



Precise and Robust Determination of Porcine DNA in Food Samples

Introduction

Analytical molecular methods are currently recognized tools for the determination of DNA in foods. To assure a high level of food and nutrition quality as well as authentication of unintended ingredients such as pork, accurate and highly sensitive animal species identification and the detection of substitutes are two of the main challenges in food industry. However, in order to gain acceptance and confidence in reliability of results obtained by such methods from different laboratories, analytical methods have to satisfy certain quality criteria. The TaqMan®-based innuDETECT Pork Assay enables a highly sensitive analysis of pork species. Further validation studies were performed in order to establish extended performance assessment to characterize the innuDETECT Pork DNA Assay.

Challenge

Reliable detection of porcine DNA in fresh and processed food.

Solution

Application of innuDETECT Pork Assay for qualitative and quantitative detection of pork DNA.

Materials and Methods

Samples and Reagents

For reference 0.1% (w/w) pork in beef meat standard (Eurofins), genomic DNA of *Sus scrofa domestica* with a concentration of 100 copies/ μ l (Eurofins) and an in-house designed plasmid with synthetic pork DNA insert (GeneArt) to be used as positive control (available as innuDETECT Animal Quantification Tool (845-IDF-0140100)) were applied using the innuDETECT Pork Assay (845-IDF-0010096).

Instrumentation

The analysis was carried out on the real-time PCR platform qTOWER³ from Analytik Jena, the CFX96 from Bio-Rad and on the Step-One Plus from ABI to allow performance assessment for the assay.

Procedure

In order to validate the innuDETECT Pork Assay and to demonstrate that the developed method corresponds with the required performance criteria of detection tests the 0.1% (w/w) pork in beef meat standard was extracted using the innuPREP DNA Mini Kit (845-KS-1040250). The protocol is based on spin filter nucleic acid extraction in conjunction with a patented chemistry based on a certain combination of chaotropic and non-chaotropic salts (Dual-Chemistry Technology). Further analysis of extracted nucleic acids was performed as described in instructions for use of the innuDETECT Pork Assay. Target amplification as well as detection was performed by real-time PCR analysis using the FAM channel for target and HEX channel for internal control. The PCR program is summarized in Table 1.

Table 1: PCR program for qTOWER³

Step	Cycle	Profile	Temperature	Holding time
1	1	Initial denaturation	95 °C	120 sec
2	35	Denaturation	95 °C	10 sec
		Annealing/Elongation *	45 °C	45 sec

* Data acquisition: Fluorescence detection (FAM; HEX)

The **analytical sensitivity** was ascertained by analyzing clarified samples with minimal amounts of DNA. Extracted nucleic acids from 0.05% pork in beef meat, one genome equivalent of genomic DNA of *Sus scrofa domestica* and 10 copies of synthetic plasmid DNA served as templates. At least 30 replicates of each sample type were analyzed and a minimum of 95% should be detected positive.

The **linear range** for the innuDETECT Pork Assay was determined by analyzing dilution series of porcine DNA ranging from 3×10^0 – 3×10^5 genome equivalents per reaction as well as plasmid DNA ranging from 3×10^0 – 3×10^7 copies per reaction.

The **robustness** was tested for the PCR setup. At least 10 samples from 0.1% pork in beef meat were amplified using different PCR instruments, with varying annealing temperatures and with deviations in MasterMix compilation.

Results and Discussion

In order to apply a detection kit for routine analysis different performance parameters have to be ascertained. Comparative analysis was performed for Limit of Detection, amplification efficiency as well as robustness. The application of different food samples was also conducted.

Detection of minimal DNA amounts per sample

In order to determine the analytical sensitivity of the innuDETECT Pork Assay different starting materials with minimal concentrations of porcine DNA per sample (n=30) were analyzed. First results demonstrate that 0.05% (w/w) pork in beef meat can be detected. Positive results for all replicates (30/30) were determined. In addition to that, less than one genome equivalent per reaction could be verified using the innuDETECT Pork Assay. Pork DNA fragments are sufficient for positive test results, as it was shown with amplification of synthetic plasmids. Using 10 copies of porcine DNA per reaction, 96% could be detected positively. All results are summarized in the table below.

Table 2: Results of analysis of samples with defined concentration of porcine DNA

Device	Pork DNA detection	Minimal detected amount of DNA
Pork in beef meat		
qTOWER ³	30/30	≤ 0.05% (w/w) pork in beef meat
CFX96	30/30	
ABI Step-One Plus	30/30	
Genomic DNA of <i>Sus scrofa domestica</i>		
qTOWER ³	30/30	≤ 1 genome equivalent*
CFX96	30/30	
ABI Step-One Plus	30/30	
Synthetic plasmid		
qTOWER ³	28/30	10 copies
CFX96	30/30	
ABI Step-One Plus	29/30	

* Amplification of mitochondrial gene, i.e. several copies per genome

The detection range of the innuDETECT Pork Assay

The linear range of the detection assay was determined by analyzing porcine DNA ranging from 3×10^0 – 3×10^5 genomic equivalents per reaction and synthetic plasmid ranging from 3×10^0 – 3×10^7 copies per reaction using the qTOWER³, the CFX96 and the ABI Step-One Plus. Obtained results, shown in Figure 1, cover a linear range over 7 \log_{10} steps. Based on the Ct value mean, the slope of the regression line was used for determination of the PCR method efficiency. For qTOWER³ using the positive control plasmid as template an efficiency of 94% could be detected. For qualitative singleplex qPCR methods no criteria of acceptance are defined. By the measure of a quantitative PCR method, the PCR efficiency should range from 90 – 110%. Meanwhile, the innuDETECT Pork Assay reaches an optimal result (Broeders et al., 2014). The calculated linearity $R^2 = 0.9955$, an additional criterion for method validation ($R^2 \geq 0.98$), emphasizes the assay performance.

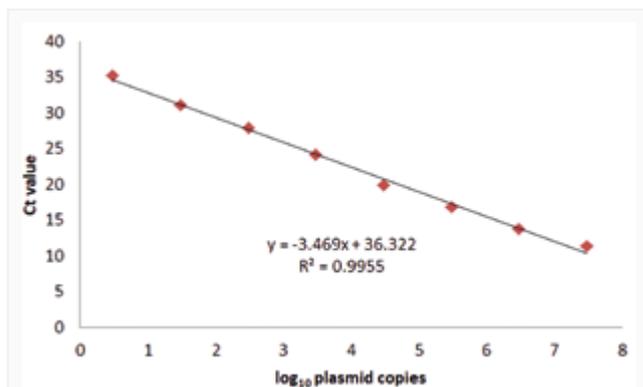


Figure 1: Linearity of the innuDETECT Pork Assay on qTOWER³ using plasmid DNA. The linear regression coefficient (R^2) is 0.9955.

Requirement of a robust qPCR setup

To evaluate the robustness of the innuDETECT Pork Assay different experimental conditions were changed. Therefore, 0.1% pork in beef meat was analyzed (n=8) using different PCR instruments (qTOWER³, CFX96, ABI Step-One Plus), with different annealing temperatures (+ 2 °C/- 4 °C) as well as with variations in MasterMix compilation (+/- 30%).

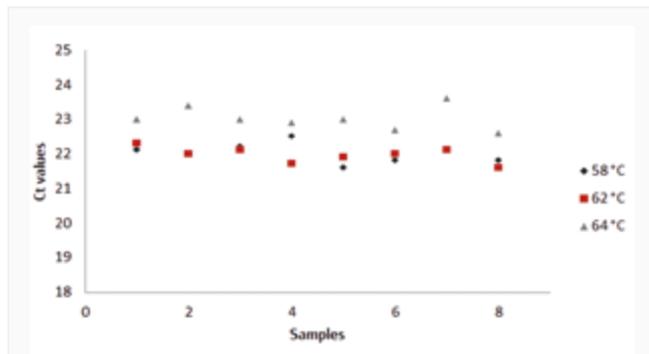


Figure 2: Influence of varying annealing temperature (black: 58 °C, red: 62 °C, grey: 64 °C) on the assay performance, analysis of 0.1 % pork in beef meat (n=8) on qTOWER³ using the innuDETECT Pork Assay

The changed experimental conditions had no influence in positive assay results. Figure 2 demonstrates the effects of different annealing temperatures on DNA detection. Deviation of the annealing temperature and MasterMix compilation for all qPCR instruments has an impact on real-time PCR results less than 5%. Regarding these experimental conditions the performance of the innuDETECT Pork Assay is convincing.

Analytical specificity and application of innuDETECT Pork Assay

Fresh and processed food was analyzed using the innuDETECT Pork Assay. For declared mixed, minced meat pork DNA could be detected. The comparative analysis of halal and non-halal marshmallows was also performed. It was demonstrate that the halal certified marshmallows are free of porcine DNA. Besides proof of principle the specificity of the innuDETECT Pork Assay was investigated. Therefore, kosher beef with and without contamination of pork DNA was utilized as sample material to verify the workflow. Even the use of contaminated knives for sample preparation is sufficient to get false positive results in the case of the innuDETECT Pork Assay.

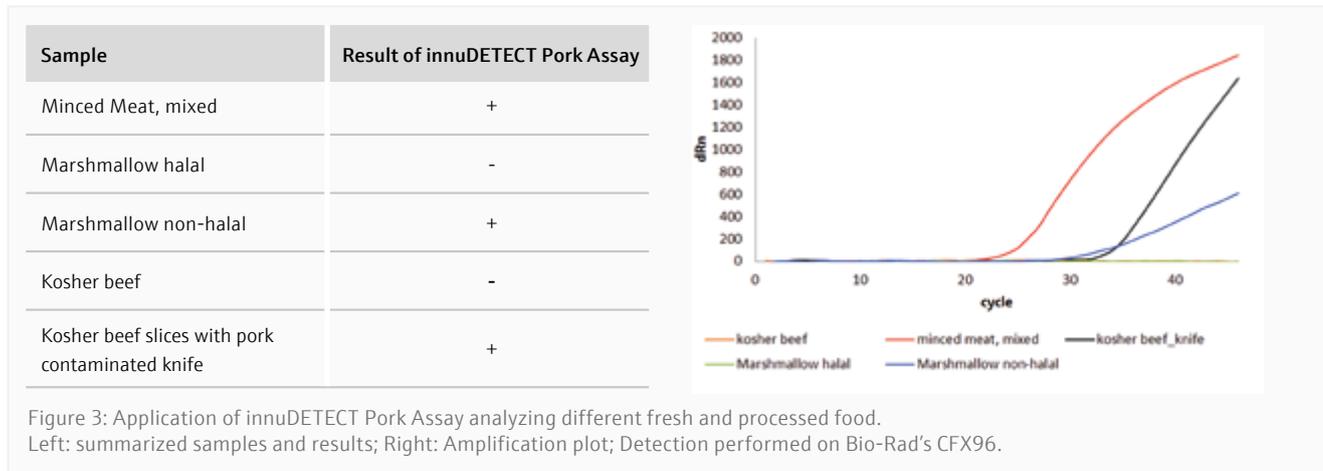


Figure 3: Application of innuDETECT Pork Assay analyzing different fresh and processed food. Left: summarized samples and results; Right: Amplification plot; Detection performed on Bio-Rad's CFX96.

Conclusion

Real-time PCR has been established as reliable, precise and fast application for animal species identification. Analytik Jena offers a solution for the detection of porcine DNA applying the innuDETECT Pork Assay on qTOWER³ as well as on other qPCR instruments. In order to validate the detection assay different performance criteria were analyzed. The robust innuDETECT Pork Assay convinced with a limit of detection $\leq 0.05\%$ (w/w) pork in beef meat. In addition to that, the assay was used for successful detection of porcine DNA in fresh and processed food.

References

Broeders, S.; Huber, I.; Grohmann, L.; Berben, G.; Taverniers, I.; Mazzara, M.; Roosens, N. and Morisset, D.; GUIDELINES FOR VALIDATION OF QUALITATIVE REAL-TIME PCR METHODS. Trends Food Sci Technol. 2014, 37, pages 115-126

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