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#859 #859

April 29, 2019

Dr. Paulette Gaynor
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Subject: GRAS Notification –
2'-Fucosyllactose
As a Food Ingredient

Dear Dr. Gaynor,

On behalf of Advanced Protein Technologies, Corp. (APTech), we are submitting a GRAS notification for 2'-fucosyllactose (2'-FL) as a food ingredient. The enclosed document provides the notice of a claim that a food ingredient, the 2'-FL, described in the enclosed notification is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be generally recognized as safe (GRAS), based on scientific procedures, as a food ingredient. We believe that this determination and notification are in compliance with Pursuant to 21 C.F.R. Part 170, subpart E.

We enclose an original copy of this notification and a CD Rom for your review. Please feel free to contact me if additional information or clarification is needed as you proceed with the review. We would appreciate your kind attention to this matter.

Sincerely,

[redacted] 4/29/2019

Susan Cho, Ph.D.
Susanscho1@yahoo.com
Agent for APTech

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF 2'-FUCOSYLLACTOSE AS A FOOD INGREDIENT

Prepared for
Advanced Protein Technologies, Corp. (APTech)

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PART 1. SIGNED STATEMENTS AND A CERTIFICATION

Pursuant to 21 CFR Part 170, subpart E, Advanced Protein Technologies, Corp. (hereinafter referred to as ‘APTech’) submits a Generally Recognized as Safe (GRAS) notice and claims that the use of 2’-fucosyllactose (2’-FL) in infant formula and selected conventional foods, as described in Parts 2 through 7 of this GRAS notice, is not subject to premarket approval requirements of the FD&C Act based on its conclusion that the substance is GRAS under the conditions of its intended use.

1.A. Name and Address of the Notifier

Contact: Dr. Jong-Won Yoon
Company: Advanced Protein Technologies, Corp.
Address: 5 Mosan-gil, Jeongnam-myeon, Hwasung City, Gyeonggi-do, 18516, Republic of Korea (South Korea)
Tel: 82-31-888-6245

1.B. Common or Trade Name

2’-fucosyllactose (2’-FL)

1.C. Applicable Conditions of Use of the Notified Substance

1.C.1. Foods in Which the Substance is to be Used

The intended use and use levels of 2’-FL are the same as those described in GRN 735 (pages 5 and 29 to 31), except in medical food application which was withdrawn from this GRAS notice. As shown in Table 1, APTech proposes to use 2’-FL as an ingredient in whey-, milk- and soy-based infant formula for full term infants, in toddler formulas, and in selected conventional foods. No uses in pre-term infants are proposed at this time. To be consistent with the revised intended uses specified in GRN 735, APTech does not intend to apply 2’-FL to the medical food category.

1.C.2. Levels of Use in Such Foods

Table 1 lists the proposed conventional food categories, intended uses, and use levels for 2’-FL. APTech’s 2′-FL is intended to be used as an ingredient in whey-, milk-, and soy-based, non-exempt infant formulas for term infants and in toddler formulas at a maximum level of 2.4 g/L of formula as consumed; infant and toddler foods at levels of 0.24-1.2 g/serving; and in the following food categories at levels of 0.28-1.2 g/serving: beverages and beverage bases; breakfast cereals; dairy product analogs; frozen dairy desserts and mixes; gelatins, puddings, and fillings; grain products and pastas; jams and jellies; milk and milk products; processed fruits and fruit juices; and sweet sauces, toppings, and syrups. The addition of 2′-FL to term infant formulas is consistent with efforts to produce infant formula that closely matches the nutrient composition of human milk.

Table 1. Proposed Conventional Food Categories and Intended Use of 2'-FL

Proposed Food Category	Food Uses	Maximum Use Level (mg/serving)	RACC (g or mL)	Maximum Use Level (mg/100 g)
Beverages and beverage bases	Energy drinks	280	360	80
	Fitness water and thirst quenchers, sports and isotonic drinks	280	360	80
Breakfast cereals	Ready-to-eat breakfast cereals for adults and children	1,200	15 (puffed) 40 (high-fiber) 60 (biscuit-types)	8,000 3,000 2,000
	Hot cereals for adults and children	1,200	40 (dry) ~250 prepared	480 (as consumed)
Dairy product analogs	Milk substitutes such as soy milk and imitation milks	280	240	120
Frozen dairy desserts and mixes	Frozen desserts including ice creams and frozen yogurts, frozen novelties	1,200	~70	1,700
Gelatins, puddings, and fillings	Dairy-based puddings, custards, and mousses	1,200	~70	1,700
	Fruit pie filling	1,200	85	1,410
	"Fruit pre" such as fruit filling in bars, cookies, yogurt, and cakes	1,200	~40	3,000
Grain products and pastas	Bar, including snack bars, meal-replacement bars, and breakfast bars	480	40	1,200
Jams and jellies, commercial	Jellies and jams, fruit preserves, and fruit butters	1,200	~20	6,000
Milk, whole, and skim	All Acidophilus or fortified milks, non-fat and low-fat fluids, including fluid milk and reconstituted milk powder	280	240	120
Milk products	Flavored milks, including milk, coffee drinks, coca, smoothies (dairy and fruit-based), other fruit and dairy combinations, yogurt drinks, and fermented milk drinks including kefir	280	240	120

	Milk-based meal replacement beverages or diet beverages	280	240	120
	Yogurt	1,200	225	530
	Formula intended for pregnant women ("mum" formulas, -9 to 0 months)	1,200	200	600
Processed fruits and fruit juices	Fruit drinks, including vitamin and mineral fortified products	280	240	120
	Fruit juices	280	240	120
Sweet sauces, toppings, and syrups	Syrups used to flavor milk beverages	280	40	700
Other Categories				
Non-exempt infant and follow-on formula	Infant formula (0 to 6 months), including ready-to-drink formula or formula prepared from powder	240	100	240 (400 mg/100 kcal)
	Follow-on formula (6-12 months), including ready-to-drink formula or formula prepared from powder	240	100	240 (400 mg/100 kcal)
	Infant meal replacement products such as PediaSure®	240	100	240 (400 mg/100 kcal)
Baby foods	Growing up (toddler) milks (12-36 months)	240	100	240
	Ready-to-eat, ready-to-serve, hot cereals	1,200	15 (dry) 110 (ready-to-serve)	1,090 (as consumed)
	Yogurt and juice beverages identified as "baby" drinks	1,200	120	1,000
	Desserts including fruit desserts, cobblers, yogurt/fruit combinations ("junior type" desserts)	1,200	110	1,090
	Baby crackers, pretzels, cookies, and snack items	400	7	5,700

Adopted from GRN 735 (pages 30 to 31), except medical foods which have been withdrawn from the original submission.
RACC= Reference Amounts Customarily Consumed per Eating Occasion in the U.S. CFR (21 CFR §101.12);
The proposed maximum use level is presented on g/kg basis for solids and g/L basis for liquids, and forms the basis for the calculation of the Estimated Daily Intake.

1.C.3. Purpose for Which the Substance is Used

2'-FL is intended for use as a food ingredient in infant formulas and conventional foods in the United States at the use levels described in Part 1.C.2.

1.C.4. Description of the Population Expected to Consume the Substance

The population expected to consume the substance consists of term infants, toddlers, and members of the general population who consume at least one of the products described above.

1.D. Basis for the GRAS Determination

This GRAS conclusion is based on scientific procedures in accordance with 21 CFR 170.30(a) and 170.30(b).

1.E. Availability of Information

The data and information that are the basis for this GRAS conclusion will be made available to FDA upon request by contacting Susan Cho at NutraSource, Inc. at the address above. The data and information will be made available to FDA in a form in accordance with that requested under 21 CFR 170.225(c)(7)(ii)(A) or 21 CFR 170.225(c)(7)(ii)(B).

1.F. Availability of FOIA Exemption

None of the data and information in Parts 2 through 7 of this GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. §552.

1.G. Certification

We certify that, to the best of our knowledge, this GRAS conclusion is based on a complete, representative, and balanced dossier that includes all relevant information, available and obtainable by us, including any favorable or unfavorable information, and pertinent to the evaluation of the safety and GRAS status of the use of APTech's 2'-FL.

1.H. Name, Position/Title of Responsible Person Who Signs Dossier, and Signature

Name: Dr. Jong-Won Yoo  Date: April 29, 2019
Title: Vice president

Address correspondence to
Susan S. Cho, Ph.D., NutraSource, Inc.
301-875-6454; susanscho1@yahoo.com
Agent for APTech

1.I. FSIS/USDA Statement

APTech does not intend to add 2'-FL to any meat and/or poultry products that come under USDA jurisdiction. Therefore, 21 CFR 170.270 does not apply.

PART 2. IDENTITY, MANUFACTURING, SPECIFICATIONS, AND TECHNICAL EFFECTS

2.A.1. Identity of the Notified Substance

2.A.1.1. Common Name
2’-fucosyllactose or 2’-*O*-fucosyllactose (2’-FL, 2-FL, 2FL)

2.A.1.2. Chemical Names of Main Component
Chemical Name: α-D-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose

Synonyms:
2'- *O*-fucosyllactose;
2'- *O*-L-fucosyl-D-lactose;
fucosyl-α -1,2-galactosyl-β-1,4-glucose;
Fuc-α-(1→2)-Gal-β-(1→4)-Glc.

2.A.1.3. Chemical Abstract Service (CAS) Registry Number
41263-94-9

2.A.1.4. Empirical Formula
$C_{18}H_{32}O_{15}$

2.A.1.5. Structural Formula
2'-FL is a trisaccharide composed of L-fucose and lactose (D-galactose and D-glucose). The monosaccharide L-fucose is linked to the disaccharide D-lactose by an α-(1→2) bond. Figure 1 shows the structure of 2’-FL.

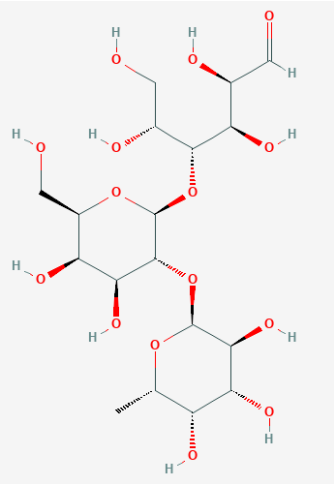

Figure 1. Chemical Structure of 2’-FL

2.A.1.6. Molecular Weight
488.44 daltons

2.A.1.7. Background
2’-FL is a trisaccharide, a type of oligosaccharide, consisting of fucose and lactose (Figure 1). Human milk oligosaccharides (HMOs) all contain lactose at their reducing end. Of the over 200 HMO that have been identified, 2’-FL is the most abundant (Castanys-Munoz et al., 2013). 2’-FL is a functional HMO that exists in small amounts in beestings (cow’s foremilk), but not in commercialized milk products, whereas it is abundant in human milk. Approximately 200 molecular species of milk oligosaccharides have been identified, based on the extension of lactose. The presence of HMOs in breast milk has been associated with a variety of nutritional effects, including the establishment and maintenance of healthy intestinal bacterial microflora that is rich in bifidobacteria, reducing the adhesion of pathogens to the intestinal wall, and providing nutritional support to the neonatal immune system (ten Bruggencate et al., 2014).

2.A.2. Potential Toxicants in the Source of the Notified Substance
No toxicant production is expected in the manufacture of 2’-FL. The final product is highly purified through several steps during production.

2.A.3. Particle Size
To check the particle size for the three batches, APTech analyzed with LA-950 lase scattering particle size distribution analyzer, and the medium for a volume distribution (DV50) had a particle size of 38~39 μm (Appendix A).

2.B. Method of Manufacture
The main production process of APTech’s 2’-FL consists of two steps. The first step is fermentation for the production of 2’-FL using genetically engineered *Corynebacterium glutamicum* APC199. Fermentation is performed in a well-defined, complex medium that excluded yeast extract and antibiotics, and uses glucose as the carbon source and lactose as the substrate of 2’-FL. The major components of the fermentation media are potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4), magnesium sulfate heptahydrate ($MgSO_4\ 7H_2O$), ammonium sulfate [$(NH_4)_2SO_4$], and urea [$(NH_2)_2CO$]. During the fermentation of *Corynebacterium glutamicum* APC199, 2’-FL is biosynthesized inside the cells and exported into the culture broth. Upon completion of fermentation, microbial cells are completely removed by micro-filtration systems. Culture supernatant containing 2’-FL is subjected to downstream purification processes.

The second step is purification. Macromolecules (e.g. proteins or nucleic acids) are removed by ultrafiltration with a 1.5 kDa MWCO membrane. Decolorization of the filtered solution is performed using activated carbon. Large molecular weight substances are further removed by nanofiltration. Ionic impurities and remaining colorants are removed by strong cation and anion exchange resins. Inorganic salts

smaller than 2’-FL are eliminated by nanofiltration with suitable size of molecular weight cut of membrane. Ion exchange chromatography and activated carbon treatment is performed to remove the remaining ionic salts and colorants. The 2’-FL solution is filter sterilized by a 0.2 μm membrane filter. The filtered solution is concentrated for the crystallization process. The crystallization step employs acetic acid as an antisolvent. The 2’-FL crystals are washed with fresh acetic acid and dried under vacuum to obtain a high purity white powder. Figure 2 presents the flow diagram of the manufacturing process.

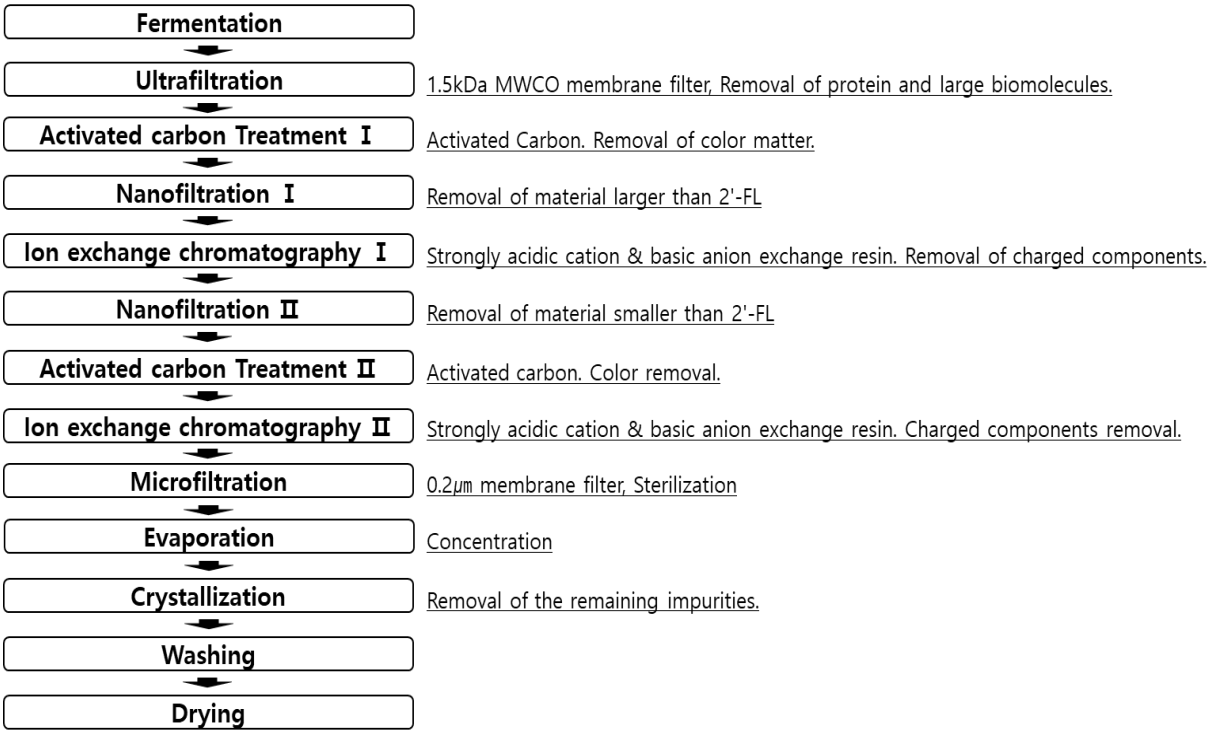

Figure 2. Flow Diagram of Manufacturing Process

2'-FL can be synthesized through the enzymatic fucosylation of lactose with GDP-L-fucose by alpha-1,2-fucosyltransferase (Figure 3). The 2'-FL producing genetically modified *C. glutamicum* was constructed by overexpressing genes encoding for heterologous GDP-L-fucose biosynthetic enzymes, lactose permease and fucosyltransferase. Figure 3 presents the enzymatic reactions during the fermentation process.

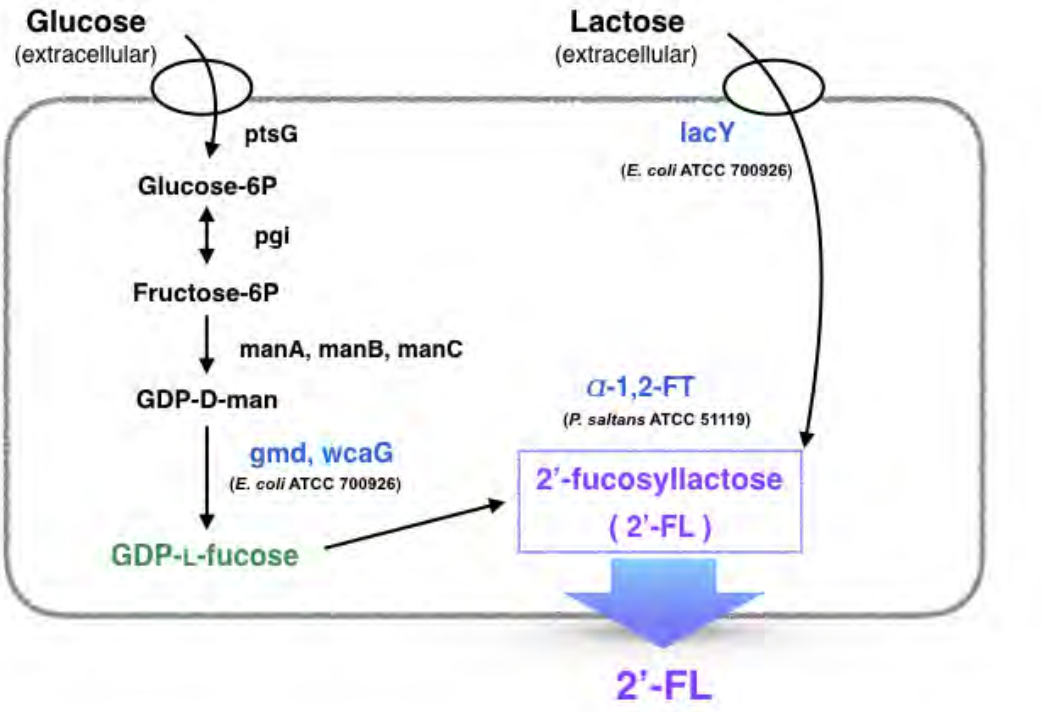

Figure 3. 2'-Fucosyllactose Biosynthesis in Genetically Modified *C. glutamicum*

Where:
ptsG = PTS system glucose-specific EIICB component,
pgi = Glucose-6-phosphate isomerase,
manA = mannose-6-phosphate isomerase,
manB = phosphomannomutase,
manC = mannose-1-phosphate guanylyltransferase,
gmd = GDP-D-mannose-4,6-dehydrogenase,
wcaG = GDP-L-fucose synthase,
lacY = lactose permease,
α -1,2-FT = α -1,2-fucosyltransferase,
Glucose-6P = Glucose-6-phosphate,
Fructose-6P = Fructose-6-phosphate,
GDP-D-man = GDP-D-mannose,
2'-FL = 2'-fucosyllactose

C. glutamicum produces GDP-D-mannose for the cell wall biosynthesis (Jackson and Brennan, 2009; Mishra et al., 2011). For the production of GDP-L-fucose from

GDP-D-mannose in *C. glutamicum*, heterologous GDP-D-mannose-4,6-dehydrogenase (gmd) and GDP-L-fucose synthase (wcaG) of *E. coli* K12 ATCC 700926 were introduced by overexpression vector plasmid. The import of lactose was also required for synthesis of 2'-fucosyllactose in *C. glutamicum.*

Because *C. glutamicum* is unable to utilize lactose, the lactose permease (lacY) of *E. coli* K12 ATCC 700926 was heterologously expressed in APC199. Lactose and GDP-L-fucose are efficiently and specifically converted into 2'-FL by α-1,2-fucosyltransferase (α-1,2-FT), first identified by APTech in the genome of *P. saltans* ATCC 51119. The expression of the heterologous genes is expressed by the polycistronic gene cassette controlled by the tuf promoter (the strong constitutive promoter of *C. glutamicum* tuf gene encoding the translational elongation factor EF-Tu) from the pFP110 vector plasmid.

As shown in Table 2, α -1,2-fucosyl-transferase is originated from a non-pathogenic, non-toxigenic strain of *P. saltans* ATCC 51119 (biosafety level 1). The three enzymes, GDP-D-mannose-4,6-dehydratase, GDP-L-fucose synthase, and lactose permease, are originated from a non-pathogenic, non-toxigenic strain of *E. coli* ATCC 700926 strain (biosafety levels 1).

Table 2. Introduced Genes in FP110 Vector Plasmid

Gene	Origin	Function	Position on plasmid
Tuf promoter	*C. glutamicum* ATCC 13032	Promoter (transcription start)	2182-2381, 200 bp
α -1,2-FT	*P. saltans* ATCC 51119	α -1,2-fucosyl-transferase	2382-3188, 807 bp
Gmd	*E. coli* ATCC 700926	GDP-D-mannose-4,6-dehydratase	3220-4341, 1122bp
wcaG	*E. coli* ATCC 700926	GDP-L-fucose synthase	4367-5332, 966 bp
lacY	*E. coli* ATCC 700926	Lactose permease	5366-6619, 1254 bp
T7 terminator	pET21a vector	Transcription termination	6747-6794, 48 bp

Tables 3-1 and 3-2 summarize the list of raw materials and processing aids, respectively.

Table 3-1. List of Raw Materials Used in the Fermentation Medium

Fermentation media ingredient	CAS Number	Regulatory Status
Yeast extract	8013-01-2	21CFR 184.1983
Tryptone	91079-40-2	
Sodium chloride	*7647-14*-5	21CFR 182.70
Glucose anhydrous	50-99-7	21CFR 168.110
Lactose	9004-34-6	21CFR 168.122
Ammonium sulfate	7783-20-2	21CFR 184.1143
Potassium phosphate monobasic	7778-77-0	21CFR 175.105
Potassium phosphate dibasic	7758-11-4	21CFR 182.6285

Magnesium sulfate heptahydrate	10034-99-8	21CFR 184.1443
Urea	57-13-6	21CFR 184.1923
Calcium chloride	10043-52-4	21CFR 184.1193
Ferrous sulfate (heptahydrate)	7720-78-7	21CFR 184.1307
Zinc sulfate	7733-02-0	21CFR 182.8997
Cupric sulfate	7758-98-7	21CFR 184.1261
Manganese (II) sulfate	10034-96-5	21CFR 184.1443
Hydrochloric acid	7647-01-0	21CFR 182.1057
Ammonia water	1336-21-6	21CFR184.1139 (ammonium hydroxide)

Table 3-2. Processing Aids for Purification of 2'-FL

Materials	Function
Activated carbon	Discoloration
1.5kDa MWCO membrane	Removal of large molecular weight impurities
Nanofiltration membrane I	Removal of molecules larger than 2'-FL
Nanofiltration membrane II	Removal of small molecules below 400 MW
Strongly acidic action exchange resin	Removal of positively charged impurities
Strongly basic anion exchange resin	Removal of negatively impurities
Glacial acetic acid	Anti-solvent for crystallization reaction and washing
0.2 μm membrane filter	Sterilization

Quality Assurance Procedure

Manufacturing process of APTech's 2'-FL meets the current Good Manufacturing Practices (cGMP) requirements. APTech observes the principles of Hazard Analysis and Critical Control Point (HACCP)-controlled manufacturing process and rigorously tests its final production batches to verify adherence to quality control specifications. All processing aids used in the manufacturing process are food grade. Process tanks and lines are cleaned with sodium hydroxide and hydrogen peroxide following standard procedures common to the dairy industry.

Safety of Microorganism

Table 4 shows the taxonomic classification of the production microorganism.

Table 4. Taxonomic Classification of *Corynebacterium glutamicum*

Kingdom	*Bacteria*
Phylum	*Actinobacteria*
Class	*Actinobacteria*
Order	*Actinomycetales*
Family	*Corynebacteriaceae*
Genus	*Corynebacterium*
Species	*Corynebacterium glutamicum*
Strain	*Corynebacterium glutamicum* APC199

The comparative genome analysis of *C. glutamicum* APC199 (test strain) and *C. glutamicum* ATCC13032 also was performed to understand the taxonomic similarity of the two strains. DNA-DNA hybridization (DDH) values have been used by bacterial taxonomists since the 1960s to determine the relatedness between strains and are still the most important criterion in the delineation of the bacterial species. Most recently, the average nucleotide identity (ANI), calculated from pair-wise comparisons of all sequences shared between any two strains, has been proposed as the new metrics for bacterial species classification. Goris et al. (2007) reported 95% similarity of calculated ANI based on whole genome sequencing corresponding to 70% of DDH which is considered to be the gold standard value of species delineation. The comparative ANI value of the test strain and *C. glutamicum* ATCC13032 was calculated using whole genome sequence ANI calculating algorithm. The result showed a 99.99% match, indicating a strong similarity between these two strains.

The analysis of whole genomic sequencing of APTech's *Corynebacterium glutamicum* APC199 revealed that the strain is absent of virulence genes and antibiotic resistance genes (except quinolone and vancomycin). In addition, *Corynebacterium glutamicum* APC199 was shown to have the following characteristics:

(1) has no hemolytic activities,
(2) has no gelatinase activities, or
(3) does not produce biogenic amines

Details are presented in Appendix B.

2.C. Specifications and Composition of 2’-FL

Tables 5 and 6 show the specifications and analytical values of 3 independent batches of APTech’s 2’-FL, respectively. The data demonstrated that the manufacturing process produces a consistent product that is in compliance with the established specifications. All methods of analyses are nationally or internationally recognized, or have been validated by APTech. The product is ≥94% pure on a dry weight basis, as measured by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Appropriate limits for heavy metals and microbial impurities have been established. The certificates of analysis (COAs) are attached to Appendix C.

Table 7 presents the specifications of APTech’s 2’-FL in comparison with those described in other GRAS notices. As shown in Table 7, the specification and composition of APTech’s 2’-FL are comparable to those presented in other GRAS notices, i.e., GRN 546 (FDA, 2015a - pages 7 and 8; Glycom, produced via chemical synthesis), GRN 571 (FDA, 2015b – stamped page 28; Jennewein Biotechnologie; 2’-FL produced via fermentation), GRN 650 (FDA, 2016a - stamped page 21; Glycom A/S; 2’-FL via fermentation), GRN 735 (FDA, 2018a - pages 22 to 24; Glycosyn LLC and FrieslandCampina Domo B.V.; 2’-FL via fermentation), and GRN 749 (FDA, 2018b - pages 20 to 21; DuPont; 2’-FL via fermentation).

Table 5. Specifications of APTech's 2'-FL

Parameters	Specification	Method
Appearance (Color)	White to off white/ivory	Visual
Appearance (Form)	Dry powder	Visual
Solubility in water	500 g/L (25°C)	Visual
Appearance in solution	Clear, colorless to slightly yellow	Visual
Chemical		
Water content, %	≤ 9.0	Karl Fischer titration
Protein content, µg/g	≤ 100	Bradford assay
Total ash, %	≤ 0.5	MFDS No.2018-98, 8.2.1.2
Arsenic, mg/kg	≤ 0.1	KS C IEC 62321-4 (2014), KS C IEC 62321-5 (2014), KS I ISO 17294:2014
Cadmium, mg/kg	≤ 0.01	
Lead, mg/kg	≤ 0.02	
Mercury, mg/kg	≤ 0.05	
Aflatoxin M1, µg/kg	≤ 0.025	MFDS No.2018-98, 8.9.2.3
Carbohydrate content		
2'-Fucosyllactose, %	≥ 94	HPAEC-PAD
Lactose, %	≤ 5 (Area)	
3-Fucosyllactose, %	≤ 5 (Area)	
Difucosyllactose, %	≤ 5 (Area)	
Fucosyl-Galactose, %	≤ 3 (Area)	
Glucose, %	≤ 3 (Area)	
Galactose, %	≤ 3 (Area)	
Fucose, %	≤ 3 (Area)	
Microbiology analysis		
Standard Plate Count, cfu/g	≤ 500	MFDS No.2018-98, 8.4.5.1
Yeast and Mold, cfu/g	≤ 100	MFDS No.2018-98, 8.4.10
Coliform, cfu/g	≤ 10	MFDS No.2018-98, 8.4.7.2
E. coli	Absent in 1 g	MFDS No.2018-98, 8.4.8.2
Cronobacter spp.	Absent in 60 g	MFDS No.2018-98, 8.4.21
Staphylococcus aureus	Absent in 1 g	MFDS No.2018-98, 8.4.12.2
Salmonella	absent in 25 g	MFDS No.2018-98, 8.4.11
Endotoxins, EU/g	≤ 100	Ph. Eur. 2.6.14
Abbreviations: MFDS = Ministry of Food and Drug Safety; KS = Korean Industrial Standards; IEC = International Electrotechnical Commission; ISO = International Organization for Standardization; HPAEC-PAD = High Performance Anion Exchange Chromatography Pulsed Amperometric Detection; cfu = colony forming units; Ph. Eur = European Pharmacopoeia		

Table 6. Analysis of Production Batches of 2'-FL

Parameters	Specification	Batch Number		
		2'-FL-CG-011	2'-FL-CG-012	2'-FL-CG-013
Appearance (Color)	White to off white/ivory	Pass	Pass	Pass
Appearance (Form)	Dry powder	Pass	Pass	Pass
Solubility in water	500 g/L (25°C)	Pass	Pass	Pass
Appearance in solution	Clear, colorless to slightly yellow	Pass	Pass	Pass
Chemical				
Water content, %	≤ 9.0	1.67	1.74	1.64
Protein content µg/g	≤ 100	< 10	< 10	< 10
Total ash, %	≤ 0.5	0.17	0.15	0.14
Arsenic, mg/kg	≤ 0.1	< 0.01	< 0.01	< 0.01
Cadmium, mg/kg	≤ 0.01	< 0.01	< 0.01	< 0.01
Lead, mg/kg	≤ 0.02	< 0.01	< 0.01	< 0.01
Mercury, mg/kg	≤ 0.05	< 0.01	< 0.01	< 0.01
Aflatoxin M1, µg/kg	≤ 0.025	ND	ND	ND
Carbohydrate content				
2'-Fucosyllactose, %	≥ 94	96.67	95.93	96.24
Lactose, %	≤ 5 (Area)	0.10	0.09	0.10
3-Fucosyllactose, %	≤ 5 (Area)	ND	ND	ND
Difucosyllactose, %	≤ 5 (Area)	0.24	0.86	0.58
Fucosyl-Galactose, %	≤ 3 (Area)	ND	ND	ND
Glucose, %	≤ 3 (Area)	1.13	1.28	1.22
Galactose, %	≤ 3 (Area)	0.78	0.78	0.78
Fucose, %	≤ 3 (Area)	ND	ND	ND
Microbiology analysis				
Standard Plate Count, cfu/g	≤ 500	0	0	0
Yeast and Mold, cfu/g	≤ 100	0	0	0
Coliform, cfu/g	≤ 10	0	0	0
E. coli, cfu/g	ND in 1 g	0	0	0
Cronobacter spp. cfu/60 g	ND in 60 g	Negative	Negative	Negative
Staphylococcus aureus, cfu/g	ND in 1 g	0	0	0
Salmonella, cfu/25 g	ND in 25 g	Negative	Negative	Negative
Endotoxins, EU/g	≤ 100	< 7.2	< 5.7	< 5
ND: Not Detected				

Table 7. Comparison of Purified 2'-FL Specifications

Physical and Chemical Parameters	2'-FL Produced by Fermentation					Synthetic
	APTech	Glycosyn/ FrieslandCampina (GRN 735)	DuPont (GRN 749)	Glycom (GRN 650)	Jennewein (GRN 571)	Glycom (GRN 546)
Appearance, Form	Dry powder	Homogenous powder	Dry powder	Powder	Spray-dried powder	Powder
Appearance, Color	White to off-white/ivory	White	White to off-white/ivory	White to off white	White to ivory-colored	White to off white
Assay	≥ 94% (HPAEC-PAD area; dry wt basis)	≥ 90% (HPAEC)	≥ 82% (AUC) (HPAEC-PAD)	≥ 94.0% (HPLC, water free)	≥ 90% (HPAEC-PAD area)	≥ 95.0% (HPLC, water free)
Water, %	≤ 9.0%	≤ 5	≤ 9.0%	≤ 5.0%	≤ 9.0%	≤ 9.0%
Ash, %	≤ 0.5%	≤ 0.2 (sulfated)	≤ 0.5%	≤ 1.5%	≤ 0.5%	≤ 0.2% (Sulphated)
Acetic acid (as free acid and/or sodium acetate)	NS	NS	NS	≤ 1.0%	NS	≤ 0.3%
Residual proteins	≤ 100 µg/g	≤ 0.01%	≤ 100 µg/g	≤ 0.01%	≤ 100 µg/g	0.1 %
Aluminum, ppm	NS	≤ 4.8	NS	NS	NS	NS
Lead, ppm	≤ 0.02	≤ 0.05	≤ 0.05	≤ 0.1	≤ 0.02	≤ 0.8
Arsenic, ppm	≤ 0.1	≤ 0.1	≤ 0.2	NS	≤ 0.2	NS
Cadmium, ppm	≤ 0.01	≤ 0.01	≤ 0.05	NS	≤ 0.1	NS
Mercury, ppm	≤ 0.05	≤ 0.05	≤ 0.1	NS	≤ 0.5	NS
Lactose, %	≤ 5 (Area)	≤ 3%	< 8 (AUC)	≤ 3%	≤ 5% (Area)	NS
Difucosyllactose, %	≤ 5 (Area)	NS	< 7 (AUC)	≤ 1.0	≤ 5% (Area)	NS
Other carbohydrates	NS	NS	< 6 (AUC)	NS	NS	NS
3-FL, %	≤ 5 (Area)	NS	< 0.1	NS	≤ 5% (Area)	NS
Fucosyl-galactose, %	≤ 3 (Area)	NS	0.72	NS	≤ 3% (Area)	NS
2'-Fucosyl-D-lactulose, %	NS	NS	0.8	≤ 1.0	NS	NS
Allo-lactose, %	NS	≤ 2%	NS	NS	NS	NS
Glucose, %	≤ 3 (Area)	≤ 2%	1.1	NS	≤ 3% (Area)	NS

Galactose, %	≤ 3 (Area)	≤ 2%	1.1	NS	≤ 3% (Area)	NS
Fucose, %	≤ 3 (Area)	≤ 2%	0.18	≤ 1.0	≤ 3% (Area)	NS
Total HMOs (2'-FL, lactose, DiFL, fucose), %	NS		95.9	≥96	NS	
Aerobic mesophilic total count, CFU/g	≤ 500	≤ 3,000	NS	≤ 500	≤ 10,000	500
Yeast, CFU/g	≤ 100 (Yeast and Mold)	≤ 10	≤ 100	≤ 10	≤ 100 (Yeast and Mold)	≤ 10
Mold, CFU/g			≤ 100	≤ 10		≤ 10
Salmonella	ND in 25 g	ND in 25 g	ND in 100 g	ND in 25 g	ND in 100 g	ND in 25 g
Enterobacteriaceae	NS	ND in 10 g	ND in 10 g	ND in 10 g	ND in 11 g (w/ Coliform)	ND in 10 g
Cronobacter (*Enterobacter*) *sakazakii*	ND in 60 g	ND in 25 g	ND in 100 g	ND in 10 g	ND in 100 g	ND in 10 g
Listeria monocytogenes	NS	NS	ND in 25 g	ND in 25 g	NS	ND in 25 g
Bacillus cereus, cfu/g	NS	Max. ≤ 100 (presumptive)	≤ 10	≤ 50	NS	≤ 50
E. coli, cfu/g	ND in 1 g	ND in 10 g	NS	NS	NS	NS
S. aureus, cfu/g	ND in 1 g	ND in 1 g	NS	NS	≤ 10 cfu/g	NS
Sulphite reducing *clostridia* spores, cfu/g	NS	≤ 30	NS	NS	NS	NS
C. perfringens, cfu/g	NS	ND in 1 g	NS	NS	NS	NS
Residual Endotoxins, EU/g	≤ 100	≤ 0.01	≤ 300	NS	≤ 300	≤ 0.05
Aflatoxin M_1, ug/kg	≤ 0.025	≤ 0.2	< 0.025	NS	≤ 0.025	NS
GMO detection	NS	Negative	Negative	NS	Negative	NS

Expanded from GRN 735. ND=not detected; NS=not specified; w/=with. Data Source - GRN 546 (FDA, 2015a - pages 7 and 8; Glycom, produced via chemical synthesis), GRN 571 (FDA, 2015b – stamped page 28; Jennewein Biotechnologie; 2'-FL produced via fermentation), GRN 650 (FDA, 2016a - stamped page 21; Glycom A/S; 2'-FL via fermentation), GRN 735 (FDA, 2018a - pages 22 to 24; Glycosyn LLC and FrieslandCampina Domo B.V.; 2'-FL via fermentation), and GRN 749 (FDA, 2018b - pages 20 to 21; DuPont; 2'-FL via fermentation).

2.C.1. Chemical Identity and Potential Impurities

Impurities may include lactose (0.1%), difucosyllactose (0.56%), glucose (1.21%), and galactose (0.78%). However, the concentrations may result in quantitatively insignificant carry-over into the finished infant formula.

Absence of Host Organism, Introduced Antibiotic Resistant Genes, and Enzyme Residues

The microorganism used in the manufacturing process is efficiently removed by the ultrafiltration step. Additionally, during downstream processing, various sequential purification processes are also applied to ensure microbiological purity.

The absence of the microorganism and residual protein in the ingredient is supported by the analysis of residual DNA in batches of the final ingredient. The absence of residual DNA from the microorganism is confirmed by validated PCR methods. In the PCR reaction, residual DNA could not be detected from the final ingredient. The PCR results demonstrated that the microorganism and residual protein are absolutely removed from the final ingredient (Appendix D).

Microbial Endotoxins

Regulatory threshold levels for food, regarding endotoxin contamination, currently do not exist. Typical ranges of endotoxin load have been reported for cow's milk (Gehring et al., 2008) and infant formula powder (Townsend et al., 2007). The endotoxin specification for 2'-FL is set to not contribute additional exposure to endotoxins that would result in exposures above the usual levels that are expected for infant formula powder currently on the market. Batch analyses of 2'-FL demonstrate compliance to the endotoxins specifications.

Chemical Identity of APTech's 2'-FL

APTech's purified 2'-FL powder was compared with the reference material (Carbosynth) using liquid chromatography tandem mass spectrometry (LC-MS/MS) and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) method.

The mass-to-charge ratio (m/z) value of 2'-FL was 487.3 (m/z) as a result of the multiple reaction monitoring (MRM) ion scan using ESI-negative operation mode. The values were identical to those of the reference material (Carbosynth), and all three batches showed similar results. Details are presented in Appendix E.

The HPAEC-PAD also showed identical retention time as the reference material, and details of the method are described in Appendix F.

2.C.2. Stability

2.C.2.1. Bulk Stability

As shown in Table 8, stability tests were conducted by various companies. Since the purity and composition of APTech’s 2’-FL preparation is similar to those described in previous GRAS notices, it is recognized that the information and data in other GRAS notices are pertinent to the stability of the APTech’s 2’-FL in this GRAS determination.

In GRN 546 (pages 11 to 13 - FDA, 2015a), Glycom reported the bulk shelf-stability of 36 months with a 97.0% recovery for its 2'-FL, prepared via chemical synthesis (≥95% purity), when stored at 25°C and a relative humidity of 60%. Under accelerated conditions (at 40°C and a relative humidity of 75%), the shelf-stability of 6 months with a 98.2% recovery was reported.

Jennewein indicates that its 2'-FL (≥90% purity), produced by genetically engineered *E. coli*, has a shelf-life of at least 2 years; 106.2-106.6% of the baseline values were recovered when stored at 25°C and a relative humidity of 65%. At an accelerated condition (40°C and a relative humidity of 75%), 102.9 -103.5% of the baseline values were recovered after 26 weeks of storage (GRN 571, stamped pages 29 to 30 - FDA, 2015b).

In GRN 650 (stamped pages 26 to 29 - FDA, 2016a), Glycom indicated that its fermentation-produced 2'-FL, manufactured via genetically engineered *E. coli* (≥94% purity), has a calculated stability of 5 years when protected from light and stored at room temperature under ambient humidity. At accelerated conditions (80°C or 60°C and ambient humidity), 99.8 to 101.5% recovery was reported when compared to the baseline value.

Glycosyn and FrieslandCampina Domo reported the stability of 98% (as compared to the baseline) after 6 months in the accelerated and room temperature storage conditions for its 2’-FL manufactured via genetically engineered *E. coli* K12 (>90% purity; GRN 735, pages 27 to 29 - FDA, 2018a).

DuPont Nutrition (GRN 749, pages 17 to 19 - FDA, 2018b) reported that its 2’-FL, manufactured via genetically engineered *E. coli* K12 (≥ 82% purity), was shelf stable for up to 26 weeks at 40°C and 75% relative humidity. Compared to the baseline, an average of 99.6% recovery was reported at 26 weeks in accelerated conditions.

APTech is currently conducting a 6-month accelerated storage and 36-month shelf stability study on its 2'-FL produced via genetically engineered *C. glutamicum* APC199. At accelerated conditions (40°C at a relative humidity of 75%), 100.5% recovery was reported when compared to the baseline value.

<u>Stability in Infant formula and conventional foods</u>

GRN 546 (pages 13 to 17 - FDA, 2015a) reported that chemically synthesized 2'-FL was stable under intended conditions of use in conventional foods and infant formula. No significant loss of 2’-FL was observed under any of the storage conditions

for up to 900 days. Briefly, three independently formulated, commercially representative infant formula powders containing a target concentration of 0.90 g 2'-FL and 0.45 g LNnT per 100 g (dry matter) of infant formula, respectively, were subjected to typical production processing steps and stored in gassed (N_2/CO_2) tin cans (1 can per time and temperature point) at 4, 20, 30, or 37°C. The 2'-FL content was measured at regular time intervals for up to 900 days of storage.

Furthermore, Glycom noted that chemically synthesized 2'-FL was stable under the intended conditions of use in other food applications, including yoghurt, citrus fruit drinks, and ready-to-drink chocolate-flavored milk when prepared and stored under the recommended conditions (Table 8; GRN 546, pages 15 to 17 - FDA, 2015a).

Overall, the stability data reported for 2'-FL in previous GRNs (FDA, 2015a; 2015b; 2016a; 2018a; 2018b) support that all purified 2'-FL preparations, regardless of methods of manufacture, are shelf stable and well-suited for the intended food uses. Since APTech's 2'-FL has a purity of ≥ 94%, the shelf stability is expected to be similar to those of other 2'-FL preparations.

Table 8. Stability of 2'-FL in Bulk Powder and Powdered Infant Formula at Room Temperature

GRN	Food matrix	Test Conditions	
		Accelerated, 40°C 75% RH	**Shelf-stability, 25°C 60% RH**
749	Bulk powder	99.6% at week 26	
735	Bulk powder	97.8% at 6 mo	98.4% at 6 mo
650	Bulk powder	**Accelerated, 60°C and ambient humidity**	
		101.5% at 3 mo	
		Accelerated, 80°C and ambient humidity	
		99.8% at 3 mo	
571	Bulk powder	102.9-103.5% at week 26	106.2-106.6% at week 104
546	Bulk powder	98.2% at 6 mo (40°C, 75% RH)	97.0% at 36 mo; 99.9% at 24 mo (25°C, 60% RH)
	Infant formula	**37°C**	**4°C, 20°C, and 30°C**
		103.15% at day 540	-3.57 to -5.84% at day 540
	Other foods		**Typical Storage Conditions***
	Yogurt		103.9-111.86% at day 21
	Citrus fruit drink		106.3% at day 28
	Milk, ultra-high temperature processing		98.6% at day 28

All the recovery values are in comparison with those at initial points; *Typical storage conditions not specified in detail.

2.D. Intended Technical Effects

2’-FL will be used as a food ingredient in conventional foods as well as infant formulas (whey-, milk-, or soy-based) for full term infants. As described in GRNs 735 and 749 (FDA, 2018a, 2018b), the intended effect is as a nutrient necessary for the body’s nutritional and metabolic processes, serving as a non-digestible carbohydrate or as a prebiotic for establishment of healthy gut microflora in infants.

Dietary fiber (or non-digestible carbohydrates) has been identified as a shortfall nutrient that is low in American diet, leading to public concerns (USDA, 2015). Increased intake of dietary fiber or non-digestible carbohydrates would help normalize the functions of the large intestine by promoting intestinal regularity and alleviating constipation, and help reduce the risk of heart disease and diabetes (IOM, 2002). The Food and Nutrition Board (FNB), the Institute of Medicine (IOM), has established Adequate Intakes for Americans (IOM, 2005). The adequate intake values for fiber range from 19 to 25 g/day for children aged 1 to 8 years, 26 to 38 g/day for children and adolescents aged 9 to 18 years, and 21 to 38 g/day for adults, 19 years or older (IOM, 2005). Recently, US FDA has raised the Daily Value of dietary fiber from 25 to 28 g to encourage Americans to consume more fiber-rich foods (FDA, 2016b). However, average Americans consume only approximately one half of the recommended intakes; the mean fiber intake for children/adolescents and adults, over 19 years, were 13.2 and 16.1 g/day, respectively (McGill et al., 2015). Addition of 2’-FL to the diet may help improve the dietary fiber intake status in Americans.

PART 3. EXPOSURE ESTIMATES

3.A. Estimated Dietary Intakes (EDIs) of 2'-FL Under the Intended Use

Since 2'-FL will be added to the same food categories at the same use levels described in GRN 735, the EDIs are expected to be the same as or similar to those found in GRN 735. 2'-FL is intended for use as a food ingredient in term infant formulas, toddler formulas, and selected conventional foods at the levels listed in Table 1.

Based on the food consumption data reported in a recent National Health and Nutrition Examination Survey (NHANES; 2013-2014) dataset compiled by the U.S. Department of Health and Human Services, National Center for Health Statistics, and the Nutrition Coordinating Center, the EDIs of 2'-FL were calculated from the food code list and the survey database of diet recalls.

EDIs of Infant Formula

Table 9 presents the data on infant formula intakes by age, which range from 1,077 to 1,219 g/person/day. On a body weight basis, these intakes correspond to 118 to 226 g/kg body weight (bw)/day.

EDIs of 2'-FL from the Proposed Use in Infant Formula Only

The estimates for the daily intake of 2'-FL from its use in only term-infant formulas are summarized in Table 10. From the use of 2'-FL in only infant formula (a maximum level of 2.4 g/L of formula), in all-user infants aged 0 to 11.9 months old, the estimated mean and 90th percentile intakes of 2'-FL were determined to be at 1.87 and 2.78 g/person/day, respectively. On a body weight basis, these intakes were determined to be 258.7 and 431.3 mg/kg bw/day, respectively. The all-user estimated mean and 90th percentile intakes of 2'-FL were greatest in infant aged 3 to 5.9 months old at 2.04 and 2.93 g/person/day, respectively (Table 10). On a body weight basis, the greatest intake was observed to occur in infants aged 0-2.9 months at 347.8 and 541.9 mg/kg bw/day, respectively.

Table 9. EDIs of Infant Formula

Population Group	All-Person Intake		All-Users Intake			
	Mean	90th Pctl	% Users	n	Mean	90th Pctl
g/person/day						
0-2.9 mo	509	1095	66.5	140	766	1212
3-5.9 mo	609	1128	71.8	151	849	1219
6-8.9 mo	629	1069	81.2	162	775	1077
9-11.9 mo	495	1012	68.6	115	721	1156
0-11.9 mo	563	1096	72.2	568	780	1157
g/kg bw/day						
0-2.9 mo	96.3	204.4	66.5	140	144.9	225.8
3-5.9 mo	85.6	170.4	71.8	151	119.2	175.5

6-8.9 mo	74.0	133.4	81.2	162	91.1	140.8
9-11.9 mo	52.8	76.6	68.6	115	76.6	118.3
0-11.9 mo	77.9	168.3	72.2	568	107.8	179.7

Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; bw = body weight; mo = months; pctl = percentile.

Table 10. EDIs of 2'-FL from the Proposed Use in Infant Formula Only

Population Group	All-Person Intake		All-Users Intake			
	Mean	90th Pctl	% Users	n	Mean	90th Pctl
g/person/day						
0-2.9 mo	1.22	2.63	66.5	140	1.84	2.91
3-5.9 mo	1.46	2.71	71.8	151	2.04	2.93
6-8.9 mo	1.51	2.57	81.2	162	1.86	2.58
9-11.9 mo	1.18	2.43	68.6	115	1.73	2.77
0-11.9 mo	1.35	2.63	72.2	568	1.87	2.78
mg/kg bw/day						
0-2.9 mo	231.1	490.6	66.5	140	347.8	541.9
3-5.9 mo	205.4	409.0	71.8	151	286.1	421.2
6-8.9 mo	177.6	320.2	81.2	162	218.6	337.9
9-11.9 mo	126.7	183.8	68.6	115	183.8	283.9
0-11.9 mo	187.0	403.9	72.2	568	258.7	431.3

Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; bw = body weight; mo = months; pctl = percentile.
Intended use of 2'-FL in infant formula=2.4 g/L.

<u>EDIs of 2'-FL from the Combined Use in Infant Formula and Other Foods and Beverages</u>

Tables 11 and 12 present the EDIs of 2'-FL from the combined use of infant formula and other foods and beverages in all infants (combining infant formula-fed and breast-fed) and all-users as well as in all population by age. Table 11 presents the data on a per person basis by population group. Table 12 presents these data on a per kilogram body weight basis. The mean and 90th percentile EDIs of 2'-FL in all-users of all ages were determined to be 1.70 and 3.54 g/person/day, respectively. Infants aged 6 to 11.9 months were determined to have the highest mean consumer only intakes at 2.28 g 2'-FL per person per day. The highest intake was observed to occur in male teenagers with the highest 90th percentile intake at 4.29 g/person/day.

On a body weight basis, the mean and 90th percentile EDIs were determined to be 36 and 80 mg/kg bw/day, respectively, in all-users. Of all-users, infants aged 0 to 5.9 months were estimated to have the highest mean and 90th percentile EDIs of 315 and 532 mg/kg bw/day, respectively. The lowest mean and 90th percentile EDIs for 2'-FL

were determined for adult females and females of childbearing age at 20 and 43 mg/kg bw/day, respectively.

Table 11. Summary of the EDI of 2'-FL from Proposed Uses by Population Group

Population Group	Age Group	All-person (or per capita) Intake (g/d)		All-users Intake (or consumers only, g/d)			
		Mean	90th Pctl	%	n	Mean	90th Pctl
Infants	0-5.9 mo	1.10	2.75	57.5	107	1.91	3.00
	6-11.9 mo	2.14	3.86	94.1	160	2.28	3.86
Toddlers	12-35 mo	1.83	2.97	100.0	348	1.83	2.97
Children	3-11 y	1.96	3.53	99.7	1,277	1.97	3.53
Female teenagers	12-19 y	1.47	2.95	94.7	544	1.55	2.95
Male teenagers	12-19 y	1.85	4.16	92.5	526	2.00	4.29
Women of child-bearing age	16-45 y	1.22	2.82	89.9	1,219	1.36	2.87
Female adults	20+ y	1.32	2.96	91.9	2,169	1.44	3.05
Male adults	20+ y	1.59	3.81	86.8	1,842	1.84	3.97
Elderly	65+ y	1.76	3.74	92.8	939	1.90	3.91
Total population	All ages	1.55	3.41	91.2	6,973	1.70	3.54

Adopted from GRN 735, page 32. Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percentile; mo = months; y = years.

Table 12. Summary of the Estimated Daily Per Kilogram Body Weight Intake of 2'-FL from Proposed Uses by Population Group

Population Group	Age Group	All-person Intake (mg/kg bw/d)		All-users Intake (mg/kg bw/d)			
		Mean	90th Pctl	%	n	Mean	90th Pctl
Infants	0-5.9 mo	181	477	57.5	107	315	532
	6-11.9 mo	244	441	94.1	160	259	447
Toddlers	12-35 mo	148	243	100.0	346	148	243
Children	3-11 y	75	147	99.7	1,268	76	147
Female teenagers	12-19 y	24	52	94.7	536	26	52
Male teenagers	12-19 y	29	67	92.5	524	31	67

Women of child-bearing age	16-45 y	18	42	89.9	1,209	20	43
Female adults	20+ y	19	42	91.9	2,156	20	43
Male adults	20+ y	19	46	86.7	1,833	22	48
Elderly	65+ y	24	53	92.6	928	26	54
Total population	All ages	32	76	91.1	6,930	36	80

Adopted from GRN 735, pages 32-33. Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percentile; mo = months; y = years.

3.B. Food Sources of 2'-FL

The primary source of 2'-FL in the human diet is from human milk. Table 13 summarizes 2'-FL concentrations of human milk collected from various cohorts (Asakuma et al., 2008, 2011; Austin et al., 2016; Balogh et al., 2015; Bao et al., 2013; Castanys-Munoz et al., 2013; Chaturvedi et al., 1997, 2001a; Coppa et al., 1999, 2011; Donovan and Comstock, 2016; Erney et al., 2000, 2001; Gabrielli et al., 2011; Galeotti et al., 2012, 2014; Goehring et al., 2014; Grollman and Ginsburg, 1967; Hong et al., 2014; Kunz et al., 1999; Leo et al., 2009, 2010; Marx et al., 2014; McGuire et al., 2017; Morrow et al., 2004; Musumeci et al., 2006; Nahkla et al., 1999; Similowitz et al., 2013; Sumiyoshi et al., 2003; Thurl et al., 1996, 2010; Wang et al., 2015). The mean concentrations of 2'-FL in human milk range from 0.22 to 8.4 g/L, depending on the genotype of the mother and stage of lactation, as indicated by the studies summarized in Table 13. In GRN 650 (FDA, 2016a), the dietary intake of 2'-FL in the human milk samples that have been reported in literature was summarized.

Based on the mean levels of 2'-FL present in mature human milk, a 6.5-kg infant drinking 1 L of milk per day would be expected to consume 170 to 660 mg/kg bw/day of 2'-FL. Among infants from secretor mothers, the intake of 2'-FL from mature breast milk may be up to 1,150 mg/kg bw/day. For newborn infants, the average intake of 2'-FL from colostrum is approximately 80 to 360 mg/kg bw/day based on a 3.4-kg newborn infant drinking an average of 250 mL of breast milk per day during the first 5 days. However, in newborns from secretor mothers, the intake of 2'-FL from colostrum may be up to approximately 620 mg/kg bw/day.

Table 13. 2'-FL Content in Human Milk

Location	Days or months after postpartum	2'-FL content (g/L)	References
Ethiopia – Rural	71 d	1.11	McGuire et al., 2017
Ethiopia – Urban	59 d	1.39	
Gambia – Rural	65 d	1.44	
Gambia - Urban	62 d	2.06	
Ghana	58 d	0.70	
Kenya	73 d	1.65	
Peru	60 d	3.19	
Spain	70 d	1.91	
Sweden	49 d	2.77	
Washington, USA	68 d	2.03	
California, USA	62 d	3.44	
China - Urban	5-11 d	2.00	Austin et al., 2016
	12-30 d	1.90	
	1-2 mo	1.70	
	2-4 mo	1.30	
	4-8 mo	1.10	
Not specified	Not specified	2.7	Donovan and Comstock, 2016
Not specified	14 d	2.87	Goehring et al., 2014
Samoa	5-10 d	0.22	Leo et al., 2010
	22-155 d	0.69	
Not specified	3 mo	14.5%	Wang et al., 2015
Japan	1 d	2.49	Asakuma et al., 2008
	2 d	2.01	
	3 d	1.58	
Not specified	1 wk of lactation	4.53-6.27	Balogh et al., 2015
		2.69-3.55	
Asia	Not specified	2.1	Castanys-Munoz et al., 2013
Europe		2.6	
Latin America		2.48	
USA		2.0	
Mexico City	30-60 d	1.21	Chaturvedi et al., 1997
Not specified	1-3 d	0.24-0.36	Grollman and Ginsburg, 1967
	5 wk	0.46	
	6 wk	0.031	
California, USA	Not specified	2.40-3.70	Marx et al., 2014
Japan	4 d	0.20	Sumiyoshi et al., 2003
	10 d	0.34	
	30 d	0.29	
	100 d	0.05	

Burkinabe, Africa	1 d	1.80	Musumeci et al., 2006
	2 d	4.50	
	3 d	8.40	
Italy	1 d	1.00	
	2 d	2.10	
	3 d	4.20	
Samoa	5-10 d	0.22	Leo et al., 2009
	>10 d	0.69	
Italy	4 d	7.3	Gabrielli et al., 2011
	5-10 d	6.05	
	>10 d	5.25	
US	1 d	2.8	Chaturvedi et al., 2001a
	2 d	3	
	3 d	3.5	
	>10 d	3.6	
Asia	0-2 d	2.29	Erney et al., 2000
	3-10 d	2.26	
	11-30 d	2.36	
	>31 d	1.50	
Europe	0-2 d	3.40	
	3-10 d	2.69	
	11-30 d	2.38	
	>31 d	2.36	
Latin-America	3-10 d	2.79	
	11-30 d	2.61	
	>31 d	1.91	
US	3-10 d	2.78	
	11-30 d	2.56	
	>31 d	1.69	
Mixed geographies		2.4	
	2 d	2.8	
	4 d	2.6	
	>10 d	2.25	
Germany	2-28 d	0.45	Kunz et al., 1999
Europe	4 d	3.93	Coppa et al., 1999
	10 d	3.02	
	30 d	2.78	
	60 d	1.84	
	90 d	2.46	
Germany	5-10 d	3.37	Thurl et al., 2010
	>10 d	2.96	
Europe	Mature milk	1.84	Thurl et al., 1996
US	0-33 d	1.13	Nahkla et al., 1999
	4-128 d	1.27	

America and Europe	1-100 d	2.38	Erney et al., 2001
Latin America	1-100 d	3.95	Morrow et al., 2004
Japan	30-120 d	1.48	Asakuma et al., 2011
Europe	25-35 d	0-2.66	Coppa et al., 2011
Europe	4-30 d	0-7.15	Galeotti et al., 2012
US	3 d	1.12	Bao et al., 2013
	14-29 d	1.08	
US	90 d	1.22	Smilowitz et al., 2013
Europe	4-30 d	0-7.80	Galeotti et al., 2014
US	35 d	0.48-2.50	Hong et al., 2014

3.C. EDIs of 2'-FL from Diet

2'-FL level in each food is not listed in the USDA food composition tables or the National Health and Nutrition Examination Survey (NHANES) databases. Thus, the EDIs from the diet were not estimated.

3.D. Total EDIs of 2'-FL from Diet and Under the Intended Use

As mentioned in 3.C., 2'-FL level in each food is not listed in the USDA food composition tables or the National Health and Nutrition Examination Survey (NHANES) databases. Thus, the EDIs from the diet were not estimated.

3.E. EDIs of Other Nutrients Under the Intended Use

No other substances are expected to be formed in or on the food under the intended conditions of use of the 2'-FL preparation.

<u>Summary of Consumption Data</u>

<u>Infants: EDIs of 2'-FL from Infant Formula Use Only</u>

From the use of 2'-FL in only infant formula (a maximum level of 2.4 g/L of formula), in all-user infants aged 0 to 11.9 months old, the estimated mean and 90th percentile intakes of 2'-FL were determined to be at 1.87 and 2.78 g/person/day, respectively. On a body weight basis, these intakes were determined to be 258.7 and 431.3 mg/kg bw/day, respectively. The all-user estimated mean and 90th percentile intakes of 2'-FL were greatest in infant aged 3 to 5.9 months old at 2.04 and 2.93 g/person/day, respectively (Table 10). On a body weight basis, the greatest intake was observed to occur in infants aged 0-2.9 months at 347.8 and 541.9 mg/kg bw/day, respectively.

EDIs of 2’-FL from the Use of Infant Formula and Other Foods

The mean and 90th percentile EDIs of 2'-FL in all-users of all ages were determined to be 1.70 and 3.54 g/person/day, respectively. Infants aged 6 to 11.9 months were determined to have the highest mean consumer only intakes at 2.28 g 2'-FL per person per day. The highest intake was observed to occur in male teenagers with the highest 90th percentile intake at 4.29 g/person/day. On a body weight basis, the mean and 90th percentile EDIs were determined to be 36 and 80 mg/kg bw/day, respectively, in all-users. Of all-users, infants aged 0 to 5.9 months were estimated to have the highest mean and 90th percentile EDIs of 315 and 532 mg/kg bw/day, respectively. The lowest mean and 90th percentile EDIs for 2'-FL were determined for adult females and females of childbearing age at 20 and 43 mg/kg bw/day, respectively.

These EDIs are within safe intake levels (details are described in Part 6). The EDI assessments are based on the assumption that APTech’s 2’-FL will replace currently marketed 2’-FL. Thus, cumulative exposures are not expected to change. In addition, the EDIs presented in this notice are highly amplified estimates since it is not likely that 2’-FL will be used at the maximum levels for all intended use food categories. In addition, short-term surveys, such as the typical 2-day dietary surveys, may overestimate the consumption of food products that are consumed relatively infrequently. More importantly, the intended use and use levels of 2’-FL will be the same as outlined in GRN 735, except in medical food application which was withdrawn from the original submission. Consequently, APTech notes that its uses will not result in any exposure beyond what was previously estimated in GRN 735.

PART 4. SELF LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with 2’-FL.

PART 5. HISTORY OF CONSUMPTION

The statutory basis for the conclusion of GRAS status of 2’-FL in this document is not based on common use in food before 1958. The GRAS determination is based on scientific procedures. 2’-FL is present naturally in human milk. It is reasonable to conclude that infants were exposed to 2’-FL prior to 1958.

PART 6. BASIS FOR GRAS DETERMINATION

6.A. Current Regulatory Status

USA

Three sources of human milk oligosaccharides (HMOs) have been evaluated by the FDA over the past 5 years for incorporation of HMO products in infant formulas for consumption by term infants. Relevant U.S. GRAS notifications include 2'-O-fucosyllactose (GRN 546, FDA, 2015a; GRN 571, FDA, 2015b; GRN 650, FDA, 2016a; GRN 735, FDA, 2018a; GRN 749, FDA, 2018b), lacto-*N*-neotetraose (GRN 547, FDA, 2015c; GRN 659, FDA, 2016c) and 3'-sialyllactose (GRN 766, FDA, 2018c). FDA had no questions on the use levels of these HMOs similar to those found in human milks. Table 14 summarizes the regulatory status of 2'-FL in the USA.

These HMOs (degree of polymerization [DP] unit of 3) are considered as dietary fiber or total fiber. The Institute of Medicine has recommended that Americans increase the consumption of dietary fiber and has not established tolerable upper intake levels of dietary fiber for any age/gender groups or special populations (IOM, 2002).

Table 14. Regulatory Status of 2'-FL in USA

GRN	Substance	Intended Food Uses	Company
546	2'-FL manufactured using chemical synthesis (≥ 95% purity)	Term infant formula and toddlers (2.4 g/L); various foods and beverages for children and adults (0.28-2.4 g/RACC, 0.084-2.5 g/L)	Glycom A/S (FDA, 2015a)
571	2'-FL manufactured using a GMO *E. coli* (≥ 90% purity)	As a nutrient for the body's nutritional and metabolic processes in term infant and toddler formulas (2 g/L)	Jennewein Biotechnologie GmbH (FDA, 2015b)
650	2'-FL manufactured using a GMO *E. coli* (≥ 94% purity)	Term infant and toddler formula (2.4 g/L); other baby foods for infants (12 g/kg); other drinks for young children (1.2 g/L); various foods for children and adults (0.084-2.5 g/L)	Glycom A/S (FDA, 2016a)
735	2'-FL manufactured using a GMO *E. coli* K12 (> 90% purity)	Term infant and toddler formulas (0.24 g/RACC or 2.4 g/L); various foods and beverages for children and adults (0.28 - 4.0 g/RACC or 0.84 - 80 g/kg); medical foods for older children and adults at age 11 or older (up to 4 g/serving and 12 g/day)	Glycosyn, LLC and FrieslandCampina Domo (FDA, 2018a)

749	2'-FL manufactured using a GMO *E. coli* K12 (≥ 82% purity)	Term infant and toddler formulas (0.24 g/RACC or 2.4 g/L); other baby foods and drinks (0.14-2.04 g/RACC or 1.2 g/kg)	DuPont Nutrition (FDA, 2018b)
Current notice	2'-FL manufactured using a GMO *Corynebacterium glutamicum* (≥ 94% purity)	Same as GRN 735; Term infant and toddler formulas (0.24 g/RACC or 2.4 g/L); various foods and beverages for children and adults (0.28 - 4.0 g/RACC or 0.84 - 80 g/kg); medical foods for older children and adults at age 11 or older (up to 4 g/serving and 12 g/day)	APTech

European Union

In the EU, 2'-FL has been authorized as a novel ingredient (EU, 2018). The European Food Safety Authority (EFSA) panel concluded that 2'-FL is safe for infants, toddlers, and adults:

1) Infants when added to infant and follow-on formula, in combination with another oligosaccharide, lacto-N-neotetraose (LNnT), at concentrations up to 1.2 g/L of 2'-FL and up to 0.6 g/L of LNnT, at a ratio of 2:1 in the reconstituted formula.

2) Young children (older than one year of age) when added to follow-on and young-child formula at concentrations up to 1.2 g/L of 2'-FL alone or in combination with LNnT, at concentrations up to 0.6 g/L, at a ratio of 2:1.

3) Adults when added to dairy and milk products, dairy analogs, cereal bars, table top sweeteners, dietary foods for weight control diets, beverages, and food supplements at concentrations of 1.2 g/L for beverage products, 1.2-2.4 g/ serving for food products, and 3.0 g/day for food supplements (EFSA, 2015).

6.B. APTech's 2'-FL is Structurally Identical to that Present in Human Milk

As presented in Parts 2.A and 2.C, APTech's 2'-FL is chemically and structurally identical to the 2'-FL which is found in human milk, and therefore, the safety of APTech's 2'-FL for all intended uses is supported by the known consumption of 2'-FL from human breast milk in infants. A summary of the 2'-FL levels in human breast milk is provided in Part 3. The safety of APTech's 2'-FL is further supported by the results from animal toxicological studies and human clinical studies, which are summarized in Parts 6.C to 6.F.

6.C. Review of Safety Data

This section comprises of the pivotal studies for the safety assessment of APTech's 2′-FL. To identify other data and information relevant to the safety of infant

formula and food uses of 2′-FL, a comprehensive search of the published scientific literature was conducted through March 2019. Published studies identified during the literature search consisted of studies relating to the metabolic fate and safety of 2’-FL. Most of the studies that form the basis of this safety assessment have been reviewed in GRN 749 (pages 30 to 39 - FDA, 2018b), GRN 735 (pages 50 to 65 - FDA, 2018a), GRN 650 (stamped pages 34 to 40), GRN 571 (stamped pages 39 to 53 - FDA, 2015b), and GRN 546 (pages 29 to 35 - FDA 2015a). As the 2’-FL in this GRAS determination has similar specifications compared to those discussed in previous GRAS notices (Table 12), it is recognized that the information and data in other GRAS notices are pertinent to the safety of the APTech’s 2’-FL in this GRAS determination. Therefore, this notice incorporates, by reference, the safety and metabolism studies discussed in the previous GRAS notices, and will not discuss previously reviewed references in detail. Additionally, this notice discusses additional studies that have been published since the FDA’s last review in 2017 - 2018 (GRNs 735 and 749). The subject of the present GRAS notice is 2’-FL produced via microbial fermentation.

6.C.1. Absorption, Distribution, Metabolism, and Elimination (ADME)

It is generally accepted that most of the HMOs, including 2’-FL, resist the pH of the stomach and are resistant to enzymatic hydrolysis in the small intestine to reach the large intestine intact. In the colon, they are either fermented by the intestinal microflora or excreted unchanged in the feces (Brand-Miller et al., 1998; Gnoth et al. 2000; Newburg et al., 2000). From a breath hydrogen test, Brand-Miller et al. (1998) estimated that, on average, all of the load of purified HMO isolated from their mothers’ milk (113% ± 18%) reached the large intestine and was fermented in infants aged 3 to 8 months. This study suggests that HMO resist digestion in the small intestine of most breast-fed infants and undergo fermentation in the colon. An *in vitro* study by Gnoth et al. (2000) demonstrated that less than 5% of the HMO are digested in a simulated intestinal tract condition. Thus, the majority of 2’-FL will pass through the intestinal tract and enter the colon intact, and will be transported intact to the large intestine and subjected to partial fermentation by the indigenous microbiota populations within the gastrointestinal tract (Brand-Miller et al., 1998). Thus, HMOs are considered as non-digestible carbohydrates or dietary fiber ingredients.

HMOs are the preferred substrate for *B. infantis* and other bifidobacteria strains, and may reduce the nutrients available for potentially harmful bacteria and keep their growth under control (Ellison et al., 2016; Rudloff et al., 2019; Thongaram et al., 2017; Weiss et al., 2014).

A study by Steenhout (2016) reported that the microbial alpha diversity and comparison of the global microbiota composition of 2’-FL supplemented infant formula group was closer to those of the breast-fed group but not to those of the unsupplemented control group. The influence of secretor status and breast feeding on gut microbiota composition persists up to two to three years (Smith-Brown et al., 2016). Newburg et al. (2000) and Chaturvedi et al. (2001b) also reported that the pattern of oligosaccharides in the urine and feces of the breast-fed infants resembles that in their mothers’ milk, suggesting that their origin is primarily human milk. Oligosaccharides in

the urine and feces of artificially fed infants have a different pattern from breast milk and from the urinary and fecal patterns of breast-fed infants. Overall, HMOs, including 2’-FL, can be considered as prebiotic dietary fibers.

Gnoth et al. (2001) have suggested that small quantities of 2’-FL may be transported transcellularly across the intestinal epithelium by receptor-mediated transcytosis and/or by paracellular means, and low quantities of 2’-FL have been detected unchanged in the urine of breast-fed infants (Goehring et al., 2014). Marriage et al. (2015) reported that the relative absorption of 2’-FL in the plasma is in the region of 0.05 and 0.07% for newborn infants receiving formula supplemented with 0.2 and 1.0 g 2’-FL/L, respectively. The relative excretion was similar among the groups fed 2’-FL: 1.26 to 1.50% for the formula fed infants (supplemented with 0.2 or 1.0 g 2’-FL/L) and breast-fed infant groups, respectively.

Studies showed that HMOs already appear in maternal urine and blood during pregnancy and as early as the first trimester (Jantscher-Krenn et al., 2019; Wise et al., 2018). Wise et al. (2018) determined whether or not HMOs also appear in amniotic fluid. Women during pregnancy were enrolled, and their urine and amniotic fluid were collected at birth as well as their milk 4 days postpartum. Several HMOs, including 2'-FL, 3’-FL, difucosyllactose, and 6'-SL, were present in different relative abundancies in all three samples (urine, milk, and amniotic fluid). The data indicate that HMOs appear in amniotic fluid and that the fetus is already exposed to HMOs *in utero.*

Rodents seems to absorb 2’-FL more effectively than humans. A rodent study by Vasquez et al. (2017) also reported that rats are able to effectively absorb a portion of HMOs from the intestine into the plasma and to excrete them in the urine. Single oral dose of 0.2, 0.1, or 5 g/kg bw 2’-FL, 0.2, 1, or 3.75 g/kg bw 6’-SL, 0.2 or 1 g/kg bw LNnT were administered to adult female Sprague-Dawley (SD) rats (8-10 weeks old; n=8 per group). The time course of HMO absorption into the bloodstream and their appearance in urine was studied. The results showed that after a single oral dose in adult rats, 2’-FL appeared in the serum as early as 30 minutes in all 2’-FL dosed animals. The lowest dose had a maximum peak in the serum at 60 minutes, and the higher doses had maximum peaks between 90 and 120 minutes. The urinary excretion of 2’-FL began after 120 minutes. In a specific kinetic absorption study with 2’-FL, 9-11 days old SD pups (n=10 per group, 5 males and 5 females per group) were intragastrically administered 1, 2.5, 5, or 10 g/L 2’-FL. Significant amount of 2’-FL were absorbed into the systemic circulation and subsequently excreted in the urine in a dose-dependent manner. During the 4 hours after a single oral dose of 2’-FL in rat pups, 2’-FL was absorbed from the intestine into the plasma. The maximum absorption occurred at 180 minutes. The serum fucose increased proportionally to 2’-FL concentration. The urinary excretion of 2’-FL is dose-dependent and constantly increasing over time. The authors also found basal levels of these HMO in plasma and urine of adult rats as well as rat pups as a natural result of nursing.

6.C.2. Mutagenicity and Genotoxicity Studies

As summarized in Table 15-1, APTech's 2'-FL was found to be non-mutagenic or genotoxic (Biotoxtech., 2019a; 2019b; 2019c). Other sources of 2'-FL also did not show any mutagenicity and genotoxicity in bacterial reverse mutation test or in human peripheral blood lymphocytes (Table 15-1; Phipps et al., 2018).

As shown in Table 15-2, previous GRAS notices also reported that 2'-FL was not mutagenic or genotoxic in bacterial reverse mutation test, micronucleus test in cultured human lymphocytes, L51784 tk+/- mouse lymphoma cells, *in vitro* mammalian cell mutation assay, or micronucleus test in bone marrow cells of the (Crl:CD(SD)) rats (Coulet et al., 2014; GRN 571, FDA, 2015b; van Berlo et al., 2018; Verbaan, 2015a, 2015b; Verspeek-Rip, 2015).

The unpublished mutagenicity/genotoxicity studies of APTech's 2'-FL confirmed the findings reported in other studies of 2'-FL reporting that the substance was not mutagenic or genotoxic under the test conditions. Thus, the unpublished status of these studies has no impact on the overall conclusion of this GRAS determination if qualified experts do not have access to such data and information.

Table 15-1. Summary of Mutagenicity or Genotoxicity Studies of 2'-FL First Reviewed in This GRAS Notice

Test System	Concentration/Dose	Result	Reference
Mutagenicity and Genotoxicity of APTech's 2'-FL			
Bacterial reverse mutation test: *S. typhimurium* (TA98, TA100, TA1535, and TA1537) and *E. coli* WP2*uvrA* (pKM101)	313, 625, 1,250, 2,500, and 5,000 µg/plate (Purity, 97.56%)	Not mutagenic	Biotoxtech, 2019a
In vitro chromosome aberration test: Chinese Hamster Lung (CHL/IU) cells	1,250, 2,500, and 5,000 µg/mL (Purity, 97.56%)	Not clastogenic	Biotoxtech, 2019b
In vivo micronucleus test: ICR mice	2,500, 5,000, and 7,500 mg/kg bw (Purity, 97.56%)	Not genotoxic	Biotoxtech, 2019c
Studies First Reviewed in This GRAS Determination			
S. typhimurium TA98, TA100, TA1535, TA1537, and *E. coli* WP2*uvr*A (pKM101)	5, 15, 50, 150, 500, 1,500, or 5,000 µg/plate 2'-FL (purity NS)/difucosyllactose (DFL) (8:1 ratio) ± S9	No genotoxicity	Phipps et al., 2018
Human peripheral blood lymphocytes	500, 1,000, or 2,000 µg/mL 2'-FL (purity NS)/DFL ± S9		

Table 15-2. Summary of Mutagenicity or Genotoxicity Studies of 2'-FL Reviewed in Previous GRAS Notices

Test System	2'-FL Conc./Dose	Result	Reference
S. typhimurium TA1535, TA1537, TA98, TA100, and *E. coli* WP2*uvr*A ± S9	62 to 5,000 µg/plate 2'-FL (Source-Friesland Campina, purity 94%)	Not mutagenic	van Berlo et al., 2018 (the same study was reported in GRN 735, page 56 - FDA, 2018a)
Micronucleus test in cultured human lymphocytes ± S9	3.9 to 2,000 µg/mL 2'-FL (Source-Friesland Campina, purity 94%); cytotoxicity test, 500, 1,000 or 2,000 µg/mL	Not clastogenic and/or aneugenic; marginally cytotoxic	
S. typhimurium TA98, TA100, TA102, TA1535, TA1537 ± S9	Plate incorporation method - 52, 164, 512, 1,600, or 5,000 µg 2'-FL/plate; pre-incubation method 492 to 5,000 ug/plate (source-Glycom, synthetic; purity >99%, dw basis)	Not mutagenicity at concentration up to 5,000 µg/plate or 5,000 µg/mL	Coulet et al., 2014 (the study was reported in GRN 546, pages 32 to 33 FDA, 2015a)
L51784 tk+/- mouse lymphoma cells ± S9	-S9, 1.7 to 5,000 µg/mL; ± S9, 492 to 5,000 µg/mL (source-Glycom, synthetic; purity >99%, dw basis)		
In vitro mammalian cell mutation assay ± S9	Up to 2,000 µg/plate (source-Glycom, produced by chemical synthesis; purity 99%)	Not mutagenic; no signs of cytotoxicity genotoxicity	Verbaan, 2015a (unpublished; GRN 650, stamped page 42)
S. typhimurium TA98, TA100, TA1535, TA1537, and *E. coli* WP2uvrA ± S9	Plate incorporation method - 52, 164, 512, 1,600, or 5,000 µg 2'-FL/plate; pre-incubation method 492 to 5,000 ug/plate (source-Glycom, produced via fermentation; purity 97.6%)	Not mutagenic	Verspeek-Rip, 2015 (unpublished; GRN 650, stamped page 42)
In vitro micronucleus test with cultured human blood peripheral lymphocytes ± S9	Up to 2,000 µg/plate (source-Glycom, produced via fermentation; purity 97.6%)	Not clastogenic or aneugenic	Verbaan, 2015b (unpublished; GRN 650, stamped page 42)
S. typhimurium TA98, TA100, TA102,	Up to 5,000 µg/plate (purity 92.4%)	Not mutagenic and cytotoxic	GRN 571 (unpublished;

TA1535, and TA1537 ± S9			pages 40 to 41 - FDA, 2015b)
Micronucleus test in bone marrow cells of the (Crl:CD(SD)) rats	A single dose of 0, 500, 1,000, or 2,000 mg/kg bw (purity 92.5%)	Not clastogenic	

NS= not specified

6.C.2.1. Bacterial Reverse Mutation Test of APTech's 2'-FL

The potential mutagenicity of APTech's 2'-FL (purity of 97.56%) was evaluated in histidine requiring *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) strains and tryptophan requiring *Escherichia coli* (WP2*uvrA*(pKM101)) strain in the presence or absence of metabolic activation (S9) (Biotoxtech, 2019a). In order to determine the high dose level of the main study, a dose range finding study was conducted. The high dose was selected at 5,000 µg/plate, and it was sequentially diluted by applying a geometric ratio of 4 to produce 5 lower dose levels (1,250, 313, 78.1, 19.5, and 4.88 µg/plate). As a result, growth inhibition and precipitation of the test substance were not evident at any dose level of the test substance in all strains in the presence and absence of the metabolic activation.

In the main study, the bacterial strains were treated with 2'-FL at concentrations of 0, 313, 625, 1,250, 2,500, and 5,000 µg/plate. Also, the negative and positive control groups (2-nitrofluorene for TA98, sodium azide for TA100 and TA1535, 9-aminoacridine for TA1537, or 4-nitroquinoline N-oxide for WP2*uvrA* (pKM101) in the absence of metabolic activation; 2-aminoanthracene for all strains in the presence of metabolic activation) were used in both experiments. The growth inhibition and deposition of the test substance was not evident at any dose levels of all strains in the absence and presence of metabolic activation. In the positive control group, the mean number of revertant colonies was markedly increased more than twice when compared to the negative control group. Thus, 2'-FL was determined to be non-mutagenic in the Ames test at concentrations up to 5,000 µg/plate under the test conditions.

6.C.2.2. *In Vitro* Chromosome Aberration Test of APTech's 2'-FL

This study was designed to evaluate the potential of 2'-FL (purity, 97.56%) to induce chromosomal aberrations in Chinese Hamster Lung (CHL/IU) cells (Biotoxtech, 2019b). To evaluate the ability of 2'-FL to induce chromosomal aberrations in cultured CHL/IU cells with and without S9 metabolic activation, two separate *in vitro* chromosome aberration assay tests were conducted. DMSO served as both the diluent for 2'-FL and the negative control substance. Mitomycin C and benzo[a]pyrene were used for the positive controls in the absence or presence of S9 metabolic activation, respectively. In order to determine the high dose level of the main study, a dose range finding study was conducted. The high dose was selected at 5,000 µg/mL, and it was sequentially diluted by applying a geometric ratio of 2 to produce lower dose levels (2,500, 1,250, 625, 313, 156, 78.1, 39.1, and 19.5 µg/mL). As a result, cytotoxicity and precipitation of the test substance were not evident in the short time treatments with and

without metabolic activation and in the continuous treatment without metabolic activation.

Therefore, the dose levels of the main study were selected as follows: 1,250, 2,500, and 5,000 ug/mL for both short time (+/-S9) and continuous treatment (-S9). In addition, the positive and negative control groups were set. As a result of the main study, the frequency of cells with chromosome aberrations in the short time treatments with and without metabolic activation and in the continuous treatment without metabolic activation was not statistically significantly different compared to the negative control group. In the positive control group, the frequency of cells with structural chromosome aberrations in the short time treatments with and without metabolic activation and in the continuous treatment without metabolic activation was statistically significantly increased compared to the negative control group. Thus, it was concluded that 2'-FL was not clastogenic under the conditions of this study.

6.C.2.3. *In Vivo* Mouse Micronucleus Test of APTech's 2'-FL

This study was designed to evaluate the potential of the test substance, 2'-FL (97.56%), to induce micronuclei in bone marrow cells of CrlOri:CD1(ICR), SPF mice when the test substance was orally administered via gastric intubation twice at 24-hour intervals (Biotoxtech, 2019c). In order to determine the high dose level of the main study, a dose range finding study was conducted. The high dose was set at 7,500 mg/kg, and it was sequentially diluted to produce 3 lower dose levels (5,000, 2,500, and 1,250 mg/kg). As a result, there were no clinical signs or mortality at any dose level of the test substance in male and female mice.

Therefore, the high dose level of the main study was set at 7,500 mg/kg and two additional lower dose levels (5,000 and 2,500 mg/kg) were produced. In addition, the positive and negative control groups were set. Since there was no mortality in either sex as a result of the dose range finding study, the main study was conducted with only males, which are known to be susceptible to micronucleus induction. Twenty-five male mice aged 8 weeks were treated by oral gavage with 2'-FL dissolved in saline over 2 consecutive days before being sacrificed. Saline was used as a vehicle control. Mitomycin C (2 mg/kg, i.p.) was administered as the positive control. Clinical signs were recorded on Day 0 (immediately and at 2 hours after the 1st dosing), Day 1 (before the 2nd dosing, immediately and at 2 hours after the 2nd dosing), and Day 2. All doses were well tolerated, and no clinical signs were observed. Immediately following sacrifice, femurs were dissected from each animal and trimmed, and bone marrow cells were collected to evaluate the frequency of micronuclei. No statistically significant increases in the incidence of micronucleated polychromatic erythrocytes (MNPCE) in polychromatic erythrocytes (PCE) were observed in any test substance groups compared with the negative control group. A significant increase in the incidence of MNPCE in PCE was observed in the positive control group compared with the negative control group. There were no statistically significant differences in the ratio of PCE to total erythrocytes in any test substance groups compared with the negative control value. Body weights of mice were comparable among the groups before and after

treatment with the test substance. It was concluded that 2'-FL did not induce micronuclei in the bone marrow cells of mice under the conditions of this study.

6.D. Animal Toxicity Studies

Since the 2'-FL in this GRAS determination has similar specifications compared to those described in previous GRAS notices (Table 7), it is recognized that the information and data in those GRAS notices are pertinent to the safety of the APTech's 2'-FL in this GRAS determination.

6.D.1. Animal Toxicity Studies First Reviewed in This GRAS Notice

Table 16-1 summarizes the toxicity studies of 2'-FL first reviewed in this GRAS determination, and Table 16-2 presents the summary of toxicity studies of 2'-FL reviewed in previous GRAS notices. As shown in Tables 16-1 and 16-2, various purified 2'-FL preparations showed similar toxicology profiles, regardless of methods of manufacture. For all 2'-FL preparations, the no observed adverse effect levels (NOAELs) were determined to be over 5,000 mg/kg bw/day in rats, indicating all purified 2'-FL preparations were considered safe. The NOAEL of APTech's 2'-FL was shown to be 7,500 mg/kg bw/day, the highest dose tested.

The unpublished animal toxicity studies of APTech's 2'-FL confirmed the findings discussed in earlier studies reporting that the NOAEL values are at least 5,000 mg/kg bw/day. Thus, the unpublished status of APTech's 2'-FL studies has no impact on the overall conclusion of this GRAS determination if qualified experts do not have access to such data and information.

Table 16-1. Toxicity Studies of 2'-FL First Reviewed in This GRAS Determination

Animal	Dose	Duration	Results	Reference
Animal Toxicity Studies of APTech's 2'-FL				
Rat (M25, F25)	0, 2.5, 5, or 7.5 g/kg bw (purity, 97.56%)	Single dose; 14 d observa-tion	Mean lethal dose (LD_{50}) was greater than 7.5 g/kg bw; lower bw in the high dose group	Biotoxtech, 2019d
Crl:CD(SD) rats	0, 2.5, 5, or 7.5 g/kg bw	90 d with 4 wk follow-up	NOAEL=7,500 mg/kg bw/d	Biotoxtech, 2019e
Animal Toxicity Studies of Other Sources of 2'-FL				
Crl:CD®(SD) neonatal rats (n=20/group)	0, 1,000, 3,000, or 5,000 mg/kg bw/d 2'-FL (purity NS)/ difucosyllactose (DFL) (8:1 ratio)	90 d	NOAEL=5,000 mg/kg bw/d	Phipps et al., 2018

Table 16-2. Toxicity Studies of 2'-FL Reviewed in Previous GRAS Notices

Animal	Dose	Duration	Results	Reference
Female Crl:CD(SD) rats (n=10)	0 or 10% (purity 96.0%) in diet	7 d	No differences in food consumption and no mortality, changes in behavior, or changes in appearance	GRN 571 (unpublished; stamped pages 42-44; FDA, 2015c)
Crl:CD(SD) rats, 4-wk-old (n=10/sex/group)	0 or 10% (purity 96.0%) in diet	90 d	NOAEL= 7,660 mg/kg bw/d (females); 8,720 mg/kg bw/d (males)	
Wistar Crl:WI(Han) rats (n=10/group)	0, 3, 6, or 10% in diet (or 0, 2.56, 5.08, or 7.99 g/kg bw (source-Friesland Campina, purity 94%)	13 wk	NOAEL= ≥ 7,250 mg/kg bw/d (males); ≥ 7,760 mg/kg bw/d (females) or 10% in the diet	van Berol et al., 2018 (published; also described in GRN 735, pages 54-55; FDA 2018a)
Wistar Crl:WI(Han) rats, 7-d-old (n=5 rats/sex/group)	0, 2,000, 5,000, or 7,500 mg/kg bw/d (source-Glycom, synthetic 2'-FL, 99% purity on a dry weight basis)	14 d	5,000 and 7,500 mg/kg bw/d doses: lower bw on day 0 to 3 than control, liquid and/or yellow feces; highest suitable dose was lower than 7,500 mg/kg bw/d	Coulet et al., 2014 (the study was described in GRN 546, pages 29-32; FDA, 2015a)
Wistar IGS:Crl:WI juvenile rats (n= 20/group)	0, 2,000, 5,000, or 6,000 mg/kg bw/d (synthetic 2'-FL, 99% purity on a dry weight basis	90 d	NOAEL= 5,000 mg/kg bw/d for male and female rats	
Wistar Crl:WI (Han) rats (n=10 or 15/sex/group)	0, 2,000, 4,000, or 5,000 mg/kg bw/d (source-Glycom produced by fermentation; 97.6% purity)	90 d with 4 wk recovery period	NOAEL= 5,000 mg/kg bw/d	Penard, 2015 (Unpublished; GRN 650, stamped pages 37-39; FDA, 2016a)
Piglet Study				
Domestic Yorkshire crossbred swine – farm neonatal	Control, 200, 500, or 2,000 mg/L 2'-FL (source:	21 d	Well tolerated up to 2,000 mg/L/d; no treatment-related effects; equivalent maximum doses=	Hanlon and Thorsrud, 2014

piglets (n=4-8 pigs/group)	Jennewein; purity 97.9%)		291.7 mg/kg bw/d (males), 298.9 mg/kg bw/d (females)	

NS= not specified.

An Acute Toxicity Study of APTech's 2'-FL

Table 16-1 summarizes the results from an acute oral toxicity study conducted with APTech's 2′-FL (97.56% purity) in juvenile (7 days old) male and female Sprague-Dawley rats (Biotoxtech, 2019d). The test groups consisted of three dose groups at dose levels of 2,500, 5,000, and 7,500 mg/kg bw and a control group (water for injection), with 5 animals of each sex per group. All animals were monitored for clinical signs and body weight changes during the 14-day observation period after dosing. They were euthanized and subjected to gross necropsy at the end of the observation period. One female was found dead at 7,500 mg/kg bw on day 2 after dosing. However, there were no test substance-related clinical signs and body weight changes in the other female pups in the 7,500 mg/kg bw dosing group. It was not considered to be test substance-related mortality since it was a natural death of the rat pup. In clinical signs, there were no abnormalities in the control and test groups although the body weight gain was significantly suppressed in the high dose male group. At necropsy, there were no test substance-related gross findings in either sex at 2,500, 5,000, and 7,500 mg/kg. It was concluded that the mean lethal dose (LD_{50}) was greater than 7.5 g/kg bw, the highest dose tested. A compound which has a LD_{50} value of over 5 g/kg bw in rats is classified as 'practically nontoxic' (Altug, 2003).

Subchronic Oral Toxicity Study of APTech's 2'-FL

This study was conducted to assess the potential toxicity and safety of the test substance, 2'-FL, when administered by oral gavage once daily to Sprague-Dawley [Crl:CD(SD)] rats of both sexes for 90 days. A total of 4 groups were assigned to one of the three test groups (2,500, 5,000, and 7,500 mg/kg bw/day) in addition to a control group (water). Each group consisted of 10 males and 10 females. Extra 5 animals of each sex were added to the control group and 7,500 mg/kg bw/day group for the recovery groups to assess the reversibility of toxicity during the 4-week recovery period. During the observation period, evaluated parameters included clinical signs, detailed examinations, body weight, food consumption, functional observations, ophthalmological examinations, urinalysis, hematological and clinical chemistry examinations, organ weights, gross post mortem examinations, and histopathological examination were performed after the observation period.

One male of the 5,000 mg/kg/day group was found dead on day 72. It was considered to be a sudden death of the rat showing no morphological changes, and it occurs often in Sprague-Dawley rats. There was no test substance-related effect on the gross findings at necropsy or histopathological lesions in this dead male. One female of the 7,500 mg/kg/day group was found dead on day 26. Serous fluid-filled thoracic cavity (clear with red color) and pulmonary congestion/edema were noted in the dead female. These findings might be due to a technical gavage error.

No test substance-related toxic effects were noted in clinical signs, detailed examinations, body weights, food consumption, functional observations, ophthalmological examination, urinalysis, hematology, clinical chemistry, organ weights, and gross postmortem examinations in males and females in the 2,500, 5,000, and 7,500 mg/kg bw/day groups. No test substance-related toxic effect was noted in the histopathological examination in males and females in the 7,500 mg/kg bw/day group. On the basis of these results, the NOAEL of APTech's 2'-FL was considered to be 7,500 mg/kg bw/day in both male and female rats after repeated oral administration for 90 days under the conditions of this study.

Subchronic Toxicity Studies of Other Sources of 2'-FL

A Study by Phipps et al. (2018)

In the subchronic study by Phipps et al. (2018), 2′-FL/difucosyllactose (DFL; 8:1 ratio) was administered to neonatal rats at doses up to 5,000 mg/bw/day, once daily for 90 days, followed by a 4-week recovery period. A concurrent reference control group received 5,000 mg/kg bw/day of fructooligosaccharide (FOS) already used in infant formula for direct comparison with the high-dose 2′-FL/DFL group. In the absence of compound-related adverse effects in the 90-day study, the NOAEL was determined to be 5,000 mg/kg bw/day.

6.D.2. Animal Toxicity Studies Reviewed in Previous GRAS Notices (Adopted from GRNs 546, 571, 650, 735, and 749)

Subchronic oral toxicity studies of 2'-FL showed that the NOAEL were in the range of 5,000 to 8,720 mg/kg bw/day for male and 5,000 to 7,760 mg/kg bw/day for female rats (Coulet et al., 2014; Phipps et al., 2018; van Berol et al., 2018).

A 90-Day Oral Toxicity Study by van Berol et al. (2018; the same study was presented in GRN 735, pages 54 to 55 - FDA, 2018a)

In a study by van Berol et al. (2018), 2′-FL (source, FrieslandCampina; 94% purity; produced through fermentation by genetically modified *E. coli* K12 GI724/ATCC 55151) was administered to Wistar Han IGS rats [Crl:WI(Han)] for 13 weeks starting post-natal days 25 to 115. The concentrations of 2'-FL in the diet were 0% (control), 3%, 6%, and 10%. These levels correspond to 2,170, 4,270, and 7,250 mg/kg bw/day for males and 2,450, 5,220, and 7,760 mg/kg bw/day for females from the low-, mid-, and high-dose groups, respectively. The exposure to 2′-FL was well tolerated at all dose levels, and did not induce any relevant changes in general condition, growth, water intake, neurobehavioral observations, ophthalmoscopy, hematology, clinical chemistry, urinalysis, organ weights, or in macroscopy and microscopy of organs and tissues.

Only a few observed changes were attributed to the administration of 2′-FL. In female rats of the high-dose group, overall food consumption was slightly decreased. In males, there was no statistically significant effect, but there was a similar trend in the high-dose group. Since the relative difference with controls was small (less than 10%)

and no clear corroborative changes were observed in any of the other parameters investigated (especially growth), this finding, although likely treatment related, was considered to be of little, if any, toxicological significance. The relative weight of the liver was increased by 8.25% in males in the high-dose group. This increase was not accompanied by changes in clinical chemistry (aspartate aminotransferase [ALT] and aspartate aminotransferase [AST] in particular, which are indicators for liver damage), and microscopic examination of the liver did not reveal any histopathological changes. Thus, it was not considered of toxicological concern. Cecal enlargement was noted in mid- and high-dose males and females, and in low-dose males. This finding is ascribed to the fact that the test substance is a non-digestible carbohydrate. It is well established that cecal enlargement in rats may arise from the feeding of large amounts of a heterogeneous family of products, referred to as 'dietary fiber' or 'poorly digestible carbohydrates.' In the absence of such histopathological correlates, the authors interpreted the cecal enlargement as a physiological response rather than a toxic effect. Thus, the NOAEL is placed at the highest level tested: ≥ 7,250 mg/kg bw/day for males and ≥ 7,760 mg/kg bw/day for females. The same study was described in GRN 735 when this study was not published.

A 90-Day Oral Toxicity Study Presented in GRN 571 (pages 42 to 43 - FDA, 2015b)

GRN 571 describes a 90-day study (unpublished) in which 4-week-old CD® rats [Crl:CD(SD), n=10/sex/group] were fed a standard rat diet *ad libitum* (control) or the standard rat diet that was supplemented with 10% of 2'-FL prepared via fermentation using genetically modified *E. coli* (source - Jennewein; 94.1% purity; specification >90%) (GRN 571; FDA, 2015c). An additional 3 animals per sex in the control group and nine animals per sex in the treatment group were used exclusively for blood sampling. No treatment-related abnormalities were observed in feed intake, clinical signs, body weight, organ weights, behavior, appearance, hematology, clinical biochemistry, urinalysis, ophthalmological examination, and histopathological examinations. Pale stools were observed in 7 of 10 males and 4 of 10 females between days 9 and 69 of the study in the 2'-FL group. This effect was attributed to the amount of undigested test item in the feces and was not considered by the authors to be adverse. In addition, one male rat had soft stools starting on day 14 for a 15-day period. This effect was not thought to be related to 2'-FL consumption. The study authors concluded that the NOAEL of 2'-FL was determined to be 7,660 and 8,720 mg/kg bw/day in female and male rats, respectively.

A 90-Day Oral Toxicity Study Presented in GRN 650 (stamped pages 37 to 39; summary in page 68 - FDA, 2016a)

Penard (2015; unpublished) conducted a 90-day oral toxicity study with an additional 28 day recovery period in Wistar [Crl:WI(Han)] rats on 2'-FL (source- Glycom; 97.6% purity, produced through fermentation using genetically modified *E. coli*; FDA, 2016a). In the main study, seven-day old neonatal Wistar rats were administered 2,000, 4,000, or 5,000 mg/kg bw of Glycom's 2'-FL or 5,000 mg/kg bw/day of FOS (reference group) for 90 days. Animals in the recovery group (5 rats per sex) were also administered control, 2'-FL, or FOS for 90 days after which they remained untreated for 28 days. One dam was then housed with a reconstituted litter of 5 pups per sex, fed a

standard diet, and the pups were treated with the same dose of 2'-FL until weaning on day PND 21. No deaths of animals that were associated with the test item occurred. Liquid feces were noted for most rats that were treated with FOS and for animals in the mid- and high-dose 2'-FL groups. No treatment-related abnormalities were observed in food intakes, body weights, organ weights, clinical chemistry, urinalysis, and macroscopic or histological observations. The authors determined a NOAEL of 5,000 mg/kg bw/day for 2’-FL produced by fermentation.

Studies by Coulet et al. (2014)

This study was presented in GRN 546 (pages 29 to 32 - FDA, 2015a) and also reviewed in GRN 571 (stamped pages 45 to 47 – FDA, 2015b), GRN 650 (stamped pages 37 to 39 - FDA, 2016a), GRN 735 (pages 50 to 52 -FDA, 2018a), and GRN 749 (pages 32 and 36 – FDA, 2018b).

In a 14-day oral tolerability and dose-range finding study, 2’-FL produced by chemical synthesis (source – Glycom’s synthetic 2’-FL; >99% purity on a dry weight basis) was administered by gavage to 7-day-old Wistar IGS:Crl:WI (Han) rats (n = 5/sex/group) at doses of 0, 2,000, 5,000, or 7,500 mg/kg bw/day (GRN 546, FDA, 2015a; Coulet et al., 2014). A reference control group was administered at 7,500 mg oligofructose (OF) per kg bw per day during the 14-day study. Observations included food intake, general health, clinical signs, mortality, and morbidity. All animals in the 5,000 and 7,500 mg per kg bw per day groups and in the OF control group had lower body weight gains between days 0 to 3 as compared with the vehicle control group. The authors concluded that the highest suitable dose of 2'-FL for the 90-day study that followed was lower than 7,500 mg per kg body weight per day and, therefore, set a high dose of 6,000 mg per kg per body per day in the subchronic toxicity study that followed.

Subsequently, a 90-day subchronic oral toxicity study of 2'-FL with a 4-week recovery period was conducted starting with 7-day-old Wistar [Crl:WI(Han)] rats (Coulet et al., 2014). 2’-FL was administered via gavage in a juvenile adapted sub-chronic rat study at dose levels of 0, 2,000, 5,000, or 6,000 mg/kg bw/day. Fructooligosaccharide (FOS) was used as a reference high-dose control at 6,000 mg/kg bw/day. No treatment-related adverse effected were noted. The exception was that one male and one female rat in the 6,000 mg/kg bw/day 2'-FL dose group, and two males and one female in the 6,000 mg/kg bw/day FOS dose group died during the treatment period. One female in the 6,000 mg/kg bw/day FOS group died during the recovery period. Since the deaths of two animals in the 6,000 mg mg/kg bw/day dose group could not be excluded, the authors concluded that the NOAEL for 2'-FL was 5,000 mg/kg bw/day in Wistar [Crl:WI(Han)] rats. Oral administration up to 5,000 mg/kg bw/day to rats over 90 days was not associated with any adverse effects based on clinical observations, body weight gain, food consumption, ophthalmoscopy, clinical pathology, organ weights, and histopathology findings.

A Piglet Study by Hanlon and Thorsrud (2014); the same study was summarized in GRN 571 (stamped pages 43 and 44 - FDA, 2015b), GRN 735 (pages 52 to 53 - FDA,

2018a), GRN 650 (stamped pages 39 to 40 - FDA, 2016a), and GRN 749 (page 35 - FDA, 2018b).

In a piglet study, 2'-FL produced by fermentation using *E. coli* (source: Jennewein Biotechnolgies; 94.1% purity) was administered by gavage to neonatal pigs (n=27 male and n=21 female) at concentrations of 0, 200, 500, or 2,000 mg/L for 20 days from day 2 of lactation (Hanlon and Thorsrud, 2014). These levels corresponded to dose levels of 29.4, 72.2, or 292 mg/kg bw/day in males and 29.3, 74.3, or 299 mg/kg bw/day in females, respectively. There were no test article-related effects on growth and development (clinical observations, body weight, and food consumption), clinical pathology parameters (hematology, clinical chemistry, coagulation, and urinalysis), or any histopathologic changes. Therefore, the authors concluded that dietary exposure to 2′-FL at concentrations up to 2,000 mg/L (up to 292 mg/kg bw/day in males and 299 mg/kg bw/day in females) were well tolerated and supported normal growth patterns in neonatal piglets with no adverse effects (Hanlon and Thorsrud, 2014). The same study was summarized in GRN 571, submitted by Jennewein Biotechnologies (FDA, 2015b).

Conclusion of Animal Toxicity Studies

Based on these studies, for purposes of this evaluation, the NOAEL of 7,500 mg/kg bw/day was chosen for APTech's 2'-FL. This value is about 20 times higher than the anticipated exposure in the human newborn infant target population. Additionally, the addition of 2'-FL concentrations of up to 2,000 mg/L (corresponding to up to 292 mg/kg bw/day in males and 299 mg/kg bw/day in females) were well tolerated and supported normal growth patterns in neonatal piglets with no adverse effects. It should be noted that various purified 2'-FL preparations showed similar toxicology profiles regardless of methods of manufacture. For all 2'-FL preparations, the NOAELs were determined to be at least 5,000 mg/kg bw/day in rats, indicating all purified 2'-FL preparations were considered safe.

2'-FL, like other oligosaccharides, belongs to the group which has the lowest toxicity rating. Thus, the unpublished status of the APTech's toxicity studies has no impact on the overall conclusion of this GRAS determination if qualified experts do not have access to such data and information.

6.E. Animal Efficacy Studies

Since the FDA's last review of 2'-FL (GRN 650, stamped pages 40 to 41 - FDA, 2016a; GRN 735, pages 59 to 60 - FDA 2018a; GRN 749, pages A110 to A113 - FDA, 2018b), two animal efficacy studies were published. Although animal efficacy studies were designed to investigate the efficacy of 2'-FL on various health parameters, several safety-related endpoints were obtained during the experiments. Therefore, these studies are reviewed below as additional supporting information. These efficacy studies showed that 2'-FL did not cause any adverse effects on immune responses (van den Elsen et al., 2019; Xiao et al., 2018). The results are summarized in Table 17. None of these studies reported adverse effects of 2'-FL on measured outcomes.

Studies First Reviewed in This GRAS Determination

Since the FDA’s last review of 2’-FL in April 2018, two animal study were identified reporting the repeat dose administration of 2’-FL at high dietary concentrations (Table 17). Any studies using modified genes or chemically- or biologically-induced disease models were not included in this review since the data from these induced disease conditions may not be relevant when evaluating the safety of 2’-FL. Newly published study did not report results inconsistent with the FDA’s prior reviews of 2014-2018.

Xiao et al. (2018) determined the effect of 2’-FL on vaccination responsiveness (both innate and adaptive) in a murine influenza vaccination model and elucidated the mechanisms involved. A dose range of 0.25-5.0% (w/w) dietary 2’-FL was provided to 6-week-old female C57BL/6JOlaHsd mice 2 weeks prior to primary and booster vaccination until the end of the experiment. Intradermal (i.d.) challenge was performed to measure the vaccine-specific delayed-type hypersensitivity. Measurements included vaccine specific cellular and humoral response (serum vaccine-specific IgG1 and IgG2a), B-cell activation and frequency, activated splenic dendritic cells and mesenteric lymph nodes, Th1 and Tregs frequency in spleen, and vaccine-specific CD4+ and CD8+ T-cell proliferation. No adverse effects were reported on measured outcomes.

In a study by van den Elsen et al. (2019), mice were supplemented with a mixture of 2'-FL (>90% purity, produced by bacterial fermentation; source - Friesland Campina), short-chain galacto-oligosaccharides (scGOS), and long-chain fructo-oligosaccharides (lcFOS) from different stages in early life. BALB/c breeding pairs were fed either control diet (AIN93G) from the day of timed mating or a prebiotic diet, AIN93G containing the prebiotic mixture, 2’-FL/GOS/FOS (the ratio of each prebiotic, not specified). To make up the prebiotic diet, 2% (w/w) of carbohydrates present in the control diet were replaced with the 2’-FL/GOS/FOS mixture. A third of the breeding pairs that received the control diet from the day of mating, were switched to the prebiotic diet within 24 h after birth and after weaning their litters were maintained on the prebiotic diet throughout the course of the experiment. The litters from another third of the control breeding pairs were provided the prebiotic diet at weaning and maintained on this diet throughout the experiment. At 6 weeks of age, male and female offspring of all 4 dietary groups were immunized subcutaneously with a fifth of the human adult dose of trivalent influenza vaccine (TIV). Both development of the gut microbiota and antibody-mediated vaccine responses were followed over time. No adverse effects of the prebiotic mixture were observed on measured outcomes.

Conclusions from Animal Efficacy Studies

Doses up to 5% dietary 2’-FL were well tolerated in mice with no adverse effects.

Table 17. Animal Efficacy Studies of 2'-FL Published since October 2017

Objective	Animal	Dose	Duration	Measurements	Reference
The Studies Reviewed in this GRAS Notice					
To determine the effect of 2'-FL on vaccination responsiveness (both innate and adaptive) in a murine influenza vaccination model	C57BL/6JOlaHsd (7 wk old; N= 9/group), vaccinated with inactivated influenza virus vaccine	Control, 0.25, 0.5, 1, 2.5, or 5% 2'-FL in diet; 2'-FL source-SSNIFF Spezialdiäten; purity >90%	31 d	Vaccine specific cellular and humoral response (serum vaccine-specific IgG1 and IgG2a); B-cell activation and Frequency; activated splenic dendritic cells and mesenteric lymph nodes; Th1 and Tregs frequency in spleen; vaccine-specific CD4+ and CD8+ T-cell proliferation	Xiao et al., 2018
To determine the effect of 2'-FL on the gut microbiota and antibody-mediated vaccine responses	BALB/c mice, vaccinated with trivalent influenza vaccine	AIN 93G control diet or a mixture of 2'-FL/short-chain GOS/ and long-chain FOS, 2% (w/w) of the diet	Different stages in early life	Development of the gut microbiota and antibody-mediated vaccine responses	van den Elsen et al., 2019

N= number of animals per group; FOS= fructo-oligosaccharide; GOS= galacto-oligosaccharide.

6.F. Human Clinical Studies

Since the FDA's last review of 2'-FL (GRN 546; GRN 571; GRN 650, stamped pages 43 to 47 - FDA, 2016a; GRN 735, pages 60 to 63 - FDA 2018a; GRN 749, pages 37 to 39 - FDA, 2018b), one new human study was published (Storm et al., 2019). Since the specifications and composition for APTech's 2'-FL in this notice are substantially equivalent to those described in previous GRAS notices, the safety data and discussion presented in previous GRAS notices are also applicable to the safety of APTech's 2'-FL. This information is hereby incorporated, by reference, in this document and will not be discussed in detail. For these 'pivotal' studies, the levels of consumption represent the maximum dose consumed rather than absolute safety endpoints.

Human Study First Reviewed in This GRAS Determination

Storm et al. (2019) evaluated the feeding tolerance of 2'-FL (0.25 g/L) in a 100% whey, partially hydrolyzed infant formula (0.67 kcal/mL and 2.2 g protein/L) with the probiotic *Bifidobacterium animalis* ssp. *lactis* strain Bb12 (*B. lactis* $1x10^6$ CFU/g powder; test) as compared with the same formula without 2'-FL (control) in healthy, full-term infants enrolled at 2 weeks of age (±5 days). After 6 weeks of feeding the assigned formula, safety parameters were assessed including tolerance (Infant Gastrointestinal Symptom Questionnaire), stooling, vomiting, spit-up, crying, and fussing. Seventy-nine infants were enrolled and 63 completed the study per protocol (30 test, 33 control). Infant Gastrointestinal Symptom Questionnaire scores were similar between groups (test: 20.9 ± 4.8, control: 20.7 ± 4.3, $P = 0.82$). There were no serious AEs reported in the study. Seventy-two AEs occurred in the study, 36 in the test group and 36 in the control group, corresponding to 17 and 19 subjects in the test and control groups, respectively. Spit-up reported as an adverse event was of interest due to the finding that there were more subjects with spit-up noted as "frequent" in the test group compared with the control group; however, only one subject in each group reported "mild" spit-up as an AE, and no subjects had reports of more extreme spitting up. Partially hydrolyzed infant formula with 2'-FL and *B. lactis* is tolerated well, as confirmed by a validated multi-symptom index.

Previous GRAS Notices Summarized the Following Studies

As shown in Table 18, infant studies evaluated the effects of 2'-FL on various measurement outcomes including growth and tolerance (Marriage et al., 2015; Puccio et al., 2017), global average microbial composition profile (Steenhout et al., 2016), and markers of immune function (Goehring et al., 2016). Healthy infants received daily dose of up to 1.0 g/L 2'-FL (Marriage et al., 2015; Goehring et al., 2016; Puccio et al., 2017; Steenhout et al., 2016) for up to 6 months. No adverse effects of 2'-FL were reported on the measured outcomes listed above.

Human studies in adults evaluated the effect of 2'-FL on safety including gastrointestinal symptoms, clinical chemistry, hematology, and gut microbiota (Table 19; Elison et al., 2016). Healthy adults received 2'-FL or lacto-*N*-neotetraose (LNnT) doses up to 20 g/d, either alone or in combination for up to 2 weeks. Hematological and blood biochemistry analyses obtained at the 2-week time-point remained within the normal

range for all subjects, and any minor changes over the course of the study compared to the baseline values were not considered clinically relevant. Adverse events reported related mainly to gastrointestinal symptoms, particularly gas/flatulence, and were characterized as mild. In this study, compared with the baseline, the changes in Gastrointestinal Symptom Rating Scale scores within an intervention group were generally not significant, with a few exceptions: volunteers taking the high 20 g dose of 2′-FL reported increased bloating and passing of gas, increased rumbling, increased nausea, diarrhea, loose stools and urgency to pass stools. Despite statistical significance, mean scores remained low (mean score <3; mild discomfort or below). Consumption of dietary fibers in large amounts often associated with gastrointestinal discomforts. The IOM has recognized that consumption of high doses of dietary fiber ingredients causes gastrointestinal discomfort. However, the IOM (2002) has not established Tolerable Upper Intake levels (UL) for fibers since most of the symptoms are usually transient and do lead to serious chronic health concerns. The IOM states as follows: ‘While occasional adverse gastrointestinal symptoms are observed when consuming one of the above isolated or synthetic fibers, serious chronic adverse effects have not been observed. Furthermore, due to the bulky nature of fibers, excess consumption is likely to be self-limiting. Therefore, a UP was not set for these individual fibers.”

Summary of Human Clinical Studies

Purified 2’-FL preparations, regardless of method of manufacture, were proven safe in both infants and adults: formula supplemented with 1.0 g/L 2’-FL and up to 10 g/day were well tolerated in infants and adults, respectively.

Table 18. Summary of Infant Studies of 2′-FL

Subject	Dose	Duration	Measurements	Reference
Studies with Infants First Reviewed in This GRAS Notice				
79 healthy, full-term infants (N=39-40)	0 or 0.25 g 2'-FL/L (2'-FL source-NA, purity-NA)	6 wk	Tolerance (Infant Gastrointestinal Symptom Questionnaire), stooling, vomiting, spit-up, crying, and fussing	Storm et al., 2019
Studies with Infants Reviewed in Previous GRNs				
175 healthy infants (mean age 7.7-8.6 d; birth wt, 2,500-4,500 g; N= 87-88)	2 groups: Control formula; or formula supplemented with 1.0 g/L 2'-FL + 0.5 g/L LNnT (2'-FL source, Glycom, purity-NA)	Test and control formula, from day 1-14 to 6 mo of life; Follow-up formula with no HMOs from 6 to 12 mo; P	Growth (anthropometric measures); formula intake and digestive tolerance; stool characteristics, behavioral patterns (restlessness, colic, nighttime awakening; morbidity (parent-reported adverse events, concomitant medications)	Puccio et al., 2017
161 healthy term infants (N= 38-65)	3 groups: Control formula; test (formula + 1.0 g/L 2'-FL + 0.5 g/L LNnT); or breast-fed (2'-FL source-NA)	From day 0-14 to 3 mo of age	Global average microbial composition profile	Steenhout et al., 2016 (abstract)
420 healthy, full-term infants (mean age 3.4-3.8 d; N= 101-109)	3 groups: 2.4 g/L GOS control (CF); 2.2 g/L GOS + 0.2 g/L 2'-FL; 1.4 g/L GOS + 1.0 g/L 2'-FL; or breast-fed reference group (2'-FL source-NA, probably fermentation, purity-NA)	From day 0-5 to 119 d of life; P	Growth (weight, length, head circumference); gastrointestinal tolerance (stool frequency, consistency, and color); 2'-FL absorption and excretion	Marriage et al., 2015
201 healthy term infants (mean age 3.4-3.8 mo; N=101-111; N for blood analysis = 37-42		From day 5 to 4 mo of life; P	Blood sample collected at 6 wk of age from the cohort of Marriage et al. (2015) - Inflammatory cytokine profiles in plasma and PBMCs; immune cell proliferation; circulating lymphocyte populations	Goehring et al., 2016

LNnT= lacto-N-neotetraose; N= the number of subjects in each group; NA= not available; PBMCs=peripheral blood mononuclear cells.

Table 19. Summary of Human Adult Clinical Studies of 2′-FL Reviewed in Previous GRNs

Subject	Dose	Duration	Measurements	Reference
Studies with Adults				
Healthy adults (mean age 29.3-39.9 y; N= 10)	10 groups: Placebo (2 g/d glucose); 2'-FL (source-Glycom chemically produced; 99.9% purity, dw basis), LNnT, or 2'-FL + LNnT (2:1 mass ratio) at 5, 10, or 20 g/d	2 wk; P	Safety and tolerance (Gastrointestinal Symptom Rating Scale; clinical biochemistry and hematology); fecal microbiota and bacterial metabolites	Elison et al., 2016

dw= dry weight; LNnT=lacto-N-neotetraose; N= the number of subjects in each group.

6.G. Other Considerations for Children and Adults

HMOs including 2'-FL (degree of polymerization [DP] unit of 3) are considered as dietary fiber or total fiber. While establishing adequate intake values for total fiber, Food and Nutrition Board, the Institute of Medicine, has recognized that dietary fiber improves laxation (i.e., promotes intestinal regularity), reduces risk of coronary heart disease, and assists in maintaining normal blood glucose levels. The IOM (2002) states as follows: "Recommended intake level for Total Fiber based on prevention of CHD should be sufficient to reduce constipation in most normal people given adequacy of hydration of the large bowel," and

"There is evidence on risk of reduction of diabetes as a secondary endpoint to support a recommended intake level for Total Fiber that is primarily based on prevention of CHD."

The Adequate Intake (AI) values for fiber range from 19 to 25 g/day for children aged 1 to 8 years, 26 to 38 g/day for children and adolescents aged 9 to 18 years, and 21 to 38 g/day for adults 19 years or older (IOM, 2005). Recently, US FDA has raised the daily value of dietary fiber from 25 to 28 g to encourage Americans to consume more fiber-rich foods (FDA, 2016c). However, intakes of total fiber in the United States (US) were low enough to be of public health concern. Total fiber was identified as a nutrient of concern by the 2015 Guidelines for Americans (USDA, 2015). Most children, adolescents, and adults do not consume the recommended amount of total dietary fiber. Average Americans consume only one half of the recommended intakes: mean fiber intake for children/adolescents and adults, over 19 years, were 13.2 and 16.1 g/day, respectively (McGill et al., 2015). Reicks et al. (2014) also reported that the mean fiber intake for American children/adolescents aged 2 to 18 y was 13.6 g/day and for adults, 19+ years, was 17.0 g/day based on the National Health and Nutrition Examination Survey (NHANES) 2009–2010 dataset. Addition of 2'-FL to diet may help improve the dietary intake status in Americans.

6. H. Safety of Production Microorganism

Safety of Production Microorganism

Corynebacterium glutamicum has been safety used in the industrial production of an amino acid, such as L-leucine (GRN 523 – FDA, 2014), and a carbohydrate, such as D-psicose (GRN 400 - FDA, 2012; GRN 693 - FDA, 2017).

Safety of Introduced Proteins

As recommended by FAO/WHO (2001), the allergenic potential for introduced proteins, was screened using the database, http://allergenonline.org/databasefasta.shtml (March 23, 2018 version).

None of introduced proteins (GDP-L-fucose synthase [WcaG], GDP-D-mannose 4,6-dehydratase [Gmd], lactose permease [LacY], and fucosyltransferase [FT]) have homology in amino acid sequences with those of allergenic proteins. Details are presented in Appendix G.

6.I. Safety Determination

The following safety evaluation fully considers the composition, intake, and microbiological and toxicological properties of 2'-FL as well as appropriate corroborative data.

1. Analytical data from multiple lots indicate that APTech's 2′-FL ingredient comply reliably with the established specifications and meet all applicable purity standards.

2. The intended use and use levels of 2'-FL are the same as those described in GRN 735, except in medical food application which has been withdrawn from the original submission. APTech's 2′-FL is intended to be used as an ingredient in whey-, milk- and soy-based, non-exempt infant formulas for term infants and in toddler formulas at a maximum level of 2.4 g/L of formula consumed; infant and toddler foods at levels of 0.24 -1.2 g/serving; and in the following food categories at levels of 0.28 - 1.2 g/serving: beverages and beverage bases; breakfast cereals; dairy product analogs; frozen dairy desserts and mixes; gelatins, puddings, and fillings; grain products and pastas; jams and jellies; milk and milk products; processed fruits and fruit juices; and sweet sauces, toppings, and syrups. The addition of 2′-FL to term infant formulas is consistent with efforts to produce infant formula that closely matches the nutrient composition of human milk.

3. Since the intended use and use levels of 2'-FL will be the same as outlined in GRN 735, APTech notes that its uses will not result in any exposure beyond what was previously estimated in GRN 735. From the use of 2'-FL in only infant formula (2.4 g/L of reconstituted formula), in all-user infants aged 0 to 11.9 months old, the estimated mean and 90th percentile intakes of 2'-FL were determined to be at 1.87 and 2.78 g/person/day, respectively. On a body weight basis, these intakes were determined to be 258.7 and 431.3 mg/kg bw/day, respectively.

4. Under the intended use of 2'-FL from the use of infant formula and other foods, the mean and 90th percentile EDIs of 2'-FL in all-users of all ages were determined to be 1.70 and 3.54 g/person/day, respectively. On a body weight basis, the mean and 90th percentile EDIs were determined to be 36 and 80 mg/kg bw/day, respectively, in all-users. The highest intake was observed to occur in male teenagers with the highest 90th percentile intake at 4.29 g/person/day. Of all-users, infants aged 0 to 5.9 months were estimated to have the highest mean and 90th percentile EDIs of 315 and 532 mg/kg bw/day, respectively. These EDIs are within safe intake levels (details are described in Part 6). More importantly, the intended use and use levels of 2'-FL will be the same as outlined in GRN 735, except in medical food application which was withdrawn from the original submission. Consequently, APTech notes that its uses will not result in any

exposure beyond what was previously estimated in GRN 735. Since APTech's 2'-FL will replace other 2'-FL ingredients in the marketplace, an increase in cumulative intake is not expected.

5. Since the specifications and composition for APTech's 2′-FL in this notice are essentially identical to those described in previous GRAS notices, the safety data and discussion in these GRAS notices are also applicable to the safety of APTech's 2′-FL. Various purified 2'-FL preparations showed similar toxicology profiles regardless of methods of manufacture. For all 2'-FL preparations, the NOAELs were determined to be over 5,000 mg/kg bw/day in rats, indicating all purified 2'-FL preparations were considered safe. In particular, the NOAEL of APTech's 2'-FL was considered to be 7,500 mg/kg bw/day in both male and female rats after repeated oral administration for 90 days under the conditions of this study. The addition of 2'-FL at doses up to 2,000 mg/L was well tolerated and supported normal growth patterns in neonatal piglets.

6. APTech's 2'-FL is chemically and structurally identical to the 2'-FL which is found in human milk, and therefore, the safety of APTech's 2'-FL for all intended uses is supported by the known consumption of 2'-FL from human breast milk in infants.

7. Purified 2'-FLs, regardless of method of manufacture, were proven safe in both infants and adults: formula supplemented with 1.0 g/L 2'-FL and up to 10 g/day were well tolerated in infants and adults, respectively.

8. HMOs, including 2'-FL (degree of polymerization [DP] unit of 3), are considered as dietary fibers or total fibers. Average Americans consume only one half of the recommended intakes. Total fiber was identified as a nutrient of concern by the 2015 Guidelines for Americans (USDA, 2015). Addition of 2'-FL to diet may help improve the dietary intake status in Americans.

6.J. Conclusions and General Recognition on the Safety of 2'-FL

6.J.1. Common Knowledge Element of the GRAS Determination

2′-FL is a naturally occurring trisaccharide found in human milk, and is therefore typically referred to as a human milk oligosaccharide (HMO). The presence of HMOs in breast milk has been associated with a variety of nutritional effects including the establishment and maintenance of healthy intestinal bacterial microflora. The addition of 2'-FL to term infant formulas is consistent with efforts to produce infant formula that closely matches the nutrient composition of human milk. APTech's 2'-FL is chemically and structurally identical to that which is found in human milk, and therefore, the safety of APTech's 2′-FL for all intended uses is supported by the known consumption of 2′-FL from human breast milk in infants. Additionally, in all the studies summarized in these GRAS determinations, there were no significant adverse effects/events or tolerance issues attributable to 2'-FL in both adults and infants. Because this safety evaluation was based on generally available and widely accepted data and information, it satisfies the so-called "common knowledge" element of a GRAS determination.

6.J.2. Technical Element of the GRAS Determination (Safety Determination)

In addition, the intended uses of 2'-FL have been determined to be safe though scientific procedures as set forth in 21 CFR 170.3(b), thus satisfying the so-called "technical" element of the GRAS determination. The 2'-FL that is the subject of this GRAS determination is produced by genetically engineered, non-toxigenic *Corynebacterium glutamicum*, and its purity is over 94%. The 2'-FL is manufactured consistent with cGMP for food (21 CFR Part 110 and Part 117 Subpart B). The raw materials and processing aids used in the manufacturing process are food grade and/or commonly used in food manufacturing processes.

The literature search did not identify safety or toxicity concerns related to 2'-FL. Toxicity studies of APTech's 2'-FL include acute and subchronic toxicity in rats, subacute toxicity in piglets, and a battery of mutagenicity and genotoxicity studies. The NOAEL of APTech's 2′-FL was determined to be 7,500 mg/kg bw/day, the highest dose tested. Thus, 2′-FL, like other non-digestible oligosaccharides or carbohydrates, belongs to the group which has the lowest toxicity rating. The addition of 2'-FL at the dose of up to 2,000 mg/L was well tolerated and supported normal growth patterns in neonatal piglets. The literature also contains a wealth of publicly available studies on the safety of 2'-FL in infants and other human age groups. APTech's 2'-FL is chemically and structurally identical to the 2'-FL which is found in human milk, and therefore, the safety of APTech's 2'-FL for all intended uses is supported by the known consumption of 2'-FL from human breast milk in infants. Purified 2'-FLs, regardless of method of manufacture, were proven safe in both infants and adults. This evidence is sufficient to support the safety and GRAS status of the proposed use of 2'-FL in these infants and other human populations.

We have concluded that APTech's 2'-FL is GRAS under the intended conditions of use on the basis of scientific procedures, and other experts qualified to assess the safety of food ingredients would concur with these conclusions. Therefore, it is excluded

from the definition of a food additive and may be marketed and sold for its intended purpose in the US without the promulgation of a food additive regulation under Title 21 of the CFR.

We have reviewed the available data and information and are not aware of any data and information that are, or may appear to be, inconsistent with our conclusion of GRAS status.

PART 7. REFERENCES

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7.B. References that are Not Generally Available

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Biotoxtech. 2019c. In vitro micronucleus test of 2’-fucosyllactose in ICR mice. Study No. B18676.

Biotoxtech. 2019d. Single oral dose toxicity study of 2’-fucosyllactose in juvenile Sprague-Dawley rats Study No. B18672.

Biotoxtech. 2019e. Ninety-day repeated oral dose toxicity study with a four - week recovery period of 2’-fucosyllactose in juvenile Sprague-Dawley rats. Study No. B18673.

APPENDIX A.
Particle Size Analysis of 2'-FL

2019.02.08 10:26:30

LA-950 Laser Scattering Particle Size Distribution Analyzer

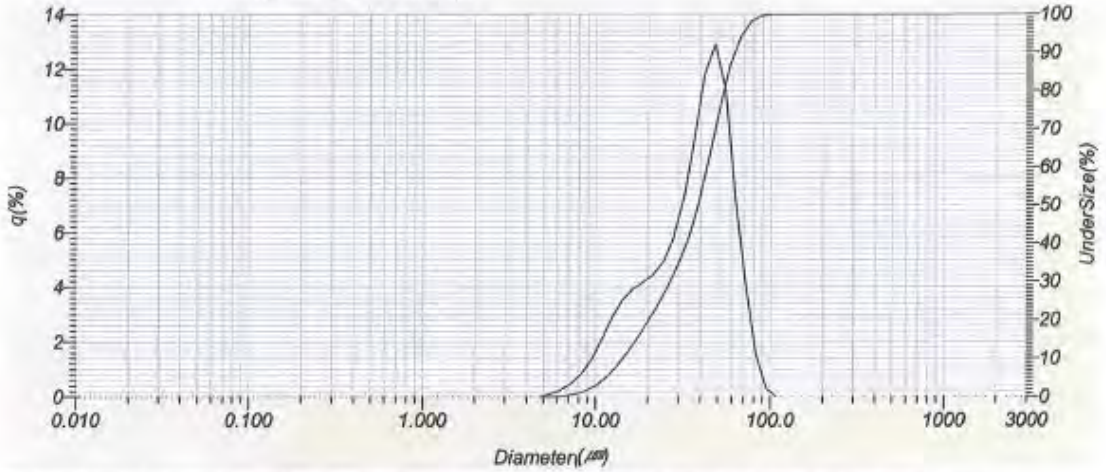
Horiba LA950 for Windows IWet Ver5.20

Sample Name : 2'-FL-CG-011
Data Name : 20190208102662 2

Transmittance(R)	: 98.4(%	Median Size	: 38.57700(μm)
Transmittance(B)	: 95.8(%	Mean Size	: 38.48777(μm)
Circulation Speed	: 8	Std.Dev.	: 17.9787(μm)
Agitation Speed	: 8	Mode Size	: 47.9573(μm)
Distribution Base	: Volume		

Refractive Index (R) : 에이피테크놀로지[AP(1.520 - 0.000i),Ethanol(1.360)]
Refractive Index (B) : 에이피테크놀로지[AP(1.520 - 0.000i),Ethanol(1.360)]

Diameter on Cumulative % : (2)10.00 (%)- 14.3886(μm)
: (6)50.00 (%)- 38.5770(μm)
: (8)90.00 (%)- 62.3035(μm)
: (10)100.0 (%)- 101.4157(μm)



No.	Diameter(μm)	q(%)	UnderSize(%)	No.	Diameter(μm)	q(%)	UnderSize(%)	No.	Diameter(μm)	q(%)	UnderSize(%)	No.	Diameter(μm)	q(%)	UnderSize(%)
1	0.011	0.000	0.000	26	0.339	0.000	0.000	51	10.097	1.334	2.981	76	300.518	0.000	100.000
2	0.013	0.000	0.000	27	0.389	0.000	0.000	52	11.565	2.060	5.041	77	344.206	0.000	100.000
3	0.015	0.000	0.000	28	0.445	0.000	0.000	53	13.246	2.836	7.877	78	394.244	0.000	100.000
4	0.017	0.000	0.000	29	0.510	0.000	0.000	54	15.172	3.484	11.361	79	451.556	0.000	100.000
5	0.020	0.000	0.000	30	0.584	0.000	0.000	55	17.377	3.903	15.264	80	517.200	0.000	100.000
6	0.022	0.000	0.000	31	0.669	0.000	0.000	56	19.904	4.160	19.424	81	592.387	0.000	100.000
7	0.026	0.000	0.000	32	0.766	0.000	0.000	57	22.797	4.426	23.850	82	678.504	0.000	100.000
8	0.029	0.000	0.000	33	0.877	0.000	0.000	58	26.111	4.894	28.744	83	777.141	0.000	100.000
9	0.034	0.000	0.000	34	1.005	0.000	0.000	59	29.907	5.760	34.504	84	890.116	0.000	100.000
10	0.039	0.000	0.000	35	1.151	0.000	0.000	60	34.255	7.232	41.736	85	1019.515	0.000	100.000
11	0.044	0.000	0.000	36	1.318	0.000	0.000	61	39.234	9.438	51.175	86	1167.725	0.000	100.000
12	0.051	0.000	0.000	37	1.510	0.000	0.000	62	44.938	11.791	62.966	87	1337.481	0.000	100.000
13	0.058	0.000	0.000	38	1.729	0.000	0.000	63	51.471	12.875	75.840	88	1531.914	0.000	100.000
14	0.067	0.000	0.000	39	1.981	0.000	0.000	64	58.953	11.253	87.093	89	1754.613	0.000	100.000
15	0.076	0.000	0.000	40	2.269	0.000	0.000	65	67.523	7.139	94.232	90	2009.687	0.000	100.000
16	0.087	0.000	0.000	41	2.599	0.000	0.000	66	77.339	3.945	98.176	91	2301.841	0.000	100.000
17	0.100	0.000	0.000	42	2.976	0.000	0.000	67	88.583	1.514	99.690	92	2636.467	0.000	100.000
18	0.115	0.000	0.000	43	3.409	0.000	0.000	68	101.460	0.310	100.000	93	3000.000	0.000	100.000
19	0.131	0.000	0.000	44	3.905	0.000	0.000	69	116.210	0.000	100.000				
20	0.150	0.000	0.000	45	4.472	0.000	0.000	70	133.103	0.000	100.000				
21	0.172	0.000	0.000	46	5.122	0.000	0.000	71	152.453	0.000	100.000				
22	0.197	0.000	0.000	47	5.867	0.116	0.116	72	174.616	0.000	100.000				
23	0.226	0.000	0.000	48	6.720	0.244	0.360	73	200.000	0.000	100.000				
24	0.259	0.000	0.000	49	7.697	0.468	0.828	74	229.075	0.000	100.000				
25	0.296	0.000	0.000	50	8.816	0.820	1.648	75	262.376	0.000	100.000				

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2019.02.14 10:05:27

LA-950 Laser Scattering Particle Size Distribution Analyzer

Horiba LA950 for Windows IWet Ver5.20

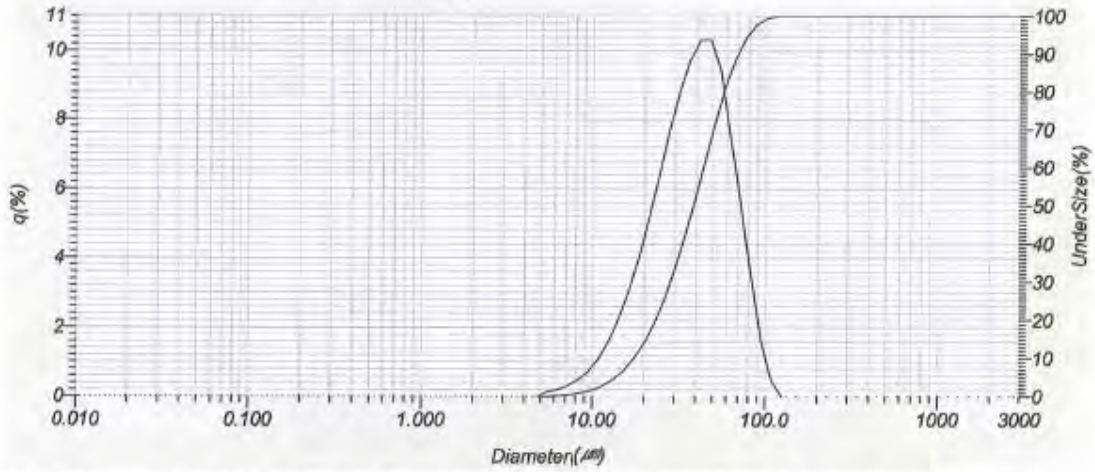
Sample Name : 2'-FL-CG-012
Data Name : 201902141005647

Transmittance(R) : 91.7(%
Transmittance(B) : 92.6(%
Circulation Speed : 8
Agitation Speed : 8
Distribution Base : Volume

Median Size : 38.49589(μm)
Mean Size : 41.29750(μm)
Std.Dev. : 19.9879(μm)
Mode Size : 47.8149(μm)

Refractive Index (R) : 에이피테크놀로지[AP(1.520 - 0.000i),Ethanol(1.360)]
Refractive Index (B) : 에이피테크놀로지[AP(1.520 - 0.000i),Ethanol(1.360)]

Diameter on Cumulative % : (2)10.00 (%)- 17.5746(μm)
(6)50.00 (%)- 38.4959(μm)
(8)90.00 (%)- 69.3470(μm)
(10)100.0 (%)- 116.1774(μm)



No.	Diameter(μm)	q(%)	UnderSize(%)	No.	Diameter(μm)	q(%)	UnderSize(%)	No.	Diameter(μm)	q(%)	UnderSize(%)	No.	Diameter(μm)	q(%)	UnderSize(%)
1	0.011	0.000	0.000	26	0.339	0.000	0.000	51	10.097	0.702	1.834	76	300.518	0.000	100.000
2	0.013	0.000	0.000	27	0.389	0.000	0.000	52	11.565	1.051	2.885	77	344.206	0.000	100.000
3	0.015	0.000	0.000	28	0.445	0.000	0.000	53	13.246	1.548	4.434	78	394.244	0.000	100.000
4	0.017	0.000	0.000	29	0.510	0.000	0.000	54	15.172	2.208	6.641	79	451.556	0.000	100.000
5	0.020	0.000	0.000	30	0.584	0.000	0.000	55	17.377	3.026	9.667	80	517.200	0.000	100.000
6	0.022	0.000	0.000	31	0.669	0.000	0.000	56	19.904	4.000	13.667	81	592.387	0.000	100.000
7	0.026	0.000	0.000	32	0.766	0.000	0.000	57	22.797	5.128	18.795	82	678.504	0.000	100.000
8	0.029	0.000	0.000	33	0.877	0.000	0.000	58	26.111	6.377	25.172	83	777.161	0.000	100.000
9	0.034	0.000	0.000	34	1.005	0.000	0.000	59	29.907	7.652	32.824	84	890.116	0.000	100.000
10	0.039	0.000	0.000	35	1.151	0.000	0.000	60	34.255	8.809	41.634	85	1019.515	0.000	100.000
11	0.044	0.000	0.000	36	1.318	0.000	0.000	61	39.234	9.728	51.362	86	1167.725	0.000	100.000
12	0.051	0.000	0.000	37	1.510	0.000	0.000	62	44.938	10.312	61.674	87	1337.481	0.000	100.000
13	0.058	0.000	0.000	38	1.729	0.000	0.000	63	51.471	10.322	71.996	88	1531.914	0.000	100.000
14	0.067	0.000	0.000	39	1.981	0.000	0.000	64	58.953	9.428	81.424	89	1754.613	0.000	100.000
15	0.076	0.000	0.000	40	2.269	0.000	0.000	65	67.523	7.485	88.909	90	2009.687	0.000	100.000
16	0.087	0.000	0.000	41	2.599	0.000	0.000	66	77.339	5.555	94.464	91	2301.841	0.000	100.000
17	0.100	0.000	0.000	42	2.976	0.000	0.000	67	88.583	3.463	97.928	92	2636.467	0.000	100.000
18	0.115	0.000	0.000	43	3.409	0.000	0.000	68	101.460	1.583	99.510	93	3000.000	0.000	100.000
19	0.131	0.000	0.000	44	3.905	0.000	0.000	69	116.210	0.490	100.000				
20	0.150	0.000	0.000	45	4.472	0.000	0.000	70	133.103	0.000	100.000				
21	0.172	0.000	0.000	46	5.122	0.000	0.000	71	152.453	0.000	100.000				
22	0.197	0.000	0.000	47	5.867	0.143	0.143	72	174.616	0.000	100.000				
23	0.226	0.000	0.000	48	6.720	0.209	0.352	73	200.000	0.000	100.000				
24	0.259	0.000	0.000	49	7.697	0.312	0.663	74	229.075	0.000	100.000				
25	0.296	0.000	0.000	50	8.816	0.468	1.132	75	262.376	0.000	100.000				

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LA-950 Laser Scattering Particle Size Distribution Analyzer

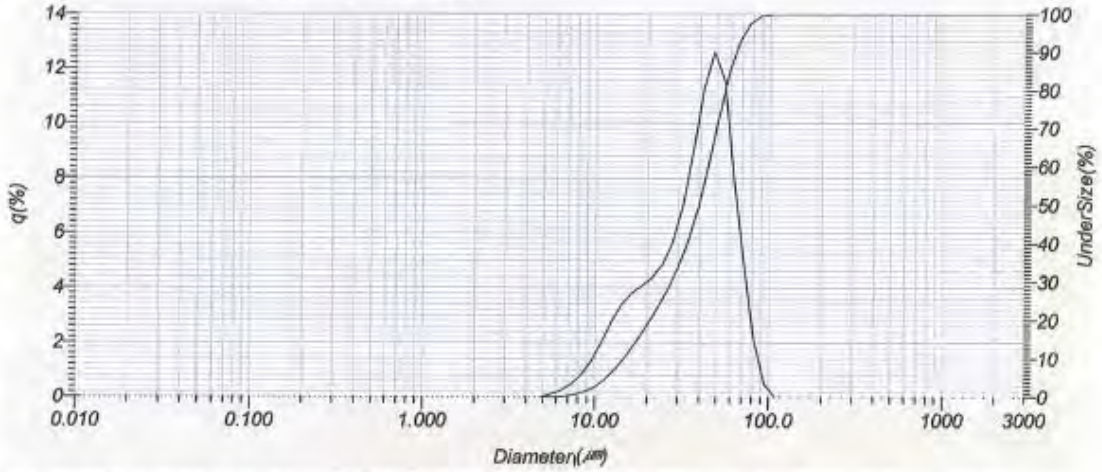
Horiba LA950 for Windows IWet Ver5.20

Sample Name : 2'-FL-CG-013
Data Name : 201902081058628

Transmittance(R)	: 93.6(%	Median Size	: 39.47268(μm)
Transmittance(B)	: 92.6(%	Mean Size	: 39.49289(μm)
Circulation Speed	: 8	Std.Dev.	: 18.6002(μm)
Agitation Speed	: 8	Mode Size	: 48.1712(μm)
Distribution Base	: Volume		

Refractive Index (R) : 에이피테크놀로지[AP(1.520 - 0.000i),Ethanol(1.360)]
Refractive Index (B) : 에이피테크놀로지[AP(1.520 - 0.000i),Ethanol(1.360)]

Diameter on Cumulative % : (2)10.00 (%)- 14.6590(μm)
: (6)50.00 (%)- 39.4727(μm)
: (8)90.00 (%)- 64.3645(μm)
: (10)100.0 (%)- 101.4312(μm)



No.	Diameter(μm)	q(%)	UnderSize(%)	No.	Diameter(μm)	q(%)	UnderSize(%)	No.	Diameter(μm)	q(%)	UnderSize(%)	No.	Diameter(μm)	q(%)	UnderSize(%)
1	0.011	0.000	0.000	26	0.339	0.000	0.000	51	10.097	1.266	2.830	76	300.518	0.000	100.000
2	0.013	0.000	0.000	27	0.389	0.000	0.000	52	11.565	1.959	4.789	77	344.206	0.000	100.000
3	0.015	0.000	0.000	28	0.445	0.000	0.000	53	13.246	2.709	7.498	78	394.244	0.000	100.000
4	0.017	0.000	0.000	29	0.510	0.000	0.000	54	15.172	3.350	10.849	79	451.556	0.000	100.000
5	0.020	0.000	0.000	30	0.584	0.000	0.000	55	17.377	3.787	14.636	80	517.200	0.000	100.000
6	0.022	0.000	0.000	31	0.669	0.000	0.000	56	19.904	4.074	18.709	81	592.387	0.000	100.000
7	0.026	0.000	0.000	32	0.766	0.000	0.000	57	22.797	4.366	23.075	82	678.504	0.000	100.000
8	0.029	0.000	0.000	33	0.877	0.000	0.000	58	26.111	4.833	27.908	83	777.141	0.000	100.000
9	0.034	0.000	0.000	34	1.005	0.000	0.000	59	29.907	5.643	33.551	84	890.116	0.000	100.000
10	0.039	0.000	0.000	35	1.151	0.000	0.000	60	34.255	6.975	40.526	85	1019.515	0.000	100.000
11	0.044	0.000	0.000	36	1.318	0.000	0.000	61	39.234	8.972	49.498	86	1167.725	0.000	100.000
12	0.051	0.000	0.000	37	1.510	0.000	0.000	62	44.938	11.253	60.751	87	1337.481	0.000	100.000
13	0.058	0.000	0.000	38	1.729	0.000	0.000	63	51.471	12.598	73.349	88	1531.914	0.000	100.000
14	0.067	0.000	0.000	39	1.981	0.000	0.000	64	58.953	11.552	84.901	89	1754.613	0.000	100.000
15	0.076	0.000	0.000	40	2.269	0.000	0.000	65	67.523	7.881	92.782	90	2009.687	0.000	100.000
16	0.087	0.000	0.000	41	2.599	0.000	0.000	66	77.339	4.728	97.510	91	2301.841	0.000	100.000
17	0.100	0.000	0.000	42	2.976	0.000	0.000	67	88.583	2.013	99.524	92	2636.467	0.000	100.000
18	0.115	0.000	0.000	43	3.409	0.000	0.000	68	101.460	0.476	100.000	93	3000.000	0.000	100.000
19	0.131	0.000	0.000	44	3.905	0.000	0.000	69	116.210	0.000	100.000				
20	0.150	0.000	0.000	45	4.472	0.000	0.000	70	133.103	0.000	100.000				
21	0.172	0.000	0.000	46	5.122	0.000	0.000	71	152.453	0.000	100.000				
22	0.197	0.000	0.000	47	5.867	0.110	0.110	72	174.616	0.000	100.000				
23	0.226	0.000	0.000	48	6.720	0.232	0.342	73	200.000	0.000	100.000				
24	0.259	0.000	0.000	49	7.697	0.444	0.786	74	229.075	0.000	100.000				
25	0.296	0.000	0.000	50	8.816	0.778	1.564	75	262.376	0.000	100.000				

APPENDIX B

Safety Evaluation of

Corynebacterium glutamicum APC199

Safety evaluation of

Corynebacterium glutamicum

APC199

March 27, 2019

Subin Yeo, Sanghun Oh, Yosep Ji,

Wilhelm H. Holzapfel

Holzapfel Effective Microbes

Introduction

Corynebacterium glutamicum is a Gram-positive, non-pathogenic bacterium in the Phylum Actinobacteria, and is referred to as the industrial workhorse for amino acid production. It was first isolated in the 1950s from Japanese soil during a quest for natural L-glutamate producers (Vertès *et al*., 2012). Since then it has been thoroughly investigated and used as a generally-regarded-as-safe (GRAS) organism in the fermentation industry for more than 50 years. Today it is used for the annual production of 2,160,000 tons of L-glutamate and 1,480,000 tons of L-lysine (Kinoshita *et al*., 1958).

Much research has been done on modifying *C. glutamicum* in various ways to make it more useful for humans. Previously, this bacterium has been mainly used for amino acid production, but more recently the focus has been on gene modification or mutations for improved production of useful amino acids and other metabolites (Schneider *et al*., 2011).

The "Ausschuss für Biologische Arbeitsstoffe" (ABAS) ["Committee for biological agents" under the "Bundesanstalt für Arbeitsschutz und Arbeitsmedizin" ("Federal Institute for Occupational Safety and Health", Berlin), regularly issues and updates "Technical Rules for Biological Agents (TRBA)". With regard to the safety of prokaryotes (bacteria and archaea) their classification system recognizes three risk groups (ABAS, 2018). Bacteria classified in risk group 1 are considered as safe. This group also includes *C. glutamicum*, suggesting that this species is generally considered as a safe biological agent for use in the industry (ABAS, 2018).

The study presented here was conducted on *C. glutamicum* (test strain) with the purpose of providing information on its safety which was deemed necessary for its application in food biotechnology. The studies focused on determination of the detection for major virulence genes and safety issues.

Materials and methods

16S rDNA sequencing

Pure cultures of *C. glutamicum* was grown on BHI agar at 30°C for 24 hours. The plate was sent to Solgent Inc. (Daejun, South Korea) for bi-directional 16S rDNA sequencing. Bi-directional sequencing results were assembled using Codon Code Aligner (Codon Code Corporation, USA) and compared with reference sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/Blastn/).

Hemolysis Test

C. glutamicum was grown at 30°C for 24 hours in BHI broth and then streaked onto 5% sheep blood agar (Hanil Komed) and incubated for 24 hours at 30 °C. Alpha (α) hemolysis was considered as the partial decomposition of the hemoglobin of the red blood cells (but does not represent true hemolysis), beta (β) hemolysis as the complete breakdown of the hemoglobin of the red blood cells observed as a clear zone in the agar plate, while gamma (γ) hemolysis as the lack of hemolysis. *Staphylococcus aureus* ATCC 6538 was used as a positive control.

Biogenic Amine Test

C. glutamicum, grown at 30°C for 24 hours in BHI broth, were streaked out onto special medium with lysine, tyrosine, histamine and ornithine as precursor amino acids according to Bover-Cid and Holzapfel (Bover-Cid & Holzapfel, 1999) and incubated for 48 hours at 30°C. to detect biogenic amine production such as cadaverine, tyramine, histamine and putrescine, respectively. *E. coli* ATCC 25922 was used as a positive control.

Gelatinase test

The basic protocol was followed according to ASM Science Recommendation (Dela Cruz & Torres, 2012). *C. glutamicum* strain, grown at 30°C for 24 hours in BHI broth, was inoculated in a gelatin medium with a loop and incubated at 30°C, for up to 1 week, and checked daily for gelatin liquefaction. Gelatin normally liquefies at 28°C and above, so to confirm that liquefaction was due to gelatinase activity, the tubes are immersed in a refrigerator for 15 to 30 minutes. Afterwards, tubes are tilted to observe if gelatin has been hydrolyzed. Hydrolyzed gelatin will result in a liquified medium even after exposure to cold temperature. *Bacillus cereus* ATCC 11778 was used as a positive control.

Whole genome sequencing

DNA of *C. glutamicum* was extracted using MagListo Genomic DNA Extraction Kit (Qiagen). DNA was extracted after full growth of *C. glutamicum* and then the instructions of the manufacturer were followed. For whole genome sequencing of the extracted DNA, shearing was by using AMPure XP magnetic beads with vortexing (Theragenetex, Korea). The size and quality of purified DNA was evaluated using Nanodrop and Bioanalyzer, and quality assured DNA was annealed on SMRTbell templates (PacBio) and primers for analysis of whole genome sequencing using PacBio RS II system. The raw data was pre-assembled using SMRTpipe HGAP, while further assembling and polishing were performed using SMRTpipe Celera Assembler and SMRTpipe Quiver. Bioinformatics analysis including Genbank annotation was performed using RAST with matching the database of NCBI.

Results

16S rDNA sequencing

After incubating the *C. glutamicum* strain, the results of sequencing 16s rDNA and analyzing it at BlastN are as shown in Figure 1. The colony taken from the cultured bacterium was found to have the same 16s rDNA sequence as the *C. glutamicum* ATCC strain when compared in blastN.

Reference:
Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000),
"A greedy algorithm for aligning DNA sequences", J Comput Biol 2000;
7(1-2):203-14.

Database: 16S Microbial Sequences
20,470 sequences; 29,764,718 total letters

Query= sb1

Length=1370

Sequences producing significant alignments:		Score (Bits)	E Value
NR_041817.1	Corynebacterium glutamicum strain ATCC 13032 16S rib...	2519	0.0
NR_074663.1	Corynebacterium glutamicum strain ATCC 13032 16S rib...	2508	0.0

Figure 1. The 16s rDNA sequencing result of *C. glutamicum.*

In-vitro safety evaluation of hemolysis activity

C. glutamicum showed negative reaction for hemolysis (Table 1).

Table 1. Hemolysis activity of *C. glutamicum* and *S. aureus* ATCC 6538.

Strain	Hemolysis activity
Corynebacterium glutamicum	Gamma
***Staphylococcus aureus* ATCC 6538 (positive control)**	Beta

In-vitro safety evaluation of biogenic amines production

C. glutamicum as shown to be negative for four different biogenic amine productions (histamine, cadaverine, tyramine and putrescine) at 30°C (Table 2).

Table 2. Biogenic amines production activity of *C. glutamicum* and *E. coli* ATCC 25922.

Strain	Histamine	Cadaverine	Tyramine	Putrescine
Corynebacterium glutamicum	Negative	Negative	Negative	Negative
***Escherichia coli* ATCC 25922 (positive control)**	positive	positive	positive	positive

Gelatinase test

C. glutamicum showed negative reaction for the gelatinase test (Table 3).

Table 3. Gelatinase test for *C. glutamicum* strain and *B. cereus* ATCC 11778.

Strain	Gelatinase test
Corynebacterium glutamicum strain	Negative
***Bacillus cereus* ATCC 11778 (positive control)**	Positive

Whole genome sequence information of C. glutamicum test strain

Whole genome sequencing results show *Corynebacterium glutamicum* test strain to contain one circular chromosomal DNA (Table. 4); it was taxonomically identified as *C. glutamicum* test strain according to closest related neighborhood match. We analyzed the whole genome sequence of *C. glutamicum* regarding known major virulence genes of the pathogenic *Bacillus cereus*. Virulence genes include Aggregation substance, Cytolysin, Cytotoxin K, Enterococcal surface protein, Endocarditis antigen, adhesion of collagen, Enterotoxin, Gelatinase, Hemolysin, Hyaluronidase, and Cereulide (Ramarao and Sanchis, 2013; Perin et al., 2014). As we compared the whole genome sequencing results of *C. glutamicum* with *B. cereus* ATCC14579, no toxigenic genes were found in *C. glutamicum* while various toxigenic genes were detected in *B. cereus* ATCC14579 which implies the safety of *C. glutamicum* as well as the absence of genetic toxigenic potencial.

Table 4. Whole genome sequence overview of *C. glutamicum* test strain

Genome	*C. glutamicum*
Taxonomy ID	6666666 (*Corynebacterium glutamicum*)
Domain	Bacteria
Taxonomy	Bacteria; Terrabacteria group; Actinobacteria; Corynebacteriales; Corynebacteriaceae; Corynebacterium; *Corynebacterium glutamicum*
Closest neighbor	*Corynebacterium glutamicum ATCC 13032*
Size (bp)	3,331,472
GC Content in the DNA	53.8 mol% G+C
Number of Contigs	1 circular (one chromosomal DNA)
Number of Coding Sequences	3224
Number of RNAs	78

Table 5. List of major virulence genes in *B. cereus* compared to *C. glutamicum* strain

Potential virulence genes	***C. glutamicum*** strain	***B. cereus* ATCC14579**
Aggregation substance (*asa1*)	**Negative**	**Negative**
Cytolysin (*CylA*)	**Negative**	Positive
Cytotoxin K (*cytK*)	**Negative**	Positive
Enterococcal surface protein (*esp*)	**Negative**	**Negative**
Endocarditis antigen (*efa*A)	**Negative**	**Negative**
Adhesion of collagen (*ace*)	**Negative**	Positive
Enterotoxin	**Negative**	Positive
Non-hemolytic enterotoxin (*nhe*)	**Negative**	Positive
Gelatinase (*coccolysin, gelE*)	**Negative**	**Negative**
Hemolysin (*hbl*)	**Negative**	Positive
Hyaluronidase (*hyl*)	**Negative**	**Negative**
Cereulide (*ces*)	**Negative**	**Negative**

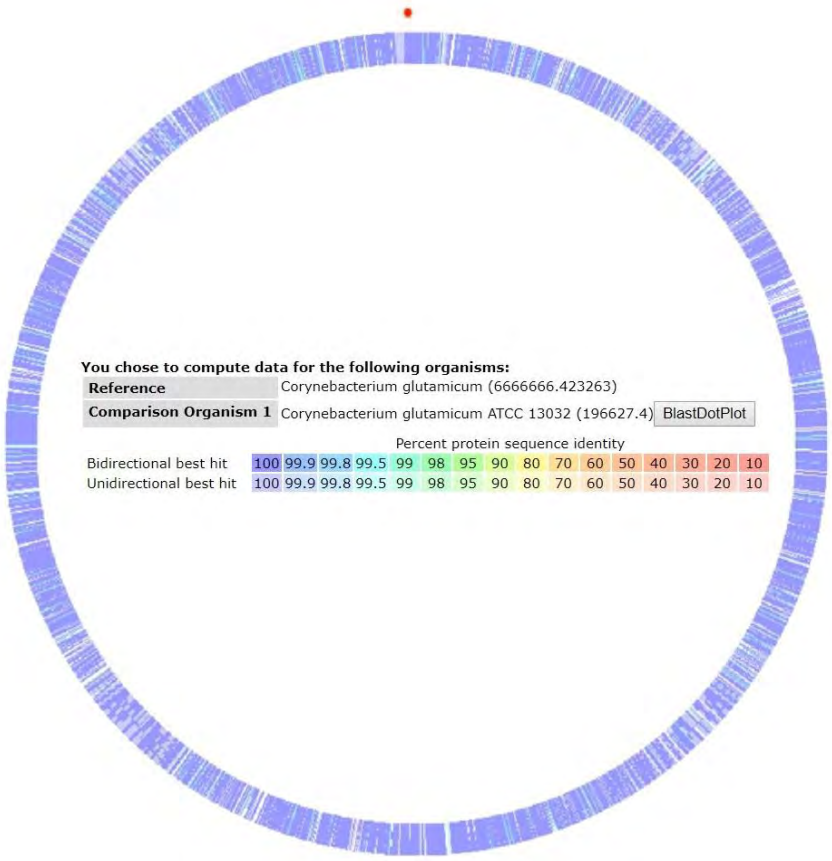


Figure 2. Comparative genomic analysis of *C. glutamicum* test strain and *C. glutamicum* ATCC13032.

Table 6. Comparative genomic analysis of *C. glutamicum* test strain (A) and *C. glutamicum* ATCC13032 (B).

Metric	Value
ANI comparative value of A and B (%)	**99.99 %**
Genome A length (bp)	3,331,472
Genome B length (bp)	3,316,624

***Comparative genomic analysis of C. glutamicum* (test strain) *and C. glutamicum* ATCC 13032**

We have performed comparative genome analysis of *C. glutamicum* (test strain) and *C. glutamicum* ATCC13032 to understand the taxonomic similarity of the two strains. DNA-DNA hybridization (DDH) values have been used by bacterial taxonomists since the 1960s to determine relatedness between strains and are still the most important criterion in the delineation of the bacterial species. Most recently, the average nucleotide identity (ANI), calculated from pair-wise comparisons of all sequences shared between any two strains, has been proposed as the new metrics for bacterial species classification. Goris et al. (2007) reported 95% similarity of calculated ANI based on whole genome sequencing corresponding to 70% of DDH which is considered to be the gold standard value of species delineation. The comparative ANI value of test strain and *C. glutamicum* ATCC13032 was calculated using whole genome sequence ANI calculating algorithm (Yoon et al., 2017) and showed a 99.99% match which proves a strong similarity between these two strains (Figure 2, Table 6). The whole genome sequence of *C. glutamicum* (test strain) has similarities over 99% with that of *C. glutamicum* ATCC13032. The similarities confirm that our test strain is not only a member strain of the species *C. glutamicum*, but it also has a close relationship with an ATCC standard strain that has been widely used and recognized for its safety.

Conclusions

The major objective of this study was to evaluate the safety of *C. glutamicum* APC199 (test strain). First, we confirmed the identity of the strain as *C. glutamicum* according to the National Center for Biotechnology Information (NCBI) database. In the hemolysis test *C. glutamicum* showed gamma activity, meaning that this strain is hemolysis negative and can be regarded as safe based on this test. *C. glutamicum* was also showed to be safe based on the absence of biogenic amine production. In the biogenic amine media, the positive results (purple pigments) of the control strain (*E. coli*) were clearly visible but not of *C. glutamicum*. According to whole genome sequencing information, *C. glutamicum* APC199 was negative for major toxicity genes including aggregation substance (*asa1*), cytolysin (*cylA*), cytotoxin K (*cytK*), enterococcal surface protein (*esp, efaA*), endocarditis antigen (*efaA*), adhesion of collagen (*ace*), enterotoxin (*NHE*), non-hemolytic enterotoxin (*nhe*), gelatinase (coccolysin, *gelE*), hemolysin (*hbl*), hyaluronidase (*hyl*) and cereulide (*ces*) while the positive control, *B. cereus* ATCC14579, was positive for some toxigenic genes. The test strain has antibiotic resistance genes against quinolone and vancomycin. Based on the overall results of the tests, it can be considered as safe by all the other safety analyses.

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Wilhelm H. Holzapfel (Dr. rer. nat.)

Chief Executive Officer of Holzapfel Effective Micr

Chair Professor of Handong Global University

Some affiliations, W. Holzapfel (present and former):

- Since 1996: President of the ICFMH of IUMS (International Committee on Food Microbiology and Hygiene of the international Union of Microbiological Societies)
- Until 2007:
    - Head (Director & Professor) of the Institute of Hygiene and Toxicology of the Federal Research Centre for Nutrition in Karlsruhe, Germany;
    - Hon. Professor for Industrial Microbiology at the Technical University (Karlsruhe Institute of Technology - KIT), in Karlsruhe, Germany;
    - Extraordinary Professor for Microbiology at the University of Stellenbosch, South Africa.
- From 1989 to 2015: Member of the Advisory Council of the German Food and Nutrition Industry (BLL, Berlin).

Appendix C. Certificate Analysis

Certificate of Test

KICET	Certificate No. : 2018-3855	KICET
388, Songnae-daero, Bucheon-si, Gyeonggi-do, Korea (Tel: +82 032 210 5110, Fax: +82 032 210 5115)	Page(1)/(1)Pages	

1. Client
   - ㅇ Company : Advanced Protein Technologies corp., / Chul-soo, Shin
   - ㅇ Address : 7th Floor Gyenggi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City, Gyeonggi-Province
   - ㅇ Date of Receipt : Nov. 23. 2018
2. Use of Report : Reference
3. Test Sample : 2FL-CG-011 Final
4. Date of Test : Nov. 23. 2018 ~ Dec. 13. 2018
5. Test method used : KS C IEC 62321-4(2014), KS C IEC 62321-5(2014), KS I ISO 17294(2014), Instrumental analysis
6. Testing Environment
   - ㅇ Temperature : (21 ± 3)℃ , Humidity : (30 ± 5)% R.H.
7. Test Results

Sample name	Item	Results	Test Method	Remark
2FL-CG-011 Final	As (mg/kg) Pb (mg/kg) Cd (mg/kg) Hg (mg/kg)	< 0.001 < 0.001 < 0.001 < 0.001	KS C IEC 62321-4(2014), KS C IEC 62321-5(2014), KS I ISO 17294:2014, Instrumental analysis	

* used instrument : Perkin-elmer ELAN DRC II

Affirmation	Tested by Name : S. T. KIM	Technical Manager Name : O. S. AHN

Dec. 13, 2018

Korea Institute of Ceramic Engineering & Technology

Certificate of Test

KICET	Certificate No. : 2018-4210-1	KICET
388, Songnae-daero, Bucheon-si, Gyeonggi-do, Korea (Tel: +82 032 210 5110, Fax: +82 032 210 5115)	Page(1)/(1)Pages	

1. Client
   - ㅇ Company : Advanced Protein Technologies corp., / Chul-soo, Shin
   - ㅇ Address : 7th Floor Gyenggi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City, Gyeonggi-Province
   - ㅇ Date of Receipt : Dec. 19. 2018
2. Use of Report : Reference
3. Test Sample : 2FL-CG-012 Final
4. Date of Test : Dec. 19. 2018 ~ Jan. 09. 2019
5. Test method used : KS C IEC 62321-4(2014), KS C IEC 62321-5(2014), KS I ISO 17294(2014), Instrumental analysis
6. Testing Environment
   - ㅇ Temperature : (17 ± 3) ℃ , Humidity : (35 ± 5) % R.H.
7. Test Results

Sample name	Item	Results	Test Method	Remark
2FL-CG-012 Final	As (mg/kg) Pb (mg/kg) Cd (mg/kg) Hg (mg/kg)	0.003 0.001 < 0.001 < 0.001	KS C IEC 62321-4(2014), KS C IEC 62321-5(2014), KS I ISO 17294:2014, Instrumental analysis	

* used instrument : Perkin-elmer ELAN DRC II

Affirmation	Tested by Name : S. T. KIM	Technical Manager Name : O. S. AHN

Jan. 09. 2019

Korea Institute of Ceramic Engineering & Technology

Certificate of Test

KICET	Certificate No. : 2018-4349	KICET
388, Songnae-daero, Bucheon-si, Gyeonggi-do, Korea (Tel: +82 032 210 5110, Fax: +82 032 210 5115)	Page(1)/(1)Pages	

1. Client
   - ㅇ Company : Advanced Protein Technologies corp., / Chul-soo, Shin
   - ㅇ Address : 7th Floor Gyenggi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City, Gyeonggi-Province
   - ㅇ Date of Receipt : Dec. 28. 2018
2. Use of Report : Quality Control
3. Test Sample : 2FL-CG-013 Final
4. Date of Test : Dec. 28. 2018 ~ Jan. 16. 2019
5. Test method used : KS C IEC 62321-4(2014), KS C IEC 62321-5(2014), KS I ISO 17294(2014), Instrumental analysis
6. Testing Environment
   - ㅇ Temperature : (17 ± 2) ℃ , Humidity : (35 ± 5) % R.H.
7. Test Results

Sample name	Item	Results	Test Method	Remark
2FL-CG-013 Final	As (mg/kg)	0.005	KS C IEC 62321-4(2014), KS C IEC 62321-5(2014), KS I ISO 17294:2014, Instrumental analysis	
	Pb (mg/kg)	0.005		
	Cd (mg/kg)	< 0.001		
	Hg (mg/kg)	< 0.001		

* used instrument : Perkin-elmer ELAN DRC II

Affirmation	Tested by Name : M. G. LEE	Technical Manager Name : O. S. AHN

Jan. 16. 2019

Korea Institute of Ceramic Engineering & Technology

양식번호 KT-QPF-26-04(03):2018.10.11 한국세라믹기술원

No. : D2019012300

Certificate of Analysis

Date of Application : 2019-01-28	Date of Manufacture :
No. of Sample : D2019012300	Expiration Date :
Lot No. :	
Inspection Purpose : Reference only	
Commodity : 2'-FL-CG-011	
Applicant — Name : Advanced Protein Technologies corp., Chul-soo, Shin	
Applicant — Company address : 7th Floor GyeongGi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City	

Analytical Result

Test Item	Result
Ash(%)	0.17 % (MFDS No.2018-98, 8.2.1.2)
Aflatoxin M_1 (μg/kg)	Not detected (MFDS No.2018-98, 8.9.2.3)
Standard plate count(/g)	0 (MFDS No.2018-98, 8.4.5.1.Dry rehydratable film method)
Mold & Yeast plate count(/g)	0 (MFDS No.2018-98, 8.4.10)
Coliform Group(/g)	0 (MFDS No.2018-98, 8.4.7.2.Dry rehydratable film method)
Escherichia coli(/g)	0 (MFDS No.2018-98, 8.4.8.2.Dry rehydratable film method)
Cronobacter spp.(/60g)	Negative (MFDS No.2018-98, 8.4.21)
Staphylococcus aureus(/g)	0 (MFDS No.2018-98, 8.4.12.2)
Salmonella spp.(/25g)	Negative (MFDS No.2018-98, 8.4.11)

Feb . 8 . 2019

We hereby certify that the above are correct.

Korea Health Supplements Association Sub. Korea Health Supplements Institute

Director : Yang, Joo-Hong Dr. j. h. yang

B-101, Korea Bio Park,, 700, Daewangpangyo-ro, Bundang-gu, Seongnam-si, Gyeonggi-do, Republic of Korea

KHSI

No. : D2019012301

Certificate of Analysis

Date of Application : 2019-01-28	Date of Manufacture :
No. of Sample : D2019012301	Expiration Date :
Lot No. :	
Inspection Purpose : Reference only	
Commodity : 2'-FL-CG-012	
Applicant — Name : Advanced Protein Technologies corp., Chul-soo, Shin	
Applicant — Company address : 7th Floor GyeongGi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City	

Analytical Result

Test Item	Result
Ash(%)	0.15 % (MFDS No.2018-98, 8.2.1.2)
Aflatoxin M_1 (μg/kg)	Not detected (MFDS No.2018-98, 8.9.2.3)
Standard plate count(/g)	0 (MFDS No.2018-98, 8.4.5.1.Dry rehydratable film method)
Mold & Yeast plate count(/g)	0 (MFDS No.2018-98, 8.4.10)
Coliform Group(/g)	0 (MFDS No.2018-98, 8.4.7.2.Dry rehydratable film method)
Escherichia coli(/g)	0 (MFDS No.2018-98, 8.4.8.2.Dry rehydratable film method)
Cronobacter spp.(/60g)	Negative (MFDS No.2018-98, 8.4.21)
Staphylococcus aureus(/g)	0 (MFDS No.2018-98, 8.4.12.2)
Salmonella spp.(/25g)	Negative (MFDS No.2018-98, 8.4.11)

Feb . 8 . 2019

We hereby certify that the above are correct.

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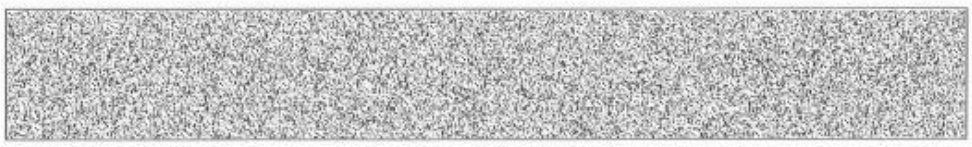
KHSI



No. : D2019012302

Certificate of Analysis

Date of Application : 2019-01-28	Date of Manufacture :
No. of Sample : D2019012302	Expiration Date :
Lot No. :	
Inspection Purpose : Reference only	
Commodity : 2'-FL-CG-013	
Applicant	Name : Advanced Protein Technologies corp., Chul-soo, Shin
	Company address : 7th Floor GyeongGi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City

Analytical Result

Test Item	Result
Ash(%)	0.14 % (MFDS No.2018-98, 8.2.1.2)
Aflatoxin M_1 (μg/kg)	Not detected (MFDS No.2018-98, 8.9.2.3)
Standard plate count(/g)	0 (MFDS No.2018-98, 8.4.5.1.Dry rehydratable film method)
Mold & Yeast plate count(/g)	0 (MFDS No.2018-98, 8.4.10)
Coliform Group(/g)	0 (MFDS No.2018-98, 8.4.7.2.Dry rehydratable film method)
Escherichia coli(/g)	0 (MFDS No.2018-98, 8.4.8.2.Dry rehydratable film method)
Cronobacter spp.(/60g)	Negative (MFDS No.2018-98, 8.4.21)
Staphylococcus aureus(/g)	0 (MFDS No.2018-98, 8.4.12.2)
Salmonella spp.(/25g)	Negative (MFDS No.2018-98, 8.4.11)

Feb . 8 . 2019

We hereby certify that the above are correct.

Korea Health Supplements Association Sub. Korea Health Supplements Institute

Director : Yang, Joo-Hong Dr. J. H. yang

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KHSI

Appendix D.

Detection of Introduced Gene in the Final 2'-FL Product

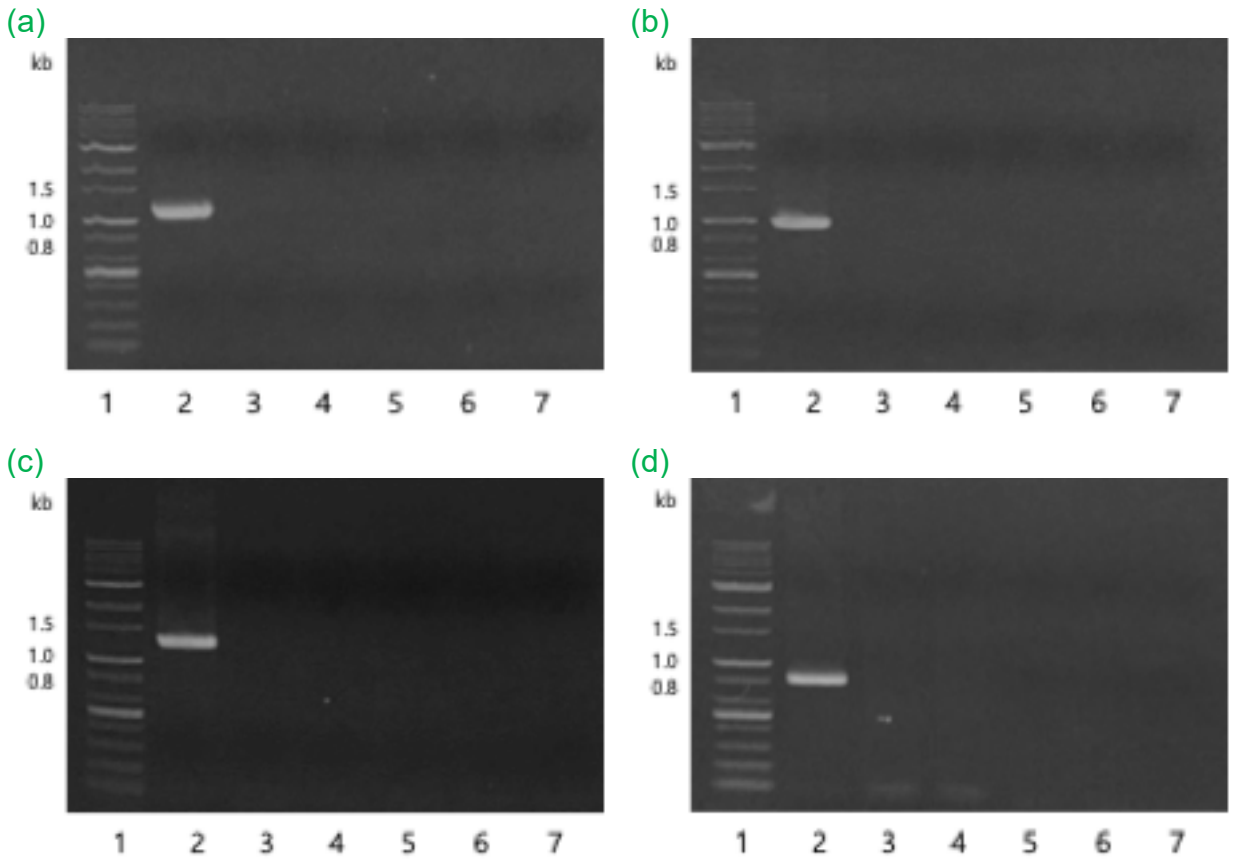
Detection of Introduced Gene in the Final 2'-FL Product

The absence of five kinds of introduced foreign gene was detected by PCR method using primers listed in Table 1.

Table 1. Primers List

Primer name	Sequence (5'→3')
Gmd	F primer - ATGTCAAAAGTCGCTCTCAT
	R primer - TTATGACTCCAGCGCGATCG
WcaG	F primer - ATGAGTAAACAACGAGTTTT
	R primer - TTACCCCCGAAAGCGGTCTT
LacY	F primer - ATGTACTATTTAAAAAACAC
	R primer - TTAAGCGACTTCATTCACCT
α -1,2-FT	F primer - ATGATATTTGTAACCGGATA
	R primer - TTAAATAATGTGTCGAAACA
NPTII	F primer - ATGATTGAACAAGATGGATT
	R primer - TCAGAAGAACTCGTCAAGAA

APTech's 2'-FL is free from contamination of introduced DNAs.



(e)

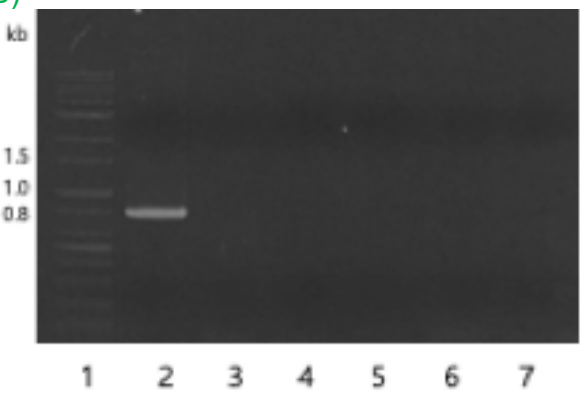


Figure 1. The Results of PCR. (a): Gmd, (b): WcaG, (c): LacY, (d): α -1,2-FT, (e): NPTII , 1: Marker, 2: Positive control, 3: 2'-FL 1 mg/ml, 4: 2'-FL 0.2 mg/ml, 5: 2'-FL 0.04 mg/ml, 6: 2'-FL 0.008 mg/ml, 7: 2'-FL 0.0016 mg/ml.

Appendix E.

LC-MS/MS Spectra; Comparison of APTech 2'-FL with Reference Material (Carbosynth)

GBSA Gyeonggido Business & Science Accelerator

Analysis Report

Sample	Carbosynth etc.	Client	Advanced Protein Technologies corp
Date of Receipt	1/28/2019		Jinhee Yu

Test Sample	Test Item	Solvent	Result
Carbosynth	MS, MRM	DW	Please refer to following page(s)
CG011	MS, MRM	DW	Please refer to following page(s)
CG012	MS, MRM	DW	Please refer to following page(s)
CG013	MS, MRM	DW	Please refer to following page(s)

Date of test	7-8/2/2019	Experimenter	Chung, Sun Ho
Date of issue	11/2/2019	Contact	031-888-6934 csh@gbsa.or.kr

*This document is a resource for research and development.

1. Material & Method

[1] Materials

1.	Solvent	DW, ACN(B&J)
2.	Reagent	Formic acid(Aldrich)

[2] Instrument Condition

① LC Method

1.	Chromatography	Nexera X2
2.	Mass spectrometry	LCMS-8050 (Shimadzu)
3.	Column	ACQUITY BEH C18, 1.7um, 50*2.1mm
4.	Solvent	A : DW(0.1% Formic acid) B : ACN(0.1% Formic acid)
5.	Elution condition	
6.	Flow rate	0.3 ml/min
7.	Injection Vol.	1 ul

Time	A	B
0.0	95	5
5.0	95	5
15.0	0	100
17.0	0	100
17.1	95	5
20.0	95	5

② MS detector condition

1.	Operation mode	ESI-negative
2.	Scan Type	MRM, Product ion scan
3.	Nebulizing Gas	3.0 L/min
4.	Drying Gas	10.0 L/min
5.	Heating Gas	10.0 L/min
6.	Interface Voltage	3.0 kV
7.	Conversion Dynode	10.0 kV
8	Interface Temp.	300 ℃
9.	DL Temp.	250 ℃
10.	Heat Block Temp.	400 ℃
11.	CID Gas	270 kPa
12.	Collision Energy	10 V

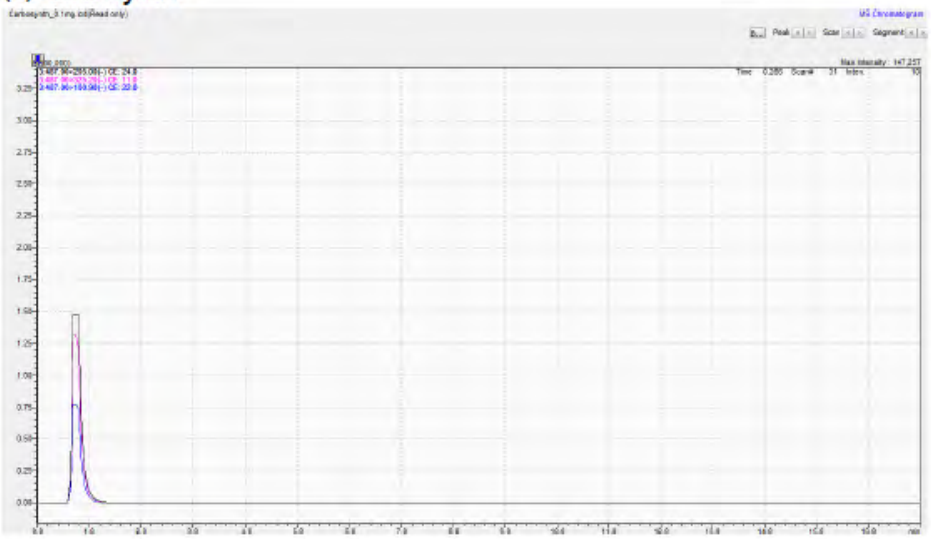
③ Transition Table

Standards	Precusor Mass	Product Mass	CE
#1	487.3[M-H]$^-$	205.0[M-H]$^-$	24
		325.2[M-H]$^-$	11
		100.9[M-H]$^-$	22

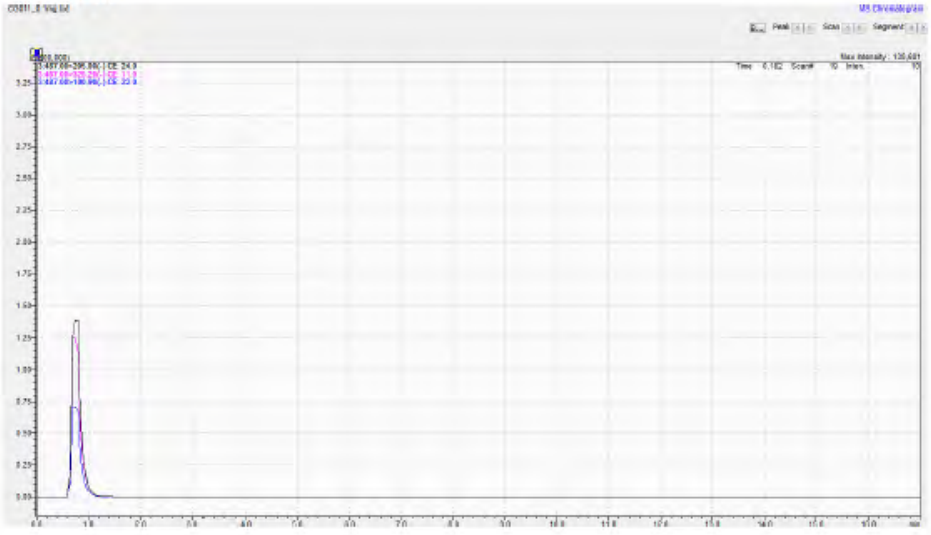
2. Results

[1] MRM

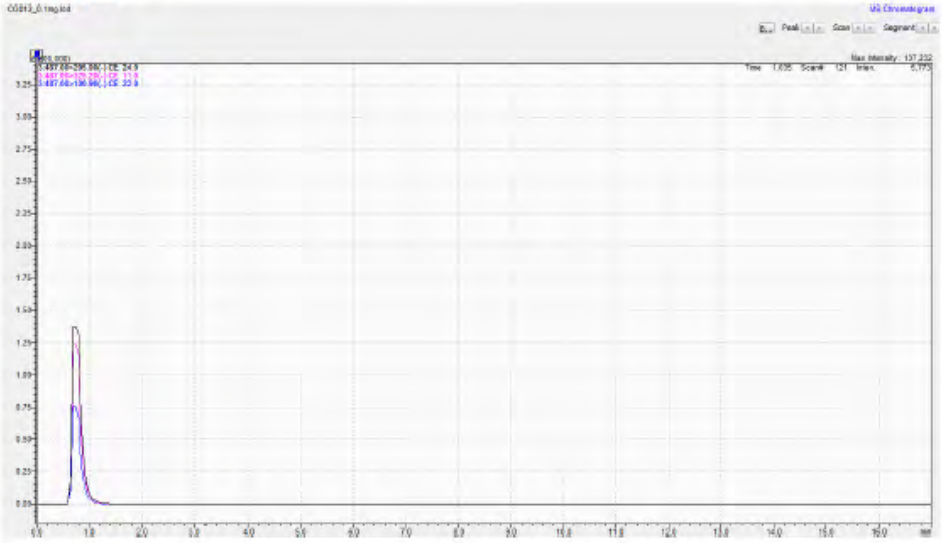
(1) Carbosynth



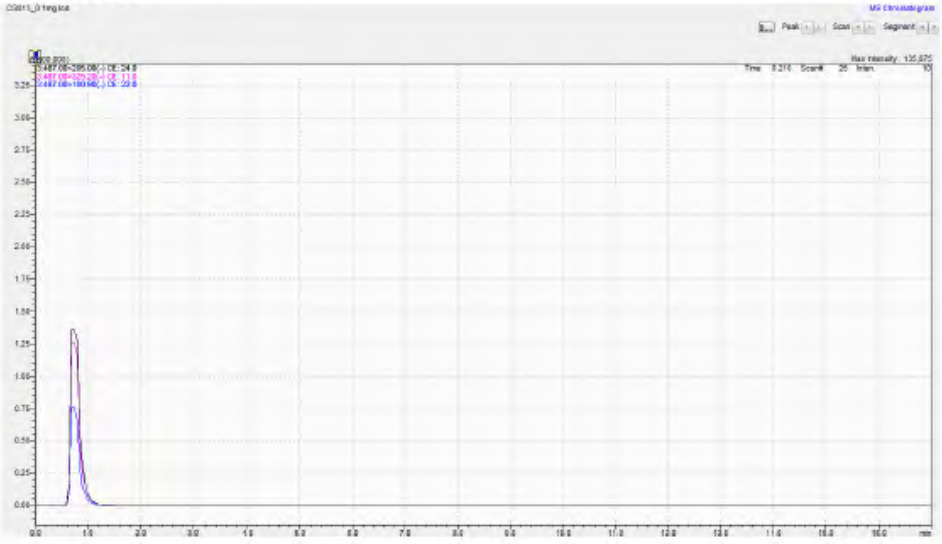
(2) CG011



(3) CG012

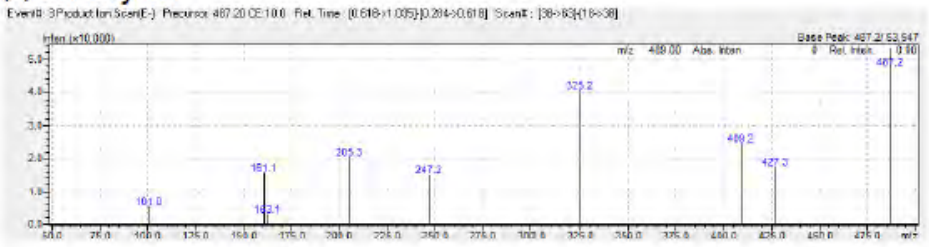


(4) CG013

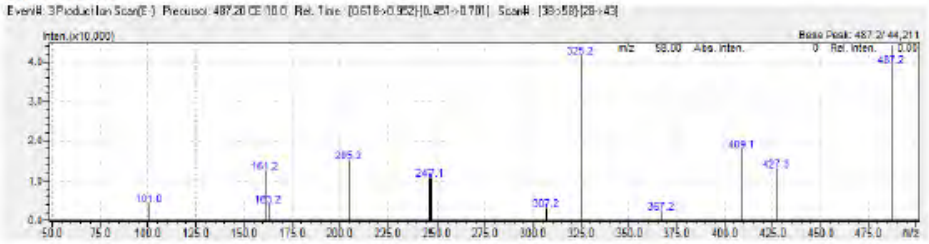


[2] MS/MS : CE:10V

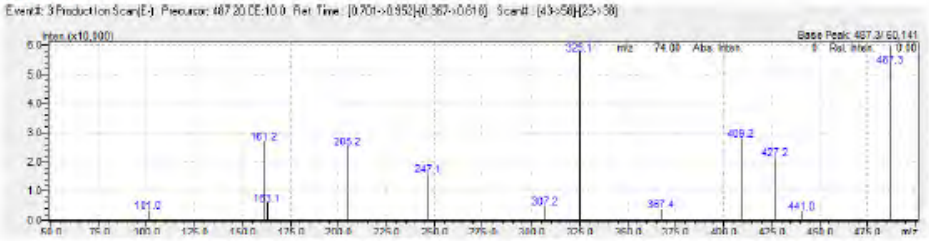
(1) Carbosynth



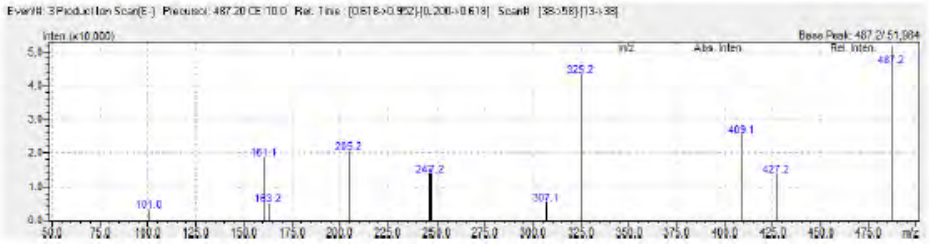
(2) CG011



(3) CG012



(4) CG013



Appendix F.

Analytical Method for 2'-FL using HPAEC-PAD with Dionex PA100 Column

1. Introduction

HPAEC-PAD equipment was used as a method to analyze 2'-FL in the fermentation and purification process. This analysis method was based on Jennewein 's method.

2. Materials and Methods

2.1 Standard Substance ; 2'-Fucosyllactose

Name	2'-Fucosyllactose ; 2'-FL ; 2FL ; 2-FL
Batch No.	OF067391501
Appearance	White crystalline powder
Molecular formula	$C_{18}H_{32}O_{15}$
Molecular weight	488.44 g/mol
Purity	> 95%
Manufacturer	Carbosynth Limited, UK

2.2 Standard Substance ; Difucosyllactose

Name	Difucosyllactose ; LDFT ; Di-FL ; DFL
Batch No.	OL065671701
Appearance	White to brown powder
Molecular formula	$C_{24}H_{42}O_{19}$
Molecular weight	634.58 g/mol
Purity	82.6%
Manufacturer	Carbosynth Limited, UK

2.3 Standard Substance ; 3-Fucosyllactose

Name	3-Fucosyllactose ; 3-FL ; 3FL
Batch No.	OF056731501
Appearance	White freeze-dried powder
Molecular formula	$C_{18}H_{32}O_{15}$
Molecular weight	488.44 g/mol
Purity	> 95%
Manufacturer	Carbosynth Limited, UK

2.4 Standard Substance ; Lactose

Name	Lactose monohydrate
Batch No.	FCI511
Appearance	White crystalline powder
Molecular formula	$C_{12}H_{22}O_{11}$
Molecular weight	360.32 g/mol
Manufacturer	Duksan Pure Chemicals Co. Ltd, KR

2.5 Standard Substance ; Glucose

Name	Dextrose anhydrous

Batch No. H6F106
Appearance White crystalline powder
Molecular formula $C_6H_{12}O_6$
Molecular weight 180.16 g/mol
Assay 99.9%
Manufacturer Duksan Pure Chemicals Co. Ltd, KR

2.6 Standard Substance ; Fucosyl-galactose
Name Fucosyl-galactose ; Blood group H disaccharide ; Fuc-(a1,2)-Gal
Batch No. OB059071101
Appearance White to off-white lyophilized solid
Molecular formula $C_{12}H_{22}O_{10}$
Molecular weight 326.3 g/mol
Purity > 95%
Manufacturer Carbosynth Limited, UK

2.7 Standard Substance ; Galactose
Name Galactose
Batch No. 060M0063V
Appearance White
Molecular formula $C_6H_{12}O_6$
Molecular weight 180.16 g/mol
Purity > 99%
Manufacturer Sigma-aldrich, USA

2.8 Standard Substance ; Fucose
Name Fucose
Batch No. SLBX2465
Appearance White
Molecular formula $C_6H_{12}O_5$
Molecular weight 164.16 g/mol
Purity > 99%
Manufacturer Sigma-aldrich, USA

3. Instrumentation and Materials

3.1 HPAEC-PAD (ICS-5000 + DC, S/N 18040897, Dionex)
3.2 Micro pipette (P1000, P200, P20, Eppendorf)
3.3 Vacuum pump (DOA-P704-AC, GAST)
3.4 Electronic balance (CAUY220, CAS)
3.5 Vortex mixer (VM-10, Wisd laboratory instruments)
3.6 Vials (AR0-9992-13, Phenomenex)
3.7 Syringe filter (0.2 μm pore, PTFE, Advantec)

3.8 Carbopac™ PA-100 (P/N.043055, 4 x 250mm, Dionex)
3.9 Carbopac™ PA-100 Guard (P/N. 043054, 4 X 50 mm, Dionex)

4. Reagents

4.1 Sodium hydroxide solution (NaOH, 50%, P/N.SS254, Fisher scientific)
4.2 Sodium acetate trihydrate (NaOAC, 99%, P/N.S7670, Sigma-aldrich)

5. Analytical Conditions

Instrument	ICS 5000, Dionex
Column	Carbopac™ PA-100, P/N.043055, 4 x 250mm
Guard column	Carbopac™ PA-100 Guard, P/N. 043054, 4 X 50 mm
Column temp.	30℃
Flow rate	1 mL / min
Injection volume	25 µL
AS/AP temperature	10℃
Eluent A	100 mM NaOH
Eluent B	100 mM NaOH + 300 mM sodium acetate
Run	

Time (min)	A(%)	B(%)
0.00	99.7	0.3
20.0	99.7	0.3
20.1	75	25
30.0	75	25
30.1	99.7	0.3
45.0	99.7	0.3

6. Preparation of the standard solutions

The standard stock solution at the highest concentration was prepared by weighing 10 mg of the standard substance and mixing with the purified water for dilution.
Each standard solution were prepared by serial dilution of the highest concentration stock solution.

7. Preparation of the sample solutions

The sample is weighed accurately into the flask, filled with purified water to the mark, and mixed well. 2'-FL samples were prepared by serial dilution with purified water.

8. Calculations

8.1 Linear regression
The calibration curve was constructed by plotting the peak area of the standard solution versus the concentration of standard solution. The linear regression equation is shown below:
$y = a\chi + b$
Where:
y = peak area generated by each standard solution
χ = concentration of the analyte in each standard solution
a = slope of the calibration curve
b = y-intercept of the calibration curve

8.2 Sample concentration
The measured concentration of the analyte in each sample was determined using the calibration curve and by solving the χ variable:
$\chi = \frac{y-b}{a}\ d$
Where:
y = peak area generated by the sample
χ = measured concentration of the analyte in each sample
a = slope of the calibration curve
b = y-intercept of the calibration curve
d = dilution factor

8.3 Accuracy
Accuracy (%) = (Mean determined concentration / Desired concentration) × 100

9. Results of validation

The test for linearity and accuracy was performed on 8 standard samples including 2'-FL. Other carbohydrates, except 2'-FL, were evaluated at five concentrations in a low concentration range to be included in specification. All samples were tested with triplicate.
The glucose and galactose, when analyzed, showed a very similar retention time, so they were found not to have a high degree of separation

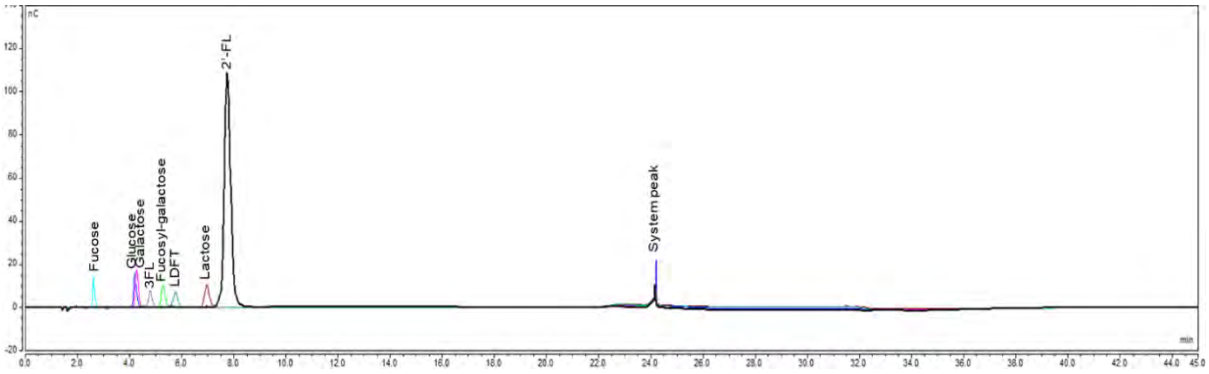
Summary of detailed results

Parameter	Fucose	Glucose	Galactose	3-FL	Fuc-gal	LDFT	Lactose	2'-FL
RT (min)	2.61	4.20	4.27	4.80	5.26	5.77	6.94	7.74
Range (ug/mL)	0.075~1.2	0.3~4.8	0.075~1.2	0.5~8	0.075~1.2	0.5~8	0.5~8	2.5~25
r^2 value	1.000	1.000	1.000	1.000	0.999	1.000	1.000	0.998
Slope	2.37	3.61	4.15	1.62	3.15	1.79	2.27	1.50
Intercept	0.04	0.16	0.07	0.12	0.02	0.15	0.20	1.37

Accuracy	102.36	97.98	102.82	97.4	99.93	97.39	100	107.58
%RSD(c=3,n=3)	0.34	0.45	0.58	0.32	0.38	0.21	0.18	0.4

Fuc-gal : Fucosyl-galactose ; LDFT : Difucosyllactose ; c=3 : three concentration

Chromatogram for standards



Results of production batches of 2'-FL

The analytical values of 3 independent batches of APTech's 2'-FL are showed purity of 94% (area) or more and these products are more than 95% pure on a dry weight basis, as measured by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

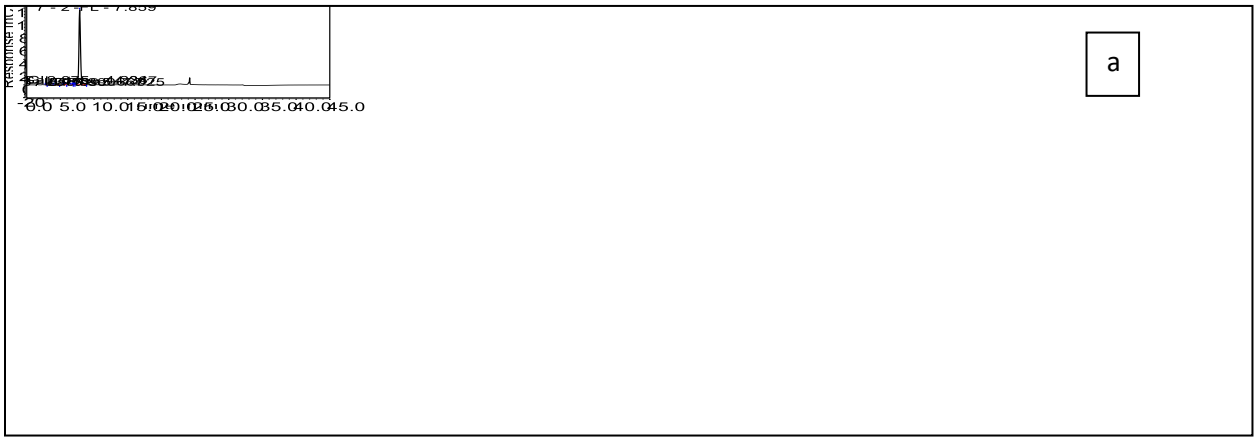


Figure 1. Chromatogram of Batch No. 2’-FL-CG-011 (a~c)

Certificate of Analysis of Standard Materials

Carbosynth

CERTIFICATE of ANALYSIS

2'-Fucosyllactose - Synthetic

Batch Number :	OF067391501
Product Code:	OF06739
Synonyms:	Fuc-a-1,2-Gal-b-1,4-Glc 2FL
CAS Number:	41263-94-9
Chemical Formula:	$C_{18}H_{32}O_{15}$
Molecular Weight:	488.44

	SPECIFICATION	RESULTS
Appearance:	White crystalline powder	White crystalline powder
Purity (^{1}H NMR):	min 95%	>95%

Carbosynth Limited
8&9 Old Station Business Park, Compton, Berkshire, RG20 6NE, UK
Tel: +44 (0)1635 578444 Fax: +44 (0)1635 579444
info@carbosynth.com www.carbosynth.com

Carbosynth

CERTIFICATE of ANALYSIS

Lactodifucotetraose

Batch Number:	OL065871701
Product Code:	OL06567
Synonyms:	LDFT Difucosyl lactose
CAS Number:	20768-11-0
Chemical Formula:	$C_{34}H_{62}O_{31}$
Molecular Weight:	934.58

	SPECIFICATION	RESULTS
Appearance:	White to brown powder	Conforms
Purity (HPEA-PAD):	min 80%	82.8%
Identity (^{1}H NMR):	Conforms to structure	Conforms

Date of issue: 23 Mar 2016

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info@carbosynth.com www.carbosynth.com

Carbosynth

CERTIFICATE of ANALYSIS

3-Fucosyllactose

Batch Number:	OF056731501
Product Code:	OF05673
Synonyms:	Galb-4(Fuca-3)Glc
CAS Number:	41312-47-4
Chemical Formula:	$C_{18}H_{32}O_{15}$
Molecular Weight:	488.44

	SPECIFICATION	RESULTS
Appearance:	White freeze-dried powder	Conforms
Purity (NMR):	min 95%	Conforms
Identity (NMR):	Conforms to structure	Conforms

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8&9 Old Station Business Park, Compton, Berkshire, RG20 6NE, UK
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info@carbosynth.com www.carbosynth.com

REAGENTS
DUKSAN

Certificate of Analysis

Lactose monohydrate

[5989-81-1]($C_{12}H_{22}O_{11} \cdot H_2O$)FW:360.32

GR Grade
Lot-FCI511

TESTS	UNIT	SPECIFICATION	RESULTS
Appearance		White crystalline powder. Some of the sweetness.	Pass
Identification		IR Spectrometry.	Pass
Solubility in water		To pass test	Pass
Solubility in dil. Ethanol	%	Max. 0.2	<0.2
Specific rotation (α) 20 D		+52.2 ~ +52.8	+52.2 ~ +52.8
Loss on drying (at. 80℃, 3hr)	%	Max. 0.5	0.1
Ignition residue (as Sulfate)	%	Max. 0.05	0.01
pH (5 w/v %, 25℃)		4.0 ~ 6.0	5.0
Total Nitrogen (as N)	%	Max. 0.005	<0.005
Heavy Metals (as Pb)	ppm	Max. 5	<5
Iron(Fe)	ppm	Max. 5	<5
Dextrine and starch		To pass test	pass

Mfg. Date : 2015-12-18
Exp. Date : 5 years after Mfg. Date
Test Method : JIS K 8728
Tested by : Min-Jung, Kim

DUKSAN PURE CHEMICALS CO., LTD
53, Sinwon-ro 133beon-gil, Danwon-gu, Ansan-si, Gyeonggi-do, Korea
TEL : +82-31-495-6886 FAX : +82-31-495-5335
World Wide Web : WWW.DUKSAN.KR
E-mail : QC@DUKSAN.KR

B. K. CHOI, Supervisor
Quality control

REAGENTS
DUKSAN

Certificate of Analysis

Dextrose anhydrous

[50-99-7] (C6H12O6) FW:180.16

Product code : 763

Extra Pure Grade
Lot-H6F106

TESTS	UNIT	SPECIFICATION	RESULTS
Appearance		White crystal or crystalline powder.	pass
Identification		IR Spectrometry.	pass
Assay	%	Min. 98.0	99.9
Specific rotation (α) 20 D		+52.5 ~ +53.2	+52.5 ~ +53.2
Solubility in Water		To pass test	pass
Loss on drying (at 105℃, 6hr)	%	Max. 1.0	0.1
Ignition residue (as Sulfate)	%	Max. 0.1	0.05
Acidity (as CH3COOH)	%	Max. 0.007	0.003
Chloride (Cl)	%	Max. 0.02	<0.02
Sulfate, Sulfite (as SO4)	%	Max. 0.025	<0.025
Lead (Pb)	ppm	Max. 5	<5
Arsenic (As)	ppm	Max. 1	<1
Dextrine & starch		To pass test	pass

Test Method : JIS K 8824
Mfg. Date : 2017-06-15
Exp. Date : 5 years after Mfg. Date
Tested by : Min-Jung,Kim

DUKSAN PURE CHEMICALS CO., LTD
53, Sinwon-ro 133beon-gil, Danwon-gu, Ansan-si, Gyeonggi-do, Korea
TEL : +82-31-495-6886 FAX : +82-31-495-5335
World Wide Web : WWW.DUKSAN.KR
E-mail : QC@DUKSAN.KR

B. K. CHOI, Supervisor
Quality control

Carbosynth

CERTIFICATE of ANALYSIS

Blood Group H disaccharide

Batch Number:	OB059071101
Product Code:	OB05907
Synonyms:	Fuc-(a1,2)-Gal 2-O-(a-L-Fucopyranosyl)-D-galactopyranose H-Disaccharide
CAS Number:	24656-24-4, 146076-26-8, 16741-18-7
Chemical Formula:	$C_{12}H_{22}O_{10}$
Molecular Weight:	326.3

	SPECIFICATION	RESULTS
Appearance:	White to off-white lyophilized solid	White lyophilized solid
Purity (TLC):	min 95%	>95%
Identity (NMR)	Conforms to structure	Conforms

Carbosynth Limited
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SIGMA-ALDRICH®

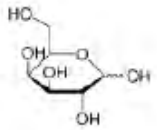
sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
D-(+)-Galactose - ≥99%

Product Number:	**G0750**
Batch Number:	**060M0063V**
Brand:	SIAL
CAS Number:	59-23-4
MDL Number:	MFCD00151230
Formula:	C6H12O6
Formula Weight:	180.16 g/mol
Quality Release Date:	24 JUN 2010
Date Retested:	07 JUL 2015
Recommended Retest Date:	JUL 2020



Test	Specification	Result
Appearance (Color)	White	White
Appearance (Form)	Powder	Powder
Solubility (Color)	Colorless	Colorless
Solubility (Turbidity) 100 mg/ml, H2O	Clear	Clear
IR Spectrum	Conforms to Structure	Conforms
% Purity (HPLC)	≥ 99	99
Impurity (by Enzymatic) as Glucose	≤ 0.1 %	0.0 %

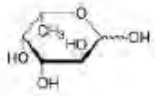
Rodney Burbach, Manager
Analytical Services
St. Louis, Missouri US

SIGMA-ALDRICH
sigma-aldrich.com
3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
L-(−)-Fucose - ≥99%

Product Number:	**F2252**
Batch Number:	**SLBX2465**
Brand:	SIGMA
CAS Number:	2438-80-4
MDL Number:	MFCD00135607
Formula:	C6H12O5
Formula Weight:	164.16 g/mol
Quality Release Date:	13 MAR 2018
Recommended Retest Date:	MAR 2020



Test	Specification	Result
Appearance (Color)	White to Off White	White
Appearance (Form)	Powder	Powder
Solubility (Turbidity) 50 mg/ml, H2O	Clear to Very Slightly Hazy	Very Slightly Hazy
Proton NMR spectrum	Conforms to Structure	Conforms
Solubility (Color)	Colorless to Faint Yellow	Very Faint Yellow
Specific Rotation (C = 4 in H2O at 20 deg C)	-76.0 - -73.0 °	-73.1 °
Purity (GC)	≥ 99 %	99 %

Rodney Burbach, Manager
Analytical Services
St. Louis, Missouri US

Appendix G. Safety of Introduced Proteins

Typically, sequence homology searches comparing the structure of introduced proteins to known allergens in a database are conducted using various algorithms, such as FASTA, to predict overall structural similarities. As recommended by FAO/WHO (2001), IgE cross-reactivity between a novel protein and a known allergen is considered a possibility when there is more than 35% identity over a segment of 80 or greater amino acids. For introduced proteins, the allergenic potential was screened using the database, http://allergenonline.org/databasefasta.shtml (March 23, 2018 version). Allergen online was used to compare FASTA sequences of each introduced protein to the protein sequences in the databases. Allergen online searches were conducted using default settings, and searches were conducted for matches to 80 amino acid sequence segments (sliding window) and 8-mer sequence alignments. In accordance with Codex guidelines, FASTA also was used to search for 80 amino acid sliding window segments aligning with a match ≥35% identity to a protein in the allergen database (Codex Alimentarius Commission, 2003). In addition, eight contiguous amino acid matches between a novel protein and a known allergen(s) are routinely used to identify sequences that may represent linear epitopes.

None of introduced proteins (GDP-L-fucose synthase [WcaG], GDP-D-mannose 4,6-dehydratase [Gmd], lactose permease [LacY], and fucosyltransferase [FT]) have homology in amino acid sequences with those of allergenic proteins.

An introduced protein, GDP-L-fucose synthase (WcaG), consists of 321 amino acids and amino acid sequences is as follows:

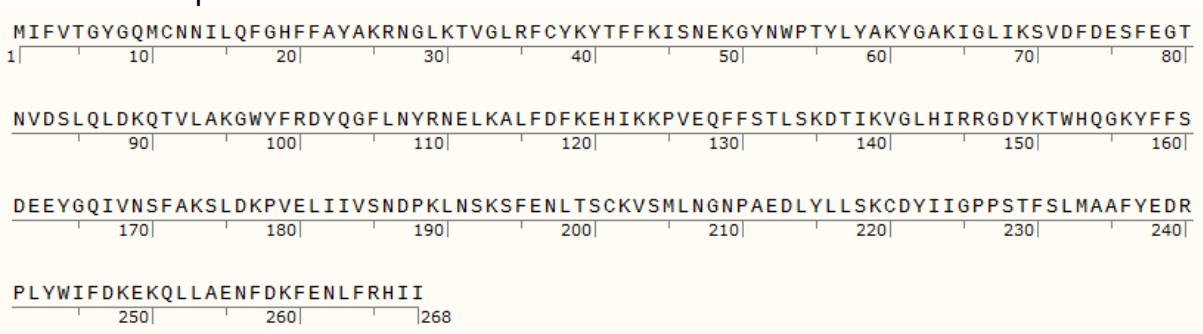
```
1      MSKQRVFIAG HRGMVGSAIR RQLEQRGDVE LVLRTRDELN LLDSRAVHDF FASERIDQVY
61     LAAAKVGGIV ANNTYPADFI YQNMMIESNI IHAAHQNDVN KLLFLGSSCI YPKLAKQPMA
121    ESELLQGTLE PTNEPYAIAK IAGIKLCESY NRQYGRDYRS VMPTNLYGPH DNFHPSNSHV
181    IPALLRRFHE ATAQNAPDVV VWGSGTPMRE FLHVDDMAAA SIHVMELAHE VWLENTQPML
241    SHINVGTGVD CTIRELAQTI AKVVGYKGRV VFDASKPDGT PRKLLDVTRL HQLGWYHEIS
301    LEAGLASTYQ WFLENQDRFR G
```

An introduced protein, GDP-D-mannose 4,6-dehydratase (Gmd), consists of 373 amino acids and amino acid sequences is as follows:

```
1      MSKVALITGV TGQDGSYLAE FLLEKGYEVH GIKRRASSFN TERVDHIYQD PHTCNPKFHL
61     HYGDLSDTSN LTRILREVQP DEVYNLGAMS HVAVSFESPE YTADVDAMGT LRLLEAIRFL
121    GLEKKTRFYQ ASTSELYGLV QEIPQKETTP FYPRSPYAVA KLYAYWITVN YRESYGMYAC
181    NGILFNHESP RRGETFVTRK ITRAIANIAQ GLESCLYLGN MDSLRDWGHA KDYVKMQWMM
241    LQQEQPEDFV IATGVQYSVR QFVEMAAAQL GIKLRFEGTG VEEKGIVVSV TGHDAPGVKP
301    GDVIIAVDPR YFRPAEVETL LGDPTKAHEK LGWKPEITLR EMVSEMVAND LEAAKKHSLL
361    KSHGYDVAIA LES
```

An introduced protein, LacY consists of 417 amino acids and amino acid sequences is as follows:

An introduced protein, fucosyltransferase (FT), consists of 268 amino acids and amino acid sequences is as follows:



From:	Susan S Cho
To:	Wafula, Denis
Subject:	Re: Information regarding GRN 000859 (2"-fucosyllactose)- Response Requested
Date:	Wednesday, August 21, 2019 4:27:44 PM
Attachments:	image005.png image001.png

Dear Dr. Wafula,

Thank you for your letter. On behalf of Aptech, we ask that FDA cease to evaluate GRN 859. We would appreciate it if you would provide us with a detailed list of deficiencies. Thank you very much.

Sincerely,
Susan
Susan Cho, Ph.D. NutraSource, Inc. 6309 Morning Dew Ct Clarksville, MD 21029 +1-410-531-3336 (O) +1-301-875-6454 (C)

On Wednesday, August 21, 2019, 01:14:20 PM EDT, Wafula, Denis <Denis.Wafula@fda.hhs.gov> wrote:

Dear Dr. Cho,

After reviewing APTech's GRAS Notice GRN 000859, our review team has identified a number of errors and deficiencies in the notice. Broadly, these include (but not limited to):

- Inaccurate or missing information on the intended use, identify, manufacturing, specifications, and exposure.
- Inaccurate descriptions or interpretation of presented studies
- Poor quality illegible chromatograms
- Direct use of language from a peer reviewed paper that could be construed as plagiarism
- Improper use of scientific terminology or making of incorrect scientific claims.

Due to the poor quality of this submission, we strongly recommend that APTech requests that we cease our evaluation of GRN 000859. After APTech requests that we cease to evaluate its notice, we will provide a detailed list of the deficiencies identified in GRN 000859. If APTech chooses not to request that we cease our evaluation of GRN 000859, then we will issue a no basis letter for this GRAS notice.

Please provide your response within 10 business days (Before COB September 4, 2019).

Sincerely,

Denis

Denis Wafula, Ph.D.
Staff Fellow

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety

U.S. Food and Drug Administration
Office: 2404021314
denis.wafula@fda.hhs.gov

U.S. FOOD & DRUG ADMINISTRATION

From: Susan S Cho <susanscho1@yahoo.com>
Sent: Thursday, June 13, 2019 6:55 PM
To: Wafula, Denis <Denis.Wafula@fda.hhs.gov>
Subject: Re: Filing Letter for GRN 000859 (2'-fucosyllactose)

Dear Dr. Wafula,

Thank you very much. Have a nice weekend!

Sincerely,

Susan

Susan Cho, Ph.D. NutraSource, Inc. 6309 Morning Dew Ct Clarksville, MD 21029 +1-410-531-3336 (O) +1-301-875-6454 (C)

On Thursday, June 13, 2019, 02:26:23 PM EDT, Wafula, Denis <Denis.Wafula@fda.hhs.gov> wrote:

Dear Dr. Cho,

Find attached the Filing Letter for GRAS Notice #GRN 000859 that you submitted to FDA. If you have any questions about the letter, do not hesitate to contact us.

Best Regards,

Denis

Denis Wafula, Ph.D.
Staff Fellow

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Office: 2404021314
denis.wafula@fda.hhs.gov

7.B. References that are Not Generally Available

Biotoxtech. 2019a. Bacterial reverse mutation test of 2'-fucosyllactose. Study No. B18674.

Biotoxtech. 2019b. *In vitro* mammalian chromosomal aberration test of 2'-fucosyllactose using mammalian cultured cell. Study No. B18675.

Biotoxtech. 2019c. In vitro micronucleus test of 2'-fucosyllactose in ICR mice. Study No. B18676.

Biotoxtech. 2019d. Single oral dose toxicity study of 2'-fucosyllactose in juvenile Sprague-Dawley rats Study No. B18672.

Biotoxtech. 2019e. Ninety-day repeated oral dose toxicity study with a four -week recovery period of 2'-fucosyllactose in juvenile Sprague-Dawley rats. Study No. B18673.

Biotoxtech is a GLP certified lab based in South Korea. To deliver the key findings of each report, the GLP statement, details of the test facility, key personnel, and individual data (usually included as appendices) were not included in these abbreviated reports. Full reports can be provided upon request.

Biotoxtech 2019a

FINAL REPORT

Bacterial Reverse Mutation Test
of 2'-Fucosyllactose

Study No.: B18674

Biotoxtech Co., Ltd.

53, Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si,
Chungcheongbuk-do, 28115, Republic of Korea

SUMMARY

This study was designed to evaluate the mutagenic potential of the test substance, 2'-Fucosyllactose, using histidine requiring *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) strains and tryptophan requiring *Escherichia coli* (WP2*uvrA*(pKM101)) strain in the presence and absence of metabolic activation.

In order to determine the high dose level of the main study, a dose range finding study was conducted. The high dose was selected at 5,000 μg/plate and it was sequentially diluted by applying a geometric ratio of 4 to produce 5 lower dose levels (1,250, 313, 78.1, 19.5 and 4.88 μg/plate). As a result, growth inhibition and precipitation of the test substance were not evident at any dose level of the test substance in all strains in the presence and absence of metabolic activation.

Therefore, the dose levels of the main study were selected as follows. In addition, the positive and negative control groups were set.

Strain	S9 mix	Dose levels of the main study (μg/plate)
TA98, TA100, TA1535, TA1537, WP2*uvrA*(pKM101)	-/+	5,000, 2,500, 1,250, 625, 313

Based on the result of the main study, the mean number of revertant colonies was less than twice when compared to the negative control group at all dose levels of the test substance in the presence and absence of metabolic activation.

In the positive control group, the mean number of revertant colonies was markedly increased more than twice when compared to the negative control group.

Based on the results of this study, the test substance, 2'-Fucosyllactose, did not exhibit any indication of mutagenic potential under the conditions of this study.

1. EXPERIMENTAL OUTLINE

1.1 Purpose

The purpose of this study was to evaluate the mutagenic potential of the test substance, 2'-Fucosyllactose, using histidine requiring *Salmonella typhimurium* strains and tryptophan requiring *Escherichia coli* strain.

1.2 Good Laboratory Practice Regulations

This study was conducted in accordance with the following Good Laboratory Practice Regulation:

- "Good Laboratory Practice Regulation for Nonclinical Laboratory Studies"

  Notification No. 2017-32, Ministry of Food and Drug Safety, Republic of Korea (May 1, 2017)

1.3 Regulatory Guidelines

This study was conducted in accordance with the following guidelines:

- "Standards for Toxicity Studies of Drugs"

  Notification No. 2017-71, Ministry of Food and Drug Safety, Republic of Korea (Aug. 30, 2017)

- "OECD Guideline for Testing of Chemicals, 471, Bacterial Reverse Mutation test"

  Organisation for Economic Co-operation and Development (Adopted: Jul. 21, 1997)

1.4 Sponsor

Name	Advanced Protein Technologies Corp.		
Address	7th Floor GyeongGi-BioCenter; 147, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16229, Republic of Korea		
TEL	+ 82-31-888-6245	FAX	+ 82-31-888-6247

1.5 Test Facility

Name	Biotoxtech Co., Ltd.		
Address	53, Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si, Chungcheongbuk-do, 28115, Republic of Korea		
TEL	+ 82-43-210-7777	FAX	+ 82-43-210-7778

2. MATERIALS AND METHODS

2.1 Test Substance

2.1.1	Name	2'-Fucosyllactose
2.1.2	Lot No.	2'-FL-CG-008
2.1.3	Appearance	Light white-yellowish powder
2.1.4	Structural formula	$C_{18}H_{32}O_{15}$
2.1.5	Molecular weight	488.44 g/mol
2.1.6	Purity	97.56%
2.1.7	Date of manufacture	Sep. 5, 2018
2.1.8	Expiration date (retest date)	Sep. 4, 2019 (one year after manufacture)
2.1.9	Storage condition	Room temperature (1–30°C)
2.1.10	Handling instructions	Wear a mask, a pair of gloves and protective equipment
2.1.11	Supplier	
	Name	Advanced Protein Technologies Corp.
	Address	7th Floor GyeongGi-BioCenter; 147, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16229, Republic of Korea
2.1.12	Disposition of test substance	Any remaining test substance is returned to the sponsor.

2.2 Negative Control

2.2.1	Name	Water for injection
2.2.2	Lot No.	17012, 18003
2.2.3	Storage condition	Room temperature
2.2.4	Manufacturer	JW Pharmaceutical Co., Ltd., Republic of Korea

2.2.5 Justification for selection — Water for injection, the vehicle of the test substance, was used as the negative control.

2.3 Positive Controls

Name	Lot No. (#: Batch No.)	Storage condition	Manufacturer
Sodium azide (SA)	#MKBX7529V	Room temperature	SIGMA-ALDRICH, Co., U.S.A.
2-Nitrofluorene (2-NF)	#S43858V	Room temperature	SIGMA-ALDRICH, Co., U.S.A.
2-Aminoanthracene (2-AA)	#STBD3302V	Room temperature	SIGMA-ALDRICH, Co., U.S.A.
9-Aminoacridine (9-AA)	BCBR5712V	Room temperature	SIGMA-ALDRICH, Co., U.S.A.
4-Nitroquinoline N-oxide (4-NQO)	#WXBC1554V, #WXBC3635V	Room temperature	SIGMA-ALDRICH, Co., U.S.A.

2.4 Preparation and Analysis of the Dosing Formulations

2.4.1 Preparation of dosing formulations of the test substance

2.4.1.1 Vehicle

2.4.1.1.1 Name — Water for injection

2.4.1.1.2 Lot No. — 17012, 18003

2.4.1.1.3 Justification for selection

In order to produce the high dose level of the dose range finding study, a preliminary solubility test was conducted. As a result, the test substance was dissolved in water for injection. Therefore, water for injection was selected as the vehicle for this study.

2.4.1.2 Preparation method

All preparations were conducted on the day of treatment of dosing formulations (dose range finding study) or on the day of analysis of dosing formulations (main study).

In order to produce the high dose level, the required amount of the test substance was weighed (CP323S, Sartorius, Germany) with a purity factor (1.025). A small amount of vehicle (water for injection) was added and the both materials were mixed using a vortex mixer until dissolved. Vehicle was added to yield the desired

dose level. The high dose formulation was serially diluted to produce lower dose levels.

In the main study, the dosing formulations were stored in a refrigerator and used within a period of stability (8 days).

2.4.1.3 Analysis of dosing formulations

2.4.1.3.1 Homogeneity and stability

As a result of analysis for homogeneity and stability conducted in the study of "An Analytical Method Validation of 2'-Fucosyllactose Dosing Formulations by HPLC (Biotoxtech Study No.: B18670)", the 0.1 and 750 mg/mL dosing solutions including the dose concentrations of the main study were confirmed to be homogenous and stable for 4 hours at room temperature and for 8 days under refrigeration.

2.4.1.3.2 Verification of dose concentrations

Analysis of the dosing formulations was conducted using a HPLC (Prominence, Shimadzu Corp., Japan).

Analysis of the dosing formulations was conducted based on the method used in the study of "An Analytical Method Validation of 2'-Fucosyllactose Dosing Formulations by HPLC (Biotoxtech Study No.: B18670)" and samples were taken three times from the middle layer of each dosing formulation prior to treatment and analyzed for verification of dose concentration.

As a result of analysis of the dosing formulations, the precision and accuracy of the dosing formulations were in the ranges of 0.75–1.79% and 93.68–98.14%, respectively. The results were considered to be acceptable because the precision was within 10% and the accuracy was in the range of 85–115% (Appendix VI).

2.4.2 Preparation of the positive controls

The dose levels of positive controls for the respective strains were determined based on the historical control data in this laboratory. The positive control, SA, was prepared in water for injection (Lot Nos.: 17006, 17012, JW Pharmaceutical Co., Ltd., Republic of Korea). 2-NF, 9-AA, 4-NQO and 2-AA were prepared in dimethyl sulfoxide (DMSO, Lot Nos.: K49393831, K49824131, Merck, Germany). The prepared positive controls were stored in a deep freezer (-80—60°C, OPR-DFU-657CEV, Operon, Republic of Korea) and thawed just prior to use.

<The type and dose of the positive controls for the respective strains>

S9 mix	Strain	Name	Dose (μg/plate)
-	TA98	2-NF	5.0
	TA100	SA	1.5
	TA1535	SA	1.5
	TA1537	9-AA	80.0
	WP2*uvrA*(pKM101)	4-NQO	0.1
+	TA98	2-AA	1.0
	TA100	2-AA	2.0
	TA1535	2-AA	3.0
	TA1537	2-AA	3.0
	WP2*uvrA*(pKM101)	2-AA	2.0

2.5 Bacterial Strains

2.5.1 Species and strains

Salmonella typhimurium TA98

Salmonella typhimurium TA100

Salmonella typhimurium TA1535

Salmonella typhimurium TA1537

Escherichia coli WP2*uvrA*(pKM101)

2.5.2 Justification for strain selection

These strains are highly sensitive to mutagens, commonly used in mutagenicity studies and recommended in the test guidelines.

2.5.3 Receipt and storage

The strains were purchased from Molecular Toxicology, Inc. (MOLTOX™, Inc., U.S.A.) on Oct. 22, 2015 (TA98, TA1535 and WP2*uvrA*(pKM101)) and Nov. 25, 2015 (TA100 and TA1537). Each strain was inoculated in the nutrient broth medium and incubated for 8 hours in a shaking water bath (37°C, 130 rpm). The genotype, spontaneous revertant colonies and sensitivity to positive control substances were confirmed following cultivation.

After those characteristics were confirmed, each bacterial strain and DMSO were mixed at a ratio of 1 to 0.09 and the mixtures were placed in cryogenic vials and stored in a deep freezer (-80—60°C).

<Genotypes of each strain>

Species	Strain	Genotype	
Salmonella typhimurium	TA98	*hisD*3052	*rfa*△*uvrB* (pKM101)
	TA100	*hisG*46	*rfa*△*uvrB* (pKM101)
	TA1535	*hisG*46	*rfa*△*uvrB*
	TA1537	*hisC*3076	*rfa*△*uvrB*
Escherichia coli	WP2*uvrA*(pKM101)	*trpE*	*uvrA*(pKM101)

2.5.4 Pre-incubation

The frozen bacterial suspensions were thawed and inoculated into the nutrient broth medium and incubated in a shaking water bath (37°C, 130 rpm, BS-31, JEIO TECH. Co., LTD., Republic of Korea). Following pre-incubation, the turbidity of the cultures was measured with a UV/VIS spectrophotometer (660 nm, V-550, Jasco, Japan). Cultures with a density greater than 1×10^9 cells/mL were used in this study.

2.6 Medium

2.6.1 Nutrient broth medium

Nutrient broth (BD, U.S.A) was weighed and mixed with a small amount of ultra pure water using a stirrer until dissolved. Ultra pure water was added to yield a concentration of 0.8% and then autoclaved.

2.6.2 Minimal glucose agar plate

Bacto agar (BD, U.S.A.) was weighed. A small amount of ultra pure water was added and then autoclaved. Sterile 10-fold Vogel-Bonner (VB) salts and sterile 20% glucose (Junsei Chemical Co., Ltd., Japan) were added. The mixed solution was transferred to petri dishes and allowed to solidify at room temperature.

<Composition of the minimal glucose agar plate>

Component	Amount of each component
Bacto agar	15 g
10-fold VB salts	100 mL
20% Glucose	100 mL
Ultra pure water	800 mL
Total volume	1 L

<Composition of the 10-fold VB salts>

Component	Used amount	Manufacturer
$MgSO_4 \cdot 7H_2O$	0.2 g	Junsei Chemical Co., Ltd., Japan
Citric acid	1.829 g	Junsei Chemical Co., Ltd., Japan
K_2HPO_4	10 g	Junsei Chemical Co., Ltd., Japan
$NaNH_4HPO_4 \cdot 4H_2O$	3.58 g	KANTO CHEMICAL CO., INC., Japan
Ultra pure water	100 mL	-

2.6.3 Top agar

NaCl and bacto agar (BD, U.S.A.) were weighed and ultra pure water was added to yield the concentrations of 0.5 and 0.6%, respectively, and then autoclaved. These mixtures were mixed with the 0.5 mM L-Histidine/D-Biotin (SIGMA-ALDRICH, Co., U.S.A.) solution at a ratio of 10 to 1 for *Salmonella typhimurium* and with the 0.5 mM L-Tryptophan (SIGMA-ALDRICH, Co., U.S.A.) solution at a ratio of 10 to 1 for *Escherichia coli*.

2.7 Preparation of S9 Mix

2.7.1 Receipt and storage

S9 and Cofactor A were purchased from ORIENTAL YEAST Co., LTD. in Japan, stored in a deep freezer (-80—60°C) and used within the expiration date.

<Characteristics of S9>

Species and Strain	Sprague-Dawley rat [Crl:CD(SD)]
Sex and Age	Male, 7 weeks old
Organ	Liver
Inducing Agent	Phenobarbital (PB) and 5,6-benzoflavone (BF)
Dose and Frequency	PB: 30 mg/kg, once (Day 1) 60 mg/kg, once daily for 3 consecutive days (Days 2–4) BF: 80 mg/kg, once (Day 3)
Route of Administration	Intraperitoneal injection

2.7.2 Composition of S9 mix

Component		Amount of each component
S9		0.1 mL
Cofactor A	0.4 mol/L $MgCl_2$	0.02 mL (8 μmol)
	1.65 mol/L KCl	0.02 mL (33 μmol)
	1.0 mol/L Glucose-6-phosphate	0.005 mL (5 μmol)
	0.1 mol/L NADPH	0.04 mL (4 μmol)
	0.1mol/L NADH	0.04 mL (4 μmol)
	0.2 mol/L Sodium phosphate buffer, pH 7.4	0.5 mL (100 μmol)
	Purified water	0.275 mL
Total volume		1 mL

2.7.3 Preparation method of S9 mix

The preparation of S9 mix was conducted immediately prior to use. The frozen S9 (Lot Nos.: 18051102 (dose range finding study), 18070604 (main study)) and Cofactor A (Lot Nos.: A18050802 (dose range finding study), A18070304 (main study)) were thawed and mixed at a ratio of 1 to 9.

2.8 Dose Range Finding Study

A dose range finding study was conducted to determine the high dose for the main study.

2.8.1 Dose levels

The high dose of the test substance was 5,000 μg/plate, which is required in the test guidelines. The high dose was sequentially diluted by applying a geometric ratio of 4 to produce 5 lower dose levels (1,250, 313, 78.1, 19.5 and 4.88 μg/plate). In addition, the positive and negative control groups were set.

2.8.2 Study method

The dose range finding study was conducted using the same method and conditions as the main study.

Two plates per dose were used in the dose range finding study.

2.8.3 Justification for selection of the dose levels in the main study

The growth inhibition and precipitation of the test substance were not evident at any dose level in the presence and absence of metabolic activation. Therefore, the high dose in the main study was selected at 5,000 μg/plate and it was sequentially diluted by applying a geometric ratio of 2 to produce 4 lower dose levels (2,500, 1,250, 625 and 313 μg/plate). In addition, the positive and negative control groups were set.

Strain	S9 mix	Dose levels of the main study (μg/plate)
TA98, TA100, TA1535, TA1537, WP2*uvrA*(pKM101)	-/+	5,000, 2,500, 1,250, 625, 313

2.9 Main Study

2.9.1 Study method

The main study was conducted according to the pre-incubation method. All treatments were divided into the presence and absence of metabolic activation.

Three plates per dose were used in the main study and the treatment was conducted in duplicate.

Each plate was labeled with an identification number which indicates the bacterial strain, dose, the positive and negative controls and the presence or absence of S9 mix.

2.9.2 Treatment method

In the presence of metabolic activation, 100 μL of each of the test substance, strain-specific positive control and negative control was placed in the respective tubes. 500 μL of S9 mix was added followed by an addition of 100 μL of pre-incubated bacterial suspension. These mixtures were incubated in a shaking water bath (37°C, 90 rpm) for 20 minutes. Then, 2 mL of warmed top agar for *Salmonella typhimurium* was added to the TA98, TA100, TA1535 and TA1537 strains and 2 mL of warmed top agar for *Escherichia coli* was added to the WP2*uvrA*(pKM101) strain. They were mixed thoroughly with a vortex mixer. Finally, these mixtures were poured on the minimal glucose agar plates and allowed to solidify at room temperature.

In the absence of metabolic activation, 500 μL of 0.1 mol/L sodium phosphate buffer (pH 7.4) instead of S9 mix was added, and the rest of procedure was carried out with the same method as above.

2.9.3 Incubation method and period

After the top agar was solidified, the plates were inverted and cultured in an incubator (DK-LI020-P, Daiki scientific Co., LTD., Republic of Korea) at 37°C for 48 hours.

2.9.4 Evaluation of microbial contamination

In order to confirm microbial contamination, 100 μL of each of the high dose formulation, 500 μL of 0.1 mol/L sodium phosphate buffer (pH 7.4) and 500 μL of S9 mix were placed in the respective tubes and incubated in a shaking water bath (37°C, 90 rpm) for 20 minutes. 2 mL of warmed top agar was added and mixed thoroughly with a vortex mixer. Then, the mixed solution was poured on the nutrient broth agar plate and the overlaid agar was allowed to solidify. After the top agar was solidified, the plates were inverted and cultured in an incubator at 37°C for 48 hours. Then, the presence or absence of colonies formed by microbial contamination in the plates was evaluated.

2.9.5 Confirmatory study

Confirmatory study was not conducted because the following conditions were not met.

- The results of gene mutagenic potential in the main studies are not reproducible.
- There are less than 4 dose levels at which growth inhibition is not observed.

2.10 Observations and Measurements

2.10.1 Observation of precipitation

The precipitation of the test substance was observed with the naked eye and recorded at the time of treatment of the test substance and colony counting.

2.10.2 Revertant colony counting

Following cultivation, the number of revertant colonies was automatically counted by a colony counter (ProtoCOL3, SYNBIOSIS, UK) or by visual counting. When automatic counting was considered to be inaccurate, the number of revertant colonies was counted by visual counting.

2.10.3 Observation of background lawn

To confirm the presence or absence of growth inhibition by the test substance, the background lawn was observed using a stereoscopic microscope (45-fold magnification, SZ61, Olympus, Japan). Growth inhibition was detected by reduction in the number of revertant colonies, or by diminution or clearing of background lawn compared to the negative control group.

2.11 Acceptance Criteria

Evaluation of the validity of the study results was conducted based on the following criteria:

- The mean number of revertant colonies for the positive and negative control groups is within the range of the historical control data or the mean number of revertant colonies in the positive control group is increased at least twice as compared to the negative control group.
- No plate shows any evidence of contamination.

2.12 Evaluation Criteria

The results of the study were considered to be positive when the following conditions were met.

- The number of revertant colonies in any strain at one or more doses is increased at least two times when compared to the negative control group. There should be dose dependency or reproducibility as dose increases.

2.13 Statistical Analysis

Individual plate was counted for revertant colonies. The average and standard deviation of the number of revertant colonies were calculated. Statistical analysis was not performed.

3. RESULTS AND DISCUSSION

3.1 Dose Range Finding Study

(Figure 1, Figure 2, Figure 3, Figure 4, Table 1, Table 2)

As a result of the dose range finding study according to the 2.8 method, the dose levels of the main study were selected as follows. In addition, the positive and negative control groups were set.

Strain	S9 mix	Dose levels of the main study (μg/plate)
TA98, TA100, TA1535, TA1537, WP2*uvrA*(pKM101)	-/+	5,000, 2,500, 1,250, 625, 313

3.2 Main Study

(Figure 5, Figure 6, Figure 7, Figure 8, Figure 9, Figure 10, Figure 11, Figure 12, Table 3, Table 4)

3.2.1 Revertant colony counting

As a result of the main study, the mean number of revertant colonies was less than twice when compared to the negative control group at all dose levels of the test substance in all strains in the presence and absence of metabolic activation, and there was no dose-related increase.

In the positive control group, the mean number of revertant colonies was markedly increased more than twice when compared to the negative control group.

3.2.2 Growth inhibition and precipitation of the test substance

The growth inhibition and precipitation of the test substance were not evident at any dose level of the test substance in all strains in the presence and absence of metabolic activation.

3.3 Acceptance of Study

The mean number of revertant colonies in the positive and negative control groups was within the range of the historical control data (Table 5) and the number of revertant colonies in each strain in the positive control groups was markedly increased at least twice when compared to the negative control group. In addition, there was no contamination. Therefore, these results indicated that this study was conducted under the suitable conditions.

4. CONCLUSION

Based on the results of this study, the test substance, 2'-Fucosyllactose, did not exhibit any indication of mutagenic potential under the conditions of this study.

Table 1. The Number of Revertant Colonies per Plate in the Presence of Metabolic Activation (Dose Range Finding Study)

Strain	Test substance	Dose (μg/plate)	Individual revertant colony counts	Mean
TA98	Water for injection	0	38 , 35	37
	2'-Fucosyllactose	4.88	40 , 43	42
		19.5	36 , 34	35
		78.1	32 , 36	34
		313	31 , 36	34
		1,250	37 , 35	36
		5,000	40 , 37	39
	2-Aminoanthracene (2-AA)	1.0	363 , 372	368
TA100	Water for injection	0	103 , 108	106
	2'-Fucosyllactose	4.88	112 , 103	108
		19.5	107 , 100	104
		78.1	112 , 118	115
		313	116 , 112	114
		1,250	104 , 108	106
		5,000	104 , 109	107
	2-Aminoanthracene (2-AA)	2.0	956 , 987	972
TA1535	Water for injection	0	13 , 12	13
	2'-Fucosyllactose	4.88	13 , 9	11
		19.5	12 , 10	11
		78.1	9 , 12	11
		313	8 , 12	10
		1,250	12 , 13	13
		5,000	10 , 12	11
	2-Aminoanthracene (2-AA)	3.0	182 , 176	179
TA1537	Water for injection	0	21 , 22	22
	2'-Fucosyllactose	4.88	23 , 25	24
		19.5	18 , 20	19
		78.1	17 , 20	19
		313	19 , 19	19
		1,250	24 , 21	23
		5,000	22 , 22	22
	2-Aminoanthracene (2-AA)	3.0	237 , 240	239
WP2*uvrA* (pKM101)	Water for injection	0	132 , 140	136
	2'-Fucosyllactose	4.88	140 , 148	144
		19.5	141 , 147	144
		78.1	150 , 157	154
		313	148 , 157	153
		1,250	149 , 152	151
		5,000	141 , 136	139
	2-Aminoanthracene (2-AA)	2.0	483 , 462	473

Table 2. The Number of Revertant Colonies per Plate in the Absence of Metabolic Activation (Dose Range Finding Study)

Strain	Test substance	Dose (µg/plate)	Individual revertant colony counts	Mean
TA98	Water for injection	0	17 , 20	19
	2'-Fucosyllactose	4.88	19 , 19	19
		19.5	22 , 20	21
		78.1	25 , 26	26
		313	24 , 28	26
		1,250	23 , 20	22
		5,000	25 , 20	23
	2-Nitrofluorene (2-NF)	5.0	737 , 708	723
TA100	Water for injection	0	105 , 99	102
	2'-Fucosyllactose	4.88	98 , 105	102
		19.5	119 , 113	116
		78.1	101 , 105	103
		313	108 , 105	107
		1,250	89 , 95	92
		5,000	110 , 103	107
	Sodium azide (SA)	1.5	727 , 753	740
TA1535	Water for injection	0	16 , 15	16
	2'-Fucosyllactose	4.88	15 , 16	16
		19.5	15 , 17	16
		78.1	15 , 18	17
		313	18 , 17	18
		1,250	18 , 15	17
		5,000	21 , 17	19
	Sodium azide (SA)	1.5	572 , 586	579
TA1537	Water for injection	0	9 , 10	10
	2'-Fucosyllactose	4.88	12 , 14	13
		19.5	10 , 8	9
		78.1	8 , 11	10
		313	12 , 9	11
		1,250	8 , 8	8
		5,000	8 , 10	9
	9-Aminoacridine (9-AA)	80.0	609 , 605	607
WP2*uvrA* (pKM101)	Water for injection	0	113 , 105	109
	2'-Fucosyllactose	4.88	96 , 94	95
		19.5	96 , 98	97
		78.1	102 , 106	104
		313	96 , 103	100
		1,250	99 , 95	97
		5,000	108 , 113	111
	4-Nitroquinoline N-oxide (4-NQO)	0.1	361 , 383	372

Table 3. The Number of Revertant Colonies per Plate in the Presence of Metabolic Activation (1st and 2nd Main Studies)

Strain	Test substance	Dose (μg/plate)	1st Main study			2nd Main study		
			Individual revertant colony counts	Mean	S.D.	Individual revertant colony counts	Mean	S.D.
TA98	Water for injection	0	35 , 36 , 35	35	1	36 , 34 , 35	35	1
	2'-Fucosyllactose	313	34 , 38 , 38	37	2	37 , 41 , 37	38	2
		625	36 , 36 , 34	35	1	39 , 38 , 41	39	2
		1,250	34 , 38 , 33	35	3	38 , 40 , 41	40	2
		2,500	33 , 36 , 38	36	3	38 , 37 , 36	37	1
		5,000	35 , 36 , 32	34	2	37 , 36 , 36	36	1
	2-Aminoanthracene (2-AA)	1.0	377 , 376 , 351	368	15	344 , 350 , 346	347	3
TA100	Water for injection	0	121 , 124 , 122	122	2	114 , 117 , 119	117	3
	2'-Fucosyllactose	313	121 , 116 , 114	117	4	104 , 106 , 109	106	3
		625	109 , 109 , 119	112	6	107 , 105 , 100	104	4
		1,250	123 , 112 , 118	118	6	110 , 109 , 118	112	5
		2,500	127 , 124 , 127	126	2	110 , 114 , 108	111	3
		5,000	120 , 116 , 112	116	4	112 , 111 , 108	110	2
	2-Aminoanthracene (2-AA)	2.0	979 , 955 , 949	961	16	931 , 949 , 947	942	10
TA1535	Water for injection	0	12 , 12 , 14	13	1	11 , 13 , 13	12	1
	2'-Fucosyllactose	313	14 , 12 , 11	12	2	15 , 12 , 15	14	2
		625	12 , 10 , 10	11	1	14 , 13 , 13	13	1
		1,250	12 , 14 , 15	14	2	14 , 13 , 13	13	1
		2,500	13 , 9 , 10	11	2	10 , 9 , 10	10	1
		5,000	14 , 17 , 14	15	2	11 , 11 , 12	11	1
	2-Aminoanthracene (2-AA)	3.0	175 , 172 , 181	176	5	169 , 174 , 180	174	6
TA1537	Water for injection	0	21 , 22 , 22	22	1	20 , 21 , 20	20	1
	2'-Fucosyllactose	313	25 , 22 , 25	24	2	19 , 20 , 21	20	1
		625	28 , 25 , 23	25	3	19 , 20 , 17	19	2
		1,250	20 , 21 , 24	22	2	19 , 20 , 17	19	2
		2,500	23 , 17 , 20	20	3	24 , 22 , 23	23	1
		5,000	19 , 19 , 22	20	2	23 , 21 , 22	22	1
	2-Aminoanthracene (2-AA)	3.0	227 , 224 , 224	225	2	235 , 242 , 238	238	4
WP2*uvrA* (pKM101)	Water for injection	0	114 , 113 , 114	114	1	120 , 115 , 117	117	3
	2'-Fucosyllactose	313	121 , 118 , 118	119	2	125 , 120 , 127	124	4
		625	121 , 120 , 113	118	4	129 , 124 , 121	125	4
		1,250	120 , 115 , 124	120	5	129 , 126 , 126	127	2
		2,500	118 , 120 , 113	117	4	121 , 125 , 128	125	4
		5,000	112 , 107 , 107	109	3	136 , 126 , 131	131	5
	2-Aminoanthracene (2-AA)	2.0	391 , 401 , 399	397	5	500 , 538 , 512	517	19

S.D.: Standard Deviation

Table 4. The Number of Revertant Colonies per Plate in the Absence of Metabolic Activation (1st and 2nd Main Studies)

Strain	Test substance	Dose (μg/plate)	1st Main study			2nd Main study		
			Individual revertant colony counts	Mean	S.D.	Individual revertant colony counts	Mean	S.D.
TA98	Water for injection	0	26 , 26 , 24	25	1	21 , 22 , 23	22	1
	2'-Fucosyllactose	313	20 , 22 , 20	21	1	20 , 22 , 23	22	2
		625	19 , 20 , 22	20	2	24 , 23 , 22	23	1
		1,250	25 , 22 , 24	24	2	24 , 22 , 21	22	2
		2,500	26 , 26 , 24	25	1	25 , 24 , 23	24	1
		5,000	20 , 20 , 22	21	1	21 , 19 , 20	20	1
	2-Nitrofluorene (2-NF)	5.0	715 , 715 , 724	718	5	730 , 739 , 749	739	10
TA100	Water for injection	0	99 , 99 , 108	102	5	91 , 100 , 96	96	5
	2'-Fucosyllactose	313	113 , 115 , 113	114	1	93 , 96 , 102	97	5
		625	114 , 114 , 106	111	5	91 , 100 , 92	94	5
		1,250	113 , 107 , 113	111	3	91 , 89 , 95	92	3
		2,500	116 , 129 , 120	122	7	107 , 106 , 103	105	2
		5,000	126 , 130 , 138	131	6	96 , 90 , 90	92	3
	Sodium azide (SA)	1.5	743 , 729 , 743	738	8	726 , 743 , 738	736	9
TA1535	Water for injection	0	15 , 14 , 16	15	1	16 , 15 , 16	16	1
	2'-Fucosyllactose	313	15 , 12 , 14	14	2	12 , 14 , 14	13	1
		625	16 , 16 , 16	16	0	17 , 19 , 16	17	2
		1,250	16 , 16 , 19	17	2	14 , 15 , 16	15	1
		2,500	15 , 12 , 17	15	3	13 , 14 , 15	14	1
		5,000	17 , 19 , 18	18	1	16 , 19 , 19	18	2
	Sodium azide (SA)	1.5	577 , 584 , 579	580	4	590 , 593 , 572	585	11
TA1537	Water for injection	0	9 , 8 , 9	9	1	9 , 9 , 10	9	1
	2'-Fucosyllactose	313	10 , 10 , 14	11	2	12 , 10 , 12	11	1
		625	11 , 10 , 13	11	2	8 , 8 , 7	8	1
		1,250	10 , 14 , 13	12	2	10 , 10 , 11	10	1
		2,500	11 , 11 , 9	10	1	11 , 9 , 8	9	2
		5,000	8 , 10 , 10	9	1	10 , 10 , 10	10	0
	9-Aminoacridine (9-AA)	80.0	573 , 565 , 559	566	7	598 , 614 , 610	607	8
WP2*uvrA* (pKM101)	Water for injection	0	97 , 98 , 100	98	2	101 , 104 , 107	104	3
	2'-Fucosyllactose	313	93 , 86 , 93	91	4	104 , 99 , 103	102	3
		625	101 , 100 , 108	103	4	95 , 92 , 94	94	2
		1,250	110 , 106 , 114	110	4	87 , 92 , 96	92	5
		2,500	100 , 101 , 106	102	3	111 , 115 , 114	113	2
		5,000	114 , 113 , 106	111	4	117 , 118 , 125	120	4
	4-Nitroquinoline N-oxide (4-NQO)	0.1	423 , 422 , 424	423	1	565 , 604 , 575	581	20

S.D.: Standard Deviation

Biotoxtech 2019b

FINAL REPORT

In Vitro Mammalian Chromosomal Aberration Test of 2'-Fucosyllactose using Mammalian Cultured Cell

Study No.: B18675

Biotoxtech Co., Ltd.

53, Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si,
Chungcheongbuk-do, 28115, Republic of Korea

SUMMARY

This study was designed to evaluate the potential of the test substance, 2'-Fucosyllactose, to induce chromosomal aberrations in Chinese Hamster Lung (CHL/IU) cells.

In order to determine the high dose level of the main study, a dose range finding study was conducted. The high dose was selected at 5,000 μg/mL and it was sequentially diluted by applying a geometric ratio of 2 to produce lower dose levels (2,500, 1,250, 625, 313, 156, 78.1, 39.1 and 19.5 μg/mL). As a result, cytotoxicity and precipitation of the test substance were not evident in the short time treatments with and without metabolic activation and in the continuous treatment without metabolic activation.

Therefore, the dose levels of the main study were selected as follows. In addition, the positive and negative control groups were set.

Treatment	S9 mix	Dose levels for the main study (μg/mL)
Short time	-/+	5,000, 2,500, 1,250
Continuous	-	5,000, 2,500, 1,250

As a result of the main study, the frequency of cells with chromosome aberrations in the short time treatments with and without metabolic activation and in the continuous treatment without metabolic activation was not statistically significantly different compared to the negative control group.

In the positive control group, the frequency of cells with structural chromosome aberrations in the short time treatments with and without metabolic activation and in the continuous treatment without metabolic activation was statistically significantly increased compared to the negative control group.

Based on the results of this study, the test substance, 2'-Fucosyllactose, did not show any indication to induce chromosome aberrations under the conditions of this study.

1. EXPERIMENTAL OUTLINE

1.1 Purpose

The purpose of this study was to evaluate the potential of the test substance, 2'-Fucosyllactose, to induce chromosomal aberrations in CHL/IU cells.

1.2 Good Laboratory Practice Regulations

This study was conducted in accordance with the following Good Laboratory Practice Regulation:

- "Good Laboratory Practice Regulation for Nonclinical Laboratory Studies"

  Notification No. 2017-32, Ministry of Food and Drug Safety, Republic of Korea (May 1, 2017)

1.3 Regulatory Guidelines

This study was conducted in accordance with the following guidelines:

- "Standards for Toxicity Studies of Drugs"

  Notification No. 2017-71, Ministry of Food and Drug Safety, Republic of Korea (Aug. 30, 2017)

- "OECD Guideline for the Testing of Chemicals, 473, *In Vitro* Mammalian Chromosomal Aberration Test"

  Organisation for Economic Co-operation and Development (Adopted: Jul. 29, 2016)

1.4 Sponsor

Name	Advanced Protein Technologies Corp.		
Address	7th Floor GyeongGi-BioCenter; 147, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16229, Republic of Korea		
TEL	+ 82-31-888-6245	FAX	+ 82-31-888-6247

2. MATERIALS AND METHODS

2.1 Test Substance

2.1.1	Name	2'-Fucosyllactose
2.1.2	Lot No.	2'-FL-CG-008
2.1.3	Appearance	Light white-yellowish powder
2.1.4	Structural formula	$C_{18}H_{32}O_{15}$
2.1.5	Molecular weight	488.44 g/mol
2.1.6	Purity	97.56%
2.1.7	Date of manufacture	Sep. 5, 2018
2.1.8	Expiration date (retest date)	Sep. 4, 2019 (one year after manufacture)
2.1.9	Storage condition	Room temperature (1–30°C)
2.1.10	Handling instructions	Wear a mask, a pair of gloves and protective equipment.
2.1.11	Supplier	
	Name	Advanced Protein Technologies Corp.
	Address	7th Floor GyeongGi-BioCenter; 147, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16229, Republic of Korea
2.1.12	Disposition of test substance	Any remaining test substance is returned to the sponsor.

2.2 Negative Control

2.2.1	Name	Water for injection
2.2.2	Lot No.	17012
2.2.3	Storage condition	Room temperature
2.2.4	Manufacturer	JW Pharmaceutical Co., Ltd., Republic of Korea
2.2.5	Justification for selection	Water for injection, the vehicle of the test substance, was used as the negative control.

2.3 Positive Controls

Name	Lot No.	Storage condition	Manufacturer
Mitomycin C (MMC)	MKBZ9075V	Refrigeration	SIGMA-ALDRICH, CO., U.S.A.
Benzo[a]pyrene (B[a]P)	SLBV8459	Room temperature	SIGMA-ALDRICH, CO., U.S.A.

2.4 Preparation and Analysis of the Dosing Formulations

2.4.1 Preparation of dosing formulations of the test substance

2.4.1.1 Vehicle

2.4.1.1.1 Name Water for injection

2.4.1.1.2 Lot No. 17012

2.4.1.1.3 Justification for selection

In order to produce a 10-fold stock (aqueous solution) of 5,000 μg/mL, which is the high dose of the dose range finding study, a preliminary solubility test was conducted. As a result, the test substance was dissolved in water for injection. Therefore, water for injection was selected as the vehicle for this study.

2.4.1.2 Preparation method

All preparations were conducted on the day of treatment of dosing formulations (dose range finding study) or on the day of analysis of dosing formulations (main study).

In order to produce a 10-fold stock of the high dose level, the required amount of the test substance was weighed (CP323S, Sartorius, Germany) with a purity factor (1.025). A small amount of vehicle (water for injection) was added and the both materials were mixed using a vortex mixer until dissolved. Vehicle was added to yield the desired dose level. The high dose formulation was serially diluted to produce lower dose levels.

In the main study, the dosing formulations were stored in a refrigerator and used within a period of stability (8 days).

2.4.1.3 Analysis of dosing formulations

2.4.1.3.1 Homogeneity and stability

As a result of analysis for homogeneity and stability conducted in the study of "An Analytical Method Validation of 2'-Fucosyllactose Dosing Formulations by HPLC (Biotoxtech Study No.: B18670)", the 0.1 and 750 mg/mL dosing solutions including the dose concentrations of the main study were confirmed to be homogenous and stable for 4 hours at room temperature and for 8 days under refrigeration.

2.4.1.3.2 Verification of dose concentrations

Analysis of the dosing formulations was conducted using a HPLC (Prominence, Shimadzu Corp., Japan).

Analysis of the dosing formulations was conducted based on the method used in the study of "An Analytical Method Validation of 2'-Fucosyllactose Dosing Formulations by HPLC (Biotoxtech Study No.: B18670)" and the samples were taken three times from the middle layer of each dosing formulation prior to treatment and analyzed for verification of dose concentration.

As a result of analysis of the dosing formulations, the precision and accuracy of the dosing formulation were in the ranges of 1.98–2.76% and 90.72–92.80%, respectively. The results were considered to be acceptable because the precision was within 10% and the accuracy was in the range of 80–115% (Appendix VI).

2.4.2 Preparation of the positive controls

MMC was dissolved in dimethyl sulfoxide (DMSO, Lot No.: K49393831, Merck, Germany) with a vortex mixer to yield a stock concentration of 10 μg/mL.

The required amount of B[a]P was weighed and dissolved in DMSO (Lot No.: K49393831) with a vortex mixer to yield a stock concentration of 2,000 μg/mL.

The prepared positive controls were stored in a deep freezer (-80—60°C, OPR-DFU-657CEV, Operon, Republic of Korea) and thawed just prior to use.

Treatment	S9 mix	Name	Stock concentration (μg/mL)	Final concentration (μg/mL)
Short time	-	MMC	10	0.1
	+	B[a]P	2,000	20
Continuous	-	MMC	10	0.1

2.5 Cell Line

2.5.1 Cell Line

CHL/IU cells

2.5.2 Justification for selection

CHL/IU cell line has high detection sensitivity, is commonly used in *in vitro* chromosome aberration studies and recommended in the regulatory guideline.

2.5.3 Receipt and storage

CHL/IU cell line was purchased from American Type Culture Collection (ATCC, U.S.A.) on Nov. 24, 2011. Cells were seeded in a 75 cm^2 flask (Nunc, Denmark) containing Eagle's Minimum Essential Medium (EMEM, Lonza Walkersville Inc., U.S.A.) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, U.S.A.) and incubated in a 5% CO_2 incubator (MCO-20AIC, SANYO, Japan) at 37°C.

<Characteristics of CHL/IU>

ATCC® Catalog No.	CRL-1935
Lot No.	3375917
Modal chromosome number	25
Organism	*Cricetulus griseus* (hamster, Chinese)
Tissue	lung
Morphology	fibroblast
Growth properties	adherent
Doubling time	approximately 15 hours

Cells were evaluated for contamination of mycoplasma using a Hoechst Stain Kit (MPBIOMEDICALS, Japan). 0.25% Trypsin-EDTA solution (SIGMA-ALDRICH, CO., U.S.A.) was added to the culture flask to detach cells from the bottom. The suspended cells were harvested, placed in a tube and centrifuged at 1,000 rpm for 5 minutes and the supernatant was removed. The pellets were resuspended with an appropriate amount of FBS to yield a concentration of 1×10^6 cells/mL and DMSO was added to a final concentration of 10%. Cell suspension was transferred into cryogenic vials, stored at -80—60°C for one day and stored in a liquid nitrogen tank until use.

2.5.4 Sub-culture

Frozen cells were thawed in a water bath at 37°C and transferred into a 50 mL tube containing EMEM supplemented with 10% FBS and centrifuged at 1,000 rpm for 5 minutes. The supernatant was removed and the pellets were resuspended with EMEM supplemented with 10% FBS. Suspended cells were transferred to a 75 cm^2 flask and incubated in a 5% CO_2 incubator at 37°C.

Cell morphology was evaluated following 70–80% proliferation on the bottom of the flask. The 0.25% Trypsin-EDTA solution (Gibco, U.S.A.) was added to detach cells. The suspended cells were harvested, transferred into a 50 mL tube and centrifuged at 1,000 rpm for 5 minutes. The supernatant was removed and the pellets were resuspended with EMEM supplemented with 10% FBS. Suspended cells were transferred into a 75 cm^2 flask and incubated in a 5% CO_2 incubator at 37°C.

2.5.5 Pre-incubation

Cells within 29 passages were used in this study.

Exponentially growing stock cultures were treated with the 0.25% Trypsin-EDTA solution (Gibco, U.S.A.) to separate cells from the bottom of the culture flask. The harvested cells were placed in a 50 mL tube and centrifuged at 1,000 rpm for 5 minutes. The supernatant was decanted and the pellets were resuspended in an appropriate volume of EMEM at 5×10^4 cells/mL. The suspended cells were placed in a 6 well plate (2 mL/well, Nunc, Denmark) for the dose range finding study and in a 60 mm plate (5 mL/plate, BD, U.S.A.) and 6 well plate (2 mL/well) for the main study. Cells were incubated in a 5% CO_2 incubator at 37°C for one day. An identification number was marked on each plate.

2.6 Culture Medium

EMEM supplemented with heat-inactivated FBS up to 10% was mixed with a penicillin-streptomycin mixture containing 10,000 units/mL penicillin G sodium and 10,000 μg/mL streptomycin sulfate (Gibco, U.S.A.) at a ratio of 100 to 1. The prepared culture medium was stored in a refrigerator (2–8°C) until use.

2.7 Preparation of S9 Mix

2.7.1 Receipt and storage

S9 and Cofactor C were purchased from ORIENTAL YEAST Co., LTD. in Japan, stored in a deep freezer (-80—60°C) and used within the expiration date.

<Characteristics of S9>

Test system	Sprague-Dawley rat [Crl:CD(SD)]
Sex and age	Male, 7 weeks old
Organ	Liver
Inducing agent	Phenobarbital (PB) and 5,6-benzoflavone (BF)
Dose and frequency	PB: 30 mg/kg, once (Day 1) 60 mg/kg, once daily for 3 consecutive days (Days 2–4) BF: 80 mg/kg, once (Day 3)
Route of administration	Intraperitoneal injection

2.7.2 Composition of S9 mix

Component		Amount of each component
S9		0.3 mL
Cofactor C	50 mmol/L $MgCl_2$	0.1 mL (5 µmol)
	330 mmol/L KCl	0.1 mL (33 µmol)
	50 mmol/L Glucose-6-phosphate	0.1 mL (5 µmol)
	40 mmol/L NADP	0.1 mL (4 µmol)
	20 mmol/L HEPES buffer (pH 7.2)	0.2 mL (4 µmol)
	Purified water	0.1 mL
Total volume		1 mL

2.7.3 Preparation method of S9 mix

Preparation of S9 mix was conducted immediately prior to use. The frozen S9 (Lot Nos.: 18070604 (dose range finding study), 18080305 (main study)) and Cofactor C (Lot Nos.: C18070404 (dose range finding study), C18080105 (main study)) were thawed and mixed at a ratio of 2 to 4.7.

2.8 Dose Range Finding Study

A dose range finding study was conducted under non-GLP conditions to determine the dose levels for the main study.

2.8.1 Dose levels

The high dose of the test substance was 5,000 µg/mL, which is required in the test guidelines. The high dose was sequentially diluted to produce 8 lower dose levels (2,500, 1,250, 625, 313, 156, 78.1, 39.1 and 19.5 µg/mL). In addition, the negative control group was set.

2.8.2 Treatment method

After pre-incubation, each plate was divided into three groups: short time treatments with and without metabolic activation and continuous treatment without metabolic activation. One well was used per dose.

All treatment mixtures were prepared and treated as follows.

Treatment	S9 mix	Treatment groups	Used volume (mL)			Treated volume (mL/well)
			EMEM with 10% FBS	S9 mix	Negative control or Dosing formulation of the test substance	
Short time	-	Negative control	2.7	-	0.3	2
		Test substance	2.7		0.3	2
	+	Negative control	2.2	0.5	0.3	2
		Test substance	2.2		0.3	2
Continuous	-	Negative control	2.7	-	0.3	2
		Test substance	2.7		0.3	2

In the short time treatments with and without metabolic activation, cells were treated with the test substance for 6 hours and each well was washed with Dulbecco's phosphate-buffered saline (D-PBS, Lonza Walkersville Inc., U.S.A.). Then, fresh medium was added and cells were cultured for 18 hours more.

In the continuous treatment without metabolic activation, cells were treated with the test substance for 24 hours. In each of the short time treatments with and without metabolic activation and the continuous treatment without metabolic activation, cells were incubated in a 5% CO_2 incubator at 37°C.

The presence or absence of precipitation of the test substance was checked immediately after the addition of the test substance, at the end of treatment and at culture completion.

After the test substance was added in the medium, the pH and osmolality were measured for the negative control group and high dose group. As a result, the pH and osmolality in the high dose group of the test substance did not change by more than 1.0 and 50 mOsm/kg, respectively, compared to the negative control group. In addition, a change in color of culture medium was not observed due to a change in pH. Therefore, the pH and osmolality in the lower dose groups of the test substance were not measured.

2.8.3 Calculation of relative population doubling (RPD)

At pre-incubation in the dose range finding study, one well was prepared for the satellite control group.

The number of cells was counted immediately after the treatment of the test substance in the satellite control group and at culture completion in the test substance group using a hemocytometer and the RPD was calculated.

$$\text{RPD (\%)} = \frac{\text{(No. of population doubling in treated cultures)}}{\text{(No. of population doubling in control cultures)}} \times 100$$

Population doubling = [log (Post-treatment cell number/Initial cell number)]/log 2

2.8.4 Justification for selection of main study dose level

2.8.4.1 Results of the dose range finding study
(Table 1)

Cytotoxicity and precipitation of the test substance were not evident in the short time treatments with and without metabolic activation and in the continuous treatment without metabolic activation.

2.8.4.2 Dose levels of the main study

The high dose level of the main study in the short time treatments with and without metabolic activation and in the continuous treatment without metabolic activation was selected at 5,000 μg/mL. It was sequentially diluted by applying a geometric ratio of 2 to produce 2 lower dose levels of the test substance. In addition, the positive and negative control groups were set.

Treatment	S9 mix	Dose levels for the main study (μg/mL)
Short time	-/+	5,000, 2,500, 1,250
Continuous	-	5,000, 2,500, 1,250

2.9 Main Study

2.9.1 Treatment method

After pre-incubation, each plate was divided into three groups: short time treatments with and without metabolic activation and continuous treatment without metabolic activation. Two plates were used per dose.

All treatment mixtures were prepared and treated as follows.

Treatment	S9 mix	Treatment groups	Used volume (mL)			Treated volume (mL/plate)
			EMEM with 10% FBS	S9 mix	Negative (Positive) control or Dosing formulation of the test substance	
Short time	-	Negative control	11.7	-	1.3	5
		Test substance	11.7		1.3	5
		Positive control	12.87		0.13	5
	+	Negative control	9.53	2.17	1.3	5
		Test substance	9.53		1.3	5
		Positive control	10.70		0.13	5
Continuous	-	Negative control	11.7	-	1.3	5
		Test substance	11.7		1.3	5
		Positive control	12.87		0.13	5

In the short time treatments with and without metabolic activation, cells were treated with the test substance for 6 hours and each plate was washed with D-PBS. Then, the fresh medium was added and cells were cultured for 18 hours more.

In the continuous treatment without metabolic activation, cells were treated with the test substance for 24 hours. In each of the short time treatments with and without metabolic activation and the continuous treatment without metabolic activation, cells were incubated in a 5% CO_2 incubator at 37°C.

The presence or absence of precipitation of the test substance was checked immediately after the treatment of the test substance, at the end of treatment and at culture completion (before colcemid treatment).

In the dose range finding study, the pH and the osmolality were not changed by more than 1.0 and 50 mOsm/kg, respectively, compared to the negative control group. Therefore, the pH and osmolality were not measured in the main study.

2.9.2 Calculation of RPD

The calculation of RPD was performed using the same method and conditions as the dose range finding study. One well per dose was used in the main study.

In the main study, suspended cells were placed in a 6 well plate (2 mL/well). The dosing formulations of the test substance and the positive and negative control substances were treated under each treatment conditions. The number of cells was counted immediately after the treatment of the test substance in the satellite control group and at culture completion in the test substance group using a hemocytometer, and the RPD was calculated.

2.9.3 Slide preparation

Two hours prior to culture completion, colcemid (Gibco, U.S.A.) was added to yield a final concentration of 0.2 μg/mL in order to arrest cells in metaphase. Following culture completion, cells were treated with the 0.25% Trypsin-EDTA solution (Gibco, U.S.A.), centrifuged at 1,000 rpm for 5 minutes (FLETA 5, Hanil Science Industrial CO., Ltd., Republic of Korea) and incubated in 5 mL of 0.075 mol/L KCl solution (pre-warmed at 37°C) at 37°C for 20 minutes. Then, cells were treated with 1 mL of ice-cold fixative (methanol:acetic acid = 3:1) and centrifuged at 1,000 rpm for 5 minutes. The supernatant was decanted and cells were fixed with 5 mL of ice-cold fixative and centrifuged at 2,000 rpm for 5 minutes. These procedures were repeated once more. Two drops of the suspension were placed on a clean dry slide to prepare one sample slide. The slides were air-dried and identified with random numbers. The slides were stained with 3% Giemsa solution for 20 minutes and washed with ultra pure water. Then, the slides were air-dried and mounted by dropping mounting medium (Entellan®new, Merck, Germany).

2.10 Observations

The observation of slides was conducted in the order of the short time treatments and continuous treatment.

Dose levels for chromosome observations were selected at 3 dose levels, at which 300 metaphases could be observed.

Three hundred metaphases per dose were observed using a microscope (600-fold magnification, BX51, Olympus, Japan).

Chromosomal aberrations were classified into structural aberration, numerical aberration and other.

Structural aberrations were classified into chromatid break (ctb), chromatid exchange (cte), chromosome break (csb), chromosome exchange (cse), chromatid gap (ctg), chromosome gap (csg) and fragmentation (frg). When several gaps and breaks were observed in metaphase, they were recorded as frg. An achromatic lesion narrower than the width of a chromatid was defined as a gap.

In addition, numerical aberrations were classified into polyploidy (pol) and endoreduplication (end).

For the aforementioned aberrations, any cell with one or more aberrations was counted as an aberrant cell. For the gap, the number of cells including and excluding gaps was scored and recorded.

Others which were not classified into structural and numerical aberrations were recorded for the type and number of cells with aberrations.

2.11 Acceptance Criteria

Evaluation of validity of the study result was conducted based on the following criteria:

- The frequency of cells with structural chromosome aberrations in the negative control groups is within the range of historical control data and the 95% control limits of the distribution of the historical control data.
- The frequency of cells with structural chromosome aberrations in the positive control group is within the range of historical control data and statistically significantly increased compared to the negative control group.
- At least 300 metaphases per dose are observed in the treatment groups, and the positive and negative control groups.
- At least three dose levels are used for the evaluation in the treatment groups.

2.12 Evaluation Criteria

The results were considered to be positive when the frequency of cells with chromosome aberrations (excluding gap) met all of the following conditions:

- The frequency of cells with chromosome aberrations shows a statistically significant increase at more than one dose level in the test substance groups compared to the negative control group.
- The cells with chromosome aberrations are increased in a dose-dependent manner.
- The frequency of cells with chromosome aberrations increases over the 95% control limits of distribution of the historical control data in the negative control group.

2.13 Statistical Analysis

Statistical analysis on the frequency of cells with chromosome aberrations (excluding gap) was performed using SAS Program (version 9.3, SAS Institute Inc., U.S.A.).

For the aberration cell data, Fisher's exact test was used for the comparison of the negative control group to the test substance group or the positive control group (significance levels: 0.05 and 0.01, two-tailed).

Table 1. Summary Results of the Dose Range Finding Study

Test substance	Dose (μg/mL)	S9 mix	Trt-Rec time (hr)	Relative Population Doubling (%)
Water for injection	0	-	6-18	100
2'-Fucosyllactose	19.5	-	6-18	98.2
	39.1	-	6-18	97.7
	78.1	-	6-18	98.2
	156	-	6-18	97.3
	313	-	6-18	96.3
	625	-	6-18	96.8
	1,250	-	6-18	96.3
	2,500	-	6-18	92.0
	5,000	-	6-18	89.5
Water for injection	0	+	6-18	100
2'-Fucosyllactose	19.5	+	6-18	94.6
	39.1	+	6-18	93.1
	78.1	+	6-18	92.6
	156	+	6-18	93.6
	313	+	6-18	93.1
	625	+	6-18	93.6
	1,250	+	6-18	92.6
	2,500	+	6-18	93.6
	5,000	+	6-18	91.0
Water for injection	0	-	24-0	100
2'-Fucosyllactose	19.5	-	24-0	97.8
	39.1	-	24-0	98.2
	78.1	-	24-0	97.3
	156	-	24-0	97.3
	313	-	24-0	97.3
	625	-	24-0	94.5
	1,250	-	24-0	94.5
	2,500	-	24-0	93.6
	5,000	-	24-0	88.2

Trt-Rec time: Treatment-Recovery times

Table 2. Summary Results of the Main Study

Test substance	Dose (μg/mL)	RPD (%)	S9 mix	Trt-Rec time (hr)	No. of cell analyzed	Number of cells with structural aberrations									Number of cells with numerical aberrations			Others[a]
						ctb	csb	cte	cse	frg	gap		total (%)		end	pol	total (%)	
											ctg	csg	gap-	gap+				
Water for injection	0	100	-	6-18	150	0	0	0	0	0	0	0	1 (0.3)	1 (0.3)	0	1	1 (0.3)	0
					150	0	0	1	0	0	0	0			0	0		
2'-Fucosyllactose	1,250	98.1	-	6-18	150	0	0	0	0	0	1	0	0 (0.0)	1 (0.3)	0	0	1 (0.3)	0
					150	0	0	0	0	0	0	0			0	1		
	2,500	96.2	-	6-18	150	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0	0	1 (0.3)	0
					150	0	0	0	0	0	0	0			0	1		
	5,000	92.2	-	6-18	150	0	0	0	0	0	1	0	2 (0.7)	3 (1.0)	0	0	0 (0.0)	0
					150	0	0	2	0	0	0	0			0	0		
MMC	0.1	59.2	-	6-18	150	7	0	22	0	0	1	0	62** (20.7)	64 (21.3)	0	0	0 (0.0)	0
					150	10	0	28	0	0	1	0			0	0		
Water for injection	0	100	+	6-18	150	1	0	0	0	0	1	0	1 (0.3)	2 (0.7)	0	0	1 (0.3)	0
					150	0	0	0	0	0	0	0			1	0		
2'-Fucosyllactose	1,250	97.1	+	6-18	150	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0	0	0 (0.0)	0
					150	0	0	0	0	0	0	0			0	0		
	2,500	95.1	+	6-18	150	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0	0	0 (0.0)	0
					150	0	0	0	0	0	0	0			0	0		
	5,000	91.0	+	6-18	150	0	0	0	0	0	0	0	1 (0.3)	1 (0.3)	0	0	1 (0.3)	0
					150	0	0	1	0	0	0	0			0	1		
B[a]P	20	51.5	+	6-18	150	4	0	27	0	0	2	0	66** (22.0)	68 (22.7)	0	0	1 (0.3)	0
					150	5	0	34	0	0	0	0			0	1		
Water for injection	0	100	-	24-0	150	0	0	0	0	0	1	0	1 (0.3)	2 (0.7)	0	0	1 (0.3)	0
					150	0	0	1	0	0	0	0			0	1		
2'-Fucosyllactose	1,250	96.8	-	24-0	150	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0	0	0 (0.0)	0
					150	0	0	0	0	0	0	0			0	0		
	2,500	94.5	-	24-0	150	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0	0	1 (0.3)	0
					150	0	0	0	0	0	0	0			0	1		
	5,000	89.1	-	24-0	150	0	0	0	0	0	1	0	0 (0.0)	1 (0.3)	0	0	1 (0.3)	0
					150	0	0	0	0	0	0	0			0	1		
MMC	0.1	52.3	-	24-0	150	10	0	54	0	0	1	0	117** (39.0)	119 (39.7)	0	0	0 (0.0)	0
					150	13	0	48	0	0	1	0			0	0		

Aberration: ctg: chromatid gap, csg: chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange, frg: fragmentation, end: endoreduplication, pol: polyploidy

MMC: Mitomycin C, B[a]P: Benzo[a]pyrene

RPD: Relative Population Doubling, Trt-Rec time: Treatment-Recovery times

gap-: Total number of cells with structural aberrations excluding gap, gap+: Total number of cells with structural aberrations including gap

a): Others were excluded from the number of cells with chromosomal aberrations

Significant difference from negative control by Fisher's exact test: ** $p<0.01$

Table 3. Historical Control Data

Historical control values of structural aberrations

Group	S9 mix	Trt-Rec time (hr)	N	Structural aberration cells excluding gap (%) (Mean±S.D.)			Range (%)		95% control limit[c] [Structural aberration cells/300 cells]	
							MIN	MAX	MIN	MAX
Negative	-	6-18	44	0.288	±	0.364	0	1.01*	0	≤3
	+	6-18	44	0.311	±	0.390	0	1.09*	0	≤3
	-	24-0	42	0.246	±	0.361	0	0.87*	0	≤2
Positive	-	6-18[a]	39	23.44	±	5.667	11.09*	35.78*		
	+	6-18[b]	39	24.64	±	4.922	12.13*	37.15*		
	-	24-0[a]	37	35.37	±	6.862	19.09*	51.65*		

Historical control values of numerical aberrations

Group	S9 mix	Trt-Rec time (hr)	N	Numerical aberration cells (%) (Mean±S.D.)			Range (%)		95% control limit[c] [Numerical aberration cells/300 cells]	
							MIN	MAX	MIN	MAX
Negative	-	6-18	44	0.174	±	0.292	0	0.83*	0	≤2
	+	6-18	44	0.167	±	0.264	0	0.97*	0	≤2
	-	24-0	42	0.262	±	0.290	0	1.13*	0	≤2

Negative control: Water for injection, Dimethyl sulfoxide, Acetone

Trt-Rec time: Treatment-Recovery times

a) Mitomycin C (0.1 μg/mL)

b) Benzo[a]pyrene (20 μg/mL)

c) Poisson-based 95% control limits of the historical negative control data.

N: The total number of chromosome aberration test

The above historical control values were obtained from the data pooled from Jul. 15, 2013 to May 22, 2017.

* The range was calculated by the control limit of X derived from $\bar{X}$-$\bar{R}$-$\bar{Rs}$ value.

Biotoxtech 2019 C

FINAL REPORT

In Vivo Micronucleus Test of 2'-Fucosyllactose in ICR Mice

Study No.: B18676

Biotoxtech Co., Ltd.

53, Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si,
Chungcheongbuk-do, 28115, Republic of Korea

SUMMARY

This study was designed to evaluate the potential of the test substance, 2'-Fucosyllactose, to induce micronuclei in bone marrow cells of mice when the test substance was orally administered via gastric intubation twice at 24-hour intervals.

In order to determine the high dose level of the main study, a dose range finding study was conducted. The high dose was set at 7,500 mg/kg and it was sequentially diluted to produce 3 lower dose levels (5,000, 2,500 and 1,250 mg/kg). As a result, there were no clinical signs or mortality at any dose level of the test substance in male and female mice.

Therefore, the high dose level of the main study was set at 7,500 mg/kg and two additional lower dose levels (5,000 and 2,500 mg/kg) were produced. In addition, the positive and negative control groups were set.

Since there was no mortality in either sex as a result of the dose range finding study, the main study was conducted with only males, which are known to be susceptible to micronucleus induction.

As a result of the main study, the incidence of micronucleated polychromatic erythrocytes (MNPCE) in polychromatic erythrocytes (PCE) in the test substance groups was not statistically significantly different from the negative control group. In addition, the ratio of PCE to total erythrocytes in the test substance groups was not statistically significantly different from the negative control group.

In the positive control group, the incidence of MNPCE in PCE was statistically significantly increased when compared to the negative control group. The ratio of PCE to total erythrocytes in the positive control group was not statistically significantly different from the negative control group.

Based on these results, the test substance, 2'-Fucosyllactose, did not have any potential to induce micronuclei formation in bone marrow cells of mice under the conditions of this study.

1. EXPERIMENTAL OUTLINE

1.1 Purpose

The purpose of this study was to evaluate the potential of the test substance, 2'-Fucosyllactose, to induce micronuclei in bone marrow cells of mice.

1.2 Good Laboratory Practice Regulations

This study was conducted in accordance with the following Good Laboratory Practice Regulation:

- "Good Laboratory Practice Regulation for Nonclinical Laboratory Studies"

  Notification No. 2017-32, Ministry of Food and Drug Safety, Republic of Korea (May 1, 2017)

1.3 Regulatory Guidelines

This study was conducted in accordance with the following guidelines:

- "Standards for Toxicity Studies of Drugs"

  Notification No. 2017-71, Ministry of Food and Drug Safety, Republic of Korea (Aug. 30, 2017)

- "OECD Guideline for the Testing of Chemicals, 474, Mammalian Erythrocyte Micronucleus Test"

  Organisation for Economic Co-operation and Development (Adopted: Jul. 29, 2016)

1.4 Animal Ethics

This study was reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of Biotoxtech Co., Ltd. based on Animal Protection Act (Enactment May 31, 1991, No. 4379, Revision Mar. 20, 2018, No. 15502) (Approval No.: 180689).

1.5 Veterinary Care

All procedures in this study were in compliance with the Animal Protection Act of Republic of Korea, the Guide for the Care and Use of Laboratory Animals.

1.6 Sponsor

Name	Advanced Protein Technologies Corp.		
Address	7th Floor GyeongGi-BioCenter; 147, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16229, Republic of Korea		
TEL	+ 82-31-888-6245	FAX	+ 82-31-888-6247

1.7 Test Facility

Name	Biotoxtech Co., Ltd.		
Address	53, Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si, Chungcheongbuk-do, 28115, Republic of Korea		
TEL	+ 82-43-210-7777	FAX	+ 82-43-210-7778

1.8 Study Director

Name	Seung-Young Hong
Position	Toxicity Team 2

1.9 Study Schedule

Study initiation	Oct. 25, 2018
Experimental start	Dec. 3, 2018
Dose range finding study	
- Animal receipt	Oct. 30, 2018
- Administration	Nov. 5–6, 2018
- Observation of clinical signs	Nov. 5–7, 2018
Main study	
- Animal receipt	Nov. 27, 2018
- Completion of quarantine and acclimation	Dec. 3, 2018
- Group assignment	Dec. 3, 2018
- Administration	Dec. 3–4, 2018
- Slide preparation and staining	Dec. 5–10, 2018
- Slide observation	Dec. 17–24, 2018
Experimental completion	Dec. 24, 2018
Study completion	Feb. 14, 2019

2. MATERIALS AND METHODS

2.1 Test Substance

2.1.1	Name	2'-Fucosyllactose
2.1.2	Lot No.	2'-FL-CG-008
2.1.3	Appearance	Light white-yellowish powder
2.1.4	Structural formula	$C_{18}H_{32}O_{15}$
2.1.5	Molecular weight	488.44 g/mol
2.1.6	Purity	97.56%
2.1.7	Date of manufacture	Sep. 5, 2018
2.1.8	Expiration date (retest date)	Sep. 4, 2019 (one year after manufacture)
2.1.9	Storage condition	Room temperature (1–30°C)
2.1.10	Handling instructions	Wear a mask, a pair of gloves and protective equipment.
2.1.11	Supplier	
	Name	Advanced Protein Technologies Corp.
	Address	7th Floor GyeongGi-BioCenter; 147, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16229, Republic of Korea
2.1.12	Disposition of test substance	Any remaining test substance is returned to the sponsor.

2.2 Negative Control

2.2.1	Name	Water for injection
2.2.2	Lot No.	DKN18004
2.2.3	Storage condition	Room temperature
2.2.4	Manufacturer	JW Pharmaceutical Co., Ltd., Republic of Korea

2.2.5 Justification for selection — Water for injection, the vehicle of the test substance, was used as the negative control.

2.3 Positive Control

2.3.1	Name	Mitomycin C (MMC)
2.3.2	Lot No.	SLBR6518V
2.3.3	Storage condition	Refrigeration
2.3.4	Manufacturer	SIGMA-ALDRICH, Co., U.S.A.

2.4 Preparation and Analysis of the Dosing Formulations

2.4.1 Preparation of dosing formulations of the test substance

2.4.1.1 Vehicle

2.4.1.1.1	Name	Water for injection
2.4.1.1.2	Lot No.	DKN18004

2.4.1.1.3 Justification for selection

In order to produce a high dose level (750 mg/mL) for the dose range finding study, a preliminary solubility test was conducted. As a result, the test substance was dissolved or suspended uniformly in water for injection. Therefore, water for injection was selected as the vehicle for this study.

2.4.1.2 Preparation method

The required amount of the test substance was weighed (ENTRIS423i-1S, CP423S, Sartorius, Germany) with a purity factor (1.025). A small amount of vehicle (water for injection) was added and the both materials were mixed using a vortex mixer until dissolved or suspended uniformly. Vehicle was added to yield the desired dose levels.

The dosing formulations were confirmed to be stable for 4 hours at room temperature and for 8 days under refrigeration, and these dosing formulations were used within 8 days.

2.4.1.3 Analysis of the dosing formulations

2.4.1.3.1 Homogeneity and stability

As a result of analysis for homogeneity and stability conducted in the study of "An Analytical Method Validation of 2'-Fucosyllactose Dosing Formulation by HPLC (Biotoxtech Study No.: B18670)", the 0.1 and 750 mg/mL dosing solutions comprising the dose concentrations of the main study were confirmed to be homogenous and stable for 4 hours at room temperature and for 8 days under refrigeration.

2.4.1.3.2 Verification of dose concentrations

Analysis of the dosing formulation was conducted using a HPLC (Prominence, Shimadzu Corp., Japan).

Analysis of the dosing formulation was conducted based on the method used in the study of "An Analytical Method Validation of 2'-Fucosyllactose Dosing Formulation by HPLC (Biotoxtech Study No.: B18670)" and samples were taken three times from the middle layer of each dosing formulation prior to administration and analyzed for verification of dose concentration.

As a result of analysis of the dosing formulations, the precision and accuracy of the dosing formulations were in the ranges of 0.82–1.82% and 90.20–93.03%, respectively. The results were considered to be acceptable because the precision was within 10% and the accuracy was in the range of 85–115% (Appendix V).

2.4.2 Preparation of the positive control

Two mg of MMC was dissolved in 4 mL of water for injection (Lot No.: 17012, JW Pharmaceutical Co., Ltd., Republic of Korea). Six mL of normal saline injection (Lot No.: 18036, JW Pharmaceutical Co., Ltd., Republic of Korea) was added to yield the desired concentration of 0.2 mg/mL.

The prepared positive control was stored in a deep freezer (-80–-60°C, OPR-DFU-657CEV, Operon, Republic of Korea) and thawed just prior to use.

2.5 Test System

2.5.1 Species and strain Mouse, CrlOri:CD1(ICR), SPF

2.5.2 Producer and supplier ORIENTBIO INC., Republic of Korea

2.5.3 Justification for species and strain selection

ICR mice are commonly used in toxicity studies with a large historical control data base. In addition, the mouse is used as recommended species in the test guideline.

2.5.4 Sex, number of animals, age and body weights range (at receipt)

2.5.4.1 Dose range finding study: Male, 17 animals, 7 weeks old, 27.7–31.7g
Female, 17 animals, 7 weeks old, 23.1–26.1 g

2.5.4.2 Main study: Male, 27 animals, 7 weeks old, 27.8–31.0 g

(Females were not used in the main study because there was no sex difference in mortality in the dose range finding study.)

2.5.5 Sex, number of animals, age and body weights range (at the start of administration)

2.5.5.1 Dose range finding study: Male, 15 animals, 8 weeks old, 33.8–36.2 g
Female, 15 animals, 8 weeks old, 26.7–29.5 g

2.5.5.2 Main study: Male, 25 animals, 8 weeks old, 32.3–36.1 g

2.5.6 Quarantine and acclimation

Body weights were weighed (CP3202S, Sartorius, Germany) after visual inspection on the day of receipt. All animals were quarantined and acclimated for 7 days and observed once daily for clinical signs. The animals were moved to an animal room after they were acclimated in a quarantine room for 3 days.

After the quarantine-acclimation period, the evaluation of health condition was conducted after the examination of clinical signs and body weight changes.

2.5.7 Animal and cage identification

During the acclimation period, a temporary identification number was marked on the tail using a red indelible pen. A temporary cage card was placed on each cage during the quarantine-acclimation period.

Following group assignment, the animals were uniquely identified by a blue indelible marking on the tail. A color-coded cage card was placed on each cage describing the group and dose level.

2.5.8 Group assignment

The group assignment was conducted on animals showing no abnormal clinical signs or weight change on the last day of the acclimation period. The weight variation of animals did not exceed ±20% of the mean body weight. Subsequently, the required number of animals was selected (dose range finding study: 15 males and females, main study: 25 males). Animals were randomly assigned to groups in an attempt to equalize mean group body weights.

2.5.9 Disposition of remaining animals

Remaining animals not selected for the study were excluded from the test system.

2.6 Animal Husbandry

2.6.1	Quarantine Room No.	A314
2.6.2	Animal Room No.	A320

2.6.3 Type & size of cage

2.6.3.1 Polycarbonate cage, 260W×420D×180H (mm)

2.6.3.2 Polycarbonate cage, 200W×260D×130H (mm)

2.6.4 Number of animals per cage

2.6.4.1	Quarantine-acclimation period	8–9 mice/cage
2.6.4.2	Testing period	3–5 mice/cage
2.6.5	Temperature	20.9–22.4°C (measurement value, A314), 21.1–22.6°C (measurement value, A320) (permissible range: 19.0–25.0°C)
2.6.6	Relative humidity	49.4–58.2% (measurement value, A314), 49.5–63.3% (measurement value, A320) (permissible range: 40.0–70.0%)
2.6.7	Air changes	10–15 clean, fresh, filtered air changes per hour
2.6.8	Lighting	12 hour light/dark cycle (7 AM to 7 PM via automated timer)
2.6.9	Intensity of illumination	150–300 Lux

2.6.10 Cage replacement and washing

Cages and feeders were replaced once every two weeks, and water bottles were replaced twice a week. These were washed in a cage washer and sterilized by an autoclave.

2.7 Feed

2.7.1 Type

Pelleted rodent chow

(Teklad Certified Irradiated Global 18% Protein Rodent Diet 2918C)

2.7.2 Lot No. 2918C-072418MA, 2918C-082118MA

2.7.3 Manufacturer Envigo RMS, Inc., U.S.A.

2.7.4 Method of feeding

The feed was placed in feeders and provided *ad libitum*.

2.7.5 Analysis and confirmation of feed

The certificate of feed analysis was provided by the manufacturer, Envigo RMS, Inc. The results of feed analysis met the allowable standard of this facility.

2.8 Drinking Water

2.8.1 Type and method of water supply

Public tap water in Cheongju-si was filtered, irradiated by ultraviolet light and provided *ad libitum*.

2.8.2 Analysis and confirmation of drinking water

Samples of drinking water were analyzed for specified microorganisms once a month and all environmental contaminants once a year by the Research Institute of Health & Environment, ChungBuk (184, Osong saengmyeong1(il)-ro, Osong-eup, Heungdeok-gu, Cheongju-si, Chungcheongbuk-do, Republic of Korea) according to the Regulation of Quality Criteria for Potable Water and Test (Ministry of Environment Ordinance No. 684, Revision Dec. 30, 2016). The results of water analysis met the allowable standard of this facility.

2.9 Dose Range Finding Study

A dose range finding study was conducted under non-GLP conditions to determine the dose levels for the main study.

2.9.1 Dose levels

The high dose of the test substance was set at 5,000 mg/kg, which is required in the test guidelines. However, the high dose of the test substance was set at 7,500 mg/kg in consultation with the sponsor as the test substance is the component of the powder milk based on the information provided by the sponsor. The high dose was sequentially diluted to produce 3 lower dose levels (5,000, 2,500 and 1,250 mg/kg). In addition, the negative control group was set.

2.9.2 Method and frequency of administration

Each dose group was consisted of 3 animals of each sex.

The dosing formulation was administered twice via gastric intubation at 24-hour intervals using a disposable syringe (1 mL) fitted with a polyethylene intubation tube.

The dose volume was set at 10 mL/kg body weight. Individual dose volume was calculated based on the individual body weight recorded at the time of group assignment.

2.9.3 Clinical signs

Clinical signs were recorded on Day 0 (immediately and at 2 hours after the 1st dosing), Day 1 (before the 2nd dosing, immediately and at 2 hours after the 2nd dosing) and Day 2.

2.9.4 Body weights
(Table 3, Table 4)

Individual body weights were recorded on Day 1 after the 2nd dosing.

2.9.5 Justification for selection of dose levels in the main study
(Table 1, Table 2)

As a result of the dose range finding study, there were no clinical signs or mortality at any dose level of the test substance in both male and female mice. In addition, there was no statistically significant difference in the body weight at any dose level in the test substance group compared to the negative control group.

Therefore, 7,500 mg/kg was selected as the high dose level of the test substance for the main study. Two additional low dose levels (5,000 and 2,500 mg/kg) were produced. In addition, the positive and negative control groups were set.

Since there was no sex difference in mortality, only males, which are known to be susceptible to induced micronuclei, were used in the main study.

2.10 Main Study

2.10.1 Group assignment

Each dose group was consisted of 5 animals of each sex.

	Group	Dose (mg/kg)	Dose volume (mL/kg)	Frequency	Route	Animals (Male) (ID No.)
G1	Negative control	0	10	2	*P.O.	5 (1101–1105)
G2	Low dose	2,500	10	2	P.O.	5 (1201–1205)
G3	Middle dose	5,000	10	2	P.O.	5 (1301–1305)
G4	High dose	7,500	10	2	P.O.	5 (1401–1405)
G5	Positive control	2	10	1	#I.P.	5 (1501–1505)

*P.O.: Per Os, #I.P.: Intraperitoneal

2.10.2 Dosing

2.10.2.1 Administration of dosing formulations of the test substance

2.10.2.1.1 Route

Oral via gastric intubation

2.10.2.1.2 Justification for route of administration

The oral route was selected because it is the intended route of administration in humans.

2.10.2.1.3 Method and frequency of administration

The dosing formulation was administered via gastric intubation twice at 24-hour intervals using a disposable syringe (1 mL) fitted with a polyethylene intubation tube.

The vehicle was administered twice via gastric intubation at 24-hour intervals as negative control substance by the same method as the test substance group.

The dose volume was set at 10 mL/kg body weight. Individual dose volume was calculated based on the individual body weight recorded at the time of group assignment.

2.10.2.2 Administration of the positive control substance

2.10.2.2.1 Route

Intraperitoneal

2.10.2.2.2 Justification for route of administration

The intraperitoneal administration, which is a commonly used method in the genotoxicity studies, was selected for the positive control substance, MMC.

2.10.2.2.3 Method and frequency of administration

MMC was intraperitoneally injected once using a needle attached to a 1 mL disposable syringe (26 G).

The dose volume was set at 10 mL/kg body weight. Individual dose volume was calculated based on the individual body weight recorded at the time of group assignment.

2.10.3 Clinical signs

Clinical signs were recorded on Day 0 (immediately and at 2 hours after the 1st dosing), Day 1 (before the 2nd dosing, immediately and at 2 hours after the 2nd dosing) and Day 2.

2.10.4 Body weights

Individual body weights were recorded prior to harvesting bone marrows cells (CP3202S, Sartorius, Germany).

2.10.5 Slide preparation

All animals were sacrificed by cervical dislocation just prior to harvesting bone marrow cells.

Immediately following sacrifice, femurs were dissected from each animal and trimmed. The proximal ends of the femur were removed with a pair of scissors. 200 μL of Fetal Bovine Serum (Lot No.: 1957600, FBS, Gibco, U.S.A.) was injected into the proximal end of the bone marrow canal. Bone marrow cells were collected by rinsing the canal with FBS.

Bone marrow samples were centrifuged at 1,000 rpm for 5 minutes (4°C, Micro17TR, Hanil Science Industrial Co. Ltd., Republic of Korea). The supernatant was discarded and then, the remaining supernatant was mixed well with the precipitate. One drop of the suspension was placed and spread on a clean dry slide. Two bone marrow sample slides per animal were prepared. The slides were identified with random numbers. The slides were air-dried, fixed with methanol for 5 minutes and stained with 3% Giemsa staining solution (0.01 mol/L Sörenson phosphate buffer solutions, pH 6.8) for 30 minutes.

The stained slides were washed with the 0.01 mol/L Sörenson phosphate buffer solution (pH 6.8) and 0.004% citric acid solution. Then, the slides were air-dried and mounted by dropping mounting medium (Entellan®new, Merck, Germany).

2.11 Observations

Coded bone marrow sample slides were observed under a microscope (BX51, Olympus, Japan) at a 600-fold magnification.

A total of 4,000 PCE per animal (2,000 PCE per slide) were observed and the ratio of micronucleated polychromatic erythrocytes (MNPCE) to PCE was calculated.

A total of 500 erythrocytes per animal (250 erythrocytes per slide) were observed and the ratio of PCE to the total erythrocytes, which provides an index of bone marrow cytotoxicity, was calculated.

2.12 Acceptance Criteria

Evaluation of the validity of the study results was conducted based on the following criteria:

- The incidence of MNPCE in the negative control group is within the range of historical control data and the 95% control limits of the distribution of the historical control data.
- The incidence of MNPCE in the positive control group is within the range of historical control data and statistically significantly increased compared to the negative control group.

2.13 Evaluation Criteria

The results of the study were considered to be positive when the following conditions were met.

- At least one of the test substance groups exhibits a statistically significant increase in the incidence of MNPCE compared to the negative control group.
- The incidence of MNPCE is increased in a dose-dependent manner.
- The incidence of MNPCE is increased outside the range of the distribution of the historical control data.

2.14 Statistical Analysis

Statistical analysis on the incidence of MNPCE, ratio of PCE to total erythrocytes and body weights was performed using SAS Program (version 9.3, SAS Institute Inc., U.S.A.).

For the incidence of MNPCE data, Kruskal-Wallis test and Mann-Whitney test were used for the comparison of the negative control group to each test substance group or the positive control group (significance levels: 0.05 and 0.01, two-tailed).

For the ratio of PCE to total erythrocytes and body weight data, Bartlett's test was used for the comparison of homogeneity of variance of the negative control group to each test substance group (significance level: 0.05). One-way analysis of variance (ANOVA) was employed for homogeneous data (significance level: 0.05). In the comparison of the negative control group to the positive control group, Folded-F test was used for homogeneity of variance (significance level: 0.05). Student t-test was employed on homogeneous data for confirming significance (significance levels: 0.05 and 0.01, two-tailed).

3. RESULTS AND DISCUSSION

3.1 Clinical Signs
(Table 5)

During the observation period, there were no abnormalities of clinical signs at any dose level of the test substance.

3.2 Body Weights
(Table 6)

During the observation period, there was no statistically significant difference in the body weight at any dose level in the test substance group compared to the negative control group.

3.3 Incidence of MNPCE in PCE
(Table 7)

There was no statistically significant difference in the incidence of MNPCE in PCE in the test substance groups compared with the negative control group. In addition, there was no statistically significant difference in the ratio of PCE to total erythrocytes in any test substance group compared to the negative control group.

In the positive control group, the incidence of MNPCE in PCE was significantly increased compared to the negative control group ($p<0.01$). There was no statistically significant difference in the ratio of PCE to total erythrocytes in the positive control group compared to the negative control group.

3.4 Acceptance of Study

The incidence of MNPCE in the negative control group was within the range of historical control data and the 95% control limits of the distribution of the historical control data. The incidence of MNPCE in the positive control group was within the range of historical control data (Table 8) and statistically significantly increased compared to the negative control group. Therefore, the results indicated that this study was conducted under the suitable test conditions.

4. CONCLUSION

In conclusion, the test substance, 2'-Fucosyllactose, did not have any potential to induce micronuclei formation in the bone marrow cells of mice under the conditions of this study.

TABLES

Table 1. Clinical Signs of Dose Range finding Study in Male ICR Mice

Group		Dose (mg/kg)	Route	Animal ID	Clinical signs	1st dosing		2nd dosing			1 day after 2nd dosing
						immediately after dosing	2 hours after dosing	before dosing	immediately after dosing	2 hours after dosing	
Negative control	Water for injection	0	P.O.	P1101		-	-	-	-	-	-
				P1102		-	-	-	-	-	-
				P1103		-	-	-	-	-	-
Test substance	2'-Fucosyllactose	1,250	P.O.	P1201		-	-	-	-	-	-
				P1202		-	-	-	-	-	-
				P1203		-	-	-	-	-	-
		2,500	P.O.	P1301		-	-	-	-	-	-
				P1302		-	-	-	-	-	-
				P1303		-	-	-	-	-	-
		5,000	P.O.	P1401		-	-	-	-	-	-
				P1402		-	-	-	-	-	-
				P1403		-	-	-	-	-	-
		7,500	P.O.	P1501		-	-	-	-	-	-
				P1502		-	-	-	-	-	-
				P1503		-	-	-	-	-	-

P.O.: Per Os.
-: No observable abnormality

Table 2. Clinical Signs of Dose Range finding Study in Female ICR Mice

Group		Dose (mg/kg)	Route	Animal ID	Clinical signs	1st dosing		2nd dosing			1 day after 2nd dosing
						immediately after dosing	2 hours after dosing	before dosing	immediately after dosing	2 hours after dosing	
Negative control	Water for injection	0	P.O.	P2101		-	-	-	-	-	-
				P2102		-	-	-	-	-	-
				P2103		-	-	-	-	-	-
Test substance	2'-Fucosyllactose	1,250	P.O.	P2201		-	-	-	-	-	-
				P2202		-	-	-	-	-	-
				P2203		-	-	-	-	-	-
		2,500	P.O.	P2301		-	-	-	-	-	-
				P2302		-	-	-	-	-	-
				P2303		-	-	-	-	-	-
		5,000	P.O.	P2401		-	-	-	-	-	-
				P2402		-	-	-	-	-	-
				P2403		-	-	-	-	-	-
		7,500	P.O.	P2501		-	-	-	-	-	-
				P2502		-	-	-	-	-	-
				P2503		-	-	-	-	-	-

P.O.: Per Os.
-: No observable abnormality

Table 3. Body Weights of Dose Range finding Study in Male ICR Mice

Group		Dose (mg/kg)	Route	Animal ID	Body weight (g)	
					before 1st dosing	1 day after 2nd dosing
Negative control	Water for injection	0	P.O.	P1101	35.7	35.0
				P1102	34.4	31.7
				P1103	35.5	36.5
				Mean	35.2	34.4
				S.D.	0.71	2.45
Test substance	2'-Fucosyllactose	1,250	P.O.	P1201	35.9	36.0
				P1202	35.1	35.3
				P1203	33.8	33.9
				Mean	34.9	35.1
				S.D.	1.08	1.07
		2,500	P.O.	P1301	34.7	34.7
				P1302	33.8	33.7
				P1303	36.2	35.4
				Mean	34.9	34.6
				S.D.	1.21	0.84
		5,000	P.O.	P1401	35.7	35.5
				P1402	34.1	34.5
				P1403	35.6	35.8
				Mean	35.1	35.3
				S.D.	0.90	0.66
		7,500	P.O.	P1501	34.3	33.5
				P1502	36.1	35.0
				P1503	35.4	35.3
				Mean	35.3	34.6
				S.D.	0.89	0.93

P.O.: Per Os.
S.D.: Standard Deviation

Table 4. Body Weights of Dose Range finding Study in Female ICR Mice

Group		Dose (mg/kg)	Route	Animal ID	Body weight (g)	
					before 1st dosing	1 day after 2nd dosing
Negative control	Water for injection	0	P.O.	P2101	28.3	25.2
				P2102	28.1	27.8
				P2103	27.4	26.1
				Mean	27.9	26.4
				S.D.	0.47	1.32
Test substance	2'-Fucosyllactose	1,250	P.O.	P2201	27.0	27.3
				P2202	28.3	26.9
				P2203	28.8	28.3
				Mean	28.0	27.5
				S.D.	0.94	0.76
		2,500	P.O.	P2301	27.8	27.7
				P2302	28.0	28.1
				P2303	27.7	26.2
				Mean	27.8	27.3
				S.D.	0.13	1.01
		5,000	P.O.	P2401	29.5	27.4
				P2402	27.2	26.7
				P2403	26.7	26.2
				Mean	27.8	26.8
				S.D.	1.49	0.58
		7,500	P.O.	P2501	28.1	27.2
				P2502	28.5	28.4
				P2503	26.8	26.3
				Mean	27.8	27.3
				S.D.	0.87	1.08

P.O.: Per Os.
S.D.: Standard Deviation

Table 5. Clinical Signs of Main Study in Male ICR Mice

Group		Dose (mg/kg)	Route	Animal ID	Clinical signs	1st dosing		2nd dosing			1 day after 2nd dosing
						immediately after dosing	2 hours after dosing	before dosing	immediately after dosing	2 hours after dosing	
Negative control	Water for injection	0	P.O.	1101		-	-	-	-	-	-
				1102		-	-	-	-	-	-
				1103		-	-	-	-	-	-
				1104		-	-	-	-	-	-
				1105		-	-	-	-	-	-
Test substance	2'-Fucosyllactose	2,500	P.O.	1201		-	-	-	-	-	-
				1202		-	-	-	-	-	-
				1203		-	-	-	-	-	-
				1204		-	-	-	-	-	-
				1205		-	-	-	-	-	-
		5,000	P.O.	1301		-	-	-	-	-	-
				1302		-	-	-	-	-	-
				1303		-	-	-	-	-	-
				1304		-	-	-	-	-	-
				1305		-	-	-	-	-	-
		7,500	P.O.	1401		-	-	-	-	-	-
				1402		-	-	-	-	-	-
				1403		-	-	-	-	-	-
				1404		-	-	-	-	-	-
				1405		-	-	-	-	-	-
[A]Positive control	MMC	2	I.P.	1501				-	-	-	-
				1502				-	-	-	-
				1503				-	-	-	-
				1504				-	-	-	-
				1505				-	-	-	-

P.O.: Per Os.
I.P.: Intraperitoneal
MMC: Mitomycin C
-: No observable abnormality
A: The positive control substance was injected intraperitoneally once at 24 hours prior to sampling time.

Table 6. Body Weights of Main Study in Male ICR Mice

Group		Dose (mg/kg)	Route	Animal ID	Body weight (g)	
					before 1st dosing	1 day after 2nd dosing
Negative control	Water for injection	0	P.O.	1101	34.8	32.8
				1102	33.5	33.3
				1103	33.5	33.0
				1104	33.4	33.7
				1105	35.2	35.4
				Mean	34.1	33.6
				S.D.	0.84	1.02
Test substance	2'-Fucosyllactose	2,500	P.O.	1201	33.7	34.2
				1202	35.0	34.8
				1203	33.5	34.5
				1204	33.1	32.5
				1205	35.0	34.7
				Mean	34.1	34.1
				S.D.	0.87	0.94
		5,000	P.O.	1301	35.1	33.8
				1302	32.3	32.2
				1303	35.0	34.1
				1304	33.5	33.3
				1305	34.1	33.9
				Mean	34.0	33.5
				S.D.	1.15	0.78
		7,500	P.O.	1401	32.3	32.6
				1402	35.8	35.0
				1403	33.2	33.5
				1404	33.4	33.1
				1405	36.1	35.2
				Mean	34.2	33.9
				S.D.	1.68	1.16
[A]Positive control	MMC	2	I.P.	1501	34.4	33.6
				1502	35.0	33.8
				1503	33.2	32.3
				1504	32.9	33.0
				1505	34.6	33.2
				Mean	34.0	33.2
				S.D.	0.92	0.60

P.O.: Per Os.
I.P.: Intraperitoneal
S.D.: Standard Deviation
MMC: Mitomycin C
A: The positive control substance was injected intraperitoneally once at 24 hours prior to sampling time.

Table 7. Results of Main Study in Male ICR Mice

Group		Dose (mg/kg)	Route	Hours after dosing	Animal ID	PCE/(PCE+NCE)	MNPCE/ PCE
Negative control	Water for injection	0	P.O.	24	1101	146 / 500	1 / 4,000
					1102	155 / 500	1 / 4,000
					1103	152 / 500	2 / 4,000
					1104	165 / 500	1 / 4,000
					1105	160 / 500	2 / 4,000
					Total	778 / 2,500	7 / 20,000
					%(Mean±S.D.)	31.1 ± 1.46	0.035 ± 0.014
Test substance	2`-Fucosyllactose	2,500	P.O.	24	1201	161 / 500	0 / 4,000
					1202	175 / 500	3 / 4,000
					1203	171 / 500	2 / 4,000
					1204	152 / 500	0 / 4,000
					1205	166 / 500	1 / 4,000
					Total	825 / 2,500	6 / 20,000
					%(Mean±S.D.)	33.0 ± 1.79	0.030 ± 0.033
		5,000	P.O.	24	1301	173 / 500	2 / 4,000
					1302	158 / 500	2 / 4,000
					1303	161 / 500	2 / 4,000
					1304	166 / 500	1 / 4,000
					1305	154 / 500	1 / 4,000
					Total	812 / 2,500	8 / 20,000
					%(Mean±S.D.)	32.5 ± 1.47	0.040 ± 0.014
		7,500	P.O.	24	1401	165 / 500	3 / 4,000
					1402	150 / 500	2 / 4,000
					1403	156 / 500	3 / 4,000
					1404	157 / 500	2 / 4,000
					1405	164 / 500	2 / 4,000
					Total	792 / 2,500	12 / 20,000
					%(Mean±S.D.)	31.7 ± 1.24	0.060 ± 0.014
Positive control	MMC	2	I.P.	24	1501	149 / 500	267 / 4,000
					1502	167 / 500	270 / 4,000
					1503	162 / 500	266 / 4,000
					1504	165 / 500	274 / 4,000
					1505	161 / 500	298 / 4,000
					Total	804 / 2,500	1,375†† / 20,000
					%(Mean±S.D.)	32.2 ± 1.40	6.875 ± 0.331

P.O.: Per Os.
I.P.: Intraperitoneal
MNPCE: Micronucleated polychromatic erythrocyte
PCE: Polychromatic erythrocyte
NCE: Normochromatic erythrocyte
S.D.: Standard Deviation
MMC: Mitomycin C
Significant difference from negative control by Mann-Whitney test: †† $p < 0.01$

Table 8. Historical Control Data

Historical control values of micronucleated polychromatic erythrocytes (MNPCE)							
Group	Hours after dosing (hr)	Dose (mg/kg)	N	MNPCE/PCE (%) (Mean±S.D.)	Range [MNPCE/PCE] (%)		95% control limit[a] [MNPCE/PCE]
					MIN	MAX	
Negative control	24	0	32	0.042 ± 0.019	0.007	0.077	<13
Positive control	24	2	32	6.119 ± 1.275	4.988	7.250	

Historical control values of ratio of polychromatic erythrocytes (PCE) to total erythrocytes						
Group	Hours after dosing (hr)	Dose (mg/kg)	N	PCE/(NCE+PCE) (%) (Mean±S.D.)	Range [PCE/(NCE+PCE)] (%)	
					MIN	MAX
Negative control	24	0	32	30.66 ± 3.006	25.96	35.36
Positive control	24	2	32	29.39 ± 3.864	24.72	34.07

Negative control: Water for injection, Normal saline injection, Corn oil, 0.5% methyl cellulose 1500centipoise solution, 0.5% carboxymethylcellulose sodium salt solution, *etc*.

Positive control: Mitomycin C (2 mg/kg, I.P., single dosing)

N: The total number of micronucleus test.

The above historical control values were obtained from the data pooled from Dec. 6, 2013 to Mar. 17, 2017.

The range was calculated by the control limit of X derived from $\overline{X}$-$\overline{R}$ value.

a) Poisson-based 95% control limits of the historical negative control data.

Biotoxtech

FINAL REPORT

Single Oral Dose Toxicity Study of 2'-Fucosyllactose in Juvenile Sprague-Dawley Rats

Study No.: B18672

Biotoxtech Co., Ltd.

53, Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si,
Chungcheongbuk-do, 28115, Republic of Korea

SUMMARY

This study was conducted to assess the potential toxicity and to determine the approximate lethal dose of the test substance, 2'-Fucosyllactose, following a single oral administration to juvenile male and female Sprague-Dawley rats (7 days old).

Test groups consisted of three dose groups at dose levels of 2,500, 5,000 and 7,500 mg/kg and a control group (water for injection), with 5 animals of each sex per group. All animals were monitored for clinical signs and body weight changes during the 14-day observation period after dosing. They were euthanized and subjected to gross necropsy at the end of the observation period.

In the result of mortality, one female was found dead at 7,500 mg/kg on Day 2 after dosing. However, there were no test substance-related clinical signs and body weight changes in the other female pups in the 7,500 mg/kg dosing group. It was not considered to be test substance-related mortality since it was natural death of rat pup.

In clinical signs, there were no abnormalities in the control and test substance dosing groups.

As a result of body weight changes, a significant suppression in the body weight gain was noted in males at 7,500 mg/kg/day from Day 1 to Day 14.

At necropsy, there were no test substance-related gross findings in either sex at 2,500, 5,000 and 7,500 mg/kg.

Based on the results of this study, the approximate lethal dose of the test substance, 2'-Fucosyllactose, was greater than 7,500 mg/kg in male and female rats under the conditions of this study.

1. EXPERIMENTAL OUTLINE

1.1 Purpose

The purpose of this study was to assess the potential toxicity and to determine the approximate lethal dose of the test substance, 2' -Fucosyllactose, following a single oral administration to male and female Sprague-Dawley rats (7 days old).

1.2 Good Laboratory Practice Regulations

This study was conducted in accordance with the following Good Laboratory Practice Regulations:

- "Good Laboratory Practice Regulation for Nonclinical Laboratory Studies"
  Notification No. 2017-32, Ministry of Food and Drug Safety, Republic of Korea (May 1, 2017)
- "OECD Principles of Good Laboratory Practice"
  Organisation for Economic Co-operation and Development, ENV/MC/CHEM (98)17 (as revised in 1997)

1.3 Regulatory Guidelines

This study was conducted in accordance with the following test guideline:

- "Standards for Toxicity Studies of Drugs"
  Notification No. 2017-71, Ministry of Food and Drug Safety, Republic of Korea (Aug. 30, 2017)

1.4 Animal Ethics

This study was reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of Biotoxtech Co., Ltd. based on Animal Protection Act (Enactment on May 31, 1991, No. 4379, Revision Jan. 20, 2015, No. 13023) (Approval No.: 180641).

1.5 Veterinary Care

Veterinary treatment was conducted in accordance with the Animal Protection Act of Republic of Korea, and the Guide for the Care and Use of Laboratory Animals.

1.6 Sponsor

Name	Advanced Protein Technologies Corp.
Address	7th floor GyeongGi-BioCenter; 147, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16229, Republic of Korea
TEL	+ 82-31-888-6245 FAX + 82-31-888-6247

1.7 Test Facility

Name	Biotoxtech Co., Ltd.
Address	53, Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si, Chungcheongbuk-do, 28115, Republic of Korea
TEL	+ 82-43-210-7777 FAX + 82-43-210-7778

1.8 Study Director

Name	Chung-Tack Han
Position	Toxicity Team 1

1.9 Study Schedule

Study initiation	Nov. 6, 2018
Animal receipt	Nov. 8, 2018
Group assignment	Nov. 21, 2018
Experimental start	Nov. 22, 2018
Administration	Nov. 22, 2018
Necropsy	Nov. 24 and Dec. 6, 2018
Experimental completion	Dec. 19, 2018
Study completion	Jan. 9, 2019

1.10 Key Personnel

Evaluation of animal's health condition	Jin-Hee Lee
Test substance storage and handling	Eun-Ae Kim
Pathology	Byung-Woo Lee

1.11 Retention of Raw Data

1.11.1 Duration Three years from the approval date

(Notification No. 2017-32, Ministry of Food and Drug Safety, Republic of Korea)

2. MATERIALS AND METHODS

2.1 Test Substance

2.1.1	Name	2'-Fucosyllactose
2.1.2	Lot No.	2'-FL-CG-008
2.1.3	Appearance	Light white-yellowish powder
2.1.4	Structural formula	$C_{18}H_{32}O_{15}$
2.1.5	Molecular weight	488.44 g/mol
2.1.6	Purity	97.56%
2.1.7	Date of manufacture	Sep. 5, 2018
2.1.8	Expiration date (retest date)	Sep. 4, 2019 (one year after manufacture)
2.1.9	Storage condition	Room temperature (1–30°C)
2.1.10	Handling instructions	Wear a mask, a pair of gloves and protective equipment.
2.1.11	Supplier	
	Name	Advanced Protein Technologies Corp.
	Address	7th floor GyeongGi-BioCenter; 147, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do 16229, Republic of Korea
2.1.12	Disposition of test substance	Any remaining test substance is returned to the sponsor.

2.2 Vehicle

2.2.1	Name	Water for injection
2.2.2	Lot No.	DKN18004
2.2.3	Storage condition	Room temperature (1–30°C)
2.2.4	Supplier	JW Pharmaceutical Co., Ltd., Republic of Korea

2.3 Preparation and Analysis of the Dosing Formulations

2.3.1 Preparation of the dosing formulations

The required amount of the test substance was weighed on an electronic balance (CP423S, Sartorius, Germany) by applying a purity factor (1.0250) and placed in a bottle. A small amount of vehicle, water for injection, was added and suspended. The vehicle was gradually added to yield the desired concentrations.

The dosing formulations were stored in a refrigerator and used within 8 days.

2.3.2 Homogeneity and stability

As a result of analysis for stability and homogeneity conducted in the study of "An Analytical Method Validation of 2'-Fucosyllactose Dosing Formulations by HPLC (Biotoxtech Study No.: B18670)", the dosing formulations including the dose levels of 0.1 and 750 mg/mL were homogenous and stable for 4 hours at room temperature and for 8 days under refrigeration.

2.3.3 Analysis of the dosing formulations

Analysis for concentration of the dosing formulations was not performed.

2.4 Test System

2.4.1	Species, Strain	Rat, Sprague-Dawley (Crl:CD(SD)), SPF
2.4.2	Producer & supplier	ORIENTBIO INC., Republic of Korea

2.4.3 Justification for species selection

Sprague-Dawley rats are commonly used in toxicity studies, having a large historical control database.

2.4.4 Sex, number age and body weight range (at receipt)

Pregnant female, 15 rats, Gestation Day (GD) 15, 287.3–421.2 g

2.4.5 Sex, number, age and body weight range (at administration)

Male, 20 rats, 7 days old, 17.7–20.7 g

Female, 20 rats, 7 days old, 16.9–19.4 g

2.4.6 Quarantine and acclimation

Upon receipt, all animals were subjected to the detailed clinical examination. Body weights were recorded using an electronic balance (CP4202S, Sartorius, Germany). Dams were quarantined and acclimated for approximately 7 days and observed for mortality, general condition and clinical signs daily.

Body weights were recorded on Postpartum Day (PPD) 0 after receipt. All dams

were observed once a day until the weaning day.

2.4.7 Delivery

Females were observed for signs of parturition daily between 9:00 AM and 4:30 PM at the late stage of gestation. If parturition was confirmed, that day was defined as PPD 0. If parturition occurred after 4:30 PM, the next day was defined as PPD 0. When dams delivered pups on GD 22, only the pups were selected for this study.

Individual body weights for all pups were recorded on PNDs 0, 4 and 6. Pups were observed daily for clinical signs from birth until PND 6. Suitable pups were selected on PND 6 according to the pre-test health examination.

2.4.8 Culling

On PND 4, the litters were culled randomly to eight pups per litter (when possible, four male and four female pups per litter). The rest of pups were euthanized by hypothermia. A litter of eight pups or less was not culled. If pups were less than four in either sex, pups of the opposite sex were added to make 8 pups in total.

2.4.9 Animal and cage identification

During the acclimation period, a temporary identification number was marked on the tail using a red indelible pen on pregnant dams. A temporary cage card was attached on each cage.

From PND 4 until hair appearance, a serial number was marked on the back of pups using a blue or red indelible pen.

After hair appearance, all animals were uniquely identified by blue or red indelible marking on the tail or back.

2.4.10 Group assignment

On PND 6, 20 males and 20 females were selected and distributed into 4 groups (5 animals/sex/group). Animals were be randomly assigned to groups in an attempt to equalize mean group body weights

2.4.11 Disposition of remaining animals

The remaining pups not selected for the study were excluded from the study. After weaning (on PPD 21), dams were euthanized by CO_2.

2.5 Animal Husbandry

2.5.1	Animal room No.	A326
2.5.2	Type & size of cage	Polycarbonate cages, 260W×420D×180H (mm)

2.5.3 Number of animals per cage

Dam and pups in polycarbonate cages (pre-weaning)

2.5.4	Temperature	Measurement value: 21.2–22.7°C permissible range: 19.0–25.0°C
2.5.5	Relative humidity	Measurement value: 49.4–63.2% permissible range: 30.0–70.0%
2.5.6	Air changes	10–15 clean, fresh, filtered air changes per hour
2.5.7	Lighting	12 hour light/dark cycle 7 AM–7 PM via automated timer
2.5.8	Intensity of illumination	150–300 Lux

2.5.9 Cage replacement and washing

Water bottles and polycarbonate cages were replaced twice a week and once a week, respectively. These were washed in a cage washer and sterilized by an autoclave.

2.6 Feed

2.6.1 Type

Pelleted rodent chow

(Teklad Certified Irradiated Global 18% Protein Rodent Diet 2918C)

2.6.2	Lot No.	2918C-072918MA
2.6.3	Manufacturer	Envigo RMS, Inc., U.S.A.

2.6.4 Method of feeding

The feed was placed in feeders and provided *ad libitum*.

2.6.5 Analysis of feed

The certificate of feed analysis was provided by the manufacturer, Envigo RMS, Inc. The results of feed analysis met the allowable standard of this facility.

2.7 Drinking Water

2.7.1 Type and method of water supply

Public tap water in Cheongju-si was filtered and irradiated by ultraviolet light and provided *ad libitum*.

2.7.2 Analysis of drinking water

Samples of drinking water are analyzed for specified microorganisms once a month and all environmental contaminants once a year by the Research Institute of Health & Environment, ChungBuk (184, Osong saengmyeong 1(il)-ro, Osong-eup, Heungdeok-gu, Cheongju-si, Chungcheongbuk-do, Republic of Korea) according to the Regulation of Quality Criteria for Potable Water and Test (Ministry of Environment Ordinance No. 684, Revision Dec. 30, 2016). The results of water analysis met the allowable standard of this facility.

2.8 Dosing

2.8.1 Route

Oral via gastric intubation

2.8.2 Justification for the route of administration

The oral route was chosen because it is the intended route of administration in humans.

2.8.3 Method of administration

Individual doses were calculated based on the animals' body weight recorded just prior to dosing at a volume of 10 mL/kg body weight. Animals were dosed via gastric intubation with a 1-mL disposable syringe fitted with an intubation tube.

2.9 Group Designation and Dose Levels

2.9.1 Group designation

Group		Dose (mg/kg)	Dose volume (mL/kg)	No. of animals (Animal ID No.)	
				Males	Females
G1	Control	0	10	5 (1101–1105)	5 (2101–2105)
G2	Low dose	2,500	10	5 (1201–1205)	5 (2201–2205)
G3	Mid dose	5,000	10	5 (1301–1305)	5 (2301–2305)
G4	High dose	7,500	10	5 (1401–1405)	5 (2401–2405)

2.9.2 Justification for dose levels

Based on the information provided by the sponsor, the dose levels selected for this study were 2,500, 5,000 and 7,500 mg/kg.

The animals of the control group were dosed with the vehicle of the same volume as the test substance dosing groups.

2.10 Parameters Evaluated

2.10.1 Clinical signs

All animals were observed for mortality, general condition and clinical signs (type, severity, time of onset and recovery) at least once for 30 minutes after dosing and at 1, 2, 4 and 6 hours after dosing on Day 0 and once daily thereafter for 14 days (Day 1−Day 14).

2.10.2 Disposition of dead animal

The dead animal was stored under refrigeration and necropsied within 6 hours after storage.

2.10.3 Body weights

The body weights were recorded prior to dosing on Day 0, on Days 1, 3, 7 and on the day of necropsy (Day 14).

The body weights of animals found dead during the observation period were recorded prior to necropsy.

2.10.4 Necropsy

On Day 14, all animals were anesthetized with CO_2 and exsanguinated from the abdominal aorta. Complete gross postmortem examinations were performed on all animals in the study.

2.10.5 Histopathology

Since no gross findings were evident at necropsy, histopathological examination was not performed.

2.11 Statistical Analysis

Statistical analysis was performed using SAS Program (version 9.3, SAS Institute Inc., U.S.A.). Body weights were analyzed utilizing Bartlett's test for homogeneity of variance (significance level: 0.05). One-way analysis of variance (ANOVA) was employed on homogeneous data; then, if significant, Dunnett's test was applied for multiple comparisons (significance levels: 0.05 and 0.01, two-tailed).

3. RESULTS AND DISCUSSION

3.1 Mortality

(Table 1)

In the 7,500 mg/kg group, one female (Animal ID No.: 2403) was found dead on Day 2 after dosing. However, there were no test substance-related clinical signs and body weight changes in the other female pups in the 7,500 mg/kg dosing group. It was not considered to be test substance-related mortality since it was natural death of rat pup.

Animals in the control group (0 mg/kg) and the 2,500 and 5,000 mg/kg dosing groups survived to the scheduled necropsy.

3.2 Clinical Signs

(Table 2, Table 4)

No abnormalities were observed in clinical signs in all surviving animals in the control and test substance dosing groups.

3.3 Body Weights

(Figure 1, Figure 2, Table 3, Table 5)

A significant decrease in the body weight was noted in males at 7,500 mg/kg from Day 1 to Day 14 when compared to the control group. A suppressed body weight gain was observed in males at 7,500 mg/kg from Day 0 to Day 14. These changes were considered to be test substance-related effects.

No significant difference in the body weight was noted in males and females at 2,500 and 5,000 mg/kg, and in females at 7,500 mg/kg when compared to the control group.

3.4 Necropsy Findings

(Appendix IV)

Dead animal

There were no macroscopic findings in the dead female at 7,500 mg/kg (Animal ID No.: 2403).

Surviving animals

As a result of necropsy, there were no noticeable abnormalities in animals in the control groups, 2,500, 5,000 and 7,500 mg/kg dosing groups.

4. CONCLUSION

In conclusion, the approximate lethal dose of the test substance, 2'-Fucosyllactose, was determined to be greater than 7,500 mg/kg in male and female juvenile rats (7 days old) under the conditions of this study.

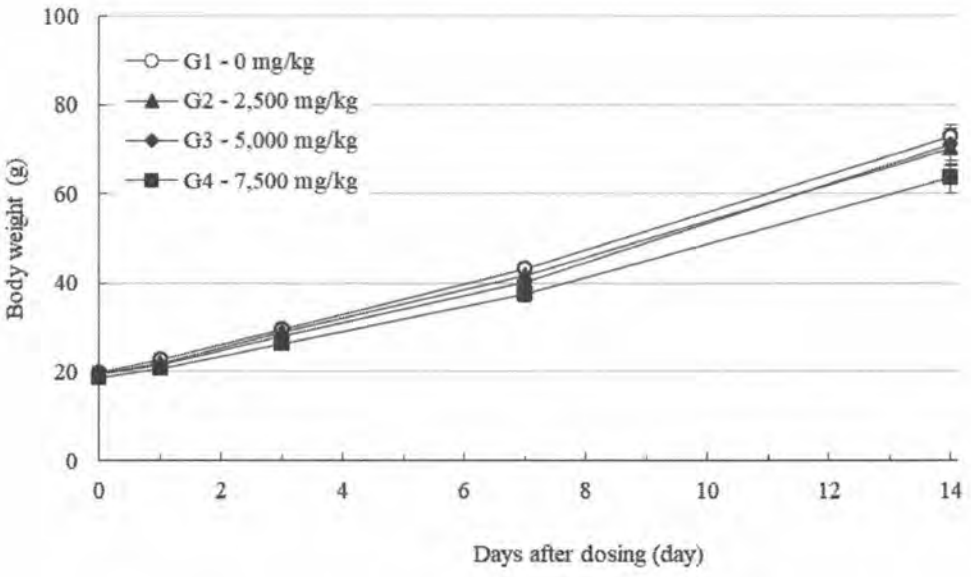


Figure 1. Body Weights in Male SD Rats

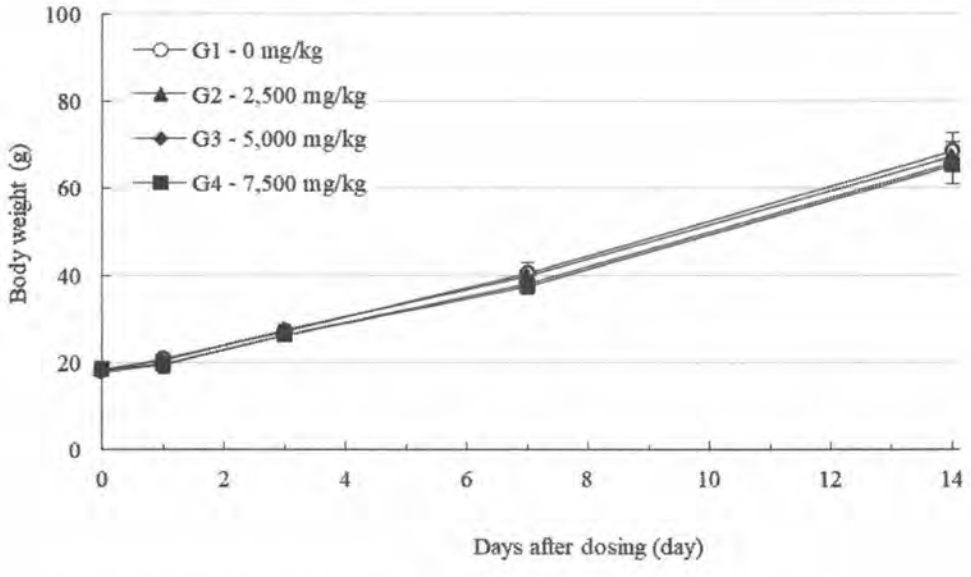


Figure 2. Body Weights in Female SD Rats

Table 1. Summary of Mortality

Sex	Group / Dose (mg/kg)	No. of animals	Days after dosing														Mortality
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Male	G1 0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
	G2 2,500	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
	G3 5,000	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
	G4 7,500	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
Female	G1 0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
	G2 2,500	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
	G3 5,000	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
	G4 7,500	5	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1/5

Table 2. Summary of Clinical Signs

Sex: Male

Group / Dose (mg/kg)	No. of animals	Clinical sign	Hours (Day 0) after dosing				
			0.5	1	2	4	6
G1 0	5	NOA	5	5	5	5	5
G2 2,500	5	NOA	5	5	5	5	5
G3 5,000	5	NOA	5	5	5	5	5
G4 7,500	5	NOA	5	5	5	5	5

Group / Dose (mg/kg)	No. of animals	Clinical sign	Days after dosing												
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
G1 0	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G2 2,500	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G3 5,000	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G4 7,500	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5

NOA: No Observable Abnormality

Table 2. (Continued)

Sex: Female

Group / Dose (mg/kg)	No. of animals	Clinical sign	Hours (Day 0) after dosing				
			0.5	1	2	4	6
G1 0	5	NOA	5	5	5	5	5
G2 2,500	5	NOA	5	5	5	5	5
G3 5,000	5	NOA	5	5	5	5	5
G4 7,500	5	NOA	5	5	5	5	5

Group / Dose (mg/kg)	No. of animals	Clinical sign	Days after dosing												
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
G1 0	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G2 2.500	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G3 5,000	5	NOA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
G4 7,500	5	NOA Death	5	4 1	4	4	4	4	4	4	4	4	4	4	4	4

NOA: No Observable Abnormality

Table 3. Mean Body Weights

(g)

Sex	Group / Dose (mg/kg)		Days after dosing					Gain
			0	1	3	7	14	0 – 14
Male	G1	Mean	19.9	22.8	29.5	43.1	72.9	53.1
	0	S.D.	0.8	1.1	1.1	1.4	1.8	1.8
		N	5	5	5	5	5	5
	G2	Mean	19.6	21.9	29.1	41.7	70.2	50.5
	2,500	S.D.	0.7	0.9	1.2	2.2	3.6	3.1
		N	5	5	5	5	5	5
	G3	Mean	19.4	21.6	28.0	40.2	70.9	51.5
	5,000	S.D.	1.1	1.7	1.4	2.6	4.5	3.7
		N	5	5	5	5	5	5
	G4	Mean	18.7	20.6 *	26.5 **	37.6 **	63.7 **	45.0 **
	7,500	S.D.	0.6	0.5	0.9	1.7	3.7	3.3
		N	5	5	5	5	5	5
Female	G1	Mean	18.0	20.7	27.1	40.5	68.5	50.5
	0	S.D.	0.6	1.1	1.0	2.4	4.3	3.9
		N	5	5	5	5	5	5
	G2	Mean	18.3	20.5	27.3	39.8	67.3	49.0
	2,500	S.D.	1.0	1.1	1.2	1.0	1.9	1.7
		N	5	5	5	5	5	5
	G3	Mean	17.9	19.7	26.2	38.2	65.9	48.0
	5,000	S.D.	0.7	1.2	1.3	2.3	4.9	4.7
		N	5	5	5	5	5	5
	G4	Mean	18.3	19.6	26.1	37.3	65.4	47.0
	7,500	S.D.	0.8	2.0	1.2	1.5	1.3	1.1
		N	5	5	4	4	4	4

Significantly different from the control by Dunnett's t-test: * $p<0.05$. ** $p<0.01$.

Biotoxtech 2019 e

DRAFT REPORT

Ninety-Day Repeated Oral Dose Toxicity Study with a Four-Week Recovery Period of 2'-Fucosyllactose in Juvenile Sprague-Dawley Rats

Study No.: B18673

Biotoxtech Co., Ltd.

53, Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si,
Chungcheongbuk-do, 28115, Republic of Korea

SUMMARY

This study was conducted to assess the potential toxicity and safety of the test substance, 2'-Fucosyllactose, when administered by oral gavage once daily to Sprague-Dawley (Crl:CD(SD)) rats of both sexes for 90 days.

A total of 4 groups were designated as follows:

Three groups of the test substance were designated at dose levels of 2,500, 5,000 and 7,500 mg/kg/day in addition to a control group (water for injection). Each group consisted of 10 males and 10 females. Extra 5 animals of each sex were added to the control group and 7,500 mg/kg/day group for the recovery groups to assess the reversibility of toxicity during the 4-week recovery period.

During the observation period, evaluated parameters included clinical signs, detailed examinations, body weight, food consumption, functional observations, ophthalmological examinations, urinalysis, and hematological and clinical chemistry examinations, organ weights, gross post mortem examinations and histopathological examination were performed after the observation period.

One male of the 5,000 mg/kg/day group was found dead on Day 72. It was considered to be sudden death of the rat showing no morphological changes and it occurs often in Sprague-Dawley rats [1], and there was no test substance-related effect on gross findings at necropsy or histopathological lesions in this dead male. One female of the 7,500 mg/kg/day group was found dead on Day 26. Serous fluid-filled thoracic cavity (clear with red color) and pulmonary congestion/edema were noted in the dead female. These findings might be due to a technical gavage error [2].

No test substance-related toxic effects were noted in clinical signs, detailed examinations, body weights, food consumption, functional observations, ophthalmological examination, urinalysis, hematology, clinical chemistry, organ weights and gross postmortem examinations in males and females in the 2,500, 5,000 and 7,500 mg/kg/day groups.

No test substance-related toxic effect was noted in the histopathological examination in males and females in the 7,500 mg/kg/day group.

On the basis of these results, the no observed adverse effect level (NOAEL) of 2'-Fucosyllactose was considered to be 7,500 mg/kg/day in both male and female rats after repeated oral administration for 90 days weeks under the conditions of this study.

1. EXPERIMENTAL OUTLINE

1.1 Purpose

The purpose of this study was to evaluate the potential toxicity of the test substance, 2'-Fucosyllactose, when administered by oral gavage to juvenile Sprague-Dawley rats (Postnatal Day (PND) 7) once daily for 90 days and to assess the reversibility of toxic effects following a 4-week recovery period.

1.2 Good Laboratory Practice Regulations

This study was conducted in accordance with the following Good Laboratory Practice Regulations:

- "Good Laboratory Practice Regulation for Nonclinical Laboratory Studies"
  Notification No. 2017-32, Ministry of Food and Drug Safety (May 1, 2017)
- "OECD Principles of Good Laboratory Practice"
  Organisation for Economic Co-operation and Development, ENV/MC/CHEM (98)17 (as revised in 1997)

1.3 Regulatory Guidelines

This study was conducted in accordance with the following guideline:

- "OECD Guideline for The Testing Of Chemicals 408, Repeated Dose 90-day Oral Toxicity Study in Rodents"
  Organisation for Economic Co-operation and Development (Adopted: 21st September 1998)

1.4 Animal Ethics

This study was reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of Biotoxtech Co., Ltd. based on Animal Protection Act (Enactment May 31, 1991, No. 4379, Revision Mar. 20, 2018, No. 15502) (Approval No.: 180571).

1.5 Veterinary Care

Veterinary treatment was conducted in accordance with the Animal Protection Act of Korea, the Guide for the Care and Use of Laboratory Animals.

2. MATERIALS AND METHODS

2.1 Test Substance

2.1.1	Name	2'-Fucosyllactose
2.1.2	Lot No.	① 2'-FL-CG-007, ② 2'-FL-CG-008, ③ 2'-FL-CG-007.5-3, ④ 2'-FL-CG-009, ⑤ 2'-FL-CG-010, ⑥ 2'-FL-CG-007-02, ⑦ 2'-FL-CG-011
2.1.3	Appearance	Light white-yellowish powder
2.1.4	Structural formula	$C_{18}H_{32}O_{15}$
2.1.5	Molecular weight	488.44 g/mol
2.1.6	Purity	① 97.05%, ② 97.56%, ③ 97.64%, ④ 97.67%, ⑤ 97.09%, ⑥ 96.32%, ⑦ 96.31%
2.1.7	Date of manufacture	① Aug. 8, 2018, ② Sep. 5, 2018, ③ Aug. 13, 2018, ④ Oct. 1, 2018, ⑤ Oct. 15, 2018, ⑥ Dec. 6, 2018, ⑦ Oct. 29, 2018
2.1.8	Expiration date (retest date)	① Aug. 7, 2019, ② Sep. 4, 2019 ③ Aug. 12, 2019, ④ Sep. 30, 2019, ⑤ Oct. 14, 2019, ⑥ Dec. 5, 2019, ⑦ Oct. 28, 2019 (one year after manufacture)
2.1.9	Storage condition	Room temperature (1–30°C)
2.1.10	Handling instructions	Wear a mask, a pair of gloves and protective equipment
2.1.11	Supplier	
	Name	Advanced Protein Technologies Corp.
	Address	7th floor GyeongGi-BioCenter; 147, Gwannggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do 16229, Republic of Korea
2.1.12	Disposition of test substance	Any remaining test substance is returned to the sponsor.

2.2 Vehicle

2.2.1	Name	Water for injection
2.2.2	Lot No.	DKN18004, DKN18007
2.2.3	Storage condition	Room temperature (1–30°C)
2.2.4	Manufacturer	JW Pharmaceutical Co., LTD., Republic of Korea

2.3 Preparation and Analysis of the Dosing Formulations

2.3.1 Preparation of the dosing formulations

The required amount of the test substance was weighed using an electronic balance (BP3100S, ENTRIS423I-1S, CP323S, CP3202S, Sartorius, Germany) with a purity factor (① 1.0304, ② 1.0250, ③ 1.0242, ④ 1.0239, ⑤ 1.0300, ⑥ 1.0382, ⑦ 1.0383) and placed in a bottle. A small amount of vehicle, water for injection, was added, mixed and suspended. The vehicle was gradually added to yield the desired concentrations.

The dosing formulations were confirmed to be stable for 8 days under refrigeration, and these dosing formulations were used within 8 days.

2.3.2 Stability and homogeneity

As a result of analysis for stability and homogeneity conducted in the study of "An Analytical Method Validation of 2'-Fucosyllactose Dosing Formulations by HPLC (Biotoxtech Study No.: B18670)", the dosing solutions comprising the dose levels of 0.1 and 750 mg/mL were confirmed to be homogenous and stable for 4 hours at room temperature and for 8 days under refrigeration.

2.3.3 Analysis of concentration of the dosing formulations

The analysis of the dosing formulations was conducted using a HPLC (Prominence, Shimadzu Corp., Japan) prior to dosing and at Week 13, and samples were taken three times from the middle layer of each dosing formulation and analyzed once each for verification of dose concentration.

As a result, the values of accuracy in the low, mid and high dose groups were 100.68, 98.18 and 100.95% prior to the first dosing and 97.04, 95.58 and 93.04% at Week 13, respectively. These results were within the acceptable range (range: ±15% of nominal value, Appendix IV).

2.4 Test System

2.4.1 Species, strain — Rat, Sprague-Dawley (Crl:CD(SD)), SPF

2.4.2 Producer & supplier — ORIENTBIO INC., Republic of Korea

2.4.3 Justification for species selection

Sprague-Dawley rats are commonly used in toxicity studies, having a large historical control database.

2.4.4 Sex and number of animals (at receipt)

Pregnant females, 30 rats, Gestation Day (GD) 15

2.4.5 Sex, number, age and body weight range of animals (at the start of administration)

Male, 50 rats, 7 days old, 16.0–22.3g
Female, 50 rats, 7 days old, 15.7–21.0g

2.4.6 Quarantine and acclimation

Upon receipt, all animals were subjected to the detailed clinical examination. Body weights were recorded using an electronic balance (Sartorius, Germany). Dams were quarantined and acclimated for approximately 7 days and observed daily for mortality, general condition and clinical signs.

Body weights were recorded on Postpartum Day (PPD) 0 after receipt. All dams were observed once a day until the weaning day.

2.4.7 Delivery

Females were observed for signs of parturition daily between 9:00 AM and 4:30 PM at the late stage of gestation. If parturition was confirmed, that day was defined as PPD 0. If parturition occured after 4:30 PM, the next day was defined as PPD 0. When dams delivered pups on GD 22, only these pups were selected for the study.

Individual body weights of all pups were recorded on PNDs 0, 4 and 6. Pups were observed daily for clinical signs from birth until PND 6. Suitable pups were selected on PND 6 according to the pre-test health examinations.

2.4.8 Culling

On PND 4, the litters were culled randomly to eight (when possible, four male and four female pups per litter). The rest of pups were euthanized by hypothermia. A litter of eight pups or less was not culled. If pups were less than four in either sex, pups of the opposite sex were added to make 8 pups in total.

2.4.9 Animal and cage identification

During the acclimation period, a temporary identification number was marked on the tail using a red indelible pen on pregnant dams. A temporary cage card was placed on each cage.

From PND 4 until hair appearance, a serial number was marked on the back of pups using a blue or red indelible pen.

After hair appearance, all animals were uniquely identified by blue or red indelible marking on the tail or back. At post-weaning on PND 21, all pups were separated individually in each cage and a color coded cage cards was placed on each cage indicating group and dose level.

2.4.10 Group assignment

On PND 6, 50 males and 50 females were selected and distributed into 4 groups (15 animals/sex/group for Groups 1 and 4, and 10 animals/sex/group for Groups 2 and 3). Animals were randomly assigned to groups in an attempt to equalize mean group body weights.

2.4.11 Disposition of remaining animals

Remaining pups not selected for the study were excluded from the study. After weaning (on PPD 21), dams were euthanized by CO_2.

2.5 Animal Husbandry

2.5.1	Room No.	A327
2.5.2	Type & size of cage	Polycarbonate cages, 260W×420D×180H (mm), Stainless wire mesh cage, 260W×350D×210H (mm)

2.5.3 Number of animals per cage
Until PND 20: Dam and pups in a polycarbonate cage (pre-weaning)
From PND 21: One pup in a stainless wire mesh cage (post-weaning)

2.5.4	Temperature	Measurement value: 20.7–23.2°C, permissible range: 19.0–25.0°C
2.5.5	Relative humidity	Measurement value: 50.9–65.3%, permissible range: 30.0–70.0%
2.5.6	Air changes	10–15 clean, fresh, filtered air changes per hour

2.5.7 Lighting — 12 hour light/dark cycle
7 AM to 7 PM via automated timer

2.5.8 Intensity of illumination — 150–300 Lux

2.5.9 Cage replacement and washing

Water bottles and polycarbonate cages were replaced twice a week and once a week, respectively. Stainless cages and fodder tubs were replaced once every two weeks. These were washed in a cage washer and sterilized by an autoclave.

2.6 Feed

2.6.1 Type

Pelleted rodent chow
(Teklad Certified Irradiated Global 18% Protein Rodent Diet 2918C)

2.6.2 Lot No. — 2918C-072418MA, 2918C-072918MA, 2918C-082118MA, 2918C-091718MA

2.6.3 Manufacturer — Envigo RMS, Inc., U.S.A.

2.6.4 Feeding

The feed was placed in feeders and provided *ad libitum*.

2.6.5 Analysis of feed

The certificate of feed analysis was provided by the manufacturer, Envigo RMS, Inc. The results of feed analysis met the allowable standard of this facility.

2.7 Drinking Water

2.7.1 Type and method of water supply

Public tap water in Cheongju-si was filtered and irradiated by ultraviolet light and provided *ad libitum*.

2.7.2 Analysis of drinking water

Samples of drinking water are analyzed for specified microorganisms once a month and all environmental contaminants once a year by the Research Institute of Health & Environment, ChungBuk (184, Osong saengmyeong 1(il)-ro, Osong-eup, Heungdeok-gu, Cheongju-si, Chungcheongbuk-do, Republic of Korea) according to the Regulation of Quality Criteria for Potable Water and Test (Ministry of

Environment Ordinance No. 684, Revision Dec. 30. 2016). The results of water analysis met the allowable standard of this facility.

2.8 Dosing

2.8.1 Route

Oral via gastric intubation

2.8.2 Justification for the route of administration

The oral route was selected because it is the intended route of administration in humans.

2.8.3 Method and frequency of administration

Dose volume was 10 mL/kg body weight. Individual doses were calculated based on the most recently recorded individual body weights. Animals were dosed once daily for 90 consecutive days via gastric intubation with 1- to 5-mL disposable syringes fitted with an intubation tube.

2.9 Group Designation and Dose Levels

2.9.1 Group designation

Group		Dose (mg/kg/day)	Dose volume (mL/kg)	Animals (ID No.)	
				Males	Females
G1	Control	0	10	10 (1101–1110) +5* (1111–1115)	10 (2101–2110) +5* (2111–2115)
G2	Low dose	2,500	10	10 (1201–1210)	10 (2201–2210)
G3	Mid dose	5,000	10	10 (1301–1310)	10 (2301–2310)
G4	High dose	7,500	10	10 (1401–1410) +5* (1411–1415)	10 (2401–2410) +5* (2411–2415)

*: Recovery group

2.9.2 Justification for dose levels

Based on the information provided by the sponsor, the dose levels selected for this study were 2,500, 5,000 and 7,500 mg/kg/day as low, mid and high doses respectively.

The animals of control group were dosed with vehicle of the same volume as the test substance dosing groups.

2.10 Parameters Evaluated

The first day of administration was defined as Day 1. The first 7-day period of

administration was defined as Week 1

2.10.1 Clinical signs

During the dosing and recovery periods, all animals were observed once daily for clinical signs and twice daily for mortality and moribundity.

2.10.2 Disposition of dead animal

Once dead animals were found, necropsies were conducted as soon as possible. However, if a necropsy was not feasible, dead animals were stored under refrigeration. Gross findings were recorded.

2.10.3 Detailed clinical sign observations

All animals were observed prior to dosing and once weekly during the observation period.

The following items were observed.

- Skin, fur, eyes, mucous membranes, occurrence of secretions, and excretions
- Autonomic activity (lacrimation, piloerection, pupil size, unusual respiratory pattern, etc.)
- Changes in gait, posture, response to handling, and the presence of clonic or tonic movements
- Stereotypies (excessive grooming, repetitive circling, etc.) or bizarre behavior (self-mutilation, walking backward, etc.)

2.10.4 Body weights

Body weights were recorded just prior to dosing on Day 1, twice weekly during the first 4 weeks of the dosing period, and once a week thereafter, the day prior to necropsy and on the day of necropsy. Body weight data on the day of necropsy were not included in the evaluation of body weights since these data were fasted body weights of main and recovery animals. Body weights of animals found dead or killed moribund were recorded before necropsy during the study.

2.10.5 Food consumption

Food consumption was be recorded once a week from PND 21 (dosing Week 3). Individual food consumption was calculated by subtracting the amount of residual feed from the amount of presented feed

2.10.6 Functional observations

Functional observations were conducted on the main group animals at Weeks 12 – 13 and on the recovery group animals at Recovery Weeks 3 – 4.

2.10.6.1 Auditory, visual and proprioceptive stimuli

Visual response, proprioceptive stimuli, auditory response, pain response, aerial righting reflex and hindlimb landing foot splay were measured once, respectively.

2.10.6.2 Grip strength

Grip strength was measured using a Push-pull gauge (RX-2, Aikoh Engineering Co., LTD., Japan). The measurement was repeated 3 times for the forelimbs and hindlimbs. Then, the maximum value was selected, respectively.

2.10.6.3 Motor activity

Motor activity was measured using an Activity Monitor (MEDD-OFA-RS, MED ASSOCIATE INC., U.S.A). Each animal was monitored every 10 minutes for one hour.

2.10.7 Ophthalmological examination

Ophthalmological examination was conducted on both eyes of all animals of the control and high dose groups in the main group at Week 13.

The examination for the pupil light reflex and anterior segment of the eye was conducted with the naked eye before instillation of mydriatic agent (ISOPTO-ATROPINE® STERILE OPHTHALMIN PREPARATION, Alcon®) and then the anterior segment of the eye, transparent media and ocular fundus were observed with the naked eye and using an ophthalmoscope (ALL PUPIL II, Keeler, U.K.) after instillation of mydriatic agent.

2.10.8 Urinalysis

Five males and females per group were randomly selected from the main group animals in addition to all recovery animals for urinalysis a few days before necropsy.

Fresh (3-hour) and 24-hour urine samples were collected from animals. When fresh urine was collected, feeding and dosing were not performed. However, drinking water was provided *ad libitum*. The following parameters were evaluated.

Urine	Parameters	Unit	Method and instrument
Fresh urine	pH	-	Combur^{10}Test®M stick (Roche, Germany), Reflectance photometry, urine chemistry analysis (cobas u 411, Roche, Germany)
	Protein	mg/dL	
	Glucose	mg/dL	
	Ketone body	mg/dL	
	Bilirubin	mg/dL	
	Occult blood	Ery/μL	

	Color and turbidity	-	Visual observation
	Sediment	-	Optical microscope
24-hour sample	Urine volume	mL	Pipette, Pipet-aid
	Specific gravity	-	Gravimeter (Vet360, Reichert, U.S.A.)

2.10.9 Hematology

All surviving animals were fasted overnight for approximately 18 hours prior to necropsy. Prior to collecting blood samples, the animals were anesthetized with isoflurane and blood samples were collected from the abdominal aorta.

Blood samples (approximately 1 mL) were collected and placed in a vacutainer containing EDTA. The following parameters were analyzed using an autoanalyzer (XN-V, SYSMEX, Japan).

Parameters	Unit	Method
Total erythrocyte count (RBC)	$\times 10^6/\mu L$	Hydrodynamic focusing DC detection
Hemoglobin (HGB)	g/dL	Cyanide-free SLS-hemoglobin method
Hematocrit (HCT)	%	Pulse height detection
Mean corpuscular volume (MCV)	fL	Calculated
Mean corpuscular hemoglobin (MCH)	Pg	
Mean corpuscular hemoglobin concentration (MCHC)	g/dL	
Platelet count (PLT)	$\times 10^3/\mu L$	Hydrodynamic focusing DC detection
Total leukocyte count (WBC)	$\times 10^3/\mu L$	Flow cytometry & Fluorescence staining
WBC differential counting ▪ Neutrophil (NEU) ▪ Lymphocyte (LYM) ▪ Monocyte (MONO) ▪ Eosinophil (EOS) ▪ Basophil (BASO)	%	
Reticulocyte (Reti)	%	Flow cytometry & Fluorescence staining

Then, approximately 2 mL of blood mixed with 3.2% sodium citrate was centrifuged at 3,000 rpm for 10 minutes to obtain plasma. The following parameters were evaluated using an automatic coagulation time meter (Coapresta 2000, SEKISUI, Japan).

Parameters	Unit	Method
Prothrombin time (PT)	sec	Coagulation time
Activated partial thromboplastin time (APTT)	sec	method

2.10.10 Clinical chemistry

Blood samples collected from the abdominal aorta in a vacutainer were centrifuged at 3,000 rpm for 10 minutes to obtain serum within one hour after blood collection. . The following parameters were analyzed using an automatic analyzer (7180, HITACHI, Japan) and electrolyte analyzer (EasyLyte, MEDICA, U.S.A.).

Parameters	Unit	Method
Alanine aminotransferase (ALT)	U/L	JSCC
Aspartate aminotransferase (AST)	U/L	JSCC
Alkaline phosphatase (ALP)	U/L	JSCC
Gamma glutamyl transpeptidase (GGT)	U/L	G5CMP
Blood urea nitrogen (BUN)	mg/dL	Urease-GLDH
Creatinine (Crea)	mg/dL	Jaffe
Total bilirubin (T-Bili)	mg/dL	Vanadate oxidation
Total bile acid (TBA)	μmol/L	Enzyme colorimetric
Total protein (TP)	g/dL	Biuret
Albumin (Alb)	g/dL	BCG
A/G ratio	-	Calculated
Total cholesterol (T-Chol)	mg/dL	Cholesterol oxidase-HMMPS
Triglycerides (TG)	mg/dL	GPO-HMMPS
Phosphorus (P)	mg/dL	Fiske Subbarow
Glucose (Glu)	mg/dL	Hexokinase-G6PDH
Calcium (Ca)	mg/dL	OCPC
Chloride* (Cl)	mmol/L	Ion-selective electrode
Sodium* (Na)	mmol/L	
Potassium* (K)	mmol/L	

*: examined using an electrolyte analyzer.

2.10.11 Necropsy (gross pathology)

All surviving animals were sacrificed by exsanguination from the abdominal aorta under isoflurane anesthesia on Day 91 and on Day 119 for the main and recovery groups, respectively. Complete gross postmortem examinations were performed on all animals including the external surface internal organs.

2.10.12 Organ weights

The following organs of terminal sacrifice animals were weighed individually. Paired organs (#) were weighed together. Organ to fasted body weight ratios were calculated.

- Brain
- Heart
- Spleen
- Adrenal gland#
- Epididymis#
- Uterus and cervix
- Thymus
- Liver
- Kidney#
- Testis#
- Ovary#

2.10.13 Histopathology

At necropsy, the following organs and tissues were harvested and preserved in 10% neutral buffered formalin except for the testes, eyes and optic nerve which were fixed in Davidson fixative, and then preserved in 10% neutral buffered formalin.

- Brain
- Pituitary gland
- Thyroid gland
- Parathyroid gland[a)]
- Thymus
- Lung including bronchi
- Trachea
- Heart
- Liver
- Spleen
- Kidney
- Adrenal gland
- Salivary glands: submandibular, sublingual and parotid glands
- Esophagus
- Stomach
- Duodenum
- Jejunum
- Ileum
- Cecum
- Colon
- Rectum
- Pancreas
- Testis
- Epididymis
- Prostate
- Seminal vesicle with coagulating gland
- Ovary
- Uterus including cervix
- Vagina
- Urinary bladder
- Submandibular lymph node
- Mesenteric lymph node
- Eye
- Optic nerve[a)]
- Harderian gland
- Mammary gland: inguinal
- Skin: inguinal
- Sternum including bone marrow
- Femur including bone marrow
- Skeletal muscle (thigh)
- Sciatic nerve
- Spinal cord
- Organs with gross lesions

a) The specimens were examined histopathologically only if present in routine sections.

For the histophatological examination, specimens of the preserved tissues were prepared according to the SOP on the preparation procedure of histopathology specimen. All residual organs and tissues were preserved in 10% neutral buffered

formalin.

Histopathological examination was performed as follows:

- Organs or tissues from control and high dose animals
- Organs or tissues from dead animals and organs and tissues with macroscopic lesions in the low and mid dose groups

2.11 Statistical Analysis

Statistical analysis of data of the body weight, food consumption, functional observations (hindlimb landing foot splay, grip strength and motor activity), urine volume, hematology, clinical chemistry and organ weights was performed using SAS Program (version 9.3, SAS Institute Inc., U.S.A.).

For the dosing period and the main group: above data were analyzed utilizing Bartlett's test for homogeneity of variance (significance level: 0.05). One-way analysis of variance (ANOVA) was employed on homogeneous data; then, if significant, Dunnett's test was applied for multiple comparisons (significance levels: 0.05 and 0.01, two-tailed). Kruskal-Wallis test was employed on heterogeneous data; then, if significant, Steel test was applied for multiple comparisons (significance levels: 0.05 and 0.01, two-tailed).

Recovery group: above data were analyzed utilizing Folded F-test for homogeneity of variance (significance level: 0.05). If the variances of two populations were assumed to be homogeneous, Student-t test was conducted (significance levels: 0.05 and 0.01, two-tailed). If the variances were heterogeneous, Aspin-Welch t-test was conducted (significance levels: 0.05 and 0.01, two-tailed).

3. RESULTS AND DISCUSSION

3.1 Clinical Signs

individual data

(Table 1-1, Table 1-2, Table 17-1, Table 17-2)

No test substance-related deaths were observed in either sex in the control group, 2,500, 5,000 and 7,500 mg/kg/day groups during the dosing and recovery periods.

One male (Animal ID No.: 1303) of the 5,000 mg/kg/day group was found dead on Day 72. It was considered to be sudden death of the rat showing no morphological changes and it occurs often in Sprague-Dawley rats [1)], and there was no test substance-related effect on gross findings at necropsy or histopathological lesions in this dead male.

One female (Animal ID No.: 2412) of the 7,500 mg/kg/day group was found dead on Day 30. Serous fluid-filled thoracic cavity (clear with red color) and pulmonary congestion/edema were noted in the dead female. These findings might be due to a technical gavage error [2)]. However, it was considered to be accidental death with no relation to the test substance since necropsy and histopathological results showed no test substance-related change.

Soft stool and diarrhea were often observed in males and females in the 7,500 mg/kg/day group from Day 26 to the end of dosing. However, these clinical signs were not observed in the recovery period except Day 91 (the day after the final dosing). It was considered to be test substance-related effects. However, there were no test substance-related changes in body weight, food consumption, gross findings at necropsy and histopathological results. Therefore, it was considered to have little toxicological significance.

Hematuria was observed in one male in the 7,500 mg/kg/day group of the recovery group. However, it was considered to be of little toxicological significance since there were no hematuria-related morphological changes at necropsy and in the histopathological examination.

3.2 Detailed Examinations of Clinical Signs

individual data

(Table 2-1, Table 2-2, Table 18-1, Table 18-2)

No clinical abnormalities were observed in males and females in the 2,500, 5,000 and 7,500 mg/kg/day groups in the detailed examinations during the dosing and recovery periods.

3.3 Body Weights

individual data

(Figure 1, Figure 2, Table 3-1, Table 3-2, Table 14-1, Table 14-2)

During the recovery period, there was no test substance-related effect in both sexes in the 2,500, 5,000 and 7,500 mg/kg/day groups.

During the dosing period, a statistically significant increase in the body weight was noted in males in the 5,000 and 7,500 mg/kg/day groups on Days 11 and 4, respectively. However, it was considered not to be a test substance-related effect since there was no dose dependency, and it was a temporary change and there was little difference compared to the control group.

During the recovery period, there was no test substance-related effect in both sexes in the 7,500 mg/kg/day group.

3.4 Food Consumption

individual data

(Table 4-1, Table 4-2, Table 15-1, Table 15-2)

During the dosing period, there was no test substance-related effect in both sexes in the 2,500, 5,000 and 7,500 mg/kg/day groups when compared to the control group.

During the recovery period, no statistically significant difference in food consumption was noted in both sexes in the 7,500 mg/kg/day group.

In addition, there was no toxicological significance since the changes observed with statistically significant decrease in females in the 7,500 group on Day 16 showed small difference and there was no change in the body weight.

3.5 Functional Observations

individual data

(Table 5-1, Table 5-2, Table 5-3, Table 5-4, Table 16-1, Table 16-2, Table 16-3, Table 16-4)

There were no test substance treatment-related effects on the visual response, proprioceptive stimuli, auditory response, pain response, aerial righting reflex, hindlimb landing foot splay, grip strength and motor activity in males and females at 2,500, 5,000 and 7,500 mg/kg/day in the main group and at 7,500 mg/kg/day in the recovery group.

In addition, there was no toxicological significance since the changes observed with a statistically significant decrease in hindlimb grip strength in the test substance dosing groups of the main group showed small difference and no dose-dependency.

3.6 Ophthalmological Examination

individual data

(Table 6-1, Table 6-2, Table 17-1, Table 17-2)

There were no ocular abnormalities in males and females in the control and 7,500 mg/kg/day groups of the main group.

3.7 Urinalysis

individual data

(Table 7-1, Table 7-2, Table 18-1, Table 18-2)

There were no test substance-related effects in males and females in the 2,500, 5,000 and 7,500 mg/kg/day groups of the main group and in the 7,500 mg/kg/day group of the recovery group.

Other changes in urinalysis were minor changes and were not dose-related changes, and there were no morphological changes or other related changes. Therefore, they were not considered to be of toxicological significance.

3.8 Hematology

individual data

(Table 8-1, Table 8-2, Table 19-1, Table 19-2)

There were no test-substance-related effects in hematological parameters in males and females in the 2,500, 5,000 and 7,500 mg/kg/day groups of the main group and in the 7,500 mg/kg/day group of the recovery group.

Other changes with statistical significance were considered not to be test substance-related changes because they were small differences and the values were within the range of historical reference data.

3.9 Clinical Chemistry

individual data

(Table 9-1, Table 9-2, Table 20-1, Table 20-2)

There were no test-substance-related effects in clinical chemistry parameters in males and female in the 2,500, 5,000 and 7,500 mg/kg/day groups of the main group and in the 7,500 mg/kg/day group of the recovery group.

Other changes with statistical significance were considered not to be test substance-related changes because they were small differences or the values were within the range of historical reference data.

3.10 Organ Weights

individual data <(Table 14-1, Table 14-2, Table 15-1, Table 15-2, Table 21-1, Table 21-2, Table 22-1, Table 22-2, Table 22-3)

There were no test substance treatment-related effects in males and females in the 2,500, 5,000 and 7,500 mg/kg/day groups of the main group and in the 7,500 mg/kg/day group of the recovery group.

3.11 Necropsy Findings

(Appendix VI) – individual data not included in this submission

Dead animal

Each one animal (Animal ID Nos.: 1303, 2412) was found dead in the 5,000 mg/kg/day group of the main group on Day 72 and in the 7,500 mg/kg/day group of the recovery group on Day 30.

Distension of cecum was noted in the dead male (Animal ID No.: 1303) in the mid dose group of the main group. White area on the hepatic surface and enlargement of liver were observed in this animal. It was also noted that the kidney was enlarged and it had two black foci.

Red discolored lung was noted in the dead female (Animal ID No.: 2412) in the high dose group of the recovery group. There was serous fluid-filled thoracic cavity (clear with red color) in this animal.

Terminal sacrifice

Macroscopic examination at necropsy did not reveal treatment-related changes in either main group or recovery group.

Black focus was observed on the mucosa of glandular stomach of one animal (Animal ID No.: 2405) in the 7,500 mg/kg/day group of the main group.

All other macroscopic findings seen in various organs and tissues were considered to be spontaneous or incidental.

3.12 Histopathological Findings

(Appendix VI) ← individual data, not included in this submission

Dead animal

Death cause-related histopathological findings were not noted in the animal (Animal ID No.: 1303) of the 5,000 mg/kg/day of the main group, which was found dead on Day 72. The gross findings seen at necropsy were considered to be postmortem or agonal

changes because there were no corresponding histopathologic lesions in the organs/tissues with alterations. It has been documented that the unexplained sudden death of a rat showing no morphological changes occurs often in Sprague-Dawley rats [1].

Pulmonary congestion and edema were noted in the female (Animal ID No.: 2412) of the high dose group of the recovery group, which was found dead on Day 30. These histopathologic alterations corresponded to the gross findings seen at necropsy. Therefore, the gross and histopathologic findings suggested that the cause of death of this animal could be associated with technical gavage error and not with the test substance treatment [2].

Terminal sacrifice

Microscopic examination did not reveal any test substance-related changes in any animal in the main and recovery groups in this study.

The presence of renal mesenchymal tumor was noted in the left kidney in one male (Animal ID No.: 1412) in the 7,500 mg/kg/day group of the recovery group.

A small tumor mass was located within outer medulla of the left kidney and the tumor tissues were dispersed between the renal parenchymal tissues of outer medulla. The cells, characterized by basophilic cytoplasm and elongated nucleus including zero to two distinct nucleoli, formed the heterogenous range of tissues, which looked like storiform and/or fascicular patterns with little connective tissues. Taken together, the histopathological examination suggested that the tumor would be renal mesenchymal tumor (RMT). It has been documented that RMT is the most common spontaneous nonepithelial tumor in the rat kidney[5] and can occur with low probability[5, 6], but there were no references that the test substance is associated with carcinogenic effect on the experimental animals[3, 4]. Therefore, RMT in the kidney was considered to be incidental or spontaneous.

All other macroscopic findings seen in various organs and tissues were considered to be spontaneous or incidental.

4. CONCLUSION

Based on the results of this study, the no observed adverse effect level (NOAEL) of 2'-Fucosyllactose was considered to be 7,500 mg/kg/day in both male and female rats after repeated oral administration for thirteen weeks under the conditions of this study.

5. REFERENCES

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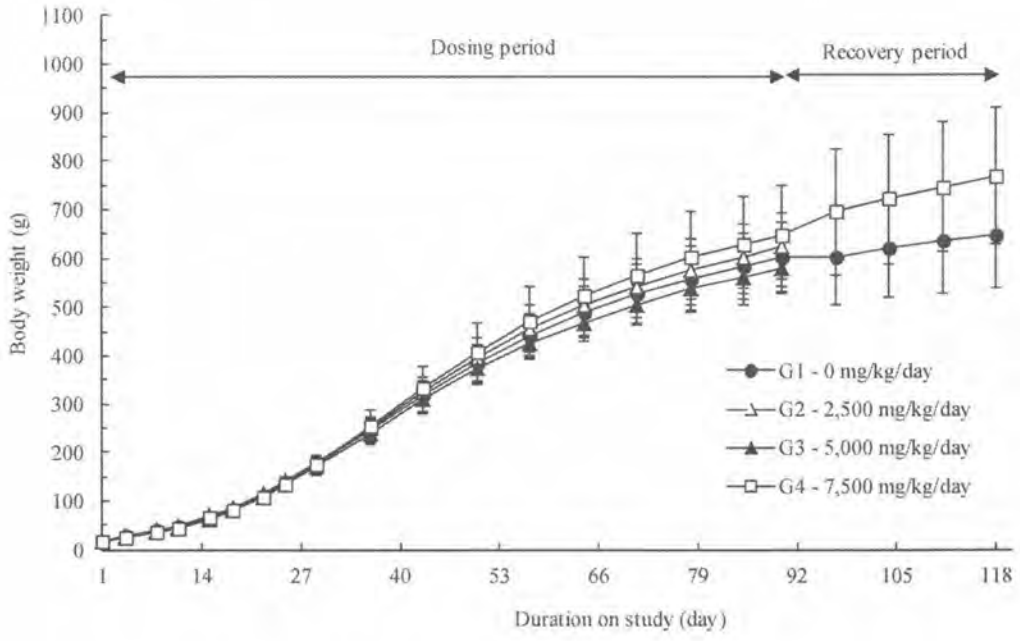

Figure 1. Body Weights in Male SD Rats

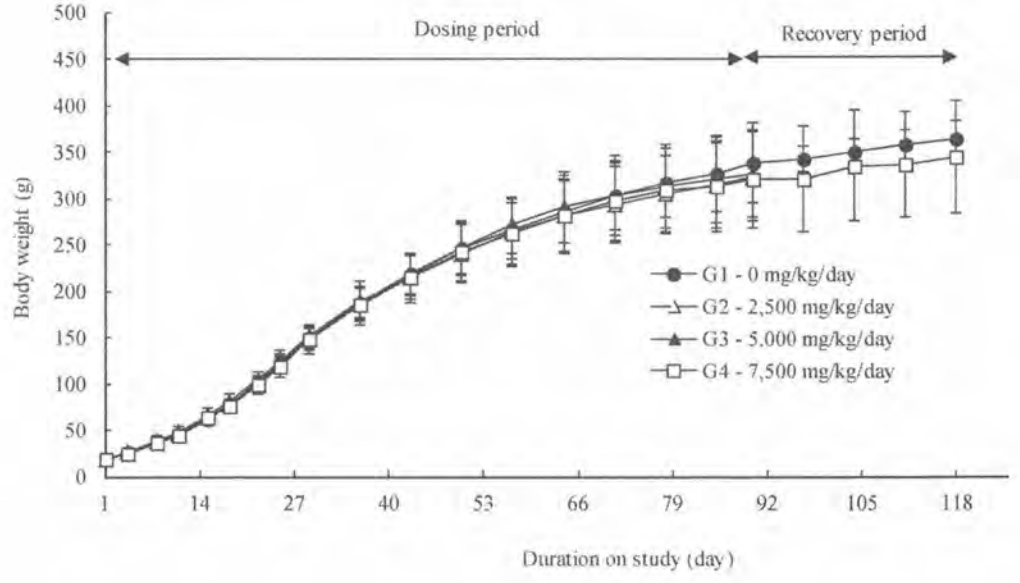

Figure 2. Body Weights in Female SD Rats

Table 1-1. Summary of Clinical Signs (Dosing period)

Sex: Male

Group / Dose (mg/kg/day)	No. of animals	Clinical sign	No. of animals affected
G1 0	15	No observable abnormality	15
G2 2,500	10	No observable abnormality	10
G3 5,000	10	No observable abnormality Death	9 1
G4 7,500	15	Diarrhea Soft stool	14 15

Sex: Female

Group / Dose (mg/kg/day)	No. of animals	Clinical sign	No. of animals affected
G1 0	15	No observable abnormality	15
G2 2,500	10	No observable abnormality	10
G3 5,000	10	No observable abnormality	10
G4 7,500	15	Diarrhea Soft stool Death	13 15 1

Table 1-2. Summary of Clinical Signs (Recovery period)

Sex: Male

Group / Dose (mg/kg/day)	No. of animals	Clinical sign	No. of animals affected
G1 0	5	No observable abnormality	5
G4 7,500	5	Diarrhea Soft stool Hematuria	2 3 1

Sex: Female

Group / Dose (mg/kg/day)	No. of animals	Clinical sign	No. of animals affected
G1 0	5	No observable abnormality	5
G4 7,500	4	No observable abnormality Diarrhea Soft stool	2 1 1

not included in this submission (pages 35-47)
Table 2-1 Detailed Examinations of Clinical Sign (Dosing period)
Table 2-2 Detailed Examinations of Clinical Sign (Recovery period)

Table 3-1. Mean Body Weights (Dosing period)

Sex: Male (g)

Group / Dose (mg/kg/day)		Day 1	4	8	11	15	18
G1	Mean	19.2	27.6	39.3	47.5	66.6	83.3
0	S.D.	1.3	1.7	2.4	2.8	4.6	5.9
	N	15	15	15	15	15	15
G2	Mean	19.5	28.6	40.9	49.8	69.3	85.7
2,500	S.D.	2.0	2.4	3.4	3.5	3.3	4.6
	N	10	10	10	10	10	10
G3	Mean	18.8	25.9	37.5	44.7 *	64.7	80.1
5,000	S.D.	1.2	1.5	2.0	2.6	4.7	5.2
	N	10	10	10	10	10	10
G4	Mean	19.4	26.0 #	37.8	45.2	65.7	80.7
7,500	S.D.	0.8	0.8	1.1	1.8	2.8	4.6
	N	15	15	15	15	15	15

Group / Dose (mg/kg/day)		Day 22	25	29	36	43	50
G1	Mean	111.1	136.5	174.9	245.5	316.2	384.8
0	S.D.	7.6	10.0	14.5	22.6	31.8	39.3
	N	15	15	15	15	15	15
G2	Mean	114.2	140.6	177.7	251.7	325.7	397.2
2,500	S.D.	5.8	9.0	12.3	20.1	28.6	38.8
	N	10	10	10	10	10	10
G3	Mean	107.6	132.7	170.0	239.8	309.4	373.2
5,000	S.D.	8.4	11.0	15.2	22.4	27.3	32.3
	N	10	10	10	10	10	10
G4	Mean	109.3	136.1	176.0	253.4	331.8	406.9
7,500	S.D.	8.0	12.8	19.7	32.7	46.9	59.8
	N	15	15	15	15	15	15

Group / Dose (mg/kg/day)		Day 57	64	71	78	85	90
G1	Mean	440.9	489.7	527.0	558.1	583.9	602.2
0	S.D.	45.9	53.0	60.2	65.4	69.0	72.5
	N	15	15	15	15	15	15
G2	Mean	457.0	505.2	543.9	578.3	604.1	625.1
2,500	S.D.	47.7	51.6	55.4	60.5	64.4	68.3
	N	10	10	10	10	10	10
G3	Mean	427.7	469.4	505.4	538.6	560.6	581.5
5,000	S.D.	33.5	37.6	41.3	49.1	55.2	53.3
	N	10	10	10	9	9	9
G4	Mean	471.1	522.7	565.6	601.4	628.6	647.2
7,500	S.D.	71.0	81.0	87.4	95.1	99.5	103.2
	N	15	15	15	15	15	15

Significantly different from control by Dunnett's t-test: * $p<0.05$.
Significantly different from control by Steel test: # $p<0.05$.

Table 3-1. (Continued)

Sex: Female (g)

Group / Dose (mg/kg/day)		Day 1	4	8	11	15	18
G1	Mean	18.1	26.2	37.0	44.9	62.7	77.9
0	S.D.	1.1	1.4	2.0	2.9	4.3	5.0
	N	15	15	15	15	15	15
G2	Mean	18.2	27.3	38.9	47.3	66.4	81.0
2,500	S.D.	1.7	2.9	4.5	5.7	7.4	7.8
	N	10	10	10	10	10	10
G3	Mean	18.6	25.9	37.5	45.2	63.8	77.8
5,000	S.D.	1.0	1.3	1.9	3.0	4.0	4.4
	N	10	10	10	10	10	10
G4	Mean	18.6	25.2	37.1	44.5	63.3	75.6
7,500	S.D.	1.4	1.7	1.9	2.4	4.1	5.9
	N	15	15	15	15	15	15

Group / Dose (mg/kg/day)		Day 22	25	29	36	43	50
G1	Mean	102.5	123.4	149.4	187.9	218.0	245.4
0	S.D.	6.7	9.4	11.7	16.3	21.3	27.3
	N	15	15	15	15	15	15
G2	Mean	104.7	125.5	151.9	189.2	215.0	240.4
2,500	S.D.	8.8	10.3	12.2	20.7	23.7	30.5
	N	10	10	10	10	10	10
G3	Mean	100.1	120.3	147.8	186.7	217.0	246.3
5,000	S.D.	5.8	8.6	12.1	16.7	22.0	28.8
	N	10	10	10	10	10	10
G4	Mean	98.4	119.0	147.1	184.6	214.4	241.8
7,500	S.D.	9.0	11.6	15.4	20.8	26.5	32.1
	N	15	15	15	14	14	14

Group / Dose (mg/kg/day)		Day 57	64	71	78	85	90
G1	Mean	265.1	284.8	302.1	316.9	326.4	337.7
0	S.D.	30.8	33.1	35.5	37.6	40.4	43.1
	N	15	15	15	15	15	15
G2	Mean	263.0	281.9	293.9	304.6	313.9	323.4
2,500	S.D.	35.4	39.4	39.7	41.7	46.1	47.4
	N	10	10	10	10	10	10
G3	Mean	270.6	290.3	303.0	313.3	320.1	325.9
5,000	S.D.	30.8	37.7	43.0	44.8	46.2	46.0
	N	10	10	10	10	10	10
G4	Mean	262.7	282.3	296.1	308.9	312.8	321.5
7,500	S.D.	35.7	41.6	44.2	48.0	48.7	53.0
	N	14	14	14	14	14	14

Table 3-2. Mean Body Weights (Recovery period)

Sex: Male					(g)
Group /		Day			
Dose (mg/kg/day)		97	104	111	118
G1	Mean	602.5	621.7	636.3	649.3
0	S.D.	97.4	103.0	107.7	111.5
	N	5	5	5	5
G4	Mean	695.9	722.4	747.1	769.1
7,500	S.D.	129.6	132.9	132.0	139.5
	N	5	5	5	5

Sex: Female					(g)
Group /		Day			
Dose (mg/kg/day)		97	104	111	118
G1	Mean	342.2	349.3	357.6	364.6
0	S.D.	14.1	14.0	15.8	18.8
	N	5	5	5	5
G4	Mean	320.6	334.7	336.6	343.9
7,500	S.D.	57.0	59.7	56.5	61.3
	N	4	4	4	4

Table 4-1. Mean Food Consumption (Dosing period)

Sex: Male (g/day)

Group / Dose (mg/kg/day)		Day 16	22	29	36	43	50
G1	Mean	11.8	19.8	27.3	33.1	37.5	38.5
0	S.D.	3.3	1.4	2.4	3.4	5.2	5.4
	N	15	15	15	15	15	15
G2	Mean	11.1	20.2	29.4	35.0	39.1	41.2
2,500	S.D.	2.8	1.6	3.6	4.7	6.0	6.7
	N	10	10	10	10	10	10
G3	Mean	10.2	18.9	27.5	32.3	35.8	37.8
5,000	S.D.	1.1	1.5	3.6	4.6	3.6	3.4
	N	10	10	10	10	10	10
G4	Mean	10.0	19.5	28.3	34.3	38.3	40.0
7,500	S.D.	1.3	2.2	4.0	5.6	7.0	7.3
	N	15	15	15	15	15	15

Group / Dose (mg/kg/day)		Day 57	64	71	78	85	90
G1	Mean	40.1	40.1	37.7	35.3	37.4	37.9
0	S.D.	5.9	6.3	6.0	5.8	6.1	5.8
	N	15	15	15	15	15	15
G2	Mean	41.4	40.7	38.3	38.5	37.7	39.9
2,500	S.D.	6.6	4.7	5.2	4.5	5.4	4.0
	N	10	10	10	10	10	10
G3	Mean	38.4	35.4	34.8	38.0	37.0	36.1
5,000	S.D.	2.8	8.1	2.7	5.0	5.6	5.1
	N	10	10	10	9	9	9
G4	Mean	40.7	41.4	40.0	39.4	40.1	39.4
7,500	S.D.	7.2	7.8	7.1	7.8	5.2	6.2
	N	15	15	15	15	15	15

Table 4-1. (Continued)

Sex: Female (g/day)

Group / Dose (mg/kg/day)		Day 16	22	29	36	43	50
G1	Mean	11.0	18.4	24.1	25.3	25.9	27.3
0	S.D.	1.4	1.8	2.5	3.3	3.7	4.2
	N	15	15	15	15	15	15
G2	Mean	11.1	19.1	23.9	25.2	24.8	24.0
2,500	S.D.	1.6	1.5	1.8	4.2	4.2	3.9
	N	10	10	10	10	10	10
G3	Mean	10.3	18.3	23.6	24.6	25.1	26.3
5,000	S.D.	1.6	2.0	3.3	2.9	3.8	4.2
	N	10	10	10	10	10	10
G4	Mean	9.5 *	17.3	23.6	25.1	25.0	25.5
7,500	S.D.	0.9	2.5	3.1	3.5	4.0	5.3
	N	15	15	15	14	14	14

Group / Dose (mg/kg/day)		Day 57	64	71	78	85	90
G1	Mean	25.0	26.4	26.4	25.5	24.0	27.8
0	S.D.	3.9	3.7	3.7	3.3	4.6	3.8
	N	15	15	15	15	15	15
G2	Mean	24.7	24.9	25.5	22.8	23.1	25.1
2,500	S.D.	5.6	6.1	4.2	4.8	3.7	4.1
	N	10	10	10	10	10	10
G3	Mean	27.2	26.5	25.1	24.2	23.1	24.5
5,000	S.D.	4.5	5.1	5.1	5.0	3.6	1.9
	N	10	10	10	10	10	10
G4	Mean	25.4	24.5	23.7	24.0	22.4	24.7
7,500	S.D.	4.6	4.9	5.6	5.9	5.5	7.8
	N	14	14	14	14	14	14

Significantly different from control by Dunnett's t-test: * $p<0.05$.

Table 4-2. Mean Food Consumption (Recovery period)

Sex: Male					(g/day)
Group /		Day			
Dose (mg/kg/day)		97	104	111	118
G1	Mean	34.8	36.1	32.2	33.4
0	S.D.	8.2	8.5	5.6	6.7
	N	5	5	5	5
G4	Mean	46.2	44.7	38.5	43.8
7,500	S.D.	10.7	9.0	5.5	9.6
	N	5	5	5	5

Sex: Female					(g/day)
Group /		Day			
Dose (mg/kg/day)		97	104	111	118
G1	Mean	25.0	26.1	21.6	24.8
0	S.D.	2.1	3.0	4.9	4.4
	N	5	5	5	5
G4	Mean	26.0	26.1	19.0	22.0
7,500	S.D.	5.0	4.5	3.7	5.6
	N	4	4	4	4

Table 5-1. Summary of Functional Observations; Perception and Motor Function Observations (Main group)

Sex: Male

Group / Dose (mg/kg/day)		Visual response	Touch response	Click response	Tail pinch response	Aerial righting reflex
G1	Mean	3	3	3	3	0
0	S.D.	0	0	0	0	0
	N	10	10	10	10	10
G2	Mean	3	3	3	3	0
2.500	S.D.	0	0	0	0	0
	N	10	10	10	10	10
G3	Mean	3	3	3	3	0
5.000	S.D.	0	0	0	0	0
	N	9	9	9	9	9
G4	Mean	3	3	3	3	0
7,500	S.D.	0	0	0	0	0
	N	10	10	10	10	10

Sex: Female

Group / Dose (mg/kg/day)		Visual response	Touch response	Click response	Tail pinch response	Aerial righting reflex
G1	Mean	3	3	3	3	0
0	S.D.	0	0	0	0	0
	N	10	10	10	10	10
G2	Mean	3	3	3	3	0
2,500	S.D.	0	0	0	0	0
	N	10	10	10	10	10
G3	Mean	3	3	3	3	0
5,000	S.D.	0	0	0	0	0
	N	10	10	10	10	10
G4	Mean	3	3	3	3	0
7,500	S.D.	0	0	0	0	0
	N	10	10	10	10	10

Visual response - 3: The animal approaches slowly and smells a stimulating bar

Touch response - 3: The animal turns around slowly

Click response - 3: Twitching of body

Tail pinch response - 3: Squeaking, turning back

Aerial righting reflex - 0: Normal (Landing on four limbs)

Table 5-1. (Continued)

Sex: Male

Group / Dose (mg/kg/day)		Hindlimb landing foot splay (mm)	Forelimb grip strength (kgf)	Hindlimb grip strength (kgf)
G1	Mean	65.78	1.127	0.584
0	S.D.	23.10	0.160	0.061
	N	10	10	10
G2	Mean	70.69	1.095	0.603
2,500	S.D.	15.90	0.162	0.065
	N	10	10	10
G3	Mean	65.49	1.235	0.552
5,000	S.D.	19.57	0.088	0.055
	N	9	9	9
G4	Mean	64.09	1.187	0.569
7,500	S.D.	19.57	0.113	0.075
	N	10	10	10

Sex: Female

Group / Dose (mg/kg/day)		Hindlimb landing foot splay (mm)	Forelimb grip strength (kgf)	Hindlimb grip strength (kgf)
G1	Mean	54.69	0.809	0.484
0	S.D.	16.94	0.070	0.068
	N	10	10	10
G2	Mean	53.81	0.774	0.408 *
2,500	S.D.	20.00	0.078	0.060
	N	10	10	10
G3	Mean	52.78	0.809	0.412 *
5,000	S.D.	12.50	0.074	0.058
	N	10	10	10
G4	Mean	46.45	0.811	0.405 *
7,500	S.D.	11.22	0.084	0.062
	N	10	10	10

Significantly different from control by Dunnett's t-test: * $p<0.05$.

Table 5-2. Summary of Functional Observations; Spontaneous Motor Activity (Main group)

Sex: Male

Group / Dose (mg/kg/day)		Ambulatory counts (minutes interval)						
		0-10	10-20	20-30	30-40	40-50	50-60	Total
G1	Mean	1480	1115	1182	1188	384	375	5724
0	S.D.	673	267	1489	1435	210	272	3265
	N	10	10	10	10	10	10	10
G2	Mean	1363	1143	802	725	496	574	5103
2,500	S.D.	332	442	539	298	354	341	1716
	N	10	10	10	10	10	10	10
G3	Mean	1428	1071	953	729	386	490	5057
5,000	S.D.	362	307	381	455	268	474	1593
	N	9	9	9	9	9	9	9
G4	Mean	1376	1109	720	676	468	415	4764
7,500	S.D.	542	494	309	362	353	114	1820
	N	10	10	10	10	10	10	10

Group / Dose (mg/kg/day)		Vertical counts (minutes interval)						
		0-10	10-20	20-30	30-40	40-50	50-60	Total
G1	Mean	93	71	48	44	30	28	314
0	S.D.	26	26	24	18	15	14	95
	N	10	10	10	10	10	10	10
G2	Mean	85	66	54	48	26	31	311
2,500	S.D.	13	19	20	19	17	14	71
	N	10	10	10	10	10	10	10
G3	Mean	93	60	47	33	29	33	295
5,000	S.D.	31	17	20	18	26	24	105
	N	9	9	9	9	9	9	9
G4	Mean	89	59	41	38	26	25	278
7,500	S.D.	22	26	14	28	21	11	99
	N	10	10	10	10	10	10	10

Table 5-2. (Continued)

Sex: Female

Group / Dose (mg/kg/day)		Ambulatory counts (minutes interval)						
		0-10	10-20	20-30	30-40	40-50	50-60	Total
G1	Mean	2411	1873	1499	1295	1174	1027	9280
0	S.D.	715	488	393	561	499	500	2375
	N	10	10	10	10	10	10	10
G2	Mean	2682	2167	1945	1769	1809	1462	11833
2,500	S.D.	693	729	469	783	750	442	3302
	N	10	10	10	10	10	10	10
G3	Mean	2498	1832	1843	1654	1411	1450	10688
5,000	S.D.	699	535	612	668	674	635	3218
	N	10	10	10	10	10	10	10
G4	Mean	2336	2006	1822	1637	1430	1304	10535
7,500	S.D.	407	614	640	676	606	326	2890
	N	10	10	10	10	10	10	10

Group / Dose (mg/kg/day)		Vertical counts (minutes interval)						
		0-10	10-20	20-30	30-40	40-50	50-60	Total
G1	Mean	109	82	66	46	50	51	405
0	S.D.	17	18	13	16	15	25	65
	N	10	10	10	10	10	10	10
G2	Mean	116	81	82	64	63	76	481
2,500	S.D.	24	25	27	15	23	35	135
	N	10	10	10	10	10	10	10
G3	Mean	106	82	59	53	53	53	406
5,000	S.D.	41	29	28	26	27	35	157
	N	10	10	10	10	10	10	10
G4	Mean	118	90	68	60	50	55	440
7,500	S.D.	30	28	27	31	25	28	146
	N	10	10	10	10	10	10	10

Table 5-3. Summary of Functional Observations; Perception and Motor Function Observations (Recovery group)

Sex: Male

Group / Dose (mg/kg/day)		Visual response	Touch response	Click response	Tail pinch response	Aerial righting reflex
G1	Mean	3	3	3	3	0
0	S.D.	0	0	0	0	0
	N	5	5	5	5	5
G4	Mean	3	3	3	3	0
7,500	S.D.	0	0	0	0	0
	N	5	5	5	5	5

Sex: Female

Group / Dose (mg/kg/day)		Visual response	Touch response	Click response	Tail pinch response	Aerial righting reflex
G1	Mean	3	3	3	3	0
0	S.D.	0	0	0	0	0
	N	5	5	5	5	5
G4	Mean	3	3	3	3	0
7,500	S.D.	0	0	0	0	0
	N	4	4	4	4	4

Visual response - 3: The animal approaches slowly and smells a stimulating bar
Touch response - 3: The animal turns around slowly
Click response - 3: Twitching of body
Tail pinch response - 3: Squeaking, turning back
Aerial righting reflex - 0: Normal (Landing on four limbs)

Table 5-3. (Continued)

Sex: Male

Group / Dose (mg/kg/day)		Hindlimb landing foot splay (mm)	Forelimb grip strength (kgf)	Hindlimb grip strength (kgf)
G1	Mean	82.74	1.099	0.651
0	S.D.	10.44	0.078	0.055
	N	5	5	5
G4	Mean	77.71	1.119	0.626
7.500	S.D.	25.49	0.055	0.037
	N	5	5	5

Sex: Female

Group / Dose (mg/kg/day)		Hindlimb landing foot splay (mm)	Forelimb grip strength (kgf)	Hindlimb grip strength (kgf)
G1	Mean	54.00	0.812	0.484
0	S.D.	8.15	0.051	0.032
	N	5	5	5
G4	Mean	40.59	0.791	0.482
7.500	S.D.	9.31	0.100	0.025
	N	4	4	4

Table 5-4. Summary of Functional Observations; Spontaneous Motor Activity (Recovery group)

Sex: Male

Group / Dose (mg/kg/day)		Ambulatory counts (minutes interval)						
		0-10	10-20	20-30	30-40	40-50	50-60	Total
G1	Mean	1623	1520	989	571	355	348	5405
0	S.D.	673	915	500	403	321	250	2860
	N	5	5	5	5	5	5	5
G4	Mean	1043	1303	508	302	230	305	3691
7,500	S.D.	383	794	527	365	203	120	1763
	N	5	5	5	5	5	5	5

Group / Dose (mg/kg/day)		Vertical counts (minutes interval)						
		0-10	10-20	20-30	30-40	40-50	50-60	Total
G1	Mean	99	47	26	15	19	15	222
0	S.D.	29	17	15	10	8	13	80
	N	5	5	5	5	5	5	5
G4	Mean	84	46	38	56	18	16	257
7,500	S.D.	22	27	39	73	9	11	104
	N	5	5	5	5	5	5	5

Sex: Female

Group / Dose (mg/kg/day)		Ambulatory counts (minutes interval)						
		0-10	10-20	20-30	30-40	40-50	50-60	Total
G1	Mean	1673	1298	1079	1148	780	760	6738
0	S.D.	359	427	515	655	682	576	3101
	N	5	5	5	5	5	5	5
G4	Mean	2310	1580	1515	782	502	1809	8498
7,500	S.D.	427	279	209	528	450	2189	2136
	N	4	4	4	4	4	4	4

Group / Dose (mg/kg/day)		Vertical counts (minutes interval)						
		0-10	10-20	20-30	30-40	40-50	50-60	Total
G1	Mean	85	64	32	38	20	23	264
0	S.D.	17	25	12	17	18	19	90
	N	5	5	5	5	5	5	5
G4	Mean	91	56	59	22	26	24	277
7,500	S.D.	20	17	30	17	32	11	105
	N	4	4	4	4	4	4	4

Table 6. Summary of Ophthalmological Examination (Main group)

Sex: Male

Group / Dose (mg/kg/day)	No. of animals	Findings	Right eye				Left eye			
			Pupil light reflex	Anterior segment	Trans-parent media	Fundus	Pupil light reflex	Anterior segment	Trans-parent media	Fundus
G1 0	10	Normal	10	10	10	10	10	10	10	10
G4 7,500	10	Normal	10	10	10	10	10	10	10	10

Sex: Female

Group / Dose (mg/kg/day)	No. of animals	Findings	Right eye				Left eye			
			Pupil light reflex	Anterior segment	Trans-parent media	Fundus	Pupil light reflex	Anterior segment	Trans-parent media	Fundus
G1 0	10	Normal	10	10	10	10	10	10	10	10
G4 7,500	10	Normal	10	10	10	10	10	10	10	10

Table 7-1. Summary of Urinalysis Results (Main group)

Sex		Male				Female			
Group /		G1	G2	G3	G4	G1	G2	G3	G4
Dose (mg/kg/day)		0	2,500	5,000	7,500	0	2,500	5,000	7,500
No. of animals		5	5	4	5	5	5	5	5
Volume (mL)	Mean	11.6	14.8	13.6	14.0	9.3	5.5 *	5.9	5.2 *
	S.D.	2.7	6.2	5.3	4.3	2.4	1.3	1.8	2.8
Color	Pale yellow	1	2		1				
	Yellow	4	3	4	4	5	5	5	5
	Amber								
	Brown								
	Red								
Transparency	Clear	5	5	3	3	5	5	4	5
	Mild turbidity								
	Turbidity			1	2			1	
pH	5								
	6						1		3
	6.5		1		1				1
	7	3	2	4	3	2	2	2	
	8	2	2		1	3	2	3	1
	9								
Protein	-		1			3	2	2	
(mg/dL)	25	4	1	2	3	2	3	3	5
	75		3	2	2				
	150	1							
	500								
Glucose	Normal	5	5	4	5	5	5	5	5
(mg/dL)	50								
	100								
	300								
	1,000								
Ketone body	-					3	2		
(mg/dL)	5	4	2	1	3	1	3	4	3
	15	1	3	2	2	1		1	2
	50			1					
	150								
Bilirubin	-	4	4	3	5	5	5	5	5
(mg/dL)	1	1	1	1					
	3								
	6								
Occult blood	-	4	4	3	1	5	5	4	5
(Ery/μL)	10	1	1		2			1	
	25								
	50								
	150			1	1				
	250				1				

Significantly different from control by Dunnett's t-test: * $p<0.05$.

Table 7-1. (Continued)

Sex		Male				Female			
Group /		G1	G2	G3	G4	G1	G2	G3	G4
Dose (mg/kg/day)		0	2.500	5.000	7.500	0	2.500	5.000	7.500
No. of animals		5	5	4	5	5	5	5	5
Cast^	0	5	5	4	5	5	5	5	5
	1 ~ 5								
	6 ~ 10								
	>10								
Epithelial cell^	0	5	5	4	5	5	5	5	5
	1 ~ 5								
	6 ~ 10								
	>10								
Leukocyte^	0	5	5	4	5	4	4	5	4
	1 ~ 10					1	1		1
	11 ~ 50								
	51 ~ 100								
	>100								
Erythrocyte^	0	5	5	3	4	5	5	5	5
	1 ~ 10			1	1				
	11 ~ 50								
	51 ~ 100								
	>100								
Specific gravity	1.000 ~ 1.010								
	1.011 ~ 1.020					1			
	1.021 ~ 1.030		1						
	1.031 ~ 1.040		1			3			
	1.041 ~ 1.050	2	1	1	1		1	2	1
	1.051 ~ 1.060	3	1	3	3		1	1	1
	>1.060		1		1	1	3	2	3

^: Sediment

Table 7-2. Summary of Urinalysis Results (Recovery group)

Sex		Male		Female	
Group /		G1	G4	G1	G4
Dose (mg/kg/day)		0	7,500	0	7,500
No. of animals		5	5	5	4
Volume (mL)	Mean	13.1	16.9	7.4	9.6
	S.D.	0.8	3.5	2.6	3.0
Color	Pale yellow	2	1	5	4
	Yellow	3	3		
	Amber				
	Brown				
	Red		1		
Transparency	Clear	5	5	5	4
	Mild turbidity				
	Turbidity				
pH	5				
	6				
	6.5	1			
	7	2	3	2	1
	8	2	2	3	3
	9				
Protein	-	1		5	4
(mg/dL)	25	2	1		
	75	1			
	150		3		
	500	1	1		
Glucose	Normal	5	5	5	4
(mg/dL)	50				
	100				
	300				
	1,000				
Ketone body	-	2	1	5	4
(mg/dL)	5	3	4		
	15				
	50				
	150				
Bilirubin	-	5	5	5	4
(mg/dL)	1				
	3				
	6				
Occult blood	-		1	5	4
(Ery/μL)	10	4	2		
	25	1			
	50				
	150				
	250		2		

Table 7-2. (Continued)

Sex		Male		Female	
Group /		G1	G4	G1	G4
Dose (mg/kg/day)		0	7,500	0	7,500
No. of animals		5	5	5	4
Cast^	0	5	5	5	4
	1 ~ 5				
	6 ~ 10				
	>10				
Epithelial cell^	0	5	5	5	4
	1 ~ 5				
	6 ~ 10				
	>10				
Leukocyte^	0	5	5	5	4
	1 ~ 10				
	11 ~ 50				
	51 ~ 100				
	>100				
Erythrocyte^	0	5	4	5	4
	1 ~ 10				
	11 ~ 50				
	51 ~ 100				
	>100		1		
Specific gravity	1.000 ~ 1.010				
	1.011 ~ 1.020				
	1.021 ~ 1.030				
	1.031 ~ 1.040	1	1		2
	1.041 ~ 1.050	1	2	3	1
	1.051 ~ 1.060	2	2	1	1
	>1.060	1		1	

^: Sediment

Table 8-1. Mean Hematological Parameters (Main group)

Sex: Male

Group / Dose (mg/kg/day)		RBC ($\times 10^6$ /μL)	HGB (g/dL)	HCT (%)	RBC Indices			PLT ($\times 10^3$ /μL)	Reti (%)
					MCV (fL)	MCH (pg)	MCHC (g/dL)		
G1	Mean	8.50	15.8	44.2	52.0	18.6	35.8	982	3.81
0	S.D.	0.33	0.4	1.2	0.9	0.4	0.3	97	0.29
	N	10	10	10	10	10	10	10	10
G2	Mean	8.47	15.8	44.2	52.2	18.6	35.7	978	3.77
2,500	S.D.	0.41	0.7	1.7	1.6	0.7	0.3	105	0.43
	N	10	10	10	10	10	10	10	10
G3	Mean	8.54	15.7	44.0	51.6	18.4	35.7	974	3.45
5,000	S.D.	0.30	0.5	1.5	0.4	0.2	0.4	90	0.45
	N	9	9	9	9	9	9	9	9
G4	Mean	8.48	15.7	43.9	51.8	18.5	35.7	960	3.39
7,500	S.D.	0.55	0.7	2.1	1.2	0.5	0.4	67	0.39
	N	10	10	10	10	10	10	10	10

Group / Dose (mg/kg/day)		WBC ($\times 10^3$ /μL)	WBC Differential Counting (%)					PT (sec)	APTT (sec)
			NEU	LYM	MONO	EOS	BASO		
G1	Mean	11.21	21.6	69.2	7.6	1.2	0.3	18.5	15.2
0	S.D.	3.00	6.5	6.4	1.6	0.4	0.2	0.8	1.7
	N	10	10	10	10	10	10	10	10
G2	Mean	10.86	18.1	70.7	9.7	1.1	0.4	17.5	14.7
2,500	S.D.	1.77	3.9	4.5	2.8	0.3	0.2	1.1	2.2
	N	10	10	10	10	10	10	10	10
G3	Mean	11.36	18.2	72.3	8.4	0.9	0.2	17.6	15.3
5,000	S.D.	3.20	5.4	6.5	2.4	0.2	0.1	0.9	0.9
	N	9	9	9	9	9	9	9	9
G4	Mean	10.74	17.6	70.2	10.6 *	1.2	0.3	17.9	15.3
7,500	S.D.	2.44	4.5	4.6	2.3	0.5	0.1	0.9	1.3
	N	10	10	10	10	10	10	10	10

Significantly different from control by Dunnett's t-test: * $p<0.05$.

Table 8-1. (Continued)

Sex: Female

Group / Dose (mg/kg/day)		RBC ($\times 10^6$ /μL)	HGB (g/dL)	HCT (%)	RBC Indices			PLT ($\times 10^3$ /μL)	Reti (%)
					MCV (fL)	MCH (pg)	MCHC (g/dL)		
G1	Mean	8.11	15.5	42.8	52.8	19.1	36.1	930	3.32
0	S.D.	0.20	0.3	1.2	0.6	0.3	0.4	106	0.53
	N	10	10	10	10	10	10	10	10
G2	Mean	7.88	15.4	42.4	53.8	19.5	36.3	869	3.38
2,500	S.D.	0.23	0.3	1.0	1.1	0.4	0.3	80	0.34
	N	10	10	10	10	10	10	10	10
G3	Mean	8.00	15.4	42.8	53.6	19.3	36.0	872	3.46
5,000	S.D.	0.35	0.5	1.5	1.7	0.6	0.2	99	0.52
	N	10	10	10	10	10	10	10	10
G4	Mean	7.91	15.2	42.3	53.6	19.3	36.0	947	3.37
7,500	S.D.	0.24	0.3	1.0	1.3	0.4	0.6	103	0.52
	N	10	10	10	10	10	10	10	10

Group / Dose (mg/kg/day)		WBC ($\times 10^3$ /μL)	WBC Differential Counting (%)					PT (sec)	APTT (sec)
			NEU	LYM	MONO	EOS	BASO		
G1	Mean	7.24	15.6	75.4	7.4	1.3	0.3	18.5	14.6
0	S.D.	3.50	5.2	6.3	2.3	0.4	0.2	0.7	1.1
	N	10	10	10	10	10	10	10	10
G2	Mean	5.67	15.8	75.9	6.9	1.2	0.2	18.0	14.4
2,500	S.D.	2.00	4.6	6.3	2.0	0.4	0.1	0.8	1.1
	N	10	10	10	10	10	10	10	10
G3	Mean	5.18	15.8	76.4	6.7	0.9 *	0.2	17.7 *	15.0
5,000	S.D.	1.63	7.9	7.4	2.4	0.2	0.1	0.7	1.0
	N	10	10	10	10	10	10	10	10
G4	Mean	5.28	19.6	72.6	6.5	1.1	0.2	17.5 *	14.8
7,500	S.D.	1.81	8.1	7.9	0.9	0.4	0.1	0.7	0.9
	N	10	10	10	10	10	10	10	10

Significantly different from control by Dunnett's t-test: * $p<0.05$.

Table 8-2. Mean Hematological Parameters (Recovery group)

Sex: Male

Group / Dose (mg/kg/day)		RBC ($\times 10^6$ /μL)	HGB (g/dL)	HCT (%)	RBC Indices			PLT ($\times 10^3$ /μL)	Reti (%)
					MCV (fL)	MCH (pg)	MCHC (g/dL)		
G1	Mean	8.68	15.5	43.7	50.4	17.9	35.5	1033	3.68
0	S.D.	0.47	0.4	0.6	2.0	1.0	0.7	135	0.77
	N	5	5	5	5	5	5	5	5
G4	Mean	8.08	14.8	41.9	52.0	18.4	35.4	1027	3.95
7,500	S.D.	0.53	0.7	1.4	2.3	0.8	0.8	121	0.69
	N	5	5	5	5	5	5	5	5

Group / Dose (mg/kg/day)		WBC ($\times 10^3$ /μL)	WBC Differential Counting (%)					PT (sec)	APTT (sec)
			NEU	LYM	MONO	EOS	BASO		
G1	Mean	8.77	16.9	72.9	8.7	1.3	0.2	17.9	15.4
0	S.D.	2.12	2.6	3.0	1.6	0.4	0.1	1.2	0.7
	N	5	5	5	5	5	5	5	5
G4	Mean	8.13	16.2	73.1	9.3	1.2	0.2	17.3	15.8
7,500	S.D.	2.09	2.3	3.4	1.3	0.3	0.1	1.2	1.5
	N	5	5	5	5	5	5	5	5

Sex: Female

Group / Dose (mg/kg/day)		RBC ($\times 10^6$ /μL)	HGB (g/dL)	HCT (%)	RBC Indices			PLT ($\times 10^3$ /μL)	Reti (%)
					MCV (fL)	MCH (pg)	MCHC (g/dL)		
G1	Mean	8.22	15.8	43.9	53.4	19.2	35.9	925	2.43
0	S.D.	0.22	0.2	0.7	1.0	0.4	0.4	34	0.14
	N	5	5	5	5	5	5	5	5
G4	Mean	7.71	14.8	41.5	53.9	19.2	35.6	1012	2.81
7,500	S.D.	0.14	0.3	0.6	0.4	0.4	0.4	81	0.21
	N	4	4	4	4	4	4	4	4

Group / Dose (mg/kg/day)		WBC ($\times 10^3$ /μL)	WBC Differential Counting (%)					PT (sec)	APTT (sec)
			NEU	LYM	MONO	EOS	BASO		
G1	Mean	4.68	18.4	72.8	7.3	1.4	0.1	17.9	14.0
0	S.D.	1.92	4.2	3.2	1.6	0.7	0.1	0.6	1.4
	N	5	5	5	5	5	5	5	5
G4	Mean	3.54	20.4	71.2	6.6	1.7	0.2	17.8	13.4
7,500	S.D.	1.46	4.1	3.7	1.9	0.3	0.2	0.5	1.0
	N	4	4	4	4	4	4	4	4

Table 9-1. Mean Clinical Chemistry (Main group)

Sex: Male

Group / Dose (mg/kg/day)		ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)	Glu (mg/dL)	BUN (mg/dL)	Crea (mg/dL)	T-Bili (mg/dL)	T-Chol (mg/dL)
G1	Mean	24.6	72.1	305.6	0.27	160	12.8	0.45	0.08	90
0	S.D.	6.7	15.7	63.8	0.17	25	1.7	0.06	0.03	13
	N	10	10	10	10	10	10	10	10	10
G2	Mean	23.5	68.4	329.9	0.18	159	12.1	0.45	0.06	97
2.500	S.D.	4.9	10.6	36.7	0.11	15	0.9	0.04	0.02	26
	N	10	10	10	10	10	10	10	10	10
G3	Mean	22.9	69.7	306.2	0.24	152	11.8	0.41	0.06	75
5.000	S.D.	5.3	13.5	61.2	0.13	12	1.6	0.03	0.01	14
	N	9	9	9	9	9	9	9	9	9
G4	Mean	32.4	85.5	314.4	0.22	151	10.9	0.40 *	0.06	74
7,500	S.D.	25.6	36.2	68.9	0.08	16	1.8	0.04	0.02	21
	N	10	10	10	10	10	10	10	10	10

Group/ Dose (mg/kg/day)		TG (mg/dL)	TP (g/dL)	Alb (g/dL)	A/G ratio	P (mg/dL)	Ca (mg/dL)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)
G1	Mean	32	5.9	2.3	0.64	6.25	10.0	136.3	3.69	106.2
0	S.D.	15	0.2	0.1	0.06	0.26	0.4	2.1	0.33	0.8
	N	10	10	10	10	10	10	10	10	10
G2	Mean	69 #	6.1	2.4	0.64	6.61	10.4	135.8	3.79	105.8
2,500	S.D.	35	0.3	0.1	0.04	0.50	0.3	1.3	0.20	1.0
	N	10	10	10	10	10	10	10	10	10
G3	Mean	58	5.9	2.3	0.63	6.79 *	10.1	135.5	3.80	105.8
5,000	S.D.	59	0.2	0.1	0.05	0.38	0.4	1.9	0.19	1.9
	N	9	9	9	9	9	9	9	9	9
G4	Mean	66	5.9	2.3	0.64	6.82 *	10.1	135.5	3.97	104.5 *
7,500	S.D.	56	0.2	0.1	0.05	0.63	0.6	3.7	0.16	1.1
	N	10	10	10	10	10	10	10	10	10

Significantly different from control by Dunnett's t-test: * $p<0.05$.

Significantly different from control by Steel test: # $p<0.05$.

Table 9-1. (Continued)

Sex: Female

Group / Dose (mg/kg/day)		ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)	Glu (mg/dL)	BUN (mg/dL)	Crea (mg/dL)	T-Bili (mg/dL)	T-Chol (mg/dL)
G1	Mean	23.1	62.5	214.1	0.53	148	13.0	0.47	0.07	70
0	S.D.	6.0	11.2	67.4	0.24	13	1.8	0.03	0.02	14
	N	10	10	10	10	10	10	10	10	10
G2	Mean	18.4	63.0	197.6	0.51	147	14.3	0.51	0.07	88 *
2,500	S.D.	4.7	7.9	40.6	0.20	17	1.9	0.04	0.02	16
	N	10	10	10	10	10	10	10	10	10
G3	Mean	16.4 *	55.4	213.8	0.42	157	12.9	0.46	0.05	94 **
5,000	S.D.	2.6	6.1	83.1	0.30	17	1.6	0.04	0.01	21
	N	10	10	10	10	10	10	10	10	10
G4	Mean	19.7	59.2	236.7	0.24 *	163	13.8	0.47	0.06	98 **
7,500	S.D.	6.0	11.4	123.3	0.11	18	2.2	0.02	0.02	14
	N	10	10	10	10	10	10	10	10	10

Group / Dose (mg/kg/day)		TG (mg/dL)	TP (g/dL)	Alb (g/dL)	A/G ratio	P (mg/dL)	Ca (mg/dL)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)
G1	Mean	17	6.0	2.6	0.74	5.18	9.6	136.5	3.66	108.8
0	S.D.	7	0.3	0.2	0.02	0.67	0.5	1.1	0.29	1.1
	N	10	10	10	10	10	10	10	10	10
G2	Mean	18	6.0	2.6	0.76	5.57	9.9	136.7	3.52	108.2
2,500	S.D.	10	0.3	0.2	0.04	0.41	0.4	1.0	0.30	0.9
	N	10	10	10	10	10	10	10	10	10
G3	Mean	20	6.0	2.6	0.79	5.33	10.0	136.1	3.75	107.8
5,000	S.D.	7	0.4	0.2	0.08	0.40	0.5	2.2	0.32	1.7
	N	10	10	10	10	10	10	10	10	10
G4	Mean	40	6.1	2.7	0.82	4.95	9.8	135.2	3.65	107.2
7,500	S.D.	43	0.3	0.1	0.07	0.45	0.5	1.5	0.23	1.5
	N	10	10	10	10	10	10	10	10	10

Significantly different from control by Dunnett's t-test: * $p<0.05$, ** $p<0.01$.

Table 9-2. Mean Clinical Chemistry (Recovery group)

Sex: Male

Group / Dose (mg/kg/day)		ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)	Glu (mg/dL)	BUN (mg/dL)	Crea (mg/dL)	T-Bili (mg/dL)	T-Chol (mg/dL)
G1	Mean	30.4	70.4	282.7	0.22	160	12.0	0.52	0.06	90
0	S.D.	4.5	13.8	37.4	0.03	11	1.3	0.01	0.01	20
	N	5	5	5	5	5	5	5	5	5
G4	Mean	22.7	69.6	243.0	0.47	162	13.2	0.52	0.07	125
7,500	S.D.	5.2	14.1	18.8	0.37	23	2.4	0.08	0.02	30
	N	5	5	5	5	5	5	5	5	5

Group / Dose (mg/kg/day)		TG (mg/dL)	TP (g/dL)	Alb (g/dL)	A/G ratio	P (mg/dL)	Ca (mg/dL)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)
G1	Mean	88	5.8	2.4	0.71	5.95	10.0	135.0	4.05	105.6
0	S.D.	17	0.2	0.1	0.05	0.08	0.0	1.0	0.14	0.9
	N	5	5	5	5	5	5	5	5	5
G4	Mean	142	5.9	2.3	0.66	6.24	10.0	134.8	4.17	105.3
7,500	S.D.	85	0.3	0.2	0.07	0.30	0.3	0.9	0.30	1.5
	N	5	5	5	5	5	5	5	5	5

Sex: Female

Group / Dose (mg/kg/day)		ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)	Glu (mg/dL)	BUN (mg/dL)	Crea (mg/dL)	T-Bili (mg/dL)	T-Chol (mg/dL)
G1	Mean	32.7	94.0	164.1	0.51	140	14.1	0.58	0.06	73
0	S.D.	13.5	13.7	29.1	0.20	14	1.8	0.08	0.00	16
	N	5	5	5	5	5	5	5	5	5
G4	Mean	23.8	84.7	150.1	0.35	151	14.6	0.57	0.07	78
7,500	S.D.	8.8	24.0	24.6	0.06	18	1.3	0.05	0.02	9
	N	4	4	4	4	4	4	4	4	4

Group / Dose (mg/kg/day)		TG (mg/dL)	TP (g/dL)	Alb (g/dL)	A/G ratio	P (mg/dL)	Ca (mg/dL)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)
G1	Mean	27	6.1	2.6	0.76	4.86	9.6	135.9	3.94	107.2
0	S.D.	8	0.1	0.1	0.04	0.27	0.2	1.1	0.17	1.5
	N	5	5	5	5	5	5	5	5	5
G4	Mean	26	5.9	2.7	0.84	5.12	9.4	135.7	3.72	107.4
7,500	S.D.	16	0.3	0.2	0.06	0.31	0.4	1.2	0.25	0.6
	N	4	4	4	4	4	4	4	4	4

Table 10-1. Mean Absolute Organ Weights (Main group)

Sex: Male (g)

Group / Dose (mg/kg/day)		B.W.	Brain	Thymus	Heart	Liver	Spleen
G1	Mean	576.2	2.16	0.43	1.64	15.88	0.93
0	S.D.	62.0	0.08	0.08	0.13	2.95	0.12
	N	10	10	10	10	10	10
G2	Mean	591.4	2.12	0.51	1.66	18.43	0.98
2,500	S.D.	66.8	0.09	0.08	0.14	3.04	0.12
	N	10	10	10	10	10	10
G3	Mean	547.3	2.11	0.40	1.57	15.95	0.92
5,000	S.D.	53.1	0.07	0.09	0.15	2.02	0.09
	N	9	9	9	9	9	9
G4	Mean	599.1	2.09	0.43	1.59	18.33	0.96
7,500	S.D.	94.1	0.10	0.07	0.17	3.46	0.11
	N	10	10	10	10	10	10

Group / Dose (mg/kg/day)		Kidney	Adrenal gland	Testis	Epididymis
G1	Mean	3.28	0.0806	3.46	1.39
0	S.D.	0.47	0.0214	0.79	0.15
	N	10	10	10	10
G2	Mean	3.43	0.0708	3.85	1.48
2,500	S.D.	0.53	0.0175	0.33	0.08
	N	10	10	10	10
G3	Mean	3.32	0.0637	3.79	1.51
5,000	S.D.	0.30	0.0121	0.29	0.11
	N	9	9	9	9
G4	Mean	3.53	0.0620	3.96	1.54
7,500	S.D.	0.40	0.0088	0.41	0.18
	N	10	10	10	10

Table 10-1. (Continued)

Sex: Female (g)

Group / Dose (mg/kg/day)		B.W.	Brain	Thymus	Heart	Liver	Spleen
G1	Mean	317.1	1.99	0.37	1.00	7.99	0.58
0	S.D.	51.5	0.09	0.10	0.14	1.45	0.06
	N	10	10	10	10	10	10
G2	Mean	304.0	1.96	0.36	0.99	8.66	0.56
2,500	S.D.	44.0	0.10	0.14	0.12	2.79	0.12
	N	10	10	10	10	10	10
G3	Mean	307.4	1.97	0.36	0.97	8.47	0.54
5,000	S.D.	47.3	0.10	0.07	0.12	1.45	0.11
	N	10	10	10	10	10	10
G4	Mean	308.4	1.96	0.38	1.02	9.01	0.55
7,500	S.D.	53.7	0.09	0.10	0.14	2.10	0.10
	N	10	10	10	10	10	10

Group / Dose (mg/kg/day)		Kidney	Adrenal gland	Ovary	Uterus and cervix
G1	Mean	1.93	0.0724	0.0862	0.66
0	S.D.	0.23	0.0138	0.0140	0.21
	N	10	10	10	10
G2	Mean	1.86	0.0656	0.0912	0.62
2,500	S.D.	0.21	0.0105	0.0174	0.23
	N	10	10	10	10
G3	Mean	1.93	0.0646	0.0952	0.61
5,000	S.D.	0.22	0.0120	0.0120	0.25
	N	10	10	10	10
G4	Mean	1.97	0.0630	0.0815	0.73
7,500	S.D.	0.29	0.0051	0.0223	0.25
	N	10	10	10	10

Table 10-2. Mean Absolute Organ Weights (Recovery group)

Sex: Male (g)

Group / Dose (mg/kg/day)		B.W.	Brain	Thymus	Heart	Liver	Spleen
G1	Mean	619.1	2.19	0.37	1.77	17.75	0.98
0	S.D.	109.6	0.16	0.05	0.31	2.96	0.17
	N	5	5	5	5	5	5
G4	Mean	726.7	2.17	0.36	1.80	20.57	1.01
7.500	S.D.	134.1	0.08	0.08	0.17	5.03	0.22
	N	5	5	5	5	5	5

Group / Dose (mg/kg/day)		Kidney	Adrenal gland	Testis	Epididymis
G1	Mean	3.60	0.0809	4.03	1.70
0	S.D.	0.69	0.0182	0.38	0.14
	N	5	5	5	5
G4	Mean	3.75	0.0697	4.15	1.75
7.500	S.D.	0.64	0.0125	0.27	0.15
	N	5	5	5	5

Sex: Female (g)

Group / Dose (mg/kg/day)		B.W.	Brain	Thymus	Heart	Liver	Spleen
G1	Mean	342.8	2.02	0.32	1.08	8.30	0.59
0	S.D.	16.8	0.06	0.06	0.16	1.08	0.09
	N	5	5	5	5	5	5
G4	Mean	325.2	1.89	0.30	1.08	8.44	0.56
7.500	S.D.	56.6	0.07	0.10	0.20	1.69	0.10
	N	4	4	4	4	4	4

Group / Dose (mg/kg/day)		Kidney	Adrenal gland	Ovary	Uterus and cervix
G1	Mean	2.06	0.0715	0.0862	0.67
0	S.D.	0.24	0.0146	0.0097	0.23
	N	5	5	5	5
G4	Mean	2.00	0.0796	0.0893	0.65
7.500	S.D.	0.29	0.0148	0.0309	0.21
	N	4	4	4	4

Table 11-1. Mean Relative Organ Weights (Main group)

Sex: Male (g/100 g body weight)

Group / Dose (mg/kg/day)		B.W. (g)	Brain	Thymus	Heart	Liver	Spleen
G1	Mean	576.2	0.38	0.08	0.29	2.74	0.16
0	S.D.	62.0	0.05	0.02	0.02	0.27	0.02
	N	10	10	10	10	10	10
G2	Mean	591.4	0.36	0.09	0.28	3.10	0.17
2,500	S.D.	66.8	0.03	0.01	0.02	0.24	0.03
	N	10	10	10	10	10	10
G3	Mean	547.3	0.39	0.07	0.29	2.91	0.17
5,000	S.D.	53.1	0.03	0.02	0.03	0.24	0.02
	N	9	9	9	9	9	9
G4	Mean	599.1	0.36	0.07	0.27	3.05	0.16
7,500	S.D.	94.1	0.05	0.01	0.02	0.27	0.02
	N	10	10	10	10	10	10

Group / Dose (mg/kg/day)		Kidney	Adrenal gland	Testis	Epididymis
G1	Mean	0.57	0.0141	0.61	0.24
0	S.D.	0.06	0.0038	0.16	0.03
	N	10	10	10	10
G2	Mean	0.58	0.0122	0.66	0.25
2,500	S.D.	0.05	0.0034	0.09	0.03
	N	10	10	10	10
G3	Mean	0.61	0.0118	0.70	0.28
5,000	S.D.	0.06	0.0031	0.09	0.03
	N	9	9	9	9
G4	Mean	0.60	0.0106	0.67	0.26
7,500	S.D.	0.05	0.0025	0.11	0.04
	N	10	10	10	10

Table 11-1. (Continued)

Sex: Female (g/100 g body weight)

Group / Dose (mg/kg/day)		B.W. (g)	Brain	Thymus	Heart	Liver	Spleen
G1	Mean	317.1	0.64	0.12	0.32	2.52	0.19
0	S.D.	51.5	0.08	0.03	0.03	0.19	0.02
	N	10	10	10	10	10	10
G2	Mean	304.0	0.65	0.12	0.33	2.87	0.18
2,500	S.D.	44.0	0.07	0.04	0.02	0.95	0.02
	N	10	10	10	10	10	10
G3	Mean	307.4	0.65	0.12	0.32	2.75	0.18
5,000	S.D.	47.3	0.08	0.02	0.02	0.18	0.03
	N	10	10	10	10	10	10
G4	Mean	308.4	0.65	0.13	0.33	2.91	0.18
7,500	S.D.	53.7	0.11	0.03	0.03	0.27	0.03
	N	10	10	10	10	10	10

Group / Dose (mg/kg/day)		Kidney	Adrenal gland	Ovary	Uterus and cervix
G1	Mean	0.61	0.0233	0.0276	0.21
0	S.D.	0.04	0.0053	0.0050	0.08
	N	10	10	10	10
G2	Mean	0.62	0.0218	0.0302	0.20
2,500	S.D.	0.07	0.0031	0.0056	0.07
	N	10	10	10	10
G3	Mean	0.63	0.0217	0.0314	0.20
5,000	S.D.	0.04	0.0064	0.0047	0.09
	N	10	10	10	10
G4	Mean	0.65	0.0209	0.0268	0.24
7,500	S.D.	0.05	0.0030	0.0068	0.07
	N	10	10	10	10

Table 11-2. Mean Relative Organ Weights (Recovery group)

Sex: Male (g/100 g body weight)

Group / Dose (mg/kg/day)		B.W. (g)	Brain	Thymus	Heart	Liver	Spleen
G1	Mean	619.1	0.36	0.06	0.29	2.88	0.16
0	S.D.	109.6	0.06	0.01	0.03	0.24	0.02
	N	5	5	5	5	5	5
G4	Mean	726.7	0.31	0.05	0.25	2.82	0.14
7.500	S.D.	134.1	0.05	0.01	0.03	0.39	0.03
	N	5	5	5	5	5	5

Group / Dose (mg/kg/day)		Kidney	Adrenal gland	Testis	Epididymis
G1	Mean	0.58	0.0133	0.66	0.28
0	S.D.	0.07	0.0030	0.10	0.03
	N	5	5	5	5
G4	Mean	0.52	0.0099	0.58	0.25
7.500	S.D.	0.05	0.0027	0.11	0.03
	N	5	5	5	5

Sex: Female (g/100 g body weight)

Group / Dose (mg/kg/day)		B.W. (g)	Brain	Thymus	Heart	Liver	Spleen
G1	Mean	342.8	0.59	0.09	0.31	2.42	0.17
0	S.D.	16.8	0.02	0.02	0.04	0.25	0.02
	N	5	5	5	5	5	5
G4	Mean	325.2	0.59	0.09	0.33	2.60	0.17
7.500	S.D.	56.6	0.09	0.02	0.03	0.22	0.02
	N	4	4	4	4	4	4

Group / Dose (mg/kg/day)		Kidney	Adrenal gland	Ovary	Uterus and cervix
G1	Mean	0.60	0.0208	0.0252	0.20
0	S.D.	0.06	0.0040	0.0037	0.06
	N	5	5	5	5
G4	Mean	0.62	0.0247	0.0268	0.21
7.500	S.D.	0.07	0.0034	0.0057	0.08
	N	4	4	4	4

INDIVIDUAL ANIMAL DATA

- skipped
- not submitted in this GRAS determination

From:	Susan S Cho
To:	Wafula, Denis
Subject:	Re: Information regarding GRN 000859 (2"-fucosyllactose)- Response Requested
Date:	Wednesday, August 21, 2019 4:27:44 PM
Attachments:	image005.png image001.png

Dear Dr. Wafula,

Thank you for your letter. On behalf of Aptech, we ask that FDA cease to evaluate GRN 859. We would appreciate it if you would provide us with a detailed list of deficiencies. Thank you very much.

Sincerely,
Susan
Susan Cho, Ph.D. NutraSource, Inc. 6309 Morning Dew Ct Clarksville, MD 21029 +1-410-531-3336 (O) +1-301-875-6454 (C)

On Wednesday, August 21, 2019, 01:14:20 PM EDT, Wafula, Denis <Denis.Wafula@fda.hhs.gov> wrote:

Dear Dr. Cho,

After reviewing APTech's GRAS Notice GRN 000859, our review team has identified a number of errors and deficiencies in the notice. Broadly, these include (but not limited to):

- Inaccurate or missing information on the intended use, identify, manufacturing, specifications, and exposure.
- Inaccurate descriptions or interpretation of presented studies
- Poor quality illegible chromatograms
- Direct use of language from a peer reviewed paper that could be construed as plagiarism
- Improper use of scientific terminology or making of incorrect scientific claims.

Due to the poor quality of this submission, we strongly recommend that APTech requests that we cease our evaluation of GRN 000859. After APTech requests that we cease to evaluate its notice, we will provide a detailed list of the deficiencies identified in GRN 000859. If APTech chooses not to request that we cease our evaluation of GRN 000859, then we will issue a no basis letter for this GRAS notice.

Please provide your response within 10 business days (Before COB September 4, 2019).

Sincerely,

Denis

Denis Wafula, Ph.D.
Staff Fellow

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety

U.S. Food and Drug Administration
Office: 2404021314
denis.wafula@fda.hhs.gov

U.S. FOOD & DRUG ADMINISTRATION

From: Susan S Cho <susanscho1@yahoo.com>
Sent: Thursday, June 13, 2019 6:55 PM
To: Wafula, Denis <Denis.Wafula@fda.hhs.gov>
Subject: Re: Filing Letter for GRN 000859 (2'-fucosyllactose)

Dear Dr. Wafula,

Thank you very much. Have a nice weekend!

Sincerely,

Susan

Susan Cho, Ph.D. NutraSource, Inc. 6309 Morning Dew Ct Clarksville, MD 21029 +1-410-531-3336 (O) +1-301-875-6454 (C)

On Thursday, June 13, 2019, 02:26:23 PM EDT, Wafula, Denis <Denis.Wafula@fda.hhs.gov> wrote:

Dear Dr. Cho,

Find attached the Filing Letter for GRAS Notice #GRN 000859 that you submitted to FDA. If you have any questions about the letter, do not hesitate to contact us.

Best Regards,

Denis

Denis Wafula, Ph.D.
Staff Fellow

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Office: 2404021314
denis.wafula@fda.hhs.gov

On Thursday, August 22, 2019, 09:18:49 AM EDT, Wafula, Denis <Denis.Wafula@fda.hhs.gov> wrote:

Dear Dr. Cho,

Attached is the promised list of deficiencies identified by our reviewers in GRN 859. Because we have ceased the evaluation of the notice at your request, you are not required to respond to these questions.

In the meantime, I will be preparing the Cease-to-Evaluate letter and will send that to you as soon as possible.

Sincerely,

Denis

Denis Wafula, Ph.D.
Staff Fellow

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Office: 2404021314
denis.wafula@fda.hhs.gov

GRN 859 Comments and Questions to Notifier

1. The notice uses terms such as "growing-up (toddler) milks" and "follow-on formula". We note that we don't have a definition of "toddler formula" in the infant formula regulations, but we use that terminology to refer to formula for children over 12 months of age. We define infant formula in the infant formula regulations to refer to infants 0-12 months. Please revise appropriately.

2. Please specifically state the amount of the ingredient intended for use in the infant formula. APTech states in Table 1 that the intended amount for infant formula is 240 mg/serving (400mg/100 kcal). Does this equate to 2.4 g/L as the intended amount for infant formula? If so, this should be stated separately for infant formula.

3. On page 7, APTech states 2'FL is intended for "ready-to-drink" formula and powder. APTech should state whether this ingredient intended for use in infant formula that must be reconstituted (i.e. concentrated) or formula that is ready to use without further preparation.

4. On page 24 of the notice, you state:
   '...the intended effect is as a nutrient necessary for the body's nutritional and metabolic processes, serving as a non-digestible carbohydrate or as a prebiotic for establishment of healthy gut microflora in infants…'

   Given that the majority of the existing infant formulas on the market do not contain 2'-FL and that breastmilk by non-secreting mothers contains little or no 2'-FL, this would suggest that 2'-FL does not serve a necessary function for infants' "nutritional and metabolic processes." Please clarify what is meant by this statement.

5. Please provide an explanation why significantly reduced body weight and body weight gain in male rats at 7500 mg/kg was reported (>10%, Table 3 of Study Report No. B18672) while under the same dose in a subchronic study, a similar reduction of body weight gain was not observed.

6. The notice includes a section (7.B.) of "References that are not Generally Available" (page 137). This section includes information from unpublished studies conducted by Biotoxtech in 2019. If this information is pivotal to the conclusions of general recognition of safety of GRN 859 2'-fucosyllactose, it should be published in a peer-reviewed journal or otherwise publicly available for consideration by qualified experts and demonstration of general consensus. Please confirm the status of these references (i.e., are they in press?).

7. The notifier states "no toxicant production is expected in the manufacture of 2'-FL. The final product is highly purified through several steps during production." This statement does not address the (in)ability of the production organism to produce toxicants under

the conditions of fermentation, although this topic is addressed in the March 27, 2019 Holzapfel unpublished report (Appendix B). Section 2 of the notice should include a summary of the publicly-available information supporting the absence of toxigenicity or pathogenicity of the production organism, including citation to relevant studies and reviews. There is mention of antibiotic resistance genes, but no supporting discussion or context is provided.

8. The notifier does not provide a statement regarding the safety and suitability of food contact materials (i.e., the ultrafiltration membranes and cation and anion exchange resins). The notifier should provide a statement about their suitability.

9. Specifications:
    1. It is unclear why there is a specification for aflatoxin M1;
    2. It is unclear why there is not a specification for Enterobacteriaceae, while showing in Table 7 that all other 2'-FL notifications have provided limits for Enterobacteriaceae.

10. The method of manufacture is not clearly explained. The purification steps include filtration and ion exchange steps, but these are only generally described (e.g., "Large molecular weight substances are further removed by nanofiltration. Ionic impurities and remaining colorants are removed by strong cation and exchange resins.") For us to evaluate the safety of the ingredient, the method of manufacture should provide enough information to identify impurities of concern and the ability of the processing steps to remove them.

11. The discussion of estimated daily intake of 2'-FL in the diet is not comprehensive. While it appears to be substitutional for the subject of GRN 735, it does not address some uses of 2'-FL not covered in GRN 735, such as dietary supplements. The statements regarding exposure should address all dietary sources (see 21 CFR 170.235) of 2'-FL. APTech should also clarify if the intended use of the ingredient is alone or in combination with other HMO ingredients?

12. On page 52 ('Human Study First Reviewed in This GRAS Determination'), APTech copies the entire paragraph partly from the abstract and from page 7 ('Adverse Events') of Storm et al., 2019. Please re-write the section in your own words to avoid the appearance of plagiarism.

13. In section 2.C.1. (Chemical Identity and Potential Impurities) APTech states that:
    'The absence of the microorganism and residual protein in the ingredient is supported by the analysis of residual DNA in batches of the final ingredient. The absence of residual DNA from the microorganism is confirmed by validated PCR methods. In the PCR reaction, residual DNA could not be detected from the final ingredient. The PCR results demonstrated that the microorganism and residual protein are absolutely removed from the final ingredient (Appendix D).'

Please note that there are errors in this paragraph. For example, PCR does not detect the presence of proteins. In your study, PCR only detected the presence of genetic material from the expression vector, indeed, in (Appendix D) you state that the results presented were for the 'Introduced Gene' which we assume are the genes found on the expression vector. If the detection of the genes from the vector was used as a proxy for the presence of the host organism, please state so. Please revise this paragraph for accuracy.

14. On Page 10, (2.B. Method of Manufacture) APTech states that: 'Fermentation was performed in a well-defined, complex medium…'
    'A well-defined, complex' medium is incorrect terminology. Microbial media can be 'defined' i.e. containing known proportions of components or 'undefined' when it contains components that are of complex composition or uncertain proportions e.g. yeast extract. Please correct the terminology
    Additionally, there is inconsistent information regarding the medium. On p. 10, you note that yeast extract and antibiotics are excluded from the medium. On p. 13, yeast extract is listed in the table of medium components.

15. The Chromatograms supplied on pages 100-102 and 108 -109 (Appendices E and F) are of poor quality and are impossible to read. Please provide legible chromatograms.

16. On page 104 (Appendix F) APTech states that they used 'Jennewein's method'. If APTech intends to state that the method used was similar to the one used by Jennewein in GRN 571, please cite the notice or the actual method in the notice.

17. One page 22 (2. C.2.1. Bulk Stability) you state that:
    'APTech is currently conducting a 6-month accelerated storage and 36-month shelf stability study on its 2'-FL produced via genetically engineered *C. glutamicum* APC199. At accelerated conditions (40°C at a relative humidity of 75%), 100.5% recovery was reported when compared to the baseline value.'

    Please note that we cannot comment on ongoing/incomplete studies. The same observation applies to Table 14 (page 36) where you imply that the current notice has been evaluated by the FDA. This notice is still under evaluation and cannot be used as part of the information supporting the safety on of the ingredient in the same notice.

18. On page 29 (Table 13) the 2'-FL content is provided as g/L for all locations apart from Wang et al., 2015 where it is provided at a percentage. Please clarify.

19. On page 34 (Part 5 History of Consumption) you state that: 'The statutory basis for the conclusion of GRAS status of 2'-FL in this document is not based on common use in food before 1958. The GRAS determination is based on scientific procedures. 2'-FL is present

naturally in human milk. It is reasonable to conclude that infants were exposed to 2'-FL prior to 1958.'

If your GRAS conclusion is based on scientific procedures, then the last statement is not relevant to this submission. Your notice is better served by focusing on the relevant safety information instead of filler material. Another example of such writing is found in the last sentence in Section 6.C (Review of Safety Data) you state that: 'The subject of the present GRAS notice is 2'-FL produced via microbial fermentation.' This sentence is random and serves no purpose in this section, because at this point in the notice, the reader already knows the subject of the notice and the methods of production.

20. On the page 37 APTech states:

'HMOs are the preferred substrate for *B. infantis* and other bifidobacteria strains and may reduce the nutrients available for potentially harmful bacteria and keep their growth under control (Ellison et al., 2016; Rudloff et al., 2019; Thongaram et al., 2017; Weiss et al., 2014).'
We disagree with APTech that the sentence reflects the correct conclusion from the cited papers. The cited studies have vastly different objectives and conclusions and lumping the studies together can lead to incorrect conclusions. Briefly, only Thongaram et al. 2017 attempts at studying substrate utilization by bifidobacteria (and lactobacilli) and more importantly, they restrict their study to experimenting on the differential utilization (by the select bacteria) of the selected HMO and HMO constituent monomers only. Therefore, we cannot conclude that B. *infantis* and other bifidobacteria prefer HMO over other substrates because other substrates (non-HMO) were not tested. While the other studies cited might present interesting findings, they do not reach the conclusions that you have stated. The studies also use different animals as test subjects and therefore lumping them together without explanation is not appropriate. Please revise the sentences to reflect the correct conclusions of the cited papers.

21. In Table 17 (page 51) APTech states that the objective of the van den Elsen et al. (2019) study was 'To determine the effect of 2'-FL on the gut microbiota and antibody-mediated vaccine responses.' However, this contradicts what is stated on page 50 and in the actual study; which utilized 2'-FL mixed with short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) and not 2'-FL alone. Please revise for consistency and accuracy.

22. On Page 13 you use the term 'biosafety level 1' to describe *P. saltans* ATCC 51119. Biosafety relates to the biocontainment of an organism during laboratory work and has no relevance to our safety evaluation. Additionally, '*P. saltans*' is mentioned for the first time without the species being fully spelled out as scientific writing convention dictates. This observation applies to all other scientific names in your notice.

23. On page 13 you use the term ‘vector plasmid’; this is redundant because in molecular biology, a plasmid is considered a vector. This comment is illustrative of the numerous instances where imprecise/unnecessary language has been used in the notice. Please strive for conciseness.