



Challenge

Extraction and detection of genetic modifications (GM) in food and feed samples is of increasing importance. As labelling even of trace amounts is legally required in various countries worldwide, sensitive and reliable detection and quantification methods are needed.

Solution

The combination of Analytik Jena's extraction workflow and qTOWER³ with SureFood[®] Assays from R-Biopharm is the perfect fit for precise detection of various GMOs.

Real-time PCR-based Screening and Quantification of Genetically Modified Organisms

Introduction

The global number of cultivated genetically modified plants has been ever increasing over the last decades. Genetically modified soy was first cultivated in 1997 and now accounts for 80% of the total soybean cultivation in the US. Globally, canola, maize, cotton, and soybean are the major GM crops. According to Regulation (EC) No 1830/2003, food products involving GMOs in their production process need to be labeled in the EU^[1]. The threshold for labeling food and feed with authorized GMO content in EU countries was set to 0.9% in April 2004. With regards to the US market, the FDA requires since January 2022 that food products (or ingredients in the food product) are labelled as bioengineered food if they contain certain types of GMOs^[2]. To detect genetic modifications, several issues need to be considered: from sampling and DNA extraction to the choice of reference materials and the possibility to detect several modifications at once. The extraction of DNA especially from plant materials can be difficult, due to secondary plant

metabolites as well as energy storing molecules such as starch or oil. Also, the yield of the extracted DNA per sample weight can vary widely due to genome size, ploidy, and general DNA content of the examined tissue. Detection is most commonly done using PCR methods^[3]. Quantitative real-time PCR employing several fluorochromes (multiplex) can be used to detect and quantify several genetic modifications at once. Plant-specific genes are used as a control for DNA extraction efficiency and as a reference in qPCR detection assays.

Detection of possible gene modifications is generally done via screening for DNA sequences that do not naturally occur in plants. These include viral and/or bacterial promoters and transcription terminators, as well as genes for bacterial enzymes. Most genetically modified plants (more than 95%) contain the 35S promoter of the cauliflower mosaic virus (CaMV) and the 3' untranslated region (terminator) of the nopaline synthase (NOS) gene of *Agrobacterium tumefaciens*.

R-Biopharm offers several kits for the detection of organisms modified genetically in such a way. Here, we employed the SureFood® GMO Quant Roundup Ready Soya real-time PCR assay as well as the SureFood® GMO Screen4plex 35S/NOS/FMV+IAC real-time PCR kit.

Analytik Jena provides solutions for the whole instrumentation workflow involved in the detection of GM materials. Here, we show an applicative example starting with the extraction of DNA from corn flour to the detection

or quantitation of genetic modifications using the two R-Biopharm assays mentioned above. It is shown that InnuPure C16 *touch* by Analytik Jena is a reliable and easy-to-use platform for automatically extracting DNA from plant materials for up to 16 samples in parallel. The qTOWER³, Analytik Jena's real-time thermocycler, proves to be a reliable platform to detect GM materials using these assays. With its flexible set of up to six color modules, various other assays can also be performed using the qTOWER³.

Materials and Methods

Instrumentation

- InnuPure C16 *touch* (845-00020-2)
- ScanDrop² (e.g. 844-00204-2)
- qTOWER³ including color modules for the detection of FAM (color module 1) (e.g. 844-00553-2) as well as modules for the detection of VIC (color module 2; 844-00521-0), ROX (color module 4; 844-00523-0), and Cy5 (color module 5; 844-00524-0)

Samples and Reagents

- Corn flour samples provided by R-Biopharm (1 – containing Roundup Ready (RR) Soya, 2 – untested for soya)
- innuPREP Plant DNA I Kit – IPC16 (845-IPP-1516096, IST Innuscreen GmbH)
- SureFood® GMO Quant Roundup Ready Soya (S2014, R-Biopharm)
- SureFood® GMO Screen4plex 35S/NOS/FMV+IAC (S2126, R-Biopharm)
- Optional: RIDA®GENE Color Compensation Kit IV (PG0004, R-Biopharm)

Methods

- **Sample Preparation**
100 mg of corn samples 1 and 2 were incubated with lysis buffers OPT and CBV, respectively. Both lysis buffers are included in the innuPREP Plant DNA I Kit - IPC16. Samples were further processed as described in the kit manual. In brief, upon lysis Precipitation buffer P was added and the samples were centrifuged on a Prefilter to clear them mainly of secondary plant metabolites. The filtrate was simply added to the third cavity in the Reagent Plate used for the extraction with the InnuPure C16 *touch*. The subsequent binding, washing and drying steps are carried out in a fully automated manner. In the final step, the DNA was eluted in 100 µL Elution Buffer and transferred to the elution vessels by the InnuPure C16 *touch*.

- **Determination of DNA concentration**
The DNA concentration of the extracted samples was determined via measurement of the absorption at 260 nm using the ScanDrop². In order to determine the purity of the DNA, absorption was also measured at 230 nm (salts, phenolic substances) and 280 nm (proteins).
- **SureFood® GMO Quant Roundup Ready Soya**
The assay was performed as per the instructions given in the assay's user manual. The reference system (Soya, unmodified sample) and the system specific for Roundup Ready Soya (RR Soya, GMO sample) both require the set-up of at least 4 reactions for a standard curve and 3 reactions for controls (1 no-template control, NTC, and 2 positive controls) as well as duplicate reactions per extracted sample DNA. The amplification reactions were carried out with the instrument settings stated in Figure 1 and Figure 2A.
- **SureFood® GMO Screen4plex 35S/NOS/FMV+IAC**
The assay was performed as per the instructions given in the assay's user manual. The amplification reactions were carried out with the instrument settings stated in Figure 1 and Figure 2A.
- **Color Compensation**
To reduce spectral overlap between signals of the various reporter dyes, the "Standard2" color compensation of the qPCRsoft was applied. This color compensation has been developed specifically for multiplex applications. Alternatively, the RIDA®GENE Color Compensation Kit IV (PG0004, R-Biopharm) can be used.
- **qPCR Instrument Settings**
The temperature-time protocols were programmed on the qTOWER³ according to the instructions of the manuals. Both assays run with the temperature-time protocol shown in Figure 1.

3 steps		scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	ΔT(°C/s)
45x[1		95,0	05:00	--	---	---	---	8,0
	2		95,0	00:15	--	---	---	---	8,0
	3	◆	60,0	00:30	2	44	---	---	6,0
	4								
	5								
	6								
	7								
	8								
	9								
	10								

Figure 1: Temperature-time protocol for Sure Food GMO assays.

Temperature-time protocol programmed on qTOWER³ for both, the SureFood[®] GMO Quant Roundup Ready Soya and the SureFood[®] GMO Screen4plex 35S/NOS/FMV+IAC qPCR detection assay.

A

Pos.	Channel	Dye	Gain	Measurement	Pass. Ref.
1	Blue	FAM	5	◆	
2	Green	JOE	5		
3	Yellow	TAMRA	5		
4	Orange	ROX	5		
5	Red	Cy5	5		
6	NIR1	Cy5.5	5		

Meas. repeats: 3 Color compensation: Off

B

Pos.	Channel	Dye	Gain	Measurement	Pass. Ref.
1	Blue	FAM	5	◆	
2	Green	VIC	5	◆	
3	Yellow	TAMRA	5		
4	Orange	ROX	5	◆	
5	Red	Cy5	5	◆	
6	NIR1	Cy5.5	5		

Meas. repeats: 3 Color compensation: Off

Figure 2: Detection channel set-up

A Channel set-up for the SureFood[®] GMO Quant Roundup Ready Soya assay. Signals are detected in the FAM channel,

B Channel set-up for the SureFood[®] GMO Screen4plex 35S/NOS/FMV+IAC assay. Targets are detected in FAM (35S promotor), VIC/HEX (amplification control), ROX (FMV promotor), and Cy5 (NOS terminator).

Results and Discussion

As plant samples are very diverse the innuPREP Plant DNA I Kit – IPC16 contains three lysis buffers with the corresponding extraction protocols. Preliminary tests showed that the OPT and CBV buffers were most efficient for the lysis of the corn meal samples. To determine if the extracts of samples lysed with either buffer yield similar results when analyzed via real-time PCR, both buffers were used to generate samples for further analysis.

DNA was extracted from the sample corn 1 with better yields than from corn 2 (see Table 1). The purity of the extracted nucleic acids was within the same range for both samples as well as both lysis buffers. The ratio of A260/280, is a measure of protein contamination of the DNA sample. Ideally, this ratio should be between 1.8 and 2.0. The ratio of A260/230 reflects the presence of salts, polysaccharides or phenolic substances within the sample. In samples with good purity this value is greater than 2.0. As can be seen from the absorbance ratios, the nucleic acids were extracted from both samples with good purity using the InnuPure C16 *touch*.

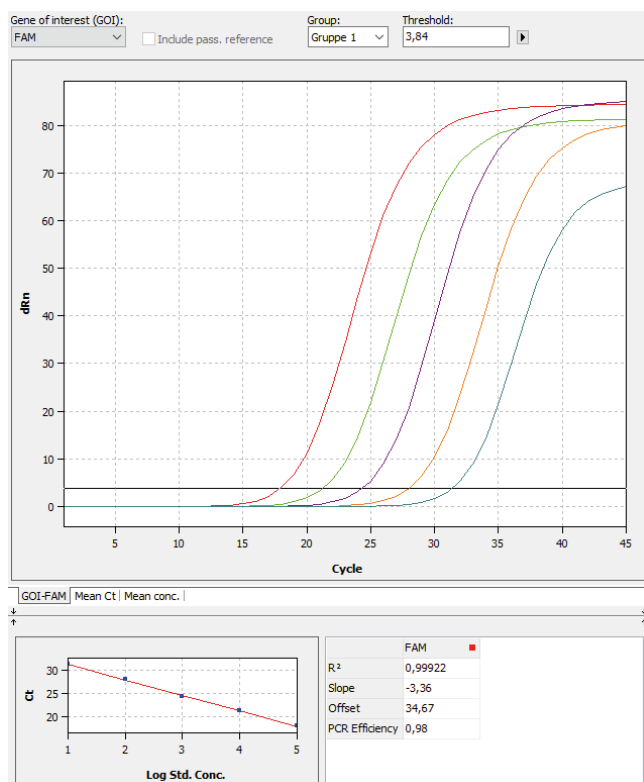
Table 1: Determination of DNA yield and purity

The eluates of the extraction of 100 mg samples with the innuPREP Plant DNA I Kit - IPC16 kit on InnuPure C16 *touch* were analyzed using ScanDrop². The absorption of the individual samples was measured at 230 nm, 260 nm and 280 nm. The DNA concentration is calculated by the FlashSoft Pro software using the absorption of the sample at 260 nm.

sample	lysis buffer	dsDNA [ng/μL]	A260/280	A260/230
Corn 1_100	OPT	168	1.99	2.27
	CBV	143	1.96	2.20
Corn 2_100	OPT	39.5	1.93	2.08
	CBV	37.5	1.88	2.14

SureFood® GMO Quant Roundup Ready Soya

Soya



RR-Soya

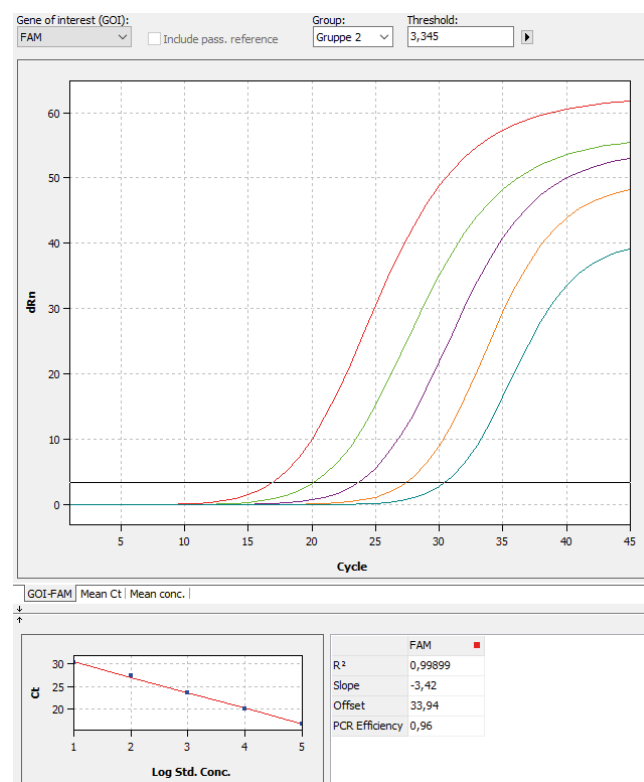


Figure 3: Standard curves and linear regression of serially diluted DNA standards

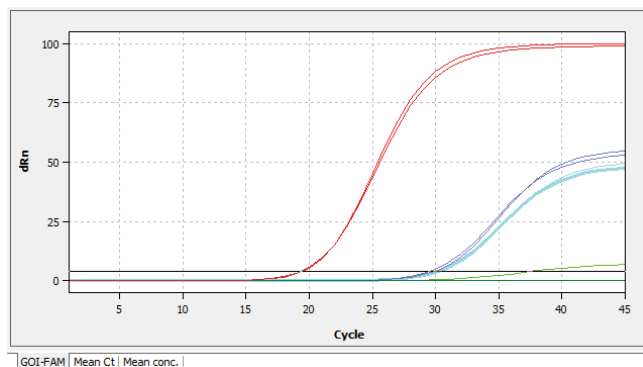
Serial dilution of DNA Standards for a natural sample (Soya) and modified sample (RR-Soya): red – 100,000 copies/μL; green – 10,000 copies/μL; purple – 1,000 copies/μL; orange – 100 copies/μL; grey – 10 copies/μL included with the SureFood® GMO Quant Roundup Ready Soya assay were amplified on the qTOWER³ and detected in the FAM channel. The calculated Ct values were used to generate a standard curve.

Table 2: Ct values of the SureFood® GMO Quant Roundup Ready Soya assay standard DNA

standard	Copies / μL – Soya/RR-Soya	Ct – Soya	Ct – RR-Soya
standard 1	100,000	17.94	16.87
standard 2	10,000	21.25	20.13
standard 3	1,000	24.31	23.57
standard 4	100	28.02	27.37
standard 5	10	31.37	30.38

SureFood® GMO Quant Roundup Ready Soya

Soya



RR-Soya

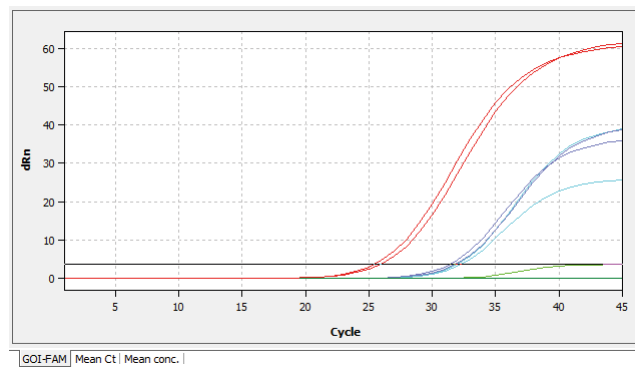


Figure 4: Amplification curves of corn samples with the SureFood® GMO Quant Roundup Ready Soya assay

The curves generated from the corn 1 sample extracts are shown in shades of blue, whereas the corn 2 samples are shown in shades of green. The lighter curves represent samples lysed with OPT, whereas the darker curves represent samples lysed with CBV. The resulting Ct-values are listed in Table 3. The positive control is shown in red, the NTC in brown.

Using the standard curve derived from the Ct values of the provided DNA standard (Figure 3, Table 2) the content of Soya and RR-Soya was determined in the corn meal samples (Table 3). The relative content of genetically modified material within the samples was calculated from the copy numbers of Soya and RR-Soya (%GM, Table 3). Using the calculated and the true GM percentage of the positive control, a correction factor can be determined:

Positive control calculated %GM = 0,85

Positive control true %GM = 1.0

Correction factor, $K = 1\% / 0.85\% = 1.17$

Applying this factor to the calculated GM percentage of the samples, results in corrected GM percentage (True %GM, Table 3).

In corn 2 samples, no significant amounts of soya were detected with this assay. Corn meal 1 contains modified (RR-Soya) and unmodified soya (Soya). About 22% of the soya found in corn 1 samples were genetically modified, namely, containing Roundup Ready resistance conferring sequences. Both of the tested lysis buffers (OPT, CBV) yielded similar results. Ct values obtained from corn 1 samples lysed with OPT were slightly higher than those of the CBV-lysed specimen.

Table 3: Determination of GM content within samples

Using the measured Ct values, the copy numbers of total soya (Soya) and genetically modified soya (RR-Soya) within the samples, was determined. From the copy numbers the percentage of GM content was calculated. The correction factor applied to get the true percentage is 1.17.

Sample name	Ct - Soya	Copies/ μ L - Soya	Ct - RR-Soya	Copies/ μ L - RR-Soya	%GM	True %GM (%GM x 1.17)																																						
Corn 1_100_OPT	30.17	20.7	32.06	4.0	19.3	22.7																																						
	30.31		32.31				Corn 1_100_CBV	29.45	31.2	31.46	5.7	18.3	21.5	29.84	31.91	Corn 2_100_OPT	No Ct	0.1	No Ct	0	0	0	37.47	No Ct	Corn 2_100_CBV	No Ct	0	No Ct	0	0	0	No Ct	No Ct	NTC	No Ct	0	No Ct	0	0	0	Positive control	19.27	36376.8	25.99
Corn 1_100_CBV	29.45	31.2	31.46	5.7	18.3	21.5																																						
	29.84		31.91				Corn 2_100_OPT	No Ct	0.1	No Ct	0	0	0	37.47	No Ct	Corn 2_100_CBV	No Ct	0	No Ct	0	0	0	No Ct	No Ct	NTC	No Ct	0	No Ct	0	0	0	Positive control	19.27	36376.8	25.99	309.6	0.85	1.0	19.38	25.48				
Corn 2_100_OPT	No Ct	0.1	No Ct	0	0	0																																						
	37.47		No Ct				Corn 2_100_CBV	No Ct	0	No Ct	0	0	0	No Ct	No Ct	NTC	No Ct	0	No Ct	0	0	0	Positive control	19.27	36376.8	25.99	309.6	0.85	1.0	19.38	25.48													
Corn 2_100_CBV	No Ct	0	No Ct	0	0	0																																						
	No Ct		No Ct				NTC	No Ct	0	No Ct	0	0	0	Positive control	19.27	36376.8	25.99	309.6	0.85	1.0	19.38	25.48																						
NTC	No Ct	0	No Ct	0	0	0																																						
Positive control	19.27	36376.8	25.99	309.6	0.85	1.0																																						
	19.38		25.48																																									

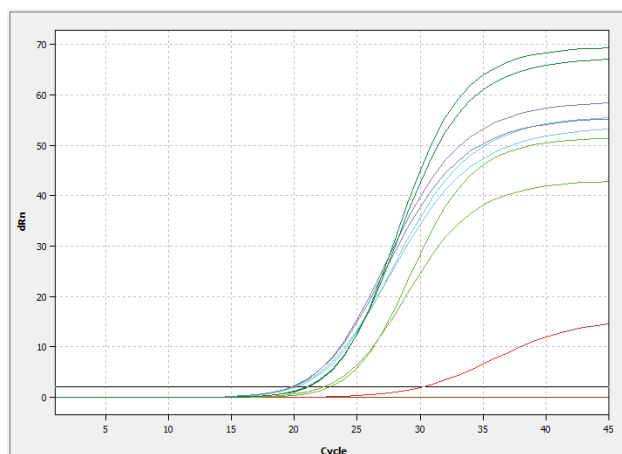
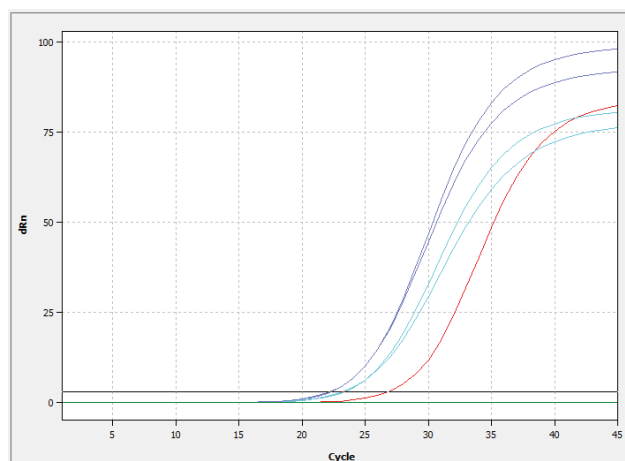
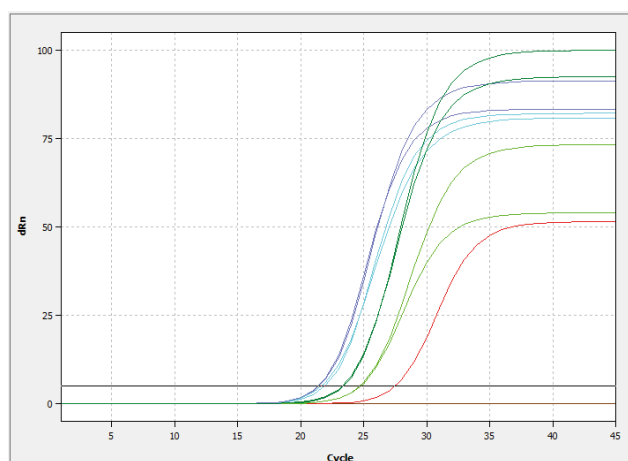
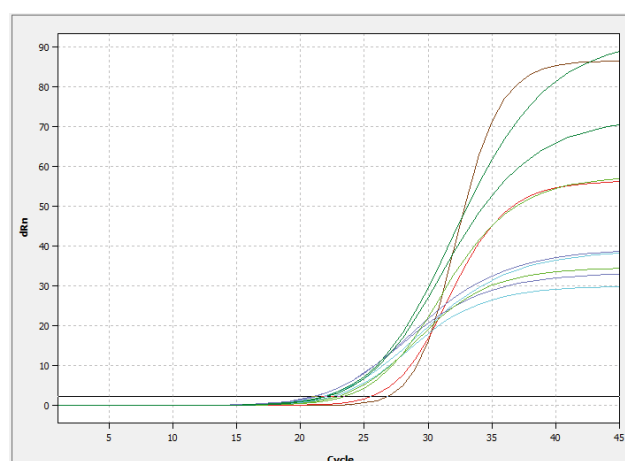
SureFood® GMO Screen4plex 35S/NOS/FMV+IAC**FAM – 35S promoter****ROX – FMV promoter****Cy5 – NOS terminator****VIC – Internal amplification control**

Figure 5: Amplification curves of corn samples with the SureFood® GMO Screen4plex 35S/NOS/FMV+IAC assay

The curves generated from the corn 1 sample extracts are shown in shades of blue, whereas the corn 2 curves are shown in shades of green. The lighter curves represent samples lysed with OPT, whereas the darker curves represent samples lysed with CBV. The resulting Ct values are listed in Table 4. The positive control is shown in red, the NTC in brown.

Corn meal 1 was positive for all three of the tested markers (35S promoter – FAM, NOS terminator – Cy5, FMV promoter - ROX) for genetically modified organisms (Table 4). In corn meal 2 only the 35S promoter and the NOS terminator were detected. The FMV promoter was not present in this sample. The markers were detected irrespective of the lysis buffer used. On average samples lysed with OPT show slightly higher Ct values than those lysed with CBV, indicating that the extraction of the target nucleic acids is marginally more efficient using the CBV lysis buffer. The internal amplification control (VIC channel) was detected in all samples, confirming the absence of inhibitory substances within the PCR and the validity of the qPCR runs.

Table 4: qPCR results of the SureFood® GMO Screen4plex 35S/NOS/FMV+IAC assay

Targets were detected as duplicates in the FAM, ROX and Cy5 channel, while the internal amplification control was detected in the VIC channel using the qTOWER³. Samples (100 mg) were extracted with either CBV or OPT buffer using the InnuPure C16 *touch*.

Sample name	FAM – 35S promoter		ROX – FMV promoter		Cy5 – NOS terminator		VIC – Internal Control	
	Ct	Mean Ct	Ct	Mean Ct	Ct	Mean Ct	Ct	Mean Ct
Corn 1_100_OPT	18.40	18.55	22.62	22.74	21.63	21.80	23.27	22.72
Corn 1_100_OPT	18.69		22.87		21.97		22.17	
Corn 1_100_CBV	18.31	18.30	21.79	21.81	21.38	21.41	21.39	21.36
Corn 1_100_CBV	18.29		21.84		21.44		21.32	
Corn 2_100_OPT	20.87	21.17	No Ct		24.79	24.74	22.95	23.22
Corn 2_100_OPT	21.48		No Ct		24.70		23.48	
Corn 2_100_CBV	19.45	19.54	No Ct		23.28	23.33	22.42	22.40
Corn 2_100_CBV	19.62		No Ct		23.38		22.38	
NTC	No Ct		No Ct		No Ct		26.47	26.47
Positive control	26.52	26.52	26.67	26.67	27.50	27.50	25.22	25.22

Conclusion

The results presented here show that the InnuPure C16 *touch* can extract nucleic acids efficiently even from plant materials. Secondary plant metabolites were eliminated as shown by the purity measurements using the ScanDrop². This provides the foundation for the specific detection and quantitation of target sequences using real-time PCR. The qTOWER³ in combination with the qPCRsoft can be used to gather and analyze amplification data of the SureFood® GMO assays. This allows fast and reliable determination of GM contents of plant materials, as well as screening for the most common genetic modifications used to generate genetically modified crops.



Figure 6: InnuPure C16 *touch* with qTOWER³

References

- [1] Regulation (EC) No 1831/2003 on the traceability and labelling of genetically modified organisms (GMOs) and the traceability of food and feed products produced from GMOs
- [2] <https://www.fda.gov/food/agricultural-biotechnology/how-gmos-are-regulated-food-and-plant-safety-united-states> (latest check November, 5th, 2021)
- [3] Marmiroli, N., Maestri, E., Gulli, M., Malcevski, A., Peano, C., Bordoni, R., De Bellis, G., Methods for detection of GMOs in food and feed, *Anal Bioanal Chem* (2008) 392:369–384

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