JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2011, 59, 513–520 513
DOI:10.1021/jf103702s

DNA-Based Identification of Spices: DNA Isolation, Whole Genome Amplification, and Polymerase Chain Reaction

Felix Focke, Ilka Haase,* and Markus Fischer

Institute of Food Chemistry, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany

Usually spices are identified morphologically using simple methods like magnifying glasses or microscopic instruments. On the other hand, molecular biological methods like the polymerase chain reaction (PCR) enable an accurate and specific detection also in complex matrices. Generally, the origins of spices are plants with diverse genetic backgrounds and relationships. The processing methods used for the production of spices are complex and individual. Consequently, the development of a reliable DNA-based method for spice analysis is a challenging intention. However, once established, this method will be easily adapted to less difficult food matrices. In the current study, several alternative methods for the isolation of DNA from spices have been developed and evaluated in detail with regard to (i) its purity (photometric), (ii) yield (fluorimetric methods), and (iii) its amplifiability (PCR). Whole genome amplification methods were used to preamplify isolates to improve the ratio between amplifiable DNA and inhibiting substances. Specific primer sets were designed, and the PCR conditions were optimized to detect 18 spices selectively. Assays of self-made spice mixtures were performed to proof the applicability of the developed methods.

KEYWORDS: Spice; herb; DNA isolation; PCR; whole genome amplification; multiple displacement amplification

INTRODUCTION

A spice is defined as a product of a single plant organism, whereas spice mixtures are defined as products made of at least two organisms. For spice production, all different parts of a plant like the fruit (e.g., pepper), seed (e.g., mustard), bud or blossom (e.g., caper or clove), rhizome (e.g., ginger), root (e.g., horseradish), bark (e.g., cinnamon), spear (e.g., angelica), or the leaf (e.g., oregano) are used.

Usually spices are identified and valued on the basis of their morphological differences, for example, characteristic cells or tissues. For that purpose, trained staff is mandatory, and the quantity of a certain spice can only be roughly estimated. Especially in fine cut products or in processed food, characteristic attributes are hardly detected, making identification difficult or impossible.

Generally, the polymerase chain reaction (PCR) is a DNA-based method enabling the control of food authenticity or the detection of genetically modified plants, allergens, or pathogens in food (*1*−*4*). Currently used PCR-based methods for the authentication of meat products, seafood products, dairy products, and foods of plant origin are summarized by Mafra et al. (*4*). For the quality control of spices, only a few DNA-based methods have been published yet, for example, for celery and mustard, which are known to contain proteins with allergenic potential (*5*, *6*) and for the detection of adulterations of spices (*7*, *8*). Apart from that, the identification of spices might be important due to the fact that some of them contain harmful substances. One recent example, where an identification of a certain spice came in the field of interest, was the different coumarin content in the two cinnamon species *Cinnamomum verum* and *Cinnamomum cassia*. High concentrations of coumarin could only be detected in *C. cassia* (*9*).

Another question is the detection of toxic plant-derived contaminants in herbs. For example, undomesticated ramsons (*Allium ursinum*) can be contaminated or confused with lily of the valley (*Convallaria majalis*) or meadow saffron (*Colchicum autumnale*) (*10*, *11*). Also possible is a contamination of roquette (*Diplotaxis tenuifolia*) with toxic ragwort species (*Senecio* spp.), which could be detected via PCR.

The most important prerequisite for a reliable PCR method is the isolation of amplifiable DNA from the sample. As described above, spices are made of very heterogenic parts of plants. Furthermore, in most cases, spices are dried and/or fermented. Consequently, the DNA of spices is embedded in different matrices and might be degraded during processing. Consequently, the first step in the analysis of spices is the development of effective DNA isolation methods, which crack the plant cell wall and carefully separate proteins, lipids, and carbohydrates in any of the spices under study. Additionally, phytochemicals should also be removed during the isolation procedure, due to their potential to act as inhibitors for the PCR (*12*−*14*).

Furthermore, spices are very different in terms of their genotypic and phenotypic relationships. Some are members of the same genus, and some are even different on the family level (**Table 1**). The former is challenging regarding the identification of specific primer hybridization regions. As a prerequisite, the respective sequence must be known from every spice under study to avoid false positive reactions. In the rDNA region, which is part of each eukaryotic genome, internal transcribed spacers

*To whom correspondence should be addressed. Tel: +49-40-428384379. Fax: +49-40-428384342. E-mail: ilka.haase@chemie.uni-hamburg.de.

Published on Web 12/23/2010

Table 1. Spices Used for Isolation of DNA

spice	family
chive (*Allium schoenoprasum* L.) garlic (*Allium sativum* L.) leek (*Allium porrum* L.) onion (*Allium cepa* L.) ramson (*A. ursinum* L.)	*Alliaceae*
anise (*Pimpinella anisum* L.) caraway (*C. carvi* L.) celery (*Apium graveolens* L.) chervil [*Anthriscus cerefolium* (L.) Hoffm.] coriander (*Coriandrum sativum* L.) cumin (*Cuminum cyminum* L.) dill (*Anethum graveolens* L.) lovage (*Levisticum officinale* W. Koch) parsley [*Petroselinum crispum* (Mill.) Nym.]	*Apiaceae*
mugwort (*Artemisia vulgaris* L.) tarragon (*A. dracunculus* L.)	*Asteraceae*
black mustard [*Brassica nigra* (L.) Koch] white mustard (*Sinapis alba* L.)	*Brassicaceae*
saffron (*Crocus sativus* L.)	*Iridaceae*
basil (*O. basilicum* L.) marjoram (*Origanum majorana* L.) oregano (*Origanum vulgare* L.) rosemary (*Rosmarinus officinalis* L.) sage (*S. officinalis* L.) thyme (*Thymus vulgaris* L.)	*Lamiaceae*
cassia (*Cinnamomum aromaticum* Nees) cinnamon (*C. verum* J. S. Presl) laurel (*Laurus nobilis* L.)	*Lauraceae*
nutmeg (*Myristica fragrans* Hout)	*Myristicaceae*
allspice [*P. dioica* (L.) Merr.] clove [*S. aromaticum* (L.) Merr. and Perry]	*Myrtaceae*
pepper (*Piper nigrum* L.)	*Piperaceae*
star anise (*I. verum* J. D. Hook f.)	*Schisandraceae*
cayenne (*Capsicum frutescens* L.) paprika (*Capsicum annuum* L.) tomato (*Lycopersicon esculentum* Mill.)	*Solanaceae*
cardamom [*E. cardamomum* (L.) Maton] curcuma (*Curcuma longa* L.) ginger (*Zingiber officinale* Boehm.)	*Zingiberaceae*

(ITS) are located between coding regions (**Figure 1a**). These noncoding ITS regions are highly diverse and are therefore well suited as organism-specific primer binding sites for the differentiation of families, genera, and also species (*15*). Another advantage of the rDNA region is the high copy number in the genome. The rDNA is a repeated sequence that occurs from a hundred up to a thousand times in every genome (*16*), decreasing the limit of detection in the amplification process.

The current paper presents the development of methods that enable DNA isolation from different food matrices, especially different spices. Additionally, a PCR method for the species specific amplification using sequence differences in the rDNA region is described. Furthermore, whole genome amplification (WGA) by multiple displacement amplification (MDA) is introduced to improve the ratio between isolated DNA and inhibiting substances where necessary (*17*).

MATERIALS AND METHODS

Spice Samples. Commercially available samples of 39 spices from 13 families were used in this study. An overview is given in **Table 1**. A contamination with other spices, plants, or microorganisms is possible but unlikely.

DNA Isolation Method 1: Precipitation (Modified CTAB Protocol, § 64 LFGB L 00.00-31). A sample amount of 50−100 mg was mixed with 1 mL of extraction buffer [55 mM cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 0.1 M tris(hydroxymethyl)-aminomethan (Tris/HCl), and 20 mM ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA), pH 8.0] in a 2 mL cap. Two steel balls (1/8″) were added, and the sample was mechanically ground for 5 min at 30 Hz using the TissueLyser system (Qiagen, Hilden, Germany) followed by an incubation at 65 °C for 30 min (water bath). A 500 μL amount of chloroform was added, and the suspension was mixed. After centrifugation (5 min, 10000*g*), 700 μL of the aqueous supernatant was carefully removed.

For DNA precipitation, the solution was mixed with 1 mL of precipitation buffer (14 mM CTAB and 40 mM NaCl) and incubated for 1 h at room temperature. To pelletize the precipitated DNA, a centrifugation step (5 min, 10000*g*) was performed, and the supernatant was carefully removed. The DNA pellet was resolved in 350 μL of NaCl solution (1.2 M) and mixed with 350 μL of chloroform. After centrifugation (2 min, 10000*g*), the aqueous supernatant was mixed with 200 μL of isopropanol. After incubation (30 min, 4 °C) and centrifugation (5 min, 10000*g*), the supernatant was removed, and the DNA pellet was washed with ethanol (70% v/v). Remaining ethanol was removed by incubation of the sample at 300 mbar for 5 min. The DNA pellet was finally resolved in 50 μL of water.

DNA Isolation Method 2: Adsorption on Silica. The first steps for the isolation of DNA by adsorption on silica were performed as described above. However, the DNA was not precipitated in the aqueous solution but was adsorbed on a silica containing EconoSpin column (Epoch Biolabs, Sugar Land, United States). After 2 min of centrifugation (10000*g*), the bound DNA was washed with 500 μL of washing buffer [50 mM NaCl, 20 mM Tris/HCl, and 1 mM Na_2-EDTA, in ethanol (50% v/v), pH 7.4] and subsequently with 500 μL of ethanol (70% v/v). Ethanol should be removed completely by an additional centrifugation step (1 min, 10000*g*).The DNA was eluted with 50 μL of water by centrifugation (2 min, 10000*g*).

Spectroscopic Characterization of DNA Isolates. The DNA purity was determined photometrically. The absorbance of 20 μL of DNA solution was measured at 260 and 280 nm in 384 well plates in a plate reader (SpectraMax M2, Molecular Devices, Ismaning, Germany). The concentration of the DNA solution was calculated fluorometrically (SpectraMax M2) using SYBR Green I and an external calibration curve (dilution series of plasmid standard).

MDA (*18*, *19*). MDA was performed in a total reaction volume of 25 μL in a thermocycler (T3000, Biometra, Göttingen, Germany) or a real-time thermocycler (IQ5, Biorad, München, Germany). An initial denaturation (96 °C for 4 min) and a prehybridization (20 °C for 10 min) were performed in a 10 μL mixture 1 containing 125 μM random heptamers as primers (Invitrogen GmbH, Karlsruhe, Germany) and 1 μL of DNA template (derived by method 1 or 2) in reaction buffer [20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, and 0.1% Triton X-100, pH 8.8]. After it was cooled (−3 °C), a 15 μL aliquot of mixture 2 was added, which contained 0.7 mM of each dNTP (Bioline, Luckenwalde, Germany) and different amounts of *Bst* DNA polymerase (large fragment, New England Biolabs, Ipswich, United States) in reaction buffer. The final activity of the polymerase was varied as follows: 8, 24, or 40 U, respectively. The MDA reaction was continued at 50 °C for 16 h. Finally, the polymerase was heat-inactivated (80 °C for 20 min). For real-time applications, 1 μL of SYBR Green I (Invitrogen, GmbH) 1:1600 solution was added to 15 μL of an aliquot of mixture 2.

PCR. One microliter of DNA template, respectively, MDA products, 0.1 U *Taq* polymerases (in house production), 0.5 μM of each primer (**Tables 2** and **3**), 0.2 μM of each dNTP (Bioline Luckenwalde, Germany), 3 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, and 0.1% Triton X-100 were combined in a total volume of 20 μL. After an initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 15 s, 58 °C for 25 s, and 72 °C for 25 s were performed. For a terminal elongation, the reaction batches were heated for 5 min at 72 °C. Reactions were performed in a thermocycler (T3000).

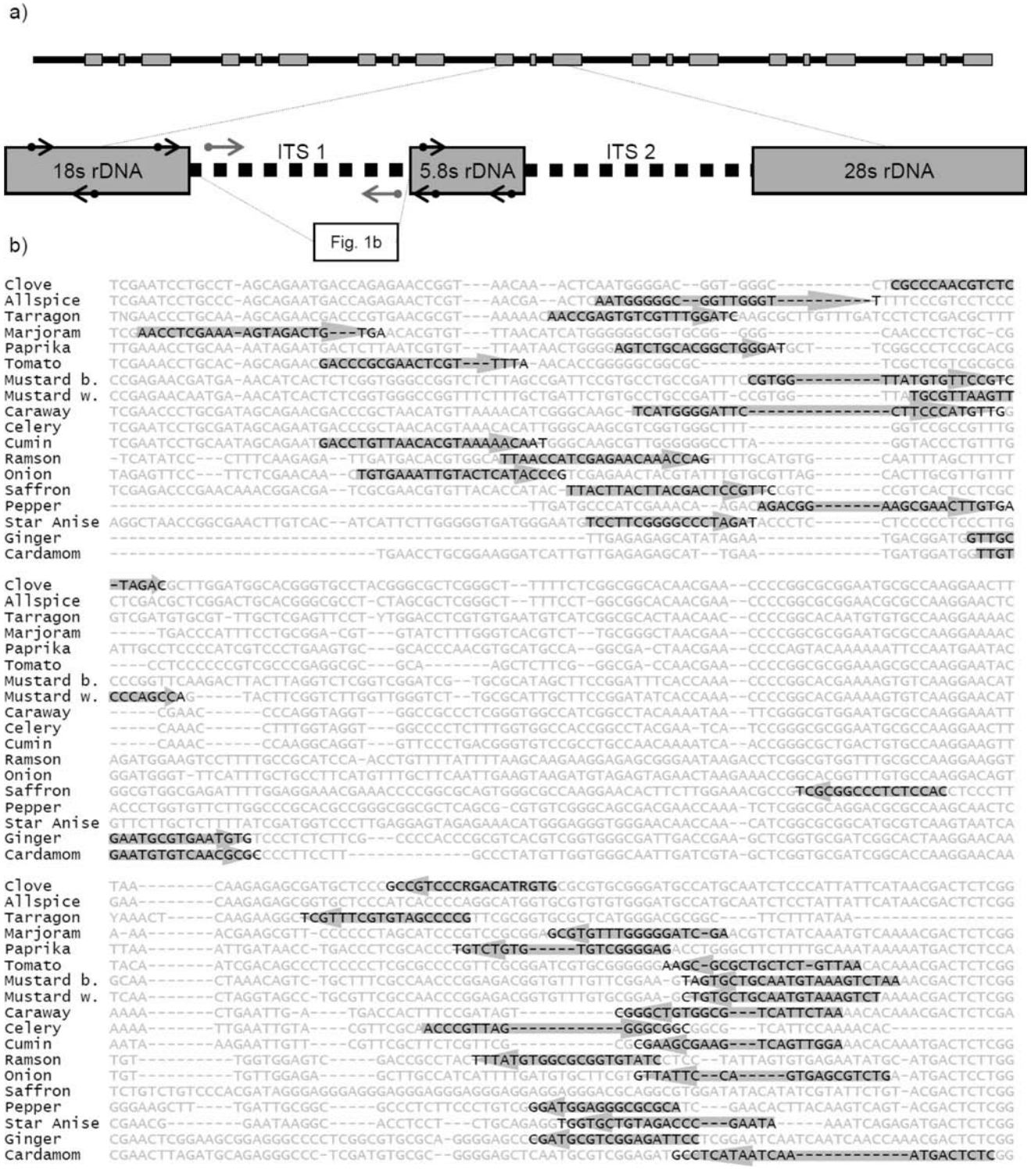

Figure 1. rDNA sequences and primer binding sites. (**a**) Gray boxes: rDNA genes (18s, 5.8s, and 28s), dotted lines ITS 1 and 2; arrows: primer binding sites (gray, specific; black, universal). (**b**) Sequence alignment of ITS1 created with ClustalX v. 2.0.9 (*20*) with primer binding sites. Gray arrows with black font: primer binding sites. Reverse primer binding sites for allspice and celery are located in ITS2; they are not shown in **b**.

Ten microliters of reaction products were separated by agarose gel electrophoresis (3%), stained with ethidium bromide solution (0.01%), and detected with UV light (gel documentation system 1040, Biostep, Jahnsdorf, Germany). The peqGold DNA marker (Peqlab, Erlangen, Germany) was used.

Sequencing. In the case of cardamom, marjoram, and paprika, the ITS1-region was amplified using universal primers (**Table 2**) and sequenced (custom sequencing service of GATC Biotech, Konstanz, Germany). The sequences were deposited in GenBank (accession numbers GQ166165.1, GQ166166.1, and GQ166167.1).

RESULTS AND DISCUSSION

DNA was isolated from 39 spices using two different methods. Method 1, a DNA precipitation procedure, was compared with method 2 based on DNA adsorption on a silica matrix. Isolated DNA was characterized accordingly: (i) quantity (fluorimetric measurements), (ii) purity (photometric measurements), and (iii) amplifiability (control PCR). In contrast to the CTAB protocol of § 64 LFGB L 00.00-31, both methods started from a mechanically grounding step using the TissueLyser system

(Qiagen, Hilden, Germany), followed by mixing the prepared suspension with chloroform. This initial step assured an efficient cracking of the plant cell wall and an early separation into solid, lipid, and aqueous compounds.

Quantity and Purity of DNA Isolates. Remarkably, larger amounts of DNA could be isolated with method 1 as compared to method 2 (mean value of method 1, 135 ng/μL; mean value of method 2, 27.5 ng/μL). The limiting binding capacity of silica spin columns as compared to the nearly infinite capacity of precipitation might be the reason for the better scoring of method 1. In parallel, the purity of the DNA isolated with method 1 was higher. The photometric analysis showed an $OD_{260/280}$ of 1.7 ± 0.3 for method 1 as compared with an $OD_{260/280}$ of 1.5 ± 0.3 for method 2. The ratio of pure DNA in solution should be in the range of $OD_{260/280}$ of 1.7−1.9. Lower values may be caused by proteins or phenolic substances that absorb at 280 nm. It should be noted that the standard deviations of both methods are very high. This was caused by very variable purity degrees of the DNA isolated from the very different spice matrices.

Amplifiability of DNA Isolates. The parameters mentioned above are only indicators for the DNA quality. They do not permit reliable predictions if the isolated DNA can serve as template in PCR assays. Consequently, isolates were tested for their amplifiability using universal binding primer sets in a control PCR (**Table 2**). The isolate was defined as amplifiable if one of the primer sets showed a positive result. Eighty-nine percent (of 39 spices in duplicates) of the isolates obtained by method 2 and 95% of the isolates obtained by method 1 gave positive PCR results.

DNA isolates from basil (*Ocimum basilicum*) and sage (*Salvia officinalis*) showed only positive PCR results using method 1 (precipitation). In the case of clove (*Syzygium aromaticum*) and allspice (*Pimenta dioica*), none of the studied methods were able to provide amplifiable DNA. The fermentation process during the spice production might be the reason for degraded DNA, resulting in shortened fragments that were not suitable for amplification by conventional PCR. Additionally, also inhibitors like phenolic substances might have avoided the amplification

Table 2. Universal Primer Sets

	forward primer 5′→3′ reverse primer 5′→3′	length (bp)[a]	target
universal-1	ACGGAGAATTAGGGTTCGATT	∼120	18s rDNA gene
	TGTTATTTATTGTCACTACCTCC		18s rDNA gene
universal-2	TGAACCTGCGGAAGGATCATTGT	∼320	18s rDNA gene
	CGAGAGCCGAGATATCCGTTG		5.8s rDNA gene
universal-3	GCAACGGATATCTCGGCTCT	∼100	5.8s rDNA gene
	TTCAAAGACTCGATGGTTCACG		5.8s rDNA gene
universal-4	ACCATCGAGTCTTTGAACGCAAGT	∼330	5.8s rDNA gene
	TATTGATATGCTTAAACTCAGCGGGT		28s rDNA gene

[a] PCR product length varies with different templates.

Table 3. Specific Primer Sets

spice	forward primer 5′→3′ reverse primer 5′→3′	length (bp)	accession[a]	abbreviation
allspice[b] (*P. dioica*)	AATGGGGGCGGTTGGGTT CCCTGGCCGTGGCTTC[c]	333	AM234081	All
black mustard[b] (*Brassica nigra*)	CGTGGTTATGTGTTCCGTC TTAGACTTTACATTGCAGCACTA	184	DQ340645	MuB
caraway[b] (*C. carvi*)	GGGATTCCTTCCCATGTTG TTAGAATGACGCCACAGCC	151	AF077878	Car
cardamom (*E. cardamomum*)	TTGTGAATGTGTCAACGCGC GAGAGTCATTTGATTATGAGGC	163	GQ166167	Card
celery[b] (*Apium graveolens*)	ACCCGTTAGGGGCGGC CTCCTTAGATGACACAATTACG[c]	∼370[c]	U30552.1 U30553.1[c]	Cel
clove (*S. aromaticum*)	CGCCCAACGTCTCTAGAC CACCATGTCTGGGACGGC	142	EF026622	Clo
cumin[b] (*Cuminum cyminum*)	GACCTGTTAACACGTAAAAACAAT TCCAACTGACTTCGCTTCG	190	CCU78362	Cum
ginger[b] (*Zingiber officinale*)	GTTGCGAATGCGTGAATGTG GGAATCTCCGACGCATCG	157	DQ064590	Gin
marjoram (*Origanum majorana*)	AACCTCGAAAAGTAGACTGTGA TCGATCCCCCAAACACGC	207	GQ166166	Maj
onion[b] (*Allium cepa*)	TGTGAAATTGTACTCATACCCG CAGACGCTCACTGGAATAAC	215	AJ411944	Oni
paprika (*Capsicum annuum*)	AGTCTGCACGGCTGGGAT CTCCCCGACACACAGACA	169	GQ166165	Pap
pepper[b] (*Piper nigrum*)	AGACGGAAGCGAACTTGTGA TGCGCGCCCTCCATCC	164	EF060077	Pep
ramson[b] (*A. ursinum*)	TTAACCATCGAGAACAAACCAG GATACACCGCGCCACATAAA	184	AJ412744	Ram
saffron[b] (*Crocus sativus*)	TTACTTACTTACGACTCCGTTC GTGGAGAGGGCCGCGA	128	DQ094185	Saf
star anise[b] (*I. verum*)	TCCTTCGGGGCCCTAGAT TATTCGGGTCTACAGCACCA	182	AF163724	StA
tarragon[b] (*A. dracunculus*)	AACCGAGTGTCGTTTGGATC CGGGGCTACACGAAACGA	173	AF045401	Tarr
tomato[b] (*Lycopersicon esculentum*)	GACCCGCGAACTCGTTTTA TTAACAGAGCAGCGCGCTT	196	AF244747	Tom
white mustard[b] (*Sinapis alba*)	TGCGTTAAGTTCCCAGCCA AGACTTTACATTGCAGCACAG	169	AY722486	MuW

[a] Accession number of sequence used for primer design. [b] Primer sets used to optimize PCR conditions. [c] Reverse primer hybridizes in ITS2.

(spiking experiments were performed; one example is given in **Table 5**, sample number 22).

Specific Detection of Spices. Species-specific primer sets were designed using the ITS 1 stretch of the rDNA sequences of 18 spices. The sequence alignment was created with ClustalX v. 2.0.9 (*20*). **Figure 1b** shows the location of each primer set; sequences are given in **Table 3**.

Fourteen of these primer sets were selected (marked by a "b", **Table 3**) and tested with isolates of 38 spices by PCR in triplicate reactions (clove was excluded because of negative PCR results in preliminary tests as described above). From 1596 reactions that were carried out, only 12 assays (0.8%) showed a false negative result. On the other hand, 263 (16.5%) assays showed false positive results. Eighty-five (5.3%) of these false positive results were characterized by amplicons of the expected size, and 178 (11.2%) PCR reactions resulted in amplicons of a different size than expected caused by miss-priming of the oligonucleotides prior optimization. To further minimize false results, reaction conditions were optimized. Optimized parameters (final concentration, temperature, or time, respectively) are summarized in **Table 4**. Templates and primer sets that had shown false positive results were retested under optimized PCR conditions. In all cases, the expected amplicon could be detected except for allspice and clove (**Figure 2**).

The three specific primer sets amplifying cardamom, marjoram, and paprika were designed after optimizing the conditions of PCR based on the other 14 specific primer sets. The performance

Table 4. PCR Optimization Parameters

parameter	conditions	optimum
salt condition	KCl/$(NH_4)_2SO_4$	KCl
magnesium concentration (mM)	1.5−9	3
primer concentration (μM)	0.1−1.0	0.5
polymerase concentration (U)	0.1−1.5	0.1
annealing temperature (°C)	53−72	58
denaturation time (s)	15−30	15
annealing time (s)	15−30	25
elongation time (s)	15−30	25
cycle number	25−40 (5/step)	35

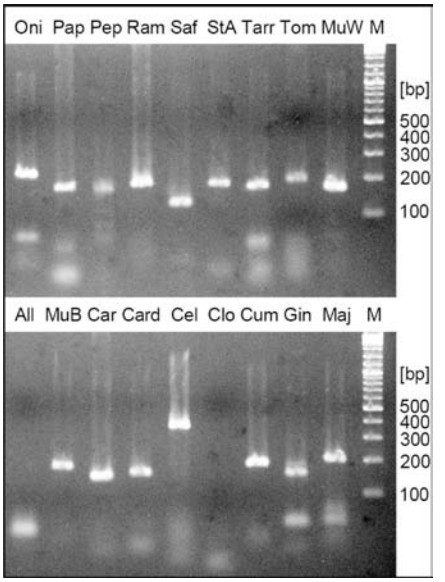

Figure 2. Amplification products of specific PCR (without MDA treatment) using specific primer pairs given in **Table 3**. M, DNA marker (amplicons with sizes <100 bp are interpreted as primer dimers).

Table 5. Results of Experiments with Spice Mixtures

sample no.	composition of the sample[a]	primer sets used for PCR analysis	primer set with positive PCR	result[b]
1	caraway	Car, Cum, MuW, Oni, Ram, StA, Tar	Car	c
2	cardamom	Card, StA	Card	c
3	clove	Car, Cum, MuW, Oni, Ram, StA, Tar		c
4	cumin	Car, Cum, MuW, Oni, Ram, StA, Tar	Cum	c
5	cumin	Cum, Saf	Cum	c
6	ginger	Car, Cum, MuW, Oni, Ram, StA, Tar		c
7	mustard black	Oni		c
8	mustard black	Gin, Ram		c
9	mustard white	Car, Cum, MuW, Oni, Ram, StA, Tar	MuW	c
10	onion	Car, Cum, MuW, Oni, Ram, StA, Tar	Oni	c
11	oregano	Car, Cum, MuW, Oni, Ram, StA, Tar		c
12	pepper	Car, Cum, MuW, Oni, Ram, StA, Tar		c
13	ramson	Car, Cum, MuW, Oni, Ram, StA, Tar	Ram	c
14	anise	MuW		c
15	star anise	Car, Cum, MuW, Oni, Ram, StA, Tar		i
16	tarragon	Car, Cum, MuW, Oni, Ram, StA, Tar	Tar	c
17	caraway, cumin	Car, Cum, MuW, Oni, Ram, StA, Tar	Car, Cum	c
18	caraway, cumin	MuB, Tar, Tom		c
19	cardamom, mustard black	Car, Cum, MuW, Oni, Ram, StA, Tar		*c*
20	cardamom, star anise	Card, StA	Card	i
21	clove, pepper	Car, Cum, MuW, Oni, Ram, StA, Tar		c
22	clove, caraway	Car, Cum, MuW, Oni, Ram, StA, Tar		i
23	cumin, saffron	Cum, Saf	Cum, Saf	c
24	mustard black, mustard white	Car, Cum, MuW, Oni, Ram, StA, Tar	MuW	c
25	mustard black, mustard white	Cel		c
26	onion, star anise	Car, Cum, MuW, Oni, Ram, StA, Tar	Oni, StA	c
27	onion, anise	MuW		c
28	pepper, tarragon	Car, Cum, MuW, Oni, Ram, StA, Tar	Tar	c
29	basil, oregano, tarragon	Car, Cum, MuW, Oni, Ram, StA, Tar		i
30	cumin, caraway, tarragon	Car, Cum, MuW, Oni, Ram, StA, Tar	Cum, Car, Tar	c

[a] Equal amount of each spice. [b] c, correct result; i, incorrect result.

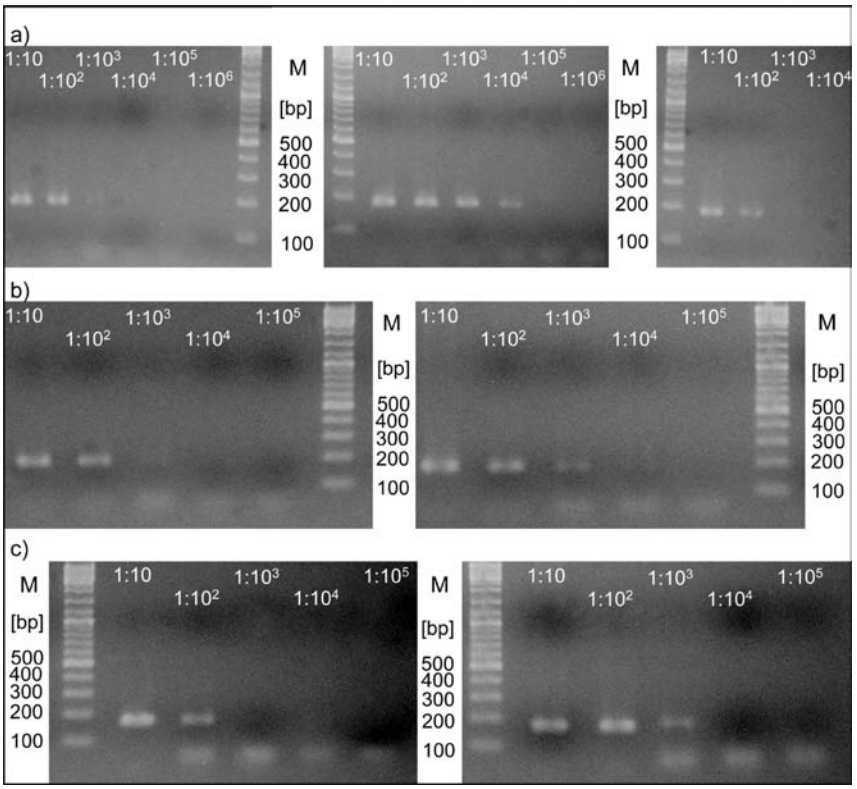

Figure 3. PCR products of DNA solutions from mustard before and after MDA treatment using primer MuB and MuW. (**a**) First run: left, black mustard isolates (1:10−1:10^6 DNA dilutions) before MDA; center, after MDA with 24 U *Bst* polymerase (1:10−1:10^6 DNA dilutions); and right, after MDA with 8 U *Bst* polymerase (1:10−1:10^4 DNA dilutions). (**b**) Second run: left, black mustard isolates (1:10−1:10^5 DNA dilutions) before MDA; and right, after MDA with 24 U *Bst* polymerase (1:10−1:10^5 DNA dilutions). (**c**) Second run: left, white mustard isolates (1:10−1:10^5 DNA dilutions) before MDA; and right, after MDA with 24 U *Bst* polymerase (1:10−1:10^5 DNA dilutions). M, DNA marker.

of the second generation of primer sets was comparable with the initially constructed and optimized sets indicating that this system of specific amplification is generally expendable. Specific primer binding sites can be found at the ITS in rDNA in many other spices or plants.

Validation. For final validation, PCR experiments with self-made spice mixtures were performed. The samples contained one, two, or three different spices in equal amounts. After DNA isolation (method 2), the samples were randomized, and PCR experiments with different primer sets were performed. For 26 of 30 samples, the PCR results showed the correct amplification pattern (**Table 5**). In the one single compound sample 15, star anise (*Illicium verum*), could not be identified. In further tests, it could be shown that star anise DNA was only amplified if parts of the seed were used for isolation (data not shown). Too much parts of pod could induce false negative results. This assumption is well in line with the findings in the two component sample 20, in which also star anise could not be detected, whereas the second component, cardamom (*Elettaria cardamomum*), gave a positive PCR result.

Regarding the multicomponent samples, caraway (*Carum carvi*) was not identified in a two component sample mix (sample 22; second component, clove) and tarragon (*Artemisia dracunculus*) showed a false negative PCR result in a three component sample mix (sample 29; second and third component, basil and oregano). It was assumed, correlating to false negative PCR results with universal primers, that inhibiting substances were coisolated with the DNA from the other spices (basil or clove).

As compared to traditional morphological methods, DNA-based analyses have the advantage to give reliable and unequivocal results. On the other hand, microscopic spice identification strongly depends on the experience and interpretation by an operator.

WGA by MDA. WGA should be able to increase the concentration of amplifiable DNA and consequently increase the ratio of DNA amount vs inhibiting substances. WGA could be carried out by MDA. The method was performed using random heptamers as primers that randomly bind at several positions of the whole genome. For the amplification reaction, a polymerase with strand displacement activity but without exonuclease activity was used (e.g., *Bst* DNA polymerase, large fragment) (*21*). To determine the method parameters, spice samples were chosen that gave reliable results in the PCR experiments described above. In **Figure 3**, the results of PCR experiments (black and white mustard) are shown using either isolated DNA or MDA products as a template. The starting solution contained 0.25 ng/μL of isolated mustard DNA (= DNA_0). One microliter of this DNA was used for a MDA reaction (= MDA_0) in a total volume of 25 μL. Both solutions DNA_0 and MDA_0 were diluted in a 10-fold dilution series (1:10−1:10^6) and were used as templates for PCR experiments using specific primer pairs (**Table 3**). In a first experiment, different *Bst* polymerase activities (8 and 24 U) were tested for the MDA reaction (**Figure 3a**). It was observed that without MDA treatment, positive PCR results could be obtained with a template dilution up to 100-fold (**Figure 3a**, left gel). The same result was observed using MDA with 8 U *Bst* polymerase (**Figure 3a**, right gel). In this case, the MDA reaction did not give any benefit because the increase of DNA amount obtained by MDA only compensates the dilution step (1:25) of the MDA. However, using 24 U *Bst* polymerase in the MDA reaction, the subsequent PCR showed positive results until a dilution of 1:10^4 (**Figure 3a**, central gel). This increase in DNA amount by about

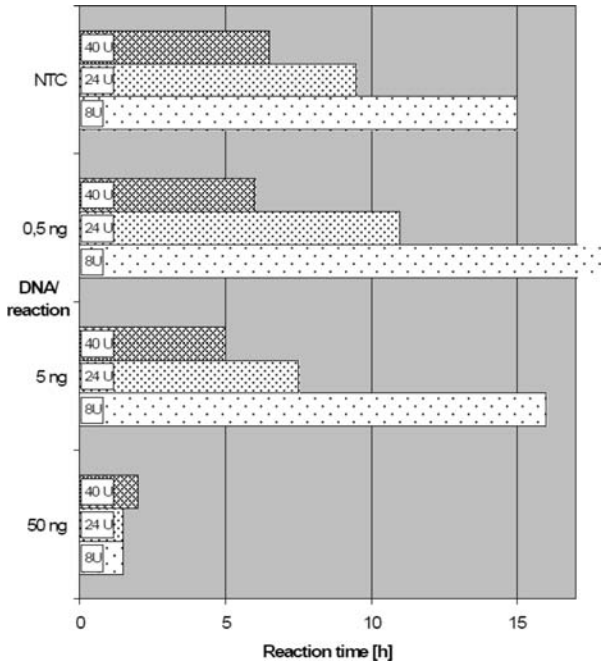

Figure 4. Time needed to complete MDA reactions under real-time conditions (using isolated DNA from black mustard as template). Completion is defined as reaching of the plateau phase (mean of triplicates). NTC, no DNA (reaction with 8 U polymerase and an amount of 0.5 ng of DNA was not completed after 16 h).

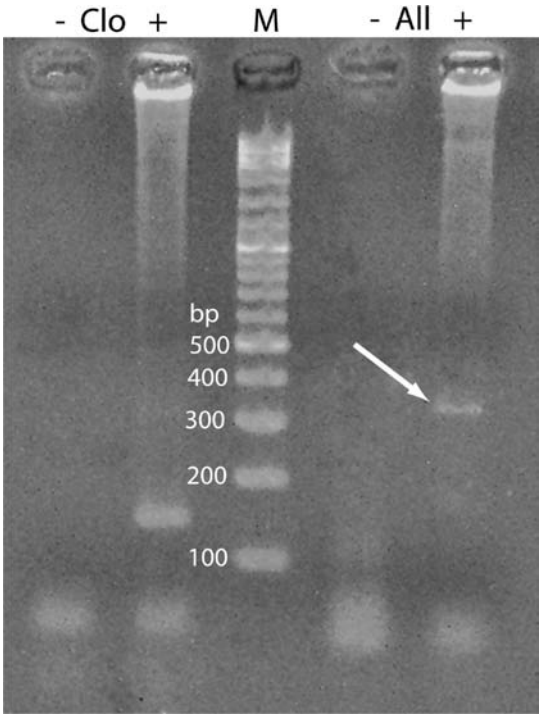

Figure 5. PCR carried out with isolates from clove (Clo) and allspice (All) before (−) and after (+) MDA. M, DNA marker.

two orders of magnitude indicates a positive effect of the MDA reaction.

To test the reproducibility of the MDA reaction, the dilution experiment was repeated (with 24 U *Bst* polymerase in the MDA reaction) using (i) DNA of the same origin (**Figure 3b**, black mustard) and (ii) from a different origin (**Figure 3c**, white mustard). In these second runs, a benefit of just one order of magnitude can be reached (**Figure 3b**,**c**, right gel) as compared to the PCR experiments with diluted DNA as template (**Figure 3b**,**c**, left gel). Replications of this experiment using exactly the same conditions resulted in DNA amount increase of the same order of magnitude (data not shown).

To determine the observed effect of polymerase activity and to optimize reaction time, MDA experiments were performed under real-time conditions using SYBR Green I for detection and isolated DNA from black mustard as template. The bars in **Figure 4** represent the required reaction time until the MDA reaction reaches the plateau phase (no more increase of fluorescence can be observed) in dependency (i) on the polymerase activity (8, 24, or 40 U, respectively) and (ii) the amount of template DNA (no template control, 0.5, 5, or 50 ng, respectively). As expected, a reduction of the DNA amount led to an extension of the reaction time. In samples with weak DNA yields (e.g., 0.5 ng DNA per reaction), a polymerase activity of 8 U was not enough to complete the reaction within 16 h. This unfinished MDA reaction confirmed the observations based on an agarose gel (right gel in **Figure 3a**). With polymerase activities of 24 and 40 U, the reactions were completed after 11 and 7 h, respectively. Regarding the no template control experiments, also an increase of the fluorescence signal could be observed. In cases of the MDA reactions with 8 and 24 U polymerase activities, these signals reached their plateaus earlier than the corresponding reactions with a template DNA amount of 0.5 ng. The formation of reaction products and concomitant fluorescence increases in DNA free blank samples could be observed in most reactions. Nevertheless, these reaction (side) products showed no amplifiability in subsequent PCR experiments. Additionally, the increase of the fluorescence signal showed two or three exponential phases separated by transient plateau phases (data not shown). Observed differences in the reaction kinetics, in the overall duration of the reaction and the lacking of amplifiable products, indicated that these products might be caused by an unspecific polymerization of the dNTPs to artifacts. In cases where DNA—even of minor concentration—is present in reaction batches with low *Bst* polymerase activity, this artifact formation was probably reduced by product formation resulting in longer reaction times but a normal reaction kinetic and amplifiable DNA.

In consideration of the described observations for further MDA reactions, *Bst* polymerase activity was adjusted to 24 U and the reaction duration to 16 h. In **Figure 5**, the result of a PCR is shown that was performed using MDA-enriched clove and allspice DNA as template and a clove/allspice-specific primer set. The expected amplification products (clove, 142 bp; and allspice, 333 bp) of the PCR reaction could be detected by agarose gel electrophoresis only after MDA treatment. The very weak band from allspice indicated that the PCR worked near to the limit of detection. The low reproducibility of MDA (shown above) also caused a lot of negative results in cases of allspice and clove.

In conclusion, a reliable method for isolation of DNA from spices could be developed, which supplies amplifiable templates for PCR in most cases. DNA isolation with method 2 (silica adsorption) is a timesaving and effective method for DNA preparation from spices. It is easier in handling and needs less time than method 1 (precipitation), and in almost all cases, accuracy and sensitivity of method 2 are sufficient for further DNA-based analysis. Consequently, if time saving is an important criterion for the application (e.g., in routine analysis), method 2 should be preferred. In comparison, for DNA isolation from 24 samples, the expenditure of time using method 2 is 1−2 and 4−5 h using method 1. If the isolated DNA gives negative results in control PCR, either a DNA precipitation should be performed or the MDA should be used as a preamplification method to increase the ratio between amplifiable DNA and inhibitors. The designed primer sets

combined with optimized PCR conditions afford a specific amplification for selective detection of 18 spices via DNA by PCR.

ABBREVIATIONS USED

WGA, whole genome amplification; ITS, internal transcribed spacer; MDA, multiple displacement amplification; CTAB, cetyltrimethylammonium bromide; Tris/HCl, tris(hydroxymethyl)-aminomethan; Na_2-EDTA, ethylenediaminetetraacetic acid disodium salt.

LITERATURE CITED

(1) Marmiroli, N.; Maestri, E.; Gulli, M.; Malcevschi, A.; Peano, C.; Bordoni, R.; Bellis, G. Methods for detection of GMOs in food and feed. *Anal. Bioanal. Chem.* **2008**, *392*, 369−384.

(2) Ehlert, A.; Hupfer, C.; Busch, U. Overview of detection methods of allergens in foods. *Fleischwirtschaft* **2008**, *88*, 89−91.

(3) Lambertz, S. T.; Nilsson, C.; Hallanvuo, S.; Lindblad, M. Real-time PCR method for detection of pathogenic *Yersinia enterocolitica* in food. *Appl. Environ. Microbiol.* **2008**, *74*, 6060−6067.

(4) Mafra, I.; Ferreira, I. M. P. L. V. O.; Oliveira, M. B. P. P. Food authentication by PCR-based methods. *Eur. Food Res. Technol.* **2008**, *227*, 649−665.

(5) Mustorp, S.; Engdahl-Axelsson, C.; Svensson, U.; Holck, A. Detection of celery (*Apium graveolens*), mustard (*Sinapis alba*, *Brassica juncea*, *Brassica nigra*) and sesame (*Sesamum indicum*) in food by real-time PCR. *Eur. Food Res. Technol.* **2008**, *226*, 771−778.

(6) Hupfer, C.; Waiblinger, H. U.; Busch, U. Development and validation of a real-time PCR detection method for celery in food. *Eur. Food Res. Technol.* **2007**, *225*, 329−335.

(7) Dhanya, K.; Sasikumar, B. Molecular Marker Based Adulteration Detection in Traded Food and Agricultural Commodities of Plant Origin with Special Reference to Spices. *Food Biotechnol. Curr. Trends Biotechnol. Pharm.* **2010**, *4*, 454−489.

(8) Dhanya, K.; Syamkumar, S.; Sasikumar, B. Development and application of SCAR marker for the detection of papaya seed adulteration in traded black pepper powder. *Food Biotechnol.* **2009**, *23*, 97−106.

(9) Lungarini, S.; Aureli, F.; Coni, E. Coumarin and cinnamaldehyde in cinnamon marketed in Italy: A natural chemical hazard? *Food Addit. Contam., Part A* **2008**, *25*, 1297−1305.

(10) Sundov, Z.; Nincevic, Z.; Definis-Gojanovic, M.; Glavina-Durdov, M.; Jukic, I.; Hulina, N.; Tonkic, A. Fatal colchicine poisoning by accidental ingestion of meadow saffron-case report. *Forensic Sci. Int.* **2005**, *149*, 253−256.

(11) Wehner, F.; Musshoff, F.; Schulz, M. M.; Martin, D. D.; Wehner, H.-D. Detection of colchicine by means of LC-MS/MS after mistaking meadow saffron for Bear's garlic. *Forensic Sci., Med., Pathol.* **2006**, *2*, 193−197.

(12) Dhanya, K.; Kizhakkayil, J.; Syamkumar, S.; Sasikumar, B. Isolation and amplification of genomic DNA from recalcitrant dried berries of black pepper (*Piper nigrum* L.)—A medicinal spice. *Mol. Biotechnol.* **2007**, *37*, 165−168.

(13) Syamkumar, S.; Jose, M.; Sasikumar, B. Isolation and PCR amplification of genomic DNA from dried capsules of cardamom (*Elettaria cardamomum* M.). *Plant Mol. Biol. Rep.* **2005**, *23*, 417/1−417/5.

(14) Syamkumar, S.; Lowarence, B.; Sasikumar, B. Isolation and amplification of DNA from rhizomes of turmeric and ginger. *Plant Mol. Biol. Rep* **2003**, *21*, 171/1−171/5.

(15) Chiou, S.-J.; Yen, J.-H.; Fang, C.-L.; Chen, H.-L.; Lin, T.-Y. Authentication of medicinal herbs using PCR-amplified ITS2 with specific primers. *Planta Med.* **2007**, *73*, 1421−1426.

(16) Rogers, S. O.; Bendich, A. J. Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. *Plant Mol. Biol.* **1987**, *9*, 509−20.

(17) Spits, C.; Le Caignec, C.; De Rycke, M.; Van Haute, L.; Van Steirteghem, A.; Liebaers, I.; Sermon, K. Optimization and evaluation of single-cell whole-genome multiple displacement amplification. *Hum. Mutat.* **2006**, *27*, 496−503.

(18) Dean, F. B.; Hosono, S.; Fang, L.; Wu, X.; Faruqi, A. F.; Bray-Ward, P.; Sun, Z.; Zong, Q.; Du, Y.; Du, J.; Driscoll, M.; Song, W.; Kingsmore, S. F.; Egholm, M.; Lasken, R. S. Comprehensive human genome amplification using multiple displacement amplification. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5261−5266.

(19) Lage, J. M.; Leamon, J. H.; Pejovic, T.; Hamann, S.; Lacey, M.; Dillon, D.; Segraves, R.; Vossbrinck, B.; Gonzalez, A.; Pinkel, D.; Albertson, D. G.; Costa, J.; Lizardi, P. M. Whole genome analysis of genetic alterations in small DNA samples using hyperbranched strand displacement amplification and array-CGH. *Genome Res.* **2003**, *13*, 294−307.

(20) Larkin, M. A.; Blackshields, G.; Brown, N. P.; Chenna, R.; McGettigan, P. A.; McWilliam, H.; Valentin, F.; Wallace, I. M.; Wilm, A.; Lopez, R.; Thompson, J. D.; Gibson, T. J.; Higgins, D. G. Clustal W and Clustal X version 2.0. *Bioinformatics* **2007**, *23*, 2947−2948.

(21) Aliotta, J. M.; Pelletier, J. J.; Ware, J. L.; Moran, L. S.; Benner, J. S.; Kong, H. Thermostable *Bst* DNA polymerase I lacks a 3′ -> 5′ proofreading exonuclease activity. *Genet. Anal.: Biomol. Eng* **1996**, *12*, 185−95.

Received for review September 24, 2010. Revised manuscript received December 2, 2010. Accepted December 4, 2010.