Fluorescence based strategies for genetic analysis

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Synthetic chemistry has been central to the design of modern methods of genetic analysis. In this article, we discuss the underlying chemistry and biophysical principles that have been used in the development of robust methods for the analysis of DNA in the diagnostic laboratory.

Introduction

In the post-genomic era a huge quantity of DNA sequence information is being generated. This is potentially of great value in many fields, including proteomics, metabolomics, population genetics and human medicine. There is a concomitant need to develop methods for the rapid and routine analysis of specific nucleic acid sequences so that this information can be understood and interpreted. The Sanger dideoxy sequencing method that was used in the elucidation of the human genome is a robust and accurate method for determining the sequence of nucleotides (nt) in DNA. However, the vast majority of the genome is identical between individuals, so the detailed information generated by DNA sequencing is often unnecessary. In particular, genotyping of single nucleotide polymorphisms (SNPs) requires the identification of specific nucleotides separated by over 1000 invariant base pairs throughout the genome. Consequently, less laborious and higher throughput methods for determining nucleic acid sequences are currently the subject of considerable research effort, and methods that utilise short oligonucleotide probes (15–30 nt) to reveal the presence of complementary sequences offer an attractive solution.

Such probes are prepared by chemical rather than biochemical methods. Solid phase oligonucleotide synthesis by the phosphoramidite method, developed by Beaucage and Caruthers,[1] is the key contribution of synthetic chemistry to genetic analysis. It is widely used to rapidly and efficiently synthesise oligonucleotide probes of any sequence up to around 200 nucleotides in length. Harnessing the selectivity of Watson–Crick base pairing allows recognition of specific DNA sequences by synthetic oligonucleotide probes, but in addition to this a signal must be generated upon hybridisation (duplex formation) to confirm the presence of the complementary (target) sequence. Nucleic acids themselves possess no readily detectable functionality, so probes are normally labelled with organic fluorescent dyes. Several oligonucleotide probe formats have been developed and these are discussed in detail below. In most of these formats, a fluorescence quencher is included in the probe design such that it is not fluorescent until hybridised to the target DNA. Probes of this design are termed ''fluorogenic''. The simplicity of oligonucleotide synthesis chemistry has led to a wide variety of fluorescent dye and quencher phosphoramidite monomers becoming commercially available (Fig. 1), covering much of the visible spectrum. Researchers have also developed their own labels to fit the requirements of their probes.[2] In some applications fluorophores (*e.g.* TAMRA) can also be used as fluorescent quenchers.

Although fluorescence is a sensitive technique, the quantity of DNA available for analysis is normally very small. Therefore amplification of the nucleic acid sequence of interest is necessary prior to detection. This is almost always done by PCR (the polymerase chain reaction), which serves to amplify the specific region of DNA under investigation, and therefore eliminates problems caused by interference from other DNA sequences. Real time, or kinetic PCR is a method in which the accumulation of amplicon (PCR product) is monitored throughout the PCR.[3] This allows detection in a 'closed-tube' format, where no manipulation is required during or after PCR, removing the risk of cross contamination between amplified samples. This addresses a major issue in biochemical laboratories as PCR is carried out by humans whose DNA is a potential contaminant. In real time PCR fluorescence is measured in each cycle to give an amplification curve, the intensity of which is proportional to the amount of PCR product (Fig. 2a). In addition to this, post-PCR melting curves can be obtained by heating the sample to melt the probe-PCR product duplex whilst monitoring fluorescence. When specific oligonucleotide probes are used, this can provide further information. Probes specific for wild type genotypes form duplexes containing mismatches with mutant genotypes. These

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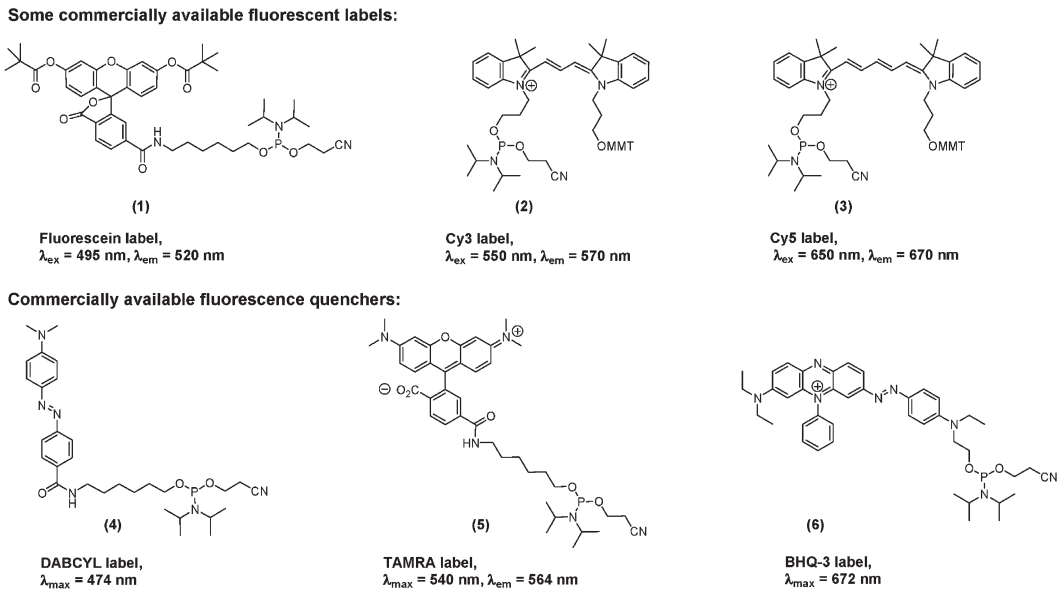


Fig. 1 Commercially available phosphoramidite monomers for fluorescent-labelling of oligonucleotides. MMT = 4-methoxytrityl protecting group. BHQ = black hole quencher.

melt at lower temperatures than perfectly matched duplexes. The T_m (the temperature at which 50% of the duplex is melted) can be determined from the position of a maximum in the first derivative of the melting curve (Fig. 2b). From its inception in the mid 1990s, real time PCR represented a major advance in quantification of DNA. Quantification of RNA is also possible by reverse transcription-PCR (RT-PCR), where RNA is first transcribed to cDNA (complementary DNA), which can be used as the template in PCR.[4] This method is particularly important as it allows quantification of mRNAs, allowing measurement of viral RNA loads and estimates of levels of protein expression.[5,6] Such measurements can be clinically significant. Real time PCR can be used for genotyping—wild type, heterozygous and mutant genotypes give rise to different amplification curves if suitable allele-specific probes or primers are used. Real time PCR is useful for genotyping SNPs.[7]

The use of real time PCR to detect specific DNA sequences is applicable in various fields such as parasitology,[8] oncology[9] and food technology.[10,11] In such applications it is necessary to establish the presence or absence of specific DNA sequences. In principle this could be accomplished by endpoint (as opposed to real time) detection but the speed and convenience of real time PCR makes it a commonly used technique. In addition, the cycle at which the fluorescent signal emerges from the background (C_t value) provides a method of quantifying the amount of DNA present at the beginning of PCR (copy number). This can be clinically important when detecting the presence of pathogens. Real time PCR has progressed beyond the research laboratory, and will soon be used for point-of-care diagnosis of *Chlamydia* and other infectious diseases.

Other methods of DNA analysis such as microarrays[12] are also of value in biomedical applications. They depend upon signals generated from immobilised unlabelled probes when hybridised to fluorescently labelled complementary sequences, such as PCR products. This is a combination of positional and fluorescence information, conceptually simple and not

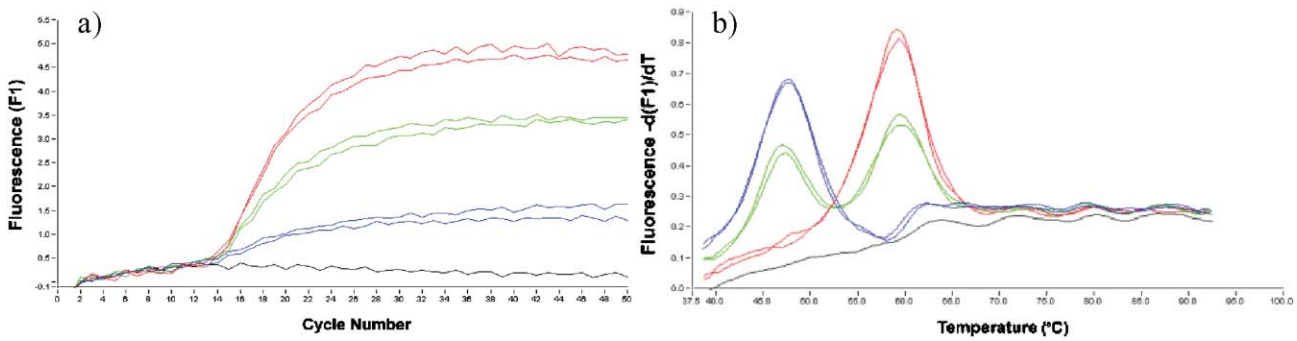


Fig. 2 (a) Real time PCR amplification curves and (b) first derivative of post-PCR melting curves. Red—homozygous wild type template, green—heterozygote, blue —homozygous mutant template, black—no template control. Fluorescence of a HyBeacon probe (see below) complementary to the wild type allele is monitored in these experiments.

discussed in detail in this review. DNA array technologies have the potential for automation and very high throughput, but the initial financial investment can be high.

Non sequence-specific detection of PCR products

The simplest way to generate a signal during real time PCR is to include a fluorescent DNA-binding agent in the reaction mixture.[13] Small molecule DNA-binders such as SYBR Green I,[14] ethidium bromide[15] and YO-PRO-1[16] bind to double-stranded DNA (dsDNA) with a very large increase in quantum yield, and are used to detect accumulation of the amplicon during real time PCR. SYBR Green I is the most commonly used dye because it has minimal binding to single-stranded DNA, a high quantum yield when bound to dsDNA and similar spectral properties to fluorescein (FAM). This is convenient as almost all genetic analysis real time PCR platforms are designed to excite fluorescein at 495 nm and detect its emission at 520 nm. This approach has been used for DNA quantification with high sensitivity, as many molecules of a DNA-binding agent can bind to one DNA duplex.[17] While this is the cheapest and simplest available signalling method, it does have major disadvantages. Most importantly the fluorophore binds indiscriminately to any sequence of dsDNA. Therefore any amplicon will generate a signal and the assay is liable to score false positives for samples where primer dimers are formed, or mispriming at incorrect sites occurs. Primer dimers are short amplicons formed between PCR primers independently of the template DNA. They are surprisingly common, even when primers have very little complementarity. When DNA-binding dyes are used there is no facility for allelic discrimination unless allele-specific (ARMS, amplification refractory mutation system) primers[18] are used.

Sequence-specific probe systems

To avoid non-specific accumulation of the fluorescent signal, labelled oligonucleotides are used to *probe* the PCR product for the presence of the complementary sequence. Several methods have been developed and the most common systems are those in which the probe is non-fluorescent until bound to its complement. Typically, either fluorescence resonance energy transfer (FRET) or Dexter (collisional) quenching is used to attenuate fluorescence in the unbound or '*off*' state. An increase in separation between fluorophore and quencher upon hybridisation leads to a more brightly fluorescent '*on*' state. The design and synthesis of new fluorescent labels, quenchers and other nucleic acid modifiers by chemists has been instrumental in the development and refinement of the probe technologies now available.

Primer-probes

Fluorescently labelled "primer-probes" can be used to generate a signal with the accumulation of PCR product. They are labelled in such a way that extension of the primer leads to enhanced fluorescence.

An early example of this methodology was "Sunrise primers", later called Amplifluor primers.[19] The Amplifluor primer is designed to fold into a stable hairpin-loop (stem-loop) structure, with a fluorophore at one end of the stem and a quencher at the other. Extension of the Amplifluor primer leads to production of one strand of the amplicon. This strand then becomes a PCR template, which is copied and in the copying process the polymerase copies-through the stem-loop. This produces a fully double-stranded PCR product in which the stem-loop structure no longer exists. Consequently the fluorophore and quencher are separated and a large increase in fluorescent signal is observed. Subsequently the assay was refined by the use of tailed unlabelled locus-specific primers. The 3′-portion of such a primer is designed to hybridise to the DNA to be amplified and the 5′-portion contains a universal sequence or "tail" (the A-sequence).[20] During PCR this universal sequence is incorporated into the amplicon. It is complementary to the primer portion of the fluorescently-labelled *universal* Amplifluor primer, which will therefore bind to the amplicon and be extended and copied, thus generating fluorescence (Fig. 3). The fluorescently-labelled universal primer is called the Amplifluor UniPrimer I, and the same labelled primer can be used in any PCR, as long as the correct tailed sequence is incorporated into the locus-specific primers. This has the obvious advantage of lowering the cost of the assay as only a limited number of fluorescently-labelled primers have to be synthesised, but is more complex than the

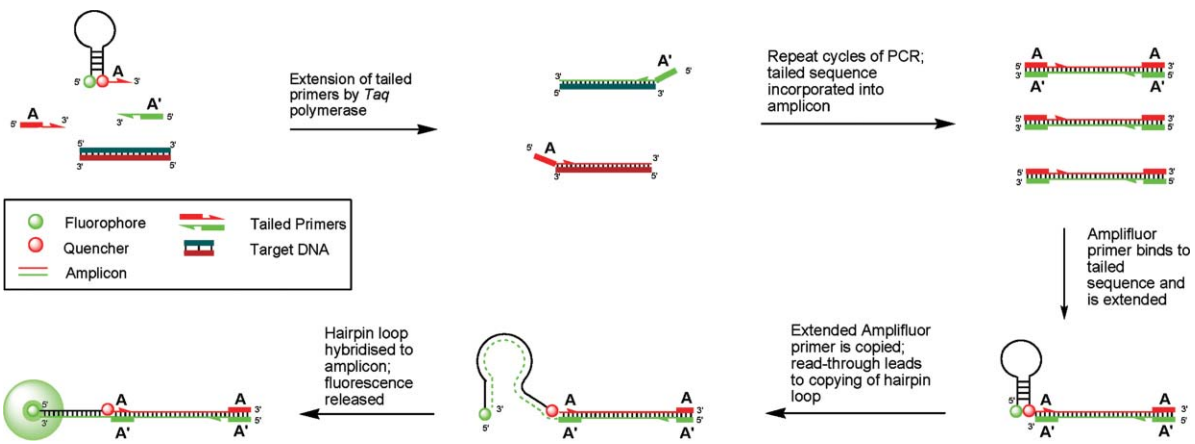


Fig. 3 Mode of action of Amplifluor UniPrimers.[20]

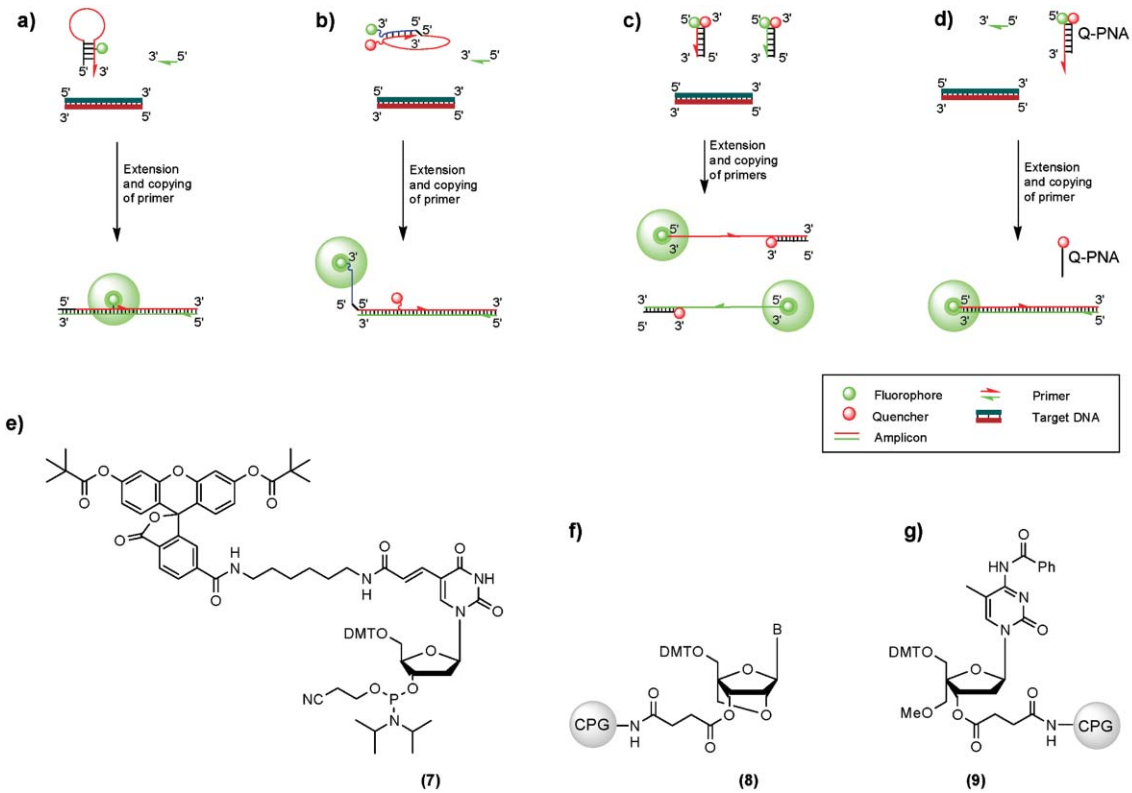


Fig. 4 Mode of action of (a) hairpin primers labelled with a single fluorophore;[22] (b) cyclicons;[23] (c) double-stranded primers;[24] (d) self-reporting PNA/DNA primers;[25] (e) 'fluorescein dT' phosphoramidite monomer used to label hairpin primers; (f) LNA-functionalised controlled pore glass (CPG) used to synthesise 3′-modified allele-specific primers;[28] and (g) 4′-methoxymethylene-dC-functionalised CPG used to synthesise allele-specific primers.[29] DMT = 4,4′-dimethoxytrityl, B = nucleobase (G, C, A or T).

original format. Allele-specific primers can be used to distinguish point mutations.

Subsequently it was found that primers with blunt-ended hairpin-loop structures and a dye attached at the C5-position of thymidine (Fig. 4e) exhibited enhanced fluorescence upon incorporation into PCR products without the necessity of a quencher moiety (Fig. 4a). The design rules, established in a biophysical study, dictate that the terminal base pair of the hairpin-loop must be CG or GC and that the thymine base bearing the fluorophore must be 2 or 3 nucleotides from the 3′-end.[21] The application of these hairpin primers to real time quantitation and end-point SNP detection has been demonstrated.[22]

Cyclicons,[23] 5′–5′ linked pseudocyclic oligonucleotides bearing a fluorophore and quencher (Fig. 4b), and double-stranded primers, with dye-labelled primers and quenchers attached to either a complementary oligonucleotide (Fig. 4c)[24] or PNA (peptide nucleic acid) oligomer (Fig. 4d),[25] can be used in similar assays. PNA is a synthetic DNA analogue that hybridises to DNA according to the normal Watson–Crick base pairing rules, exhibits enhanced duplex specificity and stability, but is not a substrate for DNA processing enzymes.

All primer-probes can generate false positive signals as a result of primer dimer formation and mispriming. However, the probability of this is reduced when the oligonucleotides have secondary structure, as this would be expected to inhibit primer dimer formation. Indeed, unlabelled primers with hairpin-loop[26] and duplex[27] structures have already been shown to enhance the specificity of PCR. Locked nucleic acid (LNA), (Fig. 4f)[28] and 4′-methoxymethylene-modified nucleotides at the 3′-end of allele-specific PCR primers (Fig. 4g)[29] have been shown to confer greater selectivity due to their enhanced mismatch discrimination, and could be applied to fluorogenic primer-probes. In any case, PCR must be optimised for all real time assays, because if reagents and primers are used up in synthesising spurious amplicons a loss of sensitivity and false negatives can occur.

Signal generation by probe cleavage

The primer-probes described above generate a signal upon incorporation into PCR products, but are vulnerable to the production of spurious amplicons resulting from primer dimers or mispriming. Probes that become fluorescent during the PCR due to *hybridisation with the specific DNA sequence under investigation* are inherently more specific. In this context PCR-induced probe cleavage was one of the first methods developed to generate a fluorescent signal in real time PCR. The fluorogenic probe is quenched in its single-stranded form, but hybridisation to the target DNA strand leads to enzymatic cleavage of the probe and generation of fluorescence.

The TaqMan® assay, as it is known commercially, exploits the 5′-nuclease activity of DNA polymerase enzymes.[30] A TaqMan® probe is an oligonucleotide, labelled with two fluorescent dyes, normally one at each end.[31] In the single-stranded form fluorescence is quenched by FRET from the

donor to the acceptor dye. The TaqMan® probe is designed to be complementary to a specific region of DNA which is amplified by two unmodified primers. As the primers are extended by the polymerase during PCR, replication is obstructed by the TaqMan® probe. The polymerase has an inherent 5′ to 3′ exonuclease activity at the point of obstruction and hydrolytically cleaves the probe. Since the donor and acceptor dyes are no longer in the same molecule, the interfluorophore distance is now greatly increased, and FRET is eliminated (Fig. 5). This leads to an accumulation of the fluorescent signal from the donor fluorophore which eventually reaches a plateau.[32] TaqMan® has been used extensively as a tool in literally hundreds of applications in genetic analysis, for example the detection of Hepatitis C virus,[33] *Salmonella* in raw meat[34] and cancer susceptibility in humans.[35]

Several improvements to the TaqMan® method involving chemical modification of the probe structure have been presented. Terbium chelates can be used as the reporters, leading to an increase in sensitivity. Low background fluorescence from these luminescent labels can be achieved by internal quenching by the probe,[38] or by the use of a complementary oligonucleotide bearing a quencher.[39] An intramolecular version of TaqMan® has been described (Fig. 6a).[40] A minor groove binder (MGB) has been developed for attachment to the 3′-end of TaqMan probes® (Fig. 5b).[41] The incorporation of the groove binder significantly increases the T_m of the TaqMan®–MGB probe–target duplex so that shorter probes can be used. This is particularly important for "AT-rich" TaqMan® probes which would otherwise have to be very long to have a suitable hybridisation temperature. Long TaqMan® probes give poor allelic discrimination and exhibit inefficient fluorescence quenching due to the increased inter-fluorophore distance. A similar approach involves the incorporation of locked nucleic acid (LNA) nucleotides into TaqMan® probes (Fig. 5c), again leading to stabilisation of the probe–target duplex and enhanced sensitivity and selectivity.[37] TaqMan®–MGB probes are commercially important and Applied Biosystems recently introduced 'Assays-On-Demand'—pre-formulated kits containing probes and primers for >146 000 SNPs.

A universal probe based on an expanded genetic information system (AEGIS) has been described recently. This utilises the unnatural nucleosides isoC (iC) and isoG (iG),[42] and the double-flap gap (DFG) nucleolytic activity of *Taq* polymerase. One normal primer, and one tailed primer, containing consecutive iC and iG residues between the allele-specific primer and the universal tail, are used. During extension of the standard primer, one nucleotide is inserted opposite iG of the tailed primer then replication stops at iC. This creates a duplex with a 5′-single-stranded overhang and a 3′-flap (single unpaired base). Hybridisation of the universal fluorescently-labelled probe to the 5′-overhang gives the double-flap gap structure. This is recognised by the polymerase and is cleaved, increasing the fluorescence of the cleaved fluorophore (Fig. 6b).[43] While the universal probe is not extended and is therefore not a primer-probe, the assay is still sensitive to mispriming and primer dimer formation, as production of any amplicon will lead to the cleavage of the probe. As with Amplifluor UniPrimers, though, the requirement for only a small number of fluorescently-labelled probes to be synthesised for a large number of loci is appealing.

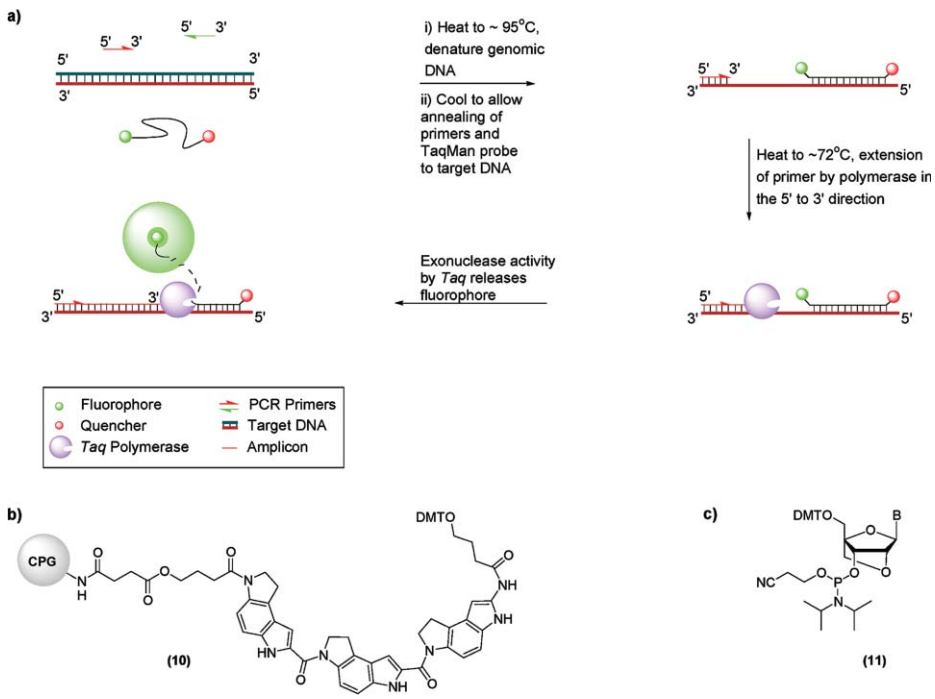


Fig. 5 (a) The TaqMan® assay;[32] (b) oligonucleotide synthesis support functionalised with the peptidic dihydropyrroloindole minor groove binder DPI_3 for 3′ labelling of TaqMan® probes;[36] and (c) general structure of locked nucleic acid (LNA) phosphoramidite monomers used for construction of TaqMan® probes.[37]

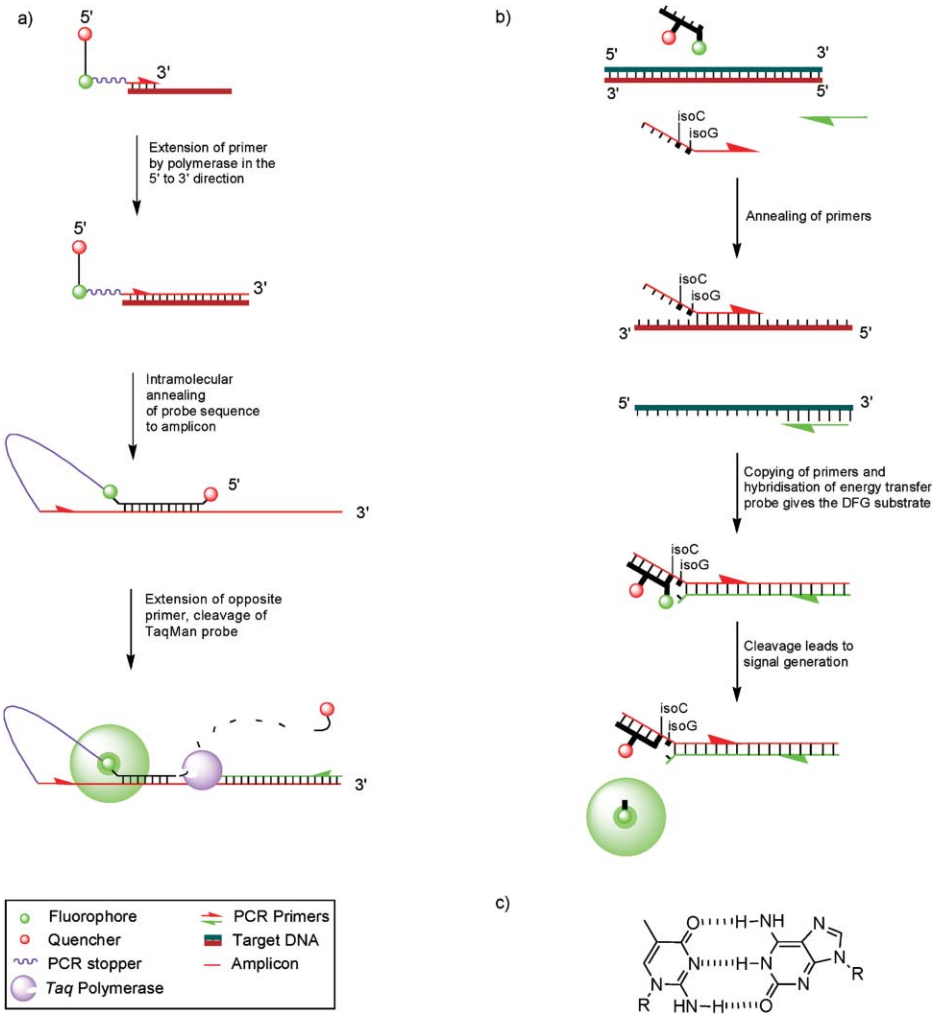


Fig. 6 Mode of action of (a) intramolecular TaqMan®;[40] (b) AEGIS probes;[43] and (c) the structure of the isoC:isoG base pair.

There is one major disadvantage with probes cleaved upon hybridisation; the probes are destroyed in order to generate a signal, so no further information in the form of probe-target melting temperature (T_m data) can be obtained after PCR. Such data can be very useful, particularly in SNP analysis and mutation detection.

Competitive hybridisation probes

Competitive hybridisation requires the use of a probe system with two dyes, one a fluorophore and the other a fluorescence quencher, which are held in close proximity by the formation of a duplex. The duplex may be inter- or intramolecular. PCR is used to amplify a fragment of DNA to be interrogated. A dye-labelled probe, which is complementary to a region of the amplicon is required. This is designed so that it can form a duplex with the amplicon that is more stable than the quenched probe system. Upon heating and cooling in PCR the probe hybridises to the amplicon, the fluorophore and quencher become distal, and a fluorescent signal is obtained. In the absence of the target DNA (amplicon) the probe refolds into its quenched form. In contrast to systems in which the probe oligonucleotide is cleaved, competitive hybridisation offers the advantage that fluorescence melting curves can be obtained at the end of the PCR.

Molecular Beacons are the most well studied example of competitive hybridisation probes. They are designed so that at either end there is a small region of DNA (~5–8 bases), which forms a double-stranded stem. The fluorophore and quencher are covalently attached to either end of the stem (*i.e.* at the 5′- and 3′-ends of the oligonucleotide). When the stem is intact the two dyes are held close together and fluorescence quenching occurs. Molecular Beacons are designed to form a stable hairpin-loop at the temperature where fluorescence is measured during real time PCR (~60 °C).

When the amplicon is present, the probe sequence will anneal to the target, in preference to formation of the hairpin-loop. To ensure this the probe sequence is much longer than the stem (~25–30 nt), so that formation of the beacon–target hybrid is thermodynamically favoured over the reformation of the stem-loop. Since in this open conformation the fluorophore and quencher do not lie in close proximity, an increase in emission from the fluorophore occurs—indicating that the target DNA sequence is present (Fig. 7a). Molecular Beacons have been used in many homogenous nucleic acid detection assays—DNA in genotyping of human alleles,[44] and drug

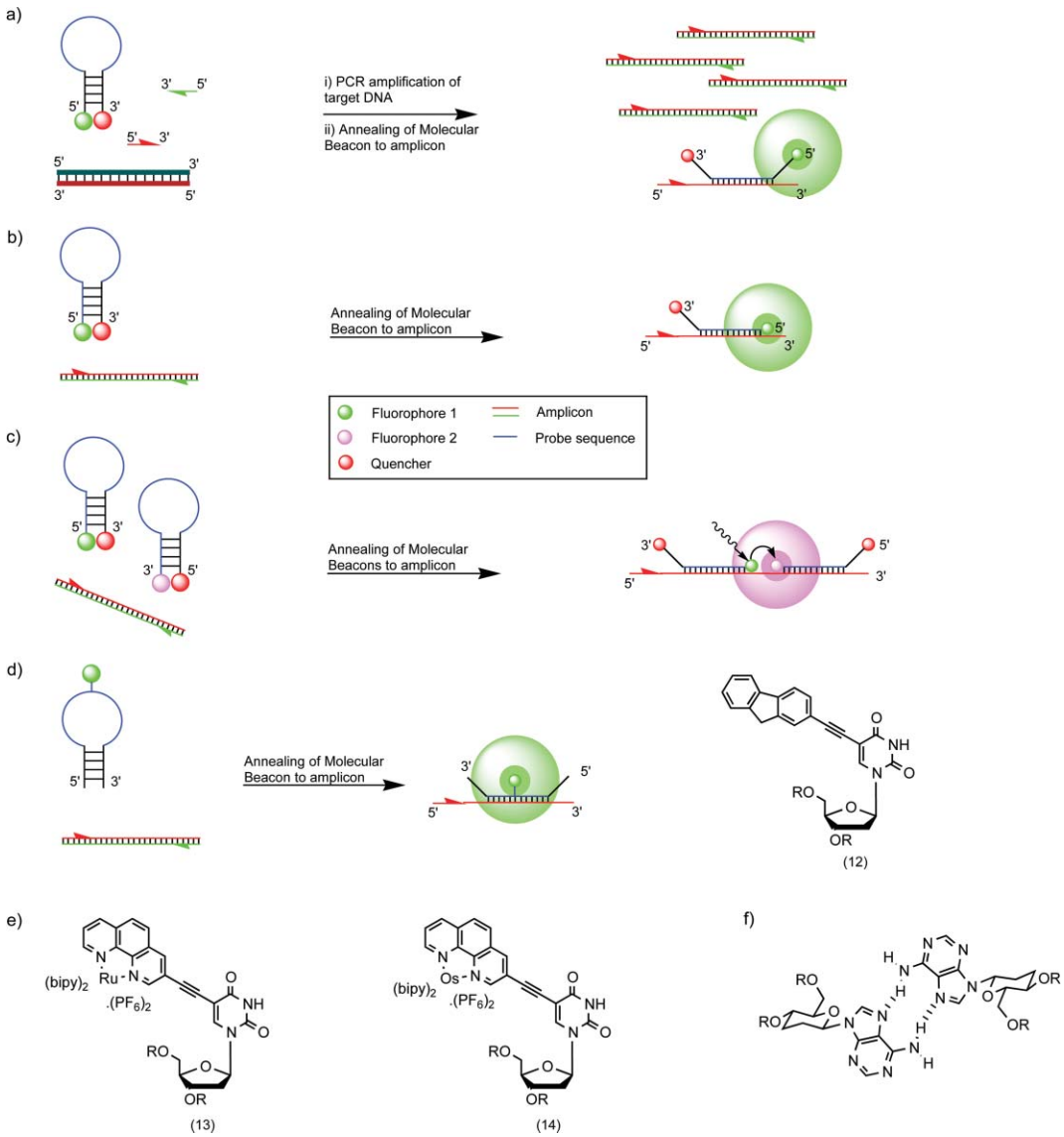


Fig. 7 The mechanism of action of (a) Molecular Beacons;[54] (b) shared stem Molecular Beacons;[52] (c) energy transfer Molecular Beacons;[53] (d) quencher-free Molecular Beacons labelled with fluorene-derivatised fluorescent nucleoside **12**;[55] (e) metallated nucleosides **13** and **14**, used to label 'metallo-beacons';[56] and (f) homo-DNA A:A base pair, used to synthesise Molecular Beacon stems which are orthogonal in pairing nature to the probe sequence.[50]

resistance in *Mycobacterium Tuberculosis*,[45] and RNA in detection of splice variants,[46] quantitation of viral loads[47] and detection of *Potato leafroll virus* and *Potato virus Y*.[48]

This assay has one particular drawback: the formation of a probe–target hybrid is a *bimolecular* process whereas reformation of the beacon stem-loop is *intramolecular* and thus is kinetically and entropically favoured.[49] Consequently there is a tendency for the fluorescent signal to decay rapidly as the beacon falls off its target and refolds. In addition, since the fluorophore and quencher remain in the same oligonucleotide in the open form, residual quenching is observed, so the intensity of the fluorescent signal is limited. Furthermore, if not carefully designed, the stem flanking the probe sequence can hybridise to the target, negating destabilisation by single mismatches and leading to false positive results. In order to eliminate the likelihood of stem sequences binding to the target, Molecular Beacons containing homo-DNA stems have been synthesised. Homo-DNA nucleotides form reversed-Hoogsteen A:A pairs with each other (Fig. 7f), but do not base pair with natural DNA, so do not hybridise to target sequences. This leads to greater hybridisation specificity.[50] Various modifications have been made to Molecular Beacons to improve their sensitivity. Molecular Beacons constructed from PNA have been used to enhance hybridisation.[51] "Shared stem" Molecular Beacons, where one half of the stem hybridises to the target, have been used to improve hybridisation and disfavour stem reformation (Fig. 7b),[52] and energy transfer Molecular Beacons have been used, where a donor and acceptor beacon hybridise adjacent to each other (Fig. 7c).[53]

As with other formats, synthetic chemistry has provided access to a range of new fluorescent labels, which in turn have

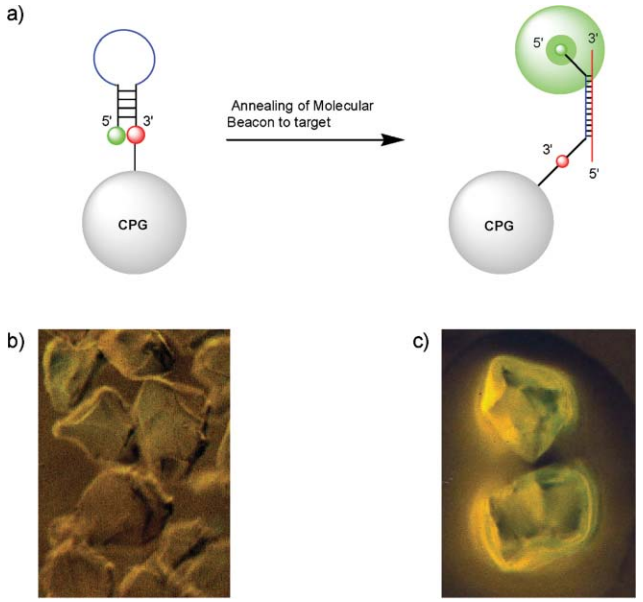


Fig. 8 Molecular Beacons attached to CPG. (a) Representation of fluorescence enhancement on hybridisation; (b) fluorescence micrograph of CPG-immobilised Molecular Beacon in the absence of target; and (c) after the addition of complement. Adapted from ref. 58.

allowed further refinement of genetic probes. The fluorene-labelled nucleoside **12** has allowed the design of quencher-free beacons. When these molecules are hybridised to matched targets, fluorescence is enhanced relative to when they are hybridised to mismatched targets (Fig. 7d).[55] The fluorescence of the fluorene moiety is quenched in mismatched duplexes, relative to the single strand, and enhanced in fully complementary duplexes. Joshi and Tor have utilised nucleosides containing a 1,10-phenanthroline moiety to co-ordinate Ru^{II} and Os^{II} cations as reporters and quenchers in 'metallo-beacons' (Fig. 7e).[56] The large Stokes shifts of co-ordination compounds such as these make them potentially very valuable tools in multiplex assays, where several dyes must be discretely monitored. Molecular Beacons have been labelled with CdSe quantum dots, which are theoretically superior to organic fluorophores due to their high quantum yields, stability to photobleaching and tunable emission wavelengths.[57]

The simple concept of Molecular Beacons has found use outside the field of homogenous genetic analysis. Molecular Beacons immobilised on an array have the potential to probe unlabelled nucleic acids, allowing the fluorescently-labelled oligonucleotide (*i.e.* the Molecular Beacon) to be re-used. Detection by Molecular Beacons immobilised on controlled pore glass (CPG, Fig. 8), and on arrays, has been demonstrated.[58,59] Cell-permeability of Molecular Beacons has been shown, and tRNA-linked Molecular Beacons have been used to image complementary mRNAs in cytoplasm.[60]

"Scorpion" is the name given to a detection system that combines a probe sequence and a PCR primer element in a single oligonucleotide.[61] Scorpions are not considered to be primer-probes due to the inclusion of a PCR stopper, which prevents copying of the stem upon extension of the reverse primer. This results in specific probing of the amplicon, as distinct from primer-probes, where accumulation of the signal is not strictly sequence-specific.

Scorpions incorporate a stem-loop section, as in Molecular Beacons. They are designed such that PCR extension of the Scorpion results in the formation of a DNA strand containing a sequence that is complementary to the probe sequence. This sequence is longer than that of the stem and hence its binding is favoured over reformation of the stem. Consequently the Scorpion undergoes a conformational reorganisation, and a fluorescent signal is produced (Fig. 9).

Since the probe and target sequences are in the same molecule, issues regarding unimolecular *vs.* bimolecular kinetics are eliminated and the Scorpion system has advantages over other assays. The speed of the unimolecular probing event enables rapid signal generation and high signal to noise ratios because probe–target binding is kinetically favoured over duplex reannealing and thermodynamically favoured over stem reformation. Also, unlike TaqMan®, the assay does not rely on an enzymatic cleavage for signal generation. ARMS PCR can be used to effect allelic discrimination,[63] but the polymorphic sequence of interest is commonly located within the probe sequence, enabling thermodynamic discrimination of SNPs.[62] The disadvantages associated with Scorpions are that the probes are long and complex (~65 nt), and rely on secondary structure for their operation. Both of these factors introduce complications into the design and synthesis of Scorpions. Despite these, Scorpions have been used to effect the quantitation of HIV-1 DNA/RNA,[64] and mRNAs linked to breast cancer[65] and muscular dystrophy.[46]

To overcome some of the difficulties associated with Scorpions, open (or "duplex") Scorpions have been developed. In this embodiment the Scorpion is comprised of a 5′-fluorophore, PCR blocker and primer sequence. A separate quencher oligonucleotide is used which is complementary to the probe element of the Scorpion and has a suitable quencher moiety attached. Upon PCR extension, the probe element of the

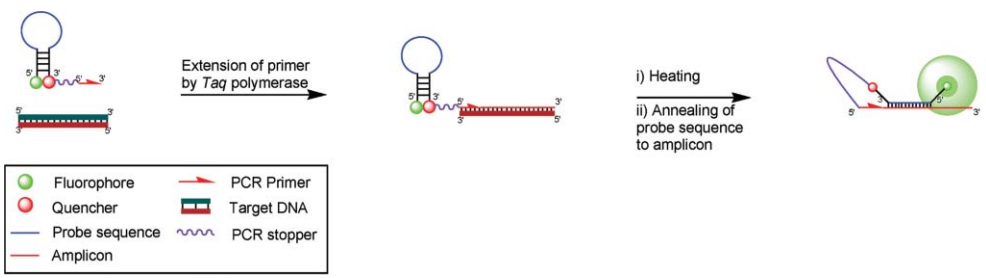


Fig. 9 Generation of a fluorescent signal from a Scorpion primer.[62]

Scorpion binds to its complement in the amplicon, as in stem-loop Scorpions. However, this *intramolecular* structure has only to compete with the reformation of an *intermolecular* duplex between the Scorpion and the quencher oligonucleotide. As the quencher moiety is in a separate oligonucleotide, Open Scorpions have the potential to produce more intense fluorescent signals than conventional or "closed" Scorpions (Fig. 10a). Open Scorpions are simpler to synthesise than closed Scorpions: the Scorpion contains a single fluorescent dye and has no inherent secondary structure; the quencher oligonucleotide contains one quencher and the total length of each oligonucleotide is reduced to 10–40 nt as opposed to 60 nt or more for closed Scorpions.[66]

Subsequent to the development of duplex Scorpions, related detection systems 'Yin–Yang'[24] and duplex probes have been reported. 'Yin–Yang' probes (Fig. 10b) differ from duplex Scorpions in that they have no primer element. They have some similarity to Molecular Beacons, so hybridisation is an intermolecular process. The quencher oligonucleotide is shorter than the dye-labelled probing strand, so hybridisation can occur spontaneously, facilitating its use in isothermal amplification protocols. Duplex probes (Fig. 10c) are similar to 'Yin–Yang' probes, except that the two probing oligonucleotides are of similar length and the cycling conditions are designed to promote exonucleolytic cleavage of the probe.[67]

Competitive hybridisation is a powerful technique, less susceptible to spurious amplicons than primer-probes and not requiring enzymatic cleavage for signal generation. However, purification of oligonucleotides with secondary structure (as in Molecular Beacons and closed Scorpions) can be difficult, although the 'two-oligonucleotide' versions of these described above alleviate this problem. Sensitivity can be limited by the nature of competitive hybridisation, as binding to the target is disfavoured by the presence of a complementary sequence elsewhere in the system. This has a hidden benefit though—specificity of hybridisation by hairpin oligonucleotides is enhanced over their linear counterparts,[68] and this is likely to also be the case for duplex structures.

Linear probes that fluoresce upon hybridisation

While primer-probes, cleaved oligonucleotides and competitive hybridisation probes have been used extensively in real time PCR, linear probes that fluoresce upon hybridisation have potential advantages over all of these techniques. Non-competitive hybridisation offers the potential for shorter and more specific oligonucleotides to be used, and non-destructive signal generation allows extra information to be obtained from post-PCR melting curves. A PCR stopper, such as a phosphate group is required at the 3′-end to prevent the probe from acting as a PCR primer and giving rise to a non-specific signal. This is a very simple and inexpensive chemical modification.

Labelling of a linear oligonucleotide with a single fluorophore is a conceptually attractive approach, since the probe can be synthesised and purified cheaply and easily. Fluorescence polarisation is observed to increase upon hybridisation of the fluorescent probe to the target DNA due to the formation of high molecular weight species which have restricted molecular motion, and this phenomenon has been used in genotyping.[69] This technique requires sophisticated equipment, which is not as widely available as thermocyclers that simply measure fluorescence intensity. Simple fluorescently-labelled probes exhibit changes in fluorescence upon hybridisation to target DNA that are potentially exploitable in genetic analysis. However, the fluorescence changes are unpredictable, with varying degrees of quenching or fluorescence enhancement depending on the base sequence, nature of the fluorescent dye and the dye attachment chemistry. HyBeacons (Fig. 11a)[70] circumvent this problem as they exploit modes of dye-incorporation that reliably lead to fluorescence *enhancement* on hybridisation. Fluorescein is positioned in the major or minor groove by conjugation to

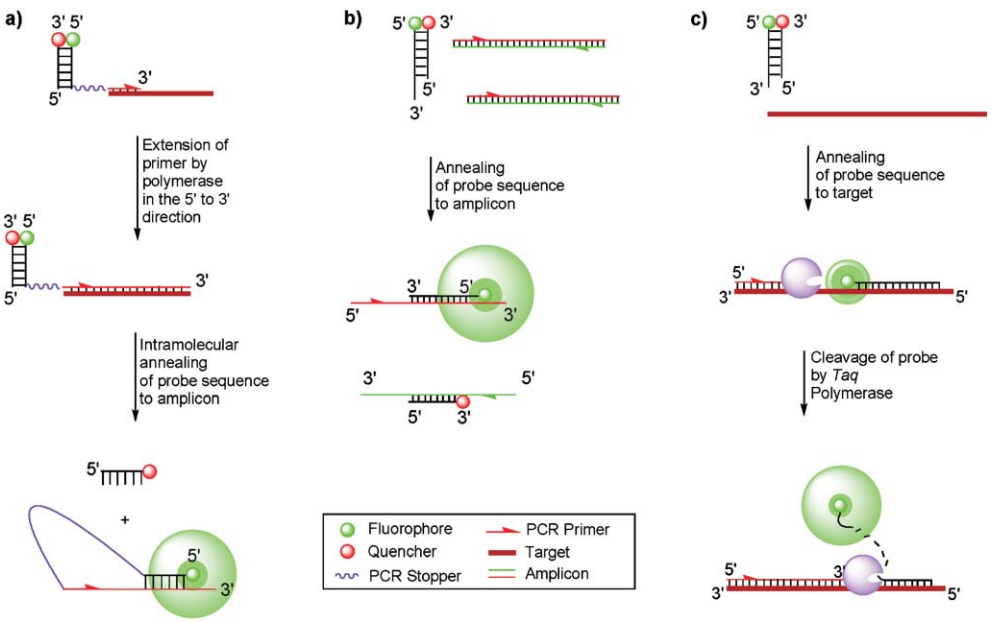


Fig. 10 (a) The duplex Scorpion;[66] (b) 'Yin–Yang';[24] and (c) duplex probe[67] formats.

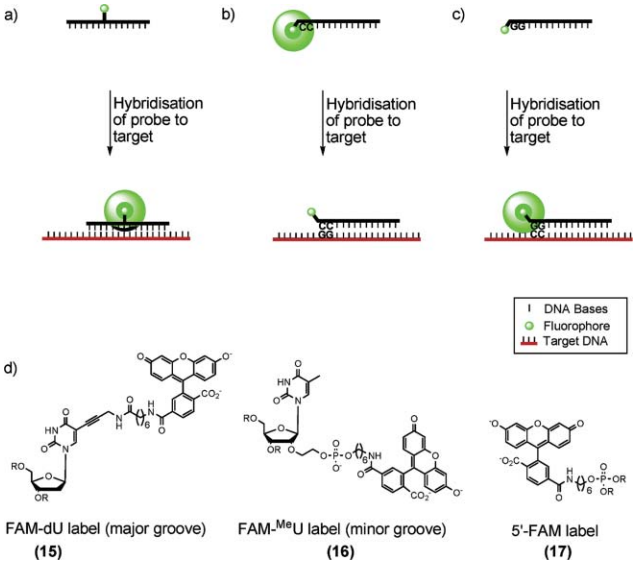


Fig. 11 Mode of action of (a) HyBeacons;[70,71] (b) fluorescein quenching probes;[74] (c) dequenching probes;[75] and (d) fluorescein labels used in these probe formats.

either the base[70] or the 2′-position of ribose.[71] HyBeacons have been used to detect and discriminate polymorphic targets amplified directly from saliva.[72]

Fluorescence quenching by nucleobases is a well known phenomenon, with guanine quenching particularly effective due to its good electron-donating properties.[73] Two fluorescent probing technologies utilise this to achieve reliable quenching or dequenching upon hybridisation. Fluorescein quenching can be achieved by positioning two contiguous cytosines next to the fluorophore in the probe. Hybridisation leads to the fluorophore being in close proximity to two guanines in the complementary strand, leading to a decrease in fluorescence (Fig. 11b). This methodology has been used in real time PCR to detect and discriminate between polymorphic amplicons by amplification curves and melting curve (T_m) analysis.[74] Target sequences with one guanine in the 5′-overhang position also give rise to detectable quenching, enabling this method to be applicable to almost all sequences. Inversion of the real time fluorescence trace is necessary for calculation of C_t, but this is a simple operation. Dequenching can also be accomplished, by placing two guanines next to the fluorophore in the probe. Internal quenching is relieved upon hybridisation, leading to signal generation (Fig. 11c). Using this methodology, melting curve analysis was used to score a number of SNPs.[75] As with the fluorescein quenching technique, the oligonucleotides are relatively cheap and easy to synthesise, using widely available reagents (Fig. 11d).

FRET can be used as a signalling mechanism in oligonucleotide probes. The donor dye must be brought close to the acceptor upon hybridisation to initiate energy transfer.[76]

A "hybridisation probe" assay of this type has been developed using a pair of labelled oligonucleotides. One probe is labelled with a fluorescent dye at its 3′-end (the donor dye), and the other is labelled with an acceptor dye at its 5′-end. The oligonucleotides are designed so that they hybridise to the amplified DNA fragment at adjacent sites, and thus the dyes are conveniently placed for FRET to occur (Fig. 12). The fluorophores can be organic dyes such as fluorescein and LC Red 640,[77] but the use of luminescent europium[78,79] or terbium[80] chelates as LRET (luminescence resonance energy transfer) donors has also been described.

The donor dye does not have to be attached to an oligonucleotide. It can be a DNA intercalator, which in the presence of single-stranded DNA remains free in solution, but binds to the duplex formed by hybridisation of the probe to its target. This gives rise to FRET, and an increase in fluorescence is observed from the reporter (acceptor) dye. Methods that utilise this principle are known as ResonSense®[81] or iFRET[82] (Fig. 13a). This methodology has the advantage that just one labelled probe has to be synthesised. An intramolecular version of ResonSense®, Angler®, has also been reported (Fig. 13b).[81]

Similarly, if one primer is labelled with a donor fluorophore, an acceptor-labelled probe can be excited by hybridisation to the labelled strand of the amplicon. This approach is used in PriProEt probes (Fig. 13c), which have been used to detect the Foot and Mouth disease virus.[83]

Intercalator-labelled probes

More elaborate chemistry can also provide probes whose secondary structure does not change upon hybridisation, but whose fluorescence is enhanced. Intercalators often display enhanced fluorescence quantum yields upon binding to dsDNA, and many probes contain tethered intercalators in order to harness this effect.[84–87] These properties have been exploited in light-up probes, PNA oligomers functionalised with the asymmetric cyanine dye, thiazole orange (Fig. 14).[84,88,89]

Thiazole orange (TO) in free solution binds to calf thymus DNA with an enhancement of quantum yield from 2×10^{-4} to 0.11, a 550-fold increase.[90] To minimise electrostatic interaction of the cationic dye with the phosphates of oligonucleotides the probe is assembled from PNA, leading to a 2.2 to 6.0-fold increase in quantum yield upon hybridisation.[88] Light-up probes have been used to detect PCR products from the human β-actin gene and the *invA* gene of *Salmonella*.[89] More recently, PNA-oligomers internally labelled with TO

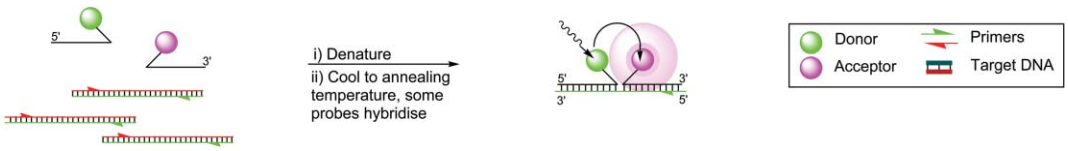


Fig. 12 Mode of action of hybridisation probes.

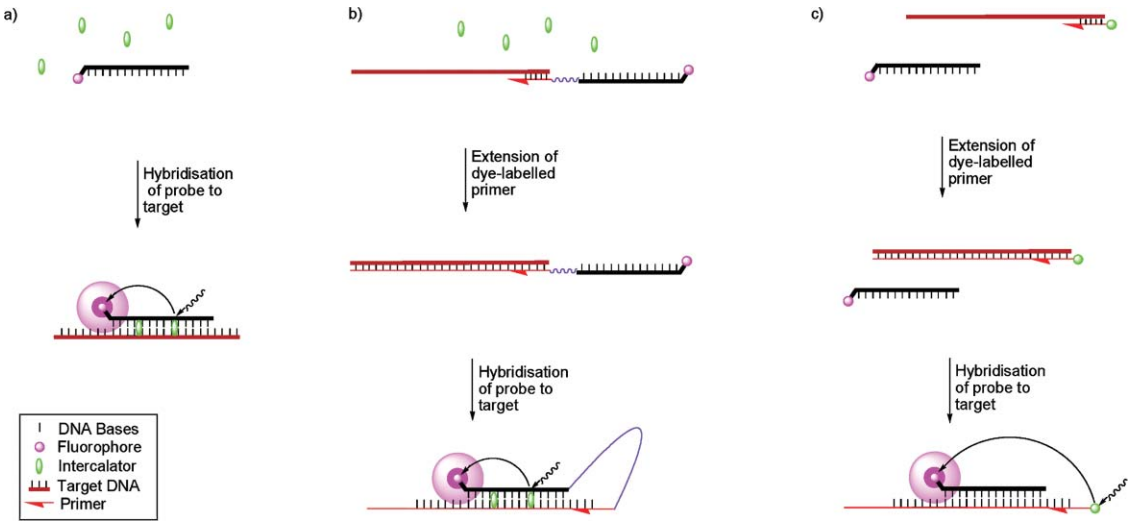


Fig. 13 (a) ResonSense®[81]/iFRET;[82] (b) Angler®;[81]and (c) PriProEt;[83] formats.

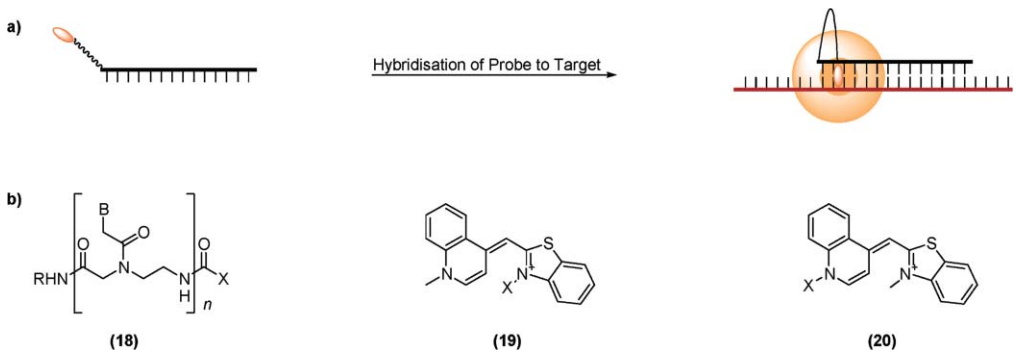


Fig. 14 (a) Mode of action and (b) chemical structure of light-up probes. B denotes nucleobases and R is H, lysinyl amide, or lys-lysinyl amide. X denotes the linker, which is either $(CH_2)_5NHCO(CH_2)_5$, or $(CH_2)_y$ where y is 5 or 10. The linker is attached either to the quinoline or benzothiazole nitrogen of thiazole orange.

have been used to detect hybridisation under non-stringent conditions. Placing TO adjacent to the polymorphic site led to enhanced fluorescence when the PNA probe was fully complementary to the target, but to a lesser degree when mismatched, the effect being attributed to disruption of TO intercalation next to mismatches.[91,92]

Yamana and co-workers have described a series of intercalator-labelled oligonucleotides to detect DNA and RNA sequences.[85,86,93–97] The fluorophores pyrene and anthracene were attached to the 2′-oxygen of RNA probes by a methylene bridge (Fig. 15a and 15b). Quenching of pyrene fluorescence by the nucleobases is relieved upon formation of an RNA–RNA duplex, leading to an increase in quantum yield of 14 to 33.9-fold.[95] Importantly, the phenomenon does not occur on hybridisation of an RNA probe to DNA sequences, or the hybridisation of a DNA probe to DNA or RNA sequences. This means that probes labelled in this way cannot be used in PCR based genetic analysis techniques. Anthracene-labelled oligodeoxynucleotides also showed an increase in fluorescence upon duplex formation with RNA but not DNA.[97]

Bis-pyrene modified oligonucleotides labelled at the 5′-terminus (Fig. 15c) *do* show enhanced fluorescence upon hybridisation to the complementary DNA sequence. The authors attributed this to excimer formation upon duplex formation, which is interrupted by one or both pyrene residues interacting with nucleobases in the single strand.[86]

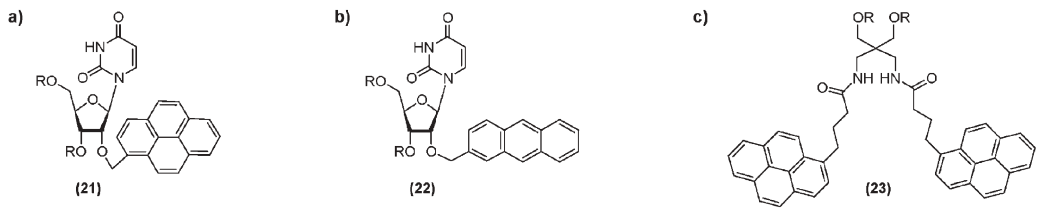


Fig. 15 Intercalator-labelled probes described by Yamana *et al.*[85,93,95–98]

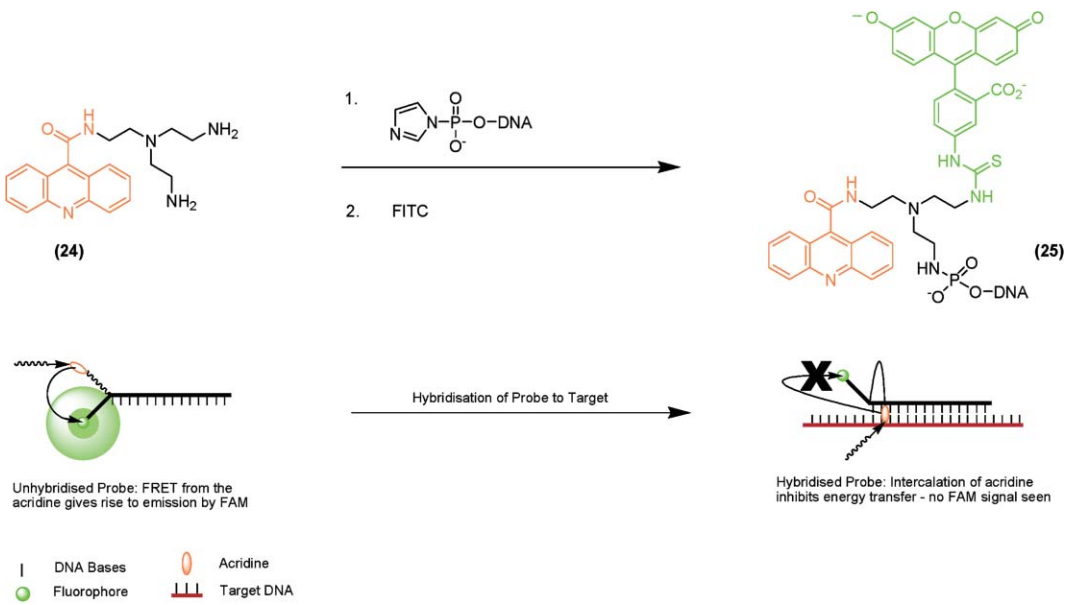


Fig. 16 (a) Labelling; and (b) mode of action of FRET fluorophore-intercalator probes.[99]

Intercalators can also be used in combination with conventional fluorophores. Shinozuka *et al.* post-synthetically modified DNA probes with an acridine–fluorescein pair (Fig. 16).[99] The acridine is a donor in a FRET system, exciting the reporter, fluorescein. Upon hybridisation, the acridine becomes less efficient in energy transfer and the fluorescence of fluorescein is reduced (Fig. 16).

Two probes were subsequently described that use intercalators as fluorescence quenchers. Interaction between fluorescein and either an acridine or pyrene quencher gives rise to quenching of fluorescein in the single strand, which is removed upon hybridisation by intercalation of the quencher (Fig. 17).[100,101]

Fluorescent nucleobase analogues

Oligonucleotide probes labelled with traditional organic fluorophores typically rely on thermodynamic (T_m) discrimination of

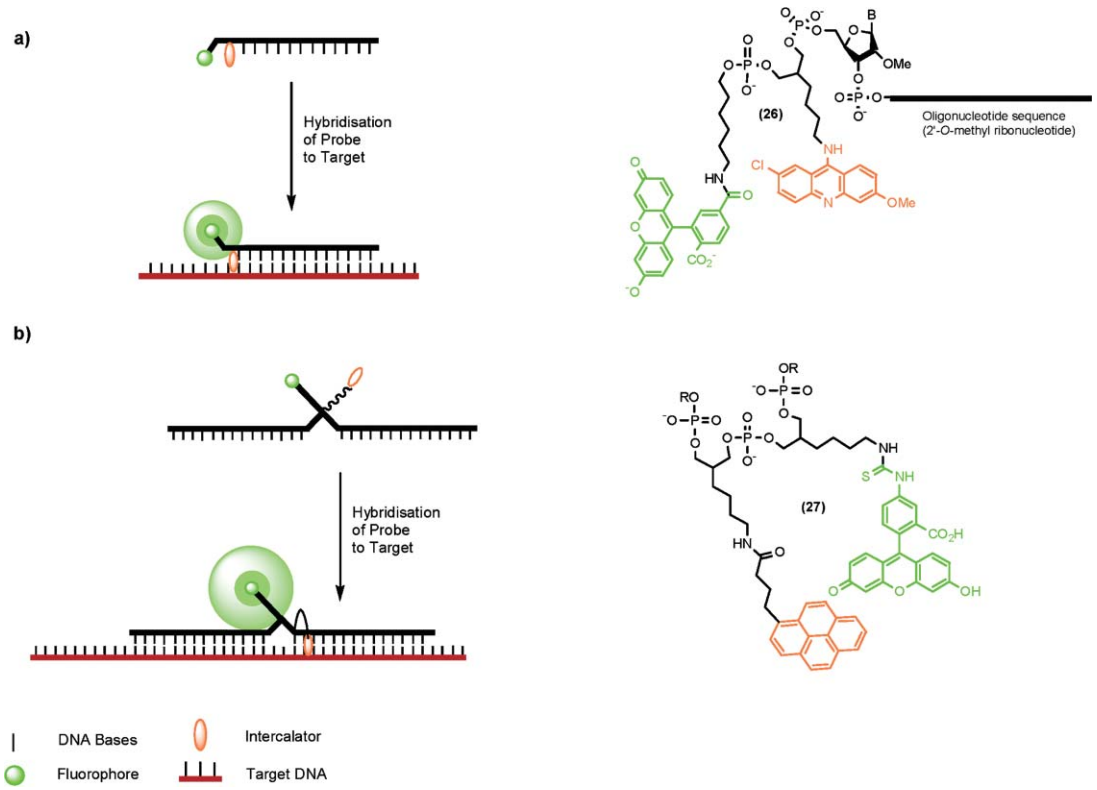


Fig. 17 Mode of action and chemical structures of (a) fluorophore-intercalator probes;[100] and (b) MagiProbe.[101]

perfectly matched sequences from mismatched complements. Fluorescence is measured at a temperature above the T_m of mismatched probe–target duplexes, but below that of the fully complementary probe–target duplex, ensuring a signal is emitted only from the latter. This can sometimes be difficult to achieve in cases where a single mismatch is present in the duplex. If the signalling moiety is a fluorescent nucleobase, the emission intensity may depend only on the local environment of the nucleobase and may therefore allow discrimination under non-stringent hybridisation conditions where both matched and mismatched duplexes are stable. This property would be important for arrays of immobilised probes, since conditions under which all mismatched probe–target duplexes are less stable than Watson–Crick duplexes are unlikely to be obtained. Comprehensive reviews of the properties and applications of fluorescent nucleobase analogues are available.[102] Here we concentrate on the analogues most applicable to genetic analysis (Fig. 18).

A series of base-discriminating fluorescent (BDF) nucleobases have been synthesised for use in genetic analysis. Purine analogues methoxybenzodeazainosine (^{MD}I, **28**) and methoxybenzodeazaadenosine (^{MD}A, **29**) become brightly fluorescent when mispaired in a duplex with C or T respectively, but not when involved in Watson–Crick base pairs or other mismatches. These nucleosides have been incorporated into oligonucleotides and can be used to detect synthetic oligonucleotides.[103] Although they are potentially useful labels, the emission wavelengths of ~430 nm are not detectable by standard genetic analysis platforms which are designed to detect fluorescein at 520 nm. The extended chromophore of naphthodeazaadenine (NDA, **30**) allowed excitation of fluorescein by FRET, although as with ^{MD}I and ^{MD}A, significant quenching by adjacent G:C base pairs was observed, limiting its applicability to genetic analysis methodologies.[104]

The sensitivity of pyrene fluorescence emission to the polarity of the local environment has been used in this context. Pyrene fluorescence is quenched in low polarity environments, and enhanced in high polarity environments, such as aqueous solution. The pyrenyl-modified 7-deazaadenine nucleotide ^{Py}A (**31**)[105] exhibits drastically quenched fluorescence when matched to thymidine due to intercalation into the resultant duplex, leading to a low polarity environment, whereas the fluorescence of ^{AMPy}U (**32**),[106] ^{Py}C (**34**) and ^{Py}U (**33**)[107] are enhanced upon formation of a Watson–Crick base pair, since the fluorophore is projected into the solvated major groove by the rigid acetylenic linker. Similarly, 1-ethynylpyrene substituted analogues of A (**35**), G, C and T have been shown to become more brightly fluorescent upon hybridisation.[108] These compounds were prepared on the DNA synthesis column by Sonogashira coupling between 1-ethynylpyrene and the appropriate 8-bromopurine or 5-iodopyrimidine nucleosides, which were in turn incorporated using their commercially available phosphoramidite monomers.

The fluorescent cytosine analogue 6-methyl-3*H*-pyrrolo[2,3-*d*]pyrimidin-2-one (pyrrolo dC, **36**)[109] is quenched when paired with G relative to single-stranded DNA and when mismatched, and therefore displays the properties required of a BDF nucleobase. The pyrrolo dC chromophore is unsuitable for use with equipment designed for fluorescein monitoring (λ_{ex} 345 nm, λ_{em} 473 nm),[109] but synthetic derivatisation has yielded the red-shifted *p*-nitrophenyl-substituted compound **37** (λ_{ex} 403 nm, λ_{em} 572 nm), although its hybridisation properties are as yet unknown.[110] The applicability of BDF nucleobases to genetic analysis may depend on their being modified to emit in this more spectroscopically useful region. However, in the

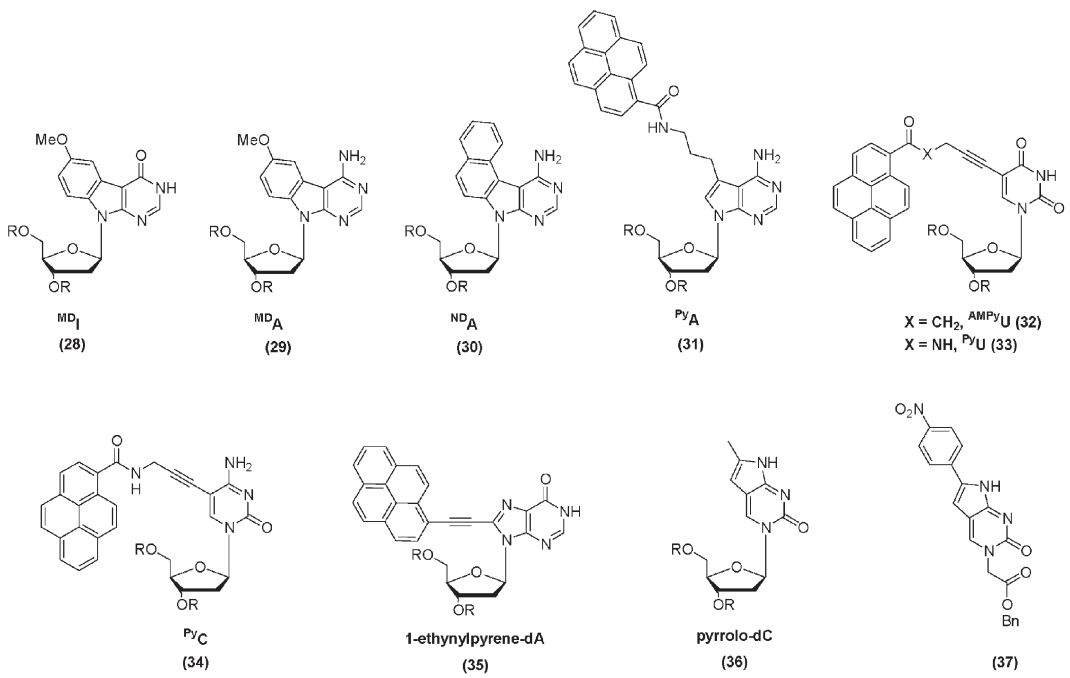
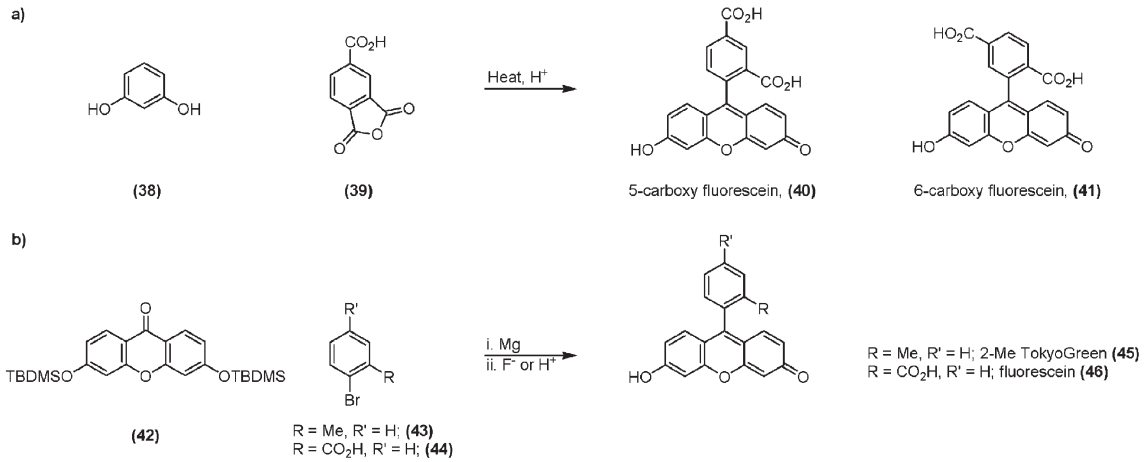


Fig. 18 Fluorescent nucleobase analogues.



Scheme 1 (a) Traditional method of xanthene dye synthesis; (b) method reported by Urano *et al*.[112]

future it is likely that inexpensive genetic analysis platforms will be developed to exploit the spectral region from 350 nm to 500 nm. If this is the case a whole range of existing fluorescent dyes will be exploited.

Conclusions

This review has highlighted the many ways by which fluorescently-labelled synthetic oligonucleotides can be used as molecular signalling devices in genetic analysis and related applications. The proliferation of such devices is testimony to the research efforts that have been expended in their development. Fluorescent PCR based methods of DNA sequence analysis have been widely used in research, and are now accepted technologies in clinical diagnostic laboratories. In the future the underlying synthetic and physical chemistry will find applications in other fields, in particular nanotechnology.

Research into new methods of DNA analysis continues, and synthetic chemistry is key to these advances. Improvements in synthetic methods for the synthesis of fluorescently-labelled oligonucleotides will reduce the cost of genetic analysis. Some reduction in cost will result from the economies of scale: synthetic unlabelled oligonucleotides have fallen in price from ~£200 per synthesis in the late 1980s to less than £10 currently. During this period, only minor changes have been made to the chemical processes involved in oligonucleotide synthesis, although more sustainable, 'green' methodologies have been sought after for production of antisense oligonucleotides on the kilogram scale.[111] In addition to the application of these principles improved syntheses of dyes may reduce the cost of fluorescent labels, and provide access to spectroscopically 'tunable' reporter molecules useful for multiplexing. A recent report of an improved synthesis of xanthene dyes such as fluorescein yielded the new dye 2-Me TokyoGreen (**45**), in which a methyl group replaces the carboxy group of fluorescein. This method produces isomerically pure xanthene dyes in high yields, and is therefore superior to the condensation of resorcinol and substituted phthalic anhydrides currently used (Scheme 1).[112] This is a fascinating example of a 21st century improvement on synthetic methods which have their origins in the 19th century.

In the future useful novel probe technologies are most likely to be the product of highly innovative chemistry, as with the fluorescently-labelled non-nucleotidic polyamide minor groove binding probe described by Rucker *et al*.[113] These probes are truly non-competitive, since they bind to double-stranded DNA, and do not require displacement of one strand of a PCR amplicon for binding.

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