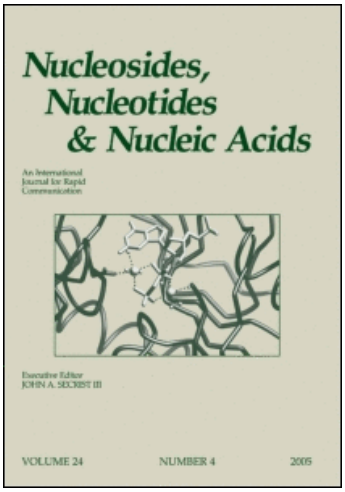

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EUROPIUM CRYPTATE LABELED DEOXYURIDINE-TRIPHOSPHATE ANALOG: SYNTHESIS AND ENZYMATIC INCORPORATION

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ABSTRACT: The synthesis of an europium tris-bipyridine cryptate labeled 2'-deoxyuridine-5'-triphosphate analog (K-11-dUTP) is described. This labeled triphosphate was incorporated into DNA through enzymatic reactions with terminal transferase and DNA polymerases. The enzymatic reactions were monitored by TRACE (Time Resolved Amplification of Cryptate Emission), a homogeneous method using Fluorescence Resonance Energy Transfer (FRET) from an europium cryptate as donor to a modified allophycocyanine as acceptor.

INTRODUCTION

As an alternative to the use of classical radioactive methods, Fluorescent Resonance Energy Transfer (FRET) between a fluorescent donor and a suitable fluorophore as acceptor is a simple and efficient way to probe either the hybridization or enzymatic reactions on nucleic acids substrates. This was exemplified respectively by molecular beacons[1] and the taqMan technique[2]. Nevertheless the steady state fluorescence FRET between conventional organic fluorophores (CY5 and CY3) was shown[3], as anticipated, to be less sensitive than the corresponding assay based on Time Resolved FRET (TR-FRET) using a lanthanide chelate as donor. The luminescent properties of lanthanides allow to work in time resolved mode and to eliminate most of the background fluorescence (unavoidable using conventional steady state fluorescence in complex media) to reach high sensitivities. Lanthanide chelates however are not fully stable in presence of competing ions or ligands as EDTA[4,5] (concentration should be kept below 0.1 mM) and at high temperature[6] (16 % metal exchange within 30 mn at 95°C and neutral pH). This represents a drawback in view of some applications regarding nucleic

acids and particularly due to the increasing use of various thermostable enzymes. The development of a nucleoside triphosphate labeled with a highly stable lanthanide complex was thus of tremendous importance. In this respect the europium cryptate, we developed in the past was attractive. This cryptate ($Eu^{3+}\subset$ tris-bipyridine) was obtained by the incorporation of an europium ion in the cavity of a tris-bipyridin ligand[7] and could be manipulated as any traditional organic fluorophore in the labeling of biomolecules[8]. As acceptor, matching the europium cryptate emission (620 nm) and ensuring an optimum spectral overlap integral, we selected a modified allophycocyanine (XL665) emitting at 665 nm. Furthermore the simultaneous double wavelength (620 and 665 nm) measurement[8] and the use of the ratio (signal 665nm / signal 620 nm) is of the uppermost importance to compensate for the presence of light absorbing compounds (inner filter effect) particularly for application in drug screening. This is the basic concept of TRACE technology (Time Resolved Amplification of Cryptate Emission) enabling the homogeneous detection of molecular interactions (as hybridization) by a simple mix and measure procedure. Its advantage over radioactive methods has already been shown in the field of molecular screening[9]. The labeling of a nucleoside triphosphate analogue with an activated ester derived from europium tris-bipyridine cryptate is presented. The use of such cryptate-labeled triphosphate, referred as to K-11-dUTP throughout the article, will be exemplified by the labeling of oligonucleotides and polymerase extension products and the design of semi-direct assays using biotinylated primer and modified allophycocyanine-streptavidin conjugate (SA-XL665) as generic acceptor.

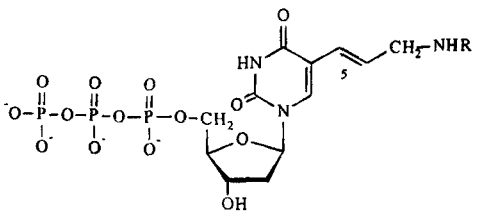
MATERIALS AND METHODS

The Reading Buffer (acronym RB) used below is 100 mM phosphate buffer pH 7.4 containing 0.4 M KF and 0.1 % BSA.

Oligodeoxynucleotides synthesis : The oligodeoxynucleotides (ODN) were synthesized and 5' end labeled with a N-4(6-biotinamidohexyl)-5-methyl-2'-deoxycytidine phosphoramidite as described [10]. The unlabeled ODN were synthesized using the DMT-on option. All ODN were purified by RP-HPLC (CH_3CN gradient in 25 mM TEAAc). The sequence of the primer used for terminal transferase (TdT) experiments was d(C^{bio} GGA GCT AGT GGC GT).

Synthesis of K-11-dUTP: The 5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate **2** was prepared according to literature[11]. N-Hydroxysuccinimide trifluoroacetamidocaproate was synthesized as described[12]. Compound 2 (15 μmol) in 3.2 ml sodium carbonate buffer (1 M, pH 9) was treated with NHS-trifluoracetamidocaproate (100 mg, 300 μmol) in 0.8 ml CH_3CN for 30 mn, the reaction mixture was diluted to 50 ml and loaded on DEAE-sepharose (Pharmacia) equilibrated in 0.1 M TEAB. The desired compound 3 was eluted with 0.26 M TEAB (linear gradient). Yield 68% based on 2. HPLC (20 mn linear gradient from 0 to 50% CH_3CN in 25 mM TEAAc on 125 X 4 RP-18E Lichrospher 5 μm, 1 ml/mn) Rt = 15.5 mn. Compound 3 was deprotected with 10% aqueous ammonia (3 h, RT), after evaporation the residue was diluted with water, loaded on DEAE-sepharose (0.02 M TEAB), compound 4 was eluted with 0.11 M TEAB. Yield 74%. HPLC (above conditions): Rt = 11.2 mn

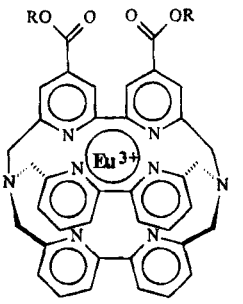
The $Eu^{3+}\subset$ tris-bipyridine dimethyl ester **5** (Fig. 1) (150 mg , 0.15 mmol) in 5 ml methanol was treated by 3 molar equivalents of sodium hydroxide at reflux for 2 h, after addition of 6 molar equivalent of 5 N aqueous HCl and cooling (-20°C, for 1 h), the white precipitate was collected and was further purified on preparative RP-HPLC (VYDAC-C18, 10 μ, 250 x 22 mm) 5% CH_3CN in 1% aqueous trifluoracetic acid for 5 mn, then 15% CH_3CN for 4 mn, then gradient from 15% to 35% in 20 mn at 32 ml/mn. The desired compound was eluted between 23 to 29 % CH_3CN. The eluate was evaporated to dryness under high vacuum to give $Eu^{3+}\subset$ trisbipyridine-4,4'-dicarboxylic acid **6** as its trifluoracetate. Yield 141 mg (80%). ES-MS (positive mode): $[M-2H]^+$= 812, $[MH+2CF_3CO_2]^+$ = 1041. $Eu^{3+}\subset$ trisbipyridine-4,4'-dicarboxylic acid in dry acetonitrile was treated with N-hydroxysuccinimide and dicyclohexylcarbodiimide. The reaction mixture containing the activated cryptate was concentrated, filtered and used extemporaneously without further purification. AH-dUTP **4** (0.4 μmole) in 0.1 M borate buffer pH 8 was treated with 320 μl of activated cryptate (4 mg/ml in acetonitrile) under efficient stirring. After 30 mn, 15 μl of TEAB (Fluka 1 M) were added and the reaction mixture loaded on a HR 10/30 superdex 30 column (Pharmacia) eluted with 25 mM TEAAc pH 7 (Fluka) containing 10% acetonitrile (1 ml/mn). The compound **1** eluting around 17 mn was collected and concentrated (speed-vac) down to 300 μl. Yield 8 UA_{305} (57 % based on **4**) UV (H_2O): λ max 241 nm (ε = 42 000), λ max 304 nm (ε = 35 000).



2 R = H

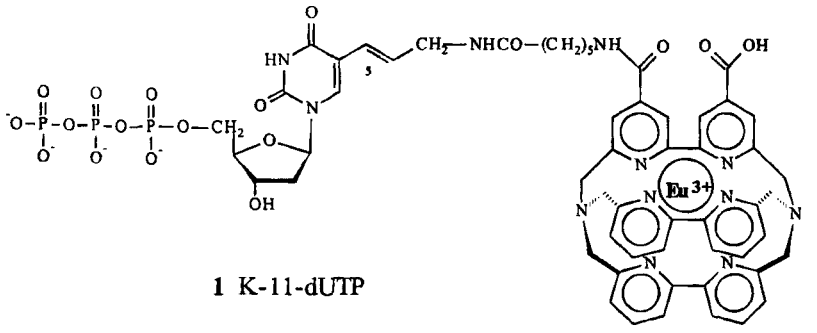
3 R = $CO(CH_2)_5NHCOCF_3$

4 R = $CO(CH_2)_5NH_2$



5 R = CH_3

6 R = H



1 K-11-dUTP

FIG. 1 : Synthetic scheme.

HPLC (A: 20 mM sodium acetate pH 7, CH_3CN 10%. B: 20 mM sodium acetate pH 7, LiCl 1 M, CH_3CN 10%. Linear gradient 0% to 20% B in 22 mn then to 100% B in 5 mn. Mono Q HR 5/5 Pharmacia, 1ml/mn) Rt = 19.7 mn (Rt = 24.1 mn for **4**). ES-MS (positive mode):$[M-2H]^+$= 1431, $[M-2H+CH_3CO_2H]^+$ = 1491.

K-11-dUTP incorporation with terminal deoxynucleotidyl transferase (TdT): A 5'-biotinylated 15-mer ODN (10 pmol) was incubated with various K-11-dUTP / dTTP ratio keeping a final dNTP concentration of 6 µM in a final volume of 50 µl of 100 mM tris-acetate pH 7.2 containing 2 mM $CoCl_2$ and TdT (EC 2.7.7.31 Sigma, 1µl, 22 units). Immediately after mixing and after the indicated elapsed time, 2 µl of the reaction mixture were withdrawn, mixed with 4 µl of 60 mM EDTA and diluted to 250 µl with the Reading Buffer (RB). A "cryptate blank" was made from a reaction mixture depleted in TdT, treated as above and 50 µl were further diluted to 200 µl with RB. In parallel, to

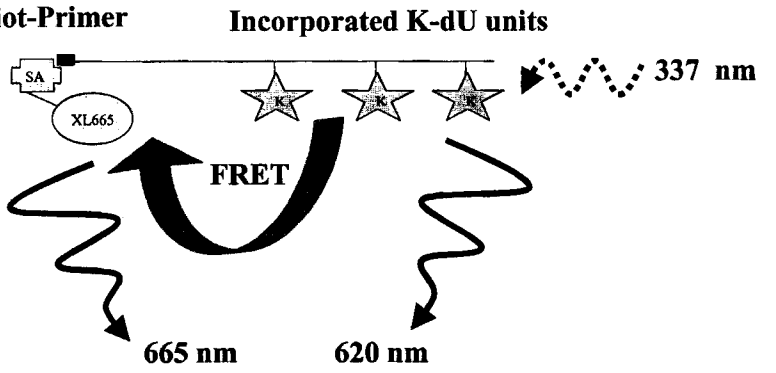


FIG. 2 TRACE detection of K-11-dTTP enzymatic incorporation

each sample (50 µl), were added 50 µl SA-XL665 (CIS-bio international, 15 nM in RB) and 100 µl of RB in 96 wells black microtiter plates (HTRF plates, Packard). The blanks and assays were made in duplicate. After incubation (15 mn , 37°C) the readings were done on a dual wavelength (620 nm/ 665 nm) time resolved (delay 50 µs / gate 400 µs) fluorimeter (Discovery, Packard) with laser (337 nm) excitation. For each point of time course the ratio[8] R = F665/F620 was computed (Ro corresponding to the ratio for cryptate blank). The relative corrected ratio [8,13] ΔF = (R–Ro)/Ro was calculated and the ΔF values were plotted against reaction time (Fig. 3). In a preparative run, 25 pmol of primer was elongated in presence of a K-11-dUTP / dTTP ratio of 1/4 as described, after 80 mn the reaction was blocked with 0.4 M EDTA (12 µl) and loaded on a NAP5 column (pharmacia) equilibrated in 10 mM phosphate buffer pH 7.4 and the first 600 µl exclusion fraction was collected. A 50 µl sample was diluted up to 250 µl with RB and the F620, compared with a serial dilution in RB from a calibrated standard cryptate solution, showed that the above fraction contained 20 pmol of cryptate, the excess of K-11-dUTP being retained. Simultaneously a TRACE assay was performed as above and showed that a strong ΔF signal (3180 %) was detected only in the exclusion fraction and not in the fractions further eluted.

Polymerase extension assay with K-11-dUTP

Preparation of the DNA template

The template DNA was obtained by PCR of a fragment from human K-*ras* exon I gene. The amplimer (117 bp length) was delineated by the forward primer $^{5'}$d(GGC CTG

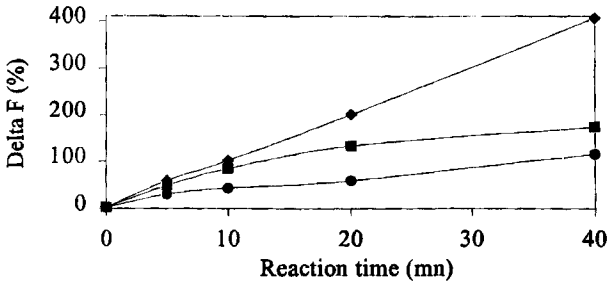


FIG. 3 : TRACE assay of K-11-dUTP incorporation by terminal deoxynucleotidyl transferase K11-dUTP alone (●) and K-11-dUTP/dTTP ratio of 1/1 (■) and 1/4 (◆).

CTG AAA ATG ACT GAA TAT) and the biotinylated reverse primer $^{5'}$d(C^{bio} TGT TGG ATC ATA TTC GTC CAC AAA ATG). The PCR mix (100 µl) contained 10 µl amplification buffer (Appligene), 34 pmol of each primer, 6 units of *Taq* DNA polymerase (EC 2.7.7.7, Appligene), 0.4 µg of human placenta DNA (Sigma) and dNTPs 200 µM each (Appligene). After 5 mn at 95°C the PCR tubes were submitted to thermocycling (1 mn / 94°C, 1 mn / 60°C, 1 mn / 70°C) for 31 cycles. Double stranded biotinylated amplimer (corresponding to 13 pmol of biotin) was captured (37°C 20 mn) on M-280 streptavidine-Dynabeads (30 µl) in binding buffer (10 mM tris-HCl pH 7.5, 1 mM EDTA, 2 M NaCl), after washing with the same buffer, denaturation with 0.1 M NaOH (10 mn), the supernatant was neutralized (dilute HCl) and concentrated (speed-vac) down to a 60 µl volume.

Primer extension with K-11-dUTP

The extension assay was carried out using either a ^{32}P labeled primer followed by PAGE analysis or the 5'-biotinylated reverse primer followed by TRACE analysis. The reaction mixture (50 µl) contained 5 µl amplification buffer (Appligene), 1.25 U *Taq* DNA polymerase (EC 2.7.7.7, Appligene), biotinylated reverse primer (21 pmol), 30 µl template, dATP, dCTP, dGTP (30 µM each), dTTP (15 µM) and K-11-dUTP (15 µM). For PAGE analysis similar reactions were carried out adding 0.5 pmol of ^{32}P-labelled primer with various dTTP / K-11-dUTP ratios and with dTTP alone as reference. The reaction mixtures were incubated at 70°C and samples (9 µl) were withdrawn at initial time, 5, 10, 15 and 20 mn reaction time. The samples were mixed with 2 µl EDTA 0.5 M pH 8 and diluted up to 275 µl with RB. Each sample was further diluted tenfold and 20 µl

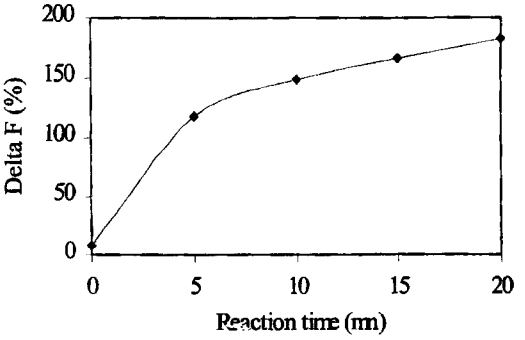


FIG. 4 : TRACE assay for K-11-dUTP / dTTP (1/4 ratio) incorporation by *Taq* polymerase.

of the later dilution were transferred to a black 96 wells microtiter plate. Either 230 µl of RB (for cryptate blank) or 30 µl of 20 nM SA-XL665 followed by 200 µl of RB were then added. After 15 mn incubation (37°C) the relative corrected ratios were calculated and the ΔF values were plotted against reaction time (Fig. 4). The same protocole was performed using the above dNTPs conditions and DNA polymerase I klenow fragment (1U, Pharmacia) in 50 mM Tris-HCl pH 7.7, 10 mM $MgCl_2$ 50 mM NaCl for 45 mn at 37°C, and gave essentially the same results.

RESULTS AND DISCUSSION

The non-isotopic labeling of nucleic acids through the incorporation of various fluorescent reporters, among them fluorescein and more recently cyanines[14], using a suitably labeled nucleoside triphosphate by nick-translation, random priming, terminal deoxynucleotidyl transferase (TdT) or polymerase chain reaction, is well documented [15]. The tethering with fluorophore is possible in several ways on purine or pyrimidine nucleoside analogs although the labeling of pyrimidine is more straightforward. Pyrimidines could be functionalized either on the N-4 position (N-substituted dCTP analogs) or on the C-5 position (5-substituted dC or dU analogues). The C-5 substitution being preferred on the basis of steric assumption. A higher incorporation of fluorescein C-5 labeled dUTP compared to N-4 labeled dCTP in nick translation experiments was recently demonstrated [16] using a general analytical method. A possible approach for the labeling of nucleic acids with europium cryptates would be the preliminary incorporation

of a aminoalkyl-dNTP analog followed by a post labeling step with activated cryptate. Due to the poor stability of the active ester derived from cryptate **6**, the synthesis of a stable cryptate labeled dUTP analog seems more practical.

The synthesis scheme used herein is depicted in Fig. 1. In this work we selected as starting material the 5-(3-aminoallyl)-2'-deoxyuridine **2** (AA-dUTP) previously described[11]. In the course of this work this compound became readily available (Sigma). A longer linker was introduced on AA-dUTP **2** by reaction with N-hydroxysuccinimide trifluoroacetamidocaproate to give compound **3** which was deprotected with ammonia to give 5-[N-(6-aminocaproyl)-3-aminoallyl)]-2'-deoxyuridine **4** (AH-dUTP). The europium tris-(bipyridine) cryptate **6** was tethered through carboxylic functions on the 4,4' positions of one of the bipyridine units and was synthesized from the ester **5** described earlier[17]. The cryptate **6** was treated with N-hydroxysuccinimide and dicyclohexyl carbodiimide in dry acetonitrile, the activated cryptate was not isolated due to its high reactivity and was used directly for coupling. The activated cryptate was reacted with AH-dUTP **4** giving the labeled triphosphate K-11-dUTP **1** with ca 50 % yield based on AH-dUTP. The cryptate labeled triphosphate derivative was freed from unbound fluorophore by size exclusion chromatography on Superdex-30. The mass spectrum of K-11-dUTP **1** displayed an isotopic profile typical of europium derivatives.

A homogeneous TR-FRET polymerase assay could be designed by following the incorporation of an acceptor-labeled nucleotide into a donor labeled primer. On this scheme a reverse transcriptase assay [18] and a terminal transferase assay [19] were investigated through the incorporation of a biotin-16-dUTP into a Eu^{3+}cryptate labeled primer followed by the addition of SA-XL665 conjugate. Since it would be suitable to incorporate several cryptate residues in order to increase the sensitivity of detection, the use of cryptate labeled triphosphate seemed attractive.

Terminal deoxynucleotidyl transferase (TdT) assays using TRACE.

TdT has a polymerase activity [15] which does not need any template, adding nucleotides (tailing) at the 3' end of an ODN primer. In order to test its ability to incorporate K-11-dUTP , a 5'-biotinylated primer was elongated in the presence of K-11-dUTP alone or in admixture with dTTP [20]. For the TdT reaction a tris-acetate buffer was preferred to the cacodylate buffer originally used [15]. For kinetic studies, at given

incubation times, a part of the reaction mixture was withdrawn and diluted in a microplate well in presence of a SA-XL665 conjugate. The enzymatically incorporated K-dU units brought in the nearby of the acceptor itself bound to the biotinylated strand give rise to resonance energy transfer (Fig. 2). Due to the high concentration of K-dUTP present in the medium the 620 nm signal (F620) does not decrease significantly, the energy transfer is evaluated by the 665 nm acceptor signal (F665). A blank from a reaction mixture without added enzyme was either diluted without adding SA-XL665 referred as "cryptate blank" used to compute Ro used below or diluted in presence of SA-XL665 ("XL665 blank") to check the level of dynamic transfer. For each well the ratio R = F665/F620 was computed, the extent of energy transfer was evaluated by the relative corrected ratio (ΔF) defined as ΔF = (R – Ro)/Ro in percent. The dynamic transfer is directly dependent on the acceptor concentration and on the mutual diffusion factor of the donor-acceptor couple (which is decreasing with the molecular weight). The low final concentration (nanomolar range) and the high molecular weight for streptavidin-allophycocyanine conjugate used here should ensure a low background. For instance the assays for the incorporation of K-11-dUTP gave a dynamic transfer ΔFo which in average ranged from 3% to 7%.

Influence of the K-dUTP/ dTTP ratio on the energy transfer signal.

Kinetics were carried-out using K-11-dUTP alone and with a K-dUTP/ dTTP ratio of 1/1 and 1/4. After 40 mn reaction time the ΔF values (duplicates) were respectively of 116%, 174% and 406% (after 80mn the ΔF was 900% in the latter condition). The lower ΔF value observed for the higher K-11-dUTP concentration (6 μM) can be explained by an inhibition of the enzyme occurring at this K-11-dUTP concentration. Such phenomenon was reported in the incorporation of DNP-11-dUTP [21] and was also observed in the *Taq* polymerase assay described bellow if concentrations above 30 μM are used. Elimination of the excess of K-dUTP from the reaction mixture (1/4 ratio) by size exclusion on sephadex and measuring the energy transfer as described gave a ΔF value above 3000 %. The separation of the cryptate labeled primer followed by F620 measurement showed that, in average, one cryptate residue was incorporated in the primer under the reported condition. As comparison, using similar dTTP concentration and α-[^{32}P]-dTTP labeling, a similar incorporation was observed using reference method[15]. For analytical purpose using TRACE a micromolar range and a K-dUTP/ dTTP ratio of 1/4 or eventually less would be preferred.

DNA polymerase extension assays.

The copy of a single stranded DNA by extension of a ^{32}P labeled primer in presence of K-dUTP and either DNA polymerase (Klenow fragment, 37°C, 45 mn) or *Taq* DNA polymerase (70°C, 15 mn) gave essentially the same results. The full extension product had an electrophoretic mobility close to the reference obtained with the four natural dNTPs and no pausing was observed. The single stranded template, a fragment from the K-*ras* gene, was obtained by PCR (biotinylated primer), captured on avidin-dynabead followed by alkaline denaturation.

DNA polymerase extension assay using TRACE.

This is a semi-direct assay using a 5'-biotin-ODN primer and a single stranded DNA as template depicted in fig. 2. The K-dU units incorporated in the complementary strand primed by the 5'-biotin-ODN will be localized at proximity of the biotin residue, after addition of SA-XL665 energy transfer will occur between cryptate and XL-665. The kinetic measurement of the K-dUTP incorporation with *Taq* DNA polymerase was accomplished essentially as described above for TdT. The ΔF values were computed as above and plotted against reaction time. The ΔF value was increasing with reaction time showing the incorporation of K-dU residues in the growing strand. The kinetics had qualitatively the feature of such enzymatic elongation monitored by the incorporation of α[^{32}P]-dTTP into acid precipitable product as reference method. Nevertheless, since the contribution of the incorporated K-dU units remote from the primer would give less energy transfer (the transfer rate vary as $1/d^6$, d being the distance between donor and acceptor) the kinetics displayed a plateau (for the ^{32}P assay and copy of a long template, this plateau arises much latter). Again in this assay, K-11-dUTP can be used in admixture with dTTP in order to limit its consumption. In summary this assay showed that K-dUTP was efficiently incorporated into DNA. The applications are twofold: the design of a simple homogeneous non-radioactive polymerase assay without the need of any separation as electrophoresis and a simple direct labeling of a DNA probe with a long lived fluorophore.

CONCLUSION

An europium cryptate labeled dUTP analog (K-11-dUTP) has been synthesized. It was shown to be a good substrate for polymerases and could be used in the preparation of

probes labeled with luminescent cryptate as reporter. The incorporation of this labeled analog could be followed by TRACE method as an alternative for the classical radioactive determination of a polymerase activity. The distance at which the FRET is 50% efficient is defined by the Förster radius (R_o), for the europium cryptate / XL665 couple used here R_o is in the 90 Å range which is substentialy higher than in FRET using conventional dyes[8] (R_o = 55 Å for fluorescein/tetramethylrhodamine). This interesting feature bring more flexibility in the design of TRACE assays using nucleic acids and this methodology can very well be expended to detect other enzymatic activity concerning nucleic acids as substrate and be used for the biomolecular screening towards DNA protein interaction. The protocol described herein allowed for the first time the direct incorporation into DNA of a lanthanide complex showing resistance to *Taq* polymerase conditions and high EDTA concentrations. Moreover the resistance of lanthanides cryptates to extreme acidic and alkaline conditions illustrates their versatility. The incorporation of K-11-dUTP, particularly using *Taq* DNA polymerase opens new possibilities in the design of bioassays. Furthermore TR-FRET techniques in association with a suitable equipment allowing on-line lifetime measurement and signal processing, would allow a better use of the time domain and extract more data from the assays.

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