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Strawberry and Raspberry Fruit Differentiation Using the Agilent CE 2100 Bioanalyzer

Application Note

Food

Author

Malcolm Burns, Rebecca Sanders, and Amy Burrell
Bio-Molecular Innovation Team, LGC, Queens Road, Teddington
Middlesex, TW11 0LY
UK

Abstract

Food authenticity is an important and rapidly expanding area that requires development of molecular approaches to help avert fraudulent replacement of expensive food ingredients, and to ensure correct ingredient levels are present in prepacked foods. We report here on the development of a method that uses polymerase chain reaction (PCR) and the DNA 1000 chip to distinguish between DNA derived from the fruits of strawberry and raspberry. Using this approach, characteristic profiles from strawberry and raspberry DNA are generated on the Agilent 2100 capillary electrophoresis system, which may prove useful in food authentication studies.

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Introduction

Food authentication is an important and rapidly expanding analytical area that is needed to ensure that food conforms to current international legislation and that policies on food labeling and ingredients are enforced.

Raspberry (genus: *Rubus*) is used in many foods, including purées, jellies, jams, pies, cakes, pastries, dessert toppings, juices, wines, and dairy products such as ice cream and yogurt, as well as being eaten fresh or stored frozen for consumption later. The leaves of raspberry are often used in herbal teas, and the fruit is also used for potential health benefits. Strawberry (genus: *Fragaria*) is also a common type of fruit that is cultivated worldwide and is of global economic significance.

However, recent studies [1, 2] report that some food and drinks (fruit juices in particular) labeled as containing a particular fruit, contain little or no fruit of that particular species, or may have substituted or mixed that fruit with other edible fruits. This may occur through either deliberate adulteration or unintentional processing errors (via contamination through inefficient washing procedures or coprocessing of fruits). Such instances are in contravention of the law, and stakeholders (food retailers, enforcement agencies, etc.) all require access to methods that allow the accurate identification of food ingredients to ensure regulatory compliance and protect consumers. Additionally, correct identification of ingredients in food products is needed to support the authentic composition of food, especially in relation to the declared presence of allergens in a food product, and also in the fraudulent replacement of more expensive food ingredients.

A novel application of the Agilent 2100 capillary electrophoresis (CE) system to differentiate between DNA derived from strawberry and raspberry fruits, is reported here. This approach makes use of microsatellite markers that allow differentiation of strawberry and raspberry DNA based on presence/absence or size differentiation of PCR products, easily measured using the DNA 1000 chip.

Experimental

PCR Primers

Microsatellite markers that have been previously described [3] were used to differentiate between strawberry and raspberry DNA:

Fvi11 Forward: GCATCATCGTCATAATGAGTGC
Fvi11 Reverse: GGCTTCATCTCTGCAATTCAA

Fvi20 Forward: GAGTTTGTCATCCTCAGACACC
Fvi20 Reverse: AGTGACCCAGAACCCAGAA

Samples

Authenticated DNA for Samples A and B were kindly provided by the SCRI (Dundee, UK) and consisted of strawberry samples derived from a numbered selection from a commercial breeding program, and Glen Moy (raspberry), respectively.

Raspberries (Sample D) were bought from a UK supermarket chain as prepacked fruit (225 g) labeled as raspberries (produce of Spain). Strawberries (Sample C) were purchased from the same UK supermarket store at the same time, as prepacked fruit labeled as strawberries. Two individual DNA extractions were taken from each fruit batch and labeled as Samples C1 and C2, and Samples D1 and D2.

DNA Extraction

For Samples C and D, DNA was extracted from strawberry and raspberry fruits using a cetyl trimethylammonium bromide (CTAB) buffer (50 mM tris HCl; 4 M NaCl, 1.8% CTAB; 25 mM EDTA). Approximately 100 mg fruit samples were weighed and homogenized, DNA was extracted using the above CTAB buffer, resuspended in 100 µL of 1x TE buffer, and quantified using a spectrophotometer.

Thermal Cycle Conditions

25 µL PCR reaction mixes were made based on the following components: 12.5 µL of 2x Fast Start PCR Master mix (Product number 04710436001, Roche); 300 nM of appropriate forward primer; 300 nM of appropriate reverse primer; 15 ng of extracted genomic DNA; and sterile distilled water to make a final volume of 25 µL.

Thermal cycle conditions (MJ Research Tetrad #2 PCR machine) consisted of 95 °C for 6 min; 40 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; followed by 72 °C for 10 min and hold at 4 °C.

Use of the DNA 1000 Chips on the Agilent 2100 CE System

All chips were prepared according to the instructions provided with the Agilent DNA 1000 LabChip kit. The gel-dye mix was prepared by mixing 400 µL of the gel matrix with 20 µL of the dye concentrate, then filtering the mixture through a spin filter. The separation chip was filled with the gel matrix/dye mixture, and 5 µL of the markers was added to each sample well. After adding samples (1 µL each) to the sample wells and the DNA sizing ladder (1 µL) to the assigned ladder well, the chip was vortexed and run on the Agilent 2100 bioanalyzer.

Results and Discussion

Fvi11 Assay

According to published literature [3, 4], Fvi11 is based on a $(GA)_{16}$ repeat motif and should give an amplicon of around 137 bp in length with strawberry, but also exhibits polymorphism in amplicon size between *Fragaria* varieties. However, Fvi11 should not cross-react with raspberry, and so no amplicon should be present.

The results from this preliminary study shown in Figures 1A and 1B indicate that Fvi11 gives an amplicon of around 122 bp in the samples that contained strawberry (A and C). Additional amplicons were also sometimes observed at 282 and 290 bp, but within the confines of the limited experimental data presented here, not on a repeatable basis. In line with expectations, Figures 1A and 1C show that Fvi11 did not cross-react with samples that contained raspberry (B and D).

Negative controls showed no detectable amplification. Additionally, Fvi11 showed a positive result and amplified the same 122-bp fragment when tested on a commercially available strawberry sauce sample with a listed ingredient of 40 percent whole strawberries (Figure 1D), inferring the assay's applicability to processed food samples containing fruit.

Fvi20 Assay

According to published literature [3, 4], microsatellite marker

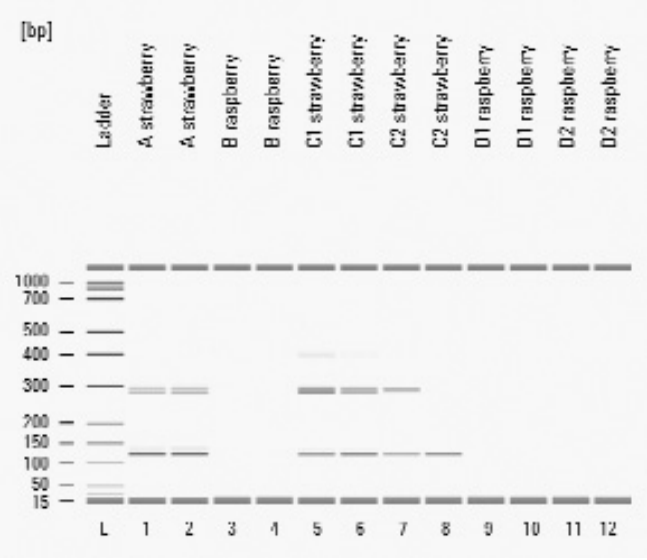


Figure 1A: Gel-like image based on Fvi11 assay on all samples.

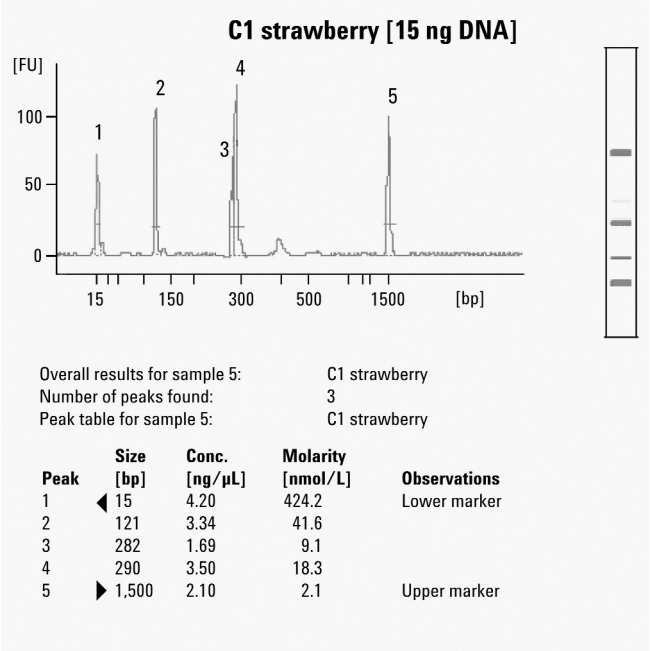


Figure 1B. Electropherogram to show profile generated using Fvi11 with strawberry DNA.

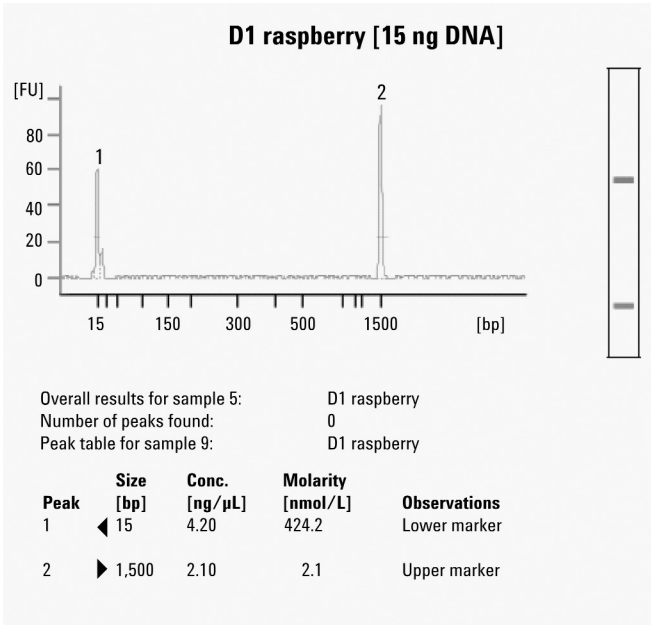
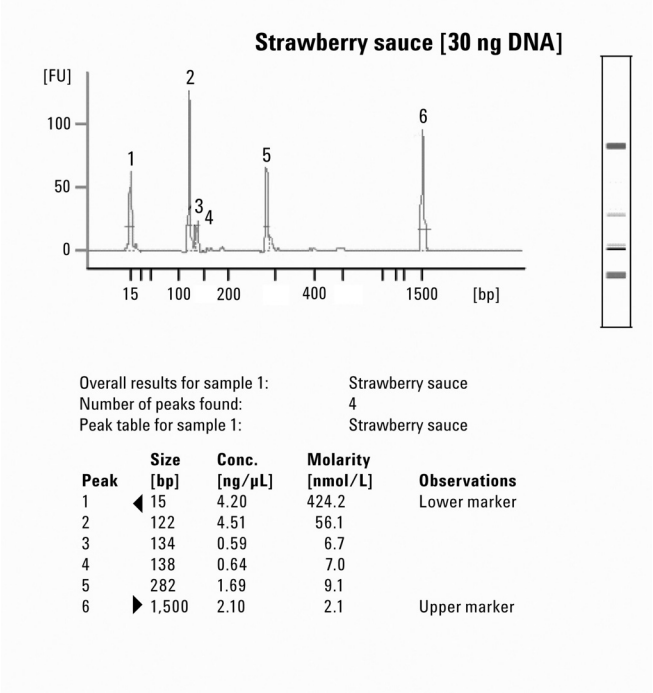


Figure 1C. Electropherogram to show absence of bands when using Fvi11 with raspberry DNA.



Overall results for sample 1: Strawberry sauce
Number of peaks found: 4
Peak table for sample 1: Strawberry sauce

Peak	Size [bp]	Conc. [ng/µL]	Molarity [nmol/L]	Observations
1	◀ 15	4.20	424.2	Lower marker
2	122	4.51	56.1	
3	134	0.59	6.7	
4	138	0.64	7.0	
5	282	1.69	9.1	
6	▶ 1,500	2.10	2.1	Upper marker

Figure 1D. Electropherogram to show characteristic strawberry DNA profile generated following application of Fvi11 to a strawberry sauce sample.

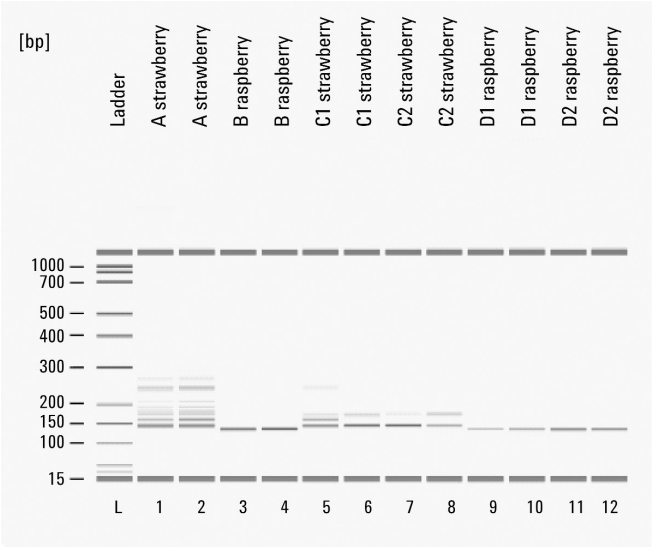


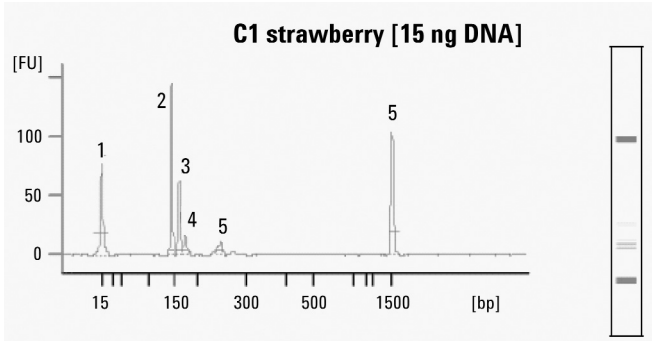
Figure 2A. Gel-like image based on Fvi20 assay on all samples.

Fvi20 is based on a $(GA)_{20}$ simple sequence repeat motif sequence, and has been shown to give a single or multiple bands around 162 bp in length for *Fragaria* varieties, but only a single amplicon with raspberry (*Rubus*) varieties.

Based on the results from this preliminary study, shown in Figures 2A and 2B, the application of the Fvi20 assay to samples that contained strawberry (A and C) gave single or multiple amplicons at around 144, 162, and/or 175 bp. In the limited strawberry samples tested in this study, the 144-bp fragment was present and predominated, while the occurrence of the other bands was less repeatable.

The application of the Fvi20 marker locus to samples that contained raspberry (B and D), shown in Figures 2A and 2C, showed the presence of a single band at 136 bp, which was easily distinguished from the 144-bp amplicon characteristic of strawberry cultivars.

Negative controls and extraction blanks showed no detectable amplification. Furthermore, the application of Fvi20 to the commercially available strawberry sauce sample showed bands around 143 and 161 bp, characteristic of strawberry DNA being present (Figure 2D).



Overall results for sample 5: C1 strawberry
Number of peaks found: 3
Peak table for sample 5: C1 strawberry

Peak	Size [bp]	Conc. [ng/µL]	Molarity [nmol/L]	Observations
1	◀ 15	4.20	424.2	Lower marker
2	144	4.13	43.6	
3	161	2.01	18.9	
4	174	0.76	6.6	
5	248	0.60	3.7	
6	▶ 1,500	2.10	2.1	Upper marker

Figure 2B. Electropherogram to show profile generated using Fvi20 with strawberry DNA.

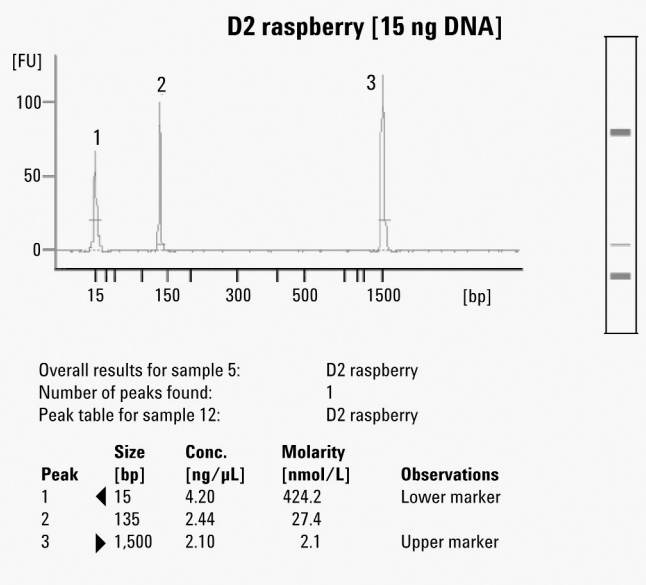


Figure 2C. Electropherogram to show presence of one band when using Fvi20 with raspberry DNA.

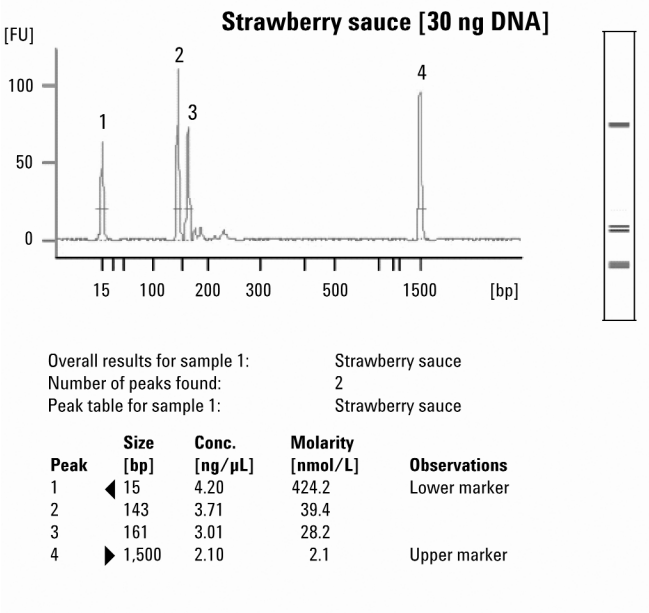


Figure 2D. Electropherogram to show characteristic strawberry DNA profile generated following application of Fvi20 to the strawberry sauce sample.

Conclusions

Originally, the microsatellite markers of Fvi11 and Fvi20 were used to assess genetic variability in strawberry (*Fragaria*) varieties. We have shown the novel application of these primer pairs using PCR and the Agilent CE 2100 system, to differentiate between samples that contain strawberry and raspberry DNA. Based on initial studies and amplicon sizes, these may prove useful in food authentication studies. The results from this small study are only representative of specific varieties of *Fragaria* and *Rubus*, but demonstrated clear differentiation between strawberry and raspberry DNA using traditional PCR followed by resolving the PCR products on the Agilent 2100 CE system.

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Acknowledgements

We gratefully acknowledge provision of strawberry Sample A and raspberry Sample B as authenticated samples from Julie Graham, SCRI, for the purposes of this study.

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Agilent Technologies

Rapid wheat varietal identification using the Agilent 2100 bioanalyzer and automated pattern-matching

Application Note

Dr Dhan Bhandari

Abstract

Accurate identification of wheat varieties is of paramount importance to the milling industry in many countries. This Application Note describes how the Agilent 2100 bioanalyzer and the Protein 230 assay can be used in conjunction with a third-party software, to analyze wheat proteins for varietal identification.

Agilent Equipment:

• 2100 Bioanalyzer Protein 230 Kit

Application Area:

• Food analysis

Agilent Technologies

Introduction

Authentication of varieties is important for the cereal industry for maintenance and testing of grain quality to meet market requirements. The charge-based separation of proteins by the acid-PAGE (polyacrylamide gel electrophoresis) technique is widely used for wheat varietal identification. However, this requires highly skilled operators to prepare, run and scan the gels and interpret band patterns. Also, there are safety concerns regarding the toxicity of unpolymerised acrylamide. While acid-PAGE is effective in analytical laboratories, the routine method can take up to two days. This is too slow for use at mill intake, which requires assessment of the wheat shipment during the period of delivery – typically under one hour. In this Application Note we demonstrate the use of the Agilent 2100 assay, with bioanalyzer and Protein 230 the Nonlinear Dynamics' Phoretix 1D Advanced (TotalLab TL120 DM) computerized pattern-recognition software to provide a better alternative to acid-PAGE. The aim of this study was to develop a robust, automated method for rapid identification of wheat varieties.

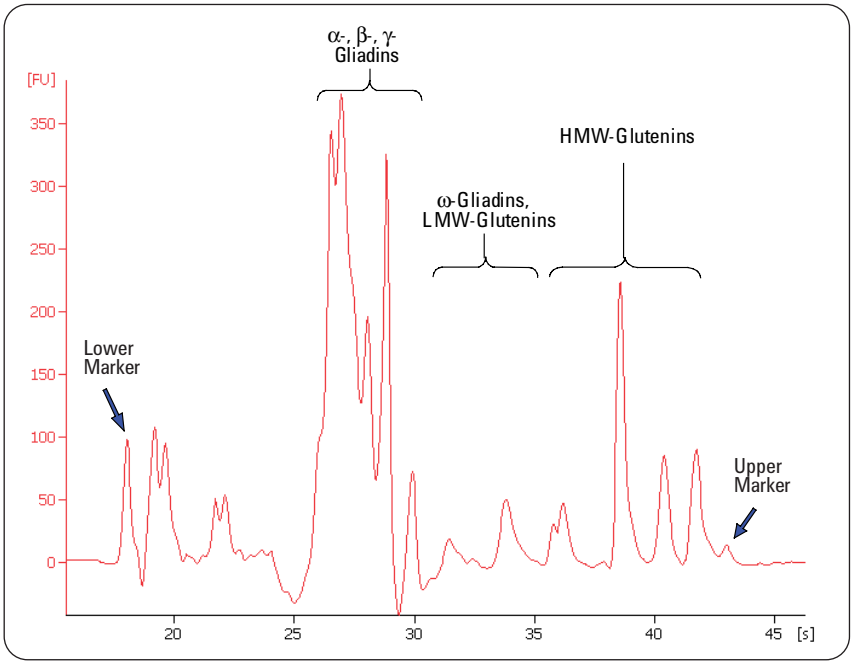

Figure 1
Typical wheat protein separation by the Protein 230 assay.

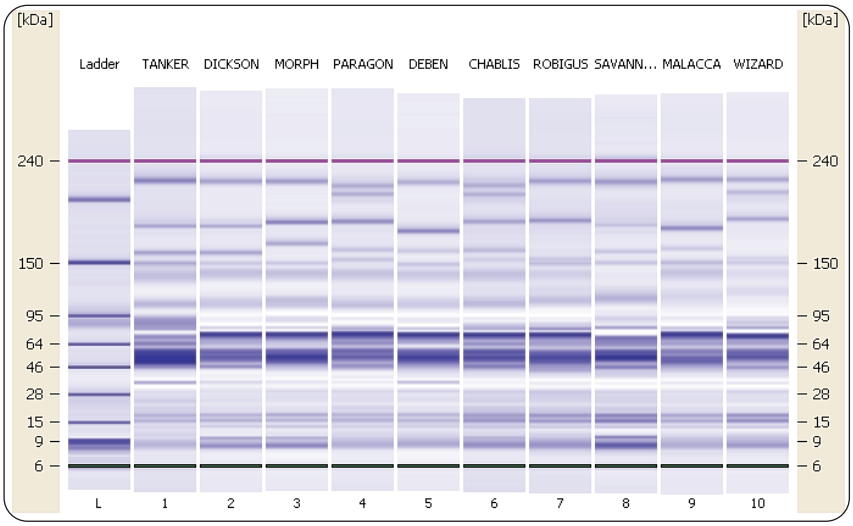

Figure 2
Bioanalyzer gel-like image of MW standards and 10 different wheat varieties.

Methods

Total wheat proteins (including glutenins) were extracted from individual grains in 0.4 mL of 2M urea, 15 % glycerol, 0.1 M DTT and 0.1 M Tris/HCl, pH 8.8, using an ultra-sonic water bath for 15 minutes. Extracts were centrifuged at 11,000 g for 5 minutes and treated with the Protein 230 assay reagents in according to the assay protocol. The samples were separated on the Agilent 2100 bioanalyzer, with each analysis of 10 samples plus ladder taking less than 25 minutes. Replicates of 34 wheat cultivars representing the 2004/5 UK Recommended List varieties were analyzed. The electropheromram profiles were processed using the Phoretix 1D Advanced and 1D Database (Nonlinear Dynamics) software for pattern-matching purposes.

Results

The Protein 230 assay produced well-resolved protein profiles, suitable for varietal discrimination (figures 1 & 2). The Phoretix software was able to compare the electropherogram profiles. Figure 3 shows an example of a dendrogram where all the replicates of three different varieties are correctly grouped. A prototype wheat library was developed by selecting the most representative varietal profile. Results showed that 90 % of test samples could be identified within the top three matches. Work is in progress to optimize the performance of this library. The practicality of the method and the robustness of the system is bourne out by the fact that the system is now in routine use in UK commercial mill intake laboratories.

Conclusions

Our study has demonstrated that using the Agilent 2100 bioanalyzer with the Phoretix system offers a standardized, objective method for rapid varietal discrimination. The ease of use and total analysis time of less than 50 minutes makes it most suitable for mill intake use. The optimized system will enable millers to make more confident decisions in accepting grain consignments, and could become widely adopted as an effective policing tool within the grain industry. A number of UK mills have purchased the combined systems for screening wheat deliveries at intake.

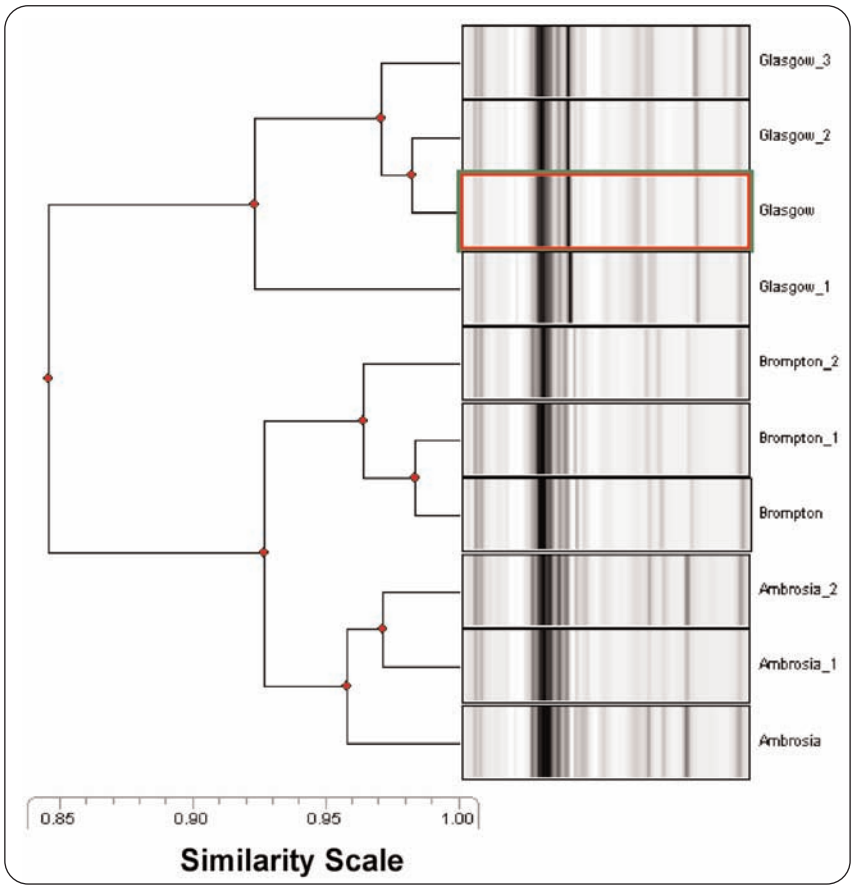

Figure 3
Dendrogram illustrating pattern-matching of replicate profiles of 3 wheat varieties.

Acknowledgements

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Dr Dhan Bhandari is Senior Scientist at Campden & Chorleywood Food Research Association (CCFRA), Chipping Campden, Gloucestershire, GL55 6LD, UK.

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Use of the Agilent 2100 Bioanalyzer for Basmati Rice Authenticity Testing

Application

Food

Author

Steve Garrett and Marie-Anne Clarke
Molecular Biology Group
Dept. Chemistry & Biochemistry
Campden & Chorleywood Food Research Association
Chipping Campden
Gloucestershire
GL55 6LD
UK

Abstract:

Ensuring integrity of raw food materials, ingredients, and products is both a product quality and regulatory compliance concern. Food suppliers and manufacturers may suffer economic and legal damages if proven to be supplying incorrectly labeled products. For example, EU Commission 1549/04 grants lower import tax on nine basmati rice varieties. A quick and cost-effective analytical method utilizing the Agilent 2100 bioanalyzer and DNA 1000 assay is shown as an alternative method to establish the authenticity of basmati rice products and to estimate the level of some varieties of non-basmati rice in ground rice products.

Introduction

The integrity of raw food materials, ingredients, and products must be maintained to ensure that they meet appropriate quality and legislative requirements. Food ingredient suppliers, manufacturers, and retailers can face legal action if proven to be supplying materials or products that are incorrectly labeled due to substitution or contamination.

One food area which has been under the spotlight in recent years is the supply of basmati rice to the UK from India and Pakistan. In Europe, Commission Regulation 1549/04 grants a lower import tax on nine basmati varieties: Basmati 370, Dehradun (Type 3), Basmati 217, Taraori, Ranbir Basmati, Kernel, Basmati 386, Pusa Basmati, and Super Basmati. Other basmati rice varieties approved by India, Pakistan, and the UK include Basmati 198, Basmati 385, Haryana Basmati, Kasturi, Mahi Suganda, and Punjab Basmati. In a Code of Practice developed by Indian, Pakistani, and UK industry and enforcement organizations that came into effect for products packed and labeled after January 2006, the level of non-basmati rice in a basmati rice product must not exceed 7% (see www.riceassociation.org.uk).

In order to check the supply of basmati rice, a DNA variety testing method using PCR amplification of eight rice microsatellite sequences has been developed for UK compliance (www.foodstandards.gov.uk/multimedia/pdfs/fsis4704basmati.pdf). During 2003, the UK Food Standards Agency carried out a surveillance exercise on basmati rice products using this method and revealed that 74% of them contained > 7% non-basmati varieties.

This study evaluates the use of the Agilent 2100 bioanalyzer to differentiate approved and non-approved varieties using three primer sets and to estimate the level of non-basmati using reference rice admixtures.

Experimental

Method

All reference basmati rice samples were obtained from the UK Food Standards Agency via the University of Wales (Bangor, UK). Other samples were from in-house basmati rice sample collections.

PCR amplification was performed using either a PE9600 or PE2400 PCR machine (Applied Biosystems).

Extraction

DNA was extracted from ground rice grains using Qiagen's DNeasy Plant Mini Kit or Promega's Maxwell 16 automated DNA extractor

PCR

DNA extracts were diluted 1 to 1 in sterile distilled water (SDW) to produce template DNA prior to use in PCRs. Amplification was performed in 25-µL PCRs containing 1x Amplitaq Gold PCR buffer (Applied Biosystems), 60 nM of each primer, 200 nM dNTPs, 3 mM $MgCl_2$, 0.05 U/µL of Ampli-Taq Gold (Applied Biosystems), and 2 µL of template DNA.

Marker	Forward Primer
RM201	CTC gTT TAT TAC CTA CAg TAC C
RM212	CCA CTT TCA gCT ACT ACC Ag
RM339	GTA ATC gAT gCT gTg ggA Ag
Marker	**Reverse Primer**
RM201	CTA CCT CCT TTC TAg ACC gAT A
RM212	CAC CCA TTT gTC TCT CAT TAT g
RM339	gAg TCA TgT gAT AgC CgA TAT g

Amplification profiles (95 °C for 15 minutes [denaturation]; 50 cycles of: 95 °C for 1 minute, 60 °C for 1 minute [amplification]; 72 °C for 10 minutes [final extension]) were used in all PCR reactions. DNA amplification was confirmed by separating PCR products using the Agilent 2100 bioanalyzer.

Capillary Gel Electrophoresis on 2100 Bioanalyzer

Reagents were prepared following manufacturers' instructions. Batches (~500 µL) of gel matrix (used to fill LabChip capillaries) were prepared as required or at 4 weekly intervals. All reagents were stored at 4 °C and allowed to reach room temperature for 1 hour before use. PCR products (1 µL)

Table 1. Analysis of Authenticated Basmati Rice Varieties Using Three Microsatellite Primer Sets

Rice variety	List of microsatellite amplification product sizes obtained with primer set*		
	RM201	RM212	RM339
Varieties listed in Commission Regulation 1549/04			
Basmati 370	162 (**162**)	134 (**139**)	200 (**193**)
Dehra Dun (Type 3)	162	134 (**139**)	200 (**195**)
Basmati 217	162	134	200
Ranbir	162	134	200
Taraori	162	134 (**139**)	200 (**195**)
Basmati 386	162	134 (**138**)	200 (**195**)
Kernel	162	134	200
Pusa	162	134 (**139**)	200 (**194**)
Super	162	134 (**140**)	204 (**196**)
Other varieties approved as basmati by UK Food Standards Agency			
Basmati 198	162	152	200
Basmati 385	162	152	200
Kasturi	162	132	166
Haryana Basmati	162	152	166
Mahi Sugandha	176	152	166
Punjab Basmati	162	152	200
Non-approved varieties			
Basmati 2000	162	152	204
Shaheen Basmati	162	152	200
Sherbati	178 (**176**)	130 (**135**)	166 (**167**)
Mugad Sugandha	178	132	166
Pak 386	178	130 (**135**)	166 (**167**)
Superfine	178	132	166
Pusa Sugandha	162	132	178
Yamini	162	134	200

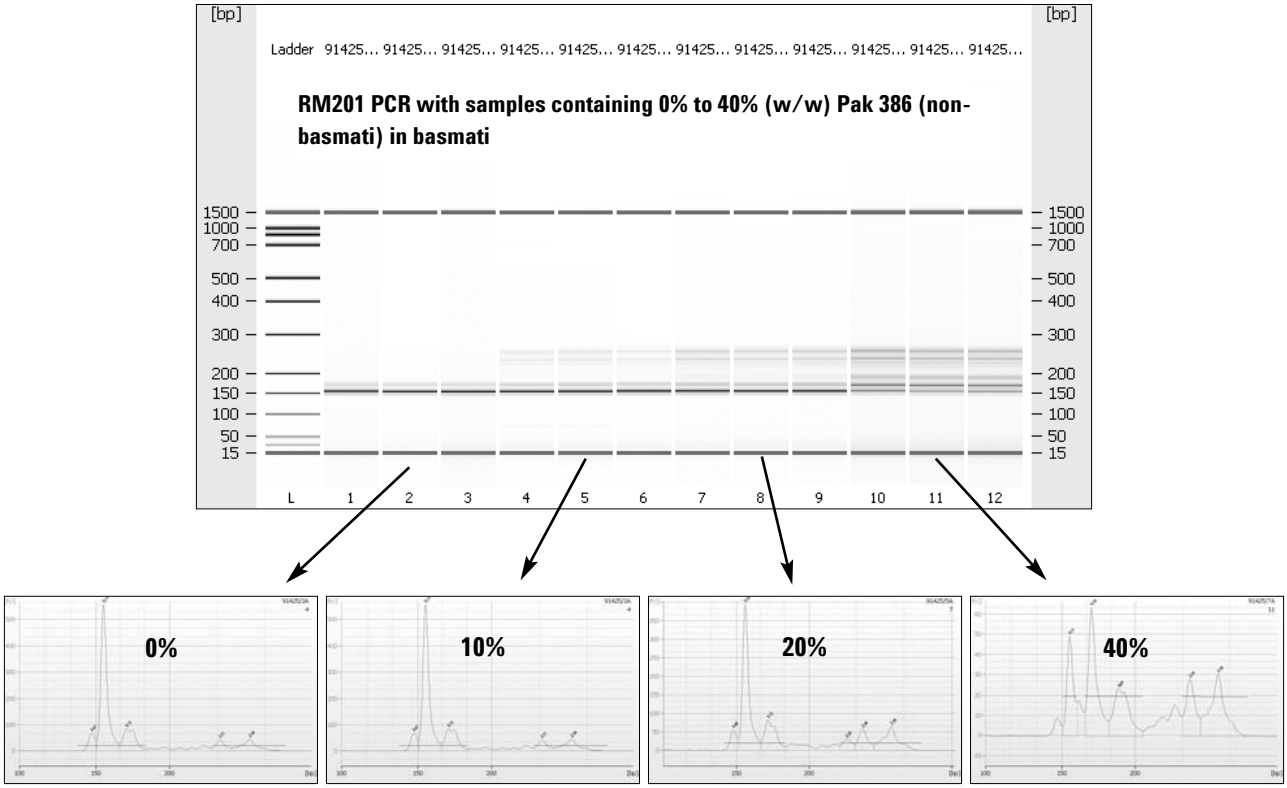
*These are from the FSA method developed by the University of Wales, Bangor. Actual size of fragments determined by the bioanalyzer using a DNA 500 chip kit are shown in brackets. The variation in bioanalyzer-determined fragment sizes can be about 5%. Shaded cells show how the varieties can be grouped using the three primer sets with analysis performed on the bioanalyzer.

were loaded directly onto prepared Series I DNA 500/DNA 1000 or Series II DNA 1000 labchips. All analysis was performed on the 2100 bioanalyzer, as per the manufacturers' instructions.

Results and Discussion

The different-sized PCR products generated when these three primer sets are applied to the different varieties are easily resolved on the bioanalyzer using the DNA 500 or DNA 1000 chip. RM 212 primers produce a 139 bp product with varieties listed in Commission Regulation 1549/04 and a 154 bp product with FSA-approved varieties apart from Kasturi and a few non-basmati varieties. The other primer sets enable separation of these varieties apart from the Yamini variety, which produces fragments similarly sized to the EC-approved varieties for all three primer sets. This variety is also difficult to distinguish from approved varieties using the standard method. The other FSA-approved basmati varieties could be distinguished from the EC-approved varieties but not from Basmati 2000 or Basmati 386 using these microsatellite primer sets. Use of further microsatellites that give PCR products that can be separated on the bioanalyzer will give improved differentiation of non-basmati rice varieties.

a) Primer set RM201



b) Primer set RM339

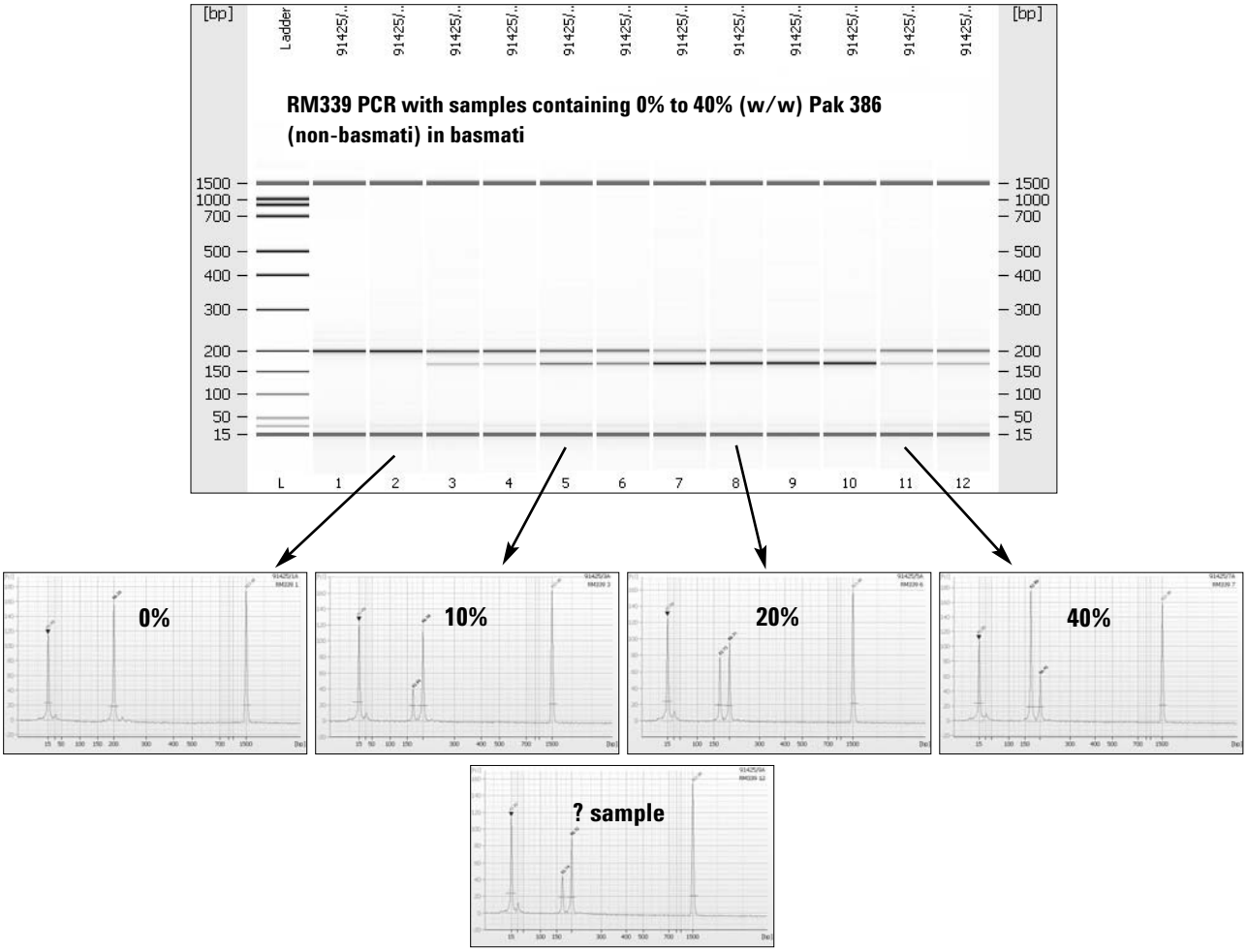


Figure 1. Analysis of non-basmati/basmati rice admixtures using two microsatellite primer sets. Estimation of percentage non-basmati variety (Pak 386) in an unknown sample.

Table 2. Experimental Summary

% Pak 386 in basmati rice	Primer RM201 mean ratio 156 bp/170 bp PCR fragments (n = 3)	Primer RM339 mean ratio 199 bp/169 bp PCR fragments (n = 2)
0	9.6	0
10	3.5	3.1
20	2.7	1.1
40	0.6	0.4
50	0.7	0.3
Unknown sample estimated to contain between 10–20% Pak 386	3.1	2.1

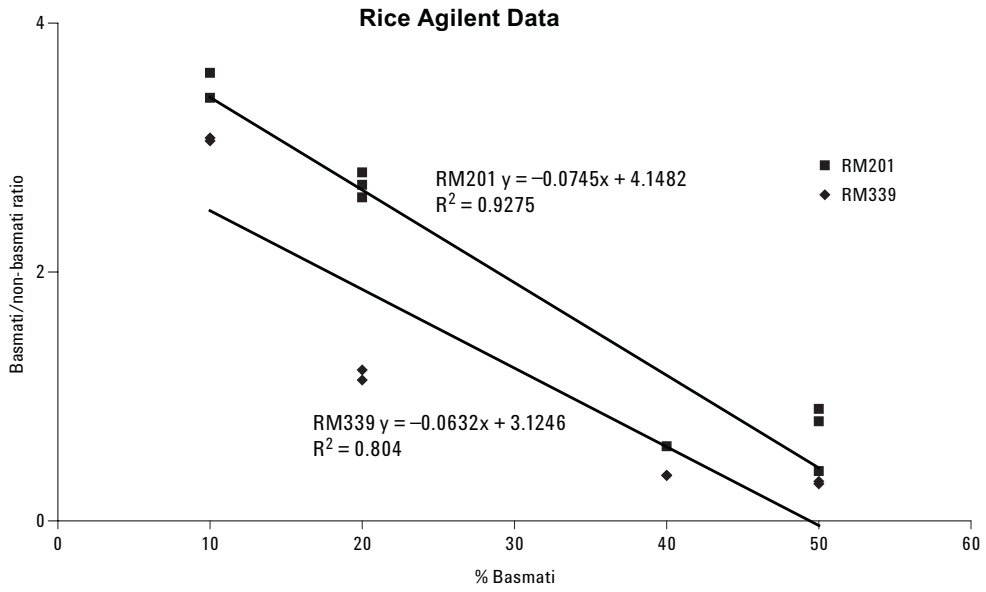


Figure 2. Standard curves based on the PCR product concentration ratios.

Both the RM201 and RM339 primer sets (Figure 1 and Table 2) could be used to produced separate and measurable PCR products with the PAK 386 basmati rice admixtures. The RM201 primers set gave a 170 bp product generated from the basmati rice and a 156 bp product from the Pak 386 variety, whereas RM339 primers gave 199 bp and 169 bp products. RM281 also gave other larger PCR products, which seemed to correlate with the presence of Pak 386 in the admixture. Standard curves (Figure 2) were produced using the PCR product concentration ratios. An unknown admixture sample was also analyzed and estimated to contain between 10 and 20% non-basmati rice in basmati.

Results show that the bioanalyzer can be used to estimate the level of non-basmati rice (Pak 386) in a basmati rice which produces different sized microsatellite PCR products from the Pak 386.

Conclusions

The Agilent 2100 bioanalyzer can be used as a quick and cost-effective alternative to establish the authenticity of basmati rice products and to estimate the level of some varieties of non-basmati rice. It should be feasible to develop further primer sets that would allow the bioanalyzer to be used to identify individual varieties. Simple manual and automated DNA extraction followed by fast PCR and post-PCR analysis on the bioanalyzer would allow rapid screening of rice materials prior to export from India and Pakistan and also allow enforcement bodies to efficiently test for microsatellite markers from non-approved basmati rice varieties in imported products.

Acknowledgements

Thanks to Mark Woolfe at UK Food Standards Agency and John Gorham and Katherine Steele at The University of Wales, Bangor, for the supply of primer set details and authentic basmati rice materials.

For More Information

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Agilent in Life Sciences > Genomics > Proteomics > Drug Discovery > Development > QA/QC

Agilent 2100 bioanalyzer

Application compendium

Agilent Technologies

Agilent 2100 bioanalyzer

Application compendium

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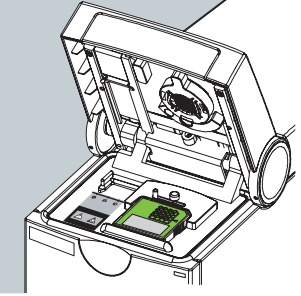
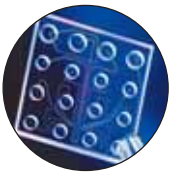




The Agilent 2100 bioanalyzer is a unique analysis tool capable of handling nucleic acids, proteins and cells on one platform. When combined with any one of our kits, you will discover how, lab-on-a-chip technology can revolutionize your laboratory.

One of the many benefits the Agilent 2100 bioanalyzer has over conventional bioanalytical methods is the elimination of time consuming procedures – you enjoy standardized handling and interpretation of data. And as our portfolio of microfluidics continues to expand, you will benefit from the wide range of applications to which the technology can be used. Lab-on-a-Chip technology simplifies the process of data gathering and analysis down to three quick and easy steps:

Load sample, run analysis, view data.

The Agilent 2100 bioanalyzer utilizes micro-fabricated chips with up to 12-wells requiring minimum sample consumption, in the low µl-range. Prepackaged reagents included with the chip kits help to speed up the entire process.

The Agilent 2100 bioanalyzer with lab-on-a-chip technology will increase the efficiency of your analysis and the productivity of your day.

- With it's single, compact system architecture the Agilent 2100 bioanalyzer integrates sample handling, separation, detection and data analysis, all in the name of speed.

- Eliminate potential mistakes that can occur while interpreting and storing data. The Agilent 2100 bioanalyzer automatically incorporates steps some researchers might otherwise ignore in the interest of time.

- The Agilent 2100 bioanalyzer helps to optimize PCR reactions for gene expression, sequencing, cloning and typing. When used in conjunction with the **DNA kits**, it provides higher sensitivity, improved sizing accuracy and automated, reproducible quantitation, compared with regular slab gel electrophoresis, which is crucial for RT-PCR and any type of multiplex PCR.

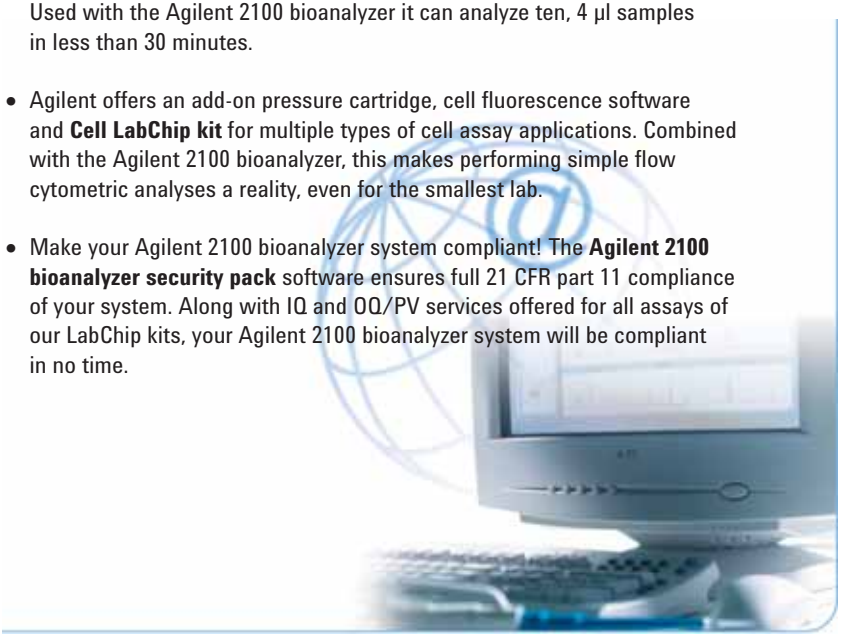
- **RNA 6000 Pico kit:** catch RNA degradation with sample amounts as low as 200 pg of total RNA and automatically detect ribosomal RNA contamination in mRNA. The **RNA 6000 Nano kit** is the industry standard for sample QC in the context of microarray analysis. With the introduction of **RIN (RNA Integrity Number**), each RNA electropherogram receives a RIN assigned by the software. The RIN unambiguously assesses RNA quality in terms of degradation.

- DNA and RNA LabChip kits enable you to check the quality of probes and targets in your **microarray gene expression analysis**. Agilent also provides the full solution for gene expression analysis with its high performance microarray scanner and the suite of off-the-shelf microarrays.

- The **Protein 200 Plus LabChip** kit is a fast and reliable assay capable of quantifying and sizing a multitude of different protein samples. Used with the Agilent 2100 bioanalyzer it can analyze ten, 4 µl samples in less than 30 minutes.

- Agilent offers an add-on pressure cartridge, cell fluorescence software and **Cell LabChip kit** for multiple types of cell assay applications. Combined with the Agilent 2100 bioanalyzer, this makes performing simple flow cytometric analyses a reality, even for the smallest lab.

- Make your Agilent 2100 bioanalyzer system compliant! The **Agilent 2100 bioanalyzer security pack** software ensures full 21 CFR part 11 compliance of your system. Along with IQ and OQ/PV services offered for all assays of our LabChip kits, your Agilent 2100 bioanalyzer system will be compliant in no time.



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I. Cell fluorescence analysis

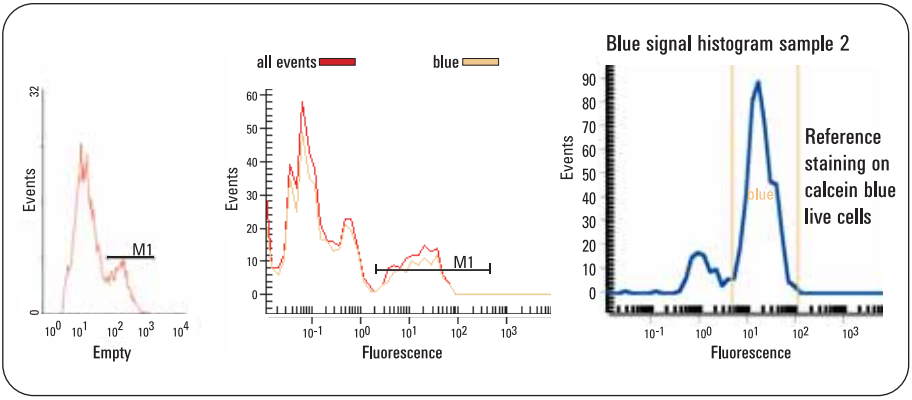
	Agriculture/Food	Drug Discovery/ Drug Development	Drug Manufacturing QA/QC	Genomics	Pharmaceuticals	Proteomics	Forensics/Homeland Security
Protein expression monitoring							
Cell surface antibody staining - CD4 in CCRF CEM T-cells		●			●	●	
Cell surface antibody staining - CD3 in T-cell leukemia		●			●	●	
CD3 expression in T-cell leukemia via on-chip staining		●			●	●	
Intracellular glucocorticoide receptor (GR) antibody staining in H4 hepatocytes		●			●	●	
Analyzing a limited number of cells		●			●	●	
Baculovirus titre determination		●	●		●	●	
Upregulated gene expression in primary cells		●		●	●	●	
Transfection efficiency monitoring							
Green fluorescent protein in CHO cells		●	●			●	
On-chip staining of GFP expression for optimizing transfection conditions with different DNA:lipid ratios		●	●			●	
Verification of stable transfected cell clones by on-chip antibody staining		●	●			●	
Transfection of primary cells		●				●	
Apoptosis detection							
Detection of phosphatidylserine on the cell surface via Annexin V binding		●			●	●	
Intracellular Caspase-3 antibody staining assay					●		
Fast Annexin protocol for time course of apoptosis induction via anti-FAS antibody		●			●	●	
Apoptosis detection in primary cells		●			●		
Gene silencing in cell culture							
siRNA transfection optimization		●		●	●	●	
Monitoring of gene silencing experiments		●		●	●	●	

Protein expression monitoring

Cell surface antibody staining - CD4 in CCRF CEM T-cells

Flow cytometer (10,000 Events)

Agilent 2100 bioanalyzer (500 Events)



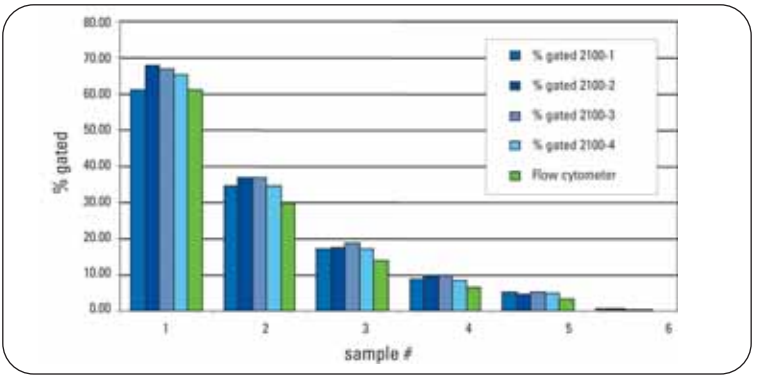
Kit: Cell fluorescence kit
Assay: Antibody staining assay
Application: CCRF-CEM cells were stained with hCD4-APC labeled antibodies and calcein live dye. 65% of all CCRF-CEM live cells (yellow curve) are expressing CD4 protein which is good in comparison to conventional flow cytometer results.

Corresponding application note: 5988-4322EN

Protein expression monitoring

Cell surface antibody staining - CD3 in T-cell leukemia

Averaged data per instrument



Mean % CD3+ cells				
2100-1	2100-2	2100-3	2100-4	Flow cyt.
60.9	67.8	66.6	65.0	60.9
34.4	36.7	36.7	34.3	29.8
17.3	17.6	18.7	17.2	13.8
8.9	9.4	9.9	8.3	6.5
5.1	4.4	5.3	4.9	3.2
0.8	0.6	0.3	0.3	0.0

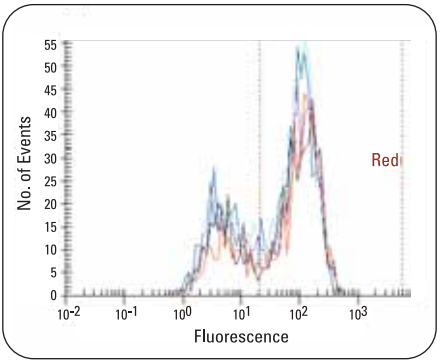
Kit: Cell fluorescence kit
Assay: Antibody staining assay
Application: Jurkat (T-cell leukemia) cells were stained with calcein alone or with calcein and APC-labeled anti-CD3 antibody. To mimic different subpopulation sizes, mixtures of both populations were prepared at various ratios.
Samples were analyzed with 4 Agilent 2100 bioanalyzer instruments on 5 chips and compared to a flow cytometer reference instrument. Interestingly, small subpopulations (like 10 - 20%) could be analyzed with good accuracy and reproducibility.

Corresponding application note: 5988-4322EN

Protein expression monitoring

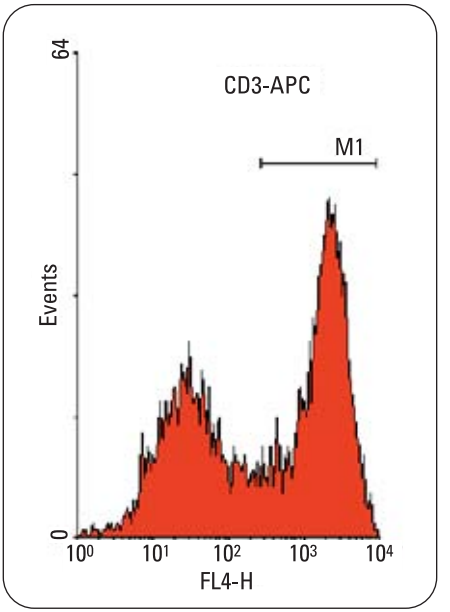
CD3 expression in T-cell leukemia via on-chip staining

A. On-chip Agilent 2100 bioanalyzer



Sample	% of Gated	Sample	% of Gated
1	-	4	64.6
2	66.9	5	66.7
3	67.2	6	72.0

B. Conventional flow cytometry



Kit: Cell fluorescence kit
Assay: Antibody staining assay
Application: Jurkat cells were stained on-chip with anti hCD3-APC prediluted 1:5.5 in cell buffer and Calcein (1:50 in cell buffer). After an incubation time of 25 minutes in the chip, samples were measured in the Agilent 2100 bioanalyzer. The faster and easier on-chip staining procedure has the advantage here of reducing cell consumption 17 fold and antibody reagent costs 80 fold.

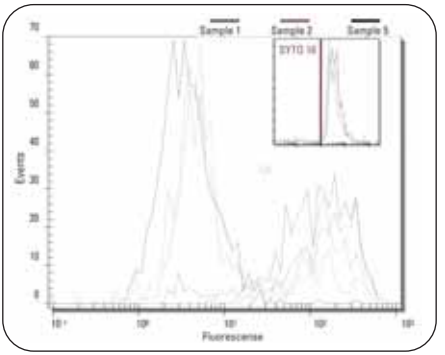
A) Overlay of representative histograms of calcein and antibody treated cells.
B) Comparison between on-chip staining data and data obtained by measuring cells stained by conventional staining on a flow cytometer.

Corresponding application note: 5988-7111EN

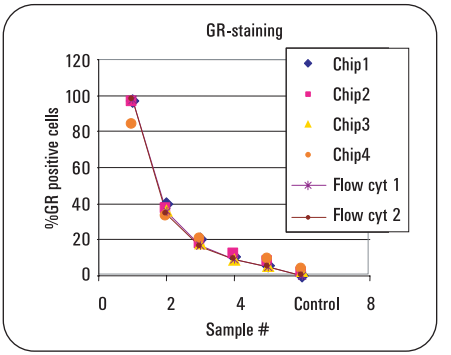
Protein expression monitoring

Intracellular glucocorticoide receptor (GR) antibody staining in H4 hepatocytes

Chip histogram overlay from 700 cells/sample



Correlation of chip vs. flow cytometer results

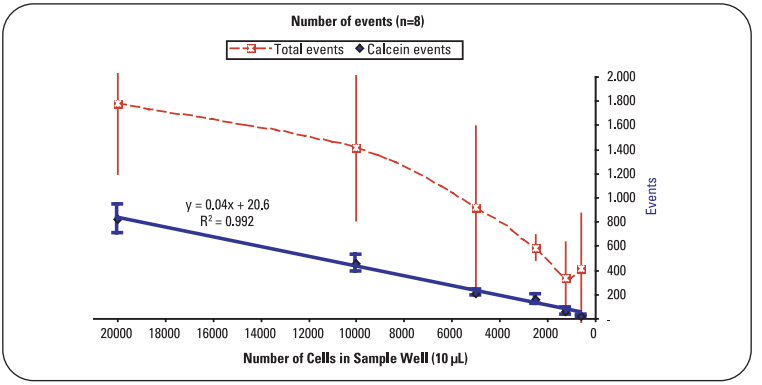


Kit: Cell fluorescence kit
Assay: Generic assay
Application: H4 hepatocytes cells were stained with SYTO16 DNA dye alone or with SYTO16 and GR primary antibody. After washing, both cell preparations were stained with APC-labeled secondary antibody. Mixtures of both populations were prepared at various ratios.
The insert in the left picture shows the overlay of all six cell samples in the blue reference color. The black histogram represents data from the control sample, no GR detected.
All other 5 samples have significant staining above marked fluorescence intensity in the red.
Good chip to chip reproducibility and comparison to flow cytometer is demonstrated.

Corresponding application note: 5988-4322EN

Protein expression monitoring

Analyzing a limited number of cells



Cells	Live-CD3+	STD(n=4)
20,000	83.7%	3.5%
10,000	85.6%	4.1%
5,000	87.7%	4.2%
2,500	84.0%	3.0%
1,250	89.8%	6.5%
625	90.0%	9.3%

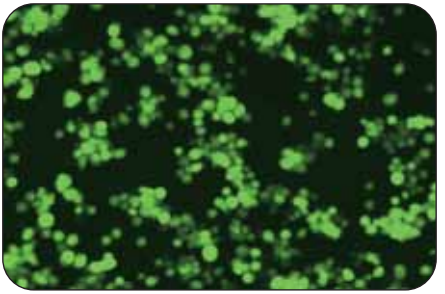
Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: The direct comparison of different input numbers of cells (down to 625 cells in 10 µl) for the on-chip staining protocol reveals that even with a much lower number than the recommended 20000 cells/10 µl for the standard protocol reliable and meaningful results can be achieved with good reproducibility. The data shown were generated with CD3-positive Jurkat cells stained with an anti-CD3 antibody for the CD3 protein and counterstained with the live cell stain Calcein AM. Similar results were obtained with primary human dermal fibroblasts (PHDF) indicating the usefulness of this method for scarce specimen. The lack of sensitivity, automation and convenient quantitation found with other methods can be circumvented easily by using the Agilent 2100 bioanalyzer.

Corresponding application note: 5989-0746EN

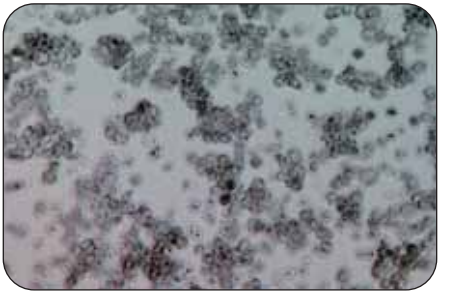
Protein expression monitoring

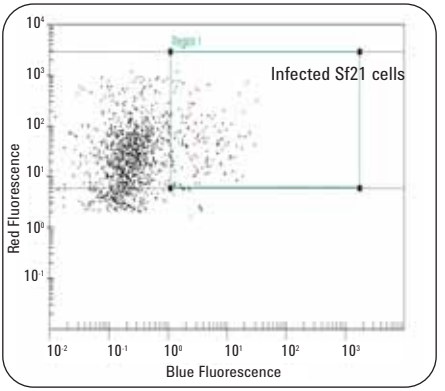
Baculovirus titre determination

Fluorescent Light



Transmitted Light



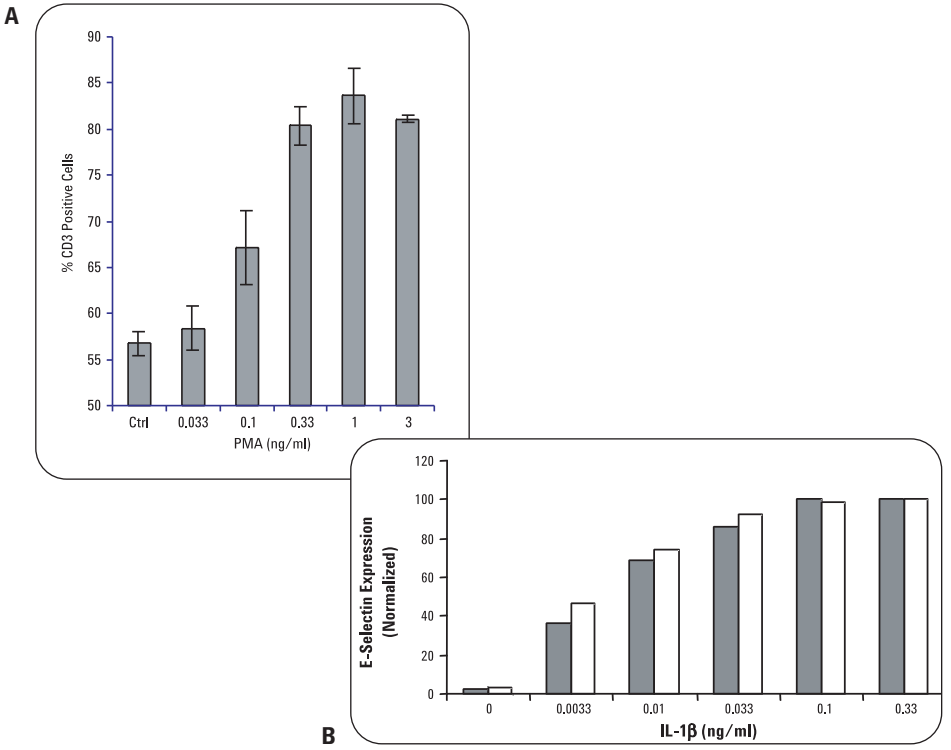


Kit: Cell fluorescence kit
Assay: GFP Assay
Application: A fast and convenient method exists for the calculation of baculovirus titre for expression systems facilitating insect cells. Using GFP-linked co-expression plasmids, the Agilent 2100 bioanalyzer and the flow cytometry set allows the calculation of the viral titre for six samples in approximately 90 minutes. It is superior to traditional plaque assays in terms of labor time, automation and user-to-user variability.

Corresponding application note: 5989-1644EN

Protein expression monitoring

Upregulated gene expression in primary cells



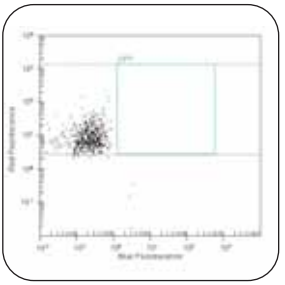
Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: Flow cytometric analysis of primary cells can present a challenge for researchers due to limited availability and life span of primary cells. A dose-respondent upregulation of protein expression in primary cells using only a minimum number of cells in a fast on-chip-staining approach is shown here. Activation of peripheral blood lymphocytes by phorbol-12-myristate-13-acetate (PMA) leads to increased expression of the T cell receptor CD3 (Figure A, mean from 3 experiments).
For HUVECs (human umbilical vein endothelia cells) the induction of E-selectin (CD62E) expression upon IL-1β treatment is shown (Figure B, white bars) in comparison to results from a conventional flow cytometer (white bars).

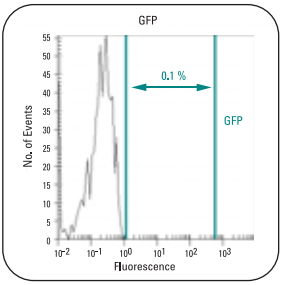
Corresponding application note: 5989-2718EN

Transfection efficiency monitoring

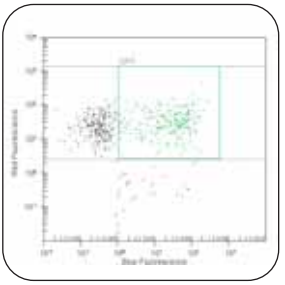
Green fluorescent protein in CHO cells

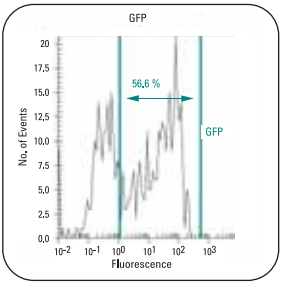
Mock transfected cells

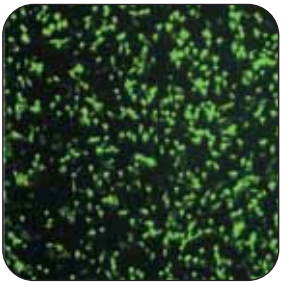




GFP transfected cells





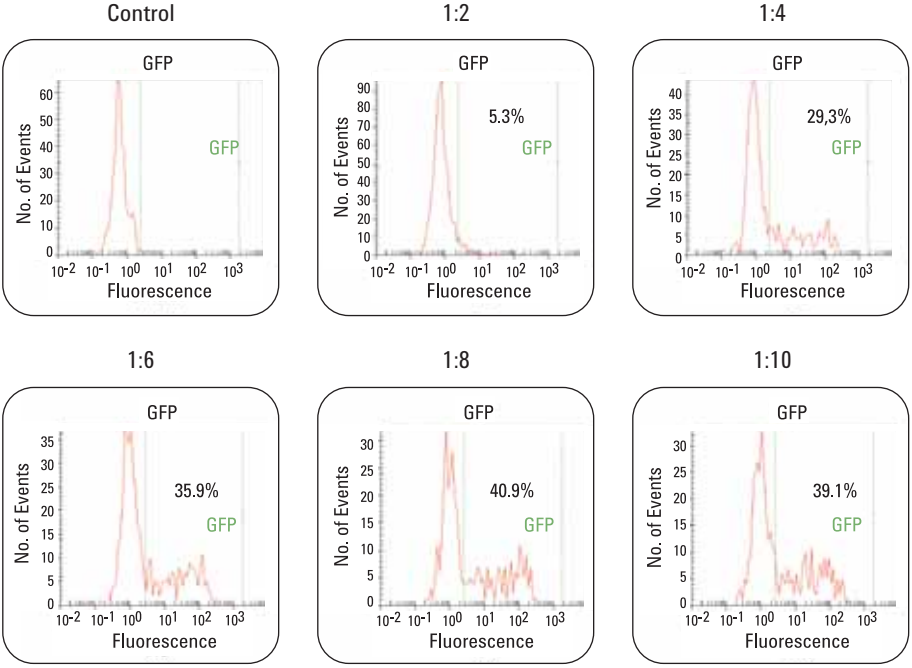


Kit: Cell fluorescence kit
Assay: GFP assay
Application: Chinese hamster ovary (CHO-K1) cells were transfected with EGFP DNA by a lipofection method. The upper panel shows the control mock transfection; here cells don't express GFP. Examples for data evaluation in dotplot view and histogram view are shown in comparison to the microscopy view. For analysis on the Agilent 2100 bioanalyzer, cells were stained with a red dye for live cells (reference stain). The transfection efficiency of 56% can be easily determined with the Agilent 2100 bioanalyzer.

Corresponding application note: 5988-4320EN

Transfection efficiency monitoring

On-chip staining of GFP expression for optimizing transfection conditions with different DNA:lipid ratios



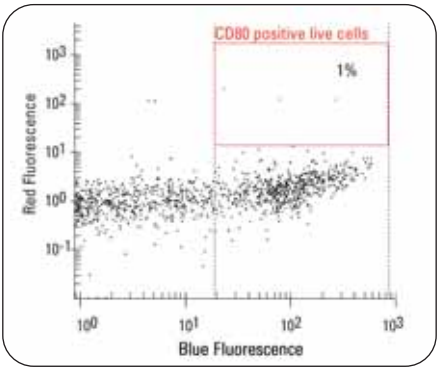
Kit: Cell fluorescence kit
Assay: On-chip GFP assay
Application: Chinese hamster ovary (CHO-K1) cells were transfected with EGFP DNA by alipofection method. Optimization of transfection conditions were done on one chip. Several DNA:lipofectamine ratios were tried. A ratio of 1:8 gave the best transfection efficiency. All cells were reference stained with a red live dye. On-chip staining was applied, minimizing the staining time, reagent usage and cell consumption.

Corresponding application note: 5988-7296EN

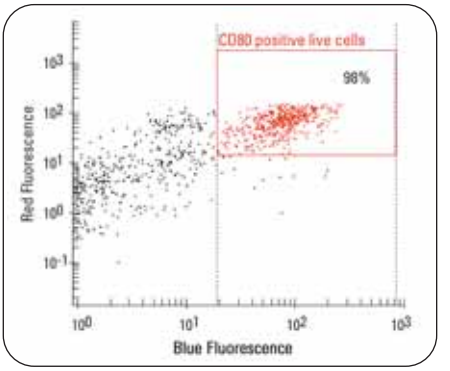
Transfection efficiency monitoring

Verification of stable transfected cell clones by on-chip antibody staining

Hek 293 control cells



CD 80 stable clone

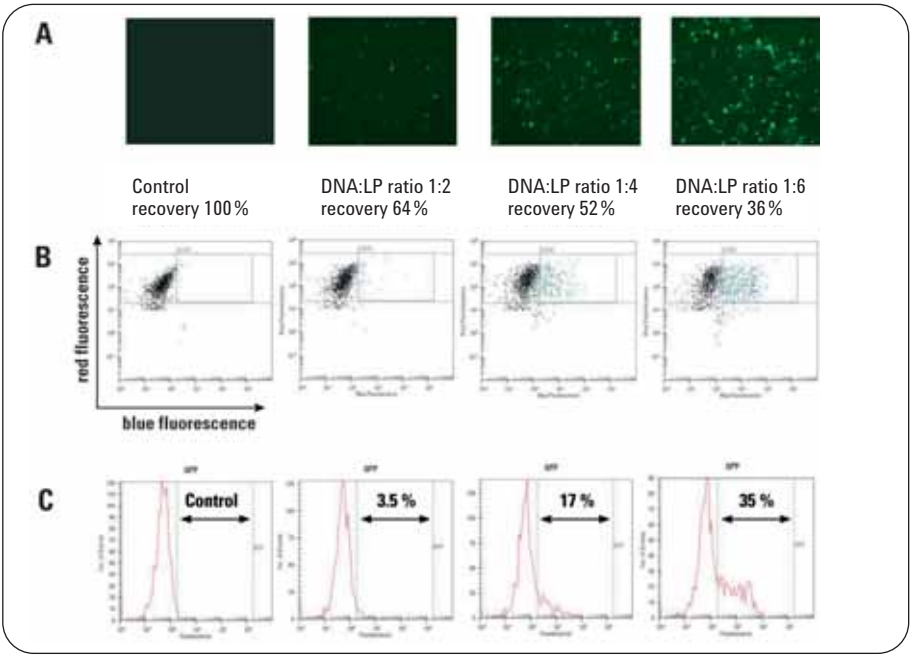


Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: Verification of CD80 protein expression in stable transfected Hek 293 cells with the Agilent 2100 bioanalyzer. Control (left dot plot) and CD80 transfected cells (right) are stained on-chip with blue calcein live dye and anti-CD80-CyChrome antibody. Red region marks CD80 protein expressing 293 cells within live cell population - confirming expression in the CD80 stable clone Hek 293 cells.

Corresponding application note: 5988-7111EN

Transfection efficiency monitoring

Transfection of primary cells

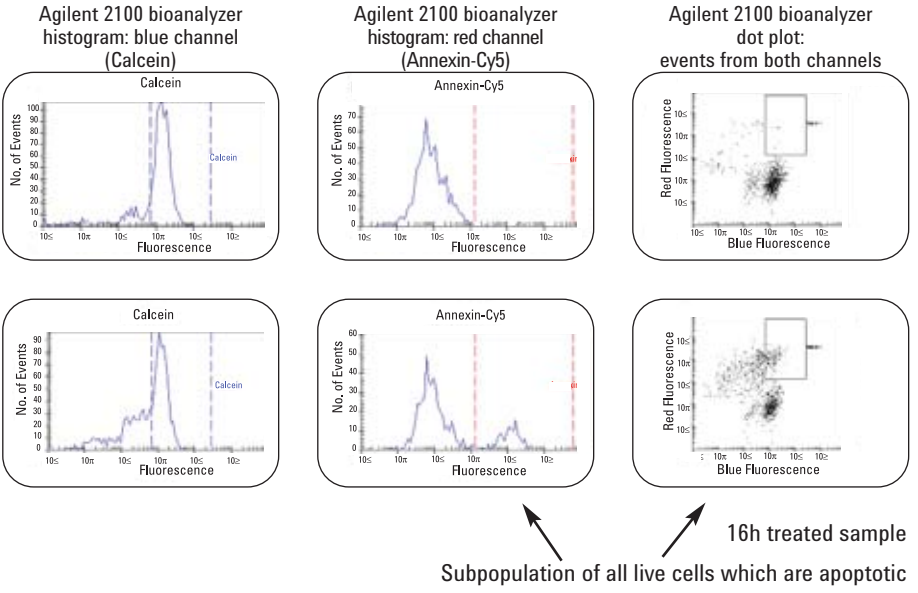


Kit: Cell fluorescence kit
Assay: GFP assay
Application: Monitoring the transfection efficiency in primary cells requires low cell consumption, high reproducibility of results, a fast on-chip staining procedure and ease-of-use all provided by the Agilent 2100 bioanalyzer. The transfection efficiency using a GFP-coding plasmid (pEGFP-C2) at varying plasmid:lipofectamine ratios (DNA:LP ratio) obtained with human umbilical vein endothelial cells (HUVEC) is measured in this optimization series. Images from a fluorescence microscope (A) and dot plots (B), as well as histograms (C) of control- and GFP-transfected cells are shown. Using increasing ratios, better transfection efficiency was achieved, whereas the toxicity of LP caused decreased recovery of living cells. Such data facilitates optimizing transfection conditions.

Corresponding application note: 5988-8154EN

Apoptosis detection

Detection of phosphatidylserine on the cell surface via Annexin V binding

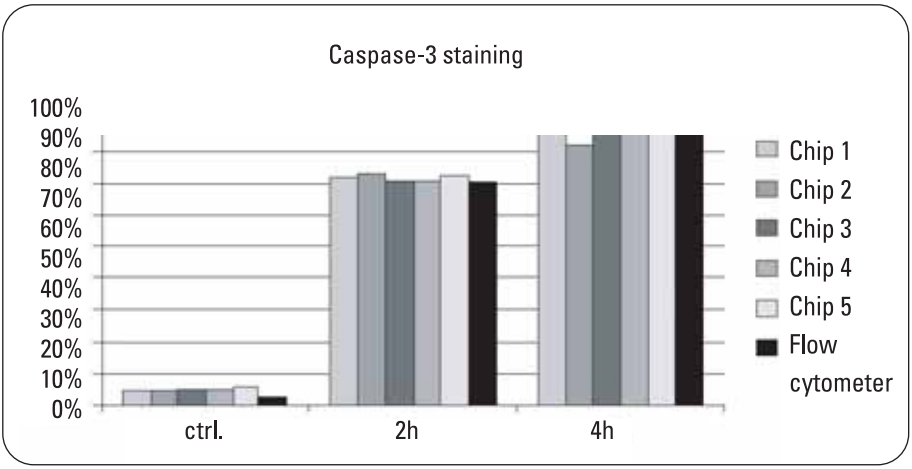


Kit: Cell Fluorescence kit
Assay: Apoptosis assay
Application: Apoptosis (programmed cell death) in Jurkat cells was induced with camptothecin. Cells treated for 16 hours and untreated cells were stained with calcein and Annexin-Cy5. Annexin-V binds to phosphatidylserine - a membrane lipid which is kept to the inner leaflet of the cell membrane of intact cells. Exposure of phopshatidylserine on the outer leaflet is an early indicator of apoptotic processes. Annexin-V binding is made detectable by Cy5 staining of the Annexin-V via a biotin-streptavidin interaction. Calcein staining of cells is used as a live control to distinguish living and apoptotic cells from dead cells. Calcein enters the cell via the membrane as a non-fluorescent ester. The ester is cleaved inside the cell which results in fluorescence.

The histograms on the left show the number and intensity value of all events which generated a signal in the blue channel, corresponding to calcein-stained cells. The histograms on the right shows all events which generated a signal in the red channel, corresponding to Annexin-V binding to apoptotic cells. While the control shows only low intensity values (background noise), the treated sample shows high intensity values (within the red markers) corresponding to apoptotic cells. The dot plot of the treated sample nicely shows the subpopulation of all live cells which are apoptotic.
Corresponding application note: 5988-4319EN

Apoptosis detection

Intracellular Caspase-3 antibody staining assay



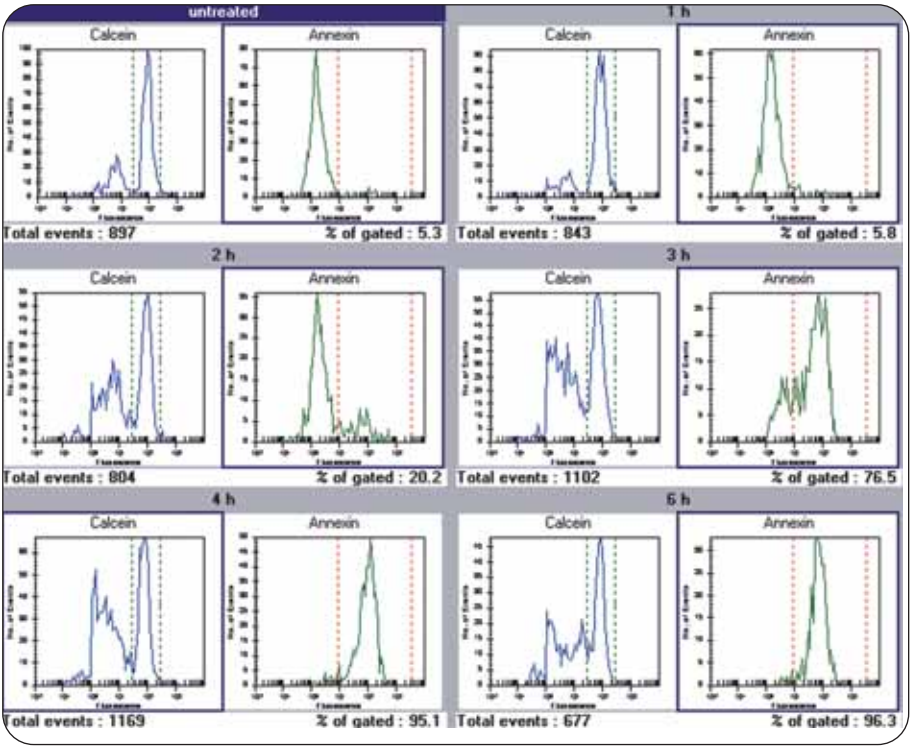
Kit: Cell fluorescence kit
Assay: Generic assay
Application: Induction of apoptosis in Jurkat cells was done with anti-FAS antibody treatment. Intracellular staining with specific antibodies against 'active' Caspase-3 were performed. Reference staining was done with SYTO16 DNA dye. Good chip to chip reproducibility and good comparison to conventional flow cytometer results were obtained.

Corresponding application note: 5988-4319EN

Apoptosis detection

Fast Annexin protocol for time course of apoptosis induction via anti-FAS antibody

A

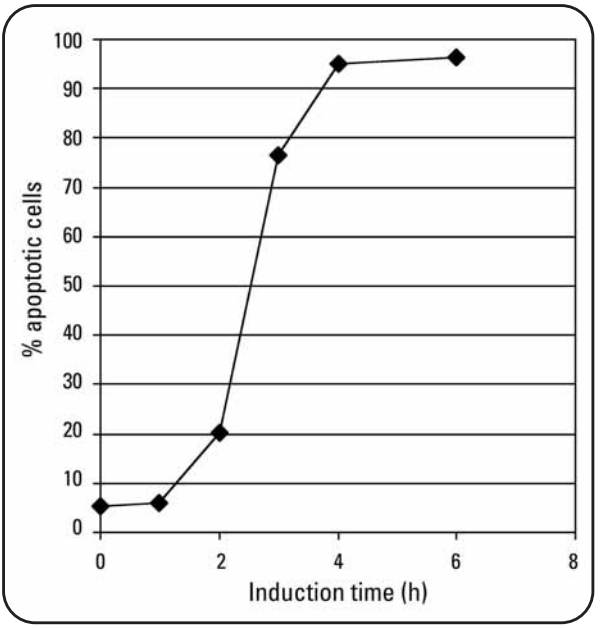


Kit: Cell fluorescence kit

Assay: Apoptosis assay

Application: Apoptosis (programmed cell death) in Jurkat cells was induced with anti-FAS antibody. Cells treated for 0,1,2,3,4 and 6 hours were stained with calcein and Annexin-Cy5. Annexin-V binds to phosphatidylserine - a membrane lipid which is kept to the inner leaflet of the cell membrane of intact cells. Exposure of phopshatidylserine on the outer leaflet is an early indicator of apoptotic processes. Annexin V binding is detectable by Cy5 staining of the Annexin-V via a biotin-streptavidin interaction. Calcein staining of cells is used as a live control to distinguish living and apoptotic cells from dead cells. Calcein enters the cell via the membrane as non-fluorescent ester. The ester is cleaved inside the cell which results in fluorescence and indicates apoptosis.

B

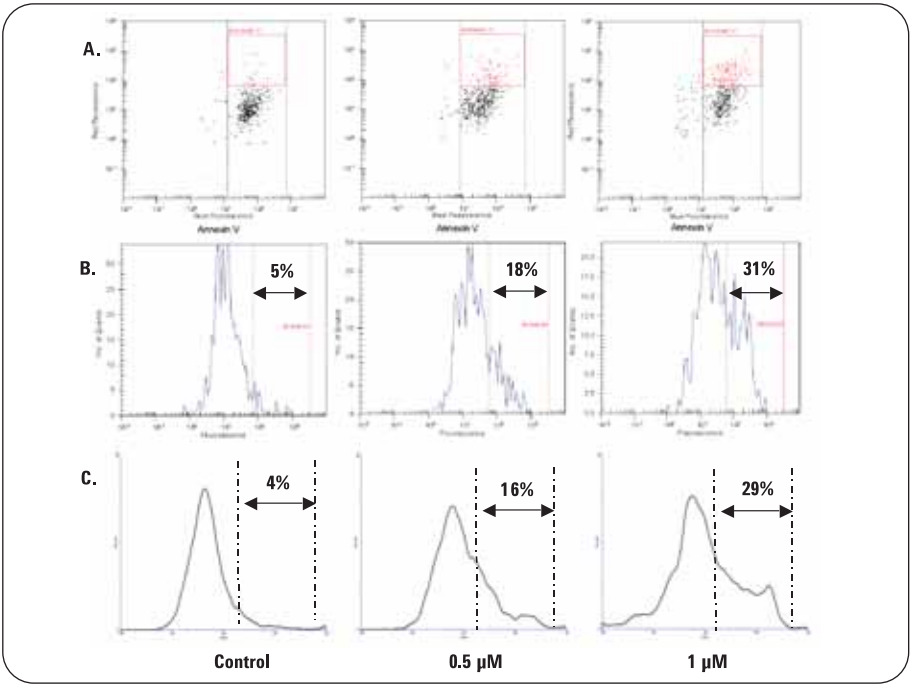


The histograms on page 18 (A) show the number and intensity value of all events which generated a signal in the blue channel, corresponding to calcein-stained cells.
The histograms on the right show all events which generated a signal in the red channel, corresponding to Annexin-V binding to apoptotic cells. While the control shows only low intensity values (background noise), the treated sample shows high intensity values (within the red markers) corresponding to apoptotic cells.
(B) Time course of the induction of apoptosis by anti-FAS antibody in Jurkat cells. Apoptosis is detectable in a significant amount of cells after 2 hours. Following a treatment of 4 hours, approximately 95% of the cells are apoptotic.

Corresponding application note: 5988-4319EN

Apoptosis detection

Apoptosis detection in primary cells

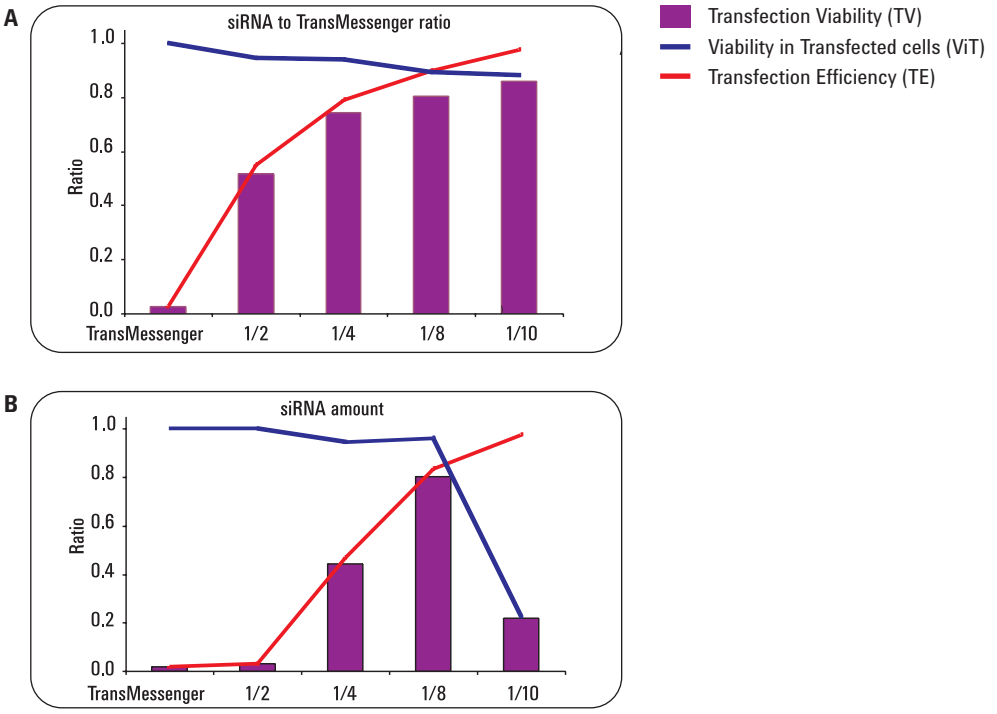


Kit: Cell fluorescence kit
Assay: On-chip antibody staining assay
Application: The Agilent 2100 bioanalyzer has been used to study induced apoptosis by monitoring annexin V-binding in primary human endothelial cells (HUVEC, not shown) and human dermal fibroblasts (NHDF, shown). A simple and fast assay protocol was used on cells left untreated or treated for 5 hours with different concentrations of staurosporine, which induces apoptosis. See row A for dot blots and B for histograms at different concentrations. Evaluation of the same samples on a conventional flow cytometer (row C) yielded similar results.

Corresponding application note: 5989-2934EN

Gene silencing in cell culture

siRNA transfection optimization



Kit: Cell fluorescence kit

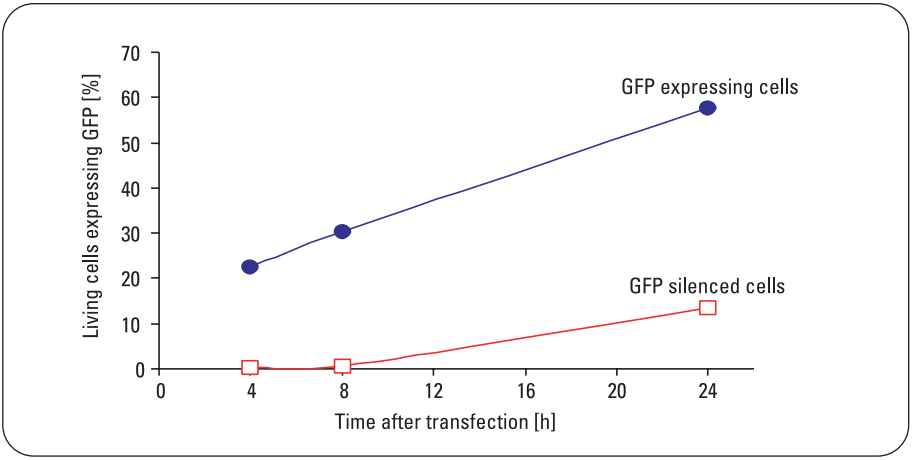
Assay: On-chip antibody staining assay

Application: In gene silencing experiments (HeLa cells) we found that increasing amounts of transfection reagent (TransMessenger™)to a constant amount of siRNA leads to a plateau of transfection viability (panel A). Transfection viability reflects the product of the viability of the transfected cells and the transfection efficiency. With a constant siRNA/transfection reagent ratio of 1:4 and increasing total amounts of introduced siRNA (panel B) the viability of transfected cells decreases at a certain point although the transfection efficiency increases. Thus, there are experimental conditions where the number of living and transfected cells are at a maximum. The Agilent 2100 bioanalyzer features on-chip staining and leads to excellent results with a minimal consumption of cells and reagents.

Corresponding application note: 5988-9872EN

Gene silencing in cell culture

Monitoring of gene silencing experiments



Kit: Cell fluorescence kit
Assay: GFP Assay
Application: After co-transfection of a GFP plasmid and Cy5-labeled siRNA (GFP-specific), GFP expression and viability of cells were detected. The course of GFP expression in control (GFP only) and siRNA/GFP transfected cells was measured on the Agilent 2100 bioanalyzer. Accurate results were obtained fast and in an automated manner. They easily allow the efficiency and reliability of a given protocol and transfection reagents to be judged. Thus, such an experiment provides efficient monitoring and optimization of any gene silencing experiment.

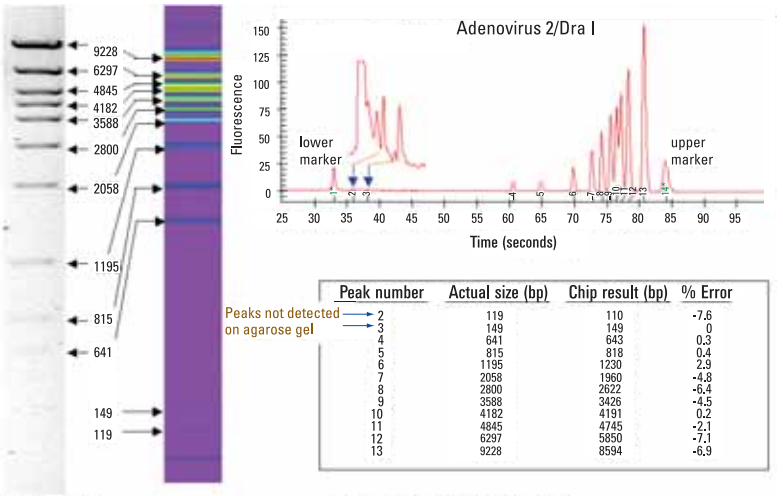
Corresponding application note: 5989-0103EN

II. DNA analysis

	Agriculture/Food	Drug Discovery/Drug Development	Drug Manufacturing QA/QC	Genomics	Pharmaceuticals	Proteomics	Forensics/Homeland Security
Restriction digest analysis							
Sizing range exemplified by the separation of Adenovirus 2/Dra I				●			
Detection of single base mutations (I)				●			●
Detection of single base mutations (II)				●			●
PCR product analysis							
Separation of 3 different mixtures of PCR products				●			●
Determination of PCR product impurity				●			●
Multiplex PCR analysis of bacteria in chicken	●			●			●
Multiplex PCR with 19 products	●			●			●
Gene expression analysis							
mRNA expression study by comparative multiplex PCR	●	●		●			
Standardized end-point RT-PCR	●	●		●			
Co-amplification of GAPDH and hsp72				●			
Co-amplification of GAPDH and hsp72 - response curves				●			
Competitive PCR	●			●			●
Food analysis							
Development of meat specific assays (I)	●			●			
Development of meat specific assays (II)	●			●			
Fish species identification by RFLP	●			●			
GMO detection							
Development of a multiplex assay for soya	●			●			
DNA stability during food processing	●			●			
GMO detection by nested multiplex PCR	●			●			
Oncology							
Tumor cell detection from carcinoma patient blood		●		●			
SNP analysis in cancer related P16 gene		●		●			
K-ras gene SNP detection		●		●			
METH-2 downregulation in lung carcinomas		●		●			
Label-free analysis of microsatellite instability in carcinoma		●		●			
Diagnostic research							
Genotyping of H. pylori	●	●		●			●
Duplications and deletions in genomic DNA		●		●			
Forensic testing							
Optimization of PCR on mtDNA				●			●
Pitfalls in mtDNA sequencing				●			●

Restriction digest analysis

Sizing range exemplified by the separation of Adenovirus 2/Dra I



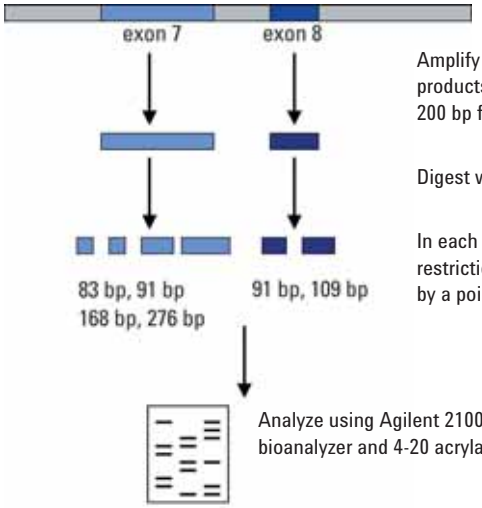
Peak number	Actual size (bp)	Chip result (bp)	% Error
2	119	110	-7.6
3	149	149	0
4	641	643	0.3
5	815	818	0.4
6	1195	1230	2.9
7	2058	1960	-4.8
8	2800	2622	-6.4
9	3588	3426	-4.5
10	4182	4191	0.2
11	4845	4745	-2.1
12	6297	5850	-7.1
13	9228	8594	-6.9

Kit: DNA 12000 kit
Assay: DNA 12000 assay
Application: Restriction digest analysis of Adenovirus 2/Dra I. For restriction fragment analysis the large linear dynamic range of the lab-on-a-chip approach is very advantageous. Analyzing samples with large and short fragments on slab gels can be difficult because of bands running off the gel and insufficient staining (or over-staining) of bands.

Corresponding application note: 5968-7501EN

Restriction digest analysis

Detection of single base mutations (I)

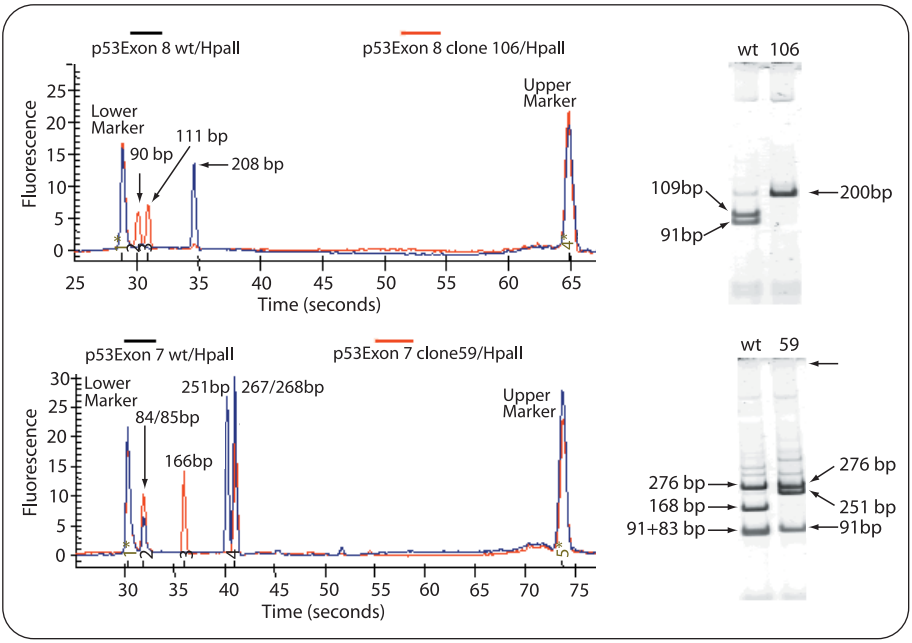


Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Mutation detection by RFLP highlights the use of the Agilent 2100 bioanalyzer. Two different regions of the p53 gene were amplified with specific primers and digested with Hpa II, which cuts in a location that is prone to mutations. In the presence of a point mutation, the enzyme Hpa II does not cleave the DNA, leaving larger fragments that can be revealed by gel electrophoresis or by analysis with the DNA 7500 LabChip kit (see next page).

Corresponding application note: data not published

Restriction digest analysis

Detection of single base mutations (II)

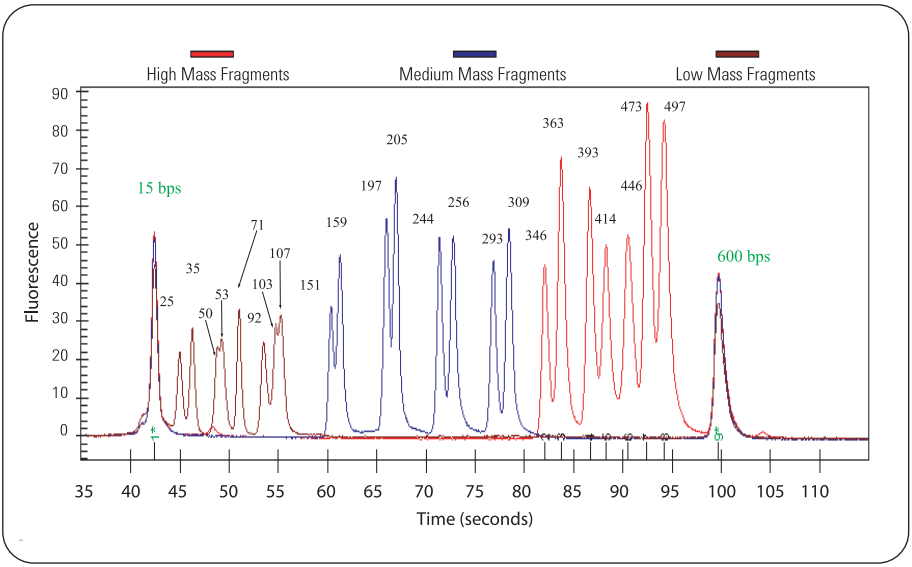


Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Analysis on the chip showed an identical pattern of digest fragments as seen on the slab gel for the wildtype and Exon 7 & 8 PCR products. Comparison of the calculated sizes of the bands shows 1-2% variance with the LabChip assay, which allows fast and accurate detection of point mutations.

Corresponding application note: 5968-7496EN

PCR product analysis

Separation of 3 different mixtures of PCR products

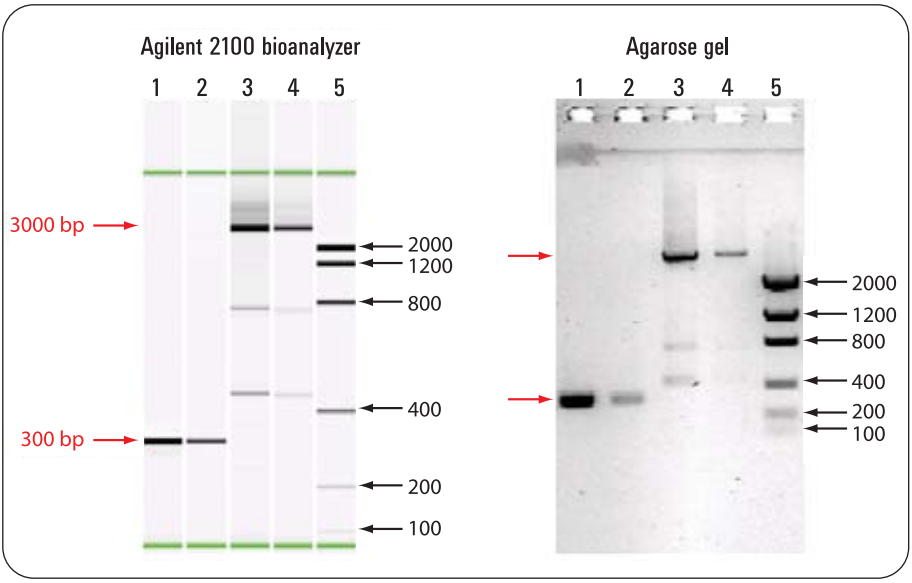


Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Overlay of three different electropherograms, which are mixtures of PCR samples ranging from 25 to 500 base pairs in size. The two closest eluting bands (50 bp and 53 bp) are partially separated and identified by the software as two separate peaks. The DNA 500 assay achieves a resolution of five base pairs from 25 to 100 base pairs and a 5% resolution from 100 to 500 base pairs where the sizing error is less than 10% over the entire size range.

Corresponding application note: 5988-3041EN

PCR product analysis

Determination of PCR product impurity

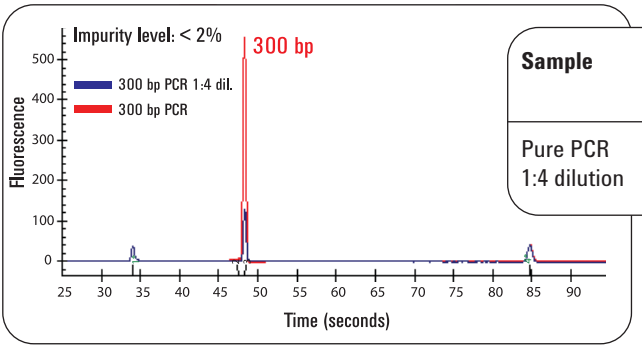


Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Comparison between the analysis of two PCR reactions (300 and 3000 bp products) using the DNA 7500 LabChip kit vs. an agarose gel. Two different concentrations are shown side by side for each PCR reaction (undiluted and 1:4 dilution). The Agilent 2100 bioanalyzer shows superior performance in locating impurities over a broader concentration range than the gel. The 300 bp fragment appears to be uncontaminated in both the gel and on the Agilent 2100 bioanalyzer. The 3000 bp fragment shows few impurities on the gel, which become invisible at the 1:4 dilution. These impurities can easily be detected with the Agilent 2100 bioanalyzer.

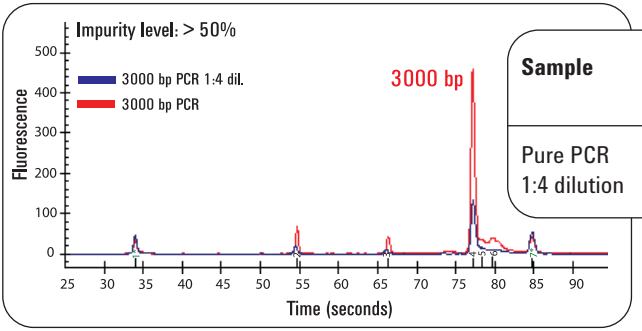
Corresponding application note: 5968-7496EN

PCR product analysis

Determination of PCR product impurity



Sample	c (DNA) All peaks	Main peak (300bp)
Pure PCR	41.4 ng/µl	40.7 ng/µl
1:4 dilution	9.6 ng/µl	9.6 ng/µl



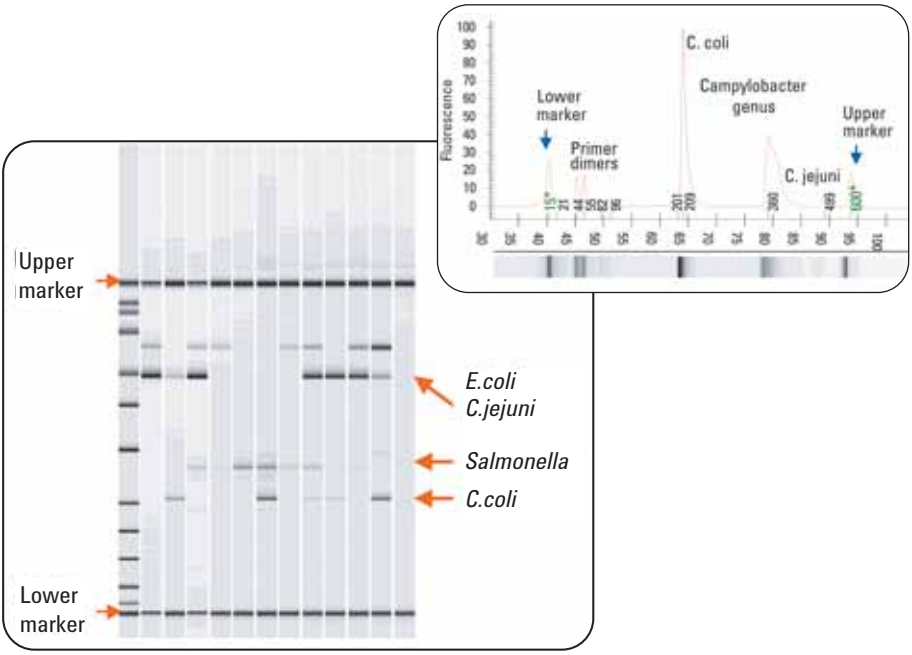
Sample	c (DNA) All peaks	Main peak (3000bp)
Pure PCR	61.9 ng/µl	40.7 ng/µl
1:4 dilution	14.8 ng/µl	9.8 ng/µl

Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: The quantitative data generated by the Agilent 2100 bioanalyzer indicate the amount of impurity or non-specific products in the PCR reactions from the previous page. Even in the 300 bp fragment a small impurity can be detected, while the 3000 bp fragment shows more than 50% impurities.

Corresponding application note: 5968-7496EN

PCR product analysis

Multiplex PCR analysis of bacteria in chicken



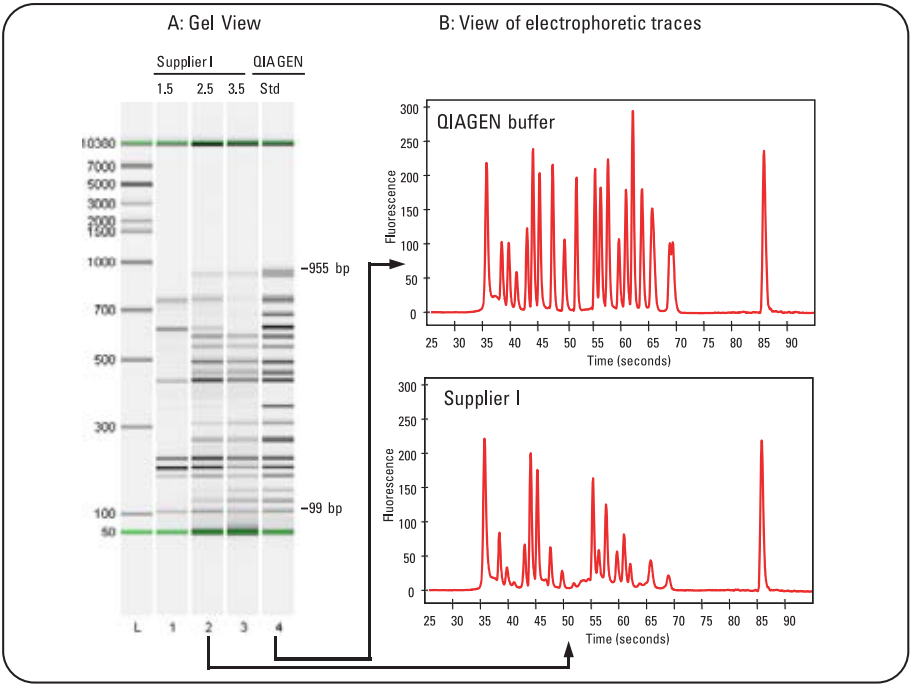
Data kindly provided by GenPoint, NL

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Multiplex PCR with four primer pairs, each one specific for a certain DNA sequence from one of the 4 bacteria to be tested for. Total DNA was extracted from chicken and subjected to PCR. The gel-like image shows traces from different chicken samples with bands showing up when an amplicon could be detected. The electropherogram is one example where bacterial DNA from two species of the *Campylobacter* genus could be detected.

Corresponding application note: data not published

PCR product analysis

Multiplex PCR with 19 products



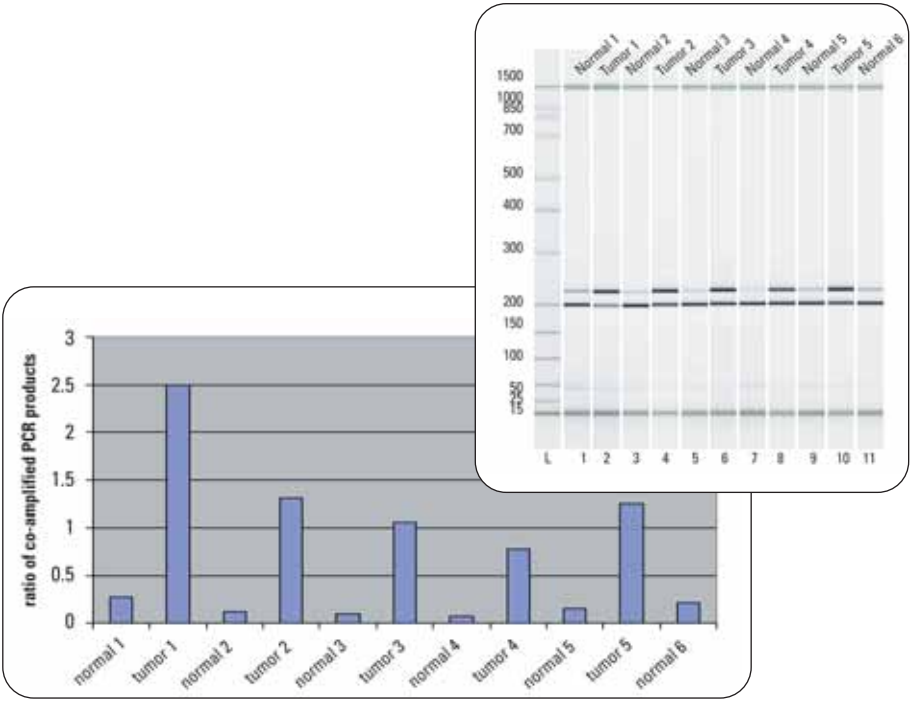
Data kindly provided by Qiagen, Germany

Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Many molecular applications include PCR multiplexing as shown above with a PCR that yields 19 products. Applications are genotyping of transgenic organisms, detection of pathogens or GMs and microsatellite genotyping (e.g. short tandem repeat (STR) and variable number tandem repeat (VNTR) analyses). The sample shows optimization of PCR conditions (Mg^{2+} concentration) performed to ensure annealing of the multiple primers under identical conditions. Visualization and evaluation of the results can be performed efficiently with the Agilent 2100 bioanalyzer because of the high resolution, the accurate sizing, quantitation and extended linear range.

Corresponding application note: 5988-9342EN

Gene expression analysis

mRNA expression study by comparative multiplex PCR



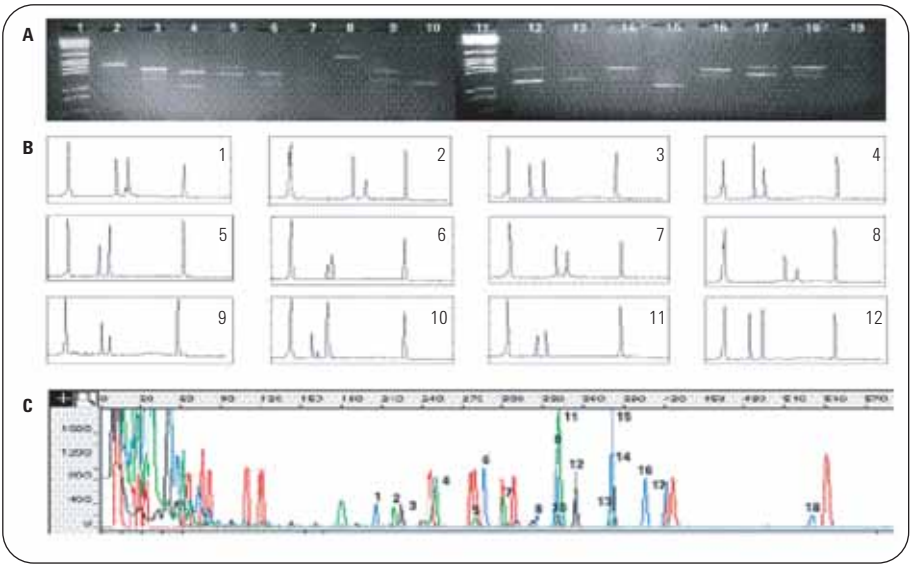
Data kindly provided by the Roy Castle Centre

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Two genes were co-amplified in this study. A tumor specific gene (upper band) along with a housekeeping gene (lower band). The upregulation of the tumor gene is visualized via analysis on the Agilent 2100 bioanalyzer. Building the ratio of the concentration values obtained from the Agilent 2100 bioanalyzer, numerical values are obtained that are normalized with regard to the RT-PCR amplification efficiency. This way tumor tissue can be distinguished from normal tissue more unambiguously.

Corresponding application note: data not published

Gene expression analysis

Standardized end-point RT-PCR



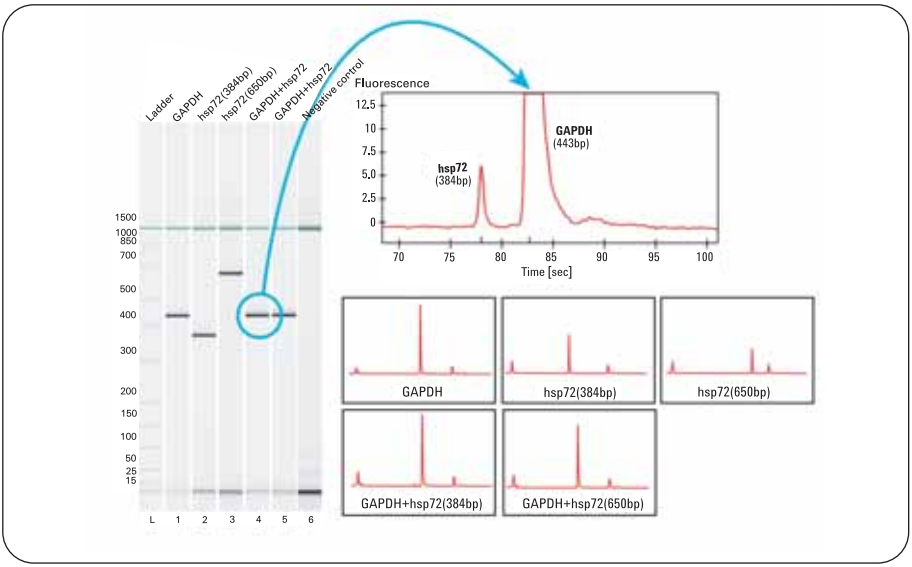
Data kindly provided by the Medical College of Ohio

Kit: DNA 7500 kit
Assay: DNA 7500 assay
Application: Complementary DNA from bronchial epithelial cells (BEC) was analyzed by a Standardized RT-PCR (StaRT) for the expression of 15 different genes. This analysis can be performed at the end-point of PCR without the need for real-time measurement at each cycle of PCR. Three methods for evaluation of representative results were compared (see above). The coefficient of variance (CV) from at least 3 measurements was calculated. The direct comparison of the reproducibility for agarose gel analysis (A, CV = 0.50) and the ABI Prism310 Genetic Analyzer (C, CV = 0.39) with the Agilent 2100 bioanalyzer (B, CV = 0.29) reveals that the Agilent 2100 bioanalyzer is superior. It is a reliable and valuable tool in quantitative gene expression analysis.

Corresponding application note: 5988-3674 EN

Gene expression analysis

Co-amplification of GAPDH and hsp72



Data kindly provided by Dr. Eric Gottwald, Forschungszentrum Karlsruhe, Germany

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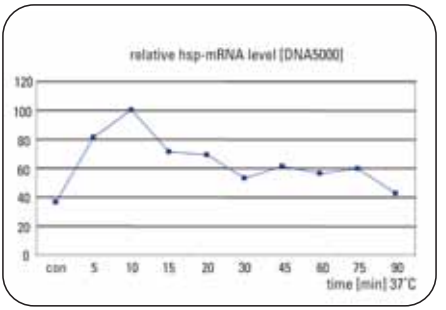
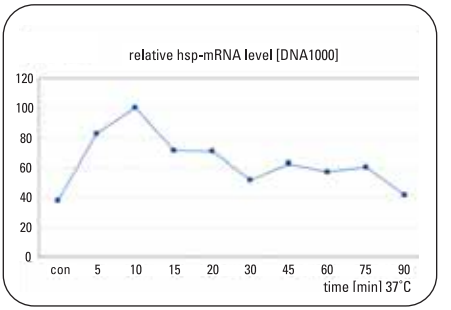
Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Gel-like image and electropherograms showing the results of separate amplifications and co-amplifications of GAPDH and hsp72 in unstimulated HepG2 cells. Primers for GAPDH yield a PCR product of 443 bp (lane 1), primers for hsp72 yield PCR products of 384 and 650 bp (lane 2 and 3). Lane 4 and 5 show the results of the co-amplification reactions. Due to the competitiveness of the reaction, very little hsp72 products could be detected in lane 4 (insert) and no product was detected in lane 5 (lane 6 = negative control). The broad linear dynamic range of the analysis allows detection of weak bands next to strong bands and helped in the determination of gene expression in this case.

Corresponding application note: 5988-4556EN

Gene expression analysis

Co-amplification of GAPDH and hsp72 - response curves





Data kindly provided by Dr. Eric Gottwald, Forschungszentrum Karlsruhe, Germany

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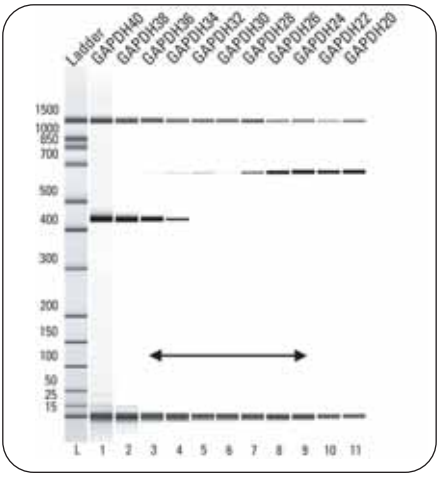
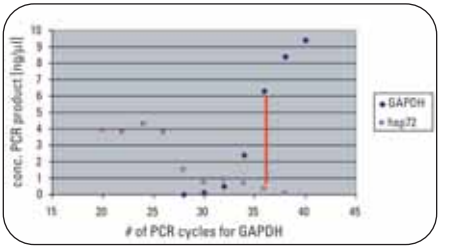
Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: The optimized PCR conditions were used to monitor the response of a stimulus to hsp. Gene expression was monitored by comparing the RT-PCR amplification of a housekeeping gene with the co-amplification of hsp. In the current case, the highest gene expression was measured after about 10 minutes. As a comparison, the same set of samples was analyzed using the DNA 500 kit. Virtually identical results are obtained with both kits, demonstrating thatlab-on-a-chip technology can serve as a standardized approach to gel electrophoresis.

Corresponding application note: 5988-4556EN

Gene expression analysis

Competitive PCR





Data kindly provided by Dr. Eric Gottwald, Forschungszentrum Karlsruhe, Germany

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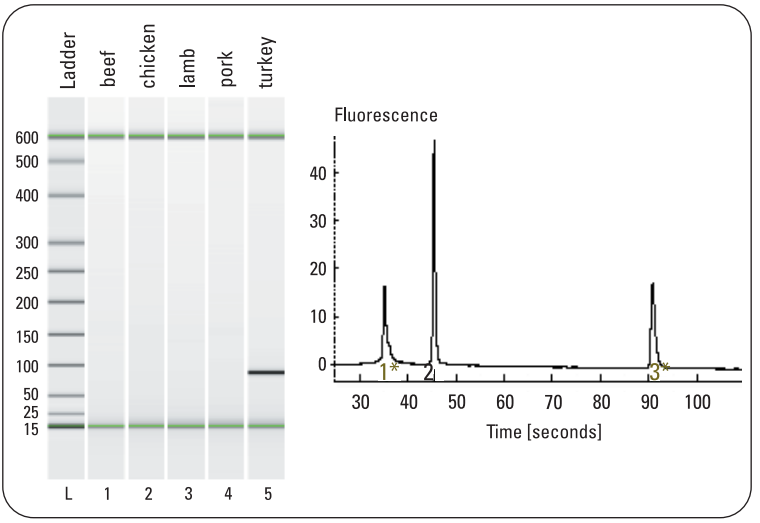
Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Two genes were reverse transcribed and co-amplified in one reaction tube. The PCR products were analyzed using the DNA 1000 LabChip kit. Primers for hsp72 were present from the beginning of the PCR reactions, while primers for GAPDH were added after various cycle numbers ranging from 20 to 40 cycles (primer dropping method). This allowed optimization of this competitive PCR reaction. The left graph displays the dynamic range (arrow) in the gel like view, whereas the right graph indicates conditions with greatest sensitivity (red line).

Corresponding application note: 5988-4556EN

Food analysis

Development of meat specific assays (I)



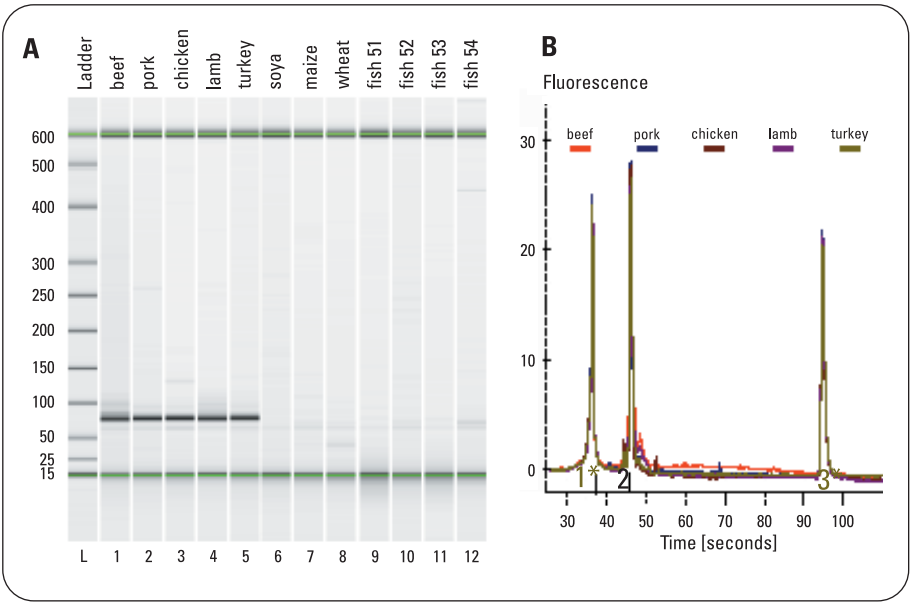
Data kindly provided by CCFRA, UK

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: For detection of individual species in processed food, PCR assays with specific sets of primers can be developed. Example: turkey specific primers do not amplify any other meat species, including beef, chicken, lamb, or pork (see lane 5 and respective electropherogram).

Corresponding application note: 5988-4069EN

Food analysis

Development of meat specific assays (II)



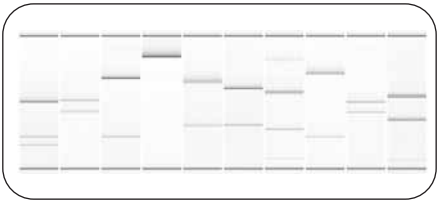
Data kindly provided by CCFRA, UK

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: For detection of individual component types in processed food, PCR assays with specific sets of primers can be developed. Example: Primers that amplify any type of meat, but do not amplify other food constituents, including soya, maize, wheat or fish.

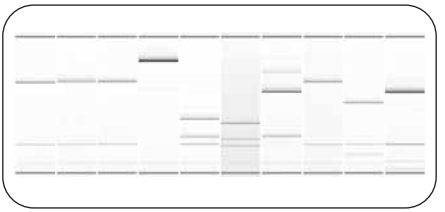
Corresponding application note: 5988-4069EN

Food analysis

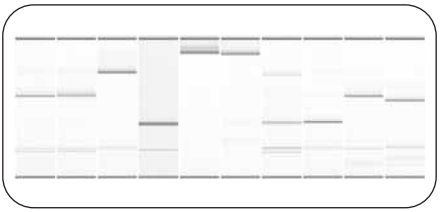
Fish species identification by RFLP



Restriction enzyme: DdeI



Restriction enzyme: HaeIII



Restriction enzyme: NlaIII

Common name (UK)	Latin name
Atlantic Cod	Gadus morhua
Pacific Cod	Gadus macrocephalus
Coley (Saithe)	Pollachius virens
Haddock	Melanogrammus aeglefinus
European Hake	Merluccius merluccius
South African Hake	Merluccius paradoxus
European Plaice	Pleuronectes platessa
Whiting	Merlangus merlangus
Alaskan (Walleye) Pollock	Theragra chalcogramma
Hoki	Macruronus novaezelandiae
Atlantic Salmon	Salmo salar
Red / Sockeye Salmon	Oncorhynchus nerka
Pink / Humpback Salmon	Oncorhynchus gorbuscha
Chinook Salmon	Oncorhynchus tschawytscha
Coho / Silver Salmon	Oncorhynchus kisutch
Keta / Chum Salmon	Oncorhynchus keta
Cut-throat Trout	Oncorhynchus clarki clarki
Dolly Varden	Salvelinus malma malma
Cherry Salmon	Oncorhynchus masou masou

Data kindly provided by CCFRA, UK

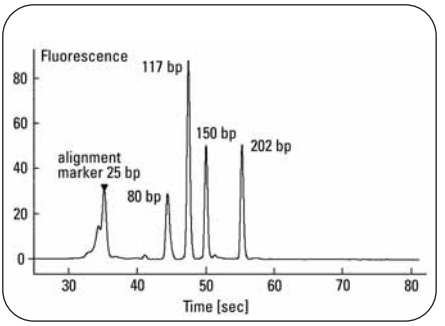
Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Identification of white fish and salmon species in the processed state presents a challenge. However, evaluation of PCR-RFLP profiles (PCR-restriction fragment length polymorphism) of a 464 bp region from the cytochrom b gene cut separately with three restriction enzymes facilitated the differentiation of 19 commercially important species. Analysis of the restriction digests was performed with the Agilent 2100 bioanalyzer. This approach was successfully tested in an interlaboratory study.

Corresponding application note: 5989-2982EN

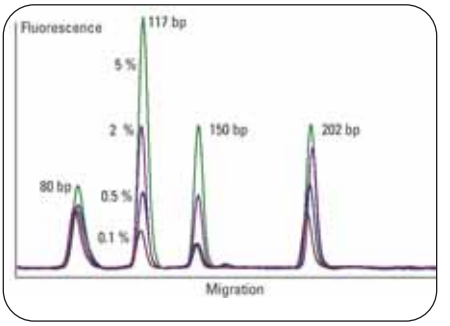
GMO detection

Development of a multiplex assay for soya

A



B



Data kindly provided by CCFRA, UK

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Multiplex assay for genetically modified (GM) soya. The aim was to develop a model assay that could be used to assess the quality of DNA extracted from heat-processed soya flour samples, in particular, to investigate differences in PCR amplification between small DNA targets. A single multiplex PCR assay was developed that enabled three GM soya targets and one control to be analyzed in a single reaction mix. Primer concentration was optimized in order to obtain four PCR products resolved by gel electrophoresis which corresponded in size to the soya lectin gene target of 80 bp, and the EPSPS (5-enolpyruvyl-shikamate- 3-phosphate synthase) gene targets of 117 bp, 150 bp and 202 bp respectively. These latter targets are only found in Roundup Ready GM soya. Figure A: Peaks produced by the four PCR products when analyzed with the Agilent 2100 bioanalyzer and DNA 500 LabChip kit. Figure B: Analysis of certified reference materials containing known amounts of GM soya.

Corresponding application note: 5988-4070EN

GMO detection

DNA stability during food processing

Time at 100°C and pH 3.3 (min)	Amount of PCR product*			
	80 bp	118 bp	150 bp	202 bp
0	100	100	100	100
3	74	77	73	67
6	57	58	21	6
9	36	23	24	15
12	67	33	47	21
15	48	27	16	0
18	0	0	0	0
21	0	0	0	0

* % product determined relative to the amount at 0 minutes

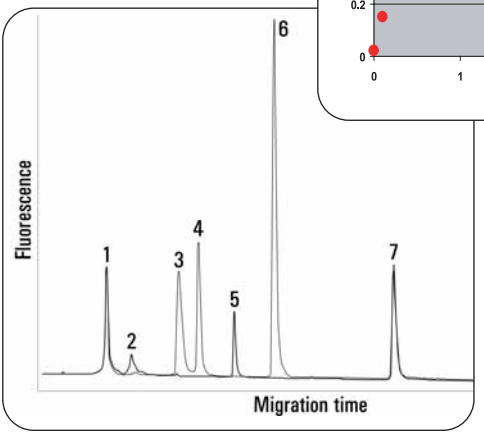
Data kindly provided by CCFRA, UK

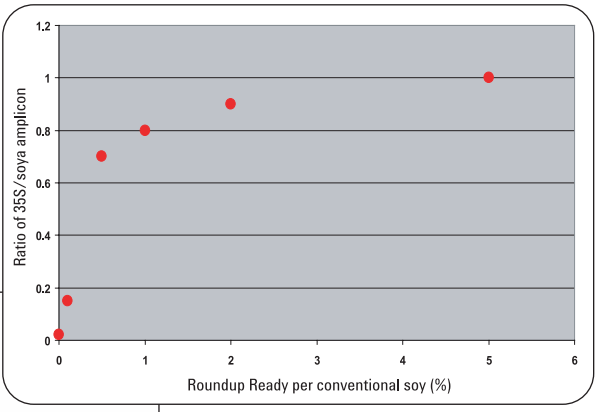
Kit: DNA 500 kit
Assay: DNA 500 assay
Application: The multiplex PCR assay was applied to soya flour samples containing approx. 1.3 % GM soya and boiled at either pH 3.3, 4.3 or 6.7 for up to 21 minutes. For accurate determination of the quantity of each PCR product, the samples were applied to the DNA 500 LabChip. The concentration of each PCR product was calculated using the Agilent 2100 bioanalyzer software. At pH 3.3 where an effect of heating time was observed, the amount of each PCR product at each time point was compared to the amount of each product at 0 minutes (Table 2). At pH 3.3, the relative amount of the 80 bp product was reduced to 48 % after 15 minutes and no product was detected at 18 or 21 minutes. After 15 minutes, the relative amounts of products of 118 bp and 150 bp were reduced to 27 % and 16 % respectively and the 202 bp product was not detected. None of the products were detected after 18 or 21 minutes.

Corresponding application note: 5988-4070EN

GMO detection

GMO detection by nested multiplex PCR





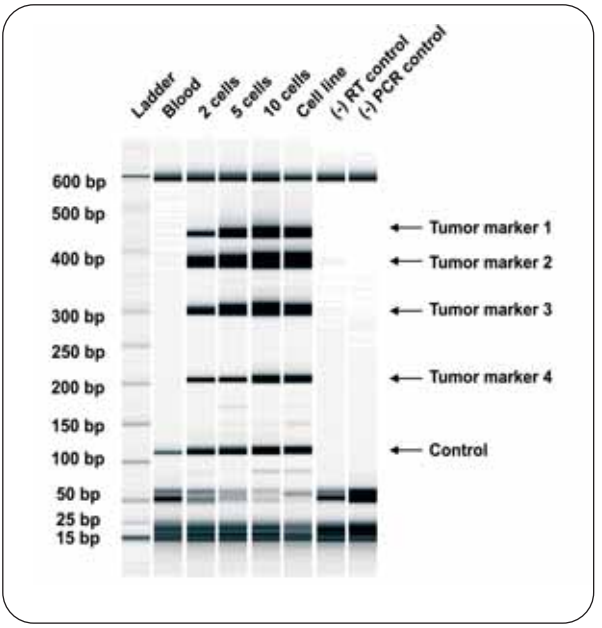
1 Low molecular weight marker
2 Primer-dimer in negative control
3 Soy lectin - 118 bp
4 35S GMO - 153 bp
5 Internal control - 217 bp
6 Corn zein - 278 bp
7 High molecular weight marker

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: GMO detection by multiplex PCR is widely used for soy and corn. Often sequences from the transgene and species specific controls or internal standard are co-amplified by endpoint PCR in a screening procedure. Multiple products can be analyzed with the Agilent 2100 bioanalyzer at high resolution and sensitivity. Quantification and comparison of product amounts may already lead to qualification of a positive screening result prior to analysis by expensive quantitative real time PCR.

Corresponding application note: 5989-0124EN

Oncology

Tumor cell detection from carcinoma patient blood



Data kindly provided by AdnaGen

Kit: DNA 500 kit
Assay: DNA 500 assay
Application: A combined method of specific tumor cell enrichment and a high sensitivity tumor cell detection by multiplex PCR allows analysis of several tumor marker genes. The method is so sensitive that it allows the detection of only a few tumor cells per 5 ml EDTA-blood. The Agilent 2100 bioanalyzer provides the performance to detect the PCR products with high sensitivity and automated result flagging. This method offers new possibilities for monitoring and prognosis in routine diagnosis, and may facilitate an appropriate selection of patients for adjuvant therapy.

Corresponding application note: 5988-9341EN

Oncology

SNP analysis in cancer related P16 gene

Sample	1	2	3	4	5	6	7	8	9	10	11
Genotype316	C	C	CG	CG	CG	CG	CG	C	C	C	G
Genotype356	C	C	CT	CT	CT	C	C	CT	CT	T	C
gel-like view Agilent 2100 bioanalyzer											
Main Band	198	198	197	198	198	198	198	198	199	198	195
Extra Band 1			204	204	204						
Extra Band 2			216	215	215	214	214				

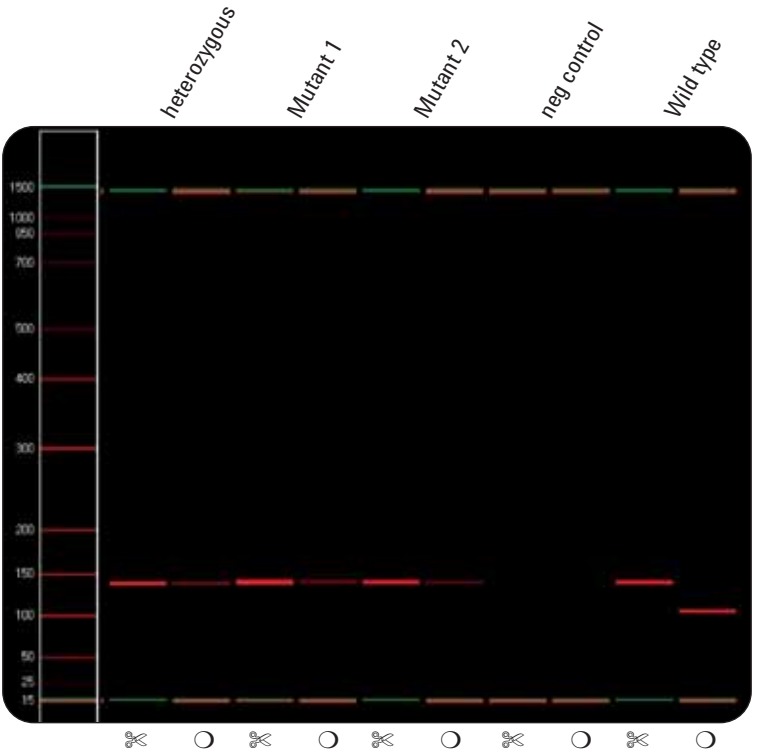
Data kindly provided by SAIC-Frederick

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Mutations in the exon 3 region of P16 gene are closely related to human cancer. A PCR yields 198 bp fragments with single, expected bands or additional, multiple bands in the Agilent 2100 bioanalyzer analysis. These observations correspond perfectly to genotyping sequencing data of normal and mutant tissues. The pattern of bands is visible due to slower mobility of the heteroduplex formed by heterozygote mutant of the samples. The method provides fast and reliable acquisition of genetic diagnostic data from cancer patients, also on single nucleotide polymorphisms (SNP).

Corresponding application note: 5989-0487EN

Oncology

K-ras gene SNP detection



Data kindly provided by SAIC-Frederick

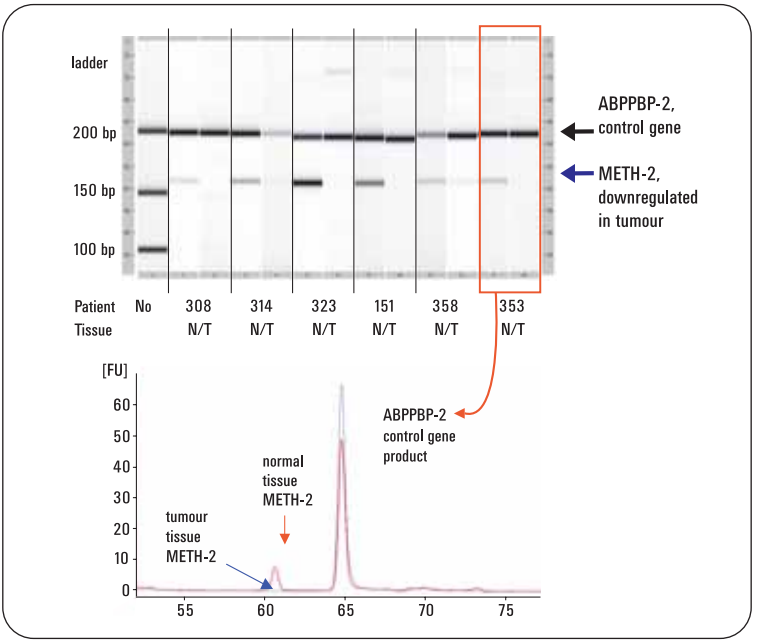
✂ - PCR product
❍ - Restriction

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Mutations in the K-ras gene coding 12 region can lead to cancer in different human tissues. A dedicated combination of PCR and specific restrictions (BstNI digest) reveals the underlying single nucletide polymorphisms (SNPs). The integral element within this test is the rapid and precise analysis of short amplicons (135 bp, see PCR-product lanes above) and fragments (106 bp, visible in lanes labeled with restriction) with the lab-on-a-chip technique. The test was used to ultimately determine a cancer patient's eligibility for a clinical trial for a peptide vaccine.

Corresponding application note: 5989-0487EN

Oncology

METH-2 downregulation in lung carcinomas



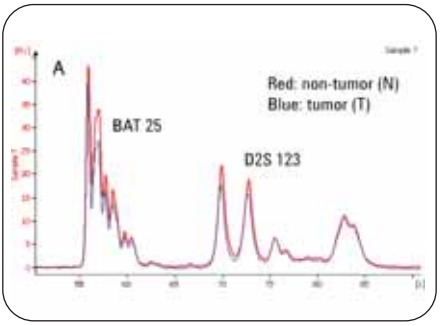
Data kindly provided by Roy Castle Lung Cancer Research Programme, University of Liverpool, UK

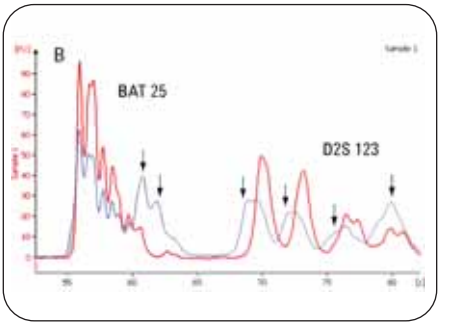
Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Microarray analysis reveals under- or over-representations of transcripts. Screening of several cell lines for independent validation of such observations can be done with different techniques such as comparative multiplex PCR. This application shows the downregulation of a characteristic antiangiogenetic factor (METH-2) for a series of patient samples. Expression in normal tissue and tissue from the non small lung carcinomas is compared. Results from the array experiments were confirmed on a broad basis. Fast and convenient analysis with the Agilent 2100 bioanalyzer with given quantitation capability fit perfectly in such analytical workflow.

Corresponding application note: 5989-3514EN

Oncology

Label-free analysis of microsatellite instability in carcinoma



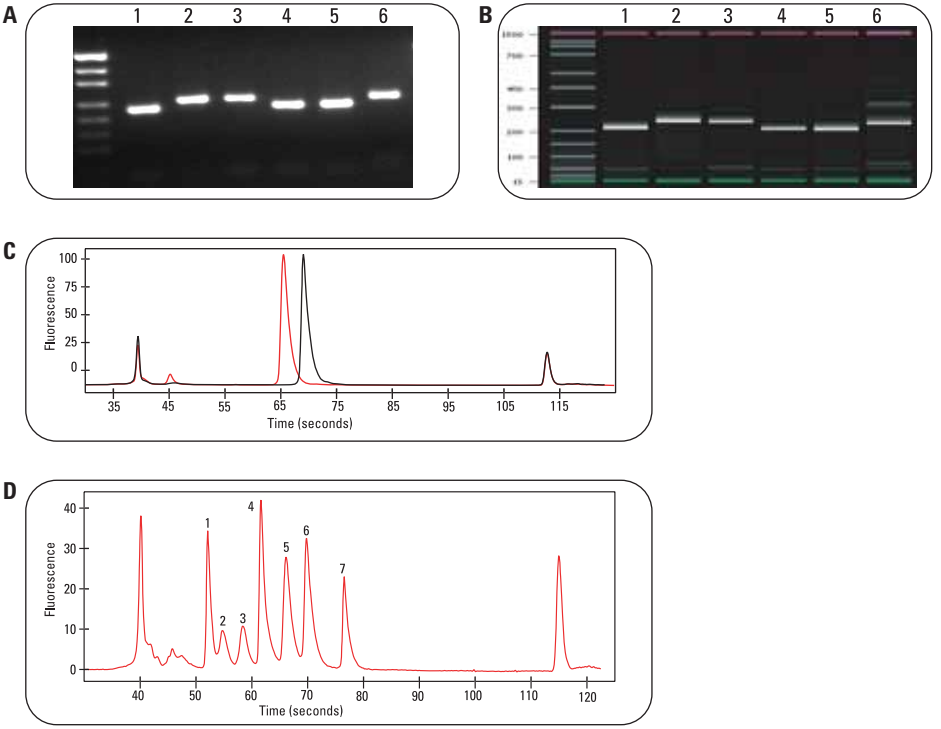


Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Microsatellite instability (MSI) is caused by a failure of the DNA mismatch repair system and occurs frequently in various types of cancer. Given that conventional techniques used for MSI detection, for example, polyacrylamide gel electrophoresis (PAGE) or capillary electrophoresis, turned out to be laborious or expensive, this study aimed to develop a simple and efficient procedure of MSI detection. Detection of MSI could be demonstrated by microsatellite loci-associated, well defined deviations in the electropherogram profiles of tumor and non-tumor material and confirmed the classification of the MSI cases performed by conventional technology (95% concordance rate). Whereas the results of the MSI detection were comparable to conventional techniques, the on-chip electrophoresis on the Agilent 2100 bioanalyzer was superior in terms of speed, usability and data management.

Corresponding application note: 5989-2626EN

Clinical research

Genotyping of H. pylori



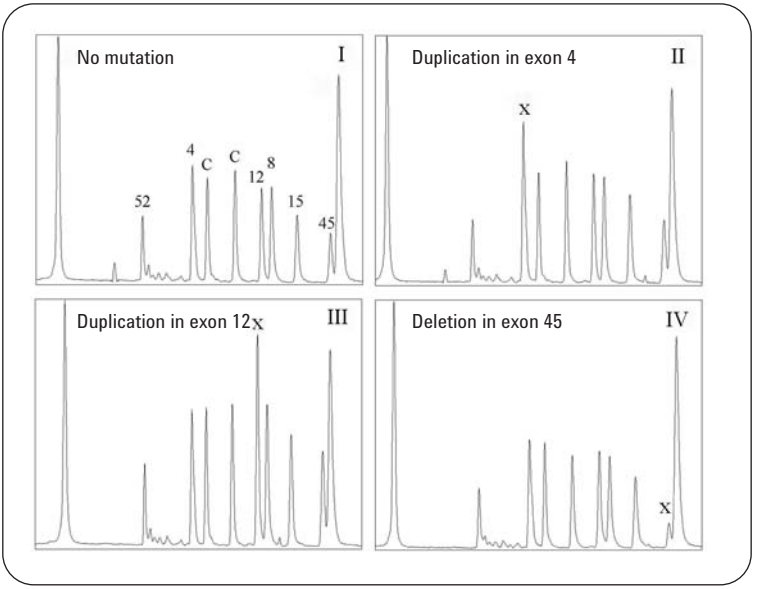
Data kindly provided by Institute for Pathology, Cologne

Kit: DNA 1000 kit
Assay: DNA 1000 assay
Application: Different allelic variants are associated with different stages of H. pylori virulence. Multiplex PCR on five alleles with products in the range of 102 to 301 bp were used to analyze DNA from paraffin embedded tissues. Agarose gel (A) yields only limited distinctiveness, whereas gel-like images (B) and electropherograms (C) show good resolution and superior reproducibility allowing convenient analysis of all desired products in parallel (D). An extended spectrum of prognostic or therapeutic relevant information is now routinely accessible for simultaneous analysis.

Corresponding application note: 5989-0078EN

Clinical research

Duplications and deletions in genomic DNA



Data kindly provided by Center for Human and Clinical Genetics, Leiden

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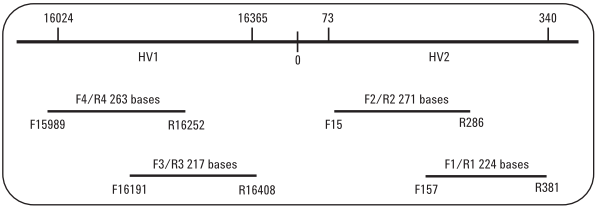
Kit: DNA 500 kit
Assay: DNA 500 assay (in expert software)
Application: Multiplex amplifiable probe hybridization (MAPH) and multiplex ligation-dependent amplification (MLPA) are high throughput techniques for the detection of reordered genomic segments. These methods include hybridization of amplifiable probes with either stringent washing or ligation events prior to amplification. Exact and reproducible sizing and quantitation of multiple products are important prerequisites which are delivered by the Agilent 2100 bioanalyzer and lead to quick and simple analysis of genetically related diseases.

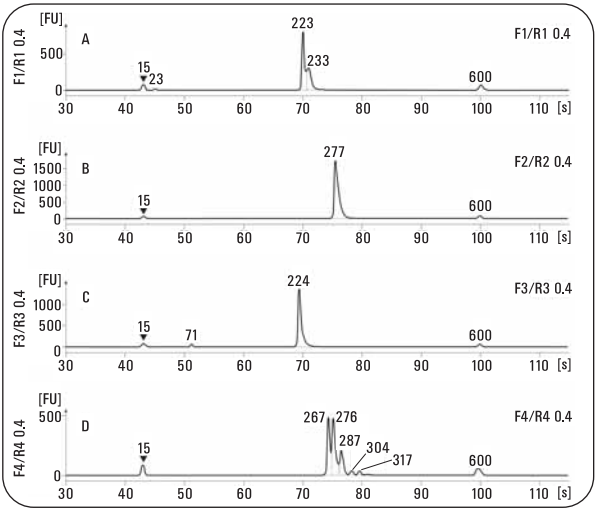
Corresponding application note: 5989-0192EN

Forensic testing

Optimization of PCR on mtDNA



Amplified areas
in human mtDNA

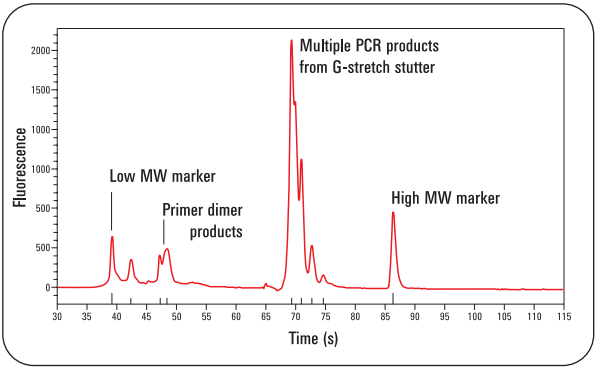


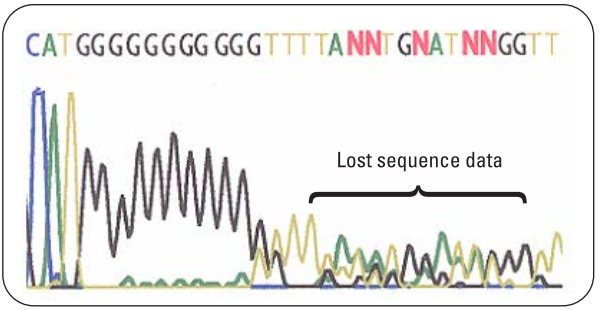
Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Human mitochondrial DNA (mtDNA) is amplifiable even from small or badly degraded samples, even if genomic DNA is not available. Lanes B and C show homogenous PCR products which can subsequently be sequenced for identification. However, careful optimization of PCR parameters, like pH, Mg2+ concentration or polymerase amount is necessary and shown in detail in this application note. For example, a high Taq concentration increased the yield but also increased the level of byproducts. PCR for samples in lane D (impurities) and lane A (C-heteroplasmy) need to be improved. The Agilent 2100 bioanalyzer provides a rapid quantitative analysis over the broad size and concentration range needed for optimization and QC. It has proven to be an indispensable tool for forensic labs.

Corresponding application note: 5989-3107EN

Forensic testing

Pitfalls in mtDNA sequencing





Kit: DNA 500 kit
Assay: DNA 500 assay
Application: Analysis of the non-coding sequence of human mitochondrial DNA (mtDNA) is performed for the purpose of identification in forensics. PCR amplification of limited or degraded mtDNA is done prior to sequencing. Quantitation and quality control of these PCR products (10-100 ng/ml, homogenous fragment in the range of 200-500 bp) was performed. Difficult PCR templates may cause G-stutters or other unintended byproducts of higher or lower mass (left). This may lead to indistinct sequence readings (right). Therefore, e.g. FBI guidelines enforce a 10% impurity level at the most. Fulfillment of this prerequisite can be satisfactorily verified with the Agilent 2100 bioanalyzer.

Corresponding application note: 5989-0985EN

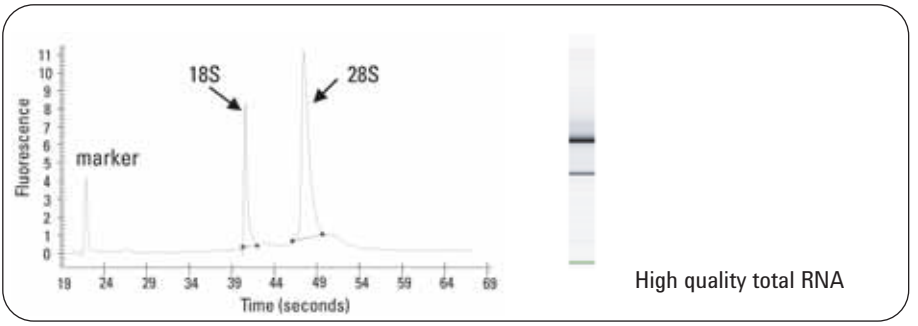
III. RNA analysis

	Agriculture/Food	Drug Discovery/Drug Development	Drug Manufacturing QA/QC	Genomics	Pharmaceuticals	Proteomics	Forensics/Homeland Security
Analysis of total RNA							
RNA integrity		●	●	●			
Standardization of RNA Quality Control		●	●	●			
Reproducibility of quantitation		●	●	●			
Genomic DNA contamination		●	●	●			
Low amounts of total RNA							
Detection of low levels of RNA				●			
RNA integrity with the RNA 6000 Pico kit		●	●	●			
RNA quality after staining and microdissection				●			
Analysis of minimum RNA amounts		●		●			
Genomic DNA in low concentrated RNA extracts		●		●			
Low RNA amounts from kidney sections		●		●			
Analysis of mRNA							
RNA integrity		●		●	●		
Ribosomal RNA contamination in mRNA samples		●		●			
Analysis of Cy5-labeled samples							
Analysis of cRNA with and without dye in gel matrix		●		●	●		
Optimization of labeling reactions		●		●	●		
cRNA fragmentation		●		●	●		
Analysis of T7-RNA transcripts							
Size estimation		●					

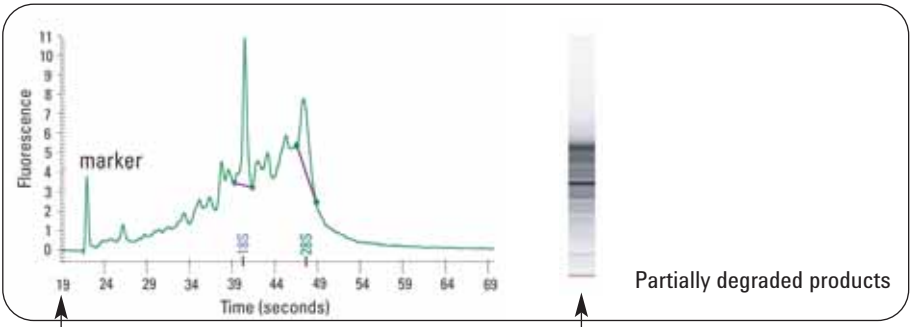
Analysis of total RNA

RNA integrity

A



B



Agilent 2100 bioanalyzer: electropherogram

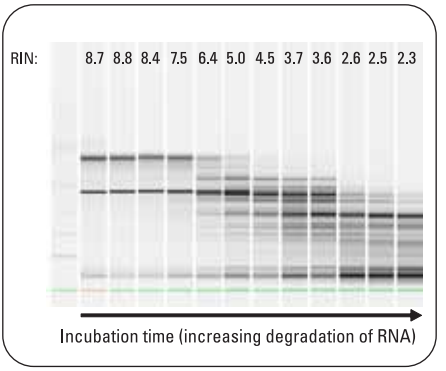
Agilent 2100 bioanalyzer: single lane gel-like image

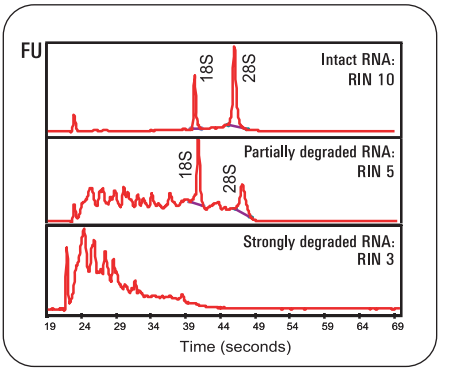
Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: Analysis of total RNA integrity - a typical first QC step during cDNA or cRNA sample prep for microarrays. In Figure A the upper electropherogram and gel-like image show the analysis of high quality total RNA with the 18S and 28S subunit as two distinct bands. Figure B shows the analysis of a partially degraded total RNA sample. Many degradation products appear between the two ribosomal bands and below the 18S band. With the help of the Agilent 2100 bioanalyzer and the RNA 6000 Nano kit the important sample QC step prior to an expensive microarray experiment can be easily and quickly achieved.

Corresponding application note: 5968-7493EN

Analysis of total RNA

Standardization of RNA Quality Control





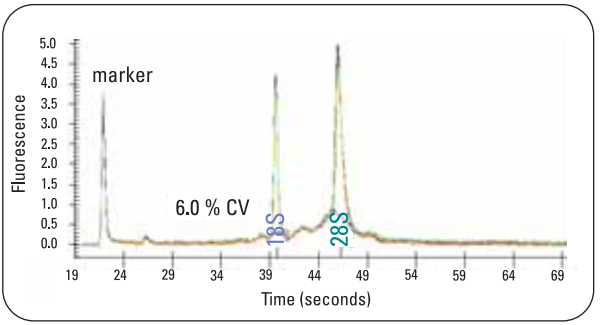
Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: The RNA integrity number (RIN) is calculated by a dedicated software algorithm (expert 2100 software, starting with Rev 02.01) to assess the quality of RNA preparations. The RIN tool is a major step in the standardization of user-independent RNA evaluation and delivers more meaningful information than simple ratio calculations for ribosomal RNA peaks. It is not influenced by instrument, sample integration and most important, concentration variability, thereby facilitating the comparison of samples and avoiding cost-intensive experiments with low quality RNA preparations. The RIN algorithm is based on a large collection of RNA data of various tissues and qualities. Furthermore, anomalies like genomic DNA contaminations are indicated with weighted error messages (critical/non-critical) to achieve a maximum of reliability.

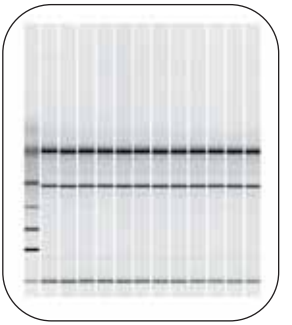
Corresponding application note: 5989-1165EN

Analysis of total RNA

Reproducibility of quantitation

Reproducibility for 12 consecutive runs



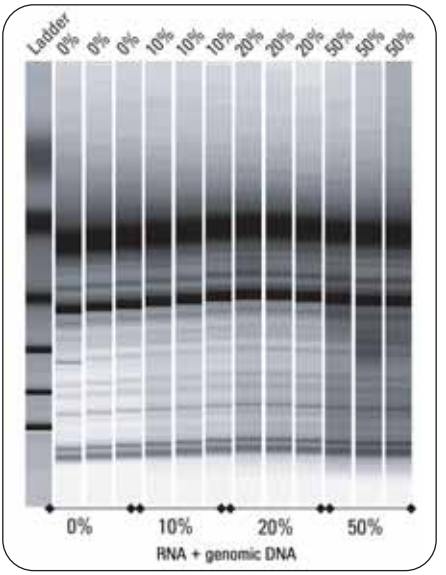


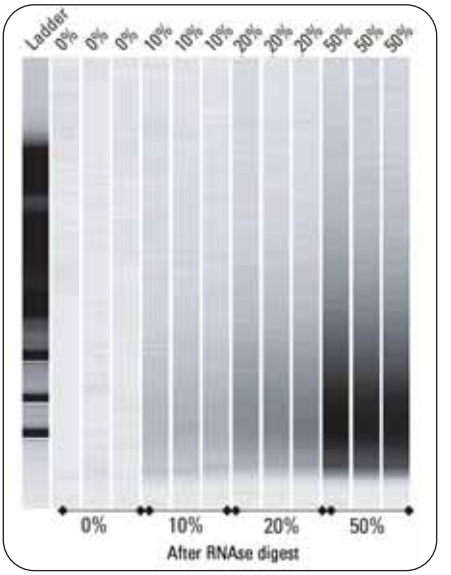
Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: Alongside the quality control of RNA samples, measurement of RNA concentration is important for (bio-)chemical reactions, such as labeling reactions in the context of microarray experiments. With the RNA 6000 Nano kit good reproducibility can be achieved (here 6% CV), which is little affected by sample contaminants, such as phenol.

Corresponding application note: 5988-7650 EN

Analysis of total RNA

Genomic DNA contamination



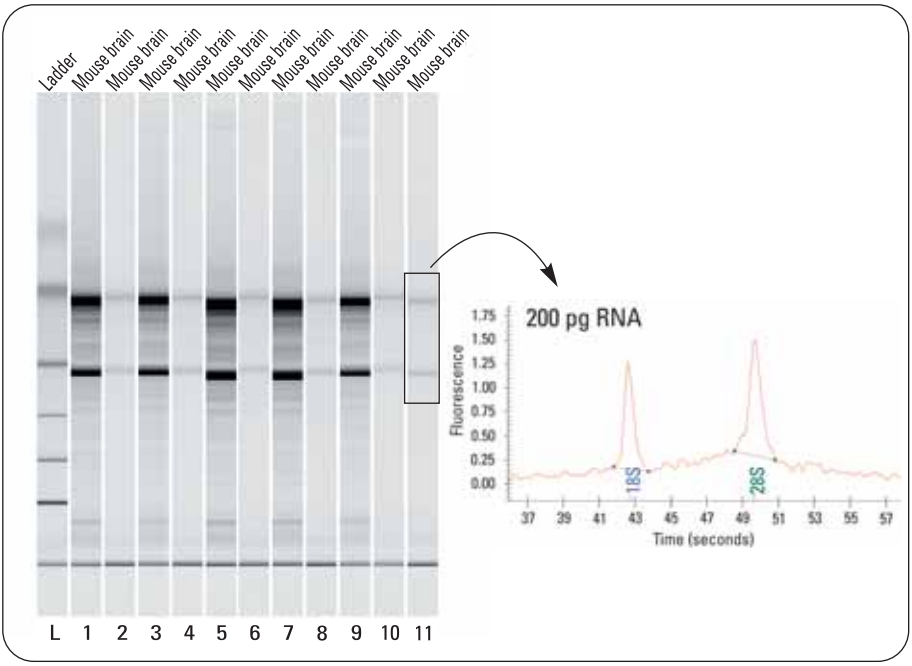


Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: Gel representation of a chip run with total RNA samples (mouse brain) spiked with varying amounts of herring sperm genomic DNA before and after treatment with RNase. The left panel shows the intact RNA with broad bands in the low MW region stemming from the genomic DNA. After the RNase digest (right panel) only the DNA bands remain, ranging in intensity according to the amount of DNA spiked into the sample.

Corresponding application note: data not published

Low amounts of total RNA

Detection of low levels of RNA



Analysis of mouse brain RNA at two different concentrations

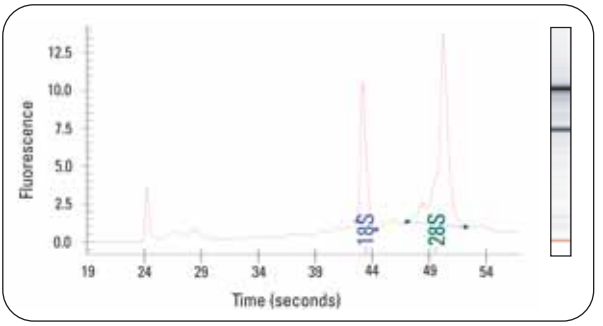
Kit: RNA 6000 Pico kit
Assay: Eukaryote total RNA Pico assay
Application: The RNA 6000 Pico kit is complementary to the RNA 6000 Nano kit and is suitable for all applications where the amount of RNA (or cDNA) is limited, e.g. for biopsy samples, samples from microdissection experiments, QC of cDNA made from total RNA, microarray samples, etc. Here Agilent 2100 bioanalyzer results obtained from mouse brain RNA (Ambion) at 200 and 1000 pg/il are shown. By analysis in repetitions the reproducibility of quality control is demonstrated. Detection of 200 pg total RNA could be achieved without problems.

Corresponding application note: data not published

Low amounts of total RNA

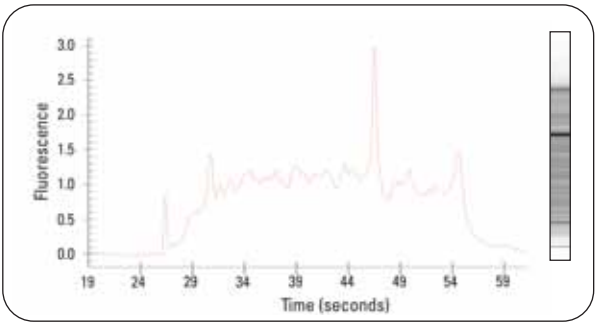
RNA integrity with the RNA 6000 Pico kit

A



Intact RNA

B



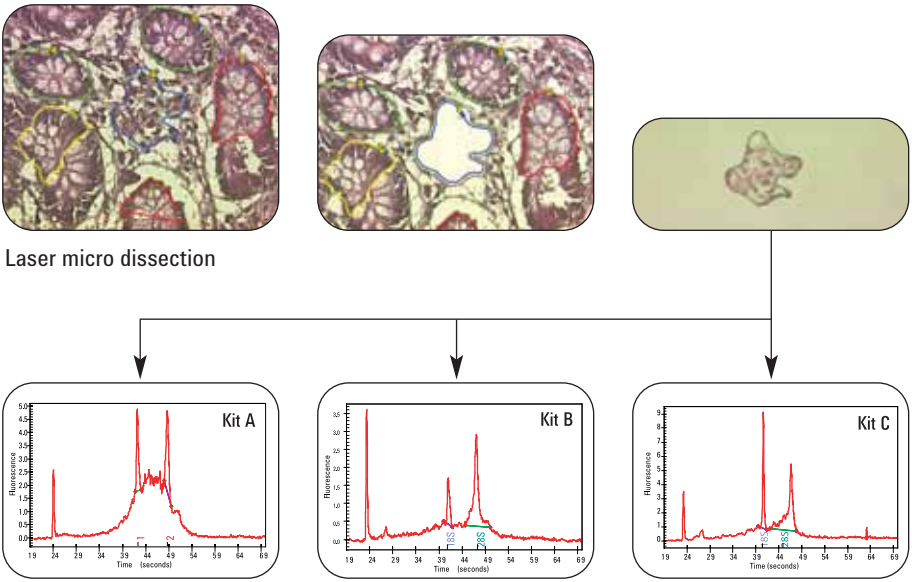
Degraded RNA

Kit: RNA 6000 Pico kit
Assay: Eukaryote total RNA Pico assay
Application: Detection of RNA degradation with the RNA 6000 Pico kit. Sample: mouse liver total RNA (Ambion) concentration: 1 ng. Degradation was accomplished by adding a low amount of RNase. In Figure A the upper electropherogram and gel-like image show the analysis of high quality total RNA with the 18S and 28S subunit as two distinct bands. Figure A shows the analysis of a partially degraded total RNA sample. Many degradation products appear between the two ribosomal bands and below the 18S band.

Corresponding application note: data not published

Low amounts of total RNA

RNA quality after staining and microdissection



Laser micro dissection

Check and optimize RNA quality and yield

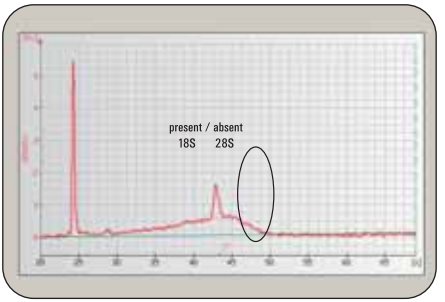
Kit: RNA 6000 Pico kit
Assay: Eukaryote Total RNA Pico assay
Application: RNA derived from laser-microdissected tissue isolated by the PALM®MicroBeam system was shown to be of high quality by convenient analysis with the RNA 6000 Pico assay. RNA-purification kits from different manufacturers and various common staining procedures have been tested and yielded 130-700pg/µl RNA from 1000 cells with different quality (see above). The RNA 6000 Pico kit was well suited to show differences in RNA quality and yield and, therefore, is an ideal tool to optimize and adapt experimental conditions to individual tissue. The experiments were accompanied by a more laborious real time PCR that revealed similar results. Due to its unprecedented sensitivity, the RNA 6000 Pico assay is an indispensable tool for quality control in the context of microdissection experiments, ensuring successful gene expression profiling experiments.

Corresponding application note: 5988-9128EN

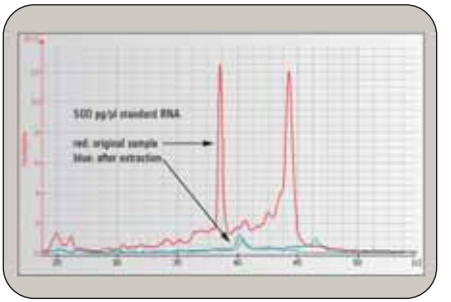
Low amounts of total RNA

Analysis of minimum RNA amounts

A



B



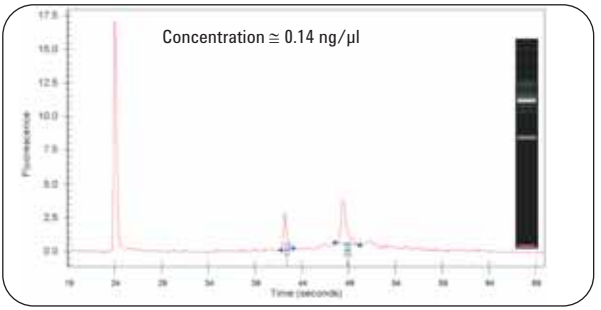
Kit: RNA 6000 Pico kit
Assay: Eukaryote Total RNA Pico assay
Application: The challenge of analysis of minimal amounts of RNA from e.g. laser micro dissections calls for detailed knowledge of extraction conditions. Some commonly used RNA isolation kits and buffer components were assessed in detail. The majority of the kits had no negative effect on the performance of the analysis, whereas, some kits include buffers which lead to shifted, missing and diminished RNA-peaks. In figure A, RNA isolated after microdissection shows lack of the 28S-peak due to high salt concentration introduced during the isolation process. In figure B, a standard RNA was diluted in water and subsequently extracted with a commercially available RNA extraction kit. The original samples (red) and the eluates after extraction are shown. These data show the importance of evaluating the individual method used for RNA extraction to exclude misleading conclusions.

Corresponding application note: 5989-0712EN

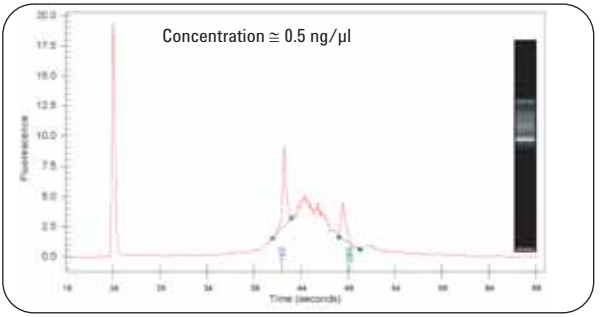
Low amounts of total RNA

Genomic DNA in low concentrated RNA extracts

A



B

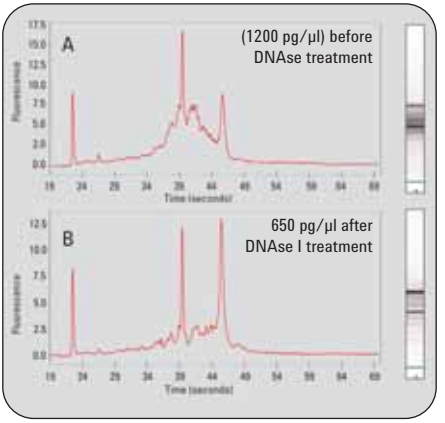


Kit: RNA 6000 Pico kit
Assay: Eukaryote Total RNA Pico assay
Application: Laser capture microdissection enables collection of cells from small tissue areas. A low RNA yield is in the nature of the extraction method from such a specimen that usually complicates quality assessment – a fact that can be circumvented by taking advantage of the Agilent 2100 bioanalyzer capabilities. A comparative study using mouse kidney cryosections showed that on-column DNase digestion is indispensable to obtain a reasonable result for integrity and yield (figure A). Experiments with omitted on-column DNA digestion confirmed that the peak visible in the inter-region consists of genomic DNA which caused overestimation of extracted RNA amounts (figure B).

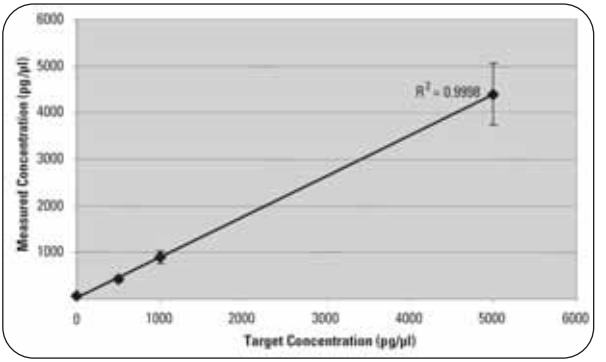
Corresponding application note: 5989-0991EN

Low amounts of total RNA

Low RNA amounts from kidney sections



Renal medulla RNA

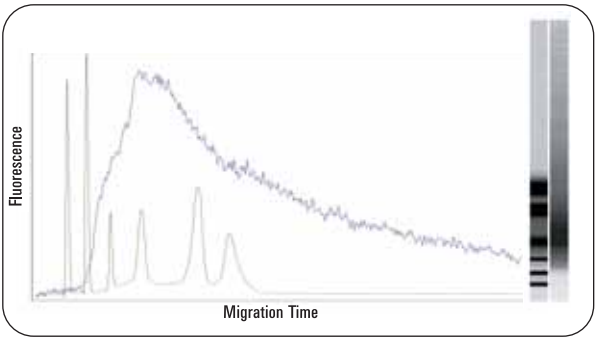


Kit: RNA 6000 Pico kit
Assay: Eukaryote Total RNA Pico assay
Application: High sensitivity quality control of RNA samples using the RNA 6000 Pico LabChip kit are demonstrated for microdissected samples (0.1 mm^3). DNAse I digestion revealed that DNA contamination was present in the sample. Removal of DNA revealed total RNA with a low degree of degradation. Under ideal conditions, the RNA Pico assay has a linear response curve and, therefore, allows estimation of RNA concentrations.

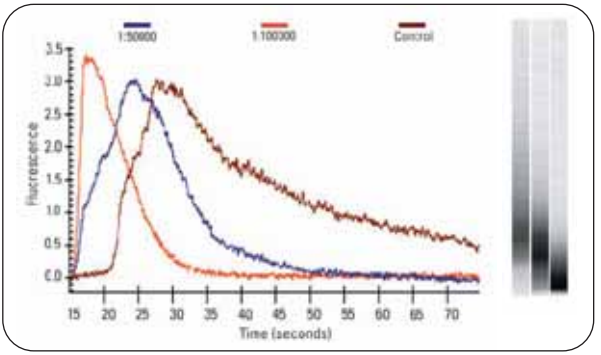
Corresponding application note: 5988-8554EN

Analysis of mRNA

RNA integrity



Highly enriched
Poly (A)+ RNA



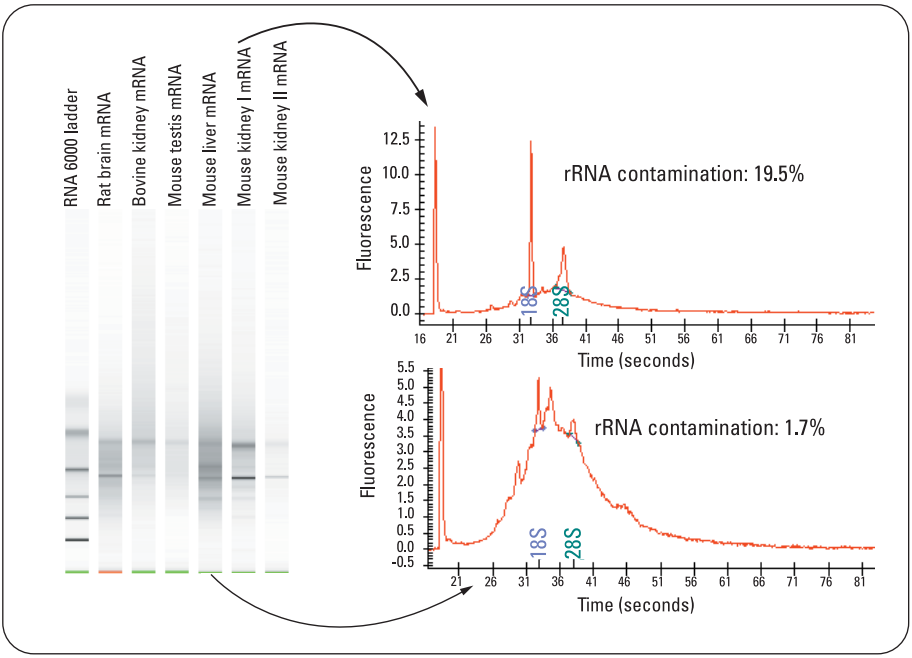
Progressive degradation
of Poly (A)+ RNA

Kit: RNA 6000 Nano kit
Assay: mRNA Nano assay
Application: Progressive degradation of Poly (A)+ RNA. Poly (A)+ RNA (60 ng/µL) from cultured Jurkat cells was incubated for 15 minutes at room temperature with very dilute RNase A (1 x 10-6 and 2 x 10-6 mg/mL, respectively). A progressive shift towards shorter fragment sizes can be observed. Even with a mild degradation, the absence of very long transcripts can be noticed.

Corresponding application note: 5968-7495EN

Analysis of mRNA

Ribosomal RNA contamination in mRNA samples

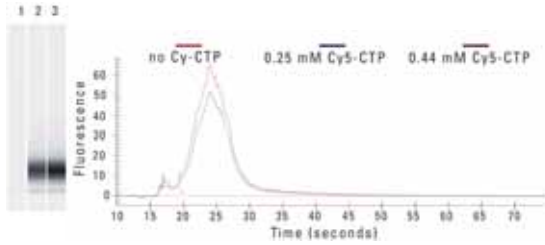


Kit: RNA 6000 Nano kit
Assay: mRNA Nano assay
Application: Ribosomal contamination in mRNA samples. During the isolation of mRNA, varying amounts of ribosomal RNA can remain in a sample. Since the purity of mRNA is of importance for a number of downstream applications, samples should be checked on the Agilent 2100 bioanalyzer. This slide shows the analysis of 6 commercially available RNA samples from different suppliers. Analysis on the Agilent 2100 bioanalyzer reveals large differences in the purity of the mRNA samples.

Corresponding application note: 5968-7495EN

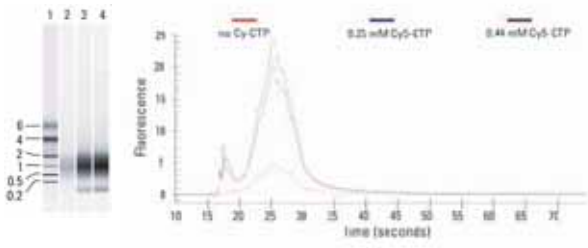
Analysis of Cy5 labeled samples

Analysis of cRNA with and without dye in gel matrix



Assay conditions:
- Cy5 labeled nucleic acids
- no intercalating dye used
- 5nM Cy5 dCTP added to gel matrix and sample buffer for focusing

Lanes:
1. Unlabeled cRNA
2. Cy5 labeled cRNA
3. Cy5 labeled cRNA



Assay conditions:
as above, but intercalating dye included in gel matrix

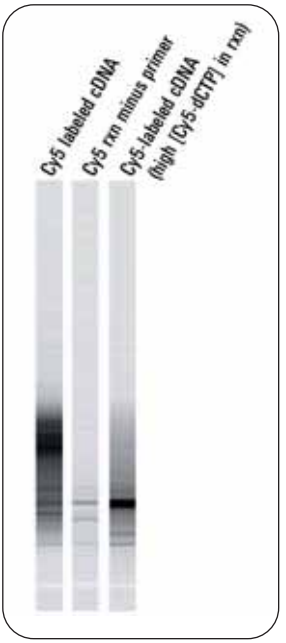
Lanes:
1. RNA transcript ladder
2. Unlabeled cRNA
3. Cy5 labeled cRNA
4. Cy5 labeled cRNA

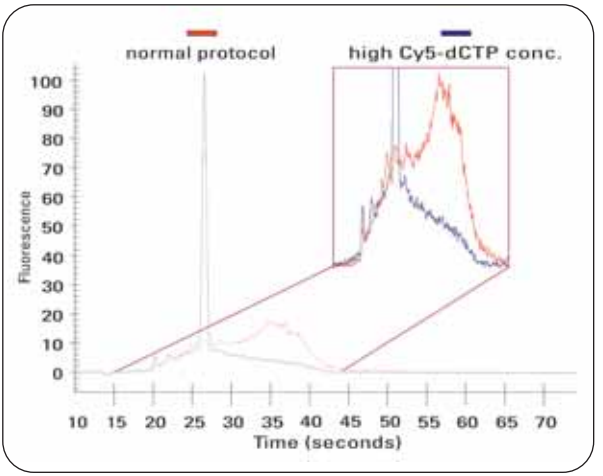
Kit: RNA 6000 Nano kit
Assay: mRNA Nano and Cy5 labeled nucleic acids Nano assay
Application: Analysis of Cy5 labeled and non-labeled cRNA samples. Cy5-labeled samples show the combined signals of the fluorescent label and the RNA signal created by the fluorescence of the RNA 6000 dye. If the RNA 6000 dye is omitted from the gel matrix, only the signal created by Cy5 is detected, allowing the determination of dye incorporation after a labeling reaction. Please note that for Cy3 labeled samples the intactness of the sample can be verified, but the dye incorporation can not be checked.

Corresponding application note: 5980-0321EN

Analysis of Cy5 labeled samples

Optimization of labeling reactions



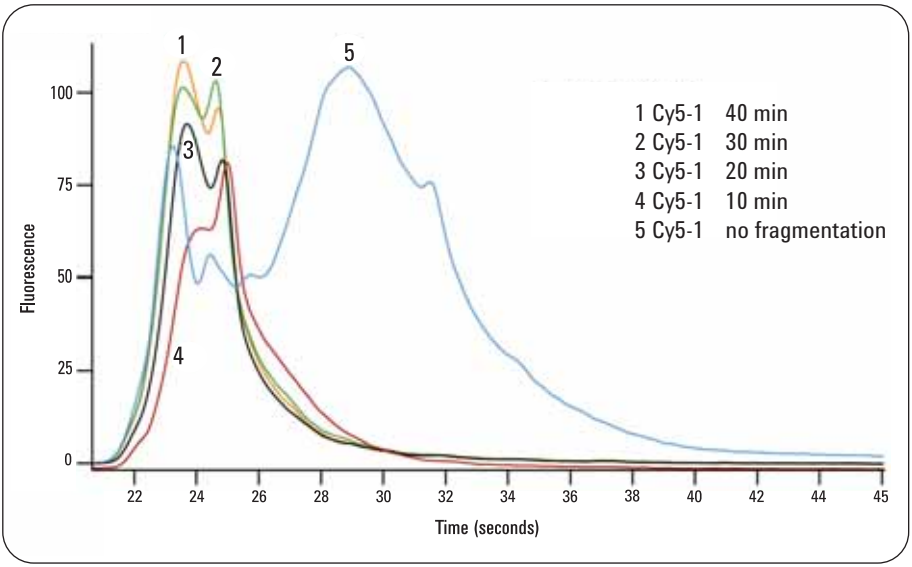


Kit: RNA 6000 Nano kit
Assay: Cy5 Labeled Nucleic Acids Nano assay
Application: An experiment was designed to check the influence of Cy5 dCTP concentration on labeling efficiency. Lane 2 represents the negative control (primer ommitted from the reaction mixture), while lane 3 shows the analysis of a reaction with a 6-fold increased Cy5 dCTP concentration. A look at the electropherograms reveals that the high Cy5 dCTP concentration not only gave a high peak of unincorporated Cy5, but also the labeling efficiency for longer fragments was very low. This approach allows the optimization of labeling reactions.

Corresponding application note: 5980-0321EN

Analysis of Cy5 labeled samples

cRNA fragmentation

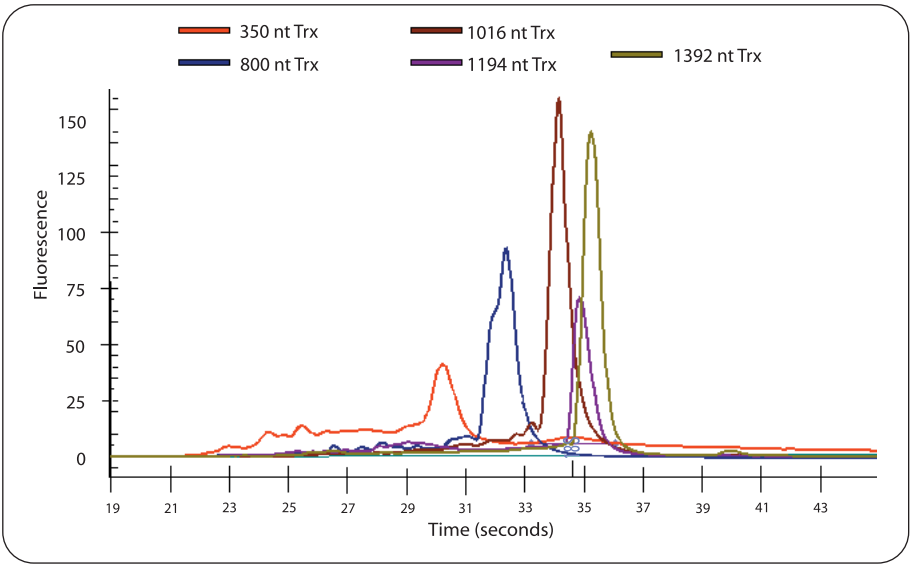


Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: The RNA 6000 Nano LabChip kit can be used to monitor completion of a cRNA fragmentation reaction. In this example, the profile of a Cy5 labeled cRNA sample was monitored at different time points during a fragmentation reaction. It can be seen that after 10 minutes most of the fragments are in the desired size range. After 20 minutes, no further shift of fragmentation can be observed indicating completion of the fragmentation reaction.

Corresponding application note: 5988-3119EN

Analysis of T7 RNA transcripts

Size estimation



Kit: RNA 6000 Nano kit
Assay: Eukaryote total RNA Nano assay
Application: A number of RNA transcripts, ranging from 350 to 1400 nt in size, were analyzed on the RNA 6000 Nano LabChip kit. Although the assay runs under native conditions and the transcripts exhibit a certain degree of secondary structure, a good size estimation can be achieved.

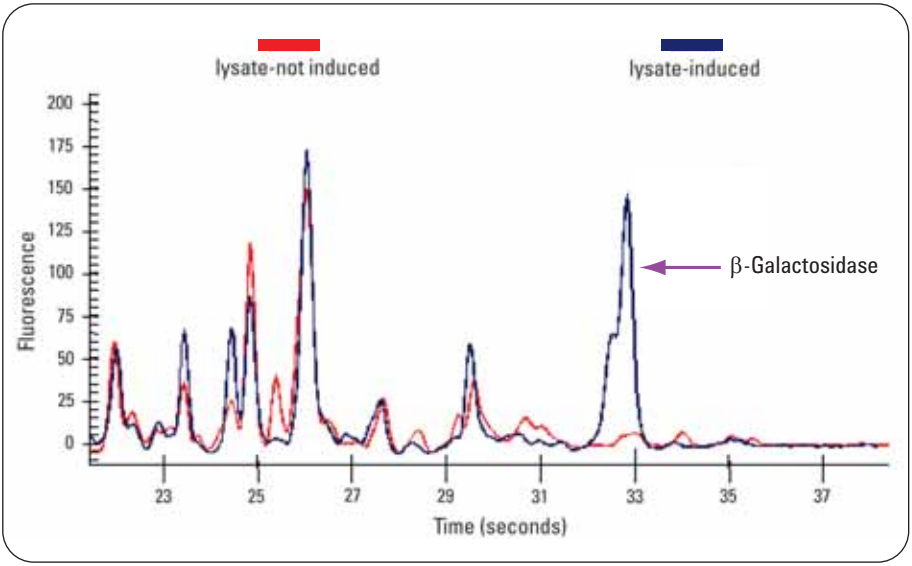
Corresponding application note: data not published

IV. Protein analysis

	Agriculture/Food	Drug Discovery/Drug Development	Drug Manufacturing QA/QC	Genomics	Pharmaceuticals	Proteomics	Forensics/Homeland Security
Protein expression							
Analysis of cell lysates - protein induction			●			●	
Protein purification:							
Comparison between lysate and flow through		●			●		
Analysis of protein purification		●		●		●	
GFP Streptag fusion protein purification		●				●	
Analysis of column capacity						●	
Analysis of column fractions to optimize conditions		●	●			●	
His-tag protein purification with Ni++ ZipTips®		●	●			●	
Enzymatic removal of His-tags from recombinant proteins			●			●	
Complementing RP-HPLC protein purification		●			●	●	
Antibody analysis							
Analysis of antibodies under reducing and non-reducing conditions		●	●				
Quantitation of the half-antibody content in IgG_4 preparations		●	●		●		
Comparison of SDS-PAGE, CGE and Agilent 2100 bioanalyzer for humanized monoclonal antibody analysis		●	●			●	
Absolute quantitation of IgG		●	●			●	
Quality control of stressed antibodies		●	●				
Separation of bispecific antibodies chains		●	●		●	●	
Food analysis							
Bovine milk analysis	●					●	
Protein pattern of different transgenic seedlines	●					●	
Protein - others							
Absolute protein quantitation	●		●		●	●	
Glycoprotein sizing	●		●			●	
Protein quality control prior to MS-analysis			●			●	
Depletion of high abundant proteins from blood samples		●			●		
Increased sensitivity by desalting protein samples	●	●			●	●	

Protein expression

Analysis of cell lysates - protein induction

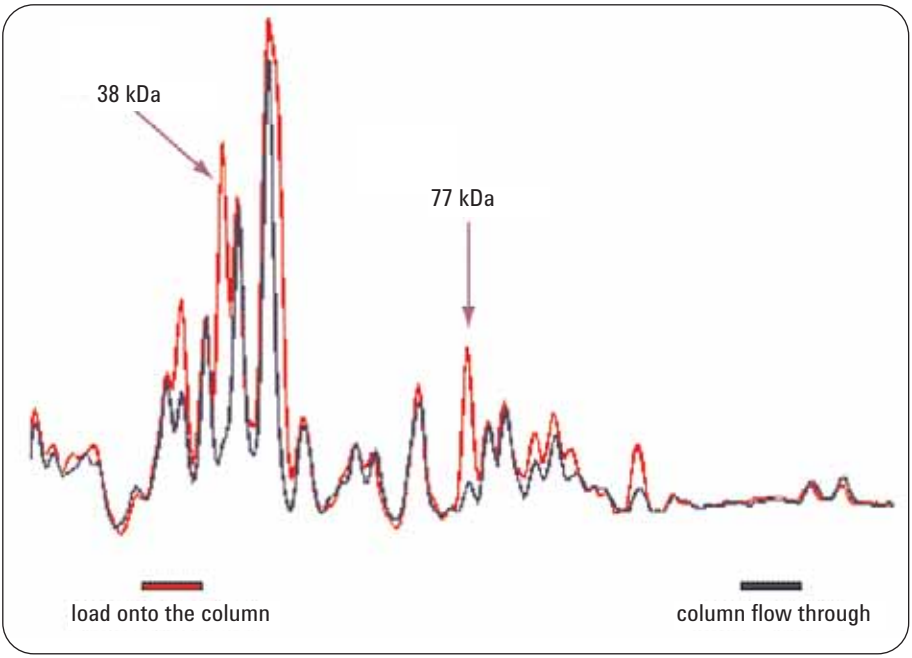


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Two cell lysates, induced and non-induced were compared to verify the induction of protein expression. The overlay feature of the bioanalyzer software allows quick sample comparison. The blue electropherogram trace shows the cell lysate highly expressing β-galactosidase (128 kDa).

Corresponding application note: data not published

Protein purification

Comparison between lysate and flow through

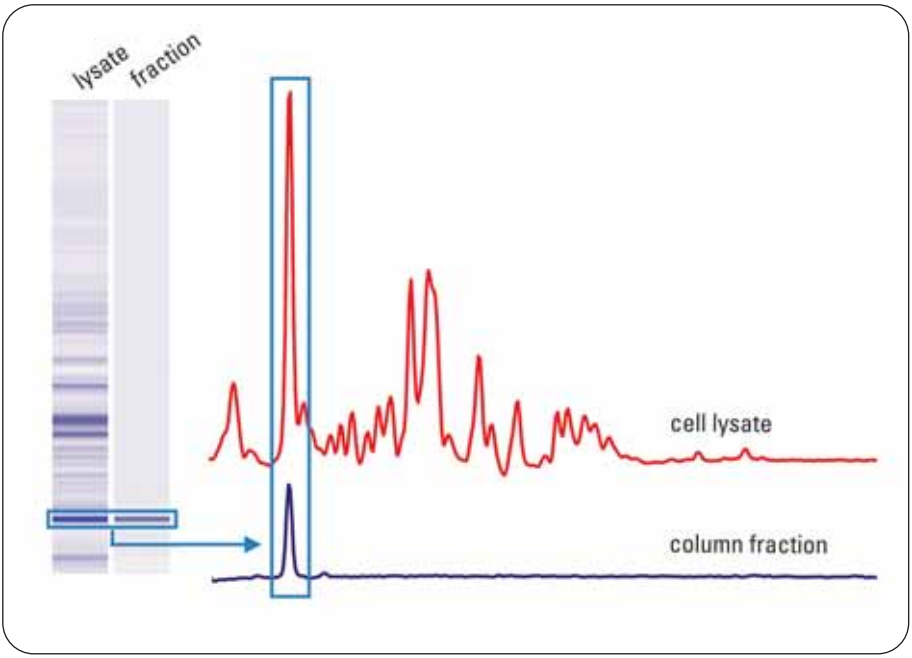


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Cells were lysed using the Pierce B-Per kit and then loaded onto an affinity column. The protein of interest, a 38 kDa protein, should bind to the column and not show up in the flow through. By overlaying the 2 electropherograms from both samples, the lysate and the flow through, it is visible that the protein of interest has bound to the column as expected. In addition, a 77 kDa protein has bound to the column, which could be attributed to unspecific binding or the binding of a dimer.

Corresponding application note: data not published

Protein purification

Analysis of protein purification



Courtesy of P. Sebastian and S.R. Schmidt GPC-Biotech AG, Martinsried, Germany

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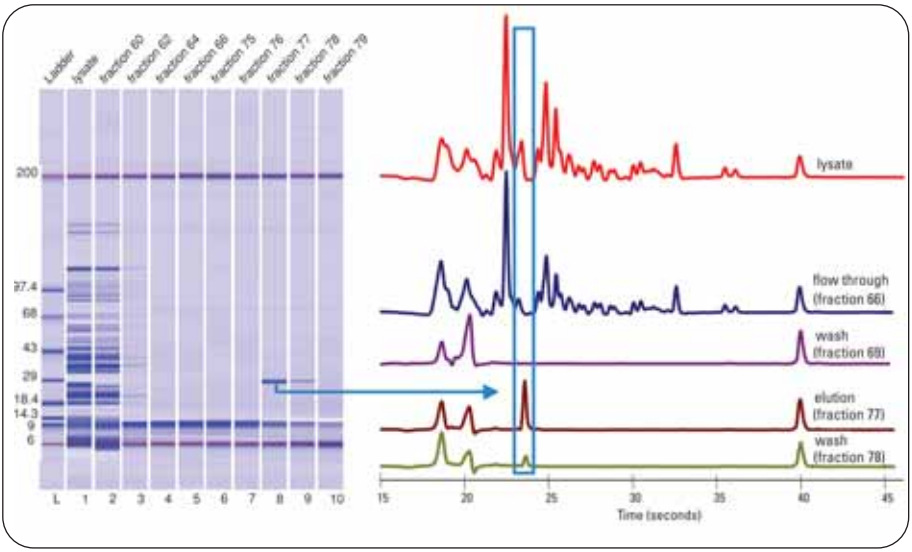
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: A 18 kDa protein was purified using affinitiy chromatography. The starting material and the column fraction were analyzed with the protein assay. The protein of interest was determined to be 99% pure and the concentration in the column fraction was 167 ng/µl.
The protein assay allows protein purity and concentration to be determined in one step, in addition it calculates protein size for reconfirmation.

Corresponding application note: data not published

Protein purification

GFP Streptag fusion protein purification



Courtesy of P. Sebastian and S.R. Schmidt GPC-Biotech AG, Martinsried, Germany

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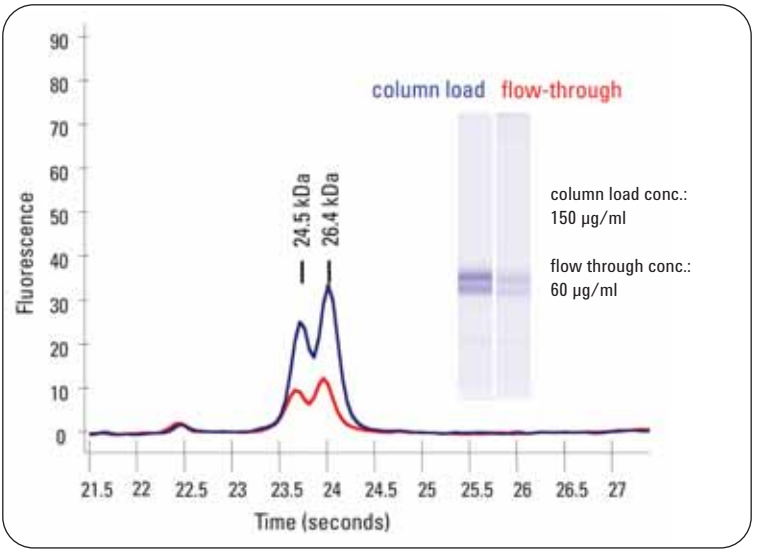
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: This example shows the analysis of various steps during the purification workflow of a GFP Streptag fusion protein (28 kDa). The protein was expressed in *E.coli* and purified via affinity chromatography with Strep Tactin Poros as the column matrix. The protein assay allows each purification step from the cell lysis to the elution of the purified protein to be monitored and optimized.

Corresponding application note: 5988-5025EN

Protein purification

Analysis of column capacity

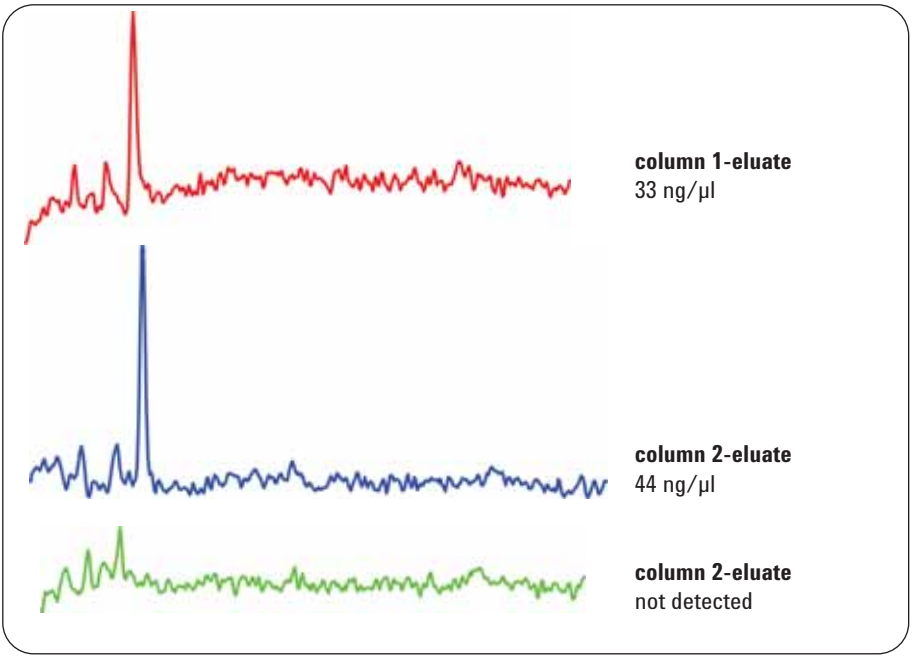


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: The binding of a recombinant antibody Fab fragment to a Sepharose column with immobilized Protein G was analyzed to determine the column capacity and prevent column overloading. The protein assay allows this purification step to be monitored and quickly optimized.

Corresponding application note: 5988-4022EN

Protein purification

Analysis of column fractions to optimize conditions



Courtesy of P. Sebastian and S.R. Schmidt GPC-Biotech AG, Martinsried, Germany

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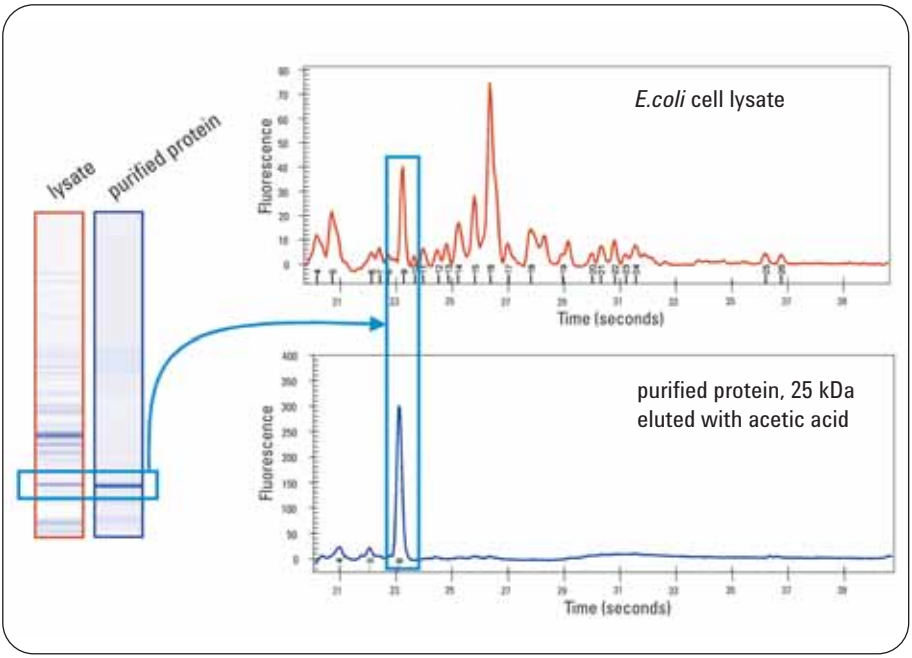
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Different column conditions were tested to optimize the purification conditions for a 30 kDa protein. The column fractions were analyzed for protein purity and concentration to identify the optimal conditions providing a highly purified protein in a good yield. Using the protein assay it was possible to determine the optimum purification conditions in a short time frame.

Corresponding application note: data not published

Protein purification

His-tag protein purification using Ni++ZipTips®

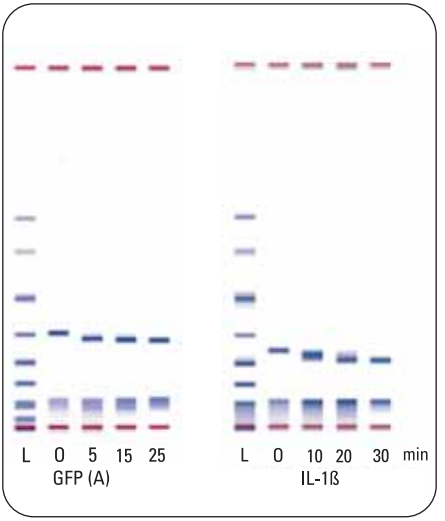


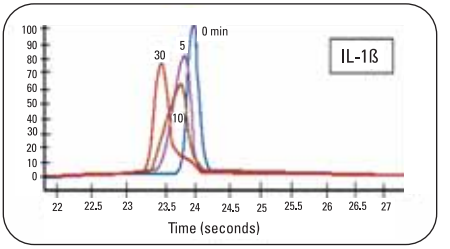
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: ZipTips loaded with a Ni^{2+}-resin (in development by Millipore) were used to purify a His-tagged protein expressed in *E.coli.* Both the cell lysate and the purified protein were analyzed with the Agilent 2100 bioanalyzer to demonstrate the performance of the tips.
The purification with the tips takes approximately 5 minutes, usually followed by the analysis of the samples with SDS-PAGE analysis which takes a further 2 hours. The SDS-PAGE analysis was substituted by the much faster Protein 200 Plus assay run on the Agilent 2100 bioanalyzer.

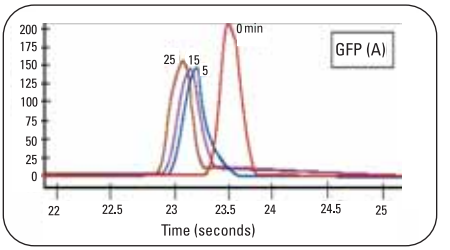
Corresponding application note: data not published

Protein purification

Enzymatic removal of His-tags from recombinant proteins







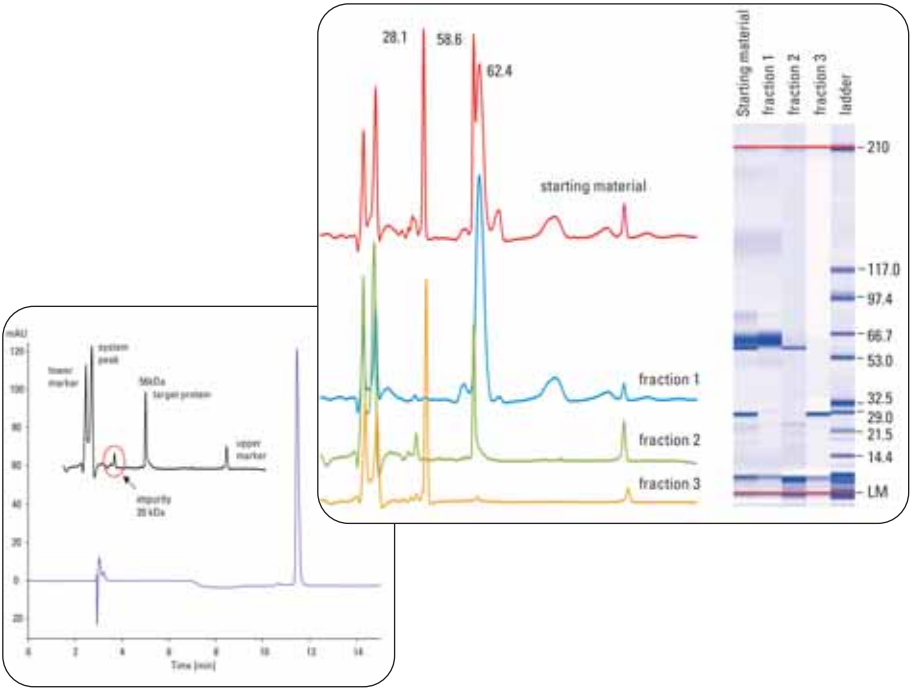
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: For some applications, it might be necessary to remove the His-tag after the protein purification because of its effects on enzymatic activity or protein structure. Here the TAGZyme system (Qiagen) was used to remove the N-terminal His-tag from two different proteins, a GFP variant and a recombinant Interleukin 1β. Samples were taken at different time points to study the kinetics of the enzymatic cleavage. The dipeptide cleavage can be detected by a size shift on the gel-like images and the electropherograms. The fast analysis with the bioanalyzer allows multiple kinetic studies to done in one day instead of waiting until the next day for the results from SDS-PAGE analysis.

Poster presented at ABRF Conference, March 2002 by
F. Schäfer, K. Steinert, C. Feckler, J.Drees, and J.Ribbe, QIAGEN GmbH, Hilden, Germany

Corresponding application note: 5988-8144 EN

Protein purification

Complementing RP-HPLC protein purification

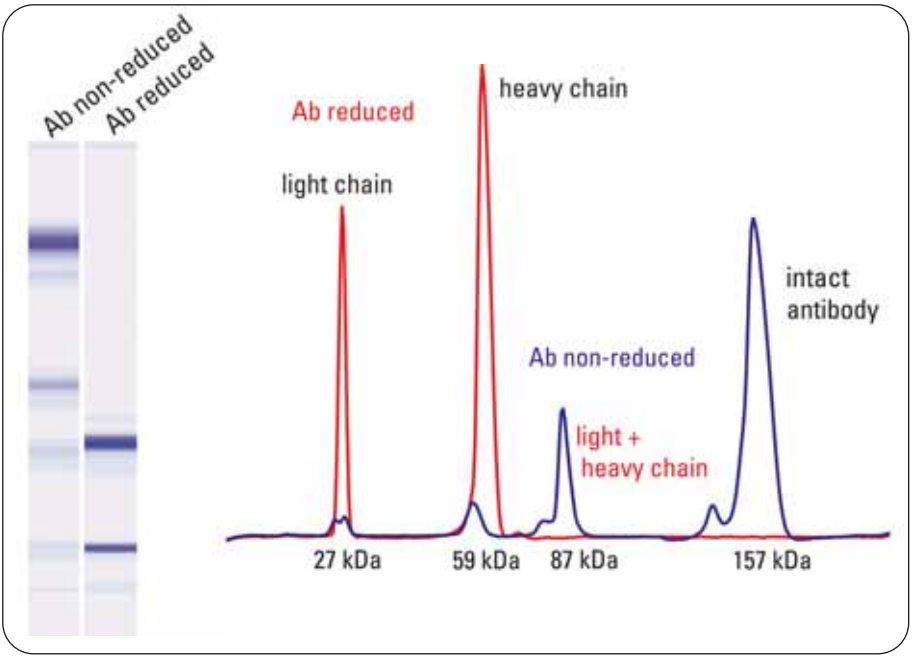


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Protein purification and characterization was carried out facilitating an Agilent 1100 Series purification system for reverse phase HPLC assisted by the Agilent 2100 bioanalyzer. The final polishing of a 56 kDa protein by RP HPLC from a pre-purified sample (starting material, right: red electropherogram and gel) and the analysis of three HPLC-fractions containing the major components are shown (fractions 1-3). No impurity is visible by RP HPLC reanalysis (left chromatogram, fraction 2) of the fraction containing the target protein. However, because the Agilent 2100 bioanalyzer is an orthogonal technique compared to reverse phase HPLC a 20 kDa protein could be found as an impurity (see insert). The reverse phase HPLC purification leads to a purity of only 76% for the protein of interest and the Agilent 2100 bioanalyzer reveals the necessity of further purification.

Corresponding application note: 5988-8630EN

Antibody analysis

Analysis of antibodies under reducing and non-reducing conditions

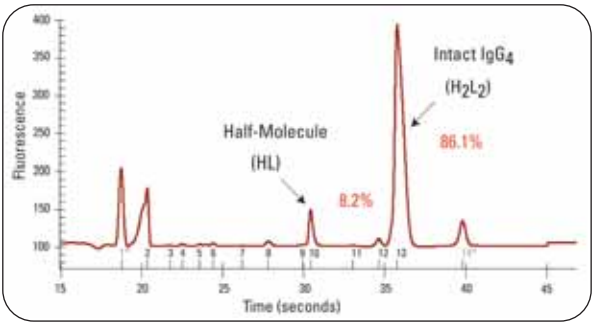


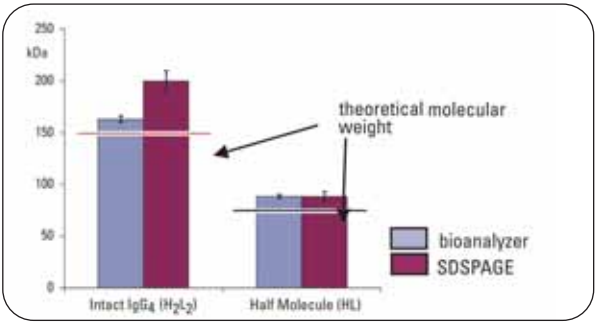
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: The protein kit allows analysis of both reduced and non-reduced antibodies on the same chip. This is not possible using SDS-PAGE, as the reducing agent will diffuse within the gel and will also reduce other samples. Under non-reducing conditions, it is expected to detect the intact antibody around 160 kDa. Here the single light and heavy chains and half-antibodies are also visible. Under reducing conditions this is all completely reverted to single light and heavy chains, due to the reduction of the disulfide bonds.

Corresponding application note: data not published

Antibody analysis

Quantitation of the half-antibody content in IgG_4 preparations





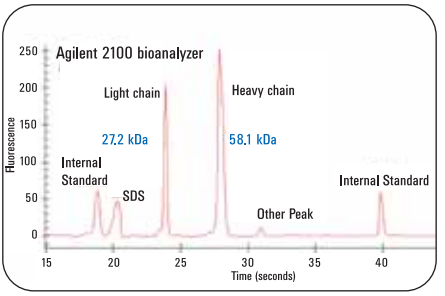
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: In the given host cell line for antibody production usually up to 30% of IgG_4 is secreted as half molecule (half antibody). The half-molecule has only a single disulfide bond between the heavy and light chains, the inter-heavy chain disulfide bonds are absent. The protein assay allows the half-antibody content in IgG_4 preparations to be determined automatically.
In addition, the sizing provided by the Agilent 2100 bioanalyzer compares very well to the theoretical size and is superior to SDS-PAGE in terms of accuracy and reproducibility.

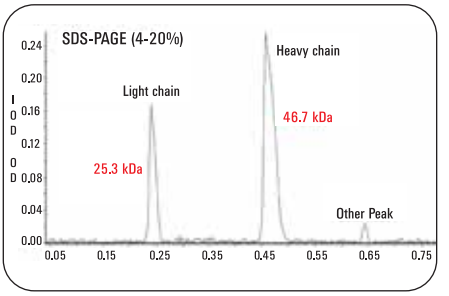
Poster presented at WCBP Conference, January 27-30, 2002 by E. Vasilyeva, H. Fajardo, P. Bove, F. Brown and M. Kretschmer. BIOGEN, Cambridge, MA , USA

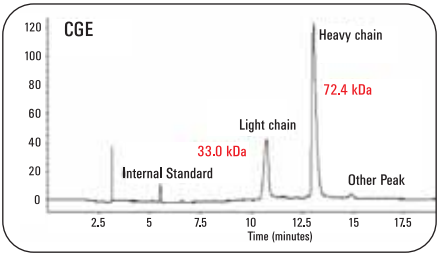
Corresponding application note: data not published

Antibody analysis

Comparison of SDS-PAGE, CGE and Agilent 2100 bioanalyzer for humanized monoclonal antibody analysis







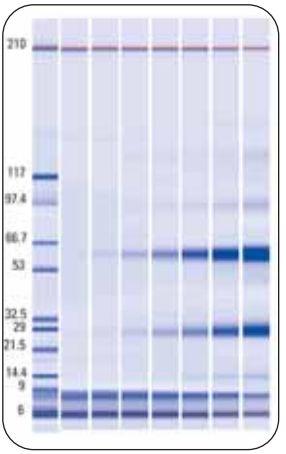
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: The analysis of a humanized monoclonal antibody under reducing condition was compared using 3 different techniques, the Agilent 2100 bioanalyzer, 4-20% SDS-PAGE, stained with Coomassie, and capillary gel electrophoresis. All 3 techniques result in a similar separation pattern showing the light and the heavy chain of the antibody. In addition, the determined sizes of the light and heavy chain were comparable for all 3 techniques and compared well to the molecular weights determined by MALDI-TOF (light chain: 23762 Da, heavy chain: 51003 Da). However, the Agilent 2100 bioanalyzer provides significant time saving compared to the other techniques.

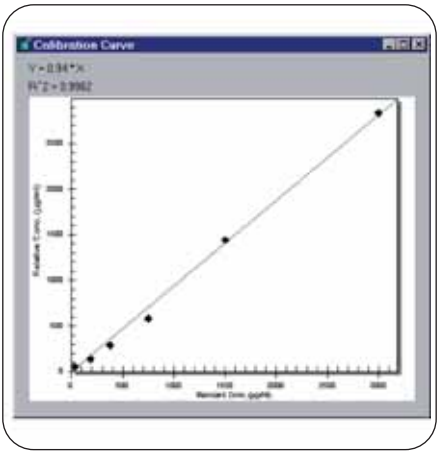
Poster presented at WCBP Conference, January 2002 by
S.H. Bowen, M. Chan, P. McGeehan, J. Smith, L. Inderdass, R. Strouse, M. Schenerman
MedImmune Inc., Gaithersburg, MD, USA

Corresponding application note: data not published

Antibody analysis

Absolute quantitation of IgG



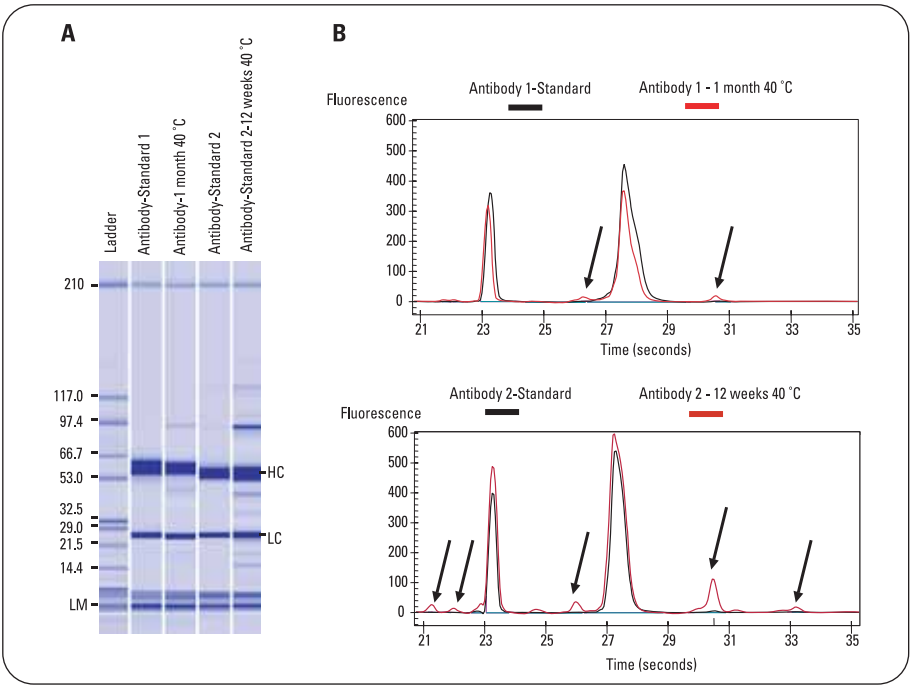


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: The calibration feature of the software allows determination of the absolute antibody concentration in comparison to user defined standards with known concentration, accurate determination of IgG concentrations and carrying out batch comparison during antibody QA/QC.

Corresponding application notes: 5988-4021EN and 5988-6576EN

Antibody analysis

Quality control of stressed antibodies

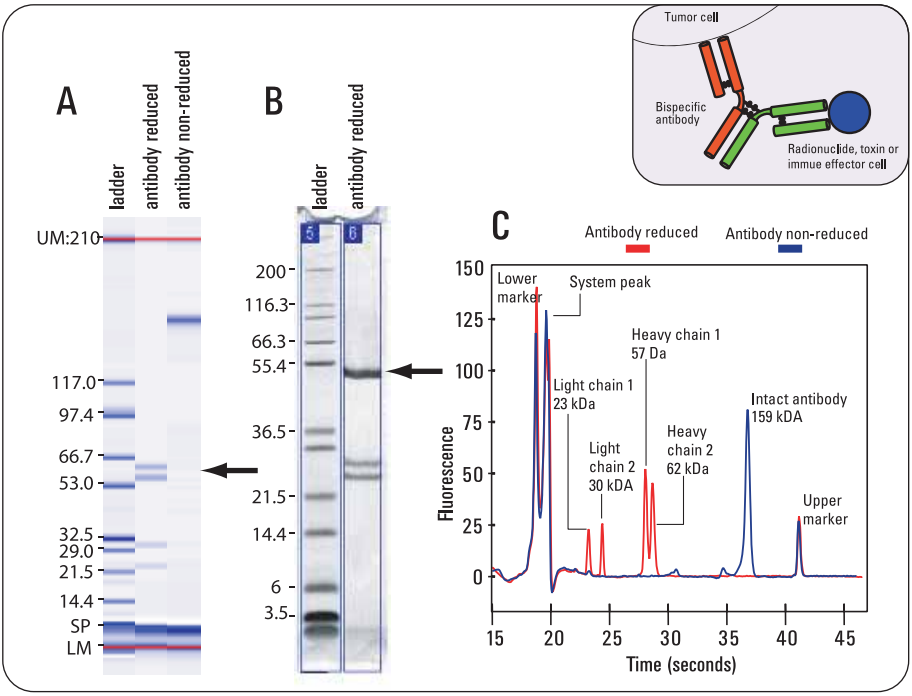


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: A quality control step in pharmaceutical QA/QC departments is to trigger typical degradation and aggregation patterns for a specific antibody. The given samples from heat stress stability studies show expected protein byproducts after aging at elevated temperatures. The content of heavy and light chain, representing the intact antibody, is reduced by 5% or 13% within 1 month or respectively 12 weeks. Excellent reproducibility in the range from 0.6 to 1.7% CV for this quantification was achieved in a validation study with three different users and two bioanalyzer instruments over several days.

Corresponding application note: 5988-9648EN

Antibody analysis

Separation of bispecific antibodies chains

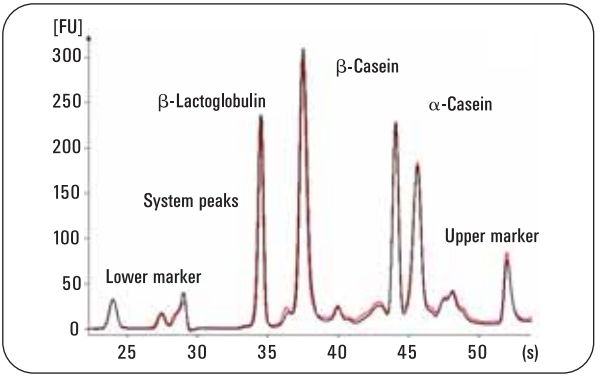


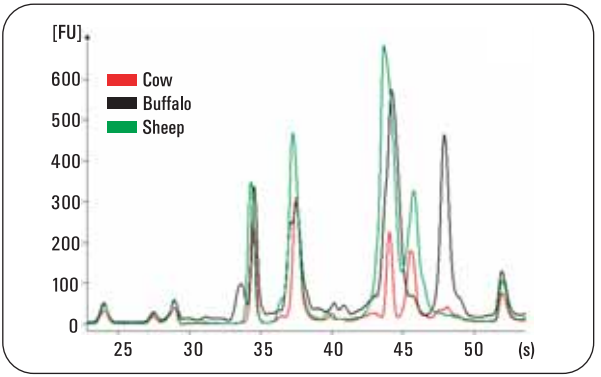
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: In general, antibodies are biopharmaceuticals of great interest. Especially bispecific antibodies often require high resolution to allow analysis of both sets of chains (Agilent 2100 bioanalyzer: A, gel like view, resolved heavy chains; C electropherogram). A labor intensive SDS-PAGE could not resolve the heavy chains (B, marked by an arrow) in the given sample. In contrast, the Agilent 2100 bioanalyzer is a superior tool for antibody quality control since it is a convenient, fast and easy to standardize method which additionally enables quantitative analysis.

Corresponding application note: 5988-9651EN

Food analysis

Bovine milk analysis



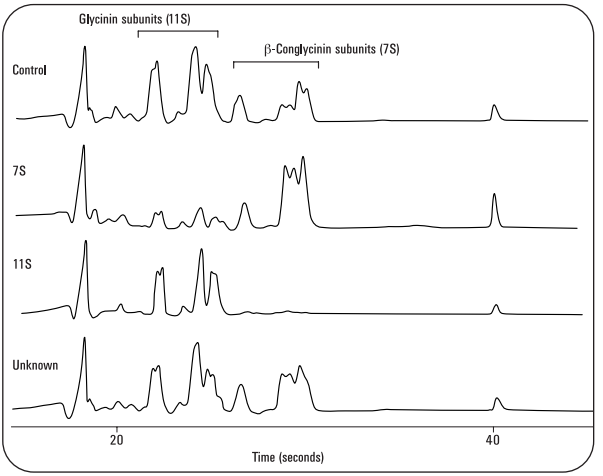


Kit: Protein 50 kit
Assay: Protein 50 assay
Application: The Protein 50 LabChip is suitable for analysis of diary products such as milk. The kit delivers an excellent reproducibility, as shown in this example (A, bovine milk diluted 1:10). Here the main protein fractions can be identified running the individual purified proteins for comparison (not shown). The overlay of the electropherograms from two separate runs under reducing conditions demonstrates the high reproducibility of the assay. Furthermore, milk from different animals could be distinguished based on their protein pattern (B) which facilitates a fast incoming inspection in routine labs.

Corresponding application note: data not published

Food analysis

Protein pattern of different transgenic seedlines



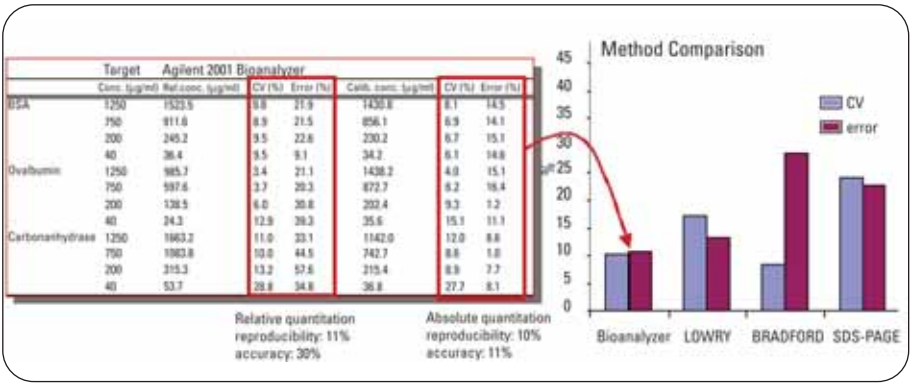
SeedLine	Extracted protein level µg/ml	7S/11S Ratio
Control	14,000	0.39±0.004(n=5)
7S	5,200	3.4
11S	14,000	0.04
Unknows	13,000	0.72±0.1(n=20)

Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Determination of protein size and concentration with sufficient accuracy and precision allows the highly efficient characterization of transgenic seed lines. Expressed protein was available after grinding, extraction of seeds and dilution with buffer. Electropherograms were evaluated by integration of regions specific for 7S or 11S seed storage proteins.
The elevated ratio of 7S/11S for the analyzed unknown line shows significant changes in the expression profile in comparison with the control.

Corresponding application note: 5988-9441EN

Protein - others

Absolute protein quantitation

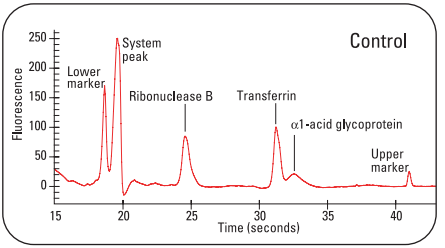


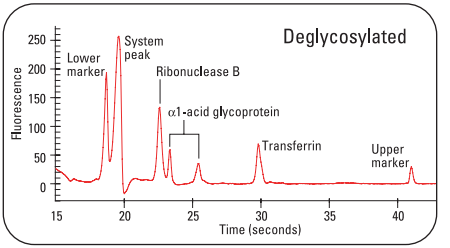
Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: A comparative analysis of different techniques used for absolute protein quantitation was performed analyzing 3 different proteins (CA, BSA, OV) in 4 different concentrations (40 - 1250 ug/ml). The same samples were quantitated using the Agilent 2100 bioanalyzer, two commonly used total protein quantitation assays, Lowry and Bradford, and SDS-PAGE, stained with Coomassie. The relative standard deviation (CV) and the error compared to the target concentration were determined. A comparison shows that the CV and error for the Agilent 2100 bioanalyzer are better than for the SDS-PAGE by a factor of 2. This data demonstrates that the Agilent 2100 bioanalyzer is a viable alternative for protein quantitation. It allows the quantitation of individual proteins and simultanous determination of protein purity and size.

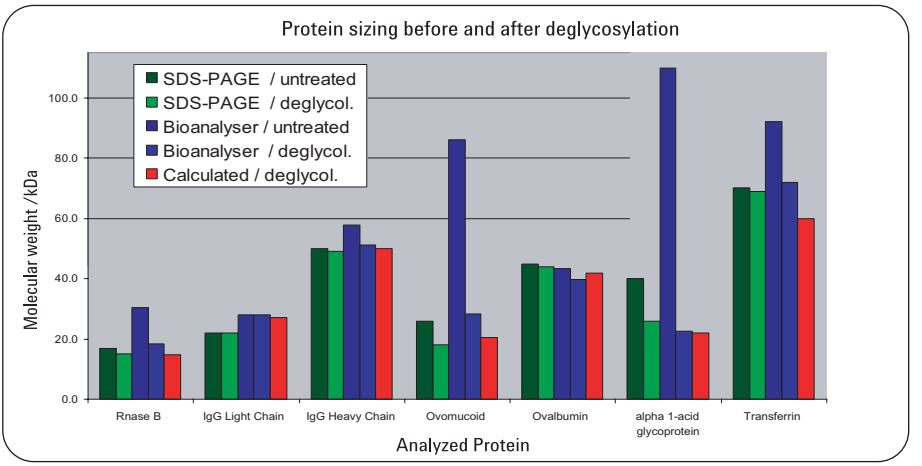
Corresponding application notes: 5988-4021EN and 5988-6576EN

Protein - others

Glycoprotein sizing





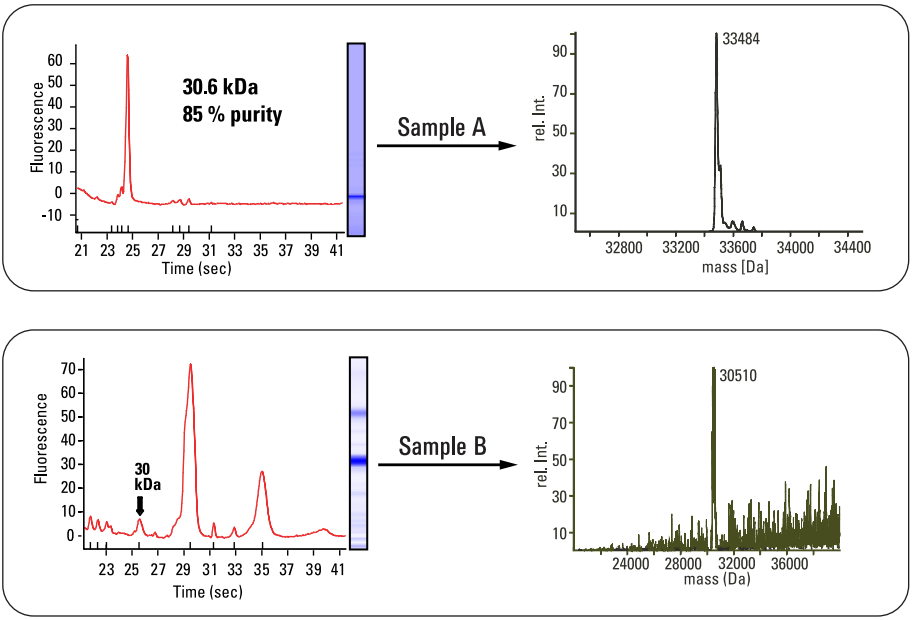


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Due to large carbohydrate moieties glycosylated proteins can differ in amount of incorporated SDS and shape of the protein/SDS-complex from non-glycosyslated proteins. This may lead to different migration times in SDS-PAGE, as well as in the Protein 200 Plus assay run on the Agilent 2100 bioanalyzer. The data compare deglycosylation of a mixture of three proteins (electropherogram on the left) with a commercial N-Glycosidase F Deglycosylation kit. Sizing experiments comparing glycosylated and non glycosylated states for additional proteins are compared and summarized on the right. Such an approach avoids misinterpretation of sizing due to glycosylation and allows detection of a posttranslational modification of unknown proteins.

Corresponding application note: 5989-0332EN

Protein - others

Protein quality control prior to MS-analysis

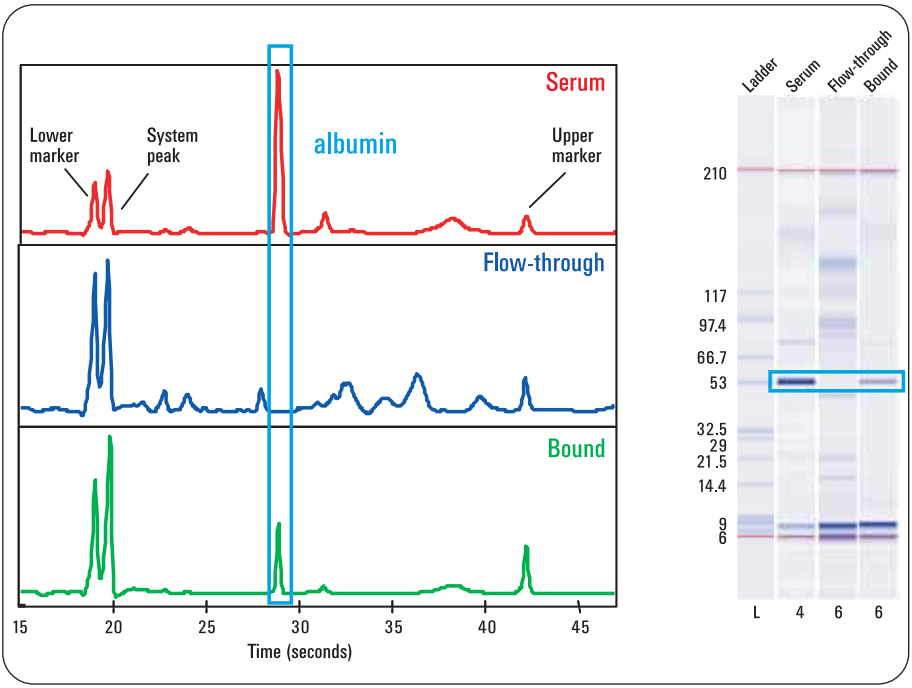


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: By applying soft ionization methods like MALDI or ESI mass spectroscopy (MS), mass information from proteins up to 300kDa can be obtained. However, proper sample preparation is an important precondition. Concentration, purity and assumed size are valuable ex ante information usually given by biochemists to MS-analysis services. Two different examples for proteins analyzed by an LC/MS-method (right panel) with good results for sample A and discrepancies for sample B are shown. An analysis of the samples with the Protein 200 Plus assay (left panel) showed an impure protein preparation for sample B. Here, two major peaks at higher masses (66 and 132kDa) potentially caused by aggregates were encountered. The protein of interest (30kDa) yielded high noise background in the MS. A quality check of the sample with the Protein 200 Plus assay, therefore, may avoid an unproductive MS analysis or data evaluation.

Corresponding application note: 5989-0771EN

Protein - others

Depletion of high abundant proteins from blood samples

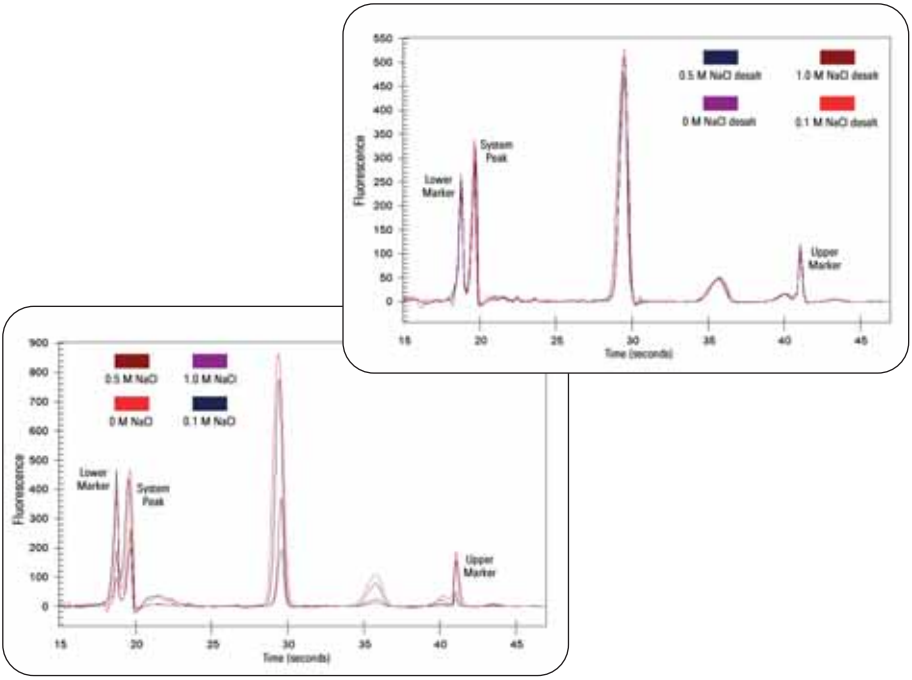


Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Depletion of high abundant proteins in human blood plasma was facilitated by the Agilent Multiple Affinity Removal System. Unprocessed serum, the flow through (i.e. the immunodepleted serum) and the bound proteins after specific elution were analyzed on the Agilent 2100 bioanalyzer. Equivalent amounts were analyzed and resulting electropherograms (left) and gel like images (right) show in comparison the completeness of separation. The Agilent 2100 bioanalyzer, in combination with the Protein 200 Plus LabChip kit, proves to be an excellent method for evaluation of serum processed with albumin removal kits. The system offers a rapid and accurate method to detect proteins both quantitatively and qualitatively.

Corresponding application note: data not published

Protein - others

Increased sensitivity by desalting protein samples



Kit: Protein 200 Plus kit
Assay: Protein 200 Plus assay
Application: Protein purification steps, such as ion exchange chromatography, often implicate high salt concentrations. Nevertheless, quantitation of these samples is effective since the upper marker serves as internal protein standard and is subjected to the same conditions. However, under high salt conditions lower amounts of protein are injected into the microfluidic channels for analysis. Therefore, the sensitivity can be increased by usage of convenient desalting spin columns. Comparably higher and homogeneous peak intensities are obtained while the recovery after such treatment is good.

Corresponding application note: 5989-0228EN

Literature

Microfluidics application notes from Agilent Technologies

To download an application note visit the library section at:
www.agilent.com/chem/labonachip

Description	Publication number
DNA	
Quantitative analysis of PCR fragments with DNA 7500 LabChip kit	5968-7496EN
High precision restriction fragment sizing with DNA 12000 LabChip kit	5968-7501EN
Comparing the Agilent 2100 bioanalyzer performance to traditional DNA analysis	5980-0549EN
Agilent 2100 bioanalyzer replaces gel electrophoresis in prostate cancer research	5988-1086EN
High resolution DNA analysis with the DNA 500 and DNA 1000 LabChip kits	5988-3041EN
Quantitative end-point RT-PCR gene expression using DNA 7500 LabChip kit	5988-3674EN
Development of meat speciation assays using the Agilent 2100 bioanalyzer	5988-4069EN
Analysis of genetically modified soya using the Agilent 2100 bioanalyzer	5988-4070EN
Detecting genetically modified organisms with the Agilent 2100 bioanalyzer	5988-4847EN
Sensitive detection of tumor cells in peripheral blood of carcinoma patients by a reverse transcription PCR method	5988-9341EN
Highly efficient multiplex PCR using novel reaction chemistry	5988-9342EN
Microfluidic analysis of multiplex PCR products for the genotyping of Helicobacter pylori	5989-0078EN
Nested multiplex PCR for the determination of DNA from genetically modified corn and soy beans using the Agilent 2100 bioanalyzer	5989-0124EN
Rapid detection of genomic duplications and deletions using the Agilent 2100 bioanalyzer	5989-0192EN
Mutation detection for the K-ras and P16 genes	5989-0487EN
Use of the Agilent 2100 bioanalyzer and the DNA 500 LabChip in the analysis of PCR amplified mitochondrial DNA	5989-0985EN
Assessing genomic DNA contaminations of total RNA isolated from kidney cells obtained by Laser Capture Microdissection using the Agilent RNA 6000 Pico assay	5989-0991EN
Integrating high-throughput, on-chip electrophoresis analysis into PCR diagnostics projects	5989-1870EN
DNA QC for Oligonucleotide Array CGH (aCGH) with the Agilent 2100 bioanalyzer	5989-2487EN
Label-free analysis of microsatellite instability in colorectal carcinoma by on-chip electrophoresis	5989-2626EN
Determination of PCR-RFLP Profiles for Fish Species Using the Agilent 2100 bioanalyzer	5989-2982EN
Using the Agilent 2100 bioanalyzer to Optimize the PCR Amplification of Mitochondrial DNA Sequences	5989-3107EN
Analysis of Cy5-labeled cRNAs and cDNAs using the Agilent 2100 bioanalyzer and the RNA 6000 LabChip kit	5980-0321EN
Measuring the METH-2 promoter hypermethylation and transcript dwonregulation in non-small cell lung carcinomas with the Agilent 2100 bioanalyzer	5989-3514EN

RNA

Analysis of total RNA using the RNA 6000 LabChip kit	5968-7493EN
Analysis of messenger RNA using the RNA 6000 LabChip kit	5968-7495EN
Analysis of Cy5-labeled cRNAs and cDNAa using the RNA 6000 LabChip kit	5980-0321EN
Quantitation comparison of total RNA using the Agilent 2100 bioanalyzer, ribogreen analysis and UV spectrometry	5988-7650EN
Characterization of RNA quality using the RNA 6000 LabChip kit	5980-0472EN
Comparing performance of the Agilent 2100 bioanalyzer to traditional RNA analysis	5980-2206EN
The total RNA story	5988-2281EN
Interpreting mRNA electropherograms	5988-3001EN
Optimizing cRNA fragmentation for microarray experiments using Agilent 2100 bioanalyzer	5988-3119EN
High sensitivity quality control of RNA samples using the RNA 6000 Pico LabChip kit	5988-8554EN
Quality assurance of RNA derived from laser microdissected tissue samples obtained by the PALM MicroBeam system using the RNA 6000 Pico LabChip kit	5988-9128EN
Advancing the quality control methodology to assess isolated total RNA and generated fragmented cRNA	5988-9861EN
siRNA transfection optimization with the Agilent 2100 bioanalyzer	5988-9872EN
Successful analysis of low RNA concentrations with the Agilent 2100 bioanalyzer and the RNA 6000 Pico	5989-0712EN
Stringent RNA quality control using the Agilent 2100 bioanalyzer	5989-1086EN
Gene Expression Profiling of Esophageal Cancer Using Laser Capture Microdissected Samples	5989-1088EN
RNA Integrity Number (RIN) - Standardization of RNA Quality Control	5989-1165EN
Confirming gene silencing mechanism by pGFP/GFP22 - siRNA co-transfection	5989-0103EN
Sensitive detection of tumor cells in peripheral blood of carcinoma patients by a reverse transcription PCR method	5988-9341EN
Assessing genomic DNA contaminations of total RNA isolated from kidney cells obtained by Laser Capture Microdissection using the Agilent RNA 6000 Pico assay	5989-0991EN
High-Purity RNA isolation from a wide range of plant species and tissue types	5989-2271EN
Isolation of high purity total cellular RNA from muscle tissues	5989-2312EN

Cells

Apoptosis detection by annexin V and active caspase-3 with the Agilent 2100 bioanalyzer	5988-4319EN
Detection of cell surface proteins with the Agilent 2100 bioanalyzer by on-chip antibody staining	5988-7111EN
Monitoring transfection efficiency in cells using an on-chip staining protocol	5988-7296EN
A fast protocol for apoptosis detection by Annexin V with the Agilent 2100 bioanalyzer	5988-7297EN
Cell fluorescence assays on the Agilent 2100 bioanalyzer - general use	5988-4323EN
Monitoring transfection efficienency by green fluorescence protein (GFP) detection	5988-4320EN
Detection of antibody-stained intracellular protein targets with the Agilent 2100 bioanalyzer	5988-4322EN
Measuring multiple apoptosis parameters with the Agilent 2100 bioanalyzer	5988-8028EN
Flow cytometric analysis of human primary cells using the Agilent 2100 bioanalyzer and on-chip staining	5988-8154EN
Confirming gene silencing mechanism by pGFP/GFP22 - siRNA co-transfection	5989-0103EN
Flow cytometric analysis of a limited number of cells using the Agilent 2100 bioanalyzer	5989-0746EN
A new method for the calculation of baculovirus titre using the Agilent 2100 bioanalyzer and the flow cytometry kit	5989-1644EN
Cytometric analysis of Upregulated Functional Gene Expression in Primary Cells by On-Chip Staining	5989-2718EN
Detection of Apoptosis in Primary Cells by Annexin V Binding Using the Agilent 2100 bioanalyzer	5989-2934EN
siRNA transfection optimization with the Agilent 2100 bioanalyzer	5988-9872EN

Proteins

Protein sizing and analysis using the Protein 200 LabChip kit	5988-0975EN
Differences and similarities between Protein 200 Assay and SDS-PAGE (technical note)	5988-3160EN
Comparison of different protein quantitation methods	5988-6576EN
Using the Agilent 2100 bioanalyzer for analysis of His-tag removal from recombinant proteins	5988-8144EN
Absolute quantitation with the Protein 200 LabChip kit	5988-4021EN
Optimization of protein purification using the Agilent 2100 bioanalyzer	5988-4022EN
Comparison of different methods for purification analysis of a green fluorescent strep-tag fusion protein	5988-5025EN
Fast analysis of proteins between 5-50 kDA using the Agilent 2100 bioanalyzer and Protein 50 assay	5988-8322EN
Using the Agilent 2100 bioanalyzer for analysis of His-tag removal from recombinant proteins	5988-8144EN
Protein purification and characterization using the 1100 Series purification system and the Agilent 2100 bioanalyzer	5988-8630EN
Characterization of Transgenic Soybean Seedlines by Protein Expression with the Agilent 2100 bioanalyzer	5988-9441EN
Quality control of antibodies using the Agilent 2100 bioanalyzer and the Protein 200 Plus assay	5988-9648EN
Analysis of bispecific antibodies using the Agilent 2100 bioanalyzer and the Protein 200 Plus assay	5988-9651EN
Evaluation of albumin removal using the Agilent 2100 bioanalyzer	5988-9911EN
Increased sensitivity by desalting protein samples prior to analysis on the Agilent 2100 bioanalyzer	5989-0228EN
Glycoprotein sizing on the Agilent 2100 bioanalyzer	5989-0332EN
Using the Agilent 2100 bioanalyzer for quality control of protein samples prior to MS-analysis	5989-0771EN
Quality Control for the Agilent 2100 Bioanalyzer Protein 200 Plus LabChip Kits (technical note)	5989-3336EN

Scientific publications

The Agilent 2100 bioanalyzer is used in virtually every kind of scientific laboratory. A list of existing scientific publications with more than 2000 citations from well-known and high- impact journals is continuously updated and can be viewed at: **http://www.agilent.com/chem/2100publications**.

Agilent 2100 bioanalyzer

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Agilent 2100 bioanalyzer system OQ/PV

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Notes

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Printed in Germany, June 1, 2007
Publication Number: 5989-3542EN

5989-3542EN

Agilent Technologies

One platform, endless possibilities

The Agilent 2100 Bioanalyzer is an indispensable tool for food chemists and biologists.

Uniquely, the 2100 Bioanalyzer performs both electrophoretic separation and flow cytometric analysis of cell fluorescence parameters. It is rapidly replacing gel electrophoresis for DNA fragment analysis and SDS-PAGE analysis of protein samples.

Now you can have a multi-purpose platform that streamlines your workflows from product development to QA/QC of bulk materials and finished food products. The Agilent 2100 Bioanalyzer can handle all your needs, whether you want to measure protein content in seeds, bulk material or finished product; measure DNA or RNA for molecular detection of genetically modified organisms, allergenic species or pathogens; or count cells.

Versatile, fast and mess-free

The Agilent 2100 Bioanalyzer is the industry's *only* platform that can cover your entire workflow with a single compact system. The first commercial, analytical instrument based on lab-on-a-chip technology, the 2100 Bioanalyzer has proven to be an excellent alternative to antibody-based, labor-intensive gel electrophoresis. This technology replaces subjective, time-consuming techniques associated with agarose or SDS-PAGE slab gels with fast, automated, high quality digital data.

Advantages of miniaturization

Miniaturization of analytical instrumentation has a number of advantages over conventional techniques:

- Data precision and reproducibility
- Short analysis times
- Minimal sample consumption
- Improved automation
- Integration of complex workflows

Answers within minutes

The 2100 Bioanalyzer provides you with a convenient and productive way to gather and store experimental and routine test data. Automation and standardization of different processes on a chip give you high quality digital data fast, increasing lab productivity. *You get answers within 30 minutes.*

Pre-packaged LabChip kits, which include sample-specific reagents and chips, let you analyze specific sample classes. A variety of kits for RNA, DNA, protein and cell assays are currently available to meet your needs.

Agilent Technologies

Why you need to test-drive the **Agilent 2100 Bioanalyzer**

Advantages of the Agilent 2100 Bioanalyzer

- Faster than gels—digital data for up to 12 samples within 30 minutes
- Reproducible and complete digital data
- Compliance Service options allow you to work within regulated environments (particularly QA/QC labs)
- 2100 Expert Software for easy digital handling and storage of all bioanalyzer data
- Multiplex detection capabilities

Advantages of Agilent's lab-on-a-chip technology

- Minimal sample consumption and fast results
- Improved assay accuracy and precision
- Digital data for convenient archiving and storage
- Various data display options
- Ease-of-use with simplified sample-to-sample comparison
- Minimum exposure to hazardous materials

Digital data

The 2100 Bioanalyzer provides fully digital data easily shared with colleagues worldwide. The fully functioning data analysis software is available free at http://www.chem.agilent.com/cag/wad/registration/2100expert.asp

1 Fast and easy operation

Add sample

- Ready-to-use reagent kits
- Quick-start instructions
- Chip preparation in less than 5 minutes
- Minimal use of hazardous chemicals and waste disposal
- Sample volumes in the µL-range

2 Automation

Start chip run

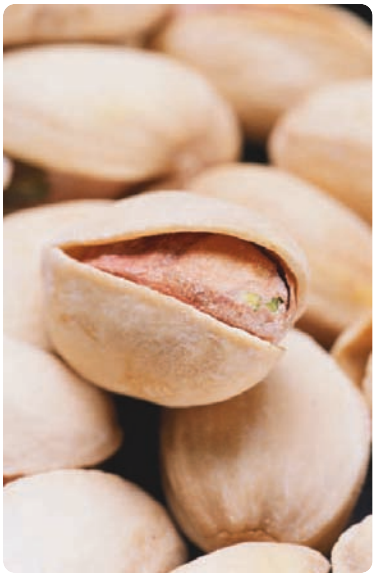
- Start analysis at the press of a button
- Predefined protocols
- System uses internal standards to calculate results

3 Digital data in 30 minutes

Watch real-time data display

- Automated data analysis
- Digital data can be filed in a database or shared
- No user-dependent data interpretation

Food safety applications



Nuts and allergens

The 2100 Bioanalyzer is more cost effective and reduces labor compared to other test methods. Recent regulatory and policy changes in the United States (US), European Union (EU), Japan, Canada and Australia require labeling of multiple allergens in food products. EU regulations, for instance, identify eight nut species that must be declared on the ingredient list.

Currently available allergen content tests for food products and materials often have difficulty in complex matrices and usually detect only a single allergen per test. In contrast, the 2100 Bioanalyzer coupled with molecular detection techniques (PCR) can cost-effectively screen for multiple allergens using a single test.

The electropherograms shown in Figure 1 demonstrate multiplex detection of three nut species—Brazil nut *(Bertholletia excelsa)*, pistachio nut *(pistacia vera)* and macadamia nut *(macadamia integrifolia)*—in a single run. Furthermore, the bioanalyzer, which uses only 1 µl of reaction mixture to load onto the LabChip, can reduce PCR volumes by up to 75 percent compared to standard gel-electrophoresis, which requires 10 to 15 µl, saving reagent costs.

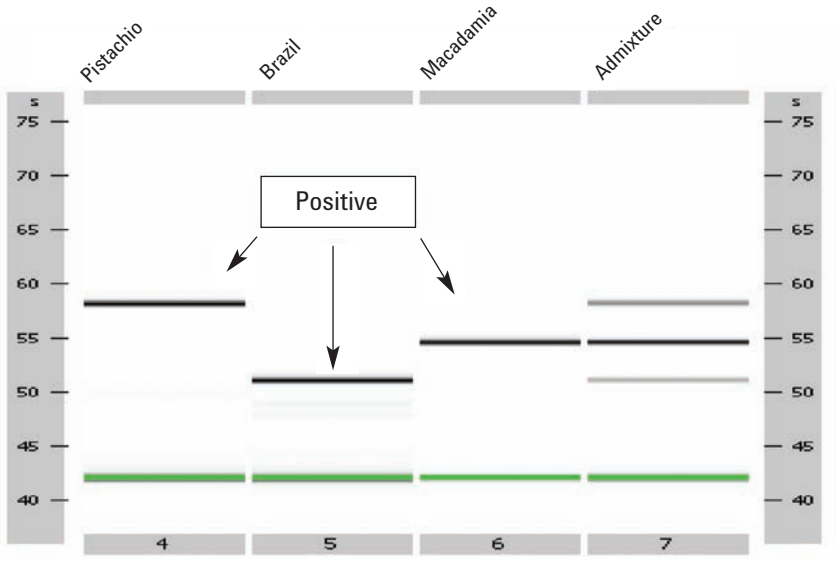


Figure 1: Cost-effective screening for multiplex PCR reaction product for nut allergen species in food using a single test. The 2100 Bioanalyzer's gel-like image shows positive test results for pistachio nut (lane 4), Brazil nut (lane 5) and macadamia nut (lane 6), as well as for a mixture of these three nuts (lane 7) in wheat.

Source: Campden and Chorleywood Food Research Association Group, Gloucestershire, UK.

Food safety applications

Genetically modified organisms

The number of genetically modified organisms (GMO) and regulatory requirements for testing and labeling continue to increase. Many countries now require GMO content labeling—or ban GMO materials altogether. Antibody tests to detect GMO proteins often require individual tests for each suspected GMO organism/event. Test accuracy can be problematic in processed materials where detected proteins may become denatured or damaged.

The 2100 Bioanalyzer, in conjunction with an appropriate PCR kit, offers multiplex screening to detect and identify multiple GMO events in processed foods, improving test accuracy and reducing the number of tests, which in turn reduce costs. Figure 2 shows multiplex detection of GMO in corn meal and soya powder.

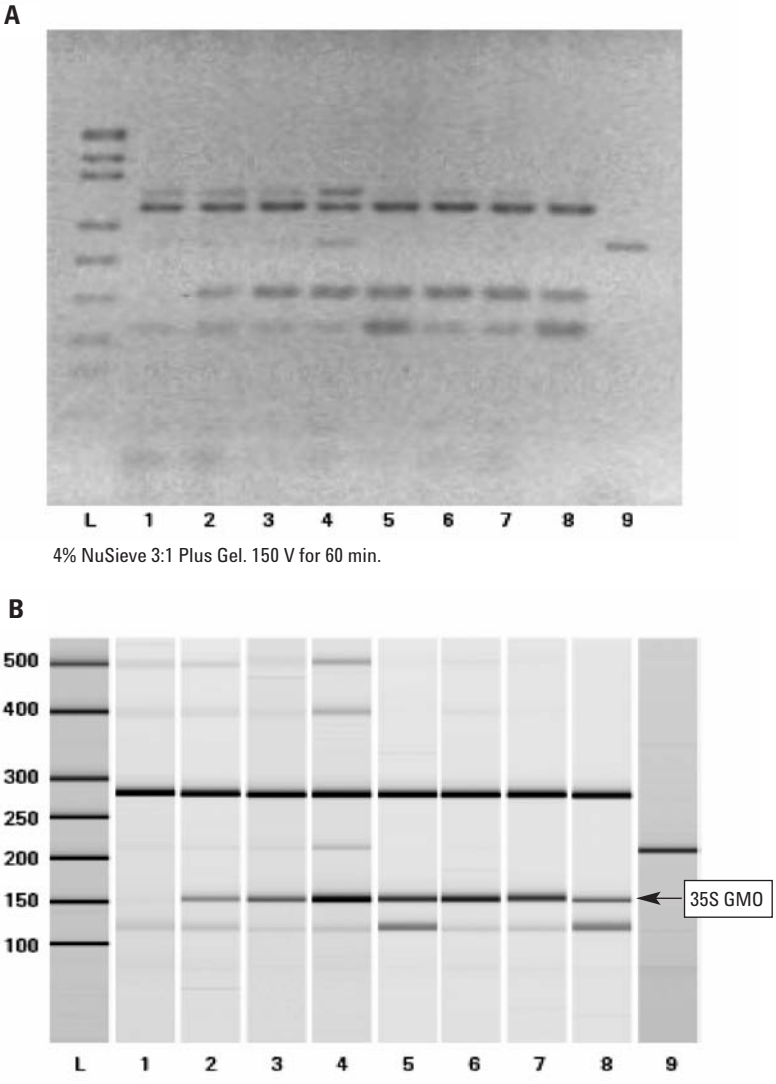


Figure 2. Comparison of slab gel electrophoresis (A) with Agilent 2100 Bioanalyzer gel-like image (B) in detecting multiple GMO events in corn meal and soybean powder. Samples: L) molecular weight ladder (26, 34, 67, 89, 110, 147, 190, 242, 353, 404, 489 and 501 bp); 1) 0% MON810 corn; 2) 0.1% MON810 corn; 3) 0.5% MON810 corn; 4) 1.0% MON810 corn; 5) 2.0% MON810 corn; 6) 5.0% MON810 corn; 7) commercial corn meal; 8) Allin positive control—0.5% Bt 176 maize and 0.5% Roundup Ready soybean; and 9) negative control (de-ionized water). Band identification: 118 bp—soy lectin; 153 bp—35S GMO; 217 bp—PCR reaction internal control; 278 bp—corn zein.

Meat and fish

The multiplex detection capability of the 2100 Bioanalyzer can also determine food product authenticity, its source and homogeneity. For example, meat authenticity is an important factor for economic as well as religious and cultural reasons.

Identifying authenticity and homogeneity of the meat source is difficult for processed meats in which proteins may be damaged by heat, rendering antibody-based tests inaccurate. Using molecular detection methods (PCR), the 2100 Bioanalyzer successfully determines meat species and sample homogeneity in one test, reducing the number of tests required and thereby reducing labor and testing costs.

Figure 3 shows the bioanalyzer's ability to detect turkey, lamb or pork in a variety of meat samples while discriminating for other various matrices such as fish or grain.

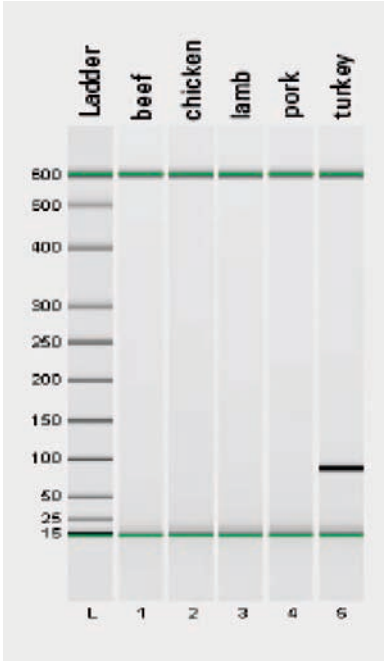


Figure 3. The 2100 Bioanalyzer can detect multiple targets at one time, including turkey, lamb or pork in a variety of meat samples. The dark band (lane 6) indicates a positive molecular test (PCR) for turkey. Negative tests (lanes 1 through 4) demonstrate test discrimination for other meat products (beef, chicken, lamb and pork).

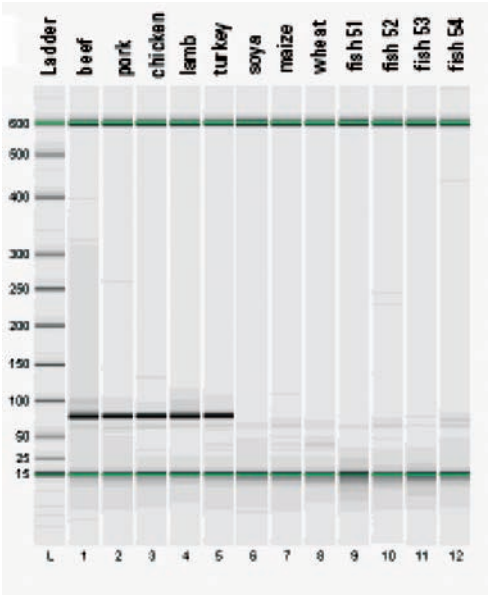


Figure 4. Agilent 2100 Bioanalyzer gel-like image showing PCR test designed to discriminate meat. Positive results are evident in lanes 1 through 5 for various meat products. Lanes 6 through 12 show negative results for grain and fish.

Source: Campden and Chorleywood Food Research Association Group, Gloucestershire, UK.

Food safety applications

Dairy: Milk protein

In addition to DNA analysis for molecular detection applications, the 2100 Bioanalyzer can detect and quantify proteins to determine protein source, quality and content. Figure 5 demonstrates determination of protein content to check the authenticity of the milk source (such as goat, sheep or cow) as well as determine the protein content and quality for in-process or finished product QA/QC.

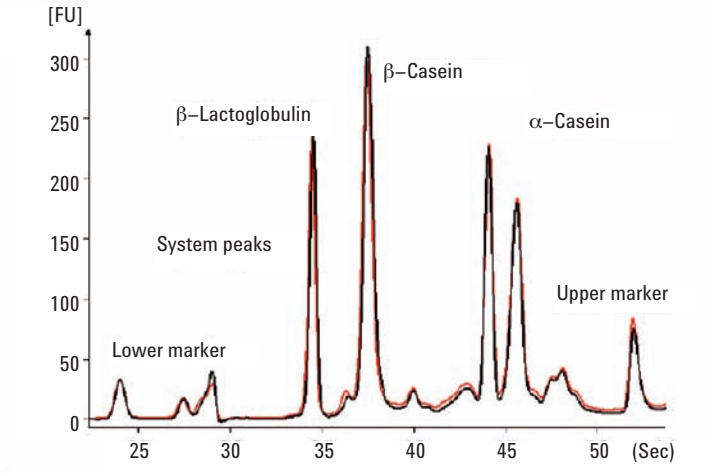


Figure 5. Milk protein analysis. The 2100 Bioanalyzer protein assay provides rapid analysis of milk proteins. Shown here are two overlaid electropherograms of bovine milk (dil. 1:10; peaks identified by separately analyzing individual protein standards).

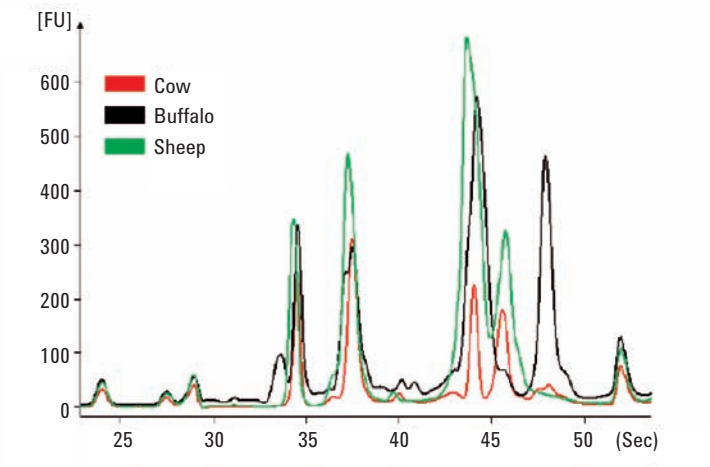


Figure 6. Milk authenticity. The 2100 Bioanalyzer protein assay also provides a rapid means to determine product authenticity quickly. Shown here are three overlaid electropherograms of milk samples (dil. 1:10) from different sources. In this example, milk samples from cow, buffalo and sheep are differentiated quickly based on relative milk protein content.

Wheat

Because the 2100 Bioanalyzer can electrophoretically separate complex protein samples, providing both protein molecular weight and quantity, you can use pattern matching of the resulting electropherogram to determine product authenticity as an alternative (or complement to) molecular detection methods. For example, verifying seed variety and quality is important economically because high quality and desirable seeds usually command a premium price. For many milled materials such as wheat flour, protein quality and quantity determine the suitability of the flour for the intended finished product. In a single test, the 2100 Bioanalyzer can determine both the genetic identity of various wheat varieties (DNA analysis) and protein content for either variety identification or product quality control. Figure 7 shows wheat variety identification based on protein pattern.

Pattern matching can identify the particular strain of wheat, in this case Halberd.

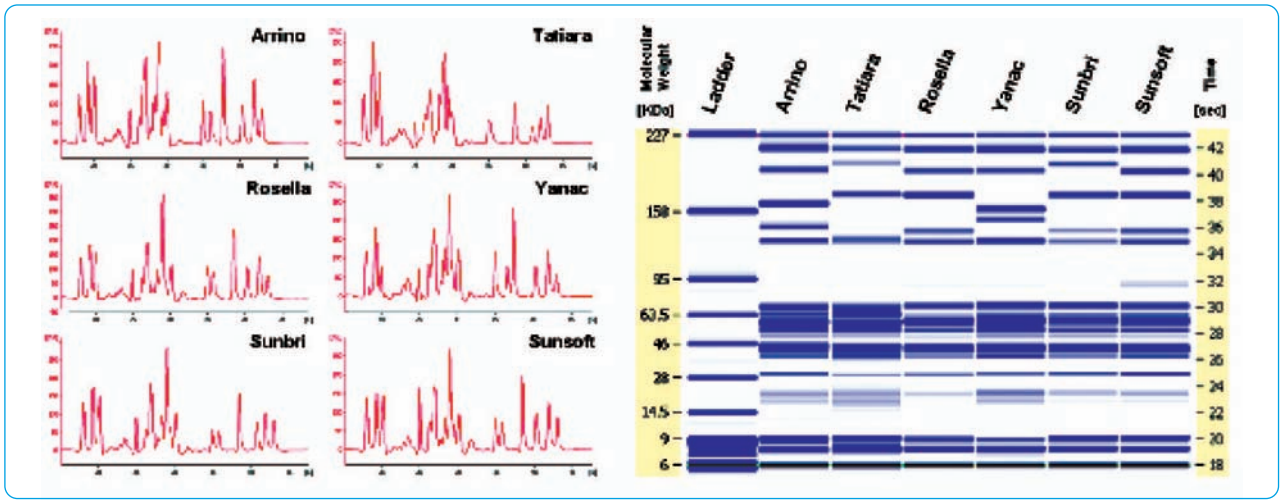
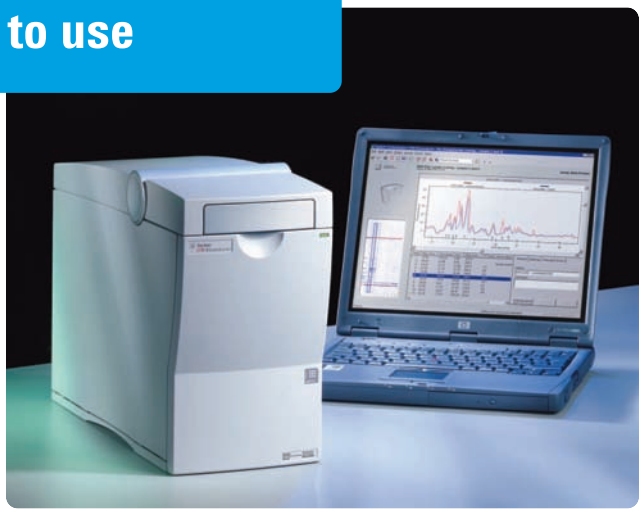


Figure 7. Protein patterns obtained by the 2100 Bioanalyzer's protein chip identify various kinds of wheat. This figure covers extracts (1% SDS + 1% DTT) of six wheat varieties: Arrino, Tatiara, Rosella, Yanac, Sunbri and Sunsoft. The elution profiles from size-based capillary electrophoresis appear on the left, corresponding to the simulated gel patterns at right.

Source: S. Uthayakumaran, I.L. Batey, C.W. Wrigley, Value Added Wheat CRC and Food Science Australia, North Ryde, Australia.

Compact, robust and simple to use



For more information

To learn more about the Agilent 2100 Bioanalyzer and additional food applications, go to:

- http://www.agilent.com/chem/labonachip
- http://www.agilent.com/chem/foods and click “Bioanalysis” and “Applications”

You can also call 1-800-227-9770 (in the U.S. and Canada) or contact your local Agilent representative or Agilent Authorized Distributor.

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Agilent Technologies

Determination of PCR-RFLP Profiles for Fish Species Using the Agilent 2100 Bioanalyzer

Application

Food Safety

Authors

Steve Garrett and John Dooley
Molecular Biology Group, Dept. of Chemistry and Biochemistry
Campden & Chorleywoood Food Research Association, Chipping Campden
Gloucestershire, GL55 6LD
UK

Abstract

This application note shows how the Agilent 2100 Bioanalyzer was used in polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) fragment analysis for fish species identification. A 464bp cytochrome-b target sequence, found in all vertebrate fish, was first amplified and then digested with restriction enzymes. The fragments were resolved on the DNA 500 LabChip®, allowing simple comparison with authentic species profiles due to the accuracy of fragment size determination. Use of the Bioanalyzer offered significant benefits over traditional gel electrophoresis and DNA staining techniques for PCR-RFLP fragment analysis.

Introduction

The diversity of fish products available to consumers has increased significantly in recent years. Products can range from premium grade fish steaks to low cost fish fingers. As fish are caught, processed, and distributed by a global network of operators, there is a need to ensure the authenticity and the origin of fish used in the products.

There is, therefore, a need to have reliable and simple species identification methods to support enforcement and compliance with labelling legislation (EC Council Regulation No. 104/2000 and EC Commission Regulation No. 2065/2001).

Methods of fish species identification based on morphological characteristics are suited to whole fish; however, fish species identification becomes more problematic once it is processed. Protein profiling is used for fish identification; however, this method requires the analysis of species reference materials alongside the samples and is less reliable when applied to processed food products as the proteins become denatured. DNA based methods offer an alternative approach to species identification as DNA remains detectable in all but the most heavily processed samples.

Direct sequencing is the most definitive method of identification; however, it cannot easily be applied to samples suspected or known to contain more than one species. Alternative techniques, using polymerase chain reaction (PCR), were developed to identify fish species based on DNA fingerprint patterns. Methods used include RAPDs (random amplified polymorphic DNA), SSCP (single strand conformation polymorphism) and PCR-RFLPs.

A PCR-RFLP technique, which involved digesting an amplified 464bp region of the cytochrome b gene with restriction enzymes to generate DNA profiles, was previously developed for the identification of salmon species [1, 2].

The aim of this work was to improve the method for identification of salmon and other species by replacing conventional gel electrophoresis and staining steps with the Agilent 2100 Bioanalyzer. The generation of species-specific PCR-RFLP profiles on the 2100 Bioanalyzer combined with accurate sizing and quantification of individual DNA fragments, offered significant advantage over gel-based approaches in terms of ease-of-use, speed, and accuracy of identification.

Materials and Methods

All chemicals used for this work, unless otherwise stated, were supplied by Sigma-Aldrich and were of molecular biology grade or equivalent. PCR primers were supplied by MWG-Biotech UK Ltd. PCR-RFLP profiles were generated using a DNA500 LabChip and the Agilent 2100 Bioanalyzer. AmpliTaq® Gold DNA polymerase from Applied Biosystems was used in all PCR reactions. Restriction enzymes were obtained from New England Biolabs and used per the manufacturer's instructions.

Fish Samples

Commercially important salmon and white fish species samples were obtained from appropriate fishery research laboratories in the UK, Canada, Alaska, New Zealand, and Japan. Five individuals were used to minimize the effects of polymorphic variation within the population. Additional samples of each fish species were obtained from local UK fishmongers and retailers.

DNA Extraction

DNA extraction was performed using a modification of the CTAB method. Samples (2 g wet weight) were suspended in 5 mL of CTAB buffer (2% CTAB [hexadecyltrimethylammonium bromide], 100-mM Tris-HCl, 20-mM EDTA, 1.4-M NaCl, pH 8.0) and 40 µL of Proteinase K solution (20 mg/mL) was added. Samples were mixed thoroughly and then incubated overnight at 60 °C. After incubation, 1 mL of supernatant was transferred to a 2.0-mL Eppendorf tube, cooled to room temperature (RT), and centrifuged at 13,000g for 10 minutes. The clear supernatant was recovered and an equal volume of chloroform was added. The solution was vortexed and then centrifuged at 16,000g for 15 minutes before the upper aqueous layer was transferred to a clean 1.5-mL Eppendorf tube. An equal volume of isopropanol was added and the DNA precipitated at RT for 30 minutes. DNA was pelleted by centrifugation at 16,000g for 15 minutes, washed in 70% ethanol and air dried for 30 minutes at RT. The DNA pellet was resuspended in 100 µL of sterile distilled water (SDW) and purified using Promega's Wizard® Purification Resin per the manufacturer's protocol. DNA extracts were recovered in 50 µL of 1×TE (10-mM Tris-HCl, pH 7.4, 1-mM EDTA, pH 8.0) buffer. Final DNA concentrations (ng/µL) were determined using a GeneQuant pro DNA calculator (Pharmacia).

DNA Amplification

PCR products (464bp target from the cytochrome b gene) were produced by amplification of DNA extracts (50 ng) in 20-µL reactions containing 1× AmpliTaq Gold PCR buffer (Applied Biosystems), 300 nM of each primer (L14735: 5'-AAA AAC CAC CGT TGT TAT TCA ACT A-3' and H15149: 5'-GCI CCT CAR AAT GAY ATT TGT CCT CA-3'), 200-nM dNTPs, 5-mM $MgCl_2$ and 0.05-U/µL of AmpliTaq Gold (Applied Biosystems). Amplification profiles (94 °C for 5 minutes [denaturation]; 40 cycles of: 94 °C for 40 s, 50 °C for 80 s, 72 °C for 80 s [amplification]; 72 °C for 7 minutes [final extension] were applied using a PE9600 PCR machine (Applied Biosystems). Unpurified PCR products (1 µL) were applied to the 2100 Bioanalyzer to confirm amplification.

PCR-RFLP Profiling

Unpurified PCR product (2.5 µL) was digested for 3 or more hours with two to five units of enzyme in a total volume of 5 µL. Reactions were terminated by incubation at 65 °C for 10 minutes. Digested PCR products (5 µL) were mixed with 5 µL of 20-mM EDTA, to achieve a final concentration of 10-mM EDTA, prior to loading on to DNA500 LabChips. Aliquots (1 µL) of the reaction mix were analyzed on the 2100 Bioanalyzer, per manufacturers' instructions.

Results

Evaluation of PCR-RFLP Profiles Generated on the 2100 Bioanalyzer for Species Identification

An initial evaluation of the PCR-RFLP method was performed using salmonid species.

Following cleavage of the amplified DNA fragment with restriction enzymes, species-specific PCR-RFLP patterns were resolved on the 2100 Bioanalyzer. An example of a PCR-RFLP pattern is given in Figure 1, which shows PCR-RFLP profiles for salmon and trout samples generated with enzymes DdeI and HaeIII.

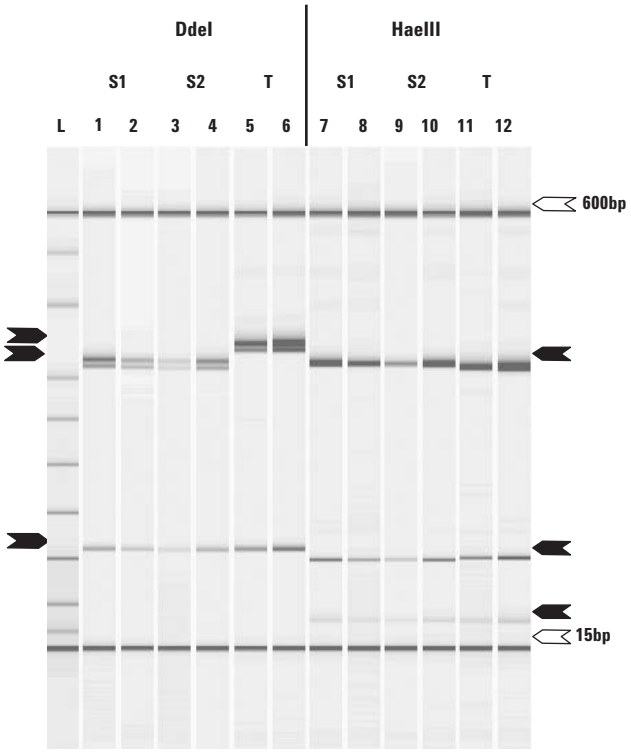

Figure 1. PCR-RFLP patterns obtained from salmon and trout with enzymes DdeI and HaeIII. PCR-RFLP patterns obtained when amplified DNA from two salmon (S1, S2) and one trout (T) samples were digested with DdeI (lanes 1–6) or HaeIII (lanes 7–12). A 15bp–600bp ladder (L) is shown. All wells contain 15bp and 600bp size markers. DNA fragments of note are indicated (arrows).

Observed and expected fragment sizes for a selection of five enzymes and four salmonids are shown in Table 1. As can be seen, patterns were similar to those reported previously [1, 2].

Expected DNA fragments of greater than about 25bp were readily detected; however, some smaller fragments were not detected because they could not be distinguished from the lower 15bp size marker or were outside the detection range (25bp–500bp) of the LabChip. Small DNA fragments (25bp–100bp), which were not reported previously, were observed in some digests [2]. This highlights the improved band resolution of this method in comparison to gel electrophoretic methods. This improved resolution is also highlighted by profiles generated with DdeI, where all four species have an extra fragment that is about 9bp smaller than the expected larger fragment. This is due to an extra DdeI site situated in primer H15149

Table 1 shows that *O. gorbuscha* and *O. mykiss* profiles generated with NlaIII have only two fragments when three are expected. This is believed to be due to the comigration of the two larger fragments. An analysis of the sequence of the *O. mykiss* 464bp amplicon indicated that cleavage of the amplicon with NlaIII should produce fragments of 192bp, 180bp and 91bp. These respectively equate to the 210bp, 190bp and 100bp fragments reported by Russell *et al.* (2000) and appear in Table 1. From the sequence data there is

Table 1. Expected and Observed PCR-RFLP Fragment Sizes Obtained with Five Restriction Enzymes and Four Salmonid Species

		Expected* (E) and observed (O) fragment sizes for each enzyme (bp)				
Species		**DdeI****	**Bsp1286I**	**HaeIII**	**NlaIII**	**Sau3AI**
O. nerka						
(Red salmon)	E	360, 130	300, 200	350, 130	310, 180	390, 120
	O	353, 346, 114	289, 172	320, 102, 35 or 421	272, 160	340, 115
O. gorbuscha						
(Pink salmon)	E	360, 130	U/C***	U/C	210, 190, 100	390, 120
	O	349, 343, 112	464	421	181, 92	338, 115
S. salar						
(Atlantic salmon)	E	350, 130	300, 200	350, 130	U/C	410, 110
	O	321, 312, 110	287, 172	318, 98, 35	438	370, 88
O. mykiss						
(Rainbow trout)	E	360, 130	300, 200	350, 130	210, 190, 100	U/C
	O	348, 339, 111	279, 174	313, 100, 33	185, 92	451

*Sizes as reported by Russell *et al.* (2000).

**Extra fragments in observed DdeI profiles are due to restriction site introduced by primer H15149.

***U/C Uncut with enzyme.

no evidence that the smaller 180bp fragment contains a higher proportion of heavier A or G bases or the larger 192bp fragment a higher proportion of lighter C or T bases, which could cause their respective molecular weights to converge. The calculated molecular weight difference (3277 Daltons) between the two fragments is equivalent to the difference in the number of bases. This makes it unlikely that comigration is due to molecular weight similarities between the two fragments. The comigration of these two fragments as a single fragment is consistently observed and does not detract from the identification of these species.

Overall, the profiles generated by the 2100 Bioanalyzer matched those expected or previously reported [1, 2], which supports the use of this approach for the identification of fish species. Further work was performed using Atlantic salmon and trout and a smaller number of enzymes to confirm the application of this approach.

Experimental Repeatability

In order to determine the experimental repeatability (LabChip-to-LabChip variability) of the complete assay, duplicate PCRs were produced from two salmon and one trout sample. Amplified fragments were cleaved with DdeI and digests stored at 4 °C until required. PCR-RFLP patterns were separated on four occasions using different DNA500 LabChips primed with two different batches of gel matrix, A and B. Two LabChips were run using a freshly prepared gel matrix (matrix A) while a third LabChip was run when gel matrix A was 1-week old. The fourth LabChip was run on the same date as the third LabChip but using a second, fresh batch of gel matrix (matrix B). Overall variation (encompassing variation due to LabChip-to-LabChip, PCR and gel matrix) appears in Table 2, which shows the results of analysis with the four LabChips following digestion with enzyme DdeI. Results are the mean fragment sizes observed on each LabChip from two PCR replicates of each species. Absolute fragment size variations within a single LabChip, that is, for PCR replicates of the same sample or between the two salmon samples, were less than 2bp and were only observed between the larger (>300bp) fragments. The overall absolute size variation for each fragment, which included variation due to different LabChips, PCRs and gel matrices, was slightly greater; the biggest variation was 6bp for the 320bp fragment in salmon (321bp to 327bp).

Fish Species Identification

Using sequence data generated from 10 different white fish species, theoretical PCR-RFLP profiles were generated from a range of restriction enzymes. Closer examination of these theoretical profiles indicated that only three enzymes would be needed to differentiate all the white fish species. Experimental profiles were generated to confirm that species identification was possible using just these three enzymes. Results of this analysis are shown in Figure 2, which shows that 9 of the 10 species could be identified based on profiles generated with only one enzyme. The remaining two species were differentiated using the other two enzymes.

Table 2. PCR-RFLP Fragment Sizes Obtained Following Separation of DNA Cleaved with DdeI on Four Different DNA500 LabChips

	Observed fragment sizes on each LabChip (bp)					
Expected band size (bp)	LabChip 1 (Fresh matrix A)	LabChip 2 (Fresh matrix A)	LabChip 3 (Week old matrix A)	LabChip 4 (Fresh matrix B)	Mean	%RSD
Salmon analysis*						
117	111	111	110	111	111	0.34
312	314	316	314	317	315	0.43
320	323	325	323	326	324	0.51
Trout analysis						
116	111	111	110	111	111	0.45
339	338	341	338	343	340	0.72
348	347	349	347	352	349	0.61

*An expected 27bp fragment from salmon is not detected by the 2100 Bioanalyzer.

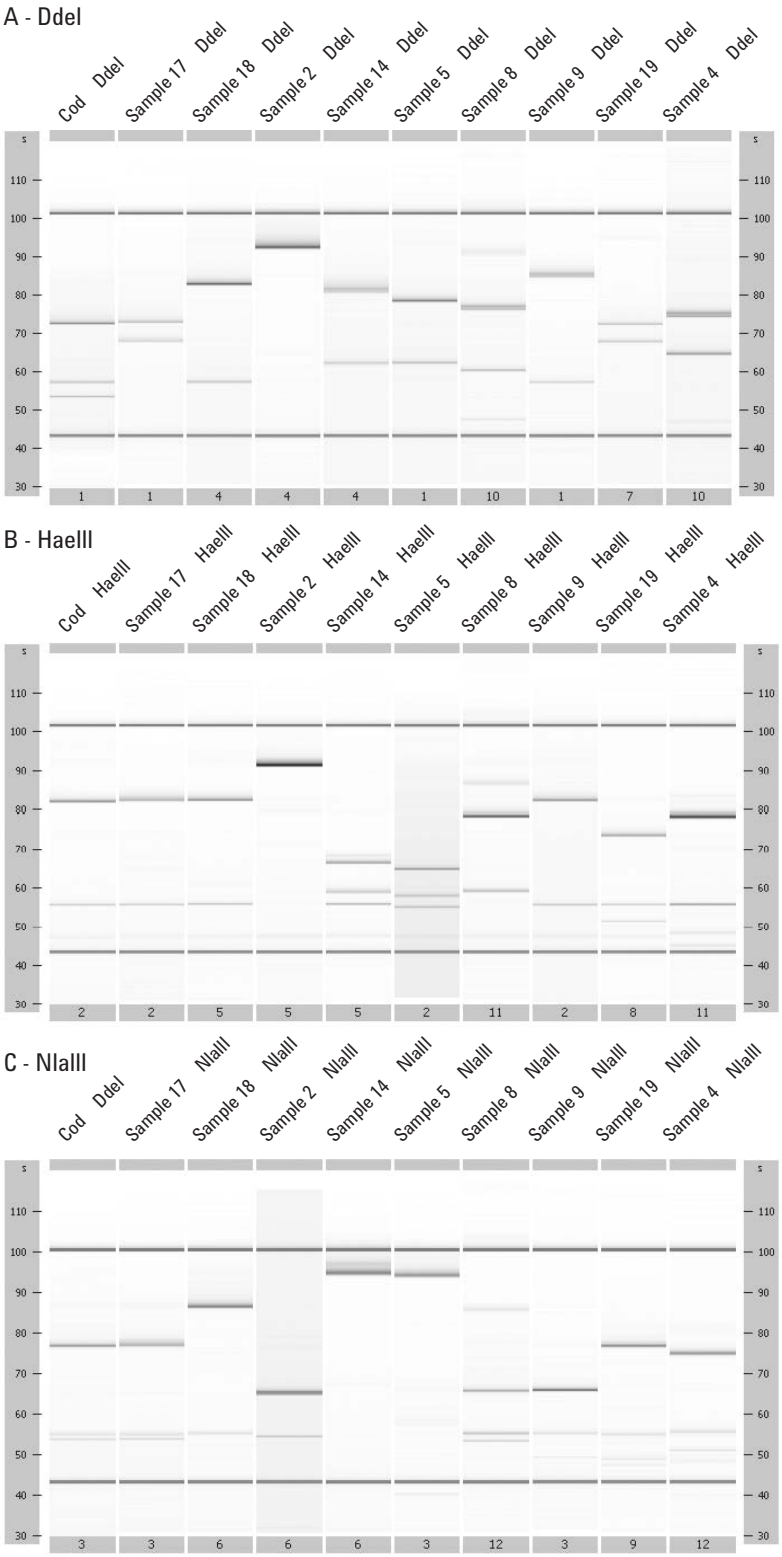

Figure 2. PCR-RFLP profiles from the 10 white fish species used in this study. Profiles were generated using enzymes DdeI (A), HaeIII (B) or NlaIII (C). Each sample number indicates a different fish species.

Analysis of a further nine salmon species was performed using these three enzymes only (data not shown). Again it was found that the salmonids could be differentiated using the three enzymes. A list of all the species studied is found in Table 3.

Table 3. Fish Species that Could be Differentiated Using PCR-RFLP with Enzymes Dde I, Hae III and NlaIII

Common name (UK)	Latin name
Atlantic cod	*Gadus morhua*
Pacific cod	*Gadus macrocephalus*
Coley (Saithe)	*Pollachius virens*
Haddock	*Melanogrammus aeglefinus*
European hake	*Merluccius merluccius*
South African hake	*Merluccius paradoxus*
European plaice	*Pleuronectes platessa*
Whiting	*Merlangus merlangus*
Alaskan(Walleye) Pollock	*Theragra chalcogramma*
Hoki	*Macruronus novaezelandiae*
Atlantic salmon	*Salmo salar*
Red / Sockeye salmon	*Oncorhynchus nerka*
Pink / Humpback salmon	*Oncorhynchus gorbuscha*
Chinook salmon	*Oncorhynchus tschawytscha*
Coho / Silver salmon	*Oncorhynchus kisutch*
Keta / Chum salmon	*Oncorhynchus keta*
Cut-throat trout	*Oncorhynchus clarki clarki*
Dolly Varden	*Salvelinus malma malma*
Cherry salmon	*Oncorhynchus masou masou*

Discussion

To identify species present in a sample when no prior knowledge of the sample is available requires a universally applicable method. PCR-RFLP profiling of a common region of the vertebrate cytochrome b gene, which is present in all fish species, enabling comparison with profiles in a database is one such universal approach.

The conventional PCR-RFLP fragment analysis involves gel electrophoresis, on large (over 30 cm^2), thin (<2 mm) acrylamide gels, to resolve the PCR-RFLP patterns. This makes handling and staining difficult and requires the use of large equipment and volumes of solution. All this makes these methods potentially hazardous and time consuming and can sometimes produce variable results. This type of detection is, therefore, not suited to use in enforcement and quality control laboratories where a rapid, robust detection method is required.

As an alternative, the 2100 Bioanalyzer incorporates conventional capillary electrophoresis (CE) technology into an easy-to-use chip-based format, which enables accurate sizing and quantification of individual DNA fragments. Coupled with the small (2 cm^2) size of the LabChip, this gives the system a significant advantage over conventional gel-based approaches in terms of ease-of-use, speed, and safety. This makes the 2100 Bioanalyzer ideally suited to the analysis of multiple small DNA fragments such as those found in PCR-RFLP profiles.

Using the 2100 Bioanalyzer it was possible to generate PCR-RFLP profiles that resembled those previously published for salmon species. However, generating PCR-RFLPs on the 2100 Bioanalyzer produced profiles with improved fragment resolution and detection, especially of smaller fragments that were not detected using conventional gel electrophoresis. PCR-RFLP profiles generated on the 2100 Bioanalyzer were also more consistently produced compared to gel electrophoresis.

Consequently, profiles based on three enzymes DdeI, NlaIII and HaeIII were developed for 19 commercially important species. Further studies should increase the range of species in the database, making it an important tool for the study of the authenticity of fish products.

Complete details of the development of the assays, application to fish species identification in products, and an interlaboratory study were recently published [3, 4, 5].

Acknowledgements

This work formed part of the UK's Food Standards Agency project Q01069 and the authors wish to acknowledge the FSA for their financial support.

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Nested Multiplex Polymerase Chain Reaction for the Determination of DNA From Genetically Modified Corn and Soy Beans Using the Agilent 2100 Bioanalyzer

Application

Agriculture

Author

Mark Jensen
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

This application note describes how the Agilent Technologies 2100 bioanalyzer and the DNA 500 LabChip can be used to detect polymerase chain reaction products corresponding to genetically modified elements and endogenous sequences in corn and soy beans. The DNA extraction protocol used in the preparation of polymerase chain reaction samples was characterized using the Protein 200 Plus LabChip.

Introduction

Six years after the introduction of genetically modified organisms (GMO), consumer concerns about the presence of such modified organisms in food remains an ongoing issue. There has been a continuous debate surrounding issues of how food products that contain GMO ingredients should be regulated and labeled. This debate has been further complicated by disagreements over how GMOs should be detected and the significance of the detected levels. In spite of these issues, the number of available transgenic events has continued to grow. At the current time there are 14 transgenic varieties of corn and soy beans that have been deregulated by the Animal and Plant Health Inspection Service of the United States Department of Agriculture (USDA).

Although enzyme immunoassay is an efficient means for detecting transgenic proteins in raw products, only DNA analysis has proved to be effective for the entire range of sample matrices from raw materials to highly processed foods. The polymerase chain reaction (PCR) has been widely accepted as a method for the detection of DNA from genetically modified ingredients such as soya or corn [1, 2]. PCR detection of GMOs can be done either as a screening test using endpoint PCR or as a quantitative test using real-time fluorescence detection of the PCR product.

Quantitation by real-time PCR is an expensive analysis requiring assay calibration for each sample lot and multiple replicates of each unknown sample. Typical service charges for a single analysis are between $150–$300. Since each transgenic event must be evaluated individually, the cost of rigorously testing an unknown food sample for all the possible current transgenic events is cost prohibitive.

These cost constraints make it necessary to screen samples for DNA components that are present in

most GMOs prior to any quantitative analysis. This type of screening analysis can be carried out with a commercially available PCR test kit, such as the Biosmart Allin 1.0 GMO Screening System from Promega (Madison, Wisconsin). This kit provides a protocol and reagents for a nested multiplex PCR assay for the detection of DNA from modified organisms containing the 35S promoter. This genetic element is derived from the cauliflower mosaic virus and is found in most transgenic crops. In addition to the 35S promoter, the multiplex PCR reaction also detects sequences for soya (lectin), corn (zein) and an internal positive control. These multiplex PCR products are all easily resolved and detected using the DNA 500 LabChip and the Agilent 2100 bioanalyzer.

Experimental

DNA Extraction

Three DNA extraction protocols were evaluated prior to the PCR analysis. These protocols were the DNeasy Plant Mini Kit from Qiagen N. V. (Venlo, Netherlands), the Wizard Genomic DNA Purification Kit from Promega (Madison, Wisconsin) and a cetyltrimethylammonium bromide (CTAB) precipitation procedure that was developed in-house. Detailed descriptions of the DNeasy and Wizard Genomic kit protocols can be found in the technical manuals that accompany these products. The CTAB protocol is described below.

CTAB Extraction protocol:

1. A 50-mg sample was added to a 1.0-mL aliquot of CTAB extraction buffer (0.055 M CTAB (cetyltrimethylammonium bromide), 0.1 M Tris, 1.4 M NaCl, 0.2 M disodium EDTA pH 8.0), heated at 65 °C for 15 min and then placed on ice.
2. The extract mixture was centrifuged at 15,000 g for 10 min to remove particulates.
3. The supernatant was removed and stored in a new microfuge tube at 4 °C. (Samples with high starch levels, for example corn, were treated with 0.2 µL of α-amylase and incubated at 37 °C for 30 min.)
4. The recovered supernatant was combined with an equal volume of chloroform mixed for 30 s and then centrifuged for 10 min at 15,000 g. The upper (aqueous) phase was transferred to a new tube.
5. Two volumes of the CTAB precipitation solution (0.014 M CTAB, 0.04 M NaCl) were added to aqueous extract and mixed.
6. The mixture was incubated at 25 °C for 30 min and then centrifuged for 10 min at 15,000 g. The supernatant was discarded.
7. The precipitate was redissolved by adding 250 µL of the 1.2 M NaCl and incubating 37 °C for 10 min. The mixture was centrifuged for 5 min at 15,000 g and the supernatant was transferred to a new tube.
8. One volume of isopropanol was added to the mixture and then the mixture was stored at 4 °C for at least 30 min.
9. The solution was centrifuged for 10 min at 15,000 g and then the supernatant was discarded.
10. The pellet was washed with 500 µL of cold 70% ethanol, the mixture was centrifuged for 5 min at 15,000 g, and then the ethanol solution was discarded.
11. After drying the tube, the DNA pellet was redissolved in 25 µL of Tris buffer (0.01 M Tris, 0.001 M EDTA pH 8.0).

The soya reference standards were used in the evaluation of the DNA extraction because soya contains a high level of protein that can be accurately tracked through each step of the extraction procedure. Although the soya protein is not problematic for the PCR assay, it serves as a useful indicator of overall DNA purity. Protein levels in the final DNA extracts were determined by a protein measurement using the Protein 200 Plus LabChip and the Agilent 2100 bioanalyzer. Absorbance measurements were made with the ND-1000 spectrophotometer from NanoDrop Technologies, Inc. (Wilmington, Delaware).

PCR Protocol

Samples used in the GMO analysis consisted of Institute for Reference Materials and Measurements (IRMM) Certified Reference GMO Soy and GMO Corn, as well as commercial corn meal and soya powder. The PCR analysis was carried out using the Biosmart Allin 1.0 GMO Screening System from Promega (Madison, Wisconsin). A modified PCR cycling protocol was developed for use with the PTC 200 Peltier Thermal Cycler from MJ Research, Inc. (Waltham, Massachusetts). The PCR protocol is described below.

Multiplex PCR Protocol

1. Dilute the DNA sample to an initial concentration of 5–50 ng/µL. (This corresponds to an optical density, (OD), at 260 nm of 0.1–1.0 for a 1-cm cell.)
2. For the first stage of the multiplex PCR combine the following per reaction:

   17-µL 2X Qiagen Multiplex PCR Master Mix

   27-µL Allin Mix 1

   5-µL Internal control

   1-µL Extracted DNA
3. Vortex the solution.
4. Perform first stage of PCR amplification using the following cycling program.

Step	Time	Temperature (°C)
1	15 min	95
2	15 s	95
3	60 s	55
4	30 s	72
5	Repeat steps 2–4 an additional 39 times	
6	3 min	72

5. For the second stage of the Multiplex PCR combine the following per reaction

   24.5-µL 2X Qiagen Multiplex PCR Master Mix

   24.5-µL Allin Mix 2

   1.0-µL Product from first stage PCR
6. Perform second stage of PCR amplification using the following cycling program.

Step	Time	Temperature (°C)
1	15 min	95
2	15 s	95
3	60 s	57
4	30 s	72
5	Repeat steps 2–4 an additional 39 times	
6	3 min	72

Analysis of PCR Products

The PCR products were analyzed using the DNA 500 LabChip and the Agilent 2100 bioanalyzer. For comparison, the same PCR products were also characterized by gel electrophoresis in a 4% NuSieve 3:1 Plus Gel. The electrophoretic separation conditions were 150 V for 60 min in a 1X Tris-borate-EDTA (TBE) buffer.

Results

DNA Extraction

DNA extraction protocols were evaluated for both yield and purity of DNA. Protein levels in the DNA extracts were determined using the Protein 200 Plus LabChip [3]. The results of this comparison are summarized in Table 1.

Table 1. Summary of DNA Extraction

Method	Yield (%)	DNA/Protein w/w	A_{260}/A_{280}
CTAB	0.008	2.7	1.82
DNeasy	0.004	2.5	1.89
Wizard Genomic	0.008	0.3	1.62

The CTAB and Wizard Genomic protocols showed the highest levels of DNA recovery with final DNA yields of 0.008% of the initial sample weight. The DNA/protein ratio of 0.3 found in the Wizard DNA extract indicated that this extract still contained a high level of protein. The corresponding ratio for CTAB was 2.7, indicating that the protein content of the CTAB extract was only 1/9 of the Wizard protocol.

The A_{260}/A_{280} ratio is also a useful indicator of DNA purity, with high purity DNA having an absorbance ratio of 1.8–1.9. A comparison of the A_{260}/A_{280} ratios for the CTAB and Wizard extracts, 1.82 and 1.62 respectively, confirmed the higher DNA purity in the CTAB process. The DNeasy extraction process produced DNA of comparable quality to the CTAB process, but at a significantly lower yield. Since the CTAB extraction protocol gave the highest yield of high purity DNA, this protocol was used to prepare the DNA extracts used in the PCR analysis.

Analysis of PCR Products

The Biosmart Allin 1.0 GMO Screening System is a multiplex PCR kit that is capable of generating four PCR products. These PCR products include the following:

Base pairs (bp)	Product
118	Soy lectin gene
150	35S Promoter
217	Internal control (corn zein sequence added to the PCR mix)
278	Corn zein gene

All four of these products can be found in either the positive or the negative control reactions using the Biosmart GMO Screening System. The DNA 500 LabChip has ample separation to resolve all of these PCR products. This resolution is illustrated in Figure 1, which shows a composite of the bioanalyzer electropherograms for the positive and negative controls.

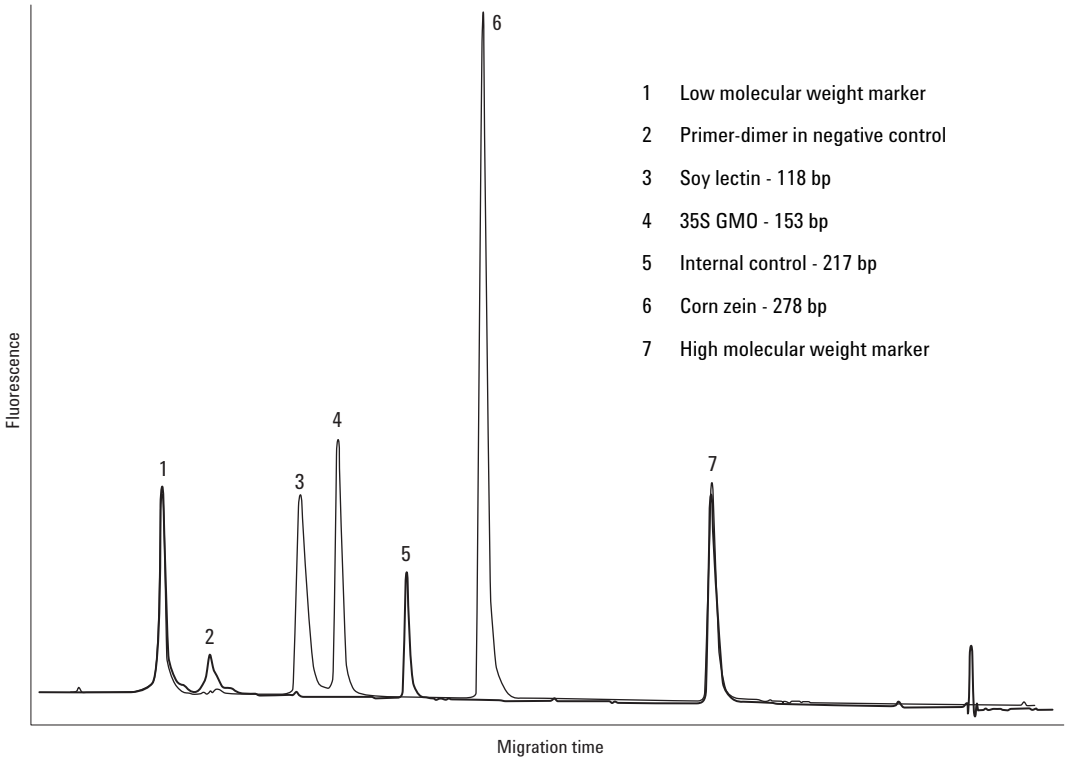

Figure 1. Composite of bioanalyzer electropherograms for positive and negative controls.

The PCR analysis was carried out on corn and soya sample sets. The corn sample set consisted of IRMM Certified Reference MON810™ corn standards at the following levels: 0% MON810, 0.1% MON810, 0.5% MON810, 1.0% MON810, 2.0% MON810, and 5.0% MON810, and commercial corn meal. The soya sample set was made of IRMM Certified Reference Roundup Ready® soya samples at the following levels: 0% Roundup Ready, 0.1% Roundup Ready, 0.5% Roundup Ready, 1.0% Roundup Ready, 2.0% Roundup Ready, and 5.0% Roundup Ready and commercial soya powder. Both sample sets contained a positive control with 0.5% each Bt176 corn and Roundup Ready soya, and deionized water as a negative control.

Figure 2 shows the electrophoretic gel separation and a gel-like bioanalyzer image for all of the samples listed above. The PCR products for the corn samples are shown in Figures 2A and 2C. Figures 2B and 2D show the soya results.

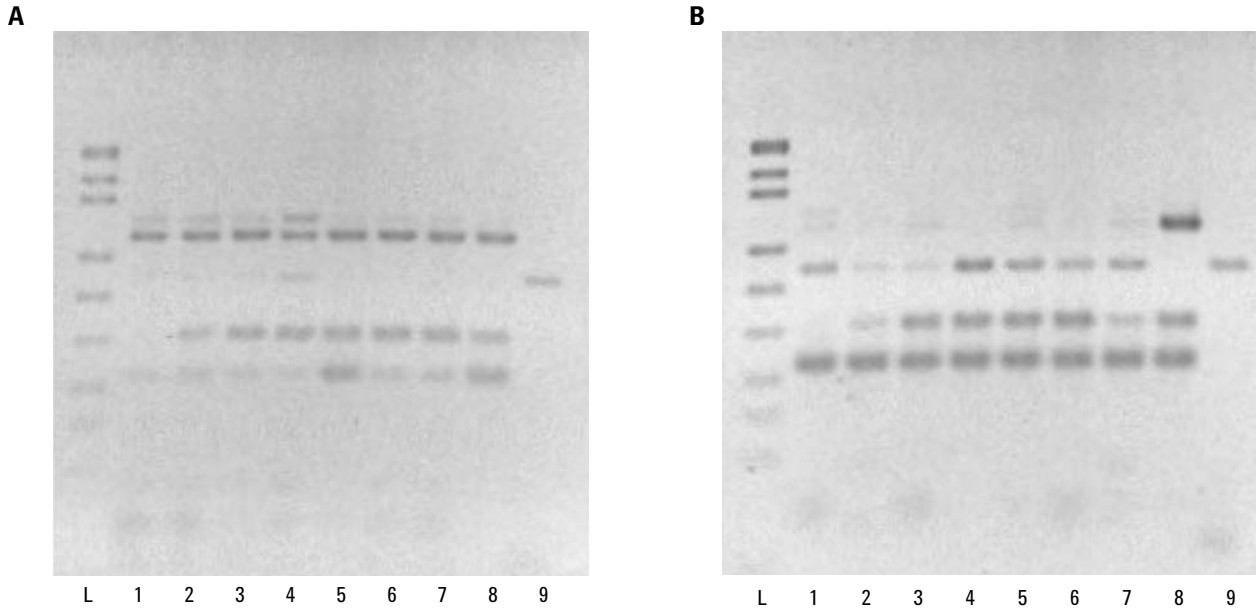

Figure 2A and 2B. 4% NuSieve 3:1 Plus Gel, 150 V for 60 min.

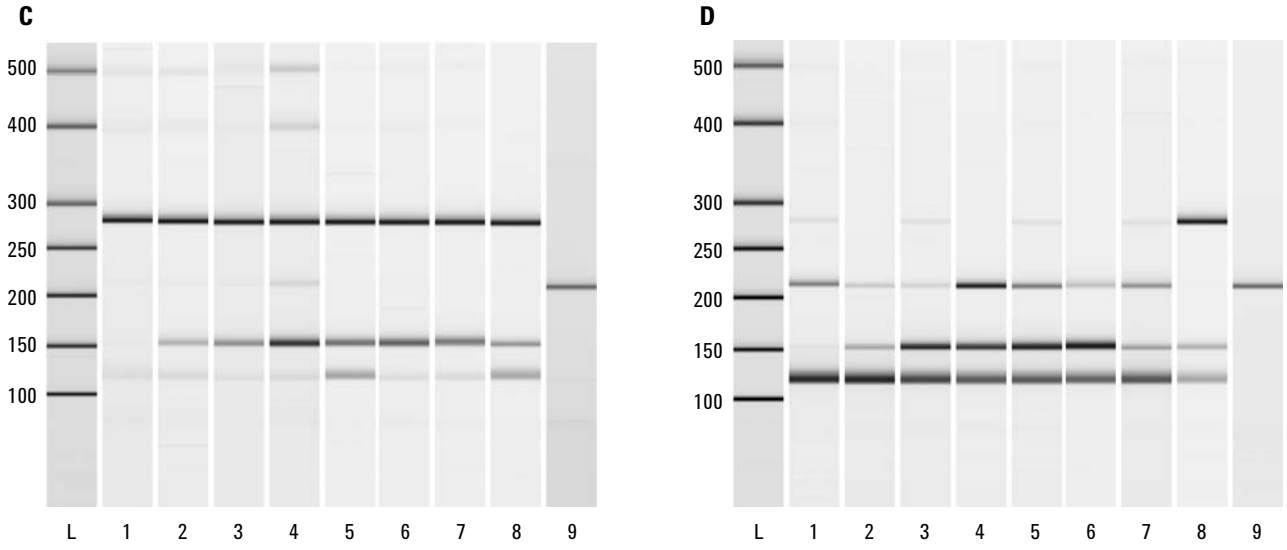

Figure 2C and 2D. Gel-like bioanalyzer image.

Figure 2. 2A (gel) and 2C (bioanalyzer)
L) Molecular weight ladder: 501, 489, 404, 353, 242, 190, 147, 110, 89, 67, 34, 34, and 26 bps **1**) 0% MON810 corn, **2**) 0.1% MON810 corn, **3**) 0.5% MON810 corn, **4**) 1.0% MON810 corn, **5**) 2.0% MON810 corn, **6**) 5.0% MON810 corn, **7**) Commercial corn meal, **8**) Allin positive control (0.5% Bt 176 maize and 0.5% Roundup Ready soybean), and **9**) Negative control (deionized water).

2B (gel) and 2D (bioanalyzer)
L) Molecular weight ladder, **1**) 0% Roundup Ready soya, **2**) 0.1% Roundup Ready soya, **3**) 0.5% Roundup Ready soya, **4**) 1.0% Roundup Ready soya, **5**) 2.0% Roundup Ready soya, **6**) 5.0% Roundup Ready soya, **7**) Commercial soya powder, **8**) Allin positive control (0.5% Bt 176 maize and 0.5% Roundup Ready soybean), and **9**) Negative control (deionized water).

Comparison of Gel and Bioanalyzer Response

The bioanalyzer DNA 500 LabChip clearly shows superior resolution and uniformity of band location compared to the 4% NuSieve gels. The enhanced reproducibility of band location in the gel-like bioanalyzer image is readily apparent in a visual comparison of Figures 2C and 2D to Figures 2A and 2B. Both the 4% gel and the bioanalyzer have sufficient sensitivity to visualize the 35S PCR product at the minimum corn and soy reference standard levels of 0.1% GMO.

A comparison of the initial 35S level in the sample to the amount of 35S PCR product shows that the 35S GMO PCR band increases as the GMO content increases. The PCR response in both corn and soy samples appears to saturate before the maximum GMO standard level of 5% is reached. This effect can be clearly demonstrated using the bioanalyzer's ability to measure PCR product concentrations. Tables 2 and 3 show the concentrations of the corn, soy, and 35S PCR products. For the corn samples, the PCR product response saturates at 1.0% GMO. In the soy samples, the saturation occurs at 0.5% GMO.

Table 2. GMO Corn Response - Corn and 35S PCR Products

Sample	278 bp-Corn amplicon (ng/µL)	153 bp-35S amplicon (ng/µL)	Ratio of 35S/ corn amplicons
0% MON810 Corn	2.5	0.03	0.01
0.1% MON810 Corn	3.4	1.2	0.4
0.5% MON810 Corn	6.2	3.8	0.6
1.0% MON810 Corn	3.5	4.3	1.2
2.0% MON810 Corn	5.2	4.4	0.9
5.0% MON810 Corn	4.7	4.7	1.0
Corn meal	6	5.2	0.9

Table 3. GMO Soya Response - Soya and 35S PCR Products

Sample	118 bp-Soya amplicon (ng/µL)	153 bp-35S amplicon (ng/µL)	Ratio of 35S/ soya amplicons
0% Roundup Ready soy	5.1	0.1	0.02
0.1% Roundup Ready soy	5.4	0.8	0.15
0.5% Roundup Ready soy	4.9	3.4	0.7
1.0% Roundup Ready soy	5.3	4.0	0.8
2.0% Roundup Ready soy	5.1	4.4	0.9
5.0% Roundup Ready soy	4.8	5.0	1.0
Soya powder	4	0.9	0.2
Positive control	4.8	2.2	0.5

Although concentrations of PCR products can be determined quite accurately, some care must be exercised in the interpretation of these quantitative results. Under conditions where a correlation can be seen between the GMO content and the concentration of GMO amplicon, for example, GMO <0.5%, a rough estimate of GMO concentration can be made. However, such an estimate is only reliable if the same PCR master mix and thermocycler are used for all the amplification reactions. In addition, sample and calibration standard matrices must also be highly similar.

For the Biosmart GMO Screening System, quantitative conclusions cannot be made at concentrations >0.5% GMO because the PCR response is saturated. Once the PCR product concentrations have reached this level, small differences in amplicon concentrations are not useful in making quantitative estimates. An example of this can be seen in Table 3. The 0.5% GMO soy reference has a 35S/soy amplicon ratio of 0.7. In the positive control, where the sample has 0.5% GMO soy and 0.5% GMO corn, the corresponding ratio is only 0.5. Since the positive control contains at least twice as much 35S GMO marker as the 0.5% soy reference, this ratio would be expected to be greater than 0.7. It is difficult to explain why the ratio is lower in the positive control. This behavior could be the result of the complex reaction kinetics in a multiplex nested PCR assay or may simply reflect the imprecision in endpoint PCR amplicon concentrations.

PCR Product Composition

The internal control in the Biosmart Allin 1.0 GMO Screening System is a 217 bp corn sequence that uses the same primer sequences as the corn PCR product. According to the manufacturer, when high levels of corn DNA are present, competition for primer may result in the loss of the internal control PCR product. An examination of the corn PCR products in Figure 2C shows this effect in that the 217 bp is either absent or visible only at trace levels. However, in the soy PCR assay in Figure 2D, the 217 bp fragment can easily be seen in all the samples except the positive control that contains corn DNA.

In the corn sample set, a weak band can be seen at around 120 bp. This suggests that during the DNA isolation step, trace amounts of soya DNA were introduced into the corn samples. Likewise, the presence of a band at 280 bp in some of the soy samples indicates low levels of corn DNA were present in several of the soy samples. It is not surprising that trace levels of cross contamination should be apparent in a nested PCR assay. Since all of the samples undergo a net 80 cycles of amplification, even a few copies of soy or corn DNA will be sufficient to produce a detectable PCR product.

In PCR assays using a large number of amplification cycles, it is not uncommon for amplicons of similar sequence to cross hybridize. In the case of the Biosmart Allin 1.0 GMO Screening System, both the corn PCR product and the internal control share a region of common DNA sequence. When these two amplicons cross-hybridize, the resulting product has both single-stranded and double-stranded regions. These structures, known as heteroduplexes, have substantially lower mobility than a corresponding double-stranded structure. The relative mobility shift depends on such factors as gel composition, ionic strength, and gel temperature [4]. In Figure 2, PCR products that are larger than the 278 bp corn amplicon are observed. These bands occur at about 320 bps in the 4% gel and at 400 and 500 bps in the bioanalyzer electropherogram. Cross hybridization of the corn and the internal control amplicons is probably responsible for these products.

Conclusion

This application note described the use of the Agilent 2100 bioanalyzer with the Protein 200 Plus and DNA 500 LabChip Kits in the evaluation of sample preparation and the analysis of multiplex PCR products. The Protein 200 Plus was used to determine which DNA extraction procedure was most effective in removing residual protein. The DNA 500 LabChip was used to characterize the Biosmart Allin 1.0 GMO Screening System, a nested multiplex PCR assay for the genetically modified corn and soy beans. Resolution and sensitivity in these assays was sufficient to identify all of the targeted multiplex PCR amplicons and to differentiate these targets from PCR artifacts. Sensitivity of the assay was sufficient to detect GMO content even at the minimum GMO standard level of 0.1% in both corn and soy reference standards.

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Agilent Technologies

Characterization of Transgenic Soybean Seedlines by Protein Expression with the Agilent 2100 Bioanalyzer

Application

Food

Authors

Debra Dempsey
E.I. DuPont de Nemours
DuPont Experimental Station
Wilmington, DE 19880
USA

Mark Jensen
Agilent Technologies
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

This application note describes how the Agilent Technologies 2100 Bioanalyzer can be used to analyze protein extracts from transgenic seedlines. Accuracy and precision in the determination of protein size and concentration was sufficiently good to allow for the characterization of experimental seedlines based solely on expressed protein profiles.

Introduction

ß-conglycinin(7S) and glycininin(11S) are the primary seed storage proteins in soybean, comprising about 70% of the total storage proteins. Because these proteins make up such a large portion of soya protein, they are of critical economic importance. Characterizing the expression of these proteins in various soybean seed lines is also essential in expanding the range of soy protein applications in food. The relative levels of these two proteins have been shown to significantly impact the nutrition, taste, and texture of food products derived from soy protein extracts [1]. For this reason, soybean lines that preferentially express the 11S or 7S proteins continue to be an active target in the efforts to improve seed quality.

Both conglycinin and glycinin are complex aggregates made of smaller protein subunits. β-conglycinin is a 7S protein with a trimeric structure and is composed of 53, 70, and 76 kDa units. Glycinin is an 11S hexameric protein consisting of six monomer units, where each monomer is made up of 40 and 20 kDa subunits [2].

Given the sizes of protein subunits, it is relatively straightforward to characterize the levels of these two proteins by electrophoresis. The Agilent 2100 Bioanalyzer, an automated microfluidic electrophoresis platform, is well suited for the analysis of proteins in this size range. The Protein 200 LabChip has a size range of 14-200 kDa. Samples of soy protein isolate can be loaded, separated, and analyzed for relative protein composition in less than 45 minutes. In this application we describe the use of the Agilent 2100 Bioanalyzer in the analysis of soybeans, to determine the level of expression of 7S and 11S proteins.

Experimental

Extraction Protocol

Grind the seed into a fine powder. Place a 30 mg sample in an Eppendorf® tube and add 1000 µL of extraction buffer (50 mM Tris [pH 7.5], 10 mM 2-mercapto-ethanol, 0.1% SDS). Agitate the mixture on a rotary shaker for 30 minutes and then centrifuge at 15,000 g for 10 minutes. Remove the supernatant and introduce the extract directly into the Protein 200 LabChip to begin the assay protocol.

If the extracts contain an excessive amount of oil, the supernatant may be removed and further diluted prior to beginning the Protein 200 protocol.

Methodology

To determine if a transgenic line of soybeans preferentially expressed the β-conglycinin or glycinin protein groups, seed extracts were compared to extracts made from wild-type seed lines that strongly expressed either the 7S or the 11S group. Twenty extracts from the unknown seedline were prepared as described above. The protein profiles were determined by separating the proteins in the Agilent 2100 Bioanalyzer. Because of the size range of the proteins, the Protein 200 Plus chip (14-200 kDa) was used for the separation. The concentration of the individual proteins was determined by a comparison with an internal concentration marker (myosin). The ratio of 7S/11S proteins was then calculated from those values and the ratio was compared to the ratio of wild-type seedlines that preferentially expressed either 7S or 11S protein groups. Figure 1 shows the electropherogram for a control seedline, a 7S wild-type, an 11S wild type, and a representative sample from the unknown transgenic seedline. A simulated gel image of the same traces is shown in Figure 2.

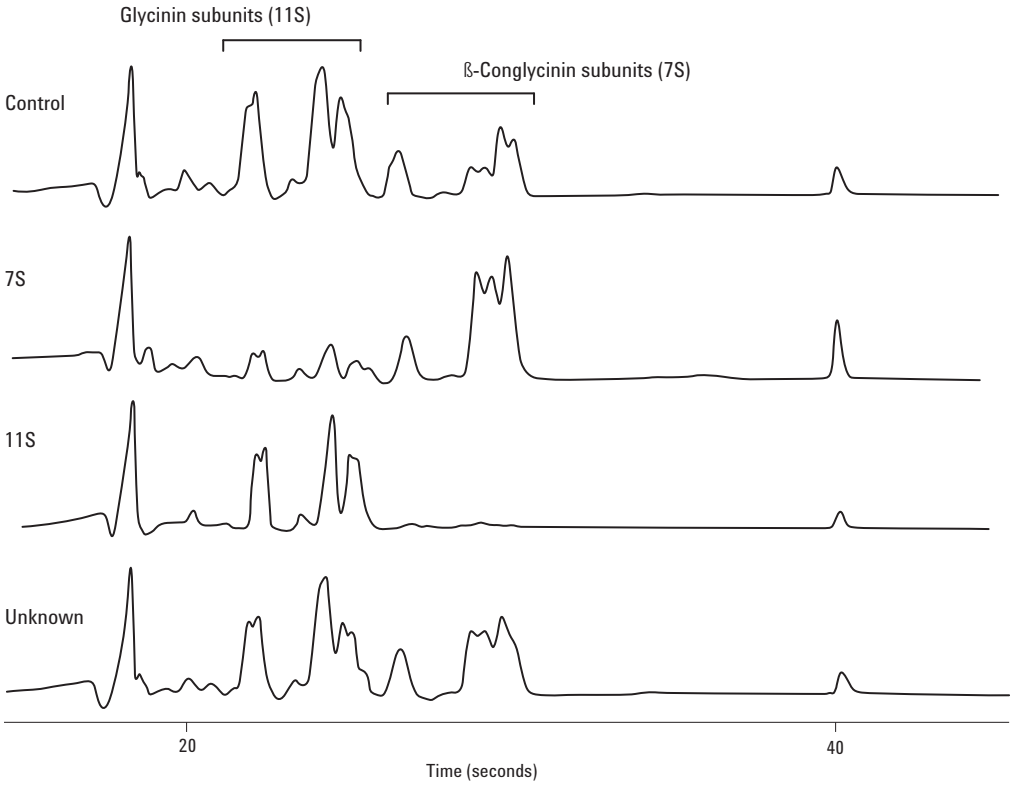

Figure 1. Electropherograms of soya protein extracts.

The ratio of 7S to 11S for the 20 sample extracts was calculated by integrating the individual components comprising the 7S and 11S groups and then determining the summed areas of the two groups. The levels of extracted protein and the 7S/11S ratios for the control 7S, 11S, and unknown groups are summarized in Table 1.

The ratios determined for the high 11S and high 7S seedlines indicate the range of expected 7S/11S ratios should fall between 0.04-3.4. The ratios determined for both the controls and unknown extracts both fall within this range. All 20 unknown samples showed a higher 7S/11S ratio than the control. Average ratio values for 20 unknown extracts and 5 control extracts was 0.72 and 0.39, respectively. Measurement precision was excellent for both sample sets.

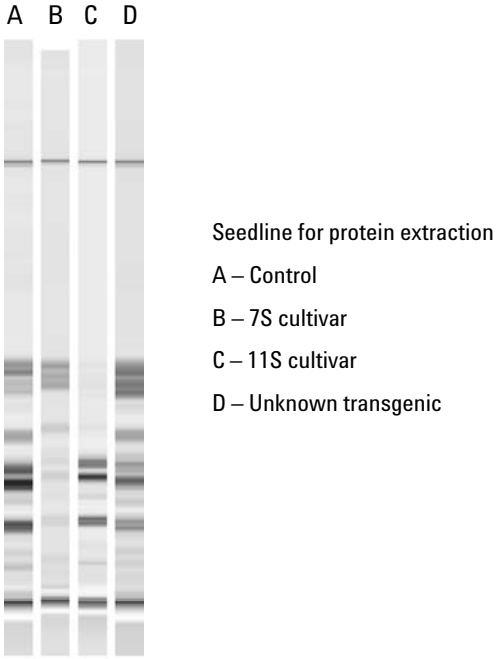

Figure 2. Gel simulation of electropherograms for soya protein extracts.

Table 1. Summary of Extracted Protein Levels and 7S/11S Protein Ratios

SeedLine	Extracted protein level µg/mL	7S/11S Ratio
Control	14,000	0.39 ±0.004 (n=5)
7S	5,200	3.4
11S	14,000	0.04
Unknowns	13,000	0.72 ±0.1 (n=20)

Conclusions

This application note describes the use of the Agilent 2100 Bioanalyzer and the Protein 200 LabChip Kit for evaluating the relative expression of β-conglycinin and glycininin in unknown seedlines. In the 20 protein extracts taken from the unknown seedline, the average ratio was 0.72 ±0.1. This ratio lays in range that is characteristic of high 7S expression seedlines. Given the precision of the ratio determination, it is clearly apparent that the assignment of this unknown seedline to the high 7S group is statistically significant. This conclusion is further supported by a comparison to a normal control seedline where the ratio of 7S/11S is 0.39 ±0.004.

The Agilent 2100 Bioanalyzer together with the Protein 200 LabChip Kit are quick and efficient tools for the determination of relative protein expression. The resulting protein expression profiles are in turn a highly effective means for the characterization of new transgenic seedlines.

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Analysis of genetically modified soya using the Agilent 2100 bioanalyzer

Application

Steve Garrett
Özge Arun
John Dooley

Abstract

In this Application Note we describe how the Agilent 2100 bioanalyzer was used to analyze multiple PCR products from Roundup Ready soya DNA. The multiplex assay assessed the effects of heat and low pH on subsequent amplification of genetically modified DNA and estimated levels of Roundup Ready soya within a sample.

Agilent Technologies

Introduction

Genetically modified organisms (GMOs) and derived food ingredients are regulated throughout the European Union (EU). Legislation requires appropriate labeling of products containing GM DNA. Whilst DNA methods based on the polymerase chain reaction (PCR) are suitable for monitoring known GMOs in raw materials and processed foods, there is concern that the analytical methods are less reliable for quantification purposes. Particular concern is for processed foods, where soya and maize ingredients, which are most likely to contain GMOs, are only a minor component of the finished product.

Food processing has a significant effect on the quality of DNA. Physical and chemical factors such as shear forces, heat treatment, nuclease activity and low pH will lead to degradation of the DNA. Soya is a common component of a wide range of foods, used as flour, protein isolate or concentrate. Soybeans are usually defatted by pressing and/or solvent extraction. In both processes, the soybeans are heated to 60–80 °C and the resulting protein meal can then be concentrated by extraction using weak acid (pH 4.5). These processes combined with further processing during product manufacture significantly reduce the quality of the soya DNA in the final product. This fragmentation of DNA reduces the probability of PCR detection particularly if the fragment sizes are smaller than the DNA sequence that is amplified by the primers.

Studies indicate that small sequences of DNA remain detectable following all but the most extreme processing conditions. In routine screening analysis for GMOs the use of small targets (<200 bp) is common. However, if there is differential degradation in these small targets (some sequences will be more susceptible to degradation than others) quantifying GMOs in processed foods using amplification of two similar but slightly differently sized targets may affect the accuracy of the results.

The aim of this project was to study the effect of food processing on PCR-based amplification and quantification of GM DNA. The approach was to develop a simple model assay system to observe differences in detection when using small targets in Roundup Ready (RR) soya heat treated at low pH. The Agilent 2100 bioanalyzer was used in post-PCR analysis to measure the concentration and number of differently sized PCR products.

Results

Assay development

The aim was to develop a model assay that could be used to assess the quality of DNA extracted from heat-processed soya flour samples, in particular, to investigate differences in PCR amplification between small DNA targets. A single multiplex PCR assay was developed that enabled four GM soya targets to be analyzed in a single reaction mix. Primer concentration was optimized in order to obtain four PCR products resolved by gel electrophoresis which corresponded in size to the soya lectin gene target of 80 bp, and the EPSPS (5-enolpyruvyl-shikamate-3-phosphate synthase) gene targets of 117 bp, 150 bp and 202 bp respectively. These latter targets are only found in Roundup Ready GM soya (Monsanto).

Although gel-based analysis enables sizing of PCR products, it cannot be used to provide accurate information on the quantity of a PCR product. Therefore, post-PCR analysis was performed using the Agilent 2100 bioanalyzer, which can accurately size and quantify PCR products. Initially, this was carried out using the DNA 7500 LabChip® kit. Four peaks were observed, corresponding to the PCR products within the lectin and EPSPS genes. However, the 80 bp peak from the lectin gene was not completely resolved from the alignment marker so quantification was not possible. Subsequently, post-PCR analysis was performed using the DNA 500 LabChip® when it was made available and resolution of all four peaks was observed (figure 1).

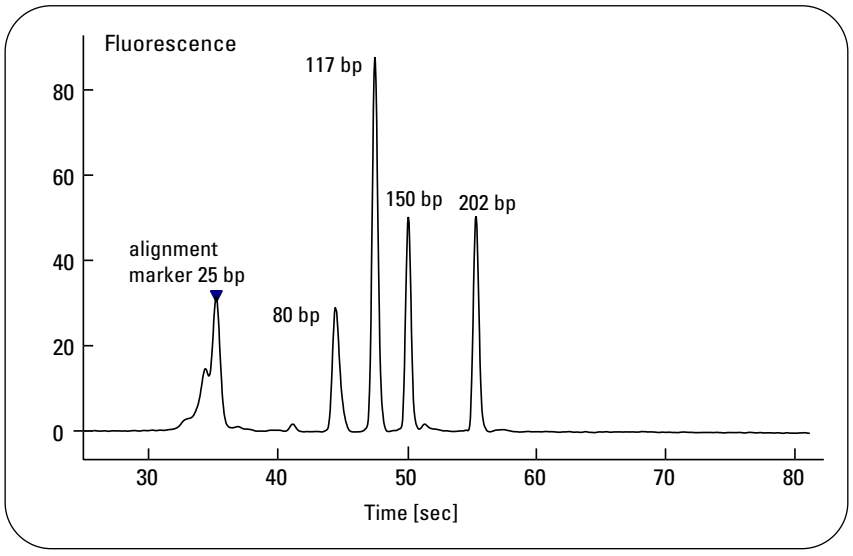

Figure 1
Multiplex assay for GM soya. Peaks produced by the four PCR products when analyzed with the Agilent 2100 bioanalyzer and DNA 500 LabChip kit.

The multiplex PCR assay was applied to DNA from RR soya flour reference materials (figure 2). The results show that there is an increase in PCR product concentration of the 117 bp, 150 bp and 202 bp products and little change in the concentration in the 80 bp product. This increase corresponds to the increase in RR content of the soya flour. No increase in the product from the lectin gene was expected, as it is common to both the GM- and non-GM soya. It should therefore be possible to estimate levels of GM soya in an unknown material by applying the multiplex assay and comparing the ratio of lectin product to the other products in the sample with ratios produced from certified reference materials (CRMs). The assays would have to

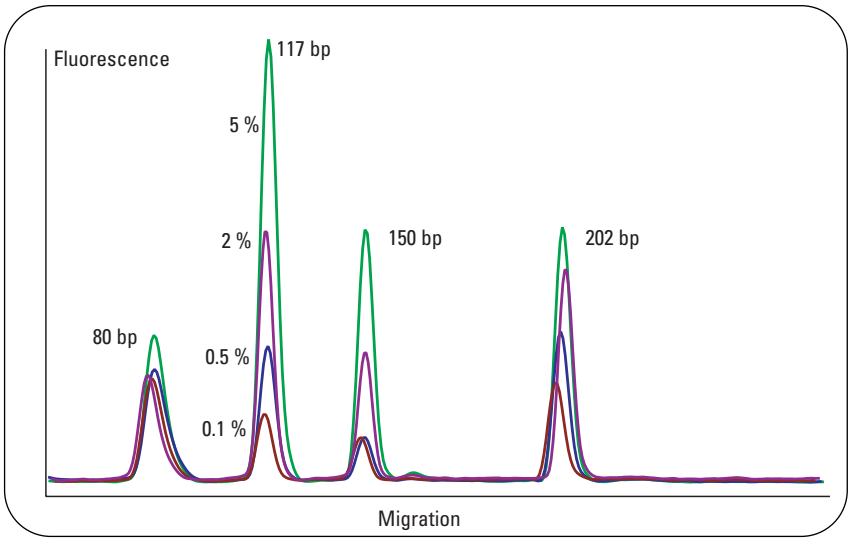

Figure 2
Peaks produced by the Agilent 2100 bioanalyzer using the multiplex assay on CRMs containing different levels of GM soya

Sample	Ratio 117/80	Ratio 150/80	Ratio 202/80
Extract 1a	1.50	0.68	1.04
Extract 1b	1.45	0.68	1.04
Extract 2a	1.40	0.46	0.71
Extract 2b	1.54	0.65	1.10
5% CRM	2.10	1.00	0.94
2% CRM	0.74	0.21	0.68
1% CRM	0.41	0.18	0.43

Table 1
Analysis of a soya flour

be performed using a limited number of PCR cycles in order to perform the end-point detection during linear stages of amplification. The reference materials and the unknown samples would also have to be similar in nature.

Analysis of a soya flour containing GM soya

The multiplex assay was applied in duplicate to two DNA extracts prepared from a soya flour sample which had given a positive result when screened for a common GM promoter sequence (CaMV 35S promoter). The assay was also applied to 1 %, 2 %, and a 5 % CRMs. Ratios of each EPSPS product compared to the lectin product were calculated (table 1). The same extracts were analyzed using a real-time PCR method for quantitative determination of GM soya. Results from the real-time analysis indicated that the sample contained approximately 5 % GM soya while the multiplex assay gave ratios indicating that the sample contained between 2 % and 5 % GM soya.

The effect of heating time and pH on detection and quantification of GM-DNA

The multiplex PCR assay was applied to soya flour samples containing approximately 1.3 % GM soya and boiled at either pH 3.3, 4.3 or 6.7 for up to 21 minutes. For accurate determination of the quantity of each PCR product, the samples were applied to the DNA 500 LabChip. The concentration of each PCR product was calculated using the Agilent 2100 bioanalyzer software. At pH 3.3 where an effect of heating time was observed, the amount of each PCR product at each time point was compared to the amount of each product at 0 minutes (table 2). At pH 3.3, the relative amount of the 80 bp product was reduced to 48 % after 15 minutes and no product was detected at 18 or 21 minutes. After 15 minutes, the relative amounts products of 118 bp and 150 bp were reduced to 27 % and 16 % respectively and the 202 bp product was not detected. None of the products were detected after 18 or 21 minutes.

Time at 100 °C and pH 3.3 (min)	Amount of PCR product*			
	80 bp	118 bp	150 bp	202 bp
0	100	100	100	100
3	74	77	73	67
6	57	58	21	6
9	36	23	24	15
12	67	33	47	21
15	48	27	16	0
18	0	0	0	0
21	0	0	0	0

* % product determined relative to the amount at 0 minutes

Table 2
The effect of heating time on RR flour held at pH 3.3, determined using the multiplex PCR method.

To eliminate any variation due to amount of DNA in each PCR reaction, the ratio of the lectin 80 bp product to each of the other three products was determined for all experiments (table 3), that is, normalized with respect to the 80 bp product. The ratios of each would be expected to remain constant if no degradation of the target DNA occurred or if the degree of degradation between the 80 bp target and the other targets was comparable. At pH 3.3 the ratios tended to increase with increasing heating time. This suggests that at low pH there were differences in the detectability of the three EPSPS targets compared to the smaller lectin target, with the

Time at 100 °C (min)		Ratio lectin 80bp/ RR-117bp	Ratio lectin 80bp/ RR-150bp	Ratio lectin 80bp/ RR-202bp
pH 3.3	0	1.8	3	1.9
	3	1.8	3	2.1
	6	1.7	7.8	17.5
	9	3	5	5
	12	3.6	4.4	6
	15	3.8	9	NP
	18	NP	NP	NP
pH 4.3	0	2	4.4	1.9
	3	2.2	2.9	1.8
	6	1.3	2	1.9
	9	1.3	2.2	2.3
	12	1.5	2.6	2.6
	15	1.8	3.7	2.7
	18	1.9	3.9	3
pH 6.7	0	1.8	4.2	1.7
	3	1.7	3.9	1.6
	6	1.2	2.3	1.5
	9	1.5	2.4	1.7
	12	1	1.9	1.3
	15	1.2	2.1	1.3
	18	1.4	2	1.4
	21	1.6	2.4	1.4

NP= no PCR products observed

Table 3
The effect of heating time on RR flour held at pH 3.3, 4.3 and 6.7, determined using the multiplex PCR method

80 bp target being degraded at a slower rate compared with the other targets. At pH 4.3, the 80/118 bp and 80/145 bp ratios decreased during the first 3–9 minutes of heating, then increased returning to the their original value, whereas the 80/202 bp ratio increased with heating time. Similar trends were observed at pH 6.7 except for the 80/202 bp ratio where little change occurred. However, further analyses are required to replicate these observations and focus around the pH where an effect is observed. These initial results indicate that the different targets used in PCR are not detected equally in these experiments.

Other studies show similar results. In 1998 Hüpfer et al.[1] demonstrated that PCR detection of GM maize in polenta could be influenced by pH during thermal treatment of the product. They showed that detection of a 1,914 bp segment of the cry1A(b) gene was not possible after boiling at neutral pH for 30 minutes, whereas a 211 bp fragment was detected after boiling for 105 minutes. At pH 2-3, the larger segment was not detected after boiling for 5 minutes and the smaller fragment was not detected after 15 minutes. As a result of such observations, it is common practice to use small target sequences in screening methods for GMOs. However, the work reported here suggests that at low pH, degradation of DNA results in differences in detection of very small target sequences. This may not be important for qualitative analysis, however, it is likely to have significance for the accuracy of quantitative analysis of processed foods with low levels of GM-DNA, when two target sequences are analyzed simultaneously as in real-time PCR.

Conclusion

The RR multiplex assay was used to quantify the amount of GM soya in a soya flour and assess the effects of pH and heat on the detection of GM soya DNA. A key component of the assay is the Agilent 2100 bioanalyzer which is used to accurately quantify the four PCR products simultaneously. This user-friendly instrument replaces gel based analysis and offers enormous potential for the routine screening of raw materials for levels of genetically modified organisms.

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Steve Garett and John Dooley are scientists in the Molecular Biology Group, Dept. of Chemistry and Biochemistry, CCFRA Technology Ltd., Chipping Campden, Glouchester, UK.

Özge Arun is a scientist at the Tubitak Marmara Research Center, Food Science and Technology, Research Institute, Kocaeli, Turkey.

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Agilent Technologies

Detecting genetically modified organisms with the Agilent 2100 bioanalyzer

Application

Anne-Céline Minvielle, Ph.D.
Hafida Oujati
Séverine Dameron

Genolife

Abstract

Labeling of food containing more than 1% of genetically modified organisms (GMOs) has been obligatory in Europe since January 2000. To guarantee transparency and labeling, methods to distinguish between transgenic food and their traditional counterparts must be available. Genolife developed a method to detect Ready RoundUp soy (RRS) and a multiplex PCR to detect five corn transgenes (Bt176, Bt11, MON810, T25 and GA21). The Agilent 2100 bioanalyzer and DNA 500 LabChip® kit provided a simple, high throughput and standardized way to analyze multiplex PCR products. The methods developed by Genolife allow detecting 0.01% of RRS in food ingredients on the one hand and 10 copies of transgene MON810, GA21 and Bt11, and 100 copies of transgene Bt176 and T25 on the other.

Agilent Technologies

caliper LabChip®

Introduction

The rapid development of biotechnology has launched products and ingredients derived from genetically modified organisms (GMOs) into the food market. The general public, however, has shown anxiety about this new technology. Information and transparency regarding these products are essential in order to become accepted by the consumers. In Europe, labeling of GMOs is regulated by the Novel Food directives 258/97/EEC[1] and 1139/98/EEC[2]. More recently, the "threshold regulation" 49/2000/EEC[3] has been approved, specifying that foodstuffs are subject to labeling. When the proportion of an individual food component is higher than 1 % manufacturers must label their products. Moreover, the presence of GMOs must be adventitious and therefore, food manufacturers must be able to supply evidence that they have taken appropriate steps to avoid using GMOs. A key factor to guarantee transparency and labeling is the availability of methods to distinguish between transgenic food and their traditional counterparts, not only in raw materials but also in food products.

Several analytical methods using polymerase chain reaction (PCR) technology have been developed to qualitatively detect the presence of a modified sequence of nucleic acid in transgenic food.[4,5] But these analytical methods detect only one modified sequence of one genetically modified organism. It would be advantageous to detect more than one sequence per genetically modified organism (one endogenous gene and several transgenic markers) or to screen several GMOs in one analysis. We therefore developed a method to detect RoundUp Ready soy (RRS) from Monsanto in one part, and another method to detect one endogenous maize gene and five genetically modified maize genes – four are authorized in Europe (Bt176, Bt11, MON810 and T25) and one is non-authorized (GA21). The Agilent 2100 bioanalyzer and DNA 500 LabChip® kit provided a simple, rapid and standardized alternative to analyze multiplex PCR products.

Results and discussion

RRS detection in food ingredients

DNA was extracted from commercial transgenic soybean reference standards (Fluka) and different food samples (lecithin, soybean proteins, soybean flour) with specific protocol developed by Genolife. For each sample, four PCR reactions were done — one for amplification of an endogenous gene (ACC1, 115 bp) to check the quality of the extracted DNA, and three PCR reactions for specific RR soybean sequences (T1: 167 bp, T2: 141 bp and T3: 189 bp). After amplification, PCR products were mixed and 1 µl of each mixed PCR was analyzed on the Agilent 2100 bioanalyzer using the DNA 500 LabChip kit, which allows analysis of DNA fragments ranging in size from 25 to 500 bp. Twelve samples were analyzed simultaneously and the 2100 bioanalyzer produced raw data and analysis in multiple formats. It displayed a simulated gel view an electropherogram. A data table labels each of the peaks and furnishes information about the size and concentration for each fragment. Results are shown in figure 1. Non transgenic soy gives

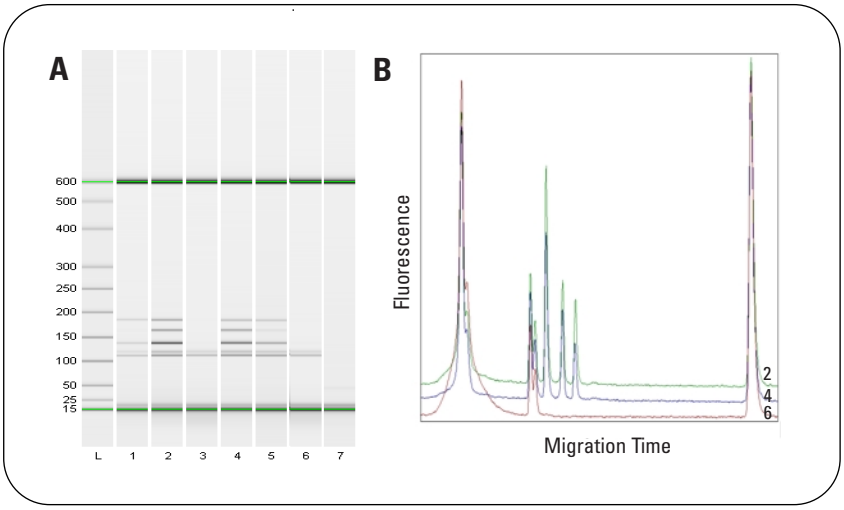

Figure 1
RR soybean detection
A) Gel view: 1-soya protein, 2- lecithin, 3-soybean flour, 4-RR soy 1 %, 5-RR soy 0.1 %, 6-non transgenic soy, 7-PCR blank.
B) Overlay of the electrophoretic traces of lanes 2, 4 and 6.

one band corresponding to the endogenous gene (115 bp) while RR soy 1 % and RR soy 0.1 % show four bands corresponding to the endogenous gene (115 bp) and the three specific RR soybean sequences (141, 167 and 189 bp). The Agilent 2100 bioanalyzer software compares unknown samples with commercial transgenic soybean reference standards. Soybean proteins and lecithin are transgenic (four bands at 115, 141, 167 and 189 bp) and soybean flour is not transgenic (only one band at 115 bp corresponding to endogenous gene). The 2100 bioanalyzer performs quantification using an internal standard (marker) added to each sample before loading, and the software calculates the DNA concentration in each band. Soybean protein contains less than 0.1 % and lecithin more than 1 % RR soy, compared to concentration DNA in transgenic band obtained with commercial transgenic soybean reference standards (table 1). The detection limit of this RR soy PCR is 0.01 %.

	RR1 %	RR 0.1 %	Soya protein	Lecithin
141 bp	1	0.24	0.12	1.3
167 bp	0.1	-	-	0.34
189 bp	1	0.56	0.48	1.3

Table 1
DNA concentration (ng/µl) of the band corresponding to the transgenic markers

GMO maize detection in food ingredients by multiplex PCR

In Europe, four GMOs maize were authorized — two insect-resistant corn species from Novartis (Bt11 and Bt176), one insect-resistant corn from Monsanto (MON810) and one glufosinate-tolerant corn developed by Agrevo (T25). We developed a PCR multiplex to detect these four corn lines and one endogenous gene to check the integrity of the extracted DNA. We also added a couple of primers to detect a glyphosate-tolerant corn GA21 produced by Monsanto. This glyphosate-tolerant corn is authorized in the USA and can be exported in Europe with authorized corn. The PCR multiplex amplified a 152-bp fragment for endogenous gene, a 343-bp fragment for Bt176 corn, a 149-bp fragment for T25, a 199-bp fragment for MON810, a 110-bp fragment for Bt11 and a 270-bp fragment for GA21. Results are presented in figure 2. The endogenous gene was amplified in all lanes except PCR blank. Only Bt176 fragment (343 bp) was obtained when only Bt176 corn was present in PCR tube (lane 4). Specificity was checked for each corn (lane 5: Bt11, lane 6: T25, lane 7: GA21 and lane 8: MON810). Lanes 1 to 3 presented PCR products when all corn lines were analyzed together. This multiplex PCR allows detection of five corn lines present at 0.2 % each (lane 3). The detection limit of this multiplex PCR is 10 copies for transgene MON810, GA21 and Bt11 and 100 copies of transgene Bt176 and T25.

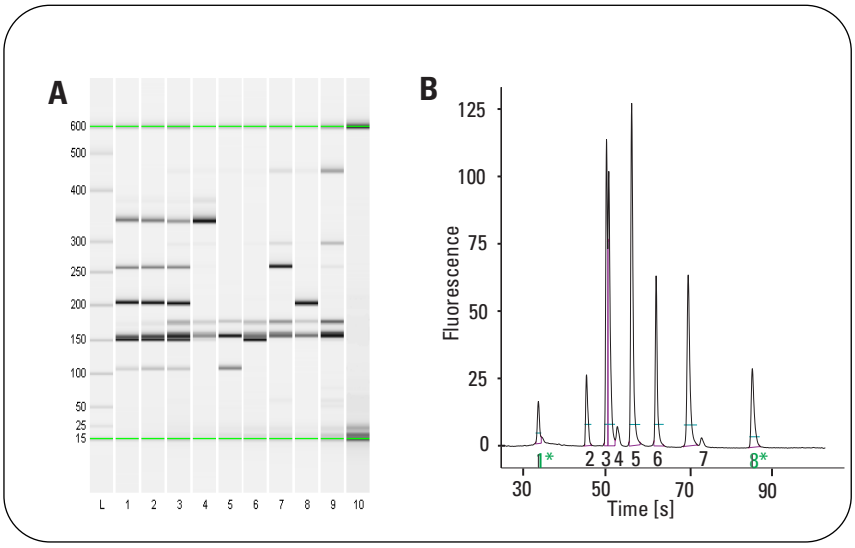

Figure 2
Multiplex PCR to detect GMO corn
A) 1-Bt176 5 %, Bt11 2 %, T25 5 %, GA21 5 %, MON810 5%, 2-Bt176 2 %, Bt11 2 %, T25 2 %, GA21 2 %, MON810 2 %, 3-Bt176 0.2 %, Bt11 0.2%, T25 0.2 %, GA21 0.3 %, MON810 0.3%, 4-Bt176 2 %, 5-Bt11 2 %, 6-T25 2 %, 7-GA21 2 %, 8-MON810 2 %, 9-non GMO corn, 10-PCR blanc
B) Electrophoregram of lane 2 (multiplex PCR with mix of 2 % corn). The peaks are: Bt11 corn (2), T25 corn (3), endogenous gene (4), MON810 corn (5), GA21 corn (6) and BT176 corn (7).

Conclusion

We developed detection methods for GMO soy and corn in food ingredients. The Agilent 2100 bioanalyzer performed the analysis of multiplex PCR product and allowed a semi quantification of GMO content. Two detection methods were presented— one for RR soy and one for GMO corn detection. The sensibility of the soya detection method is 0.01 % and 10 to 100 copies of transgene for corn mulitplex detection.

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Anne-Céline Minvielle, Ph.D, Hafida Oujati and Séverine Dameron are Research Scientists at Genolife, Clermont-Limagne, France.

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Agilent Technologies

Development of meat speciation assays using the Agilent 2100 bioanalyzer

Application

John Dooley
Steve Garrett

Abstract

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

Introduction

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

Materials and Methods

Design of PCR assay

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

Performance of PCR reaction

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

DNA 500 LabChip preparation

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

Results and Discussion

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

Species-specific assay development

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

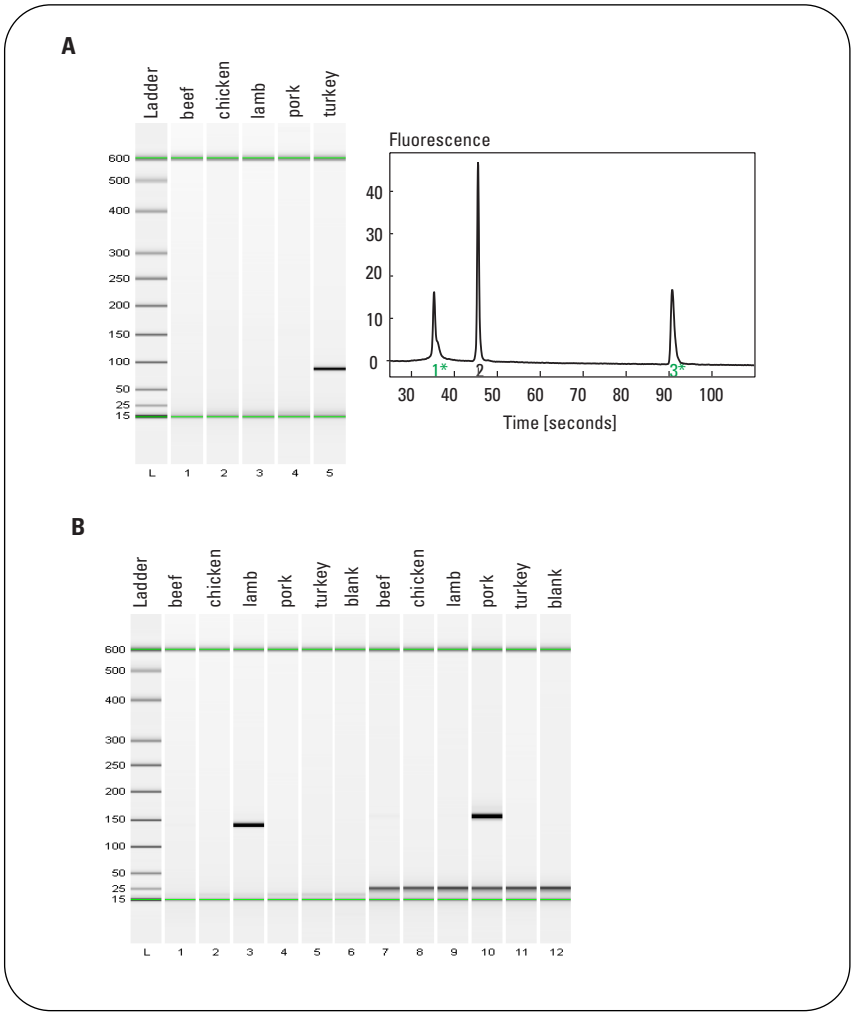

Figure 1
Species specific PCR amplification of meat samples.
A) With turkey specific primers only turkey samples are amplified. The electrophoretic trace confirms the high purity of the fragment.
B) Different sets of primers can be designed that allow the species specific PCR amplification of lamb (lanes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

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

Total meat assay development

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

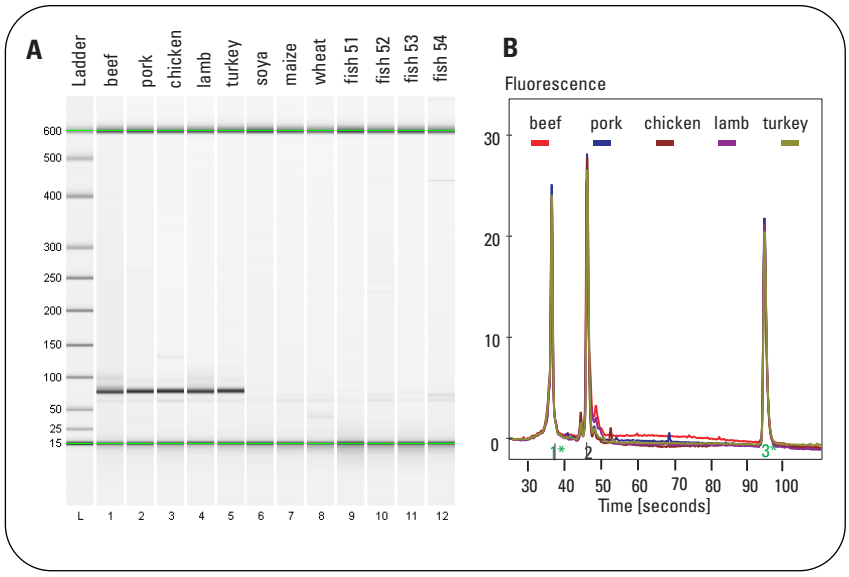

Figure 2
Development of a meat specific assay.
A) A set of primers can be designed that amplifies specifically all meat samples but does not amplify grain or fish.
B) The overlay of the electrophoretic traces reveals uniform amplification levels for different meat species.

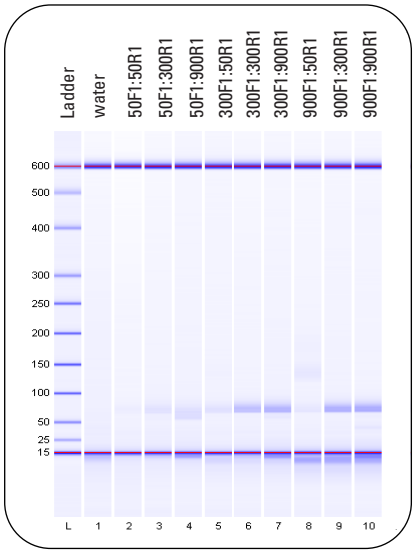

Figure 3
PCR products using TaqMan probes. Results from assy optimization test using pork DNA. Primers (forward [F] or reverse [R]) were used at 50, 300 or 900 nM concentration. Results show that at least 300 nM of F or R primer is required for amplification. 300 nM of each primer was found to be optimal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

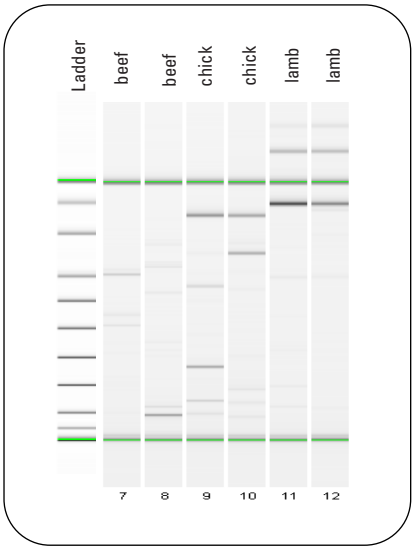

Figure 4
Non-specific amplification using non-optimal sets of primers.

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

Conclusion

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

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

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