D4.1 Report on performance and cost effectiveness of ddPCR for detection of GMOs authorised and un-authorised in the EU

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1. Introduction

In Europe the labeling threshold for authorized genetically modified organisms (GMOs) is specified in Regulation 1829/2003 [1]. This regulation states that labeling: "*shall not apply to foods containing material which contains, consists of or is produced from GMOs in a proportion no higher than 0.9 per cent of the food ingredients considered individually or food consisting of a single ingredient...*" Thus, the labeling threshold is on a *per ingredient* basis, not on a *per GMO* basis. In analytical terms, ingredient in this context is interpreted as species. So, it is the cumulative concentration, e.g. of all authorized maize GMOs relative to the total quantity of maize that determines if the threshold is exceeded or not. The technical guidance for sampling and detection of GMOs [2] states that the GMO content should be expressed as ratio of GMO in relation to the taxon (species), meaning that GMO content can either be measured as additive concentration of individual authorized GMO events or it can be measured as the total concentration of this group directly. The latter appears as a more cost-efficient approach, but until 2015 it has not been possible to implement due to lack of suitable analytical methods.

Standard curve based quantitative real-time PCR (qPCR) is still regarded as the gold standard for GMO analysis. With increase in the number of authorized GMOs over time, the required number of qPCR analyses has increased correspondingly. The use of individual event specific qPCRs is no longer cost efficient. Thus many laboratories now employ one of several alternative screening approaches to testing for presence of genetic elements commonly found in GMOs [3]. Recently a multiplex version of one of these screening approaches [4] was developed as a single qualitative pentaplex PCR [5]. The observed screening results can already exclude all or a number of GMOs, therefore less reactions are needed in subsequent steps.

To improve the cost efficiency of analyses, multiplex PCR is the most obvious option, but the complexity of multiplex systems renders these more prone to potential interference between oligonucleotides and amplification products (target and non-target). The development of quantitative high level multiplex has proven difficult. Several multiplex qPCRs already exist, but only two (duplex) are inter-laboratory validated: one in Europe [6] and one in Japan [7].

Digital PCR is a technological modification of real-time PCR. The basis of digital PCR (dPCR) is to quantify the absolute number of targets present in a sample using limiting dilutions, PCR and Poisson

statistics, which is a concept that was first described by Sykes et al. (1992) [8]. An important advantage of dPCR over qPCR is that it can be used to quantify the absolute concentration of targets without the need for calibration. This simplifies both experimentation and data comparability [9]. Digital PCR (dPCR) has been successfully applied to routine testing as well as to research analysis. Morisset et al. showed that droplet dPCR (ddPCR) is useful for routine analysis of food and animal feed samples for the presence of DNA from GMOs [10]. They concluded that for genetically modified organisms where both detection and quantification are desirable, ddPCR has the potential to provide improved throughput and cost-effectiveness compared to qPCR.

Several new ddPCR assays were developed for detection and quantification of GMOs authorised or not in the EU, to evaluate the suitability of ddPCR in terms of performance and cost effectiveness. Additionally, to the transfer of qPCR methods to a ddPCR platform, different multiplex qPCR assays were developed within the Decathlon project. In short, the new multiplex options enable quantification of GM events per ingredient, multiplex quantification of a group of events after qPCR screening and multiplex quantification of individual GM events. This document, a report for the Decathlon sub-task T4.1.3, addresses the newly developed ddPCR strategies for GMO detection, identification and quantification, and presents the final outcomes of task T4.1 (Application of ddPCR protocols for detection and quantification of GMOs).

2. Transfer of qPCR assays to dPCR and comparison of different dPCR platforms

2.1. Simplex reactions for quantification of single GM events or endogenes

First, the existing qPCR assays for detection and quantification of GMOs were transferred to three currently most commonly used commercial dPCR platforms: Fluidigm chamber dPCR (cdPCR), Bio-Rad droplet dPCR (ddPCR), and Life Technologies QuantStudio3D dPCR (3D-dPCR). The performance of these platforms was compared in DNA quantification employing genetically modified (GM) plants and animal samples as examples, including the quantitative effect of DNA inhibitors and degradation, dynamic range, limit of quantification (LOD), reproducibility, accuracy, and precision. The applications of these three platforms for GM contents quantification, CRM characterization, genotype identification, and transgene copy number evaluation were also tested. The results suggest that the absolute DNA amounts with high accuracy can be obtained from all three platforms, with better precision and accuracy than with qPCR. The results from ddPCR and 3D-dPCR are more accurate than that of cdPCR although the cdPCR has shown the best repeatability. The ddPCR and 3D-dPCR platforms show wide dynamic range in DNA quantification without any special diluting on template DNAs. It was shown that simplex dPCR is a suitable tool for GMO analysis, including accurate GM contents quantification without any calibrators, characterization of certified reference materials and estimation of the transgene copy numbers.

3. ddPCR and multiplexing

3.1. Duplex assay for quantification of individual events

There is currently only one ddPCR platform available (Bio-Rad), and this is equipped with only two fluorescence detection channels. This limits the wavelength based resolution to the detection of only two fluorophores (FAM and VIC/HEX), making it ideal for duplex assays, but not for higher levels of multiplexing. This combines well with the GMO quantification as final GM content is determined as a ratio between concentration (number) of a GM target and concentration (number) of an endogene target (reference gene). Therefore, the multiplexing capabilities of duplex ddPCR were tested first. The starting points for the work were the published results for quantification of the MON810 GM maize line with ddPCR in duplex fashion (MON810 event and hmgA endogene) [10]. The initial ddPCR protocol (first version of M4.1), which was prepared based on this study, was tested in three different labs (NIB, Eurofins GeneScan and SJTU). The same DNA sample was used in all three labs in this transferability study (dilutions were prepared in each lab separately). The results of the transferability study have shown that the protocol was clear and was successfully transferred from NIB to other labs. The relative standard deviation (RSD) of the results from different labs in terms of GM % or absolute copy numbers were within the minimum performance parameters (RSD<25%) set in the Deliverable 6.1 by WP6. The RSD between labs in terms of absolute copy numbers was ranging from 4 to 14% (Table 1), with variability of 4.5% in terms of GM% (Table 2).

Table 1 – Target concentration (copies/µl in stock DNA) determined in three laboratories

Target	Dilution	Eu-Gs	SJTU	NIB	RSD %
MON810	1	2768	2775	2951	4
	2	2282	2409	2599	7
hmgA	1	76874	71213	80616	6
	2	58566	62665	76312	14

Table 2 – GM quantity (GM%) determined in three laboratories

Laboratory	Average GM%	RSD %
EuGs	3.73	4.5
SJTU	3.87	
NIB	3.54	

3.2. Multiplex quantification of individual GM maize lines (quantification per event)

Currently, the golden standard for GMO detection and quantification is qPCR, which in most cases offers reliable and sensitive results at reasonable costs. But quantification in multiplex qPCR faces a number of challenges to which dPCR offers attractive solutions mainly because dPCR is less sensitive for competitive PCR effects. Nevertheless, qualitative multiplex (duplex to 5-plex) screening qPCRs are routinely used in official control laboratories.

The basic idea behind the multiplexing described in this chapter came from the work in laboratories performing routine GMO diagnostics. Many GMO positive samples contain RoundUp Ready (GTS 40-3-2) soybean and the qPCR screening in such samples yields positive signal for the Cauliflower 35S promotor and the *Agrobacterium* derived *nos* terminator. To exclude the presence of GM maize lines in such samples, additional reactions for identification of several GM maize lines have to be performed. At the time of assay development this number was 7, but it has increased since then.

For the purpose of simultaneous identification and quantification of the 7 GM maize lines, which contain the 35S promoter and *nos* terminator, two multiplex ddPCR assays were developed for multiplex event quantification (MEQ). The targets of each of the assays are presented in Table 3. In the MEQ1 assay one of the targets is the endogene hmgA that is needed for relative quantification of GM content. The MEQ2 does not contain an endogene target, thus both assays must be run simultaneously.

Table 3 – Division of GM maize targets into two multiplex assays

Event/gene	Multiplex group
hmgA	MEQ1
MON810	MEQ1
MON863	MEQ1
DP98140	MEQ1
MON89034	MEQ2
MIR604	MEQ2
MIR162	MEQ2
GA21	MEQ2

To be able to distinguish between the positive results for individual targets, the system uses optimized concentrations of primers and probes in order to get a separation of positive droplet clusters when two of the targets are labelled with the same fluorophore.

The performance of the assays was tested against the minimum performance parameters set in the Deliverable 6.1 by WP6. The performance was compliant with the set minimum performance parameters for absolute LOQ (aLOQ) and absolute LOD (aLOD) (Table 4). However, there were some limitations with the specificity. Since the absolute quantification relies in this case on counts of positive droplets, and because there are several possible clusters of droplets in each dimension, the effect of "rain" (positive droplets with lower, intermediate fluorescence intensity) causes some false positive results. More specifically, these droplets with intermediate fluorescence fall within the supposed cluster of positive droplets for other targets. The false positive results disappear when the concentration of the target is below 300 copies per reaction. This fact, together with the offset threshold for scoring a positive reaction = 3 droplets, limits the theoretical relative LOD (rLOD) at 1%. Such a rLOD is not suitable for GMO detection, as it is non-compliant with both the EU labelling threshold requirements and the minimum performance parameters defined in Deliverable 6.1. Nevertheless, if all of the four targets are present at high concentration, the rain droplets are masked within the clusters of positive droplets and do not have any significant effect on the absolute quantification.

Table 4 – Absolute LOQ and LOD of two multiplex ddPCR assays for quantification of 7 GM maize lines

Parameter	hmgA	MON863	MON810	DP98140	MIR604	GA21	MON89034	MIR162
aLOQ (copies/reaction)	15	44	23	18	12	11	13	49
aLOD (copies/reaction)	15	13	23	18	12	11	13	14

A manuscript for publication of the described multiplex system is prepared. But since all the data are yet not available, they are included and discussed below. For the purpose of comparison of ddPCR results, which are reported in copy number ratio, to those of qPCR reported in mass ratio a conversion factor was used. When quantification of each event with multiplex ddPCR in a DNA mix of 7 GM maize events was compared to qPCR, the results are comparable and are within the accepted ±25% range (Table 5). The conversion factor was determined for reference material of each individual GM line. There might be bias in this conversion when testing for samples not originating from reference material, thus the accepted ±25% range when comparing the results might be wider when using real life samples.

Table 5. Comparison of quantification result of multiplex ddPCR to qPCR

GM event	GM%[a]		% bias of ddPCR to qPCR	Factor used for conversion[d]
	qPCR[b]	ddPCR[c]		
MON810	1.6	1.7	7.99	0.39
MON863	1.4	1.2	-12.9	0.62
DP98140	1.6	1.8	13.4	0.79
MIR604	1.0	1.2	19.7	0.38
MON89034	1.2	1.3	8.4	0.61
GA21	1.0	1.0	4.5	0.34
MIR162	1.2	1.3	6.38	0.59

[a] in copy number ratio (copies of transgene/copies of endogene)
[b] values represent an average of measures of 2 replicates from 3 dilutions
[c] values represent an average of measures of 5 replicates from all dilutions within aLOQ range
[d] conversion factors determined based on experimental results (certified reference materials were subjected to absolute quantification with ddPCR and then copy number result was compared to the certified mass/mass %)

The ddPCR multiplex assays were also tested on two real life samples and the results were compared to qPCR. The determined GM content was comparable (Table 6) between the systems, there was only a problem of false positive result for one event (DP98140) due to the high concentration of other event that is present in same assay and labelled with the same fluorophore (MON810).

Table 6. Quantification with both multiplex assays on real life samples in comparison to qPCR

Sample	GM event	GM% qPCR quantification	ddPCR quantification[a]	Bias of ddPCR to qPCR [%]
G187/14	MON810	27.0	25.6	-5.2
	MON89034	54.0	66.6	23.4
	DP98140		0.1[b]	
G189/14	MON810	62.0	73.4	18.4
	MON89034	36.0	34.6	-3.9
	DP98140		0.4[b]	

[a] conversion factors determined based on experimental results (certified reference materials were subjected to absolute quantification with ddPCR and then copy number result was compared to the certified mass/mass %; for factors see Table 5) were used to convert ddPCR results from copynumber ratio to mass/mass ratio

[b] false positive results

To check, how the multiplex ddPCR will behave on the proficiency test samples a set of three samples was tested and z-score was calculated for the results. Bias of the results to robust mean was a bit higher than expected, most probably due to the use of conversion factor, which might not be completely suitable for these specific samples, as high bias was also observed in the case of the qPCR results that were gathered for these samples. Nevertheless, the absolute z-scores were below 2, what is the limit for passing the proficiency test. With the proficiency samples it was also shown that these multiplex ddPCR assays are suitable for samples with low relative GM content.

Table 7. Comparison of multiplex ddPCR and qPCR quantification on proficiency test samples

Sample[a]	GM event	Robust mean	GM% qPCR quantification	Bias to robust mean [%]	Z-score for qPCR	GM% ddPCR quantification[b]	Bias to robust mean [%]	Z-score for ddPCR
181/08	MON863	0.56	0.50	-10.2	-0.3	0.37	-32.8	-0.9
	MON810	0.13	<0.10			0.05	-59.3	-0.5
	MIR604	1.10	0.60	-45.2	-1.0	1.23	12.2	0.3
	GA21	0.35	0.20	-42.6	-1.0	0.32	-7.4	-0.2
188/09	MON810	0.63				0.70	11.5	0.3
	MIR604	0.39	0.30	-23.7	-0.9	0.52	32.5	1.2
	GA21	0.19	0.14	-27.4	-0.8	0.31	58.3	1.8
190/09	MON863	0.62				0.56	-9.1	-0.3
	MON810	0.53				0.55	3.4	0.1
	GA21	0.29	0.09	-68.9	-1.7	0.50	72.6	1.8

[a] samples of maize flour from USDA proficiency tests

[b] conversion factors determined based on experimental results (certified reference materials were subjected to absolute quantification with ddPCR and then copynumber result was compared to the certified mass/mass %; for factors see Table 5) were used to convert ddPCR results from copynumber ratio to mass/mass ratio

Finally the multiplex ddPCR assays were also assessed in terms of cost effectiveness. The screening phase was left out of the calculations, as these assays are always performed (invariant/constant cost). After screening, the tests with qPCR usually include identification of the GM lines that might be present in the samples and later on the quantification of the identified GM lines. From Figure 1 it can be seen that the qPCR identification of 7 GM maize lines (covered by ddPCR multiplex) is approximately 40% cheaper than multiplex ddPCR quantification of these 7 GM maize lines. So in the case of negative samples (not including GM maize) the qPCR system will be more cost effective, but whenever there is at least 1 GM maize line that needs to be quantified further on, the multiplex ddPCR assays are comparable or slightly cheaper than qPCR (Figure 1). In case additional GM maize lines would need to be quantified the multiplex ddPCR assays would be more cost effective.

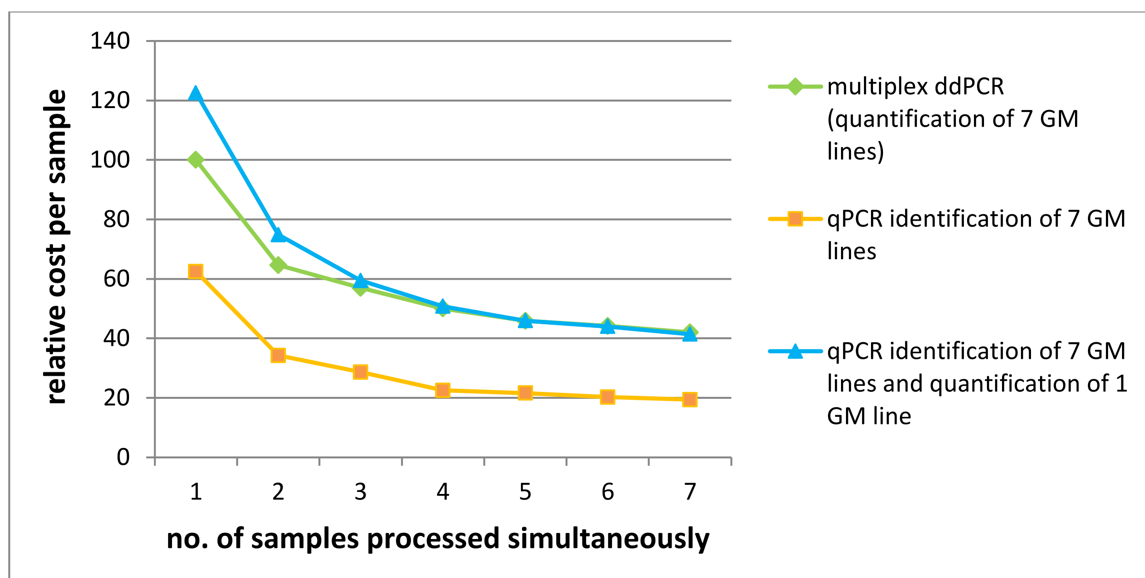

Figure 1 – Cost effectiveness of multiplex event quantification with ddPCR compared to qPCR. Relative value of 100% was set to multiplex ddPCR with one sample.

3.3. Multiplex quantification of selected GM maize events after initial qPCR screening

Another approach, which employs the combination of qPCR screening and ddPCR quantification, is a pentaplex (5-plex) ddPCR system, which was developed within T4.1.2. This analysis strategy starts with a conventional qPCR to screen all maize samples for the presence of the *nos* terminator and 35S promotor. Only if the sample is positive for one or both screening elements the sample has to be quantified with a suitable quantitative ddPCR multiplex assay. With the developed assay it is possible to quantify all nos positive and 35S negative EU authorized GM maize events (3272, GA21, MIR604, MIR162) and the endogene reference gene adh1 in only one ddPCR reaction. The GM maize line 5307 (having the same screening signal) could not be included in the ddPCR method because the validation of the 5307 PCR system was still in progress during the development of the ddPCR method and thus the primer and probe sequences had not been published yet by the EU. This method is compliant with the EU legal requirements and the minimum performance parameters (RSD<25%) set in the Deliverable 6.1 by WP6, with relative LOQ (rLOQ) of 0.03 % (Table 8).

Table 8 – determining the rLOQ of 5-plex ddPCR multiplex assay

GM%	RSD %
0.96	3
0.1	14
0.07	16
0.03	20

The combination of this newly developed multiplex ddPCR system and an upstream screening for nos and 35S based on a conventional qualitative multiplex qPCR assay also offers a more cost efficient analysis strategy compared to existing ones consisting of screening for and identification of GM events with several qualitative single and multiplex qPCRs and a subsequent quantification of all positive GM events with additional quantitative qPCRs (Table 9 and Table 10).

Table 9 – Practicability and cost-effectiveness of the combination of qPCR screening with the developed multiplex assay in comparison to existing qPCR approaches where only half of the samples are positive

Testing pipeline	Total number of parallel analysed samples	Number of samples with positive screening result	Estimated hands-on time (hours)	Relative final price per sample (%)
Screening + 5-plex ddPCR	1	1	2	100
	5	2	2	100
	11	5	2	100
Screening + Identification of 4 GM maize lines + quantification of 1 GM maize lines	1	1	2	161
	5	2	2	164
	11	5	3	178
Screening + Identification of 4 GM maize lines + quantification of 4 GM maize lines	1	1	5	390
	5	2	5	371
	11	5	8	476
Screening	1	all samples negative	1	32
	5		1	37
	11		1	39

Table 10 – Practicability and cost-effectiveness of the combination of qPCR screening with the developed multiplex assay in comparison to existing qPCR approaches where all of the samples are positive

Testing pipeline	Total number of parallel analysed samples	Number of samples with positive screening result	Estimated hands-on time (hours)	Relative final price per sample (%)
Screening + 5-plex ddPCR	1	all samples positive	2	100
	5		2	100
	11		2	100
Screening + Identification of 4 GM maize lines + quantification of 1 GM maize lines	1		2	161
	5		3	156
	11		7	252
Screening + Identification of 4 GM maize lines + quantification of 4 GM maize lines	1		5	390
	5		8	451
	11		19	674
Screening	1	all samples negative	1	32
	5		1	30
	11		1	29

3.4. Multiplex quantification of GM maize lines (quantification per ingredient)

To achieve better cost-effectiveness of the quantitative methods and to reduce the overall hands-on time, new multiplex GMO quantification methods were developed. These included high multiplexing levels with amplification of up to 11-targets per reaction.

To fulfil the regulatory requirements on labelling, where the final result of GM content is given per ingredient, the approach of quantifying a group of targets was considered. First work was done on the GM maize group. At the time of development twelve GM maize lines were authorized in the EU. The idea behind the multiplex method for quantification per ingredient was to quantify all twelve GM maize lines simultaneously. The developed method was published in a scientific journal in 2015 [11] and detailed results can be found in the publication. The Standard operating procedure for this method was prepared as the final version of M4.1. Due to interactions between some targets, the final method was divided into two multiplex assays, one quantifying a group of 9 GM maize lines together with the hmgA endogene (10-plex) and the second quantifying a group of 3 GM maize lines together with the hmgA endogene (4-plex) (Table 11).

Table 11 – Division of GM maize targets into two multiplex assays

Event/construct/gene	Multiplex group
GA21	10-plex
MON88017	10-plex
MON89034	10-plex
MIR162	10-plex
MIR604	10-plex
T25	10-plex
MON810	10-plex
MON863	10-plex
DAS1507	10-plex
NK603	4-plex
Bt11	4-plex
DAS59122	4-plex
hmgA	10-plex and 4-plex

Both multiplex assays were in-house validated, where the performance was tested against minimum performance parameters (limits of quantification and detection, RSD, false positive and negative rate...) as set in the Deliverable 6.1 by WP6. The performance was compliant with the set minimum performance parameters (Table 12).

Table 12 – Performance parameters of two ddPCR multiplex GM maize assays

Parameter	Experimental value
aLOQ	42 cp/reaction (4-plex) 29 cp/reaction (10-plex) 24 cp/reaction (hmgA)
aLOD	7 cp/reaction (4-plex) 17 cp/reaction (10-plex) 10 cp/reaction (hmgA)
rLOQ	0.068 % (4-plex) 0.058 % (10-plex)
rLOD	0.027 % (4-plex) 0.025 % (10-plex)
Specificity	no false positive reactions
Trueness	all results within ±25% of “true” value |z-scores| below 1.4

As specified for the task T4.1.2, it was shown that the method is suitable for quantification of GMOs per ingredient at both thresholds mentioned in the regulations (0.1% for un-authorised/LLP regulated events and 0.9% for authorised GMOs). Notably, the LLP regulation [12] refers to per event, not per ingredient quantification. However, historically, the presence of LLP regulated events is only exceptionally detected, and thus all samples for which presence of LLP regulated events is < 0.1% do not require further analysis. Samples exceeding this threshold will require further analysis by single event quantification. For GMOs falling outside these two categories, there is presently zero tolerance in the EU, and therefore their verified presence by qualitative detection is sufficient to ban the relevant product from marketing.

Based on this performance it was decided by WP1 that the method was suitable to enter into a full collaborative trial validation lead by WP6. Additional to the performance parameters, the cost-effectiveness of the multiplex ddPCR method was assessed in line with current GMO quantification procedure with qPCR. It was shown that the multiplex ddPCR for quantification of twelve maize lines was more cost effective than qPCR in all cases but one, which is when all tested samples are negative for the targets detectable by pentaplex screening with qPCR (Table 13).

Table 13 – Practicability and cost-effectiveness of the developed ddPCR multiplex GM maize assays in comparison to existing qPCR approaches

Testing pipeline	Steps prior quantification	Number of tested samples	Estimated hands-on time (hours)	Working days until final result (days)	Relative final price per sample (%)
Direct quantification of twelve authorised GM maize lines with 4-plex and 10-plex multiplex assays with ddPCR	/	1	3	0.8	100
		5	4	0.9	100
		11	5	1.0	100
Direct quantification of twelve authorised GM maize lines in simplex reactions with qPCR	/	1	8	2.0	272
		5	16	4.3	292
		11	25	7.5	300
Initial screening with qPCR, all samples negative	5x simplex screening elements	1	2	0.5	83
		5	6	1.3	105
		11	8	1.5	105
Initial screening with qPCR, all samples negative	5-plex screening elements	1	1	0.4	31
		5	3	0.6	39
		11	5	1.0	39
Initial screening, identification of six GM maize lines, quantification of one GM maize line with qPCR	5-plex screening elements , 6 specific lines	1	8	1.5	175
		5	14	2.8	167
		11	20	4.4	159
Direct identification of twelve authorised GM maize lines, quantification of two GM maize lines with qPCR	12 specific lines	1	10	1.8	226
		5	14	3.1	195
		11	18	4.8	185
Initial screening with qPCR and quantification of twelve authorised GM maize lines with 4-plex and 10-plex multiplex assays with ddPCR	5-plex screening elements	1	4	1.1	131
		5	7	1.5	139
		11	10	2.0	139

After the multiplex ddPCR assays for quantification of all EU-authorised GM maize lines have been developed and characterized, another GM maize line has been authorised in EU (MON87460). As it was already decided that the ddPCR multiplex assays for maize will enter the full collaborative trial validation and the work on that was already initiated, only preliminary tests were performed with addition of the new line to the 4-plex assay (resulting in new 5-plex assay). The preliminary results

have shown that this GM maize line can readily be added to the multiplex assay. However, a bigger set of experiments would need to be performed to evaluate all of the performance parameters of this new multiplex assay.

The dynamic process of authorising new GM lines is the major drawback of the described multiplex approach, as the assay performance after inclusion of primers and probes for the additional GMO lines needs to be verified/validated whenever a new GM line is added to the system. This drawback is partly addressed in the next chapter of the present report.

3.5. Multiplex quantification of GM soybean lines (quantification per ingredient)

The maize multiplex ddPCR assays were shown to be cost effective compared to qPCR quantification. Therefore, it was decided to continue the development of such assays for other plant species. In terms of global market share and number of GM events developed, commercialised and authorised or not in the EU, soybean and maize are the two top species. Thus the development of a ddPCR multiplex, covering GM soybean lines was an obvious next step.

Opposite to the GM maize, where prior screening with qPCR still seemed as a good complement to the ddPCR system (also from the point of cost effectiveness), the inclusion of screening in the GM soybean system is not advantageous anymore. There are currently 5 EU-authorised GM soybean lines that do not possess any of the common screening elements, therefore for each sample additional identification reactions for those individual events must be performed. Thus the ddPCR multiplex has a chance of having even bigger advantage in terms of cost effectiveness.

Also to address the problem of un-authorised GM soybean lines that are expected to become authorised in the EU in the near future, it was decided to develop such multiplex ddPCR GM soybean assays that would already have the un-authorised GM lines included. The individual assays for un-authorised GM soybeans could then be omitted from the testing assay when testing for labelling compliance, and added in the future when needed, without the need for additional verifications/validations.

The simplex GM soybean assays were first evaluated for the interactions between primers and probes. Based on these, also with the soybean multiplex, the division of simplex assays into two multiplex assays was done. One multiplex assay for quantifying a group of 5 GM soybean lines together with the soybean lectin (lec) endogene (6-plex) and the second quantifying a group of 10 GM soybean lines together with the lec endogene (11-plex) (Table 14).

Table 14 – Division of GM maize targets into two ddPCR multiplex GM soybean assays

Event/gene	Multiplex group
DP305423	6-plex
MON87708	6-plex
CV127	6-plex
MON87769	6-plex
MON87701	6-plex
MON40-3-2	11-plex
A2704	11-plex
MON89788	11-plex
A5547	11-plex
MON87705	11-plex
DP356043	11-plex
DAS-68416 *	11-plex
FG72 *	11-plex
DAS-44406 *	11-plex
DAS-81419 *	11-plex
lec	6-plex and 11-plex

* - Not authorised in the EU (per 23.12.2015)

It was decided to in-house validate three multiplex ddPCR assays: 6-plex, 11-plex and 7-plex. The 7-plex corresponds to the 11-plex assay without the un-authorised GM soybean lines. By the time of the preparation of this document the assays were not yet fully in-house validated. Nevertheless, the results that were already obtained are promising, as the aLOQ and aLOD (Table 15) were found to be compliant with minimum performance parameters set in Deliverable 6.1 by WP6.

Table 15 – Performance parameters of three ddPCR multiplex GM soybean assays

Parameter	Experimental value
aLOQ	38 cp/reaction (6-plex) 22 cp/reaction (7-plex) 39 cp/reaction (11-plex) 39 cp/reaction (lec)
aLOD	8 cp/reaction (6-plex) 10 cp/reaction (7-plex) 12 cp/reaction (11-plex) 18 cp/reaction (lec)

Up to date the final calculations of cost effectiveness for the soybean multiplex assays were not prepared, as not all of the parameters were evaluated. However, the two multiplex assays target a similar number of GM lines as in the maize multiplex. Additionally, the qPCR screening phase for soybean is more expensive due to necessary identification of the lines not covered by the screening. Thus, the cost effectiveness for the soybean multiplex is expected to be even greater than for the maize multiplex.

4. Conclusions

The number of authorized GMOs has increased over time and with that the required number of analyses with qPCR has also increased. This increase in number of analyses is making the detection and quantification of GMOs in different samples more and more expensive. To address the issue of cost effectiveness, new multiplex ddPCR methods were developed within the Decathlon project. The performance parameters of the new multiplex methods were evaluated according to the MPPs set in Deliverable D6.1.

As reported, the newly developed multiplex ddPCR methods are more cost effective than qPCR, whenever there are GMOs present in the sample. Individual laboratories can make their own estimations on cost effectiveness based on the number of positive samples they analyse, and then decide whether or not to implement these methods in their routine work. The newly developed methods were presented at several international conferences, meetings and workshops, where the GMO detection community has shown a great interest in the methods.

Based on the results and the development stage, the multiplex ddPCR method for quantification of 12 lines of GM maize fulfils all the requirements and is currently under collaborative trial validation organized by WP6 of the Decathlon project.

Although the majority of the methods were tested on GMOs authorised in the EU, the results on transfer of methods from qPCR to ddPCR and the performance parameters of multiplex ddPCR methods imply that the same system can also be applied to testing for unauthorised GMOs. These developments could serve as a starting point for additional new multiplex ddPCR methods, which would in the end cover the whole spectrum of GMOs.

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