

1 **Editing of the urease gene by CRISPR-Cas in the diatom *Thalassiosira pseudonana***

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9

10 **Abstract**

11 **Background:** CRISPR-Cas is a recent and powerful edition to the molecular toolbox which allows  
12 programmable genome editing. It has been used to modify genes in a wide variety of organisms, but  
13 only two alga to date. Here we present a methodology to edit the genome of *T. pseudonana*, a  
14 model centric diatom with both ecological significance and high biotechnological potential, using  
15 CRISPR-Cas.

16 **Results:** A single construct wa assembled using Golden Gate cloning. Two sgRNAs were used to  
17 introduce a precise 37nt deletion early in the coding region of the urease gene. A high percentage of  
18 bi-allelic mutations ( $\leq 61.5\%$ ) were observed in clones with the CRISPR-Cas construct. Growth of bi-  
19 allelic mutants in urea led to a significant reduction in growth rate and cell size compared to growth  
20 in nitrate.

21 **Conclusions:** CRISPR-Cas can precisely and efficiently edit the genome of *T. pseudonana*. The use of  
22 Golden Gate cloning to assemble CRISPR-Cas constructs gives additional flexibility to the CRISPR-Cas  
23 method and facilitates modifications to target alternative genes or species.

24

25 **Keywords:** CRISPR-Cas, diatom, genome editing, urease, Golden Gate, *Thalassiosira pseudonana*

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## 28 **Background**

29 Diatoms are ecologically important microalgae with high biotechnological potential. Since their  
30 appearance about 240 million years ago [1], they have spread and diversified to occupy a wide range  
31 of niches across both marine and freshwater habitats. Diatom genomes have been shaped by  
32 secondary endosymbiosis and horizontal gene transfer resulting in genes derived from heterotrophic  
33 hosts, autotrophic endosymbionts and bacteria [2, 3]. They play a key role in carbon cycling [4], the  
34 food chain, oil deposition and account for about 20% of the world's annual primary production [5,  
35 6]. However, they are perhaps best known for their intricate silica frustules which give diatoms a  
36 range of ecological advantages and play a key role for carbon sequestration and silica deposition.

37 Several aspects of diatom physiology including the silica frustule, lipid storage and photosynthesis  
38 are being applied to biotechnology. Areas of high interest include nanotechnology [7], drug delivery  
39 [8], biofuels [9], solar capture [10] and bioactive compounds [11].

40 Given the ecological importance of diatoms and their applications for biotechnology, it is pivotal that  
41 the necessary tools are available to study and manipulate them at a molecular level. This includes  
42 the ability to replace, tag, edit and impair genes. A recent addition to the genetic tool box, CRISPR-  
43 Cas, allows double strand breaks (DSBs) to be introduced at specific target sequences. This adapted  
44 mechanism, used by bacteria and archaea in nature as a defence system against viruses, facilitates  
45 knock-out by the introduction of mutations through repair by error prone non-homologous end  
46 joining (NHEJ) or homologous recombination (HR). This requires both a Cas9 to cut the DNA and a  
47 sgRNA to guide it to a specific sequence. Further information on the history and application of  
48 CRISPR-Cas can be found in several excellent reviews [12–14]. Zinc-finger nucleases (ZFNs),  
49 meganucleases and transcription activator-like effector nucleases (TALENs) have also been used to  
50 induce double strand breaks. TALENs and CRISPR-Cas both bring flexibility and specificity to gene  
51 editing, however CRISPR-Cas is also cheap, efficient and easily adapted to different sequences by  
52 simply changing the 20nt guide sequence in the sgRNA.

53 So far, within the diverse group of algae, the diploid, pennate diatom *Phaeodactylum tricoratum*  
54 [15] and the haploid, green alga *Chlamydomonas reinhardtii* [16] have been subject to gene editing  
55 by CRISPR-Cas. NHEJ and HR have been used to repair DSBs following CRISPR-Cas or TALENs in *P.*  
56 *tricoratum*, introducing mutations into a nuclear coded chloroplast signal recognition particle [15],  
57 the urease gene [17] and several genes associated with lipid metabolism [18]. *Thalassiosira*  
58 *pseudonana* is a logical choice for CRISPR-Cas development. It is a model centric diatom with a  
59 sequenced genome (first eukaryotic marine phytoplankton to be sequenced [2]) and well-  
60 established transformation systems [19, 20]. The genus has multiple biotechnology applications [8,  
61 21, 22], and although gene silencing has been established, a method to easily and efficiently knock-  
62 out and edit genes and the entire genome would be highly advantageous. The genus *Thalassiosira* is  
63 among the top 10 genera of diatoms in the World's Ocean in terms of ribotype (V9 of 18S) diversity  
64 and abundance [23] and the species *T. pseudonana* is a model for understanding the mechanisms  
65 behind silicification [24–26].

66 Golden Gate cloning can add further flexibility to CRISPR-Cas methods as demonstrated in higher  
67 plants [27]. As a modular cloning system it allows different modules, including the sgRNA, to be  
68 easily interchanged or added [28]. As a result, new constructs can be made quickly, cheaply and  
69 efficiently for new or multiple targets. This extends to any aspect of the construct, including  
70 promoters, Cas9 variants and their nuclear localisation signals (NLS). As a result, construct  
71 alterations such as replacing constitutive promoters for inducible ones, exchanging the wildtype

72 Cas9 for a Cas9 nickase or changing the localisation signal to target other organelles can be easily  
73 carried out.

74 An increasing range of software tools are available for CRISPR-Cas, including programs that facilitate  
75 sgRNA target searches in a genetic locus of interest, estimate efficiencies of sgRNAs [29] and  
76 perform off-target predictions.

77 While off-target prediction tools tend to be species specific, there are tools that accept requests for  
78 a genome to be added to the list, or allow for a genome to be directly uploaded [30, 31]. The latter is  
79 particularly useful for less studied organisms, such as diatoms. The ability to combine several  
80 different aspects of sgRNA design can help to make an informed decision when choosing target sites  
81 for gene editing.

82 Our paper represents a proof of concept to demonstrate the feasibility of gene editing in the model  
83 diatom *T. pseudonana* using two sgRNAs to induce a precise deletion in the urease gene. Methods  
84 combine a flexible Golden Gate cloning approach with sgRNA design, which draws on several  
85 available online tools. This takes into account multiple factors, such as position within the gene in  
86 terms of both early protein disruption and presence in the coding region, DNA cutting efficiency and  
87 presence of restriction enzyme sites at the cut site. The latter, in combination with inducing a large  
88 deletion by targeting with two sgRNAs, allows easy screening of mutants through either the  
89 restriction enzyme site loss assay [32] or the PCR band-shift assay [33], respectively.

90

## 91 **Method**

### 92 **Strains and growth conditions**

93 *Thalassiosira pseudonana* (CCMP 1335) was grown in 24h light (100-140  $\mu$ E) at 20°C in half salinity  
94 Aquil synthetic seawater [34]. For routine growth, a 1mM nitrate concentration was used.

### 95 **5'RACE U6 promoter**

96 To identify the U6 small nuclear RNA (snRNA) in *T. pseudonana*, an NCBI blastn search was  
97 performed on the genome against the central conserved region of the U6 sequence. Two potential  
98 guanine (G) start sites were found downstream of a TATA box in the promoter. To identify the start  
99 site of the U6 snRNA and empirically determine the end of the promoter, 5' RACE was carried out as  
100 follows: 400ml of culture was grown to exponential phase ( $1 \times 10^6$  cells ml<sup>-1</sup>) and harvested. Small  
101 RNAs were extracted and enriched using a miRNeasy kit (Qiagen). 5' template switching oligo RACE  
102 was performed according to Pinto and Lindblad [35]. For oligos used see Table 1 (ref. numbers 1-3).  
103 RACE products were sequenced and results aligned to the genome to determine the end of the  
104 promoter.

105

### 106 **Plasmid construction using Golden Gate cloning**

107 Golden Gate cloning was carried out according to Weber et al. [28] and Belhaj et al. [33]. Bsal and  
108 Bpil sites were removed in a so-called "domestication" procedure using a Q5 site-directed  
109 mutagenesis (SDM) kit (NEB). For oligos used in SDM see Table 1 (ref. numbers 17-20). Bsal sites and  
110 specific 4nt overhangs for Level 1 (L1) assembly were added through PCR primers (Table 1). Plasmid  
111 DNA was extracted using a Promega mini-prep kit.

### 112 Golden Gate reactions

113 Golden Gate reactions for L1 and Level 2 (L2) assembly were carried out using the method specified  
114 in Weber et al. [28]. Forty fmol of each component was included in a 20 $\mu$ l reaction with 10 units of  
115 Bsal or Bpil and 10 units of T4 DNA ligase in 1x ligation buffer. The reaction was incubated at 37°C for

116 5 hours, 50°C for 5 minutes and 80°C for 10 minutes. Five µl of the reaction was transformed into  
117 50µl of NEB 5-alpha chemically competent *E. coli*.

#### 118 Level 0 assembly

119 The endogenous FCP promoter and terminator were amplified with GoTaq flexi (Promega) from  
120 domesticated pTpFCP/NAT [19] and the U6 promoter from gDNA (extracted with an Easy-DNA gDNA  
121 purification kit (Thermo Fisher)). Both promoters are associated with high expression levels. The U6  
122 promoter was amplified from the position -470 to -1 (the end of the promoter), cutting off a Bpil site  
123 and removing the need for additional SDM. For oligos, see Table 1 (ref. numbers 6-7 and 10-13).

124 Products were cloned into a pCR8/GW/TOPO vector (Thermo Fisher).

125 Domesticated human codon bias Cas9 from *Streptococcus pyogenes* with an N-terminal SV40 NLS  
126 and a C-terminal YFP tag was PCR-amplified using Phusion DNA polymerase (NEB) and L1 Cas9:YFP  
127 plasmid as a template. The PCR product was purified with a GFX PCR DNA and gel purification kit (GE  
128 Healthcare) and incubated for 20 minutes with Taq to add adenine overhangs before cloning directly  
129 into a pCR8/GW/TOPO vector. For oligos, see Table 1 (ref. numbers 8-9).

#### 130 Level 1 assembly

131 The FCP:NAT cassette was PCR-amplified using Phusion polymerase and the domesticated  
132 pTpFCP/NAT as a template, purified and inserted into a L1 pICH47732 destination vector. FCP  
133 promoter, Cas9 and FCP terminator L0 modules were assembled into L1 pICH47742. For oligos, see  
134 Table 1 (ref. numbers 4-5).

135 The sgRNA scaffold was amplified from pICH86966\_AtU6p\_sgRNA\_NbPDS [32] with sgRNA guide  
136 sequences integrated through the forward primers. Together with the L0 U6 promoter,  
137 sgRNA\_Urease 1 and sgRNA\_Urease 2 were assembled into L1 destination vectors pICH47751 and  
138 pICH47761, respectively. For oligos, see Table 1 (ref. numbers 14-16).

#### 139 Level 2 assembly

140 L1 modules pICH47732:FCP:NAT, pICH47742:FCP:Cas9YFP, pICH47751:U6:sgRNA\_Urease 1,  
141 pICH47761:U6:sgRNA\_Urease 2 and the L4E linker pICH41780 were assembled into the L2  
142 destination vector pAGM4723. Constructs were screened by digestion with EcoRV and sequenced.  
143 For oligos used in sequencing, see Table 1 (ref. numbers 27-35). See Figure 1 for an overview of the  
144 Golden Gate assembly procedure and the final construct.

145

#### 146 **sgRNA design for the urease gene knockout**

147 Two sgRNAs were designed to cut 37nt apart early in the coding region of the urease gene (JGI ID  
148 30193) to induce a deletion and frame-shift. Several programmes, explained below, were used to  
149 collect data and make an informed decision on sgRNA choice. Excel was used to combine, process  
150 and compare data.

#### 151 Selecting CRISPR-Cas targets and estimating on-target score

152 Twenty bp targets with an NGG PAM were identified and scored for on-target efficiency using the  
153 Broad Institute sgRNA design programme ([www.broadinstitute.org/rnai/public/analysis-tools/sgrna-](http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design)  
154 [design](http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design)), which utilises the Doench et al. [29] on-target scoring algorithm calculated from >1800  
155 empirically tested sgRNAs.

#### 156 Determining cut positions and cross referencing to restriction recognition sites

157 All restriction sites and their positions within the urease gene were identified using the Emboss  
158 restriction tool (<http://emboss.bioinformatics.nl/>). As the Broad Institute sgRNA design programme  
159 does not give the location of CRISPR-Cas targets within a gene, this was determined using Primer  
160 map ([http://www.bioinformatics.org/sms2/primer\\_map.html](http://www.bioinformatics.org/sms2/primer_map.html) [36]). The cut site position (3nt  
161 upstream of the start of the PAM sequence) was calculated for each sgRNA depending on sense or  
162 anti-sense strand placement. All predicted CRISPR-Cas cut sites were cross-referenced to restriction  
163 recognition sites.

#### 164 Reverse complement of antisense strand CRISPR-Cas targets

165 The reverse complement (RC) was found for each CRISPR-Cas target using the programme:  
166 [http://www.bioinformatics.org/sms2/rev\\_comp.html](http://www.bioinformatics.org/sms2/rev_comp.html) [36]. In the final spreadsheet (Supplementary  
167 Figure 1), if a target was located on the anti-sense strand, the RC was shown for the 'sense strand  
168 sequence' column. This allows the sgRNA to be easily searched within the original gene sequence.

#### 169 Determine position of CRISPR-Cas cut sites in relation to coding region

170 An array was made with start and end positions for each exon/intron. Cut site positions were  
171 compared to exon/intron ranges and the relevant exon/intron returned if the data overlapped.  
172 The final spreadsheet gives data on CRISPR-Cas target sequences and their sense sequence (if  
173 located on the antisense strand), location of target (relative to the sense strand), predicted CRISPR-  
174 Cas cut site, first nucleotide of the target, PAM sequence, location (i.e. exon, intron), strand, sgRNA  
175 score and restriction recognition sites overlapping the cut site. The table (Supplementary Figure 1)  
176 was sorted to prioritise sgRNAs by starting base prioritising guanine, sgRNA score, position within  
177 the gene and interaction with restriction recognition sites.

#### 178 Predicting off-targets

179 The full 20nt target sequences and their 3' 12nt seed sequences were subjected to a nucleotide  
180 BLAST search against the *T. pseudonana* genome. Resulting homologous sequences were checked  
181 for presence of an adjacent NGG PAM sequence at the 3' end. The 8nt sequence outside of the seed  
182 sequence was manually checked for complementarity to the target sequence. In order for a site to  
183 be considered a potential off-target the seed sequence had to match, a PAM had to be present at  
184 the 3' end of the sequence and a maximum of three mismatches between the target and sequences  
185 from the blast search were allowed outside of the seed sequence.

186 Off-targets were also checked using the EuPaGDT program [31], which checks for up to 5  
187 mismatches in the 20nt target sequence and the CasOT program [30], which uses flexible  
188 parameters for identifying off-target sequences. Parameters were set to check for an NGG PAM,  
189 complete complementarity within the 12nt seed sequence and up to 3 mismatches outside of the  
190 seed region.

191

#### 192 **Transformation and selection**

193 Using the Poulsen et al. [19] method, transformations were carried out in triplicate with the CRISPR-  
194 Cas construct, pTpfcp/nat (positive control) and water (negative control). Five x 10<sup>7</sup> cells in  
195 exponential phase were used per shot with a rupture disc of 1350psi and a 7cm flight distance.  
196 Following transformation, cells were rinsed into 25ml of media and left to recover for 24 hours  
197 under standard growth conditions. Cells were counted using a Coulter counter (Beckman) and 2.5 x  
198 10<sup>7</sup> cells from each transformation were spread onto 5, ½ salinity Aquil 0.8% agar plates (5 x 10<sup>6</sup>  
199 cells/ plate) with 100µg ml<sup>-1</sup> nourseothricin. Plates were incubated under standard conditions for  
200 two weeks. Remaining sample was diluted to 1 x 10<sup>6</sup> cell ml<sup>-1</sup> in media and supplemented with  
201 nourseothricin to final a concentration of 100µg ml<sup>-1</sup> for liquid selection. Liquid selection cultures  
202 were maintained under standard growth conditions with 100µg ml<sup>-1</sup> nourseothricin. Colonies were  
203 picked and transferred to 20µl of media. Ten µl from each colony was transferred to 1ml of selective  
204 media for further growth. The remaining sample was used in screening.

205 To isolate sub-clones from colonies which screened positive for mutations, 100µl of cells at  
206 exponential phase were streaked onto ½ salinity Aquil 0.8% agar plates with 100µg ml<sup>-1</sup>  
207 nourseothricin.

#### 208 **Screening clones and cultures**

209 Ten µl from each colony or culture from liquid selection, was spun down and supernatant removed.  
210 Cells were re-suspended in 20µl of lysis buffer (10% Triton X-100, 20mM Tris-HCl pH8, 10mM EDTA),

211 kept on ice for 15 minutes then incubated at 95°C for 10 minutes. One  $\mu$ l of lysate was used in Taq  
212 PCR to amplify the CRISPR-Cas targeted fragment of the urease gene. Clones were also screened for  
213 Cas9 and NAT. For PCR primers, see Table 1 (ref. numbers 21-26). PCR products were run on an  
214 agarose gel to check for the lower MW band associated with a double-cut deletion in the urease  
215 gene and for the presence of Cas9 and NAT. Urease PCR products were also digested with BsaI and  
216 HpaII to determine if the restriction recognition sites, which overlap the cut sites, had been mutated.  
217 PCR products were sent for sequencing to confirm mutations.

## 218 **Growth experiments**

219 Knockout and wild-type (WT) cultures were nitrate depleted by growing cells in nitrate free media  
220 until cell division stopped and quantum yield of photosynthesis (Fv/Fm measured on the Phyto-  
221 PAM-ED) dropped below 0.2. Cultures were then transferred in triplicate at a final concentration of  
222  $2.5 \times 10^4$  cells  $\text{ml}^{-1}$  into 25ml of media with either 1mM sodium nitrate or 0.5mM urea. Cell count  
223 and mean cell size were measured once a day using a Coulter counter. Fv/Fm measurements were  
224 also taken daily. Growth rates were calculated using  $\mu = \frac{\ln_2 - \ln_1}{T_2 - T_1}$ , where T is a time point  
225 corresponding to exponential growth and  $\ln$  is the natural log of cell counts  $\text{ml}^{-1}$ . Analysis of variance  
226 with Tukey's pairwise comparison was used to compare both growth rates and cell size at the end of  
227 exponential phase between samples.

228

## 229 **Results and discussion**

### 230 **sgRNA design**

231 The two CRISPR-Cas targets with the highest on target scores (0.5 and 0.79), containing a predicted  
232 cut site over a restriction site and occurring early in the coding region, were chosen. sgRNAs were  
233 designed to cut 37nt apart at positions 138 and 175 within the urease gene. Both targets started  
234 with a G for polymerase III transcription (Figure 2). No off-target sites were predicted for sgRNAs  
235 designed for either of the two CRISPR-Cas target sequences.

236

### 237 **Constructing the CRISPR-Cas plasmid using the Golden Gate cloning method**

238 A single CRISPR-Cas construct was made using Golden Gate cloning (Figure 1). The construct  
239 included the NAT selectable marker and Cas9:YFP driven by an endogenous FCP promoter for high  
240 expression and two U6 promoter-driven sgRNAs. RNA polymerase III U6 promoters are a popular  
241 choice for expression of sgRNAs in CRISPR-Cas [15, 27, 37–39]. RACE products showed that the U6  
242 promoter ended 23nt after the TATA box. As a standardised, efficient, modular system, Golden Gate  
243 cloning gives a high level of flexibility to the CRISPR-Cas method and bypasses the need for co-  
244 transformation as it enables assembly of multiple expression units, such as Cas9 and sgRNAs, into a  
245 single vector backbone. Multiple sgRNA modules can be incorporated into the construct to target  
246 several genes or whole pathways. In human cells, up to 7 sgRNAs have been successfully assembled  
247 and expressed from a single construct created using the Golden Gate cloning method [39]. Golden  
248 Gate has also proved successful for building constructs for genome editing in higher plants using  
249 both TALENs [41] and CRISPR-Cas [33, 37].

250 In this study, only the promoters and target sequences are specific to *T. pseudonana*, which  
251 demonstrates how simple it can be to apply this method to a new species using the Golden Gate  
252 system. The *S. pyrogenes* Cas9 with a human codon bias, shown previously to work in higher plants  
253 [32, 33, 37], carries a SV40 NLS, which follows a canonical sequence found throughout eukaryotes,  
254 including *T. pseudonana*.



255 The long term effects from off-target mutations introduced through CRISPR-Cas are currently  
256 unknown, therefore it may be advantageous for future work to remove CRISPR-Cas constructs from  
257 mutants. Adding a yeast CEN6-ARSH4-HIS3 sequence to plasmids allows autonomous replication in  
258 diatoms and expression of genes without random integration into the genome [20]. Furthermore,  
259 removing selection leads to plasmids being discarded. By expressing CRISPR-Cas genes and selective  
260 markers on a removable episome, mutations could be introduced without integration of the  
261 plasmid. CRISPR-Cas constructs could then be expelled by removing selection. As well as  
262 considerations for long term off-target effects, this could also be advantageous for studies and  
263 applications which are sensitive to the presence of transgenes.

#### 264 **Selecting and screening for mutations in the urease gene**

265 The transformation efficiency with the CRISPR-Cas construct was on average 41.5 colonies  $\mu\text{g}^{-1}$   
266 plasmid (13.35-66.65 colonies  $\mu\text{g}^{-1}$  plasmid). Thirty three colonies were screened by PCR and  
267 sequencing of the targeted urease gene fragment.

268 Four colonies showed mutations in the urease gene. All colonies screened positive for NAT but only  
269 the four colonies with mutations screened positive for Cas9, suggesting that once the Cas9 and  
270 sgRNAs are present there is a high chance of inducing mutations in the target gene. The lack of Cas9,  
271 which accounts for a third of the construct, in the majority of colonies was potentially caused by  
272 shearing of the plasmid during microparticle bombardment [38] from either mechanical force or  
273 chemical breakdown [42].

274 Of the four primary colonies which screened positive for mutations (Figure 2), one (M4) showed a  
275 single band with a 37nt deletion between the two sgRNA cut sites which suggests that both copies of  
276 the urease gene contain the deletion giving a bi-allelic mutant. Two colonies (M2 and M3) produced  
277 two bands following PCR: a WT higher MW band and a lower MW band with the 37nt deletion,  
278 confirmed by sequencing (Figure 2). The fourth colony (M1) showed a single band associated with  
279 the WT urease, however sequencing showed two products: a WT urease and a mutant urease with a  
280 4nt deletion at the first sgRNA cut site. A mixture of PCR products may be due to a mono-allelic  
281 mutation, in which one allele is WT and the other displays a mutation. It can also be due to colony  
282 mosaicism where a colony contains a mixture of cells with WT and mutant alleles due to mutations  
283 occurring after transformed cells have started to divide. Both mono-allelic mutants and mosaic  
284 colonies have been observed in *P. tricornutum* [15, 18].

285 To determine if the colonies were mosaic or mono-allelic, cells from mutant clones producing mixed  
286 PCR products were spread onto selective plates to isolate single sub-clones. Thirty four sub-clones  
287 from each clone were screened by PCR (a few examples are presented in Figure 2). Two clones (M2  
288 and M3) were mosaic with a mixture of sub-clones showing either a single band corresponding to  
289 the expected deletion (61.5% and 25%, respectively), two bands associated with the WT and  
290 expected deletion (25.5% and 28.1%, respectively) or a single band corresponding to the WT urease  
291 fragment (13% and 46.9% respectively). For each of the two clones PCR amplicons from three  
292 putative bi-allelic sub-clones were sequenced (Figure 2). Four out of six (M2\_9, M2\_10, M3\_10 and  
293 M3\_11) showed the expected 37nt 'clean' deletion without any additional mutations. Precise  
294 deletions, such as this, using 2 sgRNAs have previously been generated with high efficiency [37, 43],  
295 and allow a large degree of control over the mutation. Two of the sub-clones (M3\_9 and M2\_12)  
296 showed one allele with the expected 37nt deletion and the other with an additional deletion at the  
297 2<sup>nd</sup> sgRNA cut site. In addition, M2\_12 showed a C->G SNP within the sgRNA1 target site. Sub-clones  
298 derived from the M1 clone showed WT and 4nt deletion PCR amplicons as seen in the original clone,  
299 suggesting that this clone may have a mono-allelic mutation.

300 Using CRISPR-Cas with one sgRNA can introduce a variety of indels into a locus of interest via the  
301 error-prone NHEJ DNA repair mechanism [15]. Cas9 preferentially cuts DNA three nucleotides  
302 upstream of the PAM sequence in the seed region [44] and the NHEJ mechanism either repairs a  
303 double strand break perfectly or indels are introduced. If cut sites are not cleaved at the same time,  
304 when using two sgRNAs, mutations at each site rather than removal of the fragment in between  
305 target sites may occur [37]. In this study, however, we report a high occurrence of bi-allelic mutants  
306 with precise deletions between the CRISPR-Cas cut sites, suggesting that the Cas9/sgRNA complex is  
307 cutting efficiently and DNA ends tend to be repaired perfectly. This allows control over the  
308 introduced mutations and gives the chance to avoid introducing in-frame indels.

309 Restriction digest (results not shown) and sequencing (Figure 2) demonstrated loss off the BclI site  
310 in all knock-out clones and HpaII in M2\_12 and M1 as a deletion downstream of the cut site is  
311 required to remove the HpaII site. This demonstrates that restriction screening can be a valuable  
312 tool, however in this case screening for a deletion based band shift by PCR was an efficient way of  
313 identifying bi-allelic mutants especially given the limited sgRNA/restriction site interactions available  
314 for this gene.

315 As well as clones from plate selection, one culture from liquid selection (LM1; population of cells  
316 transferred to liquid selective media after transformation), showed a single band associated with the  
317 bi-allelic 37nt deletion following PCR. This was confirmed by sequencing (Figure 2). PCR screening  
318 following growth of LM1 in urea showed only the lower MW band product (results not shown),  
319 giving further evidence for a bi-allelic mutation from a population of cells. As small volumes of cells  
320 are transferred to fresh media when passaging this may have isolated bi-allelic mutants.

#### 321 **Growth experiments with mutants**

322 Urease catalyses the breakdown of urea to ammonia allowing it to be used as a source of nitrogen  
323 [45]. Sub-clones from different cell-lines with 37 or 38nt deletions were tested for knock-out of the  
324 urease gene by looking for a lack of growth when supplemented with urea as the sole nitrogen  
325 source.

326 Cells were nitrogen starved and then transferred to media with either nitrate or urea. Cell counts,  
327 cells size and Fv/Fm were measured daily for 7 days. Negative controls to account for any  
328 background nitrate in the media were also run in which no nitrate or urea was added for WT  
329 cultures.

330 Four putative bi-allelic mutants (LM1, M4, M2\_10 and M3\_9) were tested along with WT and the  
331 mono-allelic M1\_10 over two growth curve experiments. Both LM1 from liquid selection ( $p=0.0029$ )  
332 and the sub-clone M3\_9 ( $p=0.0000001$ ) showed a significant decrease in growth rate in urea  
333 compared to nitrate (Figure 3) as well as a significant 13-18% decrease in cell size (Figure 4;  
334  $p=0.0029$  and  $p=0$ , respectively). The latter was also apparent with light microscopy (results not  
335 shown). Mutants in urea could be easily discerned even without cell counts, as cultures appeared  
336 much paler in colour. M4 did not show a difference in growth rate but did show a significant  
337 decrease in cell size ( $p=0.038$ ). The mono-allelic mutant M1\_10, displayed higher growth in urea and  
338 similar growth to the WT control (Figure 3). This correlates with results from Weyman et al. [17]  
339 which showed that despite a reduced protein concentration, a mono-allelic urease knock-out was  
340 able to grow in urea. M2\_10 which screened as a bi-allelic mutant prior to growth experiments  
341 showed a smaller but still significant decrease in growth rate ( $p=0.0014$ ) (Figure 3) and cell size  
342 ( $p=0.0039$ ) (Figure 4). PCR screening of the urease gene following growth in nitrate and urea showed  
343 the expected bi-allelic mutation for LM1, M3\_9 and M4, however M2\_10 also showed a faint WT  
344 band in nitrate and a strong WT band in urea (Figure 5). This suggests that M2\_10 was mosaic, with  
345 cells containing a functional urease out-competing those with a mutant urease. Given that only a



346 faint WT band was present after growth in nitrate this suggests that the majority of the cells from  
347 the sub clone contained the mutant urease, initially accounting for the majority of growth and  
348 resulting in a lower but still significant decrease in growth rate.

349 Knock-out of the urease gene in the diatom *P. tricornutum* prevents growth in urea [17]. Urease  
350 mutants in this study still grew in urea but with a lower growth rate and reduced cell-size,  
351 characteristics which are associated with nitrogen limitation in diatoms [46, 47] rather than nitrogen  
352 starvation. Mutant cell-lines in urea grew to the same density as the same cell-lines in nitrate, but at  
353 a lower rate (Figure 3). As nitrogen is an essential nutrient for growth, this suggests that mutant cells  
354 in urea still have access to nitrogen, but lower growth rates and cell-size indicates that nitrogen may  
355 not be as readily available compared to cells grown with nitrate. Controls in nitrogen free media  
356 showed very little growth which suggests that growth of mutants in urea was not due to residual  
357 nitrate in the culture. It is unlikely that random integration of the CRISPR-Cas plasmid is responsible  
358 for reduced growth rate in mutants as all four individual mutant cell-lines display increased growth  
359 rates when grown in nitrate. Therefore it seems likely that impaired growth of urease mutants in  
360 urea is due to a reduction in function of the urease gene.

361 There are a few possible reasons why a mutation in the urease gene appears to lead to nitrogen  
362 limitation rather than nitrogen starvation as seen in *P. tricornutum*. Cells may be able to access  
363 nitrogen from another source, separate to the breakdown of urea via urease. Some algae have an  
364 alternative pathway for breakdown of urea but this has only been found in Chlorophyceae [48] and  
365 blast searches show no evidence of urea carboxylase or allophanate hydroxylase, the enzymes  
366 involved in this pathway, in *T. pseudonana*.

367 The urease gene may still be active but with lower functionality. In *T. pseudonana* urease is  
368 modelled to be 807 amino acids. Urease consists of multimers of three sub-units: gamma, beta and  
369 alpha, which in TP are translated as one protein. The alpha sub-unit contains the active site which  
370 catalyses the breakdown of urea to ammonia [45]. The gamma subunit has no known enzymatic  
371 function [49] but may play a role in quaternary structure and stability [45, 50].

372 Translations of urease sequences with both 37 and 38nt deletions show frame shifts and early stop  
373 codons after the deletion in the gamma sub-unit, leading to major disruption of the gamma sub-unit,  
374 nonsense down-stream and short products of 24 or 44 amino acid residues (Figure 6). Since all  
375 mono-clonal bi-allelic mutants tested for growth in urea had either two alleles with a 37nt deletion  
376 or both a 37 and 38nt deletion, it was predicted that the urease gene would no longer be functional.  
377 However, several mechanisms exist in eukaryotes which can allow translation of the protein from  
378 start codons later in the coding region. These include leaky initiation, re-initiation of ribosomes and  
379 internal ribosome entry sites (IRES) [51]. IRES have been shown to become active in yeast following  
380 amino acid starvation [51]. If an in-frame translation can occur after the deletion at an IRES or via a  
381 mechanism such as re-initiation then the active site located in the alpha-subunit could still be  
382 present. The first in-frame ATG after the deletion would start translation of the protein just before  
383 the beta sub-unit, leading to an N-terminal truncated protein without the gamma sub-unit but with  
384 both the beta and alpha sub-units (Figure 6). Earlier start codons are predicted to result in non-sense  
385 and early stop codons.

386 The 5' end of the urease coding region was targeted to induce a frame shift and disrupt the protein  
387 early on, however it may be better to target the active site or entirely remove the gene. Precise  
388 deletions larger than a gene using CRISPR-Cas and two sgRNAs have been previously demonstrated  
389 [43].

## 390 **Conclusions**

391 CRISPR-Cas can precisely and efficiently edit the genome of the diatom *Thalassiosira pseudonana*.  
392 Twelve percent of initial colonies and 100% which screened positive for Cas9 showed evidence of a  
393 mutation in the urease gene, with many sub-clones showing precise bi-allelic 37nt deletions from  
394 two sgRNA DSBs. Screening for the deletion by PCR allowed efficient identification of bi-allelic  
395 mutants and Golden Gate cloning allowed easy assembly of a plasmid for CRISPR-Cas. This included  
396 adapting the system for *T. pseudonana* by including endogenous promoters and two specific sgRNAs.  
397 Due to the flexible modular nature of the cloning system, this can be easily adapted for other genes  
398 in *T. pseudonana*. A variety of available online tools were used to design two sgRNAs that would  
399 target the early coding region of the urease gene. A reduced growth rate and cell-size phenotype  
400 was seen in mutant cell-lines grown in urea compared to nitrate, suggesting that function of the  
401 urease may have been impaired rather than removed or an alternative source of nitrogen was  
402 available.  
403 As potentially the most important tool in gene editing to date, CRISPR-Cas is fast becoming a key  
404 method in the molecular toolbox for a large variety of organisms. This efficient method has huge  
405 potential for future work from both an ecological and biotechnology perspective in *T. pseudonana*  
406 and can potentially be easily adapted for many other algal species.

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411 RACE for identifying the U6 promoter.

412

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532

533 **Figure captions**

534 **Table 1.** Oligonucleotides used in this study. Ref. N<sup>o</sup> 1-3: oligos used in 5' RACE [35]. Ref. N<sup>o</sup> 4-16:  
535 primers for Golden Gate cloning, BsaI sites are underlined, 4nt overhangs are shown in *italics*, and  
536 sgRNA targets are shown in **bold**. Upper case indicates complement to the template. Ref. N<sup>o</sup> 17-20:  
537 primers for SDM, lower case indicates base change. Ref. N<sup>o</sup> 21-26: primers for screening  
538 transformants. Ref. N<sup>o</sup> 27-35: primers for sequencing the CRISPR-Cas construct.

539 **Figure 1.** Overview of level 1 and level 2 Golden Gate cloning for assembly of the CRISPR-Cas  
540 construct pAGM4723:TpCC\_Urease. Level 1 assemblies of pICH47742:FCP:Cas9YFP and  
541 pICH47751:U6:sgRNA\_Urease 1 are shown. BsaI or BpiI restriction enzymes cut outside the  
542 recognition site leading to specific 4nt overhangs which are complementary to adjacent modules,  
543 allowing several modules to be accurately assembled in one reaction. Complementary 4nt  
544 sequences are colour coded to indicate adjacent modules.

545 **Figure 2.** Screening by PCR and sequencing. Expected sgRNA cut indicated by ↓. PCR of targeted  
546 urease fragments from primary clones show a single higher MW band for M1 and WT, a lower MW  
547 band associated with the expected 37nt deletion for M4 and LM1 and two bands for M2 & M3.  
548 Sequence alignments of urease products with mutations are shown for primary clones. A few  
549 examples of PCR products from sub-clones are shown. Primary clones M2 and M3 appear to be  
550 mosaic with sub-clones containing bi-allelic and mixed products corresponding to full length urease  
551 and the lower MW band associated with the deletion. Sequence alignments from mono-allelic sub-  
552 clone M1\_9 and bi-allelic sub-clones from M2 and M3 are shown.

553 **Figure 3.** Growth rate of WT and mutant urease cell lines from two separate growth experiments (1)  
554 & (2). The WT cell line was grown in nitrate free (white), nitrate (dark grey) and urea (light grey)  
555 enriched media. Mutant cell lines were grown in nitrate or urea enriched media. Growth rate  
556 (division day<sup>-1</sup>) was measured in exponential phase and rates compared using analysis of variance  
557 with Tukey's pairwise comparisons.

558 **Figure 4.** Mean cell size (μm) measured at the end of exponential phase for WT and mutant cultures  
559 across two growth experiments (1) & (2). Cells were grown with nitrate (dark grey) or urea (light  
560 grey) as the sole nitrogen source. Cell size was compared using analysis of variance with Tukey's  
561 pairwise comparisons.

562 **Figure 5.** PCR of the targeted urease fragment following growth of WT and mutant cell lines in  
563 nitrate or urea. NEB 100bp ladder (1), WT in nitrate (2) and urea (3), M2\_10 in nitrate (4) and urea  
564 (5) and M3\_9 in nitrate (6) and urea (7).

565 **Figure 6.** Translated WT urease (a), frame 3 (b) and frame 1 of urease with the expected 37nt  
566 deletion (c) and frame 1 of urease with a 38nt deletion (d). Position of deletion indicated by ↓. The  
567 model WT protein contains 807aa. The figure shows the initial 260 amino acids for a and c including  
568 the start of the alpha sub-unit. Translations are identical for the unshown segments. Gamma (pink),  
569 Beta (green) and Alpha (blue) sub-units are highlighted in order. Expected start codon (red) and  
570 upstream out-of-frame start codons (grey) are highlighted.

571 **Supplementary Figure 1.** Screenshot of the final spreadsheet for choosing sgRNAs.



Name	Sequence	Ref. No
GS U6 R	AGGTTTGCTTCTCTTCGATTATG	1
TSO	GTCGCACGGTCCATCGCAGCAGTCACAGGGGG	2
U sense	GTCGCACGGTCCATCGCAGCAGTC	3
Fcp:Nat F	<u>tggtctcaggag</u> CTCGAGGTCGACGGTATC	4
Fcp:Nat R	<u>aggtctcaagcg</u> CGCAATTAACCCTCACTAAAGG	5
FCP prom F	<u>tggtctcaggag</u> AGCTTGCGCTTTTCCGAG	6
FCP prom R	<u>aggtctca</u> catTTTGGTATTGGTTTGGTAAATCAG	7
Cas9:YFP F	<u>aggtctca</u> aATGGACAAGAAGTACTCCATTGG	8
Cas9:YFP R	<u>aggtctca</u> aagcTCACTTGTACAGCTCGTCCATG	9
FCP term F	<u>aggtctcagctt</u> ATACTGGATTGGTGAATCAATG	10
FCP term R	<u>tggtctcaagcg</u> GAGAACTGGAGCAGCTAC	11
U6 prom F	<u>cggctcaggag</u> CTTCATCAAGAGAGCAACCA	12
U6 prom R	<u>aggtctca</u> ACAATTTTCGGCAAACCGT	13
Urease sgRNA1 F	<u>aggtctcattgtgtcgtaatcaagtattgccg</u> GTTTTAGAGCTAGAAATAGCAAG	14
Urease sgRNA2 F	<u>aggtctcattgtgttccgatctaattgccat</u> GTTTTAGAGCTAGAAATAGCAAG	15
Urease sgRNA R	<u>tggtctcaagcg</u> TAATGCCAACTTTGTACAAG	16
FCP prom SDM F	TCCGCGGCAGaTCTCTGTCG	17
FCP prim SDM R	AGAAGTACCGTGTGTTGTCAGTG	18
NAT SDM F	CGACACCGTaTTCCGCGTCAC	19
NAT SDM R	GTGGTGAAGGACCCATCCAG	20
Cas9 screen F	CCGAGACAAGCAGAGTGGAAG	21
Cas9 screen R	AGAGCCGATTGATGTCCAGTTC	22
NAT screen F	ATGACCACTCTTGACGACAC	23
NAT screen R	TTGATTCACCAATCCAGTATGC	24
Urease screen F 1	AAACAGACCACCTTCACCTC	25
Urease screen R	CTCCACCTGTACGTCTCG	26
Fcp seq F	CCATAAGTCAACGGCTCCAATC	27
NAT seq F	CTCTTGACGACACGGCTTAC	28
Cas9 seq 1 F	CATTACGGACGAGTACAAGGTG	29
Cas9 seq 2 F	TGAACACGGAGATCACCAAAG	30
Cas9 seq 3 F	CTTCTTGACAATGAGGAGAAC	31
Cas9 seq 4 F	CAAACCTGATCACACAACGGAAG	32
YFP FcpT seq F	ACTACCTGAGCTACCAGTCC	33
sg2 seq R	GTTTCCGATCTAATGTCCAT	34
sg1 seq F	TGTGTCGTAATCAAGTATTGC	35

Table 1

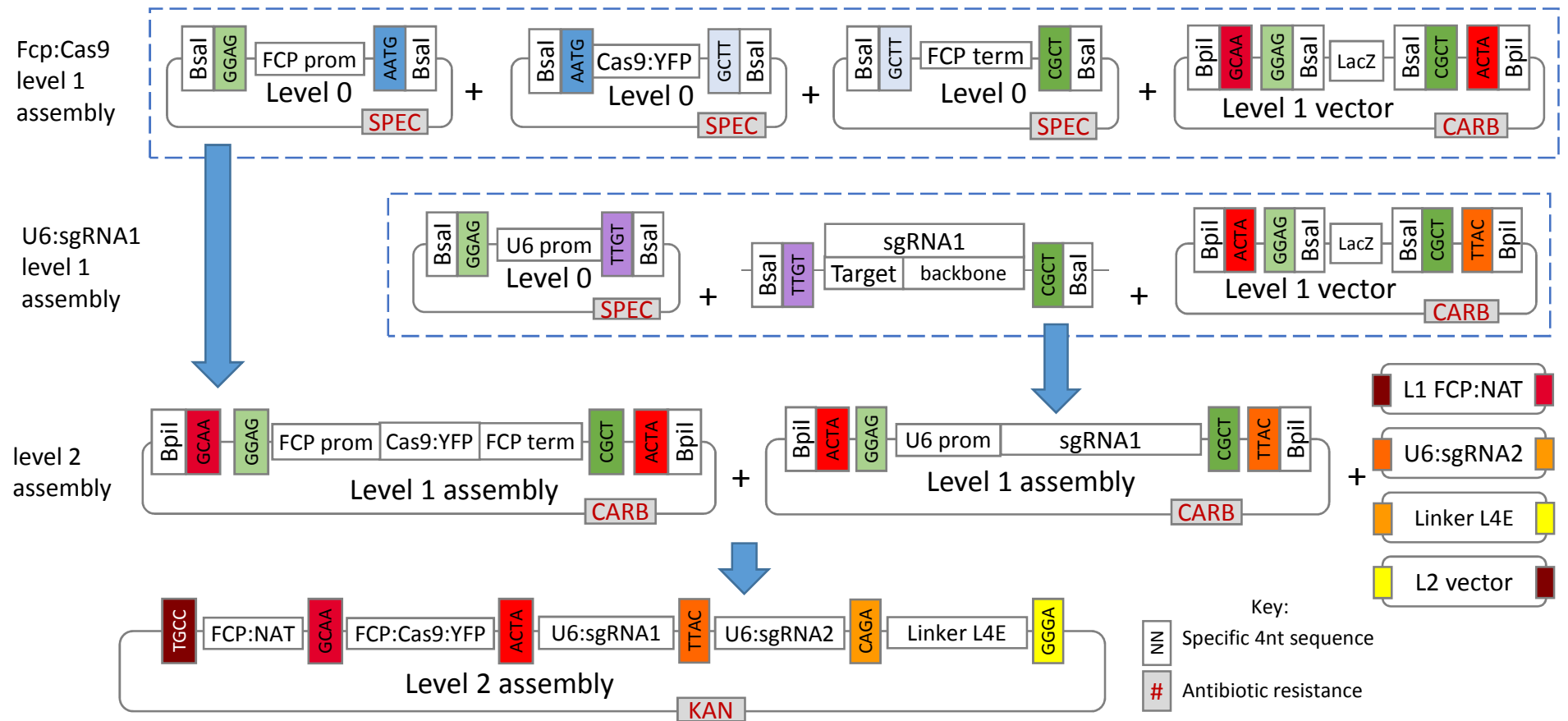
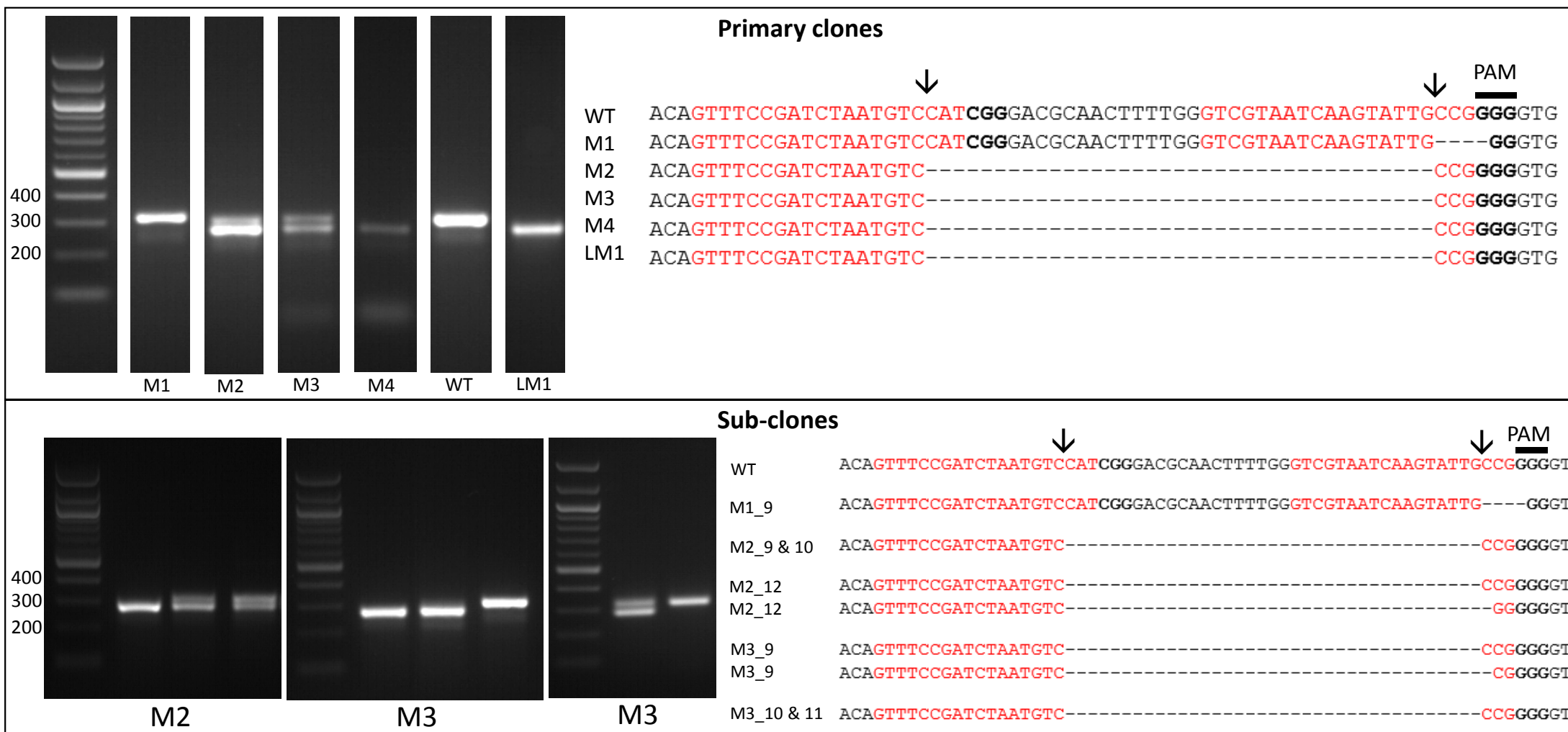


Figure 1



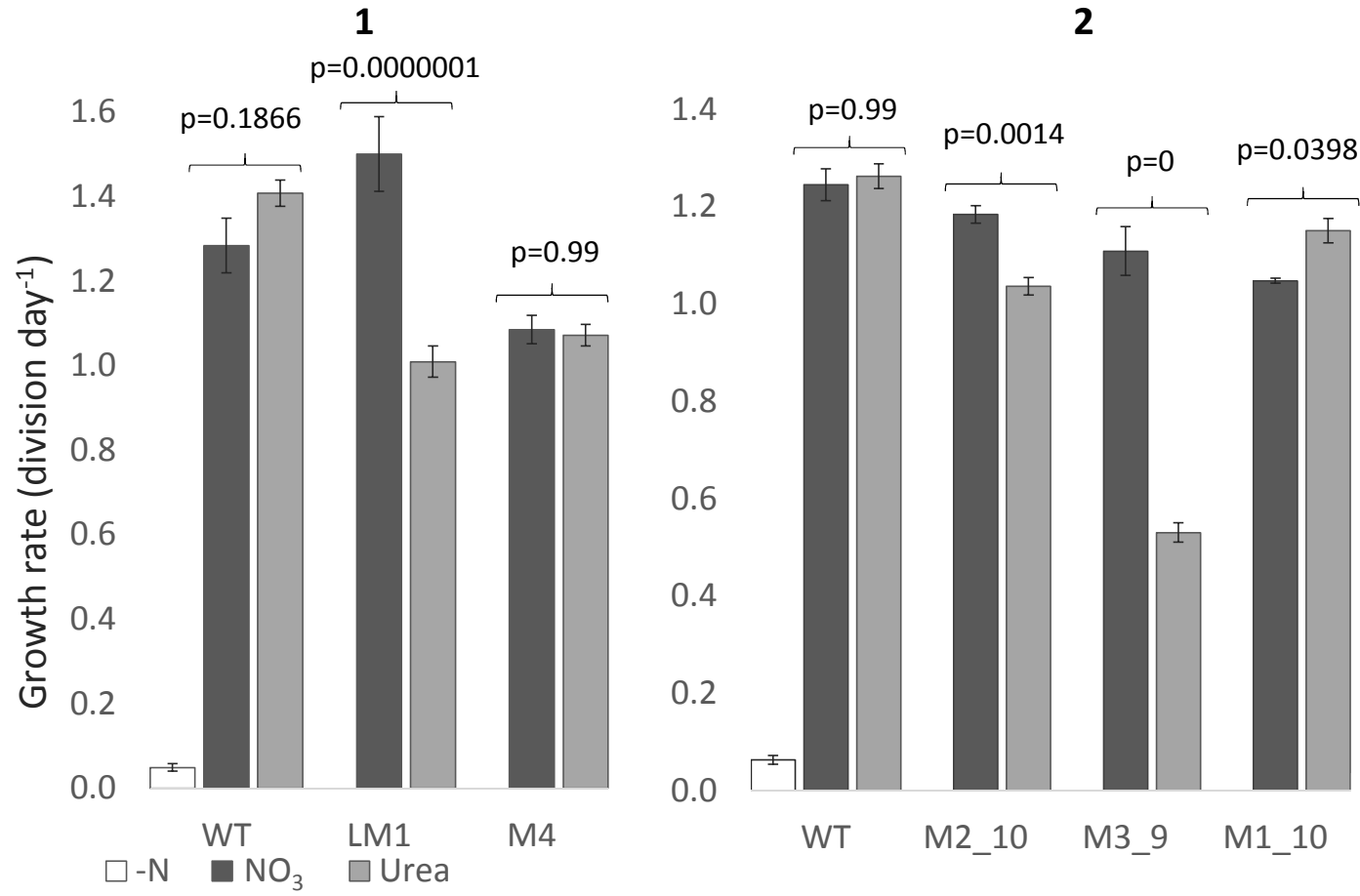


Figure 3

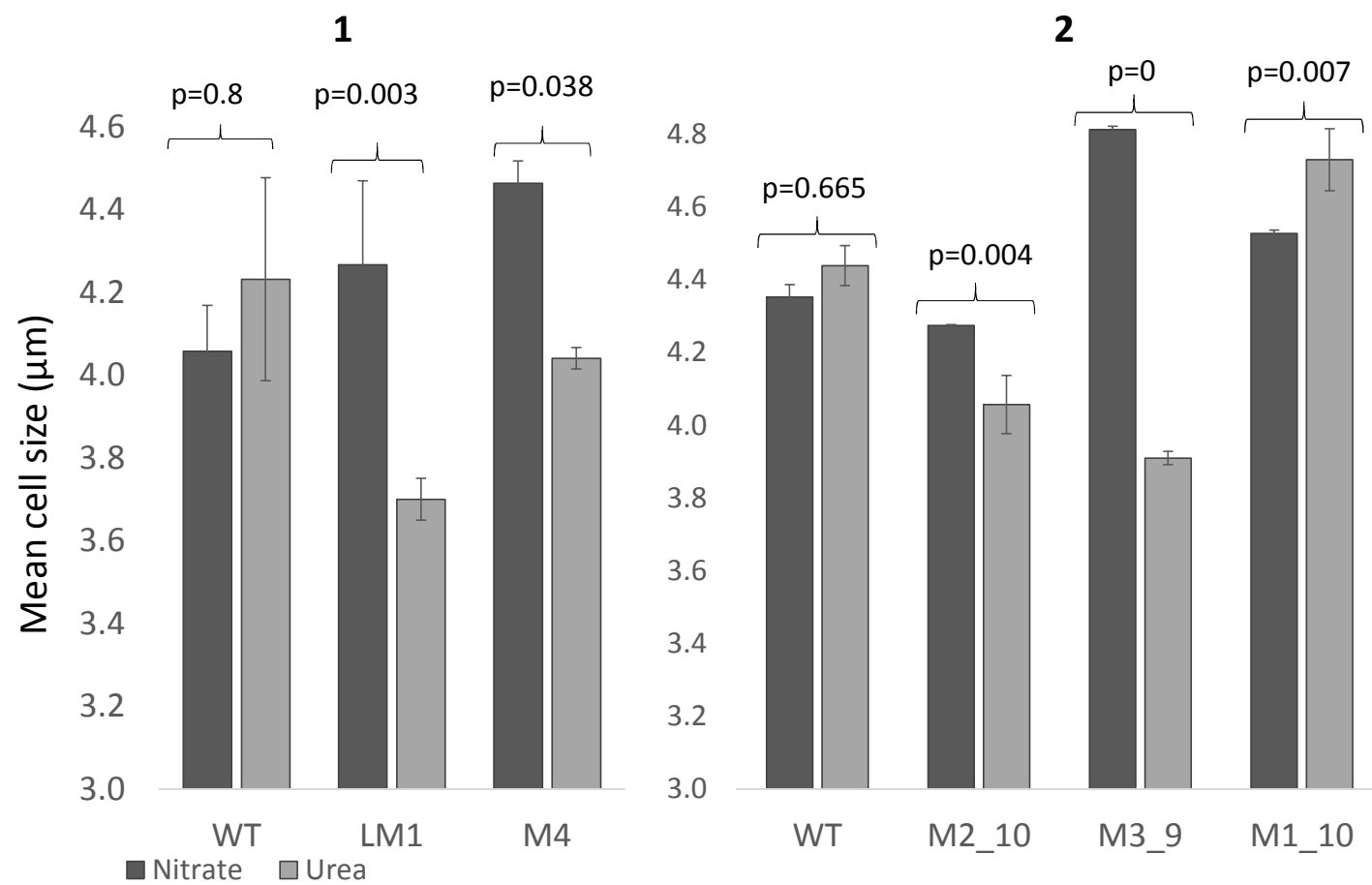


Figure 4

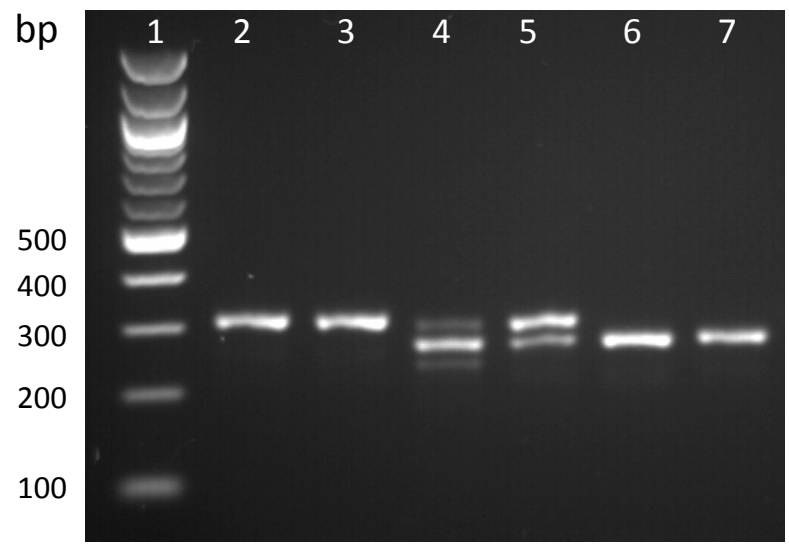


Figure 5



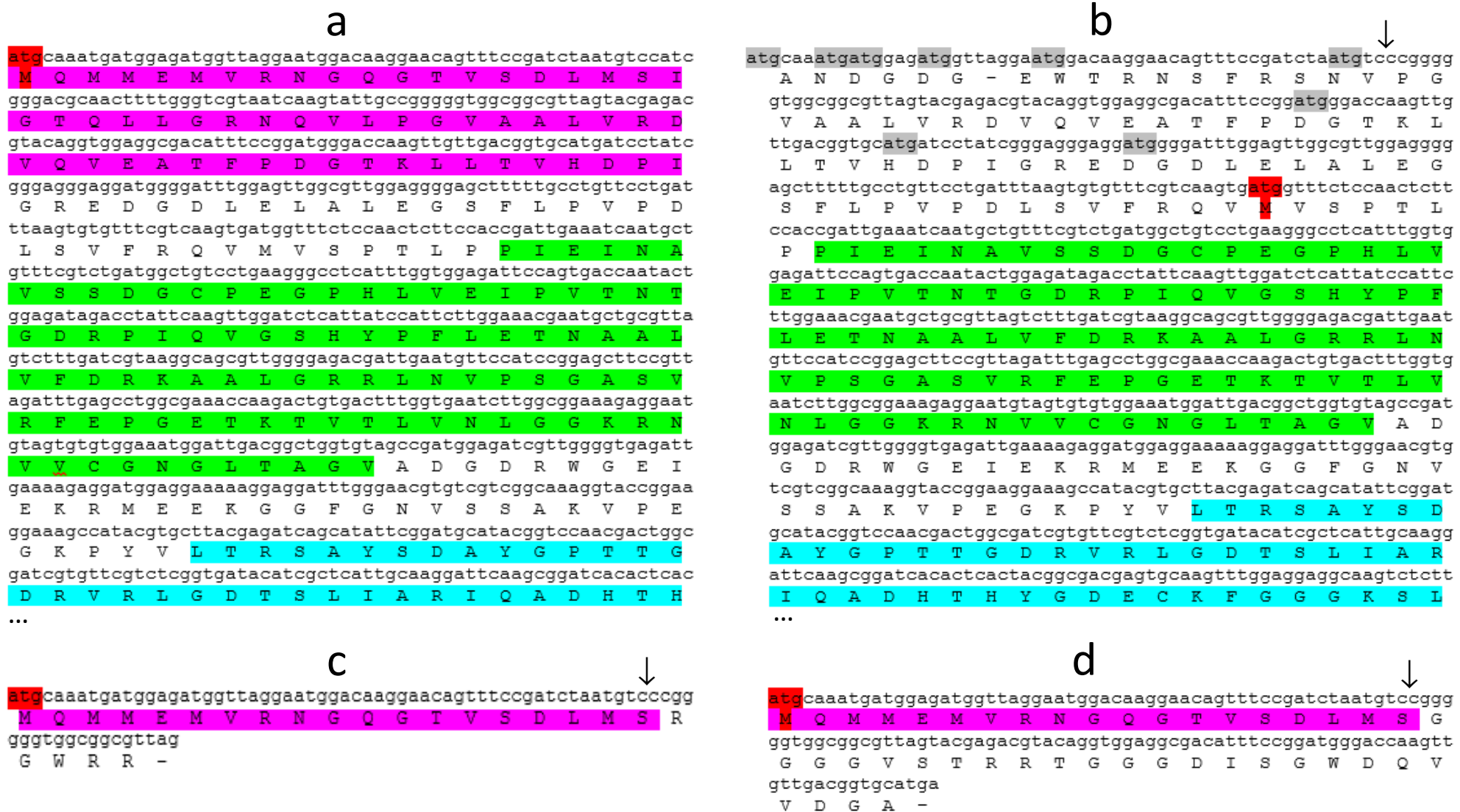


Figure 6

Target sequence	bp No	Cas9 cut	GC	5'	PAM	Location	Strand	Sense sequence	sgRNA score	Restriction sites
GCCTCGAGTAGAAGTCACCG	41	44	40%	G	TGG	Intron1	-	CGGTGACTTCTACTCGAGGC	0.8898	HphI
GCTCATTGCAAGGATTCAAG	915	932	45%	G	CGG	Exon3	+	GCTCATTGCAAGGATTCAAG	0.8388	
GTCGTAATCAAGTATTGCCG	158	175	40%	G	GGG	Exon2	+	GTCGTAATCAAGTATTGCCG	0.7908	HpaII
GTTGGGGTGAGATTGAAAAG	755	772	40%	G	AGG	Exon3	+	GTTGGGGTGAGATTGAAAAG	0.7898	
GACTATTCATGCTTACCACA	1659	1676	55%	G	CGG	Exon3	+	GACTATTCATGCTTACCACA	0.7731	
GCATCATTTCCACATGACCA	1380	1383	30%	G	GGG	Exon3	-	TGGTCATGTGGAAATGATGC	0.6920	
GTGACTTTGGTGAATCTTGG	676	693	35%	G	CGG	Exon3	+	GTGACTTTGGTGAATCTTGG	0.6834	
GTAGCCGATGGAGATCGTTG	739	756	60%	G	GGG	Exon3	+	GTAGCCGATGGAGATCGTTG	0.6533	
GATTTAAGTGTGTTTCGTGG	319	336	45%	G	TGG	Exon2	+	GATTTAAGTGTGTTTCGTGG	0.6478	
GGATGGGACCAAGTTGTTGA	225	242	45%	G	CGG	Exon2	+	GGATGGGACCAAGTTGTTGA	0.5997	
GAGAGGTCATCACTCGTACG	2048	2065	35%	G	TGG	Exon4	+	GAGAGGTCATCACTCGTACG	0.5634	Maell Csp6I SplI
GGAGTCACTACAATGTTTGG	1306	1323	40%	G	AGG	Exon3	+	GGAGTCACTACAATGTTTGG	0.5336	
GGTGCATGATCCTATCGGGA	246	263	65%	G	GGG	Exon2	+	GGTGCATGATCCTATCGGGA	0.5331	
GGTAATCCGGATAACAATGAA	1138	1155	50%	G	TGG	Exon3	+	GGTAATCCGGATAACAATGAA	0.5016	TspDI
GTTTCCGATCTAATGTCCAT	121	138	55%	G	CGG	Exon2	+	GTTTCCGATCTAATGTCCAT	0.5004	BclI
GAATGGATGGATCAAGGTGG	1905	1908	55%	G	TGG	Exon4	-	CCACCTTGATCCATCCATTC	0.4847	
GATTGTACCAGGTCAAGTGA	411	428	40%	G	TGG	Intron2	+	GATTGTACCAGGTCAAGTGA	0.4808	
GGAGATAGACCTATTCAAGT	529	546	50%	G	TGG	Exon3	+	GGAGATAGACCTATTCAAGT	0.4788	
GCAACTACTGATGTAATTGC	1198	1215	50%	G	GGG	Exon3	+	GCAACTACTGATGTAATTGC	0.4781	MluCI
GATGGTATCGGAGAACGATT	2467	2484	45%	G	GGG	Exon4	+	GATGGTATCGGAGAACGATT	0.4765	
GCGATAGCGTGGGCTCAGAT	2317	2334	50%	G	GGG	Exon4	+	GCGATAGCGTGGGCTCAGAT	0.4762	BclI BseMII
GTGGAGGCGACATTTCCGGA	208	225	40%	G	TGG	Exon2	+	GTGGAGGCGACATTTCCGGA	0.4649	BseGI HpaII BspMII

Supplementary Figure 1