Supporting information for:

Development, optimization, and evaluation of a duplex droplet digital PCR assay to quantify the *T-nos/hmg* copy number ratio in genetically modified maize

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Table S1. Primers and probes sequences used in the *T-nos* and *hmg* amplification

Name	Sequence	Amplicon lenght	Reference
Fw T-nos	5'-CATGTAATGCATGACGTTATTTATG-3'	84 bp	Reiting et al., 2007
Rv T-nos	5'-TTGTTTTCTATCGCGTATTAAATGT-3'		
T-nos probe	6FAM-ATGGGTTTTTATGATTAGAGTCCCGCAA-MGBNFQ		
Fw hmg	5'-TTGGACTAGAAATCTCGTGCTGA-3'	79 bp	ISO 21570:2005
Rv hmg	5'-GCTACATAGGGAGCCTTGTCCT-3'		
hmg probe	VIC-CAATCCACACAAACGCACGCGTA-MGBNFQ		

Fw =Forward, Rv = Reward

Table S2. Central composite design for optimization of a duplex ddPCR assay. DMR 447 IIa: NK603 100% mass fraction

Order	Run	Pt	Block	Coded values			Natural values		
				T-nos	*Hmg*	Ta	*Tnos* (nM)	*hmg* (nM)	Ta (°C)
10	1	-1	1	1.682	0.000	0.000	1002 / 384	60 / 110	60
18	2	0	1	0.000	0.000	0.000	750 / 300	60 / 110	60
6	3	1	1	1.000	-1.000	1.000	900 / 350	40 / 80	61
20	4	0	1	0.000	0.000	0.000	750 / 300	60 / 110	60
13	5	-1	1	0.000	0.000	-1.682	750 / 300	60 / 110	58
4	6	1	1	1.000	1.000	-1.000	900 / 350	80 / 140	59
9	7	-1	1	-1.682	0.000	0.000	498 / 216	60 / 110	60
7	8	1	1	-1.000	1.000	1.000	600 / 250	80 / 140	61
8	9	1	1	1.000	1.000	1.000	900 / 350	80 / 140	61
14	10	-1	1	0.000	0.000	1.682	750 / 300	60 / 110	62
3	11	1	1	-1.000	1.000	-1.000	600 / 250	80 / 140	59
17	12	0	1	0.000	0.000	0.000	750 / 300	60 / 110	60
19	13	0	1	0.000	0.000	0.000	750 / 300	60 / 110	60
12	14	-1	1	0.000	1.682	0.000	750 / 300	95/160	60
2	15	1	1	1.000	-1.000	-1.000	900 / 350	40 / 80	59
1	16	1	1	-1.000	-1.000	-1.000	600 / 250	40 / 80	59
16	17	0	1	0.000	0.000	0.000	750 / 300	60 / 110	60
5	18	1	1	-1.000	-1.000	1.000	600 / 250	40 / 80	61
11	19	-1	1	0.000	-1.682	0.000	750 / 300	25 / 60	60
15	20	0	1	0.000	0.000	0.000	750 / 300	60 / 110	60

Pt: point type; *T-nos*: *T-nos* primer/probe concentration; *hmg*: *hmg* primer/probe concentration; Ta: annealing temperature. Alpha = 1.68

Table S3. Central composite design for optimization of a duplex ddPCR assay. DMR 447 Va: NK603 10% mass fraction.

Order	Run	Pt	Block	Coded values			Natural values		
				T-nos	*Hmg*	Ta	*Tnos* (nM)	*hmg* (nM)	Ta (°C)
15	1	0	2	0.000	0.000	0.000	750 / 300	60 / 110	60
9	2	-1	2	-1.682	0.000	0.000	498 / 216	60 / 110	60
7	3	1	2	-1.000	1.000	1.000	600 / 250	80 / 140	61
20	4	0	2	0.000	0.000	0.000	750 / 300	60 / 110	60
19	5	0	2	0.000	0.000	0.000	750 / 300	60 / 110	60
6	6	1	2	1.000	-1.000	1.000	900 / 350	40 / 80	61
12	7	-1	2	0.000	1.682	0.000	750 / 300	95/160	60
16	8	0	2	0.000	0.000	0.000	750 / 300	60 / 110	60
4	9	1	2	1.000	1.000	-1.000	900 / 350	80 / 140	59
13	10	-1	2	0.000	0.000	-1.682	750 / 300	60 / 110	58
3	11	1	2	-1.000	1.000	-1.000	600 / 250	80 / 140	59
18	12	0	2	0.000	0.000	0.000	750 / 300	60 / 110	60
1	13	1	2	-1.000	-1.000	-1.000	600 / 250	40 / 80	59
8	14	1	2	1.000	1.000	1.000	900 / 350	80 / 140	61
11	15	-1	2	0.000	-1.682	0.000	750 / 300	25 / 60	60
14	16	-1	2	0.000	0.000	1.682	750 / 300	60 / 110	62
2	17	1	2	1.000	-1.000	-1.000	900 / 350	40 / 80	59
5	18	1	2	-1.000	-1.000	1.000	600 / 250	40 / 80	61
7	19	0	2	0.000	0.000	0.000	750 / 300	60 / 110	60
10	20	-1	2	1.682	0.000	0.000	1002 / 384	60 / 110	60

Pt: point type; *T-nos*: *T-nos* primer/probe concentration; *hmg*: *hmg* primer/probe concentration; Ta: annealing temperature. Alpha = 1.68

Table S4. Linear regression models built for optimized response variables in a duplex ddPCR method for *T-nos/hmg* analysis. DMR 447 IIa (100% *T-nos/hmg*).

Response variable	Coefficient	p value	Regression model	Adjusted R^2
T-nos/hmg				
Constant	52.53	0.000	***T-nos/hmg*** *= 52.53 – 30.99**Ta***	70.34 %
Ta	-30.99	0.000		
Lack of fit	-	0.828		
hmg cp				
Constant	21,100	0.000	***cp hmg*** = 21,100	-
Lack of fit	-	0.580		
***T-nos* ΔF**				
Constant	3694.5	0.000	**ΔF *T-nos*** *= 3694.5 + 862.7**T-nos** – 2428.5**Ta** + 443.8**TaxTa***	90.69 %
T-nos	862.7	0.000		
Ta	-2428.5	0.000		
TaxTa	443.8	0.028		
Lack of fit	-	0.024		
***hmg* ΔF**				
Constant	995.75	0.000	**ΔF *hmg*** *= 995.8 + 432.0**hmg** – 63.4**Ta** + 45.4**hmgxhmg***	94.85 %
hmg	432.02	0.000		
Ta	-63.43	0.015		
hmgxhmg	45.39	0.061		
Lack of fit	-	0.981		

T-nos/hmg: *T-nos/hmg* copy number ratio. ***hmg cp*:** *hmg* copy number. ***T-nos* ΔF:** Fluorescence difference between *T-nos* positive and *T-nos* negative droplets. ***hmg* ΔF:** Fluorescence difference between *hmg* positive and *hmg* negative droplets**.** ***T-nos*:** *T-nos* primer/probe concentration*.* ***hmg*:** *hmg* primer/probe concentration. ***Ta*:** annealing temperature. **ddPCR:** digital droplet polymerase chain reaction. **R^2:** coefficient of determination. **DMR 447 IIa**: certified reference material for NK603 (100% mass fraction).

Table S5. Linear regression models built for optimized response variables in a duplex ddPCR method for *T-nos/hmg* analysis. DMR 447 Va: 10% *T-nos/hmg*

Response variable	Coefficient	p value	Regression model	Adjusted R^2
T-nos/hmg				
Constant	5.06	0.000		
T-nos	0.76	0.060	***T-nos/hmg*** *= 5.06 + 0.76****T*-nos** *– 2.58****Ta***	71.71 %
Ta	-2.58	0.000		
Lack of fit	-	0.504		
hmg cp				
Constant	21968.3	0.000		
hmg	-876.6	0.066	***cp hmg*** *= 21968.3 – 876.6****hmg***	13.02%
Lack of fit	-	0.872		
***T-nos* ΔF**				
Constant	3483.77	0.000		
T-nos	805.92	0.000		
hmg	29.41	0.864	**ΔF *T-nos*** *= 3483.8 + 805.9****T-nos*** *– 2546.5****Ta*** *+ 394.6****hmgxhmg*** *-759.6****T-nosxTa*** *+ 512.2****hmgxTa***	93.43 %
Ta	-2546.45	0.000		
hmgxhmg	394.56	0.030		
T-nosxTa	-759.63	0.004		
hmgxTa	512.23	0.036		
Lack of fit	-	0.693		
***hmg* ΔF**				
Constane	1086.5	0.000		
hmg	445.6	0.000	**ΔF *hmg*** *= 1086.5 + 445.6****hmg*** *– 125.7****Ta***	95.16 %
Ta	-125.7	0.000		
Lack of fit	-	0.234		

T-nos/hmg: *T-nos/hmg* copy number ratio. ***hmg cp***: *hmg* copy number. ***T-nos* ΔF*:*** Fluorescence difference between *T-nos* positive and *T-nos* negative droplets. ***hmg* ΔF*:*** Fluorescence difference between *hmg* positive and *hmg* negative droplets**.** ***T-nos***: *T-nos* primer/probe concentration*.* ***hmg***: *hmg* primer/probe concentration**. Ta:** annealing temperature. **ddPCR:** digital droplet polymerase chain reaction. **R^2:** coefficient of determination. **DMR 447 Va:** certified reference material for NK603 (10% mass fraction).

Table S6. *T-nos* copy number, *T-nos/hmg* copy number ratio and relative standard deviation at different annealing temperatures.

Annealing temperature	*T-nos cp*		*T-nos/hmg* copy number ratio		RSD for *T-nos/hmg* copy number ratio	
	DMR IIa	DMR Va	DMR IIa	DMR Va	DMR IIa	DMR Va
58°C	17,207	1747	90.5 %	8.0 %	0.1 %	0.2 %
59°C	18,334	1715	87.9 %	7.8 %	1.2 %	5.4 %
60°C	10,579	1193	52.8 %	5.2 %	40.6 %	37.7 %
61°C	3,802	506	20.1 %	2.4 %	76.2 %	39.6 %
62°C	10	0	0.1 %	0.0 %	0.1 %	0.0 %

Average values at every temperature level. DMR IIa: NK603 (100% mass fraction). DMR Va: (10% mass fraction). cp: copies. RSD: relative standard deviation.

Table S7. Average values and variability for *hmg cp* at different *hmg* primer/probe concentrations.

hmg primer/probe concentration		*hmg cp*		RSD for *hmg cp*	
Coded level	**Natural level**	DMR 447 IIa	DMR 447 Va	DMR 447 IIa	DMR 447 Va
-1.68	25/40 nM	18890	24,663	3%	14%
-1.0	40/80 nM	20934	22,627	10%	15%
0	60/110 nM	21058	21,820	18%	10%
1.0	80/140 nM	19074	21,393	4%	4%
1.68	95/160 nM	21112	20,570	4%	4%

Average values at every *hmg* primer/probe concentration level. Data obtained from optimization of a duplex ddPCR method for *T-nos/hmg* analysis. DMR 447 IIa: 100% mass fraction. DMR 447 Va: 10% mass fraction. cp: copies. RSD: relative standard deviation.

Table S8. Specificity test of a duplex ddPCR assay

Species	*Material*	*Transformation event*	*Mass fraction*	*T-nos*	*hmg*
Maize					
Popcorn	*	---	---	-	+
Red	*	---	---	-	+
Blue	*	---	---	-	+
White	*	---	---	-	+
Yellow	DMR 436 Ia	MON 810	No GM	-	+
	DMR 453 Ia	MON 88017	No GM	-	+
	DMR 451 Ia	MON 863	No GM	-	+
	DMR 482 Ia	---	No GM	-	+
	DMR 436 Vb	MON 810	10%	-	+
	DMR 447 IIIa	NK603	1%	+	+
	DMR 451 Va	MON 863	10%	+	+
	DMR 452 IIa	MON 89034	100%	+	+
	DMR 453 Va	MON 88017	10%	+	+
Soybean	DMR 495 IIa	MON 04032-6	100%	+	-
	DMR 495 IIIa	MON 04032-6	1%	+	-
Wheat	DMR 496 IIa	DREB-1ª	100%	+	-
	DMR 496 IIIa	DREB-1ª	1%	+	-
Rice	*	---	---	-	-

* Materials acquired as seeds at local markets.
The sign showed in the *T-nos* result and *hmg* result columns, specifies if there was (+) or not (-) amplification of the corresponding target.

Table S9. Applicability assay of the *T-nos/hmg* ddPCR method, on several corn varieties.

Tested variety	*Expected T-nos/hmg copy number ratio*	*Measured T-nos/hmg copy number ratio*	*Relative standard deviation*	*Accuracy*
Popping corn	0.91%	1.0%	5.0%	111.43%
Blue corn	0.85%	0.93%	3.5%	108.72%
Red corn	0.99%	1.13%	5.5%	113.56%
White corn	0.84%	0.90%	1.2 %	108.08%

Average from three replicates. Tested samples were prepared as a mix of DNA extracted from non-GM corn and GM corn for every variety. The analyzed variety represented at least 97.5% of the final mix.

Table S10. Comparison of quantification using singleplex and duplex ddPCR assays

Material	*Measurand*	*Duplex (Dx)*	*Singleplex (Sx)*	*% CV duplex*	*% CV singleplex*	*% Bias Dx/Sx*
Dilution from DMR 447 IIIa	*T-nos* cp	20	18	3.2	13.0	6.6
	hmg cp	27819	28235	0.8	2.0	-1.3
	T-nos/hmg %	0.070	0.062	2.5	13.8	**6.5**
DMR 447 IIIa	*T-nos* cp	185	174	5.9	7.8	6.3
	hmg cp	20486	20872	1.1	0.9	-1.8
	T-nos/hmg%	0.90	0.84	5.0	8.1	**8.2**
DMR 447 Va	*T-nos* cp	1563	1675	2.4	1.7	-6.7
	hmg cp	20553	20630	1.9	1.7	-0.4
	T-nos/hmg %	7.6	8.1	2.7	0.3	**-6.3**
DMR 452 IIa	*T-nos* cp	17001	17572	1.6	1.9	-3.2
	hmg cp	32023	32122	1.8	1.3	-0.3
	T-nos/hmg %	53.1	54.7	1.8	1.2	**-2.9**
DMR 447 IIa	*T-nos* cp	14242	14069	4.0	0.3	1.2
	hmg cp	16040	15819	4.5	1.6	1.4
	T-nos/hmg %	88.8	89.0	2.0	1.9	**-0.2**

Average from three replicates. ***This material was prepared as a dilution from DMR 447 IIIa (1% mass fraction), so the final concentration was not the same for duplex and singleplex assay (0.074 and 0.07%, respectively). To address this issue, the bias was estimated taking into account the accuracy achieved in every case.

Table S11. DNA restriction enzyme digestion. DMR 447 Va (10% *T-nos/hmg*)

Measurand	***Non-digested DNA***	***BamHI digestion***	***EcoRI digestion***	***Multiple digestion***	***XhoI digestion***
T-nos cp	1491	1739	1645	1625	1336
hmg cp	18895	17844	17398	17815	17552
T-nos/hmg%	7.9[bc]	9.8[a]	9.5[ab]	9.1[abc]	7.6[c]

Average from three replicates. Treatments not sharing a letter are significantly different. (Tukey method, P≤0.05).

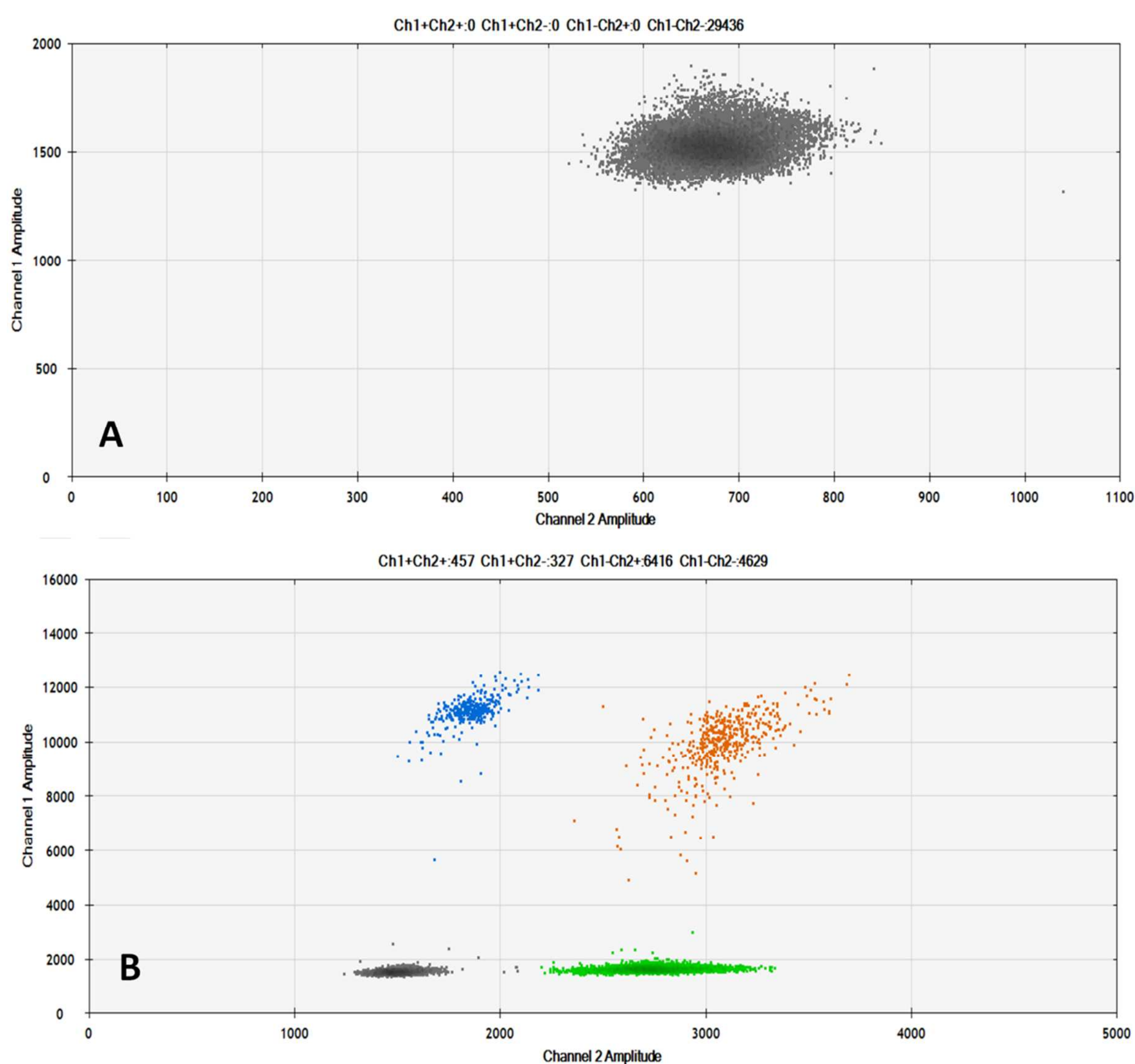

Figure S1. Example of negative and positive results. Results from a non-template control (A) and a genetically modified maize sample (B). Each point represents a droplet with a given fluorescence level. Droplet colors indicate which target was amplified: *T-nos* (blue), *hmg* (green), none of the two (gray) or both (orange).

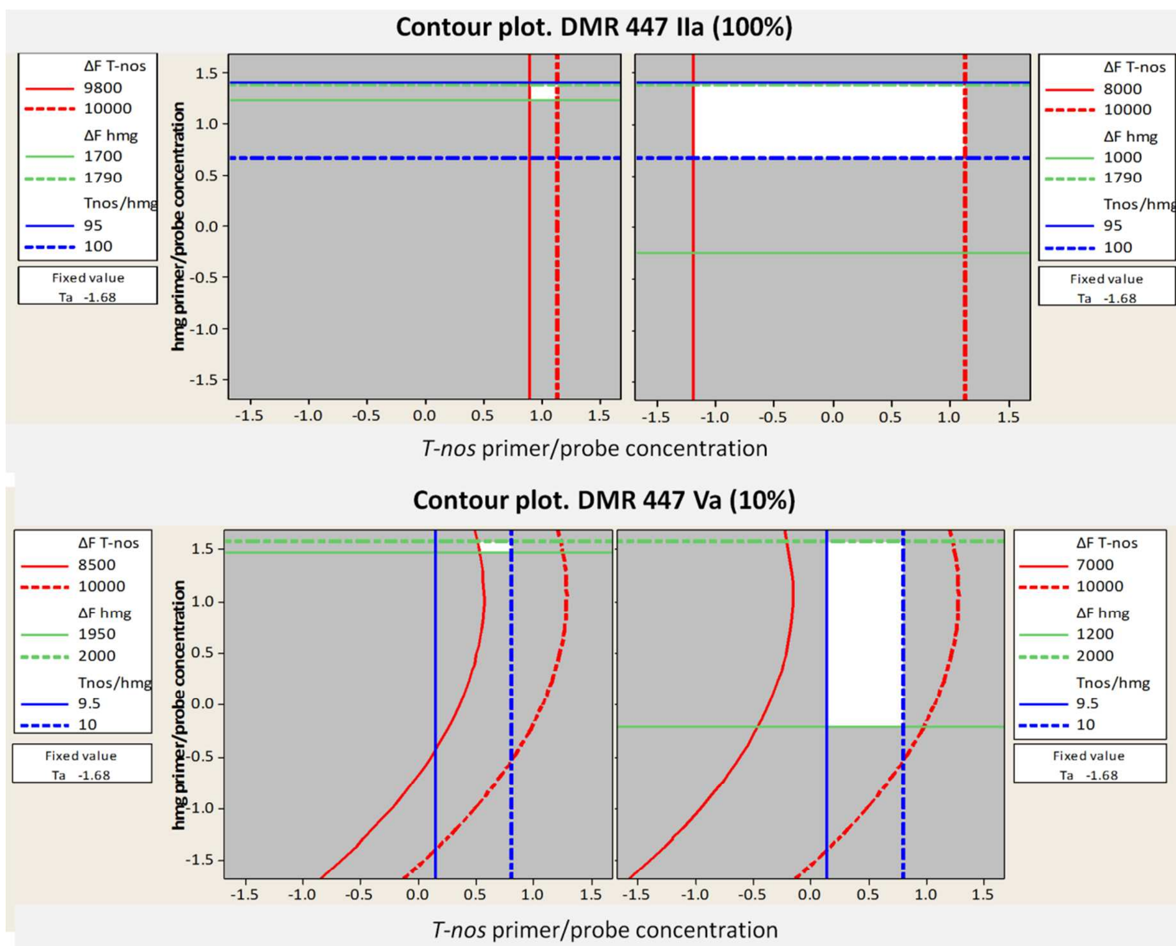

Figure S2. Overlaid contour plots for ddPCR optimization. 1) DMR 447 IIa (100%). 2) DMR 447 Va (10%). The white region shows the operating range, where the response variables are predicted to be the ones shown in the lateral boxes.

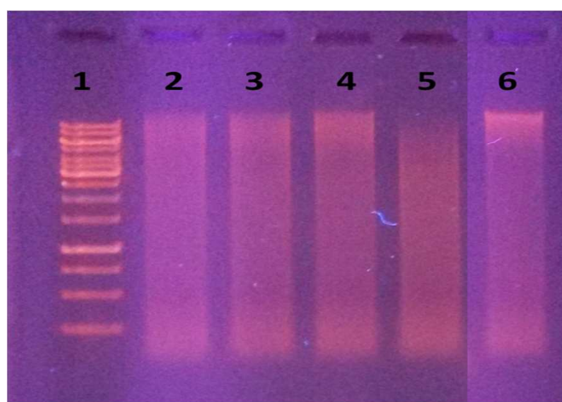

Figure S3. DNA enzymatic restriction digestion. 1) DNA ladder; 2) EcoRI; 3) BamHI; 4) XhoI; 5) Multiple digestion; 6) Non-digested DNA.

Annex S1. DNA extraction protocol

The genomic DNA was extracted using the Genomic DNA extraction kit (FAST ID, USA). 500 µL of Genomic Lyse buffer were added to 100mg of maize seed-powder flour, followed by 10 µL of proteinasa K (10 mg/mL). The sample was shacked on termomixer (Bioer, China) for 30 min (16°C; 1,500 rpm) and briefly on vortex at the end. It was centrifuged (labtron scientific, UK) for 12 min (16°C; 12,000 rpm); the supernatant was collected and 400 µL of Genomic Bind buffer were added. The sample was shaked on termomixer (1 min, 20°C, 1,500 rpm) and loaded on a DNA Binding Column. A centrifugal force (5min; 16°C; 10,000 rpm) was applied to pass the sample through the column, and the eluates were discarded. 400µL of Genomic Wash Buffer were added to the column and it was centrifuged again for 5 min (16°C; 10,000 rpm), discarding the eluates. The column was washed three times with 400 µL of 75% ethanol; at the end of every wash the column was centrifuged for 1 min (16°C; 5,000 rpm). The extraction column was transferred to a new vial and it was added with 100 µL of Extraction buffer (1XTE). The samples were incubated for 10 min at 65°C, and later centrifuged for 2 min (16°C; 5,000 rpm). The eluted DNA was collected and stored at -40°C.

Annex S2. Workflow for digital droplet PCR analysis

A mix containing the following reagents was prepared: 10 µL of supermaster mix (Bio-Rad, USA), 1 µL (or the volume needed according to starting and final concentrations desired) of *T-nos* and *hmg* primers and probes, and the volume needed of water for a final volume of 19 µL. This solution was mixed with 1 µL of template DNA. 20 µL of this mix were placed on the sample wells of an 8-well cartridge; the oil wells were filled with 70 µL of droplet generator oil. Droplets were generated using the QX200 droplet generator (Bio-Rad, USA). Water-in-oil emulsions were transferred to a 96-well plate and amplified in a GeneAmp 9500 PCR system (Applied BioSystems, USA). Thermal cycling conditions were: 15 min at 95°C for enzyme activation (1 cycle), followed by 15 s at 94°C for DNA denaturation, and 1 min at the temperature indicated for the experimental design (or the defined optimal temperature: 58°C) for annealing and extension (45 cycles). Products were cooled to 4°C. After amplification, plates were placed on the QX200 droplet reader (Bio-Rad, USA) for data acquisition. FAM signal (*T*-nos) was registered in channel 1, and VIC signal (*hmg*) was measured in channel 2. Only samples with a minimum of 8,000 accepted droplets were considered for subsequent analysis.

Annex S3. Enzymatic restriction digestion of genomic DNA

For restriction digestion assay, a mix was prepared adding the following reagents in the order stated: 12 µL of water (molecular biology grade), 2 µL of 10X FastDigest buffer (Thermo scientific, USA), 4 µL of DNA and 2 µL of enzyme (Thermo scientific, USA); it was gently homogenized. The mix was incubated for 5 min (*Bam*HI), 10 min (*Xho*I) or 20 min (*Eco*RI and multiple digestion) in a bath water at 37°C. This solution was used as template DNA for later droplet generation, amplification and analysis by ddPCR.