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Divide and Control: Comparison of Split and Switch Hybridization Sensors

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Abstract

Hybridization probes have been intensively used for nucleic acid analysis in medicine, forensics and fundamental research. Instantaneous hybridization probes (IHPs) enable signalling immediately after binding to a targeted DNA or RNA sequences without the need to isolate the probe-target complex (e. g. by gel electrophoresis). The two most common strategies for IHP design are conformational switches and split approach. A conformational switch changes its conformation and produces signal upon hybridization to a target. Split approach uses two (or more) strands that independently or semi independently bind the target and produce an output signal only if all components associate. Here, we compared the performance of split vs switch designs for deoxyribozyme (Dz) hybridization probes under optimal conditions for each of them. The split design was represented by binary Dz (BiDz) probes; while catalytic molecular beacon (CMB) probes represented the switch design. It was found that BiDz were significantly more selective than CMBs in recognition of single base substitution. CMBs produced high background signal when operated at 55°C. An important advantage of BiDz over CMB is more straightforward design and simplicity of assay optimization.

Keywords

Deoxyribozyme sensors; split hybridization probes; Catalytic molecular beacons; single nucleotide polymorphisms; instantaneous hybridization probes; fluorescent sensors

Hybridization probes for nucleic acid analysis have been used since 1961.^[1] The approach takes advantage of selective recognition of a targeted nucleic acid fragment of known sequence by short complementary DNA or RNA probes. The resulting hybrid can be detected by a variety of techniques including blot, fluorescent in situ hybridization or gel electrophoresis. One major step in the evolution of hybridization probes is the development of instantaneous hybridization probes (IHPs) that can produce a signal (e. g. fluorescence)

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Conflict of Interest

The authors declare no conflict of interest.

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immediately upon hybridization to the target.^[2] IHPs eliminate the need for time-consuming downstream analysis of hybridization mixtures, like gel-based separation of probe-analyte complex from the excess amount of the unbound probe. Most common designs for IHPs are conformational switches or split (multicomponent design).^[3] Switch IHPs (e. g. molecular beacon probes^[4] and their variations^[4b,5]) change conformations upon hybridization to the target sequence and produce a signal. Split probes (e. g. adjacent hybridization probes^[6]) consist of several parts, which produce output signal when all the components and the analyzed sequence form an associate.^[7] In this study, we directly compare the performance of the two designs.

One of the most promising IHPs are deoxyribozyme (DZ)-based probes, for which both the switch^[11] and the split^[12] designs were explored earlier. DZs are short catalytic DNA sequences derived by *in vitro* selection.^[13] Most of the DZs used in analytical applications catalyze cleavage of RNA phosphodiester bonds,^[13] which can be conveniently converted to a fluorescent response, as shown in Scheme 1A: DZ binds a fluorophore- and a quencher-labeled DNA/RNA chimera substrate (**F_sub**) and cleaves the internal RNA phosphodiester bond (Scheme 1A), thus separating the fluorophore from the quencher. We and others take advantage of RNA-cleaving DZ in developing IHPs.^[14] One major advantage of DZ probes over other IHP (e. g. MB probes) is improved sensitivity and reduced limits of detection (LOD) due to the catalytic amplification of the output signal: one analyte bound to a DZ probe triggers cleavage of multiple **F_sub** molecules (Scheme 1A). Another advantage is that expensive **F_sub** can be used for multiple analytes, given that DZa and DZb are adjusted to each new analyte sequence, which reduces the cost if multiple sequences need to be analyzed.

The allosteric switch DZ probe named in the original publication ‘catalytic molecular beacon’^[11] (CMB) is shown in Scheme 1B. The CMB design uses a monolith construct, in which the DZ catalytic site is inhibited by hybridization within the complementary fragment: the strategy akin to the intrasteric regulation in proteins.^[15] Binding of the complementary DNA/RNA analyte reduces the inhibition and releases the catalytic core, which cleaves fluorogenic substrate (**F_sub**) and produces the detectable signal.^[11] In the split strategy, the parent DZ is divided into two parts with analyte binding arms appended to each part (DZa and DZb in Scheme 1C).^[12] In the presence of a complementary analyte, the two strands assemble the catalytic site, which cleaves **F_sub** followed by increase in fluorescence. In this study, we compared the two designs in terms of selectivity (the ability to differentiate analytes with a single nucleotide substitution (SNS)), limit of detection (LOD) and optimization simplicity. We assume that any two sensors can be compared using the aforementioned parameters demonstrated by each sensor under near optimal conditions. Up to the best of our knowledge, this is the first direct experimental comparison of the two designs.

The following analytes were used in this study (Table S1): **T1–21** and **T1–86** represented 21 and 86 nucleotide-long fragments of the *TWIST1* gene coding for a transcription factor involved in cancer development.^[16] For testing selectivity of DZ sensors, SNS sites were introduced into the analytes to make **T2–21** and **T2–86**. The second target, **A1–70** represents the sequence from amelogenin gene and is used as a genetic marker for sex determination.

[17] **A2–70** analyte contained a SNS for selectivity study. The choice of the two practically significant but otherwise random targets would ensure more general applicability of the conclusions made in this study. We designed two variants of CMB and BiDz probes based on the most catalytically efficient deoxyribozyme 10–23^[18,14f] – one designed to recognize the analyte at 30°C, another optimal at 55°C, indicated by numbers 1 and 2, respectively. Lower temperature was chosen to compare our results with the earlier reported data collected under similar conditions^[4,5] and because of the importance of point-of-care diagnostic assays operating at ambient temperature.^[19] Both switch and split designs were accomplished according to the strategies reported earlier for optimal performance of each design under given conditions.^[11,12–14]

For detecting TWIST1 gene-related analytes at 30°C, we tested three CMB constructs (**CMB_T1**, **CMB_T1_a**, and **CMB_T1_b** see Table S1) with different lengths of the inhibitory fragments, which was progressively elongated to suppress CMB response in the absence of **T1** analyte. The three CMB probes were also HPLC purified prior testing to reduce the undesired background response. The initially designed **CMB_T1_a**, and **CMB_T1_b** with short inhibitory fragments demonstrated high background response in the absence of analyte (Figures S1 and S2). Finally, **CMB_T1** produced low background fluorescence and signal-to-background ratio (S/B) of ~ 3 after 1 hr of incubation with 5 nM analyte (Figure 1 A). Importantly, the differentiation of single base mismatched analyte **T2** was only moderate (Figure 1 A, bar graphs), which was quantified using the selectivity factor (SF, Table 1). Importantly, **CMB_T1** recognized short **T1–21** analyte with greater selectivity than the 40 nucleotide-long fragment of **T1–86**, which is in agreement with the affinity-specificity dilemma described by Demidov and Frank-Kmenetskii.^[19] This data also correlates well with earlier observations that CMB constructs can unambiguously discriminate only analytes with two mismatched bases, but not with SNS.^[11a,14a] In contrast, BiDZ probe demonstrated near perfect selectivity (Figure 1B). Importantly, no HPLC purification of **DZa_T1** and **DZb_T1** was needed, since contamination with shorter DNA products arising from incomplete oligonucleotide synthesis do not contribute to the elevated background as in the case of CMB design. This further reduces the synthetic cost of BiDz constructs. Furthermore, the low background for BiDZ can be achieved by optimizing the concentrations of DZa and DZb strands: we achieved near-optimal performance of the initially designed **DZa_T1** and **DZb_T1** simply by reducing their concentrations to 5 nM. Similar probe design and optimizations were performed for CMB and BiDZ probes that recognize **A1–70** and **A2–70** analytes. The obtained results demonstrate similar trend in terms of probe selectivity and LODs (Table 1 and Figures S9, S10).

It was shown earlier that BiDZ based on deoxyribozyme 10–23 has higher catalytic rates and lower LODs at elevated temperatures.^[14f] We, therefore, designed CMB and BiDZ probes for the detection of **T1–86** and **A1–70** analytes at 55°C. For both BiDZ and CMB designs, substrate-binding arms were elongated by 3 nucleotides each to enable binding of **F_sub** by the DZ constructs even at 55°C. Figure 2 A demonstrates the design and the selectivity data for one of the CMB constructs, **CMB_A2**. Importantly, the probe experienced leakage, as reflected by the high background (Figure 2B, ‘no analyte’ bar). We attempted to redesign the CMB with increased length of the inhibitory fragments to increase the stability of the folded

structure in the absence of the analyte, but without success (Figure S3 and S4). As a result of the high background, the signal for both the matched and mismatched analytes was only slightly above the background; the differentiation between fully matched **A1–70** and SNS containing **A2–70** was not observed (Figure 2 A, bar graph, Table 1). The BiDZ probe, on the other hand, demonstrated high analyte-dependent turn-on ratio (S/B), as well as impressive selectivity (see SF in Table 1). The LOD of 8 pM was achieved after 3 hrs of incubation, which is consistent with the previously reported data.^[14f,j] The lowest LOD for CMB was about 7-fold higher than the best value for BDZ. Similar design, optimization experiments and results were obtained for *TWIST1* gene-related analytes and their corresponding sensors (Table 1 and Figure S11, S12).

Among the enzyme-free IHP approaches, switch probes have received the most attention for the last 20 years and now dominate in majority of applications and the original developments.^[2,4,5] For example, allosteric switch probes based on spinach aptamer have been reported recently.^[21] In parallel, binary and multicomponent probes have been introduced.^[6,7,22] In this study, we directly compared the two designs using one of the most promising IHPs, DZ probes as a representative example. Our main conclusions are the following. (i) The detection limits for both design are comparable and consistent with the data published by different research groups. (ii) Split design is simpler and less expensive to optimize. (iii) Switch probes fail to work at 55°C. (iv) Split probes have significantly high selectivity toward single base substitutions at both 30°C and 55°C. Our results taken together with the prior observations strongly suggest that split is advantageous over switch design especially if SNS differentiation is required. At the same time, switch probes can still be useful if the formation of multistranded associates is hampered by a crowded environment e. g. found inside living cells. In this case the monolith design can facilitate sensor response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hall BD, Spiegelman S. Proc Natl Acad Sci U S A. 1961; 47:137. [PubMed: 13710749]
2. a) Navarro E, Serrano-Heras G, Castaço MJ, Solera J. Clin Chim Acta. 2015; 15:231–250. b) Armitage BA. Curr Opin Chem Biol. 2011; 15:806–812. [PubMed: 22055496] c) Østergaard ME, Hrdlicka PJ. Chem Soc Rev. 2011; 40:5771–5788. [PubMed: 21487621] d) Khakshoor O, Kool ET. Chem Commun. 2011; 47:7018–7024.
3. Gerasimova YV, Kolpashchikov DM. Chem Biol. 2010; 17:104–106. [PubMed: 20189100]
4. a) Tyagi S, Kramer F. R Nat Biotechnol. 1996; 14:303–308. b) Kolpashchikov DM. Scientifica. 2012; 2012:928783. [PubMed: 24278758] c) Zheng J, Yang R, Shi M, Wu C, Fang X, Li Y, Li J, Tan W. Chem Soc Rev. 2015; 44:3036–3055. [PubMed: 25777303]
5. a) Wang Q, Chen L, Long Y, Tian H, Wu J. Theranostics. 2013; 3:395–408. [PubMed: 23781286] b) Junager NP, Kongsted J, Astakhova K. Sensors (Basel). 2016; 16 pii: E1173. c) Kuang T, Chang L, Peng X, Hu X, Gallego-Perez D. Trends Biotechnol. 2016 pii: S0167-7799(16)30152-4.

6. a) Cardullo RA, Agrawal S, Flores C, Zamecnik PC, Wolf DE. *Proc Natl Acad Sci U S A*. 1988; 85:8790–8794. [PubMed: 3194390] b) Guo J, Ju J, Turro NJ. *Anal Bioanal Chem*. 2012; 402:3115–3125. [PubMed: 22086400] c) Dark P, Blackwood B, Gates S, McAuley D, Perkins GD, McMullan R, Wilson C, Graham D, Timms K, Warhurst G. *Intensive Care Med*. 2015; 41:21–33. [PubMed: 25416643] d) Didenko VV. *Biotechniques*. 2001; 31:1106–1116. [PubMed: 11730017]
7. Kolpashchikov DM. *Chem Rev*. 2010; 110:4709–4723. [PubMed: 20583806]
8. a) Breaker RR. *Nature*. 2004; 432:838–845. [PubMed: 15602549] b) Schlosser K, Li Y. *Chem Biol*. 2009; 16:311–322. [PubMed: 19318212] c) Silverman SK. *Trends Biochem Sci*. 2016; 41:595–609. [PubMed: 27236301]
9. a) Xiang Y, Wu P, Tan LH, Lu Y. *Adv Biochem Eng Biotechnol*. 2014; 140:93–120. [PubMed: 24026635] b) Lan T, Lu Y. *Met Ions Life Sci*. 2012; 10:217–248. [PubMed: 22210341] c) Willner I, Shlyahovsky B, Zayats M, Willner B. *Chem Soc Rev*. 2008; 37:1153–1165. [PubMed: 18497928] d) Zhang H, Li F, Dever B, Li XF, Le XC. *Chem Rev*. 2013; 113:2812–2841. [PubMed: 23231477]
10. a) Stojanovic MN, de Prada P, Landry DW. *Nucleic Acids Res*. 2000; 28:2915–2918. [PubMed: 10908354] b) Singh KK, Parwaresch R, Krupp G. *RNA*. 1999; 5:1348–1356. [PubMed: 10573125]
11. Stojanovic MN, de Prada P, Landry DW. *Chembiochem*. 2001; 2:411–415. [PubMed: 11828471]
12. Kolpashchikov DM. *Chembiochem*. 2007; 8:2039–2042. [PubMed: 17924377]
13. a) Silverman SK. *Trends Biochem Sci*. 2016; 41:595–609. [PubMed: 27236301] b) McManus SA, Li Y. *Molecules*. 2010; 15:6269–6284. [PubMed: 20877222] c) Emilsson GM, Breaker RR. *Cell Mol Life Sci*. 2002; 59:596–607. [PubMed: 12022469] d) Ward WL, Plakos K, DeRose VJ. *Chem Rev*. 2014; 114:4318–4342. [PubMed: 24730975] a) Lan T, Lu Y. *Met Ions Life Sci*. 2012; 10:217–248. [PubMed: 22210341] b) Schlosser K, Li Y. *Chem Biol*. 2009; 16:311–322. [PubMed: 19318212]
14. a) Sando S, Sasaki T, Kanatani K, Aoyama Y. *J Am Chem Soc*. 2003; 125:15720–15721. [PubMed: 14677943] b) Tian Y, Mao C. *Talanta*. 2005; 67:532–537. [PubMed: 18970201] c) Song P, Xiang Y, Xing H, Zhou Z, Tong A, Lu Y. *Anal Chem*. 2012; 84:2916–2922. [PubMed: 22400799] d) Xu J, Li H, Wu ZS, Qian J, Xue C, Jia L. *Theranostics*. 2016; 6:318–327. [PubMed: 26909108] e) Fu R, Li T, Lee SS, Park HG. *Anal Chem*. 2011; 83:494–500. [PubMed: 21158421] f) Mokany E, Bone SM, Young PE, Doan TB, Todd AV. *J Am Chem Soc*. 2010; 132:1051–1059. [PubMed: 20038095] g) Gerasimova YV, Cornett E, Kolpashchikov DM. *ChemBioChem*. 2010; 11:811–817. [PubMed: 20301161] h) Ruble BK, Richards JL, Cheung-Lau JC, Dmochowski JJ. *Inorg Biochem*. 2012; 380:386–391. i) Zagorovsky K, Chan WCW. *Angew Chem Int Ed*. 2013; 52:3168–3171. j) Gerasimova YV, Kolpashchikov DM. *Angew Chem Int Ed Engl*. 2013; 52:10586–10588. [PubMed: 24038733] k) Gerasimova YV, Cornett EM, Edwards E, Su X, Rohde KH, Kolpashchikov DM. *Chembiochem*. 2013; 14:2087–2090. [PubMed: 24106198] l) Bone SM, Hasick NJ, Lima NE, Erskine SM, Mokany E, Todd AV. *Anal Chem*. 2014; 86:9106–9113. [PubMed: 25157928] m) Gerasimova YV, Yakovchuk P, Dedkova LM, Hecht SM, Kolpashchikov DM. *RNA*. 2015; 21:1834–1843. [PubMed: 26289345] n) Zhang L, Zhu J, Li T, Wang E. *Anal Chem*. 2011; 83:8871–8876. [PubMed: 22017597] o) Cox AJ, Bengtson HN, Gerasimova YV, Rohde KH, Kolpashchikov DM. *Chembiochem*. 2016; 21:2038–2041. p) Cox AJ, Bengtson HN, Rohde KH, Kolpashchikov DM. *Chem Commun*. 2016; 52:14318–14321.
15. Kobe B, Kemp BE. *Nature*. 1999; 402:373–376. [PubMed: 10586874]
16. Dollfus H, Kumaramanickavel G, Biswas P, Stoetzel C, Quillet R, Denton M, Maw M, Perrin-Schmitt F. *J Med Genet*. 2001; 38:470–472. [PubMed: 11474656]
17. a) Nakahori Y, Takenaka O, Nakagome Y. *Genomics*. 1991; 9:264–269. [PubMed: 2004775] b) Frances O, Portol3s F, GonzQlez JI, Coltell O, Verdú F, Castell+ A, Corella D. *Clin Chim Acta*. 2007; 386:53–56. [PubMed: 17716640]
18. a) Santoro SW, Joyce GF. *Proc Natl Acad Sci U S A*. 1997; 94:4262–4262. [PubMed: 9113977] b) Santoro SW, Joyce GF. *Biochemistry*. 1998; 37:13330–13342. [PubMed: 9748341]
19. Demidov VV, Frank-Kamenetskii MD. *Trends Biochem Sci*. 2004; 29:62–71. [PubMed: 15102432]
20. a) Henihan G, Schulze H, Corrigan DK, Giraud G, Terry JG, Hardie A, Campbell CJ, Walton AJ, Crain J, Pethig R, Templeton KE, Mount AR, Bachmann TT. *Biosens Bioelectron*. 2016; 81:487–494. [PubMed: 27016627] b) Alladin-MustaN BS, Mitran CJ, Gibbs-Davis JM. *Chem Commun*.

- 2015; 51:9101–9104.c) Du TE, Wang Y, Zhang Y, Zhang T, Mao X. *Anal Chim Acta*. 2015; 861:69–73. [PubMed: 25702276] d) Lingam S, Beta M, Dendukuri D, Krishnakumar S. *Microna*. 2014; 3:18–28. [PubMed: 25069509]
21. a) Bhadra S, Ellington AD. *RNA*. 2014; 20:1183–1194. [PubMed: 24942625] b) Bhadra S, Ellington AD. *Methods Enzymol*. 2015; 550:215–249. [PubMed: 25605388] c) Akter F, Yokobayashi Y. *ACS Synth Biol*. 2015; 4:655–658. [PubMed: 25354355]
22. a) Kolpashchikov DM. *J Am Chem Soc*. 2005; 127:12442–12443. [PubMed: 16144363] b) Kolpashchikov DM. *J Am Chem Soc*. 2006; 128:10625–10628. [PubMed: 16895431] c) Kolpashchikov DM. *J Am Chem Soc*. 2008; 130:2934–2935. [PubMed: 18281995] d) Kolpashchikov DM, Gerasimova YV, Khan MS. *Chembiochem*. 2011; 12:2564–2567. [PubMed: 22006680] e) Cornett EM, O'Steen MR, Kolpashchikov DM. *PLoS One*. 2013; 8:e55919. [PubMed: 23441157] f) Bengtson HN, Kolpashchikov DM. *Chembiochem*. 2014; 15:228–231. [PubMed: 24339354] g) Kikuchi N, Kolpashchikov DM. *Chembiochem*. 2016; 17:1589–1592. [PubMed: 27305425] h) Kikuchi N, Kolpashchikov DM. *Chem Commun (Camb)*. 2017; 53:4977–4980. [PubMed: 28425510]

Figure 1.

Predicted structures of the probe-analyte complexes and selectivity data for CMB (A) and BiDZ (B) recognizing *TWIST1* gene-related analytes at 30°C. For structures: catalytic core nucleotides are in green; single base substitution site is red underlined; ribonucleotides are in low case. For right panels: all samples contained 200 nM **F_sub** and either 5 nM **CMB_T1** or 5 nM each **DZa_T1** and **DZb_T1** in the reaction buffer: 50 mM HEPES, 50 mM MgCl₂, 20 mM KCl, 120 mM NaCl, 0.03% Triton X-100, 1 % DMSO. Samples **T1** and **T2** contained 5 nM of fully complementary **T1-21**, **T-86** or 5 nM single base mismatched **T2-21** or **T2-86** analytes, respectively (for full sequences see Table S1). Fluorescence intensity

at 517 nm (emission at 485 nm) was measured after 1 hr of incubation. The data is average values of 3 independent experiments.

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Scheme 1.

Design of deoxyribozyme (DZ) probes that produce fluorescent signal upon hybridization to specific nucleic acid analytes. A) Parent RNA-cleaving DZ can cleave a fluorophore- and quencher-labelled substrate (F_{sub}), thus producing high fluorescence. B) Switch design for DZ hybridization probes: the catalytic core and/or substrate binding arms of DZ are inactivated by binding to the 'inhibitory fragment'; hybridization of the analyte to the analyte binding domain releases the substrate binding arms of the DZ construct and enables cleavage of F_{sub} ; C) Split design: two DNA strands DZa and DZb hybridize to the analyte sequence and form catalytically active DZ, which cleaves F_{sub} .

Table 1
Selectivity factor (SF) and limits of detection (LOD) for DZ probe used in this study

	Analytes	T°C	Assay time, hr	SF	LOD, pM
CMB	T1-86/T2-86	30	1	0.31	89
			3		80
	T1-86/T2-86	55	1	0.38	31
			3		ND ^[c]
A1-80/A2-80	A1-80/A2-80	30	1	0.18	74
			3		59
	A1-80/A2-80	55	1	0.07	ND
			3		ND
BiDZ	T1-86/T2-86	30	1	0.83	164
			3		26
	T1-86/T2-86	55	1	0.65	133
			3		98
A1-80/A2-80	A1-80/A2-80	30	1	0.84	47
			3		15
	A1-80/A2-80	55	1	0.89	11
			3		8

^[a] SF was calculated according to the formula: $SF = 1 - F_{mm}/F_m$, where F_{mm} and F_m are average fluorescence of samples in the presence of mismatched and fully matched analytes, respectively, using data presented in Figure S1–S5).

^[b] LOD was determined by 3 individual trials after at 1 and 3 hrs or assay (Figure S6–S9).

^[c] ND - the LOD was not determined due to the high background signal.