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REVIEW

Comparison of nine different real-time PCR chemistries for qualitative and quantitative applications in GMO detection

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Abstract Several techniques have been developed for detection and quantification of genetically modified organisms, but quantitative real-time PCR is by far the most popular approach. Among the most commonly used real-time PCR chemistries are TaqMan probes and SYBR green, but many other detection chemistries have also been developed. Because their performance has never been compared systematically, here we present an extensive evaluation of some promising chemistries: sequence-unspecific DNA labeling dyes (SYBR green), primer-based technologies (AmpliFluor, Plexor, Lux primers), and techniques involving double-labeled probes, comprising hybridization (molecular beacon) and hydrolysis (TaqMan, CPT, LNA, and MGB) probes, based on recently published experimental data. For each of the detection chemistries assays were included targeting selected loci. Real-time PCR chemistries were subsequently compared for their efficiency in PCR amplification and limits of detection and quantification. The overall applicability of the chemistries was evaluated, adding practicability and cost issues to the performance characteristics. None of the chemistries seemed to be significantly better than any other, but certain features favor LNA and MGB technology as good alternatives to TaqMan in quantification assays. SYBR green and molecular beacon assays can perform equally well but may need more optimization prior to use.

Keywords Real-time PCR · GMO detection · TaqMan · SYBR green · Molecular beacons · Alternative chemistries

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Introduction

Detection and quantification of genetically modified (GM) components in compound samples is a challenging task. Labeling threshold for food and feed ranges from for example 5% in Japan to as low as 0.9% in the European Union [1] and this requires exact quantification and the ability to detect trace amounts of GM material in many different sample types. In addition to this, a large fraction of the samples are highly processed materials for which the DNA extraction yield is generally quite low. At the same time, the number of GM organisms (GMOs) released to the market is rapidly increasing [2]. This makes it even more complicated to develop comprehensive routine detection systems for GMOs, so a methodological upgrade would be welcome.

In a GMO, a transgenic construct containing one or more trait genes has been inserted in the species' genome. To detect or to quantify GMOs routine diagnostic laboratories most commonly rely on quantitative real-time PCR (Q-PCR). In the first step of analysis screening for GMOs is performed, followed by identification and quantification of GMOs present in the sample. The general purpose of GMO quantification as defined in the legislation is then to calculate the fraction of a certain species that comes from GM materials. For this, reference gene quantification is needed to estimate the number of haploid target genomes that are present in the sample. To determine the number of haploid GM genome copies the sample contains event specific assays are used. On the other hand, only qualitative analysis is performed in the screening and identification steps of GMO detection procedures [3, 4]. There are currently about 20 different real-time PCR chemistries on the market [5], only few are however, applied in internationally validated GMO detection methods [6]. Mostly they are based on the use of either TaqMan/MGB probes or SYBR green-based procedures, and large amounts of data can be found on the performance of these. On the other hand only few comparisons have been made on the performance of alternative chemistries [7, 8].

This article gives an overview of some options available on the market for which experimental data in the context of GMO detection have recently been published [9–11]. Special emphasis was put on comparative evaluation of performance characteristics such as specificity, sensitivity, repeatability, and dynamic range for nine different real-time PCR chemistries, and robustness, cost efficiency, and practicability were also considered.

Intercalating dyes

The first demonstration of real-time PCR was done by simply adding ethidium bromide to the PCR mix and monitoring the amplification via the fluorescent properties of the reaction volume [12]. To increase sensitivity, ethidium bromide was later replaced by another intercalating dye—SYBR green [13]. SYBR green, like all other intercalating dyes, binds any double-stranded DNA (Fig. 1), so in order to separate non-specific from specific amplicons, it must be assumed that different PCR products will have different melting temperatures. Under this assumption, a so-called melting ("dissociation") curve can be generated by carefully monitoring the fluorescence properties of the PCR amplification products during a melting phase. This might aid the user in ensuring that non-specific amplification has not taken place [14]. Despite its popularity, SYBR green has several disadvantages, for example high tendency to inhibit PCR at higher concentration and preferential binding to specific DNA sequences [15, 16]. Low affinity to single-stranded DNA has also been noted [17] and interpretation of the DNA dissociation curves is not always straightforward.

Primer-based chemistries

Primer-based chemistry assays are more reliable than those based on intercalating dyes. Here, a dye does not have to be added directly to the reaction mixture because a fluorescently labeled PCR primer is used instead. These assays are relatively inexpensive to run and also fairly straightforward to design. In the Lux technology one of the primers is labeled with a fluorophore close to the 3′ end which is quenched by the hairpin structure of the primer. On integration of labeled primer into a PCR product, its fluorescence increases up to eightfold because of extension of the hairpin structure [18]. Plexor technology differs from the other chemistries in the decrease of fluorescence signal in proportion to the increase in the number of PCR products during the course of amplification. One of the primers contains a synthetic base, isocytosine, linked to the fluorophore at the 5′ end. During the amplification, this isocytosine pairs to iso-dGTP from the reaction solution and thus becomes incorporated in the newly synthesized strand. Iso-dGTP is linked to a quencher, and when incorporated in the PCR product, it is close enough to the fluorophore to quench its fluorescence. Thus with increased number of PCR products we measure a decrease in the fluorescent signal [19]. Both Lux and Plexor technologies enable dissociation curves to be analyzed and it should thus be possible to distinguish between specific and non-specific amplicons. AmpliFluor is based on three primers—two target-specific and a universal dual-labeled hairpin primer (UniPrimer). One of the specific primers includes a so-called Z sequence at the 5′ end, and this sequence is also present as a tail on the hairpin primer. As PCR products are formed and the complementary strand of the Z sequences is synthesized, the hairpin primer's tail can anneal to the newly formed amplicons and be elongated. When the complementary strand of such an elongated hairpin primer tail is synthesized (primed by a target-specific primer), the hairpin structure will be pulled open thus eliminating the quenching. Because of technology characteristics, AmpliFluor specificity cannot be monitored using a dissociation curve [20].

Probe-based chemistries

To increase the assay's specificity one can use a third oligonucleotide, a fluorescence probe, which is comple-

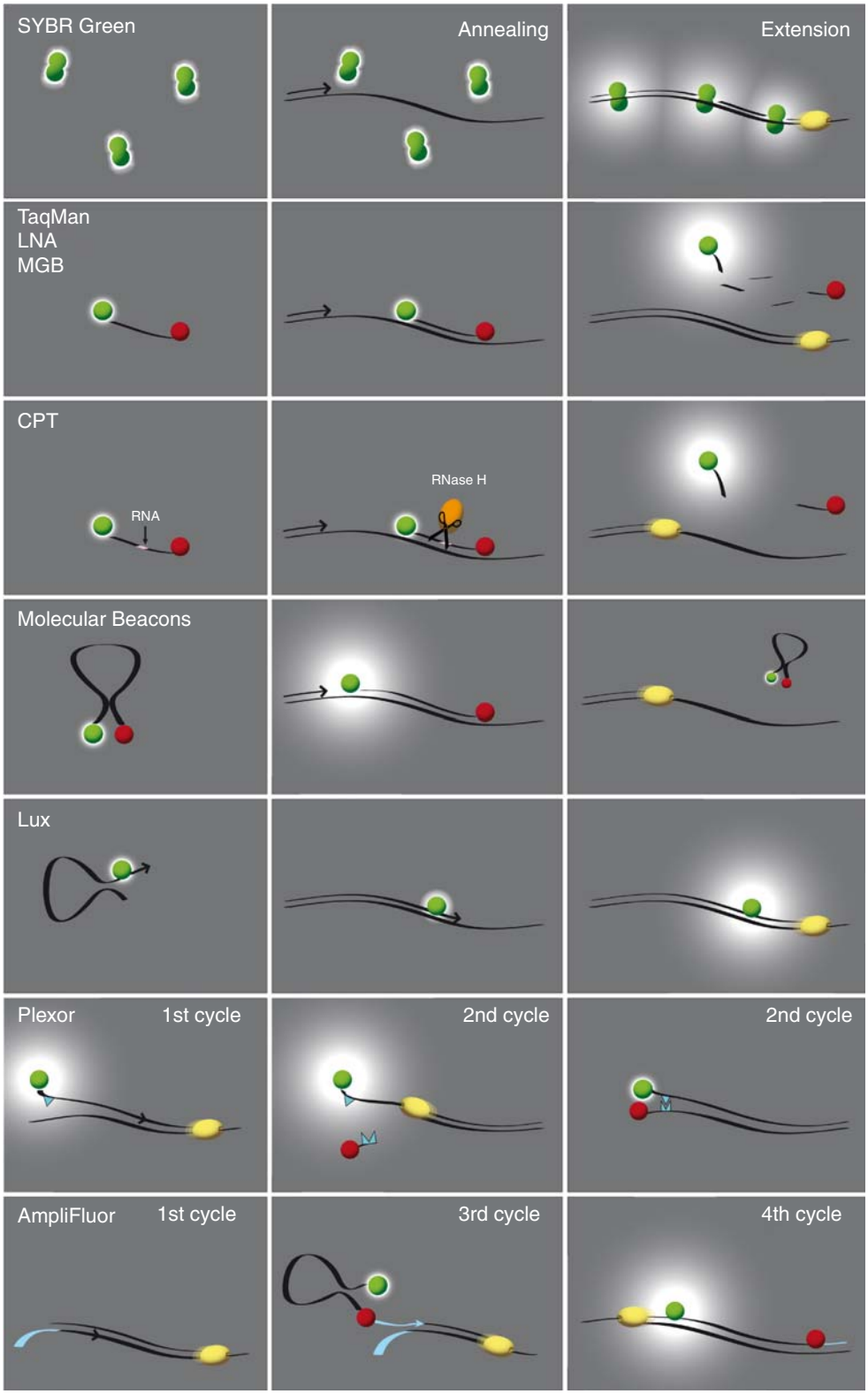

Fig. 1 Schematic representation of real time PCR with different chemistries. Principles of TaqMan, minor groove binding (MGB), locked nucleic acid (LNA) probes, cycling probe technology (CPT), and molecular beacons probes are presented together with non-specific SYBR green dye (*in green*) and labeled primer based Lux, Plexor, and Ampliflour. Chemistries are presented during the primer annealing step and after the extension, when the new strand of DNA is synthesized by DNA polymerase (*in yellow*) with a reporter fluorophore *shown in green* and a quencher *shown in red*. In the case of Plexor the dyes are linked to isocytosine (*blue triangle*) and iso-dGTP (*also in blue*). AmpliFluor is based on three primers—two target-specific and a universal dual-labeled hairpin primer (UniPrimer). One of the specific primers includes a so-called Z sequence at the 5′ end, which is also present as a tail on the hairpin primer (both marked here as a *blue line*)

mentary to a target sequence lying between the PCR primers. Each probe has a reporter fluorophore covalently attached to one end and a quencher attached to the other. As long as both dyes stay in close proximity the signal is quenched, and it is released only when dyes become physically separated [21]. The original TaqMan probe design has been further extended to include probes such as minor groove binding (MGB) and locked nucleic acid

(LNA) probes. Because of their increased melting temperatures, these probes can be designed to be shorter and have higher hybridization specificity. In MGB probes a higher melting temperature is achieved by conjugation with a minor groove binding group [22], while LNA nucleotides have a modified ribose moiety with an extra 2′-O,4′-C-methylene bridge. LNA nucleotides "lock" the probe on the target, therefore the LNA-modified probes are known to exhibit enhanced hybridization affinity toward complementary DNA [23, 24]. The cycling probe technology (CPT) probe in contrast includes a modified RNA nucleotide forming a RNA–DNA duplex after hybridization to the target. In the next step this duplex is recognized and cut by RNaseH, resulting in separation of the quencher from the reporter, accompanied by a fluorescence increase. In this case no exonuclease activity of Taq DNA polymerase is needed to get an increase in the signal [25]. Molecular beacons (MB), on the other hand, are not hydrolysis probes but hybridization probes. They consist of a sequence-specific loop region flanked by two inverted repeats which form a hairpin structure. To bind to a complementary target sequence, the beacon unfolds, leading to separation of the fluorophore from the quencher and an increase in fluorescence [26].

Comparative evaluation of real-time PCR chemistries

This manuscript is an integrative analysis of experiments recently published [9–11]. Nine different real-time PCR

Table 1 Performance of different chemistries, compared as LOD, LOQ, dynamic range, efficiency, and repeatability

	Probe-based chemistries					Primer-based chemistries			Intercalating dyes
	TaqMan[a]	MGB	MB	LNA	CPT	Plexor	Lux	AmpliFluor	SYBR green
Detection limit (PFU)	+++	+++	+++	+++	++	+++	+++	+++	+++
Assay 1	8[b]	8	8	4	20	20	4	2	8
Assay 2	5	5	/	20	20	4	20	/	5
Quantification limit (PFU)	+++	+++	+++	+++	++	+++	++	+	+++
Assay 1	31	31	31	20	100	100	100	500	125
Assay 2	50	50	/	100	400	100	200	/	50
Dynamic range	+++	+++	++	+++	++	+++	++	+	++
Assay 1	250	250	60	200	40	40	80	5	10
Assay 2	200	200	/	400	100	400	200	/	200
Amplification efficiency in dynamic range (%)	+++	+++	+++	+++	+++	++	+++	+++	++
Assay 1	95	94	95	101	98	86	93	103	81
Assay 2	88	89	/	97	93	91	90	/	98
Repeatability in dynamic range-Cv (%)	+++	+++	++	+++	+++	+++	+++	+++	++
Assay 1	13	11	16	10	5	13	11	11	21
Assay 2	12	9	/	11	14	13	10	/	16

Experimental data were taken from Refs. [9–11]. LOD is defined as a concentration that yields ≥95% probability of detecting the target if present. For the purpose of this comparison LOD was set at the PFU (PCR forming units; corresponding to amplifiable target copies) for which more than half of PCR reactions gave a positive result. This definition corresponds to "Results interpretation" section of ISO 21569 standard [33]. PCR efficiency (E) was calculated from the slope of the standard curve, using the equation $E=10(-1/\text{slope})-1$, with 1 corresponding to 100% efficiency [34]. To determine LOQ the relative standard deviation (RSDr) ≤ 25% was the primary measurement [35]. In addition, to estimate the dynamic range of the method, PCR reactions were regarded as not inhibited when the PCR efficiency was in the range $75\% < E < 120\%$ [10]. The dynamic range was expressed as a ratio between the highest and the lowest number of DNA copies within the range. PCR efficiency within the dynamic range of the assay was used in comparative analysis. An average coefficient of variation (Cv) was calculated within each dynamic range to describe repeatability between parallels

[a] TaqMan-based amplicons were optimized in previous publications [36, 37] and were analyzed here in parallel as a reference system

[b] Assays 1 for TaqMan, MGB, MB, AmpliFluor, and SYBR green were designed on the MON 810 3′ junction and tested on 2, 4, 8, 31, 125, 500, and 2,000 PFU [11]. Assays 2 for TaqMan, MGB, and SYBR green were designed on the RRS 3′ junction and tested on 5, 10, 50, 100, 1,000, and 10,000 PFU [9]. Assays 1 for LNA, CPT, Plexor and Lux were designed on the MON 810 5′ junction and tested on 0.4, 4, 20, 100, 200, 400, 4,000, and 8,000 PFU. Assays 2 for LNA, CPT, Plexor, and Lux were designed on maize invertase gene and tested on 0.4, 4, 20, 100, 200, 400, 4,000, and 40,000 PFU [10]. Assays developed on RRS junction were run on five total replicates while all other assays were run on ten

chemistries were systematically tested, including SYBR green I (Applied Biosystems, USA/Sigma–Aldrich Quimica, Spain), AmpliFluor universal detection system (Chemicon International, USA), Light upon extension (Lux) fluorogenic primers (Invitrogen, USA), Plexor technology (Promega, USA), TaqMan and MGB probes (both DNA Technology, Denmark/Applied Biosystems, USA), molecular beacons, LNA probes (Sigma Proligo, France), and CPT probes (Takara, Japan).

To make a target-independent evaluation of these chemistries, for each of the detection chemistries two assays were designed on selected loci of MON 810 (YieldGuard) maize or GTS 40-3-2 (Roundup Ready) soybean (RRS). AmpliFluor and MB, however, were an exception with only one assay included in this evaluation. All the assays were individually optimized before comparison with each other. In all three articles assay designs are explained in detail [9–11].

Other factors that could influence performance were harmonized in different setups. FAM was used as a fluorophore in all chemistries (the assay designed for AmpliFluor was an exception with JOE used as a fluorophore) and PCRs were run on two similar apparatuses, the ABI 7300 Real Time PCR and the ABI Prism 7900HT sequence detection system [9–11].

Chemistries were compared for their performance characteristics, practicability, and cost effectiveness. In an attempt to compare the chemistries in a unified way, the performance characteristics were calculated in the same manner from all three sets of raw data (see supplemental data).

Performance characteristics

For performance evaluation limit of detection (LOD), quantification (LOQ), dynamic range, PCR efficiency, and repeatability were compared. The evaluation was based on the "acceptance criteria" compiled by the European Network of GMO Laboratories (ENGL) [27].

All the developed assays show rather similar LOD values, between 2 and 20 PFU (PCR forming units; corresponding to amplifiable target copies) (Table 1). The LOQ results on the other hand were more varied with the highest LOQ values determined for AmpliFluor (500 PFU), CPT assay 2 (400 PFU) and Lux assay 2 (200 PFU). Other LOQ values were around or below 100 PFU. It should be noted, however, that LOD and LOQ could have been better for LNA, CPT, Plexor and Lux assays, but additional sample dilutions with PFU between 20 and 100 had not been tested. Because of the very high LOQ value, the narrow dynamic range for AmpliFluor was not surprising. Another assay with a narrow dynamic range was SYBR green assay 1, which also had the lowest amplification efficiency (81%). This indicates general problems with this assay's performance. On the other hand, the other assay employing SYBR green performed well, which proves that

Table 2 Practicability and overall evaluation of different chemistries

	Probe-based chemistries					Primer-based chemistries			Intercalating dyes
	TaqMan	MGB	MB	LNA	CPT	Plexor	Lux	AmpliFluor	SYBR green
Practicability and cost-effectiveness	+++	+++	++	+++	+	++	+	+	++
Ease of design	+++	+++	++	+++	++	++	+	+	++
Ease of operation	+++	+++	+++	+++	+++	++	++	++	++
Price[a] to establish a new assay	High	High	High	High	High	Middle	Middle	Middle	Low
Price[b] for 100×20 μL reactions (€)	100	120	100	105	>200	85	80	130	75
Run duration (min)	120 (50)[c]	120 (50)[c]	120 (50)[c]	120	84	87	144	140	120
Suitable for quantitative analysis	+++	+++	++	+++	++	++	+	+	++
Suitable for qualitative analysis	+++	+++	+++	+++	++	+++	++	++	+++

The ease of design was evaluated as the time required to design and validate a new method by trained personnel. The ease of operation, on the other hand, evaluates the personnel effort for setup and post analysis interpretation. The price of assays was calculated by assessing the costs of chemicals needed for 100 reactions with a 20 μL reaction volume. Estimated costs include PCR reagents, primers, and probes but exclude plastics and optical covers since they are the same for all tests. Time needed for a PCR run was also included in practicability issue

[a] For research purposes when few samples only are planned for analysis

[b] Reagents used in calculations were ABI TaqMan Universal PCR Master Mix for TaqMan, MGB, MB and LNA, ABI Power SYBR green PCR Master Mix for SYBR (both Applied Biosystems, USA) and recommended reagents for other chemistries. Prices are stated for Slovenia (CPT, Plexor, Lux) and Spain (TaqMan, MGB, MB, LNA, AmpliFluor, SYBR), both members of European Union.

[c] Number in parentheses stands for a fast cycling mode (where tested)

this chemistry can enable good detection and quantification, but it is less robust and designing a good assay is more difficult. The same was observed for MB, of which the assay included in the review showed great performance, while the authors failed to optimize the other MB assay [9]. Despite several attempts, the assay was not sufficiently optimized and for this reason it was excluded from the review. Another disadvantage of SYBR green and MB is the rather low repeatability between parallels, observed in all three assays.

CPT was less sensitive than the other chemistries and performed somewhat worse than other hydrolysis probes, considering LOQ and dynamic range. In performance of TaqMan, LNA and MGB probes there was no major difference. Low LOD and LOQ values and mainly wide dynamic range prove suitability of TaqMan, LNA, and MGB for both qualitative and quantitative analysis. Differences in performance between Plexor and Lux chemistries are not clearly visible in Table 1; however, there were fewer problems with non-specific amplification using Plexor [10].

Practicability and cost effectiveness

Practicability of the assays was evaluated according to the criteria ease of design and of operation, the first meaning the time required to design and validate a new method and second focusing on personnel effort for setup and post-analysis interpretation. In addition the respective costs were also included in the evaluation (Table 2). There is no major difference in applicability of TaqMan, LNA and MGB probes, which suggests that the latter two are good alternatives to TaqMan when designing assays for quantitative analysis. Because LNA and MGB probes can be designed much shorter they are more sensitive to a non-perfect match in target sequence and thus are appropriate for use where high specificity is needed (e.g. only one nucleotide difference in the sequence). They also offer more possibilities when the target locus is so short that the design of a satisfactory TaqMan-based assay is difficult, for example in detecting some junctions between GM insert and plant DNA. CPT probes can be designed even shorter, but moderate performance score and significantly higher costs make them inappropriate for routine use. Practicability of molecular beacons was similar to CPT and both have more advantages in nucleic acids detection under isothermal conditions and low temperatures [28] compared with real-time PCR.

Among primer-based chemistries easier design and shorter run time favors Plexor when compared to Lux and AmpliFluor. Despite being less expensive than probe-based chemistries, personnel effort when introducing this chemistry in the laboratory can be its disadvantage. SYBR green is appropriate for qualitative and quantitative detection, but careful design and some optimization is needed to obtain a good assay.

Conclusions and future perspectives

To summarize, several real-time PCR chemistries generally perform as well as the currently most broadly applied TaqMan chemistry. Certain features of LNA and MGB technologies even favor their use in quantification, because they are more flexible in design if the target sequence is narrowly defined. Also, some primer based chemistries, for example Plexor, can become the method of choice if both performance and cost are taken into account. Therefore, when designing new real-time PCR assays for routine diagnostics, these alternatives should be taken into consideration.

Some recently developed chemistries were not included in this comparison. Among improved intercalating dyes, SYTO9 and EVA green have both been reported to be less inhibitory to PCR than SYBR green [29, 30]. Probe-based technologies AllGlo and EasyBeacons may also be of special interest. The EasyBeacons contain two (or more) identical reporter dyes capable of quenching their own fluorescence. After the enzymatic degradation of the probe, two fluorophores are released per probe so, in principle, AllGlo probes should be brighter than conventional single-label probes [31]. EasyBeacons on the other hand are composed of normal and intercalating pseudo nucleotides and are thought to be particularly suitable for single nucleotide polymorphism (SNP) detection [32]. More detailed experimental evaluation would be needed to check their potential in routine GMO diagnostics.

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References

1. Gruere GP, Rao SR (2007) AgBioForum 10:51–64
2. James C (2009) Global status of commercialized biotech/GM crops 2008. ISAAA brief 39, 1–20. Ithaca, NY
3. Holst-Jensen A, Ronning SB, Lovseth A, Berdal KG (2003) Analytical and Bioanalytical Chemistry 375:985–993
4. Holst-Jensen A (2007) Sampling, detection, identification and quantification of genetically modified organisms (GMOs). In: Food Toxicants Analysis. Techniques, Strategies and Developments. Elsevier, Amsterdam, Netherlands
5. Gene Quantification [http://gene-quantification.info/]. 24-7-2009
6. Bustin SA (2005) Expert Rev Mol Diagn 5:493–498

7. Hernandez M, Esteve T, Prat S, Pla M (2004) J Cereal Sci 39:99–107
8. Terry CF, Shanahan DJ, Ballam LD, Harris N, McDowell DG, Parkes HC (2002) J AOAC Int 85:938–944
9. Andersen CB, Holst-Jensen A, Berdal KG, Thorstensen T, Tengs T (2006) J Agric Food Chem 54:9658–9663
10. Buh GM, Cankar K, Zel J, Gruden K (2008) BMC Biotechnol 8:26
11. La Paz JL, Esteve T, Pla M (2007) J Agric Food Chem 55:4312–4318
12. Higuchi R, Fockler C, Dollinger G (1993) Watson R. Biotechnology (NY) 11:1026–1030
13. Schneeberger C, Speiser P, Kury F, Zeillinger R (1995) PCR Methods Appl 4:234–238
14. Ririe KM, Rasmussen RP, Wittwer CT (1997) Anal Biochem 245:154–160
15. Giglio S, Monis PT, Saint CP (2003) Nucleic Acids Res 31:e136
16. Karsai A, Muller S, Platz S, Hauser MT (2002) Biotechniques 32:790–796
17. Zipper H, Brunner H, Bernhagen J, Vitzthum F (2004) Nucleic Acids Res 32:e103
18. Nazarenko I, Pires R, Lowe B, Obaidy M, Rashtchian A (2002) Nucleic Acids Res 30:2089–2195
19. Sherrill CB, Marshall DJ, Moser MJ, Larsen CA, Daude-Snow L, Jurczyk S, Shapiro G, Prudent JR (2004) J Am Chem Soc 126:4550–4556
20. Nazarenko IA, Bhatnagar SK, Hohman RJ (1997) Nucleic Acids Res 25:2516–2521
21. Holland PM, Abramson RD, Watson R, Gelfand DH (1991) Proc Natl Acad Sci USA 88:7276–7280
22. Kutyavin IV, Afonina IA, Mills A, Gorn VV, Lukhtanov EA, Belousov ES, Singer MJ, Walburger DK, Lokhov SG, Gall AA, Dempcy R, Reed MW, Meyer RB, Hedgpeth J (2000) Nucleic Acids Res 28:655–661
23. Costa JM, Ernault P, Olivi M, Gaillon T, Arar K (2004) Clin Biochem 37:930–932
24. Koshkin AA, Singh SK, Nielsen P, Rajwanshi VK, Kumar R, Meldgaard M, Olsen CE, Wengel J (1998) Tetrahedron 54:3607–3630
25. Duck P, Varado-Urbina G, Burdick B, Collier B (1990) Biotechniques 9:142–148
26. Tyagi S, Kramer FR (1996) Nat Biotechnol 14:303–308
27. European Network of GMO Laboratories (ENGL). Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing (2008). http://gmo-crl.jrc.ec.europa.eu/ 2009
28. Santangelo P, Nitin N, Bao G (2006) Ann Biomed Eng 34:39–50
29. Mao F, Leung WY, Xin X (2007) BMC Biotechnol 7:76
30. Monis PT, Giglio S, Saint CP (2005) Anal Biochem 340:24–34
31. AlleLogic Biosciences Corp [http://www.allelogic.com/]. 1-8-2009
32. Christensen UB (2008) Methods Mol Biol 429:137–160
33. ISO 21569:2005. Foodstuffs—Methods of analysis for the detection of genetically modified organisms and derived products—Qualitative nucleic acid based methods. 1–69. 20-6-2005. Geneva, Switzerland; 2005
34. Pfaffl MW (2001) Nucleic Acids Res 29:e45
35. ISO 24276:2006. Foodstuffs—Methods of analysis for the detection of genetically modified organisms and derived products—General requirements and definitions. 1–16. 20-6-2005. Geneva, Switzerland; 2005
36. Berdal KG, Holst-Jensen A (2001) Eur Food Res Technol 213:432–438
37. Hernandez M, Pla M, Esteve T, Prat S, Puigdomenech P, Ferrando A (2003) Transgenic Res 12:179–189