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6 **A Simple and Cost-Effective Method to Synthesize an Internal Size Standard**

7 **Amenable to Use with a 5-Dye System**

8

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21 **ABSTRACT**

22 Fluorescent end-labeling joint to capillary electrophoresis has become a common
23 practice for PCR fragment analysis. Besides its sensitivity/reproducibility, the major
24 advantage of this method is the possibility of detect simultaneously fragments
25 overlapping in size by the use of different labels. However, even thought multiplexing
26 approaches are usually presented as a way to reduce genotyping costs, the price of the
27 commercial size standards still represents a substantial deterrent. As an example, a new
28 generation of dye-systems allowing fragments labeled up to 5 different colors is now
29 commercially available but the price of the pertinent internal standard can easily
30 represent the 50% of the project budget. Here, we describe a simple and cost-effective
31 method to synthesize a fluorescently labeled size standard amenable to use with a 5-dye
32 system, the SM594. Its performance in fragment size determination is demonstrated
33 over a total of 63 alleles covering different molecular markers, species, and dyes. In
34 summary, allele calling using the SM594 and the equivalent commercial standard shows
35 identical results and maximum divergence between lectures from both standards never
36 exceed 0.64 bp. Since genotyping costs can be reduced by a 40 factor, the use of the
37 SM594 might allow researchers to address projects covering more
38 species/individuals/loci.

39 INTRODUCTION

40 Microsatellite markers are widely used in several research fields and they
41 usually represent the marker of choice for population genetic analysis (1). Reducing the
42 economic cost of microsatellite development/genotyping represents a technical
43 challenge, particularly in nonmodel organisms for which fewer resources are usually
44 available (2). In this regard, different authors have recently demonstrated the time- and
45 cost- effectiveness of next-generation sequencing technology to develop microsatellite
46 markers (3). By the contrary, and despite commonly used multiplexing assays,
47 genotyping costs continue still to be high or even limiting.

48 Since their description 20 years ago, the way that microsatellite alleles are
49 visualized/sized has changed according to technological advances. Allele sizing was
50 initially based on agarose gels. However, because microsatellite alleles can differ by as
51 little as 2 bp size, agarose gels were soon replaced by more accurate polyacrylamide gel
52 electrophoresis (PAGE). Semiautomated PAGE platforms and fluorescent dye
53 chemistry greatly improved microsatellite resolution/accuracy. Still, the major shift in
54 microsatellite genotyping arose with capillary electrophoresis (CE) technology which
55 allows higher sensitivity and reproducibility while reduces processing time.

56 Unfortunately, this increase in throughput genotyping rates also involved a considerable
57 increase of the running costs by the need to include a fluorescently labeled size standard
58 for each sample. In fact, the price of such size standards which are available from
59 relatively few vendors can in many cases exceed that of the genotyping assay itself (4).

60 In an attempt of lowering microsatellite genotyping costs, a handful of studies
61 have reported over the last decade the in-house production of fluorescently labeled size
62 standards (4-7). Although the cost-effectiveness of these methods has been clearly

63 established, two of them propose the use of specific DNA templates complicating their
64 use elsewhere (5,6). Moreover, all these protocols describe the synthesis of size
65 standards for a four-dye system. An advantage of a fluorescently-based detection system
66 is that DNA fragments overlapping in size range can be labeled with different dyes and
67 therefore be simultaneously detected in a single lane on a CE instrument. Thus,
68 although 4-dye technology has allowed a significant increase in throughput rates over
69 traditional methods, a third generation of dyes combining 5 different labels has been
70 introduced in the market in 2005.

71 In the present work, we describe a simple and cheap method to produce a
72 fluorescently labeled size standard (the SM594) amenable to use with 5-dye systems
73 and we show its accuracy and reproducibility in a range of different alleles and species.
74 Moreover, its suitability for other techniques that involve fragment separation as
75 Amplified Fragment Length Polymorphism (AFLP) is also demonstrated.

76 **MATERIAL & METHODS**

77 In order to amplify known-length fragments using the universal cloning vector
78 pGEM®-3Z(+) (Promega Corporation, Madison, WI, USA) as DNA template, 19
79 primers were designed with the software Primer3 (8). The resulting primer set was
80 composed of a single forward primer with a 5'-ATTO 633 label and 18 reverse primers
81 (Table 1), and it amplified a total of 18 single-stranded, fluorescently labeled fragments
82 (theoretical/expected sizes: 60, 100, 140, 200, 214, 220, 240, 260, 314, 340, 380, 414,
83 420, 440, 480, 500, 520, and 600 bp) (Fig. 1).

84 Size standard synthesis was carried out by amplifying individually the 18
85 fragments. PCR reactions (50 µl) were identical for all fragments apart from the reverse
86 primer identity and they contained 1x GoTaq® Flexi buffer (Promega Corporation), 1

87 mM MgCl₂, 150 μM of each dNTP (Thermo Fisher Scientific Inc., Waltham, MA,
88 USA), 400 nM of each primer (Eurofins MWG Operon, Ebersberg, Germany), 1.75 U
89 GoTaq® Polymerase (Promega Corporation), and 20 ng of pGEM®-3Z(+). Likewise,
90 the same amplification profile was applied to all reactions and it included an initial
91 denaturing step at 94°C for 5 min, followed by 30 cycles at 94°C for 30s, 65°C for 30s,
92 and 72°C for 30s, and a final extension at 72°C for 1 hour (in order to allow a consistent
93 *Taq* polymerase terminal transferase activity and guarantee that all PCR products match
94 to the +A state). Finally, individual amplifications were tested on standard agarose gels
95 and equal volumes of each PCR product were combined into a single tube and stored at
96 -20°C until their use as size standard.

97 SM594 calibration and validation were performed as follow. First, actual size of
98 the synthesized fragments was estimated by co-electrophoresis with the commercial size
99 standard GeneScan™ 600LIZ® (Life Technologies Corporation, Carlsbad, CA, USA).
100 Briefly, 0.5 μl of SM594 were mixed with 0.5 μl of the commercial size standard and
101 11 μl of Hi-Di™ Formamide (Life Technologies Corporation), the obtained mixture
102 was run on a 3130XL Genetic Analyzer (Life Technologies Corporation), and
103 size/fluorescence intensity of each SM594 fragment was determined in the
104 GENEMAPPER version 4.0 (Life Technologies Corporation). Second, 21 microsatellite
105 loci from 4 different algae species: *Chondrus crispus* (9), *Gracilaria gracilis* (10,11),
106 *Lessonia nigrescens* (12), and *Sargasum muticum* (Daguin-Thiébaud C., unpublished
107 data), as well as 10 AFLP markers from the gastropod *Crepidula fornicata* (Riquet F.,
108 unpublished data), were amplified using conventional protocols and size estimation of
109 the 63 resulting alleles was done over 8 independent runs of a 3130XL Genetic
110 Analyzer using both the SM594 size standard and the commercial size standard
111 GeneScan™ 600LIZ®. Moreover, in order to check the reproducibility and consistency

112 of the obtained sizes, 8 additional runs using the SM594 as internal size standard were
113 repeated over a different 3130XL Genetic Analyzer instrument. Allele size comparisons
114 between different internal standards (i.e. GeneScan™ 600LIZ® vs. SM594) as well as
115 between different instruments (i.e. SM594-1 vs. SM594-2) were based on the range of
116 differences in size estimates and the mean size values. Highest divergence for allele size
117 among both treatments was also calculated.

118 **RESULTS AND DISCUSSION**

119 The designed primers amplified 18 unique PCR products (Fig. 1). Estimated
120 size of the synthesized fragments differed from theoretical/expected size by 3-7
121 nucleotides (Fig. 2), probably due to the influence of the ATTO 633 fluorescent label on
122 the electrophoretic mobility (13). However, those differences were stable over different
123 SM594 batches showing its reproducibility, stability and accuracy (mean sizes \pm
124 standard deviation for the 18 fragments over 8 independent batches were 56.76 \pm 0.14,
125 95.42 \pm 0.10, 136.02 \pm 0.09, 195.44 \pm 0.11, 209.49 \pm 0.12, 215.46 \pm 0.10, 235.33 \pm 0.12,
126 255.38 \pm 0.10, 309.04 \pm 0.12, 335.40 \pm 0.07, 375.39 \pm 0.10, 409.31 \pm 0.09, 415.22 \pm 0.10,
127 434.40 \pm 0.11, 474.41 \pm 0.13, 494.48 \pm 0.08, 513.48 \pm 0.06, and 594.30 \pm 0.11). These results
128 show that the use of a universal cloning vector like the pGEM®-3Z(+) as DNA template
129 not only renders the protocol accessible to any interested laboratory but also guarantees
130 a robust amplification and avoids any subsequent purification step (6). Likewise,
131 differences in fluorescence intensity among fragments were low (at most 3-fold; Fig. 2)
132 and constant making unnecessary any further optimization (e.g. pooling unequal rates of
133 each fragment, diluting fragment pool to target an optimum peak height range) (6,7).

134 The comparison of allele sizes using both the SM594 and the GeneScan™
135 600LIZ® corroborated the reproducibility and accuracy of the in-house synthesized

136 standard (Table 2). Differences in size estimates over 8 independent runs for 53
137 microsatellite alleles ranged from 0.07 to 0.64 bp and from 0.06 to 0.51 bp for the
138 SM594 and the GeneScan™ 600LIZ®, respectively. The range of differences in size
139 estimates was also similar for both internal standards in the case of the 10 AFLP
140 markers (from 0.05 to 0.43 bp and from 0.08 to 0.23 bp for the SM594 and the
141 GeneScan™ 600LIZ®, respectively). By the other hand, although statistical differences
142 between mean sizes obtained with both standards were found in 40 alleles, maximum
143 divergence never exceed 0.64 bp guaranteeing their right calling (6,7). In fact, the
144 highest difference among independent lectures of the same allele was lower than 0.5 bp
145 in the 90% of the cases. Briefly, 4 out of the 6 alleles showing maximum divergence
146 values ≥ 0.5 bp were found in *G. gracilis* (Gg121-205, 2CT-230, 2CT-203, and Gg173-
147 170) while the 2 remaining ones belonged to *L. nigrescens* (Less1T3-138 and Less1T3-
148 141). However, the range of maximum divergence values was quite similar among the 5
149 species: 0.16-0.44 for *C. crispus*, 0.20-0.64 for *G. gracilis*, 0.28-0.46 for *C. fornicata*,
150 0.27-0.52 for *L. nigrescens*, and 0.14-0.37 for *S. muticum*. Likewise, any relationship
151 could be established between maximum divergence values and fluorescent dye (0.24-
152 0.50 for 6-FAM, 0.16-0.44 for VIC, 0.20-0.52 for NED, and 0.14-0.64 for PET).
153 Finally, a similar range of differences in size estimates was also observed when samples
154 were genotyped on a different Genetic Analyzer (from 0.06 to 0.42 bp for microsatellite
155 alleles and from 0.23 to 0.38 for AFLP markers) and, as it was the case for the
156 comparisons between both standards, the highest difference between lectures obtained
157 from different instruments never exceed 0.64 bp (Table 2).

158 Unlike genotyping using an equivalent commercial size standard (i.e. an internal
159 size standard amenable to use with a 5-dye system), allele sizing with the SM594 can be
160 easily afford by any laboratory furnished with basic molecular equipment. First, their

161 synthesis is a simple and fast procedure; in fact, any person familiarized with the PCR
162 technique can produce large volumes of this size standard in a few hours. Second, cost
163 analysis reveals that genotyping is 40 times more economical when comparing with an
164 equivalent commercial size (estimated price includes primer and cloning vector
165 synthesis, PCRs and working hours). In fact, the economic expenditure needed to
166 acquire a batch of the commercial size standard GeneScan™ 600LIZ® (approximately
167 800 samples) allows to buy an amount of primers and cloning vector enough to
168 synthesize 110 SM594 batches (approximately 990000 samples). This reduction of
169 genotyping costs is agree with previous reports of in-house synthesized size standards
170 (from 10 to 128 times more economical, 4-7). However, the economic advantage of the
171 SM594 might go further since it can be used to size fragments labeled with up to 4
172 different colors and, therefore, it increases multiplexing options.

173 In conclusion, the present work describes a simple, robust and cheap procedure
174 to synthesize a fluorescently labeled size standard amenable to use with a 5-dye system.
175 Accuracy in fragment size determination comparable to that obtained using the
176 equivalent commercial size standard is demonstrated in a wide range of microsatellite
177 loci covering different motifs, labels, and species but also in AFLP markers. Since the
178 use of this size standard reduces genotyping costs by a 40 factor, it should allow
179 researchers to address projects covering a larger number of species/individuals/loci.

180 **ACKNOWLEDGMENTS**

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182 *muticum* and *C. fornicata* samples. Funding for this work was provided by XXX; L.C.
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185 **COMPETING INTERESTS STATEMENT**

186 The authors declare no competing interests.

187 TABLES

188 Table 1. Primer sequences for SM594 synthesis.

Primer name	Direction	Primer Sequence (5' → 3')
SMF*	Forward	CGCTAGGGCGCTGGCAAGTG
57R	Reverse	AGCGCGGCGGGTGTGGTG
95R	Reverse	GCCTGAATGGCGAATGGACGC
136R	Reverse	GAAGAGGCCCGCACCGATCGC
195R	Reverse	GGCGTTACCCAACTTAATCGCCTT
209R	Reverse	ACTGGGAAAACCCTGGCGTTACCC
215R	Reverse	GTCGTGACTGGGAAAACCCTGGC
235R	Reverse	ACTGGCCGTCGTTTTACAACGTC
255R	Reverse	AGTGAGTCGTATTACAATTCAGTGGCCG
309R	Reverse	GCCTGCAGGTCTGACTCTAGAGGAT
335R	Reverse	ACACTATAGAATACTCAAGCTTGCATGC
375R	Reverse	GAAACAGCTATGACCATGATTACGC
409R	Reverse	TGTGGAATTGTGAGCGGATAACAATTTC
415R	Reverse	ATGTTGTGTGGAATTGTGAGCGGATAAC
434R	Reverse	ACTTTATGCTTCCGGCTCGTATGTTG
474R	Reverse	AATGTGAGTTAGCTCACTCATTAGGC
494R	Reverse	GCAGTGAGCGCAACGCAATTAAT
513R	Reverse	GTTTCCCGACTGGAAAGCGGGC
594R	Reverse	GAGGAAGCGGAAGAGCGCCC
*SMF has a 5' ATTO 633 label.		

189 **Table 2. Microsatellite/AFLP allele sizing using a commercial size standard (GeneScan™ 600LIZ®) and the in-house synthesized size**
 190 **standard SM594.**

Species	Locus ^b	Allele	GeneScan™ 600LIZ®		SM594-GA1 ^a		SM594-GA2 ^a		MDM ^c	MDS ^d
			Mean	SD	Mean	SD	Mean	SD		
<i>C. crispus</i>	Chc40 ^{6-FAM}	160	160.33	0.06	160.35	0.07	160.22	0.09	0.25	0.36
		176	175.69	0.04	175.64	0.09	175.61	0.09	0.24	0.29
	Chc24 ^{VIC}	175	174.84	0.04	174.74	0.05	174.63	0.10	0.25	0.33
		219	218.82	0.05	218.58	0.09	218.62	0.09	0.38	0.30
	Chc31 ^{NED}	210	210.09	0.06	209.81	0.06	209.89	0.06	0.44	0.25
		216	215.86	0.06	215.64	0.05	215.68	0.05	0.39	0.19
	Chc03 ^{6-FAM}	248	247.40	0.02	247.27	0.05	247.16	0.09	0.24	0.35
		250	250.55	0.05	250.40	0.07	250.33	0.06	0.37	0.23
	Chc02 ^{VIC}	250	249.75	0.03	249.59	0.08	249.47	0.07	0.28	0.31
		276	276.26	0.06	276.26	0.02	275.27	0.07	0.16	0.20

		279	279.21	0.03	279.21	0.08	279.17	0.07	0.17	0.23
	Chc04 ^{PET}	290	290.42	0.06	290.43	0.09	290.45	0.06	0.33	0.33
		294	294.24	0.05	294.27	0.05	294.22	0.09	0.19	0.24
<i>G. gracilis</i>	Gg173 ^{6-FAM}	162	162.11	0.03	162.09	0.10	161.86	0.05	0.29	0.40
		170	170.25	0.04	170.24	0.18	169.95	0.05	0.50	0.64
	Gg155 ^{PET}	201	200.76	0.06	200.51	0.08	200.53	0.04	0.40	0.24
		203	202.68	0.05	202.49	0.12	202.41	0.06	0.36	0.27
	2CT ^{NED}	171	171.11	0.06	171.09	0.07	171.22	0.10	0.20	0.34
		193	192.79	0.12	192.61	0.06	192.50	0.04	0.39	0.25
		199	198.70	0.13	198.56	0.13	198.51	0.03	0.44	0.32
		201	200.63	0.14	200.34	0.05	200.38	0.03	0.48	0.16
		203	202.58	0.14	202.33	0.08	202.32	0.03	0.51	0.20
		219	218.45	0.13	218.36	0.06	218.26	0.05	0.44	0.18
		228	228.24	0.15	228.21	0.10	228.22	0.09	0.44	0.31
		230	230.14	0.18	230.16	0.05	230.21	0.05	0.51	0.15

	Gg216 ^{6-FAM}	166	165.80	0.04	165.73	0.10	165.55	0.09	0.31	0.44
	Gg182 ^{VIC}	121	121.17	0.05	121.05	0.07	120.88	0.06	0.32	0.31
		131	130.64	0.07	130.69	0.08	130.59	0.07	0.38	0.40
		135	134.44	0.12	134.60	0.07	134.49	0.06	0.36	0.31
	Gg121 ^{PET}	203	203.65	0.02	203.45	0.13	203.46	0.04	0.4	0.37
		205	205.59	0.04	205.54	0.19	205.43	0.03	0.64	0.64
	Gg202 ^{NED}	168	166.99	0.05	166.91	0.12	166.75	0.11	0.35	0.40
<i>C. fornicata</i>	E14M1 ^{6-FAM}	157	157.50	0.04	157.36	0.12	157.46	0.11	0.33	0.41
		225	224.82	0.05	224.65	0.14	224.55	0.12	0.41	0.48
		383	383.37	0.04	383.17	0.12	383.11	0.08	0.46	0.43
	E15M2 ^{VIC}	114	113.99	0.03	113.67	0.02	113.71	0.08	0.35	0.24
		149	148.45	0.04	148.49	0.12	148.56	0.12	0.32	0.38
		310	310.41	0.08	310.34	0.11	310.43	0.10	0.36	0.43
	E2M11 ^{PET}	154	154.17	0.03	154.10	0.10	154.19	0.12	0.28	0.4
	E1M12 ^{NED}	228	228.32	0.03	228.10	0.10	228.12	0.12	0.39	0.39

		281	281.59	0.05	281.47	0.14	281.48	0.11	0.42	0.42
		319	319.11	0.05	318.92	0.05	318.90	0.10	0.33	0.29
<i>L. nigrescens</i>	Les1P16 ^{6-FAM}	307	307.24	0.05	307.35	0.09	307.50	0.10	0.35	0.36
		309	309.23	0.06	309.34	0.08	309.47	0.08	0.35	0.30
		326	326.47	0.13	326.21	0.09	326.34	0.10	0.48	0.34
		328	328.52	0.10	328.32	0.10	328.36	0.08	0.48	0.3
	Les2D25 ^{VIC}	222	221.77	0.08	221.51	0.06	221.60	0.07	0.44	0.25
		226	225.61	0.08	225.42	0.10	225.54	0.08	0.39	0.30
		228	227.47	0.05	227.43	0.09	227.51	0.10	0.27	0.36
		232	231.40	0.07	231.26	0.06	231.40	0.11	0.29	0.35
		234	233.37	0.09	233.25	0.08	233.39	0.09	0.40	0.43
	Les2D26 ^{PET}	135	134.81	0.06	134.95	0.10	135.04	0.07	0.37	0.29
		139	138.72	0.05	138.86	0.12	138.96	0.06	0.41	0.34
	Les1T3 ^{NED}	122	122.21	0.06	121.95	0.08	122.07	0.11	0.49	0.43
		135	134.76	0.06	135.03	0.05	135.05	0.06	0.46	0.20

		138	137.63	0.18	137.90	0.09	137.93	0.06	0.52	0.28
		141	140.50	0.11	140.73	0.07	140.78	0.07	0.50	0.28
		152	152.08	0.10	152.31	0.07	152.31	0.06	0.49	0.23
<i>S. muticum</i>	D10 ^{6-FAM}	212	211.99	0.07	211.78	0.13	211.75	0.11	0.37	0.37
		213	212.97	0.07	212.82	0.12	212.76	0.15	0.32	0.42
	C15 ^{VIC}	87	87.43	0.03	87.42	0.11	87.29	0.11	0.32	0.52
	D33 ^{PET}	289	288.84	0.04	288.83	0.05	288.72	0.09	0.14	0.31
	T29 ^{NED}	175	175.49	0.05	175.35	0.07	175.28	0.09	0.30	0.37

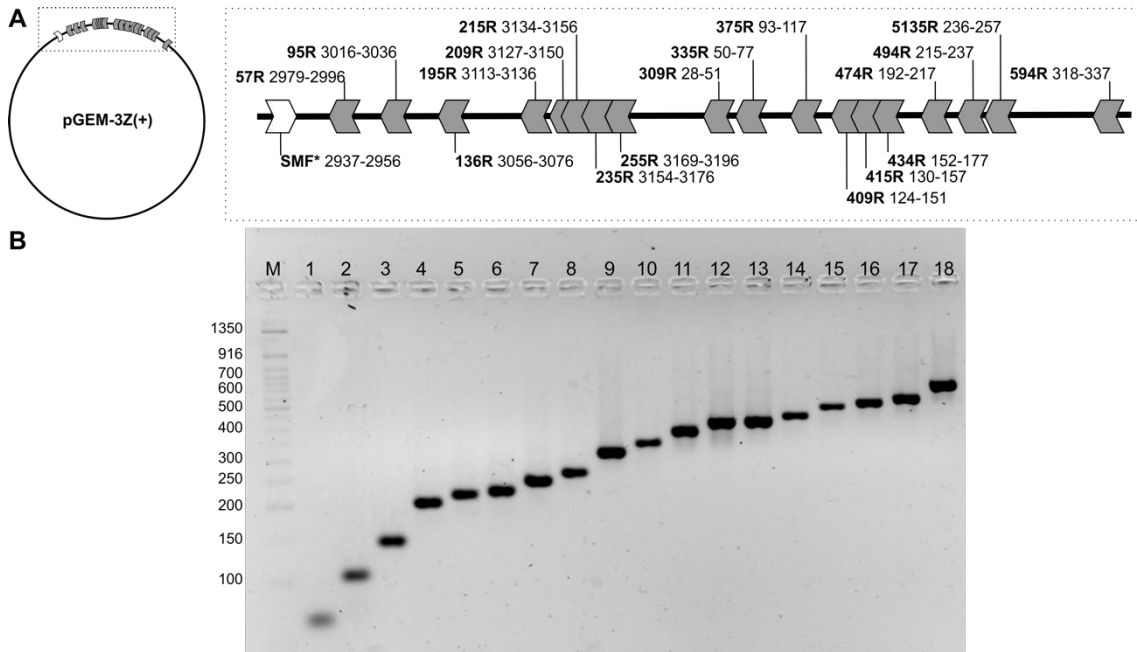
^a Allele size estimation was carried out on two different 3130XL Genetic Analyzer instruments (GA1 and GA2) using the SM594. Values in bold for GA1 denote mean sizes that are not statistically significant from those obtained with GeneScanTM 600LIZ; values in bold for GA2 denote mean sizes that are not statistically significant from those obtained using also the SM594 but on a different 3130XL Genetic Analyzer instrument (GA1).

^b Fluorescent label for each locus is indicated in the right superscript position.

^c Maximum difference among allele size estimates using the commercial size standard GeneScanTM 600LIZ and the in-house synthesized standard SM594.

^d Maximum difference among allele size estimates obtained from two different 3130X Genetic Analyzer instruments using the in-house synthesized standard SM594.

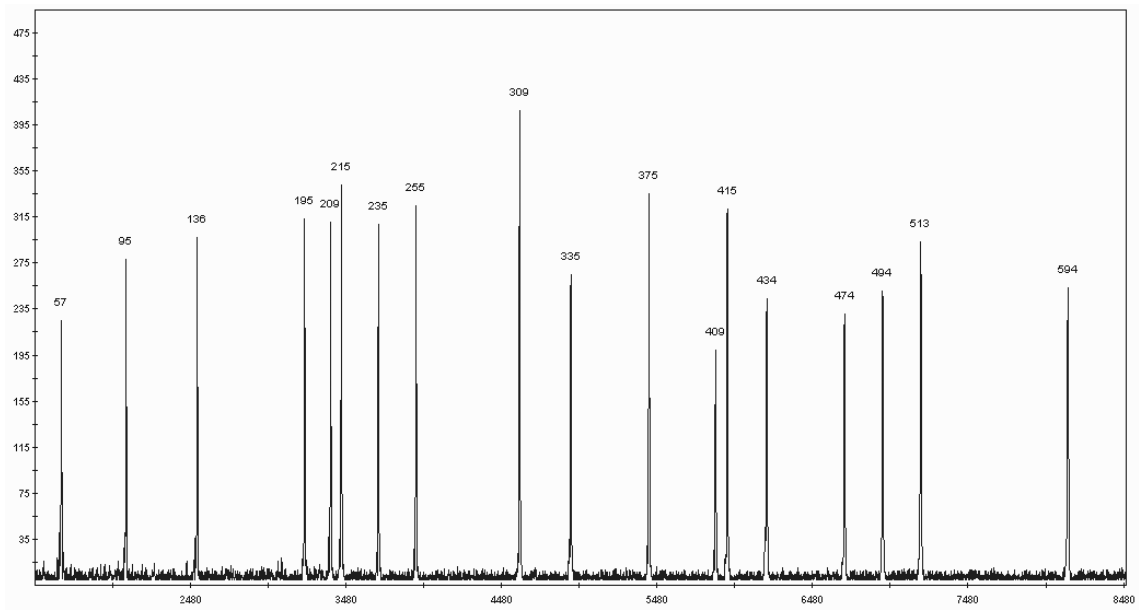
191 **FIGURE & LEGENDS**



192

193 Figure 1. (A) Map of the universal cloning vector pGEM®-3Z(+) showing the position
 194 of the 19 primers (white and grey arrows represent forward and reverse primers
 195 respectively). (B) Agarose gel of the 18 amplified PCR products; lane M corresponds to
 196 the commercial molecular weight standard 50 bp DNA Ladder (New England BioLabs
 197 Inc., Ipswich, MA, USA).

198



199

200 Figure 2. Chromatogram of the SM594 size standard on a 3130XL Genetic Analyzer.

201 Fragment size (bp) is indicated above each peak and it was estimated by co-

202 electrophoresis with the commercial size standard GeneScan™ 600LIZ®.

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