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Review

Guidelines for validation of qualitative real-time PCR methods☆

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As for many areas of molecular testing, detection of Genetically Modified Organisms (GMO) relies on the real-time Polymerase Chain Reaction (qPCR) technology. Due to the increasing number of GMO, a screening approach using qualitative screening methods has become an integrated part of GMO detection. However, specific guidelines for the validation of these methods are lacking. Here, a pragmatic approach to conduct in-house and inter-laboratory validation studies for GMO screening methods, is proposed. Such guidelines could be adapted to other areas where qualitative qPCR methods are used for molecular testing allowing to implement easily a more reliable screening phase where necessary.

Background

In many countries, GMO commercialisation is strictly regulated (Gruère & Rao, 2007; Zel *et al.*, 2012). This implies that each biotech company wishing to bring a GM event on the market needs to file a dossier for authorisation/deregulation. In addition, in the European Union (EU) for example, biotech companies need to make available the GM material and its conventional counterpart as well as a reliable detection/quantification method (Commission Regulation EC/641/2004). Currently, in the EU the "Golden Standard" for such detection methods is qPCR. The European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF) validates these methods through ring trials with the help of the National Reference Laboratories (NRL). If the method complies with the criteria set up by the European Network of GMO laboratories (European Network of GMO laboratories [ENGL], 2008), it is made publicly available (http://gmo-crl.jrc.ec.europa.eu/). Subsequently, it can be implemented by the GMO detection laboratories (ENGL, 2011a).

In the last years, the number of GM events being commercialised worldwide has been increasing steadily (James, 2013; Stein & Rodriguez-Cerezo, 2009a). To date, the International Service for the Acquisition of Agri-biotech Applications (ISAAA) has counted a total of 336 GM events in 27 crop species being approved for commercialisation, planting and/or for food/feed use (http://www.isaaa.org/gmapprovaldatabase/ (24/02/2014); James, 2013). In the EU only, to date, 42 single GM events, 20 double stacked events, 4 triple stacked and 1 quadruple stack events have been authorised under the legislations

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EC/1829 (2003) and EU/619 (2011) (http://ec.europa.eu/food/dyna/gm_register/index_en.cfm; 24/02/2014). Additionally, the presence of events that are unauthorised in one country but authorised in another for food and feed use (further referred to as UGM) may increase in the coming years (Holst-Jensen *et al*., 2012; James, 2013; Stein & Rodriguez-Cerezo, 2009b). A one-by-one basis for the identification of each GM event using event-specific assays becomes therefore unrealistic. Hence many enforcement laboratories have established a screening approach which allows saving time and costs as it aims at drawing conclusions on the presence/absence of as many GM events as possible using a minimum set of screening qPCR methods (*i.e*. taking into account the coverage and discriminative power of each target).

Hereto the official European laboratories develop their screening methods in house as they are not taken into consideration by the EU regulations. Most of the screening qPCR methods developed at the present time are qualitative and use SYBR®Green or TaqMan® chemistry. They aim at detecting one element (singleplex) or multiple sequences simultaneously (multiplex) and can target a taxon-specific sequence or a GM element (generic and trait) present in the transgenic construct(s). In addition, construct-specific qualitative qPCR methods (*i.e*. targeting the junction between two GM elements within the transgenic insert) can be regarded as suitable for screening purposes. Several such screening methods have been developed by different laboratories (Bahrdt, Krech, Wurz, & Wulff, 2010; Barbau-Piednoir *et al*., 2012; Broeders *et al*., 2013; Debode, Janssen, & Berben, 2013; Dinon *et al*., 2011; Pansiot *et al*., 2011) and are listed in various databases (http://gmo-crl.jrc.ec.europa.eu/gmomethods/; Dong *et al*., 2008). Some screening methods have been validated in collaborative trials (Barbau-Piednoir *et al*., in press; International Standard ISO 21569:2005; Jiang *et al*., 2009), with notable effort of the Federal Office of Consumer Protection and Food Safety (Berlin, Germany) and the German working group "Development of methods for identifying foodstuffs produced by means of genetic engineering techniques" (*e.g*. Grohmann, Brünen-Nieweler, Nemeth, & Waiblinger, 2009; Waiblinger, Ernst, Anderson, & Pietsch, 2008). However, as most of the screening assays are developed by a single laboratory and as no validation guidelines exist for qualitative qPCR methods, the development and validation of these methods lacks harmonisation in contrast to what is the case for quantitative qPCR methods. An important consequence thereof is the fact that the specificity as well as the robustness of screening qPCR assays is generally not fully verified, with possible important consequences on the efficacy of the screening phase (Holden, Levine, Scholdberg, Haynes, & Jenkins, 2010; Morisset *et al*., 2009). In the case of multiplex methods, the asymmetric LOD is a valuable parameter to be addressed in view of the possible low presence of EU authorised (Regulation EU/619, 2011) and unauthorised events in a sample. There is thus a need for guidelines for the validation of qualitative screening methods based on qPCR (ENGL, 2011b; Holst-Jensen *et al*., 2012; Morisset *et al*., 2009).

Strategy to develop validation guidelines

When developing and validating in-house qualitative qPCR methods for GMO screening, most laboratories use procedures based on general guidelines (Codex Committee on Methods of Analysis and Sampling [CCMAS], 2010; European Analytical Chemistry [EURACHEM], 1998; Food and Agricultural Organization [FAO], 1998; Thompson, Ellison, & Wood, 2002) or those existing for quantitative qPCR methods (ENGL, 2008, 2011a). However, these may not be adequate or respond to the needs for qualitative methods and mainly comprise theoretical information (*i.e*. parameters to be evaluated and their definition, evaluation criteria) but a precise experimental setup (material, number of replicates, DNA amount,…) is often missing. In this study, a team of experts, gathered within the SAFEFOOD ERANET GMO-seek project, has analysed the existing documents and evaluated their usefulness in view of establishing guidelines for qualitative methods.

The compilation of studied documents included the Codex Alimentarius Guidelines (CCMAS, 2010), the AFNOR norms (Association Française de Normalisation [AFNOR], 2003, 2008), several ISO standards, both general and GMO-specific ones (International Standard ISO 17025:2005; International Standard ISO 21569:2005; International Standard ISO 21570:2005; International Standard ISO 24276:2006; International Standard ISO 5725-2:1994), existing documents for quantitative qPCR methods (ENGL, 2008, 2011a) as well as a review from Taverniers, De Loose, and Van Bockstaele (2004). Each parameter was discussed and an evaluation of its inclusion in the list of required parameters was made based on the practical expertise of the partners, the feasibility of the experiments, the use of different chemistries, the time, the costs and the need for singleplex *versus* multiplex methods. Some parameters can be taken over from the quantitative methods while others need to be adapted, added or are not of application. A comparison of the parameters to be considered and the criteria to which they need to comply for qualitative and quantitative qPCR methods are given in Table 1.

As it is the case for the ENGL Minimal Performance Requirements document (ENGL, 2008), the parameters to be evaluated were divided in two groups: method acceptance parameters (to be tested by the developer during in-house validation) and method performance parameters (to be evaluated *via* inter-laboratory and collaborative trials). The different types of screening methods that are being used by the enforcement laboratories (*i.e*. singleplex, multiplex, SYBR®Green, TaqMan®) were taken into account. Additionally, a practical way to perform this evaluation and the materials to be used are given.

Table 1. Comparison of parameters to be evaluated during validation of quantitative and qualitative qPCR methods. The criteria are given between brackets.

Parameter	Quantitative qPCR method	Qualitative qPCR method
Method acceptance parameters		
Applicability	+	+
Practicability	+	+
Specificity	+	+ (no false pos/neg)
Sensitivity (LOD)	−	+ (≤20 HGE)[a]
Sensitivity (LOQ)	+ (0.9%[b] or 0.1%[c])	−
PCR efficiency (ε)	+ (90–110%)[a]	Only for multiplex (80–120%)
Linearity (R^2)	+ ($R^2 \geq 0.98$)	Only for multiplex ($R^2 \geq 0.98$)
Accuracy	+	−
Trueness	+ (±25%)	−
Precision (repeatability)	+ ($RSD_r \leq 25\%$)	−
Robustness	+ (≤30%)	+ (correct pos/neg classification)
Method performance parameters		
False positive/ negative rate	−	+ (correct pos/neg classification)
Precision (reproducibility)	+ ($RSD_R \leq 25\%$)	−
Measurement uncertainty	+ (≤50%)	−

+: parameter to be evaluated; −: parameter not to be evaluated; HGE: haploid genome equivalents; LOD: limit of detection, LOQ: limit of quantification; RSD_r: relative repeatability standard deviation; RSD_R: relative reproducibility standard deviation.

[a] These values will be adapted to ≤25 HGE for the LOD and 75–110% for PCR efficiency (personal communication).

[b] LOQ required for GMO approved according to Regulation EC/1829 (2003).

[c] LOQ required for GMO pending for authorisation according to Regulation EU/619 (2011).

It should be noted that the implementation of collaborative trial validated qualitative methods into the enforcement laboratories has not been included in this manuscript. The document established by the ENGL (ENGL, 2011a) can hereto be used as a guidance.

Practical evaluation of parameters and acceptance criteria

To evaluate the fitness for purpose of a method and its performance, several parameters need to be tested. Only when they comply with the predetermined criteria, a method can be adopted for routine analysis and can be considered for further full validation. The definitions of the different parameters mentioned below can be found in the glossary.

Method acceptance parameters

The following parameters need to be evaluated for both singleplex and multiplex qualitative qPCR methods during method development and in-house validation.

Applicability

The applicability statement should contain complete information on the scope of the method *i.e.* which target, which matrix and DNA amount have been tested by the developer. It can be evaluated using different matrices (raw/processed material, food/feed, genomic (gDNA)/plasmid (pDNA) DNA). Furthermore, different DNA amounts can be tested allowing the detection of possible PCR inhibitors. The results of these tests need to be reported and should give similar results for as many matrices as possible. Additionally, warnings on the interference with other analytes and its inapplicability to certain matrices and conditions should be included when identified.

Practicability

To test the practicability, blind samples can for example be analysed by the routine laboratory. It allows to check if the new method can easily be combined with other methods that are already used in the laboratory and can be run under the same conditions. Furthermore, the evaluation of the costs and needs for training of the staff has to be reported. The practicability can further be evaluated by transferring the method to a second laboratory where the experiment can be performed under reproducibility conditions.

A method can be considered as practical when eventual additional costs are low, training of staff is limited, routine equipment can be used and the operations are easy to carry out.

Specificity

The specificity of a set of oligonucleotides (*i.e.* primers and/or probe) should be checked during method development to guarantee that the method only reacts with the targeted sequence. Firstly, this needs to be done *in silico* by performing searches against publicly available DNA sequence databases. Based on this outcome, a first selection of the designed oligonucleotides can be made. Secondly, the chosen oligonucleotide sets can be evaluated experimentally by testing them against a set of plant materials, preferentially (Certified) Reference Materials (CRM). This set should consist of 20 non-target materials (including the most important non-transgenic food crops, as well as all available CRM for GM events that do not contain the targeted sequence) and 20 target materials if possible. For taxon-specific screening qPCR methods, materials from closely related species as well as other species commonly found in food/feed samples should be included. Each material should at least be tested in duplicate resulting in 40 results for each type of sample. According to the simplified Cochran approach (Cochran, 1977) such test sample sizes should provide 95% confidence. It should however be noted that it may not always be possible to obtain 20 positive materials due to lack of availability of CRM and/or because the targeted element is only present in a few GMO. In this case the highest possible number of positive materials should be tested. This should be mentioned in the specificity report.

The non-target materials should contain at least 1000 haploid genome equivalents (HGE) total DNA. In case the non-target material is a non-pure GM CRM (*i.e.* a CRM with a certified GM% below 100%), at least 100 HGE of the GMO should be present in the reaction to ensure that cross-reaction will be identified if it occurs. For the positive materials, the target should be present in at least 100 HGE to ensure that the element is detectable. If wished, the target can be measured in a background of DNA of the same taxon (at for example 1000 HGE). The HGE can be estimated based on the GM% of the used CRM, the species genome size (Arumuganathan & Earle, 1991), the ploidy status and the number of inserted copies in the genome. Alternatively, the absolute copy number of the targeted sequence can be measured using an event-specific assay adapted to digital PCR technology (Morisset, Stebih, Milavec, Gruden, & Zel, 2013).

For methods developed using the SYBR®Green chemistry, the melting temperature (*T*m) forms beside the *Cq* value (quantification cycle) an additional parameter that needs to be taken into account. Each amplicon has, due to its sequence, a specific *T*m (Ririe, Rasmussen, & Wittwer, 1997). To determine the *T*m of the amplicon, the amplified sequence can be cloned into the pUC18 vector and the nominal *T*m can be determined (*e.g.* Broeders *et al.*, 2013). The *T*m of the amplicon obtained on the gDNA during the specificity test, using the same running conditions, should be within the interval nominal *T*m ±1 °C to be regarded as specific.

The specificity of a qualitative method can further be checked by confirming the identity of the targeted/amplified sequence by using appropriate techniques (*e.g.* amplicon sequencing, gel electrophoresis, restriction enzyme analysis, hybridization techniques; International Standard ISO 21569:2005).

For multiplex methods, special attention needs to be given to the generation of additional amplicons (coming from the fact that for example the forward primer of one method reacts with the reverse primer of another method) when designing the primers. This can for instance be evaluated by running the obtained PCR products on an agarose gel or on the Bioanalyzer (Agilent Technologies) or by performing a melting curve analysis.

In this context, it should be noted that the available CRM are intended for use as calibrants and positive control materials for a specific GM event and not to conduct specificity tests (Institute for Reference Materials and Measurements [IRMM], 2006). As a consequence, they are certified for the content of a certain GM event and not for the absence of other events nor for the absence/presence of a specific GM element. Contamination of the used materials may thus be seen during the specificity test (false positives). Further confirmation of the presence of another event can be done by using the validated event-specific qPCR methods (http://gmo-crl.jrc.ec.europa.eu). In the case false negative results are observed, a closer analysis of the targeted sequence should be carried out. It may be possible that for example a mutation is present at the annealing site of a primer which impairs amplification (Broothaerts *et al.*, 2008; Morisset *et al.*, 2009).

The results of the specificity test can be expressed as a percentage of false positives or negatives (CCMAS, 2010) using the formulae given below. No false positive or negative results should be seen. If unexpected signals are observed, they should be further investigated.

$$\text{False neg rate} = \frac{100 \times \text{number misclassified known pos samples}}{\text{tot number known pos samples}}$$

$$\text{False pos rate} = \frac{100 \times \text{number misclassified known neg samples}}{\text{tot number known neg samples}}$$

Sensitivity

The sensitivity of a qPCR method is an important parameter to evaluate especially in view of the regulation for GM events pending for authorisation (so called Low level Presence (LLP) Regulation EU/619, 2011) and for the detection of UGM which may also be present in low amounts. The sensitivity of a qualitative qPCR method can be expressed as the limit of detection (LOD) and two types of LOD are proposed herein.

The LOD_6 is determined experimentally for each qPCR method by preparing a serial dilution of a positive CRM and analysing each dilution point in 6-fold (AFNOR, 2008). Optionally, the dilutions can be made in background DNA. The dilution series should for example cover at least the following range: 100, 50, 20, 10, 5, 2, 1 and 0.1 HGE. The last dilution where all six replicates give a positive and specific amplification (*Cq* for TaqMan® methods, *Cq* and *T*m for SYBR®Green methods) can be considered as the LOD_6. At least three runs need to be performed under repeatability conditions, *i.e.* before each run the dilution series needs to be prepared freshly and tested in 6-fold

resulting in a total of 18 results per dilution point. In this case the LOD_6 of the method is determined as the highest HGE over all runs. The 0.1 HGE level is tested to verify that the HGE of the dilution series are approximately correct. Hereto, not more than one positive result out of the six replicates should be obtained (AFNOR, 2008). If this is not the case, the target concentration must be revised.

In addition to the LOD_6, the $LOD_{95\%}$ needs to be determined for further confidence of the analytical sensitivity of the method. Hereto, the HGE corresponding to the LOD_6 is tested in 60 replicates (EU-RL GMFF, 2009) together with a higher and a lower HGE level. The lowest HGE level at which all 60 replicates show a specific positive amplification is considered as the $LOD_{95\%}$ with a 95% confidence level.

The LOD of a qualitative qPCR method should be equal or below 20 HGE (AFNOR, 2003). The *Cq* value corresponding to the LOD can further be introduced in decision support systems to be used in routine analysis of food and feed samples (*e.g.* Broeders, Papazova, Van den Bulcke, & Roosens, 2012; Van den Bulcke *et al.*, 2010).

Robustness

To evaluate the robustness of a qPCR method, different experimental conditions can be slightly changed and their impact on the results studied. Such a test is important in view of the use of the qPCR methods in routine analysis (*e.g.* a slight deviation in annealing temperature, small pipetting errors,…). In addition, the use of different qPCR instruments and master mixes should be evaluated. Such test can be conducted using for example the fractional factorial design approach (Youden & Steiner, 1975). The proposed scheme does not study one alteration at a time, but introduces several changes at once in such a manner that the effects of the individual changes can be ascertained. This allows evaluating the robustness in a time- and cost-effective manner. An example of the way to combine the different studied factors is given in Table 2.

In each combination, the method is tested using 20 HGE of the target in at least triplicates in at least one run. To be considered robust, the method should give the expected result in terms of presence of the target. If this is not the case, the factors that affect the result should be studied in detail and eventually adapted to improve the method or to enable giving the additional information to the users. The data obtained from this robustness test are mainly informative and should be reported in the validation dossier to acknowledge the possible impact of certain factors. Further, this test needs to be performed on a method-to-method basis.

Table 2. Proposal of a robustness test according to Youden and Steiner (1975).

Combination No.								
Factor value	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
A or a	A	A	A	A	a	a	A	a
B or b	B	B	b	b	B	B	B	b
C or c	C	c	C	c	C	c	C	c
D or d	D	D	d	d	d	d	D	D
E or e	E	e	E	e	e	E	E	E
F or f	F	f	f	F	F	f	f	F
G or g	G	g	g	G	g	G	G	g

A, B, C, D, E, F and G denote the nominal values for seven different factors that might influence the results if their values were varied slightly, their alternative values are denoted by a, b, c, d, e, f and g.

Method acceptance parameters specifically for multiplex methods

In addition to the above mentioned parameters, it is recommended to test some additional parameters when developing and validating qualitative multiplex qPCR methods. The PCR amplification efficiency and linearity can optionally also be determined for singleplex methods.

Amplification efficiency (ε)

To calculate the PCR efficiency of a qPCR method, a dilution series (*e.g.* 5000, 2500, 1000, 500, 100, 50, 20, 10, 5, 2, 1 and 0.1 HGE) is prepared from a specific positive material. In the case of a multiplex assay, all the targets considered in the assay should be set at the same HGE. This allows to check if competition occurs between the targets and to evaluate if all perform equally well. Setting all targets at equal HGE can for example be achieved by mixing different target-containing materials. For practical reasons, a plasmid containing all targeted sequences can be used to prepare the dilution series. For singleplex methods, the evaluation can be done using a dilution series of a positive CRM.

Each dilution point is analysed in 6-fold and four runs are performed under repeatability conditions, *i.e.* before each run the dilutions series needs to be prepared freshly and each dilution is measured in 6-fold resulting in 24 data for each dilution point. The average *Cq* values obtained for each point at the upper end of the dilution series (*i.e.* 5000 to 100 HGE) are plotted against the $\log_{10}$HGE and a linear regression analysis is performed. The outcome of this analysis is an equation of the type $y = ax + b$ in which 'y' is the plotted *Cq* value, 'a' is the slope of the regression line, 'x' is the $\log_{10}$HGE and 'b' is the intercept of the regression line. Using the slope of the regression line, the PCR efficiency can be calculated using following formula:

$$\varepsilon = 100 \times \left(10^{-1/\text{slope}} - 1\right)$$

For each target, the slope of the regression curve, when using the $\log_{10}$ transformation of the HGE, should be between −3.9 and −2.9 corresponding to PCR efficiencies ranging from 80% to 120%. This broader range has been allowed as the methods are intended for qualitative use, *i.e.* present/absent response, and not for quantitative

purposes. Additionally, the difference between the PCR efficiencies obtained for each target of the multiplex assay should not exceed 15% to limit the impact of the possible competition between the different targets (Huber *et al.*, 2013). For qualitative singleplex methods, no specific criterion of acceptance for the PCR efficiency is defined. One can use the criterion as set for quantitative qPCR methods (Table 1).

Linearity (R^2)

The experimental setup to determine the linearity for each qPCR target in a multiplex method is the same as the one used to determine the PCR efficiency. When performing a linear regression analysis on the obtained data, excel provides the correlation coefficient R^2 of the curve which is a measure of the linearity of the PCR reaction. The R^2 for each target should be $\geq$0.98.

If wished, the linearity for a singleplex method can be determined in the same way on a positive CRM and using the same acceptance criterion.

Asymmetric LOD ($LOD20_{asym}$)

The asymmetric LOD needs to be determined for each target of the multiplex assay. As it might be difficult to prepare such mixes using gDNA extracted from CRM (*e.g.* due to unavailability of single target CRM), this analysis can also be performed using a mix of single target plasmids. Each target needs to be tested at 20 HGE in a background of all other targets present at 20,000 HGE totally. Each of the background targets is set at the same HGE. Twenty HGE have been chosen for the target under investigation in relation to the required sensitivity of the methods (*i.e.* the LOD should be equal or below 20 HGE) to ensure that it can still reliably be detected. At least one PCR run using six replicates is performed for each target. If the six replicates show a specific amplification, the ratio of 1/1000 is set as the $LOD20_{asym}$. If some of the six replicates are negative, a lower background can be tested, *e.g.* 20 HGE in a total of 10,000 HGE, and the ratio set as the $LOD20_{asym}$ should be adapted (Huber *et al.*, 2013).

For example, in the case three targets are present in the multiplex assay, in a first experiment the one under investigation for the $LOD20_{asym}$ is set at 20 HGE and the other two are each set at 10,000 HGE. If one or more replicates are negative, a new experiment should be set up containing 20 HGE of the target under analysis and the two other targets at 5000 HGE each.

One can continue to adapt the background (*e.g.* to ratios of 1/400, 1/200, 1/100, 1/50) until all six replicates give a positive signal. If the background needs to be adapted further and a LOD of 1/50 is reached, it is strongly recommended to find out which of the targets in the multiplex assay is causing this competition. It will be necessary to recheck the presence of that target using singleplex qPCR. No acceptance criterion is set for this parameter. The final measured $LOD20_{asym}$ should be indicated by the method developer.

Method performance parameters

To enable the developed qPCR screening method to be recognized internationally, in-house method validation is not sufficient. A full validation involving different laboratories (collaborative trial) needs to be organised. An intermediate step, to obtain an indication on the performance of the method, can be performed in which the method is first transferred to at least one laboratory (transferability study).

Method transferability

When a method is in-house validated and complies with the set criteria, its transferability to a second laboratory can be tested. This will allow making a first evaluation of the performance of the method under different conditions (other operator, other equipment, etc). Based on the obtained results, the laboratory can decide to go on with the full validation or not and thus save costs compared to the direct organisation of the collaborative trial.

For this aim, a false positive/negative test can be performed, as described for the specificity test, using at least one laboratory and samples containing different levels of the targeted element. The results can be compared with those obtained by the developing laboratory. When the method is found to be performing well, it can be considered for full validation.

Method full validation in a collaborative trial

Further evaluation of the performance of the method concerning the detection (*i.e.* presence/absence) of the specific target needs to be done *via* a collaborative trial. As for the transferability step, the parameter to be studied in this case is the false positive/negative rate. For this aim, the procedure as for example used in collaborative studies conducted by the German Federal Office of Consumer Protection and Food Safety and its working group and from Grohmann *et al.* (2009) can be applied.

In this experimental setup, several blind samples need to be prepared: *e.g.* three positive samples and one negative sample. Hereto, a target-containing CRM and its non-transgenic counterpart can be used, respectively. The negative sample (0%) should contain for example 200 ng of taxon-specific DNA. The positive samples should contain the target (i) at the HGE corresponding to the $LOD_{95\%}$, (ii) at 0.5× $LOD_{95\%}$ (but not below 5 HGE), and (iii) at 2 times $LOD_{95\%}$ (but not above 20 HGE), in a background of for example salmon-sperm DNA (*e.g.* at 20 ng/µl). Each blind sample is prepared in 10-fold and analysed in duplicate by at least 12 independent laboratories to ensure obtaining a minimum of 100 acceptable results per sample allowing to calculate the false positive/negative rates with a 95% confidence level (Macarthur & von Holst, 2012). Results are considered as acceptable when no deviation to the

procedure or technical problems have been reported and when appropriate statistical tests have been used to exclude deviating results (*e.g.* the use of a parametric bootstrap to check how well the beta binomial model fits the observations). If wished, additional samples can be prepared and the experiment can be performed on more than one target-containing GM event.

It should be noted that when validating taxon-specific screening methods, the used materials need to be adapted. The negative sample can be a material from a non-related species, while the positive samples need to contain the taxon-specific target as mentioned above.

The false negative and false positive rates can be calculated as done for the specificity evaluation and both should be zero. If this is not the case, the reason for it should be further investigated. In addition, seen the high amount of data that will be gathered based on this setup, the LOD of the method can be determined with sufficient confidence (Macarthur & von Holst, 2012).

Conclusion

The total number of GMO that needs to be detected by enforcement laboratories is steadily increasing. To reduce the number of identifications, many GMO detection laboratories have established a screening approach in which in-house developed and validated qualitative qPCR methods are being used based on a matrix approach (Holst-Jensen *et al.*, 2012; Kralj Novak *et al.*, 2009; Van den Bulcke *et al.*, 2010; Waiblinger, Grohmann, Mankertz, Engelbert, & Pietsch, 2010). These methods are further valuable tools in the possible detection of UGM. However, their development, validation conditions and use are not standardised, and still remain the subject of individual research and choice of testing laboratories. This can have important consequences on the correctness of the screening phase and therefore on the quality of GMO testing.

To remediate to this problem of lack of standardisation, a group of experts gathered available documents that deal with validation of qPCR methods. Based on their expertise, a number of parameters were distilled out of these documents and adapted to come to a pragmatic and harmonised way of validating qualitative qPCR methods. In addition, the acceptance criteria that need to be met during in-house validation, to enable declaring the new method as fit for purpose, have been listed as well as an experimental setup and the most appropriate material(s) to be used. The established guidelines also contain the method performance parameters to be studied when performing transferability studies and further going to inter-laboratory validation. An overview is given in Table 3. The proposed scheme will readily be implemented in the GMOval project (UK Food Standards Agency contract FS244027) for inter-laboratory validation of qualitative methods for GMO testing. This will provide a first indication of its usefulness and application.

Similar to the MIQE guidelines that state the minimum information for publication of quantitative real-time PCR and digital PCR experiments (Bustin *et al.*, 2009; Huggett *et al.*, 2013), a template table (Table 4) is provided in which developers of qualitative qPCR methods can find all relevant experimental conditions and assay characteristics. Such table should help reviewers evaluating the validity of their proposed assay for publication and/or collaborative trial validation.

The current guidelines were designed to provide the necessary information enabling enforcement laboratories to develop and validate screening qPCR methods in a harmonised way (as is done for quantitative qPCR methods). This is of great value for GMO testing laboratories in view of the integration of a screening approach in routine analysis and thus reducing the cost and time of the analysis. Additionally, the validation of screening methods in a standardised way throughout different laboratories will ease the adaptation and implementation of these methods and will allow putting together a set of necessary methods covering all GM events to be detected. Furthermore, it enables comparing more easily different developed methods and will lead to the improvement of the screening phase in GMO detection.

This type of guidelines could also serve as a basis for other domains of molecular testing where no precise instructions exist, to help evaluating the performance and fitness for purpose of qualitative qPCR methods which are becoming more and more important.

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Glossary

The parameters and definitions listed in the glossary are adopted from the International Standard ISO 24276 (2006) and the ENGL Minimal Performance Requirements document (ENGL, 2008) unless mentioned otherwise.

Method acceptance parameters

The *applicability or fitness for purpose* of a method refers to the description of the method *i.e.* the analytes or species, the matrices and concentration ranges to which the method can be applied. It means that information on the scope of the method needs to be provided and the products to which it is applicable (ISO 24276, 2006).

The *practicability* takes into account the ease of operations, in terms of sample throughput and costs, the feasibility and efficiency of implementation of a new method.

Table 3. Summary on the different method acceptance and performance parameters to be evaluated and their experimental setup.

Parameter	Singleplex methods	Multiplex methods	Material	# PCR replicates	# PCR repetitions (# runs)	Number of copies (HGE)	Comment
Method acceptance parameters							
Applicability	+	+	Different matrices	n/a	n/a	n/a	Info on scope of the method (type of material, DNA amounts, inhibition)
Practicability	+	+	Blind sample(s)	n/a	n/a	n/a	Ease of use of the method, costs, need for training/ equipment; transfer to 2nd laboratory
Specificity							
in silico	+	+	Sequence databases	n/a	n/a	n/a	At least all available GM events and plant species
Experimentally	+	+	20 target and 20 non-target-containing materials (if available); preferentially (C)RM	At least 2 per material	At least 1 run	Target-containing materials: 100 HGE; non-target containing materials: 1000 HGE[a]	To be expressed as false positive/ negative rate
Sensitivity							
LOD_6	+	+	(C)RM	6/dilution point	At least 3 runs	At least 100, 50, 20, 10, 5, 2, 1, 0.1 HGE	
$LOD_{95\%}$	+	+	(C)RM	60/level	1 run/level	LOD_6, 1 level above, 1 level below	Each level tested is a run on a separate plate
$LOD20_{asym}$	−	+	(C)RM or single target plasmids	6/target	At least 1 run	20 target HGE in a total of 20,000 HGE: ratio 1/1000	Other ratios can be tested (1/500, 1/400, 1/200, 1/100, 1/50); to be done for all targets in the multiplex
Amplification efficiency (ε)*	−	+	(C)RM or multiple-target plasmid	6/dilution point	At least 4 runs	5000, 2500, 1000, 500, 100, 50, 20, 10, 5, 2, 1 and 0.1 HGE	Upper end dilutions are used (5000–100 HGE)
Linearity (R^2)*	−	+	(C)RM or multiple-target plasmid	6/dilution point	At least 4 runs	5000, 2500, 1000, 500, 100, 50, 20, 10, 5, 2, 1 and 0.1 HGE	Upper end dilutions are used (5000–100 HGE)
Robustness	+	+	(C)RM	3	At least 1 run	20 HGE	The different conditions tested need to be taken into account to calculate the number of runs needed; only used as informative data
Method performance parameters							
False positive/ negative rate	+	+	1 blind negative sample (in 10 aliquots), 3 blind positive samples (in 10 aliquots each); preferentially (C)RM	2/sample aliquot	1 run	0 HGE, 0.5× $LOD_{95\%}$, $LOD_{95\%}$, 2× $LOD_{95\%}$	The tested positive levels should be between 5 and 20 HGE; at least acceptable results of 10 laboratories should be obtained

+: parameter to be evaluated; −: parameter not to be evaluated (or optional*); (C)RM: (Certified) Reference Material; HGE: haploid genome equivalent.

[a] If the non-target material is a GM CRM with a GM% below 100%, the non-target GM should be present at about 100 HGE.

Table 4. Checklist for validation of qualitative qPCR methods. Checklist showing the different steps to be addressed when performing validation of qualitative qPCR methods including the essential (E) and desired (D) information to be reported in the validation dossier.

	ITEM	IMPORTANCE	COMMENT
qPCR target information	name of the target	E	
	full name of the targeted sequence (accession number)	D	
	length of the amplicon	E	
	sequence of the amplicon	E	
	location of the amplicon	D	
	target containing materials	E	
	non-target containing materials	E	
	sequence alignment	D	
qPCR oligonucleotides	primer sequence	E	
	probe sequence	E	n/a for SYBRGreen qPCR methods
	probe labelling	E	n/a for SYBRGreen qPCR methods; give details on labelling (e.g. FAM, MGB, Scorpion,…)
	purification method	D	
	manufacturer	D	
qPCR reaction	qPCR cycling conditions	E	
	melting curve conditions	E	only for SYBRGreen qPCR methods
	type of chemistry (SYBRGreen, TaqMan)	E	
	singleplex/multiplex	E	
	qPCR reagents	E	
	qPCR volume	E	
	qPCR mix setup	E	
	qPCR instrument type	D	
	manufacturer qPCR instrument	D	
	DNA amount/reaction	E	
Data analysis	qPCR analysis program	E	
	outlier test	E	
	inhibition test	E	
	results for the controls (PC, NTC,..)	E	
Overview qPCR validation	in-house	E	
	- applicability	E	
	- practicability	E	
	- specificity	E	
	- sensitivity	E	
	- PCR efficiency	E	optional for singleplex qPCR methods
	- linearity	E	optional for singleplex qPCR methods
	- robustness	E	
	transferability study	D	or pre-validation step (if any)
	collaborative trial	E	
	- false pos/neg rate	E	
	- robustness	E	
Applicability	scope of the method	E	
	type of matrix	E	
	inhibition information	E	
	analyte interference	E	
Practicability	easy combination with other methods	E	
	costs	E	
	need for training	E	
	blind sample analysis	D	
	method transfer	D	
	equipment needs	D	Essential if e.g. special chemistry or conditions are used
Specificity	in silico specificity (BLAST, etc)	E	Give details: databases used, parameters used, …
	in situ specificity against target containing materials	E	Give details about the material used (CRM, RM, poorly documented material, contamination check, …)
	in situ specificity against non-target containing materials	E	Give details about the material used (CRM, RM, poorly documented material, contamination check, …)
	in situ specificity against other materials (bacteria, virus,…)	D	Give details about the material used (CRM, RM, poorly documented material, contamination check, …)
	confirmation (gel electrophoresis, sequencing,…)	E	
	results expressed as false pos/neg rate	E	
Sensitivity	LOD_6	E	
	$LOD_{95\%}$	E	
	$LOD20_{assym}$	E	n/a for singleplex qPCR methods
	PCR efficiency	E	optional for singleplex qPCR methods
	linearity	E	optional for singleplex qPCR methods
Robustness	change in primer concentration	E	
	change in probe concentration	E	only for TaqMan qPCR methods
	change in reaction volume	E	
	change in annealing temperature	E	
	change of instrument	D	
	change of mastermix/qPCR reagents	D	
Transferability study	lab choice	E	
	false pos/neg test	E	
	type of samples	E	
	level/content of each sample (HGE)	E	
	number of samples	E	
Collaborative trial	choice of laboratories	E	
	false pos/neg test	E	
	type of samples	E	
	level/content of each sample (HGE)	E	
	number of samples	E	

Here the easiness of combining the qPCR method with methods already used in the laboratory (*i.e.* performing the analysis in the same run, on the same plate, using the same reagents and conditions) should be considered. In addition, the need for specific equipment and training of the staff should be evaluated (ENGL, 2008; ISO 24276, 2006).

Under the *specificity* of a method is understood the property of a method to respond exclusively to the characteristic/analyte of interest (ISO 24276, 2006). This means that in case of a GMO detection method, it should only give a specific amplification signal with materials containing the target sequence. The evaluation of the specificity needs to be done *in silico* as well as *in situ* (ENGL, 2008).

The *sensitivity* of a qPCR method is defined as the minimal analyte concentration that can be reliably detected but not necessarily quantified (ISO 24276, 2006). It is expressed as the absolute limit of detection (LOD_{abs}). Two types of absolute LOD can be determined namely the LOD_6 and the $LOD_{95\%}$. The first one is defined as the HGE at which all six replicates give a similar and specific positive amplification signal (AFNOR, 2008). The $LOD_{95\%}$ on the other hand is the LOD at which the analyte is detected with 95% confidence, meaning that less than 5% false negatives are allowed (Bustin *et al.*, 2009). Furthermore, the asymmetric LOD ($LOD20_{asym}$) is the ratio between the HGE of the tested target in comparison to the fixed HGE level of all other targets which are considered in the multiplex PCR assay (Huber *et al.*, 2013).

The *amplification efficiency* (ε) is the amplification rate that leads to a theoretical slope of −3.32 with a 100% efficiency in each cycle (ENGL, 2008).

The *linearity* of a PCR reaction is expressed as the correlation coefficient (R^2) of the curve obtained by linear regression analysis (ENGL, 2008).

The *robustness* of a method is a measure for its capacity to remain unaffected (*i.e.* to provide a similar result) by small but deliberate changes in the experimental conditions as compared to the procedure (ENGL, 2008).

Method performance parameters

False positive and false negative rates are the probability that respectively a negative sample would be classified positive or a positive one would be regarded as negative (CCMAS, 2010).

Other definitions

A *genetically modified organism (GMO)* means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination (Commission Directive 2001/18/EC).

UGM has been used here to designate GM events that are unauthorised in one country but authorised in another for food and feed use.

Repeatability conditions are conditions under which the independent results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time (ISO 24276, 2006).

Reproducibility conditions are conditions under which test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipments (ISO 24276, 2006).

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