ITEM 7

CHEMISTRY, MANUFACTURING, AND CONTROLS

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GLOSSARY OF ABBREVIATIONS

° C	degrees Celsius
° F	degrees Fahrenheit
CAS	Chemical Abstract Service
CDC	Center for Disease Control
CFR	Code of Federal Regulations
cig	cigarette
CMC	Chemistry, Manufacturing, and Controls
CO	carbon monoxide
CTP	Center for Tobacco Products
DW	dry weight
EP	electrostatic precipitation
EPA	Environmental Protection Agency
ESI	electrospray ionization
FDA	(US) Food and Drug Administration
FTC	Federal Trade Commission
g	gram(s)
GC	gas chromatography
GMP	good manufacturing practices
H_2O	dihydrogen monoxide (water)
HPLC	high performance liquid chromatograph
ICP	inductively coupled plasma
ID	identification
IND	Investigational New Drug (Application)
ISO	International Organization for Standardization
lbs	pounds
LC	liquid chromatography
MeOH	methyl alcohol
MS	mass spectroscopy
µg	microgram(s)
µL	microliter(s)
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
mm	millimeter(s)
mmWg	millimeters of water gauge
MSD	mass selective detector
N/A	not applicable
NaOH	sodium hydroxide

NCSU	North Carolina State University
NDA	new drug application
ng	nanogram
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	*N'*-Nitrosonornicotine
ppm	parts per million
RTD	resistance to draw
SN	serial number
SPE	solid phase extraction
TCA	Tobacco Control Act
TSNA	tobacco specific n-nitrosamines
US	United States
USDA	United States Department of Agriculture
VLA	very low alkaloid
VLN	very low nicotine (cigarette)
X-22	processed very low nicotine (VLN) tobacco

7.0 OVERVIEW

22nd Century Limited, LLC is developing a smoking cessation product, X-22 Smoking Cessation Product, for use in Phase II clinical trials. X-22 is a tobacco-based botanical drug substance used in the form of a cigarette. A pre-IND meeting was held between 22nd Century Limited, LLC and the Food and Drug Administration (FDA) on January 21, 2009. Chemistry, Manufacturing, and Controls (CMC) information discussed at the meeting and committed to in the December 17, 2008 Meeting Package are provided within this Investigational New Drug Application (IND). CMC data are presented in accordance with the FDA's Guidance for Industry "Botanical Drug Products, June 2004". A summary of the raw material (tobacco leaves), drug substance, X-22 cigarette filler, and the finished product, X-22 Smoking Cessation Product, are presented below.

Drug Substance:

The botanical drug substance, X-22, is processed tobacco having a very low nicotine (VLN) content. The VLN content in X-22 cigarettes is due to the use of tobacco raw material obtained from the genetically engineered tobacco line Vector 21-41. The Vector 21-41 line has been engineered to have a very low alkaloid (VLA) content. X-22 cigarettes have a tobacco content per cigarette and tar yield comparable to typical commercial cigarettes, but a nicotine content and yield that are reduced more than 90% compared to the typical commercial cigarettes.

The same VLA tobacco variety, Vector 21-41, was used in Quest® cigarettes sold by Vector Tobacco Inc. from 2002 to 2007 and in Quest 3 menthol cigarettes from 2003 to 2007. Vector 21-41 was blended with other tobacco in Quest 1 and Quest 2 cigarettes, and it was the only tobacco in Quest 3, which is most similar to X-22.

Tobacco variety Vector 21-41 was developed at North Carolina State University (NCSU) in a research program funded by 22nd Century. Reference is made to Appendix 1: Genetic Engineering Strategy for details of the genetic engineering strategy and a comparison of Vector 21-41 to a related non-transgenic variety.

The tobacco used to make the product for the proposed clinical trial was produced in the United States (US) by tobacco growers under contract to 22nd Century.

Drug Product:

X-22 Smoking Cessation Product is a tobacco-based botanical medical product manufactured to provide a VLN cigarette.

X-22 cigarettes, like conventional cigarettes, consist of filler, filter, and wrapper components. The filler in cigarettes is comprised primarily of cured tobacco leaf. All components are supplied by providers of components for conventional cigarettes, including components of the filler. X-22 cigarettes are prepared using traditional cigarette manufacturing processes. The finished drug product specifications are similar to conventional cigarette specifications. With the exception of nicotine content, specifications for X-22 Smoking Cessation Product were established based on comparisons to the most widely used cigarette products.

In contrast to conventional low-yield ("light" or "ultra light") cigarettes that have reduced tar and nicotine yields as measured by smoking machines mainly due to smoke dilution, cigarettes containing very low levels of nicotine deliver greatly reduced ratios of nicotine to other smoke components.

Reference is made to Section 7.2.3.2 for a description of the manufacturing and packaging process, including analytical controls and available data.

7.1 DRUG SUBSTANCE

7.1.1 General Information

7.1.1.1 Nomenclature

X-22: Processed Very Low Nicotine (VLN) Tobacco

7.1.2 Manufacturer

The following facilities are used for processing and testing X-22.

Manufacturing/Suppliers	
(b) (4) [redacted]	Manufacturer of X-22
Testing Laboratories	
North Carolina State University Crop Science Tobacco Analytical Services Laboratory 3709 Hillsborough Street Box 8604 Raleigh, NC 27695-7620 USA	Alkaloid screening of incoming tobacco leaf
Arista Laboratories 1941 Reymet Road Richmond, Virginia 23237 USA	Analyses of X-22 (release testing, except for pesticide residues)
Global Laboratory Services 2107 Black Creek Road Wilson, NC 27893 USA	Analyses of X-22 (release testing for pesticide residues)

7.1.2.1 Manufacturing Process

Very Low Alkaloid (VLA) tobacco raw material is grown in the US by selected suppliers for blending to produce the very low nicotine (VLN) processed tobacco, X-22. A description of the VLA tobacco production process and X-22 VLN tobacco process is provided below.

Botanical Raw Material – Very Low Nicotine Tobacco Leaf

X-22, like conventional cigarettes, is comprised primarily of cured tobacco leaf. The characteristics of cured tobacco vary due to the type and variety of tobacco, maturity of the plant, stalk position of leaves on the plant, growing conditions, crop management practices, and the manner of curing. Characteristics that distinguish types and grades of tobacco include nicotine content, sugar content, aroma, flavor and color.

Conventional cured tobacco has a nicotinic alkaloid content of 20-40 mg/g. The tobacco used in X-22 is distinguished by a very low content of nicotine and total nicotinic alkaloids in cured, mature leaf. This is required for the mainstream smoke from a VLN cigarette to have similar characteristics to that of conventional cigarettes other than the amount of alkaloids.

Currently, Vector 21-41, a tobacco variety genetically engineered to reduce nicotinic alkaloid biosynthesis is used. Cured tobacco produced from Vector 21-41 has a nicotine content (b) (4) and total nicotinic alkaloid (b) (4) The details of the genetic engineering strategy and a comparison of Vector 21-41 to a related non-transgenic variety is provided below. Except for the use of a VLA tobacco variety, the growing, curing and processing of tobacco for X-22 are the same as for conventional cigarettes.

Tobacco is grown from transplants. In the US, tobacco transplants are typically produced in a greenhouse using a system in which multi-cell styrofoam trays are floated on beds of nutrient solution. As the VLN tobacco is produced from a proprietary specialty variety, pelleted seed of the variety Vector 21-41 is provided to greenhouse operators by 22nd Century to produce a specific number of trays of seedlings for specified tobacco growers. The seed pellets of Vector 21-41 are a distinctive color to avoid confusion or mixing at seeding.

The VLN tobacco is grown in the US under direct contracts with growers, as is a major portion of US tobacco. Transplants of Vector 21-41 are provided to the growers. The contracts specify that the growers are to deliver only tobacco grown from the seedlings provided.

The growers cure the tobacco leaf after harvest. Tobacco used in cigarettes in the US is cured by flue curing or air curing.

For air curing the entire plant is harvested at one time by cutting the stalk, and the plant is hung by the stalk. Air curing is carried out in a barn or similar structure without heating. Air curing takes several months, during the tobacco leaf changes from the green and yellowish to tan and reddish-brown aromatic leaf ready for processing. After curing is completed, leaves are removed (stripped) from the stalk by hand. The individual whole leaves are sorted into grades by stalk position and physical characteristics. Leaves of the same stalk position are baled.

Flue-cured tobacco is cured in an enclosed structure using flues or pipes that direct heat from a furnace into the barn. The temperature is increased about mid-way through a curing process of approximately one week. Flue-cured tobacco remains light in color, which is why it is also referred to as "bright" tobacco. For flue curing, leaves are harvested in "primings", in which individual leaves are harvested by stalk position a few leaves at a time, starting at the base of the plant and progressing upwards as leaves mature. The whole leaves are strung on sticks and hung in the curing barn. After curing the leaves of the same stalk position are baled.

Cured tobacco is delivered by growers as bundles of whole leaves. On the days of delivery of VLN tobacco leaf by the growers, no other tobacco is handled at the receiving location. At the time of delivery of cured tobacco leaf by the grower, each bundle of tobacco leaves (500-600 lbs) is tagged with a 4-part tag with a pre-printed, unique identification number.

The bundle of tobacco leaves is visually inspected for the presence of non-tobacco material and to determine that leaf characteristics are consistent with the tobacco type and stalk position. Bundles with excessive moisture [redacted] are rejected. The grower identification (ID), date, type, stalk position and weight are recorded on the tag. One copy of the tag from each bundle accepted is pulled and attached to a copy of the grower receipt.

Two samples are taken from each stalk position from each grower from different bundles for determination of nicotinic alkaloids in the leaf lamina. The sample is labeled with the grower ID, stalk position, and date.

The nicotinic alkaloid profile in the leaf lamina is analyzed at the Tobacco Analytical Services Laboratory at NCSU according to standard practices for evaluation of tobacco

alkaloids. Briefly, for each sample, leaf lamina is manually separated from the midrib ("stem") and placed in a paper bag labeled with the sample ID number. The lamina sample is dried and ground. A weighed portion of the sample is extracted; nicotine and minor alkaloids in the extract are quantified by gas chromatography with fluorescence ionization detection. Tobacco lots with sample nicotine results (b) (4) and total nicotinic alkaloids (b) (4) are listed as suitable for processing for X-22.

Processing of tobacco to yield X-22

The cured leaf is stemmed by (b) (4), a company dedicated to tobacco processing; no other materials are processed at the site. Stemming refers to the separation of "strips" (pieces of leaf lamina) from "stems" (the midrib of the tobacco leaf). At the start of this process, the moisture content of the leaf is adjusted by use of water and steam to reach a target of approximately (b) (4) to reduce brittleness to make the tobacco suitable for processing ("conditioning").

The thinner and lighter leaf lamina is threshed from the midrib by a series of rollers and separated by forced air. These strips are screened to remove fines.

During the stemming process, mixing takes place during the adjustment of moisture levels in the conditioning drums and separation of strips and stems. In addition, deliberate blending to increase uniformity across the batch occurs in "silos" in which material is introduced into large storage containers by spreading it in thin layers from end to end from the top, and removed from one end at the bottom.

At the end of the process, the moisture content of the strips is adjusted to about (b) (4) for storage, and the strips are packed in corrugated cases lined on the bottom and top with kraft paper. Each case contains approximately 400 lbs of strips. The cases are sealed with plastic bands. The moisture content of stems is adjusted and they are packed in the same manner.

Transportation and Processing Controls

The bundles of tobacco leaf are shipped from the receiving location to the processor, (b) (4) [redacted], in trailers containing no other materials. At the time of loading, a load number is recorded on the tag attached to each tobacco leaf bundle, and a second copy of the tag on each bundle is pulled and attached to a copy of the load log.

On receipt at the stemmery, a copy of the multi-part tag on each bundle of tobacco leaf is pulled and used to enter the bundle into the processor's inventory. The fourth copy remains on the bundle during storage. All bundles of unstemmed VLN tobacco leaf are stored segregated from other tobacco.

At the time scheduled for stemming of VLA tobacco leaf for use in X-22, no other tobacco leaf is present in the facility. Prior to stemming of leaf for use in X-22, the processing area and processing line are cleaned to remove residues of conventional tobacco.

Bundles of cured leaf of the appropriate stalk position and nicotine content are selected by bundle tag ID number, brought to the stemmery and arranged at the start of a conveyor belt. The bundles are visually inspected to verify that the leaf characteristics are consistent with the type and grade indicated on the tag. The bundle tag ID number, grower ID, and date on the tag are checked against the list of bundles to be included in the stemming batch.

The fourth copy of the tag from each bundle of tobacco leaf is collected as the bundles are opened and the leaf is placed on a conveyor belt. The tags used to generate a list of tobacco leaf bundles in the stemming batch, and retained by the processor.

As it is loaded on the initial belt the leaf is inspected for non-tobacco material and damaged tobacco. Moisture is added as the leaf is gently tumbled while rollers with rods to loosen the leaf bundles. The leaf then passes sequentially through three conditioning cylinders (large, slightly inclined, rotating cylinders) in which the moisture level is refined and made more uniform. As the whole leaf passes from the first conditioning cylinder to the second conditioning cylinder it is again inspected visually for non-tobacco material. The moisture level of whole leaf at the end of the conditioning process is monitored to achieve a target level of approximately (b) (4) [redacted] prior to the threshing of the strips from the stems.

Strips and stems exiting the stemming process are visually inspected as they are transferred to the dryers.

Cartons are labeled on both ends with the crop year, batch ID, and date processed, and are serially numbered. Samples are taken from cases throughout the production of each lot to monitor moisture content and for analysis of nicotine content. Samples are analyzed for nicotine and minor alkaloid content by an accredited laboratory (Arista). The samples are pooled and tested for pesticide residues. Reference is made to Table 1 for in-process data for the clinical lots.

Table 1 In-Process Data for Lots (b) (4) and (b) (4)

	Receiving Samples – Leaf Lamina from Unstemmed Whole Leaf (15 growers)		**Processed Tobacco - Strips**	
	Nicotine (mg/g DW) mean (range)	Total Nicotinic Alkaloids (mg/g DW) mean (range)	Nicotine (mg/g DW)	Total Nicotinic Alkaloids (mg/g DW)
(b) (4)	[redacted]	[redacted]	[redacted]	[redacted]
(b) (4)	[redacted]	[redacted]	[redacted]	[redacted]

7.1.3 Characterization

As agreed with the FDA at the January 21, 2009 pre-IND meeting, characterization data will be provided in a future IND amendment and prior to initiation of Phase III clinical trials.

7.1.4 Control of Drug Substance

7.1.4.1 Specification

X-22 will be tested according to the specifications listed in Table 2. As discussed at the pre-IND meeting, Identification is performed on the VLN tobacco raw material and X-22 processed tobacco. As development continues and prior to Phase III, the identification and analytical controls will be enhanced.

Table 2 Specifications for X-22

Test	Method	Acceptance criteria
Appearance	Visual inspection	Free from visible non-tobacco material and visible mold.
Identification	Arista AM-072 Arista AM-055	Presence of nicotine and other nicotinic alkaloids below specified limits.
Loss on Drying	Arista A AM-058	[redacted]
Nicotine and Nicotinic Alkaloids	Arista AM-072 Arista AM-055	[redacted] total nicotinic alkaloids [redacted] mg/g
Pesticide Residues	Global WI 2.01-05-02-01 Global WI 2.01-05-05-01 Global WI 2.01-05-04-01 Global WI 2.01-05-03-01	Below limits specified in 7 CFR 29-427 (See Table 3)

7.1.4.2 Analytical Procedures

Appearance:

X-22 is visually inspected to verify that the material is composed of leaf fragments of buff, tan, red orange, and yellow color consistent with the appearance of cured tobacco leaf and free from non-tobacco material and visible mold.

Identification:

X-22 is identified as VLN tobacco by the presence of nicotine and related nicotinic alkaloids at levels (< 1/mg/g nicotine and <2.5 mg/g total alkaloids) that distinguish it from conventional tobacco.

Loss on Drying (Oven Volatiles):

The method used for this study is detailed in Arista Analytical Method AM-058 - The Determination of Oven Volatiles.

Oven Volatiles in tobacco, including water and other low boiling compounds, are determined by heating samples in an oven at 100°C, for three hours.

For smokeless or cut rag tobacco, 3.0 ± 0.1 grams of tobacco are placed in a previously weighed aluminum dish and sample weight is recorded to the nearest 0.0001g. Each sample is analyzed in triplicate. The aluminum dishes with the tobacco samples are placed in an oven maintained at 100 ± 2.0°C. After 3 hours ± 5 minutes the samples are removed from

the oven and placed in a desiccator until they reach room temperature. New sample weights are recorded and % Oven Volatiles in the samples is calculated.

Nicotine in Processed VLN Tobacco:

The method used is detailed in Arista Analytical Method AM-072, The Determination of Nicotine by GC for Smokeless Tobacco Products. This method was published in the Federal Register (Center for Disease Control (CDC) Notice, Federal Register Vol. 74, No. 4 January 7, 2009).

The tobacco sample is prepared (depending on type) as recommended in the CDC method. Aqueous NaOH is added to a previously weighed sample of tobacco, swirled to mix and allowed to stand. After 15 minutes, methyl tert-butyl ether containing the internal standard (quinoline) is added and the sample is placed on a shaker for 2 hours. Once the sample has been removed from the shaker it is placed in vertical position to allow the phases to separate. An aliquot of the organic layer is transferred to a sample vial and analyzed for nicotine by gas chromatography (GC).

This method is used for determination of nicotine content of processed VLN tobacco because the content is below the limit of quantitation of the liquid chromatography-mass spectroscopy/mass spectroscopy (LC-MS/MS) method below as carried out by the contracted laboratories.

Determination of Minor Alkaloids in Processed VLN Tobacco:

The analytical methods used for this study are detailed in Arista Analytical Method AM-055, Determination of Nicotine and Minor Alkaloids in Tobacco Filler.

Nicotine and the related minor alkaloids are quantitated from tobacco filler that has been extracted with an aqueous ammonium acetate solution. LC-MS/MS (liquid chromatography coupled to a triple quadruple mass spectrometer) allows for the determination of the targeted alkaloids with minimum interference from other components in tobacco filler. The LC-MS/MS method uses a high performance liquid chromatography (HPLC) equipped with a C18 reversed phase column to resolve the alkaloids from potential interferences. Further selectivity is accomplished using a triple quadruple mass spectrometer with atmospheric

pressure chemical ionization and selective reaction monitoring of characteristic daughter ions. Deuterated internal standards (d-nicotine and d-nornicotine) are added to the extraction solution and used to correct for recovery of the alkaloids.

The concentration of the alkaloids is reported in units of mass-to-volume (i.e., µg/mL). The measured concentration, the mass of tobacco extracted, and the sample solution volume(s) are also used to calculate the total analyte mass on a per gram of filler basis.

This method is not used for the determination of nicotine content in processed VLN tobacco because the nicotine content is below the limit of quantitation for the LC-MS/MS method below as carried out by the contracted laboratories. The GC method above is used.

Pesticide Residues:

Although the Environmental Protection Agency (EPA) regulates and specifies which pesticides may be used on tobacco, it does not regulate residues of pesticides approved for use on tobacco in conventional tobacco products. Standards were set by the United States Department of Agriculture (USDA) for the maximum allowable concentration of certain pesticide residues for imported tobacco. These standards specified in 7 CFR §29.427 are listed in Table 3. Residue analysis of tobaccos to be used in the clinical trial material will be carried out to confirm that they are in compliance with these limits.

Table 3 § 29.427 Pesticide Residue Standards – Maximum Concentration of Residues Allowed (ppm)

	ppm
CHLORDANE	3.0
DIBROMOCHLOROPROPANE (DBCP)	1.0
DICAMBA	5.0
ENDRIN	0.1
ETHYLENE DIBROMIDE (EDB)	0.1
FORMOTHION	0.5
HEXACHLOROBENZENE (HCB)	0.1
METHOXYCHLOR	0.1
TOXAPHENE	0.3
2,4-D	5.0
2,4,5-T	0.1
Sum of ALDRIN and DIELDRIN	0.1
Sum of CYPERMETHRIN and PERMETHRIN	3.0
Sum of DDT, TDE (DDD), and DDE	0.4
Sum of HEPTACHLOR and HEPTACHLOR EPOXIDE	0.1

Determination of Acid Herbicide Pesticides

The analytical methods used for determination of acid herbicide pesticides are detailed in Global Laboratory Services Method WI 2.01-05-02-01. Finely ground tobacco is extracted with acetonitrile. The extract is filtered and concentrated by evaporation under nitrogen. Diazomentane, petroleum ether, and acidified sodium sulfate are added. The upper phase is loaded onto a Florisil Sep-Pak Solid Phase Extraction cartridge. The flow through is collected. The cartridge is eluted with 50:50 ethyl ether:petroleum ether and the eluate is added to the sample. The sample is concentrated by evaporation under nitrogen, diluted with iso-octane, and analyzed by gas chromatography using electron capture detection.

Determination of Organophosphate Pesticides

The analytical methods used for determination of acid herbicide pesticides are detailed in Global Laboratory Services Method WI 2.01-05-05-01. Finely ground tobacco is extracted with acetonitrile. The extract is filtered. The filtrate is analyzed by gas chromatography.

Determination of Ethylene Dibromide and Dibromochloropropane Residues

The analytical methods used for determination of acid herbicide pesticides are detailed in Global Laboratory Services Method WI 2.01-05-04-01. Tobacco is extracted with deionized water:iso-octane (100:3). The sample is heated to boiling. The water and iso-octane are collected in a distillation receiver. The iso-octane layer is collected and saturated with sodium sulfate. An aliquot of the iso-octane layer is analyzed by gas chromatography

Determination of Chlorinated Hydrocarbon Pesticides

The analytical methods used for determination of acid herbicide pesticides are detailed in Global Laboratory Services Method WI 2.01-05-03-01. Finely ground tobacco is extracted with acetonitrile. The extract is filtered and concentrated by evaporation under nitrogen. The extract is filtered and concentrated by evaporation under nitrogen. Petroleum ether and acidified sodium sulfate are added. The upper phase is loaded onto a Florisil Sep-Pak Solid Phase Extraction cartridge. The flow through is collected. The cartridge is eluted with 15:85 ethyl ether:petroleum ether and the eluate is added to the sample. The cartridge is eluted with 50:50 ethyl ether:petroleum ether and the eluate is added to the sample. The sample is concentrated by evaporation under nitrogen, diluted with iso-octane, and analyzed by gas chromatography.

7.1.4.3 Validation of Analytical Procedures

Validation of methods is not required at this stage of development.

7.1.4.4 Batch Analyses

Release testing for drug substance lots, used in the manufacture of X-22 Smoking Cessation Product batch 23149 is provided in Table 4. X-22 Smoking Cessation Product batch 23149 will be used in a Phase II clinical study.

Table 4 Batch Analysis Data of X-22

Lot Number		(b) (4)	(b) (4)
Date of Manufacture		March 2010	March 2010
Purpose		Clinical use	Clinical use
Test	**Acceptance criteria**	**Results**	**Results**
Appearance	Free from visible non-tobacco material and visible mold.	Pass	Pass
Identification	Presence of nicotine and other nicotinic alkaloids below specified limits.	Pass	Pass
Loss on Drying	[redacted]	[redacted]	[redacted]
Nicotine	[redacted] total nicotinic alkaloids [redacted] mg/g	[redacted]	[redacted]
Pesticide Residues	Below limits specified in 7 CFR 29-427	Pass	Pass

[1] One or more minor nicotinic alkaloid below LOQ (anatabine <0.100 mg/g, anabasine <0.025 mg/g, mysomine <0.025 mg/g)

7.1.5 Reference Standards or Materials

This section is not applicable for this drug substance.

7.1.6 Container and Closure System

X-22 is stored in corrugated cartons that contain approximately 400 lbs net weight. The cartons are sealed with plastic bands.

7.1.7 Stability

A stability program will be established to support the holding of X-22 drug substance prior to the manufacture of the cigarette filler. Stability information will be provided in a future IND Amendment.

7.2 DRUG PRODUCT

7.2.1 Description and Composition of the Drug Product

X-22 Smoking Cessation Product is a tobacco-based botanical medical product in the form of a cigarette containing very low nicotine (VLN) tobacco. Like conventional cigarettes, X-22 Smoking Cessation Product is comprised of cigarette filler wrapped in a paper tube (tobacco section) and a filter section. The cigarette filler accounts for 93.5% of the tobacco section, which is the part combusted in normal use. X-22 Smoking Cessation Product is manufactured with components as outlined in Table 5.

Table 5 Composition of the X-22 Smoking Cessation Product Cigarette

			% of Tobacco Section
X-22 Cigarette			
Component	**Sub Component**	**Material**	
Tobacco Section	Tobacco Filler	[redacted]	[redacted]
Tobacco Section	Cigarette Paper	[redacted]	[redacted]
Tobacco Section	Seam Adhesive	[redacted]	[redacted]
Filter Section	Filter	[redacted]	N/A
Filter Section	Tipping Paper	[redacted]	N/A
Filter Section	Tipping Adhesive	[redacted]	N/A

The contribution of each component to the complete cigarette filler and final product is provided in Table 6.

Table 6 Filler Component Table

			Weight (mg/cigarette)	% of Filler	% of Tobacco Section	% of Cigarette
(b) (4) VLN Cut Rag						
Component	**Description**	**Supplier**				
Tobacco Strips	VLN cured tobacco lamina	(b) (4)				

7.2.2 Pharmaceutical Development

Not applicable at this stage of development.

7.2.3 Manufacture

7.2.3.1 Manufacturers

X-22 Smoking Cessation Product is manufactured, packaged, labeled, and release and stability tested at the following facilities. Also provided are suppliers of the filter, papers and adhesives used to produce the final product.

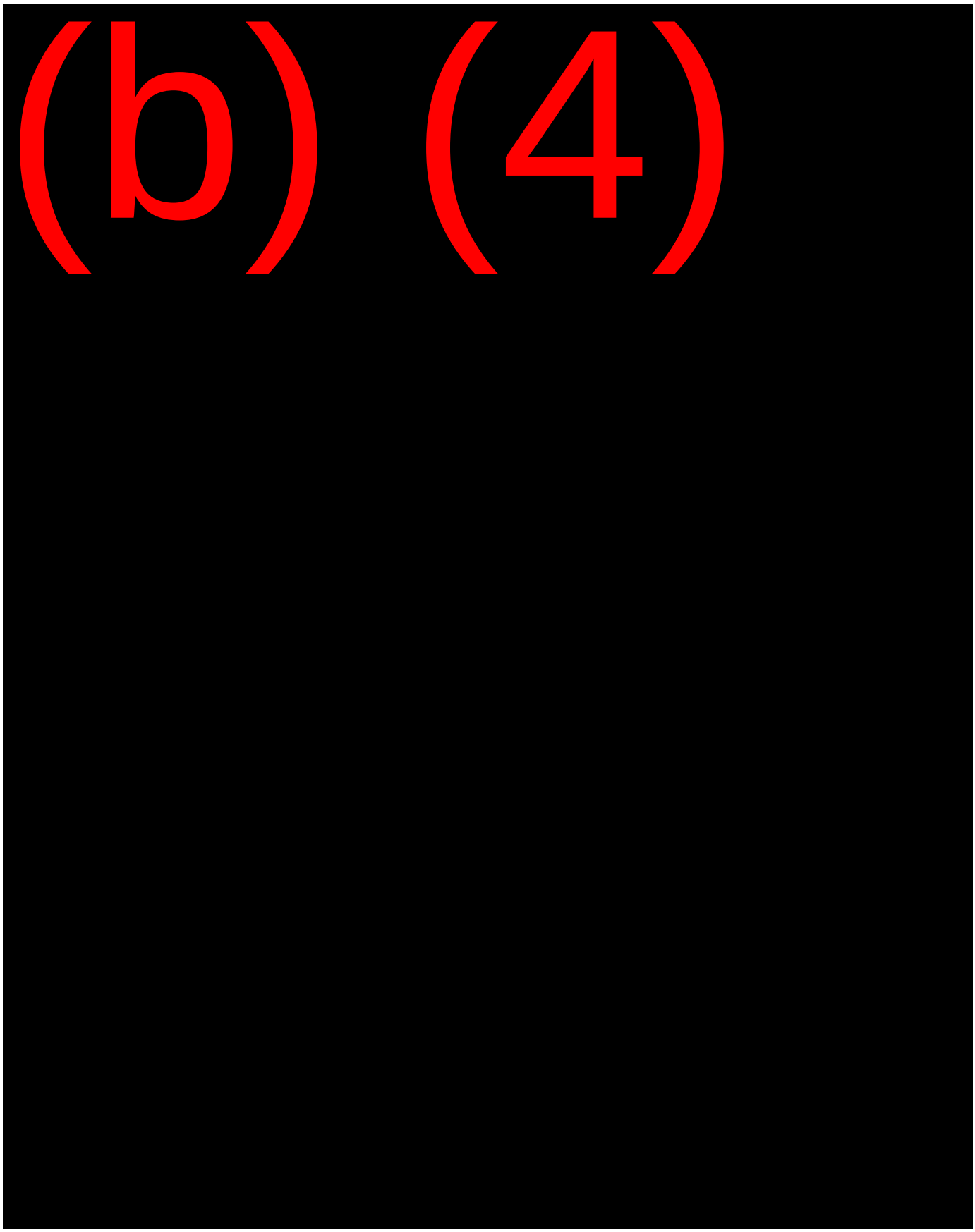
Manufacturing/Suppliers	Function
(b) (4)	Manufacturing of VLN cut rag (cigarette filler)
	Cigarette manufacturing and packing
	(b) (4)
Arista Laboratories 1941 Reymet Road Richmond, VA 23237 USA	Analyses, cigarette filler (nicotine and minor alkaloids, cigarettes (final product): weight, circumference, draw resistance drop, oven volatiles; tar, nicotine and carbon monoxide (CO) yield, heavy metals in smoke
Global Laboratory Services 2107 Black Creek Road Wilson, NC 27893 USA	Analysis of pesticide residues
Cigarette Delivery Components	
(b) (4)	Cigarette paper

Manufacturing/Suppliers	Function
(b) (4)	Filters
	Tipping paper (wraps around filter and overlaps tobacco rod to attach filter to cigarette)
	Adhesives (used to hold cigarette paper together and tipping paper to filter and cigarette)

7.2.3.2 Method of Manufacture

Except for use of a VLN tobacco variety, the materials used, and manufacturing of X-22 VLN cigarettes are carried out in the same manner as for conventional cigarettes. All processing of tobacco leaf and manufacturing of cigarette filler and cigarettes is currently carried out under contract by processors and manufacturers producing commercial tobacco products in the US.

(b) (4)


(b) (4)

(b) (4) [redacted]

Manufacturing of Cigarettes

The cigarettes for use in the clinical trial are manufactured by (b) (4) [redacted] As is required by the Family Smoking Prevention and Tobacco Control Act (TCA) of June 22, 2009, (b) (4) registered with and reports to the FDA Center for Tobacco Products (CTP). Although the CTP has not yet prescribed regulations requiring that methods used in cigarette manufacturing conform to current good manufacturing practice (GMP), as it is empowered to do under Section 906(e) of the Food Drug and Cosmetic Act as revised by the TCA, Dosal has implemented a GMP system.

The components of the cigarettes are complete filler, cigarette paper, seam adhesive, filter, tipping paper, and tipping adhesive, which are described in detail in Section 7.2.4. All components are supplied by manufacturers of components for conventional commercial cigarettes. Components for each lot of cigarettes to be manufactured are specified in a bill of materials. The bill of materials is used to generate materials lists for each lot of cigarettes to be made that include the product name and the IDs of all components (other than the adhesives, which are not product-specific).

The cigarettes are manufactured on an automated maker. (b) (4) [redacted]

Specifications for physical parameters (weight, circumference, tip dilution, resistance to draw) for each product are entered into a central quality monitoring system and displayed, along with data from sampling, on an interactive monitor at the maker.

Filler introduced into a hopper on the maker is mixed and screened to remove fines, and formed into a continuous band that is trimmed and overlaid on a continuous strip of cigarette paper. The paper is formed into a tube around the tobacco and sealed to produce a continuous rod or wrapped tobacco. Segments of the wrapped tobacco are cut to length to form the tobacco section of a cigarette.

A filter is attached to the tobacco section by abutting a tobacco section against the filter and wrapping them in a segment of tipping paper that covers the filter and overlaps the filter-tobacco section joint. On the (b) (4) maker currently used, as on many makers, two tobacco sections are joined simultaneously to a double filter rod by a double width tipping paper, and then the assembly is cut to produce two cigarettes.

The tipping paper used for the clinical trial cigarettes carries a distinctive mark, which while it is the same for the VLN cigarettes and Active Control cigarettes, labels each clinical trial cigarette in a manner that distinguishes it from all other cigarettes.

Samples are taken at intervals throughout the run to monitor physical characteristics. Test data (weight, circumference, tip ventilation, and resistance to draw) are displayed graphically on the interactive monitor at the maker.

The finished cigarettes are loaded into trays by an automated loader. Filled trays are transferred to a cart labeled with the card containing the lot specifications and cart number described above.

The clinical trial cigarettes are packaged in the same manner as conventional cigarettes, in custom packaging to distinguish them from other cigarettes. All packaging components are provided by suppliers of packaging components for commercial cigarettes. The trial cigarettes are packaged by an automated machine which forms sets of 20 cigarette and checks that each cigarette has a well-filled tip and that the overall dimension of the bundle are within pack tolerances. Bundles of 20 cigarettes are wrapped in paper-backed foil,

around which an inner frame and paper pack is formed and glued. Each pack is embossed with a numeric code that identifies the lot.

Each pack is wrapped in cellophane with a tear strip. The packs are packed 10 packs per carton. The cartons are packed in cases of 30 cartons per case. The cases are stamped with the date of manufacture and shipped for clinical labeling.

7.2.4 Control of Excipients

(b) (4)

[redacted]

7.2.4.1 (b) (4)

[redacted]

7.2.4.2 (b) (4)

[redacted]

7.2.4.3 (b) (4)

[redacted]

(b) (4)

[redacted]

7.2.5 Control of Drug Product

7.2.5.1 Specifications

The X-22 Smoking Cessation Product must conform to the Quality Specifications listed in Table 7. As agreed with the FDA at the January 2009 pre-IND meeting, further characterization of the finished drug product will be provided in a future IND amendment and prior to initiation of Phase III clinical trials.

Conformance with product specifications is assessed by an independent laboratory. Physical characteristics (weight, diameter, draw resistance), filler composition (nicotine content, pesticide residues) and smoke characteristics (nicotine, "tar," CO and heavy metals) are analyzed. Batch analyses data for the clinical batch of X-22 Smoking Cessation Product, batch 23149 is provided in Table 8.

Table 7 Specification Summary

Test	Method	Acceptance criteria
Physical Properties		
Total Weight	Arista AM-009	(b) (4)
Circumference		
Draw Resistance		
Chemical Properties:		
Smoke Yields[1]		
Tar	Arista T-001	
Nicotine		
Carbon Monoxide	Arista T-007	
Heavy Metals		
Arsenic	Arista AM-021	
Cadmium		
Lead		
Nickel		
Selenium		
Mercury	Arista AM-036	
Oven Volatiles (%)	Arista AM-058	
Pesticide Residues	Global WI 2.01-05-02-01 Global WI 2.01-05-05-01 Global WI 2.01-05-04-01 Global WI 2.01-05-03-01	

[1] Cigarettes are to be smoked using International Organization for Standardization (ISO) smoking conditions.

7.2.5.2 Analytical Procedures

Standards were set by the USDA for the maximum allowable concentration of certain pesticide residues for imported tobacco. These are specified in 7 CFR §29.427 and provided in Table 3.

Physical Properties

Total Weight

The method used for this study is detailed in Arista Analytical Method AM-009 – Physical Properties.

This measurement can either be performed automatically on the (Cerulean QTM automated cigarette analyzer (QTM stack) or manually. The QTM automatically feeds cigarettes from a hopper and analyzes for weight, circumference, pressure drop and ventilation. In the manual method, conditioned cigarettes are weighed, individually, on a calibrated analytical balance and recorded in grams to the nearest 0.0001g. The average, standard deviation and relative standard deviation are reported for multiple observations.

Circumference

The method used for this study is detailed in Arista Analytical Method AM-009 – Physical Properties.

Ten cigarettes are placed into the hopper of a QTM Stack with Module QTM 3. The Module should be calibrated before use. Circumference is reported in millimeters.

Draw Resistance

Ten previously conditioned cigarettes are placed into the hopper of a QTM Stack with module QTM 5U with filters pointing to the direction of the hopper diagram.

The QTM 5U automatically calibrates pressure drop at 17.5 mL/s. A check standard is used to verify, 1.99 mmWg for cigarettes and 803 mmWg for filter rods. Total resistance to draw (RTD) is defined as the pressure differential developed by a cigarette when air is pulled through it at the rate of 1050 mL/min.

Chemical Properties

Tar, Nicotine, Water (stability only) and Carbon Monoxide in Mainstream Smoke

The methods used for this study are detailed in Arista Analytical Method AM-001, Determination of Nicotine and Water in Smoke Condensates – Gas Chromatographic

Method, and Arista Analytical Method AM-007, Determination of Carbon Monoxide in Vapor Phase Cigarette Smoke.

This method applies to the measurements of nicotine and water in total particulate matter (TPM) from cigarette smoke collected on a 44mm Cambridge filter pad. The smoke condensate collected on the pad is dissolved in isopropanol. Subsequently, an aliquot of the sample solution is analyzed by gas chromatography (GC), and the nicotine and water content of the smoke condensate is determined. Using the nicotine and water values, and the measured wet total particulate matter, the tar content is then calculated. The resulting data is reported in milligrams per cigarette (mg/cig) for each smoke sample.

For carbon monoxide determination, the smoke is collected in gas sampling bags attached to the smoking machine. A non-dispersive infrared absorption method is used to measure the CO concentration in the vapor phase in percent by volume (%vol). Using the number of cigarettes, puff counts, puff volume and ambient conditions, the %CO is converted to milligrams per cigarette (mg/cig).

Selected Metals in Mainstream Smoke

The methods used for this study is detailed in Arista Analytical Method AM-021 - The Determination of Selected Metals in Mainstream Cigarette Smoke and Arista Analytical Method AM-036 - The Determination of Mercury in Mainstream Cigarette Smoke.

<u>Determination of Arsenic, Cadmium, Chromium, Lead, Nickel and Selenium in Mainstream Cigarette Smoke</u>

Smoking is performed on a Borgwaldt RM 20 CSR analytical smoking machine equipped with an electrostatic precipitation (EP) unit. A quartz EP tube is used in the EP unit for smoke collection.

The smoke condensate is rinsed out of the EP tube, using methanol. Next, the methanol is evaporated and the sample is digested using nitric acid, hydrogen peroxide and heat. The digested samples are subsequently analyzed by Inductively Coupled Plasma – Mass Spectroscopy (ICP-MS). The ICP-MS used in the method is a Perkin Elmer ICP-MS equipped with a dynamic reaction cell that allows for the determination of elements,

including Chromium and Selenium, which normally suffer from severe polyatomic interferences. Ammonia and methane are used as the reaction cell gas for chromium and selenium, respectively. Samples are introduced by nebulization into a radio frequency plasma where energy transfer processes cause desolvation, atomization, and ionization. The ions are extracted from the plasma through a differentially pumped vacuum interface and detected on the basis of their mass to charge ratio by a quadrupole mass spectrometer. The ions transmitted through the quadrupole are detected by an electron multiplier and the ELAN® software processes the ion information. Instrumental drift is corrected for by the use of indium and gallium as internal standards, which are added online.

The metals are reported in units of mass-to-volume (ng/mL). The measured concentration, the number of cigarettes smoked, and the sample solution volume(s) are also used to calculate the total analyte mass on a per cigarette basis. The measured concentration of the metals and the number of cigarettes smoked are used to calculate the concentration of each element in units of nanograms per cigarette (ng/cig).

Determination of Mercury in Mainstream Cigarette Smoke

Smoking is performed on a Borgwaldt RM 20 CSR. Particulate phase and gas phase mercury in mainstream smoke is trapped using two impingers connected in-series, each containing an acidic potassium permanganate solution. The impinger solutions containing the smoke are subjected to a microwave digestion in which mercury compounds (organic and inorganic) are oxidized to Hg+2. Mercury ions (Hg+2) in the digestate are reduced by stannous chloride to elemental mercury and analyzed by cold vapor atomic absorption spectrometry.

Mercury is reported in units of mass-to-volume (ng/mL). The measured concentration, the number of cigarettes smoked, and the sample solution volume(s) are used to calculate the total analyte mass in units of nanograms per cigarette (ng/cig).

Oven Volatiles (Cigarette Filler):

The method used for this study is detailed in Arista Analytical Method AM-058 - The Determination of Oven Volatiles.

Oven Volatiles in tobacco, including water and other low boiling compounds, are determined by heating samples in an oven at 100°C, for three hours.

For smokeless or cut rag tobacco, 3.0 ± 0.1 grams of tobacco are placed in a previously weighed aluminum dish and sample weight is recorded to the nearest 0.0001g. Each sample is analyzed in triplicate. The aluminum dishes with the tobacco samples are placed in an oven maintained at 100 ± 2.0°C. After 3 hours ± 5 minutes the samples are removed from the oven and placed in a desiccator until they reach room temperature. New sample weights are recorded and % Oven Volatiles in the samples is calculated.

Pesticide Residues:

Although the EPA regulates and specifies which pesticides may be used on tobacco, it does not regulate residues of pesticides approved for use on tobacco in conventional tobacco products. Standards were set by the USDA for the maximum allowable concentration of certain pesticide residues for imported tobacco. These standards specified in 7 CFR §29.427 are listed in Table 3. Residue analysis of tobaccos to be used in the clinical trial material will be carried out to confirm that they are in compliance with these limits.

7.2.5.3 Batch Analyses

Results for the clinical batch are presented below.

Table 8 Batch Analyses Results for the X-22 Smoking Cessation Product

Batch Number:		**23149**
Drug Substance Lot Used:		(b) (4)
Size:		
Use:		Phase II
Test	**Acceptance criteria**	**Results mean (range)**
Total Weight	(b) (4)	
Circumference		
Draw Resistance		
Smoke Yields		
Tar		
Nicotine		
Carbon Monoxide		
Heavy Metals		
Arsenic		
Cadmium		
Lead		
Mercury		
Nickel		
Selenium		
Oven Volatiles (%)		
Pesticide Residues	Below limits specified in 7 CFR 29-427 (See Table 3)	Pass (all below limit of quantitation)

7.2.6 Cigarette Packaging Components

The trial cigarettes will be packaged by the manufacturer, Dosal Tobacco Corporation, in a manner similar to conventional cigarettes. There are 20 cigarettes per pack. The packs are printed with the trial protocol identification, the investigational material caution ("Caution: New Drug – Limited by Federal Law to investigational use by qualified investigators only. Keep out of the reach of children. For clinical trial use only."), and the Surgeon General's Warning. The lot is indicated by an embossed alphanumeric code that appears on the bottom

of each pack. All cigarettes to be used in the planned Phase II trial are from a single lot. Active Control Cigarettes are embossed with the same number. The packs are wrapped in film and packaged 10 packs to a carton. The cartons are printed with the trial protocol identification, the investigational material caution, and the Surgeon General's Warning. The cartons are packed in cases that are labeled with the product (X-22 or Active Control) and stamped with the date. Cartons of X-22 and Active Control Cigarettes are distinguished only by the coded Subject ID number on the label applied when the trial materials are packed into the kits for distribution to the trial sites.

7.2.7 Stability

Stability of the trial materials is performed by Arista Laboratories and will be monitored throughout the course of the Phase II study. Samples will be shipped to an independent laboratory, stored at ambient conditions in their facility, and analyzed at the beginning of the study and after 3, 6, 9 and 12 months. Analyses will be carried out on cigarettes and smoke. The smoke constituents monitored will include "tar," nicotine, carbon monoxide, benzo[a]pyrene, and 4-(N- methylnitrosamino)-1-{3-pyridyl)-1-butanone (NNK).

For the clinical batches, long-term stability is conducted to support a recommended storage condition of controlled room temperature (15–30°C, 59–86°F). Long-term stability will be assessed for 12 months.

The stability protocol is provided in Table 9. Stability studies have been initiated and data will be provided in a future IND Amendment.

Table 9 Stability Protocol for the X-22 Smoking Cessation Product

Storage Condition	**Time (months)**				
	Initial	**3**	**6**	**9**	**12**
Controlled room temperature	X	X	X	X	X

X = Appearance and assays

Assays:

- Puff count and tar, nicotine, water and carbon monoxide in smoke, ISO smoking conditions
- benzo[a]pyrene in smoke, ISO smoking conditions

- NNK (4-(N-methylnitrosamino)-1-{3-pyridyl)-1-butanone in smoke, ISO smoking conditions
- Oven volatiles

Analytical procedures for methods not described in Section 7.2.5.2 are provided below.

Puff Count

Cigarettes are stored at ambient conditions until required for testing. Before smoking, cigarettes are conditioned according to Arista SOP FAC-035 Handling of Test Items. Smoking is carried out on a rotary or linear smoking machine meeting the specifications in ISO 3308, Routine Analytical Cigarette Smoking Machine – Definitions and Standard Conditions. The puffing regimen consists of 35 mL puffs initiated at 60 second intervals having a duration of 2 seconds. The cigarettes are smoked to a butt length of the tipping paper plus 3 mm.

Benzo[a]pyrene in Mainstream Smoke

The method is detailed in Arista Analytical Method AM-044 Determination of Selected Polynuclear Aromatic Hydrocarbons in Mainstream Smoke.

Mainstream smoke from test cigarettes is passed through a 44-mm Cambridge filter. After smoking, the mainstream smoke filter is weighed and transferred to an amber bottle, then 20 mL of MeOH and internal standard are added and the sample is shaken for 30 min. Type 1 water is added and the solution is passed through a C-18 solid phase extraction (SPE) cartridge that has been rinsed with 30% MeOH in Type 1 water. The eluent is discarded and toluene is added to the SPE cartridge for extraction of the B[a]P fraction using vacuum. The toluene extract from the C-18 cartridge is then poured through a Silica Si-1 SPE cartridge and collected in a disposable 15mL culture tube. The sample is transferred to an autosampler vial and diluted 1:1 with acetone for analysis by gas chromatography/mass selective detector (GC/MSD).

Individual analyte concentrations are determined by the internal standard method using gas chromatography with a DB-17ms column for chromatographic separation of the analytes and selected ion monitoring after electron impact ionization. The concentrations of the analytes,

determined by the GC/MSD are reported in units of mass-to-volume (i.e., ng/mL). The measured concentration, the number of cigarettes smoked, and the sample solution volume(s) are used to calculate the total analyte mass on a per cigarette basis.

NNK in Mainstream Smoke

The method is detailed in Arista Analytical Method T-020 Determination of Tobacco Specific N-Nitrosamines in Mainstream Smoke.

The tobacco specific n-nitrosamines (TSNAs) are quantitated from mainstream smoke collected on a 44 mm Cambridge filter as the collection media. LC-MS/MS allows for the determination of the targeted nitrosamines with minimum interference from other components in mainstream smoke. The LC-MS/MS method uses a HPLC equipped with a C18 reversed phase column to resolve TSNAs from potential interference. Further selectivity is accomplished using a triple quadrupole mass spectrometer with electrospray ionization (ESI) and selective reaction monitoring of characteristic daughter ions. Deuterated internal standards (d-NNN, and d-NNK) are added to the buffer extraction solution and are used to correct for recovery of nitrosamines.

The concentrations of the TSNAs determined by this method are reported in units of mass-to-volume (i.e., ng/mL). The measured concentration, the number of cigarettes smoked, and the sample solution volume(s) are also used to calculate the total analyte mass on a per cigarette basis.

7.3 ACTIVE CONTROL CIGARETTE

7.3.1 Description and Composition of the Active Control Cigarette

The composition of the Active Control Cigarette is provided in Table 10. The Active Control Cigarette is packaged identically to the X-22 Smoking Cessation Product. Reference is made to Section 7.2.6 for information on the packaging.

Table 10 Composition of the Active Control Cigarette

			% of Tobacco Section
Active Control Cigarette			
Component	**Sub Component**	**Material**	
Tobacco Section	(b) (4)		
Tobacco Section			
Tobacco Section			
Filter Section			
Filter Section			
Filter Section			

7.3.2 Justification of Choice of the Active Control Cigarette

There are a large number of commercial cigarette brands on sale in the US. The largest-selling brands, and the major part of all brands, are “American Blend” cigarettes. The filler in these cigarettes consists mainly of a blend of flue-cured tobacco, burley tobacco, and minor amount of oriental tobacco burley, with expanded stem and/or reconstituted tobacco. The filler typically contains complex mixtures of sugar, flavoring and humectants. Within most brands there are a number of “brand styles,” which differ mainly in characteristics other than the filler composition, such as length (king size vs. 100 mm) and machine smoking tar yield (e.g. cigarettes formerly known as “lights” and “ultra-lights). The majority of smokers

smoke king-size cigarettes with yields in the "light" range. (FTC, 2009, Federal Trade Commission Cigarette Report for 2006).

The Active Control cigarette consists of a commercially available American Blend filler produced by [redacted] in a cigarette made with the same paper, filter and tipping paper as X-22 cigarettes. This provides a cigarette that is similar to popular brand styles and identical in appearance to X-22 cigarettes.

7.3.3 Certificate of Analysis for the Active Control Cigarettes

22nd Century Limited, LLC

CERTIFICATE OF ANALYSIS

FINAL PRODUCT SPECIFICATION FOR ACTIVE CONTROL CIGARETTE

	Active Control Cigarette Specification	Clinical Trial Material Lot 23150
(b) (4), (b) (6)		

8201 Main Street, Suite 6 • Williamsville, New York 14221 • TEL 716-270-1523 • FAX 716-877-3064 • www.xxiicentury.com

7.4 PRODUCT LABELING

7.4.1 Draft Labeling for the Pack

Protocol Number: X-22-201

Caution: New Drug – Limited by Federal Law to investigational use by qualified investigators only.

Keep out of the reach of children.

For clinical trial use only.

Contains 20 cigarettes

7.4.2 Draft Labeling for the Carton

Caution: New Drug – Limited by Federal Law to investigational use by qualified investigators only. Keep out of the reach of children. For clinical trial use only.

Protocol Number: X-22-201

Store at room temperature in a secure location with limited access.

Contains 200 Cigarettes **22nd Century Limited, LLC**

7.5 ENVIRONMENTAL ASSESSMENT

Pursuant to the provisions in 21 CFR § 25.31(e), 22nd Century Limited, LLC claims a categorical exclusion from the requirements for an environmental impact assessment.

Furthermore, no extraordinary circumstances exist. X-22 Smoking Cessation Product is intended to be used in a clinical environment where waste will be controlled and the amount of waste expected to enter the environment may reasonably be expected to be nontoxic. Returned or rejected drug product will be disposed of at licensed disposal facilities. For home use, used Test Articles and their packaging components will be disposed of by the patient as household solid waste.

X-22 Smoking Cessation Product is derived from cultivated botanical material. Vector 21-41, the reduced nicotine/transgenic tobacco, was grown in Lancaster County, PA. It is not determined to be endangered or threatened under the Endangered Species Act or the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), is not entitled to special protection under Federal laws or international treaty and the areas where it is grown are not a critical habitat to an endangered or threatened species.

Additionally, the Animal and Plant Health Inspection Service (APHIS) of the USDA has prepared an environmental assessment of tobacco line Vector 21-41 and granted a petition by Vector Tobacco to designate this produce and non-regulated, based on a determination that it poses no significant impact to the environments (USDA 2002).

APPENDICES

APPENDIX 1: GENETIC ENGINEERING STRATEGY

Overview of the Genetic Modification in Tobacco Variety Vector 21-41

Low Alkaloid Tobacco

Legg and co-workers (1969) incorporated genetic loci (now called nicl and nic2) from low alkaloid Cuban cigar cultivars into cultivar Burley 21 by hybridizing the two cultivars followed by a series of twelve back-crosses to produce LA (low alkaloid) Burley 21. LA Burley 21 is not cultivated for use in commercial cigarettes. Collins and co-workers (1974) compared Burley 21 and Burley 21 LA lines and found that they did not differ in days to flower, number of leaves, leaf size, and plant height. Legg and co-workers (1970) registered the Burley 21 LA germplasm. Other low alkaloid varieties were similarly produced by crossing the nic1 nic2 loci into other tobacco cultivars. These low alkaloid varieties have 10-20% of the alkaloid content of the varieties from which they were derived.

A very low nicotine tobacco was developed by further reducing the synthesis of nicotine in the roots of the tobacco plant by specific suppression of the enzyme quinolinate phosphoribosyltransferase (QPTase) (Xie et al., 2004) Tobacco produced from this variety has 5% or less of the nicotine content of typical varieties used for cigarette tobacco, but is otherwise similar to conventional tobacco.

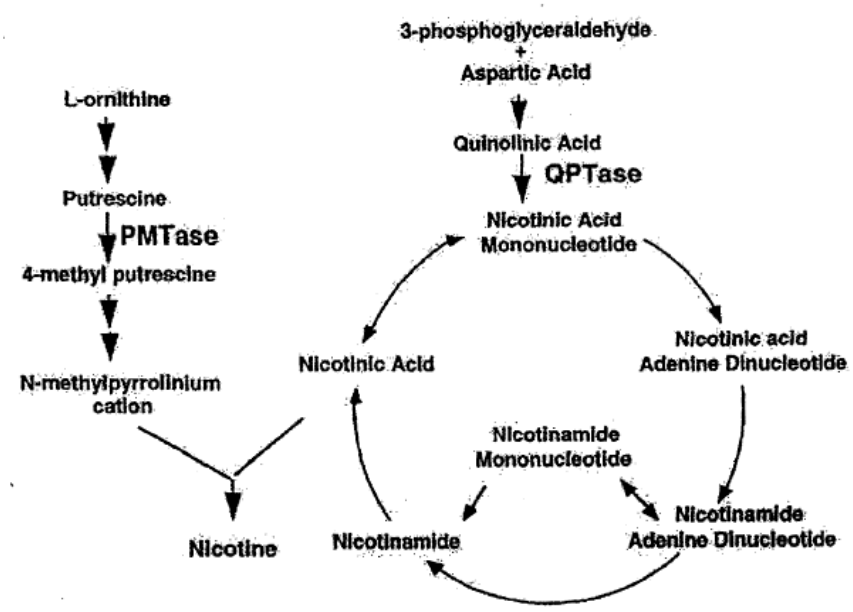
Nicotine Biosynthesis

Nicotine is the predominant alkaloid found in tobacco. During the domestication of tobacco, varieties producing high levels of nicotine were typically selected. Commercial varieties generally produce nicotine at levels between 2 and 4 percent of dry weight (20,000 to 40,000 ppm) (Tso, 1972). Nicotine has been found in other plant species and families including some non-solanaceous plants, however the amount synthesized is much lower than that of N. tabacum (Leete, 1983).

Studies have been carried out to establish the nicotine biosynthetic pathway (Hashimoto and Yamada, 1994; Leete, 1983) by administering labeled compounds, studying tissue cultured

plants and recently using molecular techniques. Nicotine is composed of pyridine and N-methylpyrrolidine rings linked together and is produced by the condensation of two metabolites, nicotinic acid and N-methylpyrroline, that are synthesized by two separate pathways (Waller and Dermer, 1981) The pathway is shown in (Figure 1). Nicotinic acid is synthesized in the pyridine-nucleotide cycle (Wagner et al.,. 1986; Wagner and Wagner, 1985), whereas N-methylpyrroline is synthesized from ornithine or arginine via putrescine (Tiburcio and Galston, 1986).

Figure 1 The Nicotine Biosynthetic Pathway in Tobacco



Quinolinic acid phosphoribosyltransferase (QPTase), which converts quinolinic acid to nicotinic acid mononucleotide (NAMN), serves as the entry-point enzyme into the pyridine-nucleotide cycle that supplies nicotinic acid (Wagner and Wagner, 1985). The pyridine-nucleotide cycle produces nicotinamide adenine dinucleotide (NAD) and nicotinic acid and is a part of primary metabolism, but also produces components for the secondary metabolism such as nicotine synthesis in the root of tobacco (Waller and Denner, 1981). The *NtQPT1* gene (originally called *TobRD2*) that encodes QPTase was isolated as part of a screen for

genes expressed specifically in roots of tobacco. Using an RNA blot, it was confirmed that *NtQPT1* is expressed almost exclusively in the roots (Conkling et al., 1990; Xie et al., 2004).

The other precursor for nicotine is the *N*-methylpyrrolinium cation. Putrescine *N*-methyltransferase (PMTase) catalyzes the formation of *N*-methylputrescine which is then oxidatively de-aminated to 4-aminobutanol by diamine oxidase (DAO). 4-aminobutanol spontaneously cyclizes to the N-methylpyrrolinium cation, a direct precursor of nicotine (Feth et al., 1986).

DNA constructs, Plasmid and Vector

Modified Nicotine Synthesis: *NtQPT* antisense construct

An antisense strategy was used to reduce expression of the endogenous *NtQPTl* gene. Antisense RNA is complementary to the mRNA transcribed by the endogenous gene. Expression of an antisense transcript in the same cell expressing sense transcripts of the endogenous gene can down-regulate the production of the gene product encoded by the endogenous gene. Antisense suppression of gene expression requires a high degree of sequence complementarities between the antisense and sense transcripts.

The full-length *NtQPT1* cDNA was cloned in the antisense orientation behind the 2.0 kb *NtQPT1* promoter. The 2.0 kb *NtQPT1* promoter directs transgene expression at high levels in the same tissues under the same conditions as the endogenous *NtQPT1* gene.

The antisense transcript was terminated by the nos termination/polyadenylation sequences. This gene cassette (*NtQPT1* promoter - *NtQPTl* cDNA antisense – *nos* terminator) was introduced into tobacco via Agrobacterium transformation as described below.

Agrobacterium transformation

The vector system, pBin19 (Bevan, 1984), used to transform genes in tobacco is a binary system based on the Ti plasmid of *Agrobacterium tumefaciens*. pBin19 has been sequenced and is in the GenBank database, accession number U09365. Although some of the DNA sequences used in the transformation process were derived from the plant pathogen *A. tumefaciens*, the genes responsible for crown gall disease were first removed; therefore,

the recipient plant does not have crown gall disease. Figure 2 illustrates the plasmid vector, pYTY32. Plasmid pYTY32 was produced by cloning the *NtQPTl* antisense expression cassette into plasmid pBin19.

In addition to the *NtQPT1* antisense gene, the *nptII* gene from the *Escherichia coli* transposon Tn5 has been introduced into the transgenic tobacco for use as a selectable marker. The gene encodes neomycin phosphotransferase that confers resistance to the common aminoglycoside antibiotic kanamycin. The DNA sequence has been determined (Beck et al. 1982). Expression of the *nptII* gene is under the control of the *nos* promoter and the *nos* terminator.

The sizes, in kb, of the genetic elements of pBin19 are shown in Figure 2. In addition, the direction of transcription of promoter elements and the direction of the sense orientation of the structural genes within the T-DNA are also shown in Figure 2. Details of the genetic elements present in PYTY32 and their origin are listed in Table 11.

Figure 2 Plasmid PYTY32

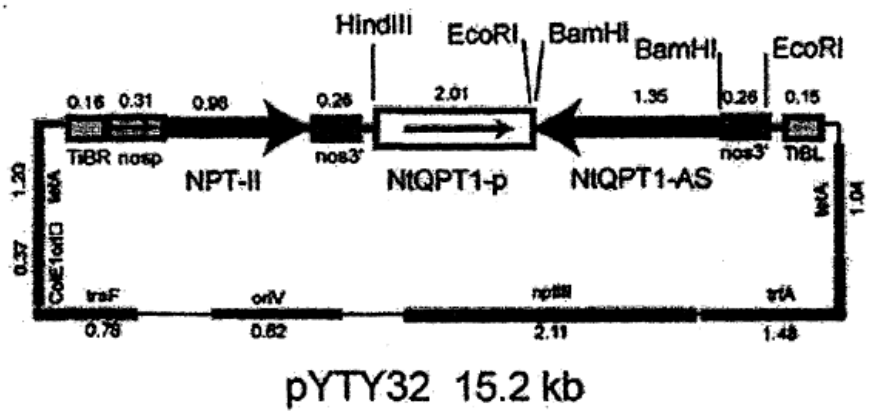


Table 11 Genetic Elements in PYTY32

T-DNA SEQUENCES

Genetic element	Origin	Size (kb)	Function
T-Border-Right (TiBR)	*A. tumefaciens*	0.16	Right border of T-DNA
Nos Promoter (nosp)	*A. tumefaciens*	0.31	Transcribe the npt-II gene
Nos Terminator (nos3')	*A. tumefaciens*	0.26	Terminate transcription
Neomycin phosphotransferase (npt-II)	*E. coli* Tn5	0.98	Kanamycin resistance/ Plant selectable marker
NtQPT1 Promoter (NtQPT1-p)	*N. tabacum*	2.01	Transcribe NtQPT1 antisense
NtQPT1 cDNA Antisense (NtQPT1-AS)	*N. tabacum*	1.35	Suppress QPTase expression
T-Border-Left (TiBL)	*A. tumefaciens*	0.15	Left border of T-DNA

pBin19 SEQUENCES OUTSIDE OF T-DNA

Genetic element	Origin	Size (kb)	Function
tetA	*P. aeruginosa*	2.24	None. T-DNA disrupts gene
trfA	*E. coli* pRK2	1.48	Plasmid transfer/ conjugation
nptIII	*S. faecalis*	2.11	Kanamycin resistance/ Bacteria selectable marker
oriV	*E. coli* pRK2	0.62	Wide host range origin of replication
traF	*E. coli* pRP4	0.78	Plasmid transfer/ Conjugation
ColE1 Ori	*E. coli* pcolE1	0.37	Origin of replication

pYTY32 was transformed into the disarmed *A. tumefaciens* strain LBA4404 (Hoekema et al. -1983) via electroporation. LBA4404 carries a Ti-plasmid that has had the T-DNA region completely removed. It provides the *vir* gene products in trans for plant transformation.

Tobacco leaf disks were transformed and transgenic plants regenerated according to the procedure of An and co-workers (1986). Transformed plant cells, callus, seedlings and plantlets were selected on MS medium containing kanamycin at 300 µg/ml.

Independent transformants were allowed to self. Progeny of the selfed-plants were screened for segregation of the transgene. T1 progeny segregating 3:1, indicating transformation at a single locus were selfed and subjected to further analysis. Alkaloid levels in homozygous and

heterozygous T2 progeny were measured. Homozygous T2 progeny of transformant #41 had the lowest nicotine levels and were renamed Vector 21-41. (Xie et al., 2004)

Characterization of Vector 21-41

Nicotinic Alkaloids and Alkaloid Precursors

Nicotine is synthesized in the roots of tobacco plants and translocated to the leaves, where it accumulates. Analyses of leaf from Vector 21-41 and the non-genetically engineered variety from which it was derived did not show any differences in alkaloid precursors in the leaves.

An independent laboratory quantified the principal biosynthetic intermediates of the nicotine biosynthesis pathway in samples of tobacco produced from the tobacco varieties Vector 21-41 and LA Burley 21 grown, harvested and cured under identical conditions, as well as filler from a reference cigarette.

The biosynthetic precursors tested were divided into two groups: 1) principal polyamine intermediates (i.e., putrescine, *N*-methylputrescine, spermidine, and spermine); and 2) quinolinic acid and nicotinic acid. These specific compounds were selected based upon their importance in the biosynthetic production of nicotine, and their probability for analytical recovery. Three minor tobacco alkaloids (anabasine, anatabine and myosmine) were also analyzed for their presence in each of the processed tobacco samples. A complete study report, including descriptions of the analytical methodology developed for analysis of polyamines, nicotinic acid, and quinolinic acid in processed tobacco, is available upon request.

Polyamine levels are considered to be semi-quantitative; mean levels reported for each processed tobacco type are provided in Table 12. Vector 21-41, has lower or comparable levels of polyamine intermediates tested when compared to its parent, LA Burley 21. Both Vector 21-41 and LA Burley 21 tobacco contain higher levels of putrescine and spermidine than the Kentucky 2R4F Reference Cigarette filler, which is comprised of flue-cured and Oriental tobacco varieties, and reconstituted tobacco, as well as burley tobacco.

Table 12 Polyamine Results, Mean (SD)

Tobacco Type	Putrescine* (µg/g)	N-Methylputrescine* (µg/g)	Spermidine* (µg/g)	Spermine* (µg/g)
Burley 21LA	23.3 (0.66)	<LOQ	10.2 (1.64)	N/A
Vector 21-41	19.1 (0.23)	<LOQ	10.2 (1.17)	N/A
KY Reference	7.72	<LOQ	3.16	N/A

* Semi-quantitative
<LOQ: Below limit of quantification
N/A: Not Applicable

Summary results of mean nicotinic and quinolinic acid levels recovered are provided in Table 13. The levels of nicotinic acid are similar in Vector 21-41 and its non-transgenic parent, LA Burley 21and are about one-third of the level in the Kentucky 2R4F reference cigarette filler. Quinolinic acid levels were below the limit of quantitation across all three processed tobacco types.

Table 13 Nicotinic and Quinolinic Acid Results; Mean (SD)

Tobacco Type	Nicotinic Acid (µg/g)	Quinolinic Acid (µg/g)
Burley 21LA	27.7 (0.8)	<LOQ
Vector 21-41	32.7 (2.6)	<LOQ
KY Reference	95.4 (1.1)	<LOQ

<LOQ: Below limit of quantification

The LOQ for quinolinic acid is 16.0 µg/g

Minor alkaloids were analyzed according to applicable standard operating procedures. The mean results for the minor alkaloids are summarized in Table 14. Vector 21-41 tobacco contains levels of minor alkaloids comparable to those in its non-transgenic parent, LA Burley 21. The levels of anabasine and anatabine are substantially lower than those in the Kentucky 2R4F reference cigarette filler.

Table 14 Minor Alkaloid Results; Mean (SD)

Tobacco Type	Anabasine (µg/g)	Anatabine (µg/g)	Myosmine (µg/g)
Burley 21LA	<LOQ	15.8 (1.7)	N/A
Vector 21-41	<LOQ	29.0 (5.0)	N/A
KY Reference	86.6 (5.1)	495 (33)	10.2 (0.7)

<LOQ: Below limit of quantification
N/A: Not Applicable

LOQ Anabasine 13.7 µg/g, Anatabine 4.4 µg/g, Myosmine 4.5 µg/g;

USDA Deregulation of Vector 21-41

Vector 21-41 does not express any novel agronomic trait such as insect resistance or herbicide tolerance. The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has prepared an environmental assessment of tobacco line Vector 21-41 and granted a petition to designate this product as non-regulated.(USDA, 2002)

The only new protein possibly expected in Vector 21-41 is neomycin phosphtransferase encoded by *nptII*. The *nptII* gene was the first selectable marker for plant transformation in products approved for human consumption (FDA 1994). The biosafety of the use of kanamycin resistance as a selectable marker in transgenic plants has been discussed extensively (Nap et al. 1992; Redenbaugh et al. 1994). The FDA evaluated the potential for horizontal transfer of the *nptII* gene to soil or intestinal (human and animal) microorganisms. They concluded that the probability of gene transfer was substantially less than the occurrence of kanamycin-resistance arising by mutation (FDA 1998). Similarly, horizontal gene transfer to the epithelial cells lining the intestinal lumen was not observed (FDA 1998; Hoskins 1978). The *nptII* gene product has no significant homology with proteins listed as food allergens or toxins and is instable to proteolytic enzymes, heat, and stomach acid conditions. For these reasons, FDA had no allergenicity or toxicity concerns regarding npt II. (FDA 1998)

Tobacco is not consumed as a food, but rather smoked. Therefore like other proteins in tobacco, neomycin phosphotransferase would be broken down during the curing, aging, processing and burning of the tobacco prior to human exposure (Leffingwell, 1999). Breakdown products and derivatives of proteins in tobacco Vector 21-41 would be similar to those derived from other proteins normally found in conventional non-transgenic tobacco leaves to which humans are exposed.

Based on this body of information, it is reasonable to conclude that Vector 21-41 tobacco poses no new hazard or health risk beyond the risks known for traditional, non-transgenic tobacco.

APPENDIX 2: (b) (4)

				% of (b) (4)	% of (b) (4)	Weight Added* (mg/cigarette)	% of Filler	% of Tobacco Section	% of Cigarette
(b) (4)									
Ingredient		**CAS #**	**Function**						
(b) (4)									

APPENDIX 3: (b) (4) [redacted]

				(b) (4) [redacted]	Weight (mg/cigarette)	% of Filler	% of Tobacco Section	% of Cigarette
(b) (4) [redacted]								

				% o (b) (4)	Weight (mg/cigarette)	% of Filler	% of Tobacco Section	% of Cigarette
(b) (4)								

APPENDIX 4: (b) (4) [redacted]

				% of (b) (4) [redacted]	Weight (mg/cigarette)	% of Filler	% of Tobacco Section	% of Cigarette
(b) (4) [redacted]								

				% of (b) (4)	Weight (mg/cigarette)	% of Filler	% of Tobacco Section	% of Cigarette
(b) (4)				(b)				
Ingredient		**CAS #**	**Function**					
(b) (4)								

APPENDIX 5: (b) (4)

				% of (b) (4)	Weight (mg/cigarette)	% of Filler	% of Tobacco Section	% of Cigarette
(b) (4)								
Ingredient		**CAS #**	**Function**					

(b) (4)

				% of (b) (4)	Weight (mg/cigarette)	% of Filler	% of Tobacco Section	% of Cigarette
(b) (4)								
Ingredient		**CAS #**	**Function**					
(b) (4)								