



Rapid and specific detection of PCR products using light-up probes

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Newly developed light-up probes offer an attractive tool for PCR product detection. The light-up probe, which consists of a thiazole orange derivative linked to a peptide nucleic acid oligomer, hybridizes specifically to complementary nucleic acids. Upon hybridization the thiazole orange moiety interacts with the nucleic acid bases and the probe becomes brightly fluorescent. This eliminates the need to separate bound from unbound probes and reduces the risk of cross contamination during sample handling.

We demonstrate here the applicability of light-up probes in two different PCR assays, one directed towards the human β -actin gene and the other towards the *invA* gene of *Salmonella*. The probes do not interfere with the PCR reaction and can either be included in the sample mixture or added after completed amplification. The specificity of the probe is found to be excellent: a single-base mismatch in the target sequence is sufficient to prevent probe binding as indicated by the lack of fluorescence increase. Furthermore, a clear correlation is found between the intensity of gel bands and the measured probe fluorescence in solution, which suggests that the amount of PCR products can be quantified using light-up probes.

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INTRODUCTION

Molecular diagnostics that involves the detection of specific nucleic acid sequences is becoming increasingly important, not only in clinical diagnostics but also, for example, in forensic sciences and quality assurance of food products. The amount of nucleic acid to be detected is often limited and is generally amplified by PCR.^{1–3} Traditionally, the PCR products are subjected to agarose gel electrophoresis and subsequent staining with ethidium bromide to determine

whether PCR amplification has succeeded. To increase assay specificity, the product DNA may thereafter be transferred to a membrane and a fluorescent or radioactively labelled DNA probe is hybridized to a specific target sequence. This heterogeneous methodology requires time-consuming and labour-intensive washing steps to separate bound from unbound probes and the risk of probes remaining non-specifically bound to the membrane is substantial. As a consequence, the background signal may vary from sample to sample, causing low reproducibility. There-

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fore, with an increasing number of analyses being conducted, there is a rising demand for faster, more specific and easily automatized test methods.

During the past few years, several homogeneous technologies, non-specific as well as specific, have been developed to overcome the drawbacks of the heterogeneous methods. Most of these assays are based on the detection of fluorescence. Methods classified as non-specific include the detection of amplified products with double-strand specific dyes⁴⁻⁶ or the use of dual-labelled primers.^{7,8} Although the latter do possess a degree of specificity by detecting only the products formed by the labelled primers, all such products, including primer dimers and non-specific amplification products, are detected.

The specific methods, which are analogous to the traditional heterogeneous hybridization methods, have the advantage that they include an additional hybridization to an internal part of the amplified product. In accordance, the use of such methods adds specificity to the detection and analysis of PCR products by generating a signal change only when the correct product is amplified. TaqMan,⁹ Molecular Beacons,¹⁰ and Scorpions primers¹¹ are all examples of this type of specific detection assay. To generate a detectable change in fluorescence, these assays and the dual-labelled primers^{7,8} are based on energy transfer and require two dyes, a donor-acceptor pair, of which the acceptor usually acts as quencher.

We have developed a novel homogeneous probe technology for the detection of specific nucleic acid sequences,¹² which is utilized, for example, in Light-Up[®] and Nicogreen products for clinical and food diagnostics, respectively (PCT Pat. WO97/45539). The light-up probe consists of a sequence-recognizing element linked to a single fluorescent dye that serves as a reporter group (Fig. 1). In contrast to all of the probe assays described above, the light-up technology utilizes a nucleic acid analogue instead of natural DNA as sequence recognizing element.

The probes studied here are constructed from the uncharged nucleic acid analogue peptide nucleic acid (PNA),¹³ in which the entire deoxyribose phosphate backbone of DNA has been replaced by an achiral pseudo-peptide backbone composed of N-(2-aminoethyl)glycine units (Fig. 2a). Another PNA based probe assay, utilising a molecular beacon that consists partly of PNA, has recently been described.¹⁴ PNA hybridizes faster to, and forms more stable sequence-specific complexes with, both DNA and RNA than the natural nucleic acids themselves do to the same target sequence.^{15,16} These features are attributed mainly to the charge-neutral nature of PNA, which eliminates the electrostatic repulsion between the

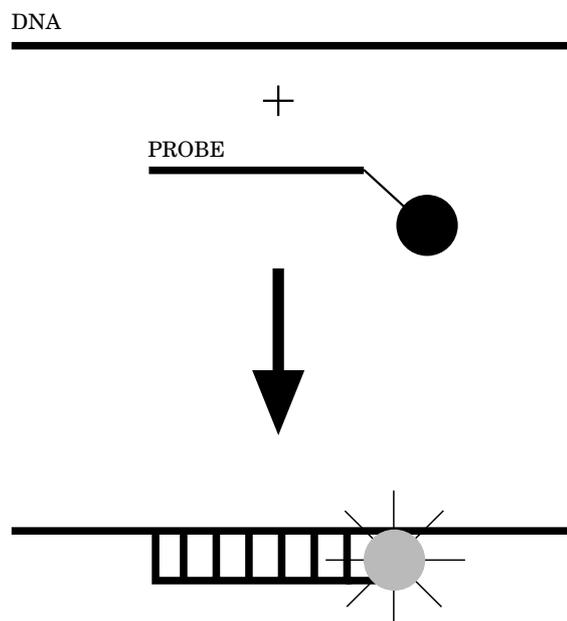


Fig. 1. Schematic showing the principle of homogeneous detection using light-up probes. The light-up probe consists of a sequence-recognizing element linked to a reporter group, here peptide nucleic acid (PNA), coupled to a thiazole orange derivative. When free in solution, the probe has low fluorescence due to the intrinsic properties of the reporter dye. Binding of the sequence-recognizing element to its complementary target allows strong interaction between the dye and the nucleobases, which causes the light-up probe to fluoresce brightly.

hybridizing strands. Depending on the sequence content of PNA, the PNA/DNA complex generated upon hybridization is either double or triple-stranded.¹⁷ A triplex forming PNA hybridizes by strand invasion to its complementary DNA strand, displacing and leaving the non-complementary DNA strand in a single-stranded loop.^{13,18} PNA hybridization is also very sensitive to mismatches: when a mismatch is introduced in the target, the decrease in thermal stability is greater for PNA/DNA hybrid complexes than for normal DNA duplexes.¹⁶ As a consequence, in the recognition of nucleic acids, a more efficient sequence discrimination is achieved by PNA than that obtained by either DNA or RNA. An additional advantage of utilizing PNA instead of natural nucleic acids in probe assays is that it is not a substrate for nucleases, proteases or peptidases.

The reporter group in the light-up probe is a derivative of the asymmetric cyanine dye thiazole orange (Fig. 2b). In this type of dye, the two aromatic systems can rotate around the interconnecting methylene bond, forming a channel for non-radiative relaxation. When bound to nucleic acids, the aromatic moieties are fixed in a co-planar geometry, and the

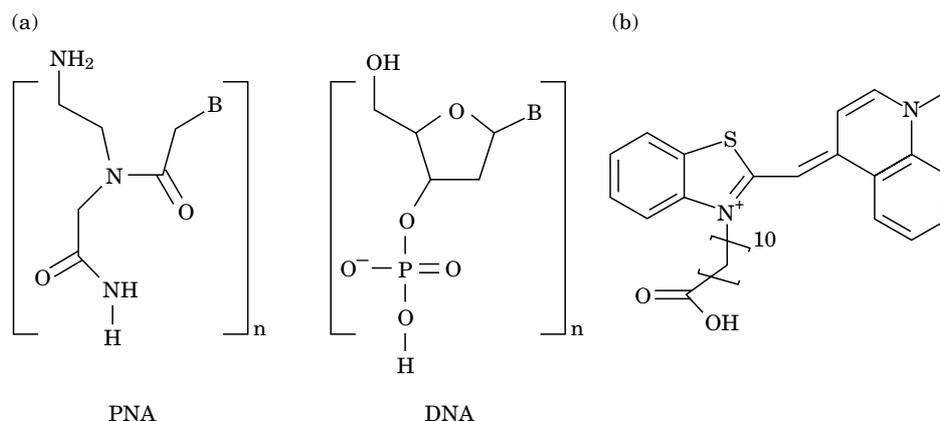


Fig. 2. (a) Chemical structures of DNA and PNA monomer units; B denotes the nucleobase. (b) Chemical structure of the thiazole orange derivative (TO-N'-10-COOH).

dye becomes strongly fluorescent.^{19,20} As a consequence, the light-up probe, which has low fluorescence in its unbound state, generates a readily detectable signal when hybridized to its target sequence. This eliminates the need to separate unbound probes from the hybridization complex prior to detection. Thus, light-up probes differ from the probes used in most other homogeneous assays,⁹⁻¹¹ since they contain a single dye instead of a fluorophore and a quencher. Although other probe assays utilizing a single fluorescent dye have been described, they are based on fluorescence polarization measurements that require far more advanced instrumentation and analysis protocols.^{21,22}

Light-up probes are shown here to be efficient tools for the detection of specific PCR products in a homogeneous format. It is also demonstrated that light-up probes offer reliable discrimination between targets differing in a single position, and generate a fluorescence signal that is proportional to the amount of PCR product formed. Hence, end-point quantification of PCR products is facilitated by the use of light-up probes.

MATERIALS AND METHODS

Probe synthesis and analysis

Two light-up probes, ACTp and SALp, directed towards PCR amplification products of human β -actin and *Salmonella invA* genes, respectively, were designed. The probes and the thiazole orange derivative, TO-N'-10-COOH (N-methyl-4-((3'-carboxydecyl-1', 3'-benzothiazol-2'-yl)methylenyl)quinolinium iodide), were synthesized and purified as described.¹² The concentrations of the probes were determined

spectrophotometrically assuming molar extinction coefficients of $63\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 500 nm for the thiazole orange moiety,²⁰ and $69\,000\text{ M}^{-1}\text{ cm}^{-1}$ and $92\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 260 nm for the PNA part of ACTp and SALp, respectively.

DNA template preparation

The β -actin template was male human genomic DNA, purchased from Sigma-Aldrich (Sweden), and used without further purification. *Salmonella* cultures were kindly provided by Diffchamb S.A., Lyon, France. Bacterial genomic DNA was isolated as described²³ and the concentrations were determined spectrophotometrically, assuming a molar absorptivity at 260 nm of $13\,200\text{ M}^{-1}\text{ cm}^{-1}$ per base pair.²⁴

PCR assays

A 295-bp fragment of the human β -actin gene²⁵ containing the ACTp target sequence was amplified. The PCR reaction mixture contained 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 0.2 mM deoxyribonucleotides, 100 nM of each primer, 1 U of *Taq* DNA polymerase (Sigma-Aldrich, Sweden), and 100 nM of the ACTp probe in a total volume of 100 μ l. The thermocycler program (Techne Progene) consisted of a pre-incubation for 5 min at 94°C followed by 35 cycles of 45 s at 94°C, 45 s at 45°C, and 1 min at 72°C, and finally, a 5-min extension at 72°C. In the *Salmonella* assay, a 409-bp fragment of the *invA* gene (GenBank accession numbers U43237 and U43241) containing the SALp target sequence was amplified. The same buffer as in the β -actin assay was employed, however there was no light-up probe

present, and 300 nM of each primer was used. The temperature program consisted of 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, and finally, 7 min at 72°C. In both assays, 10 µl-aliquots of the PCR products were analysed by electrophoresis in 2% (w/v) agarose gels, stained with ethidium bromide and made visible by U.V. illumination.

Fluorescence measurements

All fluorescence measurements were performed on an ISS PC1 instrument, and emission spectra were recorded between 505 and 650 nm using 488 nm excitation and 8 nm bandwidths. The fluorescence of the β -actin samples was recorded after amplification without further manipulation, while 100 nM SALp probe was added to the *Salmonella* samples before starting the measurement procedure. To dissociate strands, 90 µl of the post-PCR samples were heated for 5 min at 95°C and subsequently transferred to the measurement cell which was kept at the annealing temperature used in the corresponding PCR assay. All spectra shown were baseline corrected by subtracting the fluorescence of a negative PCR control sample that contained neither probe nor template. The free probe level was represented by the fluorescence of a negative PCR control with probe present, assuming that no target sequences exist in the sample and that all probes are in their unbound state.

RESULTS

Homogeneous detection of PCR samples

To demonstrate that light-up probes can homogeneously detect PCR products, a probe was designed to specifically recognize a region of the human β -actin gene. This gene is constantly expressed in most tissues and cells and is therefore frequently used as a standard in mRNA quantification assays to normalize gene expression levels and as a positive control in PCR assays in general. The present assay amplifies a 295-bp fragment,²⁵ which contains the target sequence for the ACTp light-up probe. Four samples, three positive and one negative, are compared in Fig. 3. The fluorescence intensities of the positive PCR samples, containing either 0.01, 1 or 10 ng of human genomic DNA template, are substantially enhanced compared with that of the free probe (Fig. 3a). The negative sample, on the other hand, displays a fluorescence emission spectrum that is virtually identical to that of the free probe. The

negative PCR sample contained *Salmonella* genomic DNA (0.5 ng), assumed to lack complementarity for the β -actin primers, and no amplification products were obtained with this template (Fig. 3b, lane 3). All three positive samples, however, produced readily detectable bands when analysed by agarose gel electrophoresis. It can also be noted that the strength of the gel bands correlates perfectly with the fluorescence intensities measured in solution (Fig. 3b, lanes 4, 5, and 6).

Light-up probes discriminate between target sequences differing by a single base

An assay was developed to amplify and detect a fragment of the *Salmonella invA* gene, which is highly conserved among different species of *Salmonella* and is not present in other bacteria.²⁶ Genomic DNA of three *Salmonella* serotypes was used as PCR template: *S. enteritidis* and *S. anatum* both belong to species *enterica*, subspecies *enterica*, while *S. bongori* is a separate species. According to sequence data (GenBank accession numbers U43237 and U43241), amplification should occur when using any of the three templates. Furthermore, the amplification products of *S. enteritidis* and *S. anatum* will both contain a sequence that is fully complementary to the SALp probe, while the product of *S. bongori* has one mismatch in the central region of the probe target site. As expected, all three templates produced products of high yield as visualized by agarose gel electrophoresis (Fig. 4b, lanes 3, 4 and 5). Addition of the SALp probe to the *S. enteritidis* and *S. anatum* samples gave fluorescence signals approximately six times that of the free probe. In contrast, the *S. bongori* sample had a fluorescence emission spectrum almost superimposable to that of the free SALp probe (Fig. 4a). Therefore, the single mismatch in the *S. bongori* target sequence is sufficient to prevent the probe fluorescence increases observed for positive samples containing a perfectly matched target sequence.

DISCUSSION

We have developed a novel probe technique, for which the probe is composed of a single fluorescent dye linked to a PNA-based sequence-recognizing element, to be used for the detection of PCR products. It is shown here that the presence of PCR product is a prerequisite for an increase in fluorescence intensity to occur. This strongly suggests that the dye is prevented from interacting with any arbitrary nucleobases, such as primer dimers, non-specific amplification products or background DNA, the

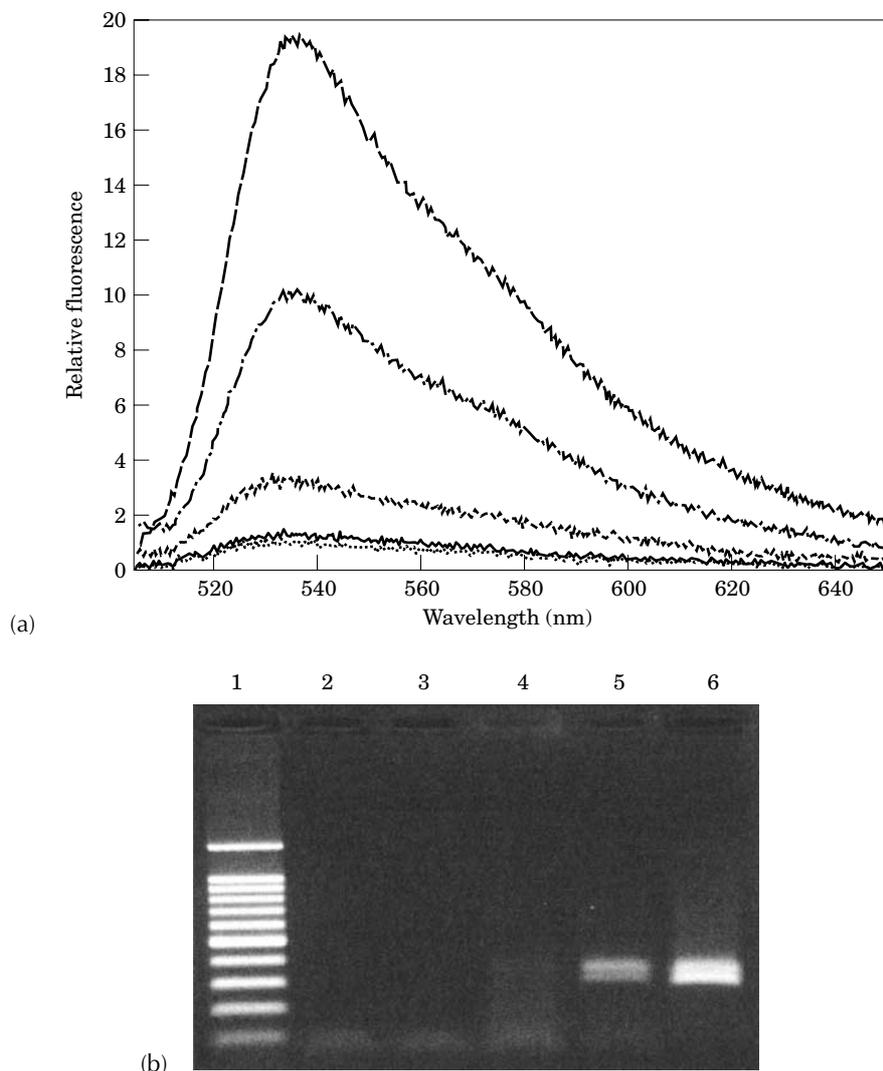


Fig. 3. Analysis of PCR products derived from the β -actin gene fragment. (a) Fluorescence emission spectra of PCR samples containing 100 nM ACTp. The free probe spectrum is represented by a negative PCR control, and its maximum fluorescence is normalized to unity (.....). The positive PCR samples contain 0.01 ng (—), 1 ng (— · — ·) and 10 ng (—) of the human genomic DNA template, while the negative PCR sample contains *Salmonella* DNA as template (—). (b) the samples in (a) as visualized by ethidium bromide stained agarose gel electrophoresis. Lane 1, 100-bp molecular mass marker; lane 2, negative PCR control; lane 3, negative PCR sample with *Salmonella* DNA template; lanes 4, 5 and 6, positive PCR samples with 0.01, 1 and 10 ng of human genomic DNA template, respectively.

amount of which might be substantial, for example, in samples utilized in food pathogen analysis³ and clinical specimen. Hence, the sequence-recognizing PNA has to identify its specific target to enable the dye to interact with the bases and cause a fluorescence increase. This is in contrast to the behaviour of double-strand specific dyes,^{4,6} which generate a signal in the presence of any double-stranded DNA, and give rise to false positives if unspecific products or primer dimers are formed. The data presented here also demonstrate that the PCR product must contain a perfectly matching target sequence for a signal increase to occur (Fig. 4). A

single-base mismatch is sufficient to prevent probe hybridization and subsequent fluorescence enhancement. It should be noted, however, that the degree of sequence discrimination could depend on the location of the mismatched base. In the case of *S. bongori*, the mismatch is in the central part of the probe target, which naturally destabilizes the PNA/DNA complex to a greater extent than if it had been at one of the ends of the complex. In the latter case, a partial fluorescence increase would probably be observed upon hybridization. Nonetheless, the present assay demonstrates excellent sequence discrimination: a single-base mismatched PCR product can easily be distinguished from the correct one.

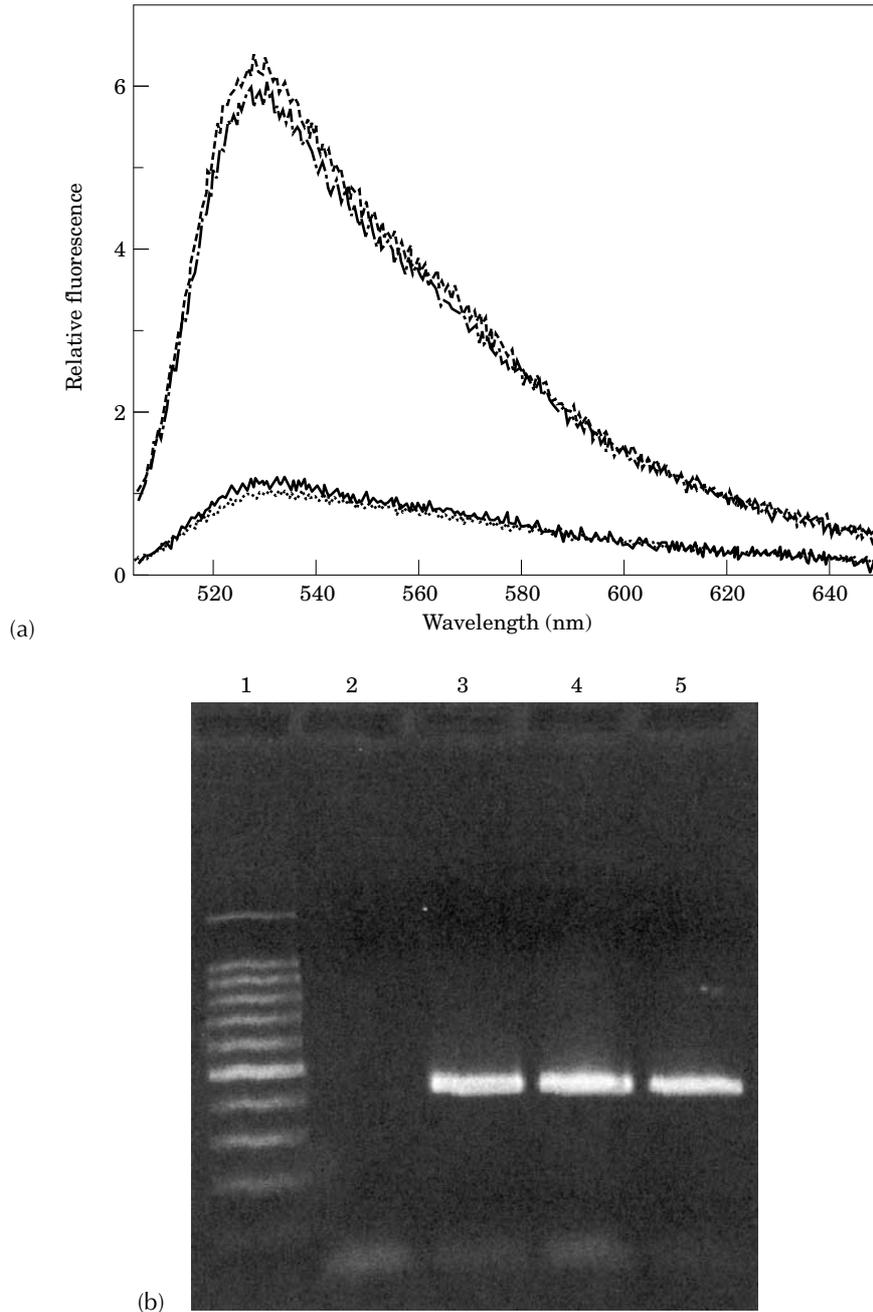


Fig. 4. Analysis of PCR products derived from the *Salmonella invA* gene fragment. (a) Fluorescence emission spectra of PCR samples containing 100 nM SALp. The free probe spectrum is represented by a negative PCR control, and its maximum fluorescence is normalized to unity (.....). PCR samples contain DNA from *Salmonella enteritidis* (----), *Salmonella anatum* (-·-·-) and *Salmonella bongori* (——) templates. (b) The samples in (a) as visualized by ethidium bromide stained agarose gel electrophoresis. Lane 1, 100-bp molecular mass marker; lane 2, negative PCR control; lanes 3, 4 and 5, positive PCR samples with *S. bongori*, *S. enteritidis* and *S. anatum* DNA templates, respectively.

Since the fluorescence intensity clearly reflects the amount of PCR product generated, it should be possible to estimate this quantity (Fig. 3). However, in end-point detection assays, the number of correct target sites is determined; this is a measure that is not directly related to the initial template amount. To ascertain the quantity of PCR template present in each

sample, the detection must be carried out in the log phase of the reaction. This is most readily achieved using a method by which the experimental reactions can be followed during amplification, i.e. real-time monitoring. Since the light-up probe can be included in the PCR reaction mixture without causing any detectable inhibition (data not shown), real-time

measurements are feasible. Accordingly, the light-up probes can be included in quantitative PCR assays, to be used in determinations of for example sample viral loads and mRNA quantification assays (unpublished data). If desired, the light-up probe can be added to the sample after amplification. This might be an attractive option to minimize the disturbance of a well optimized system, such as an assay based on the simultaneous amplification of two target sequences. When using TaqMan probes⁹ or Scorpions primers,¹¹ for example, this is not an alternative, since their respective actions rely directly or indirectly on the *Taq* polymerase activity.

The different signal increases upon hybridization obtained for the two assays examined here (Figs 3 and 4) probably have several origins. For instance, the fluorescence quantum yield of thiazole orange bound to DNA varies with the sequence, reflecting the ability of the dye to interact differently with the four nucleobases.²⁰ As a consequence, the fluorescence intensity of the hybridized probe depends on the nucleobases available for intercalation. In the present assays, the fluorescence quantum yields of the free probes are practically identical (data not shown). The ACTp system generates an approximately 19-fold increase in fluorescence intensity in the presence of generous amounts of target nucleic acid, while the maximum increase exhibited by the SALp system is only about 6-fold. This may seem surprising, since earlier work has demonstrated that the fluorescence quantum yield for the hybridized probe is relatively constant from assay to assay, while the free probe fluorescence may vary significantly, i.e. the fluorescence increase obtained is mainly determined by the free probe fluorescence signal.¹² Nevertheless, the reported variation in hybridized probe fluorescence¹² more than well accounts for the 3-fold difference between the fluorescence increases observed in the assays studied here.

A presumably more important factor that should be taken into consideration is the different detection temperatures applied in the two assays. It could be argued that, since the fluorescence quantum yield for thiazole orange decreases with increasing temperature even when quantitatively bound to DNA,²⁰ detection should be performed at as low a temperature as possible. However, it is crucial for the performance of any probe assay that the probe-binding event is monitored at a discriminating temperature, i.e. close to the melting temperature of the hybridization complex. The two PCR protocols utilized here have different optimal annealing temperatures. Therefore, to have the choice of making either a qualitative (post-PCR) or a quantitative (real-time PCR) determination without changing the probes or PCR para-

eters, the probes were designed to have melting temperatures close to the respective annealing temperatures and all measurements were conducted at these temperatures.

In conclusion, the results clearly demonstrate that the light-up probe technology is an appropriate method for PCR product detection and an attractive alternative to traditional gel electrophoresis methods. The use of light-up probes offers excellent specificity, and the sensitivity is at least as good as in ethidium bromide stained agarose gel electrophoresis (Fig. 3). Once PCR amplification has been carried out, the analysis is readily completed within a few minutes. In comparison with other homogeneous hybridization methods that are based on dual-labelled probes, the light-up probe technology has the advantage of requiring technically less demanding synthesis and less expensive instrumentation. Owing to the exceptional hybridization properties of PNA, there is no need to adopt asymmetric PCR protocols in order to get stable and intense signals when using the light-up probes. In accordance, the sample tubes do not have to be opened during or after PCR, and the risk of contamination of equipment, instrumentation, or between individual samples, is significantly reduced.

Although further work is required before the general applicability of the light-up probe technology can be assessed, the work presented here shows that the detection of PCR products can be done rapidly and specifically by using light-up probes. Therefore, light-up probes have the potential of being very useful for the routine processing of large numbers of samples, for example in the screening of viruses and foodborne pathogens.

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