Application Notes

Eu-labelled oligonucleotides are stable and sensitive as probes and primers

Simple and user friendly methods for detection of gene mutation or viruses in routine laboratories can be designed using Eu-labelled oligonucleotides. Dual or even triple-label assays can be set up using DELFIA® technology to include internal control for the sample or increase throughput of the assay. Streptavidin coated DELFIA plates as solid phase eliminate laborious sample preparation and electrophoresis procedures. Boiling is enough to prepare the sample for the assay.

The Eu-labelled oligonucleotides are stable during storage and in assay, an advantage when quality and economy are important. Labelling does not change their melting temperature or hybridization efficiency.

Table 1. Properties of a Eu labelled oligonucleotide probe (18-mer probe with 25 primary aminogroups).

Eu / oligo	Melting temperature, T 1/2	Hybridization efficiency
9	20	12 %
11	22	12 %
14	20	14 %
25	18	12 %

SIMPLE LABELLING PROCEDURE

A tail of fully protected primary aminogroups (15-25) is introduced in the 5' end of the oligonucleotide during the synthesis. After deprotection and purification of any free amino groups using standard methods the oligonucleotide is labelled with an isothiocyanate derivative of the Eu-chelate.

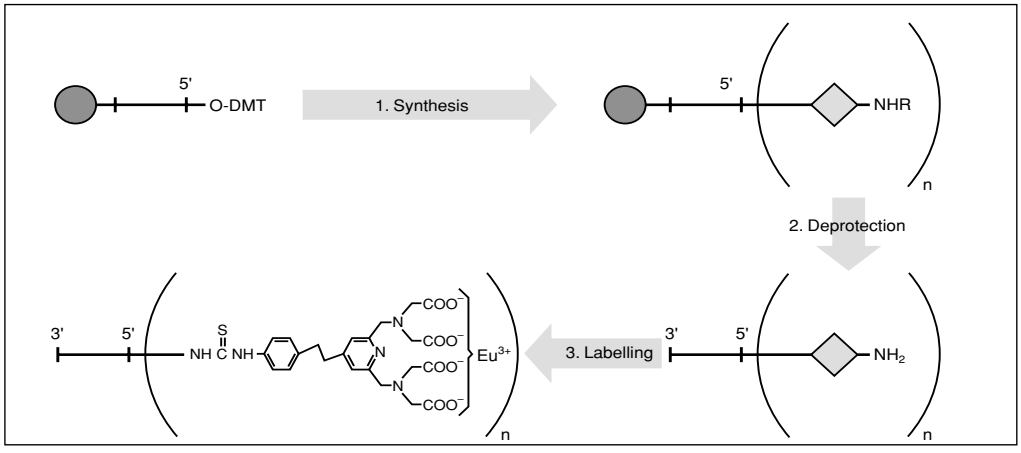

Fig. 1. Synthesis of Eu-labelled oligonucleotides.

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The reaction takes place in carbonate buffer, pH 9.5, overnight at room temperature. The labelled oligonucleotide is then purified using gel filtration (Sephadex G 50, 1 x 50 cm). Eu-labelling reagent (Product number C401-101) is commercially available and Sm- and Tb-labelled oligonucleotides can be ordered from Wallac's Labelling Service.

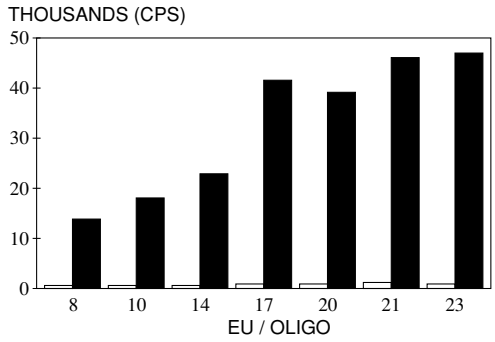


Fig. 2. Increased sensitivity in an assay by raising specific activity of the labelled probe.

Short oligonucleotides (< 20 nucleotides long) should not be labelled with more than 20 primary amino groups per oligonucleotide to avoid precipitation in the purification column. Longer oligonucleotides can be labelled with up to 25 primary amino groups to improve the specific activity of the probe.

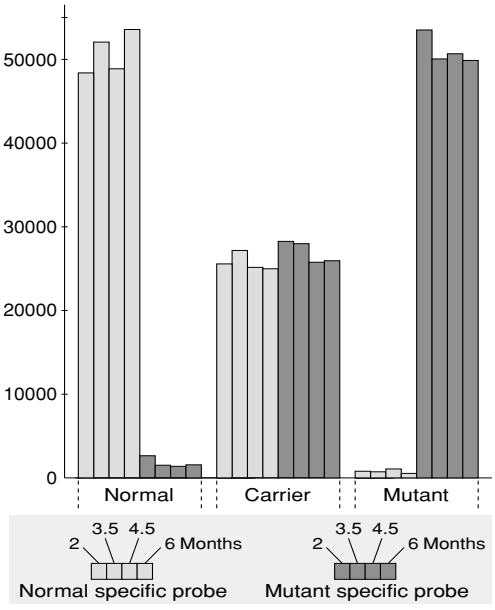


Fig. 3. Stability of Eu-labelled oligonucleotide probes stored at +4°C.

USE AS PROBES IN HYBRIDIZATION ASSAYS

Eu-labelled oligonucleotides have been applied in the diagnosis of genetic diseases, virus and oncogen detection. Simple tests have been developed for the detection of a single base change in the human genome using allele-specific oligonucleotides. Eu-labelled oligonucleotide has been used as a primer in a polymerase chain reaction (PCR*). The other primer was labelled with biotin, thus allowing direct collection of the amplified double- stranded, Eu-labelled target DNA (ref.3).

** Polymerase chain reaction (PCR) is covered by patents owned by Hoffman-La Roche Inc. If you are interested in performing PCR, please contact Hoffman-La Roche for information on obtaining an appropriate licence.*

TABLE 2.

Assay system	Probe	Target	Ref.
Modified dot blot	Eu-oligo	a1-antitrypsin deficiency	
	Eu/Sm-oligo	MCAD	
Solution hybridization	Eu-oligo	HIV 1	2
		HTLV I/II	9
		Adenovirus	4
		Rhino / enterovirus	
		α1-antitrypsin deficiency	1
		Philadelphia chromosome	
		Leber disease	5
	Eu- and Sm-oligo	ΔF508 mutation	7, 8, 11
	Eu-, Sm- and Tb-oligo	HLA alleles	13

Solution hybridization with Eu-labelled probes has been used mostly for analysis of PCR products. The nucleic acid sequences to be amplified should be ideally 100-150 base pairs long, i.e. the amplified region must be long enough for two oligonucleotides to hybridize without overlapping with the primers. In the detection of microbes the amplified region must be conserved to minimize the sequence variation in order to get reliable results. When mutations are to be detected, the position of the suspected mutation dictates the position of the amplified region. The Eu- (Sm- and Tb-) and biotin- labelled probes should have approximately the same melting temperatures to ensure maximum hybridization rates.

AN EXAMPLE OF THE USE OF EU-LABELLED PROBE FOR DETECTION OF ΔF508 MUTATION

1. The oligonucleotide having a tail of 25 primary amino groups is labelled with Eu-Oligo-labelling reagent using 20-fold excess over the amino groups following the instructions in the kit insert (product number C401-101). Gel filtration is used for purification of the labelled probe.

2. PCR of the sample is carried out according to standard procedures using 25 cycles. Boiling of dried blood spots is a practical sample preparation method for PCR when a large number of samples have to be handled (ref 7, 8).

3. Prepare two hybridization solutions. The final solutions contain 5 ng/ml biotinylated catching probe (mutant or normal specific) and 10 ng/ml detector probe in 0.5 mol/l NaCl, 25 mmol/l Tris-HCl, pH 7.8, 0.005% Tween- 40, 0.25% BSA, 0.023% bovine globulin, 10 µmol/l DTPA and 0.025% NaN_3. The hybridization solution can be very easily prepared by adding 2.92 g NaCl to 50 ml of DELFIA Assay Buffer (product number 1244-106).

4. Denature the samples 5 min. at 100° C using a heat block. Transfer the tubes onto ice immediately after boiling and incubate 5 min. Then centrifuge the samples and return to ice.

5. Pipette 10 µl of each oligonucleotide standard (normal, carrier, mutant) and PCR samples as four replicates into streptavidin coated microtitration wells (product number C122-105).

6. Add 100 µl of the normal specific hybridization solution to the first two wells and 100 µl of the deletion specific hybridization solution to the last two wells for all the samples.

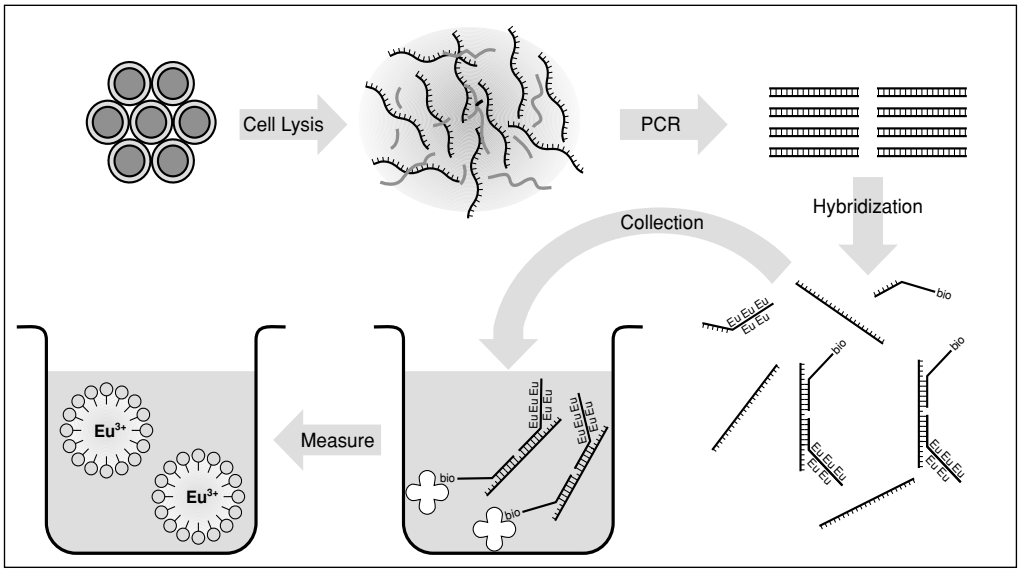


Fig. 4. DELFIA hybridization assay.

7. Cover the wells with a tape and incubate them at + 47° C for 2 hours.

8. Prepare DELFIA Wash Solution according to instructions and warm up the solution to + 50° C in a water bath for 2-3 hours.

9. Remove the cover tape of the microtitration plate. Rinse the Plate Wash with warm wash solution and immediately after that wash the plate 6 times.

10. Add 200 µl of Enhancement Solution to each well. Incubate on a DELFIA Plateshake 25 min. at room temperature using slow shaking. Count the samples using 1420 VICTOR^{2}$_{™}$ Multilabel counter.

Table 3. Easy to read results for detection of cystic fibrosis ΔF508 mutation using allele specific primers (m/m = mutant and n/n = normal allele, m/n = heterozygote).

no.	Patient	Normal specific primers/CPS	Deletion specific primers/CPS	Status
1	A 97	145990	211066	m/n
2	C 26	274029	937	n/n
3	A 117	215296	1369	n/n
4	C 25	222149	1508	n/n
5	A 104	73157	937	n/n
6	C 4	237470	1189	n/n
7	C 15	1265	231618	m/m
8	C 1	1370	272036	m/m
9	C 3	1313	269915	m/m
10	C 10	1010	206864	m/m
11	C 12	245529	273875	m/n
12	C 19	1008	285542	m/m
13	C 75	204067	288895	m/n
14	C 9	191198	170879	m/n
15	C 14	178051	239435	m/n
16	C 72	226912	289723	m/n
17	C 11	918	254797	m/m
18	C 86	245649	1319	n/n
Control DNA		65230	1564	n/n
Control DNA		51778	76079	m/n
Control DNA		1156	100794	m/m

MODIFIED DOT BLOT FOR THE ANALYSIS OF PCR PRODUCTS

A modified dot blot approach can be used to avoid reannealing of target DNA longer than 150 base pairs. In the assay, biotinylated target DNA is collected on a streptavidin-coated plate. After denaturation and washings Eu-labelled probe is allowed to hybridize to it.

1. PCR is run with two primers, one biotinylated, resulting in double-stranded PCR product.

2. Amplification product is allowed to attach to SA-plates.

3. Non-biotinylated strand is washed away with dilute alkali.

4. Hybridization with Eu-labelled probe. Stringent washes and detection.

SPECIFICITY AND THROUGHPUT USING MULTIPLE LABELS

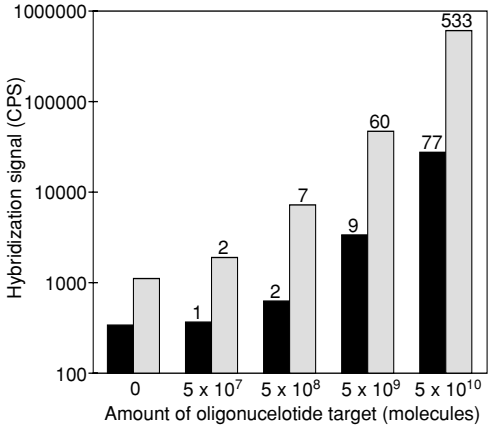


Fig. 4. Sensitivity of solution hybridization. Specific signal divided by the background fluorescence is indicated above the bars using Eu-labelled (open) or Sm-labelled (black) probes.

The properties of time-resolved fluorescence can be exploited in the simultaneous detection of different analytes in a hybridization assay. DNA probes labelled with Eu and Sm have been used for detection of two cystic fibrosis alleles simultaneously from one hybridization reaction (ref. 6). The dual or even triple-label assays are practical in different

research applications like detection of multiple virus sequences, internal control sequence or detection of different RNA molecules. Having the results in a numeric form helps the interpretation and documentation tremendously (table 4).

Table 4. Mean ratio of the specific signal to the background fluorescence is given for the europium and samarium fluorescence for patient samples.

No.of samples	**Signal-to-noise ratio**		
	Europium	Samarium	Status
21	73·7	1·0	Normal
13	42·1	9·7	Carrier
19	2·5	22·5	Mutant

AVOID EXTENSIVE CYCLING OF THE PCR

Sensitive Eu-label allows reduction of the amplification cycles from 30-40 to about 25 (Ref.3). In addition to the time saving of 10-30 minutes more important is the decreased amount of the amplified DNA which means reduced risk of contamination and more accurate quantification.

Since the PCR amplification produces double-stranded DNA, there will be a competition in the hybridization reaction between the two oligonucleotides and the complementary strand for hybridization to the target sequence. This is reflected in the linearity of the assay which can be improved by running asymmetric PCR where the target DNA will be almost exclusively single-stranded. Eu-labelled primers can also be used in restriction fragment length polymorphism (RFLP) or in the analysis of restriction enzymes to remove the need for the laborious gel electrophoresis step commonly used (Ref.12). Restriction enzymes can split biotinylated and Eu-labelled target DNA collected on streptavidin-coated plates. The cleaved label is washed away and the reduced signal is measured.

OPTIMIZATION OF ASSAY

PROBLEM	**REASON FOR THE PROBLEM**	**HOW TO AVOID THE PROBLEM?**
No or low labelling of DNA	- Labelling at low pH pH 9.5 overnight at RT	- Incubation in carbonate buffer,
	- No deprotection of amino groups	- Deprotect according to manufacturer's instructions
	- No amino groups available	- Introduce phosphoramidate or Unilink reagent to 5' end during synthesis
	- Non-specific amino groups present in the oligo solution	- Purify the oligonucleotide carefully after deprotection and gel electrophoresis
No counts or very low counts in hybridization assay	- Sample DNA has been double-stranded	- Denature the sample prior to hybridization
	- Storage of labelled DNA at low pH	- To adjust pH use 20-50 mmol/l Tris-HCl pH 8.5
	- Failure in PCR	- Check PCR
	- Low pH of hybridization solution	- To adjust pH use 20-50 mmol/l Tris-HCl pH 8.5 at 30-50° C and at higher temperatures 50 mmol/l HEPES pH 8
	- Wrong hybridization temperature	- Optimize hybridization conditions

PROBLEM	REASON FOR THE PROBLEM	HOW TO AVOID THE PROBLEM?
No counts or very low counts in hybridization assay	- Post-hybridization washings at too low salt concentration /	- Optimize washing regarding temperature and salt concentration too high temperature
	- Reannealing if > 150 nucleotides long target DNA	- Use instead asymmetric PCR (ref.4), digest competing strand with λ exonuclease or use modified dot blot
	- High concentrations of EDTA	- Use EDTA concentration no higher than 0.1 mM.
High background	- No purification of Eu -labelled probe	- Use e.g. Sephadex G50 (1 x 50 cm)
	- Background of the plates is higher than 2000 counts	- Use DELFIA Plates (1244-550), bkg <600 counts
	- Inadequate washing prior to addition of Enhancement solution	- After hybridization 5-8 stringent washes, check temperature during washings
Poor reproducibility	- Inadequate washing prior to addition of Enhancement solution	- See above
	- Too short incubation with Enhancement solution	- Incubate 25 minutes on a shaker before measurement
Very narrow dynamic range	- Low binding capacity of coated plate	- Try streptavidin-coated wells of high binding capacity
No separation between negative and positive control		- If there is no difference between the known samples and standards give normal results the possibility of PCR contamination should be considered
		- If samples are OK but standards are not separated they may have become contaminated
		- Change reagents

PRODUCTS

C401-101 DELFIA Eu-Oligolabelling reagent, 5 mg. Can be used for labelling of several aminomodified oligonucleotides. Enough for labelling of 200 µg of oligonucleotide.

1244-104/105 Enhancement Solution 50ml/ 250 ml

1244-106/111 DELFIA Assay Buffer, 50 ml/250 ml. For optimal stringency the salt concentration need to be increased.

B117-100 Wash concentrate, 8 x 250 ml. For optimal stringency dilution need to be optimized.

Labelling service: Eu-, Sm- and Tb-labelled oligonucleotides can be ordered from the Labelling service at Wallac. The service includes synthesis, labelling and purification.

As specialty products: CC13-105 Streptavidin- coated plates, 5 plates. Specially for hybridization assays due to low binding capacity.

Instrumentation specially designed for TR-fluorometry:

1420	VICTOR$^2_{TM}$ Multilabel counter
1296-026	DELFIA Platewash
1296-003/004	DELFIA Plateshake
1296-014	Multipette
1296-016	Combitips, 5 ml, pack of 100
1296-017	Combitips, 2.5 ml, pack of 100

REFERENCES

1. Dahlén, P., Carlson, J., Liukkonen, L., Lilja, H., Siitari, H., Hurskainen, P., Iitiä, A., Jeppson, J.-O., Lövgren, T. (1993) Europium-labelled oligonucleotides to detect point mutations: Application to PI Z a1-antitrypsin deficiency. Clin.Chem. 39:1626-1631.

2. Dahlén, P., Iitiä, A., Skagius, G., Frostell, E., Nunn, M., Kwiatkowski, M. (1991) Detection of human immunodeficiency virus type 1 by using the polymerase chain reaction and a time-resolved fluoroscence based hybridization assay. J. Clin. Microbiol. 29:798-804.

3. Dahlén, P., Iitiä, A., Mukkala,V-M., Hurskainen, P., Kwiatkowski, M. (1991) The use of europium (Eu^{3+}) labelled primers in PCR amplification of specific target DNA. Mol. Cell. Probes 5, 143 -149

4. Hierholzer, J., Halonen, P., Dahlén, P., Bingham, P., McDonough, M. (1993) Detection of adenovirus in clinical specimen by polymerase chain reaction and liquid-phase hybridization quantitated by time-resolved fluorometry. J. Clin. Microbiol. 31(7):1886-1891.

5. Huoponen, K., Juvonen, V., Iitiä, A., Dahlén, P, Siitari, H., Aula, P., Nikoskelainen, E., Savontaus, M-L. (1994) Time-resolved fluorometry in the diagnosis of Leber hereditary optic neuroretinopathy. Human Mutation 3:29-36.

6. Hurskainen, P., Dahlén, P., Ylikoski, J., Kwiatkowski, M., Siitari, H., Lövgren, T.(1991) Preparation of europium-labelled DNA probes and their properties. Nucleic Acids Res., 19 (5):1057-1061.

7. Iitiä, A., Liukkonen, L., Siitari, H. (1992) Simultaneous detection of two cystic fibrosis alleles using dual-label time-resolved fluorometry. Mol. Cell. Probes 6, 505-512.

8. Iitiä, A., Högdall, E., Dahlén, P., Hurskainen, P., Vuust, J., Siitari, H. (1992) Detection of Mutation DF508 in the Cystic Fibrosis Gene Using Allele-specific PCR Primers and Time-resolved Fluorometry. PCR Methods Appl. 2: 157 -162.

9. Iitiä, A., Dahlén, P., Nunn, M., Mukkala, V-M., Siitari, H. (1992) Detection of Amplified HTLV-I/II Viral Sequences Using Time-Resolved Fluorometry. Anal. Biochem. 202, 76-81.

10. Lövgren, T., Iitiä, A., Hurskainen, P., Dahlén, P. (1995) Detection of lanthanide chelates by time- resolved fluorescence. Kricka, L.J. Nonisotopic probing, blotting and sequence. Academic Press Inc.

11. Norgaard-Pedersen, B., Hogdall, E., Iitiä, A., Arends, J., Dahlén, P., Vuust, J. (1993) Immunoreactive trypsin and a comparison of two DF508 mutation analyses in newborn screening for cystic fibrosis: An anonymous pilot study in Denmark. Screening, 2:1-11.

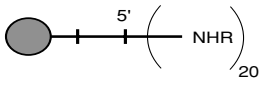
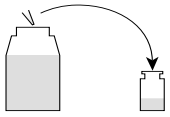
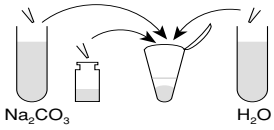
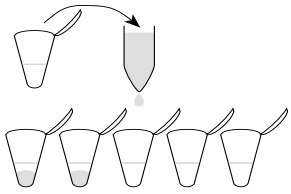
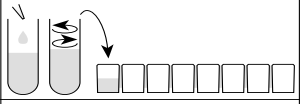
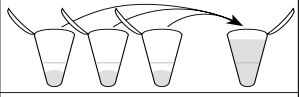
12. Petersen, N.E., Blaabjerg, O., Brock, A., Brondum-Nielsen, K., Brosen, K., Horder, M., Käehne, M., Larsen, A. (1992) Use of Europium-labelled primers in polymerase chain reactions (PCR) eliminating gelectrophoresis. Abstract.

13. Sjöroos, M., Iitiä, A., Ilonen, J., Reijonen, H., Lövgren, T., (1995) Triple label hybridization assay for type-1 diabetes-related HLA alleles. Biotechniques 18: 870-877.

Visit http://lifesciences.perkinelmer.com/referenceDB for more references.

EU-LABELLING OF OLIGONUCLEOTIDE

SUMMARY PROTOCOL SHEET

Oligonucleotide synthesis and purification.		Incorporate 15-25 free amino groups at the 5' prime end. Deprotect and purify.
Dissolve labelling reagent.		300 µl distilled water.
Calculate 20 x reagent excess: O = Amount of oligonucleotide L = Length of oligonucleotide A = No. of amin ogroups / oligo E = Amount of chelate solution to give 20 x excess		O / L / A / E 20 µg (3.03 nmol) / 20 / 20 / 48.5 µl 20 µg (3.03 nmol) / 20 / 25 / 60.6 µl 20 µg (2.02 nmol) / 30 / 25 / 40.4 µl
Start reaction.		20 µl oligonucleotide 10 µl 1mol/l Na_2CO_3, pH 9.5 48.5 µl chelate 21.5 µl distilled water 100 µl total volume
Incubate.		Overnight at room temperature.
Purify.		Use gel filtration column (1x 50 cm) packed with Sephadex G 50. Collect fractions of ~ 600 µl.
Enhance.		Dilute 2 µl of each fraction in 2 ml of Enhancement solution. Mix and transfer 200 µl in micro-titration wells. Slow shaking 25 min.
Count.		Create protocol for counts.
Pool fractions.		Pool first 2-4 fluorescent fractions.
Characterize.	Eu / Oligo	Measure A260 of the pooled fraction. Count fluorescence of pool against 1 nmol/l Eu-standard. Count Eu/oligo.

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