Quantitative PCR Analysis of Meat Speciation Data Using the $2^{-\Delta\Delta Cq}$ Method

Abstract

In recent years the food production industry has been placed under intense scrutiny. This has led to an increased pressure for advancements in the field of speciation testing. Quantitative PCR is a highly sensitive and accurate method of assaying samples for nucleic acid content. In combination with well-established DNA extraction procedures, PCR is now being used by many labs to quantify relative levels of animal DNA in total meat. Using the 2^-ΔΔCq method, calculations can be accurately applied to multiplex formats of speciation tests (including two sets of primer and probe, one for the species of interest, the other as a universal detection system), using total DNA as a positive control calibrator sample. Primerdesign now offers a complete speciation solution, using the most up-to-date methods, as part of the genesig® range of products for accurate and accessible speciation testing.

Introduction

Testing for meat speciation poses many technical challenges regardless of the approach that is used. Sensitivity of adulterating meat detection needs to be high to detect trace contamination in large manufacturing scenarios. Typically a high quality test will need to determine adulteration down to 1% of the total meat or better and this is working close to the limits of detection. The sample itself may be a single pure ingredient such as a joint of meat but more often it is a highly processed mixture of complex biological material and typically may be cooked. Cooking poses a particular challenge for all methods since it degrades both proteins and nucleic acids considerably reducing the quality of the sample. Then there are theoretical considerations such as the interpretation of data. What does a DNA test really measure? – is it really meat or a surrogate marker for meat requiring careful consideration. For example, the DNA in a yoghurt sample cannot be said to be a representation of 'meat'.

Considering the field as a whole, DNA testing is emerging as the method of choice offering rapid and sensitive testing. The mitochondrial DNA is an ideal candidate in many respects because it is a "multi-copy" being present at about 75 copies per cell. Targeting a multi-copy target enables a level of test sensitivity that would be completely impossible if a single copy genomic DNA target was selected. Given that sensitivity is required down to 1% adulteration or less, high sensitivity is an imperative for any useful test of adulteration

The usual configuration of a DNA test is to measure two targets within the test sample. One of these uses a universal primer/probe set that will detect all animal DNA present in the sample. Such a measure is taken to represent the total amount of meat in the sample. A second primer/probe targets the

DNA of a potentially adulterating meat. By comparing the relative signal of each it is possible to calculate the percentage of DNA that is from the adulterating source whilst a negative result confirms the provenance of the test sample. Furthermore, since the universal primer/probe set will give an aggregate signal based on the total meat contents of the sample, this format of testing can be applied even to samples that contain multiple different species in, for example, the same pie.

The data obtained with this approach can be used to test against a set tolerance which is usually set at 1% of the total meat. Some tolerance of low level contamination is important since factories may handle different meat products and trace unintentional contamination may be inevitable and is harmless.

genesig® q16 A complete solution

Primerdesign's new qPCR offering in the food testing space is designed to meet the above challenges providing a turnkey solution that is easy to use, with unsurpassed accuracy and with a convenient time to test. Some of the improvements Primerdesign Ltd has made are intrinsic to the work flow; such as a deskilled extraction solution that can handle almost any starting material – gone are the days when you need a mountain of different extraction kits; one for each sample type under investigation. The genesig lab-in-a-box solution includes fixed volume pipettes and colour coded solutions so it is nearly impossible to make an error during extraction.

However the real revolution lies within the q16 real time PCR instrument, which has a software package specially tailored to the needs of the food testing industry. There is no upstream programing required to run the instrument and no downstream data processing to obtain the results. Put the samples in and hit run and the machine will tell you not only if the sample is positive but also the percentage of adulterating DNA and also the limits of sensitivity of the test. Importantly the percentage calculation for a single meat adulteration remains accurate even when multiple meats are present in the same sample.

The software also includes a wide range of controls that pre-determine that the test has been soundly executed before reporting a positive test result.

genesig® q16 features

- Simple universal extraction
- No thermocycler programming
- No data analysis
- Automatic Sensitivity reporting
- Automatic Adulteration percentage reporting
- Automatic sample quality control reporting
- Improved mathematical modelling

q16 quality control

The q16 assesses multiple aspects of the test to ensure that the extraction and the reaction set up have been performed correctly and the results are sound for further analysis and presentation to the user.

The positive control is pure DNA of the species under investigation. It has to give a signal between strict tolerance limits to prove that all aspects of the test have been carried out correctly.

The global detecting primer and probe set are used to determine the abundance of meat DNA in the sample for quantification purposes but also to determine the theoretically sensitivity of the test on that particular sample.

Only when all the controls have passed the quality assurance algorithm will the software then calculate and report a positive result and sensitivity of the test.

Even an inexperienced user can report the result for quality assurance purposes with confidence. If the test fails for any reason then the software will also provide guidance linked to the handbook to facilitate rapid troubleshooting.

A more accurate mathematical model

Whenever two real time PCR tests are combined into a single calculation, there are important mathematically complexities to be considered. A food test fits this description because both the total amount of meat present, and also the amount of adulterating meat must be detected and compared to arrive at a 'percentage adulteration'. The comparison of these two signals involves assumptions that are implicit in the standard mathematics used to do the calculation. The most critical assumption, and necessary truth of the system, is that both of the detecting assays must function at exactly the same efficiency in detecting their targets. If one is more efficient than the other then this will lead to under or over reporting of adulteration depending on which assay is weakest. PCR is sensitive over many orders of magnitude making it ideal for detecting very low abundance targets and high abundance targets in the same sample. However this strength becomes a weakness when trying to deduce the small differences such as between 5% and 10% adulteration as this difference will be represented in the test by very small changes in the fluorescent signal (small delta Cq values). In practice no two assays can be perfectly balanced as they will always have slightly different efficiencies.

This is the major breakthrough that Primerdesign Ltd has made in analysing food samples. The q16 software can make an assumption free, accurate relative quantification of adulteration REGARDLESS of the relative priming efficiencies of the two assays. This problem is solved mathematically by reference to an internal calibrator which can correct for any variation in priming efficiency before reporting the percentage adulteration. The calibrator (which also functions as the positive control) is a pure 100% biological DNA sample of the species under investigation. The universal assay and test assay are compared on this sample. Intrinsically they should both give an identical signal as the sample is 100% meat and 100% the test sample – theoretically the real time PCR amplification plots should perfectly overlay each other. However they will not perfectly overlay each other and the delta Cq between these two signals can be used as a correction factor and applied to the unknown sample. Potentially under or over reporting is eliminated resulting in a much more accurate test.

The use of a universal primer set as part of the assay brings other benefits especially when examining meat that may have multiple different adulterated components. Because all meat present will contribute to the universal signal the calculated percentage of one particular contaminate is always accurately expressed as a percentage of the whole. Multiple tests can be performed for different potential adulteration events and the q16 will accurately report them all.

Application of the the 2-ΔΔCq Method in Meat Speciation Testing

Assuming approximately equal amplification efficiencies, the 2^-ΔΔCq equation is a convenient method for calculating normalised relative expression levels. The normalisation is performed relative to a reference molecule (either a gene or transcript). In the case of speciation testing this is usually a DNA locus common to all species in question (i.e. fish or meat). In this sense, a universal locus is the ideal reference molecule for quantification purposes.

In order to calculate X_N (the normalised amount of target), X_0 (the initial number of target molecules) is subjected to division by R_0 (the initial number of reference molecules). According to the equation that describes the exponential amplification of PCR (Livak and Schmittgen, 2001), this can be simplified as below:

Normalisation Formula

		Livak and Schmittgen		Speciation Parallel
1	A	$\frac{X_0}{R_0} = K \times (1+E)^{-\Delta C_q} = X_N$	B	$\frac{No.of\ target\ molecules}{No.of\ REF\ molecules} = 2^{-\Delta C_q} = X_N$
		• ΔCq equals the threshold cycle difference (Cq,X – Cq,R) • Equation assumes similar efficiences (*E*) for *X* and *R* • K is a constant related to the different amounts of each molecules at the set threshold		• ΔCq equals the threshold cycle difference (Cq,target – Cq,REF) • Equation assumes $E \geq .9$ for both target of REF assays • Equation assumes K equals 1

Under some circumstances, the speciation normalisation (equation [1A]) may provide an accurate description of the speciation ratio. This ratio can be multiplied by 100 in order to provide speciation percentage. However, the ratio implies certain assumptions regarding the constant K including: identical reporter dyes, sequence context effects on fluorescence, the efficiency of probe cleavage, purity of the probe, and the applied fluorescence threshold (Livak and Schmittgen, 2001). In order to circumvent these assumptions, an arbitrary sample can be used for calibration. The calibration formula subjects $X_{N,q}$ (the normalised amount of target in the sample) to division by $X_{N,cb}$ (the normalised amount of target molecules in the calibrator):

Calibration Formula

Livak and Schmittgen, 2001

2 A $$\frac{X_{N,q}}{X_{N,cb}} = \frac{K \times (1+E)^{-\Delta C_{q,q}}}{K \times (1+E)^{-\Delta C_{q,cb}}} = (1+E)^{-\Delta\Delta Cq}$$

- -ΔΔCq = -(ΔCq,q – Cq,cb)
- Assumptions regarding the constant K are internally controlled

Speciation Parallel

B $$\frac{X_{N,q}}{X_{N,cb}} = \frac{2^{-\Delta Cq,sample}}{2^{-\Delta Cq,calibrator}} = 2^{-\Delta\Delta C_q}$$

- -ΔΔCq = -(ΔCq,sample – ΔCq,calibrator)
- Assumption of K = 1 is internally controlled

The calibration provided by the 2^-ΔΔCq method subjects the constant *K* to cancellation. In practical terms, this demonstrates that the factors affecting *K* have been internally controlled. Also important to note is that the calibration formula represents the number of target molecules as a normalised ratio relative to the calibrator. Under normal circumstances an arbitrary calibrator sample ($X_{N,cb}$) is used. In more intuitive terms, this means that a normalised relative ratio of 1 is arbitrarily applied to the calibrator sample. A limitation stemming from this sort of analysis is that the specific normalised relative quantities (NRQs) are only relevant to the population of data from which the calibrator sample is derived. Though this does not preclude further analysis, proper experimental design will still yield meaningful results, the NRQs have limited relevance to the wider scientific community.

In terms of speciation, these concerns can be obviated wholesale by the use of a non-arbitrary calibrator sample, a known positive control. As discussed previously, when using two sets of primers and probe; one specific for the species of interest, another targeting a common DNA locus amongst meat (or fish), allows for a total DNA extract (from the species of interest) to be used as the calibrator sample. As total DNA is universally available through commercially available extraction procedures, the experimentally derived NRQs will be applicable on an inter-laboratory basis. Furthermore, not only is the calibrator sample not experimentally arbitrary, but importantly, it is also not mathematically arbitrary. As one can assume that extracts of total DNA contain the target locus and the common DNA locus in a 1:1 ratio, applying the normalised relative ratio of 1 to total DNA is mathematically sound. Therefore, the derived NRQ will provide a true calibrated ratio of target-to-reference molecules for a particular sample. This ratio can be multiplied by 100 to achieve a speciation percentage (Equation 3).

Speciation Formula

$$2^{-\Delta\Delta C_q} \times 100 = Speciation\ Percentage\ (\%)$$

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Concluding Remarks

The genesig® q16 and the work flow described above represent a significant step forward in the application of DNA testing to the problem of meat adulteration. An improved workflow, an automated software solution and normalising the test results using species-specific total DNA as a calibrator, all combine to produce a new system that is simple and accurate. The system has the potential to aid inter-laboratory collaboration efforts and thus increase the accessibility of speciation testing. It also has the added benefit of mitigating some of the pitfalls associated with PCR testing when performed by inexperienced users. A solution of this nature to accurately quantify and report meat contamination is unmatched by any other product offering in the market.

References

Kenneth J. Livak, Thomas D. Schmittgen, Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method, Methods, Volume 25, Issue 4, December 2001, Pages 402-408, ISSN 1046-2023, http://dx.doi.org/10.1006/meth.2001.1262.