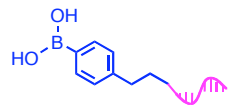
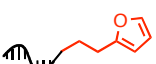
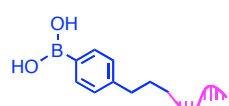
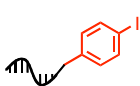
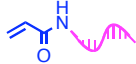
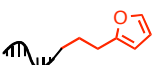
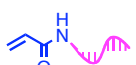
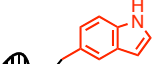
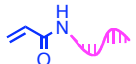
<u>substrates</u>		<u>DNA-templated yields</u>	
		<u>37$^\circ\text{C}$</u>	<u>25$^\circ\text{C}$</u>
		35	31
		28	20
		36	34
		45	42
		57	39
		30	<10
		39	14
		19	<10
		<10	<10

Fig. S2. DNA-templated yields of reactions corresponding to array positives.

MALDI-TOF Analysis of DNA-Templated Reaction Products

The products arising from reactions validated in a DNA-templated format by gel electrophoresis were further characterized by MALDI-TOF mass spectrometry. Two oligonucleotides were used for these experiments: a 20-base oligonucleotide labeled with a substrate at the 5' end and a 15-base oligonucleotide (complementary to a 15-base region of the 20-mer) with a 5' biotin group and a substrate attached to the 3' end through a base-labile sulfone linker. The 20-base oligonucleotide was synthesized with a 5' amine using the 5' Amino-Modifier 5 phosphoramidite and labeled using the procedures described in the preparation of substrate-linked oligonucleotides for pool A.

The 15-base oligonucleotide was synthesized with a 3' amine and a 5' biotin using the 3' Amino-Modifier C7 CPG and the 5' Biotin phosphoramidite. The oligonucleotide was labeled with a substrate via a cleavable linker in a two step procedure. In the first step, 5-10 nmol of oligonucleotide in 100 μ L of 0.2 M phosphate pH 8.0 was combined with 10 μ L of a 0.1 M solution of *bis*[2-(succinimidylloxycarbonyloxy)-ethyl]sulfone (BSOCOES, Pierce) in DMF and vortexed for 90 s. Immediately after vortexing, 9 μ L of a 0.2 M solution of hexanediamine dihydrochloride salt in H₂O was added and the solution was agitated at 25 °C for 1 h. The solution was then passed through a Nap-5 gel filtration column (Amersham) and the hexanediamine-labeled oligonucleotide was purified by reverse-phase HPLC. The purified oligonucleotide was then labeled with the appropriate carboxylic acid using the same procedures described above.

Substrate-linked 20-base and 15-base oligonucleotides used in these experiments were characterized by MALDI-TOF mass spectroscopy as shown in Table S6.

substrate	expected mass of labeled 20-base oligonucleotide	observed mass
6-heptynoic acid	6317.15	6315 $\pm$ 5
4-iodobenzoic acid	6439.02	6440 $\pm$ 5
3-(2-furyl)propionic acid	6331.13	6330 $\pm$ 5
4-(2-carboxyethyl)benzeneboronic acid	(citric acid adduct) 6541.2	6539

substrate	expected mass of labeled 15-base oligonucleotide	observed mass
acrylic acid	5611.2	5610 $\pm$ 5
6-heptenoic acid	5667.2	5668 $\pm$ 5
4-(2-carboxyethyl)benzeneboronic acid	(citric acid adduct) 5889.2	5888 $\pm$ 5
6-heptynoic acid	5665.2	5664 $\pm$ 5
3-hexenoic acid	5653.2	5656 $\pm$ 5

Table S6. MALDI-TOF characterization of DNA-linked substrates for characterizing reaction products in a DNA-templated format.

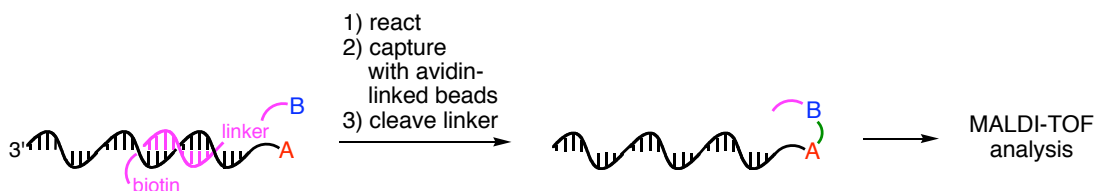


Fig. S3. Strategy used to characterize products of DTS reactions by MALDI-TOF mass spectrometry.

To obtain a product sample suitable for MALDI-TOF analysis (Fig. S3), 50 pmol of substrate-labeled 20-mer was combined with 75 pmol of substrate-labeled 15-mer in 450 μ L of 0.1 M MOPS, 1 M NaCl, pH 7.0 and allowed to hybridize for at least 20 min at 25 $^{\circ}$ C. Reactions were initiated by adding 50 μ L of 5 mM Na_2PdCl_4 in H_2O and allowed to react for either 20 min at 25 $^{\circ}$ C or 1 h at 37 $^{\circ}$ C before the Pd was quenched with 5 μ L 2 M β -mercaptoethanol. The DNA-linked material was precipitated with EtOH and the pellets were resuspended in 300 μ L 10 mM Tris, 0.1 M NaCl, 1 mM EDTA, pH 8.2 and added to an aliquot of streptavidin magnetic particles (Roche) representing 80 pmol of biotinylated oligonucleotide binding capacity. After 15 min, the supernatant was removed and the particles were washed twice with 150 μ L Milli-Q H_2O . The particles were then resuspended in 0.1 M CAPS, pH 11 and agitated for 15 min to effect sulfone linker cleavage. The supernatant was spin-filtered and subjected to EtOH precipitation. The pellet was resuspended in 0.1 M TEAA, desalted using a Zip-Tip cartridge (Millipore), and subjected to MALDI-TOF analysis, yielding the results in Fig. S4.

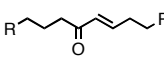
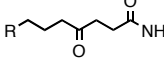
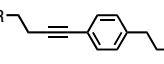
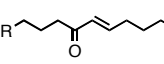
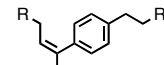
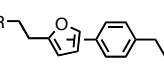
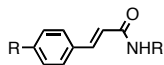
substrates	possible product	expected mass	observed mass
		6559.4	6557 $\pm$ 5
		6505.3	6501 $\pm$ 5
		6563.3	6563 $\pm$ 5
		6559.4	6557 $\pm$ 5
		6551.3	6548 $\pm$ 5
		6577.3	6579 $\pm$ 5
		6481.3	6479 $\pm$ 5

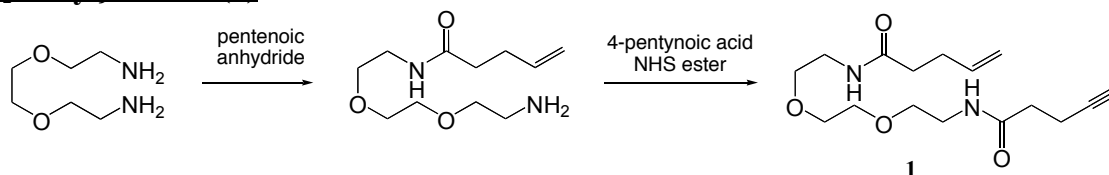
Fig. S4. MALDI-TOF characterization of products from DNA-templated reactions.

Additional relevant reaction possibilities tested in a DNA-templated format in the presence of Pd(II) include:

- Ketone (A12) + alkyne (A5): yields no product
- Alkyne (A5) + azide (B9), a borderline positive by green:red fluorescence ratio quantitation: yields only very weak product formation.
- Benzeneboronic acid (B8) homocoupling: yields product consistent with biphenyl formation (expected mass = 6587.3; observed mass = 6585 $\pm$ 5).

Copper(I)-Mediated Degradation of DNA

To assess the effects of the Cu(I)-containing conditions used above on DNA stability, we exposed 17 pmol of a DNA 30-mer in 100 μ L to 500 μ M CuSO₄, 500 μ M sodium ascorbate, 50 mM MOPS, 0.1 M Na₂SO₄, pH 7.0, at 25 °C for 10 min. The solution was subjected to gel filtration to remove small molecules (including Cu(I)) and then analyzed by denaturing PAGE. For comparison, identical reactions were also performed in the absence of CuSO₄ and sodium ascorbate. DNA staining with ethidium bromide and densitometry were used to quantitate the amount of full-length DNA remaining in each sample. The sample exposed to Cu(I) was observed to contain 52% of the full-length DNA present in the no-Cu control sample.

Synthesis and Characterization of Pent-4-enoic Acid {2-[2-(2-Pent-4-Ynoylamino-Ethoxy)-Ethoxy]-Ethyl}-Amide (1)

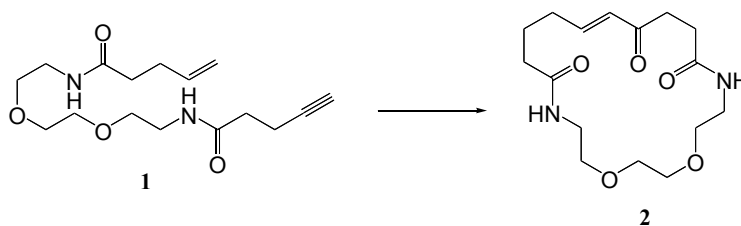
Pentenoic anhydride (789 mg, 4.3 mmol, 1.0 equiv.) in acetonitrile (10 mL) was added dropwise over the course of 30 min to a stirring solution of 2,2'-(ethylenedioxy)bis(ethylamine) (640 mg, 4.3 mmol, 1.0 equiv.) in 20 mL acetonitrile at 25 °C. At the conclusion of addition the reaction solution was dried *in vacuo*. The dried material was dissolved in 50 mM acetic acid in H₂O (20 mL) and purified by preparative HPLC (acetonitrile/50 mM aqueous acetic acid gradient) followed by lyophilization to afford the acetate salt of the monoacylated material (560 mg, 45%) as a pale yellow oil. To the monoacylated material (300 mg, 1.0 mmol, 1.0 equiv.) in acetonitrile (20 mL) was added *N*-hydroxysuccinimide activated 4-pentynoic acid (220 mg, 1.1 mmol, 1.1 equiv.) and the resulting clear solution was allowed to stir under N₂ atmosphere at 25 °C for 20 min. The reaction was dried *in vacuo*, and the material was resuspended in H₂O (20 mL) and purified via preparative HPLC (acetonitrile/water gradient). Lyophilization afforded **1** (230 mg, 74%, 33% overall) as an off-white crystalline solid.

¹H-NMR (500 MHz, CDCl₃): δ 6.20 (br, s, 1H, NHCO), δ 6.01 (br, s, 1H, NHCO), δ 5.86-5.78 (m, 1H, CHCH₂), δ 5.06 (dd, 1H, *J* = 1.5, 17.0 Hz, CHCH₂), δ 4.99 (dd, 1H, *J* = 1.5, 10.0 Hz, CHCH₂), δ 3.60 (s, 4H, OCH₂CH₂O), δ 3.55 (m, 4H, CHOCH₂), δ 3.47 (m, 4H, CH₂NH), δ 2.52 (m, 2H, CH₂CCH), δ 2.44-2.36 (m, 4H, CH₂CHCH₂, NHCCH₂), δ 2.28 (t, 2H, NHCCH₂), δ 2.00 (t, 1H, CCH).

¹³C-NMR (100 MHz, CDCl₃): δ 175, 174, 137.3, 115.8, 83.2, 70.5, 70.4, 70.2, 70.1, 69.5, 39.5, 39.4, 36.0, 35.5, 29.8, 15.1.

HRMS (TOF ES⁺) calculated for C₁₆H₂₆N₂O₄ (M+H)⁺ = 311.1972; observed = 311.1989.

Synthesis and Characterization of 1,4-Dioxo-7,18-Diaza-Cycloicos-12-Ene-8,11,17-Trione (2)



In a typical experiment, a 6 mM solution of **1** was added over the times listed in Fig. 4 to a stirring aqueous solution containing Na_2PdCl_4 and CuCl_2 in the stoichiometries listed in Fig. 4. The final concentration of **1** was typically 1.5 mM. After completion of the addition, analytical HPLC typically revealed quantitative conversion of **1** to a single product. To purify the product, the reaction was subjected to preparative reverse-phase HPLC purification with an acetonitrile/water gradient. Lyophilization of the purified material provided macrocyclic enone **2** as a yellow oil in the isolated yields reported in Fig. 4.

Figure 4, Entry a

To a solution of Na_2PdCl_4 (9.4 mg, 32 μmol , 1.0 equiv.) in 1 M NaCl in water (17 mL) stirring at 25 °C open to the air was added, at a rate of 0.34 mL/h, a solution of **1** (10 mg, 32 μmol , 1.0 equiv.) in water (5.0 mL). At the completion of addition, analytical HPLC of 1.0 mL of the reaction showed quantitative conversion of starting material to a single product. To isolate the product, 20 mL of the 22 mL total reaction volume was subjected to preparative reverse-phase HPLC purification with an acetonitrile/water gradient. Lyophilization of purified material provided macrocyclic enone **2** (8.2 mg, 86%) as a yellow oil.

Figure 4, Entry b

To a solution of Na_2PdCl_4 (0.49 mg, 1.6 μmol , 0.05 equiv.) in 100 mM NaCl in water (15 mL) was added CuCl_2 (5.4 mg, 32 μmol , 1.0 equiv.). A 6 mM solution of **1** (10 mg, 32 μmol , 1.0 equiv.) in water (5 mL) was added at 4.1 mL/h. The reaction mixture was allowed to stir at 25 °C open to the air for 1 h. Analytical HPLC of 500 μL of the reaction showed quantitative conversion of the starting material to a single product. The product was purified as described above to afford **2** (9.0 mg, 90%) as a clear oil.

Figure 4, Entry c

To a solution of Na_2PdCl_4 (0.49 mg, 1.6 μmol , 0.05 equiv.) in 6.5:1 tetrahydrofuran:water (15 mL) was added CuCl_2 (5.4 mg, 32 μmol , 1.0 equiv.). A 6 mM solution of **1** (10 mg, 32 μmol , 1.0 equiv.) in tetrahydrofuran (5 mL) was added at 2.7 mL/h. The reaction mixture was allowed to stir at 25 °C open to the air for 2 h. Analytical HPLC of 500 μL of the reaction showed quantitative conversion of the starting material to a single product. Tetrahydrofuran was removed *in vacuo*, and the product was purified as described above to afford **2** (9.0 mg, 91%) as a yellow oil.

Figure 4, Entry d

To a solution of Na_2PdCl_4 (1.4 mg, 4.8 μmol , 0.15 equiv.) in 9:1 tetrahydrofuran:water (40 mL) was added **1** (10 mg, 32 μmol , 1.0 equiv.). The reaction mixture was allowed to stir at 25 °C under 1 atm O_2 for 14 h. Following analytical HPLC of 1.0 mL of the reaction, the product was purified as described above to afford **2** (7.5 mg, 73%) as a yellow oil.

Figure 4, Entry e

To a solution of CuCl_2 (2.7 mg, 16 μmol , 1.0 equiv.) in 100 mM NaCl in water (10 mL) was added **1**. The reaction was allowed to stir at 25 °C open to the air for 4 h at which point 1.0 mL of the reaction mixture was subjected to analytical HPLC. No conversion of **1** to any product was observed.

Figure 4, Entry f

To a suspension of CuCl (1.6 mg, 16 μmol , 1.0 equiv.) in 100 mM NaCl in water (10 mL) was added **1**. The reaction was allowed to stir at 25 °C open to the air for 4 h at which point 1.0 mL of the reaction mixture was subjected to analytical HPLC. No conversion of **1** to any product was observed.

UV: 230 nm, 310 nm

HRMS (TOF ES⁺) calculated for $\text{C}_{16}\text{H}_{26}\text{N}_2\text{O}_5$ (M+H)⁺ = 327.1921; found = 327.1914.

¹H-NMR (400MHz, CD₃OD): δ 6.94-6.86 (dt, 1H, J = 7.2, 16 Hz, CHCHCO) δ 6.08 (dt, 1H, J = 1.2, 16 Hz, CHCHCO), δ 3.62 (s, 4H, OCH₂CH₂O), δ 3.56 (t, 2H, OCH₂CH₂), δ 3.54 (t, 2H, OCH₂CH₂), δ 3.38-3.35 (m, 4H, CH₂NH), δ 2.92 (t, 2H, CHCOCH₂), δ 2.41 (t, 2H, CH₂CONH), δ 2.30 (m, 2H, CH₂CH₂CH), δ 2.24 (t, 2H, CH₂CH₂CONH), δ 1.91- 1.82 (m, 2H, CH₂CH₂CH₂).

¹H-NMR (500MHz, CDCl₃): δ 6.83-6.77 (dt, 1H, J = 7.2, 16 Hz, CHCHCO), δ 6.51 (bs, 1H, NHCO), δ 6.12-6.07 (dt, 1H, J = 1.2, 16 Hz, CHCHCO), δ 6.10 (bs, 1H, NHCO), δ 3.60 (s, 4H, OCH₂CH₂O), δ 3.56 (t, 2H, OCH₂CH₂), δ 3.53 (t, 2H, OCH₂CH₂), δ 3.47-3.42 (m, 4H, CH₂NH), δ 2.88 (t, 2H, CHCOCH₂), δ 2.57 (t, 2H, CH₂CONH), δ 2.32 (m, 2H, CH₂CH₂CH), δ 2.18 (t, 2H, CH₂CH₂CONH), δ 1.91 (m, 2H, CH₂CH₂CH₂).

¹³C-NMR (100 MHz, CDCl₃): δ 199.9, 173.0, 172.4, 148.0, 132.6, 70.8, 70.6, 70.4, 69.8, 39.5, 39.2, 35.0, 34.2, 31.6, 31.0, 23.2.

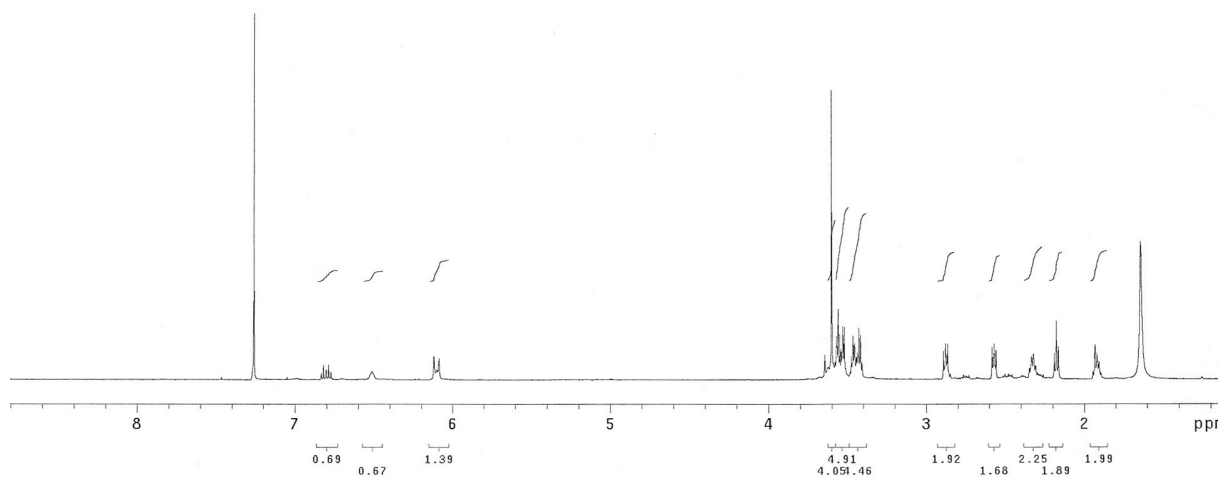
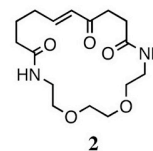
NMR spectra of **2** are shown below:

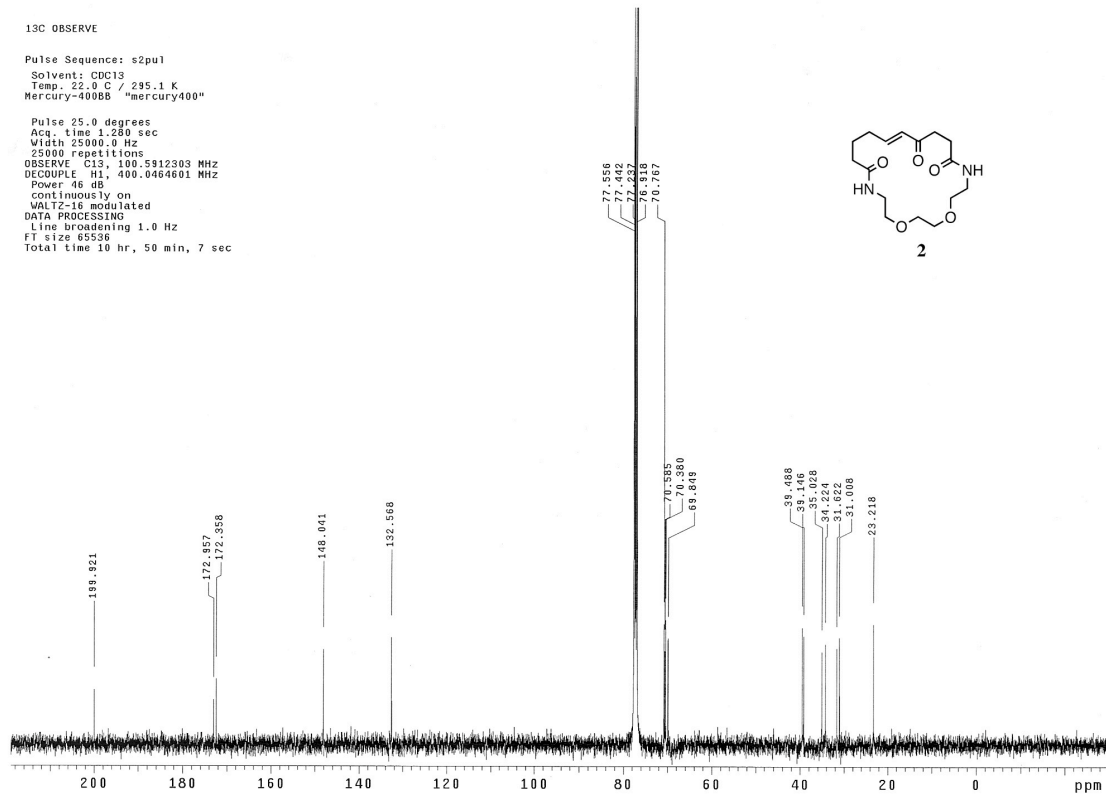
¹H-NMR spectrum:

STANDARD PROTON PARAMETERS

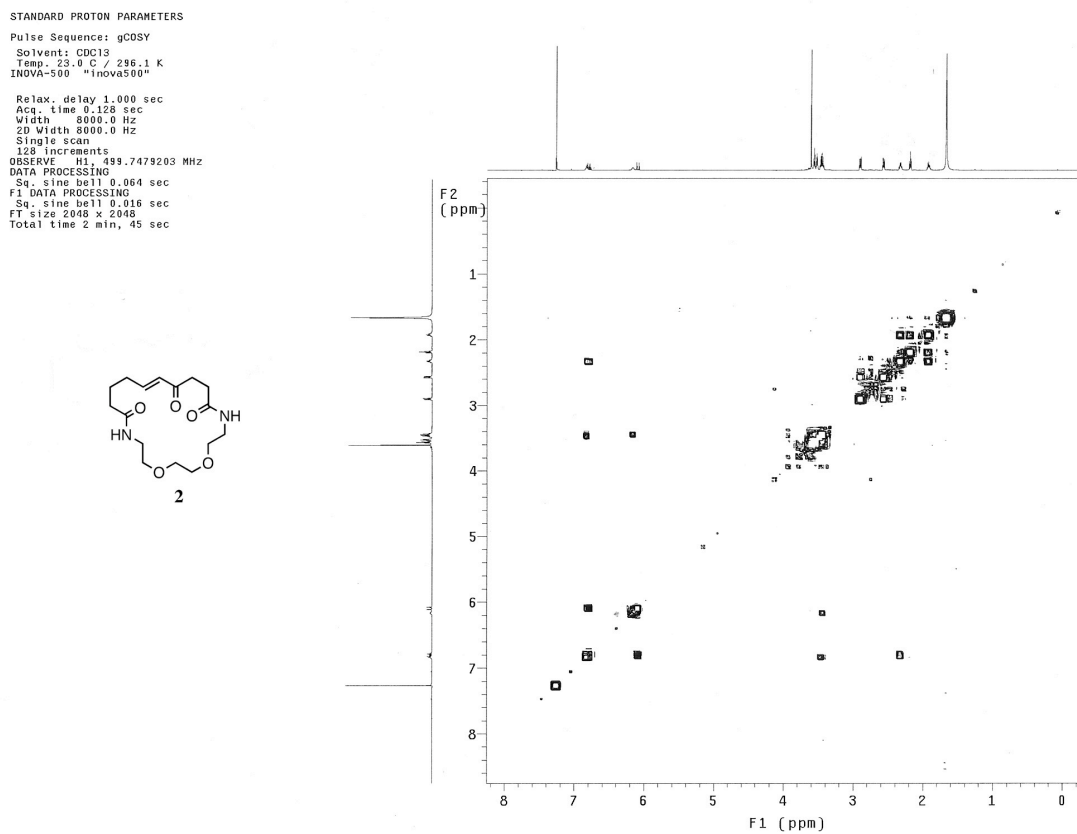
Pulse Sequence: s2pul
Solvent: CDC13
Temp: 23.0 C / 296.1 K
INOVA-500 "inova500"

Relax. delay 0.200 sec
Pulse 74.0 degrees
Acq. time 2.048 sec
Width 8000.0 Hz
16 repetitions
OBSERVE H1: 499.7479203 MHz
DATA PROCESSING
Line broadening 0.1 Hz
FT size 32768
Total time 0 min, 40 sec



^{13}C -NMR spectrum:

Homonuclear COSY spectrum:



APPENDIX G
TO
DECLARATION OF
MARIA P. GARCIA

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Supplementary Tables S1-S6 (individually numbered and integrated into the above).

DNA oligonucleotide coupling methods on a PerSeptive Biosystems phosphoramidite for DNA purification by reverse-phase HPLC and an acetonitrile/100 mM spectroscopy. Labeled oligonucleotides were purified by reverse-phase HPLC within 0.075% of expected retention times using a Milli-Q purification system.

For purification and templated reactions, analytic with a Varian Pursuit C18 re on a Varian ProStar instrui reverse-phase column. Prot (¹³C-NMR), and COSY spe INOVA500 (500 MHz) NM analyzed by electrospray mas

Preparation of Pool A DNA

Pool A Oligonucleotide Sequences
Complete pool A template annealing region comprising coding region used to identify S1). Each pool A small-molecule linkage at the 5' end of the oligonucleotide.

Preparation of Component O

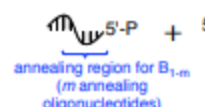
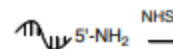
Pool A DNA-linked
types of oligonucleotides: "
description and preparation o

Coding oligonucleotides, containing a binding site, a 12-base coding sequence (the C7 amino CPG). This sequence has natural 3'-OH groups.)

Annealing oligonucleotides with Chemical Phosphorylation unique 15-base annealing region present in the coding region undergo a ligation reaction via assembly (see below).

Splint oligonucleotide
to an annealing region, 5 bas
bases complementary to the 3'

Substrate-linked oligo
(installed using the Amino-M



APPENDIX H
TO
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TI [Bringing picomolar](#) protein detection into proximity.
AU [Famulok Michael](#)
SO Nature biotechnology, (2002 May) Vol. 20, No. 5, pp. 448-9.
Journal code: 9604648. ISSN: 1087-0156. L-ISSN: 1087-0156.
DOI <http://dx.doi.org/10.1038/nbt0502-448>
CY United States
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News Announcement
LA English
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APPENDIX I
TO
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nature biotechnology

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Reprogramming fibroblasts

Picomolar protein detection

SAGE gets just a little longer

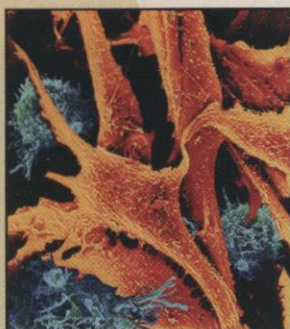
Mouse mutant shortcut

RNAi cassettes prolong the silence

Alamar Exhibit 1040
Alamar v. Olink

nature biotechnology

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Colored scanning electron micrograph of T cells (green) superimposed onto fibroblasts (orange). On p. 460, Collas and colleagues use T cell extracts to reprogram fibroblasts. Images © Photo Researchers, Inc.

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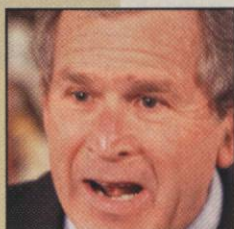
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inducible siRNA expression systems may provide an interesting alternative in such cases⁶. For example, cells could be grown on a large scale before induction of the knockdown, which may be beneficial for proteomic analysis. Nonetheless, in targeting nonessential proteins, stable "knock-down" cells may be of great value when studying inducible processes such as UV or other irradiation damage response, host-pathogen interactions, or cell differentiation.

Considering all the pros and cons of expressed versus synthetic siRNAs, it is probably most effective to begin the search for highly effective siRNAs with synthetic, ready-to-use duplex RNAs of defined sequence and length, and to select the synthetic sequences such that they are readily compatible with the sequence requirements for expression within U6 or H1 RNA expression cassettes. This will first entail ensuring that in the U6 snRNA and H1 RNA systems that the +1 position is a guanosine^{7,9} and an adenosine, respectively. In addition, it will require that uridines be present in the 3'-terminal position encoded by the oligothymidine Pol III terminator signal sequence⁹.

Ultimately, the possibility of stable expression of siRNAs may pave the road for new gene therapy applications, such as treatment of persistent viral infections.

Incorporation of siRNA expression cassettes into retroviral vectors may also allow the targeting of primary cells previously refractory to siRNA or plasmid DNA transfection⁸. Considering the high specificity of siRNAs⁴⁻⁷, the approach should allow the targeting of disease-derived transcripts with point mutations, such as *RAS* or *TP53* oncogene transcripts, without alteration of the remaining wild-type allele. Finally, because of the automation developed for high-throughput sequence analysis of the various genomes, the DNA-based methodology may also be a cost-effective alternative for automated genome-wide loss-of-function phenotypic analysis, especially when combined with miniaturized array-based phenotypic screens¹¹.

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Bringing picomolar protein detection into proximity

Combining target recognition by two aptamers, enzymatic ligation, and PCR, the proximity ligation method enables the detection of minute amounts of proteins.

Michael Famulok

Thousands of proteins with potential diagnostic and/or therapeutic applications are expected to emerge from the various genome projects. Proteins in different tissues or individual cells can be up- or downregulated in response to internal or external stimuli, signal transduction, transcriptional control, medication, disease, or

pathogens. The ability to monitor slight differences in the amounts of proteins and other biomolecules within the smallest possible detection volumes down to the single-cell level is of utmost importance not only for proteomics research but also for diagnostic and technological purposes. In this issue, Fredriksson *et al.*¹ describe a new method, proximity ligation, that allows the detection and quantification of minute amounts of a specific protein.

Methods currently in widespread use for research requiring standard protein detection include two-dimensional gel electrophoresis, mass spectrometry, and antibody-based

detection by sandwich enzyme-linked immunosorbent assays (ELISAs) or western blotting. Though sufficient for most standard applications, these techniques are not sensitive enough to meet the increasingly stringent detection limits required in the postgenomics era. For example, many proteins, such as cytokines and certain kinases, exert their functions at concentrations considerably lower than the thresholds of detection attainable by these methods. To overcome these limitations, variations of the sandwich ELISA with markedly increased sensitivity, such as the "immuno-polymerase chain reaction" (immuno-PCR)² and a technique termed "immunodetection amplified by T7 RNA polymerase" (IDAT)³, have been developed. These methods combine antigen recognition by a biotin-tagged antibody with PCR amplification of a biotinylated reporter DNA, linking the two to one another via a streptavidin molecule. Tags other than biotin, alternative bridging moieties, or covalent coupling methods have also been used in immuno-PCR experiments. When performed under "real-time PCR" conditions, in which a fluorogenic oligonucleotide probe is used to measure PCR product accumulation, these methods can provide very accurate and highly sensitive quantification of the PCR products.

Fredriksson *et al.* do not use any antibodies, tags, or linker molecules and yet achieve levels of protein detection competitive with those of immuno-PCR. They have used a clever strategy whereby an "antigen" is detected by nucleic acid-based receptor molecules, so-called aptamers⁴. As a test case, the researchers use a pair of DNA aptamers that binds to the homodimer of the target analyte protein, platelet-derived growth factor B-chain (PDGF-BB). Each aptamer has a different DNA-sequence extension that does not interfere with its folding and is not required for target recognition. Binding of the aptamer pair brings the ends of the oligonucleotide extensions into close spatial proximity (Fig. 1A) so that a "splint" oligonucleotide can hybridize to both ends, which are subsequently ligated together by T4 DNA ligase. The ligated species can then act as a PCR template and the amplified PCR product can be monitored and quantified under real-time PCR conditions, whereas no signal is obtained with unligated probes (Fig. 1B).

The sensitivity that can be achieved with the method is remarkable. As few as 24,000 molecules, or 4×10^{-20} moles, of the PDGF-BB protein could be detected, approximately 1,000-fold fewer than could be

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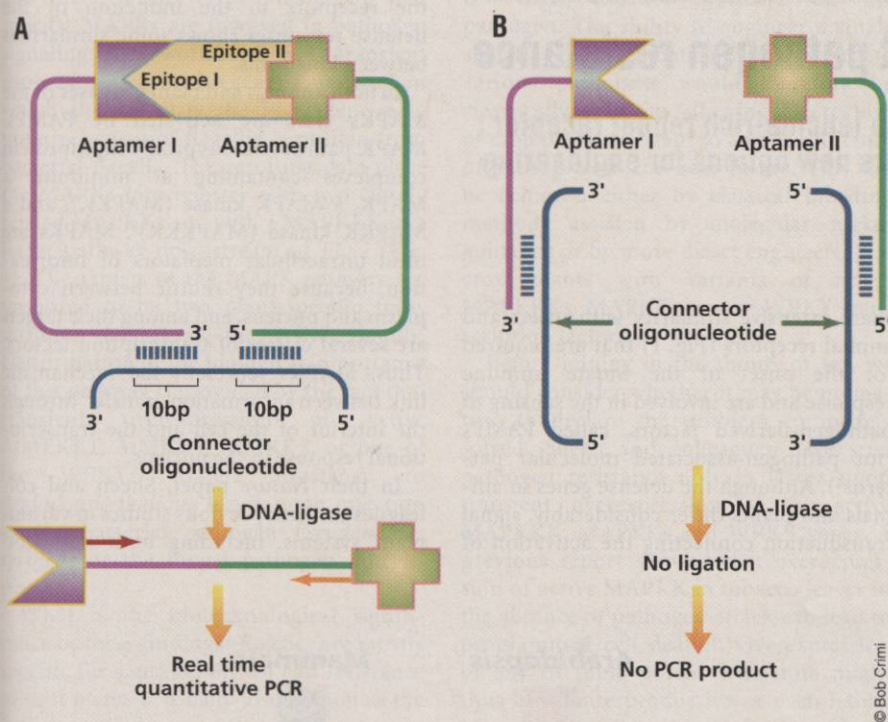


Figure 1. The proximity ligation assay can be 1,000-fold more sensitive than a conventional ELISA. (A) A pair of aptamers that bind either to distinct epitopes of the target protein or to the same epitope in a homodimer bring the free ends of their sequence extensions into close spatial proximity. This allows the core sequence of the connector oligonucleotide to hybridize to both ends, acting as a template for enzymatic ligation. Subsequently, ligated sequences can be PCR amplified using specific primer sequences (brown and orange arrows). Detection of the PCR product is achieved under conditions of real-time quantitative PCR. (B) Without the target protein, the connector oligonucleotide cannot act as a ligation template.

detected in a standard sandwich ELISA assay for the same target. Although the detection sensitivity is in the same ballpark as for immuno-PCR, a major advance of the proximity ligation assay as compared with immuno-PCR is that the whole assay can be performed in a homogenous format that does not include a single wash or separation step. This saves considerable time and should make the method suitable for automation. The assay also has potential for application in clinical laboratories or hospitals, as it can detect PDGF-BB present in crude biological samples such as fetal calf serum, cerebrospinal fluid, cell culture media, and human blood serum¹.

Like immuno-PCR, proximity ligation is prone to certain restrictions connected with the nonlinear amplification ability of PCR that may limit the approach for quantification. Rolling-circle PCR (ref. 5) or IDAT (ref. 3) may easily overcome these restrictions. A potentially more serious limitation, however, could be the difficulty of generalizing the assay. Fredriksson *et al.* obtained their most impressive result with a multimerized protein, the PDGF homodimer. To detect PDGF they used two aptamer-based proximity probes that recognize the same

site on each dimer. In their second example, detection of a monomeric protein, the human blood clotting factor α -thrombin, required a pair of two different aptamers that recognize distinct sites on the protein. This example was selected for good reason: except for α -thrombin, there is not a single additional protein target for which a set of aptamers that bind different sites on the same protein has been isolated. Although in principle it should be possible to obtain aptamers that bind to distinct sites on a target, it is unclear at present whether this can be done with the same ease as for antibodies, and, more importantly, whether very many targets exist for which such different aptamers are selectable.

However, it is now well established that aptamers can form high-affinity binding sites for almost any given target protein, with dissociation constants as low as sub-picomolar⁶ and binding specificities that can be as good as, or sometimes even better than, those of antibodies⁷. Furthermore, aptamers have the added advantage of stability because chemical modifications, such as 2'-fluoro-2'-deoxypyrimidine residues, can render them nearly resistant against nuclease degradation⁶. Aptamer researchers

will probably now look much more thoroughly at whether their selected libraries contain aptamer pairs that bind different target sites. Furthermore, with automated selection protocols available⁸, the isolation of the required aptamer pairs may become an easier task than in pre-automation days because *in vitro* selections can focus on selecting high-affinity aptamers that bind more than one site on a target. In addition, there is no reason that the approach should be limited to aptameric receptors. Proximity ligation will likely work for antibody probes that are linked to a DNA sequence, similar to immuno-PCR, and it will be interesting to see what level of sensitivity can be achieved with "immuno-proximity ligation".

The extremely sensitive detection of molecules that can be achieved with these methods raises the question of whether it will be possible, by clever technical refinement and by transformation into array formats, to build proximity ligation-based sensors that may reach the capabilities of, for example, the sensor array of the mammalian olfactory epithelium. This may seem far-fetched, but in the future it should be possible to use these or similar technologies for the identification of many different individual "odors" (i.e., minimal alterations in the composition of different sets of proteins from various tissues or cells). Unlike the mammalian olfactory system, however, sensor arrays based on the proximity ligation method may not be of use in quantifying small molecules. The fact that two different epitopes will have to be bound, regardless whether antibodies or aptamers are used, would make it difficult to adapt the method to small organic molecules or even small peptides. Even so, using the approach in combination with other methodologies, such as allosteric ribozymes⁹, may help to achieve this goal. Wherever its future lies, the proximity ligation method deserves a close look, as it represents a significant advance as compared with current methods for protein quantification.

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