Casu, A., Chiusa, G., Battilani, P., & Mehl, H. L. (). Optimization of DNA extraction and application of qPCR and ddPCR assays for detection of toxigenic *Aspergillus flavus* in hazelnut kernels. *Phytopathologia Mediterranea* 64(3): 637–652. doi: 10.36253/phyto-16897

Supplementary Table S1. Comparison of DNA quantity and quality metrics across four different extraction methods applied to ten naturally contaminated hazelnut samples.

Method	Sample	Nanodrop, (total ng)[a]	A260/A280[b]	A260/A230[c]	Qubit, (total ng)[d]	qPCR spiked, (copies *A. flavus* DNA)[e]	Measured vs expected (%)[f]
Callicott *et al*., 2015	1	8.2×10^3	1.43	0.37	1.5×10^3	< LOD	*n.d*
	2	5.9×10^3	1.58	0.47	5.9×10^2	< LOD	*n.d*
	3	6.3×10^3	1.43	0.42	5.3×10^2	< LOD	*n.d*
	4	1.0×10^4	1.43	0.36	8.5×10^2	< LOD	*n.d*
	5	9.1×10^3	1.42	0.38	7.9×10^2	< LOD	*n.d*
	6	7.9×10^3	1.57	0.51	4.3×10^2	< LOD	*n.d*
	7	6.2×10^3	1.62	0.45	4.1×10^2	< LOD	*n.d*
	8	9.0×10^3	1.61	0.48	9.8×10^2	< LOD	*n.d*
	9	3.6×10^3	1.68	0.46	3.2×10^2	< LOD	*n.d*
	10	8.6×10^3	1.55	0.46	5.8×10^2	< LOD	*n.d*
DNeasy Plant Pro Kit (Qiagen)	1	1.0×10^3	1.77	0.66	1.5×10^3	2.0×10^3	40
	2	5.5×10^2	1.65	1.2	6.3×10^2	1.5×10^3	30
	3	6.6×10^2	1.83	0.95	9.5×10^2	1.8×10^3	30
	4	7.8×10^2	2.01	0.87	1.0×10^3	5.5×10^3	110
	5	6.5×10^2	2.28	0.1	8.4×10^2	2.0×10^3	40
	6	3.5×10^2	2.91	0.57	6.6×10^2	1.8×10^3	35
	7	9.8×10^2	2.13	1.29	1.4×10^3	2.0×10^3	40
	8	5.5×10^2	2.22	0.79	9.5×10^2	1.5×10^3	30
	9	6.6×10^2	1.98	0.95	9.1×10^2	6.0×10^3	121
	10	7.5×10^2	1.87	0.17	1.0×10^3	1.8×10^3	35
FastDNA Spin Kit (MP Biomedicals)	1	3.4×10^3	1.79	0.03	2.0×10^3	1.8×10^3	35
	2	4.8×10^3	1.58	0.04	2.4×10^3	1.8×10^3	35
	3	3.2×10^3	1.67	0.03	1.7×10^3	2.0×10^3	40
	4	3.5×10^3	1.77	0.02	1.3×10^3	4.0×10^3	81
	5	3.5×10^3	1.76	0.03	1.8×10^3	2.0×10^3	40
	6	3.7×10^3	1.54	0.03	1.7×10^3	1.5×10^3	30
	7	3.3×10^3	1.68	0.03	1.7×10^3	2.0×10^3	40
	8	3.7×10^3	1.53	0.03	2.0×10^3	1.8×10^3	35
	9	3.5×10^3	1.75	0.03	1.6×10^3	4.0×10^3	81
	10	3.6×10^3	1.45	0.03	1.7×10^3	2.0×10^3	40
InstaGene Matrix (Bio-Rad)	1	3.3×10^4	1.31	0.27	*n.a*	< LOD	*n.d*
	2	3.5×10^4	1.36	0.29	*n.a*	< LOD	*n.d*
	3	4.2×10^4	1.3	0.28	*n.a*	< LOD	*n.d*
	4	5.0×10^4	1.27	0.3	*n.a*	< LOD	*n.d*
	5	5.0×10^4	1.32	0.3	*n.a*	< LOD	*n.d*
	6	5.1×10^4	1.04	0.33	*n.a*	< LOD	*n.d*
	7	5.6×10^4	1.33	0.32	*n.a*	< LOD	*n.d*
	8	5.1×10^4	1.31	0.32	*n.a*	< LOD	*n.d*
	9	8.2×10^4	1.25	0.36	*n.a*	< LOD	*n.d*
	10	4.9×10^4	1.03	0.3	*n.a*	< LOD	*n.d*

[a] DNA concentration was measured with Nanodrop and reported in total ng per sample.
[b] A260/A280 is a measure of purity of DNA in relation to protein content. An ideal value is around 1.8 for pure DNA.
[c] A260/A230 is a purity indicator in relation to salts, polysaccharides and phenolic compounds. An ideal value is around 2.0-2.2 for pure DNA.
[d] DNA concentrations were measured with Qubit and reported in total ng. n.a indicates non applicable, and it is used when Qubit analysis was not performed on samples.
[e] qPCR was performed on each sample. Since only samples spiked with 5.00×10^3 of AF13 DNA gave positive amplifications, only these data are shown on the table. All results are expressed in copies/reaction. ddPCR was also performed on each sample but amplification did not occur.
[f] Inhibition of PCR amplification based on measured copies/reaction with respect to the expected value. N.d is indicated when no copies of *A. flavus* DNA were measured.

Supplementary Table S2. Evaluation of DNA extraction performance from spiked hazelnut samples using the DNeasy Plant Pro Kit (Qiagen) and FastDNA Spin Kit (MP Biomedicals) under different contamination levels and sample fractions.

Method	Spore Concentration[a]	Fraction[b]	Nanodrop (total ng)[c]	A260/A280[d]	A260/A230[d]	Qubit (total ng)[c]	qPCR (copies/g)[f]	ddPCR (copies/g)[f]	qPCR measured –expected (%)[g]	ddPCR measured – expected (%)[g]
DNeasy Plant Pro Kit (Qiagen)	1.2×10^5	Kernel	$1.0 \times 10^3 \pm 3.1 \times 10^2$	1.85 ± 0.01	0.39 ± 0.24	$9.0 \times 10^2 \pm 3.0 \times 10^2$	$4.8 \times 10^3 \pm 1.4 \times 10^3$	$3.6 \times 10^3 \pm 8.7 \times 10^2$	4	3
		Liquid	$2.2 \times 10^3 \pm 2.8 \times 10^2$	1.85 ± 0.02	0.88 ± 0.45	$2.2 \times 10^3 \pm 2.6 \times 10^2$	$8.1 \times 10^3 \pm 2.4 \times 10^3$	$4.6 \times 10^3 \pm 3.9 \times 10^3$	7	4
	1.2×10^4	Kernel	$9.3 \times 10^2 \pm 3.3 \times 10^2$	1.85 ± 0.06	0.66 ± 0.43	$8.8 \times 10^2 \pm 3.9 \times 10^2$	$4.7 \times 10^2 \pm 6.9 \times 10^2$	$2.2 \times 10^2 \pm 2.7 \times 10^2$	4	2
		Liquid	$2.7 \times 10^3 \pm 6.3 \times 10^2$	1.88 ± 0.04	0.48 ± 0.26	$2.6 \times 10^3 \pm 7.3 \times 10^2$	$1.1 \times 10^3 \pm 4.0 \times 10^2$	$1.2 \times 10^3 \pm 5.7 \times 10^1$	9	10
	1.2×10^3	Kernel	$9.5 \times 10^2 \pm 4.9 \times 10^2$	1.89 ± 0.04	0.66 ± 0.53	$8.4 \times 10^2 \pm 4.3 \times 10^2$	$3.5 \times 10^2 \pm 4.9 \times 10^2$	$2.1 \times 10^2 \pm 3.1 \times 10^2$	29	17
		Liquid	$2.9 \times 10^3 \pm 7.4 \times 10^2$	1.88 ± 0.02	0.36 ± 0.07	$2.7 \times 10^3 \pm 5.4 \times 10^2$	$3.0 \times 10^2 \pm 3.0 \times 10^2$	$1.7 \times 10^2 \pm 1.2 \times 10^2$	25	14
FastDNA Spin Kit (MP Biomedicals)	1.2×10^5	Kernel	$2.8 \times 10^3 \pm 8.8 \times 10^2$	1.72 ± 0.09	0.03 ± 0.01	$1.4 \times 10^3 \pm 4.1 \times 10^2$	$2.1 \times 10^3 \pm 3.0 \times 10^2$	$2.2 \times 10^3 \pm 5.8 \times 10^2$	2	2
		Liquid	$4.1 \times 10^3 \pm 2.8 \times 10^2$	1.82 ± 0.07	0.05 ± 0.02	$2.6 \times 10^3 \pm 6.0 \times 10^2$	$4.3 \times 10^3 \pm 1.2 \times 10^3$	$5.5 \times 10^3 \pm 1.5 \times 10^3$	4	5
	1.2×10^4	Kernel	$2.5 \times 10^3 \pm 4.7 \times 10^2$	1.71 ± 0.10	0.03 ± 0.00	$1.3 \times 10^3 \pm 1.8 \times 10^2$	$2.3 \times 10^2 \pm 5.8 \times 10^1$	$2.3 \times 10^2 \pm 1.0 \times 10^2$	2	2
		Liquid	$3.8 \times 10^3 \pm 2.0 \times 10^2$	1.87 ± 0.03	0.05 ± 0.02	$2.5 \times 10^3 \pm 2.6 \times 10^2$	$1.2 \times 10^3 \pm 1.1 \times 10^3$	$8.7 \times 10^2 \pm 3.2 \times 10^2$	10	7
	1.2×10^3	Kernel	$3.0 \times 10^3 \pm 5.3 \times 10^2$	1.81 ± 0.13	0.03 ± 0.01	$1.7 \times 10^3 \pm 1.3 \times 10^2$	$5.4 \times 10^1 \pm 9.4 \times 10^1$	$1.5 \times 10^1 \pm 2.6 \times 10^1$	5	1
		Liquid	$3.7 \times 10^3 \pm 5.2 \times 10^2$	1.89 ± 0.09	0.04 ± 0.01	$2.4 \times 10^3 \pm 2.2 \times 10^2$	$9.7 \times 10^1 \pm 8.4 \times 10^1$	$1.5 \times 10^2 \pm 8.0 \times 10^1$	8	12

[a] Hazelnut samples were artificially contaminated with three concentrations of *A. flavus* spores (1.2×10^5, 1.2×10^4 and 1.2×10^3 conidia/g).
[b] For each contamination level, both the liquid fraction and kernel fraction were analyzed separately.
[c] DNA concentrations measured with Nanodrop and Qubit reported in total ng per sample.
[d] A260/A280 is a measure of purity of DNA in relation to protein content. An ideal value is around 1.8 for pure DNA.
[e] A260/A230 is a purity indicator in relation to salts, polysaccharides and phenolic compounds. An ideal value is around 2.0-2.2 for pure DNA.
[f] qPCR and ddPCR were performed on each samples and results are expressed in copies/g.
[g] Inhibition of PCR amplification based on measured copies/reaction with respect to the expected value.
All reported values represent the mean ± standard deviation of three independent replicate.

Supplementary Table S3. UNIANOVA of quantitative and qualitative results obtained from hazelnut samples tested with two DNA extraction methods (DNeasy, FastDNA), three spore concentrations (1.2 × 105, 1.2 × 104, and 1.2 × 103 conidia/ml) and two separated fractions (liquid, kernel).

Factors	Nanodrop (total ng)[a]	A260/A280	A260/A230	Qubit (total ng)[a]	qPCR (copies/g)[b]	ddPCR (copies/g)[b]
Method (M)	*n.s*	*n.s*	**	*	**	n.s
DNeasy Plant Pro Kit (Qiagen)	$1.8 \times 10^3 \pm 9.7 \times 10^2$	1.85 ± 0.07	0.54 ± 0.36 a	$1.7 \times 10^3 \pm 9.4 \times 10^2$ b	$2.5 \times 10^3 \pm 3.2 \times 10^3$ a	$1.7 \times 10^3 \pm 2.3 \times 10^3$
FastDNA Spin Kit (MP Biomedicals)	$3.3 \times 10^3 \pm 7.5 \times 10^2$	1.81 ± 0.10	0.04 ± 0.01 b	$2.0 \times 10^3 \pm 6.1 \times 10^2$ a	$1.3 \times 10^3 \pm 1.7 \times 10^3$ b	$1.5 \times 10^3 \pm 2.1 \times 10^3$
Concentration (C)	*n.s*	*n.s*	*n.s*	*n.s*	**	**
1.2×10^5	$2.5 \times 10^3 \pm 1.2 \times 10^3$	1.81 ± 0.07	0.34 ± 0.42	$1.8 \times 10^3 \pm 7.7 \times 10^2$	$4.8 \times 10^3 \pm 2.6 \times 10^3$ a	$4.0 \times 10^3 \pm 2.2 \times 10^3$ a
1.2×10^4	$2.5 \times 10^3 \pm 1.1 \times 10^3$	1.83 ± 0.09	0.30 ± 0.36	$1.8 \times 10^3 \pm 8.6 \times 10^2$	$7.6 \times 10^2 \pm 7.3 \times 10^2$ b	$6.4 \times 10^2 \pm 4.8 \times 10^2$ b
1.2×10^3	$2.6 \times 10^3 \pm 1.2 \times 10^3$	1.87 ± 0.08	0.28 ± 0.36	$1.9 \times 10^3 \pm 8.1 \times 10^2$	$2.0 \times 10^2 \pm 2.9 \times 10^2$ b	$1.4 \times 10^2 \pm 1.7 \times 10^2$ b
Fraction (F)	*n.s*	*	*n.s*	**	**	*
Kernel	$1.8 \times 10^3 \pm 1.1 \times 10^3$	1.80 ± 0.05 b	0.30 ± 0.35	$1.2 \times 10^3 \pm 4.3 \times 10^2$ b	$1.3 \times 10^3 \pm 1.9 \times 10^3$ b	$1.1 \times 10^3 \pm 1.4 \times 10^3$ b
Liquid	$3.2 \times 10^3 \pm 8.0 \times 10^2$	1.87 ± 0.10 a	0.31 ± 0.37	$2.5 \times 10^3 \pm 4.4 \times 10^2$ a	$2.5 \times 10^3 \pm 3.1 \times 10^3$ a	$2.1 \times 10^3 \pm 2.6 \times 10^3$ a
Interactions						
M × C	*n.s*	*n.s*	*n.s*	*n.s*	**	*n.s*
M × F	*n.s*	*	*n.s*	*	*n.s*	*n.s*
C × F	*n.s*	*n.s*	*n.s*	*n.s*	**	*n.s*
M × C × F	*n.s*	*n.s*	*n.s*	*n.s*	*n.s*	*n.s*

[a] Nanodrop and Qubit values are expressed as total ng per sample.
[b] qPCR and ddPCR results are expressed as copies/g.
** $P < 0.01$; * $P < 0.05$; n.s. stands for non-significant. Different letters indicate significant differences according to Tukey's test.
Values are expressed as mean ± standard deviation.

Supplementary Table S4. Quantification and quality assessment of DNA extracted from ten naturally contaminated hazelnut samples using the optimized DNeasy Plant Pro Kit protocol.

Sample	Nanodrop (total ng)[a]	A260/A280[b]	A260/A230[c]	Qubit (total ng)[a]	qPCR (copies/g)[d]	ddPCR (copies/g)[d]	Expected copies/g[e]	qPCR measured – expected (%)[f]	ddPCR measured -expected (%)[f]
1	$2.8 \times 10^3 \pm 1.0 \times 10^3$	1.84 ± 0.07	0.29 ± 0.11	$3.0 \times 10^3 \pm 3.9 \times 10^2$	$1.8 \times 10^2 \pm 1.6 \times 10^2$	$1.5 \times 10^2 \pm 1.1 \times 10^2$	3.2×10^2	57	46
2	$3.3 \times 10^3 \pm 6.1 \times 10^2$	1.86 ± 0.02	0.78 ± 0.25	$3.2 \times 10^3 \pm 1.0 \times 10^3$	$6.4 \times 10^1 \pm 1.3 \times 10^2$	$2.9 \times 10^1 \pm 5.8 \times 10^1$	1.2×10^2	52	23
3	$4.1 \times 10^3 \pm 1.2 \times 10^3$	1.91 ± 0.04	0.38 ± 0.47	$3.7 \times 10^3 \pm 1.2 \times 10^3$	$3.1 \times 10^2 \pm 2.7 \times 10^2$	$1.5 \times 10^2 \pm 8.0 \times 10^1$	*n.d*	*n.a.*	*n.a.*
4	$2.9 \times 10^3 \pm 9.3 \times 10^2$	1.87 ± 0.02	0.24 ± 0.25	$3.0 \times 10^3 \pm 4.6 \times 10^2$	$1.3 \times 10^2 \pm 2.5 \times 10^2$	$5.2 \times 10^1 \pm 3.8 \times 10^1$	5.2×10^2	24	10
5	$3.5 \times 10^3 \pm 6.0 \times 10^2$	1.90 ± 0.03	0.38 ± 0.35	$3.1 \times 10^3 \pm 6.6 \times 10^2$	$6.3 \times 10^1 \pm 8.8 \times 10^1$	$2.7 \times 10^1 \pm 3.3 \times 10^1$	5.0×10^1	127	54
6	$2.5 \times 10^3 \pm 3.4 \times 10^2$	1.90 ± 0.02	0.29 ± 0.24	$2.2 \times 10^3 \pm 1.5 \times 10^2$	$2.3 \times 10^2 \pm 1.3 \times 10^2$	$9.2 \times 10^1 \pm 5.2 \times 10^1$	2.0×10^1	1167	459
7	$3.4 \times 10^3 \pm 1.3 \times 10^2$	1.86 ± 0.01	0.21 ± 0.01	$2.9 \times 10^3 \pm 2.3 \times 10^2$	$2.4 \times 10^1 \pm 4.8 \times 10^1$	$2.0 \times 10^1 \pm 2.3 \times 10^1$	*n.d*	*n.a.*	*n.a.*
8	$2.8 \times 10^3 \pm 1.6 \times 10^1$	1.91 ± 0.02	1.14 ± 0.84	$2.4 \times 10^3 \pm 1.3 \times 10^2$	$1.5 \times 10^2 \pm 3.0 \times 10^2$	$1.1 \times 10^1 \pm 2.3 \times 10^1$	*n.d*	*n.a.*	*n.a.*
9	$2.8 \times 10^3 \pm 4.6 \times 10^2$	1.84 ± 0.07	0.65 ± 0.67	$2.9 \times 10^3 \pm 5.3 \times 10^1$	$1.2 \times 10^3 \pm 1.8 \times 10^3$	$1.2 \times 10^2 \pm 1.3 \times 10^1$	1.5×10^3	76	8
10	$2.3 \times 10^3 \pm 3.3 \times 10^2$	1.89 ± 0.01	0.33 ± 0.26	$2.0 \times 10^3 \pm 3.6 \times 10^2$	*n.d*	$2.1 \times 10^1 \pm 4.1 \times 10^1$	*n.d*	*n.a.*	*n.a.*

[a] DNA concentrations were measured with Nanodrop and Qubit and reported in total ng per sample.
[b] A260/A280 is a measure of purity of DNA in relation to protein content. An ideal value is around 1.8 for pure DNA.
[c] A260/A230 is a purity indicator in relation to salts, polysaccharides and phenolic compounds. An ideal value is around 2.0–2.2 for pure DNA.
[d] qPCR and ddPCR was performed on each samples and results are expressed in copies/g.
[e] Expected copies are expressed in CFU/g and derive from the plate counting analysis.
[f] Inhibition of PCR amplification based on measured copies/reaction with respect to the expected value from plate counting.
All values reported represent the mean ± standard deviation from two independent replicates. N.d is indicated when *A. flavus* was not found through plate counting or its DNA was measured through qPCR. N.a indicates values that could not be calculated because the expected concentration (based on culture-derived CFU/g) was equal to zero. In these samples, molecular quantification was detected (qPCR or ddPCR) despite the absence of culturable *A. flavus* colonies, thus preventing calculation of a measured/expected ratio.