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Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737

Re: **Request for Confirmation of Exemption** of Reduced Browning Banana with Modified Fruit Quality due to Reduced Polyphenol Oxidase (PPO) Enzyme

Dear Ms. Juarez,

Tropic Biosciences respectfully requests confirmation of exemption from regulations under 7 CFR part 340 pursuant to § 340.1(b)(1), covering a genetic modification resulting from cellular repair of a targeted DNA break in the absence of an externally provided repair template. The request for confirmation of exemption is for reduced browning banana (*Musa acuminata*, Cavendish subgroup, Grande Naine cultivar) with modified fruit quality. The bananas were developed using CRISPR/Cas9 gene editing, in which targeted DNA double-stranded breaks (DSBs) are created using the Cas9 endonuclease and are repaired by the banana plant's endogenous mechanisms, which may introduce short deletions, substitutions or additions at the repair site. The targeted gene in reduced browning banana is the polyphenol oxidase (PPO) gene [ ], which contributes to enzymatic browning in banana fruit. As described below, the resulting banana plants and harvest products will qualify for an exemption based on 7 CFR part 340 pursuant to § 340.1(b)(1). **CBI-Deleted**

In bananas, PPO enzymes are released from plastids upon mechanical damage of the fruits, including peeling, bruising and slicing. The released PPO enzyme oxidizes phenolic compounds in fruit tissues, resulting in discoloration known as enzymatic browning and ultimately lowering the quality of the bananas. [ ] is one of four PPO genes expressed in banana [ ]. **CBI-Deleted**
Among these four PPO genes, [ ] accounts for the highest mRNA abundance in [**CBI-Deleted**
], and it is predominantly expressed in [ ], with lower levels of **CBI-Deleted**
expression in [ ] and very low expression in [ ] (Tropic Biosciences **CBI-Deleted**
RNA-seq expression data). The reduced browning banana will have reduced levels of PPO enzyme in the fruit as a result of the loss of function of [ ]. **CBI-Deleted**

CRISPR/Cas9 was used to introduce a targeted DSB with two specific single guide RNAs [**CBI-Deleted**
], both directed to [ ]. Endogenous **CBI-Deleted**
banana DSB repair generated a [**CBI-Deleted**
]. In the disrupted allele, the [ ] caused a **CBI-Deleted**
frameshift in the [ ] coding sequence, thereby preventing the functional [ ] protein **CBI-Deleted**
from being produced. Figure 1 depicts the edit [ ] introduced into [**CBI-Deleted**
] the banana [ ] gene using sgRNA [ ]-guided CRISPR/Cas9-induced DSB and **CBI-Deleted**
banana endogenous cellular repair machinery.

The sgRNAs, [ ], depicted in Figure 1 were designed and selected based on several criteria to maximize target specificity and reduce the potential for secondary target edits. Young et al. (2019), demonstrated that sgRNAs, which are predicted through bioinformatics to be specific, do not cause secondary target mutations in maize. The two selected sgRNAs used to create the reduced browning banana were used as inputs for the Cas-OFFinder tool (http://www.rgenome.net/cas-offinder/) (Bae S. et al., 2014) and the tool provided potential secondary targets within the banana genome for the sgRNAs. The analyses revealed that only the intended sequence, [ ], is likely to be targeted by the Cas9 endonuclease using sgRNAs [ ]. Three potential secondary targets, located in unrelated banana genes, [ ], were identified for sgRNA [ ], whereas no secondary targets were identified for sgRNA [ ]. The reduced browning banana plant was analyzed to confirm the presence of the [ ] edit in the sgRNA [ ]-guided location and the absence of edits in the sgRNA [ ]-guided target and in the potential secondary target locations.

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Figure 1: (**A**) Partial genomic sequence from the [ ] of the banana [ ] gene. Black text indicates nucleotides from the coding sequence (nucleotides 1 to 695 from the start codon adenosine are displayed) and grey text indicates nucleotides from the upstream non-translated region (nucleotides -166 to -1 from the start codon adenosine are displayed). CRISPR/Cas9-associated DNA double-stranded break (DSB) sites are indicated with a dotted line. Two sgRNAs [ ] targeting the [ ] gene are indicated in blue and green shading, respectively, and their protospacer adjacent motif (PAM) sequences are indicated in grey shading. Red shading indicates the nucleotide deleted [ ] in reduced browning banana plants. Yellow shading indicates primers used for PCR to amplify the [ ] target site region for sequencing and confirmation of edits. (**B**) Partial alignment of [ ] protein sequences produced from non-edited and edited [ ] genes. The red box indicates changes in protein sequence brought about by the [ ] in the [ ] gene induced by sgRNA [ ]-guided CRISPR/Cas9 DSB. Whereas the full-length non-edited [ ] protein is [ ] amino acids in length, the edited [ ] protein is [ ] due to presence of [ ] (indicated with an asterisk) arising from [ ]. CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted

Summary Information on the Gene-Edited Reduced Browning Banana to Support the Exemption Confirmation Request:

- **Plant genus and species**
  Musa acuminata, Cavendish subgroup, Grande Naine cultivar
  Common name: Banana

- **Statement of regulatory exemption for which the plant qualifies**
  The reduced browning banana plants qualify for exemption under 7 CFR part 340 pursuant to § 340.1(b)(1) as the banana plants contain a genetic modification resulting from cellular repair of a targeted DNA break in the absence of an externally provided repair template.

- **Trait**
  Modified fruit quality

- **Description of Intended Phenotype**
  Reduced browning of the banana fruit due to a frameshift in [ ] gene, resulting from a [ ]. In the edited allele, the introduced sequence frameshift from the [ ] prevents functional [ ] protein from being produced and as a consequence the PPO enzyme content in the banana fruit is expected to be reduced. CBI-Deleted CBI-Deleted CBI-Deleted

- **Description of the genetic modification in the banana plant**
  Cavendish bananas have a triploid genome (AAA) and as such there are three homologs per gene, which are distinguished into three alleles based on single nucleotide polymorphisms (SNPs). As shown in Figure 1, the reduced browning banana contains [ ] in the [ ] gene. [ ] is absent in the edited allele, resulting in a frameshift that truncates [ ] and prevents the correct [ ] protein from being translated from the mRNA during protein synthesis. As a result, the protein produced from this allele is non-functional. The remaining [ ] alleles do not have changes at the sgRNA-directed DSB sites and will produce functional [ ] protein, though the overall quantity of [ ] protein in the banana fruit tissue is expected to be lower in the reduced browning bananas. CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted

- **Description of the method used to produce the modification**
  Reduced browning bananas were produced through transient T-DNA expression following *Agrobacterium*-mediated transformation of banana embryogenic cells. Selection with the aminoglycoside antibiotic [ ] was used to enrich for transformed cells transiently expressing the T-DNA from the plasmid pMOL_0019 (see Figure 2 and Figure 3). The T-DNA region contains the [ ], encoding the transiently expressed selection enzyme for [ ] resistance, as well as the *CAS9* gene and [ ]-targeting sgRNAs. This period of T-DNA transient expression allowed for the sgRNAs and Cas9 protein to be synthesized in the cells and thereby to generate targeted CRISPR/Cas9 DSBs followed by cellular repair. The embryogenic cells were regenerated into individual plants in the absence of [ ] selection and plants were screened in order to identify plant lines that contain a targeted modification in the [ ] gene but did not integrate the T-DNA, as described below. Plants containing a DNA edit at a targeted site in the [ ] gene and confirmed absence of plasmid DNA were propagated to produce banana plant clones. CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted

The following gene cassettes were included in the T-DNA region of the plasmid used during the transient selection transformation process:

Selection Cassette			
Genetic Element	**Origin**	**Function**	
NOS promoter	*Agrobacterium tumefaciens*	Promoter region of the nopaline synthase gene	
[ ] coding sequence	*Escherichia coli*	Coding sequence of the [ ] enzyme, conferring resistance to [ ] antibiotics for selection in plants	CBI-Deleted
OCS terminator	*Agrobacterium tumefaciens*	Termination region of the octopine synthase gene	
Expression Cassette 1 (Nuclease)			
Genetic Element	**Origin**	**Function**	
[ ] promoter	[ ]	Promoter region of the [ ]	CBI-Deleted
Cas9 coding sequence	*Streptococcus pyogenes*	Coding sequence of the Cas9 endonuclease (human codon-optimized), which creates a double-stranded break in the target DNA sequence	
NLS^{SV40} coding sequence	*Simian virus 40*	Coding sequence of the large T antigen nuclear localization signal	
35S terminator	Cauliflower mosaic virus	Termination region of the Cauliflower mosaic virus	
Expression Cassette 2 (sgRNA [ ])			CBI-Deleted
Genetic Element	**Origin**	**Function**	
TaU6 promoter	*Triticum aestivum*	Promoter region of the U6 spliceosomal small nuclear RNA (snRNA) gene	
sgRNA [ ]	*Musa acuminata*	Single guide RNA consisting of spacer and scaffold sequences, which bind and activate Cas9 and define the [ ] target site	CBI-Deleted
Expression Cassette 3 (sgRNA [ ])			CBI-Deleted
Genetic Element	**Origin**	**Function**	
TaU6 promoter	*Triticum aestivum*	Promoter region of the U6 spliceosomal snRNA gene	
sgRNA [ ]	*Musa acuminata*	Single guide RNA consisting of spacer and scaffold sequences, which bind and activate Cas9 and define the [ ] target site	CBI-Deleted

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Figure 2: T-DNA from plasmid pMOL_0019 that was used for transient expression to generate reduced browning banana.

- **Function of the modified gene or genetic element**
  The modified [ ] gene contains a [ ], CBI-Deleted
  introducing a frameshift in the coding sequence of [ ] and thereby preventing the CBI-Deleted
  functional protein from being produced. [ ] alleles of the [ ] gene CBI-Deleted
  contains this [ ] edit. This change in the [ ] gene acts to lower the maximal level CBI-Deleted
  of functional [ ] enzyme in banana fruit, and as a result the extent of enzymatic CBI-Deleted
  browning in banana fruit is expected to be reduced.

- **Molecular characterization – PCR amplification and sequencing of targeted [ ] gene modifications** CBI-Deleted
  In the initial screen, genomic DNA was extracted from a single leaf of banana plants regenerated from *Agrobacterium*-transformed embryogenic cells (transiently expressing the gene from the T-DNA). The two regions of the banana [ ] gene targeted by sgRNAs CBI-Deleted
  were amplified by PCR (Figure 1 and Figure 4) and analysed using Sanger sequencing. The relative height of chromatogram peaks was used to assess allelic ratios of the identified modification. To confirm genetic modification, analyses were then repeated using genomic DNA extracted from leaves from at least two distinct regions of the plants. These analyses confirmed a [ ], in [ ] the CBI-Deleted
  [ ] gene. CBI-Deleted

- **Molecular characterization – quantitative PCR analyses for absence of plasmid DNA**
  In the initial screen, genomic DNA was extracted from a single leaf of banana plants regenerated from *Agrobacterium*-transformed embryogenic cells (transiently expressing the gene from the T-DNA). Absence of T-DNA in the banana plants was assessed using quantitative PCR (qPCR) with primers designed to amplify two regions of the T-DNA (Figure 3 and Figure 4). To confirm the absence of plasmid DNA in the genome of the plants, genomic DNA extracted from leaves from at least two distinct regions of the plants and qPCR analyses were performed using primers spanning 9 regions of the T-DNA and plasmid backbone, including Cas9, sgRNA cassettes, bacterial and plant resistance markers, and left and right T-DNA borders (Figure 3 and Figure 4).

  As shown in Table 1, plasmid-specific primers failed to amplify target sequences from genomic DNA extracted from reduced browning banana plants. This was also the case for DNA from negative control wild-type plants, whereas these primers did amplify plasmid sequences from genomic DNA extracted from positive control transgenic plants. As an internal control, an endogenous banana genomic region amplified in all samples. These analyses, therefore, confirm that plasmid sequences are absent from the genome of reduced browning banana plants.

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Figure 3: Map of plasmid pMOL_0019 with qPCR amplicons numbered 1 through 9 (indicated in red in the inner circle) used to confirm the absence of DNA integration from the T-DNA and plasmid backbone in reduced browning bananas.

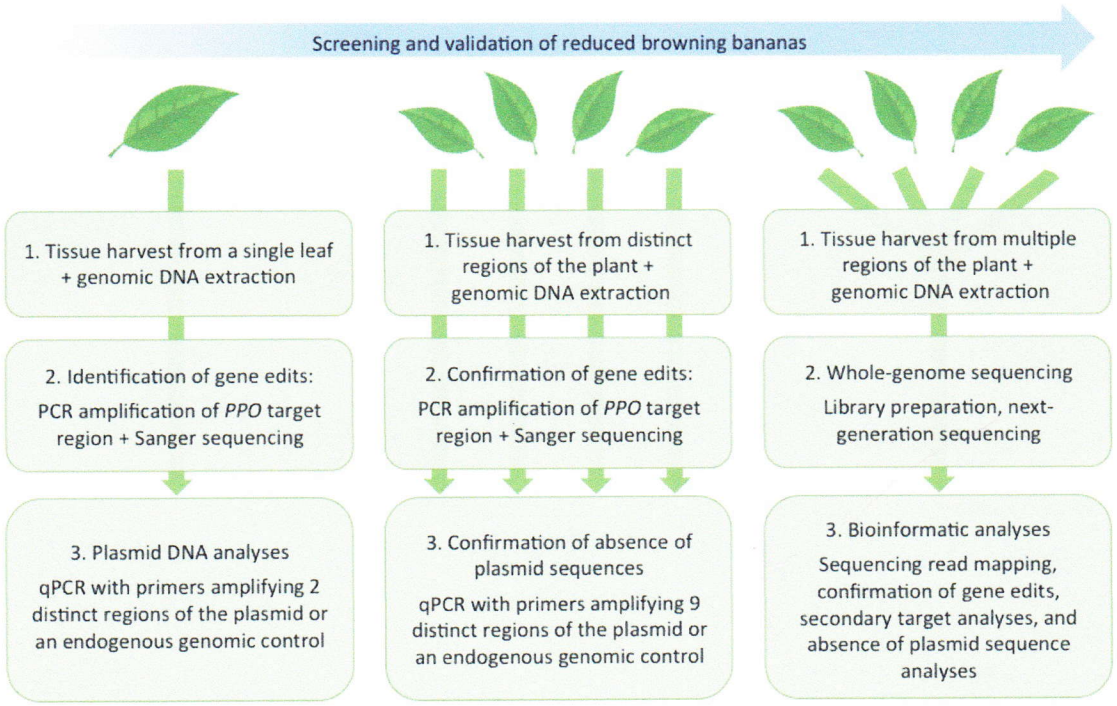

Figure 4: Molecular analyses used for molecular characterization of reduced browning banana.

Table 1: Cq values from quantitative PCR analyses

Sample	qPCR amplicons[1]								
	1	2	3	4	6	7	8	9	Endogenous genomic control
Reduced browning banana plant	NA[2]	NA	NA	NA	NA	NA	NA	NA	27.2
Negative control wild-type banana plant	NA	NA	NA	NA	NA	NA	NA	NA	28.19
Positive control transgenic banana plant	23.1	21.46	21.39	20.84	23.56	22.86	22.28	22.48	30.19

[1]Amplicon IDs from Figure 3
[2]NA = Not Amplified, DNA region not present in the sample

- **Molecular characterization – whole-genome sequencing and bioinformatic analyses**
  Genomic DNA will be extracted from the reduced browning banana plants, as well as from negative control wild-type plants, as reference comparisons. Next-generation sequencing (NGS) and bioinformatic analyses will be used to obtain precise in-depth whole-genome sequences of these plants, to further confirm the accuracy of the intended edit and the absence of edits in the three potential secondary targets and unintentionally integrated DNA from plasmid pMOL_0019 in the reduced browning banana plants used for propagation. The resulting reduced browning banana plants and harvest products will contain only the intended edit and no plasmid sequence.

- **Analyses of potential sgRNA secondary targets**
  Target specificity is built into the bioinformatic sgRNA design process, using strict parameters to ensure that there are no likely secondary target sites. Three low-likelihood potential secondary targets were identified for sgRNA [ ], whereas no potential secondary targets were identified for sgRNA [ ]. These identified potential secondary targets were examined for the absence of CRISPR/Cas9 edits compared to a negative control wild-type banana sequence, using specifically designed primer sets to selectively amplify the regions that might be targeted by sgRNA [ ] in these genes, followed by Sanger sequencing of the PCR amplicons. The sequence results confirmed the absence of edits in the potential secondary targets. Further validation will be available during the bioinformatic analyses of the whole-genome NGS data to confirm the absence of any unintended changes in the genomic sequence at identified potential secondary target sites.

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- **Measuring PPO activity to evaluate the reduced browning trait**
  PPO enzymatic activity in banana fruit may be estimated by recording the number of minutes elapsed from mechanical damage until the browning discoloration is observed (Escalante-Minakata et al., 2018). Enzymatic browning typically occurs within 15 minutes of slicing in banana flesh and between 30 minutes and 24 hours of bruising in banana peel (Tropic Biosciences, empirical observations). In reduced browning banana, the onset of enzymatic browning is expected to be delayed compared to negative control wild-type bananas.

  Biochemical approaches to quantify PPO enzymatic activity utilize specific phenolic substrates, the PPO-catalyzed oxidation reactions of which are associated with a quantifiable change in absorbance. Such substrates include for example 3,4-dihydroxyphenylalanine, characterized by a colorless-to-brown color change (Constabel and Ryan, 1998), or caffeic acid and 2-nitro-5-thio-benzoic acid, characterized by a yellow-to-colorless color change (Sullivan et al., 2004). In these assays, total proteins are extracted and mixed with PPO substrates, spectrophotometric measurements of the reaction mix are taken periodically, and the rate of absorbance change is normalized to total protein abundance, thereby giving a measurement of PPO activity. In reduced browning banana, the rate of the PPO-catalyzed reaction is expected to be lower compared to negative control wild-type bananas.

- **Agronomic assessments**
  Reduced browning banana plants with modified fruit quality will be grown for agronomic and phenotypic assessments, along with control banana varieties including the variety used

for plant transformation to evaluate performance compared to conventional banana plants. A number of agronomic endpoints will be assessed throughout the growing season to evaluate characteristics such as growth, disease susceptibility, fruit quality and fruit yield. Abiotic and biotic stressor data and environmental conditions will be recorded and evaluated to assess any impact from the [ ] modification. Banana plants that perform within established acceptable criteria, without evidence of additional traits outside of the intended reduced browning trait, will be advanced to the final product. CBI-Deleted

Conclusion and Request for Confirmation of Exemption

As described within this letter, the reduced browning banana plants (*Musa acuminata*, Cavendish subgroup, Grande Naine cultivar) with modified fruit quality developed using CRISPR/Cas9 gene editing, without an externally provided repair template, qualify for an exemption based on 7 CFR part 340 pursuant to § 340.1(b)(1). Loss-of-function of one of three alleles of the [ ] gene in bananas is expected to result in lower levels of enzymatic browning due to reduced maximal expression of polyphenol oxidase (PPO) enzymes released from plastids during damage of banana fruit. The presence of the intended genetic modification introduced by cellular repair of a targeted DNA break and the absence of plasmid DNA sequences in the reduced browning banana plants were confirmed by Sanger sequencing of target PCR fragments and quantitative PCR analyses, respectively. The absence of modifications in guide RNA potential secondary targets was confirmed by Sanger sequencing of PCR fragments. Additionally, the reduced browning banana plants will be characterized by whole-genome sequencing, to confirm the presence of the intended modification and the absence of both secondary target modifications and plasmid DNA sequences integrated into the genome. CBI-Deleted

Therefore, Tropic Biosciences respectfully requests confirmation of exemption from regulations for reduced browning banana under 7 CFR part 340 pursuant to § 340.1(b)(1), as the genetic modification is a change resulting from cellular repair of a targeted DNA break in absence of an externally provided repair template.

Sincerely,

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