Finding of No Significant Impact and Decision Notice

Animal and Plant Health Inspection Service

APHIS Permit 05-354-03r for antibody production in *Nicotiana* hybrid

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) received a permit application (APHIS number 05-354-03r) from Planet Biotechnology to conduct an environmental release with a *Nicotiana* interspecies hybrid that is genetically engineered to produce an antibody. On June 13, 2007, APHIS published a notice in the *Federal Register* (72 *FR* 32607-32608, Docket no. 2007-0029) announcing the availability of the draft environmental assessment (EA) for 30-day public comment period, ending July 13, 2007.

In the draft EA, APHIS considered two alternatives: Alternative A – Denial of the permit; Alternative B – Issue the permit with Supplemental Permit Conditions. APHIS proposed Alternative B as its preferred alternative because after review of the processes and procedures to prevent the dissemination and establishment of plant pests as describe in the permit and the additional supplemental conditions, APHIS concluded that the permit conditions would be adequate to confine the field release and prevent the release of the regulated article.

Based upon analysis described in the revised, final EA and in APHIS's response to comments, which is attached to this Finding of No Significant Impact (FONSI), APHIS has determined that the preferred alternative, to issue the permit with Supplemental Permit Conditions, will not have a significant impact on the quality of the human environment and will not pose a risk of the introduction or dissemination of a plant pest for the following reasons.

1. The genetically engineered *N. tabacum* X *N. glauca* hybrid line produces the antibody CaroRx™ that specifically binds to the bacterium *Streptococcus mutans*. In general, antibodies are non-toxic and clinical

trials with CaroRx indicated no adverse effects on humans. Antibodies are ubiquitous in nature, so many insects and animals are routinely exposed to antibodies. The selectable marker gene for kanamycin resistance (*npt*II) is not toxic and is present in many plant lines previously deregulated. The NOS (nopaline synthase) protein is naturally produced by many plants and is not expected to have significant effects on nontarget organisms.

2. The genetically engineered *Nicotiana* species hybrid does not produce viable seeds. In addition, as neither parental species of the hybrid overwinters in the state of Kentucky, even if plants produced viable seeds, these are not likely to persist in the environment. The addition of the transgenes is not likely to render the *N. tabacum* and *N. glauca* hybrid line, weedier. The addition of the transgenes is not likely to render tobacco more weedy.

3. The genetically engineered *Nicotiana* hybrid is not likely to outcross to the surrounding tobacco (*N. tabacum*) because the common practice of topping tobacco cultivars means that receptive non-regulated tobacco flowers will not be near the environmental release. In addition, *N. tabacum* X *N. glauca* hybrids are not able to fertilize tobacco plants and the field release will be at least ½ mile from all tobacco and 1-mile from tobacco that is grown for seed production.

4. The regulated *Nicotiana* hybrid is not likely to outcross to other *Nicotiana* species because *Nicotiana* interspecies hybrids rarely produce fertile plants, *Nicotiana* species are not frequently grown as ornamentals, and wild and weedy *Nicotiana* plants do not reside in Kentucky.

5. From an analysis of critical habitat and Threatened and Endangered Species, APHIS concluded that the release would have no effect on listed (or proposed) species.

6. The release site is on land that has been under agricultural cultivation for more than 10 years. The only past, present, and reasonably foreseeable actions associated with the location for the proposed release are those related to agricultural production. APHIS has determined that there are no past, present, or reasonably foreseeable actions that would aggregate with effects of the proposed action to create cumulative impacts or reduce the long-term productivity or sustainability of any of the resources (soil, water, ecosystem quality, biodiversity, etc.) associated with the release site or the ecosystem in which it is situated. No resources will be significantly impacted due to cumulative impacts resulting from the proposed action.

7. The field release is confined.
    a. Accidental transport of regulated articles from the site by humans is minimized by strict SOPs and permit conditions. All field plots will be tagged and GPS (Global Positioning System) coordinates recorded and communicated to APHIS. The field plot will be bordered on all four sides with 50 feet of perimeter fallow zone (not in production) to allow farm machinery to move around the site and yet still prevent physical mixing of the regulated plants with surrounding plants that may be used for food or feed.

    b. *Nicotiana* hybrid seeds will be germinated in a greenhouse and plantlets will be transplanted out into the field. This reduces the possibility of the small seeds being released out into the environment.

    c. An isolation distance of at least ½-mile will be maintained between the regulated plots and non-regulated tobacco. This area will be monitored throughout the field release period. At least a 1-mile distance will be maintained between the field plots and any

d. During the growing season the plants will be inspected for traits such as weediness, resistance/susceptibility to insects or disease, or unusual differences in plant growth or morphology.

e. All field equipment or vehicles entering the field, used for harvest, transport, and pest/weed control, will be cleaned prior to use and after use according to the APHIS approved Standard Operating Procedures (SOPs). Within 2 weeks following harvest and antibody extraction, the remaining plant material will be disked into the soil.

f. The regulated plants are not expected to produce viable seeds. Nevertheless, the release site and the 50-foot border area will be monitored for one year for volunteers and any volunteers will be destroyed.

g. Personnel who handle the regulated material will receive instruction in all the activities that they carry out involving the regulated material. This training will be documented and the documentation will be made available to APHIS inspectors. This training will encompass conditions stipulated in the permit, the APHIS permit conditions, the APHIS supplemental permit conditions, and the pertinent Federal regulations. Activities related to the field release and movement of the regulated article will be documented.

h. Dedicated facilities (locked or secured buildings, bins, or areas, posted as restricted to authorized personnel only) will be used for

storage of equipment and regulated articles for the duration of the field release.

i. APHIS will inspect permittee records that cover multiple aspects of the field release and inspect field releases timed to occur at critical steps in the production process.

Therefore, considering the organism and the trait introduced, the limited duration of the release, the manner in which the release must be conducted, the size and location of the proposed field release, the release is unlikely to significantly affect the quality of the human environment and will not pose a risk of the introduction or dissemination of a plant pest. Because APHIS has reached a finding of no significant impact, no Environmental Impact Statement will be prepared regarding this proposed action.

Michael C Gregoire

Michael Gregoire
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Biotechnology Regulatory Services
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Date: FEB 4 2008

Attachment to Finding of No Significant Impact
Notice Response to Comments
APHIS No. 05-354-03r

In response to a notice published in the *Federal Register* (72 *FR* 32607-32608, Docket no. 2007-0029) announcing the availability of the draft environmental assessment (EA) for public view and comment following a 30-day period ending July 13, 2007, APHIS received 6 comments from the general public and an environmental organization. All of the comments were in opposition to the permit. The issues raised regarded the inability to confine the regulated article to the field release site, the lack of protection of wildlife and humans, and the potential for horizontal transfer (transfer of transgenic DNA to other organisms). We have confined our response to the relevant issues raised by the commenters that relate to any plant pest or environmental risks posed by the confined release as described in the permit application.

APHIS reviews the permit to determine if the genetically engineered (GE) organism should be released into the environment under conditions that would confine the regulated article to the site of the field release according to regulations found at 7 CFR part 340. Prior to making a decision on this permit, APHIS prepared an EA to evaluate the significance of impacts on the environment arising from a decision to issue the permit. APHIS prepares the EA as part of its obligation, like other Federal agencies, to meet the requirements of the National Environmental Policy Act of 1969 (NEPA). As part of this process, APHIS considers public comments on the proposed permit as well as on the EA that APHIS prepares pursuant to NEPA. The commenters raised several issues and each is addressed below.

Comment: Several commenters were opposed to genetic engineering in general.
Response: APHIS disagrees with the statement that genetically engineered plants are dangerous and unwanted. First it must be noted that genetically engineered crops have a history of safe use. Over a billion acres have been planted with genetically engineered

crops, and there are no reported instances of any physical harm to humans and the environment
(Graham Brookes and Peter Barfoot, ISAAA Briefs 36, 2006, http://www.isaaa.org/). Though genetically engineered plants have been adopted on a widespread basis in the U.S. and are accepted by much of the public, APHIS acknowledges that the acceptance is not universal and that these types of plants are not wanted by some.

USDA believes that all methods of agricultural production (using conventional, organic, or genetically engineered varieties) can provide benefits to the environment, consumers, and the agricultural economy. The role of Biotechnology Regulatory Services within APHIS is to provide regulatory oversight pursuant to APHIS statutory authorities and regulations that allow for the safe development and use of genetically engineered organisms. The regulations in 7 CFR 340.4 describe the process that APHIS uses to issue permits for the confined release of regulated genetically engineered organisms. APHIS considers scientific data provided by the applicant, published in scientific journals, or provided by interested parties during the public comment period. The decision is based on whether the regulated article will be confined to the field release site in a manner that is not likely to present a risk of plant pest introduction into the environment. APHIS has found that the information submitted by Planet Biotechnology meets the requirements of 7 CFR 340.4 and is sufficient to allow the issuance of the permit according to the procedures and processes described in detail in the permit and the EA (pages 8-9, Appendix B and Appendix C of the EA).

Comment: One commenter wanted the neighbors to be informed of the field release.
Response: APHIS has no information regarding whether the neighbors will be notified when the field release is conducted. The applicant has claimed the site of the field release as Confidential Business Information (CBI). APHIS has reviewed the CBI justification provided by the applicant and is maintaining the information in a way that is consistent with our policy. While CBI information is not available to the public, APHIS has thoroughly reviewed all of the information provided by the applicant before making a decision. APHIS is required by law to protect CBI data. The APHIS policy on CBI was

published in 50 F.R. 38561. That policy requires information that is: 1) asserted to be a trade secret by the applicant; or 2) established by review to potentially cause substantial competitive harm, if made public will be released only if required by statute or court order or otherwise required by law. Information of this nature is protected under the Freedom of Information Act (FOIA) (5 U.S.C. 552). Section (b)(4) of FOIA exempts from disclosure "trade secrets and commercial or financial information obtained from a person and privileged or confidential." 5 U.S.C. 552 (b)(4). Releasing this information is a violation of the Trade Secrets Act (18 U.S.C. 1905). APHIS fully supports FOIA in all of its processes. For more information, please visit http://www.ftc.gov/foia/faq3exemptions.htm.

Comment: Several commenters were concerned about the inability to confine the regulated *Nicotiana* hybrid to the field release site without reference to a specific biological or physical process by which this would occur.
Response: APHIS disagrees with the commenter's contention that the regulated article would not be limited to the site of the release. Planet Biotechnology plantings include numerous safeguards to limit the release of the regulated article to the field release site as summarized in pages 8-9 of the EA. APHIS has evaluated the biology of regulated article (pages 7-8 and 10-11 of the EA) and has determined that the procedures described in the permit and the EA are expected to limit the regulated article to the release site. In addition, APHIS conducts inspections on pharmaceutical permits such as this timed to occur at critical steps in the production process (pages 9-10) to validate that the permit conditions specified in the permit are in fact carried out.

Comment: Several commenters indicated that the risk outweighs the benefits and that an alternative approach may be more effective than those proposed by the applicant.
Response: APHIS BRS does not determine whether an alternative research approach is more effective than the approach proposed. APHIS will leave it to the applicant to determine the viability of the alternative research direction proposed in the comment. The role of APHIS does not entail taking positions on the need or value of such plantings, but rather fulfilling its obligations of addressing safety issues under the Plant Protection Act

and other relevant statutes. APHIS considers the potential for the proposed study to impact the human environment. After careful consideration, APHIS concluded that the proposed study does not significantly impact the human environment.

Comment: One commenter indicated that the potential harm to wildlife was not evaluated.

Response: APHIS disagrees with this comment. Information presented in the EA (pages 12, 14 and Appendix A) discuss potential impacts on animals. Antibodies are ubiquitous in nature and as a class are non-toxic. Insects and animals that consume eggs and milk are routinely exposed to antibodies. The applicant provided the results of clinical trials, which indicated that the antibody is non-toxic and non-allergenic to humans. Additional information documenting APHIS' analysis of the potential for effects on listed or proposed threatened or endangered animals is included in the EA (pp. 14). APHIS reasonably concludes that the genetically engineered *Nicotiana* hybrids present no increased risk to wildlife compared to the non-GE *Nicotiana* hybrid.

Comment: One commenter stated that the possibility of horizontal transfer is not addressed.

Response: APHIS disagrees with this comment. APHIS assessed the likelihood of whether DNA transfer could occur to soil-inhabiting microbes through a process known as horizontal transfer on page 13 of the EA. In summary, FDA has concluded that the likelihood of transferring antibiotic resistance genes from plant genomes to microorganisms, in the gastrointestinal tract of humans or animals or in the environment, is remote.

Comment: One commenter asserted that the *trfA* gene (trans replication factor, involved in the replication of DNA in bacteria) and ColEI and RK2 (origins of DNA replication) would increase the chance of the transgenic DNA being replicated.

Response: APHIS disagrees with this comment. The EA (page 12) addresses the issue of the introduced *trfA* gene and ColEI and RK2 of bacterial origin. Promoters (regions of the DNA that control gene expression) in plants and promoters in bacteria are not

interchangeable. This is because the cellular machinery that regulates the expression in plants is different from that in bacteria and does not "recognize" bacterial promoters. Therefore, as discussed in the EA, the bacterial protein (*trfA*) is not expected to be expressed in plants. Furthermore, even if trfA were expressed in plants, it is not expected to lead to the replication of the bacterial DNA. This is because the transgenic DNA is integrated into the plant chromosomes and is expected to replicate at the same frequency as the plant DNA by the proteins that normally replicate plant DNA. In plants, DNA replication is initiated during cell division (Kornberg 1992). Even if the *trfA* gene was expressed in plants, other plant-produced enzymes (such as thymidine kinase, primase and polymerase) and processes (such as break down of the nuclear envelope and condensation of the chromosomes) are required for DNA replication in plants. Consequently, the introduced *trfA* gene is not expected to have any effect on DNA replication.

Comment: One commenter claimed that there were no provisions to protect water runoff.

Response: APHIS disagrees with this comment. At the termination of the field release the above ground portion of the plant is harvested, leaving mostly root material at the field release site. The roots of the engineered plant contain about 0.5 – 3 % of the total plant-produced antibody (page 7 of the EA and final report for Planet Biotechnology permit 06-037-01r). Thus, only a small percentage of the antibody produced in the plant would remain in the environment following the field release. Furthermore, the engineered product is an antibody; and antibodies are ubiquitous in nature (page 21 of the EA). Any antibody remaining in the plant root debris would have the same fate as antibodies in decaying animal tissue and would be quickly degraded by microbes (Appendix A of the EA). Antibodies in general are non-toxic and the specific antibody produced in the engineered plants was demonstrated to be safe in clinical trials (page 14 and Appendix A of the EA). APHIS therefore has no reason to believe that this field release will adversely affect the water runoff from the field and any organism that would come into contact with the water.

Kornberg A, Baker T (1991) DNA Replication. W.H. Freeman and Company, New York.

USDA/APHIS Environmental Assessment

In response to the Planet Biotechnology permit application 05-354-03r for an environmental release to produce antibodies in genetically engineered *N. tabacum* X *N. glauca* hybrid plants

U.S. Department of Agriculture

Animal and Plant Health Inspection Service

Biotechnology Regulatory Services

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I. SUMMARY

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture, has prepared an environmental assessment (EA) in response to a request for a permit (APHIS Number 05-354-03r) submitted by Planet Biotechnology for environmental release of a genetically engineered *Nicotiana* interspecies hybrid. Planet Biotechnology crossed a genetically engineered *Nicotiana tabacum* that produces an antimicrobial antibody which binds to a bacterium (*Streptococcus mutans*) associated with tooth decay in humans, with *N. glauca* (*N. tabacum* X *N. glauca*) to generate an interspecific *Nicotiana* hybrid line, 06PBCarHG1.

Prior to submission of this permit, Planet Biotechnology obtained APHIS permits for small-scale field tests of 06PBCarHG1. In permit application 05-354-03r Planet Biotechnology seeks approval to grow 06PBCarHG1 at a larger-scale of production. The mitigation measures imposed to prevent outcrossing for previous field tests included removal of flower buds. More recent data on the fertility of these hybrids have indicated that 06PBCarHG1 is sterile under certain circumstances (see Section V. AFFECTED ENVIRONMENT, part B. Biology of *Nicotiana* and of *N. tabacum* X *N. glauca* Hybrids, for a discussion of sterility factors). Therefore, during the proposed field trial, Planet Biotechnology has proposed that the *N. tabacum* X *N. glauca* hybrid 06PBCarHG1 plants be allowed to flower openly. This EA discusses the contribution of the sterility of these hybrids to the overall confinement of the regulated article. Additional physical confinement procedures and processes will also contribute to the confinement of the regulated article to the field test site. This EA examines whether there are potential adverse environmental impacts associated with the proposed action.

APHIS has reviewed the information submitted by Planet Biotechnology and is considering whether to deny the permit (Alternative A), or issue the permit with the requirements proposed in the application and the supplemental permit conditions (Alternative B). This EA and the comments received from the public will serve to inform this decisionmaking process to allow or not to allow this environmental release.

The preferred alternative that APHIS selected is "Alternative B," *Issue the Permit with Supplemental Permit Conditions*. This decision is based upon the conclusion that the mitigation measures described in the environmental assessment are adequate to prevent significant environmental impacts.

II. INTRODUCTION

A. Purpose of the Environmental Release

On December 21, 2005, APHIS received a permit application (05-354-03r) from Planet Biotechnology of Hayward, CA. The application requests permission to release a genetically engineered *N. tabacum* X *N. glauca* hybrid line, 06PBCarHG1, into the environment in a confined field trial. The purpose of the environmental release is to grow the genetically engineered *Nicotiana* hybrid line, 06PBCarHG1, which produces an antibody, designated by Planet Biotechnology as CaroRx™. The environmental release,

in Daviess County, Kentucky, will begin in June 2007 and will conclude in the fall of 2007. Following harvest of the *Nicotiana* hybrid leaves, the permittee will extract and purify the CaroRx™ antibody. Application of CaroRx™ to human teeth is intended to prevent tooth decay (Ma 1998). In the United States, CaroRx™ is an Investigational New Drug (BB-IND # 7526) and in the European Union, it is a registered Medical Device.

B. USDA-APHIS Regulatory Authority

The authorities for regulation of genetically engineered plants is the Plant Protection Act, 7 U.S.C. 7701-7772, and USDA-APHIS regulations under Title 7 of the Code of Federal Regulations, Part 340 (7 CFR part 340), "Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which are Plant Pests or Which There is Reason to Believe are Plant Pests." A genetically engineered organism is considered a regulated article if the donor organism, recipient organism, vector, or vector agent used in engineering the organism belongs to one of the taxonomic groups listed in the regulation and is also a plant pest, or if there is a reason to believe it is a plant pest. The permit application submitted to APHIS by Planet Biotechnology requests approval for environmental release of transgenic *Nicotiana* hybrid line, 06PBCarHG1, which contains regulatory genes from the cauliflower mosaic virus (CaMV). Because CaMV is listed as a plant pest under 7 CFR 340.2, the organism is deemed a regulated article.

This EA was prepared in accordance with: (1) The National Environmental Policy Act of 1969 (NEPA), as amended (42 U.S.C. 4321 *et seq*.); (2) regulations of the Council on Environmental Quality for implementing the procedural provisions of NEPA (40 CFR 1500-1508); and (3) APHIS' NEPA Implementing Procedures (7 CFR part 372).

Generally, the issuance of permits for confined field tests of regulated articles is categorically excluded from the requirements for an EA under APHIS' NEPA implementing procedures (7 CFR 372.5(c)(3)(ii)). In certain cases, when APHIS determines that a confined field release of a genetically engineered organism has the potential to significantly affect the quality of the human environment as those terms are defined in 40 CFR 1508.27 and 1508.14, an EA or environmental impact statement is prepared pursuant to 7 CFR 372.5(d). Accordingly, APHIS prepared this EA because the permit applicant intends to grow *N. tabacum* X *N. glauca* hybrid plants, which are genetically engineered to produce antibodies, at a large scale for a plant made pharmaceutical. The applicant designed the production practices for the proposed environmental release that raises new issues. The permittee proposes use of no mitigation measures to remove the flower buds because *N. tabacum* X *N. glauca* hybrids exhibit male sterility. Consequently, APHIS has prepared this EA to determine whether there are adverse environmental effects associated with the proposed release of *N. tabacum X N. glauca* hybrid line, 06PBCarHG1, genetically engineered to express an antibody as well as the marker gene, neomycin phosphotransferase (NPTII), and nopaline synthase (NOS).

III. NEED FOR THE PROPOSED ACTION

Under APHIS regulations (7 CFR 340.4(e)), the receipt of a permit application to introduce a genetically engineered organism requires a response from the Administrator:

> "*Administrative action on applications*. After receipt and review by APHIS of the application and the data submitted pursuant to paragraph (a) of this section, including any additional information requested by APHIS, a permit shall be granted or denied."

IV. ALTERNATIVES TO THE PROPOSED ACTION

A. No Action

For the purposes of this EA, the "no action" alternative would be denial of permit application 05-354-03r. This would be the preferred alternative if, after review of the processes and procedures to prevent the dissemination and establishment of plant pests as described in the permit, APHIS concluded that the permit conditions would not be adequate to prevent significant environmental impacts.

B. Issue the Permit with Supplemental Permit Conditions

If APHIS made the decision to issue this permit with supplemental permit conditions, the permittee would be allowed to proceed with the environmental release in Daviess County, Kentucky, (see Section V. AFFECTED ENVIRONMENT, part C. Description of the Field Release), but would have to adhere to supplemental permit conditions specified by APHIS in addition to the standard permit conditions and those proposed in the application. APHIS would base the supplemental permit conditions on its scientific analysis of the permit application, input from the state of Kentucky, and public comment on this EA (see Appendix C: Supplemental Permit Conditions). If warranted, APHIS would require mitigating measures, stipulated in the final supplemental permit conditions, to prevent dissemination of the organism outside the field production area.

Currently, APHIS proposes to include the following measures to promote a confined field release and to ensure no significant harm to the environment:

1. The permittee must document activities related to the field test and movement of the regulated article and make them available for APHIS inspections.
2. The permittee will report unintended releases according to timeframes and procedures provided in the supplemental permit conditions.
3. The permittee will provide reports to APHIS according to the guidance in the supplemental permit conditions.

Under APHIS regulations, compliance with all mitigating measures is required:

> "*Permit conditions.* A person who is issued a permit and his/her employees or agents shall comply with the following conditions (Standard Permit Conditions, Appendix B), and any supplemental permit conditions (Supplemental Permit Conditions, Appendix C) which shall be listed on the permit, as deemed by the Deputy Administrator to be necessary to prevent the dissemination and establishment of plant pests" 7 CFR 340.4(f)

This would be the preferred alternative if, after review of the processes and procedures to prevent the dissemination and establishment of plant pests as described in the permit and in the supplemental permit conditions, APHIS concluded that the permit conditions would adequately prevent any significant environmental impacts.

V. AFFECTED ENVIRONMENT

A. Description of the Regulated Article

The interspecific *Nicotiana* hybrid, 06PBCarHG1, is genetically engineered to produce the antibody CaroRx™ for use as a treatment to prevent tooth decay. CaroRx™ specifically binds to the bacterium, *S. mutans*, which has been identified as the major organism causing tooth decay (Loesche 1975). When the attachment of *S. mutans* to the tooth surface is blocked by an antibody, such as CaroRx™, which binds to the *S. mutans* SAI/II surface adhesion protein, subsequent infection and resulting tooth decay may be prevented (Ma 1998).

An antibody is a protein that binds specifically to a particular substance, known as an antigen. While each antibody is unique in its ability to bind to its corresponding antigen, antibodies, in general, have the same overall structure. Typically, antibodies exist as one or more copies of a Y-shaped unit composed of four polypeptide chains called immunoglobulins (Ig). Each Y-shaped Ig contains two identical copies of a heavy chain, and two identical copies of a light chain (see Appendix A, Fig.1A). An antibody can also be fragmented via enzymatic digestion into the Fab, Fab2, and Fc fragments (see Appendix A, Fig. 1B). Secretory antibodies comprise the most abundant class of antibodies produced in humans (Ma 2005). They exist in the dimeric form with two Y-shaped Ig molecules bound to a J chain and to a secretory component (see Appendix A, Fig. 2). The J chain serves to dimerize the two Ig molecules and the secretory component serves to protect the immunoglobulin from proteases (Ma 1998). The Ig molecules in CaroRx™ were derived from Guy's 13-immunoglobulin G (IgG) monoclonal antibody that specifically binds to *S. mutans* (Ma 1994). The CaroRx™ antibody differs from Guy's 13 antibody as the former is engineered to contain an Fc portion of the heavy chain from an immunoglobulin A (IgA). This chimeric IgA/IgG antibody binds to a J chain and further assembly with a secretory component (Ma 1995, Ma 1998). The secretory antibody CaroRx™ prevents bacterial colonization to protect humans against oral *S. mutans* infection (Ma 1998).

Planet Biotechnology generated the transformed *Nicotiana* interspecies hybrid line, 06PBCarHG1, by first cloning and expressing the CaroRx™ gene in *N. tabacum* line H8-105 and then crossing H8-105 with *N. glauca.* The *Nicotiana* hybrid line, 06PBCarHG1, is genetically engineered with the constituent parts of the secretory CaroRx™ antibody (light chain from mouse, heavy chain from mouse, J chain from mouse, and secretory component from rabbit), all driven by the cauliflower mosaic virus (CaMV) promoter. These constituent parts were cloned and expressed in tobacco by independent transformation events. The events were combined into a single line by classical breeding methods (Ma 1995). Hybrid line 06PBCarHG1 contains two additional protein products that are expressed under the control of a plant recognized NOS promoter (one of very few

bacterial promoters known to be expressed in plants). These proteins are NPTII (from *E. coli*), an enzyme that confers resistance to kanamycin, which is used as a selectable marker, and NOS (from *A. tumefaciens*), an enzyme that forms nopaline from the amino acid arginine and alpha-ketoglutaric acid. NOS, although present in the plasmid vector used in the production 06PBCarHG1, was not utilized as a selectable marker in the construction of 06PBCarHG1. Line 06PBCarHG1 also contains *trf*A (from *E. coli*) that encodes for a DNA-binding protein important for plasmid DNA replication and *add*3 (from *E. coli*) that encodes for resistance to the antibiotic streptomycin/spectinomycin. However, because *trf*A and *add*3 are driven by bacterial promoters that are not recognized by plants, these genes are not expressed in 06PBCarHG1. Additional non-coding sequences (sequences contained in the transformed plant, but not converted into protein products) present in 06PBCarHG1 are ColEI and RK2 origin of replication sequences (from *E. coli*) and the *nos* terminator from *A. tumefaciens.*

In the transformed line 06PBCarHG1, the expression level of CaroRx^{TM} can be as high as 35.0 mg per kilogram of fresh weight in leaves (mg/kg FW), up to 8.0 mg/kg FW in stems, and up to 4.1 mg/kg FW roots, but it is not detectable in pollen (data submitted by the applicant with the 05-354-03r permit).

B. Biology of *Nicotiana* and of *N. tabacum* X *N. glauca* Hybrids

The genus, *Nicotiana*, is composed of some 76 naturally occurring species (Chase 2003). Many *Nicotiana* species are native to South America with the remainder distributed throughout Central America, western North America, Australia, and various islands of the South Pacific. *N. tabacum* L. (tobacco) probably originated in Argentina by hybridization of *N. sylvestris* Speg. & Comes, and *N. tomentosiformis* Goods, where the progenitors are native. Most *Nicotiana* species are not cultivated, with the exception of *N. tabacum*, and to a lesser extent, *N. rustica* (Chaplan 1979). *N. glauca* Graham, also a native of Argentina, is not naturalized in Kentucky (USDA, NRCS 2006), although it does naturalize in warm temperate regions throughout the world.

The subject of this EA is a *N. tabacum* X *N. glauca* hybrid line 06PBCarHG1 that resulted from crossing *N. tabacum* cultivar Petite Havana line H8-105 (female parent) to a wild type *N. glauca* (male parent). The Kentucky Tobacco Research and Development Center (KTRDC) made all crosses at the University of Kentucky. The strategy of production of *N. tabacum* X *N. glauca* hybrid plants having various phenotypic characteristics, which are optimal for plant made pharmaceutical applications, is property of the University of Kentucky, and the subject of a U.S. patent application (20,060,236,433) by KTRDC.

Natural hybridization has frequently occurred in the evolution of the genus *Nicotiana* (Goodspeed 1954). More recently, to create improved varieties of *N. tabacum* (commonly cultivated tobacco), breeders have taken advantage of the interspecific transfer of genes from wild relatives of *N. tabacum* for traits such as disease and insect resistance. These interspecific *Nicotiana* hybrids generally have reduced fertility (therefore some level of sterility) such as reduced pollen viability and/or reduced seed production and seed viability (Al-Almad 2006, Burk 1979, Nikova 1997). When there is a large difference in the numbers of chromosomes

between the two parents, there is likely to be very little chromosome pairing at meiosis resulting in progeny that are sterile. For *N. tabacum*, which has twice the number of chromosomes as *N. glauca,* sterile progeny are expected. Interspecific breeding allowed for production of male sterile lines that lack viable pollen. Such lines are useful in breeding *Nicotiana,* species, such as *N. tabacum,* which produce flowers that are selfing (are fertilized by their own pollen) (Burk 1979). Previously, *N. tabacum* crossed with N. *bigelovii*, *N. debneyi*, *N. glutinosa, N. megalosiphon*, *N. plumbaginifolia*, *N. rustica, N. suaveolens*, and *N. undulata* also resulted in sterile *Nicotiana* hybrids (Chaplin 1964). In the present case, KTRDC produced sterile hybrids as a confinement measure for field testing genetically engineered *Nicotiana* hybrids (Mundell 2005).

As discussed in the section VI. POTENTIAL ENVIRONMENTAL IMPACTS, the only potential plants to which outcrossing could occur, that are likely present in the area surrounding the release site, are cultivated tobacco (*N. tabacum*). The sterility of 06PBCarHG1 and *N. tabacum* X *N. glauca* hybrids was analyzed in crossing tests in the greenhouse, where manual hand pollination was evaluated, and in the field, where natural bird and insect pollination was evaluated (Mundell 2005, data submitted with permit 05-354-03r). Under field conditions, 06PBCarHG1 is highly likely to be both female and male sterile.

C. Description of the Field Release

The affected environment will be limited to the release site, as the applicant proposes a confined environmental release in the permit. The applicant shall perform mitigation measures designed to confine the regulated material to the release site and apart from nonregulated material.

The applicant proposed the following measures in the submitted permit to confine the release to the field test site:

1. The applicant shall use dedicated facilities (locked or secured buildings, bins, or areas, posted as “Restricted To Authorized Personnel Only”) for storage of equipment and regulated articles for the duration of the field test.
2. The applicant shall tag all field plots and record and communicate Global Positioning System (GPS) coordinates to APHIS in order to precisely locate the field (helps to locate the field to monitor for volunteers).
3. The applicant shall border the field plots shall on all four sides with 50 feet of perimeter fallow zone (not in production) to allow farm machinery to move around the site and will prevent physical mixing of the regulated plants with surrounding plants that may be used for food or feed.
4. The applicant shall maintain an isolation distance of at least ½-mile between the regulated plots and non-regulated tobacco. The applicant will monitor this area throughout the field test period to ensure the minimum ½-mile distance to non-regulated tobacco.
5. Applicants will maintain at least a 1-mile distance between the field plots and any seed production. The applicant will monitor this area throughout the field test period to ensure the minimum 1-mile separation from seed production.

6. Applicants shall germinate seeds in a greenhouse and transplant plantlets out into the field.
7. APHIS will inspect the plants during the growing season for traits such as weediness, resistance/susceptibility to insects or disease, or unusual differences in plant growth or morphology.
8. The applicant shall clean all field equipment or vehicles entering the field that are used for harvest, transport, and pest/weed control both prior to use and after use, according to the APHIS-approved Standard Operating Procedures (SOPs).
9. The applicant will monitor all surrounding land within a ½-mile radius during the period of time when the regulated article is producing pollen to ensure that the area has no reproductively compatible plants.
10. The applicant shall disk into the soil the remaining plant material within 2 weeks following harvest and antibody extraction.
11. Harvesting equipment must be dedicated for use in the permitted test site(s).
12. The applicant shall monitor the field plot and 50-foot border area for volunteers for 12 months. Applicants will uproot any volunteers, destroy them, and incorporate them into the soil.
13. Applicants shall provide instruction to personnel who handle the regulated material so that they are aware of handling procedures. The applicant shall document this training and make it available to APHIS inspectors. This training shall encompass conditions stipulated in the permit, the APHIS permit conditions, the APHIS-supplemental permit conditions, and the pertinent Federal regulations.
14. The applicant shall document activities related to the field test and movement of the regulated article.

In addition, APHIS performs the following activities to reduce the possibility of human error:

1) Inspection of records that cover multiple aspects of the field trial.
2) Multiple inspections of field trials timed to occur at critical steps in the production process.

VI. POTENTIAL ENVIRONMENTAL IMPACTS

The proposed action is to conduct a confined environmental release with a regulated article, a genetically engineered organism. The permit application describes the procedures that the applicant has submitted to APHIS to confine the regulated organism to the release site according to the requirements under 7 CFR part 340. APHIS evaluated the proposed action based on the biology of the *N. tabacum* X *N. glauca* hybrid plants, any potential hazards associated with the transgenes, and the likelihood that the transgenes could persist and potentially harm the environment.

A. Potential for Persistence of the Engineered Plants in the Environment

1. Potential for Human Error

APHIS considered the likelihood that humans could inadvertently move the regulated article from the environmental release site. In a recent workshop hosted by APHIS dealing with gene

confinement issues in genetically engineered crops (USDA-APHIS 2004) one of the more likely mechanisms contributing to the breakdown of confinement and movement of seed was identified as human error, and the most reliable means of preventing this is to maintain and enforce stringent SOPs. APHIS requests and reviews SOPs for equipment and processes related to the movement, planting, monitoring, and harvest of genetically engineered plants. APHIS verifies implementation of these procedures during multiple inspections. In addition, APHIS requires that the personnel who handle the regulated material receive training according to APHIS-approved training processes to further ensure that the SOPs are carried out during the course of the environmental release. Therefore, APHIS believes that measures are in place to ensure that unauthorized movements resulting from human error are very unlikely.

2. **Potential for Seed Production**

APHIS evaluated the potential for the *N. tabacum* X *N. glauca* hybrid line, 06PBCarHG1, plants or their progeny to survive in the environment at the conclusion of the environmental release. *Nicotiana* hybrid production starts in the greenhouse, where seeds are germinated under controlled conditions. *Nicotiana* hybrid seedlings are then transplanted into the field and grown to maturity. Researchers at the University of Kentucky performed several years of field trials with non-genetically engineered *N. tabacum* X *N. glauca* hybrids, where the plants flowered. The fields used for these trials were surveyed for seedpods. No viable seeds were observed after these trails ended (Richard Mundell, KTRDC, University of Kentucky, personal communication to Orlando Chambers KTRDC, University of Kentucky, December 7, 2006). Additional greenhouse testing and field experiments indicated *N. tabacum* X *N. glauca* hybrids and 06PBCarHG1, when crossed with pollen from *N. tabacum* did not produce viable seeds (Mundell 2005, data submitted with permit 05-354-03r). APHIS therefore determines that the genetically engineered hybrid line 06PBCarHG1 is not likely to persist because viable seeds are not likely to be produced. Even if the hybrid *Nicotiana* produces seeds, they would likely not survive the winter and establish, because neither parental species of the hybrid overwinters in the state of Kentucky (USDA-NRCS 2006). The applicant will monitor the environmental release site for volunteers the following year. APHIS determines that viable seed production is not likely and even if seed production occurs, the resulting plants are not likely to persist in the environment.

3. **Potential for Outcrossing to Cultivated Tobacco**

APHIS further evaluated the potential for genes from 06PBCarGH1 to persist in the environment due to the possibility that the transgenic pollen would outcross to surrounding tobacco plants. In nature, pollination of *Nicotiana* occurs via honeybees (*Apis mellifera* L.), sweat flies (*Didea fasciata* Macq.), bumblebees (*Bombus* species), hawkmoths (*Theretra tersa* L.), hummingbirds (*Archilochus colubris* L), and in some cases by bats (Hodges 1952, McMurtrey 1960, Nattero 2003, Poehlman 1959). Greenhouse pollination tests are typically much more efficient than crosses in nature, especially when pollen of doubtful viability is involved, because the hand crosses involve larger quantities of pollen than would be transferred by an animal pollinator and are from a single source. When grown in the greenhouse or out in the field, *N. tabacum* X *N. glauca* hybrids were not able to fertilize *N. tabacum* plants placed in proximity when pollinated by insects or birds nor when pollinated by hand (Mundell 2005). Furthermore, less than 1 percent of the pollen germinated indicating that the hybrid pollen is predominantly non-viable. Similarly, the genetically engineered hybrid 06PBCarHG1 line was analyzed in greenhouse studies by hand pollinating 06PBCarHG1 pollen onto *N. tabacum* plants (data submitted with permit 05-354-

03r). Most attempts to pollinate *N. tabacum* using 06PBCarHG1 as the male parent also failed. In 1 experiment, out of 32 attempted crosses, a single seedpod produced viable seeds, (05-354-04r field test report). When these seeds were germinated and resulting plants were tested for CaroRx, no CaroRx was detected. The greenhouse and field test results suggest that outcrossing will not occur. Even if the 06PBCarHG1 was capable of outcrossing to tobacco plants, in nature, animal pollinators will frequent different flowers and will bring a mixture of pollen to the flower including pollen of much higher viability than that produced by 06PBCarHG1 and with a much higher probably of successfully fertilizing flowers.

Furthermore, the transgenic hybrid 06PBCarGH1 site is isolated from other tobacco plants by at least ½-mile. This distance is twice the AOSCA standard to produce tobacco seed of 0.01 percent varietal purity. The AOSCA standard assumes pollen of full viability, and 06PBCarHG1 has been shown to have pollen of only about 1% viability. Another factor that will reduce the likelihood of outcrossing is that the common practice for the production of commercial tobacco in Kentucky involves topping (flower removal) and sucker control methods (Dr. Robert Pearce, Extension Tobacco Specialist, University of Kentucky, Communicated to APHIS on 11/03/06). These production methods will reduce or eliminate the availability of receptive cultivated tobacco flowers for cross-hybridization.

APHIS believes that the field studies have shown that hybrid 06PBCarGH1 plants are sterile when the hybrid 06PBCarGH1 is grown and pollinated under natural conditions. However, because some of the greenhouse crossing experiments indicate some low level of fertility, the proposed field release will include trap plants to obtain additional field data on the sterility of the 06PBCarGH1 under field conditions. Planet Biotechnology will place male sterile/female fertile tobacco plants 8-15 feet from the edge of each field release site. The male sterile tobacco plants will be monitored for seed formation to assess the outcrossing potential from the hybrid plants. In this way, the expectation that transgenic hybrid 06PBCarGH1 plants will not outcross with surrounding cultivated tobacco will be directly tested.

4. Potential for Outcrossing to Tobacco Grown for Seed Production

Another concern that APHIS must consider is the proximity of tobacco seed production in the area of the environmental release. Such tobacco plants will not be topped and the seed will be saved for planting. The closest known tobacco seed production area is at least 10 miles from Daviess County, Kentucky (Dr. Robert Pearce, Extension Tobacco Specialist, University of Kentucky, 11/03/06). This distance is 20 times greater than the distance specified by the AOSCA standard for 0.01 percent varietal purity (AOSCA 2004). APHIS concludes there is a negligible likelihood that the transgenic hybrid 06PBCarGH1 plants will outcross to tobacco grown for seed production purposes.

5. Potential for Outcrossing to Naturalized or Ornamental Species of *Nicotiana*

APHIS evaluated the potential for the genetically engineered hybrid line 06PBCarHG1 to outcross to other *Nicotiana* species, other than that of cultivated tobacco. No wild and weedy *Nicotiana* plants that could potentially cross with 06PBCarHG1 reside in Kentucky (Kartesz 2005, USDA-NRCS 2006). Ornamental *Nicotiana* species are not frequently grown in Kentucky (Robert G. Anderson**,** Extension Professor**,** Department of Horticulture, University of Kentucky**,** Lexington, KY, personal communication). Even if ornamental *Nicotiana* species were grown near this field plot, seed production between interspecific hybrids is unlikely for reasons already

discussed above (Al-Ahmad 2006, Nikova 1997). Even if seeds were produced, APHIS does not foresee that these seeds would be collected and saved for planting the following year.

APHIS concludes that gene introgression from 06PBCarHG1 into cultivated tobacco and ornamental *Nicotiana* species and persistence of the regulated article in the environment is extremely unlikely to occur as a result of the proposed environmental release.

B. Impacts from the Presence of *npt*II, *nos, add*3, *trf*A and ColEI and RK2 Origin of Replication.

The selectable marker gene for kanamycin resistance (*nptII*) is expressed in 06PBCarHG1. Because NPTII is not toxic, shares no homology with proteins known to be toxic or allergenic (U.S. FDA 1998), and is present in many plant lines previously deregulated by USDA, the expression of NPTII in *Nicotiana* hybrid plants is not expected to have deleterious effects or significant effects on nontarget organisms, including beneficial organisms and either listed or proposed-for-listing threatened and endangered species (TES). The NOS protein, which produces nopaline, has no known sequence homology to known toxins or allergens (Canadian Food Inspection Agency, 1998), and many plant species, such as soybeans and cotton, naturally produce nopaline (Christou 1986). Similarly, the expression of NOS in *Nicotiana* hybrid plants is not expected to have deleterious effects or significant effects on nontarget organisms, including beneficial organisms and TES.

Other DNA sequences are present, but are not expected to be expressed in 06PBCarHG1. Because the genes *trf*A (encodes for a DNA-binding protein that initiates replication from the RK2 origin of replication) and *add*3 (encodes for the resistance to streptomycin/spectinomycin) are under the control of bacterial promoters, they are unlikely to be expressed in the nuclear plant genome. Similarly ColEI and RK2, origin of replication sequences (from *E. coli*), and the *nos* terminator (from *A. tumefaciens*) are not expressed in 06PBCarHG1. Therefore, APHIS has determined the presence of the NPTII and NOS proteins and the non-expressed DNA will have no significant environmental impacts.

C. Impact on animals

Animals, other than the occasional skunk foraging on insects, do not generally consume *Nicotiana* hybrids planted in an agricultural setting (Dr. Orlando Chambers, University of Kentucky, personal communication). In the rare situation that an animal might forage on a 06PBCarHG1 plant, antibodies as a class are non-toxic. Antibodies are ubiquitous in nature, so insects and animals that consume eggs and milk are routinely exposed to antibodies (see Appendix A: Antibodies). Any antibody in plant debris that was produced via genetic engineering would have the same fate as antibodies in any decaying animal tissue or animal by-product, and would be quickly degraded (see Appendix A: Antibodies, for more detailed information on the degradation of antibodies in the environment). The genetically engineered CaroRx™ has been found to be non-toxic and non-allergenic. During clinical trials where CaroRx™ was administered to animals and human volunteers, no adverse effects were detected suggesting that plant preparations containing the purified antibodies did not induce an allergic response when given orally

(Ma 1998, Ma 2005, Weintraub 2004). Therefore, APHIS believes that the genetically engineered *Nicotiana* hybrids present no increased risk to animals compared to the non-GE *Nicotiana* hybrid.

D. Alteration in susceptibility to disease or insects

The presence of CaroRx™ in *Nicotiana* hybrids is not expected to alter the susceptibility of *Nicotiana* hybrid plants to diseases or to insects, as the antibody binds specifically to the *S. mutans* SAI/II surface adhesion protein in the bacterium *S. mutans*. *S. mutans* is an organism that causes tooth decay but has no reported effects on plants and due to its specificity will not bind to plant pathogens or insect pests. In addition, previous field tests conducted with *N. tabacum* X *N. glauca* hybrids in 2005 (05-053-01r, 05-354-04r, and in 2006 (06-037-0r) did not reveal increased susceptibility to disease or insects. Therefore, APHIS believes that this environmental release will not increase *Nicotiana* hybrid disease or susceptibility of *Nicotiana* hybrids to insects.

E. Weediness

APHIS evaluated the potential for the genetically engineered *N. tabacum* X *N. glauca* hybrid line 06PBCarHG1 to be weedy. Previous field tests over a number of years have indicated that the *N. tabacum* X *N. glauca* hybrid 06PBCarHG1 does not produce viable seeds. Because *Nicotiana* plants propagate by the production of seeds and little to no seeds will be produced, the field release of hybrid line 06PBCarHG1 is not expected to result in plants that could potentially be weedy. As neither parental species of the hybrid overwinters in the state of Kentucky, even if plants produced viable seeds, the resulting plants are not likely to persist in the environment. The addition of the transgenes is not likely to render the *N. tabacum* and *N. glauca* hybrid line, weedier. None of the gene products is likely to increase the fitness, alter reproductive capacity, or affect other traits associated with weediness.

F. Impact on Existing Agricultural Practices

The transgenic *Nicotiana* hybrid plants engineered to produce antibodies will be cultivated using standard cultivation practices generally used for tobacco production in Kentucky. Additional measures will be taken to ensure that the regulated article is confined to the site of the environmental release. These measures are described in part V, section C, Affected Environment Description of the Field Release, and in Appendix C, Supplemental Permit Conditions, of this EA. Planet Biotechnology will monitor the fields throughout the growing season for deleterious effects on plants, non-target organisms, or the environment, and during the following year for volunteer plants. The use of these plants in the proposed environmental release should not affect current agricultural practices with tobacco.

G. Horizontal Gene Transfer to Other Organisms

Following harvest of the *Nicotiana* hybrid plants, some plant material will remain at the environmental release site and will be subject to natural degradation by soil-inhabiting microbes. APHIS has assessed the likelihood of whether DNA transfer could occur to soil-inhabiting microbes through a process known as horizontal transfer. Horizontal gene transfer of DNA from the *Nicotiana* hybrid plants to bacteria and expression in bacteria is unlikely to occur. First, many genomes have been sequenced from bacteria that are

closely associated with plants, including *Agrobacterium* and *Rhizobium* (Kaneko 2000, Wood 2001, Kaneko 2002). There is no evidence that these organisms contain genes derived from plants. Second, in cases where review of sequence data implied that horizontal gene transfer occurred, these events are inferred to occur on an evolutionary time scale, on the order of millions of years (Koonin 2001, Brown 2003). Third, FDA has evaluated horizontal gene transfer from the use of antibiotic resistance marker genes in genetically engineered plants and concluded that the likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms, in the gastrointestinal tract of humans or animals or in the environment, is remote (http://vm.cfsan.fda.gov/~dms/opa-armg.html), and APHIS concurs with this finding. Therefore, APHIS concludes that horizontal gene transfer is unlikely to occur.

H. Impacts on Human Health

The *Nicotiana* hybrid plants in this environmental release will be used for the processing and extraction of antibodies, but will not be used directly for other purposes. After extraction of CaroRx™, the remaining *Nicotiana* hybrid plant material will be incorporated into the soil for natural decomposition. CaroRx™ has been the subject of clinical trials where its safety was demonstrated. No adverse effects to humans were reported when CaroRx™ antibodies were applied orally (Ma 1990). Because there may be some flowers produced, and honeybees are known to pollinate *Nicotiana* species, APHIS considered the possibility that the gene product would be present in honey. APHIS concludes that the CaroRx™ will not be detectable in honey because CaroRx™ is not expressed in pollen or nectar at levels that can be measured with a sensitive antibody assay (data submitted with the 05-354-03r permit by Planet Biotechnology). No potential impact on people living in the area of the environmental release, or any other human population, can be identified. Therefore, APHIS concludes that there should not be any significant effects on the human environment as a result of this proposed field trial.

I. Impacts on Threatened and Endangered Species

APHIS evaluated the potential for impacts on TES proposed and listed with the U.S. Fish and Wildlife Service (FWS) using the U.S. Fish and Wildlife database http://www.fws.gov/endangered/wildlife.html and NatureServe database: http://www.natureserve.org/explorer/. APHIS analyzed the published data and studies supplied by the applicant and supports the applicant's conclusion that the confined release of 06PBCarHG1 would not harm any Federally listed (or proposed) TES (see Appendix D: The Threatened and Endangered Species worksheet that was prepared by the permit applicant). The analyses found that none of the listed (or proposed) TES are associated with *Nicotiana* hybrid fields in Kentucky. Even if any of the listed (or proposed) TES frequented the environmental releases, none of the species would likely be exposed to the engineered products because they do not consume tobacco plants and the engineered products are not detectable in pollen. Even if the listed species were exposed to the engineered products (antibody, NOS, NPTII), the risk would be negligible because there is no reported toxicity of the products and, therefore, the *Nicotiana* hybrids would not be any more hazardous to these organisms than unmodified tobacco.

APHIS has reached a determination that the proposed environmental release should have no effect on federally listed TES or species proposed for listing, and no effect on designated critical habitat or habitat proposed for designation in the action area. Consequently, consultation under Section 7 of the Endangered Species Act with FWS is not required for the action described in the preferred alternative of this EA.

J. Cumulative Environmental Effects

The vast majority of tobacco grown in the U.S. for leaf and seed purposes is non-transgenic. A small portion of the tobacco currently grown is a deregulated transformed line (Vector 21-41) that was engineered to produce reduced levels of nicotine. Relative to the total tobacco acreage (110,734 acres in Kentucky and 428,631 total acres in the U.S., in 2001, according to the USDA National Agricultural Statistics Service) the proposed 100 acres will represent a very small percentage of the acreage that will be grown in Kentucky in 2007. This genetically engineered line differs from non-transformed tobacco, by its ability to produce an antibody. Compared to field testing and/or large scale plantings that may raise issues for loss of effectiveness of an engineered herbicide because of the preponderance of use, or for the build up of resistance in target organisms due to plant incorporated protectants, this field release of an antibody does not raise similar issues. Any antibody in plant debris that was produced via genetic engineering would have the same fate as antibodies in any decaying animal tissue or by-product, and would be quickly degraded and incorporated into the nitrogen cycle (see Appendix A: Antibodies, for more detailed information on the degradation of antibodies in the environment). However, if any unidentified cumulative effects should be found in the future, APHIS would take the appropriate action to curtail potential significant environmental effects. There are no other past, present, or reasonably foreseeable actions, which could, in aggregation with the environmental release of 06PBCarHG1, cause any significant cumulative impacts on the environment.

K. Special Considerations: Other Environmental Statutes and Considerations

Executive Order (EO) 12898, "Federal Actions to Address Environmental Justice in Minority Populations and Low-Income Populations," requires Federal agencies to conduct their programs, policies, and activities that substantially affect human health or the environment in a manner so as not to exclude persons and populations from participation in or benefiting from such programs. It also enforces existing statutes to prevent minority and low-income communities from being subjected to disproportionately high and significant human health or environmental effects. Each alternative was analyzed in its ability to affect minority and low-income populations. None of the alternatives were found to pose disproportionately high or significant human health or environmental effects to any specific minority or low-income group.

EO 13045, “Protection of Children from Environmental Health Risks and Safety Risks,” acknowledges that children may suffer disproportionately from environmental health and safety risks because of their developmental stage, greater metabolic activity levels, and behavior patterns, as compared to adults. The EO (to the extent permitted by law and consistent with APHIS’s mission) requires each Federal agency to identify, assess, and address environmental health risks and safety risks that may disproportionately affect

children. None of the alternatives are expected to have disproportionately high or significant human health or environmental effects to children.

EO 13112, “Invasive Species,” states that Federal agencies take action to prevent the introduction of invasive species and provide for their control and to minimize the economic, ecological, and human health impacts that invasive species cause. The engineered *Nicotiana* hybrid is not expected to produce viable seeds, and is also not expected to survive Kentucky winters, so it is not thought that it will persist in the environment and become invasive. Based on the data submitted by the applicant and reviewed by APHIS, the engineered plant is not significantly different in any fitness characteristics from its parent in ways that might increase its invasive potential.

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APPENDIX A: ANTIBODIES

Introduction
The following section is an in depth description of the structure and function of antibodies as well as their uses in research and disease treatment.

The Immune System
Healthy humans are born with an innate (or natural) immunity, a type of immunity that occurs naturally as a result of a person's genetic constitution or physiology and does not arise from a previous infection or vaccination. Innate immunity also includes the external barriers of the body, like the skin and mucous membranes (like those that line the nose, throat, and gastrointestinal tract). There is also a second kind of protection called adaptive (or active) immunity. Adaptive immunity evolves as a person or animal is exposed to diseases or immunized against diseases through vaccination and generally produces long-term immunity. Passive immunity is the transfer of antibodies from another individual, as through injection or placental transfer to a fetus; it essentially is "borrowed" from another source and it lasts for a short time.

The immune system (both active and innate) is the body's defense against infectious organisms and other invaders. It is made up of a network of cells, tissues, and organs that work together to protect the body. The cells that are part of the adaptive defense system are white blood cells, or leukocytes. One type of leukocyte is called a lymphocyte, which allows the body to remember and recognize previous invaders. There are two kinds of lymphocytes: B lymphocytes (B cells) and T lymphocytes (T cells). Lymphocytes start out in the bone marrow and either stay there and mature into B cells, or they leave for the thymus gland, where they mature into T cells. One of the main jobs of B cells is the production of antibodies. The part of the immune system that involves antibodies secreted by B cells is called humoral immunity. A substance introduced into the body that stimulates the production of an antibody is called an antigen. Antigens include toxins, bacteria, foreign blood cells, and the cells of transplanted organs. When an organism detects an antigen, several types of cells work together to recognize and respond to it. These cells trigger the B cells to produce antibodies. Antibodies are specialized proteins that lock onto specific antigens. Although antibodies can recognize an antigen and lock onto it, they are not capable of destroying it without the help of T cells. T cells are part of the system that destroys antigens that have been tagged by antibodies or cells that have been infected or somehow changed. Antibodies can also neutralize toxins (poisonous or damaging substances) produced by different organisms.

Antibodies
An antibody is a protein that binds specifically to a particular substance (an antigen). While each antibody is unique in its ability to bind to its corresponding antigen, antibodies in general have the same overall structure and are referred to collectively as immunoglobulins. Antibodies exist as one or more copies of a Y-shaped unit, composed of four polypeptide chains. Each Y contains two identical copies of a “heavy” chain, and two identical copies of a “light” chain, named as such by their relative molecular weights (Fig.1A). An antibody can also be broken into two pieces (via enzymatic digestion) called the Fab, Fab2, and Fc fragments. The Fab fragment is the

portion of the immunoglobulin where the relevant antigen binds and the Fc fragment is the other section of the immunoglobulin (Fig 1B). Antibodies can be divided into five classes: IgG (IgY in birds), IgM, IgA, IgD and IgE, based on the number of Y units and the type of heavy chain. Antibodies are produced by plasma cells (B cells) in response to infection or immunization. By binding to an antigen (or pathogen), the antibody either neutralizes the antigen or prepares it (or pathogen) for uptake and destruction by phagocytes (Janeway 2001).

Polyclonal versus Monoclonal Antibodies

Antibodies produced by the immune system are, by definition, polyclonal; meaning many different B cells produced the antibodies in response to antigen stimulation. A monoclonal antibody is produced by cloning a single B cell. Large amounts of monoclonal antibodies are produced by a hybridoma; a cell line that is made by fusing a B cell with a myeloma cell (Köhler 1975). Monoclonal antibodies can also be produced in genetically transformed microbial cells. Recombinant antibodies have been successfully made in microbial expression systems and mammalian cell cultures for over two decades. The estimated cost to produce a monoclonal antibody using hybridoma technology or microbial expression systems is US $5000/gram (Institute for Laboratory Animal Research and Council 1999). The cost to produce recombinant proteins (such as antibodies) is reduced 80-98 percent in plants as compared to traditional microbial and mammalian cell systems (Institute for Laboratory Animal Research and Council 1999, Giddings 2001), with much of the costs focused on downstream purification systems.

Importance of Monoclonal Antibody Production

Some of the early applications of monoclonal antibodies were blood-group typing, pregnancy testing, and identifying viruses, cancers, blood clots, and heart disease. Today, along with multiple diagnostic test uses, monoclonal antibodies are part of many cancer treatments, as well as treatments for arthritis, a variety of viruses, diabetes, and multiple sclerosis (http://users.path.ox.ac.uk/~scobbold/tig/new1/mabth.html). The FDA has approved many monoclonal antibodies for use in cancer therapy (www.cancer.org):

Trade Name (Generic Name)	Cancer Treated	Approved
Rituxan (Rituximab)	Non-Hodgkin lymphoma	1997
Herceptin (Trastuzumab)	Breast cancer	1998
Mylotarg (Gemtuzumab ozogamicin)	Acute myelogenous leukemia (AML)	2000
Campath (Alemtuzumab)	Chronic lymphocytic leukemia (CLL)	2001
Zevalin (Ibritumomab tiuxetan)	Non-Hodgkin lymphoma	2002
Bexxar (Tositumomab)	Non-Hodgkin lymphoma	2003

Erbitux (Cetuximab)	Colorectal cancer	2004
Avastin (Bevacizumab)	Colorectal cancer	2004

Another important therapeutic use of antibodies is the post-exposure rabies treatment to prevent disease outbreak in a bitten human (considered category III exposure by WHO, http://www.who.int). The administration of antibody to an unimmunized individual is called passive immunization. One of the first monoclonal antibodies to be marketed for passive immunization was palivizumab, which is used to prevent serious lower respiratory tract infections caused by respiratory syncytial virus (RSV) in infants. Passive immunization of *Campylobacter jejuni*, infected chickens with oral monoclonal antibodies, was found to be successful as both prophylaxis and therapy (Tsubokura 1997). With rising medical costs and increasing use of monoclonal antibodies in the medical and industrial field, researchers have found ways to produce monoclonal antibodies at a reduced cost to the consumer by creating plants containing the monoclonal antibody gene construct.

Ubiquitous Presence of Antibodies in Nature

Humans consume 50-100g of protein per day as part of their normal dietary intake (USDA and HHS 2005). Foods that contain naturally present polyclonal antibodies include eggs, meat, milk, and milk products. Egg yolks contain approximately 100-150 mg of total IgY antibody per yolk, while the egg whites contain trace amounts of IgM and IgA (Polson 1990). Polyclonal antibodies are a normal component of animal blood (Tizard 2000); and therefore are present in all meat. Cow's milk (not colostrum, whose antibody content is considerably higher) contains 50-100 mg IgA/dl, 5-10 mg IgM/ dl, and 20-50 mg IgG/dl (Tizard 2000).

Antibody Degradation in the Environment

The growth of all organisms depends on the availability of nitrogen, which is required in large amounts as an essential component of proteins, nucleic acids, and other cellular constituents. Nitrogen is often the limiting factor for growth and biomass production in all environments where there is suitable climate and availability of water to support life. Along with nitrogen fixation (the conversion of atmospheric nitrogen to ammonium or nitrate ions), microbes degrade organic material and debris in the soil, releasing nitrogen for reuse by other organisms (http://soil.gsfc.nasa.gov/NFTG/nitrocyc.htm). Microbes are known to degrade many different types of complex organic and inorganic molecules such as TNT, Dioxins, and polychlorobiphenyls (PCBs) (Tiedje 1993, Wittich 1998, Lewis 2004). Antibodies are relatively simple molecules (see Figure 1) consisting of a chain of amino acids that fold into a three-dimensional shape due to amino acid interactions and disulfide bonds. Any antibody in plant debris that was produced via genetic engineering would have the same fate as antibodies in any decaying animal tissue or by-product and would be quickly degraded and incorporated into the nitrogen cycle.

Antibody Degradation in the Digestive Tract

Without degradation into smaller peptides, proteins (such as antibodies) are too large to pass intact through the intestinal wall. They need to be broken down into amino acids or small peptides before they can be absorbed. The breakdown of protein begins in the stomach where

hydrochloric acid (HCl) denatures the protein and facilitates the action of pepsin, the major gastric enzyme that splits the peptide bonds. Other proteolytic enzymes (enzymes that break down protein bonds) involved in the gastric process are trypsin, chymotrypsin, carboxypeptidase, and elastase. Most monoclonal antibody immunizations and therapies are given intravenously and not orally due to the instability of the antibody during digestion (Zeitlin 1999).

Orally administered antibodies break into F(ab)2, Fab, and Fc fragments in the digestive system (Fig. 1B). It has been found that 19 percent of these fragments retain their neutralizing activity in the ileum of healthy adults (Roos 1995). Another study (Bogstedt 1997) analyzed the amount of neutralizing antibody activity in the fecal material of healthy adults orally administered antibodies and only found minute amounts present (<0.01 percent of the ingested antibodies), suggesting the majority of the peptides are absorbed in the small intestine. When the time of passage through the gastrointestinal tract is short (as with patients who have diarrhea) antibodies can retain more activity in the lower gastrointestinal tract and be more effective in a therapeutic setting (Hammarström 1994).

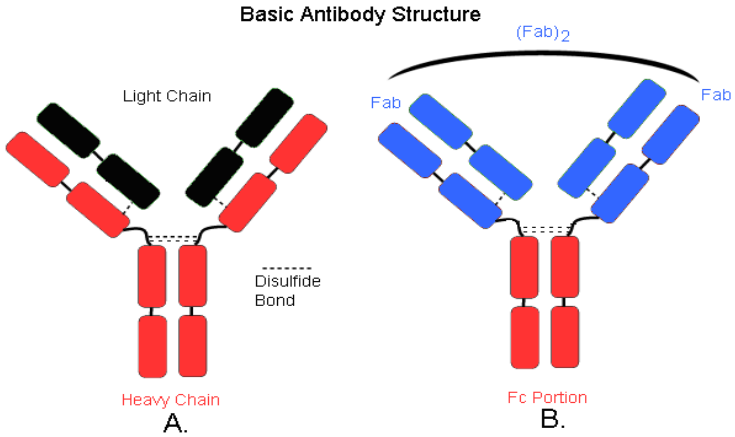

Figure 1. Basic Antibody Structure. A. Typical Y structure with two light chains (in black) and two heavy chains (in red) bound by disulfide bonds (dotted lines); **B.** Location of the Fab and Fc fragments on the antibody molecule. The two Fab antibody regions are typically indicated as $(Fab)_2$.

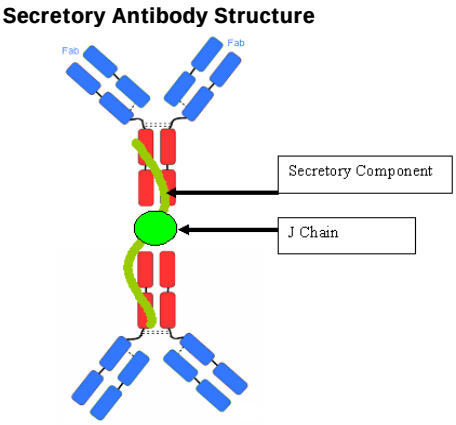

Figure 2. Secretory Antibody Structure. Complex antibody where two Y shaped antibodies are joined by a J Chain to a secretory component.

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APPENDIX B: STANDARD PERMIT CONDITIONS FROM 7 CFR 340.4

(f) *Permit conditions*. A person who is issued a permit and his/her employees or agents shall comply with the following conditions, and any supplemental permit conditions which shall be listed on the permit, as deemed by the Administrator to be necessary to prevent the dissemination and establishment of plant pests:

1. The regulated article shall be maintained and disposed of (when necessary) in a manner so as to prevent the dissemination and establishment of plant pests.

2. All packing material, shipping containers, and any other material accompanying the regulated article shall be treated or disposed of in such a manner so as to prevent the dissemination and establishment of plant pests.

3. The regulated article shall be kept separate from other organisms, except as specifically allowed in the permit;

4. The regulated article shall be maintained only in areas and premises specified in the permit;

5. An inspector shall be allowed access, during regular business hours, to the place where the regulated article is located and to any records relating to the introduction of a regulated article;

6. The regulated article shall, when possible, be kept identified with a label showing the name of the regulated article;

7. The regulated article shall be subject to the application of measures determined by the Administrator to be necessary to prevent the accidental or unauthorized release of the regulated article;

8. The regulated article shall be subject to the application of remedial measures (including disposal) determined by the Administrator to be necessary to prevent the spread of plant pests;

9. A person who has been issued a permit shall submit to APHIS a field test report within 6 months after the termination of the field test. A field test report shall include the APHIS reference number, methods of observation, resulting data, and analysis regarding all deleterious effects on plants, non-target organisms, or the environment;

10. APHIS shall be notified within the time periods and manner specified below, in the event of the following occurrences:

    - Orally notified immediately upon discovery and notify in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article;

    - In writing as soon as possible but not later than within 5 working days if the regulated article or associated host organism is found to have characteristics

substantially different from those listed in the application for a permit or suffers any unusual occurrence (excessive mortality or morbidity, or unanticipated effect on non-target organisms);

11. A permittee or his/her agent and any person who seeks to import a regulated article into the United States shall:

    - Import or offer the regulated article for entry only at a port of entry which is designated by an asterisk in 7 CFR 319.37-14(b);

    - Notify APHIS promptly upon arrival of any regulated article at a port of entry, of its arrival by such means as a manifest, customs entry document, commercial invoice, waybill, a broker's document, or a notice form provided for such purpose; and

    - Mark and identify the regulated article in accordance with 7 CFR 340.5.

APPENDIX C: SUPPLEMENTAL PERMIT CONDITIONS

I. Compliance with Regulations

1. Any regulated article introduced not in compliance with the requirements of Title 7 of the Code of Federal Regulations, Part 340, or any standard or supplemental permit conditions, shall be subject to the immediate application of such remedial measures or safeguards as an inspector determines necessary, to prevent the introduction of such plant pests. The responsible party may be subject to fines or penalties as authorized by the Plant Protection Act (7 U.S.C. 7701-7772).

2. This Permit (APHIS form 2000) does not eliminate the permittee's legal responsibility to obtain all necessary Federal and State approvals, including: (A) for the use of any non-genetically engineered plant pest or pathogens as challenge inoculum; (B) plants, plant parts or seeds which are under existing Federal or State quarantine or restricted use; (C) experimental use of unregistered chemicals; and (D) food, feed, pharmacological, biologic, or industrial use of regulated articles or their products and co-mingled plant material. In the latter case, depending on the use, reviews by APHIS, the U.S. Food and Drug Administration, or the U.S. Environmental Protection Agency may be necessary.

3. The procedures, processes, and safeguards used to prevent escape, dissemination, and persistence of the regulated article as described in the permit application, in APHIS-approved Standard Operating Procedures (SOPs) and, in the supplemental permit conditions must be strictly followed. The permittee must maintain records sufficient to verify compliance with these procedures, including information regarding who performed the activity. Persons performing such activities shall have received training as described in a training program submitted to and approved by APHIS. These records are subject to examination by APHIS. APHIS, BRS must be notified of any proposed changes to the protocol referenced in the permit application.

II. Reporting Unauthorized Releases and Unintended Effects

1. According to the regulation in 7 CFR 340.4(f)(10)(i), APHIS shall be notified orally immediately upon discovery and notified in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article.

    - For immediate oral notification, contact APHIS/BRS Compliance Staff at (301) 734-5690 and ask to speak to a Compliance and Inspection staff member.
    - In the event of an emergency and you are unable to reach the BRS Compliance Staff at the above number, you may call:

The APHIS/BRS Regional Biotechnology Coordinator assigned to the state, where the field test occurs

For Western Region, contact Ralph Stoaks by phone at (970) 494-7573 or e-mail Ralph.D.Stoaks@aphis.usda.gov

For Eastern Region, contact Ashima SenGupta by phone at (919) 855-7622 or e-mail Ashima.SenGupta@aphis.usda.gov

Or

The APHIS/PPQ Regional Biotechnology Coordinator assigned to the state where the field test occurs

For Western Region, contact Stacy Scott by phone at 970-494-7577 or e-mail Stacy.E.Scott@aphis.usda.gov

For Eastern Region, contact Susan Dublinski by phone at (919) 855-7324 or e-mail Susan.G.Dublinski@aphis.usda.gov

Or

The APHIS State Plant Health Director for the state where the field test occurs. The list of APHIS State Plant Health Director is available at http://ceris.purdue.edu/napis/names/sphdXstate.html

KY	Mike Madryga, Prospect	(502) 228-8224	(502) 228-6306	michael.b.madryga@aphis.usda.gov

2. According to 7 CFR 340.4(f)(10)(ii), APHIS shall be notified in writing as soon as possible but within 5 working days if the regulated article or associated host organism is found to have characteristics substantially different from those listed in the permit application or suffers any unusual occurrence (excessive mortality or morbidity, or unanticipated effect on non-target organisms).

3. Written notification should be sent by one of the following means:

By e-mail:

BRSCompliance@aphis.usda.gov

By mail:

Biotechnology Regulatory Services (BRS)
Compliance and Inspection Branch
USDA/APHIS
4700 River Rd. Unit 147
Riverdale, MD 20737

III. Perimeter Fallow Zone

1. To ensure that transgenic plants are not inadvertently commingled with plants to be used for food or feed, a perimeter fallow zone of at least 50 ft. must be maintained around the transgenic test site in which no crops are grown to be harvested or used for food or feed.

2. The permitted border rows of non-transgenic plants that are the same as, or sexually compatible with, the regulated article are considered part of the field test. The perimeter fallow zone shall start outside the border rows.

3. The perimeter fallow zone shall be managed in a way that allows detection and destruction of volunteer plants that are the same as, or sexually compatible with, the transgenic plants.

IV. Dedicated Planting and Harvesting

1. To ensure that the regulated article is not inadvertently removed from the site, harvesting equipment must be dedicated for use in the permitted test site(s) or used on non-regulated research tobacco that are not used for food or feed (BRS Variance 05-001) from the time of planting through the end of harvesting.

2. After harvest, you will not be required to obtain APHIS authorization to use this equipment on APHIS -permitted sites (same sites or different sites) planted with same transgenic crop, with the target protein(s) authorized under this permit, in subsequent growing seasons under an extension of this permit or a different permit.

3. Authorization is required from APHIS before harvesting equipment used during this field test can be used on sites planted to crops not included under this permit. The permittee must notify APHIS/BRS and the State Regulatory Official at least 21 calendar days in advance of cleaning this equipment for this purpose so that APHIS may schedule an inspection to ensure that the equipment has been cleaned appropriately.

V. Cleaning of Equipment

1. To minimize the risk of seed movement and commingling, equipment used for planting and harvesting, as well as other field equipment (e.g. tractors and tillage attachments, such as disks, plows, harrows, and subsoilers) used at any time from the time of planting through the post-harvest monitoring period must be cleaned in accordance with procedures submitted to and approved by APHIS before they are moved off of the environmental release site.

2. Equipment used to transport seeds or harvested material must be cleaned prior to loading and after transportation to the authorized site in accordance with procedures submitted to and approved by APHIS.

3. Seed cleaning and drying must be performed in accordance with the procedures submitted to and approved by APHIS to confine the plant material and minimize the risk of seed loss, spillage, or commingling.

VI. Use of Dedicated Storage Facilities

1. Dedicated facilities (locked or secured buildings, bins, or areas, posted as restricted to authorized personnel only) must be used for storage of equipment and regulated articles for the duration of the field test.

2. Before returning these facilities to general use, they must be cleaned in accordance with procedures submitted to and approved by APHIS. **The permittee must notify** APHIS/BRS and the State Regulatory Official at least 21 calendar days in advance to allow for APHIS to schedule an inspection to ensure that the facilities have been cleaned appropriately. APHIS authorization must be received before facilities are returned to general use.

VII. Post Harvest Monitoring

1. The field test site including the perimeter fallow zone must be monitored for the presence of volunteer ***Nicotiana*** **hybrid** plants for **one year** after termination of the field test. Viable plant material should not remain at the test site following termination. Volunteers, if found, will be uprooted by hand and destroyed by dismemberment and incorporation into the soil.
2. Fields must be checked for volunteers once every 2 weeks, over a period of 4 weeks, immediately post harvest. Then, before the Fall and Winter months arrive, (the first frost) the fields will be checked once every 6 weeks. During the Fall and Winter months the fields will be checked once every 8 weeks. During Spring and the following Summer the fields will be checked once every three weeks.

VIII. Post Harvest Land Use Restrictions

1. Production of food and feed crops at the field test site and the perimeter fallow zone is restricted during the growing season that follows harvest or termination of the field test.

2. Permission must be obtained from APHIS/BRS prior to planting any food or feed crop at the field test site and perimeter fallow zone during the post-harvest monitoring period. Requests for such permission are not encouraged and will not be granted in cases where there is a reasonable potential for plant material derived from, or originating from, the regulated articles to become mixed with the proposed food or feed crop during harvesting.

IX. Inspections

1. APHIS Biotechnology Regulatory Services (BRS) and/or an APHIS/PPQ Regional Biotechnologist, APHIS/BRS Regional Biotechnology Coordinator or APHIS State Plant Health Director may conduct inspections of the test site, facilities, and/or records at any time.

2. APHIS may invite the FDA or State Regulatory Officials to participate in these inspections.

3. Inspections will likely correspond to the beginning of the field test, mid-season or during flowering, at and/or following harvest, and during the post-harvest monitoring period.

4. Inspections will include examination of records that verify compliance with regulations and SOPs.

X. Reports and Notices

Send notices and all reports (CBI and CBI-deleted or non-CBI copies) to BRS by e-mail, mail, or fax.

BRS E-mail:
BRSCompliance@aphis.usda.gov

BRS Mail:
Biotechnology Regulatory Services (BRS)
Compliance and Inspection Branch
USDA/APHIS
4700 River Rd. Unit 147
Riverdale, MD 20737

BRS Fax:
Compliance and Inspection Branch
(301) 734-8669

In addition, fax the CBI deleted or non CBI version of the pre-planting and pre-harvest (termination) notices to the State Regulatory Official:

State Plant Regulatory Official	John Obrycki, State Entomologist
Mailing Address	Department of Entomology S-225 Ag. Science Center North University of Kentucky Lexington, KY 40546-0091
Phone	859-257-5838
Fax	859-257-3807
Email	john.obrycki@uky.edu

Web Site	http://www.KyStateEnt.org

Contact information for State Officials
http://www.nationalplantboard.org/member/index.html

1. **Pre-Planting Notice**
   At least 7 calendar days before planting, submit a Pre-Planting notice that includes the following information for each field test site:
   i. Provide APHIS with the contact information for each field test site.
   ii. Indicate if planting and harvesting equipment will be moved between authorized field test sites.
   iii. A map that clearly identifies the site location to facilitate any inspections by USDA personnel.
   iv. The planned number of acres for each gene construct.
   v. The planned planting date

2. **Planting Report**
   Within 28 calendar days after planting, submit a planting report that includes the following information for each field test site:
   i. A map of the site, with sufficient information to locate it, that includes: the state, county, address, GPS coordinates for each corner of the plot (inclusive of the border rows of any sexually compatible plants);
   ii. The location and the approximate number and/or acres of transgenic plants which were actually planted at the test site for each of the target proteins;
   iii. The total acreage of the test plot (exclude border rows, if any);
   iv. The distance from the genetically engineered plants to the nearest plants of the same crop that will be used for food, feed, or seed production. A survey should be done within the distance specified in the permit.
   v. A list of the specific confinement option(s) selected at each site if your permit allows different confinement options (e.g. bagging flowers, border rows, or isolation distance.).
   vi. The actual planting date.

3. **Pre-Harvest/ Termination Notice**
   At least 21 calendar days prior to the anticipated harvest or termination, submit a Notice indicating the planned date of harvest **or** termination and the contact information for each field test site. For multiple harvests, submit the notice prior to the initial harvest.

4. **Field Test Report**
   Within 6 months after the end of the field test (final harvest or crop destruct), the permittee is required to submit a field test report. Field test reports shall include:
   i. APHIS reference number
   ii. Methods of observation.
   iii. Resulting data.
   iv. Analysis of all deleterious effects on plants, non-target organisms, or the environment.

v. A list of the lines planted at each site

vi. Disposition table

The disposition table should contain the following information: site name (or GPS), crop, gene, harvest date, and disposition of harvested material.

The disposition table is a formal record of how the regulated material was removed from the environment. An accounting of the harvested material should be provided with regards to what material is harvested, how much material is harvested per site, what is done to devitalize residual and harvested material at the site, where the harvested material is transported, stored and further processed up to the time it is taken to a contained facility.

5. **Monitoring Report**

Within 3 months after the end of the monitoring period, submit a volunteer monitoring report. The report must include:

i. Dates when the field site and perimeter fallow zone were inspected for volunteers.

ii. Number of volunteers observed.

iii. Any actions taken to remove or destroy volunteers.

APPENDIX D: APPLICANT SUPPLIED TES WORKSHEET

12-15-05

TES WORKSHEET Page 1 CBI DELETED COPY

PLANTS EXPRESSING CARORX AND THE STATE OF KENTUCKY

RECIPIENT ORGANISM:

Transgenic tobacco.

PRODUCT:

CaroRx, a monoclonal, chimeric secretory immunoglobulin A antibody is assembled, *in planta*, from four subunits derived from mouse and rabbit gene sequences. CaroRx binds to SA I/II, a protein located on the surface of the oral, cariogenic, bacteria *Streptococcus mutans*. Using this surface protein, *S. mutans* adheres to teeth, a necessary step in the formation of cavities. The binding of CaroRx to these bacteria can prevent them from adhering to teeth and may be used to reduce, or eliminate, the infection of teeth by these cariogenic bacteria (Ma, et al., 1998; Lehner, et al., 1985; Lehner, et al., 1975; Lehner, et al., 1986; Ma, et al., 1989; Ma and Lehner, 1990; Ma, et al., 1987). CaroRx is intended for use in humans to reduce or eliminate the colonization or infection of teeth by *S. mutans*.

In the United States, CaroRx is an Investigational New Drug (BB-IND # 7526) and CaroRx is a registered Medical Device in the European Union.

LOCATION OF FIELD RELEASE:

Daviess County, KY.

LEVELS OF CARORX PRODUCED IN VARIOUS TISSUES:

Fully assembled, partially assembled and degraded forms of CaroRx are present in extracts prepared from plant tissues. [.] Functional CaroRx includes the canonically equivalent SIgA/G, dIgA/G, IgA/G and $F(ab')_2$ forms of the plantibody. CaroRx is not an enzyme and CaroRx's only function is to bind its target. CaroRx does not prevent its target organism from growing once it has bound to the target.. The free or degraded subunits are present in smaller amounts which have not been quantified in any tissues. For the forgoing reasons concerning the assembled antibody, these entities are also not expected to present any danger of toxicity to mammals, birds or to the environment as a whole.

CBI Deleted

ASSESSMENT OF CARORX PLANTS

Importantly, there were no unusual occurrences during field trials of any CaroRx-expressing plants, including no observations of any deleterious effects on the environment or on non-target organisms, as reported in the on February 25, 2003 for permit number 02-108-02r, the report submitted, in early 2005, for permit number 04-044-01r and the reports submitted, in late 2005, for permit numbers 05-053-01r and 05-087-01r.

Based on literature review and discussions with tobacco scientists, we can identify no organisms, except plant pests and possible skunks, that consume tobacco tissues (personal communication from Dr. Orlando Chambers and Mr. Richard Mundell). Earthworms are negatively impacted by nicotine in the soil.

CaroRx, or its constituent subunits, have no known toxicities.

Based on the literature, and on safety data from human clinical trials, CaroRx is not known to be toxic when applied orally and subsequently ingested (BB-IND # 7526 and Ma, et al., 1998; Weintraub et al., 2005). It is relevant to note that CaroRx is non-toxic even when applied to its target, *Streptococcus mutans* (Ma, et al., 1990 and report for permit number 04-044-01r).

Tobacco is not sexually compatible with any TES plant listed for Kentucky. Any unexpected effects from a field test of CaroRx plants would be minimized by the confinement of the plants to the test site. After harvest the plants will be destroyed in such a way as to maximize the capture the CaroRx and to minimize the possibility of the escape of any viable transgenic tissue in solid or liquid waste streams.

CONCLUSION:

A previous field trial of the CaroRx plants in Kentucky reported no unusual occurrences, and, in particular, reported no observations of any effects on the environment or on non-target organisms.

Since there is no identifiable direct effect of this field test on any plant or animal species, there will be no anticipated adverse effects on any threatened or endangered species.

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TES Listing by State accessed at:
http://ecos.fws.gov/tess_public/TESSWebpageUsaLists?state=KY
Accessed on: December 15, 2005

• Kentucky -- 51 listings

Animals -- 43

Status	Listing
E	Bat, gray (*Myotis grisescens*)
E	Bat, Indiana (*Myotis sodalis*)
E	Bat, Virginia big-eared (*Corynorhinus (=Plecotus) townsendii virginianus*)
XN	Bean, Cumberland (pearlymussel) AL; Free-Flowing Reach of the Tennessee River below the Wilson Dam, Colbert and Lauderdale Counties, AL (*Villosa trabalis*)
E	Bean, Cumberland (pearlymussel) Entire Range; Except where listed as Experimental Populations (*Villosa trabalis*)
T	Bear, grizzly lower 48 States, except where listed as an experimental population or the Yellowstone population (*Ursus arctos horribilis*)
XN	Blossom, tubercled (pearlymussel) AL; Free-Flowing Reach of the Tennessee River below the Wilson Dam, Colbert and Lauderdale Counties, AL (*Epioblasma torulosa torulosa*)
E	Blossom, tubercled (pearlymussel) Entire Range; Except where listed as Experimental Populations (*Epioblasma torulosa torulosa*)
XN	Catspaw (=purple cat's paw pearlymussel) AL; Free-Flowing Reach of the Tennessee River below the Wilson Dam, Colbert and Lauderdale Counties, AL (*Epioblasma obliquata obliquata*)
E	Catspaw (=purple cat's paw pearlymussel) Entire Range; Except where listed as Experimental Populations (*Epioblasma obliquata obliquata*)
E	Clubshell Entire Range; Except where listed as Experimental Populations (*Pleurobema clava*)
XN	Combshell, Cumberlandian AL; Free-Flowing Reach of the Tennessee River below the Wilson Dam, Colbert and Lauderdale Counties, AL (*Epioblasma brevidens*)
E	Combshell, Cumberlandian Entire Range; Except where listed as Experimental Populations (*Epioblasma brevidens*)
XN	Crane, whooping U.S.A.(AL, AR, GA, IL, IN, IA, KY, LA, MI, MN, MS, MO, NC, OH, SC, TN, VA, WI, WV) (*Grus americana*)
T	Dace, blackside (*Phoxinus cumberlandensis*)
E	Darter, duskytail Entire (*Etheostoma percnurum*)
E	Darter, relict (*Etheostoma chienense*)
T	Eagle, bald lower 48 States (*Haliaeetus leucocephalus*)
E	Elktoe, Cumberland (*Alasmidonta atropurpurea*)
E	Fanshell (*Cyprogenia stegaria*)
T	Lynx, Canada lower 48 States DPS (*Lynx canadensis*)
E	Mapleleaf, winged Entire; except where listed as experimental populations (*Quadrula fragosa*)
E	Mucket, pink (pearlymussel) (*Lampsilis abrupta*)
XN	Mussel, oyster AL; Free-Flowing Reach of the Tennessee River below the Wilson Dam, Colbert and Lauderdale Counties, AL (*Epioblasma capsaeformis*)
E	Mussel, oyster Entire Range; Except where listed as Experimental Populations (*Epioblasma capsaeformis*)
E	Pearlymussel, cracking Entire Range; Except where listed as Experimental Populations (*Hemistena lata*)
E	Pearlymussel, dromedary Entire Range; Except where listed as Experimental Populations (*Dromus dromas*)
E	Pearlymussel, littlewing (*Pegias fabula*)
E	Pigtoe, rough (*Pleurobema plenum*)

E	Pimpleback, orangefoot (pearlymussel) (*Plethobasus cooperianus*)
T	Plover, piping except Great Lakes watershed (*Charadrius melodus*)
E	Pocketbook, fat (*Potamilus capax*)
E	Puma (=cougar), eastern (*Puma (=Felis) concolor couguar*)
E	Riffleshell, northern (*Epioblasma torulosa rangiana*)
E	Riffleshell, tan (*Epioblasma florentina walkeri (=E. walkeri)*)
E	Ring pink (mussel) (*Obovaria retusa*)
E	Shiner, palezone (*Notropis albizonatus*)
E	Shrimp, Kentucky cave (*Palaemonias ganteri*)
E	Sturgeon, pallid (*Scaphirhynchus albus*)
E	Tern, least interior pop. (*Sterna antillarum*)
T	Trout, bull U.S.A., conterminous, lower 48 states (*Salvelinus confluentus*)
E	Wartyback, white (pearlymussel) (*Plethobasus cicatricosus*)
E	Wolf, gray lower 48 States, except MN and where XN; Mexico (*Canis lupus*)

Plants -- 8

Status	Listing
E	Clover, running buffalo (*Trifolium stoloniferum*)
E	Goldenrod, Short's (*Solidago shortii*)
T	Goldenrod, white-haired (*Solidago albopilosa*)
T	Potato-bean, Price's (*Apios priceana*)
E	Rock-cress, Braun's (*Arabis perstellata*)
T	Rosemary, Cumberland (*Conradina verticillata*)
E	Sandwort, Cumberland (*Arenaria cumberlandensis*)
T	Spiraea, Virginia (*Spiraea virginiana*)