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Review

DNA-based methods for food authentication

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A variety of methods exist whereby the residual DNA content of food material can be used to unequivocally identify the nature of the product. Various techniques are described and discussed, mainly with reference to meat and fish. An attempt has been made to collate the species identified, comprehensively but not exhaustively. Emerging technologies have the potential to simplify the protocols involved with food authentication testing in the future, although the uptake of these by the food industry is liable to be limited by factors such as cost.

Recent consumer concern relating to a variety of issues, such as BSE and genetic modification, has resulted in increased awareness regarding the composition of food products. The identity of ingredients in processed or composite mixtures is not always readily apparent and verification that the components are authentic and from sources acceptable to the consumer may be required.

In the past, food authentication routinely involved the detection of species-specific proteins, when attempting to discern the origins of material for human consumption [1]. Such tests employed a variety of immunological and electrophoretic methods, but they were not without their problems. As a result of heating, the processing of foodstuffs can cause denaturation of the proteins under study and, in addition, protein expression is usually tissue dependent [2]. Furthermore, as the majority of commercial methods have been designed to detect plasma proteins, it has also been argued that adventitious contamination of meat with blood from other species could lead to spurious results [3]. Attempts have been made to study processed material by raising antibodies to heat resistant antigens [4–6] or subjecting reactivated enzymes to isoelectric focusing [7]. Increasingly, however, attention has now turned to DNA as a source of information.

As DNA is more thermostable than many proteins, analyses using nucleic acid are less liable to be disrupted by processing of foodstuffs. Furthermore, DNA is present in the majority of the cells of an organism, potentially enabling identical information to be obtained from any appropriate sample from the same source, regardless of the tissue of origin. Additionally, through the acquisition of sequence data, DNA can potentially provide more information than protein, due to the degeneracy of the genetic code and the presence of many non-coding regions. Furthermore, driven by the clinical arena, nucleic acid-based technologies are developing rapidly and the informed adoption of suitable methods by the food industry has the potential to greatly simplify methods of authentication.

DNA hybridization

Initial studies using DNA to detect meat species used relatively simple methods, whereby labelled DNA probes were hybridized to samples of genomic DNA covalently attached to nylon membranes in a slot- or dot-blot format [8–11]. The experimental format for such a test is shown in Fig. 1. In this most basic regime, it was shown that under conditions of appropriate stringency and for certain species, probes comprising labelled total genomic DNA from a given species would hybridize to DNA from the same species with little cross-reactivity. The preparation of such probes did not require any prior knowledge as to the precise nature of the DNA sequences under study. Species-specific binding of the probes to targets was believed to result from the hybridization of complementary repetitive sequences [10]. These are tandemly-arranged and found throughout the genome as 'satellite' sequences.

Using such methods, samples of chicken and pork [10], DNA extracted from cooked meats and commercial products [8, 10, 11], and pork in pork/beef admixtures [9]

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were unequivocally identified. Probes for goat, sheep and beef were demonstrated albeit, with some degree of cross-reactivity [10, 12]. Such results were liable to be a reflection of the varying degrees of homology in the sequences of the satellite DNA in the different species.

Although cross-hybridization between probe and DNA sequences from closely-related species was shown to be reduced by the addition of unlabelled DNA from the cross-hybridizing species, adequate differentiation between sheep and goats remained elusive [12]. Indeed, sequence data had previously shown that the satellite DNA of these two species is highly homologous [13] and specificity was improved by using probes derived from published satellite sequence information. Winterø *et al.* [9] compared a probe based on a 2.7 kb porcine-specific satellite fragment [14] with labelled total genomic DNA, and found the former to be more specific. The use of further species-specific satellite DNA probes has subsequently been described and unequivocal identification of meat from cattle, deer, pig, chicken, turkey, rabbit, sheep and goat has been accomplished in raw products [3, 15, 16]. The presence of different species in admixtures and a wide range of commercially processed, heated and canned products has been demonstrated [3, 16]. Additionally, DNA hybridisation has also been used to identify cattle of different breeds [17], although different probes were used here. As the species-specific probes tend to be relatively short oligonucleotides of less than 100 bases, hybridization is possible even after a considerable amount of DNA degradation has occurred.

An element of quantification can also be introduced into food testing by DNA hybridisation, by determining the signal intensity associated with bound, labelled probe [3, 9, 11, 12, 16]. However, a degree of caution should be

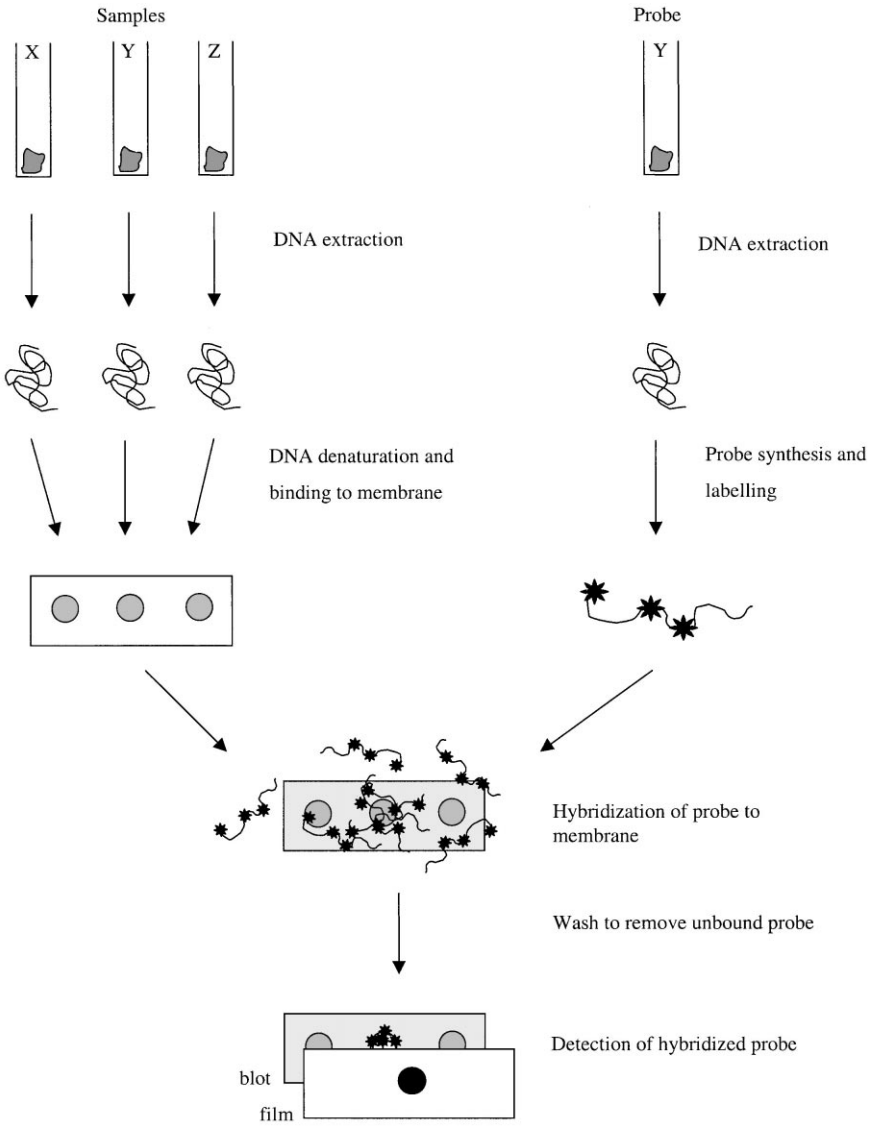

Fig. 1. The basic experimental format for testing a sample by DNA hybridization. Three samples are shown, species X, Y and Z, being tested against a probe prepared from species Y.

exercised as the signal strength can be influenced by factors such as the tissue of origin of the sample or the way in which it has been processed [15].

Methods based on the polymerase chain reaction

DNA testing by hybridization is relatively cumbersome and other DNA-based approaches to species identification have been described, relying on the use of the polymerase chain reaction (PCR) [18]. Here, short synthetic oligonucleotide primers anneal to complementary strands of the DNA duplex and direct the synthesis of millions of copies of the fragment of DNA they have been chosen to flank. The amplification products (or amplicons) can then be analysed further by a variety of methods, as described below.

Sequencing of PCR products based on (mitochondrial) DNA

The most direct means of obtaining information from PCR products is by sequencing. Information thus obtained has been used to identify the animal of origin of various meats [19–23]. Such work has tended to concentrate on the amplification of mitochondrial DNA sequences, generally the cytochrome b gene. Since its use in determining phylogenetic relationships in vertebrates was first described [24], a wealth of sequence data for this gene has been documented.

Mitochondrial DNA possesses several advantages over nuclear DNA for studies of speciation in meat products. It is relatively more abundant in total nucleic acid preparations than nuclear DNA, with the copy number of the mitochondrial genome exceeding that of the nuclear genome several fold [25]. Mitochondrial DNA tends to be maternally inherited so that individuals normally possess only one allele and thus sequence ambiguities from heterozygous genotypes are generally avoided. The relatively high mutation rate compared to nuclear genes has tended to result in the accumulation of enough point mutations to allow the discrimination of even closely-related species. It should however be noted that mitochondrial DNA also exhibits a degree of intraspecific variability and so care has to be taken when studying differences between organisms based on single base polymorphisms [26].

Restriction digestion of PCR products

Chikuni *et al*. [27] designed PCR primers based on the sheep satellite I DNA sequence [28] that were shown to amplify DNA from sheep and goat but not other species, including cattle, water buffalo, deer, horse, pig, chicken or rabbit. When the PCR products were sequenced, they showed 92% homology. However, four restriction sites were different between sheep and goat and so it was possible to distinguish the two species by restriction enzyme digestion. The 374bp sheep ovine product was cut into fragments of 236 and 138bp by the enzyme *Apa*I. As this site was absent from the goat product, this would remain uncut and the difference could be visualised by agarose gel electrophoresis. This technique, polymerase chain reaction–restriction fragment length polymorphism analysis (PCR–RFLP) has subsequently found widespread use for the successful identification of many meat [29–34] and fish [34–42] species. Furthermore, screening of PCR products without sequencing is also possible, whereby amplicons can be subjected to incubation with a wide variety of restriction enzymes and empirical observations made as to their utility for species differentiation. This makes the technique available to workers without access to direct sequencing facilities.

Species-specific PCR primers

Detailed sequence information has become available for many species and consequently phylogenetically informative single base polymorphisms may be identified that enable species-specific primers to be designed. Under suitably stringent reaction conditions, such primers generate a product only in the presence of DNA from a given species. Complete sequence information permits the size of the product to be predicted, so that identification is confirmed if an appropriately sized amplicon is seen on a gel. By pairing species-specific primers with a non-selective counterpart, it is possible to test for the presence of more than one species simultaneously. The non-selective primer tends to be based on a sequence that is common to all species under study in a given system; its precise location in the gene can be used to dictate the size of the amplification products that will be generated. Using such a regime, multiplex reactions have been described for both meat animal [43–45] and fish species [46, 47].

Single strand conformational polymorphism analysis

For the above PCR-based methodologies, knowledge of the amplified sequence in addition to that of the primers is generally required. Other methods have been used where this degree of sequence information is not necessary. These generally produce species-specific patterns when amplified products are subjected to electrophoresis and visualized on gels. Subjecting PCR products to single strand conformational polymorphism analysis (PCR–SSCP) has been used to identify a variety of fish species [48–52] and also in attempts to discriminate European pig and wild boar meat samples [53]. PCR is used to amplify the same region of DNA from different species, generally a part of the mitochondrial cytochrome b gene. The double stranded products are then denatured and the single stranded DNA adopts a secondary structure that is dependent on its sequence. After polyacrylamide gel electrophoresis under appropriate conditions, products with different secondary structures exhibit different electrophoretic mobilities and different patterns are obtained.

Using this method, it is possible to differentiate between sequences differing by as little as a single base [54].

Random amplified polymorphic DNA (RAPD) analysis

Arbitrary primers or random amplified polymorphic DNA markers [55, 56] have been used for species identification [57–62]. Short primers of approximately 10 bases are required. As the sequence of these is arbitrary, no prior knowledge relating to any DNA sequence in the species under study is necessary. Given appropriate PCR conditions, the primers amplify a series of loci, creating an electrophoretic pattern comprised of bands of different sizes. Certain patterns have been found to be species-specific. Different primer pairs will produce different patterns, whose usefulness is determined empirically. Both meat and fish have been identified using this method [57–62].

Use of information derived from the actin multigene family

An alternative technique has been described whereby generic primers based on actin gene sequences are used to produce species-specific fingerprints [63]. As the actin multigene family has a high gene copy number, with high conservation of the coding sequences but introns that vary in both position and size, species identification has proved to be possible. PCR primers have been based on a region of the protein coding sequence known to be highly conserved in different actin isoforms, but interrupted by an intron in most members of the gene family. By using these primers to amplify several actin gene loci simultaneously, it has been possible to generate electrophoretic patterns consisting of products of different sizes, a direct consequence of intron variation. These patterns were reproducible and did not vary between individuals of the same species, or between different breeds within a species. Alternatively, by using intron variation and a non-selective oligonucleotide in the adjoining exon, primers have been designed that selectively amplify DNA from chicken and turkey but not other meat species [32]. The PCR products can be distinguished using restriction enzymes, indicating that there are polymorphisms on which chicken- and turkey-specific primers could be based.

An indication as to the range of species that the various DNA-based methods of food authentication described above have been used to detect is shown in Table 1.

Comparison of DNA-based methodologies for food authentication

It is appropriate to consider the utility of the methods described above as applicable to food authentication.

DNA hybridisation tests are relatively time-consuming to perform and labelling of the probe may involve radioactivity, which has inherent drawbacks with regard to ease of handling and disposal. However, under appropriate conditions, many different meat animal species have been identified, even from relatively degraded DNA, although relatively large amounts of purified DNA are required.

Tests based on PCR can be undertaken with much lower amounts of starting material, due to the amplification that the method provides. Highly purified DNA is not generally required, although samples should be free from contaminants that may inhibit the reaction [64]. The sensitivity of the method does mean that it is susceptible to contamination, although this can be

Table 1. A summary of the types of meat studied using various DNA-based food authentication methods

Method	Meat studied
DNA hybridization	Cattle [3, 8–12, 15, 16], pig [3, 8–11, 16], sheep [3, 10, 12, 15], goat [3, 10, 12, 15], chicken [8, 15, 16], turkey [15], rabbit [3], deer [15], horse [3, 15]
PCR product sequencing	Cattle [19, 21, 23], pig [19], sheep [19, 21], goat [21], buffalo [21], donkey [21], chicken [19, 21], turkey [19, 23], emu [21], kangaroo [23], crocodile [23], frog [23], fish including mackerel [19], cod [19], herring [19], salmon [19], tuna and tuna-related related species [20, 22]
PCR–RFLP	Cattle [29, 31, 33, 34], pig [30, 31, 33, 34], sheep [27, 29, 31, 33, 34], goat [27, 29, 33, 34], chicken [29, 31, 32, 34], turkey [29, 31, 32, 34], several species of deer [29, 33, 34] and antelope [29, 33], horse [29, 34], buffalo [29, 33, 34], moose [29, 33], hare [33], kangaroo [29, 33, 34], wild boar [29, 33], duck [34], emu [34], gnu [33], cat [34], dog [34], crocodile [34], snail [30], fish including barramundi [34], John Dory [34], Nile perch [34], sole [41], halibut [41], sturgeon [40], salmon [37–39, 42], trout [37–39] and tuna [26, 35, 36]
Species-specific primers	Cattle [44, 45], pig [43–45], sheep [44, 45], goat [44], chicken [44, 45], turkey [45], horse [44, 45], donkey [45], tuna [46], bonito [46], sturgeon [47]
PCR–SSCP	harp seal [62], minke, fin and sei whales [62], fish including flatfish [52], eel [49], sturgeon [49], salmon [49], trout [49], tuna and tuna-related species [48, 50, 51]
RAPDs	Cattle [58–61], pig [57, 60, 61], sheep [58, 59, 61], goat [57, 59, 61], chicken [57, 58], ostrich [61], duck [57], rabbit [57, 60], deer [60, 61], horse [58, 60, 61], buffalo [59–61], mule [61], donkey [61], kangaroo [60, 61], wild boar [60], dog [57, 58, 60], cat [60], harp seal [62], minke, fin and sei whales [62]
Actin	Cattle [63], pig [63, 32], sheep [63], chicken [63, 32], turkey [32], horse [63]

minimised. Appropriate laboratory practice, such as having different areas for pre- and post-PCR sample manipulation, or the use of uracil-n-glycosylase [65] which prevents contamination from one series of reactions being carried over to the next, are routinely adopted in laboratories where large amounts of work are undertaken.

The use of species-specific primers in PCR is a rapid means of sample identification and it may also be advantageous to be able to screen for several species simultaneously. In addition to being able to detect the substitution of cheaper for more expensive meat, qualitative admixture detection may also be possible. Obviously, prior sequence knowledge is required in order to design primers and appropriate controls should be included to preclude the possibility of false positive or negative results being obtained. There is a possibility of the amplification being confounded by intra-specific variation, especially if the primers are based on mitochondrial genes. Also, during the course of evolution, copies of mitochondrial genes may have been transferred to the nuclear genome and these nuclear pseudogenes may be subject to coamplification by primers directed towards mitochondrial sequences. This has been reported during identification of game species and required primer repositioning in order to overcome the problem [66].

When PCR is undertaken without species-specific primers, some form of secondary discriminatory technique is required. Sequencing is time consuming and technically demanding but will produce a relatively large amount of information, requiring good data handling capacity. RFLP analysis of PCR products has been extensively used for species discrimination and a single primer pair can produce a fragment that can be used for the identification of multiple species with judicious choice of restriction enzymes. The method has the drawback that incomplete digestion may occasionally occur and intra-specific variation could delete or create additional restriction sites.

As heat processing may reduce the fragment size of DNA, analyses of canned material such as fish have tended to rely on the amplification of relatively short regions of DNA [48]. It is theoretically possible that, although inter-specific variation may be contained within these amplicons, it may not actually be associated with restriction enzyme recognition sequences, although this does not yet appear to have presented as a problem in practice.

When PCR products are cut by restriction enzymes, routine gel analysis is sufficient to resolve products of different sizes. However, the analysis of amplicons via SSCP is more demanding as it is the differences in secondary structure that show a variation on the gels. Such differences are critically dependent on the conditions under which the products are analyzed, especially the temperature [67]. Consequently, reproducibility may be a problem here. Although this has been investigated with regard to tuna [51] and been shown not to confound results, the robustness of the method could be open to question. The reproducibility of RAPD patterns has also been a major concern, with reaction conditions, including the type of DNA polymerase used, having an effect on the fingerprints produced [68]. Also, as relatively large fragments can be produced by RAPD analysis, the quality of the template DNA is important. If the DNA is badly degraded, this will influence the size of the amplicons, and hence the pattern, that is produced.

Neither SSCP nor RAPDs are inherently unreliable, but stable reaction conditions are crucial to reproducibility. Standards should be run on each gel to enable species identification to be made with confidence.

Admixture detection and quantification using PCR-related methods

With any method for which the sequence of the DNA lying between the primers is not known, a series of bands is liable to be produced. If admixtures are studied, it is possible that these patterns may become too complex for convenient analysis. Consequently, other methods have been used for the study of admixtures, of which arguably the most convenient is the use of species-specific primers. Levels of adulteration or contamination down as low as 1% have been routinely detected and amounts of less than 0.1% have been shown to produce positive results [43]. However, such methods are essentially qualitative and the presence or absence of a given species can be ascertained, but not its absolute amount. It may be necessary to differentiate contamination that is technically unavoidable from intentional adulteration. Consequently, some form of quantitative methodology, such as competitive PCR [69], is required.

To perform competitive PCR, an artificial construct is created that contains exactly the same sequences for primer binding as the target DNA, but can be differentiated from it on the basis of size [69, 70] or the presence or absence of a restriction site [71, 72]. As a single primer pair is used, it is assumed that the amplification efficiency is the same for both the target DNA and the construct. During the PCR, fixed quantities of the exogenously added artificial construct or 'competitor' are amplified along with the target DNA. Calibration involves a gel-based titration of the target DNA amplification with different amounts of competitor. Competitive PCR techniques have only recently been adopted for food-related applications, where they have been used in the detection of genetically modified crops [73] (see below). It is likely that their use will increase.

The detection of foodstuffs of plant origin and genetically modified organisms (GMOs)

The majority of the work concerned with DNA-based testing of food has involved the study of material of

animal origin. However, certain plant products have also been studied. In general these can present problems due to the increased difficulty of DNA extraction involved and in certain products, such as soya sauce, purified lecithin and glucose syrup, it may not actually be possible to detect DNA [74]. In many instances, however, DNA of sufficient quality can be obtained.

Amongst the food-related studies that have been undertaken, cultivars of wheat [75], grapevine [76, 77] and rice [78, 79] have been studied, although this is more akin to breed than species identification in animals. Microsatellite polymorphism has been used to do this: variable numbers of di- and tri-nucleotide repeats scattered throughout the genome are amplified by PCR and subsequently detected electrophoretically as length variants. Other PCR-based methods have been described for the detection of soya in processed meat products [80] and wheat-contamination in non-wheat foods [81]. Attention has also been focussed on the issue of detecting genetically modified organisms. This subject has already been recently reviewed [74] and is not discussed in detail here, except to note that PCR primers are designed to target the introduced DNA or altered gene sequences. Consequently, it is a prerequisite of such tests that these are known. Another factor worthy of consideration is that the vectors used to transfer genes into plants, such as Cauliflower Mosaic Virus and *Agrobacterium* can contaminate crops naturally [74]. This could result in false positives with regard to GMO identification.

Emerging technologies

Largely driven by the diagnostics industry, DNA-based technologies are evolving rapidly. It is desirable to simplify PCR product analysis by removing the requirement for electrophorsis. One means of doing this is through the use of array technologies and microchips [82,83]. However, from a practical point of view, these are currently in their infancy and their adoption is prohibitively expensive for the majority of laboratories. Techniques involving fluorescence have also been described and are becoming more accessible. These also potentially eliminate the requirement for electrophoretic analysis of PCR products. Closed tube methods can enable product formation to be monitored during the course of a reaction, in 'real time'. They offer advantages over more traditional systems in that results can be obtained in a shorter time, the potential for cross-contamination is minimized and quantitative data can be obtained more readily. Some of these technologies use both primers and probes simultaneously (see below). They have the additional advantage of potentially higher specificity, compared with methods that rely on primers alone for discrimination.

In the most basic fluorescence protocol, an intercalating agent can be used to bind to any double-stranded DNA that is produced during the course of an amplification reaction [84–86]. More product gives a stronger signal. Alternatively, primers can be adapted to contain a fluorescent tag, as in the Amplifluor^{TM} system [87, 88]. In this format, one of the primers of a conventional pair of oligonucleotides incorporates a 5′ extension containing a hairpin loop in which a fluorescent moiety is held in close proximity to a quenching molecule, preventing it from producing a signal. When this primer is incorporated into a PCR product, the complementary DNA strand that is synthesized reads into the loop and causes it to open up, with the subsequent production of a fluorescent signal. These techniques are capable of detecting the presence or absence of amplification products, but provide no information as to their nature. False positives could thus potentially pose a problem here, with primer dimers and undesired amplicons acting to increase fluorescence, although judicious primer design can limit this.

A variety of fluorescence methods can be used to detect the presence of specific PCR products. As with the Amplifluor^{TM} system described above, these tend to involve the processes of fluorescence reasonance energy transfer (FRET) [89], whereby the interaction of fluorophore and quencher or acceptor moities is altered during the course of a reaction by changes in their physical proximity.

In the TaqMan^{TM} assay [90,91], a probe is used that binds to a target amplicon. The probe is synthesised to contain a reporter molecule at one end and a quencher at the other. The close proximity of the two results in the quenching of emission from the reporter dye and no signal is produced. Taq DNA polymerase possesses 5′–3′ exonuclease activity and this results in bound probe being cleaved during each cycle, with the subsequent separation of the reporter and quencher. Fluorescence emitted by the reporter dye can then be detected and this increases as amplification proceeds.

An alternative approach uses 'Molecular Beacons' [92,93]. These are single-stranded oligonucleotides designed to hybridise to specific amplicons. They also incorporate a hairpin loop in which a fluorescent dye and a quencher moiety are kept in close proximity. Consequently, the fluorophore/quencher complex is unable to be excited when the probes are not bound to their target. On hybridizing to an unlabelled complementary sequence, such as a newly-generated PCR product, the probe opens up and with the movement of the fluorophore away from the quencher, fluorescence can be detected.

'Scorpion^{TM}' primers have also been described [94]. These are oligonucleotides consisting of a functional primer region and a 5′ extension. The extension comprises a probe designed to anneal to a specific product, and a pair of self-complementary stem sequences that bring a fluorophore and quencher into close proximity, thereby preventing excitation. A blocking monomer, positioned

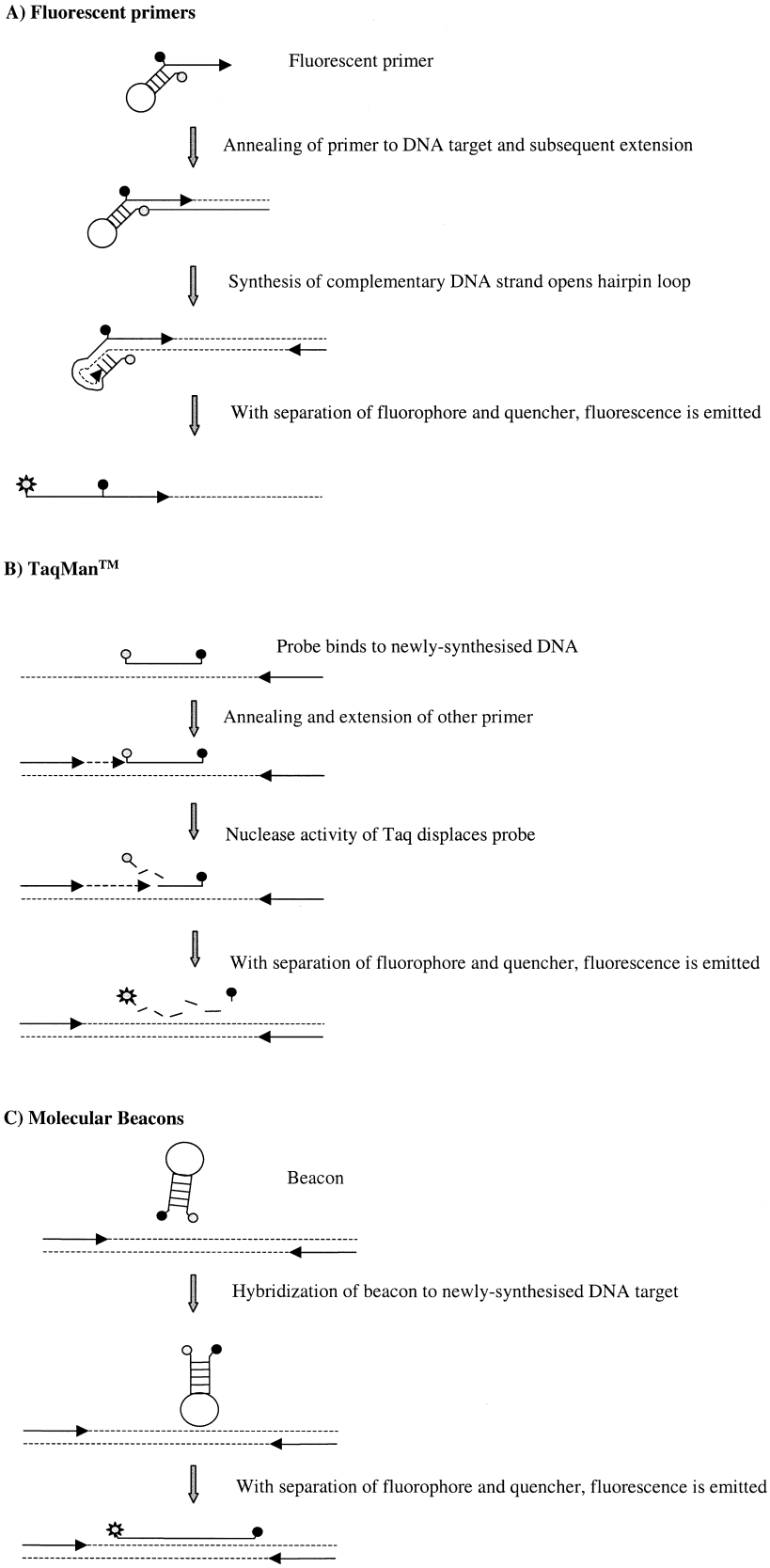

Fig. 2. The mechanisms of action of a variety of fluorescence techniques. In all of the figures, primers are represented as solid arrows, newly-synthesized DNA is shown as a dashed line, fluorophores are light grey circles and quencher molecules are black circles.

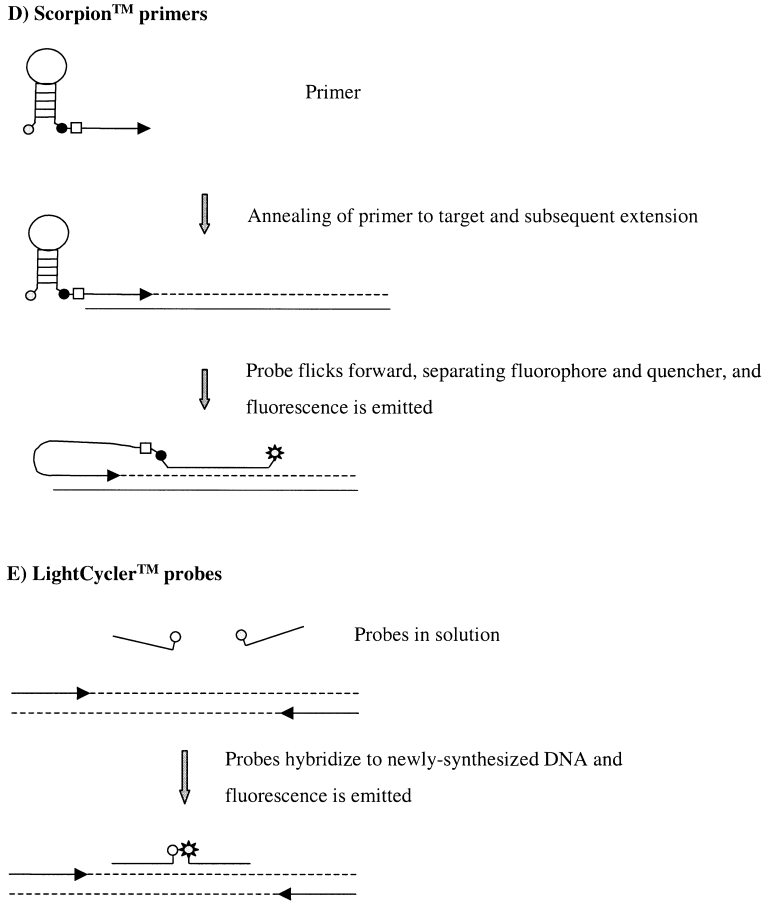

Fig. 2. (*continued*)

between the primer and its extension, prevents the latter from being copied into PCR products. When the primer participates in PCR in the presence of an appropriate target, it is extended, with the newly synthesized region containing DNA complementary to that contained in the probe. The probe can then 'flick forward' and anneal to the target. With the fluorophore consequently moving away from the quencher due to the loss of the stem-loop, fluorescence can then be detected.

In an alternative fluorescence format optimized for the use with LightCycler™ technology [91], two oligonucleotide probes are used, labelled with different dyes, one being a donor and the other an acceptor. In the presence of an appropriate template, as generated during PCR, the two probes bind in a head to tail arrangement, bringing the two dyes into close proximity, as they are attached to the probes at adjacent termini. Excitation of the donor molecule results in energy being transferred to the acceptor, and this then emits light at a different wavelength to produce a detectable signal.

The various technologies described above are illustrated in Fig. 2. Whilst none of these has yet been reported as having been used for food authentication with regard to species identification, it is likely that the future will see these fluorescence methodologies employed more widely. Note, however, that the TaqMan™ assay has been used for microbiological testing of food products, determining the presence of pathogens [95–98] and recently the detection of *Salmonella* via molecular beacons has also been described [99].

Issues related to food testing

The impetus behind many of the advances in amplification technologies has been clinical research. Food authentication may impose constraints that would not necessarily be of concern in other areas, in particular cost and DNA quality.

Budgets for food authentication testing are generally lower than for health-related research and this may influence the nature of the protocols that are undertaken. Whilst the cost of any DNA-based work is significant, access to expensive instrumentation is required for sequencing or real-time PCR analysis and this can be expected to influence the uptake of such technologies by food control laboratories.

The quality of the DNA available for food authentication is also an important factor in the design of such tests. Extensive processing, such as is involved in the canning of fish, can cause extensive degradation of the DNA, limiting the size of the products that can be successfully amplified from such samples. For routine testing of badly degraded DNA, it may prove difficult to generate products much larger than around 120bp [42]. This is an important factor to be considered when designing tests intended to be used for the analysis of processed products and amplicon size should be tailored accordingly.

Conclusions

A variety of DNA-based methods are potentially available for use in food authentication. These vary in their complexity and cost. Both of these factors are liable to influence the uptake of such tests by food control laboratories. With the likelihood of increased regulation of food products, in response to consumer concern, tests such as those described here, many of which are used routinely in a research environment, could be adopted for use in the marketplace. However, before this can occur, strict processes of validation would need to be undertaken. Indeed, examination as to the reliability and reproducibility of such protocols for more general use is already underway and some of the methods described above are currently in practice.

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