# Development of meat speciation assays using the Agilent 2100 bioanalyzer

Application

John Dooley
Steve Garrett

## Abstract

The use of real-time PCR assays for quantitative PCR is becoming more frequent. During the development of such assays it is necessary to match both the PCR primers and the fluorescent probe (used for detection) in a single reaction. The probe production costs are high compared to the primer production costs. It is, therefore, useful to know that the newly designed PCR primers are functioning in an expected manner before the cost of probe production is incurred. This Application Note describes the use of the Agilent 2100 bioanalyzer with the DNA 500 LabChip® kit to confirm that primer sets are suitable before the probe is finally produced.

## Introduction

Campden & Chorleywood Food Research Association (CCFRA) is interested in the development of PCR based methods for food authenticity, particularly in relation to the detection and quantification of meat species in meat products. The assays must be applicable to processed foods and, therefore, use small DNA targets as the extracted DNA is often degraded. One approach is to develop real-time methods based on the ABI Prism 7700 Sequence Detection System, known as TaqMan. The method is suitable for amplicon detection in the range 60–150 base pairs. It is common during the TaqMan assay development stage to find several suitable probes, each with several different primer sets. Although, theoretically, these assays should all work to consistent levels, practically there are variations between them and some assays are unlikely to work at all. Therefore, it is advisable to confirm that the primers designed are specific (produce only a single PCR product with no primer-dimerization) and will work under TaqMan conditions (high $MgCl_2$ concentration and strict cycling parameters). These conditions are fundamental to the accurate quantification of samples. Although confirmation of primers can be performed using traditional agarose gel methods, the Agilent 2100 bioanalyzer has several advantages over the agarose methods including speed of analysis, accurate quantification of PCR yield and sizing of products. In addition, safety is improved as there is a reduced risk from handling DNA staining dyes such as ethidium bromide. Using the DNA 500 LabChip kit allows accurate sizing of small PCR products. This is advantageous for real-time PCR where the amplicon size required is small (less than 150 bp). We describe the use of the Agilent 2100 bioanalyzer to assist in the development of assays suitable for sensitive detection of one meat species in another.

## Materials and Methods

### Design of PCR assay

TaqMan PCR assays (primers and probes) were designed using the Primer Express software (Applied Biosystems, Warrington, Cheshire, UK). Primers (forward and reverse) were designed to amplify single genomic DNA targets from pig, cow, sheep, turkey and chicken of less than 150 bp, in accordance with TaqMan design restraints. Primers were produced by MWG-Biotech, UK.

### Performance of PCR reaction

PCR was performed in 25-µl volumes using 300 nM of each primer, 5 mM $MgCl_2$ and 100 ng of template DNA. A TaqMan-based amplification protocol (30 cycles of a two-step reaction consisting of 95 °C for 15 seconds and 60 °C for one minute) was applied to the reactions. PCR was finished with a final 10-minute step at 72 °C.

### DNA 500 LabChip preparation

Chips were primed according to Agilent's instructions, provided with the chips. Samples (1µl) of each PCR reaction were loaded onto the DNA 500 LabChip following Agilent protocols and the chips were loaded into the Agilent 2100 bioanalyzer. The analysis of the DNA products was performed using the DNA 500 protocol of the accompanying software.

## Results and Discussion

To perform absolute quantification it was necessary to design two assay types, a species-specific assay and a total meat assay that would be suitable for all meat species.

### Species-specific assay development

Figure 1 shows examples of results. Figure 1A was obtained following amplification of different mammal or poultry species with a specific turkey assay. A single band was obtained with turkey (lane 5) only, i.e. there was no amplification with chicken, pork,

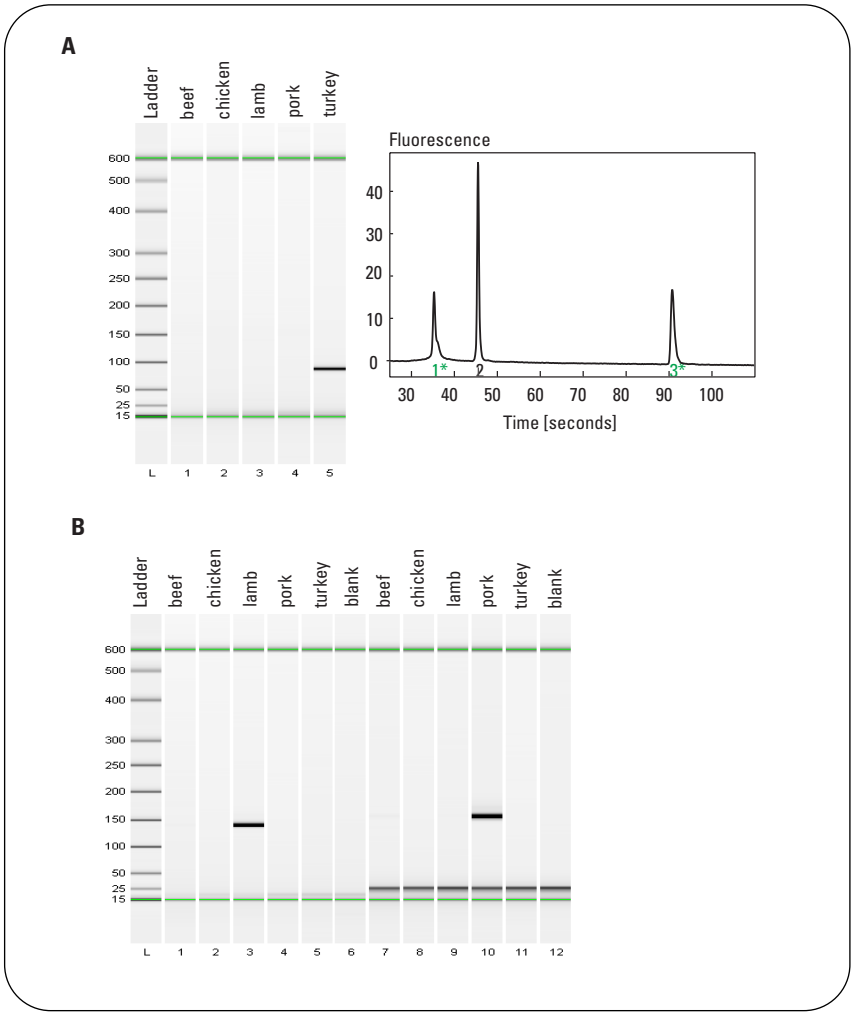


**Figure 1**
**Species specific PCR amplification of meat samples.**
**A) With turkey specific primers only turkey samples are amplified. The electrophoretic trace confirms the high purity of the fragment.**
**B) Different sets of primers can be designed that allow the species specific PCR amplification of lamb (lanes 1-6) or pork (lanes 7-12).**

beef and lamb. This band was not seen in any other poultry or mammal species. Figure 1B shows results of amplification with a set of specific mammal primers. A fragment can only be seen in the correct species, with no amplification detectable in any other species. Similar results were obtained for all five species under investigation. These results suggested that these primer sets would be suitable for individual species detection on the TaqMan. The appropriate probes were produced and the assays optimized for TaqMan usage. Species-specific amplification was observed on the TaqMan system.

### Total meat assay development

Figure 2A shows the results of designing a total meat assay. The aim was to develop an assay that would amplify all meat species with the same degree of efficiency. The assay was also designed to show no amplification with non-meat species, including fish. Figure 2A shows that a single band of equal intensity was observed in all five species, whether of mammal or poultry origin. Figure 2B shows the overlay of the electrophoretic traces for these PCR products. The yields for all species were similar (mean 5.43± 0.64 ng/µl suggesting that this assay would be suitable for developing TaqMan-based, absolute quantification protocols. No amplification was observed in non-meat samples tested, including maize, soya, wheat and fish (figure 2A lanes 6-12). Figure 3 shows results from the complete TaqMan assays where the primers and probes were combined. Poor clarity (smudging) of the bands is possibly due to the use of dUTP in the TaqMan assay as opposed to the

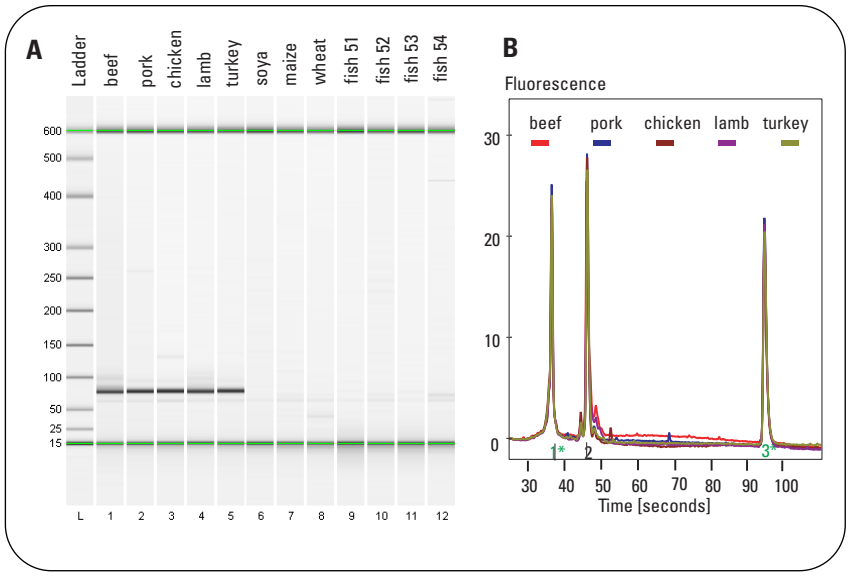


**Figure 2**
**Development of a meat specific assay.**
**A) A set of primers can be designed that amplifies specifically all meat samples but does not amplify grain or fish.**
**B) The overlay of the electrophoretic traces reveals uniform amplification levels for different meat species.**

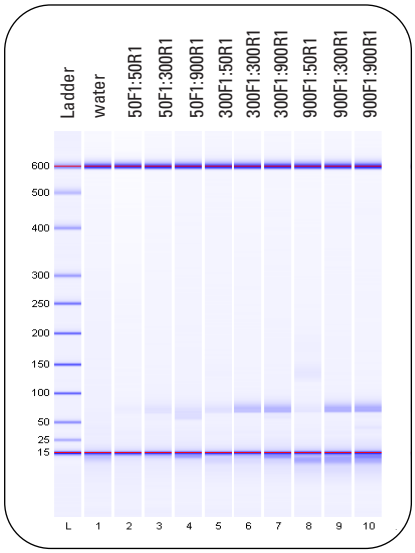


**Figure 3**
**PCR products using TaqMan probes. Results from assy optimization test using pork DNA. Primers (forward [F] or reverse [R]) were used at 50, 300 or 900 nM concentration. Results show that at least 300 nM of F or R primer is required for amplification. 300 nM of each primer was found to be optimal for this amplification.**

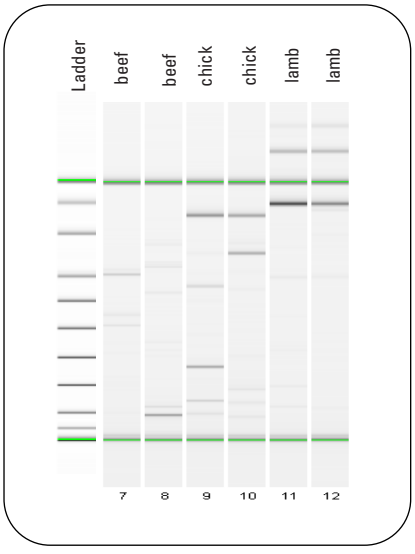


**Figure 4**
**Non-specific amplification using non-optimal sets of primers.**

use of dTTP, which was used in the conventional PCR reactions. An example of a primer set, although designed to be specific, is not specific in practice, is shown in figure 4. This assay was designed to amplify a single target in all meat species. As can be seen the number, size and yield of PCR fragments varies between the species. This primer pair was, therefore, inappropriate for use but having used the Agilent 2100 bioanalyzer to check the primers before purchasing the probe saved a considerable expense.

## Conclusion

We believe the Agilent 2100 bioanalyzer provides a quick, visual method to confirm primer specificity and suitability for use in TaqMan assays. Although it would be possible to perform similar checks using SYBR Green DNA stains in the TaqMan machine itself, it is not possible to determine if the observed fluorescent change is due to primer-dimer formation or from the target of interest. The Agilent 2100 bioanalyzer allows confirmation of this and also confirmation that only a single target of expected size is being amplified. The ability of the Agilent 2100 bioanalyzer to quantify PCR yields is useful especially if assays being designed are required for quantitative or semi-quantitative determination, or as in our case to design a single assay suitable for detecting multiple species.

*The authors are scientists in the Molecular Biology Group, Dept. of Chemistry and Biochemistry, Campden & Chorleywood Food Research Association (CCFRA), Chipping Campden, Gloucestershire, UK.*

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