Analytical Methods

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Sensing Enzymatic Activity by Exposure and Selection of DNA-Encoded Probes

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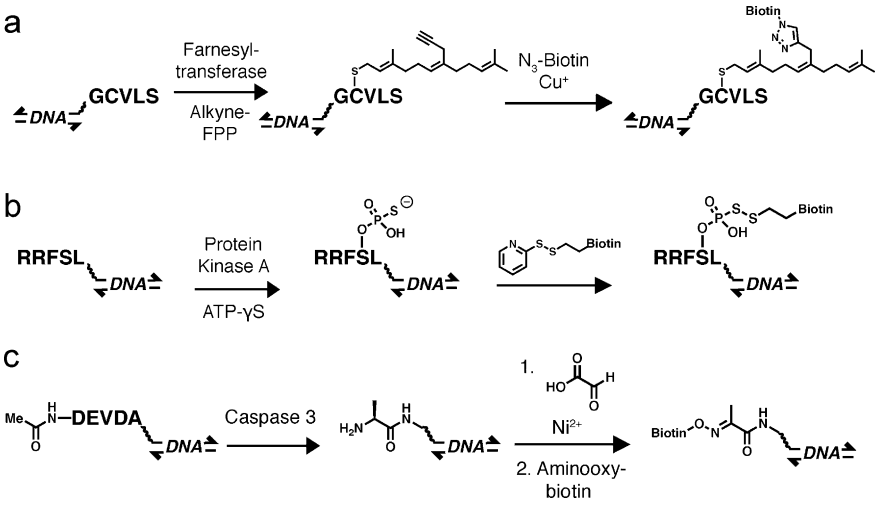
Abstract: *A sensing approach is applied to encode quantitative enzymatic activity information into DNA sequence populations. The method utilizes DNA-linked peptide substrates as activity probes. Signal detection involves chemical manipulation of a probe population downstream of sample exposure and application of purifying, selective pressure for enzyme products. Selection-induced changes in DNA abundance indicate sample activity. The detection of protein kinase, protease, and farnesyltransferase activities is demonstrated. The assays were employed to measure enzyme inhibition by small molecules and activity in cell lysates using parallel DNA sequencing or quantitative PCR. This strategy will allow the extensive infrastructure for genetic analysis to be applied to proteomic assays, which has a number of advantages in throughput, sensitivity, and sample multiplexing.*

Recent technological advances have resulted in an explosion of genomic data.[1] These achievements have been facilitated by the powerful tools available to read, write, and manipulate DNA-encoded information. Both enzymes from nature (polymerases, restriction enzymes, recombinases) and man-made technologies (DNA synthesis chemistry, DNA sequencers, thermocyclers) have made information encoded in DNA highly accessible.[2] Given the power of these techniques, additional approaches to transduce nongenomic information into DNA sequences would be broadly useful.[3]

In natural selection, nature provides a mechanism by which exogenous information is encoded within DNA sequences. Selection-induced allele frequency changes in a population serve to encode the relative function of alleles within their environment (i.e. exogenous factors). In this Communication, we describe a recreation of this encoding process in the test tube using the exposure and selection of DNA-linked molecules to detect enzymatic activity by DNA sequence analysis. As outlined in Figure 1, the approach involves DNA-linked substrates serving as sample probes whereby DNA sequences indicate the identity of a sample. Exposure to enzymes potentially alters probe phenotypes from substrates to products. The probe phenotype determines survivability in a subsequent selection on a probe pool from many samples. The change in probe frequency as detected by DNA sequence analysis allows assessment of sample enzyme activity.

We have applied this approach for the detection of farnesyltransferase (FTase), protein kinase, and protease activities. Probes for these activities consist of peptide substrates covalently attached to an encoding DNA construct (Scheme 1; see also Figure S1 in the Supporting Information). The conversion of the substrate into the product allows for the selective addition of a biotin affinity tag, using previously demonstrated schemes for enrichment of post-translationally modified peptides.[4–6] Selective purification of enzyme product–DNA conjugates using streptavidin magnetic beads applies selective pressure on the probe population and allows determination of enzymatic activity through DNA quantitation.



Scheme 1. Selective labeling of enzyme products. Chemical biotinylation approach for a) FTase, b) protein kinase a (PKA), and c) caspase 3 enzyme products. Alkyne-FPP = 7-propargyl-farnesyl pyrophosphate; ATP-γ-S = adenosine 5′-[γ-thio]triphosphate.

Peptide substrates were synthesized on commercially available, 5′-modified 20-mer oligonucleotides (see the Supporting Information for synthetic details). Conjugates were prepared either by direct peptide synthesis on amine-modified oligonucleotides[7] or by postsynthetic conjugation using copper-catalyzed azide–alkyne cycloaddition.[8] These oligo-

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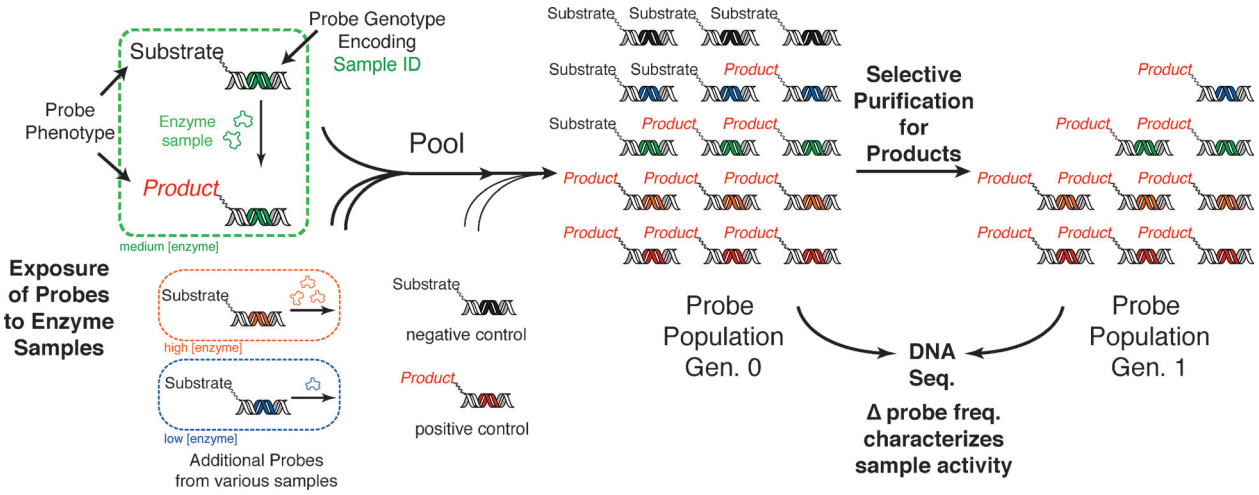

Figure 1. Detection of enzyme activity using DNA-encoded probes. The probe molecules consist of enzyme substrates linked to DNA. The DNA (probe genotype) encodes the identity of a particular enzyme sample to which a probe is exposed. This exposure potentially converts the probe phenotype from substrate into product. After quenching enzyme activity, the probes are pooled with appropriate controls and are subjected to a selection for product probes. The resulting change in probe frequency within a population quantifies the enzymatic activity of the samples.

nucleotides were then used as primers in a PCR to append substrates to specific encoding constructs (140-mer DNAs; Figure S1). Encoding constructs contained two unique 20-mer barcode regions directly within common end-priming regions, which were used for specific amplification in quantitative PCR (qPCR). The product-selective labeling schemes were validated on peptide–DNA conjugates by gel shift assays through binding to avidin (Figure 2a–c) and also by enrichment with streptavidin beads and qPCR analysis (Figure 2d).

These three enzyme assay schemes were selected because of their potential for generality in detecting activity of a number of biologically important enzymes. In addition to FTase, many transferases accept modified substrates containing click labels (azides or alkynes). Thus, this assay approach can be easily extended to other enzymes, such as glycosyltransferases[9] (several azido sugars are commercially available), methyltransferases,[10] acetyl transferases,[11] and palmitoyltransferases.[12] Similarly, numerous protein kinases have demonstrated the ability to accept thio-ATP as a substrate.[13] Also, the N-terminal labeling approach could be applied to the cleaved products of any endoprotease.

Using sets of peptide–DNA constructs with unique DNA sequences, we performed multiplexed assays with several enzyme concentrations using parallel DNA sequencing (Figure 3a,b; Figure S2a) and qPCR (Figure S2b–d). Probe frequency changes were detected among a pool of approximately 40 sequences in response to enzyme exposure, biotin labeling, and affinity purification (see Figure S3 for a representative scheme). For each of the three assays, we observed dependency of the assay signal on enzyme concentration. Results were similar quantifying DNA by read ratio with parallel DNA sequencing (Illumina) or by qPCR using construct-specific primers, although variance in signal was generally greater using qPCR. In the low turnover range (<20%) of the FTase and kinase assays, linearity was observed (Figure 3a,b, insets).

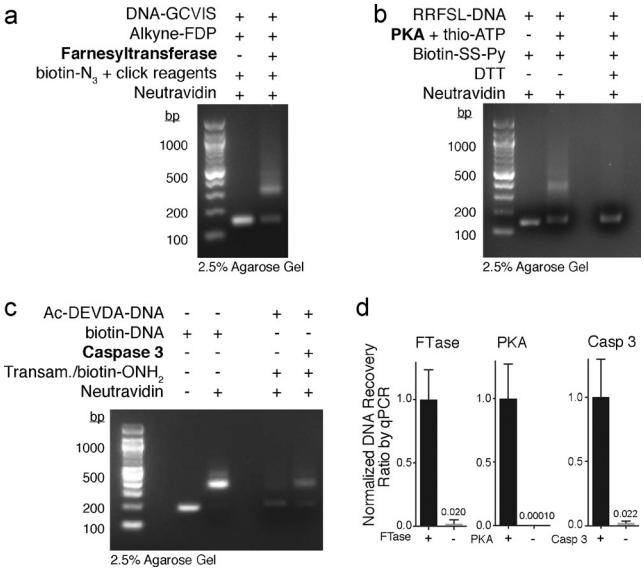

Figure 2. Enzyme-dependent biotinylation and purification of peptide–DNA conjugates. a–c) Gel shift analysis of enzyme product-selective labeling. Approximately 500 ng portions of a) 180-mer Casp 3, b) 140-mer PKA, or c) 140-mer FTase substrate peptide–DNA conjugates were treated using modification procedures (Scheme 1; see also the Supporting Information). Prior to gel analysis, purified DNAs were incubated with 4 μg neutravidin (deglycosylated avidin, ThermoFisher), as indicated. d) Normalized recovery of DNA-encoded probes in response to indicated treatments and streptavidin affinity purification. Error bars represent one standard deviation for 3–5 unique DNA constructs treated as indicated.

To investigate the utility of DNA-encoded probes in complex mixtures, we conducted the caspase assay in lysates from HeLa cells with or without staurosporine treatment to induce apoptosis. The approximate sixfold increase in caspase activity observed by qPCR (Figure 3c) was consistent with a colorimetric assay and with previous reports.[14] While some

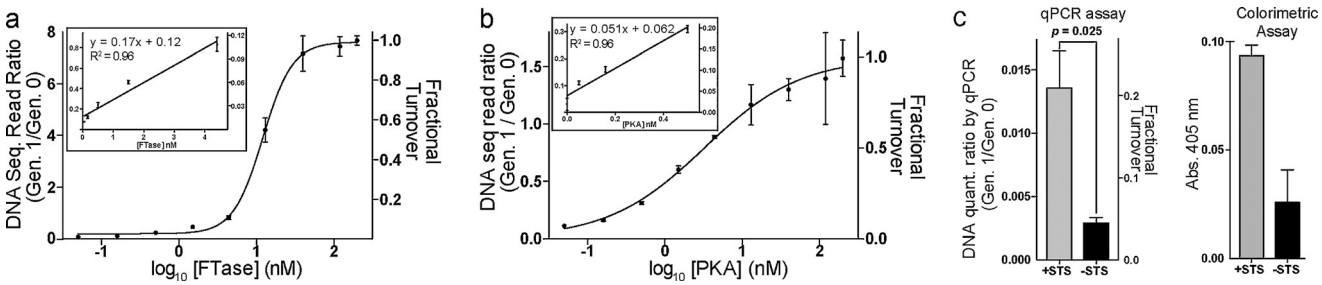

Figure 3. DNA sequence-based detection of enzymatic activity using DNA-encoded probes. a, b) Plots of relative DNA read ratio versus FTase (a) and PKA (b) concentrations. Insets in (a) and (b): the low turnover range on a linear scale. c) Detection of change in caspase 3 activity in response to apoptosis induction with staurosporine (STS) in HeLa cells using qPCR or a colorimetric assay of cell lysates. Error bars represent one standard deviation of the signal for four unique DNA constructs at a single enzyme concentration or lysate sample.

degradation of the DNA would not preclude detection, we observed minimal DNA degradation of the probes in cell lysate over a 90 min incubation period. As expression of several nucleases are induced in apoptosis,[15] this could be considered a stringent test for probe stability.

To demonstrate the utility of these assays for detection of enzyme inhibition, we measured the half maximal inhibitory concentrations (IC_{50}) for the FTase inhibitor tipifarnib[16] (Figure 4a), and the protein kinase a (PKA) inhibitor staurosporine (Figure 4b). To test the feasibility of inhibition assays for high-throughput screening (HTS), we assessed the separation of sets of partially biotinylated (10%) and non-biotinylated DNA constructs by parallel DNA sequencing (Figure 4c). This test was designed to mimic high-throughput screening conducted at 10% enzymatic turnover. The results showed little contribution of the construct sequences to the observed signal. As a mock assay, the results yielded a Z′ factor of 0.5, which is indicative of robustness.[17] Random sampling of sequencing reads indicated that the read number per sample could be reduced to approximately 600 without affecting the Z′ factor (Table S9).

The use of DNA as a signal has advantages of high sensitivity through DNA amplification. In these three assays, the limits of detection (LOD) observed were generally around 2% of the full substrate turnover (Figure 2d, Figure 3a,b; Figure S2). This background signal was consistent with the level observed in the separation of biotinylated and nonbiotinylated DNA constructs (Figures 4c; Figure S4). Improvements could be achieved through optimized streptavidin purifications or by use of alternative affinity tags. Nonetheless, these assays require a very small amount of sample for detection, which, in theory, is limited only by adequate sampling of the DNA probes. In the kinase assay for example, detection of activity by qPCR at the LOD ($\approx$50 pM) would require less than 1 nL of sample. In addition, this sensitivity allows assays to be conducted at low concentrations ($\approx$10 nM or lower) of substrate peptide–DNA probes. With these concentrations being well below enzyme Michaelis constant K_m values, these assays are highly sensitive to inhibition.

In addition to facilitating analysis of many samples, the collective manipulation and selection of a pool of DNA-encoded probes from several samples is critical for precise and quantitative activity detection. Pooled probe manipulation allows any inefficiencies or variability in chemical modification, DNA purification/precipitation, or affinity purification steps to be normalized across the pool. This normalization enables precise assays. In general, low percent coefficients of variation (%CV) were

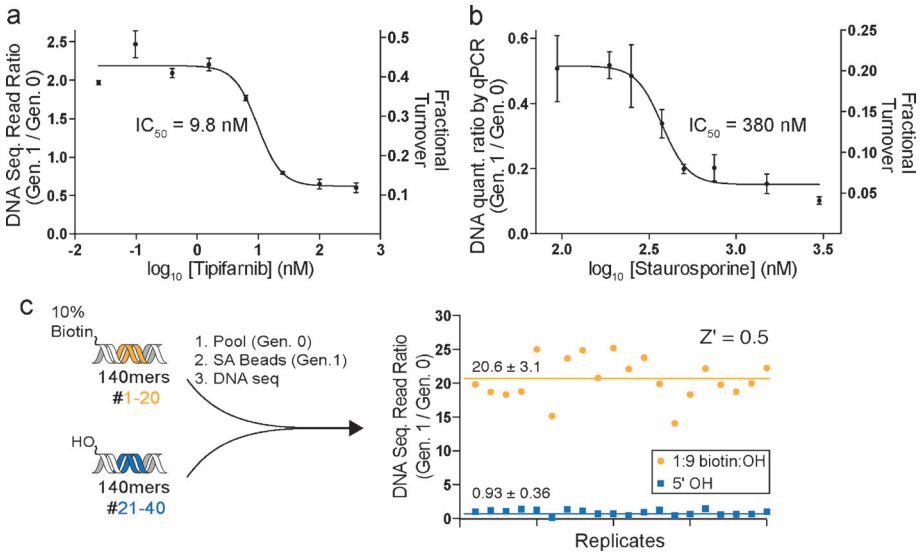

Figure 4. Application of DNA-encoded probes for detection of enzyme inhibition and in a mock multiplexed assay. a, b) Determination of IC_{50} values for a) the FTase inhibitor tipifarnib by DNA sequencing and b) for the PKA inhibitor staurosporine by qPCR. Error bars represent one standard deviation of the signal for four unique DNA constructs at a single inhibitor concentration. c) Assessing sequence variability for multiplexed activity detection. To mimic assay screening at 10% turnover, 20 unique constructs were prepared that were partially biotinylated (10%) and then mixed with 20 nonbiotinylated, control constructs. After streptavidin (SA) purification, sample pools were sequenced. Solid lines indicate sample means.

observed. For example, in the determination of the IC_{50} value for tipifarnib by DNA sequencing (Figure 4a), the overall average %CV was 10%. In addition, the recovery of a particular probe relative to a positive control within the pool allows determination of fractional turnover and a quantitative measurement of enzyme activity. In the protease assay, a DNA construct with the fully cleaved peptide product was doped into the pool. In the FTase and PKA assays, probes treated with an excess of enzyme were used as positive controls. Negative controls established the assay background and were either DNAs lacking peptide substrates or peptide–DNA conjugates that were not treated with enzyme.

The advent of DNA-encoded small-molecule libraries has enabled the application of selection-based approaches to large collections of synthetic molecules.[3d,18] Assays with DNA-encoded probes will enable many of the same benefits realized in those approaches to be applied to any molecule of interest that is free in solution, such as molecules within existing library screening collections. As probe manipulations (covalent modifications and DNA purifications) and affinity selections are conducted on a probe pool, costs and effort increase only marginally as more assays are combined. The high sensitivity achieved by DNA amplification allows assays to be massively miniaturized, conserving costly enzyme and compound stocks. The high capacity of parallel DNA sequencing would readily allow concurrent assessment of approximately 150000 samples (at present levels, allotting 1000 reads per sample).[3a]

DNA-encoded probes could also see application in activity profiling of proteomic samples. DNA-assisted protein detection approaches, such as immuno-PCR and proximity extension assays,[19] have demonstrated remarkable sensitivity and multiplexed analyte detection. Peptide substrates are commonly employed to detect protease and protein kinase activities in fluorescence, mass spectrometry, and microarray approaches.[20,21] Using sets of validated peptide substrates and additional, identifying DNA barcodes, multiple activities could be assessed concurrently in a manner analogous to a peptide microarray.

In summary, we have developed a novel method for sensing sample characteristics through exposure, selection, and DNA sequence analysis of DNA-encoded probes. We have applied the approach for detection of enzymatic activities. As the capabilities of genetic analysis techniques continue to increase rapidly, we expect the approach to find broad application in enzyme inhibitor assays and proteome activity profiling. Development of suitably responsive probe molecules will allow this general approach to be extended to the detection of other stimuli by DNA analysis.

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