DNA Detection

DOI: 10.1002/anie.200605023

# DNA Photography: An Ultrasensitive DNA-Detection Method Based on Photographic Techniques**

*David M. Hammond, Antonio Manetto, Johannes Gierlich, Vladimir A. Azov, Philipp M. E. Gramlich, Glenn A. Burley, Melanie Maul, and Thomas Carell**

The ability to manipulate and analyze the genetic information of all organisms along with the discovery that small RNA strands control critical cellular functions has given rise to the development of new, sophisticated methods for the ultrasensitive detection of DNA and RNA.[1] In the postgenomic era, it is believed that such techniques will revolutionize the diagnosis of genetically encoded diseases such as cancer. In fact, the development of more-personalized medicine is critically linked to the development of reliable ultrasensitive DNA detection methods in combination with novel methods that allow isolation of the gene of interest (analyte) from biological samples. The method reported herein provides a simple solution to the first part of the problem. Although the polymerase chain reaction (PCR) is predominantly used for DNA detection, new methods for the highly sensitive detection of DNA and RNA are constantly being developed. Most recently, for example, Mirkin and co-workers, Willner and co-workers, and Heeger and co-workers have reported the utilization of nanoparticles, aptamers, or novel electrochemical setups to detect DNA with sensitivity limits in the range of pico- and femtomoles.[1–7] Even sensitivities in the zeptomolar regime ($\approx 10$ copies in 30 μL) have been reported.[8] All these methods require sophisticated technology, which limits their widespread use. We report herein a simple and efficient method for DNA detection in the femto- to attomole ($10^{-18}$ mol) range based on the amplification process provided by black and white photography (Figure 1).

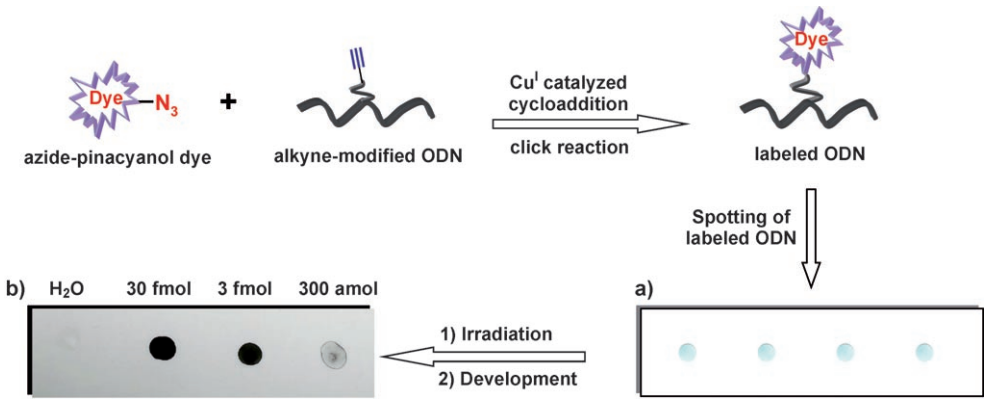



***Figure 1.*** DNA detection using the methods of black and white photography. a) Commercial photopaper loaded with different concentrations of the labeled ODN (graphical representation). b) Photopaper after irradiation and development (scanner reproduction). Visual detection of the labeled ODN at three different concentrations; 1 μL spotted (×4).

[*] Dr. D. M. Hammond,[+] Dr. A. Manetto,[+] Dipl.-Chem. J. Gierlich, Dr. V. A. Azov, Dipl.-Chem. P. M. E. Gramlich, Dr. G. A. Burley, Dipl.-Ing. M. Maul, Prof. Dr. T. Carell
Fakultät für Chemie und Pharmazie
Ludwigs-Maximilians-Universität München
Butenandtstrasse 5–13, 81377 München (Germany)
Fax: (+49) 89-2180-77756
E-mail: thomas.carell@cup.uni-muenchen.de

[+] These authors contributed equally to the work.

[**] We thank Martin Hadden of Ilford and Dr. Peter Bergthaller and Dr. Klaus Wagner for helpful discussion, and the Volkswagen Foundation, the Deutsche Forschungsgemeinschaft, and BASF AG for financial support. A.M. thanks the EU Marie Curie Training and Mobility Program under contract MRTN-CT-2003–505086 [CLUSTOXDNA] for an ESR fellowship. J.G. and P.M.E.G. thank the Fonds of the Chemical Industry and Cusanuswerk, respectively, for predoctoral fellowships. G.A.B. thanks the Alexander von Humboldt Foundation for a research fellowship.

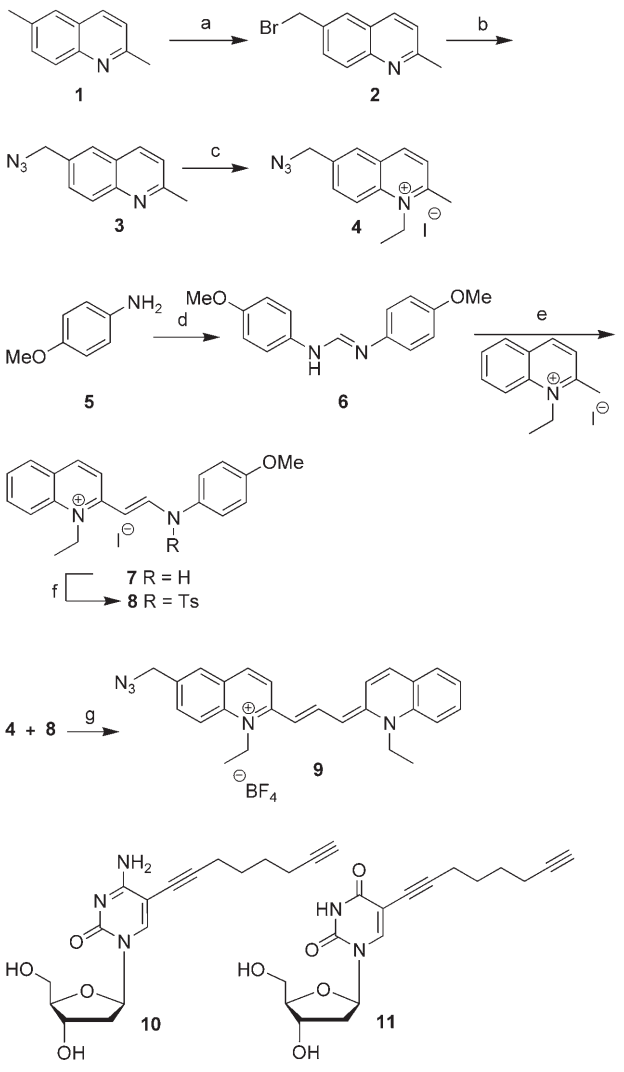
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

In the black and white photographic process, a few photons captured by a light-sensitive AgBr-crystal-containing layer (film or photopaper) induce the formation of an $Ag_n$ nucleus as a latent image center. In the subsequent development process, this $Ag_n$ cluster (for $n > 3$) catalyzes the reduction of the entire AgBr crystal to $Ag^0$, which stains the photopaper black.[9] To date, the detection of analytes based on silver deposition has been performed with Ag and Au clusters followed by Ag deposition from solution and not through photopaper.[10] The solution process is limited to amplification factors of about $10^5$. Herein, we report the application of the standard and inexpensive AgBr-crystal-based photographic process for the detection of labeled biomolecules. This process can provide, in principle, amplification factors of $10^{11}$,[9] which are similar to standard PCR reactions.

We reported recently the ability to modify oligonucleotides with the help of copper(I)-catalyzed azide/alkyne cyclo-

addition (click chemistry).[11–15] To link DNA detection to the photographic process, we aimed to couple DNA to special dyes, so-called spectral photographic sensitizers. These dyes are used in the black and white photographic process to sensitize the AgBr emulsion present in the photopaper to light of wavelengths above 520 nm, where AgBr does not absorb light.

To investigate the sensitivity limits of the method, we initially explored one of the most widespread photosensitizers used in black and white photography, the pinacyanol dye, which is modified as azide **9**. This dye has the advantage that it strongly adsorbs to AgBr crystals providing an efficient energy/electron transfer to the AgBr crystals. The synthesis of the dye azide and the structure of the 2′-deoxycytidine alkyne **10**[16] and 2′-deoxyuridine alkyne **11**,[15] building blocks needed for the click reaction, are shown in Scheme 1.




***Scheme 1.*** a) NBS, benzoyl peroxide, $CCl_4$, reflux, 47%; b) $NaN_3$, DMF, 97%; c) EtI, MeCN, 70% (based on recovered starting material); d) triethyl *ortho*-formate, 140°C, 55%; e) triethyl *ortho*-formate, BuOH, reflux, 91%; f) TsCl, DIPEA, $CH_2Cl_2$, 70%; g) 1) **4**, DIPEA, pyridine; 2) Dowex 1x8–200, $NH_4BF_4$, 47%. DIPEA = *N*,*N*-diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, Ts = toluene-4-sulfonyl.

Commercially available 2,6-dimethylquinoline (**1**) was first brominated by using *N*-bromosuccinimide (NBS) to give the monobrominated compound **2**.[17] Nucleophilic displacement of bromide by using sodium azide afforded the azido derivative **3**, which was subsequently alkylated with ethyl iodide to give the quinolinium salt **4**. In a parallel synthesis, *p*-methoxyaniline was condensed with triethylformate, yielding amidine **6**. This was then reacted with 1-ethyl-2-methylquinolinium iodide to give the hemicyanine dye **7**, which was subsequently tosylated and coupled with intermediate **4** to give the target azido cyanine **9**. We next prepared three oligodeoxyribonucleotides (ODNs) containing various alkyne-modified nucleotides as shown in Table 1. We incorporated the 5-alkynyl-2′-dC **10** (dC = deoxycytidine)[16] into ODN-1 and ODN-2 and the 5-alkynyl-2′-dU **11** (dU = deoxyuridine)[15] in ODN-3 through their corresponding phosphoramidites.

***Table 1:*** Alkyne-modified ODNs used to click **9** onto DNA.[a]

| | ODN Sequences (5′→3′) |
|---|---|
| ODN-1 | GCG CTG T**X**C ATT CGC G |
| ODN-2 | GCG CTG **XX**C ATT CGC G |
| ODN-3 | TTA ATT GAA TTC GAT T**Y**G GGC CGG A**Y**T TGT TTC |

[a] **X** = 5-alkynyl-2′-dC, **10**; **Y** = 5-alkynyl-2′-dU, **11**.

In the presence of a $Cu^I$ salt and a $Cu^I$-stabilizing ligand,[18] the cycloaddition between azide **9** and the three alkyne-modified oligonucleotides proceeded quantitatively, as evidenced by MALDI-TOF analysis; this proves that the click reaction is amenable to both the cytidine- and uridine-containing ODNs. The oligonucleotides were subsequently precipitated from ethanol for purification. Owing to the high yield of the click reaction, this simple precipitation step was sufficient to fully separate the ODNs from any excess dye azide as proven by the clean HPLC traces and MALDI-TOF spectra obtained from the precipitated ODN compounds.

To test our hypothesis of detecting dye-modified ODNs through a photographic process, we simply spotted 1 μL of a 10 μM solution of either ODN-1, -2, or -3 onto commercially available photopaper, which is sensitive only to UV and blue light, under darkroom conditions. The used photopaper contained no red-light spectral sensitizer, which makes it insensitive to light with wavelengths above 520 nm. We then illuminated the photopaper for typically less than 30 s with light, $\lambda > 570$ nm, and subsequently developed the photopaper by using commercially available development reagents (see the Supporting Information). All ODNs induced under these conditions led to deep black spots of 2–3 mm diameter on the photopaper. In contrast, control oligonucleotides, which contained no dye molecules, gave no spot at all (data not shown), showing that dye-modified ODNs can be selectively detected by using this method. For the control ODNs, only a faint spot was detected at very high concentrations of greater than 50 mM (50 nmol, see the Supporting Information).

We next studied the sensitivity of the method, the results of which are shown in Figure 2 a–c. When we spotted,

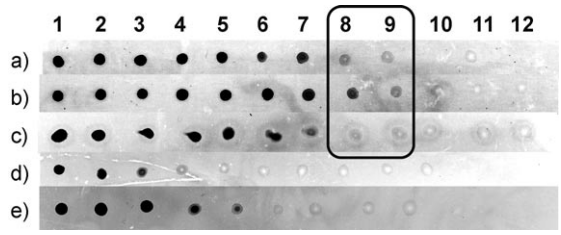



***Figure 2.*** Photostrips with sensitizer-modified ODNs. a) ODN-1; b) ODN-2; c) ODN-3; d) Cy5-ODN; e) Cy3-ODN. Amounts of DNA in $H_2O$ (1 μL was spotted): **1**: 10 pmol; **2**: 1 pmol; **3**: 300 fmol; **4**: 100 fmol; **5**: 30 fmol; **6**: 10 fmol; **7**: 3 fmol; **8**: 1 fmol; **9**: 300 amol; **10**: 100 amol; **11**: 30 amol; **12**: $H_2O$. The black box indicates the detection limit.

irradiated, and then developed a dilution series of dye-modified DNA samples onto the photopaper, we could see that all ODNs with one (ODN-1) or two (ODN-2 and ODN-3) pinacyanol dyes displayed a sensitivity of down to 300 attomoles. By using this method, the detection is purely based on visual inspection of the developed photopaper. Hybridization of ODN-3 to its counter strand revealed a higher detection limit of 30 femtomoles. To determine if a labeled ODN can be detected in a mixture with other DNA fragments, we used a primer labeled with two dye molecules and generated a 300-bp dsDNA fragment, which was than digested with restriction enzymes. This mixture too provided a positive black spot (see also the Supporting Information). The whole photographic procedure, including spotting of the DNA onto the paper, irradiation, development, and visual inspection, typically took less than 20 min. The photopaper can of course be loaded with multiple samples to create an inexpensive, time-saving, and easy-to-use photographic assay array.

With the direct detection of 300 attomoles, this method is in a sensitivity range that rivals most other non-PCR-based methods whilst having the advantage that no equipment other than a standard photographic darkroom setup is needed. Because the sensitivity limit of 300 attomoles was reached with standard photopaper, we can envision that a special paper tuned for diagnostic purposes will be able to further improve the sensitivity.

To investigate if the method is applicable for the detection of ODNs containing other fluorophores that are currently in use for DNA labeling, we also spotted Cy5- and Cy3-modified DNA onto the photopaper (Figure 2d,e). As expected, these ODNs were also detected but the sensitivity was strongly reduced (100 fmol), proving that the dyes developed for photographic purposes provide a significant advantage. The result, however, that typical dye-labeled ODNs can be detected allowed us to set up an initial experiment to clarify if the method would be suitable for the detection of health-threatening pathogens. We set the goal of detecting a short DNA sequence encoding a small part of the 16S rRNA gene (rRNA = ribosomal RNA) from the bacterium *Yersinia pestis*.[19] The commercially available DNA hairpin (molecular beacon, MB)[20] with a 5′-Cy3 fluorophore and a BHQ2 fluorescence quencher at the 3′ end was purchased to see whether the fluorescence on–off property of MB could be exploited for detection based on the photographic process. The loop region was designed to be complementary to the DNA sequence of the rRNA gene with the underlined sequence forming the stem region of the MB (5′-Cy3-CGCTGCCCCTTGAGGCGTGGCTGCAGCG-BHQ2–3′; Figure 3). In the absence of the specific *Y. pestis* gene (5′-AGCCACGCCTCAAGGG-3′), the MB is in the closed state in which the fluorescence is quenched owing to the close

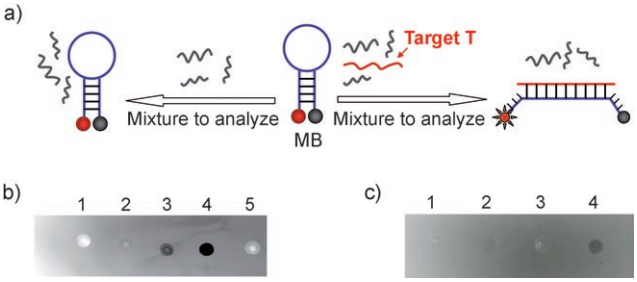



***Figure 3.*** a) The MB concept used to detect the *Y. pestis* gene. The beacon opens up in the presence of the target, which increases the fluorescence and hence the ability of the beacon to sensitize the photopaper. Scanner reproductions of two photographic experiments with the positive black spots in b4 and c4. A volume of 1 μL of target-DNA solution in buffer solution was spotted. b) Spots 1 and 5: buffer solution (5 mM Tris-HCl pH 8, 5 mM KCl, 0.5 mM $MgCl_2$; Tris = tris(hydroxymethyl)aminomethane); spot 2: 10 μM T; spot 3: 1 μM MB; spot 4: 1 μM MB and 10 μM T. c) Spot 1: buffer solution (5 mM Tris-HCl pH 8, 0.5 mM $MgCl_2$); spot 2: 0.1 μM MB; spot 3: 0.6 μM T, and spot 4: 0.1 μM MB and 0.6 μM (600 fmol) T.

proximity to the BHQ2 quencher.[21] Spotting of the non-fluorescing MB at a high concentration (1 μL of a 1 μM solution) onto the photopaper followed by irradiation and development gave only a faint spot as expected (Figure 3b, spot 3), showing that BHQ-2 can inhibit sensitization of the photographic emulsion by the Cy3 dye; The faint staining is the result of residual fluorescence of the quenched molecular beacon, showing again the impressive sensitivity of the method. If we add a mixture of various DNA strands to this hairpin solution, among them the *Y. pestis* DNA sequence, the MB opens up, thereby separating the fluorophore from the quencher and enabling it to sensitize the AgBr crystals in the photopaper. Indeed, when we spotted this mixture (1 μL of a 1 μM solution) onto the photopaper, irradiated, and developed the strip, a thick black spot was clearly visible (Figure 3b, spot 4).

We finally investigated the detection limit of this commercially available Cy3-labeled MB. As shown in Figure 3c, we could detect the presence of the *Y. pestis* gene with the naked eye down to 600 fmol (spot 4) even if we added additional genomic DNA to the solution (see the Supporting Information).

In summary, we have identified a novel yet simple technique for the detection of DNA to a detection limit of 300 attomoles by merely spotting sample solutions onto nonsensitized photopaper, irradiating for few seconds, and developing the image in a standard photographic developing solution. We applied this method for the direct detection of

600 femtomoles of a selected target, which was associated with the disease of plague, by using hybridization probes (MBs). Detection is performed purely by visual inspection without the need for expensive fluorescence detectors or scintillation counters. Optimization of the photopaper, investigation of other photographic dyes, and the development of methods that allow the efficient isolation of the to-be-detected DNA from biological samples are problems that now need to be tackled.

Received: December 12, 2006
Revised: February 27, 2007
Published online: April 25, 2007

**Keywords:** biosensors · click chemistry · DNA · fluorescent probes · photosensitizers

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