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Transfer of the real-time PCR method to detect and quantify common wheat adulteration of pasta to the Agilent 2100 Bioanalyzer capillary electrophoresis system

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Background

The authenticity of *T. durum* pastas and the detection and quantification of deliberate adulteration of *T. durum* pastas by common wheat (*T. aestivum*) continues to be an important issue throughout Europe. The geographical origin of the *T. durum* grain used for pasta production can be extremely diverse, and is dependent upon a number of factors that include, amongst others, economic trading agreements and occasional adverse harvest conditions. The EU has strict laws and agreements concerning the adventitious contamination of *T. durum* by *T. aestivum* and these require enforcing to protect both the retailers and the consumer. To achieve this, a number of analytical procedures exist, employing a wide range of techniques and technologies; however, they all have shortcomings resulting in quantificational difficulties. MAFF projects 2A015, AN0667 and FSA project Q01085 have demonstrated that it is possible to address the problems associated with the detection and quantification of common wheat (*T. aestivum*) adulteration of *T. durum* pastas by analysing the DNA extracted from the pasta. The Polymerase Chain Reaction (PCR) is commonly used to amplify specific DNA sequences prior to their analysis, initially by fractionation on the basis of size using agarose gel electrophoresis; however, this method has now been superseded by real-time PCR. Real-time PCR has been used successfully to analyse very small target DNA sequences present in processed foods and hence was chosen to address the detection of common wheat in pasta and semolina. The same technique has already been applied effectively to the qualitative and quantitative detection of genetically modified organisms (GMOs) in both ingredients and final products for which UKAS accreditation is held (ISO17025) and to the detection of meat in vegetarian products.

The results of our previous research in this area has shown that the detection of the D-genome is highly unlikely to be compromised by cross-reaction with other unrelated genomes, following the analysis of a large number of commercial cultivars of both *T. durum* and *T. aestivum* from around the world. These findings give an increased confidence in the analytical results and to the general applicability of the method for the detection of *T. aestivum* contamination from a wide geographical origin in *T. durum* pasta. The analysis of *T. aestivum* adulteration using DNA-based methods addresses some of the potential problem areas associated with convergent wheat breeding programmes that potentially may produce cultivars that do not express marker proteins assayed by other methods.

The major benefit of using the real-time PCR method was to reduce the uncertainty associated with the measurement of adulteration. While this was not formally determined for the Acid-PAGE method it was sufficiently large to raise the level at which adulteration could be reliably detected to 8%, a figure that was widely regarded as unacceptable by analysts, retailers and manufacturers alike. Project Q01085 determined that at 3% *T. aestivum* could be measured with an uncertainty at an approximate 95% confidence limit of ± 1.62%, i.e. 1.38-4.62%.

Commercial capillary electrophoresis 'Lab on a chip' systems have the potential to offer significant benefits to this area of analysis. These advantages are mainly due to the instruments flexibility, and as a result, it has been adopted as an analytical tool by many of the Public Analyst laboratories. It would therefore seem sensible and indeed practical to attempt to transfer certain existing methods of analysis such as the detection and quantification of *T. aestivum* adulteration of *T. durum* pastas, into this format. If a successful analysis could be achieved using the Agilent Bioanalyzer it would allow the regulatory authorities amongst others greater access to modern

molecular methods. The cheaper and quicker method would than allow a more regular programme of surveillance to be carried out to assess ingredient and labelling claims.

Rationale and Objectives

The scientific or technical problem that requires solving and why the Agency's support should be given.

A robust and accurate real-time PCR method for the detection and quantification of common wheat adulteration of pasta has been established as a result of funding by the FSA [2, 3,]. The method was subsequently validated [9] in-house by RHM Technology, after which it was concluded that the procedure offered a significant advance over previous analytical methods delivering improved accuracy and reduced analytical uncertainty. While more accurate, this procedure is laborious, relatively costly and time consuming, consequently there is a desire to make the method more accessible and cost effective without compromising accuracy. A commercial capillary electrophoresis chip system (The Agilent Bioanalyzer) has the potential to offer benefits in this area of analysis and due to its flexibility has been adopted by many of the Public Analyst laboratories. Transfer of methods into this format would allow the regulatory authorities amongst others to use modern molecular methods to assess ingredients and labelling claims. Consumers already exercise a number of choices which are made on the basis of product labelling, including GMO and organic status. Strict labelling regulations combined with robust, validated analytical testing to minimize misdescription are needed to support and protect this choice.

State of the art in the research area

All current published methods used to quantify the *T. aestivum* content of *T. durum* pastas employ real-time PCR [5, 6, 7, 8, and 9]. A significant contribution to current knowledge has been made as a result of FSA funding at RHM Technology (now part of Premier Foods Group Ltd) employing our cereals expertise and access to reference materials. Our standard analysis utilizes a number of specially developed reference materials to ensure the quality and accuracy of the analyses.

The scientific and technological basis of the project.

The project has investigated the transfer of the developed method [3] to the Agilent Bioanalyzer (LabChip) by making as few changes to the established procedures as possible. The Agilent Bioanalyzer capillary electrophoresis system is a means of separating and quantifying the PCR products produced by the amplification reaction. The existing real-time PCR amplicons have been designed to be as small and as similar as possible in terms of size (117 and 121bp) to reduce any discrepancy in the efficiency of PCR amplification. The small amplicon size is also important as the DNA template extracted from the sample is highly dependant upon processing. Hence, it was essential that the resolution of the Agilent Bioanalyzer capillary electrophoresis system was investigated to ensure that it pocessed the ability to resolve the amplicons and effect quantification without redesigning the PCR. Real-time PCR uses fluorescence generated at a proportional rate to amplicon production to determine the relative amounts of DNA templates present in the sample before amplification. It was therefore

necessary to investigate the generation and quantification of the amplicons using the least number of PCR cycles in an attempt to retain the proportionality of the analysis. A similar exercise was necessary when LGC transferred a GMO method from Real-time to the LabChip [11]. The performance characteristics of the Agilent Bioanalyzer method were investigated together with alternative methods of obtaining a quantitative analysis.

In what respect the project advances the state of the art in the research area

It has been established that 'real-time' PCR is an applicable tool for the analysis of dried pasta. This study has investigated the transfer of the established method [3] to the Agilent Bioanalyzer by making as few changes to the established procedures as possible. The Agilent Bioanalyzer capillary electrophoresis system is a means of separating and quantifying the PCR products produced by the amplification reaction. The real-time PCR amplicons were designed to be as small and as similar as possible in terms of size to reduce any discrepancy in amplification efficiency and to negate any effect of thermal processing. Hence, it was essential that the resolution of the LabChip capillary electrophoresis system was investigated to ensure that it possessed the ability to resolve the amplicons and effect quantification without redesigning the PCR. Real-time PCR uses fluorescence generated at a proportional rate to amplicon production to determine the relative amounts of DNA templates present in the sample before amplification. It was therefore imperative to investigate the generation and quantification of the amplicons using the least number of PCR cycles in an attempt to retain the proportionality of the analysis. There is no precedent indicating that the two methods are in any way comparable as the means of detecting and then quantifying the amplicons generated is very different indeed. Amplicons generated by real-time PCR are detected within the same sample tube at the earliest possible moment using fluorescence detection; as such this method is exceptionally sensitive. There is no attempt to quantify the amount of material present. The Agilent Bioanalyzer on the other hand is a means of separating and sizing PCR amplicons previously generated using a conventional thermocycler. Detection following electrophoretic separation is facilitated using UV light and the results are presented as the amount of amplicon present in the peak. Because of these fundamental differences between the two systems the study also investigated the performance characteristics of the transferred method.

This project, has demonstrated that it is possible, but with defined limitations to transfer the established real-time PCR method for the determination of common wheat adulteration in pasta to the lab chip. This allows a more rapid and more accessible analysis to be carried out by the regulatory authorities. The misdiscription of pasta products can be detected more easily, hence, protecting and supporting consumer preferences.

Approach

Task 1- (RHMT – now Premier Foods)
Project management throughout the duration of the project.

Task 2- (RHMT (now Premier Foods) and LGC)
The initial task will be to establish the assay in its original PCR form i.e. not the current 'real-time' conditions. While real-time PCR gives an indication of the right number of

cycles for a quantitative response at the log linear phase, transfer from real-time PCR to normal PCR is certain to change the kinetics of the reaction. An essential component of this task will be to arrange for the original assay to be carried out in a multiplex rather than simplex format. Multiplexing the assay is seen as a pre-requisite to analysis using the LabChip capillary electrophoresis system.Task 2 will also ensure that the two amplicons generated by the established 'real-time' method can be separated with sufficient resolution by the LabChip capillary electrophoresis system as simultaneous analysis would simplify the analysis considerably. All analyses will be carried out using 'pseudo-reference' materials currently available to RHMT and which are used to as 'standards and controls' within the routine real-time PCR analysis.

Task 3- (RHMT (now Premier Foods) and LGC)
Using the analytical conditions established during task 1, the relative copy number method (determining the copy numbers of the D genome and the ABD genomes of wheat) will be transferred to the LabChip capillary electrophoresis system. This will entail performing the analyses using a wide range of DNA dilutions to generate the necessary calibration curves.

Task 4- (LGC)
While the use of the LabChip capillary electrophoresis system has the potential to quicken/simplify the analysis, it makes a significant departure from the conventional wisdom associated with quantitative or semi-quantitative PCR methods in that its proposed use is to analyse PCR products; in effect an end point detector which looses its stoichiometry with respect to a quantitative response due to inhibitory effects. To redress this and in an attempt to obtain quantitative results, the least number of PCR cycles required to effect the analysis will be established.

Task 5- (RHMT (now Premier Foods) and LGC)
The calibration curves generated in task 3 will be examined with respect to their fitness for purpose (linear range and slope) and the appropriate adjustments made where necessary to the protocol.

Task 6- (RHMT (now Premier Foods) and LGC)
Once the method using the LabChip capillary electrophoresis system has been established it will be important to ensure that the results are comparable with the established real-time method. A limited inter-instrument trail will be carried out using a range of internal test materials and commercial samples.

Task 7- (RHMT (now Premier Foods) and LGC)

Following a successful comparison of the two instruments a statistically based validation exercise will be performed to establish the performance characteristics of the new method. This exercise will include repeatability, reproducibility and accuracy.

Task 8- (RHMT (now Premier Foods) and LGC)

Following the validation exercise the 'in-house' intermediate precision statement will be derived as an estimate of the uncertainty of the method.

Task 9- (RHMT (now Premier Foods))

Write SOP adhering to the new guidelines prepared by the Authenticity Methodology Working Group.

Task 10- (RHMT (now Premier Foods) and LGC)
Final report and the SOP to the FSA.

Outcome/Key Results Obtained

1. The original real-time PCR amplicons could not be separated using the Agilent LabChip 2100 or the comparable Caliper instrument.

2. Following a redesign of the original normalisation amplicons, the two new amplicons (D-genome (121bp) and normalisation (129bp)) were readily separated by the LabChip instrument.

3. It is apparent that the 'normalisation' peak, (used to detect all wheat present in the sample) exhibits heterogeneity upon fractionation, most probably as a result of heteroduplex formation.

4. Due to the extreme sensitivity of the amplification system and the general prevalence of wheat in the environment, it was necessary to instigate a range of extreme measures to eliminate any DNA contamination and DNA carry over from one analysis to another.

5. We were able to design the analysis in a multiplex format thus reducing costs and simplifying the analysis compared to the simplex PCR carried out for the real-time PCR method.

6. Linear copy number calibration curves were established for each of the D-genome and 'normaliser' amplicons.

7. The concentration range of the template DNA applicable to the LabChip instrument is comparable to that used for real-time PCR.

8. The optimum number of PCR cycles prior to LabChip analysis is 39.

9. It is essential that all data generated by the Bioanalyzer is within the linear working range of the instrument i.e. the amount of amplicon present is not below the LOD or above that which can be reliably quantified.

10. It is evident that the 'conventional' wisdom and methods of real-time PCR cannot be transferred directly to the LabChip. The two techniques use different processes to derive results and it is clear that there is no immediate relationship between the Ct of 'real-time' PCR and the amount of amplicon accumulation as measured by the LabChip.

11. The analysis based upon copy-number generates results that seem very inaccurate. This is most probably due to sensitivity issues with the Agilent Bioanalyzer. The issue of sensitivity appears to originate with the low copy number of the D-genome sequences present in each of the samples.

12. Due to the lack of proportionality between Ct and amplicon accumulation (as measured by the Labchip) it was necessary to investigate an alternative quantification method. The alternative, based upon the amplicon accumulation from co-analysed reference materials gives a practical solution to the problems of quantification. While this approach yield significantly better results, it should only be deemed to be semi-quantitative at present.

What it means and why it's important

The work carried out within this project has shown that it is possible to transfer the established real-time PCR method to the Agilent Bioanalyzer and to estimate the level of common wheat adulteration in pasta. This will allow a more rapid and more accessible analysis to be carried out by the regulatory authorities. The transferred method allows the estimation of the level of contamination without recourse to the more expensive and time consuming real-time method. The real-time method, however, remains the more accurate approach serving as a confirmatory technique in cases of dispute or uncertainty. The greater availability of the Agilent Bioanalyzer method to Public Analysts will greatly assist the detection of mis-described pasta products, protecting and supporting consumer preferences.

Objectives

01	Re establish the non-real-time PCR method in a multiplex format and determine if the LabChip capillary electrophoresis system will resolve the two amplicons generated in the PCR assay at the same time.
02	Transfer the two (D-genome specific & normalisation sequence) copy number assays to the LabChip capillary electrophoresis system.
03	Determine the minimum number of PCR cycles required to generate sufficient PCR product to effect detection and quantification.
04	Obtain adequate copy number standardisation curves.
05	Verify that the method generates results that are comparable with the established real-time method.
06	Establish the performance characteristics of the LabChip capillary electrophoresis system method.
07	Contribute towards establishing the analytical uncertainty of the method
08	Write SOP.
09	Final report and Standard Operating Procedure to the FSA.

Project milestones

Milestone	Target date	Target title
01/01	T_0 + 2months (31/8/06)	Redesign of the assay in a multiplex format and determine if the Labchip will resolve the two amplicons generated in the PCR assay at the same time.
02/01	T_0 + 3months (29/9/06)	Determine the minimum number of PCR cycles required to generate sufficient PCR product to effect detection and quantification.
03/01	T_0 + 4months (31/10/06)	Transfer the two (D-genome specific & normalisation sequence) copy number assays to the Lab-chip.
04/01	T_0 + 5months (30/11/06)	Obtain adequate copy number standardisation curves.
05/01	T_0 +6 months (29/12/06)	Verify that the method generates results that are comparable with the established real-time method.
06/01	T_0 +9 months (30/03/07)	Establish the performance characteristics of the Lab-chip method.
07/01	T_0 +11 months (31/5/07)	Contribute towards establishing the analytical uncertainty of the method.
08/01	T_0 + 12 months (30/6/07)	Submit SOP.
09/01	T_0 + 12 months (30/6/07)	Final report and Standard Operating Procedure to the FSA.

1.0 Introduction

The authenticity of *T. durum* pastas, and the detection and quantification of deliberate adulteration of *T. durum* pastas by common wheat (*T. aestivum*), continues to be an important issue throughout Europe. As a result of occasional adverse harvest conditions and economic trading agreements the geographical origin of the grain used to produce pasta may vary. However, the strict laws and agreements regarding the adventitious contamination of *T. durum* by *T. aestivum* require enforcing to protect both the retailers and the consumer. A large number of analytical procedures exist to facilitate the detection of *T. aestivum*, employing a wide range of techniques and technologies; however, most suffer from numerous shortcomings resulting in quantificational difficulties [1]. MAFF project's 2A015 [2] and AN0667 [3] have demonstrated that it is possible to address the problems associated with the detection and quantification of common wheat (*T. aestivum*) adulteration of *T. durum* pastas using molecular biology techniques. Initial research showed that a repetitive but specific D-genome DNA sequence known as Dgas44 [4] could be used to detect the presence of adulterating common wheat [5]. Multiple copies of the Dgas 44 sequence are known to be present within the D-genome of common wheat varieties; however, the exact copy number is believed to vary with the cultivar in question, making quantification impossible. To address this issue, subsequent investigations developed a 121bp specific single copy DNA sequence (PSR 128-Figure 1) to effect quantification. Quantification of the specific DNA sequences was achieved using real-time Polymerase Chain Reaction (PCR) and incorporated an amplification of a second single copy indigenous gene sequence (117bp) to effect normalisation of the signal produced. A large number of commercial cultivars of both *T. durum* and *T. aestivum* were collected from around the world during these studies and each was investigated using PCR amplification of the D-genome specific sequence. In addition to these cultivars, a large number of related species and amphiploid lines of wheat were also assessed. The results showed that the detection of the D-genome is highly unlikely to be compromised by cross-reaction with other unrelated genomes. These investigations and their conclusions gave increased confidence in the analytical results and to the general applicability of the method for the detection of *T. aestivum* contamination from a wide geographical origin in *T. durum* pasta. The analysis of *T. aestivum* adulteration using molecular methods also addresses some of the potential problem areas associated with convergent wheat breeding programmes that may potentially produce cultivars that do not express marker proteins assayed by other methods. A number of *T. aestivum* cultivars (i.e. contain the D-genome) exist but which do not express the omega-gliadins, making them undetectable by many of the currently used protein detection methods.

Since the inception of these studies other research groups also have approached the problem of detecting and quantifying *T. aestivum* contamination in pasta and semolina using 'real-time 'PCR. While their methods use the same analytical tools as we have described previously [3] the target sequences used for the analysis are very different. Alary et al [6] have used the DNA sequence from part of the purindoline b gene as a marker for the presence of *T. aestivum* and a DNA sequence from the lipid transfer protein gene as a marker and measure for the presence of all wheat varieties in a multiplex PCR to calculate the amount of *T. aestivum* present in a given mixture. In common with perceived good practice in this field of analysis the amplicon size was small (63 and 61 bp respectively) making the method amenable to the analysis of degraded DNA following processing. The authors noted that a decreased yield of DNA was obtained from heat-treated pasta and that it was necessary to dilute the DNA prior to amplification to remove the adverse effects of inhibitors that co-purified with the wheat DNA. The authors did not attempt to address issues relating to the accuracy, precision and reproducibility of their methods and simply presented the data from a limited number of analyses.

Sequence of clone PSR 128

```
cDNA          CCTCGAGAAC AAGGAGAAGG AGCTCGCCAA CG........ .......... .......... .......... .......... .......... ..........
A             CCTCGAGAAC AAGGAGAAGG AGCTCGCCAA CGGTACCTCC CGAGTCCATC GTTCTCTGCT T......... .......... .......... ..........
B             CCTCGAGAAC AAGGAGAAGG AGCTCGCCAA CGGTACGTCC ATCG...... CTCCTCTGCT T......... .......... .......... ..........
D       1     CCTCGAGAAC AAGGAGAAGG AGCTCGCCAA CGGTACCTCC ATAGTCTCTC TTCTTCTTCT TCTTTTTTCA AATATCCAGC TAGATGGCTG GCTTCTATTT

cDNA          .......... .......... .......... .......... .......... .......... .......... .......... .......GGA GACTGGCGAT
A             .......... ....GCTTCA CCATCGTTAA TTTCTAATCG GACGGCTCTT GTTAACTTTT GGATGTTTT. ....TCGATT CGTTCAGGGA GACTGGCGAT
B             .......... ....GCTTCA CCGTCGTTAA TTTCTAATCG GACGGCTCCT GTTAACTTTT GGATGTTTT. ....TCCATT CGTTCAGGGA GACTGGCGAT
D       101   CATGATCCAT CGTCTCTTCT GGACCCTCGG TTCCTAATCG GACGGCTCTT GTTAATTTTT GGATTTTTTT TTCATTCATT CATTCAGGTA GACTGGCGAT

cDNA          GCTGGCCTTC CTGGGGTTCC TGGTGCAGCA CAACGTGACC GGCAAGGGCC CGTTCGAGAA CCTGCAGGAG CACCTGGCCG ACCCATGGCA CAACACCATC
A             GCTGGCGTTC CTGGGGTTCC TGGTGCAGCA CAACGTGACC GGCAAGGGCC CGTTCGAGAA CCTGCAGCAG CACCTGGCCG ACCCATGGCA CAACACCATC
B             GCTGGCGTTC CTGGGGTTCC TGGTGCAGCA CAACGTGACC GGCAAGGGGC CGTTCGAGAA CCTGCAGCAG CACCTGGCCG ACCCATGGCA CAACACCATC
D       201   GCTGGCGTTC CCGGGGTTCC TGGTGCAGCA CAACGTGACC GGCAAGGGCC CGTTCGAGAA CCTGCAGCAG CACCTGGCCG ACCCATGGCA CACCACCATC

cDNA          ATCCAGACCA TCTCCGGCCA ATAAATCCGT CGATTTCACC GACCGGGGAG GCTTCAGGTG GTCTGAATTG TTATTTTGGG GTGCTGAGGA TGTACAAAGT
A             ATCCAGACCA TCTCCGGCCA ATAAATCCGT CGATTTCACC GACCGGGGAG GCTTCAGGTG GTCTGAATTG TTATTTTGGG GTGCTGAGGA TGTACAAAGT
B             ATCCAGACCA TCTCCGGCCA ATAAATCCGT CGATTTCACC GACCGGGGAG GTTTCAGGTG GTCTGAATTA TTATTTTGGG GTGCTGAGGA TGTACAAAGT
D       301   ATCCAGACCA TCTCCGGCCA GTAAATCCGT CGATTTCACC GACCGGGGAG GTTTCAGGTG GTCTGAATTG TTATTTTGGG GTACTGAGGA TGTACAAAGT

cDNA          GTGGAGGAGT AGGTGGACAG
A             GTGGAGGAGT AGGTGGACAG
B             GTGGAGGAGT AGGTGGACAG
D       401   GTGGAGGAGT AGGTGGACAG

cDNA
```

D-Genome specific detection

5′ AGGAGAAGGAGCTCGCCAA 3′ Forward primer (bases 12-30), 5′GCTAGATGGCTGGCTTCTATTTCATGATCCA 3′ Taqman™ probe 1 (bases 79-109)
3′ CAGAGAAGACCTGGGAGCCAA 5′Reverse primer (bases 112-132)

Normalisation

5′ CAMCACCATCATCCAGACCATCT 3′ Forward primer (bases 291-313), 5′AATCCGTCGATTTCACCGACCGG 3′ Taqman ™ probe 2 (bases 324-346)
5′ CCTCCACACTTTGTACATCCTCAG 5′ Reverse primer (bases 384 -407)

Figure 1- Sequence of PSR128 showing the position of the primers used to amplify the D-genome specific and normalisation sequences

The data relating to the copy number of the gene sequences used together with potential cross-reactivity towards closely related wheat species was not presented. The authors advised that the best analyses were obtained when 30-100 copies of the target gene were present and noted that at higher contamination levels an overestimate of the *T. aestivum* content was obtained. Similarly, Arlorio et al [7] have presented data based upon the detection, of the purindoline b gene, present on the 5D chromosome of *T. aestivum.* In this case the PCR amplification products were analysed following electrophoretic fractionation using an agarose gel. This step clearly limited the sensitivity of the method as they reported a limit of detection of 0.2%. In this instance normalisation for the amount of 'total' wheat present was carried out using a 'universal' DNA sequence from the ribosomal region in a multiplexed analysis. Again, no data was presented relating to the copy number of the gene sequences. The amplicons were particularly large (310-460bp) in stark contrast to other workers who favour smaller amplicons, due to their more general applicability for use in detecting adulteration in processed materials. The ribosomal DNA sequence employed for normalising purposes seemed to be present in other related cereals; it is also likely that the sequence used is present as a multiple copy. Terzi et al [8] have also been investigated sequence tagged sites (STS) present in gliadin and glutenin genes as a means of quantifying the *T. aestivum* content of pastas. Amplicons of 67-800bp were analysed using agarose gels and 'end point' real-time PCR using the intercalating dye, SYBR green™ to follow the PCR reaction. The authors examined the specificity of the DNA sequences employed using a wide range of *Triticum* species including *T. aestivum* and *T. durum* and other related grass and cereal species and concluded that particular combinations of primers were suitable for detecting the presence of *T. aestivum* in pasta products. The initial results obtained using flour blends and agarose gel electrophoresis had a limit of detection of 1% and was only suitable for raw materials due to the size of the amplicon (800bp). The method, however, has been further developed using 'real-time' PCR with primer and probe sets specific for a D-genome specific glutenin sequence (101bp) and a *Triticum* specific, (normalising) gliadin sequence (101bp). The results (Cts) obtained from the different cultivars show a high degree of variation, suggesting that the copy number varies significantly between the cultivars examined making quantitative comparison difficult, if not impossible. The authors did not attempt any statistical evaluation of their results.

It has been established that 'real-time' PCR is an applicable tool for the analysis of dried pasta; however, it is evident that any new method requires validation as a pre-requisite of becoming a robust method of analysis. To date, there is very little statistical data that underpins the characteristics of these new analytical methods and which can be used to appraise their fitness for purpose. However, it is well known that the coefficients of variation (cv) in similar assays that employ PCR are typically in the region of 17-42% [10]. It is also known that the cvs are not linear throughout the analytical range, increasing dramatically with increasing measured analyte concentration. We established the basic performance characteristics of the real-time PCR method in a recent FSA study [9], these characteristics included the analytical uncertainty and accuracy. The issue of generating suitable reference material was also addressed and it was determined that 'standards' generated from *T. durum* and *T. aestivum* flours were an appropriate tool for the analysis of pastas with unknown thermal histories. The role of a performance standard of known composition in assessing the quantitative PCR was also studied. Due to the highly specific nature of the analysis; this particular analysis is performed routinely only within our own laboratories, hence, the analytical uncertainty obtained [9] should be regarded as an 'intermediate' precision statement.

In this study it was proposed to investigate the transfer of the developed method [3] to the Agilent Bioanalyzer by making as few changes to the established procedures as possible. The Agilent Bioanalyzer capillary electrophoresis system is a means of separating and quantifying the PCR products produced by the amplification reaction. The real-time PCR

amplicons have been designed to be as small and as similar as possible in terms of size (117 and 121bp) to reduce any discrepancy in amplification efficiency. The small amplicon size is also important as the DNA template extracted from the sample is highly dependant upon processing. Hence, it was essential that the resolution of the LabChip capillary electrophoresis system was investigated to ensure that it posessed the ability to resolve the amplicons and effect quantification without redesigning the PCR. Real-time PCR uses fluorescence generated at a proportional rate to amplicon production to determine the relative amounts of DNA templates present in the sample before amplification. It was therefore imperative to investigate the generation and quantification of the amplicons using the least number of PCR cycles in an attempt to retain the proportionality of the analysis, if indeed this is possible. A similar exercise was necessary when LGC transferred a GMO method from real-time PCR to the Agilent Bioanalyzer [11]. There is no precident indicating that the two methods are in any way comparable as the means of detecting and then quantifying the amplicons generated is very different indeed. Amplicons generated by real-time PCR are detected within the same sample tube at the earliest possible moment using fluorescence detection; as such this method is exceptionally sensitive. There is no attempt to quantify the amount of material present. The Agilent Bioanalyzer on the other hand is a means of separating and sizing PCR amplicons previously generated using a conventional thermocycler. Detection following electrophoretic separation is facilitated using UV light and the results are presented as the amount of amplicon present in the peak. Because of these fundamental differences between the two systems the study also investigated the performance characteristics of the transferred method.

2.0 Materials and Methods

The research described in this report was not subjected to any of the formalised accreditation systems but it should be noted that the methods used were similar to those for our analytical procedures that are accredited (UKAS/ISO17025). Hence, laboratory equipment such as pipettes, balances and PCR thermocyclers were routinely maintained and calibrated. An identical approach was used also in the application of appropriate controls to check for laboratory based contamination and system effectiveness.

2.1 Reference materials

2.1.1 Wheat cultivars

An accredited sample of the French *T. durum* cultivar, Pescedou, was a generous gift from Dr Pierre Devaux of Florimond Desprez. The English *T. aestivum* cultivar, Hereward, was obtained commercially from NIAB in Cambridge, UK.

Each cultivar was ground using a small coffee mill and the flour generated passed through a 250µm sieve. Flours generated in this manner were stored at -20°C until required. *T. durum* flour (Pescedou) containing known amounts of *T. aestivum* (Hereward) were produced gravimetrically by weighing and then mixing the appropriate amounts of each component.

2.1.2 Pasta quantitative control (PQC)

The pasta quantification control (PQC) is a blend of pastas obtained from commercial sources that have been previously analysed by real-time PCR and found to contain contaminating *T. aestivum*. These pastas have been blended and sieved through a 250µm sieve to generate ground ‘pasta’ that

can be measured by real-time PCR to contain 3% *T. aestivum.* This material is used as a quantitative control in all real-time PCR analyses of pasta to determine the *T. aestivum* content. It was therefore deemed as being suitable for assessing the performance of the LabChip instrument.

2.1.3 Adulterated flour and commercial pasta samples

The following sample unknowns were included for evaluation:

- 3% *T. aestivum* flour sample made by combining 100% *T. aestivum* flour (3mg) with 100% *T. durum* flour (97mg).
- 5% *T. aestivum* flour sample made by combining 100% *T. aestivum* flour (5mg) with 100% *T. durum* flour (95mg).
- PQC Pasta sample (a 3% *T. aestivum* adulterated pasta-used as a quality control material for real-time PCR analysis).
- Pasta sample 180–a commercial pasta sample.
- Pasta sample 318–a commercial pasta sample.
- Pasta sample 319–a commercial pasta sample.

2.2 Real-time PCR primers and reagents

The PCR mastermix and all custom synthesised primers and Taqman probes were obtained from Applied Biosystems Ltd, Warrington UK.

2.3 DNA extraction

DNA was extracted from the flour blends/ground pasta and purified using the procedure detailed in the Standard Operating Procedure (SOP) presented in Appendix 1. All chemicals were as listed and specified in Appendix 1 and were of molecular biology grade or better. DNA extracts, once prepared, were stored at 4℃ until required for analysis.

2.4 Polymerase chain reaction

Traditional PCR was performed using the materials and methods detailed in the Standard Operating Procedure (SOP) – Appendix 1

2.5 Real-time PCR

The PCR primers and probes were designed using Primer Express software (Applied Biosystems). Oligonucleotide primers and fluorescent probes were synthesised by Applied Biosystems. PCR amplification was performed using a pre-mixed mastermix (Applied Biosystems) to which forward and reverse primers (300nM), fluorescent probe (100nM) and template DNA ~50ng were added (Appendix 2). All reactions were performed using an ABI 7700 Prism 'real-time' instrument. The amplification conditions used in each case were 50°C for 2 min followed by 95°C for 10 min, followed by 45 cycles of 95°C for 15 seconds then 60°C for 1 min.

The results from the ABI 7700 instrument were incorporated into an Excel spreadsheet. Following transfer and manipulation of the data, results were archived as Excel spreadsheets using a centralised computer network that was protected and backed up daily.

3.0 Results

3.1 Resolution of the 117 & 121bp amplicons generated by the real-time PCR assay using the Agilent 2100 Bioanalyzer.

A central task in transferring the real-time PCR method for the determination of *T. aestivum* contamination of *T. durum* pastas to the LabChip was the fractionation of the original amplicons, to facilitate quantification. These amplicons were 117 and 121bp in size and had been deliberately chosen to be a similar as possible in size during the real-time PCR study to minimise any difference in their amplification kinetics (*Figure 2*). It was realised that fractionating the 4bp difference between the two amplicons would be challenging as the difference approximated to the resolution of the LabChip and other 'lab on a chip' devices i.e. approximately 5%. However, before making the necessary changes to resolve the two amplicons, it was thought prudent to attempt to fractionate the original amplicons in attempt to keep the method as similar as possible.

The two amplicons were produced using singleplex reactions, mixed 50:50 and introduced to the Labchip using the manufacturer's instructions. It was evident after multiple analyses using a number of different LabChips that the two amplicons could not be separated using the DNA 500. A specimen analysis is presented as a gel (*Figure 3*) and as a chromatogram (*Figure 4*).

Parallel to the analyses carried out using the Agilent LabChip an opportunity arose to compare the Caliper LC90 which is a rival instrument to the Agilent LabChip claiming similar resolution. This instrument was also unable to separate the original amplicons despite a number of changes being made to the resolving gel within the chip. A specimen analysis is presented in *Figure 5* & *Figure 6*.

It should be noted that during the course of the project, Agilent 'improved' the gel within the Labchips to enable larger DNA fragments to be fractionated; these new LabChips are known as DNA 1000 chips. The DNA 1000 chip also failed to separate the two original amplicons, despite this version of the device having superior resolution. The DNA1000 chip was used for the remainder of the project.

Sequence of clone PSR 128

```
cDNA          CCTCGAGAAC  AAGGAGAAGG  AGCTCGCCAA  CG........  ..........  ..........  ..........  ..........  ..........  ..........
A             CCTCGAGAAC  AAGGAGAAGG  AGCTCGCCAA  CGGTACCTCC  CGAGTCCATC  GTTCTCTGCT  T.........  ..........  ..........  ..........
B             CCTCGAGAAC  AAGGAGAAGG  AGCTCGCCAA  CGGTACGTCC  ATCG......  CTCCTCTGCT  T.........  ..........  ..........  ..........
D       1     CCTCGAGAAC  AAGGAGAAGG  AGCTCGCCAA  CGGTACCTCC  ATAGTCTCTC  TTCTTCTTCT  TCTTTTTTCA  AATATCCAGC  TAGATGGCTG  GCTTCTATTT

cDNA          ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  .......GGA  GACTGGCGAT
A             ..........  ....GCTTCA  CCATCGTTAA  TTTCTAATCG  GACGGCTCTT  GTTAACTTTT  GGATGTTTT.  ....TCGATT  CGTTCAGGGA  GACTGGCGAT
B             ..........  ....GCTTCA  CCGTCGTTAA  TTTCTAATCG  GACGGCTCCT  GTTAACTTTT  GGATGTTTT.  ....TCCATT  CGTTCAGGGA  GACTGGCGAT
D       101   CATGATCCAT  CGTCTCTTCT  GGACCCTCGG  TTCCTAATCG  GACGGCTCTT  GTTAATTTTT  GGATTTTTTT  TTCATTCATT  CATTCAGGTA  GACTGGCGAT

cDNA          GCTGGCCTTC  CTGGGGTTCC  TGGTGCAGCA  CAACGTGACC  GGCAAGGGCC  CGTTCGAGAA  CCTGCAGGAG  CACCTGGCCG  ACCCATGGCA  CAACACCATC
A             GCTGGCGTTC  CTGGGGTTCC  TGGTGCAGCA  CAACGTGACC  GGCAAGGGCC  CGTTCGAGAA  CCTGCAGCAG  CACCTGGCCG  ACCCATGGCA  CAACACCATC
B             GCTGGCGTTC  CTGGGGTTCC  TGGTGCAGCA  CAACGTGACC  GGCAAGGGGC  CGTTCGAGAA  CCTGCAGCAG  CACCTGGCCG  ACCCATGGCA  CAACACCATC
D       201   GCTGGCGTTC  CCGGGGTTCC  TGGTGCAGCA  CAACGTGACC  GGCAAGGGCC  CGTTCGAGAA  CCTGCAGCAG  CACCTGGCCG  ACCCATGGCA  CACCACCATC

cDNA          ATCCAGACCA  TCTCCGGCCA  ATAAATCCGT  CGATTTCACC  GACCGGGGAG  GCTTCAGGTG  GTCTGAATTG  TTATTTTGGG  GTGCTGAGGA  TGTACAAAGT
A             ATCCAGACCA  TCTCCGGCCA  ATAAATCCGT  CGATTTCACC  GACCGGGGAG  GCTTCAGGTG  GTCTGAATTG  TTATTTTGGG  GTGCTGAGGA  TGTACAAAGT
B             ATCCAGACCA  TCTCCGGCCA  ATAAATCCGT  CGATTTCACC  GACCGGGGAG  GTTTCAGGTG  GTCTGAATTA  TTATTTTGGG  GTGCTGAGGA  TGTACAAAGT
D       301   ATCCAGACCA  TCTCCGGCCA  GTAAATCCGT  CGATTTCACC  GACCGGGGAG  GTTTCAGGTG  GTCTGAATTG  TTATTTTGGG  GTACTGAGGA  TGTACAAAGT

cDNA          GTGGAGGAGT  AGGTGGACAG
A             GTGGAGGAGT  AGGTGGACAG
B             GTGGAGGAGT  AGGTGGACAG
D       401   GTGGAGGAGT  AGGTGGACAG
```

D-Genome specific detection
5’ AGGAGAAGGAGCTCGCCAA 3’ Forward primer (bases 12-30),
3’ CAGAGAAGACCTGGGAGCCAA 5’Reverse primer (bases 112-132)

Normalisation
5’ CAMCACCATCATCCAGACCATCT 3’ Forward primer (bases 291-313),
5’ CCTCCACACTTTGTACATCCTCAG 5’ Reverse primer (bases 384 -407)

Figure 2-Sequence of PSR128 showing the position of the original primers used to amplify the D-genome specific (121bp) and normalisation sequences (117bp)

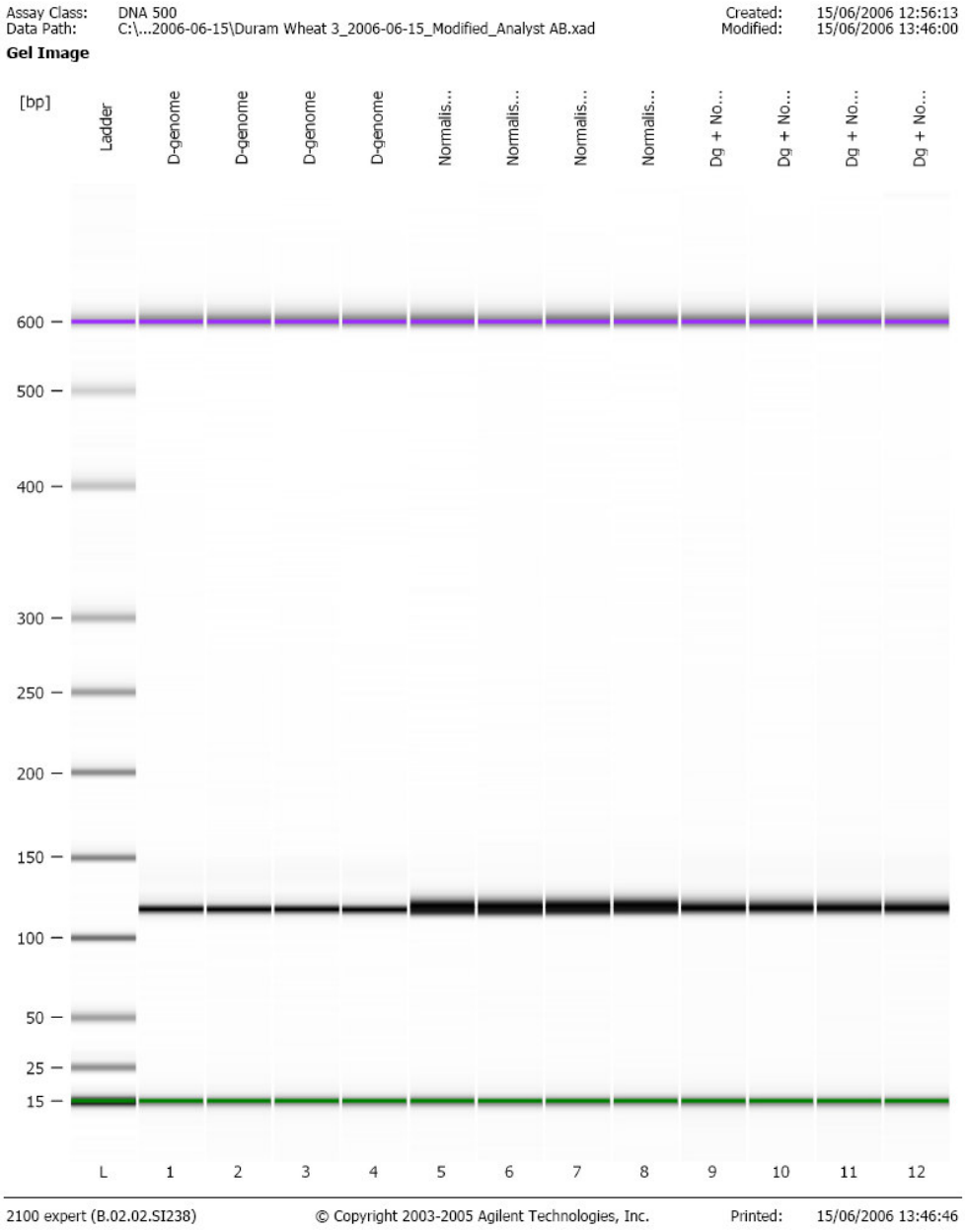

Figure 3- LabChip (DNA 500) analysis of the original D-genome and 'normaliser' amplicons

Legend

Lane L DNA calibration ladder
Lanes 1-4 121 bp D-genome specific amplicon
Lanes 5-8 117bp 'normaliser' amplicon
Lanes 9-12 50:50 mix of 117 &121 bp amplicons

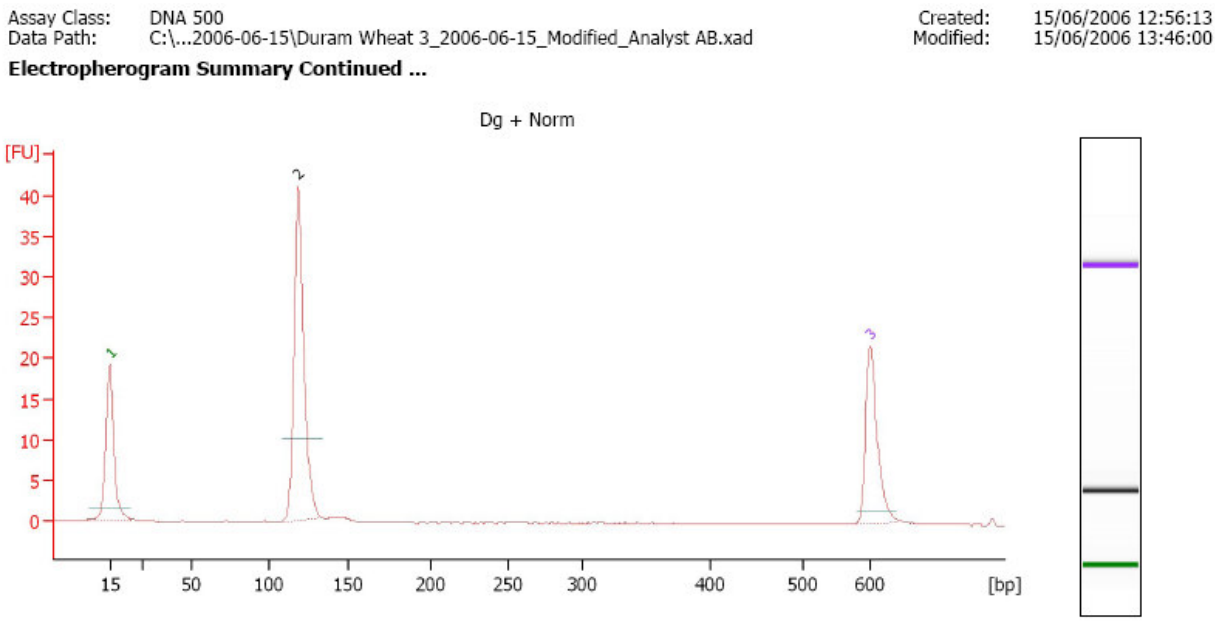

Figure 4 - LabChip (DNA 500) analysis of a (50:50) mix of the original D-genome and 'normaliser' amplicons; lane 12 from figure 3

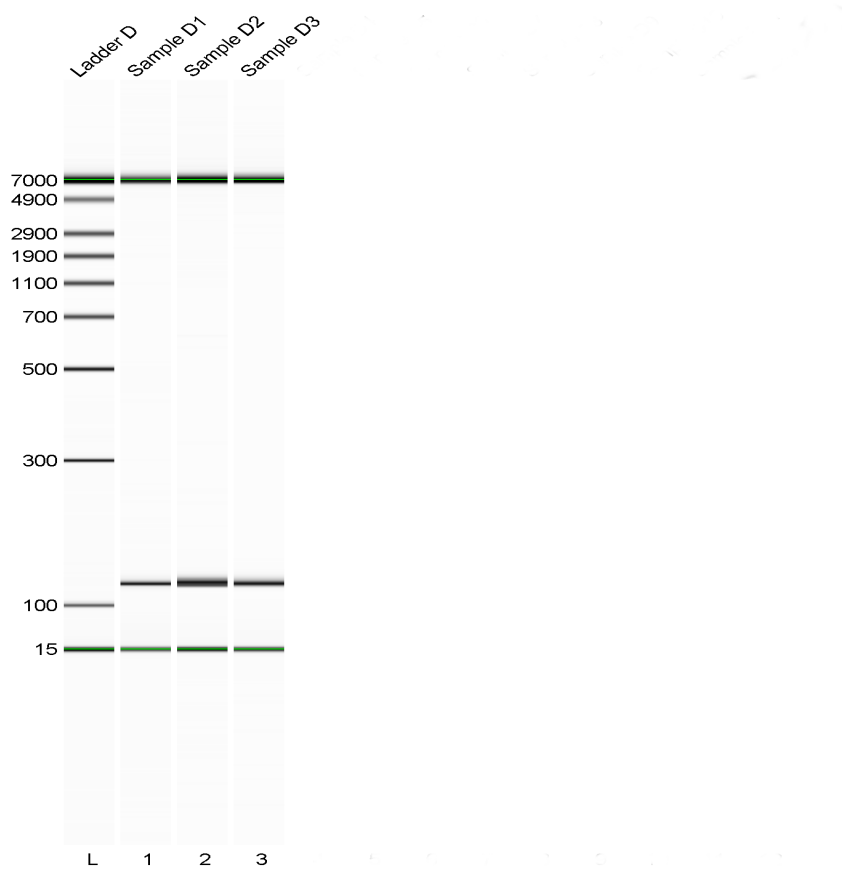

Figure 5 - Caliper LC90 analysis of the original D-genome and 'normaliser' amplicons

Legend

Lane L	DNA calibration ladder
Lanes 1	121 bp D-genome specific amplicon
Lanes 2	117bp 'normaliser' amplicon
Lanes 3	50:50 mix of 117 &121 bp amplicons

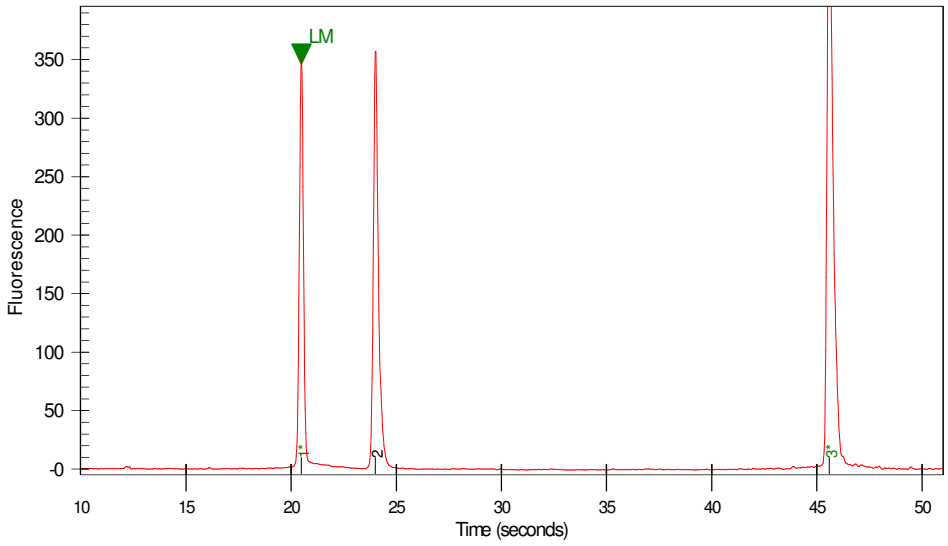

Figure 6- Caliper LC90 analysis of a (50:50) mix of the original D-genome and 'normaliser' amplicons; lane 3 from figure 5

The inability of either of the instruments to separate the two amplicons was not unexpected but it precipitated the need to redesign one of the amplicons to facilitate separation.

3.1.1. Redesign of the 'normaliser' amplicon

The unique intron sequence of the D-genome is rather small (53bp) and the use of a fluorescently-labelled probe targeted at this area greatly enhances the ability for real-time PCR to detect and quantify the D-genome amplicon. The current project requires the amplification and detection of the same DNA sequence without recourse to using a fluorescently-labelled probe, hence, it was decided not to redesign this amplicon due to the potential difficulties involved and to concentrate on re-working the 'normalisation ' amplicon. The DNA sequence of the PSR128 Exon is homologous for large portions of the sequence (*Figure 1*) allowing a degree of freedom in designing a new 'normalisation' amplicon. A new 'normalisation' amplicon (129bp -*Figure 7*) was designed using Primer Express to be as similar in size to the D-genome specific amplicon as possible in an attempt to minimise any differential kinetic effects during amplification. Following amplification using the standard PCR method, PCR product was mixed (50:50) with the 121bp D-genome specific PCR product prior to fractionation. Uasing the LabChip DNA1000 device, it can be seen (*Figure 8* & *Figure 9*) that the two amplicons are readily separated.

Sequence of clone PSR 128

```
cDNA            CCTCGAGAAC  AAGGAGAAGG  AGCTCGCCAA  CG........  ..........  ..........  ..........  ..........  ..........  ..........
A               CCTCGAGAAC  AAGGAGAAGG  AGCTCGCCAA  CGGTACCTCC  CGAGTCCATC  GTTCTCTGCT  T.........  ..........  ..........  ..........
B               CCTCGAGAAC  AAGGAGAAGG  AGCTCGCCAA  CGGTACGTCC  ATCG......  CTCCTCTGCT  T.........  ..........  ..........  ..........
D       1       CCTCGAGAAC  AAGGAGAAGG  AGCTCGCCAA  CGGTACCTCC  ATAGTCTCTC  TTCTTCTTCT  TCTTTTTTCA  AATATCCAGC  TAGATGGCTG  GCTTCTATTT

cDNA            ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........  .......GGA  GACTGGCGAT
A               ..........  ....GCTTCA  CCATCGTTAA  TTTCTAATCG  GACGGCTCTT  GTTAACTTTT  GGATGTTTT.  ....TCGATT  CGTTCAGGGA  GACTGGCGAT
B               ..........  ....GCTTCA  CCGTCGTTAA  TTTCTAATCG  GACGGCTCCT  GTTAACTTTT  GGATGTTTT.  ....TCCATT  CGTTCAGGGA  GACTGGCGAT
D       101     CATGATCCAT  CGTCTCTTCT  GGACCCTCGG  TTCCTAATCG  GACGGCTCTT  GTTAATTTTT  GGATTTTTTT  TTCATTCATT  CATTCAGGTA  GACTGGCGAT
                            CAGAGAAGA  CCTGGGAGCC  AA

cDNA            GCTGGCCTTC  CTGGGGTTCC  TGGTGCAGCA  CAACGTGACC  GGCAAGGGCC  CGTTCGAGAA  CCTGCAGGAG  CACCTGGCCG  ACCCATGGCA  CAACACCATC
A               GCTGGCGTTC  CTGGGGTTCC  TGGTGCAGCA  CAACGTGACC  GGCAAGGGCC  CGTTCGAGAA  CCTGCAGCAG  CACCTGGCCG  ACCCATGGCA  CAACACCATC
B               GCTGGCGTTC  CTGGGGTTCC  TGGTGCAGCA  CAACGTGACC  GGCAAGGGGC  CGTTCGAGAA  CCTGCAGCAG  CACCTGGCCG  ACCCATGGCA  CAACACCATC
D       201     GCTGGCGTTC  CCGGGGTTCC  TGGTGCAGCA  CAACGTGACC  GGCAAGGGCC  CGTTCGAGAA  CCTGCAGCAG  CACCTGGCCG  ACCCATGGCA  CACCACCATC

cDNA            ATCCAGACCA  TCTCCGGCCA  ATAAATCCGT  CGATTTCACC  GACCGGGGAG  GCTTCAGGTG  GTCTGAATTG  TTATTTTGGG  GTGCTGAGGA  TGTACAAAGT
A               ATCCAGACCA  TCTCCGGCCA  ATAAATCCGT  CGATTTCACC  GACCGGGGAG  GCTTCAGGTG  GTCTGAATTG  TTATTTTGGG  GTGCTGAGGA  TGTACAAAGT
B               ATCCAGACCA  TCTCCGGCCA  ATAAATCCGT  CGATTTCACC  GACCGGGGAG  GTTTCAGGTG  GTCTGAATTA  TTATTTTGGG  GTGCTGAGGA  TGTACAAAGT
D       301     ATCCAGACCA  TCTCCGGCCA  GTAAATCCGT  CGATTTCACC  GACCGGGGAG  GTTTCAGGTG  GTCTGAATTG  TTATTTTGGG  GTACTGAGGA  TGTACAAAGT
                                                                                                  GACTCCT  ACATGTTTCA

cDNA            GTGGAGGAGT  AGGTGGACAG
A               GTGGAGGAGT  AGGTGGACAG
B               GTGGAGGAGT  AGGTGGACAG
D       401     GTGGAGGAGT  AGGTGGACAG
                CACCTCC
```

D-Genome - specific detection

5' AGGAGAAGGAGCTCGCCAA 3' Forward primer (bases 12-30)
5' AACCGAGGGTCCAGAAGAGAC 3' Reverse primer (bases 112-132)

Normalisation

5'CGACCCATGGCACACCA 3' Forward primer (bases 279-295)
5'CCTCCACACTTTGTACATCCTCAG 3' Reverse primer (bases 384-407)

Figure 7-Sequence of PSR128 showing the position of the primers used to amplify the D-genome specific(121bp) and the new normalisation sequences(129bp)

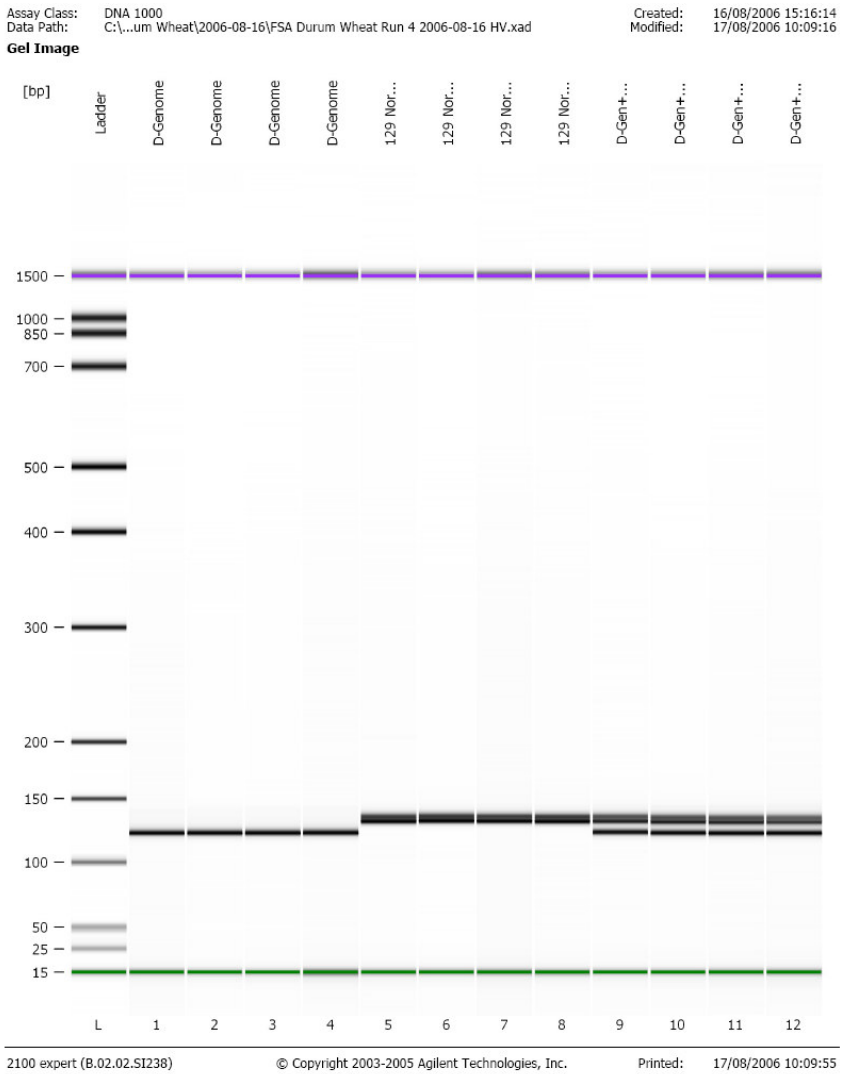

Figure 8 - LabChip (DNA 1000) analysis of the original D-genome and new 'normaliser' amplicons

Legend

Lane L	DNA calibration ladder
Lanes 1-4	121 bp D-genome specific amplicon
Lanes 5-8	129bp 'normaliser' amplicon
Lanes 9-12	50:50 mix of 121 &129 bp amplicons

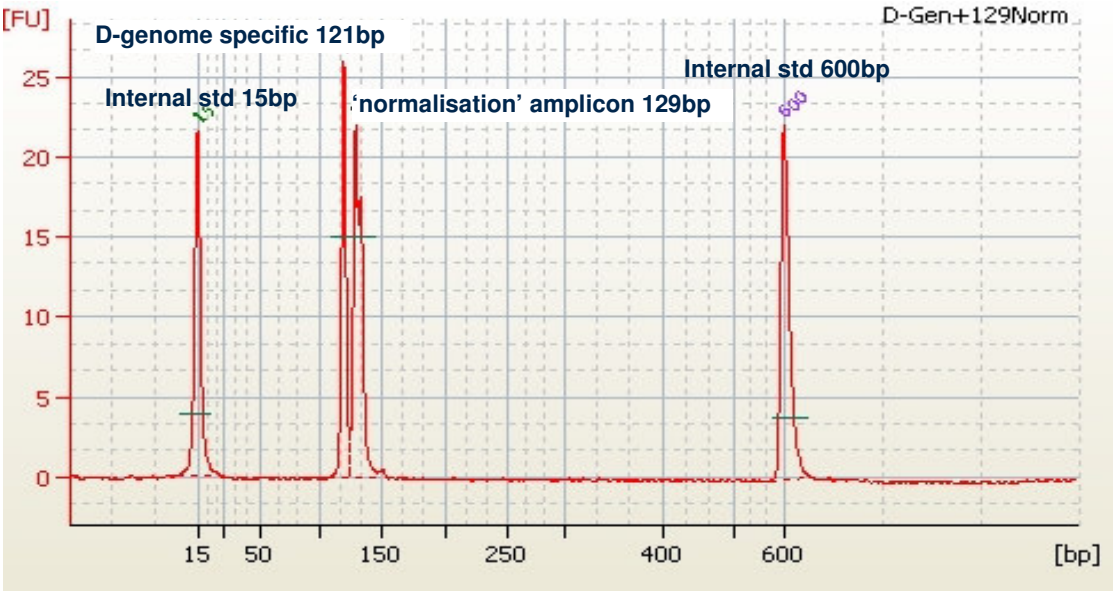

Figure 9 - LabChip1000 analysis of a (50:50) mix of the original D-genome and the new 129bp 'normaliser' amplicons;

3.1.3. Heterogeneity of the 129bp 'normaliser' amplicon

It was noticeable that the peak of PCR product generated following electrophoretic separation on the Agilent 2100 Bioanalyzer was heterogeneous (*Figure 10*). The PCR product separated into two broad peaks (*Figure 11*) with a suggestion that at least three electrophoretically distinct products were present. This effect was seen each time the 129bp PCR product was analysed. There are two possible explanations for this observation. The first and simplest explanation for this is, based on a close examination of the PSR128 exon sequence in the region that is amplified, shows that each of the three DNA sequences (A, B & D genome) contain small sequence differences (SNPs-single nucleotide polymorphisms). A total of three different 129bp sequences are possible from the wheat genomes, encompassing 4 SNPs in total (*Figure 7*-SNPs re indicated in **bold** type). The second and most probable explanation is that we are observing the separation of heteroduplex species formed by the hybridization of the three distinct amplified sequences. While each heteroduplex will be the same size (129bp), each heteroduplex species could adopt a slightly different confirmation in the separation buffer, affecting the retention time within the electrophoretic gel. This effect has not been reported in the literature but has been observed by other workers (personal communication) performing similar analyses to that described here. The ability for the LabChip to resolve such differences was totally unexpected; nevertheless, the effect was absolutely reproducible.

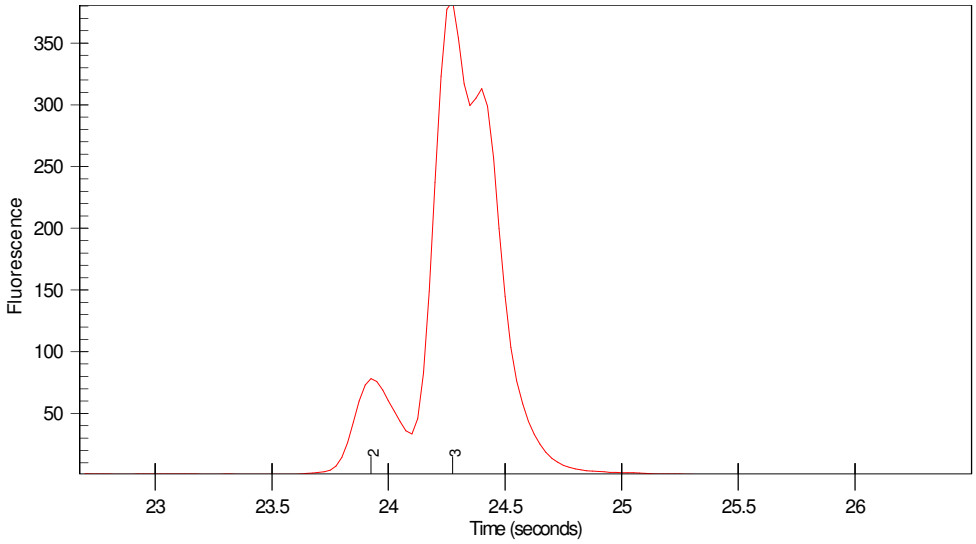

Figure 10 -Expanded graph –showing both amplicons i.e. 121 & 129bp

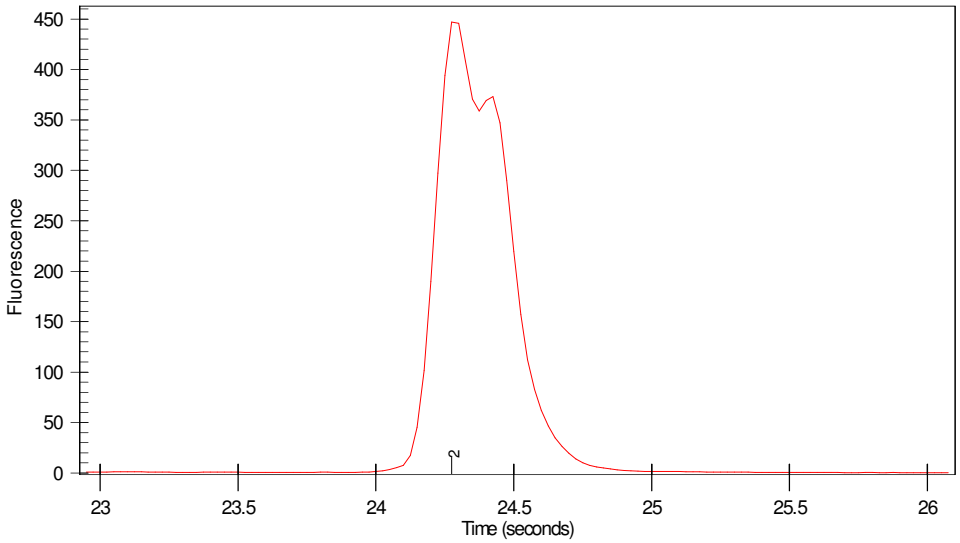

Figure 11-129bp 'normalisation' amplicon-expanded to show heterogeneity

3.2 Optimisation of the multiplex PCR

3.2.1 PCR cycle number

The Agilent 2100 Bioanalyzer was unable to separate the original PCR amplicons of 121bp (D-genome) and 117bp (normaliser: A, B and D genomes), which had been designed for real-time quantitative PCR. This necessitated a redesign of the two primer pairs to allow successful separation on the Agilent 2100 Bioanalyzer. The new primer-pairs which were designed for this assay to give successful separation on the Agilent 2100 system resulted in amplicon sizes of 121bp for the D-genome, and 129bp for the 'normaliser'.

Additionally, the throughput of the Agilent 2100 Bioanalyzer is limited to twelve samples per run / batch. In order to increase the number of comparisons and sample evaluations per run, the PCR was re-designed as a duplex reaction whereby both the D-genome and normaliser assays were conducted within the same reaction.

It was decided that the main criteria for successful duplex optimisation was the generation of linear calibration curves for both the D-genome and 'normaliser' amplicons, for the seven 1 in 2 serial dilutions of ½, ¼, 1/8, 1/16, 1/32, 1/64, 1/128 that were prepared from the 10ng/ul working stock solution of 10% *T.*

aestivum. In order to achieve this, PCR was performed using a range of cycles, so that the concentration of the two amplicons could be estimated whilst still in the log-linear phase of amplification, as opposed to being based on end-point detection. Cycle numbers of 30, 35-40 (inclusive) and 45 were used to produce a series of calibration curves (see Appendix 4). Following detailed analysis of the results cycle 39 was chosen as the optimum cycle number, based on the following criteria:-

- The Agilent 2100 Bioanalyzer responses for the D-genome and 'normaliser' amplicons from all dilutions of the standards.
- The lack of a "plateau effect" in the calibration curve (indicative of the maximum amount of product formed).
- The correlation coefficients associated with the linear fit of the model.

3.2.2 Optimisation of the PCR magnesium concentration

A comprehensive PCR optimisation experiment was also conducted. This optimisation included an initial comparison of PCR primers that had not been HPLC purified and those that had. Minimal differences were observed between the two types of primers, although overall, the HPLC purified primers resulted in less non-specific amplification. In order to select the best magnesium concentration in the final PCR reaction, a range of six magnesium concentrations 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0mM magnesium were investigated. The same primer concentrations were used throughout the magnesium optimisation. PCR products were analysed on the Agilent 2100 Bioanalyzer, with a replication factor of six per magnesium optimisation. A final concentration of 2mM magnesium was chosen as the optimum concentration based on size and repeatability of the D-genome and "normaliser" peaks, and the absence of false positives in the negative "no template" controls-(data not shown).

3.2.3 Optimisation of the primer concentrations

For the optimisation of the primer concentrations, singleplex amplifications of the 'normalisation' amplicon and D-genome were initially conducted. This optimisation included a comparison between the Applied Biosystems 7900HT Sequence Detection System, and the standard GeneAmp® PCR System 9700. The false positive rate was measured on both instruments, by checking for the occurrence of any positive amplification of PCR products in the no template controls. Both instruments showed no false positives on a test set of samples, concluding that the Applied Biosystems 7900HT Sequence Detection System and the standard GeneAmp® PCR System 9700 were performing correctly. The latter, as an industry standard for normal PCR, was taken forward as the choice instrument for further optimisations. The optimisations included testing the following final concentrations (mM) of primers (where "F" represents the forward primer and "R" the reverse primer): 50F/300R, 50F/900R, 300F/50R, 300F/300R, 300F/900R, 900F/50R, and 900F/300R. PCR was performed using these different concentrations for both the 'normalisation' and D-genome amplifications, as singleplex reactions and later combined in a duplex PCR. PCR products were analysed on the Agilent 2100 Bioanalyzer. The optimum primer concentrations were chosen on the basis of peak height (relatively high fluorescence units), repeatability associated with the amplifications, and the absence of high background, false positives or non-specific amplification occurring in the negative controls. The optimal duplex primer concentrations were 300mM Forward and 50mM Reverse for the Normalisation amplicon, and 300mM Forward and 50mM Reverse for the D-genome amplicon.

The PCR optimisation was developed further using the Agilent Bioanalyzer DNA 1000 chips. In June 2006, the original DNA 500 chips produced by Agilent technologies were no longer supported, and were replaced by the DNA 1000 chips. Different quantification and sizing accuracies exist between the two chip systems. However, all data shown in this report is associated with DNA 1000 chips.

3.3 Experimental design

3.3.1Precautions to minimise potential contamination

In order to minimise any potential contamination issues, it is suggested that the following preventative measures are taken:

- Before loading PCR samples for analysis on the Agilent 2100 Bioanalyzer, it is advised that PCR tubes/plate are subjected to centrifugation.
- Load negative controls first onto the chips
- Always use a micropipette tips fitted with a filter.
- If possible use positive displacement pipettes

3.3.2 Implementation of randomised designs

For any experimental design, randomisation of sample position provides an unbiased estimate of the sampling error. Using the LabChip 1000 system, a sample throughput of 12 can be achieved per chip. An experiment was conducted to investigate any differences between a “normal” systematic layout of samples on a chip, and a fully randomised design. A two way analysis of variance was used to test for significant differences between “normal” and randomised chips, and to test if a significant interaction of sample with chip existed (data not shown). The results showed no significant differences or interactions, showing that the samples consistently were behaving the same on both “normal” and randomised chips. As the process of generating randomised chips takes additional time, and can increase the chance of contamination / pipetting the wrong samples, it was decided to use only “normal” chips whereby samples were loaded in a systematic fashion.

3.3.3. Sample size: level of replication

Statistically, a sample replication factor of six has been shown to provide reasonably confident estimates of the original population mean and standard deviation. Increasing the replication factor above this level tends to result in marginal increases in the level of confidence of the sampling statistics. Reducing the level of replication below six can result in reduced levels of confidence that the population parameters are being estimated correctly. However, real-time PCR has been shown to be the bench-marking standard for many quantitative analyses, due to good performance characteristics inclusive of repeatability. Additionally, each chip on the Agilent 2100 Bioanalyzer is limited to a throughput of 12 sample reactions. Therefore, it was

decided that a sample level of replication of four would be used, so as to provide reasonable estimates of the population parameters whilst still allowing the assay to be performed under realistic conditions on the practical side.

3.4 Calculations and data analysis

As per section 9 of the SOP "Calculations and data analysis", PCR products were analysed on the Agilent Bioanalyzer. The fluorescent height threshold was reduced from the default setting of 20 down to 2 fluorescent units. Data was exported into Microsoft Excel, and the concentration of the 'normaliser' and D-genome amplicons (in ng/ul) were tabulated for each dilution alongside the relevant standards and sample unknowns. Note that the D-genome peak should appear around 124bp and the 'normaliser' should appear around 132bp. However, a sizing accuracy of +/-5% is acceptable for these two peaks.

An example data set for the calibrants based on the serial dilution series from the 10% *T. aestivum* standard is shown in *Table 1*.

Dilution	D-genome (ng/ul)	Normaliser (ng/ul)
1	0.81	2.12
1	1.1	2.33
1	1.06	2
1	0.76	2.13
1/2	0.54	1.74
1/2	0.78	2.05
1/2	0.62	1.95
1/2	0.72	1.96
1/4	0.55	1.82
1/4	0.37	1.39
1/4	0.32	1.43
1/4	0.41	1.64
1/8	0.5	1.86
1/8	0.48	1.04
1/8	0.42	1.54
1/8	0.32	1.51
1/16	0.4	1.33
…	…	…

Table 1- Example data set for calibration curve.

The calibrants are based on seven ½ serial dilution of a 10ng/ul 10% *T. aestivum* standard. The values in the table represent the estimated concentration (ng/ul) of the D-genome and 'normaliser' PCR amplicons, using the Agilent 2100 Bioanalyzer.

As with all experimental data, it is essential that any obvious outliers should be removed prior to the analysis. A standard curve is plotted, based on plotting the log (copies of D genome) on the x-axis, against the concentration (ng/ul) on the y-axis. *Table 2* gives the relative copy numbers of both the D-genome sequence and the 'normaliser' sequence for a dilution series based upon the 10% *T. aestivum* standard.

Dilution	Copies of D genome	Copies of Normaliser
1	1	21
½	0.5	10.5
¼	0.25	5.25
1/8	0.125	2.625
1/16	0.0625	1.3125
1/32	0.03125	0.65625
1/64	0.015625	0.328125
1/128	0.0078125	0.1640625

Table 2- The ratio of the D-genome copies to the normaliser copies based on the stock and serial dilution series of the 10% T. aestivum standard.

Excel is instructed to draw a calibration curve based on a simple linear unweighted regression, and the correlation coefficient (R^2) and the equation of the line are displayed on the graph. An example calibration curve for the D-genome is shown in *Figure 12*.

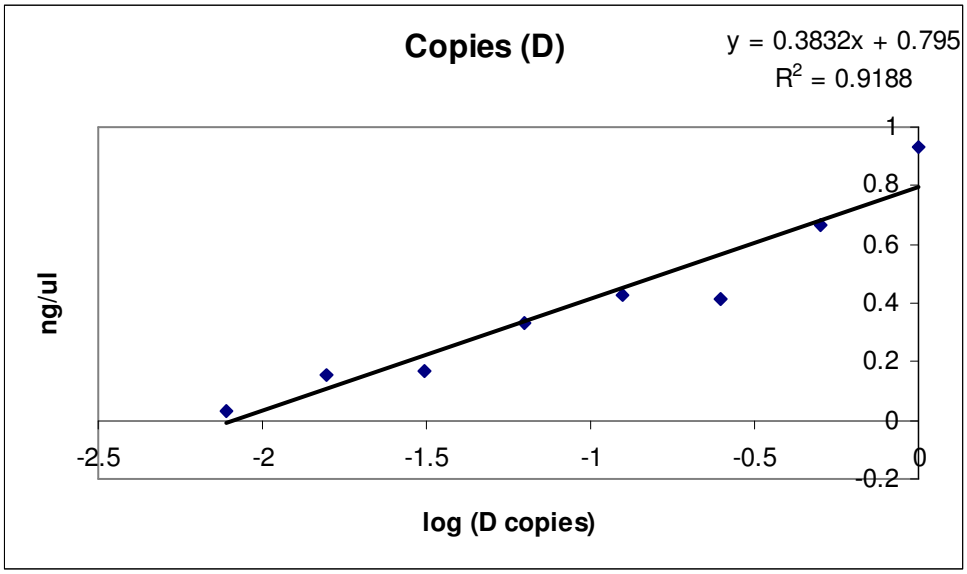

Figure 12 - D-genome calibration curve based on serial dilution series of the 10% T. aestivum standard.

The x-axis represents the logarithm of the copy numbers of the D-genome from *Table 2* and the y-axis represents the estimated concentration of the calibrants determined by the Agilent 2100 Bioanalyzer.

This process is then repeated for the 'normaliser' amplicon.

As detailed in Appendix 3, the % *T. aestivum* adulteration of the sample unknowns is estimated as follows:

- For each dilution of the sample unknown, calculate the average concentration (ng/ul) of the D-genome and the 'normaliser' for the sample unknown.
- Using the respective calibration curves, estimate the copy numbers for the D-genome and the 'normaliser'.
- Calculate the % *T. aestivum* contamination by:
  = 200 / ((Normaliser copies – D-copies)/D-copies)

A worked example is shown below: (see Appendix 3)

For a "sample unknown" (that contains 10% *T. aestivum*), the average D-genome peak concentration is 0.865 ng/ul, and the average 'normaliser' peak concentration is 2.52 ng/ul, at the ½ serial dilution.

The regression line for the D-genome calibration curve is y = 0.4947x + 0.9539, where y is the ng/ul of the D-genome peak concentration, and x is the logarithm of the number of copies of the D-genome. Based on a y value of 0.865 ng/ul, the number of D-genome copies is calculated as 0.66.

Similarly, the regression line for the 'normaliser' calibration curve is y = 0.9424x + 1.3993. Based on a y value of 2.52 ng/ul, the number of 'normaliser' copies is calculated as 15.55.

The equation 200 / ((normaliser copies – D-copies)/D-copies) is then used to calculate the % *T. aestivum* adulteration of the sample unknown, based on 'normaliser' copies = 15.55, and D-copies = 0.66. This is evaluated as 8.88%

3.5 Method verification

3.5.1. A comparison of the real-time and Agilent Bioanalyzer methods.

To provide proof of principle for the approach described above to determine the *T. aestivum* adulteration present in an unknown sample, standards and samples were amplified using PCR on two 96 well micro-titre plates. The first PCR plate consisted of a full set of the 10% *T. aestivum* standards (stock, and dilutions of ½, ¼, 1/8, 1/16, 1/32, 1/64, 1/128) and sample unknowns 180, 3% and 5% (at dilutions of stock, ½, ¼, 1/8 and 1/16). The second PCR plate also had a full set of 10% *T. aestivum* standards, with sample unknowns PQC, 318 and 319. Within each plate, each dilution of a sample/standard was replicated four times.

Results for the standards for the first PCR plate are shown in *Table 3*, the values in the table refer to the estimated concentration of the D-genome or 'normaliser' from the Agilent Bioanalyzer.

10% *T. aestivum* Dilution	D-genome ng/ul	normaliser ng/ul
1	0.88	2.61
1	1.19	2.86
1	1.1	2.52
1	1.15	2.5
1/2	0.82	2.48
1/2	1	2.75
1/2	0.85	2.21
1/2	0.79	2.65
1/4	0.52	1.74
1/4	0.46	1.86
1/4	0.49	2.1
1/4	0.47	1.81
1/8	0.38	2.23
1/8	0.47	2.04
1/8	0.28	1.81
1/8	0.33	1.67
1/16	0.51	1.6

1/16	0.3	1.31
1/16	0.28	1.36
1/16	0.25	1.29
1/32	0.26	1.21
1/32	0.58	1.24
1/32	0.36	1.23
1/32	0.61	1.22
1/64	0.04	0.92
1/64	0.33	0.96
1/64	0.66	1.2
1/64	0.11	0.66
1/128	0.05	0.76
1/128	0.05	0.3
1/128	0	0.64
1/128	0.28	0.73

Table 3- Raw data for the calibration curve associated with Plate 1.

The calibrants are based on seven ½ serial dilutions of a 10ng/ul 10% *T. aestivum* standard. The values in the table represent the estimated concentration (ng/ul) of the D-genome and 'normaliser' PCR amplicons, using the Agilent 2100 Bioanalyzer. Values highlighted in blue were regarded as outliers, and removed from further analysis. Values highlighted in yellow were also below the stated quantification range of 0.1 ng/ul of the Agilent 2100 Bioanalyzer, and were also removed.

Results for the sample unknowns for evaluation are displayed in *Table 4*, in a similar fashion:

Sample unknown	dilution	D-genome ng/ul	'normaliser' ng/ul
180	1	1.67	2.02
180	1	1.29	1.6
180	1	1.2	1.57
180	1	1.4	1.65
180	1/2	1.14	1.31
180	1/2	0.75	1
180	1/2	1.1	1.27
180	1/2	1.19	1.39
180	1/4	0.81	0.92
180	1/4	0.68	0.85
180	1/4	0.91	1.13
180	1/4	0.93	0.87
180	1/8	0.74	0.84
180	1/8	0.53	0.94
180	1/8	0.55	0.96
180	1/8	0.72	0.81
180	1/16	0.8	0.86
180	1/16	0.74	0.78
180	1/16	0.48	0.85
180	1/16	0.61	0.67
3%	1	0.38	2.69
3%	1	0.27	2.58
3%	1	0.35	2.68
3%	1	0.48	3.06
3%	1/2	0.23	2.88
3%	1/2	0.23	2.24
3%	1/2	0.21	2.04
3%	1/2	0.25	2.44

3%	1/4	0.25	2.46
3%	1/4	0.36	2.79
3%	1/4	0.21	2.42
3%	1/4	0.24	2.56
3%	1/8	0.13	1.91
3%	1/8	0.08	1.97
3%	1/8	0.1	2.14
3%	1/8	0.13	1.64
3%	1/16	0.04	1.56
3%	1/16	0.16	2.14
3%	1/16	0.05	1.56
3%	1/16	0.13	1.92
5%	1	0.85	2.93
5%	1	1.18	2.92
5%	1	0.99	2.93
5%	1	0.9	3.03
5%	1/2	0.55	2.36
5%	1/2	0.56	2.33
5%	1/2	0.73	2.21
5%	1/2	0.52	2.33
5%	1/4	0.5	2.42
5%	1/4	0.51	2.86
5%	1/4	0.61	2.68
5%	1/4	0.45	2.32
5%	1/8	0.33	2.44
5%	1/8	0.45	2.03
5%	1/8	0.31	1.74
5%	1/8	0.66	2.36
5%	1/16	0.33	1.81
5%	1/16	0.33	1.99
5%	1/16	0.34	2.31
5%	1/16	0.48	1.77

Table 4 - Raw data for the sample unknowns associated with Plate 1.

The values in *Table 4* represent the estimated concentration (ng/ul) of the D-genome and normaliser PCR amplicons, using the Agilent 2100 Bioanalyzer, for the three sample unknowns. As for *Table 3*, the values highlighted in blue were regarded as outliers, and removed from further analysis. Values highlighted in yellow were also below the stated quantification range of 0.1 ng/ul of the Agilent 2100 Bioanalyzer, and were also removed.

The concentration (ng/ul) of the D-genome and 'normaliser' were plotted against the logarithm of the respective copy numbers, according to *Table 2*. The resultant calibration curves are shown in Figure 13 and Figure 14.

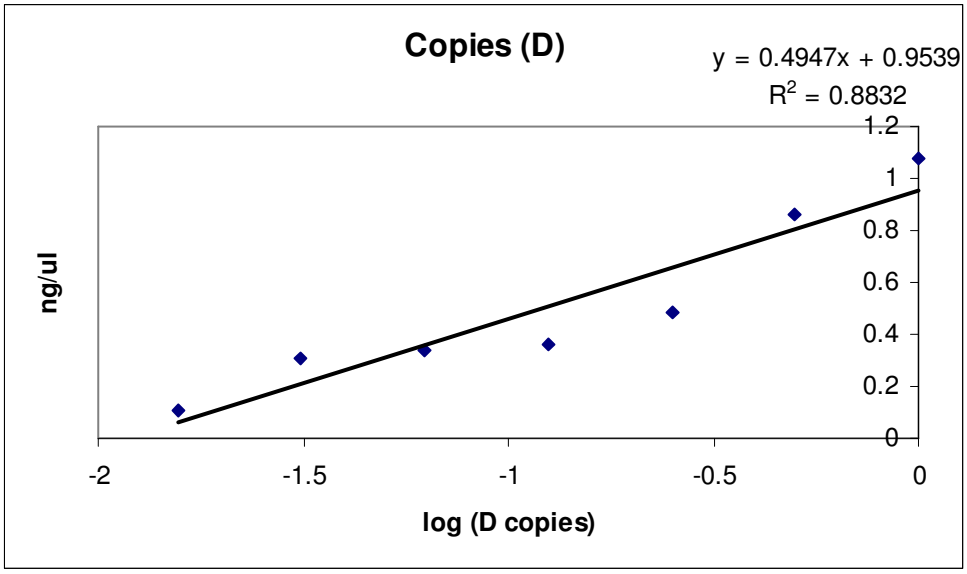

Figure 13 - D-genome calibration curve based on serial dilution series of the 10% T. aestivum standard (Plate 1).

The x-axis represents the logarithm of the copy numbers of the D-genome from *Table 2* and the y-axis represents the estimated concentration of the calibrants, from the Agilent 2100 Bioanalyzer.

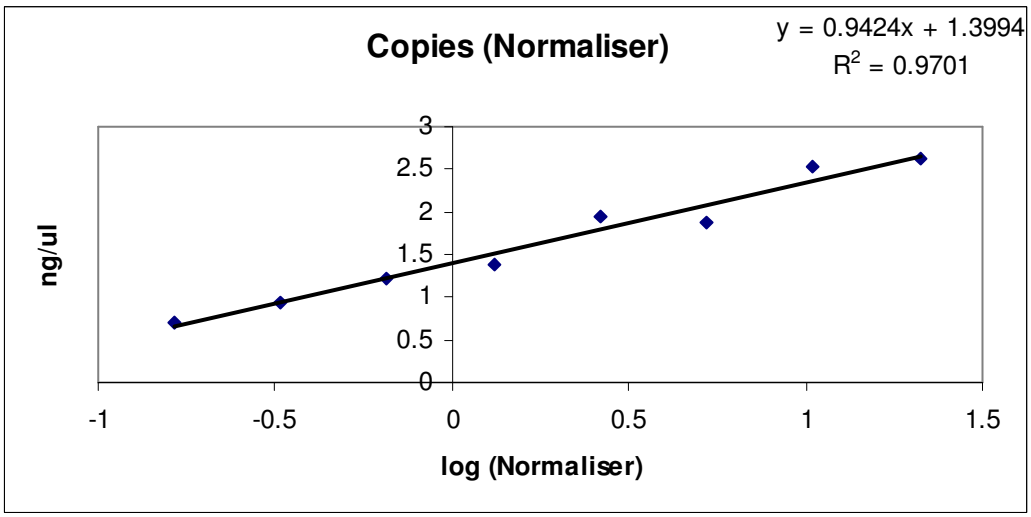

Figure 14 - 'Normaliser' calibration curve based on serial dilution series of the 10% T. aestivum standard (Plate 1).

The x-axis represents the logarithm of the copy numbers of the normaliser from *Table 2*. The y-axis represents the estimated concentration of the calibrants, from the Agilent 2100 Bioanalyzer.

The data from Plate 2 were also evaluated using a similar approach, and the raw data can be seen in *Table 8* and Table 9 (Appendix 5), along with the respective calibration curves shown in *Figure 166* and *Figure 177*. Using the procedure outlined above, the results in
Table *5* were obtained for an evaluation of sample unknowns (and the 10% standard, included as a control).

Plate	Sample	Dilution	Estimated % *T. aestivum*	Expected % *T. aestivum*
1	5%	1	3.79	5
		1/2	4.08	5
		1/4	2.48	5
		1/8	2.99	5
		1/16	3.33	5
1	10%	1	19.92	10
		1/2	8.88	10
		1/4	7.27	10
		1/8	3.53	10
		1/16	10.95	10
1	3%	1		3
		1/2	0.89	3
		1/4	0.58	3
		1/8	1.18	3
		1/16	2.36	3
1	180	1	1.11	24.7
		1/2	262.92	24.7
		1/4	-471.92	24.7
		1/8	760.10	24.7
		1/16	-1930.59	24.7
2	10%	1	20.76	10
		1/2	8.01	10
		1/4	5.01	10
		1/8	7.03	10
		1/16	12.48	10
2	318	1		2
		1/2	0.22	2
		1/4	0.22	2
		1/8	0.61	2
		1/16	3.52	2
2	319	1	1.10	2.7
		1/2	0.71	2.7
		1/4	1.16	2.7
		1/8	4.06	2.7
		1/16	9.64	2.7
2	PQC	1	0.44	3
		1/2	1.14	3
		1/4	0.35	3
		1/8	6.78	3
		1/16	0.68	3

Table 5 - Evaluation of the % T. aestivum adulteration of the sample unknowns.

The % *T. aestivum* adulteration present in the sample unknowns was evaluated across both plates using the original approach. The first column specifies which plate the sample unknown was based on. The second column specifies the sample unknown, and the third column shows the dilution factor. The fourth column shows the estimated adulteration using the original approach described in this report, and the fifth column shows the expected adulteration based on real-time PCR.

Plate 1 results

- Based on an estimation of the 5% sample unknowns were relatively encouraging. Across the range of dilutions used for evaluation, the estimated values varied from 2.48% to 4.08%.

- The 10% *T. aestivum* standard was also used as a positive control to check that calculations were being performed correctly. The variability across the range of dilutions was very large between 3.53 to 19.92 percent. The most accurate evaluation appeared to be associated with the ½ dilution, giving a value of 8.88 percent.

- The evaluation of the 3% flour sample was very imprecise, and ranged from 0.58 to 2.36 percent, always underestimating the correct *T. aestivum* concentration.

- The 180 pasta sample had an estimated value of 24.7% *T. aestivum* content based on the real-time PCR evaluation. Using the approach described here gave non-sensical results varying from -1930.59 to 760.10 percent.

Plate 2 Results

- Using a 10% *T. aestivum* standard as a positive control. The results varied significantly, between 5.01 and 20.76, with the best evaluation again being associated with the ½ dilution giving an estimated value of 8.01 percent.

- The 318 pasta sample had an assigned value of 2%, but the approach described here estimated the content between 0.22 and 3.52 percent. Equally, the value of the 319 pasta sample varied between 0.71 and 9.64 percent, whilst the assigned value was 2.7%.

- Finally, the pasta PQC sample (assigned value of 3%), gave values varying between 0.36 and 6.78 percent, depending upon the dilution of the sample unknown used.

Overall, the approach described above did not appear to evaluate the sample unknowns correctly, and cannot be considered “fit for purpose” for quantitative determinations. The results are improved when only the ½ serial dilution of the sample unknown is used in the evaluation (based on 25ng of DNA per PCR reaction) but the results remain poor.

It is suspected that the very poor accuracy associated with this approach may be due to the non-linearity associated with the D-genome calibration curve (with an r-squared of 0.88 for plate 1, and 0.89 for plate 2). Whilst the ‘normaliser’ calibration curve was linear across its entire range (an average r-squared of 0.98), the D-genome curve exhibited curvature at around the ¼ and 1/8 dilutions. The approach described was based upon an emulation of real-time PCR, where the PCR cycle number was reduced from 45 to 39 so that PCR product formation was prevented from entering the plateau phase, while retaining the amplification within the log-linear phase. If this had been achieved then there would be a clear correlation between amount of PCR product formed and the amount of DNA used as a starting template. Whilst this hypothesis appears to hold well for the ‘normaliser’, it does not appear to

be the case for the D-genome. Thus, transferring a real-time PCR system onto the Agilent CE system is not a straightforward situation.

While the above approach is unsuitable for quantitative purposes the assay does give an indication as to the relative content of *T. aestivum* DNA present in a sample, and may be thus useful for screening or semi-quantitative purposes. For example, *Table 6* shows the assigned values of the % *T. aestivum* content of the samples, whose order is the same as the order for the estimated contents, based on the results obtained from the ½ dilutions:

Rank	Sample	Assigned *T. aestivum* content (%)	Estimated *T. aestivum* content (%)
1	318	2	0.22
2	319	2.7	0.71
3	3%	3	0.89
4	PQC	3	1.14
5	5	5	4.08
6	10	10	8.88
7	180	24.7	262.92

Table 6 - Rank order of sample unknowns according to estimated adulteration.

The rank order of the sample unknowns according to the assigned and estimated % *T. aestivum* adulteration is the same, for the ½ dilutions. Thus, it may be possible to use the above approach as a screening or semi-quantitative assay.

Having reviewed the data and the assumptions made in trying to emulate the real-time method we were disappointed by the outcome. This has led to the exploration of the same raw data generated in an attempt to find a different approach to evaluate sample unknowns based on construction of a single calibration curve. This single calibration curve approach is now suggested as an alternative method with the potential of delivering a quantitative result.

3.6 An alternative approach

3.6.1 Alternative method description

The 3, 5 and 10% *T. aestivum* flour samples are treated as standards, and used to produce the calibration curve. Only the ½ serial dilutions are used for this purpose (equivalent to 25ng of DNA per PCR reaction). The average ng/ul of the D-genome and ‘normaliser’ are calculated from the raw data files. A “Ratio (D:N)” is then calculated as the D-genome concentration, divided by the ‘normaliser’ concentration *Table 7*

Standard (%)	log (% adulteration)	D-genome ng/ul	Normaliser ng/ul	Ratio (D:N)
3	0.48	0.23	2.40	0.10
5	0.70	0.59	2.31	0.26
10	1	0.86	2.52	0.34

Table 7- Table for the calibration curve for the alternative approach.

The 3, 5 and 10% *T. aestivum* standards are used to generate the calibration curve. The average estimated concentration (ng/ul) of the D-genome and 'normaliser' amplicons are shown in the table, along with the ratio of the two.

For each of the three standards, the logarithm of the *T. aestivum* percentage is plotted against the Ratio (D:N) and a linear calibration curve applied as before (*Figure 15*).

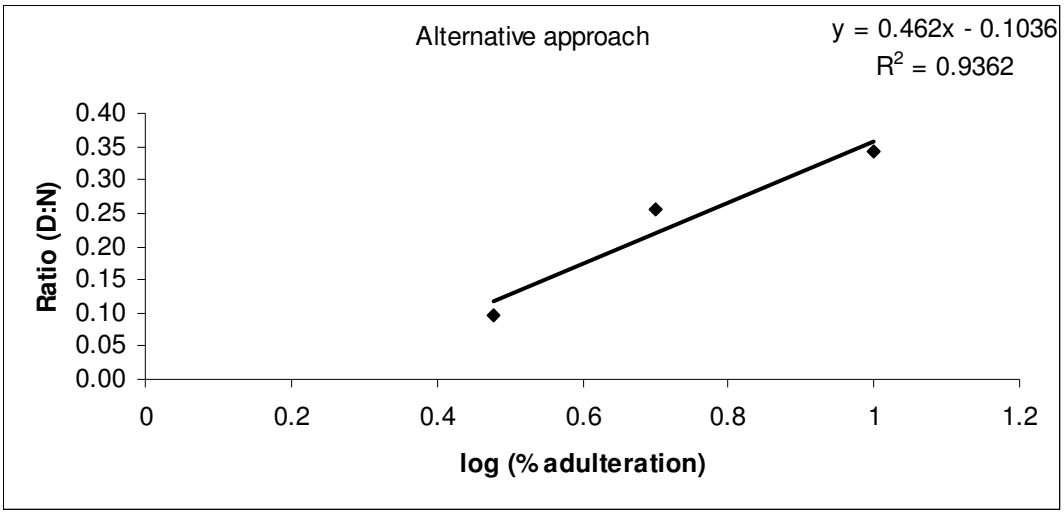

Figure 15 - Calibration curve using the alternative approach.

The logarithm of the % *T. aestivum* adulteration is shown on the x-axis, whilst the y-axis shows the ratio of the D-genome to the 'normaliser'. The above calibration curve is based on three standards, with four replicates per standard. A linear fit to this data was shown to be statistically better than a quadratic derived

3.6.2 Analysis of test samples

For the sample unknowns at the ½ dilution (25ng of DNA), the average concentration (ng/ul) of the D-genome and 'normaliser' are tabulated. The Ratio D:N is then calculated based on the D-genome concentration divided by the 'normaliser' concentration. The % *T. aestivum* content of the sample unknown is then solved using the regression equation from the calibration curve: $y = 0.462x - 0.1036$, where y is the Ratio D:N for the sample unknown, and x is the logarithm of the % *T. aestivum* content. The results from the raw data generated in this project, are shown in

Sample	Ratio (D:N)	% adulteration estimated	% adulteration expected
180	0.84	107.88	24.7
318	0.05	2.15	2
319	0.08	2.48	2.7
PQC	0.22	4.97	3
3%	0.10	2.72	3
5%	0.26	6.02	5
10%	0.34	9.32	10

Table 8 - Evaluation of the % T. aestivum adulteration of the sample unknowns.

An evaluation of the % *T. aestivum* adulteration of the sample unknowns across both plates using the alternative approach is shown above where the third column indicates the estimated adulteration using this alternative approach and the fourth column the expected adulteration based on real-time PCR.

In summary:-

- Flour samples containing 3, 5 and 10% adulteration (also used as standards) gave estimated values of 2.72, 6.02, and 9.32 percent respectively.

- The PQC pasta sample was estimated as containing 4.97% *T. aestivum*, whilst its value obtained when analysed by real-time PCR is 3%.

- Pasta sample 310 when analysed by real-time PCR gave a value of 2.7%, using this alternative approach gave an estimated value of 2.48%.

- When analysed by real-time PCR, pasta sample 318 gave a value of 2%, and when analysed using the alternative approach gave an estimated value of 2.15%.

- The pasta sample 180 remains a problem with an estimated value of >100% *T. aestivum* content.

It should be noted that for the evaluation of pasta samples 318 and 180) the ratio D:N for the two samples lie outside the linear working range of the current calibration curve. The % *T. aestivum* for these samples was obtained by extrapolation. Hence, interpretation of these results must be treated with less confidence.

Although the reliability and usefulness of this alternative approach should be investigated by using a wider range of standards for the calibration curve, at this early stage it appears to be relatively “fit for purpose” in terms of screening, semi-quantitative, and quantitative use. A potential advantage of this alternative approach would be that it uses the reliable values obtained from the D-genome calibration curve at ½ dilution.

Based on the usefulness of this alternative approach for estimation of *T. aestivum* concentration in adulterated pasta, the method described can

provide some guidance on when a sample is likely to have more than the legislative limit of 3% *T. aestivum*.

3.6.3 Analytical Uncertainty

Using the ½ dilution, the mean ratio D:N based on four replicates of the 3% *T. aestivum* standard is 0.10, with an associated standard deviation of 0.011. Based on three degrees of freedom as an appropriate coverage factor for measurement uncertainty, this translates to the 3% *T. aestivum* standard having a mean ratio D:N value of 0.10 +/- 0.018, covering a 95% confidence interval. Thus, a sample whose ratio D:N is less than 0.08 (i.e. 0.1 – 0.18) is likely to have less than 3% *T. aestivum* present. With regard to table 8, this would classify pasta samples 318 and 319 as having less than 3% *T. aestivum* content, which appears to hold true.

4.0 Conclusions

1. The original real-time PCR amplicons could not be separated using the Agilent LabChip 2100 or the comparable Caliper instrument.

2. Following a redesign of the original normalisation amplicons, the two new amplicons (D-genome (121bp) and normalisation (129bp)) were readily separated by the LabChip instrument.

3. It is apparent that the 'normalisation' peak, (used to detect all wheat present in the sample) exhibits heterogeneity upon fractionation, most probably as a result of heteroduplex formation.

4. Due to the extreme sensitivity of the amplification system and the general prevalence of wheat in the environment, it was necessary to instigate a range of extreme measures to eliminate any DNA contamination and DNA carry over from one analysis to another.

5. We were able to design the analysis in a multiplex format thus reducing costs and simplifying the analysis compared to the simplex PCR carried out for the real-time PCR method.

6. It was possible to establish linear copy number calibration curves for each of the D-genome and 'normaliser' amplicons.

7. The concentration range of the template DNA applicable to the LabChip instrument is comparable to that used for real-time PCR.

8. The optimum number of PCR cycles prior to LabChip analysis is 39.

9. It is essential that all data generated by the Bioanalyzer is within the linear working range of the instrument i.e. the amount of amplicon present is not below the LOD or above that which can be reliably quantified.

10. It is evident that the 'conventional' wisdom and methods of real-time PCR cannot be transferred directly to the LabChip. The two techniques use different processes to derive results and it is clear that there is no immediate relationship between the Ct of 'real-time' PCR and the amount of amplicon accumulation as measured by the LabChip.

11. The analysis based upon copy-number generates results that seem very inaccurate. This is most probably due to sensitivity issues with the Agilent Bioanalyzer. The issue of sensitivity appears to originate with the low copy number of the D-genome sequences present in each of the samples.

12. Due to the lack of proportionality between Ct and amplicon accumulation (as measured by the Labchip) it was necessary to investigate an alternative quantification method. The alternative, based upon the amplicon accumulation from co-analysed reference materials, gives a practical solution to the problems of quantification. While this approach yield significantly better results, it should only be deemed to be semi-quantitative at present.

5.0 Recommendations

1. A real-time PCR method for the detection of adulteration of *T. durum* samples can be transferred to the Agilent CE system in conjunction with traditional PCR using a copy number approach. However, it is recommended that this approach be used only for semi-quantitative estimation of adulteration.

2. The approach detailed in the associated SOP provides a working method and proof of principle for the quantitative nature of estimating the percentage adulteration associated with samples. This alternative approach used the same data set as the above method, but employed a different approach to data handling. It is recommended that this approach be considered for quantification of adulteration of *T. durum* samples.

3. The real-time PCR method remains the most accurate approach for quantiation of wheat samples, but the greater availability of the Agilent Bioanalyzer method to Public Analysts will greatly assist the detection of mis-described pasta products, protecting and supporting consumer preferences.

4. Transfering real-time PCR methods onto the Agilent Bioanalyzer may result in the necessity to re-design assays to take into account equipment specification associated with the Agilent system. For example, the limited throughput of 12 sample wells per chip on the Agilent, may mean that singleplex PCR assays may have to be re-designed as multiplex assays, which in turn potentially involves re-optimisation of PCR conditions (e.g. magnesium and primer concentrations). Additionally, characteristics such as assay specificity (lack of real-time PCR probes), and resolving power of the CE system in order to separate closely sized DNA amplicons, must be considered.

5. Due to the extreme sensitivity of the amplification system and the general prevalence of wheat in the environment, it was necessary to instigate a range of extreme measures to eliminate any DNA contamination and DNA carry over from one analysis to another.

6. An alternative approach for detecting and measuring the amount of adulteration in *T. durum* samples has been provided in the SOP. This description provides proof of principle of the quantitative nature of the approach. However, as is common with all initial methods resulting from research and development, we would recommend that this approach be fully validated by providing objective evidence of the performance characteristics associated with this method. To provide the “fitness for purpose” of the detailed approach in terms of being used for quantitation of the amount of adulteration in wheat, we would advocate that the performance characteristics of trueness, precision, biological sensitivity and specificity, and measurement uncertainty, be evaluated. In particular, we would advocate that the range of standards used for calibration be increased, (e.g. 1, 2, 3, 5, and 10% adulteration standards be included), the level of replication of the samples be increased up to six, and the reproducibility of the approach be verified by application to additionally generated data sets.

7. That the FSA fund a training course in conjunction with Agilent and ourselves to train Public Analysts in the method.

6.0 Acknowledgements

We would like to thank Dr Pierre Devaux of Florimond Desprez, B.P. 41-59242 Cappelle-en Pévèle, France, for his generosity in providing accredited samples of *T. durum* used in the study.

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8.0 Appendices

8.1 Appendix 1- Standard Operating Procedure (SOP)

FOOD STANDARDS AGENCY

STANDARD OPERATING PROCEDURE (SOP)
VERSION 1.0, March 2008

Detection and quantification of *T. aestivum* addition in *T. durum* pastas and semolinas by PCR followed by analysis using the Agilent 2100 Bioanalyzer capillary electrophoresis system.

Prepared by Dr G. Wiseman & Dr M. Burns **Date** 18th March 2008

Approved by _____ **Date**

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2. HISTORY/BACKGROUND

Pasta manufactured from durum wheat which is intended for export outside the EU may contain a maximum of 3% common wheat (EC Commission Regulation (1994) 1222/94 (Annex C), Official Journal of the European Community L136, 31 May 1994, page 5 and International Standard (ISO 11051:1994 (E)), to allow for adventitious cross contamination during the agricultural process. Pasta containing *T. aestivum* must also not be mis-described on the product label (UK Food Labelling Regulations 1996 (SI No. 1499). These legal requirements were examined by the Steering Group on Chemical Aspects of Food Surveillance in 1995 which examined the authenticity of dried durum wheat pasta in the UK using Acid Polyacrylamide Gel Electrophoresis (Acid PAGE). Following this survey a number of research projects were funded by MAFF/FSA, to improve the analytical methods used to detect *T. aestivum* adulteration of *T. durum* pastas. These projects, 2A015, AN0667 and Q01085 demonstrated that it is possible to address the problems associated with the detection and quantification of common wheat (*T. aestivum*) adulteration of *T. durum* pastas by analysing DNA extracted from the pasta. The resulting method employed the Polymerase Chain Reaction (PCR) to amplify two small sequences of DNA. One sequence is present in the D-genome of *T. aestivum* and is indicative of adulteration, the other is present in all three genomes (A, B & D) and can be used to 'normalise' the signal obtained and can therefore be used to determine the level of adulteration present. These two reactions are performed using real-time PCR which employs a fluorescence-based detection mechanism. The results obtained were considered to have a lower estimate of analytical uncertainty than the previous methods and that this new method was adequate to enforce the 3% limit of *T. aestivum* adulteration. Recently, other more rapid and cheaper analytical methods such as the LabChip have been devised which could quicken the analytical process. This Chip–based capillary electrophoresis (CE) instrument is highly adaptable and has gained favour with Public Analyst laboratories. Hence, the method used to detect *T. aestivum* adulterated pasta has been transferred to this instrument to make it more accessible and applicable to routine testing by Public analysts.

3. PURPOSE

The purpose of this SOP is to minimise deviation from the analytical protocol ensuring that the benefits gained from the development of the real-time method can be maximised by using the LabChip CE to analyse the PCR products formed in the initial part of the method. This will allow Trading Standards Officers to examine more pasta products in the course of their work while minimising the analytical costs, effecting a better protection for the public against those wishing to defraud.

4. SCOPE

This method is applicable to the semi-quantitative analysis of *T. aestivum* present in dried pasta, fresh, chilled or frozen pasta and ground mixtures of *T. durum* and *T. aestivum* grains and to their flours (semolinas).

5. DEFINITIONS AND ABBREVIATIONS

LabChip: a capillary electrophoresis system that facilitates fractionation and detection of DNA (or proteins) from 12 samples within a small cassette (chip)

PCR-Polymerase Chain Reaction

T. durum : tetraploid wheat, also called macaroni or pasta wheat (AABB)

T. aestivum : hexaploid wheat, also called common or bread wheat (AABBDD)

SDW : Sterile distilled water

6. PRINCIPLE OF THE METHOD

The basis of this method derives from projects, 2A015, AN0667 and Q01085 which identified a short (121bp) single copy DNA sequence specific to the D-genome of *T. aestivum* and a short (117bp) single copy DNA sequence that was present in all three wheat genomes (A,B & D). This common DNA sequence can be used to 'normalise' the generation of PCR product following DNA extraction from an analytical sample and contributing to the determination of the amount of *T. aestivum* present in the sample. These amplification reactions are carried out in simplex format to prevent non-stoichemometric accumulation of product and have until recently been performed by real-time PCR. In this variant of the original method, the size of the 'normalising' PCR product present in all three genomes has been changed to 129bp to allow separation by capillary electrophoresis using the LabChip instrument, and the reactions are carried out in PCR duplex format (both reactions carried out in the same vessel). One further change is that the PCR is terminated at 39 cycles, which ensures that amplification is still within the linear range for the production of PCR amplicon. It is essential that a detectable amount of PCR product is generated in the PCR as the LabChip uses UV-light to detect and quantify the amount of material in each peak present in the fractionated material. The relative amounts of the two PCR products (expressed in ng) are used to calculate the amount of *T. aestivum* present in the analytical sample.

7. MATERIALS AND EQUIPMENT

7.1 DNA extraction materials and equipment

Disposable, powder free, nitrile gloves (for example -Fisher p/n SAR-690-070A)

A top pan balance (reading to at least 0.01g)

A pH meter (calibrated at pH 7.0 and 10.0)

Pipette - for example (Eppendorf Biopur Multipette (Fisher p/n PMP-117-011R))

Disposable pipette tips (for example Eppendorf Biopur Multipette 10ml

tips (Fisher p/n PMP-117-523N))	
Plastic Pasteur pipettes (for example -Fisher p/n PMK-400 065A)	
waterbath (set at 65°C ± 2°C)	
Waterbath (set at 35°C ± 1°C)	
Centrifuge (to spin 8 x 50ml tubes at 12,096 x g, 20°C or 6 x 300ml pots at 12,096 x g, 20 - 30°C) (for example -MSE mistral 2000)	
Centrifuge (to spin 4 x 30ml bottles or 16 x 13ml test tubes at 2000rpm, room temperature) (for example - MSE mistral 2000)	
50ml polypropylene tubes, caps and racks (for example - Fisher p/n 05-539-7)	
30ml polystyrene bottles, caps and racks (for example - Fisher p/n DIS-080-100Q 128A)	
13ml polypropylene test tubes, caps and racks (for example - Camlab p/nPS/T406-2A)	
Measuring cylinders	
Magnetic stirrer	
Freeze drier (for example-Edwards-Super modulyo)	
Aluminium foil	
Ethylenediaminetetraacetic acid (EDTA) (for example - Sigma 99+%-E9884)	Harmful
Trizma (pre-set crystals pH 8.0) (for example - Sigma reagent grade-T8443)	Irritant
Trizma (pre-set crystals pH 8.3) (for example - Sigma reagent grade-T8943)	Irritant
2M sodium hydroxide solution (BDH -31959-445, volumetric solution)	Corrosive
Sodium chloride (for example - Sigma Ultra-S-7653)	
Sodium dodecyl sulphate (for example - BDH-product 4444647)	Harmful
Sodium acetate trihydrate (for example - Sigma-S-9513)	

Proteinase K (mandatory - Promega cat no V 3021), 100mg	Irritant
Ribonuclease A (mandatory- Sigma molecular biology reagent) cat no-R-6513	Irritant
1M hydrochloric acid (BDH-32050-602,volumetric solution)	Harmful
Isoamyl alcohol (for example - Sigma molecular biology reagent-I9392)	Harmful
Chloroform (for example – Fisher Scientific product C/4960/17)	Harmful
Propan-2-ol (for example – Fisher Scientific product P/7500/PC17)	Harmful, Flammable
Absolute ethanol (for example – Fisher Scientific product E/0650DF/17)	Flammable
Phenol (mandatory-Sigma 99+%, p/n-P1037, 500g)	Toxic,Causes burns
Deionised Water	
Liquid Nitrogen (BOC food grade)	Cryogenic liquid
Sodium hypochlorite solution (for example- Sigma 23,930-5)	Toxic, corrosive

7.2 DNA extraction reagents

7.2.1 S-Buffer

Dissolve 18.61g ± 0.01g EDTA in 700ml water, measured in a measuring cylinder, in a 1l beaker. Use a magnetic stirrer to assist dissolution. Once dissolved add 2M NaOH solution to adjust the pH to 8.0 ± 0.2. If the pH becomes too alkaline, adjust with 1M hydrochloric acid. Add to this solution 13.54g ± 0.01g Tris (pre-set crystals pH 8.3) and 5.84g ± 0.01g NaCl. Finally add 20g ± 0.10g sodium dodecyl sulphate. Stir, and gently heat if necessary, until dissolved. Transfer to a 1l measuring cylinder and make up to 1l with deionised water. Transfer to a labelled reagent bottle. Store at room temperature. Stable 2 months.

7.2.2. TE Buffer

Dissolve 0.37g ± 0.01g EDTA in 700ml water, measured in a measuring cylinder, in a 1l beaker. Use a magnetic stirrer to assist dissolution. Adjust the pH to 8.0 ± 0.1 with 2M NaOH solution. If the pH becomes too alkaline, adjust with 1M hydrochloric acid. Add to this solution 1.41g ± 0.01g Tris (pre-set crystals pH 8.0). Once dissolved, transfer to a 1l measuring cylinder and make up to 1l with deionised water. Transfer to a labelled reagent bottle. Store at 2 - 5°C. Stable 2 months

7.2.3. Proteinase K (10mg/ml)

Using an autopipette add 10ml sterile deionised water to a 100mg bottle of proteinase K. Cap the bottle and gently mix by inversion until the material has visibly dissolved. Aliquot into 1ml tubes and store below -10°C. Stable 1 year

7.2.4 Ribonuclease A

Using an autopipette add 10ml sterile deionised water to a bottle of 50mg ribonuclease A. Cap the bottle and mix gently by inversion until the material has visibly dissolved. Aliquot into 1ml tubes and store below -10°C. Stable 1 year.

7.2.5 Chloroform : isoamyl alcohol

Using a measuring cylinder, pour 10ml isoamyl alcohol and 240ml chloroform into a reagent bottle. Cap the bottle and swirl gently to mix. Store at room temperature. Stable 2 months.

7.2.6 Chloroform-phenol

Use a measuring cylinder to decant 500ml TE buffer into a bottle of 500g phenol. Leave to equilibrate. Transfer to a 1l measuring cylinder and leave until the two layers have separated. Note the volume of the bottom layer and use a measuring cylinder to decant an equal volume of chloroform : isoamyl alcohol into a 2l brown glass bottle. Add the phenol-TE mixture, shake well to mix and allow the layers to separate before use. Store the chloroform-phenol under the layer of TE buffer. Store at 2 - 5°C. Stable 2 months. Do not use if the phenol layer is coloured.

7.2.7 70% ethanol

Using a measuring cylinder, transfer 350ml absolute ethanol into a reagent bottle. Using a separate measuring cylinder, add 150ml deionised purified water to the ethanol. Mix well and store at room temperature. Stable indefinitely.

7.2.8 3M sodium acetate solution

In a beaker, dissolve 204.12g ± 0.10g sodium acetate trihydrate in approximately 400ml deionised water. Transfer to a measuring cylinder and dilute to 500ml with water. Store in a reagent bottle at room temperature. Stable 2 months.

7.3 DNA purification materials and equipment

Promega Wizard® DNA clean-up system (mandatory - Promega p/n A7280) Harmful

100µl positive displacement pipettes and tips (mandatory - Anachem p/n F148314)

1000µl positive displacement pipettes and tips (mandatory - Anachem p/n F148180)

Plastic Pasteur pipettes (for example - Fisher p/n PMK-400 065A)

1.5ml microcentrifuge tubes (for example - Anachem p/n 96.7514.9.01)

1.5ml microcentrifuge racks

Microcentrifuge (to spin 14x 1.5ml microcentrifuge tubes at 10000g, room temperature) (for example - Eppendorff 5415c)

Vacuum pump with manifold to take Wizard® minicolumns (mandatory - Promega P/N A2291)

Microwave oven (600W)

Propan-2-ol (for example-Fisher Scientific P/7500/PC17 Flammable

Deionised Water

100ml and 200ml measuring cylinders

7.4 DNA purification reagents

7.4.1 80% propan-2-ol

Using a measuring cylinder, transfer 160ml propan-2-ol into a labelled 250ml reagent bottle. Add to this 40ml deionised water, measured separately in a measuring cylinder. Mix well. Store at room temperature. Stable indefinitely.

7.4.2. Promega wizard® DNA clean-up resin

The resin is provided ready to use but must be free of crystals and aggregates. Shake the bottle well before use. If any crystals or aggregates are present, dissolve by warming at 35°C ± 2°C.

Store according to manufacturers instructions.

7.5 DNA quantification materials and equipment

Spectrofluorimeter (for example - Shimadzu RF-540)

Fluorimeter cuvettes, 4.5 ml (for example - Kartell p/n 1961)

DNA quantification kit (mandatory-Sigma p/n DNA-QF) - This kit is sufficient for 750 reactions (50 experiments of 15 reactions each, 2 ml volume)
This kit must be stored at kit at –20 ℃

7.6 DNA quantification reagents

7.7.1 DNA standard

Calf thymus DNA, 1 mg/ml solution of in 10 mM Tris HCl, 1 mM EDTA, pH 7.4 - Product No. D 48101

7.7.2 bisBENZIMIDE

bisBENZIMIDE 10 mg/ml (Hoechst 33258) solution in deionized water.

7.7.3. 10X Fluorescent Assay Buffer

Tris HCl 100 mM, 10 mM EDTA, 2 M NaCl, pH 7.

7.7 PCR materials and equipment

96 well optical plates and caps (mandatory - Abgene p/n AB0900)

1.5ml microcentrifuge tubes (for example - Anachem p/n 96.7514.9.01)

Vortex mixer

10µl positive displacement pipettes and tips (mandatory - Anachem p/n F148312)

25µl positive displacement pipettes, capillaries and tips (mandatory - Anachem p/n F148112)

100µl positive displacement pipettes and tips (mandatory - Anachem p/n F148314)

1000µl positive displacement pipettes and tips (mandatory - Anachem p/n F148180)

UV Cabinet – (for example - Herolab: Clean Cab)

Microcentrifuge – (for example - MSE Microcentaur)

Gilson Pipettes (2µl, 10µl, 20ul, 200ul and 1000µl) and appropriate Alpha tips.

1.5ml, 0.5ml and 0.2ml tubes – alpha (LW2375, LW2372, LW2130 respectively)

UV Spectrometer – (for example -Nanodrop ND1000)

Direct Thermal Tough Spots 9.5mm labels – (for example -Web Scientific Ltd, DTHE-4000)

Tube racks of various sizes – (for example - Fisher Scientific, FB71032)

Thermocycler – (mandatory - Perkin Elmer, 9700).

7.8 PCR reagents

Normalisation Primers: Forward and Reverse (Sigma) - Please see section 8.6.3 for sequence information

D-Genome Primers: Forward and Reverse (Sigma) - Please see section 8.6.3 for sequence information

Forward primer-working solution 80pM/µl

Reverse primer- working solution 80pM/µl

Standards: Working stock solutions (10ng/ul) of the following three standards:
3, 5 and 10% *T. aestivum*

Sterile distilled Water- SDW (mandatory - Sigma)

Amplitaq Gold (1U/reaction) (mandatory – Applied Biosystems)

PCR buffer (x10)-supplied with Amplitaq Gold (no Mg^{2+})

Mg^{2+} (25Mm)-supplied with Amplitaq Gold

Nucleotide mix-dNTPs (each 10mM) – (mandatory- Roche p/n 11 581 295 001)

7.9 LabChip materials and equipment

Agilent 2100 Bioanalyzer.

Agilent 2100 Expert software.

Agilent Chip priming station.

IKA vortex mixer (models MS2-S8/MS2-S9)

Microcentrifuge

Positive displacement pipettes (2µl, 10µl and 1000µl) and tips

7.10 LabChip reagents

The Agilent DNA 1000 Lab Chip Kit (p/n 5067-1504) includes enough reagents and Lab Chips to perform 25 runs:

- DNA Ladder
- DNA Marker
- Gel Matrix
- Dye
- 25 DNA Lab Chips
- 1 electrode cleaning chip

The kit contains DMSO which is a potential mutagen, therefore, it is recommended that hand and eye protection is worn.

Unless otherwise stated, all reagents and reagent mixes are stored at 4℃ in the dark when not in use to avoid photodegradation.

8. PROCEDURES

8.1 Sample preparation

8.1.1 Dried Pasta

Dried pasta (10g) should be ground using a coffee grinder being careful not to heat up the sample during the grinding process. The ground pasta must then be sieved through a 250 micron mesh. The sample then should be mixed thoroughly and stored in a labelled plastic container at 0 - 6ºC.

8.1.2 Fresh, Chilled, Tinned or Frozen pasta

Fresh, wet or chilled pasta must be washed in deionised water to remove any ‘dusting flour’ or sauce that may be present. Once washed, the pasta should be dabbed dry with laboratory roll or tissue and wrapped in aluminium foil prior to freeze drying for 12-48 hours. The sample then must be ground and treated as for dried Pasta (8.1.1)**.**

Pasta contained in tins, chilled or frozen meals (once thawed) must be washed thoroughly in several changes of distilled water till all the adhering food material is removed. The pasta then must be dabbed dry with laboratory roll or tissue, wrapped in aluminium foil prior to freeze drying for 12-48 hours. The sample then must be ground and treated as for dried Pasta (8.1.1)**.**

8.1.3 Preparation of standards

For each batch of samples it is necessary to prepare and extract *T. durum* standards containing 3%, 5 %, and 10% *T. aestivum*. Ground single species flours can be stored at -20℃ for 48 months. Flour mixtures should be prepared immediately prior to DNA extraction (8.2)

8.2 DNA extraction

a. Using an autopipette add 2x 10ml S buffer to the sample contained in a sterile polypropylene tube (50ml). DO NOT MIX. Using an autopipette, add 100µl proteinase K solution. Cap the tubes andvortex mix until the contents of the tube have thoroughly dispersed.
Carefully release any pressure from the tube by loosening the cap.

b. Place the tubes in a waterbath at 65°C ± 2°C for 90min.
Remove the tubes from the waterbath and allow to cool to room temperature. The tubes may be immersed in cold water if necessary.

c. Once cooled, use an autopipette to add 15ml chloroform-phenol to each tube. Cap the tubes and mix gently by inversion until an emulsion is formed. Carefully release any pressure from the tube before progressing.

d. Allow the tubes to stand for at least 1 minute then centrifuge at ~12,096 x g for 10 min.

e. Using a plastic Pasteur pipette transfer the top layer from each tube into a sterile polystyrene bottle (30ml). Discard the chloroform-phenol residues left in the tubes into waste containers kept in the fume cupboard.

f. Add 0.6 to 1.0 volumes of propan-2-ol to the bottles.
Cap the bottles and mix gently by inversion. Allow to stand for at least 1 minute then centrifuge at 2000 x g for 5 to 10 min, ensuring the precipitates have settled as a pellet at the bottom of the bottle. Pour off the propan-2-ol and carefully invert the bottles onto a clean piece of tissue paper to drain. If the precipitate is not firmly adhering to the bottle, remove as much of the propan-2-ol as possible using a plastic Pasteur pipette. Do not invert the bottle.

g. Use an autopipette add 5ml 70% ethanol to the bottles and swirl by hand to wash the precipitates.

h. Centrifuge the bottles again at 2000 x g for 3 to 5 min, pour off the ethanol and drain the bottles as before (see f above).

i. Pipette 5ml TE buffer into the bottles using an autopipette and allow the precipitate to dissolve with gentle inversion. Samples may be heated in a waterbath at 65°C ± 2°C for a maximum of one hour or until dissolved.

j. The samples may now be stored at 2 - 5°C for up to four days.

k. Using an autopipette add 10µl ribonuclease A solution to each sample

bottle. Use a clean tip for each sample. Cap the bottle and mix gently by hand. Place the bottles in a waterbath at 36°C ± 1°C for 60 ± 5 minutes. Remove the bottles and allow them to cool.

l. Using an autopipette, for each sample, pipette 5ml of chloroform-phenol into a 13mlpolypropylene test tube. Transfer the incubated samples from step k into the 13ml tubes containing chloroform-phenol. Cap the tube and mix gently by inversion until an emulsion is formed. Allow the tubes to stand for at least1 min then centrifuge at 2000 x g for 5 to 15 minutes, to ensure that the top layers have clarified.

m. Using a clean plastic Pasteur pipette for each sample, transfer the top layer to a fresh 13ml polypropylene test tube containing 5ml chloroform : isoamyl alcohol added by autopipette. Cap the tube and mix by inversion. Centrifuge the tube at 2000 x g for 5 to 10 minutes. Using a clean plastic Pasteur pipette for each sample, transfer the top layers to fresh 30ml polystyrene bottles. Discard the chloroform-phenol and chloroform : isoamyl alcohol residues into the waste containers in the fume cupboard.

n. Using an autopipette add 0.5ml 3M sodium acetate to each bottle solution and swirl to mix. Using an autopipette add 10ml absolute ethanol, cap the bottle and mix by inversion. Allow the bottles to stand for at least 1 minute then centrifuge at 2000 x g for 5 to 10 minutes. Pour off the ethanol and invert the bottle onto a clean piece of tissue paper to drain.

o. Using an autopipette, add 5ml 70% ethanol to each bottle to wash the residues. Centrifuge at 2000 x g for 3 to 5 minutes and drain as before (see n above).

p. Using an autopipette, add 1.8 - 2.2ml water. Allow the precipitates to dissolve. This can be aided by gentle inversion of the bottles. Samples may be heated in a waterbath at 65°C ± 2°C for a maximum of 1 hour or until the precipitates have dissolved.

q. Store the samples at 2 - 5°C ready for DNA purification.

8.3 DNA purification

a. Label one 1.5ml microcentrifuge tube per sample and using an autopipette, transfer 1ml Wizard® DNA clean-up resin into each. Taking one sample at a time, use an auto pipette to transfer 250µl of sample solutions into the microcentrifuge tubes. Use a clean tip for each sample. Mix thoroughly by inverting the tubes.

b. Attach one Wizard® minicolumn to one syringe barrel for each sample. Label the minicolumn and attach to the vacuum manifold. Using a clean plastic Pasteur pipette for each sample, transfer the sample/resin mixtures into their syringe barrels. Switch on the vacuum pump and allow the vacuum to draw the sample/resin mixtures through the minicolumns.

c. Once all of the mixtures have passed through the minicolumns, use a plastic Pasteur pipette to transfer 2ml 80% propan-2-ol into each syringe barrel. Allow the 80% propan-2-ol to pass through the minicolumns. Continue to draw a vacuum for approximately 30 seconds after the 80% propan-2-ol has passed through the minicolumns to dry them.

d. Break the vacuum to the minicolumns and switch off the vacuum pump. Discard the syringe barrels and transfer the minicolumns to clean 1.5ml microcentrifuge tubes. Centrifuge the minicolumns in their respective microcentrifuge tubes at 10,000 x g for 2 min.

e. While the miniclouns are being centrifuged heat approximately 50ml water, in a reagent bottle with a loosened cap, in the microwave oven at full power until boiling.

f. Discard the microcentrifuge tubes and transfer the minicolumns to clean, labelled 1.5ml microcentrifuge tubes.

g. Use a 100µl autopipette with a clean tip, to add approximately 80µl pre-boiled water (section e) into each minicolumn. Allow to stand for at least 60 seconds. Centrifuge the minicolumns in their microcentrifuge tubes at 10,000 x g for 20 seconds ± 3 seconds.

h. Discard the minicolumns and use an autopipette to add approximately 100 - 250µl water to each sample in its microcentrifuge tube.

i. Use a 1ml autopipette to add 1ml wizard® DNA clean-up resin to each sample.

j. Repeat, once steps a-g

k. Discard the minicolumns. Cap the microcentrifuge tubes and ensure they are labelled with the sample number, date and the number of times the purification was performed.

l. Store the samples in their microcentrifuge tubes at 2 - 5°C, ready for the PCR step.

8.4 DNA quantification by fluorescence method.

A detailed description of the DNA quantification method is given in Appendix 1

The DNA concentration present in each of the purified analytical samples must be determined along with that present in each of the pasta reference materials.

8.5 DNA standardisation

Following the determination of the DNA concentration in the samples and reference materials, standardised working solutions of each DNA sample 10ng/µl should made. This should be achieved by the appropriate dilution of each DNA sample with sterile deionised water using positive displacement pipettes.

8.5.1 Calibrant and sample preparation

Quantify calibrant DNA and sample DNA using the NanoDrop® ND1000 according to the manufacturer's protocol.

For all samples and the calibrant, prepare working stocks of 500ul at 10ng/ul.

For production of the calibration curve, the 3, 5 and 10% *T. aestivum* stock DNA solutions are prepared at 10ng/ul.

8.5.2 Primer preparation

Resuspend the PCR primers with sterile deionised water to a final concentration of 100µM following manufacturer's instructions, to give:

Normalisation Primer: Forward and Reverse 100µM Stock solution.
D-Genome Primer: Forward and Reverse 100µM Stock solution.

Dilute PCR primer 100µM stock solution to 300nM Forward and 50nM Reverse for both the Normalisation Primer set and the D-Genome Primer set:

- For both Forward primers; use a pipette (P1000) to add 727.5µl SDW to a 1.5ml eppendorf tube, then using a pipette (P100) add 22.5µl of forward primer 100µM stock solution.
- For both Reverse primers; use a pipette (P1000) to add 746.3µl SDW to a 1.5ml eppendorf tube, then using a pipette (P10) add 3.8µl of forward primer 100µM stock solution.

Dilute dNTPs to a final concentration of 5mM.

8.5.3. PCR experimental design

It is recommended that four replicates per standard (at each dilution) and per sample (at each dilution) are run.

Suitable negative controls of 2 x PCR no template controls, and 2 x Extraction Blanks, should be included

The calibrant of 10ng/µl of 10% *T. aestivum* DNA serves to function as the positive control.

8.6 PCR amplification

8.6.1. PCR master mix

Using the appropriate volume pipettes prepare Master Mix for 100 Reactions (96 reactions + sufficient to allow for pipetting errors and tube adhesion).

Multiplex MM	Final Conc.	Preferred stock conc.	1 Reaction	100 Reactions
PCR buffer (x10)-supplied with Amplitaq Gold (no Mg^{2+})	1x	10x	5.0 µl	500 µl
Mg^{2+} (25Mm)	2.0mM	25mM	4.0 µl	400 µl
dNTP (5mM)	100uM	5mM	1.0 µl	100 µl
N-Forward primer (300nM)	300nM	3uM	5.0 µl	500 µl
N-Reverse primer (50nM)	50nM	0.5uM	5.0 µl	500 µl
D-Forward primer (300nM)	300nM	3uM	5.0 µl	500 µl
D-Reverse primer (50nM)	50nM	0.5uM	5.0 µl	500 µl
Water (Sigma)	-	-	14.8 µl	1480 µl
Amplitaq Gold (1U/reactionl)	1U/reaction	1U/µl	0.2 µl	20.0 µl
Total Volume			**45.0µl**	**4500 µl**

- Using a pipette (P100) add 45µl Reaction Master Mix to 96 0.2ml Eppendorf tubes.
- Using a pipette (P10) add 5µl of the appropriate DNA (standards or samples at 10ng/µl)
- Make sure tubes are sealed.
- Vortex each tube for 5 seconds.
- Centrifuge in microcentrifuge at 13,000 x g for 10 seconds.
- Place tubes into the PE 9700 thermocycler.

8.6.2. Thermocycler conditions

Stage 1	50℃	2 min
Stage 2	95℃	10 min

Followed by 39 cycles of

95℃	15s
60℃	1min

The maximum ramp rate was used to progress between each stage.

8.6.3 PCR primer sequences

D-genome - forward primer- 5’ Agg AgA Agg AgC TCg CCA A 3’
D-genome - reverse primer- 5’ AAC CgA ggg TCC AgA AgA gAC 3’

Normalisation – forward primer- 5’ CgA CCC ATg gCA CAC CA 3’
Normalisation – reverse primer- 5’ CCT CCA CAC TTT gTA CAT CCT CAg 3’

8.6.4 PCR setup for the D-genome and ‘normalisation’ amplicons

1. This assay comprises the PCR amplification of two amplicons both of which are generated in a duplex PCR reaction. Each DNA sample is

therefore analysed for the presence of each of the two amplicons in the same reaction vessel.

2. It is essential to establish the number of PCRs required for each of the two amplicons (D-genome and normalisation) (allowing for standards, no template controls and test samples and appropriate replication), allowing for an additional 3 mixes to account for adherence of the mastermix to the mixing tube.

3. Having referred to section 8.6.1, transfer the calculated quantities of mastermix components, water, buffer, dNTPs, magnesium chloride solution, primers and AmpliTaq using the appropriate positive displacement pipettes, into a capped sterile 1.5ml microcentrifuge tubes. Ensure that the solutions are well mixed by using a vortex mixer.

4. Transfer 20µl of the mastermix solution using a 100 µl positive displacement pipette into the appropriate wells of an optical 96 well plate. Pipette into the bottom of each well to ensure no liquid sits on the sides of the well.

5. Using a 10µl positive displacement pipette with a clean tip for each sample, transfer 5µl of each standard into its designated well.

6. Using a 10µl positive displacement pipette with a clean tip for each sample, transfer 5µl of each sample extract into the appropriate wells.

7. Cap the tubes firmly and place the plate into the heating block of the thermocycler instrument.

8. Programme the thermocycler instrument to deliver the cycling programme as per section 8.6.2.

9. When the sequence has finished, remove the plate and analyse the data using the LabChip software within 24 h. If this is not possible freeze the PCR reactions at -20°C.

8.7 Agilent 2100 expert Bioanalyzer

Analysis of samples was conducted using the Agilent DNA 1000 kit, according to manufacturer's instructions (http://www.chem.agilent.com/scripts/LiteraturePDF.asp?iWHID=46764)

Remove reagents and samples from 4°C storage, and leave to equilibrate at room temperature for 30 minutes.

Using a fresh syringe with each new reagent kit.

Assemble the chip priming station with the screw syringe (supplied), and set the base to position C and the syringe stop/release clip to its lowest position.

Ensure that the Bioanalyzer electrodes are clean before use by adding 350µl water to the electrode cleaning chip and placing this inside the Bioanalyzer with the lid down for 10 seconds.

Note: For accurate results the pipette tip must be placed at the bottom of the well when dispensing liquid. Loaded chips must be used within 5 minutes of preparation.

8.7.1 Preparing the Gel-Dye Mix

Briefly vortex the reagents once they have equilibrated to room temperature.

Add 25µl DNA dye concentrate (blue) to a DNA gel matrix vial (red), vortex solution thoroughly and centrifuge (10,000 x g) briefly (5 s).

Transfer the mixture to the spin filter.

Centrifuge at 2240 x g ± 20 % for 15 minutes, protecting the solution from light.

The Gel-Dye mix can now be stored at 4 ℃ for up to 1 month.

8.7.2 Loading the Gel-Dye Mix

Allow all pre-made gel-dye mixes to equilibrate to room temperature for 30 min before use.

Put an unused DNA chip on the chip priming station.

Pipette 9.0 µl of gel-dye mix in the well marked '**G'.**

Ensure that the plunger is positioned at 1 ml and then close the chip priming station.

Press plunger until it is held by the clip.

Wait for exactly 60 s then release clip.

Wait for 5 s and then slowly pull back plunger to the 1ml position.

Open the chip priming station and pipette 9.0 µl of gel-dye mix in to the top two wells on the right, according to the manufacturer's protocol.

8.7.3 Loading the Markers

Pipette 5 µl of marker (green) in to each sample well and in to the ladder well. Do not leave any wells empty.

8.7.4 Loading the Ladder and the Samples

Pipette 1 µl of DNA ladder (yellow) in the well marked ladder.

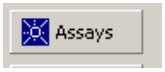
Pipette 1 µl of sample (used wells) or 1 µl of de-ionized water (unused wells) into the respective sample wells.

Place the chip in the IKA vortex and vortex for 1 min at 2400 rpm at the indicated setting.

Place lab chip inside the Bioanalyzer and place the lid down slowly.

Note: The Lab Chip **must** be run within 5 minutes of vortexing.

Initiate the 2100 expert software by double-clicking on the 2100 expert software icon.

Locate the “Assay selection”, by clicking on the Assay button 

. This will show a drop down list. Choose “electrophoresis”, and then “DNA 1000 Series II”.

Enter file name, this file will automatically be saved in the default folder; to change this check custom and change the file save location.

Once the chip has been placed inside the Bioanalyzer, the checklist on the right hand side of the screen will present all green ticks.

Click on Start button 

, to initiate the run.

Once the run is established a link will appear in blue below the Start button, 

Click on this link to access the file (do not click on the start button again as it will cancel the run).

Once the file has been opened, double click on any writing area to change/input names. This can be done for sample names and LOT numbers. Once these have been entered, save the file.

9 CALCULATIONS AND DATA ANALYSIS

Using the Agilent Bioanalyzer, open the file containing the results.

Reduce the Height Threshold (FU) value from the default setting of 20 to 2 FU.

From the File Menu, choose to export the data with the file extension *.csv

Open the file in Microsoft Excel.

Using a fresh Excel worksheet, prepare five columns for the calibrants labelled as “% standard”, “log (% adulteration)” , “D-genome (ng/µl)”, “normaliser (ng/µl)” and “Ratio D:N”

For the “% standard” column enter the 3, 5, and 10% values according to how many replicates were used. For the “log (%adulteration)“ column, calculate the logarithm of the % standard.

Copy the concentration (ng/µl) of the D-genome peak (121bp) and the "normaliser" peak (129bp) into the relevant columns on the Excel spreadsheet (A sizing accuracy of +/-5% is acceptable for the two peaks)

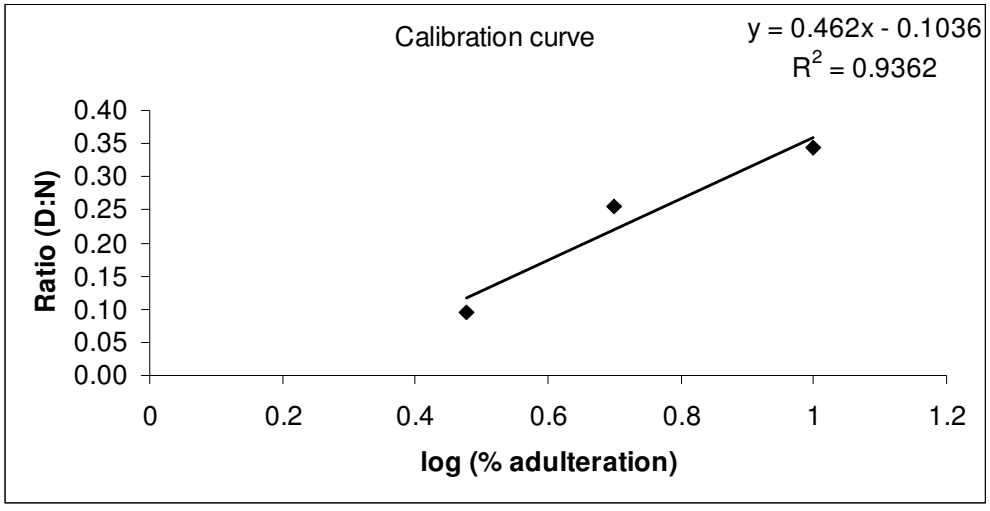
In the final "Ratio (D:N)" column calculate the "D-genome (ng/µl)" divided by the "normaliser (ng/µl)."

Repeat this process for the sample unknowns.

Example data set based on four replicates per calibrant:

% standard	log (% adulteration)	ng/µl D-genome	ng/µl Normaliser	Ratio (D:N)
3	0.477121255	0.23	2.88	0.08
3	0.477121255	0.23	2.24	0.10
3	0.477121255	0.21	2.04	0.10
3	0.477121255	0.25	2.44	0.10
5	0.698970004	0.55	2.36	0.23
5	0.698970004	0.56	2.33	0.24
5	0.698970004	0.73	2.21	0.33
5	0.698970004	0.52	2.33	0.22
10	1	0.82	2.48	0.33
10	1	1	2.75	0.36
10	1	0.85	2.21	0.38
10	1	0.79	2.65	0.30

To generate the standard curve, plot the log (% adulteration) of the standards on the x-axis, against the Ratio (D:N) on the y-axis. Instruct Excel to draw a calibration curve based on simple linear unweighted regression. Display the correlation coefficient (R^2) and the equation of the line on the graph. An example calibration curve is shown below:



To estimate the % *T. aestivum* adulteration of the sample unknowns, the regression equation for the calibration curve is used. In the example above, $y = 0.462x – 0.1036$, where y is the Ratio D:N for the sample unknown, and x is the logarithm of the % *T. aestivum* content.

A worked example is shown below:

Sample	ng/µl D-genome	ng/µl Normaliser	Ratio (D:N)	Average Ratio (D:N)	% *T. aestivum* content
PQC	0.4	2.22	0.1802	0.2181	4.97
PQC	0.44	2.3	0.1913		
PQC	0.6	2.3	0.2609		
PQC	0.55	2.29	0.2402		

10. RELATED PROCEDURES

11. APPENDICES

11.1 Appendix 1 –DNA quantification

Preparation of working solutions

1. 1 mg/ml bisBENZIMIDE:

Dilute an aliquot of the 10 mg/ml bisBENZIMIDE stock solution to a concentration of 1 mg/ml with molecular biology grade water. Store in the dark at 2-8℃. The 1 mg/ml bisBENZIMIDE solution can be stored up to 6 months.

2. 10X Fluorescent assay buffer:

After the initial thaw, mix until the solution is completely homogenous and store at 2-8 ℃.

3.100 µg/ml and 10 µg/ml DNA standard stock solutions:

Thaw the stock DNA standard (1 mg/ml) and store an aliquot at 2-8 ℃. The remaining solution should be stored at –20 ℃. More than 4 freeze/thaw cycles are **not** recommended for the Standard DNA. Dilute the DNA standard to two concentrations, 100 µg/ml and 10 µg/ml DNA as indicated below using sterile pipettes and tubes.

Mix and store at 2-8℃ for up to 6 months.

	Amount for 100 µg/ml calf thymus DNA solution	Amount for 10 µg/ml calf thymus DNA solution
Calf thymus DNA, 1 mg/ml	100 µl	10µl
10X Fluorescent assay buffer	100 µl	100 µl
Molecular biology grade water	800 µl	890µl
Total volume	1 ml	1 ml

4. bisBENZIMIDE solutions for standard curve and measurement of unknown DNA concentrations:

Add the amounts of the reagents indicated in the table below to a tube and mix. Note: These solutions should be made fresh before use each time and stored in the dark.

	Amount for 1 µg/ml bisBENZIMIDE solution	Amount for 0.1µg/ml bisBENZIMIDE solution
BisBENZIMIDE 1 mg/ml	30 µl	3 µl
10X Fluorescent assay buffer	3 ml	3 ml
Molecular biology grade water	27 ml	27ml
Total volume	30 ml	30ml

Each 30 ml bisBENZIMIDE solution is sufficient for 15 determinations, using 2 ml per sample (7 levels for the standard curve and 8 samples to be measured).

Procedure

The bisBENZIMIDE assay requires a DNA standard calibration curve in order to determine the DNA content of an unknown sample. A dye concentration of 0.1µg/ml bisBENZIMIDE is adequate for the analysis of DNA at a final concentration up to ~500 ng. A dye concentration of 1 µg/ml bisBENZIMIDE will extend the assay's range to 10 µg, but will limit the sensitivity at low concentrations. For accurate determination of the DNA concentration, two standard curves are suggested in Table 1 and Table 2. Low concentrations of DNA may give low relative fluorescence unit values that are within the linear range.

1. Turn on the fluorometer and allow it sufficient time to warm up. Set the excitation wavelength to 360 nm and the emission wavelength to 460 nm.

1. Prepare 0.1 μg /ml and 1μg /ml bisBENZIMIDE solutions in 1X fluorescent assay buffer (see Preparation Instructions, Step 4) and store in the dark until ready to use. Prepare sufficient amount of bisBENZIMIDE solution for standard curves as shown in Table 1 or Table 2 and for the sample determination. The DNA is added directly to the cuvette in the fluorometer.

Table 1

Assay with 0.1 μg/ml bisBENZIMIDE for DNA at the range of 10–500 ng/ml

Sample	Volume of 10 μg/ml DNA standard	Volume of 100 μg/ml DNA standard	0.1 μg/ml bisBENZIMIDE solution	Final amount of DNA in 2 ml
1	-	-	2 ml	Blank
2	2μ l	-	2 ml	20 ng
3	5 μ l	-	2 ml	50 ng
4	10 μ l	-	2 ml	100 ng
5	-	2μ l	2 ml	200 ng
6	-	5 μ l	2 ml	500 ng
7	-	10 μ l	2 ml	1000 ng

Table 2

Assay with 1 μg/ml bisBENZIMIDE for DNA at the range of 100 ng-5 μg/ml

Sample	Volume Of 100 μg/ml DNA standard	Volume of 1 mg/ml DNA standard	1 μg/ml bisBENZIMIDE solution	Final amount of DNA in 2 ml
1	-	-	2 ml	Blank
2	2μ l	-	2 ml	200 ng
3	5 μ l	-	2 ml	500 ng
4	10 μ l	-	2 ml	1μg
5	-	2μ l	2 ml	2 μg
6	-	5 μ l	2 ml	5 μg
7	-	10 μ l	2 ml	10 μg

3. Pipette 2 ml of the appropriate bisBENZIMIDE assay solution (prepared in step 2) into the cuvette and place in sample chamber.

4. Read the blank at 360 nm excitation and 460 nm emission at ambient temperature. Using slits set at 2.5 for the 0.1-5 μg/ml range and 5 for the 10-500 ng/ml range may increase the range of DNA detected.

5. With the cuvette still in the chamber, add the DNA standard according to Table 1 or 2 to the blank assay solution. Mix the solutions in the cuvette. Read the emission.

The sample should be in the chamber only while reading in order to reduce photo-bleaching.

Note: The maximum volume of DNA to be added to the bisBENZIMIDE assay solution should not exceed 10 µl.

6. Using a fresh cuvette and repeat steps 4 and 5 with the remaining DNA standards and the unknown samples using the fresh assay solutions.

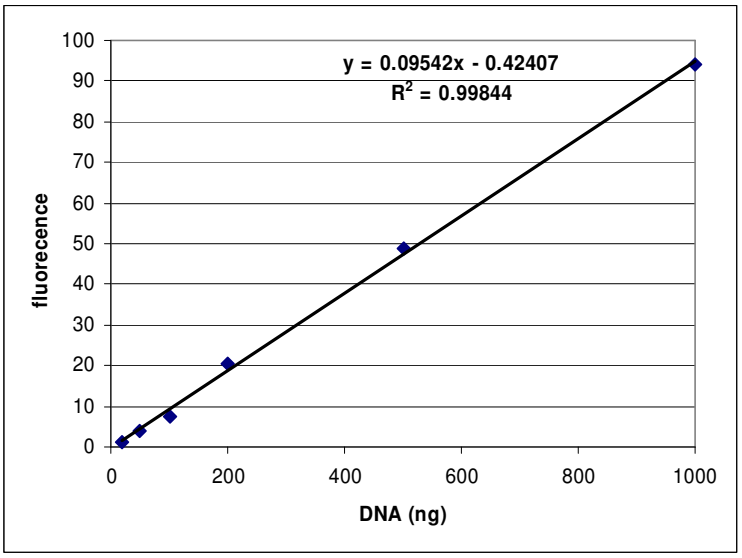
Data analysis

1. Prepare a calibration curve by plotting total DNA concentration versus relative fluorescence units- see below.

2. Determine the least squares regression equation for the line generated by the standard samples.
The linear equation is **y = mx +** b, where:
y - Emission expressed in Relative Fluorescent
Units (RFU)
m - The slope
x - DNA concentration
b - The intercept

Calculate the coefficient of determination with R^2 being >0.98

Use these derived concentrations to make dilutions of each sample and standard to 10ng.



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8.2 Appendix 2 -Real-time PCR

Resources

ABI 7700 Sequence detector

0.2ml optical tubes, caps, plate and rack

96 well optical plates and caps

1.5ml microcentrifuge tubes

10µl, 25µl, 100µl and 1000µl positive displacement pipettes with appropriate capillaries and pistons

TaqMan™ PCR mastermix (PE Corporation)

Forward and reverse primers (PE Corporation)

VIC and TET labelled probes (PE Corporation)

5.0% or 10.0% *T. aestivum* in *T. durum* standards (purified extracts)

Water

Standards and no-template controls

The standard materials are extracted in exactly the same way as samples. These extracts are purified and stored below -10°C, to be used in PCR as required

Two no-template controls must be run with each set of standards being run. These consist of 23µl of working mastermix run without the addition of any sample or standard extract.

Run type

If qualitative results only are required, samples should be run in triplicate where 5µl aliquots of sample extract (50 ng) are used in the 3 tubes respectively. A single analysis for each of the standards should also be run on the same plate.

Where quantification is to be performed, this plate type should be run initially, followed by a plate consisting of 6 analyses for each sample and triplicate analyses for the appropriate standards. The range of standards chosen should best cover the levels apparent in the samples.

Procedure

I. Using the numbered Excel spreadsheets (these are saved under a file name which is the same as the numbers of the specific primer pairs to which they apply), enter the number of optical tubes to be used for the analyte into the cell called No. of replicates. The correct quantities of mastermix, water and the required primers and probes to be used in the working mastermixes are displayed.

II. Set up a plate by placing optical tubes into a frame or use a preformed plate. Use 2 tubes for the no-template controls and 3 tubes per sample plus 1 each for the standards.

Clean Laboratory

III. Use appropriate positive displacement pipettes to prepare the working mastermix for each analyte. Transfer the calculated quantities of mastermix, water, primers and probe into a 1.5ml microcentrifuge tube. Mix well. Use a positive displacement pipette to transfer 20µl of this solution into the optical tubes. Pipette into the bottom of each tube to ensure no liquid sits on the sides of the tubes.

IV. Using a clean capillary and piston each time, use a 10µl positive displacement pipette to transfer the 2µl or 5µl of each DNA standard extract into its tube.

V. Using a clean capillary and piston each time, use a 10µl positive displacement pipette to transfer the required volume of each sample DNA (5µl) extract into the appropriate tubes.

VI Using a clean capillary and piston each time, use a 10µl positive displacement pipette to transfer a sterile water balance to each reaction tube (5, 3 µl) making the total volume in each reaction 25µl.

VII. Cap the tubes firmly and place the plate into the heating block of the ABI 7700 sequence detector. Draw the heated cover over the plate and screw into place.

GC / PCR Laboratory

VIII. Open the ABI 7700 sequence detector programme on the Mackintosh PC. Enter the positions of the NTCs, standards and unknowns. Enter the details of the standards and the sample numbers of the unknowns.

IX Use the following parameters for the thermal cycler conditions:

No. of cycles = 45

Stage 1: 50°C, 2.0 min.

Stage 2: 95°C, 10.0 min.

Stage 3: 95°C, 15 seconds - followed by

60°C, 1.0 min.

Start the sequence.

X. When the sequence has finished, remove the plate and discard the tubes/plate.

XI. Use the 7700 sequence detector programme to analyse the raw data then save the data.

Qualitative interpretation of results

If a qualitative analysis is being performed, samples producing threshold cycles (C_T) of less than 37 on the FAM side of the plate should be considered to have tested positive for the presence of *T. aestivum*. Samples producing C_T of 37 and above should be considered negative unless the value is reproduced across each analysis for that sample.

Quantification

Convert the results file from the floppy disk into an excel spreadsheet. Use the spreadsheet to subtract the TET Cts from the FAM Cts to give ΔC_T for each sample and standard. Produce a graph showing a curve for the average ΔC_T of

the standards against their *T. aestivum* content. The *T. aestivum* content of the samples is then determined by reading a value from the position of their average ΔC_T on this curve or, alternatively, their average ΔC_T values may also be entered into the spreadsheet so that they appear on the graph. Analyses producing C_T of 45 should not be included in the calculation of ΔC_T.

8.3 Appendix 3- Calculation to determine the percentage *T. aestivum* present in T. durum pasta based upon the copy number method.

When calculating the percentage of a component present in a mixture using the 'relative' copy number approach it is imperative that the number of copies of each of the sequences used is known. In the case of the quantification of Roundup Ready™ soya the ratio is normally accepted to be 1:1, making the ensuing calculation easy. In the case of the quantification of *T. aestivum* present as a contaminant in *T. durum,* the case is a little more complicated. The situation is as follows for the genome specific sequence:- *T. aestivum* has 2 copies and *T. durum* has 0 copies. The situation for the normalisation sequence is somewhat different, here; *T. aestivum* has 6 copies and *T. durum* 4 copies.

Following construction of the two calibration curves the amount of *T. aestium* present in a test sample may be calculated using the following formula:-

$$R = \frac{[(100-A) \times 4] + [6A]}{2A}$$

Hence,

$$A = \frac{200}{(R-1)}$$

Where the R= 'relative' copy number ratio and A = the % *T. aestivum*

T. durum has 4 copies of the normalisation sequence
T. aestivum has 6 copies of the normalisation sequence
T. aestivum has 2 copies of the D-genome specific sequence
T. durum has 0 copies of the D-genome specific sequence

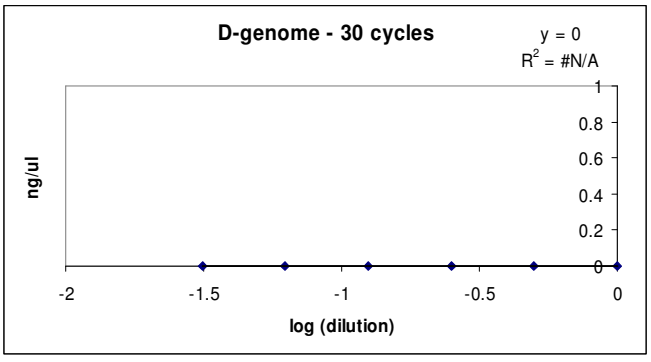
8.4 Appendix 4 PCR cycle number optimisation data

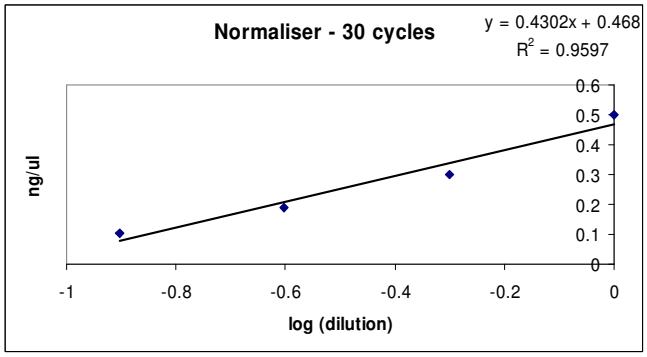
30cycles

	Results 10ng/ul D-genome	Normaliser	Ratio D:N		dilution	log (dilution)	D-genome	Normaliser
1		0.53	0.00		1	0		0.53
1		0.47	0.00		1	0		0.47
1/2		0.32	0.00		0.50	-0.301029996		0.32
1/2		0.28	0.00		0.50	-0.301029996		0.28
1/4		0.17	0.00		0.25	-0.602059991		0.17
1/4		0.21	0.00		0.25	-0.602059991		0.21
1/8		0.09	0.00		0.13	-0.903089987		0.09
1/8		0.12	0.00		0.13	-0.903089987		0.12
1/16			#DIV/0!		0.06	-1.204119983		
1/16			#DIV/0!		0.06	-1.204119983		
1/32			#DIV/0!		0.03	-1.505149978		
1/32			#DIV/0!		0.03	-1.505149978		
1/64			#DIV/0!		0.02	-1.806179974		
1/64			#DIV/0!		0.02	-1.806179974		
1/128			#DIV/0!		0.01	-2.10720997		
1/128			#DIV/0!		0.01	-2.10720997		

Averaged results

dilution	log (dilution)	D-genome	Normaliser
1	0	#DIV/0!	0.5
0.50	-0.30103	#DIV/0!	0.3
0.25	-0.60205999	#DIV/0!	0.19
0.13	-0.90308999	#DIV/0!	0.105
0.06	-1.20411998	#DIV/0!	#DIV/0!
0.03	-1.50514998	#DIV/0!	#DIV/0!
0.02	-1.80617997	#DIV/0!	#DIV/0!
0.01	-2.10720997	#DIV/0!	#DIV/0!





Summary:

30 cycles gave very poor results: no values for the D-genome and only up to 1/8 dilution for the normaliser

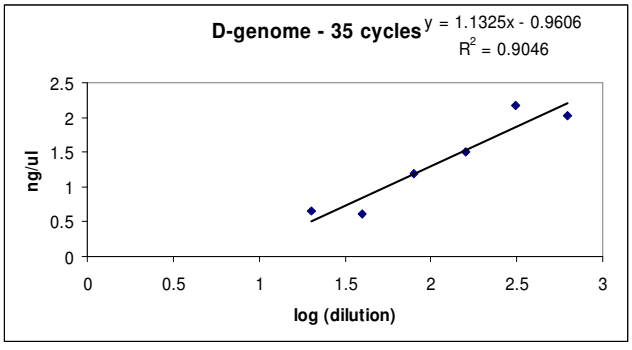
35 cycles

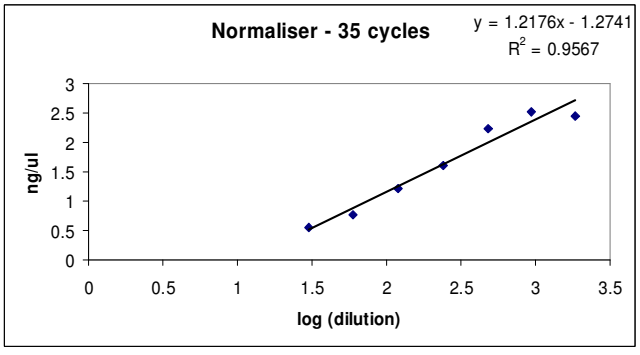
	Results 10ng/ul D-genome	Normaliser	Ratio D:N
1	0.7	2.6	0.27
1	0.65	2.3	0.28
1/2	0.8	2.56	0.31
1/2	0.65	2.46	0.26
1/4	0.53	2.18	0.24
1/4	0.47	2.27	0.21
1/8	0.35	1.46	0.24
1/8	0.44	1.76	0.25
1/16	0.19	1.1	0.17
1/16	0.22	1.33	0.17
1/32	0.22	0.83	0.27
1/32	0.22	0.71	0.31
1/64	n/a	0.5	#VALUE!
1/64	n/a	0.61	#VALUE!
1/128	0.29	0.79	0.37
1/128	n/a	0.24	#VALUE!

dilution	log (dilution)	D-genome	Normaliser
1	0	0.7	2.6
1	0	0.65	2.3
0.50	-0.301029996	0.8	2.56
0.50	-0.301029996	0.65	2.46
0.25	-0.602059991	0.53	2.18
0.25	-0.602059991	0.47	2.27
0.13	-0.903089987	0.35	1.46
0.13	-0.903089987	0.44	1.76
0.06	-1.204119983	0.19	1.1
0.06	-1.204119983	0.22	1.33
0.03	-1.505149978	0.22	0.83
0.03	-1.505149978	0.22	0.71
0.02	-1.806179974		0.5
0.02	-1.806179974		0.61
0.01	-2.10720997	0.29	0.79
0.01	-2.10720997		0.24

Average

dilution	log (dilution)	D-genome	Normaliser
1	0	0.675	2.45
0.50	-0.30103	0.725	2.51
0.25	-0.60205999	0.5	2.225
0.13	-0.90308999	0.395	1.61
0.06	-1.20411998	0.205	1.215
0.03	-1.50514998	0.22	0.77
0.02	-1.80617997	#DIV/0!	0.555
0.01	-2.10720997	0.29	0.515





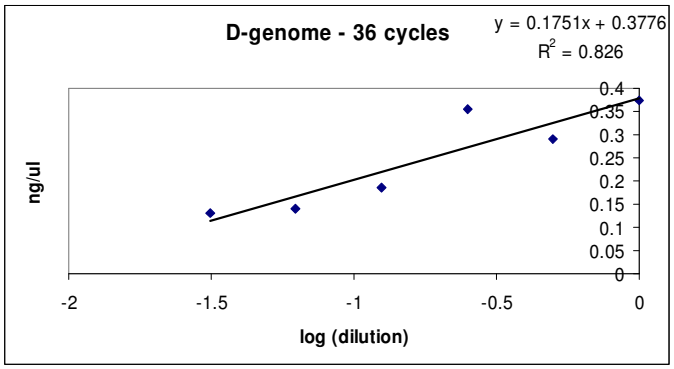
36 cycles

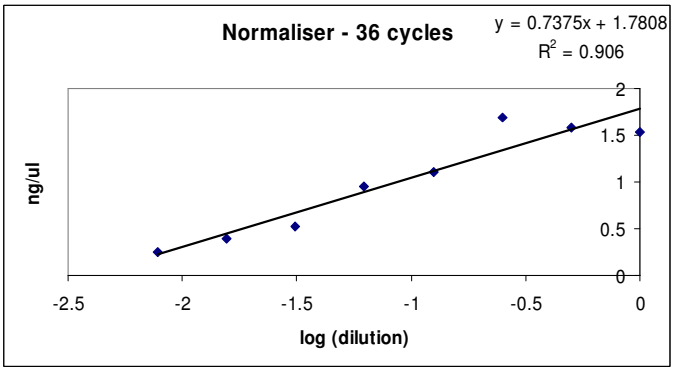
	Results 10ng/ul D-genome	Normaliser	Ratio D:N
1	0.35	1.58	0.22
1	0.4	1.49	0.27
1/2	0.21	1.42	0.15
1/2	0.37	1.75	0.21
1/4	0.34	1.68	0.20
1/4	0.37	1.7	0.22
1/8	0.21	1.17	0.18
1/8	0.16	1.04	0.15
1/16	0.15	0.99	0.15
1/16	0.13	0.92	0.14
1/32	0.15	0.55	0.27
1/32	0.11	0.5	0.22
1/64	n/a	0.43	#VALUE!
1/64	n/a	0.35	#VALUE!
1/128	n/a	0.24	#VALUE!
1/128	n/a	0.25	#VALUE!

dilution	log (dilution)	D-genome	Normaliser
1	0	0.35	1.58
1	0	0.4	1.49
0.50	-0.301029996	0.21	1.42
0.50	-0.301029996	0.37	1.75
0.25	-0.602059991	0.34	1.68
0.25	-0.602059991	0.37	1.7
0.13	-0.903089987	0.21	1.17
0.13	-0.903089987	0.16	1.04
0.06	-1.204119983	0.15	0.99
0.06	-1.204119983	0.13	0.92
0.03	-1.505149978	0.15	0.55
0.03	-1.505149978	0.11	0.5
0.02	-1.806179974n/a		0.43
0.02	-1.806179974n/a		0.35
0.01	-2.10720997n/a		0.24
0.01	-2.10720997n/a		0.25

Average

dilution	log (dilution)	D-genome	Normaliser
1	0	0.375	1.535
0.50	-0.30103	0.29	1.585
0.25	-0.60205999	0.355	1.69
0.13	-0.90308999	0.185	1.105
0.06	-1.20411998	0.14	0.955
0.03	-1.50514998	0.13	0.525
0.02	-1.80617997	#DIV/0!	0.39
0.01	-2.10720997	#DIV/0!	0.245





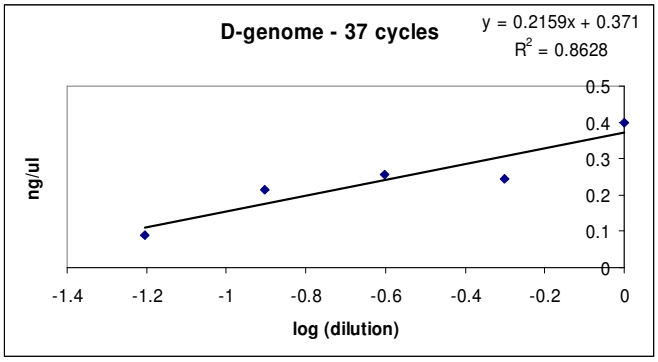
37 cycles

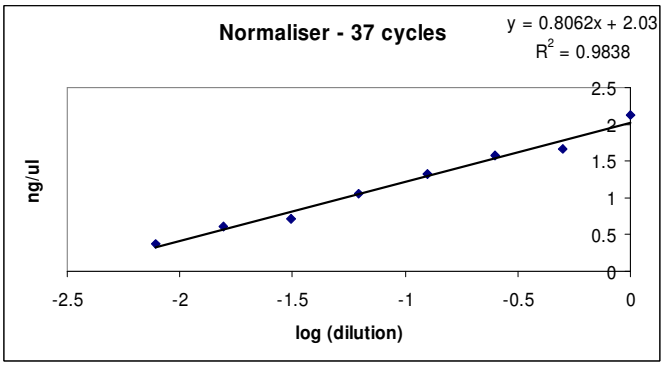
	Results 10ng/ul D-genome	Normaliser	Ratio D:N
1	0.43	2.24	0.19
1	0.37	2.03	0.18
1/2	0.27	1.73	0.16
1/2	0.22	1.59	0.14
1/4	0.24	1.46	0.16
1/4	0.27	1.68	0.16
1/8	0.13	1.08	0.12
1/8	0.3	1.56	0.19
1/16	0.08	1.1	0.07
1/16	0.1	1.01	0.10
1/32		0.74	0.00
1/32		0.7	0.00
1/64	0.26	0.57	0.46
1/64		0.65	0.00
1/128		0.37	0.00
1/128		0.38	0.00

dilution	log (dilution)	D-genome	Normaliser
1	0	0.43	2.24
1	0	0.37	2.03
0.50	-0.301029996	0.27	1.73
0.50	-0.301029996	0.22	1.59
0.25	-0.602059991	0.24	1.46
0.25	-0.602059991	0.27	1.68
0.13	-0.903089987	0.13	1.08
0.13	-0.903089987	0.3	1.56
0.06	-1.204119983	0.08	1.1
0.06	-1.204119983	0.1	1.01
0.03	-1.505149978		0.74
0.03	-1.505149978		0.7
0.02	-1.806179974	0.26	0.57
0.02	-1.806179974		0.65
0.01	-2.10720997		0.37
0.01	-2.10720997		0.38

Average

dilution	log (dilution)	D-genome	Normaliser
1	0	0.4	2.135
0.50	-0.30103	0.245	1.66
0.25	-0.60205999	0.255	1.57
0.13	-0.90308999	0.215	1.32
0.06	-1.20411998	0.09	1.055
0.03	-1.50514998	#DIV/0!	0.72
0.02	-1.80617997	0.26	0.61
0.01	-2.10720997	#DIV/0!	0.375





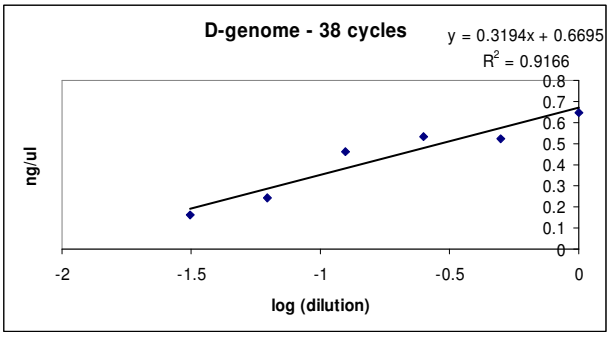
38 cycles

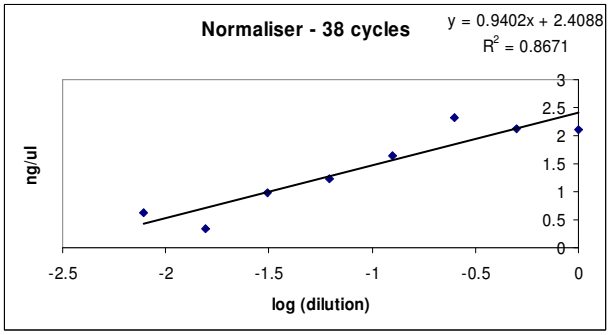
	Results 10ng/ul D-genome	Normaliser	Ratio D:N
1	0.67	2.27	0.30
1	0.63	1.93	0.33
1/2	0.43	2.1	0.20
1/2	0.62	2.15	0.29
1/4	0.32	2.07	0.15
1/4	0.75	2.58	0.29
1/8	0.45	1.61	0.28
1/8	0.47	1.67	0.28
1/16	0.32	1.34	0.24
1/16	0.17	1.11	0.15
1/32	0.19	0.97	0.20
1/32	0.13	0.98	0.13
1/64	n/a	0.3	#VALUE!
1/64	n/a	0.37	#VALUE!
1/128	n/a	0.6	#VALUE!
1/128	n/a	0.64	#VALUE!

dilution	log (dilution)	D-genome	Normaliser
1	0	0.67	2.27
1	0	0.63	1.93
0.50	-0.301029996	0.43	2.1
0.50	-0.301029996	0.62	2.15
0.25	-0.602059991	0.32	2.07
0.25	-0.602059991	0.75	2.58
0.13	-0.903089987	0.45	1.61
0.13	-0.903089987	0.47	1.67
0.06	-1.204119983	0.32	1.34
0.06	-1.204119983	0.17	1.11
0.03	-1.505149978	0.19	0.97
0.03	-1.505149978	0.13	0.98
0.02	-1.806179974n/a		0.3
0.02	-1.806179974n/a		0.37
0.01	-2.10720997n/a		0.6
0.01	-2.10720997n/a		0.64

Average

dilution	log (dilution)	D-genome	Normaliser
1	0	0.65	2.1
0.50	-0.30103	0.525	2.125
0.25	-0.60205999	0.535	2.325
0.13	-0.90308999	0.46	1.64
0.06	-1.20411998	0.245	1.225
0.03	-1.50514998	0.16	0.975
0.02	-1.80617997	#DIV/0!	0.335
0.01	-2.10720997	#DIV/0!	0.62





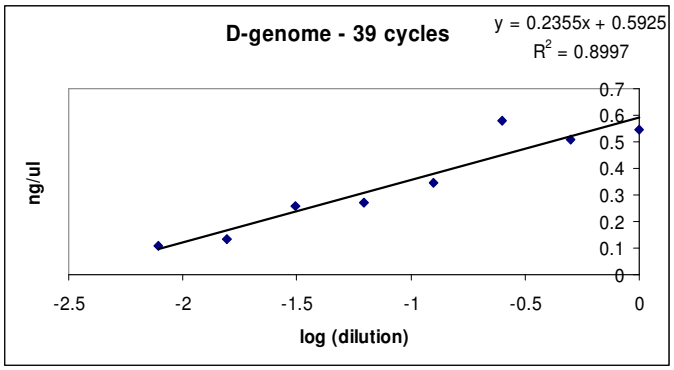
39 cycles

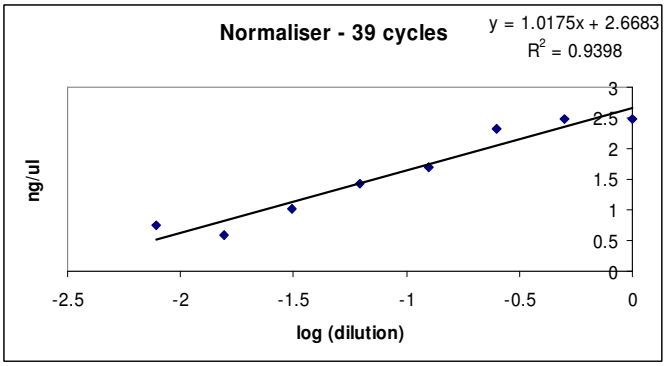
	Results 10ng/ul D-genome	Normaliser	Ratio D:N
1	0.51	2.48	0.21
1	0.58	2.48	0.23
1/2	0.51	2.48	0.21
1/2	0.51	2.48	0.21
1/4	0.49	2.1	0.23
1/4	0.67	2.55	0.26
1/8	0.38	1.75	0.22
1/8	0.31	1.64	0.19
1/16	0.27	1.43	0.19
1/16	0.27	1.43	0.19
1/32	0.24	1.06	0.23
1/32	0.28	0.98	0.29
1/64	0.22	0.73	0.30
1/64	0.05	0.44	0.11
1/128	0.11	0.7	0.16
1/128	n/a	0.81	#VALUE!

dilution	log (dilution)	D-genome	Normaliser
1	0	0.51	2.48
1	0	0.58	2.48
0.50	-0.301029996	0.51	2.48
0.50	-0.301029996	0.51	2.48
0.25	-0.602059991	0.49	2.1
0.25	-0.602059991	0.67	2.55
0.13	-0.903089987	0.38	1.75
0.13	-0.903089987	0.31	1.64
0.06	-1.204119983	0.27	1.43
0.06	-1.204119983	0.27	1.43
0.03	-1.505149978	0.24	1.06
0.03	-1.505149978	0.28	0.98
0.02	-1.806179974	0.22	0.73
0.02	-1.806179974	0.05	0.44
0.01	-2.10720997	0.11	0.7
0.01	-2.10720997	n/a	0.81

Average

dilution	log (dilution)	D-genome	Normaliser
1	0	0.545	2.48
0.50	-0.30103	0.51	2.48
0.25	-0.60205999	0.58	2.325
0.13	-0.90308999	0.345	1.695
0.06	-1.20411998	0.27	1.43
0.03	-1.50514998	0.26	1.02
0.02	-1.80617997	0.135	0.585
0.01	-2.10720997	0.11	0.755



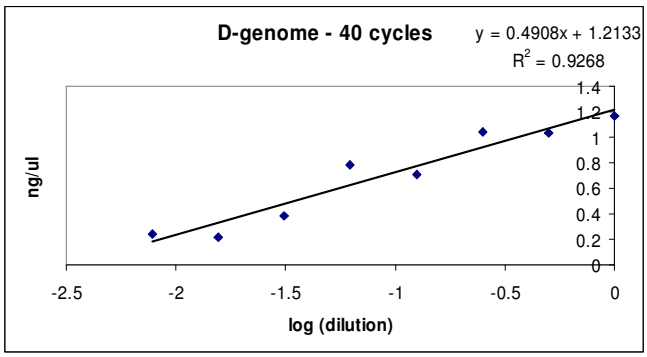


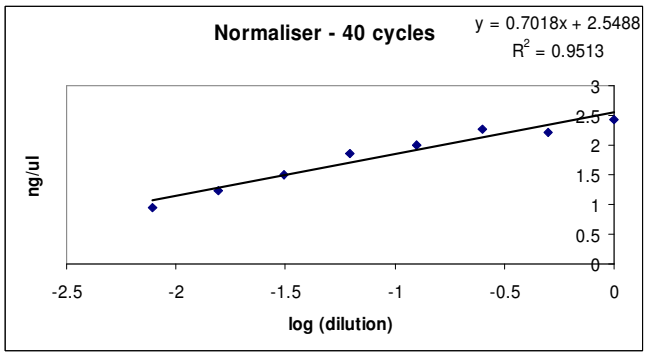
40 cycles

	Results 10ng/ul D-genome	Normaliser	Ratio D:N		dilution	log (dilution)	D-genome	Normaliser
1	1.23	2.39	0.51		1	0	1.23	2.39
1	1.1	2.48	0.44		1	0	1.1	2.48
1/2	1	2.28	0.44		0.50	-0.301029996	1	2.28
1/2	1.06	2.16	0.49		0.50	-0.301029996	1.06	2.16
1/4	0.98	2.13	0.46		0.25	-0.602059991	0.98	2.13
1/4	1.1	2.41	0.46		0.25	-0.602059991	1.1	2.41
1/8	0.66	1.97	0.34		0.13	-0.903089987	0.66	1.97
1/8	0.75	2.03	0.37		0.13	-0.903089987	0.75	2.03
1/16	0.74	1.74	0.43		0.06	-1.204119983	0.74	1.74
1/16	0.83	1.99	0.42		0.06	-1.204119983	0.83	1.99
1/32	0.27	1.51	0.18		0.03	-1.505149978	0.27	1.51
1/32	0.5	1.48	0.34		0.03	-1.505149978	0.5	1.48
1/64	n/a	1.18	#VALUE!		0.02	-1.806179974		1.18
1/64	0.22	1.29	0.17		0.02	-1.806179974	0.22	1.29
1/128	n/a	1.13	#VALUE!		0.01	-2.10720997		1.13
1/128	0.24	0.78	0.31		0.01	-2.10720997	0.24	0.78

Average

dilution	log (dilution)	D-genome	Normaliser
1	0	1.165	2.435
0.50	-0.30103	1.03	2.22
0.25	-0.60205999	1.04	2.27
0.13	-0.90308999	0.705	2
0.06	-1.20411998	0.785	1.865
0.03	-1.50514998	0.385	1.495
0.02	-1.80617997	0.22	1.235
0.01	-2.10720997	0.24	0.955





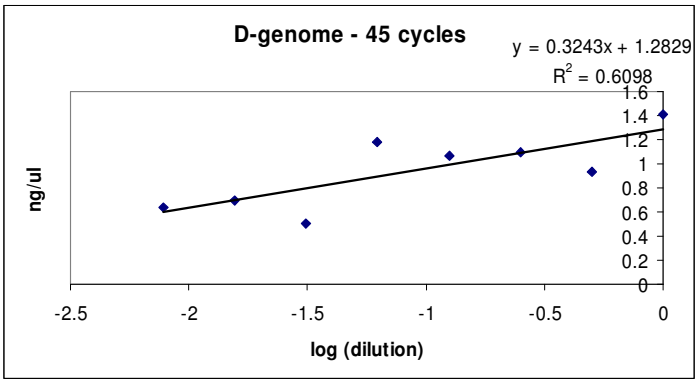
45 cycles

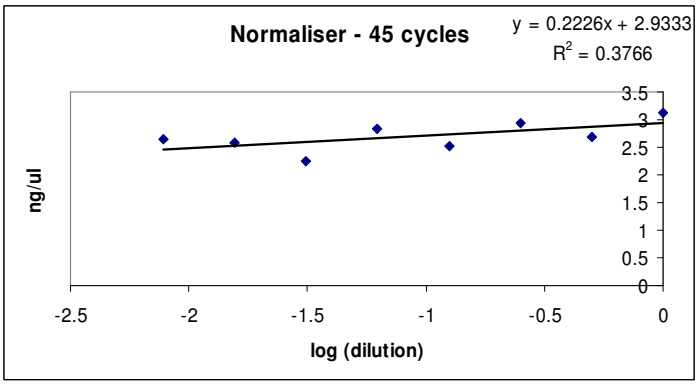
	Results 10ng/ul D-genome	Normaliser	Ratio D:N
1	1.53	3.25	0.47
1	1.29	2.99	0.43
1/2	1	2.64	0.38
1/2	0.86	2.75	0.31
1/4	1.07	2.9	0.37
1/4	1.12	2.98	0.38
1/8	0.87	2.39	0.36
1/8	1.27	2.66	0.48
1/16	1.49	2.8	0.53
1/16	0.87	2.86	0.30
1/32	0.49	2.29	0.21
1/32	0.52	2.21	0.24
1/64	0.76	2.81	0.27
1/64	0.64	2.35	0.27
1/128		2.74	0.00
1/128	0.64	2.56	0.25

dilution	log (dilution)	D-genome	Normaliser
1	0	1.53	3.25
1	0	1.29	2.99
0.50	-0.301029996	1	2.64
0.50	-0.301029996	0.86	2.75
0.25	-0.602059991	1.07	2.9
0.25	-0.602059991	1.12	2.98
0.13	-0.903089987	0.87	2.39
0.13	-0.903089987	1.27	2.66
0.06	-1.204119983	1.49	2.8
0.06	-1.204119983	0.87	2.86
0.03	-1.505149978	0.49	2.29
0.03	-1.505149978	0.52	2.21
0.02	-1.806179974	0.76	2.81
0.02	-1.806179974	0.64	2.35
0.01	-2.10720997		2.74
0.01	-2.10720997	0.64	2.56

Average

dilution	log (dilution)	D-genome	Normaliser
1	0	1.41	3.12
0.50	-0.30103	0.93	2.695
0.25	-0.60205999	1.095	2.94
0.13	-0.90308999	1.07	2.525
0.06	-1.20411998	1.18	2.83
0.03	-1.50514998	0.505	2.25
0.02	-1.80617997	0.7	2.58
0.01	-2.10720997	0.64	2.65





8.5 Appendix 5-validation data-original proposed method

10% *T. aestivum* Dilution	D-genome ng/ul	Normaliser ng/ul
1	0.81	0.48
1	1.1	2.33
1	1.06	2
1	0.76	2.13
1/2	0.54	1.74
1/2	0.78	2.05
1/2	0.62	1.95
1/2	0.72	1.96
1/4	0.55	1.82
1/4	0.37	1.39
1/4	0.32	1.43
1/4	0.41	1.64
1/8	0.5	1.86
1/8	0.48	1.04
1/8	0.42	1.54
1/8	0.32	1.51
1/16	0.4	1.33
1/16	0.21	0.62
1/16	0.37	0.97
1/16	0.36	1.46
1/32	0.15	0.71
1/32	0.05	0.7
1/32	0.19	0.81
1/32	0.08	0.83
1/64		1.05
1/64	0.15	0.89
1/64	0.35	0.56
1/64	0.16	0.42
1/128		0.44
1/128	0.17	0.65
1/128	0.03	0.25
1/128		0.32

Table 8. Raw data for the calibration curve associated with Plate 2.

The calibrants are based on seven ½ serial dilution of a 10ng/ul 10% *T. aestivum* standard. The values in the table represent the estimated concentration (ng/ul) of the D-genome and Normaliser PCR amplicons, using the Agilent 2100 Bioanalyzer. Values highlighted in blue were regarded as outliers, and removed from further analysis. Values highlighted in yellow were also below the stated quantification range of 0.1 ng/ul of the Agilent 2100 Bioanalyzer, and were also removed.

Sample unknown	Dilution	ng/ul D-genome	ng/ul Normaliser
PQC	1	0.17	1.93
PQC	1	0.16	1.55
PQC	1	0.2	2.18
PQC	1	0.2	2.21
PQC	1/2	0.4	2.22
PQC	1/2	0.44	2.3
PQC	1/2	0.6	2.3
PQC	1/2	0.55	2.29
PQC	1/4	0.31	2.32
PQC	1/4	0.13	1.96
PQC	1/4	0.29	2.11
PQC	1/4	0.38	2.57
PQC	1/8		1.43
PQC	1/8		1.18
PQC	1/8	0.17	1.07
PQC	1/8	0.32	0.8
PQC	1/16	0.08	2.04
PQC	1/16	0.35	1.84
PQC	1/16	0.2	1.8
PQC	1/16	0.19	1.65
318	1		0.59
318	1		0.81
318	1		0.64
318	1		1.24
318	1/2	0.1	2.18
318	1/2	0.11	1.88
318	1/2	0.1	2.17
318	1/2	0.1	1.95
318	1/4	0.07	2.08
318	1/4	0.11	2.35
318	1/4		1.78
318	1/4	0.11	2.05
318	1/8	0.28	1.39
318	1/8	0.03	1.32
318	1/8	0.03	1.62
318	1/8		1.57
318	1/16	0.04	0.62
318	1/16	0.61	0.72
318	1/16		1.2
318	1/16		
319	1	0.33	2.1
319	1	0.28	2.06
319	1	0.09	1.66
319	1	0.29	1.69
319	1/2	0.12	1.68
319	1/2	0.05	1.61
319	1/2	0.15	1.75
319	1/2		1.51
319	1/4	0.06	1.36
319	1/4	0.13	1.34
319	1/4	0.1	1.71
319	1/4	0.28	1.73
319	1/8		1.52
319	1/8	0.13	1.23
319	1/8	0.12	0.99
319	1/8	0.39	1.2
319	1/16		0.66
319	1/16	0.41	0.56

319	1/16	0.05	0.73
319	1/16	0.14	0.81

Table 9- Raw data for the sample unknowns associated with Plate 2.

The values in the table represent the estimated concentration (ng/ul) of the D-genome and 'normaliser' PCR amplicons, using the Agilent 2100 Bioanalyzer, for the sample unknowns. Values highlighted in blue were regarded as outliers, and removed from further analysis. Values highlighted in yellow were also below the stated quantification range of 0.1 ng/ul of the Agilent 2100 Bioanalyzer, and were also removed.

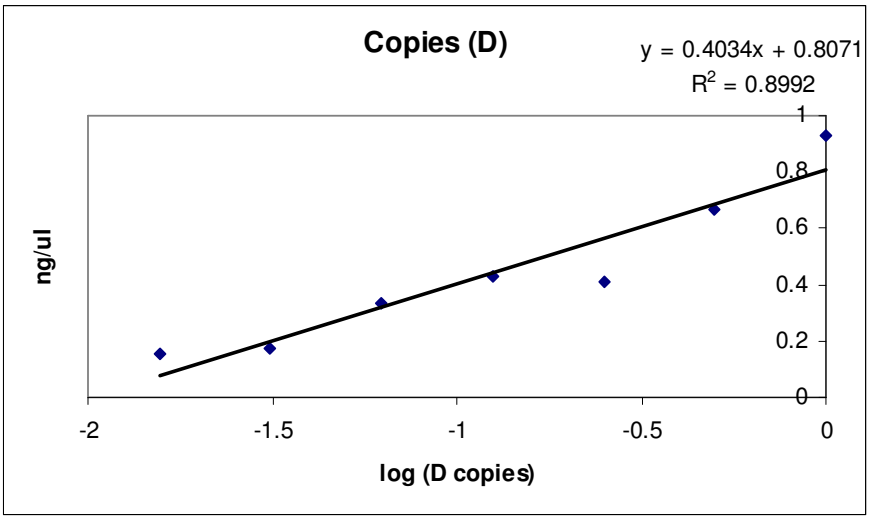


Figure 16- D-genome calibration curve based on serial dilution series of the 10% T. aestivum standard (Plate 2).

The x-axis represents the logarithm of the copy numbers of the D-genome from Table X. The y-axis represents the estimated concentration of the calibrants, from the Agilent 2100 Bioanalyzer.

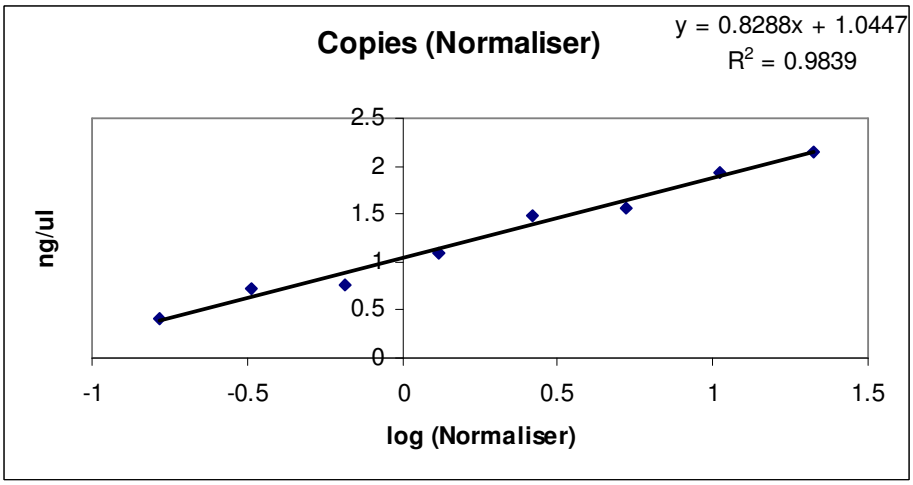


Figure 17- Normaliser calibration curve based on serial dilution series of the 10% T. aestivum standard (Plate 2).

The x-axis represents the logarithm of the copy numbers of the Normaliser from Table X. The y-axis represents the estimated concentration of the calibrants, from the Agilent 2100 Bioanalyzer.