A homogeneous europium cryptate-based assay for the diagnosis of mutations by time-resolved fluorescence resonance energy transfer

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ABSTRACT

Oligonucleotide ligation assay (OLA) is considered to be a very useful methodology for the detection and characterization of mutations, particularly for clinical purposes. The fluorescence resonance energy transfer between a fluorescent donor and a suitable fluorophore as acceptor has been applied in the past to several scientific fields. This technique is well adapted to nucleic acid analysis such as DNA sequencing, DNA hybridization and polymerase chain reaction. We describe here a homogeneous format based on the use of a rare earth cryptate label as donor: tris-bipyridine-Eu^{3+}. The long-lived fluorescence of this label makes it possible to reach a high sensitivity by using a time-resolved detection mode. A non-radiative energy transfer technology, known as time-resolved amplification of cryptate emission (TRACE®) characterized by a temporal and spectral selectivity has been developed. The TRACE® detection of characterized single nucleotide polymorphism using the OLA for allelic discrimination is proposed. We demonstrate the potentialities of this OLA–TRACE® methodology through the analysis of K-ras oncogene point mutations.

INTRODUCTION

The development of methods to detect single nucleotide polymorphisms (SNPs) such as point mutations has been rapid over recent years, especially since the advent of the PCR technique (1). Mutation scanning assays (2–5) allow the detection of unknown alterations, while known mutations are analyzed by mutation diagnosis techniques. Methodologies for the detection of specific mutations in nucleotide sequence at one or a few sites include allele-specific PCR (6), primer-introduced restriction analysis (7), mini-sequencing (8), 5′ nuclease assay (9), allele-specific oligonucleotide hybridization (ASO) (10) and oligonucleotide ligation assay (OLA) (11). Nevertheless, the last three techniques are the most suitable for high throughput analysis. In the OLA, allele discrimination is based on the ability of the enzyme DNA ligase to covalently link two immediately adjacent oligodeoxynucleotides (ODNs) on a target DNA molecule, while the ligation fails to occur if a mismatch is present at the junction of the two ODNs. Moreover, the development of new thermostable DNA ligases (12,13) allows an increase of both sensitivity and specificity. Detection of ligation products may be carried out on solid phase by using an ELISA colorimetric assay (14) or a separation by gel electrophoresis (15). The major drawback for the aforementioned methodologies is the requirement for several steps of sample purification and separation before the final detection. In order to circumvent these problems, homogeneous reaction formats have been developed using fluorescence resonance energy transfer (FRET) as the main detection method. FRET between an excitated fluorescent donor and an acceptor molecule is observed when two fluorophores are in close proximity and when the donor emission spectrum overlaps the acceptor excitation spectrum (16). This phenomenon has been applied to a variety of scientific challenges (17) including protein studies, analysis of cell surface distribution, immunoassays and nucleic acids analysis. The combination of a homogeneous test with FRET as a detection tool for the analysis of single nucleotide variations is exemplified by molecular beacons (18), the Taqman technique (19) and the dye-labeled oligonucleotide ligation assay (20). All these approaches are based on conventional fluorophores such as Cy5, rhodamine derivatives (ROX, 6-carboxyl-X-rhodamine; TAMRA, *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine) and fluorescein derivative (FAM, 5′-carboxyfluorescein). The FRET between these short-lived fluorophores does not make it possible to reach the sensitivity level of assays based on time-resolved FRET (TR-FRET) using fluorescent rare earth chelates as donors. Nevertheless, the poor stability of lanthanide chelates, considering the high temperature and the presence of competiting ions or ligands, has prompted the development of new rare earth complexes such as lanthanide cryptates (21).

Time-resolved amplification of cryptate emission (TRACE®) technology that uses a europium cryptate structure ($Eu^{3+} \subset$ tris-bipyridine) as donor has been applied in homogeneous assay formats characterized by both a spectral and a temporal selectivity (22). Applications of this new fluorophore, symbolized as [TBP(Eu^{3+})], and technology include immunoassays (22–24), biomolecule interaction analysis (25), studies on enzymatic

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activities (26) and the field of nucleic acids (27–29). Also, we have previously shown the high sensitivity of time-resolved fluorescence with [TBP(Eu^{3+})] by detecting nucleic acid hybridizations on microtiter plates or membranes as solid supports (30,31).

In this report we describe two new homogeneous assays, a semi-direct and a direct format, combining oligodeoxynucleotide ligation with TRACE® for the detection of SNPs. As acceptors matching the [TBP(Eu^{3+})] spectral features, two fluorophores were evaluated: a modified allophycocyanine (XL665) emitting at 665 nm and the conventional organic fluorophore Cy5. The feasability of such assays was exemplified by using K-ras point mutation detection as a model. Parameters allowing the discrimination between mutant and wild-type DNA were studied on human cell lines. The usefulness of this diagnostic method was then demonstrated on DNA from colorectal cancer patients.

MATERIALS AND METHODS

Cell lines and tumor samples

Lymph node metastasis of colon adenocarcinoma cell line SW620 [homozygous, GTT (Val)], pancreatic carcinoma cell line MIA PaCa-2 [homozygous, TGT (Cys)], lung carcinoma cell line A549 [homozygous, AGT (Ser)], colon carcinoma cell lines LS174T [heterozygous, GG/AT (Asp)] and SW1116 [heterozygous GG/CT (Ala)] displaying a mutation on the K-ras codon 12 sequence and the lymph node metastasis of prostate carcinoma cell line LNCaP [wild-type K-ras codon 12 sequence, GGT (Gly)], were purchased from the American Type Culture Collection (Rockville, MD). Tumor samples were obtained during surgery from patients with colorectal cancer (CRLC Val d'Aurelle, Montpellier, France) (32). Fresh samples were immediately frozen in liquid nitrogen before subsequent nucleic acid extraction.

DNA isolation

High molecular weight DNA from the cell lines and frozen biopsies was prepared by standard proteinase K digestion and phenol–chloroform extraction (33).

Synthesis of DNA sequences with base-specific K-ras mutations

Seven synthetic DNAs corresponding to all possible K-ras codon 12 alleles were prepared as previously described (32) and their genotypes checked by direct DNA sequencing.

DNA sequencing

Sequence determination was done on amplified (MD1: 5′-GTACTGGTCGAGTATTTGAT, MD2: 5′-GTCCTCCAC-GAGTAATATGC) DNA by using [α-^{33}P]dATP and P3ras1 (5′-GGCCTGCTGAAAATGACTGAATAT) as sequencing primer in the dideoxy chain termination method.

Oligodeoxynucleotides

The ODN primers, ligation and hybridization probes were synthesized on an Applied Biosystems 381 A DNA synthesizer and purified by reversed-phase HPLC (CIS Bio International, Gif-sur-Yvette, France). Cy5-ODN was purchased from Genset (France). The sequences of ODNs for the ligation assay are listed in Table 1. The common ligation probes were 5′-phosphorylated and possessed an aminohexyl linker group at the 3′-end for the subsequent [TPB(Eu^{3+})] labeling. The labeling of ODNs with [TPB(Eu^{3+})] was performed using [TPB(Eu^{3+})]–NHS ester as previously described (28). Briefly, 10 nmol of ODN in 0.125 M borate buffer pH 8 was reacted with 130 nmol of [TPB(Eu^{3+})]–NHS ester in acetonitrile. After 15 min incubation at room temperature, the conjugate was desalted through the use of a NAP-5 column (Amersham Pharmacia Biotech, Orsay, France) and 25 mM triethyl ammonium acetate pH 7, as elution buffer. The ODN–[TPB(Eu^{3+})] was then purified by anion exchange chromatography on Mono Q HR 5/5 (Amersham Pharmacia Biotech), 1 ml/min. A gradient of 0–20% buffer B in 2 min, 20–60% buffer B in 28 min then 60–100% buffer B in 5 min was used. The compositions of the buffers were: buffer A, 20 mM sodium acetate pH 5, 10% CH_3CN; buffer B, 20 mM sodium acetate pH 7, 10% CH_3CN, 1 M LiCl. The relevant fractions were collected and reduced to 200 µl, then the ODN conjugate was desalted on a NAP-5 column equilibrated in 25 mM triethyl-ammonium acetate pH 7. The labeled ODNs were stored frozen until use. In order to calculate the concentration and the labeling ratio of the conjugates we took into account the contribution at 260 nm from the [TPB(Eu^{3+})] absorption ($\varepsilon_{260\ nm}$ = 17 000 $M^{-1}cm^{-1}$), we assumed that the absorption of [TPB(Eu^{3+})] and ODNs were unchanged after coupling and also that the absorption of ODNs at 305 nm was negligible. The [TPB(Eu^{3+})]–ODN conjugates were further characterized by their fluorescence lifetime measured on a LS50 spectrofluorimeter (Perkin-Elmer, Foster City, CA).

Homogeneous hybridization assays

Hybridization reactions were carried out directly in black 96-well microtiter plates (HTRF plates, Packard, Torrance, CA) in a final volume of 200 µl in a 100 mM phosphate, 0.1% BSA, 1 M NaCl buffer by just mixing 800 fmol of each ODN. The hybridization between a [TBP(Eu^{3+})]–ODN and the 5′-biotinylated 43mer ODN: SG0 (5′ bio-GCTGTATCGTCAAGG-CACTCTTGCCTACGCCACCAGCTCCAAC) was monitored by the addition of 800 fmol of Streptavidin-Cy5 (SA-Cy5) (Amersham Pharmacia Biotech) or Streptavidin-XL665 (SA-XL665) (CIS Bio International). Reactions were performed at 37°C, then the FRET was directly measured after different incubation times.

Polymerase chain reactions

The DNA for analysis was amplified by PCR using P3ras1 and P3ras2 (5′-TGTTGGATCATATTCGTCCACAAAATG) (12 pmol each) as primers to generate a 117 bp product. The PCR was carried out in a total volume of 50 µl containing 250 ng of extracted DNA, 200 µM dNTP, 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl. After a 10 min denaturation step, 2 U of *Taq* DNA polymerase (Life Technologies, Cergy Pontoise, France) was added under hot start conditions and amplification was carried out in a Biometra TGradient thermocycler (Göttingen, Germany) with 32 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 30 s) and a 3 min at 72°C final extension step.

Ligation reactions

Ligation reactions were performed in a final volume of 45 µl containing a fraction of the K-ras exon 1 PCR product, 5 U *Taq* DNA ligase (New England Biolabs, Beverly, MA), 800 fmol of [TBP(Eu^{3+})]-labeled common probe and 800 fmol of allele-specific probe 5′-labeled either with biotin for the semi-direct detection via a streptavidin conjugate (SA-XL665 or SA-Cy5) or with Cy5 for the direct detection. Reactions in the ligation buffer (20 mM Tris–HCl, 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 1 mM NAD, 0.1% Triton X-100; pH 7.6) were processed on the Biometra TGradient apparatus for 12 cycles consisting of 93°C for 30 s and 1 min 30 s at the optimal hybridization–ligation temperature. After cycling, 5 µl of 0.1 M EDTA was added in order to stop the enzymatic reaction. For each PCR product tested a negative OLA control containing all the constituents of an assay but without the *Taq* DNA ligase was made.

TRACE® detection

The semi-direct format. The 50 µl of the ligation reactions was transferred to black 96-well microtiter plates. For the semi-direct format using 5′-biotinylated allele-specific probes, 800 fmol of SA-Cy5 or SA-XL665 conjugate dissolved in reading buffer (50 mM HEPES pH 7.4, 0.1% BSA and 50 mM KF) was added to the ligation products and the streptavidin–biotin recognition reaction was carried out for 1 h at 37°C before measurement at room temperature. For the direct format using 5′-Cy5-labeled allele-specific ODNs, the FRET was directly measured after addition of the reading buffer. All measurements were achieved in a final volume of 200 µl. For each series of analyses a ‘cryptate blank’ performed as an assay but omitting the addition of the streptavidin conjugate (semi-direct format) or the Cy5-ODN (direct format) was prepared. The blanks, negatives and assays were made in duplicate.

For each well, fluorescence emission was measured on a dual wavelength (620/665 nm) time-resolved fluorimeter equipped with a nitrogen laser as excitation source (337 nm) (Discovery, Packard, Torrance, CA) with the following gate parameters: 620 nm channel: delay = 200 µs, width = 400 µs; 665 nm channel: delay = 50 µs, width = 400 µs. The ratio R = (fluorescence 665 nm/fluorescence 620 nm) × 10^4 was computed (22,23). The relative corrected ratio $\Delta F = (R - R_{neg}/R_{neg})$ was then calculated, where R_{neg} corresponded to the ratio for the negative OLA control.

RESULTS AND DISCUSSION

The developed homogeneous 96-well assays based on TRACE® technology for the diagnosis of point mutations are described in Figure 1. The method used two sets of ODN probes (common probes, allele-specific probes) that hybridized to adjacent sequences on the DNA target. Probes were ligated by a thermostable DNA ligase if there was no mismatch at their junction. FRET between the two fluorophores will then occur only when the common probe and the allele-specific probe are in close proximity, i.e. after ligation. Two formats have been developed, a direct assay and a semi-direct assay, the latter involving the bioanalytical system Streptavidin/Biotin. The semi-direct format presents several advantages: (i) it allows the use of a generic acceptor conjugate common to all the allele-specific probes, (ii) it brings flexibility since several dye-acceptors may be tested for the detection of the same ligation product, (iii) a multi-labeling of the streptavidin by the acceptor may be reached thus increasing the detection sensitivity, and (iv) the steric bulk of the conjugate may contribute to a close proximity of donor and acceptor. The direct format based on acceptor-labeled ODN is of particular interest for an adaptation to high throughput and automation.

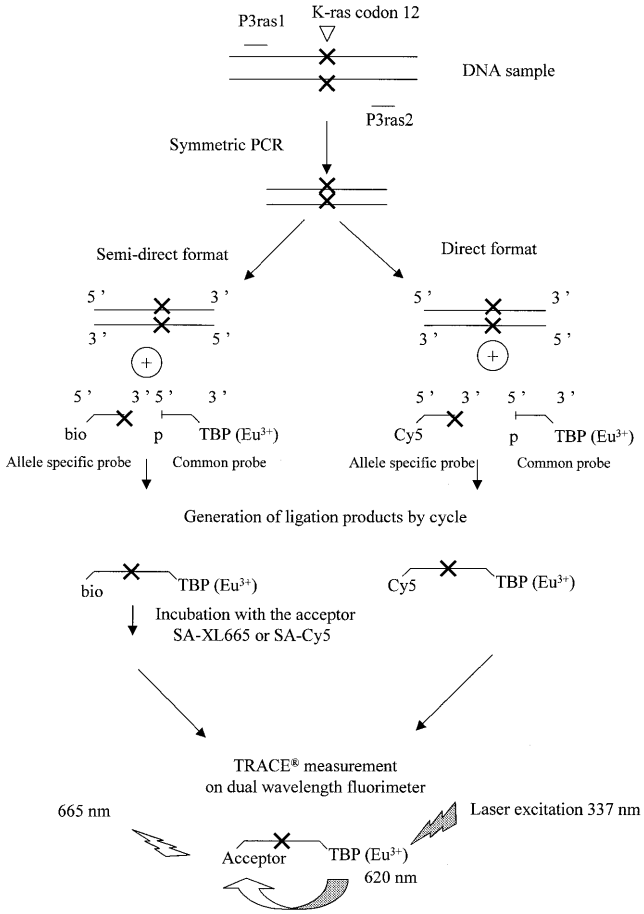

Figure 1. OLA with TRACE® detection. A symmetric PCR encompassing the bases to analyze was made. In the semi-direct format a common probe: 5′-phosphorylated (p) and 3′-[TBP(Eu^{3+})]-labeled and an allele-specific probe: 5′-biotinylated (bio) containing at its 3′-end the specific base, were reacted with the PCR products in a cycling mode. Perfect hybrids (allele-specific probe: PCR product) allow the generation of a ligation product 5′-biotinylated and 3′-[TBP(Eu^{3+})]-labeled. After the addition of the streptavidin conjugate (SA-XL665 or SA-Cy5), fluorescence energy transfer was monitored on a dual wavelength fluorimeter. In the direct format, the allele-specific probe was 5′-Cy5-labeled, then after the cycles of hybridization/ligation, the products were directly analyzed.

Lanthanide cryptates such as [TBP(Eu^{3+})] (Fig. 2) (22) are characterized by (i) kinetic and thermodynamic stabilities allowing a robust assay, (ii) long-lived emission (~1 ms) compatible with a time-resolved mode for reduced background, and (iii) narrow line emission spectra limiting the cross-talk with acceptor emission, and are therefore well suited donors for FRET detection. An optimal acceptor must fulfill several criteria such as compatibility with cryptate fluorescence emission, ease of coupling and low dynamic energy transfer

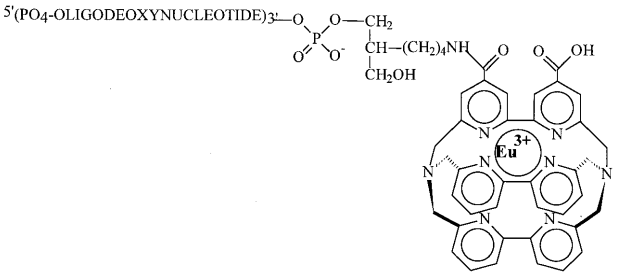

Figure 2. Chemical structure of [TBP(Eu^{3+})]-labeled common probes.

ensuring low background. The dynamic energy transfer arises between donor and acceptor which are free in solution and is related to the size and the concentration of the acceptor (34). Among the potential acceptors a large protein and a small organic dye were selected. A chemically crosslinked trimeric allophycocyanine (XL665), stable even at low concentrations (35), was first used. Then, Cy5, a cyanine structure available for the direct labeling of ODNs and as streptavidin conjugate was studied. It should be mentioned that XL665, unlike Cy5, displays photophysical properties independent of the medium and environment (36).

[TBP(Eu^{3+})]–ODN labeling

Three common probes KRCPA1 (20- and 15-base-long) and KRCPA2 (20-base-long) were synthesized in order to detect mutations of the first or the second base of the K-ras codon 12 sequence (GGT), respectively. These probes were 5′-phosphorylated, a prerequisite for the formation of a phosphodioester bond with the 3′-OH end of the allele-specific probes in the ligation process. A covalent attachment of [TBP(Eu^{3+})] to ODN was performed (Fig. 2). Starting with a 10-fold molar excess of [TBP(Eu^{3+})]–NHS per ODN molecule, the final labeling ratio, calculated using the optical densities at 305 nm {maximum absorption of [TBP(Eu^{3+})]} and 260 nm as aforementioned, was roughly equivalent to one molecule of label per molecule of ODN, demonstrating the generation of a [TBP(Eu^{3+})]–ODN free of unbound fluorophore. On HPLC (data not shown), the conjugates showed a unique peak [retention time (Rt) ≈ 22 min for the 20-base-long ODNs, Rt ≈ 18.5 min for the 15-base-long ODN] eluting slightly faster than the corresponding unlabeled ODN. The yield for the different [TBP(Eu^{3+})]–ODN was ~40%, with minimal variations between batches. The coupling process does not affect the major characteristics of [TBP(Eu^{3+})] and the fluorescence lifetime of the conjugate measured in 50 mM HEPES pH 7.4, 0.1% BSA and 50 mM KF buffer was 1.19 ms.

In order to evaluate the transfer efficiency for the various [TBP(Eu^{3+})]–ODN probes (Table 1), homogeneous liquid phase hybridizations (Table 2) were performed at 37°C with the synthetic 43-base-long and 5′-biotinylated ODN (SG0), containing the whole sequence complementary to the full-length ligation product, in the presence of SA-XL665. Simultaneously, a negative control was made omitting the SG0 ODN. The ΔF value after 1 h incubation, for KRCPA1–20 (analysing the first base of the codon 12) and KRCPA2–20 (analysing the second base of the codon 12), both 20-base-long, were in the same range for the two labeled ODNs, 809% ± 11.4% and 750% ± 9.1% of ΔF respectively. The same experiment, carried out with the KRCPA1–15, 15-base-long, gave a ΔF value of 252% ± 3.2% after 1 h incubation at 37°C, while this value reached 721% ± 11.9% after 4 h. Moreover, a direct correlation was observed for the different hybrids (Table 2) between the hybridization time, temperature and the ΔF value. It is worthwhile noticing that the dynamic transfer between the [TBP(Eu^{3+})] and SA-XL665 was negligible [ratio R(665/620) ~950], which ensures a low background.

Table 1. Oligonucleotide ligation set for detecting K-ras codon 12 mutations

Name	Sequence (5′→3′)
Common probes	
First base of codon 12	
KRCPA1–20	p-**GT**G GCG TAG GCA AGA GTG CC-K
KRCPA1–15	p-**GT**G GCG TAG GCA AGA-K
Second base of codon 12	
KRCPA2–20	p-**T**GG CGT AGG CAA GAG TGC CT-K
Allele-specific probes	
First base of codon 12	
Wild-type (Gly)	
RWB1–20	bio-ACT TGT GGT AGT TGG AGC T**G**
RWB1–15	bio-TGG TAG TTG GAG CT**G**
RWCy5–20	Cy5-ACT TGT GGT AGT TGG AGC T**G**
RWCy5–15	Cy5-TGG TAG TTG GAG CT**G**
G→A mutation (Ser)	
RM1B	bio-ACT TGT GGT AGT TGG AGC T**A**
G→C mutation (Arg)	
RM2B	bio-ACT TGT GGT AGT TGG AGC T**C**
G→T mutation (Cys)	
RM3B–20	bio-ACT TGT GGT AGT TGG AGC T**T**
RM3B–15	bio-TGG TAG TTG GAG CT**T**
Second base of codon 12	
Wild-type	
RWB2	bio-CTT GTG GTA GTT GGA GCT **GG**
G→A mutation (Asp)	
RM4B	bio-CTT GTG GTA GTT GGA GCT **GA**
G→C mutation (Ala)	
RM5B	bio-CTT GTG GTA GTT GGA GCT **GC**
G→T mutation (Val)	
RM6B	bio-CTT GTG GTA GTT GGA GCT **GT**

p = phosphate, K = [TBP(Eu^{3+})]. Bold letters indicate the bases belonging to the codon 12.

Optimization of OLA-TRACE®

The critical parameters of the assay were carefully optimized by carrying out experiments on genomic DNAs extracted from characterized cell lines. LNCaP and Mia PaCa-2 displaying respectively the wild-type K-ras codon 12 sequence and a

Table 2. Analysis of [TBP(Eu^{3+})]-labeled ODN by FRET after homogeneous liquid phase hybridization

Hybrid	Reaction temperature (°C)	Delta F (%) after indicated reaction times[d]				
		1 h	1 h 30 min	2 h	3 h	4 h
SG0/KRCPA1–20[a]	4	292 (5)	365 (2.6)	426 (4.1)	486 (2.8)	625 (5.7)
	24	424 (13.1)	574 (7)	685 (2)	818 (8.2)	1007 (12.6)
	37	809 (11.4)	954 (5.1)	1084 (12.7)	1258 (16.6)	1371 (2.3)
SG0/KRCPA2–20[b]	4	269 (1.3)	319 (2.8)	373 (1.2)	450 (0.3)	579 (5.5)
	24	394 (6.8)	543 (12.7)	654 (6.3)	805 (2.3)	960 (1.2)
	37	750 (9.1)	914 (5)	1009 (7.7)	1225 (12.6)	1417 (0.5)
SG0/KRCPA1–15[c]	4	26 (0.7)	36 (0.1)	39 (0.5)	47 (0.3)	63 (2.4)
	24	56 (1.6)	91 (3.4)	131 (0.7)	176 (1.1)	242 (10.9)
	37	252 (3.2)	332 (5.6)	421 (9.9)	562 (2.8)	721 (11.9)

[a]5′-bio-GCTGTATCGTCAAGGCACTCTTGCCTACGCCACCAGCTCCAAC-3′
3′-K-CCGTGAGAACGGATGCGGTG-5′
[b]5′-bio-GCTGTATCGTCAAGGCACTCTTGCCTACGCCACCAGCTCCAAC-3′
3′-K-TCCGTGAGAACGGATGCGGT-5′
[c]5′- bio-GCTGTATCGTCAAGGCACTCTTGCCTACGCCACCAGCTCCAAC-3′
3′-K-AGAACGGATGCGGTG-5′
[d]Each Delta F value represents the average of two consecutive experiments. Values in parentheses correspond to the standard deviations.

GGT→TGT homozygous mutation were used. The [TBP(Eu^{3+})] cryptate is compatible with thermostable enzyme operating conditions (28). The thermostable DNA ligase makes it possible to reach high specificity, perform temperature cycling which results in an increase of ligation products, and carry out analysis directly on small amounts of double-stranded PCR products. First, optimal hybridization/ligation temperature was determined by using 15-base-long ODNs: KRCPA1–15 common probe and RWB1–15 (GGT) allele-specific probe. The results in Figure 3 demonstrate that the best discrimination of 3′ mismatches was obtained at 54°C. Below this temperature a misligation was obtained with the MIA PaCa-2 cell line, and above 54°C, the specific signal (LNCaP cell line) dramatically decreased. At this optimal temperature the 665 nm-specific signal was high [9500 UF (units of fluorescence)]. This high transfer is corroborated by a simultaneous dropping of the 620 nm signal from 18 000 UF to 15 200 UF. Thus, the dual wavelength time-resolved mode measurement can make a clear discrimination between the signal of the acceptor in ligation products and the excess of acceptor label in solution. Moreover, compared to ligation using europium chelates (37) the developed homogeneous OLA–TRACE® methodology is more attractive since tedious steps of capture and washing are avoided.

Different ratios (ligation probes/PCR products) were used to determine optimal reaction conditions. Using 800 fmol of each allele-specific probe and common probe in combination with 250 fmol of amplification products, a clear discrimination was obtained between a full 3′ complementarity and a 1-bp mismatch.

In this assay, the length of the generated ligation product was 30 bases. The Förster radius (R_0) characterizes a donor/acceptor pair by the distance at which the energy transfer is 50% efficient. Since the R_0 value, ~90 Å for the [TBP(Eu^{3+})]/XL665 pair in phosphate buffer is very high (22), 20-base-long ODNs providing a 40-base-long ligation product could be tested. The temperature of ligation reactions was optimized as above for the 15-base-long ODNs. An increase from 54 to 60°C (data not shown) resulted in high specific ligation reactions. Using the allele-specific ligation probe WT (Gly) and a

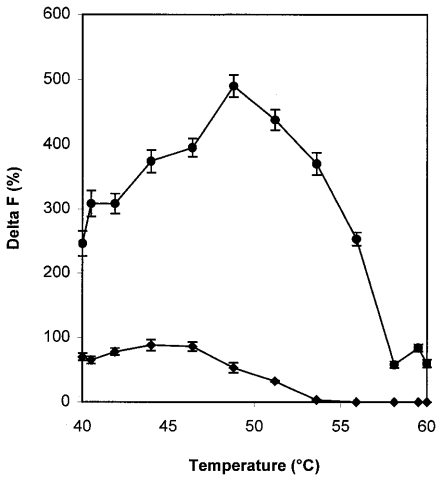

Figure 3. Temperature optimization of the OLA–TRACE®. OLA–TRACE® reactions were performed as described in Materials and Methods at various cycling temperatures by using 800 fmol of common [TBP(Eu^{3+})]-labeled probe (KRCPA1–15), 800 fmol of allele-specific probe (RWB1–15, wild-type GGT K-ras codon 12 sequence), 800 fmol of SA-XL665, and an aliquot of the PCR amplified cell line to analyze LnCaP (GGT, circles) or Mia PaCa-2 (TGT, diamonds).

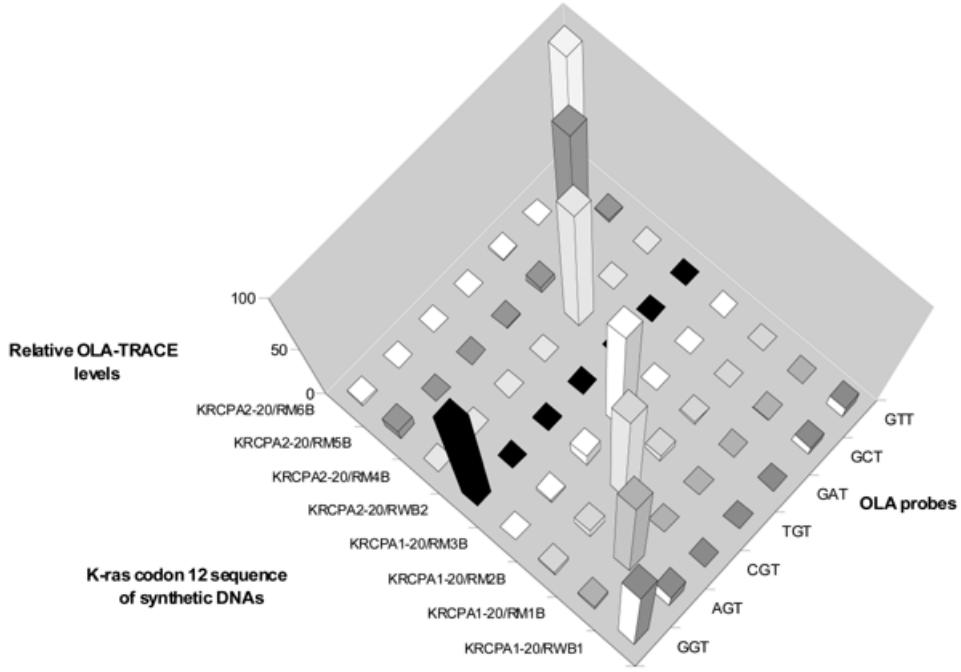

Figure 4. Specificity of the OLA–TRACE® technology by analyzing synthetic DNAs. Seven synthetic DNAs containing the seven K-ras codon 12 (GGT, GAT, GCT, GTT, GAT, GCT and GTT) possible sequences were analyzed at the same time by eight pairs of 20-base-long OLA probes in the semi-direct format. The results represent the mean of two consecutive assays. The relative Delta F value 100 was assigned to the reaction between full complementary sequences.

Table 3. Comparison of OLA–TRACE® measurements by using 20mer or 15mer-long OLA probes

ODN length		LnCaP (665/620) ratio	LnCaP Delta F	Mia PaCa-2 (665/620) ratio	Mia PaCa-2 Delta F
20[a]	Assay	6416	439% ± 7.4%	1207	5.3% ± 0.17%
	Negative	1190		1146	
15[b]	Assay	6420	379% ± 3.2%	1200	7.4% ± 0.26%
	Negative	1339		1117	

The results represent the mean of two consecutive experiments.
[a]KRCPA1–20 (common probe)/RWB1–20 (allele-specific probe).
[b]KRCPA1–15 (common probe)/RWB1–15 (allele-specific probe).
The negative ligation reaction control contains all the components excluding the *Taq* DNA ligase. The 665/620 ratio for the blank cryptate is 850.

wild-type PCR product (LnCaP cell line) the same range of FRET was obtained for the 20-base-long ODN (KRCPA1–20) and 15-base-long ODN (KRCPA1–15) as shown in Table 3. The absence of a non-specific signal for the assay with Mia PaCa2 cell line should be noticed. The equimolar amount for streptavidin conjugate and biotinylated ODN gave the better FRET signal, therefore we used it in all subsequent experiments, while optimal and stable signals were obtained by taking measurements 1 h after the addition of the streptavidin conjugate.

Characterization of the semi-direct OLA–TRACE®

In order to assess the specificity of the developed OLA–TRACE® for the detection of point mutations, we studied seven synthetic DNAs, each carrying one of the seven possible K-ras codon 12 sequences: GGT, AGT, CGT, TGT, GAT, GCT or GTT. For each DNA to be analysed, eight OLAs were performed in the same way. The set of ODNs KRCPA1–20/RWB1, KRCPA1–20/RM1B, KRCPA1–20/RM2B and KRCPA1–20/RM3B were used for the analysis of the first base of the K-ras codon 12. The pairs: KRCPA2–20/RWB2, KRCPA2–20/RM4B, KRCPA2–20/RM5B and KRCPA2–20/RM6B interrogate the second base of the codon. It appears (Fig. 4) that the highest misligation effiencies were observed for the G.T mismatches. The relative OLA–TRACE® levels for the two combinations: synthetic DNA (AGT, K-ras codon 12 sequence)/RWB1 (allele-specific probe)/KRCPA1–20 (common probe), and synthetic DNA (CGT, K-ras codon 12

sequence)/RM3B (allele-specific probe)/KRCPA1–20 (common probe) were 17.2 and 12.3%, respectively. These relatively high values may be explained by the reported thermodynamic stability of the non-Watson–Crick base opposition G.T (38) corrolated to hydrogen bonding in the G.T wobble pair (39). Moreover, the nearest-neighbor contribution of the A.T base pair may favor a conformation allowing ligation. Nevertheless, the discrimination between perfectly matched hybrids was unequivocally performed and the two-step process separating amplification from allelic discrimination by ligation led to a reduced rate of false-positive reactions.

The sensitivity [(twice the mean of the ΔF value obtained for the analysis of the LNCaP cell line in the semi-direct OLA–TRACE® with the OLA probes KRCPA1–20 and RM3B) + (2SD)] of the assay was then tested by diluting DNA from the homozygous mutated Mia PaCa-2 cell line with increasing amounts of wild-type cell line LnCaP (Fig. 5). After mixing, DNA were amplified by symmetric PCR then analyzed by the OLA–TRACE® methodology. Two series of experiments were carried out; one series with the ODN set KRCPA1–20 common probe/RM3B-20 allele-specific probe and one series with the ODN pair KRCPA1–20 common probe/RWB1–20 allele-specific probe. Using the optimized parameters, the GGT(Gly)→TGT(Cys) mutation was reproducibly and unambiguously detected even when the mutated DNA represented <5% of the total DNA in reaction. The same sensitivity level was obtained with our previously reported mutation tube assay (32) based on the use of a solid support and radioactive detection, whereas the sensitivity of the traditional sequencing methods is usually 15%. Furthermore, OLA was carried out on PCR products obtained by using as DNA target an equal amount of the DNA from two cell lines (MIA PaCa-2 and LNCaP). In this case, the same ΔF values were found with the full complementary OLA probes.

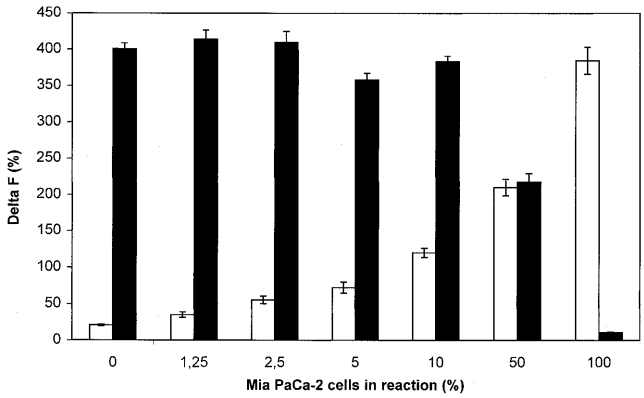

Figure 5. Sensitivity of detection of K-ras mutant alleles. DNA from Mia PaCa-2 (TGT) and LnCaP (GGT) cell lines were diluted and the OLA products which were generated by using the allele-specific TGT probe RM3B-20 (white columns) or the allele-specific GGT probe RWB1-20 (black columns), were analysed by TRACE®.

Analysis of DNA from colorectal patients

DNA samples from human colorectal cancer patients, with K-ras codon 12 genotype previously determined (32) by direct radioactive dideoxy sequencing, were analyzed by OLA–TRACE®. Each DNA was tested in seven OLAs by using one of the seven possible K-ras codon 12 allele-specific probes in combination with one of the two common [TBP(Eu^{3+})]-labeled probes. Table 4 shows typical results obtained by analysing five genomic DNAs. As K-ras is an oncogene, a maximum of

Table 4. Assay on genomic DNAs

Allele-Specific Probes		Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
		665/620	Delta F	665/620	Delta F	665/620	Delta F	665/620	Delta F	665/620	Delta F
GGT	Assay	5680	456%	5354	381%	5725	467%	6870	567%	5560	425%
	Negative	1080		1112		1009		1030		1058	
AGT	Assay	1452	10%	1365	9%	1261	10%	1256	10%	1246	8%
	Negative	1325		1254		1154		1140		1156	
CGT	Assay	1410	8%	1394	9%	1298	12%	1471	11%	1295	11%
	Negative	1313		1286		1162		1325		1169	
TGT	Assay	3158	181%	4850	282%	1213	6%	1228	7%	1384	11%
	Negative	1123		1268		1154		1156		1246	
GAT	Assay	1210	8%	1321	2%	3698	194%	1287	11%	1254	8%
	Negative	1125		1295		1259		1164		1169	
GCT	Assay	1236	6%	1298	12%	1356	6%	1398	12%	1256	8%
	Negative	1169		1163		1279		1256		1168	
GTT	Assay	1298	12%	1342	6%	1323	3%	1245	7%	4596	274%
	Negative	1157		1264		1294		1168		1230	
OLA-TRACE genotype		**G/TGT**		**G/TGT**		**GG/AT**		**GGT**		**GG/TT**	
Sequencing genotype		**G/TGT**		**G/TGT**		**GG/AT**		**GGT**		**GG/TT**	

Table 5. Comparison of OLA–TRACE® measurements by using Cy5 or XL665 as acceptor in the semi-direct format

Acceptor		LnCaP[a] (665/620) ratio	Delta F	Mia PaCa-2 (665/620) ratio	Delta F
Cy5[a]	Assay	6352	489% ± 14.1%	1190	10.2% ± 1.4%
	Negative	1078		1080	
XL665	Assay	6321	429% ± 11.2%	1167	9.9% ± 0.9%
	Negative	1194		1062	

[a]Allele-specfic probe = RWB1 wild-type (Gly).

Table 6. OLA–TRACE® measurements by using a direct format with 5′-Cy5-labeled ODNs

ODN length		LnCaP[a] (665/620) ratio	Delta F	Mia PaCa-2 (665/620) ratio	Delta F
15	Assay	5392	488% ± 10.2%	1044	4.4% ± 0.7%
	Negative	919		1000	
20	Assay	8519	600% ± 9.5%	1363	7.4% ± 1.4%
	Negative	1216		1269	

[a]Allele-specfic probe = RWCy5 wild-type (Gly).

50% of alleles can be mutated. Sample 4 displayed the wild-type K-ras codon 12 sequence since a high ΔF value was obtained with the wild-type allele-specific probe, while no other allele-specific probe gave a signal higher than the detection limit. Samples 1, 2, 3 and 5 were heterozygous. The variations in Delta F values for the different mutated alleles may be assigned to (i) the heterogeneity of the tumoral population and (ii) the contamination of tumoral cells by surrounding normal cells. Moreover, the genotypes of the 50 patients scored by the developed methodology were in perfect agreement with the reference DNA sequencing results and all calculated coefficients of variation were <5%.

Cy5 as acceptor

The photostability of Cy5 derivates is comparable to, or somewhat better than that of fluorescein, and this labeling reagent may be considered as an easy-to-use alternative to allophycocyanine. FRET intensity is highly dependent on the spacing between the two fluorophores involved. The spectral characteristics of the Cy5 dye match the [TBP(Eu^{3+})] emission. Particularly, the R_0 for this pair, ~64 Å (E.Trinquet, personal communication), is compatible with their use in a semi-direct or direct format. In comparison, the characteristic Förster length is 56 Å for the fluorescein/Cy3 pair and 50 Å for fluorescein/rhodamine. First we compared the XL665 and Cy5 acceptors through the streptavidin/biotin bioanalytical system. Twenty-base-long ligation probes with a wild-type allele-specific probe (Gly) were used on both wild-type (LnCaP) and mutated (Mia PaCa-2) cell lines. Despite differences in molecular weight, absorption coefficient and number of dye per streptavidin between the two tested molecules, the results in Table 5 demonstrate the potential of Cy5 as acceptor. In this semi-direct format, TRACE® measurements based on the [TBP(Eu^{3+})]/XL665 pair were insensitive to the buffer composition or pH and similar OLA–TRACE® results (data not shown) were obtained with various reading buffer such as 50 mM phosphate, 0.1% BSA, 50 mM KF, pH 7; 50 mM HEPES, 0.1% BSA, 50 mM KF, pH 5.4; or 50 mM Tris, 0.1% BSA, 50 mM KF, pH 7.7. On the contrary, TRACE® signals obtained by using the SA-Cy5 conjugate as acceptor were dependent on the measurement buffer and a 50% decrease of Delta F values was observed when using the above phosphate or Tris buffer instead of HEPES. These results may be assigned to the pH sensitivity of the Cy5 molecule. Moreover, as Cy5 may be easily incorporated into ODNs a direct format has been devised. Sets of 20mers or 15mers with a 5′-Cy5 allele-specific probe WT (Gly) were used for the analysis of ligation products. Table 6 demonstrates that the direct format allows the analysis of the ligation product by TRACE®, moreover the 20-base-long ODNs gave significantly better results than 15-base-long ODNs. These data may be related to a more efficient synthesis of ligation products when using longer ODNs. However, a special folding of the 40-base-long product resulting in a higher proximity between donor and acceptor may not be excluded. Compared to the semi-direct format, the latter developed assay allows the measurements of OLA products directly after cycling without any tedious manipulations. Nevertheless, using 5′-Cy5-labeled ODN no FRET was obtained when replacing the HEPES reading buffer by the aforementioned phosphate buffer or Tris buffer. In the TRACE® methodology the FRET is associated with an enhancement of the 665 nm signal

coming from the acceptor while the 620 nm signal emitted by the [TBP(Eu^{3+})] decreases. In the experiments using the direct format a 15% decrease of the 620 nm was observed after ligation in HEPES buffer. The same level of decrease was obtained whatever the media tested (phosphate or Tris-based buffer). These results demonstrate that an efficient transfer between [TBP(Eu^{3+})] and Cy5 occurs regardless of the buffer composition. It has been demonstrated that Cy5 conjugation to proteins is difficult to achieve without decrease of quantum yield and that the fluorescence efficiency of a Cy5 conjugate depends on the protocol and final Cy5/protein ratio (40). So, it may be assumed that the absence of 665 nm signal increase after ligation in phosphate or Tris buffer is related to a Cy5 emission quenching. The differences in the behaviour of Cy5 can be correlated to the differences in the chemical structures of the Cy5 used for the protein (sulfonated Cy5) and the ODN (Cy5 phosphoramidite) labeling.

CONCLUSION

A non-radioactive, homogeneous assay based on oligonucleotide ligation and TRACE® technology has been developed and applied to the detection of known SNPs. The main advantages of this methodology are (i) the absence of tedious steps of capture and washing before reading, (ii) the elimination of post-ligation processing, (iii) the sensitivity of detection of mutations in a large amount of wild-type sequence, (iv) the reduction in time required for sample analysis and (v) the high degree of discrimination for single base change. These characteristics, in conjunction with developed instrumentation, provide the basis for establishing fast, reliable and accurate formats suitable for use in routine diagnostic laboratories. Besides the OLA format described here, TRACE® technology could be applied to the diagnosis of point mutations based on different allelic discrimination principles such as mini-sequencing (8) or allele-specific PCR (6), for example.

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