RNA Detection

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# Target-Catalyzed Transfer Reactions for the Amplified Detection of RNA

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Signal amplification is key to the detection of small amounts of biological targets. In nucleic acid diagnostics the polymerase chain reaction (PCR) is the standard method for highly sensitive detection of DNA and RNA. The PCR first amplifies the target and allows the subsequent detection of formed product. Detection methods that do not require amplification of the target could lead to simpler devices and are highly desired. PCR-less detection has been approached by taking advantage of the catalyzed formation of signaling molecules[1] as well as by electrochemical[2] and nanoparticle-based assays.[3] High sensitivities have been obtained by the coupling of two amplification methods. For example, in a bar-code-based method one molecule of target DNA first recruits multiple copies of DNA tags, which are detected in a second step by means of nanoparticle-catalyzed development of silver.[4] A preamplification step was also included in a sensitive peptide nucleic acid (PNA)-based hybridization assay wherein one target DNA acquired multiple copies of horseradish peroxidase for inducing the catalytic production of dye molecules.[1b] The recruiting step typically involves binding of the target molecule to an immobilized capture oligonucleotide and a second detector oligonucleotide probe that features traceable entities (e.g. bar-code DNA or biotin) for detection of complementary sequences. However, the noncovalent nature of the formed sandwich complexes sets limits on the stringency of washing protocols required to remove nonbound detection oligonucleotides.

These sandwich assays draw upon the recruitment of a catalytically active moiety to a specific DNA structure. In an alternative design, the target itself may act as a catalyst of a chemical reaction, for example, by aligning the functional groups of modified oligonucleotides.[5–8] This alignment allows the acceleration of reactions such as ligations[5,9] and hydrolyses[6] which proceed very slowly in the absence of the template. The previous DNA-templated reactions have met these criteria with only limited success. DNA-catalyzed reactions have allowed up to $10^2$-fold signal amplification, which is not sufficient to achieve PCR-less detection of small amounts of DNA. Herein, we introduce target-catalyzed reactions for preamplification of signals that can passed on to enzyme-based detection platforms. The reaction system was designed such that the target catalyzes the covalent attachment of detectable groups to nucleic acid based probes. We show that the preamplification and the covalent mode of reporter-group attachment allow increases of the sensitivity of an enzyme-linked immunosorbant assay (ELISA)-derived detection method.

Recently, we introduced the target-catalyzed transfer of a reporter group **R** from a donating PNA probe **1** to an accepting PNA probe **2** (Figure 1 A) as a concept for the detection of nucleic acid targets. Probes **1** and **2** are designed to anneal adjacently to complementary segments of the **target**. The alignment of thioester **1** and the N-terminal isocysteine (*i*Cys) **2** in the ternary complex **target·1·2** facilitates a thiol-exchange reaction. By analogy to native chemical ligation[10] the formed thioester intermediate **4** spontaneously reacts by means of an irreversible S→N acyl migration yielding product **5**. Reactant and product probes have similar

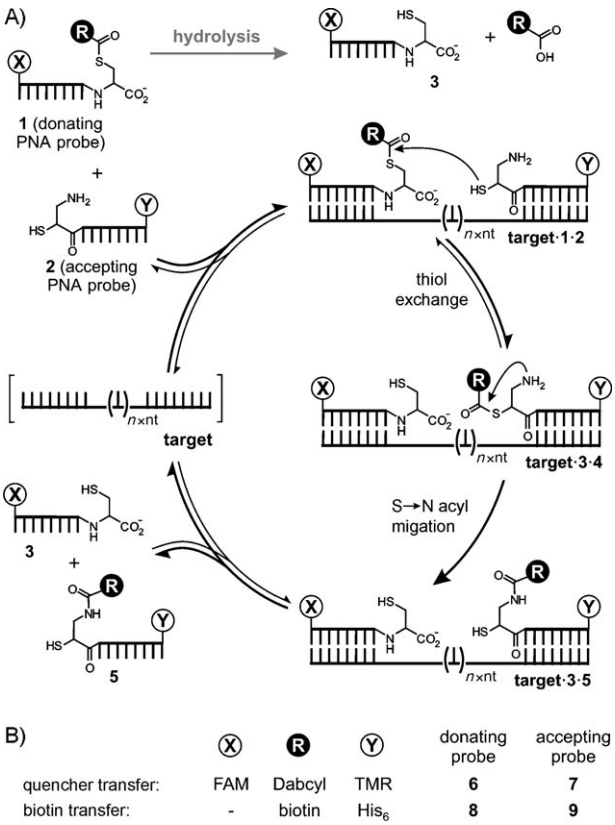


***Figure 1.*** A) Catalytic cycle of the nucleic acid catalyzed transfer of a reporter group (**R**) from donating **1** to accepting **2** PNA probe and the hydrolysis reaction of the thioester bond in probe **1**; B) type of labels for the quencher and biotin-transfer reactions (FAM: 6-carboxyfluorescein, Dabcyl: 4-[4-dimethylamino)phenylazo]benzoyl, TMR: 5-carboxytetramethylrhodamine).

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affinity to the **target,** and thus strand-exchange reactions furnish the ternary complex **target·1·2** in a dynamic equilibrium. In preliminary work, we reported the DNA-catalyzed transfer of a fluorescence quencher (Figure 1B, top).[8] The quencher (Dabcyl) was transferred from a fluorescein(FAM)- to a tetramethylrhodamine(TMR)-modified PNA probe (**6** and **7**, respectively). The relocation switched on emission of FAM while switching off emission of TMR.

The rate of nucleic acid templated reactions is dependent on the architecture of the formed target–probe complex.[11] Most DNA-directed reactions involve seamless hybridization of reactant probes. We have shown that unpaired nucleobases opposite to the ligation site can confer increases of the sequence specificity of PNA ligations at the cost of decreased reaction rates.[12] The requirements of transfer chemistry in terms of the flexibility at the reaction center were tested by varying the number ($n$) of unpaired nucleotides (nt) between the reactive groups of probes **6** and **7** and monitoring the initial rates. Previous experiments revealed high catalytic activity of DNA with $n = 1$ nt, which spanned a sequence around a known carconogenic G12V mutation of the *ras* gene, at a reaction temperature of 32 °C.[8] Interestingly, similar distance dependence was found for equimolar (gray) and substoichiometric (black) amounts of DNA (Figure 2A). The

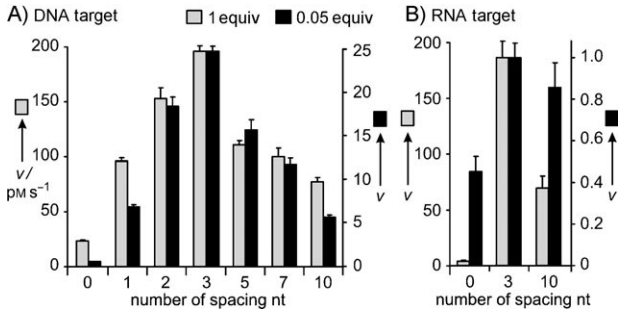


***Figure 2.*** Initial rates $v$ (experiments conducted in triplicate) of the transfer reaction of probe **6** (FAM-AEEA-tcttccccac-Cys(Dabcyl-Gly)-OH) and **7** (TMR-AEEA-Lys(*i*Cys-cctacag)-$NH_2$) in the presence of DNA (A, 32 °C) and RNA (B, 37 °C) with varying numbers of unpaired nucleotides (nt); **target** (for RNA: $T = U$): 5′GCGCTGTAGGXGTGGGGAAGAGT3′ (recognition sequence underlined); **X** ($n$): – (0), T (1), TT (2), TTA (3), TTATC (5), TTATCGA (7), TTATCGATTA (10); Conditions: 100 nM **6**, 100 nM **7**, 10 mM $K_2HPO_4$, 200 mM NaCl, 1 mM triscarboxyethylphosphine (TCEP), 0.2 mg mL$^{-1}$ Roche blocking reagent, pH 7.0 (for RNA: 0.1 u µL$^{-1}$ RiboLock).

maximum transfer rate occurred for both concentrations at a distance of 3 nt and is more than two times greater than that at the original distance of 1 nt.[13] In particular, the reactivity of probes **6** and **7** with substoichiometric amounts of target (0.05 equiv) benefits from the increased distance of 3 nt and results in a more than threefold increase in catalytic activity.

We wanted to explore the usefulness of transfer reactions in the detection of infectious or pathogen RNA and therefore assessed reactions of probes **6** and **7** on RNA templates that contained 0, 3, and 10 spacer nucleotides. PNA–RNA duplexes are more stable than PNA–DNA complexes. To enable strand exchange the reaction temperature was increased to 37 °C. Under these conditions equimolar RNA concentrations (gray, Figure 2B) provided initial rates similar to those with equimolar DNA (gray, Figure 2A). In these studies with RNA templates the fasted reactions occurred on RNA that contained three spacer nucleotides between the aligned reactant probes ($n = 3$ nt). We noticed reduced reaction rates and a decreased influence of the flexibility at the reaction center when substoichiometric amounts of RNA were used. One likely reason is that strand exchange may become rate limiting in RNA-catalyzed reactions, an issue that has commonly been observed in ribozymes.[14] However, in RNA detection chemistry this concern can likely be addressed by reducing concentrations of probes and targets, increasing temperature, curtailing probe length, and prolonging reaction times.

We selected HIV-I RNA as the target and chose a sequence (**HIV**) that is highly conserved[15] and located at an accessible site[16] in the 5′-LTR region of the HIV-I genome. In terms of sensitivity it would be advantageous if the catalytic activity of a target was combined with other signal amplification techniques employed in bioanalytics. Many sensitive detection platforms rely on noncovalent association of biotinylated probes and subsequent binding of streptavidine–enzyme conjugates to induce the catalyzed formation of signaling molecules. For example, Appella and co-workers recently used capture PNA to hybridize the DNA target to the surface of a well plate.[1b] A second biotin-labeled PNA probe enabled the recruitment of horseradish peroxidase, which catalyzed the formation of a colored quinoid compound.

We designed a target-catalyzed transfer reaction that induces the covalent attachment of biotin. The detection system (Figure 3, see also Figure 1B) employs the donating PNA probe **8**, which contains the biotinylated reporter group, and the accepting PNA probe **9** bearing a C-terminal His tag (**$His_6$**). Both probes were designed to be separated by 3 nt when hybridized with target RNA (underlined sequences in **HIV**). The transfer reaction (reaction I) was performed with 500–750 fmol probes in 50 µL buffer. In the presence of **HIV** the reporter group is transferred yielding products **10** and **11**. Initial experiments based on HPLC/MS analysis proved the potential of a $10^2$-fold preamplification (see Figure S3 in the Supporting Information). Transfer product **11** bears both a biotin label and a His tag. As His tags are capable of forming strong interactions with nickel, it is possible to immobilize probe **11** on the surface of Ni-coated well plates. After stringent washing, the wells were incubated with a horseradish peroxidase–streptavidin conjugate (HRP-SA) and washed again. Only in wells with transfer product **11** could HRP-SA be immobilized (see **12**) by the streptavidin–biotin interaction. HRP catalyzes the oxidation (reaction II) of colorless tetramethylbenzidine (TMB, **13**) to the colored quinoid compound **14**. This reaction was performed using commercially available 1-step Ultra TMB solution and stopped by the addition of 1M $H_2SO_4$. The depicted wells in absence (−**HIV**) and presence (+**HIV**, 50 fmol) of target RNA were treated as described above.

To determine signal intensities the absorbance at 450 nm was measured using a plate reader. Figure 4A shows back-

  

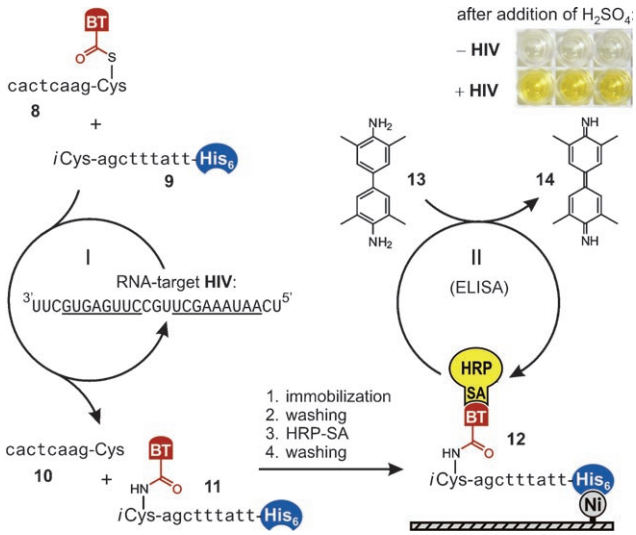


***Figure 3.*** Double signal amplification using the RNA-catalyzed biotin (**BT**) transfer reaction as preamplification (reaction I: 750 fmol **8** with 500 fmol **9** in 50 μL) and a modified ELISA as the final amplification step (reaction II). Six wells (96-well pate) are shown (identical conditions for each row) without RNA (−**HIV**) and with 50 fmol RNA (+**HIV**).

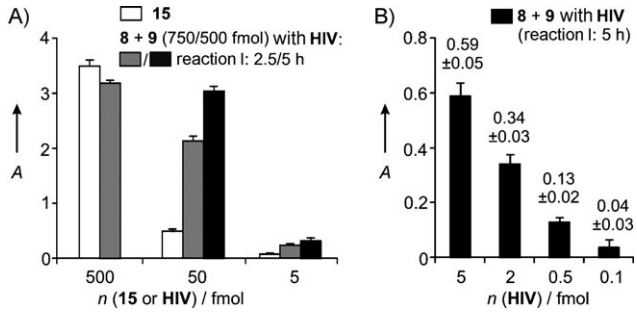


***Figure 4.*** Background-corrected absorbance at 450 nm (experiments conducted in triplicate); Conditions: reaction I: 750 fmol **8**, 500 fmol **9** in 50 μL buffer (10 mM $K_2HPO_4$, 200 mM NaCl, 1 mM TCEP, 0.2 mg mL$^{-1}$ Roche blocking reagent, 0.2 u μL$^{-1}$ RiboLock pH 7.0); reaction II: 80 μL Ultra-TMB solution for 25 min followed by addition of 40 μL 1 M $H_2SO_4$; A) incubation with 0.5 μg mL$^{-1}$ HRP-SA, control experiment: probe **15** without reaction I (white); B) incubation with 2 μg mL$^{-1}$ HRP-SA..

ground-corrected signals obtained after biotin transfer (reaction I) in the presence of 5–500 fmol **HIV**, subsequent immobilization, and incubation with 0.5 μg mL$^{-1}$ HRP-SA (gray). In the presence of 500 fmol of a random RNA sequence no significant signal was measured (Table S3 in the Supporting Information). For 50 fmol (0.1 equiv) and 5 fmol (0.01 equiv) **HIV**, the reaction time of reaction I was extended from 2.5 h to 5 h without changing other parameters. This resulted in signal intensities increased by more than 30% (black, Figure 4A) indicating the preamplifying effect of reaction I. As a control we investigated the influence of reaction I on the signal amplification by using a mimic of transfer product **11**, the biotin- and $His_6$-labeled probe **15** (**BT**-Cys-agctttatt-**$His_6$**). The wells were charged with 500, 50, and 5 fmol probe **15** and the signals (white, Figure 4A) were compared with the values determined for the same amounts of **HIV** with 750 fmol probe **8** and 500 fmol probe **9**. An amount of 500 fmol **15** provided a slightly higher signal than reactive probes **8** and **9** in the presence of 500 fmol **HIV** (1 equiv). This observation is reasonable as the transfer reaction may not proceed quantitatively. For substoichiometric amounts of **HIV** (50 and 5 fmol) the preamplified reactions (5 h, black) provided more than threefold higher signals than the corresponding amount of probe **15**. Thus, the amount of immobilizable biotin products obtained in the RNA-catalyzed transfer reaction must exceed the amount of RNA target. These results are testimony to the preamplification provided by the target-catalyzed biotin transfer.

To test the sensitivity of the reaction system **HIV** amounts ranging from 0.1–5 fmol were reacted with 750 fmol **8** and 500 fmol **9** for 5 h. The immobilization of **$His_6$**-modified probes was followed by addition of 2.0 μg mL$^{-1}$ HRP-SA. This procedure allowed the detection of **HIV** amounts as low as 500 attomol (Figure 4B). The analogous sandwich assay, in which two biotin groups were immobilized by PNA–target hybridization followed by treatment with monovalent HRP-SA conjugates, allowed the detection of 1000 fmol DNA.[1b] This substantiates the advantages provided by preamplification and covalent attachment of the biotin reporter.

For the detection of zeptomol quantities of nucleic acid targets further $10^5$-fold enhancements of sensitivity would be required. Up to $10^8$-fold increases of sensitivity have been reported when a polyvalent HRP-SA conjugate was applied rather than the monovalent HRP-SA conjugate[1b] used by us. Thus, enhancements of signal amplification may be achievable by interfacing our preamplification strategy with improved enzyme reporters and/or by using chemoluminescent substrates in smaller well plates. The transfer of multiply biotinylated reporter groups may also provide opportunities to further increases of sensitivity. None of these options would require thermal cycling.

In summary, we have demonstrated signal amplification by DNA- and RNA-catalyzed transfer of reporter groups. The RNA-catalyzed transfer of a biotin reporter allowed preamplification for an enzyme-based readout. In contrast to previous sandwich assays used in nucleic acid detection our strategy capitalized upon covalent attachment of the reporter group. By means of this double-amplification strategy (transfer + modified ELISA) it was possible to detecte 500 attomol HIV-I RNA; this exceeds the sensitivity obtained by previous nucleic acid catalyzed chemical reactions.[5–8] Of note, this sensitivity was achieved by measuring optical density with a common plate reader and using reagents that are available in any clinical diagnostics laboratory, while most other sensitive detection methods need specialized equipment.

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[13] In principle, local flexibility should always help when reactive groups are not perfectly aligned. We think that the demands of transfer chemistry are different from those of ligation chemistry. The rate-determining step probably is thiol exchange. In ligation chemistry, both probe sequences remain hybridized upon formation of the thiol-exchange product. By contrast, the probes can readily leave the complex after thiol exchange in transfer chemistry. Thus, distortions of the duplex architecture should be better tolerated in transfer chemistry, because constrain likely facilitates dissociation of the acyl donor rather than affecting the transition state. By analogy to Baldwin ring-closure rules, there will be an optimum, which apparently is in the range of 3 nt distance.

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