

## Tail-labelling of DNA probes using modified deoxynucleotide triphosphates and terminal deoxynucleotidyl transferase. Application in electrochemical DNA hybridization and protein-DNA binding assays

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Received 9th October 2010, Accepted 11th November 2010

DOI: 10.1039/c0ob00856g

A simple approach to DNA tail-labelling using terminal deoxynucleotidyl transferase and modified deoxynucleoside triphosphates is presented. Amino- and nitrophenyl-modified dNTPs were found to be good substrates for this enzyme giving 3'-end stretches of different lengths depending on the nucleotide and concentration. 3-Nitrophenyl-7-deazaG was selected as the most useful label because its dNTP was efficiently incorporated by the transferase to form long tail-labels at any oligonucleotide. Accumulation of many nitrophenyl tags per oligonucleotide resulted in a considerable enhancement of voltammetric signals due to the nitro group reduction, thus improving the sensitivity of electrochemical detection of the tail-labelled probes. We demonstrate a perfect discrimination between complementary and non-complementary target DNAs sequences by tail-labelled hybridization probes as well as the ability of tumour suppressor p53 protein to recognize a specific binding site within tail-labelled DNA substrates, making the methodology useful in electrochemical DNA hybridization and DNA-protein interaction assays.

### Introduction

Base-modified nucleic acids are of great current interest due to applications in chemical biology, bioanalysis, catalysis or nanotechnology and material science.<sup>1</sup> Recently we<sup>2</sup> and others<sup>3</sup> have developed polymerase incorporations of base-functionalized deoxynucleoside triphosphates (dNTPs) for enzymatic preparation of labelled DNA. We succeeded in the use of this approach for redox labelling of DNA by ferrocene,<sup>4</sup> amino- and nitrophenyl,<sup>5</sup> or Ru/Os(bpy)<sub>3</sub>,<sup>6</sup> tags useful for electrochemical detection and for attachment of reactive aldehyde functions<sup>7</sup> for further modification. The polymerase incorporation (either by primer extension or PCR) needs a template strand for specific incorporations of the modified dNTPs. However, for many applications, a simple end labelling by one or more modified nucleotides is sufficient. Therefore, we report here on an efficient technique of DNA tail labelling based on terminal deoxynucleotidyl transferase (TdT) that attaches nucleotides at the 3'-OH terminus of DNA using dNTPs as substrates.<sup>8</sup> A single-stranded primer can thus easily be extended by a homonucleotide (when a single dNTP is used) stretch, the length of which depends on the dNTP/primer ratio

and on the efficiency of incorporation of the given nucleotide. We focused the present study on incorporation of redox active aminophenyl- and nitrophenyl-modified dNTPs.<sup>5</sup>

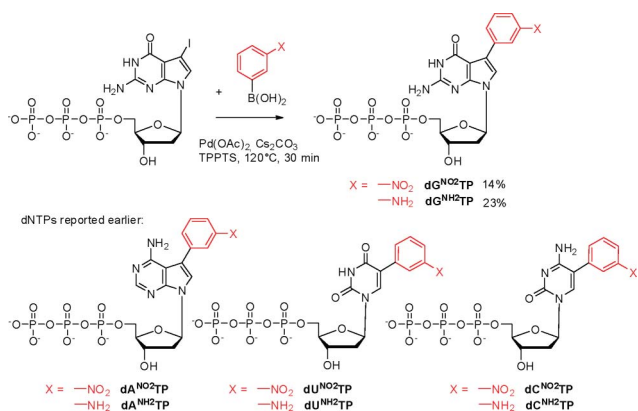
### Results and discussion

#### Synthesis and incorporation of labelled nucleotides with TdT

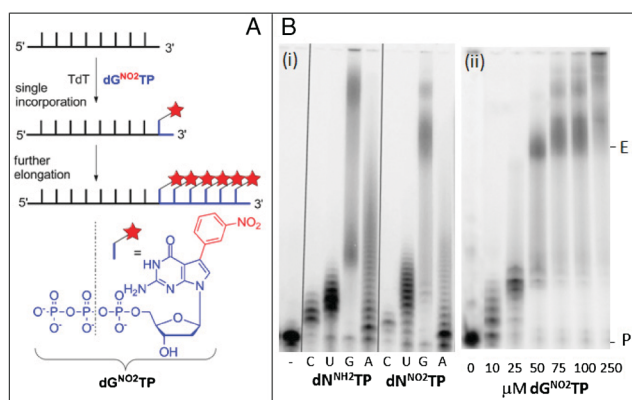
All eight combinations of NH<sub>2</sub>- and NO<sub>2</sub>Ph-modified dNTPs (dN<sup>NH<sub>2</sub></sup>TP and dN<sup>NO<sub>2</sub></sup>TP, where N = 7-deazaA, 7-deazaG, C, U) were prepared by the aqueous Suzuki–Miyaura cross-coupling of the corresponding halogenated dNTPs with 3-amino- or 3-nitrophenylboronic acid (Scheme 1). The modified dG<sup>X</sup>TPs were new compounds, while the other nucleotides were reported earlier.<sup>5</sup> All these dN<sup>X</sup>TPs were tested as substrates for TdT. In general, longer labelled tails were created with 7-substituted 7-deazaguanines than with 5-substituted pyrimidines, but remarkable differences in length distributions of the products were observed also within these two groups (Fig. 1B,i). The dC<sup>X</sup>TPs gave products elongated by 1 to 5 nucleotides, with most intense bands corresponding to 2–3 extra nucleotides. For dU<sup>X</sup>TPs the mean length of the labelled tail was 4–5 nucleotides and the maximum length of the tail was ~14 nucleotides. The most intense band observed for TdT reaction with dA<sup>X</sup>TPs corresponded to one nucleotide attached, but the products were rather polydisperse in lengths, reaching several tens of extra nucleotides. The most facile synthesis of long labelled tails was exhibited, under the given conditions, by dG<sup>X</sup>TPs (see below).

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**Scheme 1** Synthesis of  $\text{dG}^{\text{NO}_2}\text{TP}$  and  $\text{dG}^{\text{NH}_2}\text{TP}$  and structures of other dNTPs used in the study (reported earlier in ref. 5).



**Fig. 1** (A) Scheme of end- or tail-labelling of an ON probe with an electroactive marker  $\text{G}^{\text{NO}_2}$  using  $\text{dG}^{\text{NO}_2}\text{TP}$  and TdT. (B) Denaturing PAGE of products of TdT-catalyzed extension of  $^{32}\text{P}$ -labelled *prim-15* ON: (i), reactions with  $\text{dN}^{\text{NH}_2}\text{TP}$  and  $\text{dN}^{\text{NO}_2}\text{TP}$  (200  $\mu\text{M}$ ); (ii), reactions with  $\text{dG}^{\text{NO}_2}\text{TP}$  at concentrations given in the figure; P, primer; E, long extended products.

For further experiments we chose  $\text{dG}^{\text{NO}_2}\text{TP}$  with respect to (a) facile TdT extension, and (b) favourable electrochemical properties of the nitro group that undergoes multi-electron electrochemical reduction at mercury- or carbon based electrodes (see below).

The TdT extension reactions were performed with several primer oligonucleotides (ONs; see Table 1) which gave basically the same results as shown in Fig. 1B,ii for the *prim-15* ON. For 7  $\mu\text{M}$  primer and 10  $\mu\text{M}$   $\text{dG}^{\text{NO}_2}\text{TP}$ , the ON was extended by 0–5 nucleotides. For 25  $\mu\text{M}$   $\text{dG}^{\text{NO}_2}\text{TP}$ , the main product contained 4 extra nucleotides which was close to the dNTP/primer molar ratio. For  $\text{dG}^{\text{NO}_2}\text{TP}$  concentrations between 50 and 100  $\mu\text{M}$ , the mean lengths of the products were outside the region of efficient separation in the 15% polyacrylamide gel (marked by E in Fig. 1B), and for 250  $\mu\text{M}$   $\text{dG}^{\text{NO}_2}\text{TP}$ , a considerable portion of the radioactive material was unable to enter the gel. In all cases, certain amount of the relatively short extension products was detected (even in the high excesses of the  $\text{dG}^{\text{NO}_2}\text{TP}$ ), which was consistent with the non-processive mechanism of the TdT tailing reactions.<sup>8</sup> Essentially the same behavior was observed with other ON substrates used in this work (Table 1).

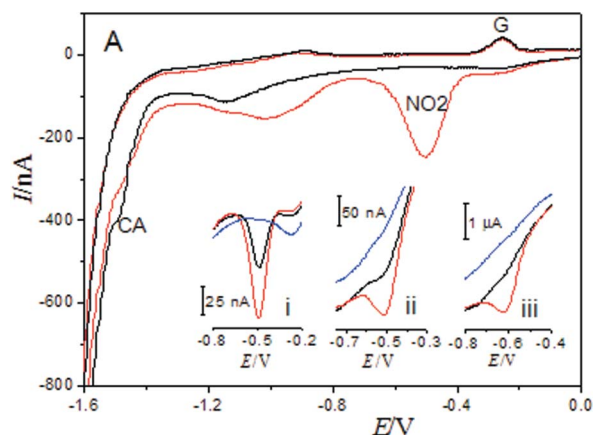
**Table 1** Synthetic ONs used in this work

Acronym	Sequence (5'→3')
<i>prim-15</i>	GAATTCGATATCAAG
<i>prim-18A20</i>	(A) <sub>20</sub> AGGCATGGGCGGCATGGG-3'
<i>target A<sup>a</sup></i>	CAGGCACAAACACGCACCTCA(A) <sub>20</sub>
<i>probe A<sup>a</sup></i>	GAGGTGCGTGTTTGTGCCTG
<i>target B<sup>a</sup></i>	GGATGGGCTCCGGTTCATGA(A) <sub>20</sub>
<i>probe B<sup>a</sup></i>	CCTACCCGGAGGCCAAGTACT
<i>PGM1<sup>b</sup></i>	GACGGTATCGATAAGAGGCATGTCTAGGCATGTCT- CTTGATATCGAATTC
<i>noCON<sup>b</sup></i>	GACGGTATCGATAAGGCATCATAGCGCATCATA- GCCTTGATATCGAATTC

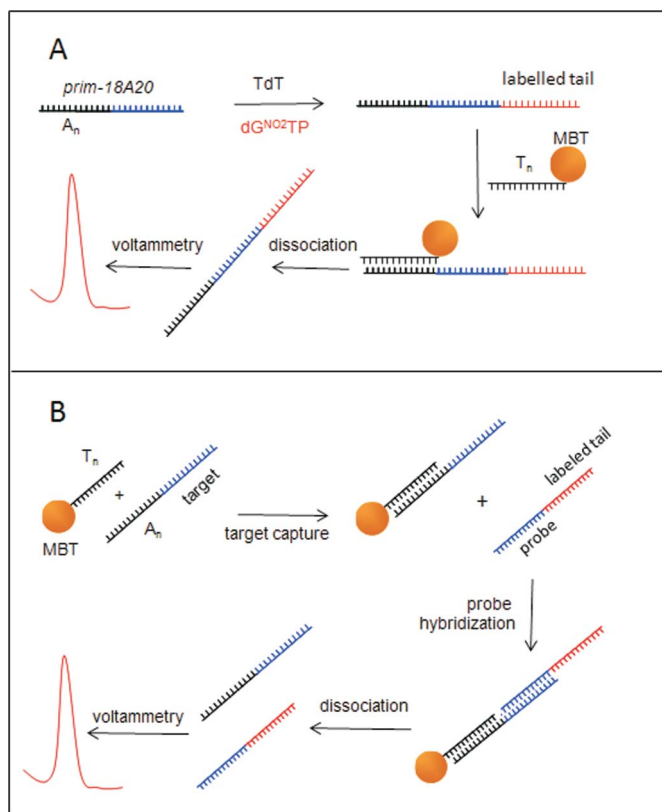
<sup>a</sup> Sequences derived from two regions of *TAp53* gene encompassing mutation hotspots in codons 273 (*target A* and *probe A*) and 248 (*target B* and *probe B*).<sup>9</sup> The (A)<sub>20</sub> adaptors are used for attachment of the targets at magnetic beads in the DNA hybridization experiments.<sup>10</sup> <sup>b</sup> Top strands of *PGM1* and *noCON* 50-mer duplexes used in the protein-DNA binding experiments. *PGM1* comprises an optimized binding site for the p53 protein<sup>11</sup> (bold), *noCON* is a non-specific control.

### Electrochemical analysis of tail-labelled ONs

Previously it was shown<sup>5</sup> that DNA labelled with nitrophenyl A, C and U conjugates yields irreversible four-electron (per nitro group) reduction detectable at carbon electrodes. Here we measured voltammetric responses for products of the TdT reactions of various ON substrates (in Fig. 2 shown for *prim-18A20*, isolated after the TdT extension from the reaction mixture using magnetic beads bearing d(T)<sub>25</sub> stretches, see Scheme 2A), with  $\text{dG}^{\text{NO}_2}\text{TP}$  at mercury (HMDE), mercury meniscus-modified solid amalgam (m-AgSAE)<sup>12</sup> and pyrolytic graphite (PGE) electrodes.<sup>13</sup> Cyclic voltammogram of unextended ON primers (Fig. 2, black curve) displayed two current signals corresponding to redox processes of natural nucleobases, a cathodic peak CA at  $\sim -1.5$  V (due to A and C) and a peak G at  $-0.25$  V (due to guanine).<sup>13</sup> For the product of the tailing reaction, another distinct cathodic peak appeared at  $-0.50$  V suggesting an efficient incorporation of the  $\text{G}^{\text{NO}_2}$  electroactive tags (peak NO<sub>2</sub>; red curve in Fig. 2). Current



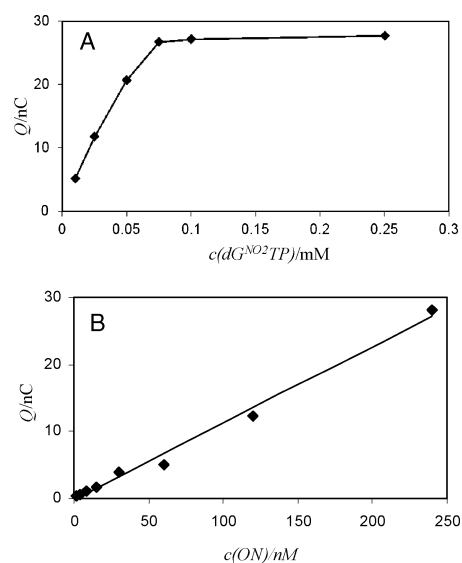
**Fig. 2** *Ex situ* cyclic voltammograms (CV) measured at HMDE for *prim-18A20* ON after TdT-catalyzed extension reaction with 75  $\mu\text{M}$   $\text{dG}^{\text{NO}_2}\text{TP}$  (red) and negative control (no TdT added; black). Insets: CV measured at HMDE (i) or m-AgSAE (ii), and square wave voltammograms measured at PGE (iii) with TdT extension products obtained for 25  $\mu\text{M}$  (red curves) or 10  $\mu\text{M}$  (black)  $\text{dG}^{\text{NO}_2}\text{TP}$  and for the negative control (blue).



**Scheme 2** (A) TdT extension and magnetic separation of *prim-18A20* ON followed by voltammetric analysis. The ON involves an A<sub>20</sub> stretch at its 5'-end. After the TdT reaction, the products are captured from the reaction mixture at magnetic beads covered with T<sub>n</sub> ONs (MBT), followed by separation, washing and release of the labelled ONs which are thus ready for *ex situ* voltammetric analysis. (B) Sandwich DNA hybridization at MBT with a tail-labelled reporter probe. Target ON possessing the A<sub>n</sub> adaptor is captured at the beads, followed by hybridization with the complementary probe. Separation, washing, dissociation and voltammetric detection are performed as above.

signals of the G<sup>NO<sub>2</sub></sup> labels in products extended in the presence of various dG<sup>NO<sub>2</sub></sup>TP concentrations were compared for different working electrodes (inset in Fig. 2). Using the HMDE (panel i), a symmetrical peak NO<sub>2</sub> was obtained for 10 μM dG<sup>NO<sub>2</sub></sup>TP per 0.7 μM ON (corresponding to incomplete labelling of the 38-mer *prim-18A20* with one or few G<sup>NO<sub>2</sub></sup> moieties, Fig. 1B). A distinct (albeit less well developed) peak NO<sub>2</sub> was observed for the same extension product with the m-AgSAE as well (panel ii), while its response at the PGE was rather poor (panel iii). Longer extension products (bearing about 4 G<sup>NO<sub>2</sub></sup> tags per ON, obtained for 25 μM dG<sup>NO<sub>2</sub></sup>TP) yielded well defined signals at all electrode types (Fig. 2). The mercury-based electrodes thus offered a higher sensitivity of the G<sup>NO<sub>2</sub></sup> tags detection, but using longer labelled tails, carbon electrodes can be used owing to the signal amplification.

Areas of the peak NO<sub>2</sub> increased with increasing concentration of dG<sup>NO<sub>2</sub></sup>TP in the TdT reaction mixture about linearly up to 75 μM and levelled off for higher dNTP concentrations (Fig. 3A). Such behaviour displayed correlation between the signal intensity and the labelled tail (as assessed by the PAGE analysis, Fig. 1B) in the former region and revealed limitations of the signal amplification through further extension of the ON. Fig. 3B shows calibration

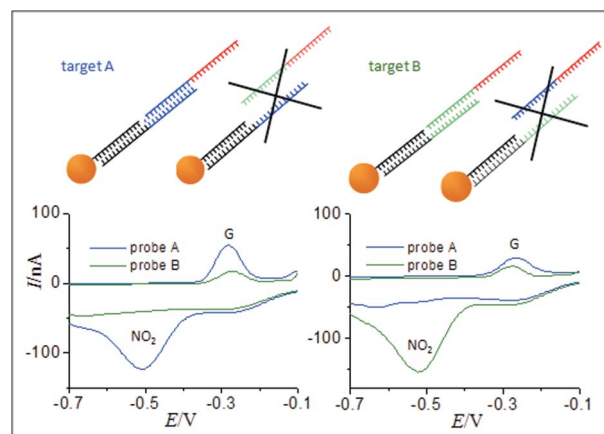


**Fig. 3** (A) Dependence of the peak NO<sub>2</sub> (see Fig. 2) area on dG<sup>NO<sub>2</sub></sup>TP concentration used for the TdT extension of *prim-18A20* (for 0.8 μM ON). (B) Dependence of the peak NO<sub>2</sub> area on the labelled ON concentration (for *prim-18A20* TdT extension of 0.8 μM ON in the presence of 100 μM dG<sup>NO<sub>2</sub></sup>TP; concentrations in the graph were reached by dilution of the reaction product).

curve for products of TdT extension of *prim-18A20* with 100 μM dG<sup>NO<sub>2</sub></sup>TP, exhibiting linearity at least between 2 and 240 nM of the tail-labelled DNA.

### Examples of bioanalytical applications

The TdT tail-labelled ONs were further tested in simple model bioassays. In particular, we were interested whether ON probes/substrates with long chemically modified overhangs retain their biorecognition features such as sequence-specific DNA hybridization or DNA-protein recognition, and whether these events are detectable by simple electrochemical assays. For this purpose, we prepared tail G<sup>NO<sub>2</sub></sup>-labelled reporter probes (RP) for magnetic beads-based sandwich hybridization assays<sup>10</sup> (Fig. 4A) and DNA

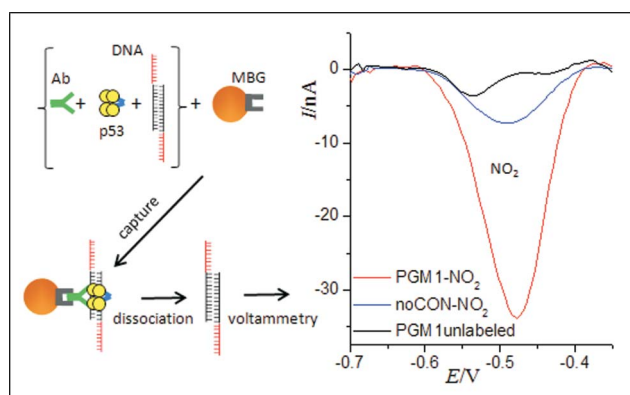


**Fig. 4** Sandwich DNA hybridization assay at magnetic beads with (G<sup>NO<sub>2</sub></sup>)<sub>n</sub> tail-labelled reporter probes. Left: results for target A; right: results for target B. For procedure, see Scheme 2B.

substrates for immunoprecipitation binding experiments with tumour suppressor protein p53<sup>14</sup> (Fig. 3B).

Hybridization experiments were performed with target sequences derived from two *TAp53* gene regions (Table 1). Both target ONs comprised dA<sub>20</sub> adaptors for immobilization at magnetic beads bearing dT<sub>25</sub> strands (MBT). The target strands were captured at the MBT and subsequently hybridized with one of the tail-labelled RPs, of which one was complementary to the given target and the other non-complementary. After magnetic separation and washing of the beads, the hybridized DNA was recovered by thermal treatment and determined by adsorptive transfer stripping (AdTS) cyclic voltammetry (CV) at the HMDE (Scheme 2B). The results perfectly correlated with complementarity between the target and RP sequences (Fig. 4): when *target A* was first immobilized at the MBT followed by hybridization with the complementary *probe A*, a strong peak NO<sub>2</sub> was observed, which was however absent when the non-complementary *probe B* was applied. When analogous hybridization experiments were performed with *target B*, positive hybridization signal (peak NO<sub>2</sub>) was observed for *probe B* and negative for *probe A* (Fig. 4). The peak G reflected presence and abundance of guanine residues in the target and/or complementary probe strands (compare nucleotide sequences in Table 1).

Protein-DNA binding experiments were conducted according to the scheme depicted in Fig. 5. Immune complex of the p53 protein with the Bp53-10.1 antibody (Ab) was first formed in solution to which the labelled DNA substrate was subsequently added, followed by incubation to allow the p53-DNA binding. The Ab-p53-DNA complexes were captured at magnetic beads covered with protein G. After magnetic separation, the labelled DNA substrate was recovered through salt-induced dissociation of the p53-DNA complex and analyzed voltammetrically. Two types of DNA substrates were used: one comprising a specific binding site of the p53 protein (*PGMI*) and the other lacking such site (*noCON*). As shown in Fig. 5, a well defined peak NO<sub>2</sub> was obtained for the labelled *PGMI* substrate (red curve), while for



**Fig. 5** MBIP assay for p53-DNA binding using (G<sup>NO<sub>2</sub></sup>)<sub>n</sub> tail-labelled DNA substrates: left, scheme of the procedure. The antibody-p53-DNA complex was formed in solution, followed by capture at magnetic beads covered with protein G (specifically binding the antibody; MBG). After separation, DNA was dissociated from the complex by heating at high salt and analyzed by *ex situ* CV at HMDE. Right: sections of voltammograms obtained for *PGMI* ON (red curve) comprising a specific p53 binding sequence and non-specific *noCON* ON (blue); black curve corresponds to the same experiment with unlabelled *PGMI*.

the same but unlabelled DNA only a weak signal at a potential by 50 mV more negative was detected (black). The latter signal was probably due to certain amount of protein released from the beads which was adsorbed at the electrode and yielded a characteristic peak due to cysteine thiol groups (this assumption was supported through measurements with free p53 protein, not shown). For the G<sup>NO<sub>2</sub></sup> tail-labelled *noCON* substrate, the intensity of peak NO<sub>2</sub> was about 4-times lower (blue), compared to the same signal obtained for the specific *PGMI* substrate.

## Conclusions

In this paper we present a facile tail-labelling of DNA probes using TdT enzyme and a modified dNTP. Unlike classical primer extension or PCR, the labelling by TdT does not require template strand and therefore it is a general method for 3'-end-labelling of virtually any DNA. Different modified dN<sup>x</sup>TPs gave tail-labels of different length depending on the nucleobase, label and concentration of the nucleotide. Long G<sup>NO<sub>2</sub></sup> tails were efficiently attached to specific ON sequences rendering them electrochemical activity of the nitrogroup and making them sensitively detectable by simple *ex situ* voltammetric measurements. Tail-labelled hybridization probes showed perfect discrimination between complementary and non-complementary target DNAs. The p53 protein-DNA binding experiments revealed reasonable distinction between sequence-specific and non-specific DNA substrates, as well as relatively weak interference of the protein electrochemical activity in the peak NO<sub>2</sub> measurements. Thus, both hybridization probes and protein binding substrates with long G<sup>NO<sub>2</sub></sup> tails retained their biomolecular recognition properties. To improve the protein binding specificity, particularly to minimize the extent of non-specific protein-DNA binding, more work (focused on *e.g.*, optimization of the tail lengths, choice of optimum labels, *etc.*) is required which is matter of our ongoing study.

## Experimental section

### Suzuki cross-coupling reactions of 7-I-7-deaza-dGTP with 3-aminophenylboronic and 3-nitrophenylboronic acid

**General procedure.** Water-acetonitrile mixture (2 : 1, 0.5 ml) was added through septum to an argon purged vial containing halogenated dNTP (0.042 mmol), 3-aminophenylboronic acid hydrochloride (14.6 mg, 0.084 mmol) or 3-nitrophenylboronic acid (14 mg, 0.084 mmol), Cs<sub>2</sub>CO<sub>3</sub> (68.5 mg, 0.21 mmol). After dissolving of the solids, a solution of Pd(OAc)<sub>2</sub> (1 mg, 0.0042 mmol) and TPPTS (12 mg, 0.021 mmol) in water-acetonitrile (2 : 1, 0.3 ml) was added and the mixture was stirred and heated up to 120 °C for 30 min. Products were isolated from crude reaction mixture by HPLC on C18 column with the use of linear gradient of 0.1 M TEAB (triethylammonium bicarbonate) in H<sub>2</sub>O to 0.1 M TEAB in H<sub>2</sub>O-MeOH (1 : 1) as eluent, followed by HPLC on POROS (Applied Biosystems) with the use of gradient of H<sub>2</sub>O to 400 mM TEAB to separate out the diphosphate derivative. Several co-distillations with water and conversion to sodium salt (Dowex 50 W X 8 in Na<sup>+</sup> cycle) form followed by freeze drying from water gave white solid products in purity *ca.* 75% (impurity: *ca.* 25% of the corresponding nucleoside diphosphate).



7-(3-Nitrophenyl)-9-(2'-deoxy- $\beta$ -D-erythro-pentofuranosyl)-7-deazaguanine-5'-O-triphosphate (**dG<sup>NO2</sup>TP**). Yield 14%, purity 75% (impurity: 25% of the corresponding nucleoside diphosphate). MS(ESI<sup>-</sup>): 648 (40, M+Na<sup>+</sup>-1), 546 (100, M-PO<sub>3</sub>H<sub>2</sub>-1), HRMS: for C<sub>17</sub>H<sub>18</sub>N<sub>5</sub>O<sub>15</sub>NaP<sub>3</sub> calculated 647.9910 found 647.9908. <sup>1</sup>H NMR (499.8 MHz, D<sub>2</sub>O, pD = 7.1, ref<sub>dioxane</sub> = 3.75 ppm): 2.43 (ddd, 1H,  $J_{gem} = 14.0$ ,  $J_{2'a,1'} = 6.2$ ,  $J_{2'b,3'} = 3.0$ , H-2'b); 2.71 (ddd, 1H,  $J_{gem} = 14.0$ ,  $J_{2'a,1'} = 7.8$ ,  $J_{2'a,3'} = 6.5$ , H-2'a); 4.14, 4.18 (2 × m, 2 × 1H, H-5'); 4.21 (m, 1H, H-4'); 4.76 (overlapped with HDO signal, H-3'); 6.47 (dd, 1H,  $J_{1'2'} = 7.8$ , 6.2, H-1'); 7.40 (s, 1H, H-8); 7.59 (t, 1H,  $J_{5,4} = J_{5,6} = 8.1$ , H-5-C<sub>6</sub>H<sub>4</sub>NO<sub>2</sub>); 8.08 (bd, 1H,  $J_{6,5} = 8.1$ , H-6-C<sub>6</sub>H<sub>4</sub>NO<sub>2</sub>); 8.11 (dd, 1H,  $J_{4,5} = 8.1$ ,  $J_{4,2} = 2.0$ , H-4-C<sub>6</sub>H<sub>4</sub>NO<sub>2</sub>); 8.70 (t, 1H,  $J_{2,4} = J_{2,6} = 2.0$ , H-2-C<sub>6</sub>H<sub>4</sub>NO<sub>2</sub>). <sup>13</sup>C NMR (125.7 MHz, D<sub>2</sub>O, pD = 7.1, ref<sub>dioxane</sub> = 69.3 ppm): 40.85 (CH<sub>2</sub>-2'); 68.35 (d,  $J_{C,P} = 7.0$ , CH<sub>2</sub>-5'); 73.96 (CH-3'); 85.67 (CH-1'); 87.78 (d,  $J_{C,P} = 9.7$ , CH-4'); 100.53 (C-5); 120.04 (CH-8); 121.72 (C-7); 124.07 (CH-4-C<sub>6</sub>H<sub>4</sub>NO<sub>2</sub>); 125.82 (CH-2-C<sub>6</sub>H<sub>4</sub>NO<sub>2</sub>); 132.07 (CH-5-C<sub>6</sub>H<sub>4</sub>NO<sub>2</sub>); 136.82 (CH-6-C<sub>6</sub>H<sub>4</sub>NO<sub>2</sub>); 137.60 (C-1-C<sub>6</sub>H<sub>4</sub>NO<sub>2</sub>); 150.48 (C-3-C<sub>6</sub>H<sub>4</sub>NO<sub>2</sub>); 155.08 (C-4); 155.67 (CH-2); 163.76 (C-6). <sup>31</sup>P (<sup>1</sup>H dec.) NMR (202.3 MHz, D<sub>2</sub>O, pD = 7.1, ref<sub>phosphate buffer</sub> = 2.35 ppm): -21.27 (b, P<sub>β</sub>); -10.29 (d,  $J = 18.8$ , P<sub>α</sub>); -6.62 (b, P<sub>γ</sub>).

7-(3-Aminophenyl)-9-(2'-deoxy- $\beta$ -D-erythro-pentofuranosyl)-7-deazaguanine-5'-O-triphosphate (**dG<sup>NH2</sup>TP**). Yield 23%, purity 75% (impurity: 25% of the corresponding nucleoside diphosphate). MS(ESI<sup>-</sup>): 618 (30, M+Na<sup>+</sup>-1), 516 (100, M-PO<sub>3</sub>H<sub>2</sub>-1), HRMS: for C<sub>17</sub>H<sub>20</sub>N<sub>5</sub>O<sub>13</sub>NaP<sub>3</sub> calculated 618.0168 found 618.0165. <sup>1</sup>H NMR (499.8 MHz, D<sub>2</sub>O, pD = 7.1, ref<sub>dioxane</sub> = 3.75 ppm): 2.39 (ddd, 1H,  $J_{gem} = 14.0$ ,  $J_{2'b,1'} = 6.3$ ,  $J_{2'b,3'} = 3.2$ , H-2'b); 2.69 (ddd, 1H,  $J_{gem} = 14.0$ ,  $J_{2'a,1'} = 7.7$ ,  $J_{2'a,3'} = 6.3$ , H-2'a); 4.14, 4.18 (2 × m, 2 × 1H, H-5'); 4.22 (m, 1H, H-4'); 4.74 (dt, 1H,  $J_{3',2'} = 6.3$ , 3.2,  $J_{3',4'} = 3.2$ , H-3'); 6.47 (dd, 1H,  $J_{1'2'} = 7.7$ , 6.3, H-1'); 6.80 (bd, 1H,  $J_{4,5} = 7.5$ , H-4-C<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>); 7.21 (m, 2H, H-2,6-C<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>); 7.24 (s, 1H, H-8); 7.26 (bt, 1H,  $J_{5,4} = J_{5,6} = 7.5$ , H-5-C<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>). <sup>13</sup>C NMR (125.7 MHz, D<sub>2</sub>O, pD = 7.1, ref<sub>dioxane</sub> = 69.3 ppm): 40.81 (CH<sub>2</sub>-2'); 68.34 (d,  $J_{C,P} = 5.7$ , CH<sub>2</sub>-5'); 73.98 (CH-3'); 85.61 (CH-1'); 87.72 (d,  $J_{C,P} = 8.8$ , CH-4'); 100.65 (C-5); 117.97 (CH-4-C<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>); 119.02 (CH-8); 119.11 (CH-2-C<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>); 122.64 (CH-6-C<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>); 123.69 (C-7); 132.13 (CH-5-C<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>); 137.19 (C-1-C<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>); 148.21 (C-3-C<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>); 154.80 (C-4); 155.53 (CH-2); 163.75 (C-6). <sup>31</sup>P (<sup>1</sup>H dec.) NMR (202.3 MHz, D<sub>2</sub>O, pD = 7.1, ref<sub>phosphate buffer</sub> = 2.35 ppm): -21.40 (t,  $J = 19.3$ , P<sub>β</sub>); -10.35 (d,  $J = 19.3$ , P<sub>α</sub>); -6.68 (d,  $J = 19.3$ , P<sub>γ</sub>).

## Other material

Synthetic oligonucleotides (ONs; Table 1) were purchased from VBC genomics (Austria). Terminal deoxynucleotidyl transferase (TdT) and T4 Polynucleotide kinase were purchased from New England Biolabs,  $\gamma$ -<sup>32</sup>P-ATP from MP Empowered Discovery (USA), unmodified nucleoside triphosphates from Sigma (USA), magnetic beads bearing oligo d(T)<sub>25</sub> (MBT) and protein G-coated magnetic beads (MBG) from Invitrogen (USA). Murine anti-p53 monoclonal antibody Bp53-10.1 was obtained from the Masaryk Memorial Cancer Institute (Brno, Czech Republic). Wild type human full length p53 protein, expressed in *E. coli* BL21/DE3 and purified as described previously,<sup>15</sup> was kindly donated by Dr Marie Brázdová. Other chemicals were of analytical grade.

## TdT-catalyzed tail-labelling of ONs

If not stated otherwise in the text, the reaction mixtures contained, per 10  $\mu$ L-sample, 4 U of TdT and (a) 0.7  $\mu$ M ON, 200  $\mu$ M dNTP (*prim-15* labeling for PAGE - Fig. 1); (b) 0.8  $\mu$ M ON, 100  $\mu$ M **dG<sup>NO2</sup>TP** (*prim-18A20* labeling for electrochemical measurements - Fig. 2); (c) 1.5  $\mu$ M probe A or probe B, 100  $\mu$ M **dG<sup>NO2</sup>TP** (labelling of reporter probes, Fig. 3A); or (c) 1.5  $\mu$ M of ss ONs forming *PGM1* or noCON duplexes, 250  $\mu$ M **dG<sup>NO2</sup>TP** (labelling of DNA substrates for DNA-protein binding, Fig. 3B). Reactions were conducted at 37 °C for 60 min. For polyacrylamide gel electrophoresis (PAGE, Fig. 1) experiments, *prim-15* was <sup>32</sup>P-prelabeled at its 5' end. For electrochemical analysis of the tail-labelled ONs, *prim-18A20* containing (A)<sub>20</sub> stretches at its 5' ends was used and the extended products were purified using magnetic beads MBT.<sup>10</sup>

## Denaturing PAGE

The TdT tail-labelled products were mixed with loading buffer (80% formamide, 10 mM EDTA, 1 mg mL<sup>-1</sup> xylene cyanol, 1 mg mL<sup>-1</sup> bromphenol blue) and subjected to electrophoresis in 15% denaturing gel containing 1 × TBE buffer (pH 8) and 7 M urea at 25 W for 50 min. Gels were dried, autoradiographed and visualized using Phosphorimager Storm.

## Separation of modified ONs with MBT

The TdT tail-labelled products of *prim-18A20* were captured at MB via (A)<sub>20</sub> adaptors. Then, 50  $\mu$ L aliquots of the reaction mixtures were added to the MBT (25  $\mu$ L of the stock suspension washed twice with 100  $\mu$ L of 0.3 M NaCl, 10 mM Tris-HCl, pH 7.4 - buffer H). The mixture was incubated on a shaker for 30 min at 20 °C. Then the beads were washed three times with 100  $\mu$ L PBS (0.14 M NaCl, 3 mM KCl, 4 mM sodium phosphate, pH 7.4) with 0.01% Tween20, three times with 100  $\mu$ L of the buffer H and resuspended in deionized water (50  $\mu$ L). The labelled ONs were recovered by heating at 75 °C for 2 min. Prior to the *ex situ* electrochemical measurements, NaCl was added to the samples to reach final concentration of 0.3 M.

## DNA Hybridization at MBT

Target ONs bearing A<sub>20</sub> stretch (20  $\mu$ L of 0.8  $\mu$ M ON in solution containing 0.3 M NaCl) were captured on MBT (20  $\mu$ L of the stock suspension washed twice with 100  $\mu$ L of buffer H). The mixture was incubated on a shaker for 30 min at 20 °C. Subsequently, the tail-labelled reporter probes (20  $\mu$ L of unpurified TdT-reaction mixture supplemented with NaCl to concentration of 0.3 M) were added to the MBT, incubated on a shaker for 30 min at 20 °C. Then the beads were washed three times with 100  $\mu$ L PBS with 0.01% Tween20, three times with 100  $\mu$ L of the buffer H and resuspended in deionized water (20  $\mu$ L). The hybridized DNA was released by heating at 75 °C for 2 min. Again, NaCl was added to each sample to a final concentration of 0.3 M prior to the *ex situ* voltammetric analysis.

## Immunoprecipitation assay of the p53-DNA binding with magnetic beads

(MBIP assay<sup>14</sup>) The p53 immune complexes were prepared by mixing of Bp53-10.1 antibody<sup>16</sup> with the protein at a molar ratio of 16:1 in binding buffer (50 mM KCl, 5 mM Tris and 0.01% Triton X-100, pH 7.6), followed by a 20-min incubation. Then, approximately 200 ng of the tail-labelled duplexes (prepared by annealing of equivalent amounts of tail-labelled and complementary strands, purified using QIAquick Nucleotide Removal Kit, Qiagen) and 300 ng of scDNA pBSK<sub>(-)</sub> were mixed with the given immune complex (p53 tetramer/scDNA molar ratio was 2.5:1) and incubated in the binding buffer for 30 min on ice. Magnetic beads MBG (12 µl of the stock suspension per sample) were washed three times with 100 µl of the binding buffer. The beads were separated from the supernatant using magnetic particle concentrator. Then the binding reaction mixture was added and incubated with the beads for 30 min at 10 °C whilst shaking mildly. Finally, after triplicate washing with the binding buffer, DNA was released from the MBG-confined p53 complexes through a 5-min incubation at 65 °C in 20 µl of 0.5 M NaCl.

## Electrochemical analysis

The TdT tail-labelled products were analyzed by using *ex situ* (adsorptive transfer stripping) cyclic voltammetry (AdTS CV) using hanging mercury drop electrode (HMDE) or meniscus-modified solid amalgam electrode<sup>17</sup> (m-AgSAE, prepared and treated as described<sup>18</sup>), or by *ex situ* square wave voltammetry (AdTS SWV) using basal-plane pyrolytic graphite electrode (PGE; prepared and treated as described<sup>19</sup>). DNA was accumulated at the electrode surface from 5 µL aliquots containing 0.3 M NaCl for 60 s. Then the electrode was rinsed by deionized water and was placed into the electrochemical cell. CV settings: initial potential 0.0 V, switching potential -1.85 V, final potential 0.0 V, scan rate 1 V s<sup>-1</sup>, step potential 5 mV, in 0.3 M ammonium formate with 0.05 M sodium phosphate, pH 6.9 as a supporting electrolyte. SWV settings: initial potential 0.5 V, final potential -1.5 V, frequency 200 Hz, amplitude 25 mV, in 0.2 M acetate buffer, pH 5. The measurements were performed at ambient temperature by using Autolab analyzer (Metrohm Autolab, The Netherlands) in connection with VA-stand 663 (Metrohm, Switzerland) in a three-electrode setup (with the HMDE, mAgSAE or PGE as working electrode, Ag/AgCl/3 M KCl as reference, and platinum wire as counter electrode). Baseline correction of chosen curves was performed using GPES 4 software (EcoChemie).

## Acknowledgements

This work was supported by the Academy of Sciences of the Czech Republic (Z4 055 0506, Z5 004 0507 and Z5 004 0702), the Ministry of Education (LC06035, LC512), Grant Agency of the Academy of Sciences of the Czech Republic (IAA400040901), and by Gilead Sciences, Inc. (Foster City, CA, U. S. A.). The authors thank Dr Marie Brázdová for donation of the p53 protein.

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