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The Application of PCR-Based Methods in Food Control Agencies – A Review

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1. Introduction

In food control laboratories the world over, molecular biological techniques play an increasingly central role in the analysis of food and food ingredients. Although the classical methods employing cultural, biochemical, cytological and immunological procedures are still being commonly practiced, molecular biological tools employing polymerase chain reaction (PCR) have become an increasingly popular alternative in many food control agencies in recent years. Factors responsible for the popularity of PCR-based detection assays include rapidity, specificity and enhanced sensitivity of the assays. With regard to the latter, often highly denatured food samples and ingredients can still be processed for PCR detection assays because the DNA may still be reliably amplified, as opposed to loss of processing material in detection methods relying on protein analytical tools.

Microbial source tracking (MST) which involves the ability to trace microbes, particularly food-borne pathogens, poses unique challenges to the food industry and food regulatory agencies (Santo Domingo and Sadowsky, 2007). Such information would assist regulatory agencies in localizing food producers or vendors responsible for supplying foods involved in human infections. Additionally, such knowledge would afford public health investigators the opportunity to track food-borne disease outbreaks to their point of origin, thereby preventing future occurrences. In providing such crucial information reliably and within the shortest possible time frame, MST employs a number of PCR-based detection assays. The recent outbreak of EHEC infections arising from verocytotoxin-producing *Escherichia coli* EHEC O104:H4, predominantly in Germany furnishes a good example of the importance of a rapid screening tool for the prompt identification of an infectious agent and surveillance monitoring. More than 16 countries in Europe and North America reported a total of 4,075 cases and 50 deaths as of July 21 2011, two months after the first reported case at the beginning of May 2011 (WHO International Health Regulations, Outbreaks of *E. coli* O104:H4 infection, Update 30) .

In this and other similar cases, PCR-based molecular biological methods are usually employed in the rapid and initial screening of samples, while complementing this approach with the classical cultural technique for reliable end-identification of the isolate. While not replacing the classical methodologies that have stood the test of time, PCR-based molecular approaches are rapidly becoming the initial screening tools in diverse food analytical processes. Commonly the molecular biological methods are supplemented with classical

diagnostic tools to reach a definitive consensus before prosecution for negligent practice or falsified declaration by food producers and processors is effected by food control agencies. This review looks at the plethora of PCR-based approaches in food control laboratories, from pathogen detection and control, food allergen and GMO detection and quantitative determination, to animal species verification.

2. Molecular biology tools for detection of foodborne pathogens

In many food control agencies worldwide, continuous effort is devoted to risk monitoring assessments and evolution of novel strategies for more rapid and reliable detection of the medically relevant enteropathogens. Although the ultimate goal is a zero-reduction of the pathogens in food, especially meat products and fresh produce, the quantitative microbiological risk-assessment has become an increasingly important parameter in predicting the infectious potential of a given food matrix (FAO/WHO, 2002). The medically relevant species are usually bacterial in origin, and include among others thermophilic *Campylobacter* spp., *Salmonella* spp., enterohaemorrhagic *Escherichia coli* (EHEC), *Listeria monocytogenes*, *Bacillus cereus*, *Clostridium* spp. and *Shigella* spp. Typical clinical symptoms include diarrhea, which could be self-limiting, invasive or bloody, and vomiting. In Europe, salmonellosis and campylobacteriosis account for the most cases of notified bacterial infections, while listeriosis, although less commonly reported accounts for the most mortalities. In the USA, bacterial pathogens like *Salmonella* and *Campylobacter* are also prevalent, but surveillance of food borne illness is complicated by underreporting (European Food Safety Authority, EFSA 2009, Mead *et al.*, 1999).

The traditional culture-based enumeration of the bacteria is often laborious and time-consuming. A typical detection assay for *Campylobacter* for example, requires up to 5 days, with enrichment. Additionally, the bacterial strain of interest can be frequently overlooked when only culture-based enumeration techniques are employed, due to a strong background of microflora that obscure the accurate detection and quantitative estimation of the pathogen. PCR-based detection of pathogens has therefore become increasingly popular in recent times. Effective PCR-detection assays have been successfully designed and implemented for a broad range of these bacterial food-borne pathogens such as *Salmonella*, *Campylobacter*, *Bacillus cereus*, pathogenic *Escherichia coli* (EHEC) and others (Anderson *et al.*, 2010, Lehmann *et al.*, 2010, Josefsen *et al.*, 2010, Fratamico *et al.*, 2011, Wang *et al.*, 2011).

2.1 PCR-based food - borne pathogen (bacteria) detection

On a global scale, the food sector remains a major player in the lives and well being of the general human population, and considerable trust and confidence is invested in it by consumers. When food-borne related illnesses or epidemics hit the headlines, the public is understandably disturbed and clamour for tighter regulations and more effective surveillance of food products. The food distribution chain is however a very complex one and tracing the origin of a food outbreak can be very difficult to achieve. In an attempt to address the challenges facing the food sector as regards protecting consumer trust and confidence, the Federation of Veterinarians of Europe (FVE) introduced the “stable to table approach” of food safety (FVE Food safety report). The concept involves a holistic approach embracing all elements, which may have an impact on the safety of food, at every level of the food chain from the stable to the table. Accordingly, the phrase is used to encompass not

only the production of all foods of animal origin (including meat, milk, eggs, fish and other products from aquaculture), but fruits and vegetables as well. Applying this approach means that food safety is not solely a matter of inspection at the slaughterhouse or processing plants as has traditionally been the case. On the contrary, this system emphasises the need for interaction between all participants in the entire food chain, from the animal feed manufacturer down to the individual consumer.

In Europe, a Rapid Alert System for Food and Feed (RASFF) was implemented in 1979, to provide food and feed control authorities an effective tool to exchange information about measures taken in responding to serious risks detected in relation to food or feed. This exchange of information helps Member States to act more rapidly and in a coordinated manner in response to a health threat caused by food or feed. In 2010, more than 3,358 notifications were transmitted through the RASFF, with cases of food poisoning accounting for 60 of such reports (Rapid Alert Systems for Food and Feed (RASFF) Annual Report 2010).

A major advantage in the application of PCR-based methodologies lies in the fact that such assays are generally more specific, sensitive, and faster than conventional microbiological assays. However the inherent complexities and composition of food matrices hampers the direct application of PCR detection assays, requiring a pre-enrichment step, thus increasing the processing time for the analysis of the food sample. Nevertheless the simplicity and time saving feature of the PCR reaction has made it increasingly applicable for detection of bacterial pathogens in food. For reliable detection of possible contaminants in the PCR reaction, it is essential to include appropriate negative controls, both during DNA extraction procedures (extraction control) and during the PCR reaction (master mix control). Additionally, it is essential to monitor or detect possible inhibitors that could hamper the efficiency of the PCR reaction. There are a number of possibilities to detect such PCR inhibitors, the commonest of which is to include in each PCR run, an inhibition control, or an internal amplifications control (IAC). The requirement for inclusion of an appropriate IAC for each PCR run is non-negotiable and is in fact jointly stipulated by the International Standard Organization (ISO) and the European Standardization Committee (CEN) in a general guiding policy for PCR reactions in food analytical procedures (EN ISO22174). The choice of the IAC may vary from an artificial DNA molecule which is co-amplified with the same primers for the target DNA (competitive IAC), to a foreign DNA molecule which is coamplified in the PCR reaction with a different primer set (non-competitive) (Hoorfar *et al.*, 2004).

An example of a typical real-time PCR based approach for the detection of *Salmonella*, against the backdrop of the traditional cultural enumeration is outlined below. For the routine or traditional culture-based enumeration, an appropriate amount of the probe is inoculated in buffered peptone water. The culture is incubated at 37 °C for 18 – 24 h, followed by subculture in parallel, on a semi-solid MSR/V plate (Rappaport-Vassilidis-Medium) and in Rappaport-Bouillon for 18-24 h at 43±1°C. On day 3, *Salmonella* suspects are then subcultured in parallel on XLD and Rambach agar, according to standard procedures. Presumptive *Salmonella* colonies are then confirmed by serotyping.

With the traditional culture enumeration, outlined above, up to 5 days must be allowed for a definite identification of the bacteria. Sometimes, *Salmonella* positive probes can be completely missed with the conventional cultural enumeration due to strong growth of accompanying flora as mentioned previously. In contrast, a real-time PCR assay for *Salmonella* detection can be completed in less than 2 days, with an initial and shortened pre-

enrichment step. In a comprehensive study by Anderson *et al.*, 2010, such a real-time PCR assay was described for the qualitative detection of *Salmonella* in several food samples. More than 1,900 natural food samples were analyzed in this study and the method was found to be robust and resulted in reliable identification of the bacteria in as little as 28 hr, in contrast to 4 or 5 days with conventional *Salmonella* diagnostics. An internal amplification control, which is co-amplified in a duplex PCR reaction, was included in the assay.

As mentioned previously, a number of real-time PCR assays have been published for several important food pathogens. Fricker *et al.*, (2007) reported on the successful application of real-time PCR in the detection of *B. cereus*, which together with the closely associated *S. aureus* are the two most important bacteria responsible for food-associated intoxications. The traditional detection of the emetic toxin associated with these bacteria is often difficult, time consuming and expensive. With the described real-time PCR assay, a first diagnosis can be achieved within 30 hours, greatly accelerating the potential for rapidly implementing risk assessment studies for different food products or matrices. In another study, the successful implementation of multiplex real-time PCR assays in the detection of neurotoxin producing *Clostridium botulinum* in clinical, food and environmental samples was described (De Medici *et al.*, 2009, Messelhäusser *et al.*, 2011a and b).

A more recent approach is the quantitative real-time PCR assay. Various possibilities exist for quantification strategies, one of which is the employment of a CFU-based standard curve for quantification. Briefly, the bacteria of interest are grown or cultivated according to standard procedures and a serial dilution of the bacteria, spanning a representative colony concentration (say 10^1 to 10^8 cells) is plotted as a standard curve. With this curve, the unknown concentration of bacteria in a food sample can be calculated. A second possibility is the employment of a serial dilution of bacterial DNA for the generation of a standard curve for quantification (see fig. 3). In a recent study by Josefsen *et al.*, 2010, a CFU-based standard curve was utilized in the quantitative determination of *Campylobacter* in chicken rinse (Josefsen *et al.*, 2010). In this work, the quantification method was compared with culture-based enumeration on 50 naturally infected chickens. The cell contents correlated with cycle threshold (C_T)* values with a quantification range of 1×10^2 to 1×10^7 CFU/ml). In a previous study, Yang *et al.*, (2003) also successfully applied a real-time PCR assay for quantitative detection of *C. jejuni* in poultry, milk and environmental water. Such quantification strategies are increasingly in demand and a number of commercial products are now available for such purposes.

Although the PCR method has evolved as a very powerful analytical tool indeed, a limitation of such methods is that the DNA analysis will generate results of all the bacteria present in the food sample or probe, irrespective of the status of the cells - whether the cells are live and viable or dead. Thus data for dead or inactivated bacteria which might not be significant from an epidemiological viewpoint are invariably included in such quantitative assays. An improvement in such analysis is the use of an appropriate DNA intercalating dye to distinguish dead from viable and viable, but non-culturable (VBNC) bacteria. Propidium monoazide (PMA) is one such chemical which selectively penetrates only into 'dead' bacterial cells with compromised membrane integrity but not into live cells with intact cell membranes (Nocker *et al.*, 2006, 2009, Pan and Breidt, 2007). PMA possesses an azide group which permits cross-linking of the dye to DNA after exposure to strong visible light. When the PMA-treated cells are subjected to DNA extraction procedures and subsequently PCR for detection of the bacteria of interest, a reduction in the number of detectable bacteria is

often observed with PMA-treated cells (Josefsen *et al.*, 2010). The PMA approach is currently being developed and validated in our laboratory for the reliable identification and quantification of viable and live bacterial pathogens in various food matrices.

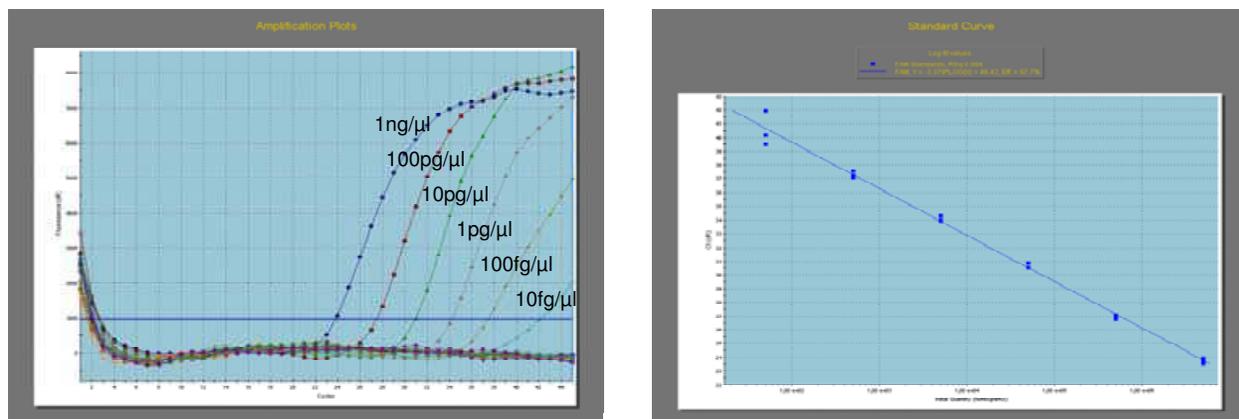


Fig. 1. Principle behind the quantitative PCR approach. A serial dilution of bacterial genomic DNA (fig. 1a) or DNA extracted from a dilution series of appropriate bacterial CFUs (fig. 1b) forms the basis for the calculation of a standard curve for quantification.*

2.2 PCR detection of food-borne viruses

A number of viruses associated with food infections are increasingly becoming important in recent years. The most relevant species are the norovirus, hepatitis-A virus, sapovirus, adenovirus, rotavirus, enterovirus and others. One category of implicated foods is those that are minimally processed, such as fresh produce and vegetables and bivalve molluscs. These are typically contaminated with viruses in the primary production environment. In addition, many of the documented outbreaks of foodborne viral illness have been linked to contamination of prepared, ready-to-eat food by an infected food handler. While in many countries viruses are now considered to be an extremely common cause of foodborne illness, they are rarely diagnosed as the analytical and diagnostic tools for such viruses are not widely available (Microbiological risk assessment series 13, 2008, WHO). Attempts have been made to implement PCR approaches in detection of food-borne viruses. While the overwhelming majority of food-associated viruses are RNA viruses, the RT-PCR (reverse transcription-PCR in which a reverse transcription step converting the viral RNA to template DNA precedes the PCR reaction) is the gold standard for analysis (Höhne and Schreier, 2004). Transferring the traditional and established methods for medical viral diagnosis to a food analytical setting is not readily implementable. While the viral particle load in human and animal tissues or organs is considerably great, the viral load in food samples is usually quite low – in some cases only 10-100 virions may be present in a food probe. Visualization of such a very low viral presence with electron microscopic means and detection of the viral protein through ELISA or latex tests would be impossible where the detection limits of such methods lie within the 10⁵ to 10⁶ virus particle range pro gram food. The PCR approach is in this regard the most promising of all techniques because the detection limit with RT-PCR lies in the 10¹ to 10³ virus particle/g food range (Koopmans und Duizer, 2004).

(* a threshold for detection of DNA-based fluorescence is set slightly above background. The number of cycles at which the fluorescence exceeds the threshold is called the cycle threshold).

Adequate care has to be however taken while subjecting the sample to extraction procedures for optimal yield of high quality nucleic acid (Crocchi *et al.*, 2003, De Husman *et al.*, 2007). Examples of successful application of the RT-PCR technique include the detection of norovirus in raspberries associated with a gastroenteritis outbreak, and the detection of the virus in oysters from China and Japan (Phan *et al.*, 2007). Other PCR-based methods that have been developed include a nested RT-PCR approach, real-time RT-PCR, and the limited application of nucleic acid sequence-based amplification, among others (Jean *et al.*, 2001, Kojima *et al.*, 2002, Nishida *et al.*, 2003, Beuret *et al.*, 2004).

3. PCR-based allergen detection and quantification in food matrices

Globally, millions of people suffer from allergic reactions to food, which fortunately in most cases range from mild to minor symptoms. In some extreme cases however, food allergies can trigger moderate to more severe life threatening reactions. In contrast to food intolerance, which is also a common form of an adverse reaction to food arising for example from an enzymatic deficiency, such as lactose intolerance, food allergies are immune-mediated. Usually a protein in the food is mistakenly recognized as harmful, triggering the recruitment of IgE antibody with a subsequent allergic reaction (Bush and Hefle, 1996). Symptoms may vary from dermatitis, gastrointestinal and respiratory distress to life-threatening anaphylactic shock. The most common food substances, accounting for almost 90 % of all allergic food reactions are milk, egg, peanut, tree nuts, fish, shellfish, soy, and wheat.

In order to protect consumer safety and health, the EU Labelling Directive (Directive 2000/13/EC) and its later amendments specifically mandate the labelling of allergenic foods. The Labelling Directive requires food manufacturers to declare all ingredients present in pre-packaged foods sold in the EU allowing very few exceptions. In order to respond to our rapidly changing times, this directive has been amended a number of times with regard to allergens. The two most important amendments are: Directive 2003/89/EC introduced Annex IIIa, which is a list of allergenic foods that must always be labelled when present as ingredient in a product, and Directive 2007/68/EC which contains the most recent amendment of Annex IIIa. The latter lists all the allergenic foods that must be labelled as well as a few products derived from those foods for which allergen labelling is not required (European Commission, 2000, 2003, and 2006).

Food allergies are present in about 1-3 % of the global adult population, while in children, a slightly higher incidence (4-6 %) has been documented (Bock *et al.*, 2001). While some of these allergies may be shed when children approach adolescence and adulthood, a few of them are present for life, such as peanut and shellfish allergies. A need for careful labelling of food and food ingredients is strongly underscored by the fact that in some cases, even very minute amounts of an allergen can trigger such life-threatening anaphylactic responses like biphasic anaphylaxis and vasodilation, requiring immediate emergency intervention. Threshold doses for peanut allergic reactions have been found to range from as low as 100 µg up to 1g of peanut protein (Hourihane *et al.*, 1997, Poms *et al.*, 2007).

A variety of techniques have evolved over the years for the detection and possible quantification of the most common food allergens. Protein-based methods that have been employed include the RAST (radio-allergosorbent test, Holgate *et al.*, 2001), RIE (rocket immuno-electrophoresis, Malmheden, *et al.*, 1994) and the ELISA (enzyme-linked

immunosorbent assay, Hefle *et al.*, 2001 and Hlywka *et al.*, 2000). The ELISA method is by far the most common and is routinely employed in various food analysis labs due to its high precision, simple handling and good potential for standardization. Additionally, quantitative data are possible with the ELISA technique (Shim and Wanasundara, 2008). However results generated with the ELISA method must be sometimes taken with caution as substantial differences in the detectable protein from the standard on which the test is based, resulting for example from variations in the processing of the food matrix, might lead to false results. Recently, PCR-based detection of allergens has become increasingly popular. A major advantage in the employment of PCR-based methods lies in the high specificity of the reaction. Additionally, proteins in foods that have been harshly processed, might not be detectable in the classical ELISA based approach for example, while the target DNA might be nevertheless efficiently extracted under such denaturing conditions. Another advantage that the PCR holds out against the classical protein-based analytical methods is its stability against the backdrop of geographical and seasonal variations in fruits and nuts for example, with accompanying variance in protein composition (Poms *et al.*, 2007).

Hupfer and colleagues have developed and validated a number of molecular-biology based methods for the detection of a number of allergens, notably celery, lupine and cashew nut (Demmel *et al.*, 2008, Hupfer *et al.*, 2006, Ehlert *et al.*, 2008). A typical scheme for the development and validation of an allergen, with celery as an example is described below (Fig. 2). Other studies have successfully identified and quantified allergens in various food matrices such as the work by Hirao and colleagues who developed a PCR method for quantification of buckwheat by using a unique internal standard. Food-labelling regulations in Japan require that buckwheat must be declared on the food label if its protein is present at concentrations higher than a few micrograms per gram, thus the relevance of this study (Hirao *et al.*, 2006). More recently, Mujico and colleagues developed a highly sensitive real-time PCR for quantification of wheat contamination in gluten-free food for celiac patients. The method compared well with the ELISA in efficiency, with a quantification limit of 20 pg DNA/mg food sample (Mujico *et al.*, 2011). In addition to the conventional singleplex PCR or real-time PCR reactions for allergenic qualitative detection, attempts have also been made to detect simultaneously more than one allergenic event in a food matrix. This multiplex approach was recently demonstrated by Köppel and colleagues and allows the parallel detection of peanuts, hazelnuts, celery and soya in one multiplex reaction, and the quantitative detection of egg, milk, almond and sesame in another multiplex reaction. The tests exhibited good specificity and sensitivity in the 0.01 % range. Due to comparatively lower DNA content in milk and eggs, the authors reported lower sensitivities for these allergens. Initial comparisons of the generated results with conventional ELISA suggested a qualitative accordance, with low correlation of quantitative data (Köppel *et al.*, 2010a).

Another PCR-based approach partly developed and validated by our laboratory is the simultaneous detection of DNA from various food allergens by ligation-dependent probe amplification (LPA). Ligation dependent PCR is a technique originally used for detection of nucleic acids (Hsuih *et al.*, 1996). Briefly this method employs the ligation of bipartite hybridization probes that bind to a target DNA derived from the foodmatrix under investigation. The target DNA is first denatured according to standard protocols, and then incubated with the LPA probes, allowing binding of the LPA probes to the DNA strand, following which the two probes are ligated in a simple ligation reaction. The resulting

oligonucleotide is then subjected to PCR amplification. The arising PCR amplicon is then subjected to capillary electrophoresis and visualized with laser-induced fluorescence. With this method, the simultaneous detection of DNA from 10 allergens, notably peanuts, cashews, pecans, pistachios, hazelnuts, sesame seeds, macadamia nuts, almonds, walnuts and brazil nuts was possible (Ehlert *et al.*, 2009). Fig. 3 below outlines the principle of the LPA methodology.

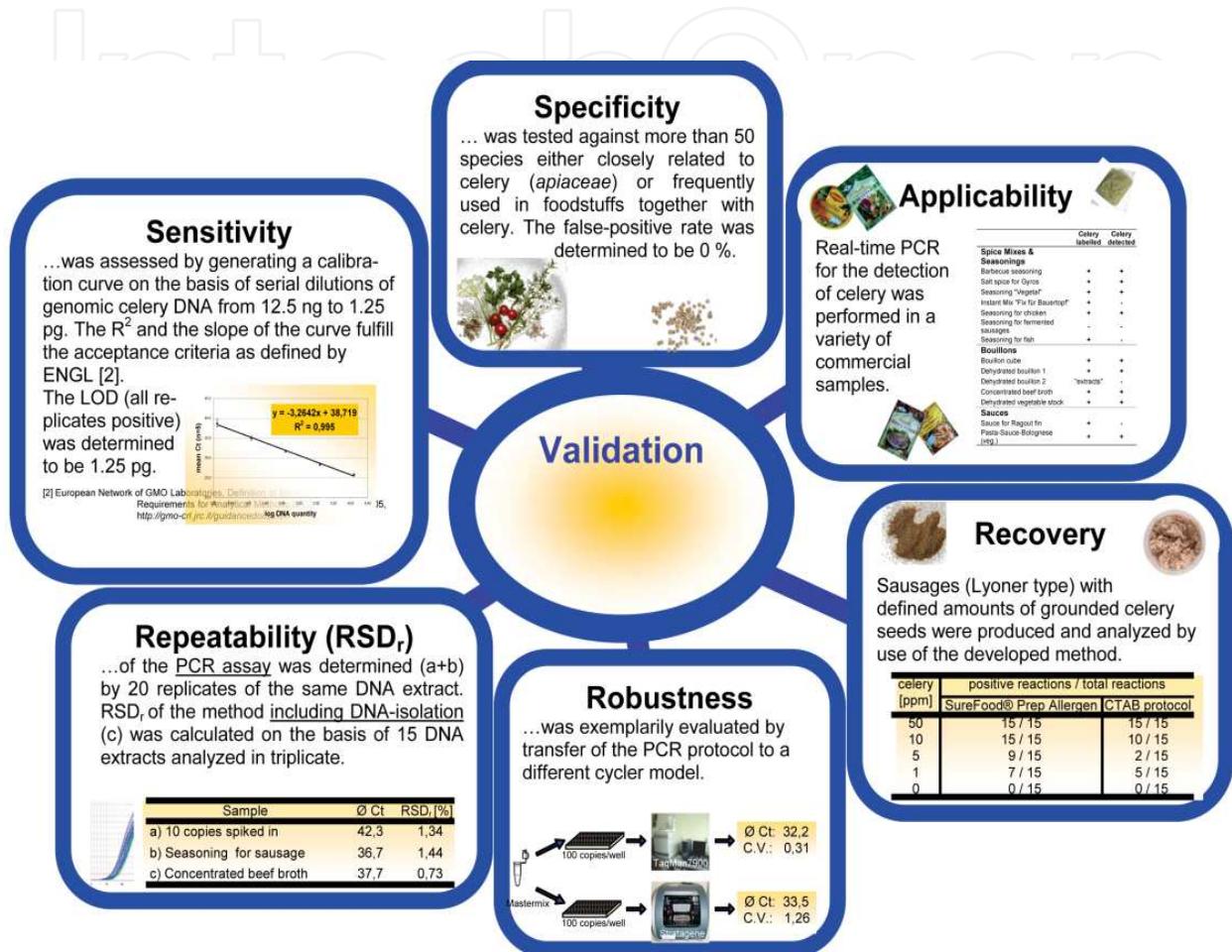
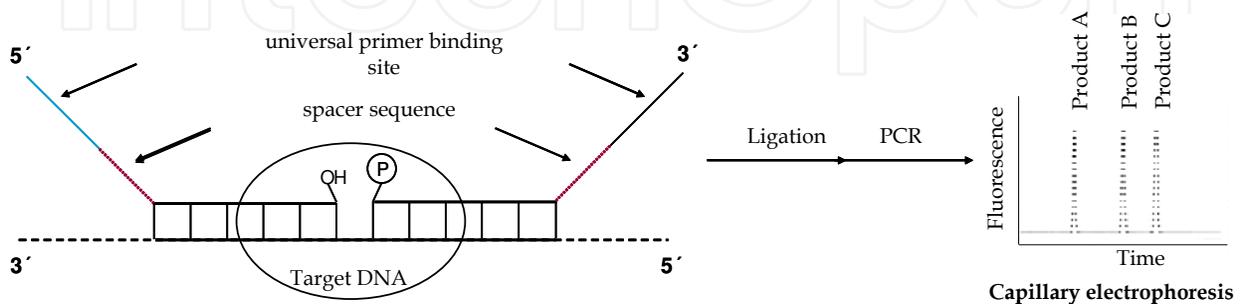


Fig. 2. Development and Validation of a Real-time PCR Detection Method for Celery in Food (Hupfer *et al.*, 2006)



(Demmel *et al.*, 2011, Personal communication)

Fig. 3. Diagrammatic representation of the ligation dependent probe amplification (LPA) approach

4. Application of PCR in animal species detection and differentiation in meat products

A major challenge for food control agencies worldwide is the accurate determination of declared meat components for food and feed ingredients. For protection of consumer trust and confidence and to ensure the quality of meat produce, the verification of declared animal species is important for the following reasons: a) ethical considerations of some might reject the consumption of certain meat products, b) the underlying health condition of some might preclude consuming certain meat products, and c) possible economic loss from the fraudulent substitution of expensive meat components with inferior products (Commission Directive 2002/86/EC, Commission Recommendation 2004/787/EC).

A rapid and dependable detection system is therefore indispensable in a food control agency for protection of consumer trust. In the past, the traditional method for determination of animal species in food relied heavily on immunochemical and electrophoretic analysis of proteins. Although these protein-based analytical methods are still important tools in the food analytical industry, a major drawback in such applications is that in the case of highly processed food, the resulting protein denaturation affects the sensitivity of the procedure. Additionally, such methods may not enable the fine discrimination between closely related animal species like chicken and turkey, or sheep and goat. DNA-based detection systems have thus become increasingly popular in recent times. The distinct advantage of DNA-based detection lies in (1) the increased specificity (generally unambiguous identification of target sequences) and (2) relative stability of the DNA molecule, allowing detection of animal species even in food that have been seriously compromised by excessive processing.

In the early stages, molecular biological methods in species identification were largely based on the use of hybridization of homologous sequences, employing genomic DNA as a species-specific probe, hybridized to DNA extracted from meat samples (Lenstra *et al.*, 2001). Later improvements saw the development of probes derived from species-specific satellite repetitive DNA sequences, making detection of admixtures that account for less than 5 % of the product possible. These methods are however time consuming and quite laborious, with reduced sensitivity in some cases. PCR-based methods have thus become increasingly important in recent times, allowing enhanced sensitivity and specificity of the assays. In most PCR-based approaches, species-specific primers are employed that bind to sequences unique to the species under investigation. Another approach is the employment of universal primers that bind to consensus sequences in all the animal species present in the meat sample. Following amplification, the resulting DNA fragments are subjected to differing analytical procedures for accurate determination of the present species. A popular approach is the use of restriction fragment length polymorphism (RFLP, Fig. 4), which commonly employs restriction digestion assays to generate fragments that are unique to the different animal species present in the sample. Each species is then recognised by its unique restriction fragment pattern (Ong *et al.*, 2007, Girish *et al.*, 2005, Gupta *et al.*, 2008, Meyer *et al.*, 1995). In order to achieve a high level of sensitivity in these assays, especially when universal primers are employed for simultaneous amplification of all present meat species, genes present in multiple copies are usually employed as targets. Prime candidate genes are usually mitochondrial rRNA (12S or 18S) or the phylogenetically robust and highly conserved *cyt b* gene (Kocher *et al.*, 1989, Jain *et al.*, 2007).

In an attempt to simultaneously detect several meat species present in a food sample, several multiplex real-time PCR assays for species differentiation have been described in recent

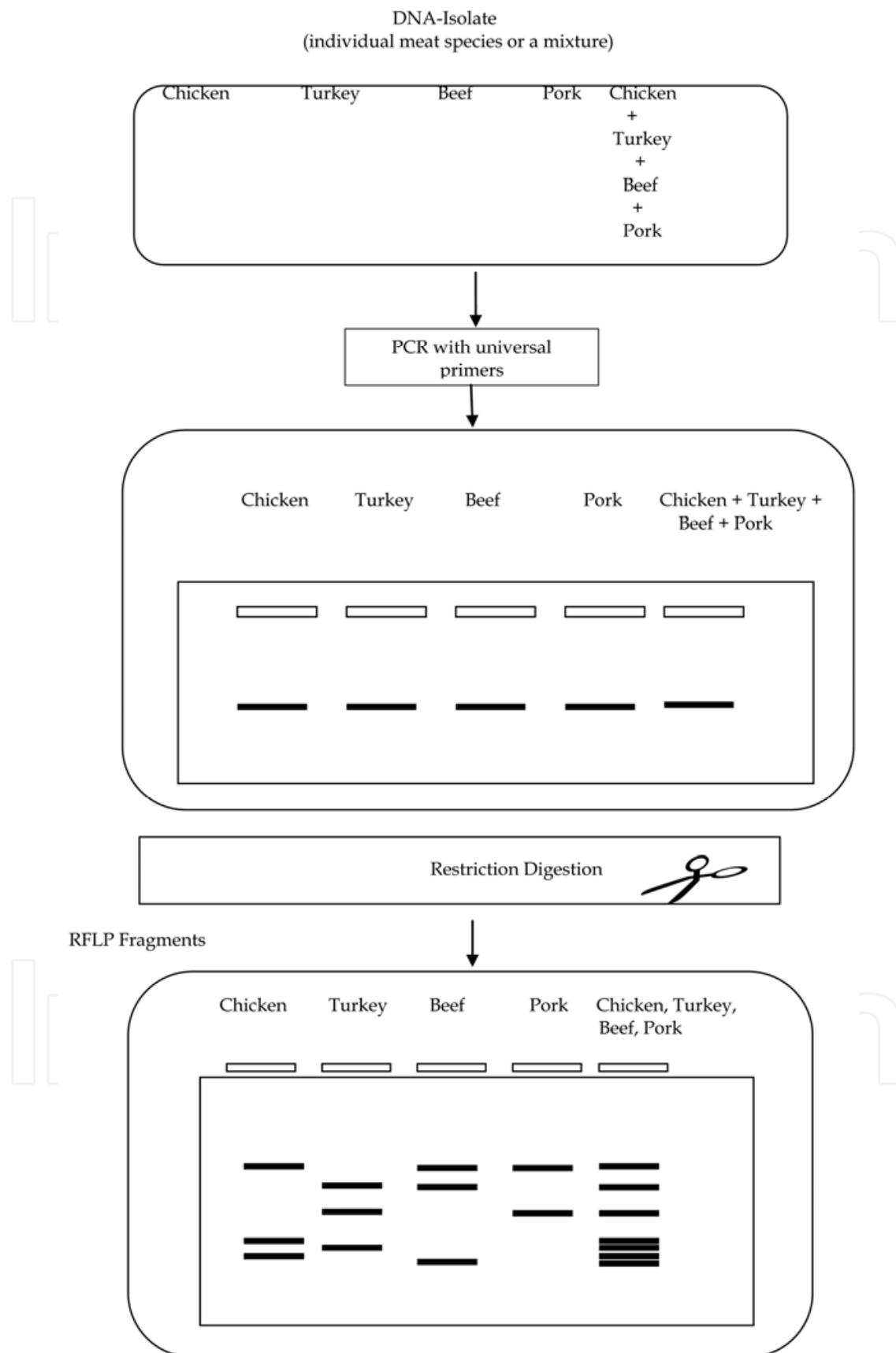


Fig. 4. PCR-Restriction Fragment Length-Polymorphism (PCR-RFLP)

times. Köppel et al. (2009) have for example described the implementation of a heptaplex Real-time PCR assay for the simultaneous identification and quantification of DNA from beef, pork, chicken, turkey, horse meat, sheep (mutton) and goat. Although such multiplex approaches will greatly accelerate meat species identification, results generated must be taken with caution as several meat products are produced with widely varying fat and tissue composition, thus the DNA extractable from similar meat products might vary greatly (Laube *et al.*, 2003).

As regards the accurate differentiation of fish species, several PCR assays have been developed. The majority of these assays rely on the application of universal primers for the generation of consensus sequences among various fish species and the subsequent use of restriction digestion to identify restriction fragments or patterns unique to various fish species. Here, as with meat species differentiation, molecular fish identification methods aim at ensuring that consumers get their money's worth when more expensive fish varieties are bought – substitution of expensive fish with much cheaper varieties can be unravelled by such techniques. Additionally, certain individuals are allergic to certain fish proteins and accurate identification of such potential fish allergens is another argument in favour of a robust fish differentiation method.

4.1 DNA Chip Technology in meat species differentiation

The 20th century saw an explosion of computer technology on all fronts. During the 1990s, molecular biology techniques met with computer electronics to see the birth of a DNA Microarray or DNA chip. One of the earliest attempts at microarray technology for global gene expression was reported by Shena et al., 1995, who designed a quantitative high-capacity system for monitoring of gene expression patterns with a complementary DNA microarray for *Arabidopsis*. Today microarray analyses are widely implemented in molecular biology laboratories, offering the unique advantage of simultaneous analysis of a variety of genetic events in an organism. In food control agencies, the biochip system has also come of age, enabling the quick and efficient analysis of meat products for answers as to their origin and composition.

The first commercial DNA-Chip for the detection of animal constituents in food products is the CarnoCheck Chip (Greiner Biosciences, <http://www.greinerbioone.com>). The chip allows the simultaneous identification of up to 8 different animal species in processed food and meat products with complex composition. The eight animal species detected by the CarnoCheck Test kit are pig, cattle, sheep, turkey, chicken, horse, donkey, and goat. Following sample homogenization and DNA extraction, a 389-bp fragment of the *cyt b* gene of all the animal species present in the food sample is amplified through polymerase chain reaction. By coupling the fluorophore Cy5 onto one of the primers, the amplified fragments are subsequently labelled in the applied PCR reaction. The labelled fragments are then hybridized to complementary oligonucleotide probes fixed as targets on the bottom of the biochip. The target probes themselves are coupled with the Cy3 fluorophore. Due to the use of fluorophore-labeled PCR primers (Cy5) and fluorophore-labeled target probes for the on-chip control system (Cy3), the analysis of the biochips is performed by microarray scanners using wavelengths of ~532 nm (Cy3) and ~635 nm (Cy5).

Another Biochip test system for species differentiation is the LCD-Array from Chipron. The LCD Array (Chipron Germany, <http://chipron.com/index.html>) allows the simultaneous detection of up to 14 animal species in food: cattle, buffalo, pig, sheep, goat, horse, donkey,

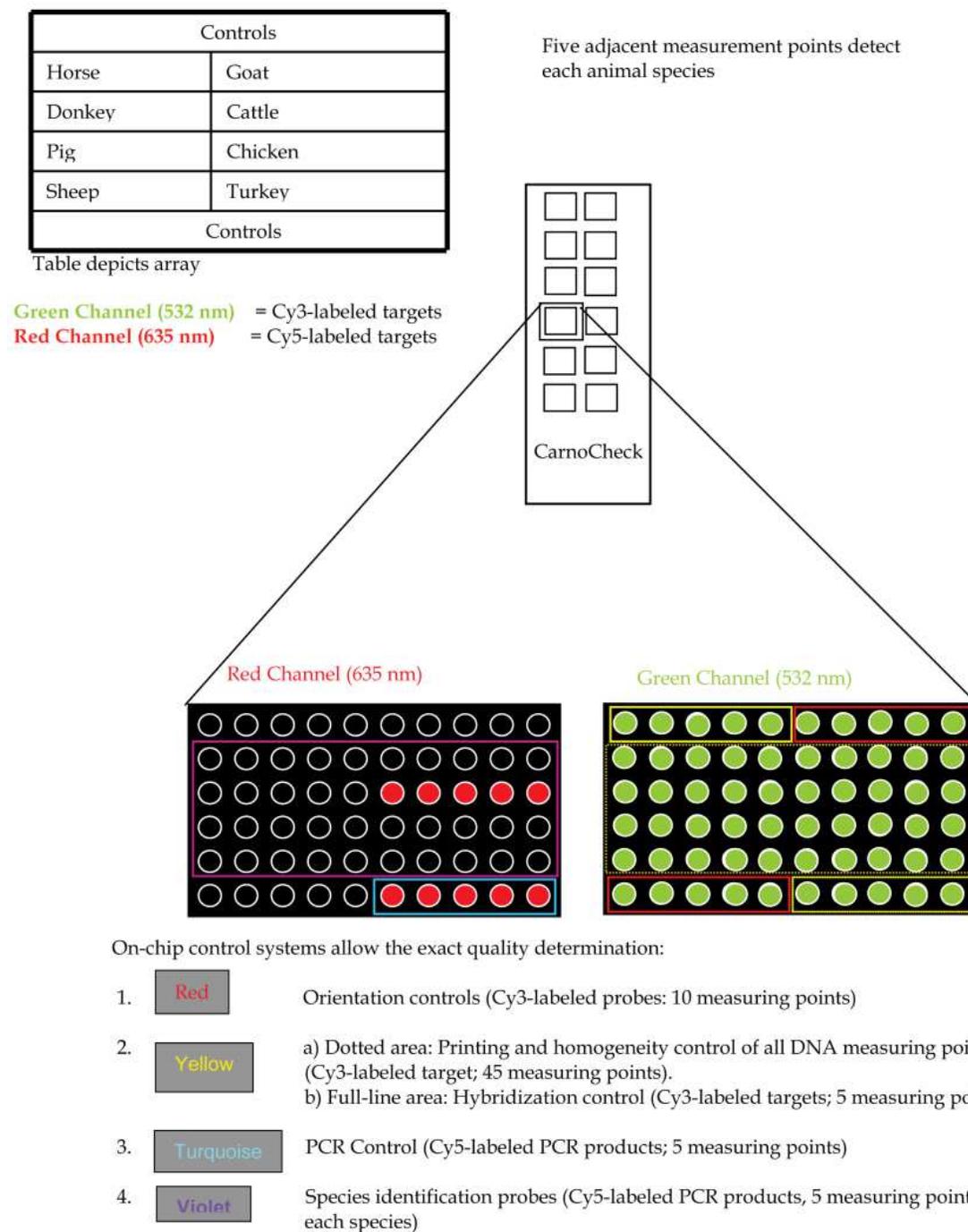
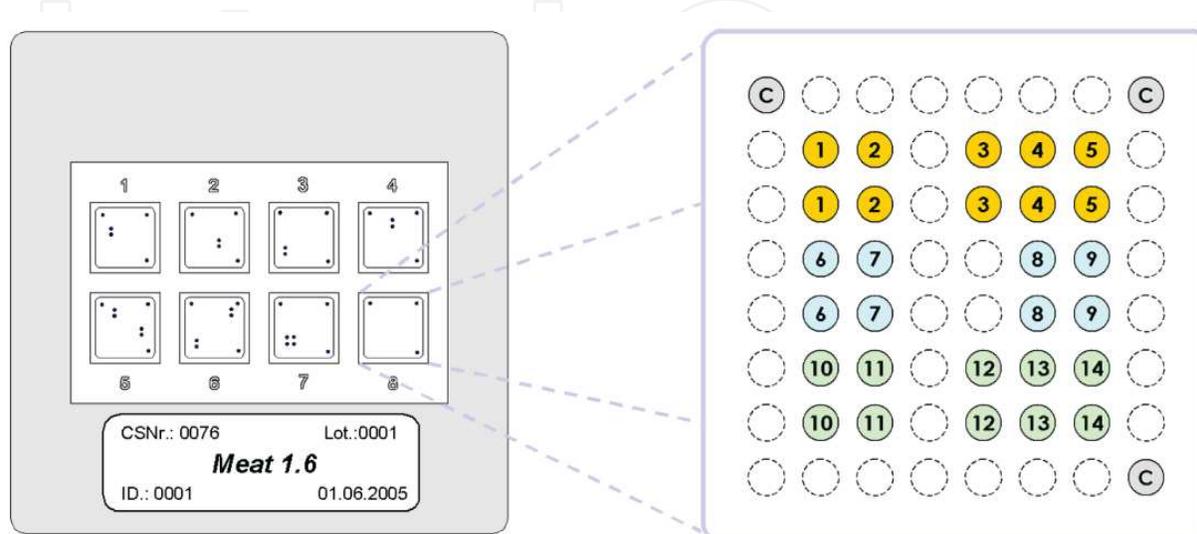


Fig. 5a. CarnoCheck Test kit for the detection of animal species in food. The small table above shows the order of the measurement points for the animal species while the figure below depicts the on-chip control systems for exact quality determination (orientation controls in red, printing controls in green). (CarnoCheck Handbook, Manual version: BQ-020-00, Greiner Bio-one).

rabbit, hare, chicken, turkey, goose, and two duck varieties. The test system here relies on the detection of specific sites within the 16S rRNA mitochondrial locus of all the meat species present in the tested food sample. Included in the test system is a consensus primer pair that amplifies the desired region of the animal species in a PCR. The pre-labeled PCR

primer mix provided with the test kit generates biotinylated amplicons of the animal mtDNA present in the food sample. The labelled PCR fragments are then hybridized to the corresponding capture sequences on the individual array fields. The strong affinity between Biotin and streptavidin is exploited by the LCD Array test principle, and positive samples can be visually identified or by employing the scanner and software provided by the kit manufacturer. Figure 5 provides a schematic representation of the two test systems.



Capture probes

No	Probe	Specificity	No	Probe	Specificity
01	Beef	<i>Bos taurus</i>	08	Rabbit	<i>Oryctolagus cuniculus</i>
02	Buffalo	<i>Bubalus bubalis</i>	09	Hare	<i>Lepus europaeus</i>
03	Pork	<i>Sus scrofa</i>	10	Chicken	<i>Gallus gallus</i>
04	Sheep	<i>Ovis aries</i>	11	Turkey	<i>Meleagris gallopavo</i>
05	Goat	<i>Capra hircus</i>	12	Goose	<i>Ansa albifrons</i>
06	Horse	<i>Equus caballus</i> 1)	13	Mall. Duck	<i>Anas platyrhynchos</i>
07	Donkey	<i>Equus asinus</i> 1)	14	Musc. Duck	<i>Cairina moschata</i>
			C	Hyb-Contr.	Functional controls (Hybridization + stain)

Fig. 5b. LCD Array Meat 1.6 Test System for meat species identification. The figure shows the spotting pattern of the array while the table lists the capture probes immobilized on each array (Data Sheet MeatSpecies 1.6, V-I-08, Chipron)

In a recent study, these two biochip test systems were thoroughly validated and approved for routine use in the meat labour of a food control agency (Iwobi *et al.*, 2011). In this study, the two animal species differentiation biochip methods compared well in efficiency and detection limits were found to be in the range of 0.1% to 0.5% in meat admixtures, with good reproducibility of results. More than 70 commercially available meat samples were analyzed in this work, with the results validated against traditional PCR methodology. Although such a simultaneous PCR approach will lead to accelerated analysis of meat species origin in food, while concomitantly revealing possible sources of deliberate adulteration or contamination, the efficiency of the approach is greatly influenced by the overall proficiency of the PCR reaction. In cases where very small amounts of a meat species is present in the

food matrix, the amplification of such sequences might be hampered by the presence of other meat species present in more abundance in the sample, leading to possible false negative results. Bai *et al.* (2009) cited the inherent complexity, low amplification efficiency, and unequal amplification efficiency on different templates as major drawbacks of currently described multiplex PCR reactions, thus precluding their commercial application. The biochips here described nevertheless hold great promise in the parallel identification of meat species in food products or samples.

5. GM Food and Feed detection using PCR methods

Genetically modified organisms (GMOs) can be defined as organisms in which the DNA has been altered in a way that does not occur naturally. The technology used is often through recombinant DNA procedures and mainly involves the transfer of genetic material, usually from a microbe as donor to another host, in the context of this review, a plant. The resulting GM plants are then used to grow GM food crops. Generally, all GM crops available on the international market today have been designed to confer one of three basic traits to the plant: resistance to insect damage, resistance to viral infections and tolerance towards certain herbicides. Less common are genetic modifications resulting in plant varieties with altered nutritional values, or longer shelf lives (Holst-Jensen, 2007).

Although the DNA elements of interest mostly derive from microbes, such as the *cry* genes from *Bacillus thuringiensis*, which confer resistance to insects and the *cp4 EPSPS* gene encoded by *Agrobacterium sp.*, other eukaryotic hosts may play a role, such as the plant *Petunia hybrida*, which is the source of a chloroplast transit peptide (CTP4). Transformation of the recipient plant cell might be characterized by one or more events or genetic rearrangements. Because current plant transformation procedures do not target specific locations in the recipient's genome, a second transformation event will be directed to a different location within the plant cell, thus making complex, detection of the genetic modification (Holst-Jensen *et al.*, 2006).

From its relatively small beginnings, GM plants have seen a recent explosion in recent times. Worldwide, more than 70 % of all soybeans cultivated are genetically modified, with genetically modified maize accounting for more than a quarter of global outputs. In 2009, for example, genetically modified corn was cultivated in approximately 91 % of all corn fields in the USA. In the most recent report on the Global Status of Commercialized Biotech/GM Crops in 2010, a total of 15.4 million farmers planted biotech crops on an estimated 148 million hectares in 29 countries (James, 2010). Detection and appropriate monitoring strategies are therefore indispensable in many food control agencies.

5.1 Regulation of GMOs

Worldwide, more than 100 genetically modified organisms (GMO) have received authorization for commercial use as food or feed.

Generally, GMOs are regulated by diverse legislation, aimed at protection of consumer safety and health. In the USA, the authorization process is simple and there is no requirement for traceability or labelling of de-regulated (approved) GMOs. In the EU, the GM legislation covering regulatory issues in the approval, detection and monitoring of GMOs is more complex. The authorization and use of genetically modified food and feed is covered by the

provisions of regulation EC no. 1829/2003 and EC No. 1830/2003 (EC 2003a and b). In the EU appropriate thresholds have been set for both unintentional presence of GMOs in non-GMO food backgrounds (0.9 % per ingredient), and zero tolerance for non-approved varieties.

5.2 PCR-based detection and quantification of GMOs

Detection of GMOs usually relies on the identification of the altered genotypic locus or the detection of the novel trait or phenotype arising from the genetic modification event. The genetic modification event will usually result in a new phenotypic trait, arising from the production of a new protein of the modified organism. In the context of plants, which account for the greatest number of GM events, such traits could include resistance to herbicides or pests. For detection of the altered phenotypic traits, a number of immunological assays, typically ELISA tests have been developed and even marketed commercially (Anklam *et al.*, 2002, Stave, 2002). For detection of the genotypic trait, the PCR reaction is the most important approach in use. In this context, real-time PCR detection is the preferred method of choice because of its high specificity, its closed amplification system, resulting in fewer contamination incidents, and its potential for quantification of GMO events.

For a reliable PCR, good quality sample DNA is a prerequisite. Adequate care must be taken to ensure that the sample to be tested is truly representative of the matrix and that it has been adequately homogenized. Failure in extraction of adequate amounts of DNA for the PCR can be most readily overcome by increasing the volume of the sampling pool. Care however has to be taken in this regard as increasing the sample pool will also lead to an increased concentration of contaminants or inhibitors that could negatively hamper the PCR (Holst-Jensen, 2007, Anklam *et al.*, 2002).

In the event of a genetic transformation in an organism, not only the gene encoding the novel and desired trait is transferred, but also other important genetic control elements such as for example the strong 35S - Promoter from cauliflower mosaic virus (CaMV), which promotes high-level expression of the encoded trait, and *Agrobacterium tumefaciens* nos terminator (*nos3'*). Additionally, for easier identification of the transformed plant cells, reporter genes are included in the design of the transformation event. Because the above-mentioned markers are commonly found in many GMOs, they are readily employed for the routine screening of GMO events in food. However, the detection of these GMO markers is only an indication that the analyzed sample contains DNA from a GM plant, but does not provide unequivocal information on the specific trait that has been transformed in the plant. To achieve this, target sequences carrying the gene of interest that are characteristic for the transgenic organism must be reliably determined at their junctures with appropriate regulatory sequences (construct-specific detection). However this complete gene construct may have been transformed into different crops. To provide unambiguous verification of the transformation event in the particular plant under study, PCR reactions targeting the junction at the integration site between the plant genome and the inserted DNA or transgene provide the highest level of specificity (event-specific detection). An example of the principle behind the PCR-based detection of genetically modified plant is depicted below (Fig 6).

Several real-time PCR reactions for the detection of GMOs in food have been published in recent times (Gaudron *et al.*, 2009, Kluga *et al.*, 2011, Pansiot *et al.*, 2011). Reiting *et al.*, (2010) for example recently published a testing cascade for the real-time PCR detection of the genetically modified rice Kefeng6 which is unauthorized in Europe. While this work was

based on the construct-specific detection of this rice line, our lab recently published and validated an event-specific detection of this rice line, allowing greater specificity in its identification (Guertler *et al.*, 2011, in Press). Additionally, we currently developed a modular approach allowing the simultaneous and parallel detection of several GMOs in a food matrix. With this approach, the detection systems for 15 transgenic maize events were combined in one setup, with additional detection of maize and soybean reference genes (see Fig. 7). The reactions are based on validated single detection systems and are run in parallel with identical temperature profiles, thereby allowing the simultaneous detection of all relevant transgenic events together with corresponding controls for DNA quality, reaction setup and contamination (Gerdes *et al.*, 2011, in Press).

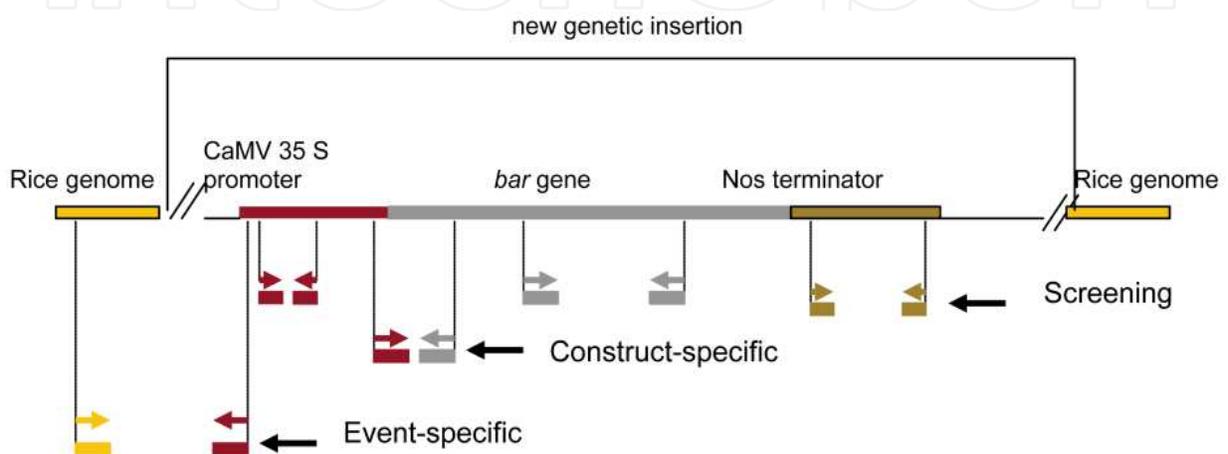


Fig. 6. Principle behind the molecular biological PCR-based detection of a genetic modification event in rice LL601. The commonly employed genetic elements CaMV 35S promoter, the bar gene (encoding herbicide resistance) and nos terminator are here depicted for rapid detection of a genetic modification event. The point of integration of the newly inserted genetic element is the basis for the event-specific detection (adapted from Waiblinger, 2010).

Presently, a major challenge in PCR approaches is the development of multiplex assays for the simultaneous quantification of several targets in the same sample. Multiplexing offers the advantage of lower costs and expenditure, and higher throughput compared to single-target assays. Kalogianni *et al.*, (2007) recently reported on a multiplex quantitative PCR based on a multianalyte hybridization assay performed on spectrally encoded microspheres. While these endpoint PCR approaches hold great promises, one major drawback is the requirement of separate steps for DNA amplification and detection of the products. Quantitative real-time PCR which allows continuous monitoring of the amplification products by a homogeneous fluorometric assay account therefore for the most widely used approach in GMO testing (Su *et al.*, 2011, Xu *et al.*, 2011,). In this regard, Köppel and colleagues reported on the development of a multiplex real-time PCR assay for the simultaneous detection and quantification of DNA from three transgenic rice species and construction and application of an artificial oligonucleotide as reference material. Their test exhibited good specificity and sensitivity for the transgenes was in the range of 0.01-1% (Köppel *et al.*, 2010b). In summary, real-time PCR assays remain the gold standard in the analysis of GMO events in food. Because of the trend toward multiple detection events, multiplexing, with microarray-based methods will most likely continue to see greater applications in the future.

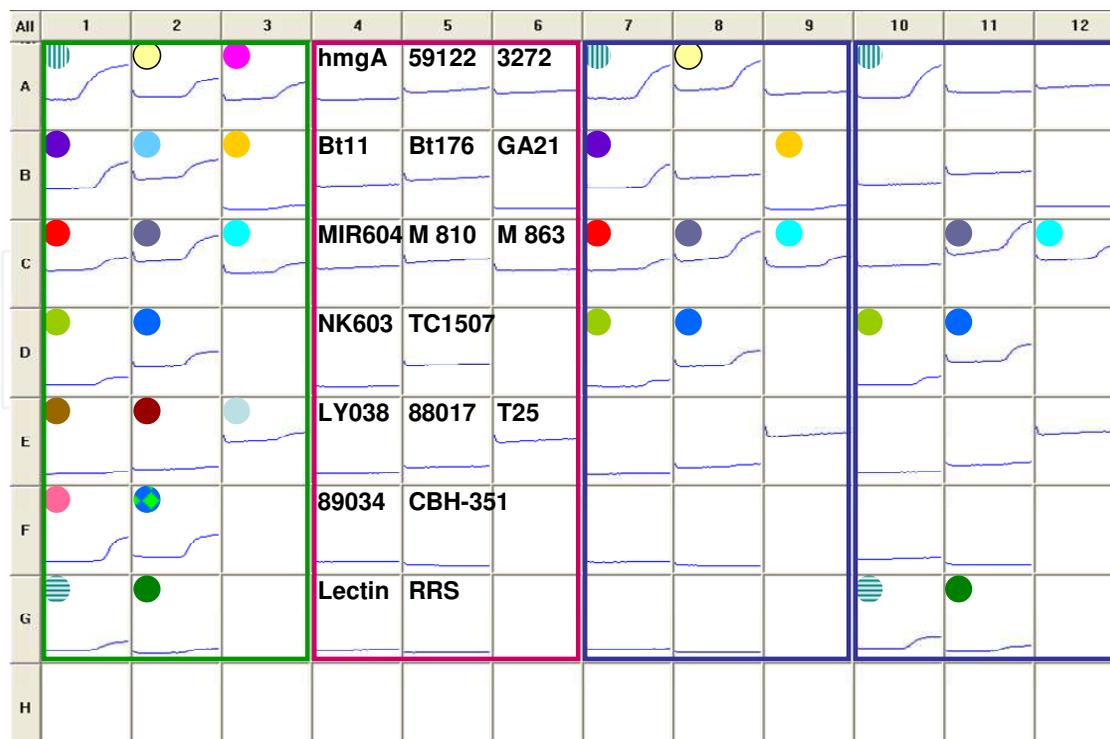


Fig. 7. Analysis of samples with the maize module

Two chocolate bar samples were analysed with the maize module on the Mx3005P. An overview of the recorded FAM fluorescence (R) of all 96 wells is shown. Positive control reactions are enclosed by green, negative control reactions by red, and samples by blue boundaries, respectively. Positive reactions were marked with a coloured dot in the upper left corner. All samples reacted positive for hmgA thus confirming that amplifiable DNA was present. One sample tested positive for eight maize events, the other was positive for four maize events, and RoundupReady soy (RRS).

6. Conclusion

PCR-based applications in food control agencies have seen a tremendous boost in recent years. The simplicity, specificity and rapidity inherent in molecular-based approaches continue to make them increasingly attractive in a wide spectrum of food analytical procedures. Multiplexing applications will continue to see an increase in the near future as the demand for simultaneous detection and quantification of various events in food matrices grows. Additionally, it is expected that increased instrumental development will push the drive toward automation of various analytical procedures commonly employed in food diagnostics.

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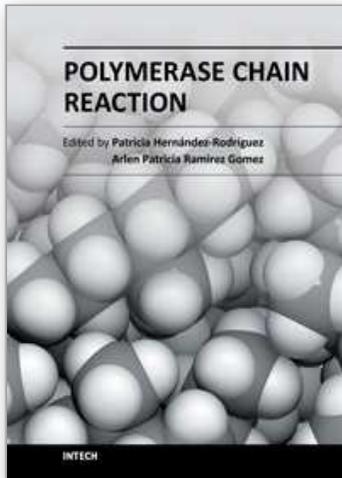
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