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# A novel real-time PCR method based on signaling-by-incorporation

**Da-Rae Kim,[a] Hee-Chul Ahn,[b] Won-Ja Lee,[c] and Dae-Ro Ahn*[a]**

[a]Biomedical Research Center, Korea Institute of Science and Technology, PO Box 131, Cheongryang, Seoul 130-650, South Korea. Fax: +82 2-958-5909; Tel: +82 2-958-6645; E-mail: drahn@kist.re.kr; fouahn@gmail.com

[b]Advanced Analysis Center, Korea Institute of Science and Technology.

[c]Division of Malaria and Parasitic Diseases, Korea Centers for Disease control and Prevention, National Institute of Health, Seoul, South Korea.

## MATERIALS AND METHODS

### Reagents and materials

All chemicals, solvents used for synthesis of the modified dGTP, and the phosphodiesterase for cleavage of phosphodiester bonds were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deoxyguanosine triphosphate (dGTP) was purchased from Promega (Madison, USA). $^{1}$H- and $^{31}$P-NMR spectra were measured using Varian VNMRS 900 spectrometer, and Varian 500MHz VNMRS spectrometers, respectively. Mass spectrum of the nucleotide was measured using Voyager DE STR™ MALDI-TOF MS (Applied Biosystems, Foster City, CA, USA). Reverse phase-HPLC was performed using a Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA) equipped with an Eclipse XBD-C18 column (4.6 mm x 250 mm, Agilent Technologies, Palo Alto, USA). Buffer A contained 20 mM triethylammonium bicarbonate (TEAB), pH 7.5, and Buffer B contained 20 mM TEAB, pH 7.5, 90% acetonitrile (v/v). Anion exchange-HPLC was performed using a a DNAPac PA100 column (4 mm x 250 mm, Dionex, Sunnyvale, CA, USA). Buffer A contained 20 mM $NaH_2PO_4$, pH 6.5, and Buffer B contained 20 mM $NaH_2PO_4$, 500 mM NaCl, pH 6.5. Denaturing polyacrylamide gel electrophoresis (PAGE) analysis was performed on Vertical Gel Electrophoresis Units (Sigma-Aldirch) with constant voltage of 1000V for 4hr. Oligonucleotides were purchased from Intergrated DNA Technologies (Coralville, USA). Polymerases and reaction buffers were purchased from New England Biolabs (Ipswich, MA, USA). Real-time PCR experiments were performed by using StepOne™ system (Applied Biosystems, Foster City, CA, USA). PAGE gel images were evaluated by using ImageQuant™ ver. 5.2

### Synthesis of γF-dGTP .

$\gamma NH_2$-dGTP (5.4 mM) in water (50 μL) was added to BODIPY-FL N-hydroxysuccinimidyl ester (SE) (10 mM) in DMSO (140 μL). The resulting solution was incubated for 3 hours at room temperature and purified by sequential

HPLC of anion exchange- and reverse phase-HPLC. $^{1}$H NMR (900 MHz, $D_2O$) δ 7.76 (s, 1H), 7.46 (s, 1H), 7.16 (s, 1H), 6.85 (d, *J* = 3.6 Hz, 1H), 6.18 (d, *J* = 2.7 Hz, 1H), 6.07 (s, 1H), 5.88 (t, *J* = 6.3 Hz, 1H), 4.49 (m, 1H), 4.03 (m, 1H), 3.94-3.99 (m, 2H), 3.17 (t, *J* = 5.4 Hz, 2H), 2.96 (t, *J* = 5.4 Hz, 2H), 2.87-2.92 (m, 4H), 2.51-2.56 (m, 1H), 2.37-2.39 (m, 1H), 2.31 (s, 3H), 2.09 (s, 3H). $^{31}$P NMR (201.6 MHz, $D_2O$) -4.71 (d, *J* = 20.9 Hz, 1P), -14.31 (d, *J* = 19.6 Hz, 1P), -25.44 (t, J = 19.6 Hz, 1P) ppm; MALDI-TOF MS calculated for $[C_{26}H_{35}BF_2N_9O_{13}P_3]^-$ : 823.1628, found: 823.1190.

**Measurement of fluorescence intensity of γF-dGTP, Bodipy-FL SE, and γF-dGTP treated with phosphodiesterase**

The fluorescence intensities of **γ**F-dGTP, Bodipy-FL SE, and hydrolyzed **γ**F-dGTP were measured at 50 nM in the PDE buffer (110 mM Tris·HCl buffer, pH 8.8, with 0.11 M NaCl and 15 mM $MgCl_2$). Hydrolysis of **γ**F-dGTP (50 nM) was carried out by treatment with phosphodiesterase (5 mg) in PDE buffer at 37°C for 30 min. The fluorescence intensities of the solutions were measured using LB50B luminescence spectrometer (Perkin-Elmer, Waltham, USA). The excitation wavelength for the measurement was 488nm.

**Incorporation of γF-dGTP by polymerases**

The polymerase incorporation reaction was performed in a 20 μL of mixture containing 1 μM of a fluorescein-labeled primer (5'-fluorescein-GCT ACG ACT CAC TAT GGA CG-3'), 1 μM of the template (5'-GCA TGC ATG CAT GCA ACG TCC ATA GTG AGT CGT AGC-3'), 25 μM each of dATP, dCTP, **γ**F-dGTP, and dTTP, 1X Reaction buffer (Thermopol™ I buffer for Taq, Vent (exo-), DeepVent (exo-) Bst; NEBuffer™ 2 for Klenow Fragment (exo-); Therminator **γ** buffer), and 4 units of DNA polymerases. The mixture was denatured at 95°C for 5 min, cooled to room temperature for 10min, and then incubated at 72°C or 37°C (Klenow Fragment) for 10 min. The reaction was quenched with 20 μL of stop solution (98% deionized formamide; 10 mM EDTA, 0.5 mg/mL bromophenol blue and xylencyanol), heated to 95°C for 5 min, placed on ice for 5 min, and then analyzed on a 20% polyacrylamide gel (7 M urea, 1× TBE). The DNA bands were visualized by using a fluorescence scanner (Typhoon9400, GE healthcare). After dilution with 700 μL Tris buffer (50 mM, pH=8.0), fluorescence intensities of the polymerase reaction mixtures were measured and compared with the intensity of the reference mixture lacking polymerases.

**Real-time PCR with SBI**

The PCR reaction was performed in a 20 μL of mixture containing forward primer (100 nM), reverse primer (100 nM), synthesized DNA targets ($10^5$ -$10^{10}$ molecules) or 0.84ng of human thymus cDNA as the templates, dATP (25 μM), dCTP (25 μM), **γ**F-dGTP (25 μM), dTTP (25 μM), 1X Therminator **γ** reaction buffer, and 0.8 unit of Therminator **γ**$^{TM}$ polymerase. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, 40 cycles consisting of the denaturation step at 95°C for 10 sec, the annealing step at 54°C for 15 sec, and the extension step at 72°C for 10 sec. Fluorescence intensity was measured at the end of the extension step in each cycle. All assays were performed in triplicate.

**Real-time PCR with SG**

The PCR reaction was performed in a 20 μL of mixture containing forward primer (100 nM), reverse primer (100 nM), synthesized DNA targets ($10^5$ -$10^{10}$ molecules) or 0.84ng of human thymus cDNA as the templates, dATP (25 μM), dCTP (25 μM), dGTP (25 μM), dTTP (25 μM), 1X Thermopol$^{TM}$ I reaction buffer, 1XSG-I and 0.4 unit of Taq DNA polymerase. The fluorescence intensity was measured by the same manner used for SBI-based PCR.

**Malaria diagnosis**

Extraction of parasite DNA from blood samples was carried out as described in the previous report[1]. The real-time-PCR assays were carried out by the similar manner used for quantification of the genes in the cDNA as described above adopting the extracted DNA as the targets and the forward (5'-CGCTTCTAGCTTAATCCACATAACTGATAC) and reverse (5'-ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA) primers designed for the target gene. The PCR conditions were as follows: initial denaturation at 95°C for 10 sec, 40 cycles consisting of the denaturation step at 95°C for 5 sec and the extension step at 60°C for 20 sec

**Table S1** Sequences of primers and target genes for real-time PCR.

| | Sequence | Accession No. |
|---|---|---|
| *gapdh* | CCACTCCTCCACCTTTGACGCTGGGGCTGGCATTGCCCTCAACGACCA CTTTGTCAAGCTCATTTCCTGGTATGACAACGAATTTGGCTACAGCAACA GGGT | NM_002046 |
| Forward primer | CCACTCCTCCACCTTTGAC | |
| Reverse primer | ACCCTGTTGCTGTAGCCA | |
| *tuba1b* | AGCGTGCCTTTGTTCACTGGTACGTGGGTGAGGGGATGGAGGAAGGCG AGTTTTCAGAGGCCCGTGAAGATATGGCTGCCCTTGAGAAGGATTATGA GG AGGTTGGTGTG | BC009314 |
| Forward primer | AGCGTGCCTTTGTTCACTG | |
| Reverse primer | CACACCAACCTCCTCATAATCC | |
| *bgb* | TGCACGTGGATCCTGAGAACTTCAGGCTCCTGGGCAACGTGCTGGTCT GTG TGCTGGCCCATCACTTTGGCAAAGAATT | AF007546 |
| Forward primer | TGCACGTGGATCCTGAGAACT | |
| Reverse primer | AATTCTTTGCCAAAGTGATGGG | |

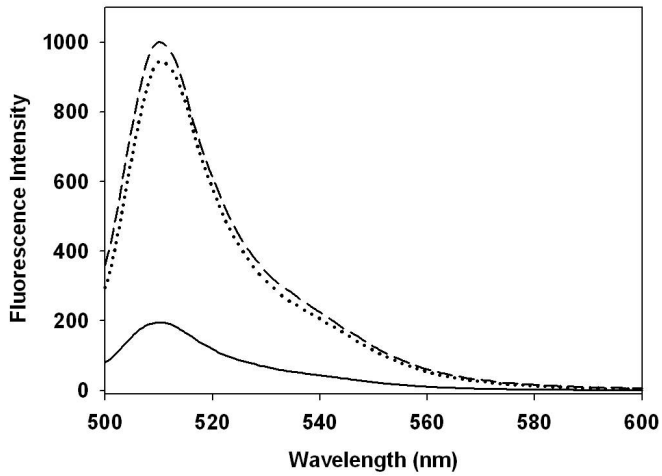


**Fig. S1** Fluorescence spectra of γF-dGTP (solid), Bodipy-FL SE (dotted), and γF-dGTP treated with phosphodiesterase (dashed). Concentration of each compound was 50 nM in PBS.

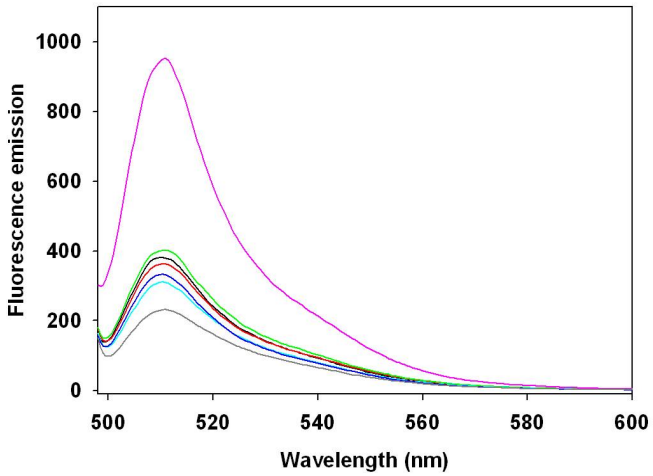


**Fig. S2** Fluorescence spectra of primer extension reactions with various polymerases: Taq (black), Vent(exo-) (red), DeepVent(exo-) (green), Bst (cyan), Klenow Fragment(exo-) (blue), and Theminator γ (pink). Fluorescence of the reaction without polymerases was also measured as the negative control (gray).

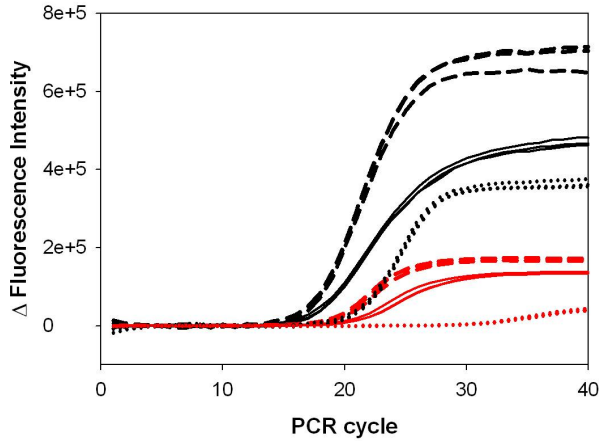


**Fig. S3** Quantification of *gapdh* (solid), *tuba1b* (dashed), and *bgb* (dotted) in cDNA from human thymus cells using the real-time PCR based on SBI (black) or SG (red).

## Reference

1. G. Snounou, S. Viriyakosol, X. P. Zhu, W. Jarra, L. Pinheiro, V. E. do Rosario, S. Thaithong, and K. N. Brown, *Mol Biochem Parasitol*, 1993, **61**, 315.