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March 27, 2019

Dr. Szabina Stice
Division of Biotechnology and GRAS Notice Review (DBGNR)
Toxicology Group
Food and Drug Administration
5001 Campus Drive – HFS 225
College Park, MD 20740

Subject: GRAS Notification 2'-Fucosyllactose

Dear Dr. Stice:

BASF SE (Carl-Bosch-Strasse 38, 7056 Ludwigshafen am Rhein, Germany) is submitting a Generally Recognized As Safe – GRAS notice in accordance with 21 CFR Part 170 Food Additives Subpart E. The enclosed paper copy of this document is the notice of a claim that BASF product: 2'-Fucosyllactose is exempt from the premarket approval requirement of the FD&C Act, because it has been determined to be generally recognized as safe (GRAS) based on scientific procedures, when used as an ingredient in foods including exempt infant formula, follow-on formula and baby foods.

If you have any questions or require any additional information regarding this notification, please do not hesitate to contact Ms. Claudia Callies-Kluepfel via Email: claudia.callies-kluepfel@basf.com or by phone: +49 621 60-58377 OR me via Email: haresh.p.madeka@basf.com or by phone: 1-973-245-6120.

Sincerely,

(b) (6) [redacted]

Haresh P. Madeka, PhD
Sr. Regulatory and External Affairs Manager
Nutrition and Health NA

BASF Corporation
Nutrition & Health NA
100 Park Ave.
Florham Park, NJ 07932

Generally Recognized as Safe (GRAS) Notice

for

2'-Fucosyllactose

Final 27.03.2019

Submitted to:

Food and Drug Administration
Center for Food Safety & Applied Nutrition
Office of Food Additive Safety
GRAS Notification Program
5001 Campus Drive
College Park, MD 20740

Notifier:
BASF SE
Ludwigshafen, Germany

Contents

1 Signed statements and certifications (Part 1)

1.1 Submission of GRAS notice

BASF SE (hereinafter BASF or the Notifier) submits in accordance with 21 CFR Part 170 Food Additives Subpart E – Generally Recognized as Safe (GRAS) Notice, this notice to support the claim that their product 2'-Fucosyllactose is generally recognized as safe (GRAS) when used as an ingredient in foods including exempt infant formula, follow-on formula and baby food levels ranging from 0.24 to 1.2 grams/serving.

1.2 Name and address of organization

BASF SE
Carl-Bosch-Strasse 38
67056 Ludwigshafen am Rhein
Germany

BASF's contact person	Claudia Callies-Kluepfel BASF SE, Carl-Bosch-Strasse 38, 67056 Ludwigshafen am Rhein, Germany Email: claudia.callies-kluepfel@basf.com Tel.: +49 621 60-58377
BASF's US representative	Haresh Madeka BASF Corporation, 100 Park Avenue, 07932 Florham Park, USA Email: haresh.p.madeka@basf.com Tel.: +1 973 245-6120

1.3 Name of the notified substance

The name and appropriate descriptive term of the notified substance is 2'-Fucosyllactose.

1.4 Intended conditions of use

2'-Fucosyllactose is intended as an ingredient in beverages and beverage bases; breakfast cereals; dairy product analogues; frozen dairy desserts and mixes; gelatins, puddings, and fillings; grain products and pastas; jams and jellies; milk, whole and skim; milk products; processed fruits and fruit juices; sweet sauces, toppings, and syrups; non-exempt infant and follow-on formula; and baby foods at levels ranging from 0.24 to 1.2 grams/serving. The detailed conditions of use are given in Part 2.5 (Technical effects).

1.5 Basis for conclusion of GRAS status

The determination of the GRAS status of 2'-Fucosyllactose is based on scientific procedures in accordance with the Code of Federal Regulations (CFR) § 170.30(a) and (b).

1.6 Not subject to premarket approval

The Notifier claims that the notified substance as described below is GRAS under the proposed conditions of use and the substance is therefore exempt from the requirement for premarket approval as defined by the Federal Food and Drug Cosmetic Act.

1.7 Availability of information

This GRAS notice is being submitted in paper and electronic format. The Notifier will retain copies of all data and information that form the basis for the conclusion of GRAS status. The Notifier agrees to provide FDA, either during or after its evaluation of the notice, complete copies of the data and information, either in electronic format accessible for evaluation or on paper.

FDA can review or copy the data and information during customary business hours at the address of BASF's US representative as mentioned in section 1.2. Requests for copies of the respective materials may be directed to BASF's contact person.

1.8 Data and information exempt from disclosure under the FOI

This submission does not contain any confidential information that is exempt from disclosure und FOIA.

1.9 Certification

This GRAS notice was compiled in accordance with the rules and regulations set out in 21 CFR Part 170, Subpart E. The Notifier certifies to the best of its knowledge that the GRAS notice is a complete, representative and balanced submission that includes unfavourable information, as well as favourable information, that is known to the Notifier, and pertinent to the evaluation of the safety and GRAS status of the use of the substance

1.10 Name and position

This GRAS notice is signed by Claudia Callies-Kluepfel, Manager Global Regulatory & External Affairs

(b) (6) [redacted]

	27.03.2019
Signature	Date

2 Identity, method of manufacture, specifications, and technical effect (Part 2)

2.1 Scientific data and information that identifies the notified substance (21 CFR 170.230 (a))

2.1.1 Identity

General information

2'-Fucosyllactose is a naturally occurring trisaccharide; it is present in mammalian milk including human breast milk.

Systematic name:	D-Glucose, O-6-deoxy-alpha-L-galactopyranosyl-(1-2)-O-beta-D-galactopyranosyl-(1-4)-
Synonyms:	2'-O-Fucosyllactose
	6-Deoxy-alpha-L-galactopyranosyl-(1→2)-beta-D-galactopyranosyl-(1→4)-D-glucose
Chemical formula:	$C_{18}H_{32}O_{15}$
Molecular weight:	488.436
CAS Number:	41263-94-9

2'-Fucosyllactose is a trisaccharide based on the monosaccharide L-fucose and the disaccharide D-lactose, they are linked by an α-(1→2) bond. The structure is given in Figure 1:

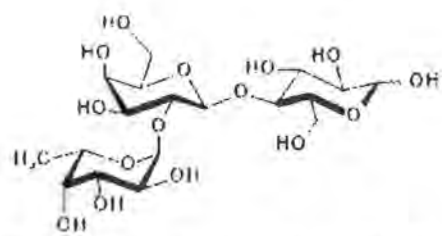

Figure 1 Chemical structure of 2'-Fucosyllactose

Identity of fermentation product

The chemical identity of the 2'-Fucosyllactose produced by the notifier's *E. coli* K12 (LU20297) was confirmed by NMR spectroscopy (Annex I) and verified by comparison with NMR-literature data (Ishizuka *et al.* 1999).

2.1.2 Characteristic properties

2'-Fucosyllactose is a white to off white powder that may show signs of agglomeration. A 5% solution in water (t=20° C) has a pH in the range from 3.5 to 7.5 (Annex II). The substance is very soluble in water.

2.1.3 Quantitative composition

Constituents including related products

The substance subject to this notification contains mainly 2'-Fucosyllactose accompanied by other minor chemically related sugars including D-Lactose, L-Fucose, 2'-Difucosyl-D-lactose, and 2'-Fucosyl-D-lactulose. Table 1 provides an overview of the content of representative batches obtained from fermentation with the Notifier's proprietary strain *E. coli* K12 LU20297.

D-Lactose, L-Fucose and 2'-Difucosyl-D-lactose originate from the fermentation broth, they are natural components of breastmilk. 2'-Fucosyl-D-lactulose can originate from the isomerization of 2'-Fucosyllactose where the terminal Glucose moiety is converted into a Fructose sugar. If D-Lactose isomerizes to D-Lactulose, 2'-Fucosyllactose can also be converted to 2'-Fucosyl-D-lactulose. These isomerizations are pH and temperature dependent and have been commonly reported for the closely related conversion of D-Lactose into D-Lactulose during heat treatment achieved during ultra-high temperature (UHT) processing and pasteurization of milk, including human donor milk (Beach and Menzies (1983), Schuster-Wolff-Bühring *et al.* (2010), Gómez de Segura *et al.* (2012)). This isomerization reaction of carbohydrates is also known as the Lobry de Bruyn–van Ekenstein transformation (Angyal (2001), Wang (2010)).

Table 1 Composition of 2'-Fucosyllactose

Constituent	**Content (%) *)**
2'-Fucosyllactose (wt/wt, dry matter)	96.7 – 100.7
D-Lactose	< 0.5 – 0.8
L-Fucose	< 0.3 - < 0.5
2'-Difucosyl-D-lactose	< 0.3 - < 0.5
2'-Fucosyl-D-lactulose	<0.3 – 0.7
Water	4.7 – 6.8

*) Ranges results for six batches (Annex II)

Analytical determination of 2'-Fucosyllactose and other minor chemically related sugars

The analytical method of choice for the determination of 2'-Fucosyllactose and secondary components is HPLC using a detector based refractive index (RI). This method is equivalent to other methods described in GRAS notices (e.g. GRN 546, 571, 650, 735) and has been validated by BASF in line with relevant guidelines (IUPAC). Representative chromatograms are given in Figure 2.

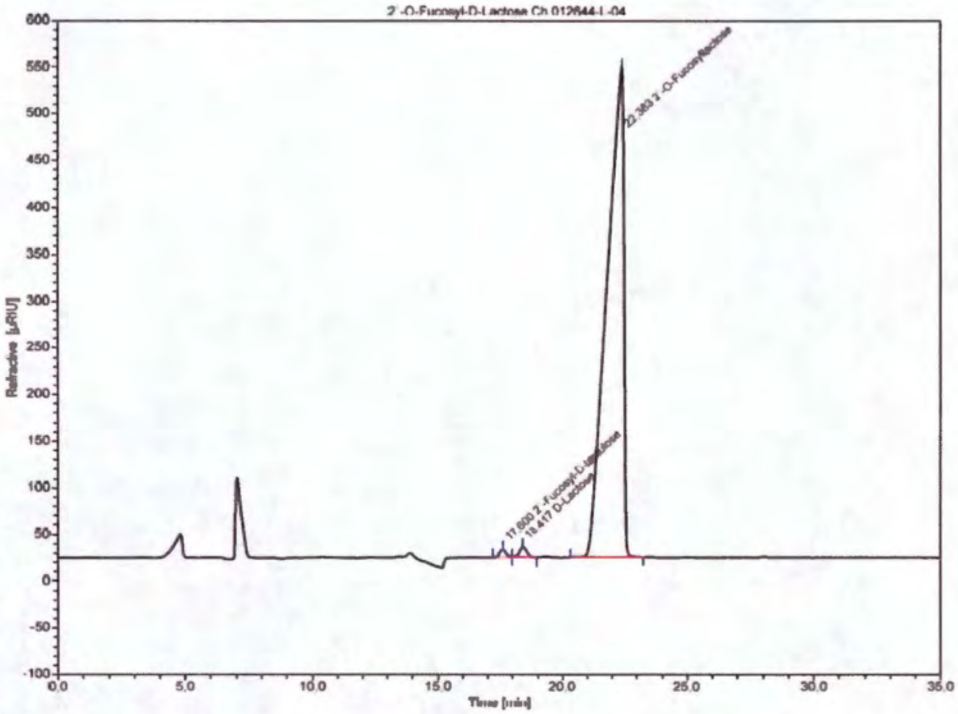

Figure 2a

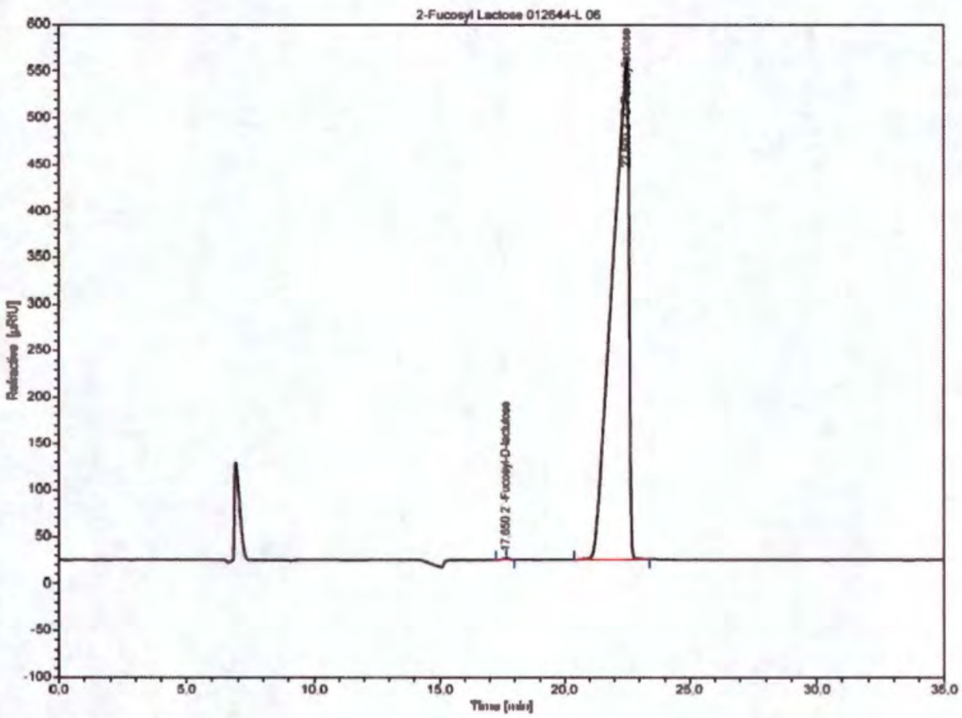

Figure 2b

Figure 2 Determination of 2'-Fucosyllactose and secondary components by HPLC using a refractive index detector. 2'-Fucosyllactose precipitated from water: acetic acid (Figure 2a), ethanol (Figure 2b)

2.1.4 Impurities

As process related impurity, acetic acid or ethanol may be present as the purification involves induction of precipitation by addition of acetic acid or ethanol. As potential impurities from the fermentation broth endotoxins (which are part of the cell wall of the producing microorganism) and residual protein could be carried over. Analytical data for these impurities, for ash, heavy metals and arsenic are reported for representative batches as indicated in Table 2.

Table 2 Impurities in 2'-Fucosyllactose

Impurity	Unit	Content	N *)
Acetic acid	%	< 0.1 – 0.75	6
Ethanol	mg/kg	< 10 – 50	5
Sulfated ash	%	< 0.05	5
Lead	mg/kg	< 0.05	5
Cadmium	mg/kg	< 0.01 – < 0.05	5
Mercury	mg/kg	< 0.05	5
Arsenic	mg/kg	< 0.05	5
Endotoxin	EU/mg	< 0.1	6
Residual Protein	%	< 0.01	5

*) Number of batches for which range in content is reported (Annex II).

2.1.5 Microbial purity

The microbial purity is defined by the levels that are required for ingredients to be suitable for use in infant formula. Test results for various bacterial species, yeasts, and moulds are summarized in Table 3.

Table 3 Microbial purity of 2'-Fucosyllactose

Microorganisms	Tolerance	Values *)
Total microbial aerobic count	<500 CFU/g	< 10 – < 100
Yeasts and moulds	<100 CFU/g	< 10 – < 100
Enterobacteria & other Gram-negative bacteria	absent in 10 g	absent in 10 g
Cronobacter sakazakii	absent in 10 g	absent in 10 g
Salmonella	absent in 25 g	absent in 25 g
Listeria monocytogenes	absent in 25 g	absent in 25 g

*) Ranges of data for six batches (Annex II)

Absence of the production microorganism in the final product is verified by determination of residual DNA using PCR (Annex III).

2.1.6 Stability

Stability testing

2'-Fucosyllactose is an oligosaccharide which, like other carbohydrates, is known to be fairly stable molecules if protected from moisture and air. This was demonstrated in several GRAS notifications before (e.g. GRN 546, 571, 650, 735 and 749). Additionally, one batch of 2'-Fucosyllactose was tested under accelerated conditions (40° C / 75% relative humidity) and found to be stable for up to 6 months when it is stored in the original unopened bag (Annex IV). The packaging in this test was a water-resistant aluminium foil bag, which is similar to the primary packaging material used for storage and distribution of the commercial product.

Table 4 Accelerated stability study: 2'-Fucosyllactose stored at 40°C ± 2°C/ 75% RH ± 5% RH

Analytical method	Batch	Start of the study	Spec.	0 m	3 m	6 m
2'-Fucosyllactose (HPLC) [%] (DM)	012545-L	08/2017	Min. 94.0	97.3	99.0	98.3
D-Lactose (HPLC) [%]	012545-L	08/2017	Max. 3.0	<0.5	0.6	0.7

Shelf life

2'-Fucosyllactose is stable over the time course of at least 6 months at 40°C (75% relative humidity) when it is stored in the original unopened bag. According to common understanding these storage conditions are equivalent to a storage time of 24 months at 25°C. Therefore, based on the abovementioned results a shelf-life of at least 24 months at 25°C is expected. Ongoing stability studies are currently conducted to further extend the future shelf life recommendation.

2.2 Developmental history of the production strain

2.2.1 Taxonomy of host strain

The host strain JM109 is a derivative of *E. coli* K12, a well characterized non-pathogenic lab strain which was isolated from a convalescent *Diphtheria* patient in 1922 (Lederberg, 1951). This lab-adapted strain is unable to colonize the human gut and is listed under the lowest biosafety level 1 (Kuhnert *et al.* (1995), Bauer *et al.* (2007)). This is also in line with the conclusions of the United States Environmental Protection Agency on the use of *E.coli* K-12 under contained conditions in fermentation facilities (U.S. EPA, 1997a, b)

The taxonomy of *E. coli* can be described as:

(a) Domain:	Bacteria
(b) Kingdom:	Eubacteria
(c) Phylum:	Proteobacteria
(d) Class:	Gammaproteobacteria
(e) Order:	Enterobacteriales
(f) Family:	Enterobacteriaceae
(g) Genus:	*Escherichia*
(h) Species:	*Escherichia coli*

For the classification of the host microorganism *E. coli* K12 the following analysis has been made. Based on the sequence of housekeeping genes, pathogenic and commensal strains can be clearly divided in phylogroups (Archer *et al.* (2011)). Safe strains like K12, B and Crooks have been found to be members of the phylogroup A, whereas pathogenic strains have been found in group B2, D and E (Archer *et al.* (2011)), see the cladogram for the taxonomic overview of sequenced *E. coli* strains in Figure 3). *E. coli* K12 and its derivatives are routinely being used for different industrial production applications i.e. to produce biopharmaceuticals like recombinant proteins (Baeshen *et al.* (2015)).

The genome of *E. coli* K12 is fully sequenced (Blattner (1997)). *E. coli* K12 JM109 is a derivative of K12 and was evolved in the lab as a standard cloning and expression strain and shows the following genotype (Yannisch-Perron *et al.* (1985)):

endA1 glnV44 thi-1 relA1 gyrA96 recA1 Δ(lac-proAB) e14- [F' traD36 proAB$^+$ lacI^q ΔlacZ (M15)] hsdR17($r_K^- m_K^+$)

EndA1:	Modification of endonuclease prevents non-specific degradation of foreign DNA.
glnV44:	Suppression of amber stop codons
Thi-1:	Mutation in thiamin metabolism; supplementation of thiamin for growth in minimal medium required
relA1:	Mutation causes modifications in cell membrane composition. Cells are more fragile and sensitive to sonication and osmotic shock. Furthermore, this mutation allows RNA synthesis in the absence of protein synthesis.
gyrA96:	DNA gyrase mutation confers tolerance to nalidixic acid
recA1:	Prevents recombination of homologous DNA.
Δ(lac-proAB):	Deletion of lac operon and first two genes of proline biosynthesis
hsdR17 ($r_K^- m_K^+$):	Inactivation of restriction system for degradation of foreign DNA.

The strain *E.coli* K12 JM109 carries a stable and well described F'-plasmid which contains the following mutations/genes:

traD36:	a mutation that prevents transfer of F-plasmid to other microorganisms, thereby preventing genetic transfer of the F-'plasmid to other microorganisms
proA$^+$B$^+$:	are the first two intact genes of the proline biosynthesis relieving the strain from proline auxotrophy caused by the Δ(lac-proAB) deletion. It also stabilizes the F'-plasmid against plasmid loss
lacI^q:	the intact lac repressor gene with a mutation in the promotor. This allele renders the strain with a functional lac repressor with increase expression for increased repression of lac regulated promotors
Δ(lacZ) M15:	partial deletion of β-galactosidase gene, rendering a strain which does not degrade lactose

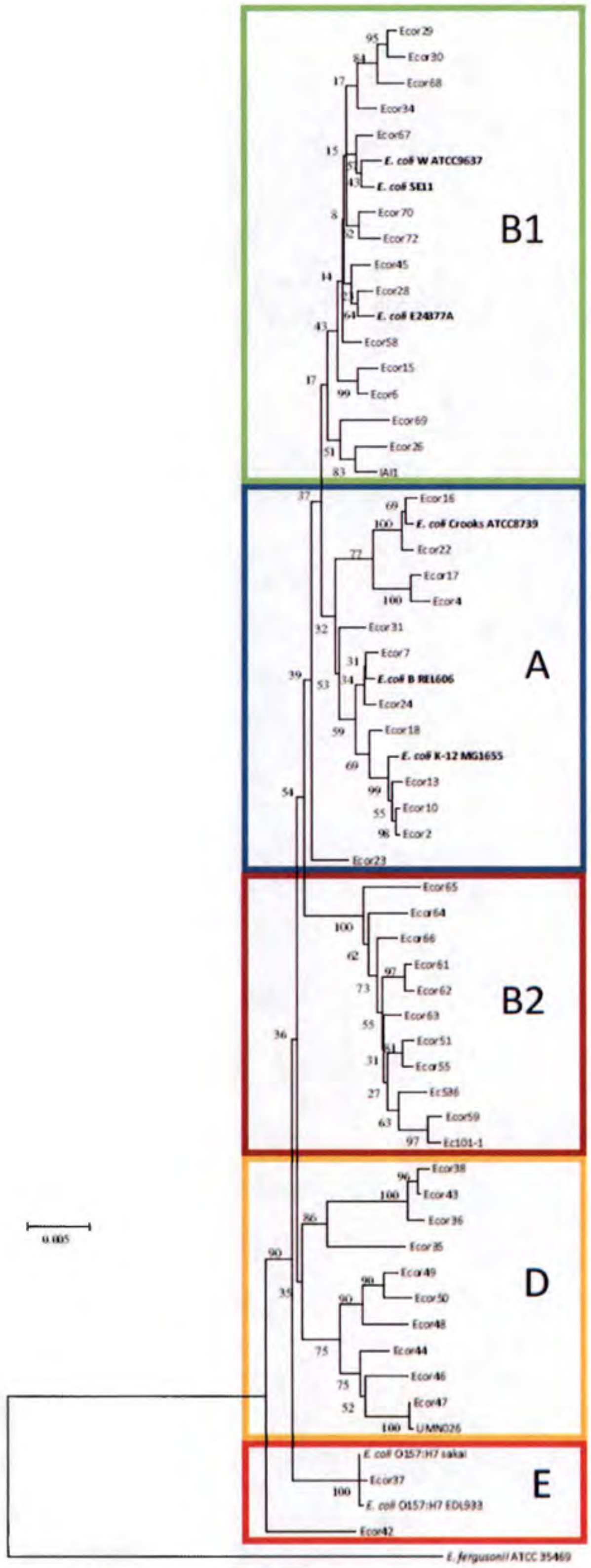

Figure 3 Phylogenetic tree of sequenced *E. coli* strains (Archer, 2011)

2.2.2 Development of producer strain

The genetically modified strain LU20297 capable of producing 2'-FL, had been derived from *E. coli* K12 JM109 by state-of-the-art molecular biological methods. The synthetic operon comprises five genes whereof four are derived from *E. coli* itself (*gmd, wcaG, manC, manB*). The fifth gene (*fucT2*) is derived from *Helicobacter pylori*. The genotype of the strain LU20297 can be described as follows:

> LU20297: endA1 glnV44 thi-1 relA1 gyrA96 rpsL(StrR) recA1 Δ(lac-proAB) e14- ΔfucIK::Ptac fucT2_gmd_wcaG_manC_manB, [F' traD36 proAB+ lacIq ΔlacZ (M15)] hsdR17(rK-mK+)

The strain LU20297 has been described internally also as N8_2 and has been deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) with the deposit number DSM 32665 (Annex V).

Origin of the inserted sequences (donor organism)

The genes *wcaG* (NP_416556, coding for a GDP-fucose synthase), *gmd* (NP_416557, coding for a GDP-mannose 4,6-dehydratase), *manB* (NP_416552, coding for a phospho-mannomutase) and *manC* (NP_416553, coding for a mannose-1-phosphate-guanylyltransferase) have been derived from *E. coli* K12 MG1655 sequences. The genes *manC* and *manB* have been cloned as codon-optimized versions of the genes, whereas the genes *gmd* and *wcaG* are coded by DNA derived and identical to the genomic content of *E. coli* K12 JM109. Codon-optimization for *manC* and *manB* was performed using a proprietary algorithm performed by a contractor (Atum (formerly DNA 2.0®), Newark, California, USA).

In the case of the fucosyltransferase *fucT2*, the protein sequence (AF076779) originated from the strain *Helicobacter pylori* UA802, isolated from the stomach of a human patient in Australia (Jiang *et al.* 1996). For improving expression/translation in the host *E. coli* K12 JM109, the sequence coding for the FucT2 enzyme was codon-optimized prior to gene synthesis. Codon-optimization for the gene *fucT2* was performed using a proprietary algorithm performed by a contractor (Atum DNA 2.0). The taxonomic description of the strain *Helicobacter pylori* UA802 is depicted here:

(a) Domain:	Bacteria
(b) Kingdom:	Eubacteria
(c) Phylum:	Proteobacteria
(d) Class:	Epsilonproteobacteria
(e) Order:	Camphylobacterales
(f) Family:	Helicobacteriaceae
(g) Genus:	*Helicobacter*
(h) Species:	*Helicobacter pylori*

By using a synthesized *fucT2* gene it can be excluded that other genes from the pathogenic strain *H. pylori* are accidently introduced to *E. coli* JM109 during construction of LU20297. Furthermore, it was shown by Yavuz and coworkers that the expression of a heterologous fucosyl-transferase in *E. coli* K12 does not lead to pathogenicity of the strain (Yavuz *et al.* (2011)).

Table 5 Genetic modifications of *E. coli* K12 Strain LU20297

Gene	Gene function	Modification introduced	New locus designation	Purpose
fucI	L-fucose-isomerase	Inactivated		Prevent fucose degradation
fucK	L-fucolo-kinase	Inactivated; remnant of 349 bp at 3'		
Ptac	Promotor	Arrangement in a synthetic operon (see chapter "*Rational for the construction of an artificial operon for the production of 2'-Fucosyllactose*")	Introduced into *fucIK* locus	Regulate expression of the operon
gmd	GDP-mannose 4,6-dehydratase			Synthesis of GDP-L-fucose
wcaG	GDP-fucose synthase			
manB	phospho-mannomutase			
manC	mannose-1-phosphate-guanylyltransferase			
fucT2	fucosyl-transferase			Glycosylation of lactose with GDP-L-fucose
TrrnB	strong *E. coli* terminator			Terminate transcription of operon

Rationale for the construction of an artificial operon for the production of 2'-Fucosyllactose

As mentioned previously it was planned to express the genes *fucT2* (F), *gmd* (G), *wcaG* (W), *manC* (C) and *manB* (B) in form of an artificial operon rendering all genes being transcribed by a single regulatable promotor. For expression of the constructed operon, consisting of the five genes, the gene order FGWCB (5'-3' orientation) was given (Figure 4) and the well-established PTac promotor was chosen. This synthetic promotor has been derived from a fusion of the lac and tac-promotor (de Boer *et al.* (1983)) and is repressed by the activity of the strain encoded lac repressor lacI but not by catabolite repression. The expression of this promotor is strongly repressed in the absence of an inducer of the lac operon since the lacI molecule blocks the activity of the RNA polymerase. Induction of this promotor can be achieved by the addition of IPTG (Isopropyl β-D-1-thiogalactopyranoside). Each gene of the constructed operon is combined with a ribosomal binding site (RBS) which is placed upstream of the gene in a defined spacing. In the case of the *wcaG* and *gmd* genes, the native genomic organization including the RBS sequences was retained. For terminating the transcription of the constructed operon, the strong transcriptional termination-inducing terminator derived from the Tr*rnB* operon was used (Orosz *et al.* (1991)). Using this well-defined termination signal prevents read through of the RNA polymerase into distal DNA sequences and ruling out the possibility that 3' of the operon sequences are transcribed.

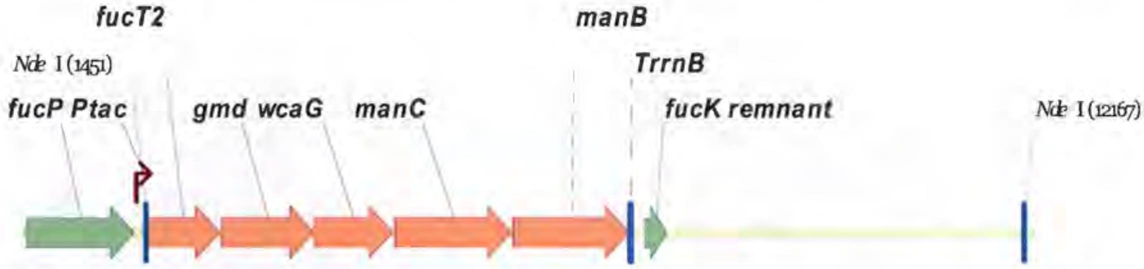

Figure 4 Map of the synthetic 2'-FL operon integrated into the fucose locus. Shown are *Nde*I restriction sites that were used in Southern hybridization experiments.

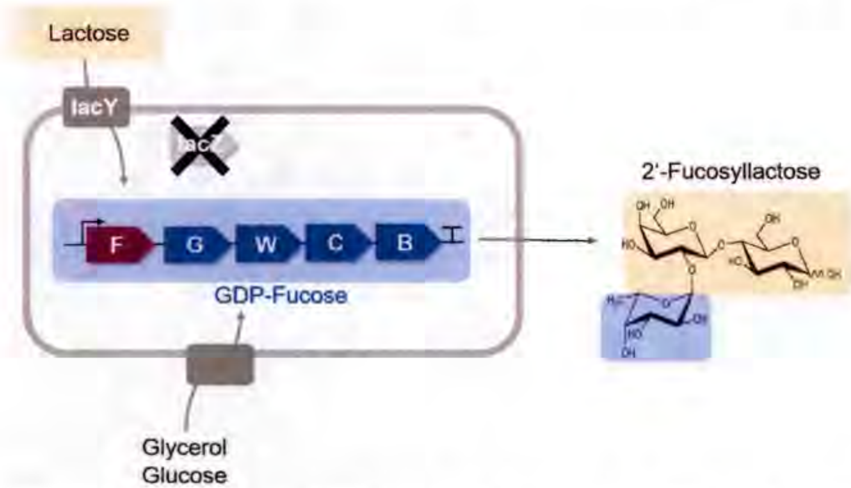

Figure 5 Biosynthetic scheme for 2'-FL biosynthesis. Lactose is supplemented into the medium and is taken up by the cells via the lactose transporter LacY. LU20297 is unable to degrade lactose as the beta-galactosidase LacZ is inactivated in the parental strain *E. coli* K12 JM109. GDP-Fucose is synthesized via a synthetic operon which consists of four *E. coli* derived genes (blue, G=*gmd*, W=*wcaG*, C=*manC*, B=*manB*) and a heterologous fucosyltransferase *fucT2* (red, F). The operon is controlled by an inducible promotor (Ptac) and terminated by a terminator. Glycerol or glucose can serve as main carbon source. GDP-Fucose is transferred to lactose via *fucT2* and 2'-FL is released from the cell.

Method of integration of the genetic elements into the genome of the strain* E. coli *K12 JM109

The strain was constructed to carry a single copy of the constructed operon in a defined locus of the genome. In order to do this, the so-called *lambda-red*-integration technology (Red/ET) was used. In short, the recombination technology is based on the transient expression of a phage *lambda* recombinase which is able to catalyze homologous recombination between two similar genetic elements (Heermann *et al.* (2008)). In the case described here, the recombination events took place between the genomic DNA of JM109 and the constructed operon, carrying the genes necessary for the production of 2'-FL.

The Red/ET technology uses *in vivo* homologous recombination mediated by the phage *lambda redα* and *redβ* genes. This recombination occurs typically between a linear and circular DNA molecule, whereas the linear molecule contains two homology regions for the desired site of recombination. The Red/ET system is encoded on a transiently expressed plasmid which carries the respective genes for recombination also including the *recA* recombination gene and the araC gene (necessary for the transient expression) of *E. coli.* In addition, the plasmid contains a suitable selection marker (e.g. an ampicillin resistance cassette) and has a temperature sensitive origin of replication which restricts replication at 37°C and leads to a loss of the plasmid after the successful recombination. Further information on the activity and mechanism of the lambda red system can be found in Heermann *et al.* (2008).

For performing a DNA recombination in *E. coli*, certain selection steps are necessary since the recombination efficiency is too low to obtain the wanted genotype without selection pressure. For achieving a successful recombination, thus either positive or negative counter-selection approaches have been used. The Zeocin resistance gene (zeo) and the Chloramphenicol resistance gene (cmR) have been used as positive selection markers. The *rpsL* gene has been used as a negative counter-selection marker. The counter-selection method is based on the fact, that mutations within *rpsL* confer tolerance to streptomycin. If a strain carries a *rpsL* gene without a mutation, the strain has a streptomycin-sensitive phenotype. If the strain carries a *rpsL* mutation (e.g. *rpsL*150) it shows a streptomycin-tolerant phenotype which is also a naturally occurring phenotype (Heermann *et al.* (2008)). In case both versions, *rpsL* as well *rpsL150*, are present in the genome, the strain shows a streptomycin-sensitive phenotype as the wildtype *rpsL* allele is dominant over the mutated gene. This fact allows for a recovery of strains which have deleted the wildtype allele at the location of the recombination and results in a streptomycin-tolerant phenotype.

In addition, methods for replacing DNA fragments by lambda red mediated homologous recombination with antibiotic resistance genes (Zeocin resistance gene (zeo) and Chloramphenicol resistance gene cmR) have been utilized to select for DNA insertion and allelic replacement and have been planned in a way that in the final step of strain development replacement of both used antibiotic markers zeo and cmR by suitable flanking DNA fragments is performed. Due to this method, all heterologous antibiotic markers are fully deleted from the genome of the strain LU20297. This deletion is confirmed by Southern hybridization (and sequencing).

Choice of integration site for the constructed operon

In case of LU20297, the fucose utilization locus (*fucPIKU*) has been chosen as target site for the insertion of the 2'-FL biosynthetic operon. The *fucPIKU* locus is responsible for the uptake and catabolism of fucose as a carbon source. Thus, integration of genes into this locus is not prone to inactivate genes which are essential for normal functions of *E. coli*. The *fucPIKU* regulon is located at 63.2 min of the *E. coli* chromosome. It consists of genes coding for proteins which are necessary for the uptake (fucP), the isomerization to fuculose (fucI), the kinase for activating the fuculose and the mutarotase fucU. Since these genes are only active in the degradation of fucose it was decided to utilize this genetic locus for integrating the genes for the 2'-FL synthesis. Furthermore, it was believed that intracellular fucose could potentially be more stable for the synthesis of 2'-FL if these genes were inactivated. Careful planning for choosing the recombination target sequences was taken to avoid the occurrence of residual open reading frames after integrating the recombinant DNA fragment. In case of LU20297 *fucI* was completely and *fucK* partially deleted. The operon is terminated by the strong *E. coli* terminator *TrrnB* which prevents transcription of the *fucK* remnant and downstream located genes.

Confirmation of all genetic modifications in the final strain E. coli K12 (LU20297)

The 4.6 Mbp genome sequence of the parental strain *E. coli* K12 (MG1655) was published in 1997 and 4288 protein-coding genes have been annotated (Blattner *et al.* (1997)). Whole genome sequencing was applied to the parental strain *E. coli* K12 JM109 (Illumina) and the production strain *E. coli* K12 LU20297 (Illumina and Pacbio). Genomic DNA was isolated with the QIAGEN "Blood & Cell Culture DNA Midi Kit".

Whole genome sequences of the parent host strain and the final strain were established as follows:

- Genome sequencing of *E. coli* K12 LU20229 was performed utilizing two modern technologies: the *Illumina Technology (Illumina)* and the *Pacific Biosciences (PacBio)*. For *Illumina*, a total of 2x 5265239 paired-end reads of 300 bps length were obtained (MiSeq methodology, V3-chemistry, 328-fold coverage). For PacBio, 21345 reads (up to 7 kbp long, 19-fold coverage) and 66404 so called subreads (68-fold coverage) were obtained.
- Quality of both datasets was verified with the program *FastQC v0.11.5* and determined to be very good. No contaminating DNA sequences or vector DNA sequences were found when checking the obtained DNA sequences with the program *Fastq_screen v0.9.2* (5% threshold).

- *Illumina* reads were quality-trimmed with the program *Trimmomatic* v0.36, and then merged with the program *Flash v1.2.11*.
- Genome assembly was performed using the program *Spades v3.9.0*, utilizing all the sequencing data together (*Illumina* and *PacBio*, hybrid assembly). Genome assembly for a circularized genome was done with the program *Circlator v1.5.2*.
- For the *de novo* prediction of coding sequences CDS, the program *Prodigal v2.6.3* was used. Functional annotations were added using in-house software *ProGAP (commit 631262b)*.
- Genome sequencing of *E. coli* K12 JM109 was performed with *Illumina* only (with 2x 4237371 paired-end reads, 260 -fold coverage). Data quality was very good, with no contaminating DNA sequences or vector DNA sequences at a 3% threshold. Processing workflow was similar to that of *E. coli* K12 LU20297.
- *E. coli* K12 LU20297 Genome assembly resulted in a complete/finished genome, with a circular chromosome (4425750 bp long, 50.78% GC content) and a circular plasmid F' ("F-prime", 231175 bp long, 50.41% GC content).

Genome comparison of E. coli K12 LU20297 to E. coli K12 JM109 and E. coli K12 MG1655

Genome structure: *E. coli* LU20297 genome structure is fully concordant with that of *E. coli* K-12 substr. JM109. The genome consists of a circular chromosome and a circular plasmid F'. In some strains (like *E. coli* K-12 MG1655) this F' plasmid is integrated into the genome (Neidhardt (1986)).

Single nucleotide polymorphisms (SNPs): A total of 2 SNP difference groups were identified between LU20297 and JM109:

- a known/expected change in the *rpsL* gene, TAAA (JM-109) => ACGT (LU20297) at positions 1382071-1382074 on LU20297 chromosome;
- a single SNP in the *hisA* gene, G (JM109) => A (LU20297) at position 2727115 on LU20297 chromosome; further comparison to K12 MG1655 indicates that it also has a guanine at this position.

Genome assembly and annotation: The sequence of LU20297 was assembled using the sequencing reads and resulted in a fully closed genome containing two contigs, one for the chromosome of LU20297 and one for the F' plasmid. Corresponding statistics are given in Table 6 and Table 7.

Table 6 Genome assembly statistics for *E. coli* K12 LU20297

Statistics without reference	*E. coli* K12 LU20297
# contigs (>= 10000 bp)	2
# contigs (>= 25000 bp)	2
# contigs (>= 50000 bp)	2
Largest contig	4425750
N50	4425750
N75	4425750
L50	1
L75	1
GC (%)	50.75

Table 7 Genome annotation statistics for *E. coli* K12 LU20297.

contigs	2
bases	4656925
rRNA	22
tmRNA	1
misc_RNA	143
CDS	4322
repeat_region	2
tRNA	88

Operon sequence coding: A sequence alignment (Figure 6) of the planned operon sequence coding for proteins producing 2'-FL and the actually occurring sequence of the operon in the sequenced locus showed no difference. The full genetic organization of the original *fuc*-locus with the integrated operon for the synthesis of 2'-Fucosyllactose is given in

Table 8.

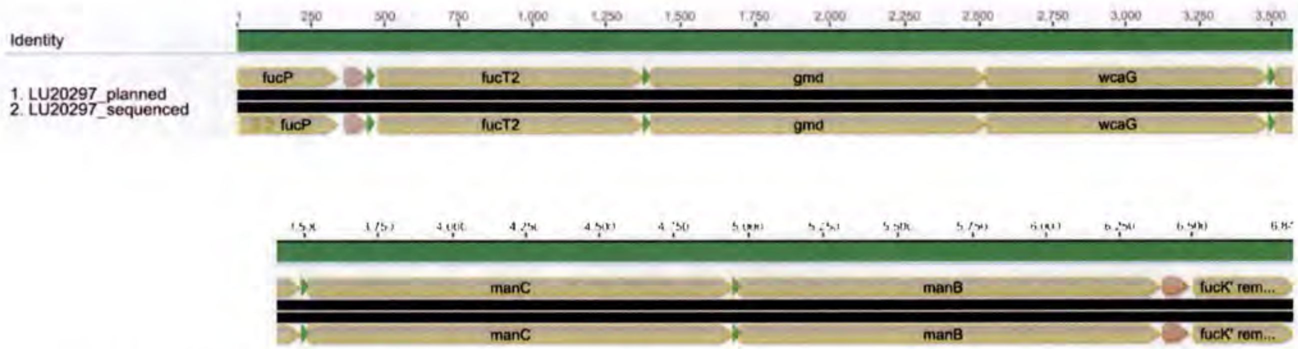

Figure 6 Alignment of planned operon sequence (LU20297_planned) with sequencing result of LU20297 (LU20297_sequenced) in the *fuc*-locus. Bar next to "identity" indicates 100% sequence identity between aligned sequences. The alignment was done using the MUSCLE algorithm (MUltiple Sequence Comparison by Log-Expectation) in Geneious (Biomatters, LTD. New Zealand software, Version 6.1.7). The detailed alignment sequence is shown in Annex VI.

Table 8 Genetic organization of the *fuc*-locus with integrated 2'-FL operon

Name	Function	Start	End	Size (bp)
~*fucP*	gene	1	338	
Ptac	promoter	360	440	81
RBS_1	RBS	443	465	23
fucT2	gene	473	1375	903
RBS_2	RBS	1382	1396	15
gmd	gene	1397	2518	1122
wcaG	gene	2521	3486	966
RBS_4	RBS	3495	3511	17
manC	gene	3512	4947	1436
RBS_5	RBS	4954	4970	17
manB	gene	4971	6389	1419
TrrnB	terminator	6401	6487	87
fucK' remnant	gene	6501	6849	349

Southern blot hybridization

Analysis using Southern blot hybridization was performed in order to verify the presence and correct insertion site of the synthetic operon as well as the absence of two resistance makers in *E. coli* K12 LU20297 (*zeo, cmR*) which had been introduced in intermediate strains but removed eventually. *FucT2* and *manB* have been chosen as representative genes of the operon as they represent the 5' prime (*fucT2*) and the 3' prime (*manB*) of the operon.

The hybridization data verified that the operon (represented by the genes *fucT2* and *manB*) were inserted at the desired *fuc*-locus shown by the expected band size after hybridization with the respective probe. The wildtype *E. coli* K12 JM109 was used as control and did not show the respective band size (only the signal for the native occurring *manB* gene in *E. coli* JM109) and in case of *fucT2* no signal at all. While the marker genes *zeo* and *cmR* were detected in the intermediate strains where they were needed for selection purposes, neither the host nor the final production strain contained any of these genes.

Sequence analysis of occurrence of the pRed/ET Amp plasmid in the genome of the strain E. coli K12 LU20297

In the process of the lambda red mediated gene recombination a helper plasmid carrying and expressing the lambda red structural genes gam beta alpha, as well as the *E. coli* gene *recA* was used (Figure 7). All these genes are under control of the additionally coded *araC* protein. In the strain construction, the recombination plasmid has to be removed from the final production strain at the end of strain construction. This was done by utilizing the temperature-sensitive replication phenotype of the Red/ET plasmid. The plasmid origin of replication confers plasmid replication only at permissive temperatures. This allows plasmid stability at 30°C and will eventually lead to a loss of the plasmid once the strain is being maintained at >37°C. The strain was re-streaked several times on non-antibiotic containing agar and incubated at 37°C. The resulting strain after strain maintenance at 37°C was found to be ampicillin sensitive and thus lost the ampicillin resistant phenotype conferred by the plasmid. In addition, a sequence analysis of the full genome of *E. coli* K12 LU20297 was performed to show absence of any of the specific DNA sequences of the helper plasmid.

The sequence analysis was done as followed. The sequence of the plasmid pRed/ET AmpR contains sequences coding for different DNA fragments and genes:

The *E. coli* K12 derived araC protein and its terminator

The *E. coli* K12 derived araBAD promotor

The phage lambda derived genes gam beta alpha, the *E. coli* K12 derived *recA* gene

The temperature sensitive plasmid origin of replication SC101

The *amp*-resistance gene

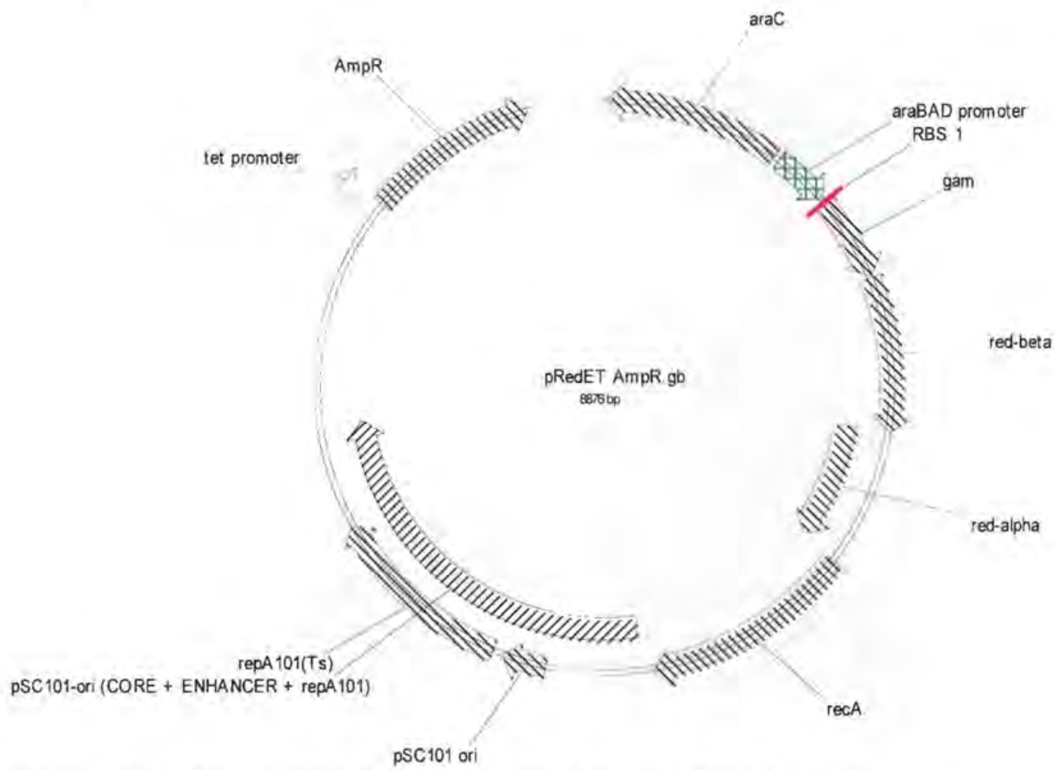

Figure 7 Map of the lambda Red recombination plasmid pRed/ET AmpR

In order to allow a defined analysis of the occurrence of the pRed/ET ampR in the genome of the strain *E. coli* K12 LU20297 the plasmid DNA sequence was divided into parts which contain only sequence which, by definition, should not occur in the genome of the strain. This sequence search analysis therefore omitted the *araC* gene-, the *araBAD* promoter- and the *recA* gene-sequence since they are already encoded in the *E. coli* K12 JM109 genome.

The sequence containing the parts between bp 1225 and bp 3016 with the phage *lambda gam, beta, alpha* genes (1882 bp length) was searched against the genome of the strain *E. coli* K12 LU20297 using the blast algorithm with the standard settings. The result was that no sequence similarity is identified between the phage lambda genes *gam, beta, alpha* and the DNA sequence of the strain *E. coli* K12 LU20297 (Annex VII).

Using the other sequence parts of the plasmid pRed/ET ampR, covering all residual sequences beyond the *E. coli* derived *recA* gene up to the *E. coli* derived terminator of the *araC* gene (covering the bases bp 4195 to bp 8786 (total of 4592 bp)) found no large similarities. One specific part of this sequence showed identity to a small number of similar sequences in the genome of *E. coli* K12 LU20297, however. This 24bp-sequence identity, originating from the terminator downstream of the plasmid encoded *recA* gene contains a 23s rRNA derived terminator sequence. The 23s rRNA can be found at the sites in the 3' region of the 23S RNA copies contained in the genome, from which it was derived originally. Thus, these short sequence identities are not reflecting an occurrence of the plasmid in the genome.

The sequence search using the transiently propagated pRed/ET plasmid in *E. coli* K12 LU20297 for similar sequences in the full genome sequence did reveal no sequences with homology beyond very short similarly sequences originating from naturally terminator sequences which had been used in the construction of the plasmid. The sequence analysis shows the complete absence of the pRed/ET ampR plasmid in the 2'-FL production strain *E. coli* K12 LU20297.

Genetic stability of the fucIK genomic locus carrying the 2'-FL biosynthetic genes

For the control of the genetic stability over the intended period of production, individual colonies of strain *E. coli* K12 LU20297 were isolated at the end of three independent 60 h fermentation runs. From every fermentation five individual colonies were selected (clones 1-5) and the integration locus was analyzed by Southern blotting to verify the identity of the strains. Primers were chosen for *fucT2* (heterologous gene, first gene of the operon) and *manB* (codon optimized *E. coli* gene, last gene of the operon).

The genomic DNA of five clones from every batch was isolated using the QIAGEN "Blood & Cell Culture DNA Midi Kit". Chromosomal DNA of LU20297 was directly prepared from the master cell bank and served as control. Southern blot hybridization followed standard routines as described by Sambrook and Russel, 2001.

The results from the hybridization experiments demonstrate that although several individual colonies are tested at the end of the fermentation process, it was not observed that an expression unit or a part of it become an integral part outside the expected locus resulting in an additional signal of a different size. Furthermore, no change to smaller signals or a complete loss of hybridization bands can be detected indicating that the integrated operon, represented by the genes *fucT2* and *manB*, is highly stable over the intended period of production.

2.3 Manufacturing Process (21 CFR 170.230 (b))

The production process (Table 9) is performed in four major activities: fermentation (steps 1 – 3), isolation (steps 4 and 5), purification (steps 6 – 19), and packaging/product release (steps 20 – 22).

Purification is performed by crystallisation from a solvent pair consisting of water and acetic acid or water and ethanol. Both solvent pairs result in the same product quality[1] with respect to content in 2'-FL and minor oligo-, di and monosaccharides, and other impurities. 2'-FL purified using acetic acid will contain residual amounts of acetic acid in the range between 0.1 and 1%.

Table 9 Schematic manufacturing process steps

Step No	Process step	Purpose
01	Media Preparation	
02	Seed cultivation	
03	Product fermentation	Production of 2'-FL
04	Cell separation	Removal of biomass and particles
05	Ultrafiltration	Removal of large molecules (e.g. protein, DNA and lipopolysaccharides)
06	*Optional Concentration*	*Removal of water, minerals and small molecules*
07	Decolorization (e.g. charcoal, polymeric resins)	Removal of color and impurities
08	*Optional Concentration*	*Removal of water, minerals and small molecules*

[1] Annex II: Batches 012644-L 01, 04 and 05 are crystallized from water/acetic acid, Batches 012644-L 02, 06 and 10 from crystallized from water/ethanol.

Step No	Process step	Purpose
09	*Optional Filtration*	*Removal of solids*
10	Demineralization (e.g. ion exchange resins, electrodialysis, nanofiltration)	Removal of charged molecules
11	*Optional Filtration*	*Removal of solids*
12	Concentration (e.g. reverse osmosis, evaporation)	Concentration
13	*Optional Filtration*	*Removal of solids*
14	Crystallization	Fine purification of product by solidification
15	Solid separation	Isolation of solid product
16	Washing & Drying	Removal of impurities
17	Re-dissolving in water	Pre-conditioning
18	Filtration	Removal of solids and large charged molecules
19	Drying	Drying, final removal of solvents
20	Sampling and Packaging	
21	Quality control	
22	Batch Release	

2.3.1 Fermentation

The biosynthesis of 2'-Fucosyllactose is performed with a well-controlled fermentation process using the production organism *Escherichia coli* K12 LU20297 (see section 2.2 for a detailed description). The fermentation process is divided into a sequence of seed stage cultures (seed train) and a production stage culture. In all cultivation stages, all relevant raw materials are of high purity in food grade (FCC recent edition or equivalent) or pharma grade (USP recent edition or equivalent) quality. A list of raw materials, their function, and the regulatory reference for the quality used is shown in Table 10.

Table 10 Growth media components and their regulatory status

Compound	CAS No.	Function	Regulatory status
Glycerin	56-81-5	Nutrient, precursor for 2'-Fucosyllactose synthesis	21 CFR 182.90
Lactose	63-42-3	Precursor for 2'-Fucosyllactose synthesis	Food grade *)
KH_2PO_4	7778-77-0	Nutrient, buffer	21 CFR 175.105
$(NH_4)_2SO_4$	7783-20-2	Nutrient	21 CFR 184.1143
$MgSO_4 \cdot 7\ H_2O$	10034-99-8	Nutrient	21 CFR 184.1443
Citric acid · H_2O	5949-29-1	Nutrient, complexation aid	21 CFR 184.1033 (citric acid, anhydrous)
Na_2SO_4	7757-82-6	Nutrient	21 CFR 186.1797
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	367-93-1	Inducer	From Plant origin galactose

Compound	**CAS No.**	**Function**	**Regulatory status**
Thiamin HCl	67-03-8	Nutrient	21 CFR 184.1875
Vitamin B_{12}	68-19-9	Nutrient	21 CFR 184.1945
Na_2-EDTA · 2 H_2O	6381-92-6	Complexation aid	21 CFR 172.135 (Disodium EDTA)
$CaCl_2$ · 2 H_2O	10035-04-8	Nutrient	21 CFR 184.1193
$FeSO_4$ · 7 H_2O	7782-63-0	Nutrient	21 CFR 184.1315
$ZnSO_4$ ·7 H_2O	7446-20-0	Nutrient	21 CFR 182.8997
$MnSO_4$ · H_2O	10034-96-5	Nutrient	21 CFR 184.1461
$CuSO_4$ · 5 H_2O	7758-99-8	Nutrient	21 CFR 184.1261
Na_2SeO_3 water free	10102-18-8	Nutrient	USP
Na_2MoO_4 · 2 H_2O	7631-95-0	Nutrient	USP
NH_3 (g)	7664-41-7	pH control agent	FEMA GRAS 24
NaOH	1310-73-2	pH control agent	21 CFR 184.1763
Alkoxylated fatty acid ester on a vegetable base	-	Defoamer	Compliant with 21 CFR 173.340
Propylene glycol	57-55-6	Defoamer	21 CFR 184.1666
Purified water	7732-18-5	Solvent	-

*) Substances Added to Food (formerly EAFUS)

Seed train: The seed train is carried out in a sequence of at least two cultivation stages, where the cells are propagated to accumulate enough biomass to start the production stage cultivation. In the seed cultivations, the cells are cultivated under conditions that suppress the biosynthesis of 2'-FL. The seed cultivation medium is a chemically defined nutrient medium mainly consisting of inorganic salts dissolved in purified water and glycerol as main carbon source. The seed cultivation stages are carried out under aerobic conditions at tightly controlled process conditions (such as temperature, pressure, oxygen availability and pH). Oxygen is provided to the culture via surface aeration and by sparging of sterile filtered, compressed air through the medium. The first seed stage cultivation usually takes place in a shake flask, inoculated with a recently thawed cell bank vial. The cultivation is carried out until a pre-defined cell density is obtained, then the seed stage culture is transferred to the production stage cultivation.

Production cultivation: In the production stage cultivation, the cells are cultivated under conditions that enable the biosynthesis of 2'-FL in a stirred tank bioreactor under aerobic conditions. The cultivation medium is a chemically defined nutrient medium mainly consisting of inorganic salts dissolved high-purity water, glycerol as main carbon source and lactose as precursor for 2'-FL synthesis. Oxygen is provided to the culture by sparging of sterile filtered, compressed air through the medium. The production of 2'-FL is triggered by addition of small amounts of isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture, thereby inducing the expression of the relevant transporter and biosynthetic genes in the production organism. Continuous feeding of nutrients is used to control the growth of the cells and to provide precursors for the 2'-FL biosynthesis. pH is controlled in the range of 6.3-7.3 by automatic addition of sterile filtered ammonia. Temperature is kept at 33-39° C. All relevant process parameters are tightly controlled to obtain a high 2'-FL concentration and purity in the fermentation broth. A range of in-process analytics and controls is used to detect and avert any negative process conditions, which may impair the quality of the fermentation broth and product. A food grade defoamer is used to suppress foam formation arising from the air sparging. 2'-FL is excreted by the cells during the cultivation and can hence be efficiently separated from the biomass by the subsequent purification steps, without need for cell disruption or permeation. The correct batch endpoint is determined by robust in-process analytics, whereby the fermentation broth is transferred to the next downstream process stage. At the end of the production stage fermentation, the main components of the fermentation broth are biomass, 2'-FL, lactose, 2'-difucosyl-D-Lactose and residual inorganic salts. In addition, minor amounts of organic acids such as acetic acid or succinic acid may be formed as byproducts during the fermentation. Minor amounts of proteins and fragments from the cells may also be present in the fermentation broth.

2.3.2 Isolation and purification

After biosynthetic production of 2'-FL, the product purification of secreted 2'-FL is carried out using several process steps.

At first, the living and intact cells are separated from fermentation broth. The separation can be done either by cross-flow microfiltration or centrifugation.

The 2'-FL containing liquid phase is further filtered by a cross-flow filtration process with an ultrafiltration membrane to remove large molecules (e.g. protein, DNA and lipopolysaccharides). Prior to or after ultrafiltration, a concentration by vacuum evaporation or filtration (e.g. nanofiltration) can be performed. During the processing of 2'FL aqueous phase, the pH may, if necessary, be adjusted by using an acid (inorganic or organic) or an inorganic base.

Color impurities and other hydrophobic impurities are removed from solution using an adsorbent (e.g activated carbon or polymeric resin). The decolored crude 2'-FL solution is then further demineralized by ion exchange adsorbent and / or electrodialysis.

Prior to final purification the crude 2'-FL solution is concentrated by filtration (nanofiltration) and / or vacuum evaporation to the desired concentration level of 2'-FL.

The 2'-FL is then purified by crystallization from concentrated solution in water using the solvent pair approach by well-controlled addition of a second solvent (acetic acid or ethanol). The solid crystalized product is removed from the mother liquor by filtration or centrifugation. Crystals are washed by suitable solvents to further remove traces of impurities and are dried to reduce the volatile content of the crystal. The obtained crystalline 2'-FL can already be used as final product form. Solid amorphous product requires removal of the liquid phase by spray drying.

Sampling and quality control measures throughout the whole process are carried out to ensure that product fulfils the product specification. All raw materials are of food or pharma grade quality. All process aids (e.g. resins, activated carbon, membranes) are certified for their use with food, in food processing (Table 11).

Table 11 Processing aids and their regulatory status

Compound	CAS No.	Function	Regulatory status
Activated carbon	64365-11-3	Adsorbens	21 CFR 177.1210 *)
Cation exchange resin	-	Ion Exchanger	21 CFR § 173.25(a)(5)
Anion exchange resin	-	Ion Exchanger	21 CFR § 173.25(a)(1)
Chromatography Resin	-	Fine purification	21 CFR § 173.25(a)(1)
Ethyl alcohol	64-17-5	Solvent	21 CFR 184.1293
Acetic acid	64-19-7	Solvent	21 CFR 184.1005
H_2SO_4 (aq)	7664-93-9	pH control, cleaning	21 CFR 184.1095
NaOH (aq)	1310-73-2	pH control, cleaning	21 CFR 184.1763
Purified water	7732-18-5	Solvent	-
Microfiltration membranes	-	Membrane	21 CFR 177.2910, EC 1935/2004
Ultrafiltration membranes		Membrane	21 CFR 177.2910, EC 1935/2004

Compound	CAS No.	Function	Regulatory status
Reverse osmosis membranes	-	Membrane	21 CFR 177.2550, 177.1655 177.2420, EC 1935/2004, EU 10/2011, TSE&BSE free

*) Substances Added to Food (formerly EAFUS)

2.4 Specifications (21 CFR 170.230 (c))

The 2'-fucosyllactose produced by fermentation followed by a downstream process that aims at removing effectively the biomass (specifically cell-walls incl. endotoxins and protein) and isolating a product of high purity with a low content of other related sugars. The analytical data presented in Sections 2.1.3, 2.1.4, and 2.1.5 show the corresponding levels that have been achieved for 2'-Fucosyllactose and related sugars (Table 1), organic and inorganic impurities (Table 2), and microbial contaminants (Table 3).

Based on these data the product specifications laid down in Table 12 were adopted. They reflect also the specifications for 2'-Fucosyllactose produced from a microbial source (genetically modified strain of *Escherichia coli* K-12) which were adopted by the European Union[2] and reflect the products which had been evaluated by the European Food Safety Authority (EFSA) and found to be safe for use in common food, dietary supplements, and foods for babies including infant and follow-on formulae. They also are in agreement with the specifications applied to 2'-FL notified previously to FDA.

Table 12 Specifications of 2'-Fucosyllactose (BASF)

Parameter	Method	Specification
Assay by HPLC	BASF-HPLC method	min. 90% (water-free)
Identification		
Appearance, visual	MSZ ISO 6658:2007	Powder or agglomerates
Color, visual	MSZ ISO 6658:2007	white to off-white powder
Identification, HPLC, Rt main component	BASF-HPLC method	Rt standard +/- 3%
Related substances		
D-Lactose	BASF-HPLC method	≤ 3.0 % (as is)
L-Fucose	BASF-HPLC method	≤ 2.0 % (as is)
2'-Difucosyl-D-Lactose	BASF-HPLC method	≤ 2.0 % (as is)
2'-Fucosyl-D-Lactulose	BASF-HPLC method	≤ 2.0% (as is)
Characteristic properties		
pH (20°C, 5% solution)	Ph. Eur. 2.2.3	3.2 - 7.5
Sulfated Ash	Ph. Eur. 6.7 04/2010:20414	≤ 1.5 %
Acetic acid (as free acid and/or sodium acetate)	Megazyme K-ACETRM 07/12	≤ 1.0%
Water, Karl-Fischer	Karl-Fischer (Ph. Eur. 2.5.12)	≤ 9.0 % (weight)
Heavy Metals / Contaminants		
Pb	ICP-MS	≤ 0.05 mg/kg
Cd	ICP-MS	≤ 0.05 mg/kg

[2] [https://ec.europa.eu/food/safety/novel_food/authorisations/union-list-novel-foods_en]

Parameter	Method	Specification
Hg	ICP-MS	≤ 0.05 mg/kg
As	ICP-MS	≤ 0.1 mg/kg
Endotoxin	Limulus amebocyte lysate kinetic chromogenic assay described in the European Pharmacopoeia	≤ 10 EU/mg
Residual Protein (Bradford)	modified Bradford Assay	≤ 0.01 %
Microbiology		
Total microbial aerobic count	MSZ-EN-ISO 4833-1:2014	<500 CFU/g
Yeasts and Molds	MSZ-ISO 7954:1999	<100 CFU/g
Enterobacteria & other Gram-neg	ISO 21528-1:2004, MSZ ISO 21528-2:2007	absent in 10 g
Cronobacter sakazakii	ISO-TS 22964:2006	absent in 10 g
Salmonella	MSZ-EN-ISO 6579:2006	absent in 25 g
Listeria monocytogenes	MSZ-EN-ISO 11290-1:1996/A1:2005, MSZ-EN-ISO 11290-1:1998	absent in 25 g

The 2'-Fucosyllactose produced by the notifier is of equivalent quality as 2'-FL produced by chemical synthesis or from other microbial sources. Relevant specifications for products notified under the GRAS route to FDA and from EU regulations are compared in Table 13. Some minor entries used by other manufacturers (such as additional tests for specific heavy metals or certain microorganisms) do not affect the conclusion that the notifier's 2'-FL is substantial equivalent to the other listed 2'-FL. Based on this equivalence it is possible to read-across from the safety data reported and published by third parties (see Part 6).

Table 13 Specifications of 2'-Fucosyllactose (BASF) compared with 2'-FL documented/specified elsewhere

Parameter	BASF (*E. coli* K12)	GRN 546 (synthetic)	GRN 650 (*E. coli* K12)	GRN 571 (*E. coli* BL21)	GRN 735 (*E. coli* K12)	GRN 749 (*E. coli* K12)	2-FL (synthetic) as adopted by the European Union [e]	2-FL (*E. coli* K12) as adopted by the European Union [e]	2-FL (*E. coli* BL21) as adopted by the European Union [e]
Assay	min. 90% (HPLC, water-free)	Min 95.0% (HPLC, water free)	Min 94.0% (HPLC, water free)	≥ 90 % (HPAEC-PAD area)	min. 90% (HPAEC)	≥ 82 %	≥ 95 %	≥ 90 %	≥ 90 %
Identification									
Appearance, visual	Powder or agglomerates	Powder	Powder	-	Homogenous powder	Powder	Powder	Powder	Powder
Color, visual	White to off-white	white to off-white	white to off-white	-	White	White to off-white	White to off-white	White to off-white	White to off-white
Related substances									
D-Lactose	≤ 3.0 % (as is)	-	Max. 3.0 w/w%	≤ 5 %	Max 3 %	≤ 8 %	≤ 1.0 %	≤ 3.0 %	≤ 5.0 %
L-Fucose	≤ 2.0 % (as is)	-	Max. 1.0 w/w%	≤ 5 %	Max 2 %	≤ 6 % [d]	≤ 1.0 %	≤ 2.0 %	≤ 3.0 %
2'-Difucosyl-D-Lactose	≤ 2.0 % (as is)	-	Max. 1.0 w/w%	≤ 5 %	-	≤ 7 %	≤ 1.0 % (isomers)	≤ 2.0 %	≤ 5.0 %
2'-Fucosyl-D-Lactulose	≤ 2.0% (as is)	-	Max. 1.0 w/w%	-	-	≤ 6 % [d]	≤ 0.6 %	≤ 1.0 %	
2'-Fucosyllactose	-					≤ 6 % [d]			≤ 5.0 %
Fucosylgalactose	-	-	-	≤ 3 %	-	≤ 6 % [d]		-	≤ 3.0 %
Allo lactose	-	-	-	-	Max 2 %	-		-	
Glucose	-	-	-	≤ 3 %	Max 2 %	≤ 6 % [d]		-	≤ 3.0 %
Galactose	-	-	-	≤ 3 %	Max 2 %	≤ 6 % [d]		-	≤ 3.0 %

Parameter	BASF (*E. coli* K12)	GRN 546 (synthetic)	GRN 650 (*E. coli* K12)	GRN 571 (*E. coli* BL21)	GRN 735 (*E. coli* K12)	GRN 749 (*E. coli* K12)	2-FL (synthetic) as adopted by the European Union [e]	2-FL (*E. coli* K12) as adopted by the European Union [e]	2-FL (*E. coli* BL21) as adopted by the European Union [e]
Characteristic properties									
pH (20°C, 5% solution)	3.2 - 7.5	3.0 – 7.5	3.2 – 5.0	-	3.0 – 7.5 (10 % solution)	-	3.2 – 7.0	3.0 – 7.5	-
Sulfated Ash	≤ 1.5 %	Max. 0.2 %	Max. 1.5 %	≤ 0.5 %	Max. 0.2%	-	≤ 0.2 %	≤ 2.0 %	≤ 0.5 %
Acetic acid (as free acid and/or sodium acetate)	≤ 1.0%	Max. 0.3 %	Max. 1.0 %	-	-	-	≤ 0.3 %	≤ 1.0 %	-
Water, Karl-Fischer	≤ 9.0 % (weight)	Max 9.0 %	Max 5.0 %	≤ 9.0 %	Max 5.0 %	≤ 9.0 %	≤ 9.0 %	≤ 9.0 %	≤ 9.0 %
Heavy Metals / Contaminants									
Pb	≤ 0.05 mg/kg	Max. 0.8 mg/kg	Max. 0.1 mg/kg	≤ 0.02 mg/kg	Max. 0.05 mg/kg	≤ 0.05 mg/kg	-	-	≤ 0.02 mg/kg
Cd	≤ 0.05 mg/kg	-	-	≤ 0.1 mg/kg	Max. 0.01 mg/kg	≤ 0.05 mg/kg	-	-	≤ 0.1 mg/kg
Hg	≤ 0.05 mg/kg	-	-	≤ 0.5 mg/kg	Max. 0.05 mg/kg	≤ 0.1 mg/kg	-	-	≤ 0.5 mg/kg
As	≤ 0.1 mg/kg	-	-	≤ 0.2 mg/kg	≤ 0.1 mg/kg	≤ 0.2 mg/kg	-	-	≤ 0.2 mg/kg
Endotoxin	≤ 10 EU/mg	Max 50 EU/mg	-	≤ 300 EU/mg	Max. 10 EU/mg	-	≤ 10 EU/mg	≤ 10 EU/mg	≤ 100 EU/mg
Residual Protein (Bradford)	≤ 0.01 %	0.1 %	0.01 %	≤ 100 µg/g	Max. 0.01 %	≤ 100 mg/kg	≤ 0.01 %	≤ 0.01 %	≤ 0.01 %
Microbiology									
Total microbial aerobic count	≤ 500 CFU/g	Max. 500 CFU/g [a])	Max. 500 CFU/g [a])	≤ 10000 CFU/g [b])	Max. 3000 CFU/g	-	≤ 500 CFU/g	≤ 3000 CFU/g	Max. 3000 CFU/g
Yeasts and Molds	≤ 100 CFU/g	Max. 10 CFU/g [c])	Max. 10 CFU/g [c])	≤ 100 CFU/g	Max. 10 CFU/g [c])	-	Max. 10 CFU/g	Max. 100 CFU/g [c])	≤ 100 CFU/g

Parameter	BASF (*E. coli* K12)	GRN 546 (synthetic)	GRN 650 (*E. coli* K12)	GRN 571 (*E. coli* BL21)	GRN 735 (*E. coli* K12)	GRN 749 (*E. coli* K12)	2-FL (synthetic) as adopted by the European Union [e]	2-FL (*E. coli* K12) as adopted by the European Union [e]	2-FL (*E. coli* BL21) as adopted by the European Union [e]
Enterobacteria & other Gram-neg	absent in 10 g	absent in 10 g	absent in 10 g	absent in 11 g (incl. coliform)	absent in 10 g	-	-	-	absent in 11 g (incl. coliform)
Cronobacter sakazakii	absent in 10 g	absent in 10 g	absent in 10 g	absent in 10 g	absent in 25 g	absent in 100 g	-	-	absent in 100 g
Salmonella	absent in 25 g	absent in 25 g	absent in 25 g	absent in 100 g	absent in 25 g	absent in 100 g	-	-	absent in 100 g
Bacillus cereus	-	Max. 50 CFU/g	Max. 50 CFU/g	-	Max. 100 CFU/g	-	-	-	-
Listeria monocytogenes	absent in 25 g	absent in 25 g	absent in 25 g	-	-	-	-	-	-

[a]) Aerobic mesophilic total (plate) count

[b]) Standard plate count

[c]) Separate specifications for yeasts and moulds

[d]) Limit of 6% for other carbohydrates that includes 3'-Fucosyllactose, 2'-Fucosyl-D-lactulose, Fucosylgalactose, Glucose, Galactose, Fucose, Sorbitol, Galactitol, Mannitol, and Trihexose

[e]) [https://ec.europa.eu/food/safety/novel_food/authorisations/union-list-novel-foods_en]

2.5 Technical effects (21 CFR 170.230 (d)

2'-Fucosyllactose is the most abundant Human Milk Oligosaccharide (HMO) present in human breast milk, together with other HMOs it is understood to play a pivotal role in establishing the intestinal microflora of the new-born baby, and to support maintaining a healthy composition of the microflora in young children and adults (see discussion and references in Part 6.6.1).

2'-Fucosyllactose will be used in infant formula at levels at which it has been determined to be present in human milk. For young children, adults, i.e. the general population 2'-Fucosyllactose will be used at levels that support digestive function and health (see discussion and references in Part 6.6.1).

The corresponding intended use levels for 2'-FL are given in Table 14 (they are the same as proposed by GRN 735).

Table 14 Intended food categories and use levels for 2'-FL

Proposed Food Category (21 CFR 170.3)	Food Uses	Maximum 2'-FL use level (g / serving)	RAAC[a]) (g or mL)	Maximum 2'-FL use levels (g / 100 g)
Beverages and Beverage Bases	Energy drinks	0.28	360	0.08
	Fitness water and thirst quenchers, sports and isotonic drinks	0.28	360	0.08
Breakfast cereals	Ready-to-eat breakfast cereals for adults and children	1.2	15 (puffed) 40 (high-fiber) 60 (biscuit-types)	8.0 3.0 2.0
	Hot cereals for adults and children	1.2	40 (dry) ~ 250 (prepared)	0.48 (as consumed)
Dairy Product Analogs	Milk substitutes such as soy milk and imitation milks	0.28	240	0.12
Frozen Dairy Desserts and Mixes	Frozen desserts including ice creams* and frozen yogurts, frozen novelties	1.2	~ 70	1.7
Gelatins, Puddings and Fillings	Dairy-based puddings, custards and mousses	1.2	~ 70	1.7
	Fruit pie filling	1.2	85	1.41
	"Fruit prep" such as fruit filling in bars, cookies, yogurt and cakes	1.2	~40	3.0
Grain Products and Pastas	Bars, including snack bars, meal-replacement bars and breakfast bars	0.48	40	1.20
Jams and Jellies, Commercial	Jellies and jams, fruit preserves*, and fruit butters	1.2	~20	6.0
Milk, Whole and Skim	All *acidophilus* or fortified milks, non-fat and low-fat milk fluids, including fluid milk and reconstituted milk powder*	0.28	240	0.12

Proposed Food Category (21 CFR 170.3)	Food Uses	Maximum 2'-FL use level (g / serving)	RAAC a) (g or mL)	Maximum 2'-FL use levels (g / 100 g)
Milk Products	Flavored milks, including chocolate milk, coffee drinks, cocoa, smoothies (dairy and fruit-based), other fruit and dairy combinations, yogurt drinks, and fermented milk drinks including kefir **	0.28	240	0.12
	Milk-based meal replacement beverages or diet beverages**	0.28	240	0.12
	Yogurt*,**	1.2	225	0.53
	Formula intended for pregnant women ("mum" formulas, -9 to 0 months)	1.2	200 b)	0.6
Processed Fruits and Fruit Juices	Fruit drinks, including vitamin- and mineral-fortified products	0.28	240	0.12
	Fruit juices*	0.28	240	0.12
Sweet Sauces, Toppings and Syrups	Syrups used to flavor milk beverages	0.28	40	0.70
		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	0.24	100 b)	0.24 (0.40 g / 100 kcal) c
	Follow-on formula (6 to 12 months), including ready to drink formula or formula prepared from powder	0.24	100 b)	0.24 (0.40 g / 100 kcal) c
Baby Foods	Meal replacement products such as Pediasure	0.24	120 b)	0.2
	Growing-up (toddler) milks (12-36 months)	0.24	120 b)	0.2
	Ready-to-eat, ready-to-serve hot cereals	1.2	15 (dry) 110 (ready-to-serve)	1.09 (as consumed)
	Yogurt and juice beverages identified as "baby" drinks	1.2	120	1.0
	Desserts including fruit desserts, cobblers, yogurt/fruit combinations ("junior type" desserts)	1.2	110	1.09
	Baby crackers, pretzels, cookies, and snack items	0.4	7	5.7

[a]) Reference Amounts Customarily Consumed per Eating Occasion (RACC), based on values established in 21 CFR 101.12. Note: when a range of values is reported for a proposed food use, particular foods within that food use may differ with respect to their RACC

[b]) No RACC value exists; therefore, approximate serving sizes are provided according to food manufacturers instructions

[c]) The intended use level in infant formula is 2.4 g per L (0.24 g per 100 mL) or 0.40g per 100 kcal. For a 100 mL formula that contains 60 kcal, the conversion is as follows:

60 kcal = 100 ml formula = 0.24 g 2'-FL

100 kcal = 166 ml formula = 0.4 g 2'-FL

* 2'-FL is intended for use in unstandardized products when standards of identity do not permit the addition

** Includes ready-to-drink and powder forms

3 Dietary exposure (Part 3)

3.1 Estimated dietary exposure of 2'-Fucosyllactose from all proposed Food Uses (21 CFR 170.235 (a))

Estimated dietary exposure for all proposed uses

The estimated intake analysis based on the proposed usage levels of 2'-FL is summarized in Table 15. As use levels are the same as proposed by GRN 735, the dietary exposure is therefore in accordance with the values reported in GRN 735 and specifically the Appendix 8 of that GRAS notice.

Food codes representative of each proposed food-use were chosen from NHANES 2013-2014 (National Health and Nutrition Examination Survey). Food codes have been put into food-use categories according to 21 CFR 170.3. If necessary, adjustment factors were developed for composite foods / mixtures based on data given in the Food and Nutrition Database for Dietary Studies (FNDDS).

The total estimated intakes of 2'-FL, determined as g per person per day and as mg per kg body weight per day are summarized in Table 15 and Table 16. In both tables, the consumer-only EDIs represent the estimated exposures in the target population.

The mean and 90th percentile consumer-only intakes of 2'-FL in the total population were calculated to be 1.70 g per person per day and 3.54 g per person per day, resp. Highest mean consumer-only intakes were found for infants 6-11 months of age (2.28g/day) and mal teenagers have highest 90th percentile consumer-only intakes (4.29 g / day). Women of child-bearing age (16-45 years) show both: lowest mean consumer-only intake (1.36 g /day) as well as lowest 90th percentile consumer-only intake (2.87g / day).

Estimated dietary intake data were also calculated on a per kg body weight – basis. On total population basis, the mean consumer-only intake was 36 mg / kg bw / day and the 90th percentile value was 80 mg/ kg bw /day. The population subgroup with highest mean and 90th percentile intakes were infants aged 0-5 months with 315 mg / kg bw / day and 532 mg / kg bw / day, resp. Lowest levels were estimated for women at child bearing age and female adults: mean consumer-only intake per kg bw / day 20 mg and 90th percentile consumer-only intake level was 43 mg / kg bw /day.

Table 15 Summary of the Estimated Daily Intake (EDI) of 2'-FL from Proposed Food-Uses in the United States by Population Group (2013-2014 NHANES Data)

Population Group	Age Group (years)	Per Capita Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0-5 months	1.10	2.75	57.5	107	1.91	3.00
Infants	6-11 months	2.14	3.86	94.1	160	2.28	3.86
Toddlers	12-35 months	1.83	2.97	100.0	348	1.83	2.97
Children	3-11	1.96	3.53	99.7	1277	1.97	3.53
Female Teenagers	12-19	1.47	2.95	94.7	544	1.55	2.95
Male Teenagers	12-19	1.85	4.16	92.5	526	2.00	4.29
Women of Child-bearing age	16-45	1.22	2.82	89.9	1219	1.36	2.87
Female Adults	20 and up	1.32	2.96	91.9	2169	1.44	3.05
Male Adults	20 and up	1.59	3.81	86.8	1842	1.84	3.97
Elderly	65 and up	1.76	3.74	92.8	939	1.90	3.91
Total Populations	All ages	1.55	3.41	91.2	6973	1.70	3.54

Table 16 Summary of the Estimated Daily Per Kilogram Body Weight Intake of 2'-FL from Proposed Food-Uses in the United States by Population Group (2013-2014 NHANES Data)

Population Group	Age Group (years)	Per Capita Intake (mg / kg bw /day)		Consumer-Only Intake (mg / kg bw / day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0-5 months	181	477	57.5	107	315	532
Infants	6-11 months	244	441	94.1	160	259	447
Toddlers	12-35 months	148	243	100.0	346	148	243
Children	3-11	75	147	99.7	1268	76	147
Female Teenagers	12-19	24	52	94.7	536	26	52
Male Teenagers	12-19	29	67	92.5	524	31	67
Women of Child-bearing age	16-45	18	42	89.9	1209	20	43
Female Adults	20 and up	19	42	91.9	2156	20	43
Male Adults	20 and up	19	46	86.7	1833	22	48
Elderly	65 and up	24	53	92.6	928	26	54
Total Populations	All ages	32	76	91.1	6930	36	80

Estimated dietary exposure by infants and toddlers

BASF's 2'-FL is intended for use in non-exempt infant formulas and toddler foods, as notified by Glycom GRN 546 and GRN 650, Jennewein in GRN 571, Glycosyn &Friesland in GRN 735 and Dupont in GRN 749. Since BASF's 2'-FL will be an alternative source of 2'-FLin the market, it is not expected that the intended use of BASF's 2'-FL will noticeably change the total intake.

To estimate the intake of 2'-FL from infant formula products, NHANES data were further analysed by removing the data for breast-fed individuals. This approach is similar to the one reported in GRN 546, GRN 571 and GRN 735.

Table 17 shows the estimated 2'-FL intakes of non-breastfed infants and toddlers who consume non-exempt formulas. Mean intakes decrease as the infant ages, reflecting the transition from infant formula and specific baby food to a regular mixed diet.

Table 17 Estimated daily intake of 2'-FL for non-breastfed infants and toddlers from non-exempt formulas.

Population Group	Age Group (Months)	Consumer-Only Intake (mg / kg bw / day) [a])					
		%	N	g / day		mg / kg bw / day	
				Mean	90th percentile	Mean	90th Percentile
Infants	0-5	43.0	79	2.14	2.88 [b])	354	498 [b]
Infants	6-11	56.6	100	1.67	2.56	192	311
Toddlers	12-35	11.7	39	0.39	1.14 [b])	40	101 [b])

[a]) Results represent the intake of 2'-FL from non-exempt infant formulas and follow-on formulas among consumers of formula (individuals consuming human milk in NHANES were left out).

[b]) The small samples sizes do not meet the minimum reporting requirements; the intake estimates may not be statistically significant

3.2 Estimated dietary exposure of substances formed (21 CFR 170.235 (b))

2'-FL is expected to be stable when added to the foods given in Table 14 at the levels mentioned. Corresponding data are presented and discussed in GRN 546. As 2'-FL described in the present notification is equivalent to the 2'-FL described in GRN 546, GRN 650, GRN 735, and GRN 749, no formation of other substances is expected under its proposed conditions of use.

3.3 Estimated dietary exposure of another substance present in 2'-FL (21 CFR 170.235 (c))

The 2'-FL described in this GRAS notice contains low amounts of mono-, di- and oligosaccharides (see Table 1) which all are naturally occurring in food and human breast milk. At the proposed conditions of use a specific dietary exposure assessment is not required.

3.4 Discussion of assumptions (21 CFR 170.235 (e))

The dietary exposure assessment discussed above is based on the assumption that a generic food ingredient produced by a different route of manufacturing but being of similar composition as others assessed previously and being subject to the same conditions of use, will result in the same dietary exposure assessment as the consumer, over the years, will be exposed to the ingredient being produced via various synthetic and fermentative routes. Different 2'-FL accepted as GRAS will replace each other in the market place and publicly available data on dietary exposure assessments for one of them are applicable to others.

4 Self-limiting levels of use (Part 4)

There are no known self-limiting levels of use for 2'-Fucosyllactose.

5 Experience based on common use in food before 1958 (Part 5)

2'-Fucosyllactose was not commonly used as a commercially available ingredient in food before 1958. Saying that, however, it should be noted that 2'-Fucosyllactose, being the major human milk oligosaccharide, is present in breast milk of women with diverse ethnic background (Table 18, based on data from published literature as discussed by GRN 735). which indicates that the common ancestor of *Homo sapiens* was already in possession of the ability to secrete 2'-FL into breast milk.

Table 18 Levels of 2'-Fucosyllactose in human breast milk

Study population (geography/number)	Concentration (g/L)	Reference
Africa (n=53)	1.8 – 8.4	Musumeci *et al.* (2006)
Italy (n=50)	1 – 4.2	Musumeci *et al.* (2006)
Italy (n=42)	5.25 – 7.3	Gabrielli *et al.* (2011)
Polynesia (n=12)	0.22 – 0.69	Leo *et al.* (2009)
Japan (n=12)	1.6 – 2.5	Asakuma *et al.* (2008)
US (n=11)	2.8 – 3.6	Chaturvedi *et al.* (2001a)
US (n=36)	2	Erney *et al.* (2000)
Asia (n=80)	2.1	Erney *et al.* (2000)
Europe (n=68)	2.6	Erney *et al.* (2000)
Latin America (n=197)	2.5	Erney *et al.* (2000)
Not specified (n= not given)	0.3 – 3.9	Castany-Munoz *et al.* (2013)

Though 2'-Fucosyllactose has not been available as an isolated ingredient, there is significant history of exposure of breast-fed infants and toddlers to it at levels in milk which resulted in significant intakes by these populations sub-groups before 1958.

GRN 650 summarized *in extenso* the likely intake of new-born babies and toddlers from breast milk as discussed by various authors (referring also to GRN 546). Based on mean levels of 2'-FL present in mature human milk samples that have been reported in the literature, a 6.5-kg infant drinking 1 L of milk per day would be expected to consume 170 to 660 mg/kg body weight/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 body weight/day.

For new born infants, the average intake of 2'-FL from colostrum is approximately 80 to 360 mg/kg body weight/day based on a 3.4-kg new-born infant drinking an average of 250 mL of breast milk per day during the first 5 days. However, in new-borns from secretor mothers, the intake of 2'-FL from colostrum may be up to approximately 620 mg/kg body weight/day.

6 Narrative (Part 6)

6.1 Introduction

This narrative provides a basis for the Notifier's conclusion that 2'-Fucosyllactose produced with the production strain *E. coli* K12 LU20297, is GRAS when used as an ingredient in foods including infant formula, follow on formula and baby food at levels ranging from 0.24 to 1.2 grams/serving.

The specifications for 2'-Fucosyllactose subject to the present notice are compared with five previous GRAS notices (GRN 546, GRN 650, GRN 571, GRN 735, and GRN 749) in Table 13. The product is similar in quality to the range of products notified previously and there are no reasons to assume that it would behave chemically and biochemically different to those. This applies also to the related substances which are mono-, di-, and trisaccharides which are present in other 2'-FL described in GRAS notifications which have not been objected by FDA. The inorganic (heavy metals and arsenic) and organic (endotoxins, residual protein) impurities are limited by similar albeit usually lower levels and do not raise concerns as the product shall be used in the same foods and at the same levels as previously notified substances.

The narrative will focus on published data available from the public domain including the five previously submitted GRAS notices. As there is already a considerable data set available further studies in animals or humans cannot be justified.

Two recently published reviews focused on the safety and utility of using human milk oligosaccharides including 2'-Fucosyllactose in infant formula and baby food. Vandenplan *et al.* (2018) confirmed previous conclusions that the addition of one or two HMO to infant formula is safe and brings infant formula closer to human milk. Reverri *et al.* (2018) concluded for 2'-FL that clinical experiences demonstrated that 2'-FL being added to infant formula was safe, well-tolerated, and absorbed and excreted with similar efficiency to 2'-FL naturally present in human milk.

6.2 Safety of production strain

E. coli is a bacterial species that normally inhabit the intestinal tract of humans and other animals. There are some pathogenic strains that may cause human illness. It is therefore important to choose a production organism as host organism that is non-pathogenic. The *E. coli* K12 strain JM109 is part of the K12 *E. coli* lineage which is the "workhorse" organism used for most recombinant DNA work in laboratories worldwide. *E. coli* K12 has a defective cell envelope that renders it incapable of colonizing or surviving in the human gut.

E. coli K12 contains no known pathogenic genes (either colonization factors or toxin genes) and is universally recognized as a safe, commercial manufacturing host. *E. coli* K12 is used on a global scale in the commercial fermentation of food additives and food ingredients (amino acids and vitamins), recombinant human proteins used in pharmaceutical applications including active pharmaceutical ingredient used as injectables (Blount (2015)).

The DNA sequence modifications were introduced using current techniques that assure that only the intended changes are made, and unintended changes are avoided (Heermann *et al.* (2008)). Next to four naturally occurring genes in *E. coli*, one heterologous gene had been used. All genes were arranged in a synthetic operon which has been synthesized and partly codon-optimized for *E. coli*. The introduction of synthetic DNA excludes the unintended introduction of other genes or gene sequences of the donor organism to LU20297. Resistance markers used during strain development were removed effectively. The presence of intended and the absence of unintended DNA sequence changes was verified by whole genome sequencing of the production strain *E. coli* K12 LU20297. Such verification may be considered "gold standard" for the safety assessment of a bacterial strain used for fermentation of food ingredients.

6.3 Toxicological studies

6.3.1 Genotoxicity studies

6.3.1.1 Published genotoxicity studies

Coulet *et al.* (2014) conducted a bacterial reverse mutation assay (Ames test) on Glycom's 2'-FL (purity of 99%) using test strains TA98, TA100, TA1535, TA1537, and TA102 of *Salmonella typhimurium* in the presence or absence of metabolic activation (S9). The study adhered to Good Laboratory Practice principles (OECD, 1998a) and was conducted in accordance with OECD (1997a). Bacterial strains were incubated at concentrations of 52, 164, 512, 1,600, or 5,000 µg 2'-FL per plate when the plate incorporation method was used. In the pre-incubation method experiment, bacterial strains were incubated at concentrations of 492, 878, 1,568, 2,800, or 5,000 µg 2'-FL per plate. Water was used as the vehicle control. In assays conducted in the absence of S9, 2-nitrofluorene was used as the negative control for strain TA98, sodium azide was used as the negative control for strains TA 100 and TA 1535, 9-aminoacridine was used as the negative control for strain TA1537, and t-butyl hydroperoxide was used as the negative control for strain TA102. In assays conducted in the presence of S9, 2-aminoathracene was used as the positive control. There was no biologically significant increase in the number of revertant colonies in the treatment with 2'-FL compared with the negative control at any concentration either in the presence or absence of S9 in both the plate incorporation and the pre-incubation methods. There were increases in the number of revertant colonies in the treatments with positive control agents. There was no cytotoxicity or precipitation observed in any strain treated with 2'-FL in the presence or absence of S9. Thus, 2'-FL was determined to be non-mutagenic in the Ames test at concentrations up to 5,000 µg per plate.

Coulet *et al.* (2014) investigated the mutagenic potential of Glycom's 2'-FL (99% purity) in an in vitro mammalian cell gene mutation test in L5178Y tk+/-mouse lymphoma cells. The study was conducted in accordance with OECD Test Guideline 476 (OECD, 1997b) and adhered to Good Laboratory Practice principles (OECD, 1998a). Cells were incubated for 24 hours with 2'-FL at concentrations ranging from 1.7 to 5,000 µg per ml in the absence of S9. In a separate experiment, cells were treated for 4 hours with concentrations of 2'-FL ranging from 492 to 5,000 µg per ml in the absence or presence of S9. There was no evidence of precipitation or cytotoxicity at any dose of 2'-FL and there were no statistically or biologically significant increases in the frequency of mutations in cells treated with 2'-FL in the presence or absence of metabolic activation. The authors concluded that 2'-FL showed no mutagenicity at doses of up to 5,000 µg per ml under the conditions described.

The notifier of GRN 735 reported results from a bacterial mutagenicity study and a micronucleus test in cultured human lymphocyte which were published meanwhile (Berlo *et al.* 2018): In the Ames test no significant increase in mutations was observed in a single assay in four tester strains of *Salmonella* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *E. coli* (WP2 uvrA) in the absence or presence of an exogenous liver extract (S9) to provide metabolic activation with five concentrations of test material up to a concentration of 5,000 µg per plate. No toxicity was observed in any strain at any dose. The study with cultured binucleated human lymphocytes included one experiment with pulse treatment with and without metabolic activation, marginal cytotoxicity and no increase in the occurrence of micronuclei were observed at 3 concentrations (500, 1,000 and 2,000 µg per ml). In a second experiment with continuous treatment at the same concentrations without metabolic activation, marginal cytotoxicity and no increase in the occurrence of micronucleated cells was seen. The investigators concluded that 2'-FL did not exhibit any clastogenic or aneugenic activity in this cell system.

6.3.1.2 Unpublished genotoxicity studies

GRN 571

The notifier of GRN 571 investigated the mutagenicity of their 2'-FL at concentrations of up to 5,000 µg per plate that was tested in a bacterial reverse mutation test using *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535, and TA 1537 in the absence and presence of metabolic activation (S9). The plate incorporation and preincubation methods were used and the study was conducted in accordance with OECD Test guideline 471 (OECD, 1997a) and adhered to current Good Laboratory Practice. There were no signs of cytotoxicity, nor were there any increases in the numbers of revertant colonies in any of the five test strains with or without activation. Significant increases in the number of revertant colonies were observed for the positive controls. The authors concluded that the test material was not cytotoxic or mutagenic at doses of up to 5,000 µg per plate under the conditions of this study.

The notifier of GRN 571 conducted also a preliminary *in vivo* micronucleus test examining rat bone marrow cells in which doses of 500, 1,000, or 2,000 mg per kg bw of 2'-FL (from fermentation) were administered to one male and one female animal per dose. No systemic toxicity was observed; therefore, the same doses were used in the main study. In the main study, Jennewein's 2'-FL was administered by gavage to groups of 5 rats (Crl:CD(SD)) per sex (Jennewein, 2015) at doses of 500, 1,000, or 2,000 mg per kg bw per day. A vehicle control group received 0.8% aqueous hydroxypropylmethylcellulose and a positive control group was administered cyclophosphamide. The study was conducted in accordance with OECD Guideline 4 7 4. The rats were sacrificed at 24-or 48-hours post administration and bone marrow smears were evaluated by observing 2,000 erythrocytes per animal. There was no increase in the incidence of micronucleated polychromatic erythrocytes (PC Es) at any of the three tested dose levels of test material compared with the control. The positive control showed a significant increase in the number of micronuclei.

GRN 650

The notifier of GRN 650 investigated the possible mutagenicity of their 2'-FL produced by fermentation (HPLC purity=97.6%) in the *Salmonella* mutagenicity assay (Verspeek-Rip (2015), as quoted by GRN 650). As described in GRN 650, *Salmonella typhimurium* strains TA98, TA100, TA 1535, and TA 1537 and *E. coli* strain WP uvrA were exposed to 2'-FL in the presence or absence of metabolic activation. For the plate incorporation method, the doses of 2'-FL used were 52, 164, 512, 1 ,600, or 5,000 µg per plate and for the preincubation method, the doses used were 492, 878, 1,568, 2,800, or 5,000 µg per plate. No cytotoxicity or precipitation occurred at any dose in any bacterial strain in the presence or absence of metabolic activation and there was no biologically significant increase in the number of revertant colonies. It was concluded that test material was not cytotoxic or mutagenic under the conditions of this study.

The notifier of GRN 650 reported also the results of an in vitro micronucleus test conducted on concentrations of up to 2,000 µg per ml of 2'-FL manufactured by chemical synthesis (GRN 546). The study was conducted in accordance with OECD Guidelines 487 (OECD, 2014) and followed Good Laboratory Practice principles (OECD, 1998a). There was no significant increase in the number of micronucleated peripheral human lymphocytes in the presence of absence of metabolic activation (Verbaan (2015) , as quoted by GRN 650).

6.3.2 Repeated oral toxicity studies

6.3.2.1 Published toxicological studies in rats

Coulet *et al.* (2014) conducted a 14-day tolerability and dose-range finding study using 7-day-old [post-natal day (PND) 7] Wistar [Crl:Wl(Han)] rats. Five animals per sex per group were administered Glycom's 2'-FL (purity of 99%) by gavage at doses of 0 (vehicle control), 2,000, 5,000, or 7,500 mg per kg body weight (bw) per day. A reference control group was administered 7,500 mg oligofructose (OF) per kg bw per day during the 14-day study. Observations were conducted two times per day for general health, mortality, and morbidity, clinical observations were conducted once per day, and detailed clinical examinations were conducted once per week. Body weights were measured on post-natal days 1, 4, 7, 10, 14, 17, and 20. All animals were euthanized at the end of the 14-day administration period and macroscopic examinations were performed. One female in the 7,500 mg per kg bw per day group died on day 12, with no significant findings at necropsy. One female rat that was partially cannibalized on day 6 in the 7,500 mg per kg bw per day group had presented clinical signs and lost body weight on days 0 to 3. No compound-related macroscopic findings were observed at necropsy. The cause of death was undetermined. In the 7,500 mg per kg bw per day group, the OF control group, and to a lesser extent, the 5,000 mg per kg bw per day group, liquid and/or yellowish liquid feces were observed in some animals from days 1 to 3 up to days 9 to 11 and were occasionally observed in conjunction with erythema in the urogenital region. 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.

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:Wl(Han)] rats (Coulet *et al.*, 2014). The period of administration of Glycom's 2'-FL occurred within the window of time when immune and sexual maturity take place in rats. 2'-FL (purity of 99%) was administered at doses of 2,000 and 5,000 (n=10 animals per sex per dose group), and 6,000 mg per kg bw per day (n=15 animals per sex). The vehicle (water) control group (0 mg per kg bw of 2'-FL per day) consisted of 15 animals per sex. A reference control group was administered 6,000 mg per kg bw per day of OF (15 animals per sex). Standard diet (A04C-10) and water were provided ad libitum. Clinical observations were conducted once per day, observations were conducted twice per day for mortality and morbidity, and detailed clinical examinations were conducted once per week. Body weights were measured prior to dosing and twice weekly during the first 8 weeks of the study and then once per week for the remainder of the study. Food intake was measured twice weekly starting at week 2 until week 8, and then once per week for the remainder of the study. During the last week of administration, ophthalmological analyses were conducted on animals from the control group, the 6,000 mg per kg bw per day 2'-FL group, and the 6,000 mg per kg bw per day OF group. Hematology, coagulation, clinical chemistry, and urinary analyses were conducted at the end of the administration period. Twenty animals from each treatment group (10 rats per sex) were euthanized and necropsied. The remaining animals, 5 rats per sex per group in the vehicle control, the 6,000 mg per kg bw per day 2'-FL group, and the reference control group of OF were observed for 4 weeks, after which all animals were euthanized, necropsies were performed, and histopathological analyses were conducted on all organs and tissues. Kidneys of all females in the 2,000 and 5,000 mg per kg bw per day groups and in all recovery, groups were microscopically inspected. Clinical pathology was performed on all animals from all groups.

One male and one female rat in the 6,000 mg per kg bw per day 2'-FL dose group, two males and one female in the 6,000 mg per kg bw per day OF dose group died during the treatment period. One female in the 6,000 mg per kg bw per day OF group died during the recovery period. The authors stated that because there was no histopathological correlation to their deaths, they could not show a relationship to treatment. Diarrhea occurred occasionally for rats of both sexes in the 2,000 mg per kg bw per day dose group and for all animals in the 5,000 and 6,000 mg per kg bw per day of 2'-FL treatment groups and the OF treatment group. The authors noted that this effect was associated with erythema in rats that were treated with 6,000 mg per kg bw per day of 2'-FL and OF. Rats of both sexes also experienced hyper salivation. There were no significant differences in food consumption or terminal body weights between any test group and the control group during the treatment or the recovery period. No compound-related ophthalmological findings were reported. Occasional significant changes in hematological parameters in female rats were attributed to low grade chronic stress related to diarrhea. A significant increase in prothrombin time for males in the 6,000 mg per kg bw per day dose group was described as slight and unrelated to the test article. In clinical chemistry analyses, significant reductions in aspartate aminotransferase in rats of both sexes in the 6,000 mg per kg bw per day 2'-FL and OF dose group and in the 5,000 mg per kg bw per day dose groups were not considered by the authors to reflect an adverse event. Other changes were of low magnitude, typically remained within the range of historical control values, and occurred in a single sex. Urinalysis revealed a significant reduction in specific gravity that appeared to be dose-dependent; however, the authors described the magnitude of the change as too small to be considered toxicologically relevant.

There were statistically significant decreases in absolute adrenal weights in males in the 5,000 and 6,000 mg per kg bw per day groups, the relative adrenal weights of males in the 6,000 mg per kg bw per day group, and in absolute brain and relative kidney weights in females in the 6,000 mg per kg bw per day group. There were statistically significant increases in heart weights in males in the 5,000 mg per kg bw per day group. These differences in organ weights were not associated with histological changes, did not occur in both sexes, had resolved by the end of the 90-day treatment period, or also occurred in control group animals. Therefore, differences in organ weights were not considered to be toxicologically significant. The animals that died during the treatment period had reduced lymphoid follicle development of the spleen. At the end of the administration period, females in the 5,000 mg per kg bw per day and 6,000 mg per kg bw per day dose groups and in the OF group exhibited an elevated incidence of minimal cortical tubular epithelial cytoplasmic vacuolation of the kidneys in the absence of renal degeneration. Similar effects were observed in females in the control group at the end of the recovery period. The authors considered these effects to be non-adverse. Because an association between the treatment and the deaths of two animals in the 6,000 mg per kg bw per day dose group could not be excluded, the authors concluded that the no-observed-adverse-effect level (NOAEL) for 2'-FL was 5,000 mg per kg bw per day in Wistar [Crl:WI(Han)] rats.

The test material used in this study is sufficiently similar to the 2'-Fucosyllactose subject to the present notice as specified in Part 2.4 (Table 12).

The notifier of GRN 735 reported a 90-day dietary study (including a dose-range finding study) on the subject 2'-FL in Wistar outbred (Crl:WI(Han)) rats according to OECD guidelines (Appendices 9 and 10 of GRN 735) which has been published meanwhile (Berlo *et al.* 2018).

Doses of 2'-FL for the study were selected by conducting a 14-day range finding study in male rats. In the range finding study, 2'-FL was added at 0, 3, 6, or 10% of the diet. The doses of 2'-FL calculated from food consumption data in this study were 0, 2.56, 5.08, and 7.99 g per kg bw. No treatment related effects were seen on clinical signs, body weights, food consumption, and macroscopic examination. Organ weights were normal with two exceptions. Relative liver weights were decreased in the mid-and high-dose groups. Elevations of absolute and relative cecal weights were observed in the mid-and high dose groups. The investigators concluded that decreases in liver weights are not usually considered toxicologically significant and the increase in cecal weights were likely a physiological adaptation to the test material as studies on other poorly digestible and fermentable sugars showed similar effects and this effect was not considered to be adverse in those studies (WHO, 1987). Based on these results, a decision was made to use the same treatment levels of 2'-FL in the 90-day rat study.

The 90-day study was conducted with the same doses of 2'-FL in the same species, using groups of 10 rats of each sex per dose group. All animals survived to the end of the study with the exception of one mid-dose female whose death was considered not related to treatment. Sporadic and slight (<10% compared to control) increases in water consumption and decreases in food consumption at some measurement points during the study were not considered to be related to treatment. Based on weekly food consumption measurements, these dietary levels provided an overall mean intake of the test substance in the low-, mid-and high-dose groups of 2.17, 4.27, and 7.25 g per kg bw per day for males and 2.45, 5.22, and 7.76 g per kg bw per day for females, respectively. Analyses of homogeneity, content, and stability of the test substance in the test diets confirmed that the rats consumed the intended amounts of the test substance. Clinical signs were considered normal for all animals. Neurobehavioral observations and motor activity assessments in a functional observational battery did not indicate any neurotoxic potential of the test substance. Ophthalmoscopy did not reveal any treatment related ocular changes.

Hematological and clinical chemistry analyses were conducted on all rats at necropsy. There were no toxicologically significant changes in red blood cell variables or in total and differential white blood cell counts. The investigators discounted an increase in thrombocytes in high-dose females because it was slight and not seen in males. There were no treatment related changes in any clinical chemistry measurements. An increase in the urea concentration in mid-dose and high-dose males was considered a chance finding in the absence of this finding in females and any corroborative histopathological findings in males. No significant changes were seen in urinalysis measurements. The relative weight of the liver was slightly (approximately 8%), but statistically significantly increased in males in the high-dose group. This elevated relative liver weight was not accompanied by changes in clinical chemistry or microscopy of the liver and did not occur in females and was therefore not considered to be adverse.

Significantly increased cecal weights were seen in males at all doses and in mid-and high-dose males. Histopathology of the cecum was considered normal in all high dose animals. The investigators concluded that the effect on the cecum was due to physiological adaptation to the nature of the test materials being an indigestible carbohydrate. This effect has been well documented in the literature as an adaptive effect (WHO, 1987) and has been seen in studies with hydroxypropyl starches (Leegwater *et al.*, 1974), fructans (Demigne *et al.*, 2008) and cellulose and glucomannan (Oku, 1995). In a more recent rat study, several forms of dietary fiber were found to increase cecal weight and improve the histomorphology of the cecal lumen. The investigators concluded that increased crypt depth as a result of dietary supplementation of low-digestible carbohydrates is a beneficial morphological effect. The crypts contain intestinal stem cells, the principal site of cell proliferation in the intestinal mucosa, and increased depth is associated with increased rate of turnover of intestinal mucosa I cells (Knapp *et al.*, 2013).

Macroscopic examination at necropsy and microscopic examination of organs and tissues did not reveal treatment-related findings.

It was concluded that the subject 2'-FL did not induce any toxicologically relevant changes in any test group, and therefore, the authors of the study decided that the NOAEL was the highest level tested, i.e., 10% in the diet (~ 7.25 g per kg body weight per day).

6.3.2.2 Unpublished toxicological studies in rats

GRN 571

The notifier of GRN 571 describes a 90-day study according to OED TG 408 (limit test) in which a total of 40 male and female CD® rats (Crl:CD(SD)) were fed a standard rat diet (ssniff-R/M-H V1530) ad libitum (control) or the standard rat diet that was supplemented with 10% 2'-FL (10 rats per sex per dose group). 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. None of the animals died during the study. There were no differences in food consumption, body weight, or body weight gain in males or females in the treatment group compared with the control group. There were no differences between treatment and control groups in clinical signs, food consumption, body weight, behavior, appearance, hematology, clinical biochemistry, urinalysis, or ophthalmological examination. Intake of 2'-FL decreased during the study from 11 .54 g per kg per day to 5.25 g per kg per day in male rats (mean=7.66±2.21 g per kg per day) and from 12.07 g per kg per day to

5.78 g per kg per day in female rats. At necropsy, there were no differences in organ weights, gross pathology, or histopathology between the treatment group and the control group. The authors stated that histopathological effects were not treatment-related. 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 test material was safe at average doses of 7.66 grams per kg per day and 8.72 grams per kg per day (the NOAEL) in female and male rats, respectively.

GRN 650

The notifier of GRN 650 submitted a 90-day oral toxicity study with an additional 28 day recovery period in Wistar [Crl:WI(Han)J rats on their own 2'-FL. The study was conducted in accordance with OECD standard of Good Laboratory Practice. In the main study seven-day old neonatal Wistar rats were administered 2,000, 4,000, or 5,000 mg per kg bw of 2'-FL (produced by fermentation, purity 97.6%) or 5,000 mg per kg bw per 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 and were killed after the 90-day time period. One dam was then housed with a reconstituted litter of 5 pups per sex, fed a standard diet (A04C-10), 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. Rats that were treated with the mid-dose of 2'-FL also had soiled urogenital regions. Beginning on day 35 of the main part of the study, the rats treated with FOS or the mid-or high-doses of 2'-FL showed hypersalivation, abnormal foraging, and/or pedaling, but this effect was not observed during the recovery period.

There were no ophthalmological effects related to test article administration observed and there were no remarkable effects on body weight, body weight gain, or food consumption. There were no toxicologically relevant changes in tibia length, reflex and physical development, time to sexual maturation, learning capacity, memory, motor activity (Morris water maze, exploratory behavior, or general movement (open-field test). Small differences in hematological parameters were not considered to be toxicologically significant. There were some significant changes in serum chemistry parameters including reductions in triglyceride concentrations for the mid and high dose 2'-FL groups in comparison to the control and reference groups and reduced concentrations of cholesterol in all males given 2'-FL and in females that were give the mid and high doses of 2'-FL. These changes were small and or remained within the normal historical control data range and did not occur during the recovery period. As a result, the study investigators concluded that these effects were not adverse. No differences in urinalysis, organ weights, macroscopic or histological observations were attributable to treatment with 2'-FL. The authors determined a NOAEL of 5,000 mg per kg bw per day.

6.3.2.3 Published toxicological studies in piglets

Twenty-seven male and twenty-one female domestic farm piglets (Domestic Yorkshire Crossbred Swine) were employed in a 20-day oral toxicity study on 2'-FL (Hanlon and Thorsrud, 2014). The 2'-FL used in this study was manufactured via a fermentation process. The pigs were fed a liquid diet that included 0 (vehicle control), 200, 500, or 2,000 mg 2'-FL per L starting when they were two days old. The diets that included 2'-FL were equivalent to 29.37, 72.22 and 291.74 mg per kg per day 2'-FL, respectively, in males and 29.30, 74.31, and 298.99 mg per kg per day 2'-FL, respectively in females. The piglets were checked twice daily for signs of morbidity, mortality, and injury, clinical examinations were conducted two times per week, and blood samples were taken on study days 7 and 21 for clinical pathological examinations. Body weights were measured daily during the first week and every other day during the remainder of the study. The animals were sacrificed on Day 22. At necropsy, organ weights were measured, and histopathological examinations were conducted on the brain, heart, kidneys, large intestine (cecum, colon, rectum), liver, small intestine (duodenum, jejunum, ileum), spleen, eyes, gall bladder, stomach, lung with bronchi, mesenteric lymph nodes, pancreas, and Peyer's patches. Watery feces were observed in 5 animals in the 2,000 mg per dl dose group (3 males and 2 females), 3 animals (1 male and 2 females) in the 500 mg per L dosing group and 4 animals (2 males and 2 females) in the 200 mg per L dosing group. The authors stated that these results were not dose-related. A reduction in appetite was observed in one male and two females in the 2,000 mg per L dose group for one day and for one female in the 200 mg per L dose group for two days but was not dose related. Adverse effects were observed, including elevated alanine aminotransferase (ALT) levels in males in the 2,000 mg per dl dose group. However, because they were not considered to be dose-related and occurred in the absence of other related pathological effects, these adverse effects were not considered by the authors to be toxicologically significant. The authors reported that no adverse effects associated with the test material were observed on growth and development, clinical pathology, and histopathology at terminal necropsy. The authors concluded that the administration of 2'-FL in a milk replacement formula to neonatal piglets, from birth to age 3 weeks, at concentrations of up to 2,000 mg 2'-FL per L per day was well tolerated by piglets.

6.4 Other animal studies

The publications discussed in this section are not safety studies per se, but try to elucidate health effects of 2'-FL. However, it may be concluded that also these more explorative investigations do not indicate any adverse effects

Vazquez *et al.* (2015) studied the effect of 2'-FL on hippocampal long-term potentiation (L TP) and learning abilities in mice and rats. Chronic oral administration of 2'-FL resulted in mice and rats exhibited improved input/output curves and L TP in alert behaving animals. The improvement in L TP was associated with improved performance. Mice that were administered 2'-FL showed better performance than control animals in spatial learning, working memory, and operant conditioning as measured using the IntelliCage system. Similarly, improved performance was observed for rats when tested in the fixed-ratio schedule in the Skinner box. Exposure to 2'-FL was associated with greater expression of molecules associated with storing newly acquired memories.

Vazquez *et al.* (2016) reported that addition of 0.625% (w/w) of 2'-FL but not 0.21 % (w/w) of fucose to the diet of male Sprague Dawley adult rats improves operant conditioning and LTP associative learning related skills. These doses provided 350 mg of 2'-FL per kg bw per day. The authors stated this amount was similar to the dose found in breastmilk. The doses of 2'-FL and L-fucose were equimolar. The effect on 2'-FL on L TP was inhibited following vagotomy.

Oliveros *et al.* (2016) investigated whether oral supplementation with 2'-FL during the lactation period affects memory and learning in rats. Rat pups were orally administered 2'-FL or water during the lactation period and were then fed a standard diet and were assessed at weaning age 4-6 weeks and at age one year. At age 4 to 6 weeks, the behavior of rats in both groups was similar in the Morris Water Maze; however, the authors stated that the rats that were fed 2'-FL appeared to do slightly better in the test. When tested at one year of age, rats that were treated with 2'-FL exhibited better performance in the Novel Object Recognition and Y Maze paradigms compared with controls and had longer and more intense L TP in young and adult rats.

Castillo-Courtade *et al.* (2015) showed that following an oral ovalbumin challenge in sensitized 8-to 9-month-old male Balb/c mice, daily oral administration of 2'-FL or sialyllactose reduced food allergy symptoms, such and diarrhea and hypothermia. In addition, there was a suppression of antigen-induced increases in mouse mast cell protease-1 in serum and mast cell numbers in the intestine, and increased CD4(+), CD25(+), and I L-10(+) in cells in the Pe ye r's patches and the mesenteric lymph nodes. Treatment with 6'-siallylactose was associated with elevated concentrations of IL-10 and reduced TNF production. While both HMOs reduced the passive cutaneous anaphylaxis response only 6'-sialyllactose directly inhibited mast cell degranulation in vitro, at high concentrations.

6.5 *In vitro* studies

He *et al.* (2016) reported that 2'-FL reduces inflammation mediated by lipopolysaccharide in human enterocytes by decreasing the induction of CD14.

6.6 Human data and studies

6.6.1 Biological function of human milk oligosaccharides and 2'-FL

Andreas *et al.* (2016) reviewed the composition and bioactivity of human breast milk. Though human milk oligosaccharides (HMO) are make up a significant fraction of breast milk carbohydrate, they are indigestible by the infant, their function instead is to nourish the gastrointestinal microbiota. HMO function as prebiotics, encouraging the growth of certain strains of beneficial bacteria, such as *Bifidobacterium infantis*, within the infant gastrointestinal tract, protecting the infant from colonisation by pathogenic bacteria. They play an important role in preventing neonatal diarrhoeal and respiratory tract infections. The excretion of HMO into mother milk is genetically determined, different profiles of milk oligosaccharide occur as a result of specific enzymes expressed in the lactocytes. Two such genes, important for determining the HMO profile a mother produces, are the Secretor (Se) and Lewis blood group (Le) genes. The Secretor gene encodes for the enzyme α[1,2]-fucosyl-transferase (FUT2), responsible for linking fucose in a α1-2 linkage to elongate the HMO chain. The enzyme FUT3 is encoded for by the Lewis blood group gene; this enzyme catalyses the reaction between fucose in a α1-3/4 linkage, creating further fucosylated oligosaccharides. As a result of the different expressions of these enzymes, there are four main phenotypes in relation to HMO profile: Se+/Le+, Se−/Le+, Se+/Le− and Se−/Le−.

Practico *et al.* (2014) investigated breast milk HMO profiles and demonstrated that Se+/Le+ mothers produced all types of fucosylated oligosaccharides, whilst Se−/Le+ mothers did not produce α1,2-fucosylated structures, such as 2'-Fucosyllactose. Se+/Le− mothers secreted α1,2- and α1,3-fucosylated oligosaccharides, but not HMO containing α1,4-fucose residues. They noted that in Se−/Le+ mothers, α1,3-fucosylated oligosaccharides, such as 3'-Fucosyllactose, were between two and fivefold higher than in Se+/Le+ mother's breastmilk. This suggested that there is an increase in FucT3 activity in non-secretor mothers, meaning that the total oligosaccharide production is relatively equal between the different groups.

Castaniz-Muñoz *et al.* (2013) focussed specifically on the attributes of 2'-FL in terms of its occurrence in mammalian phylogeny, its postulated biological activities, and its variability in human milk. The authors emphasized the ubiquitous presence of 2'-FL in many mammalian species, the notable absence in some taxa (e.g. cows), and the variability in the human population.

In a recently published "Consensus statement" on definition and scope of prebiotic a panel convened by the International Scientific Association for Probiotics and Prebiotics (ISAPP) commented on human milk oligosaccharides as follows (Gibson *et al.* (2017)):

> Among the first group of substances recognized for their ability to influence gastrointestinal health were the oligosaccharides present in human milk. Human milk oligosaccharides (HMOs)

are particularly important for the development of the newborn baby's intestinal microbiota and metabolic and immunological systems, which have consequences for health later in life. Consumption of mother's milk containing these HMOs clearly increases the proportion of HMO-consuming *Bifidobacteriaceae* and *Bacteroidaceae. Bifidobacterium longum* subsp. *infantis* (*B. infantis*) is the only *Bifidobacterium* spp. That has specifically evolved machinery to degrade the complete repertoire of HMOs. Other *Bifidobacterium* spp. predominant in adults, mainly *B. longum* subsp. *longum*, B. *adolescentis* and *B. lactis*, lack many of the enzymes necessary to directly utilize HMOs effectively.

HMOs might indirectly affect composition of the intestinal microbiota by modulating immune responses and also have metabolism-independent mechanisms of action in the infant gut40. In particular, fucosylated and sialylated HMOs can prevent adhesion of pathogens to the intestinal epithelium through a competitive mechanism that ultimately protects the neonate from infection.

The expert panel also discussed whether HMOs including 2'-FL when added as purified substances to food would also exert a prebiotic effect. Based on available clinical studies where 2'-FL had been used alone or in combination with galactooligosaccharides or lacto-N-neotetraose, they agreed that that some HMOs are candidate prebiotics (in infants and adults).

Other reviews that discuss the available evidence on the functional biology of 2'-Fucosyllactose and other human milk oligosaccharides, their importance for the new-born child, the development of its intestinal functions, and general health are available (Smilowitz *et al.* (2014); Bode (2015); Moukarzel & Bode (2017)).

6.6.2 Absorption, Distribution, Metabolism and Excretion of 2'-FL

The notifier for GRN 735 reviewed the present knowledge on metabolism of human milk oligosaccharides which are considered to be "dietary fiber" because they are poorly absorbed by the human gut (Engfer *et al.* (2000); Gnoth *et al.* (2000)). This was demonstrated in a study that used semi-quantitative methodology (Chaturvedi et al., 2001 b). As a non-digestible sugar, 2'-FL is available to act as a carbon source (a prebiotic) for commensal organisms in the lower intestine. Specifically, 2'-FL supports the growth of a variety of beneficial bacteria in vitro, including *Bifidobacteria* and *Bacteroides* species (Newburg (2009)).

2'-FL undergoes partial fermentation in the colon when infants are given a load of HMOs (a purified oligosaccharide fraction from mothers' milk) (Brand Miller *et al.* (1995); Brand-Miller *et al.* (1998)).

Despite the fact that HMOs are non-digestible, it has been shown that some HMOs can be absorbed intact and enter the circulation (Goehring *et al.* (2014), Chaturvedi *et al.* (2001b)) compared the profile of HMOs in the feces and the urine of infants that were fed mothers' milk to the profile in the feces and urine of infants that were fed formula. They reported that oligosaccharide concentrations in the feces were higher than those in mothers' milk and much higher than that in urine. According to Goehring *et al.* (2014), 2'-FL and other human milk oligosaccharides have been identified in the urine and plasma of breast-fed infants at levels that correspond to the amounts in human milk, but not formula-fed infants. They also reported that 2'-FL was not present in the circulation of infants who consumed breast milk that did not contain 2'-FL. Coppa *et al.* (2001) reported that 40-50% of HMO is present in the feces of breast-fed infants.

Multiple studies have shown that administration of a bolus of ^{13}C-galactose or ^{13}C-glucose to lactating mothers results in the presence of 13C-HMO in the urine of infants (Dotz *et al.* (2015), Obermeier *et al.* (1999), Rudloff and Kunz (2012)). Gnoth *et al.* (2001) showed that in vitro transport of neutral HMOs such as 2'-FL across the intestinal epithelium occurs via receptor-mediated transcytosis and paracellular pathways (Vazquez *et al.* (2017)).

Altogether, studies show that a minor portion of HMOs (and 2'-FL) is absorbed into the circulation, whereas the majority of HMOs (and 2'-FL) are not absorbed and function as a substrate for the growth of the intestinal microbiota.

6.6.3 Clinical studies

Marriage *et al.* (2015) conducted a prospective, randomized, controlled, growth and tolerance study in healthy, singleton infants. Over a period of 4 months, infants received either human breastmilk, a control formula containing 2.4 g per L GOS or one of the test formula containing either 0.2 g per L of 2'-FL and 2.2 g per L of GOS, or 1.0 g per L 2'-FL and 1.4 g per L of GOS. Study participants were enrolled in the study by age 5 days. There were no significant differences among any groups for weight, length, or head circumference growth during the 4-month study period. All of the formulas were well tolerated and comparable for average stool consistency, number of stools per day, and percent of feedings associated with spitting up or vomit. 2'-FL was present in the plasma and urine of infants fed 2'-FL, and there were no significant differences in 2'-FL uptake relative to the concentration fed. The authors concluded that infants fed 2'-FL-fortified formulas with a caloric density similar to human milk grew and had 2'-FL uptake were similar to the control infants receiving human milk only.

Goehring *et al.* (2016) conducted a substudy that was nested in the randomized controlled growth and tolerance trial undertaken by Marriage *et al* (2015). The objective was to investigate the effect of supplementation of infant formula with 2'-FL on markers of immune functions. . Healthy singleton infants were enrolled in the study starting on 5 days old exclusively fed formula (n=317) or breastmilk (n=107) through age four months. Feeding infants formula to which 2'-FL had been added resulted in reduced plasma inflammatory cytokines profiles that resembled the inflammatory profiles of infants who were breastfed whereas the levels in infants fed the control formula were higher.

Puccio *et al.* (2017) performed a double-blind, randomized, controlled clinical trial including 175 healthy, full term infants. Between day of life 0-14, the infants were - randomly assigned to receive formula containing a combination of 2'-FL and lacto-N-neotetraose (LNnT) (n=88, 1.0grams per L of 2'-FL and 0.5grams per L of LNnT for reconstituted formula) or formula that did not contain oligosaccharides (n'=86) for up to 6 months. There was no inferiority of the weight gain of infants receiving 2'-FL and LNnT compared with those consuming the control formula until they were 4 months old. The mean weight, length, head circumference, and body mass index (BMI) for infants through age 4 months compared well with the WHO standard growth curves. The data obtained on stool endpoints and the altered composition of the microbiota in infants that were given the oligosaccharide mixture gave no cause for concern about the safety of the mixture. Infants that were fed with 2'-FL and LNnT had significantly lower incidences of bronchitis than infants in the control group [odds ratio (OR)= 0.30; 95 % Cl 0.1 1-0.73; p = 0.004]. In addition, infants receiving the test formula with 2'-FL and LNnT had significantly lower antibiotic use than infants in the control group (25.0 % vs. 41.4 %; OR= 0.47; 95 % Cl 0.23-0.94, p = 0.025). There were no significant differences in adverse effects between test and control groups. The dose of 2'-FL in infant formula in the study was approximately half of the maximum proposed level. This concentration of 2'-FL would be equivalent to 1.27 grams per day and 209 mg per kg bw per day at the 95th percentile for an infant who is 3 months old and weighs 6 kg.

Elison *et al.* (2016) performed a placebo-controlled, double-blind, parallel, dose-response trial in 100 healthy adults (49 women and 51 men). Study participants were randomly assigned to one of 10 treatment groups (n=10 per group) in which they consumed single doses of 5, 10, or 20 grams per day of 2'-FL or LNnT alone; 5, 10, or 20 grams per day of a combination of 2'-FL and LNnT (with 2'-FL a~d LNnT in a 2:1 ratio), or glucose (placebo) each day for two weeks. In comparison with the placebo group, study participants who consumed 20 grams per d of 2'-FL experienced increased incidences of nausea, rumbling, bloating, passing gas, diarrhea, loose stools, and urgency after two weeks. No significant increases in the incidences of these effects were reported in the 5 and 10 grams per day 2'-FL dose groups compared with the placebo group; however, for the 10 grams per day and 20 grams per day groups, an increased incidence of passing gas was noted. No significant differences in stool consistency were noted between placebo and intervention groups. Seventy-eight symptoms were reported by 44 study participants. Gas/flatulence, stomach pain, and diarrhea and rumbling were reported most frequently; however, the adverse events were described as mild and no serious adverse events were noted.

Sprenger *et al.* (2017a) used logistic regression models to explore the relationship between the concentration of FUT dependent oligosaccharides in breastmilk and the risk of developing allergies at ages 2 and 5 years. To do so, data from the placebo group in a randomized, placebo-controlled study on prebiotics and probiotics were used. It was found that infants who are delivered by C-section and who have a high hereditary risk of allergy potentially have a reduced risk of IgE associated eczema at age two years but not age 5 years when fed breastmilk containing FUT2-dependent oligosaccharide.

Sprenger *et al.* (2017b) studied the relationships between FUT2 status, the concentration of major HMOs in breastmilk, and infant growth through 4 months of age in an open observatory, single center, longitudinal cohort study. Breastmilk was collected at 30, 60, and 120 days postpartum from 50 mothers, who gave birth to 25 female and 25 male singleton infants. 2'-FL concentration in breastmilk decreased over time. Mothers were placed into two categories: low 2'-FL, (mean 27 mg per L, 95% confidence interval of mean 12-42 mg per L) and high 2'-FL (mean 2,170 mg per L, 95% confidence interval of mean 1,880-2,460 mg per L). Individuals who had low concentrations of 2'-FL in their milk had lacto-N-tetraose (LNT) as the major HMO. The variation in HMOs for high and low clusters of 2'-FL showed no effect on infants of either sex for body length, body weight, BMI, and head circumference.

Steenhout *et al.* (2016) (abstract) analyzed microbiota of stool samples from healthy infants at 3 months of age who had been fed either a cow's milk-based infant formula (control, n=87) or the same formula with 1.0 g per L 2'-FL and 0.5 g per L LNnT (Test, n= 88) or were breastfed (reference group, n=38). They reported that supplementation of formula with 2'-FL and LNnT moves the microbiota and metabolic signature in stool closer to that of breastfed infants with respect to composition and function.

Lewis *et al.* (2015) compared the effects of feeding breastmilk from non-secretor mothers (milk lacking 2'-FL) with the effect of feeding breastmilk from secretor mothers (milk containing 2'-FL) on establishment of *Bifidobacteria*-rich microbiota in infants on days 6, 21, 71, and 120 of life. Infants who were fed breastmilk from non-secreter mothers showed delayed establishment of *Bifidobacteria*-rich microbiota compared with infants who were fed breastmilk from secreter mothers.

6.7 Summary and conclusions

Substantial equivalency

The 2'-Fucosyllactose described in this GRAS notification (Table 12; Annex I) is of comparable composition with respect to its main constituent (2'-FL), the minor mono-, di- and oligosaccharides, and the specified impurities as other 2'-fucosyllactoses which are authorized or recognized as GRAS (Table 13). In total five 2'-fucosyllactoses have been notified to FDA with the conclusion of being GRAS, which were not objected by FDA. BASF's 2'-FL complies also with the European Union's generic specifications for 2'-Fucosyllactose obtained from the source "genetically modified strain of *Escherichia coli* K-12". The product is therefore substantially equivalent, and the biological data obtained for 2'-fucosyllactoses from other sources apply to BASF's product.

Genotoxicity (6.3.1)

2'-FL from four different sources have been investigated in bacterial reverse mutation tests and found to be not-mutagenic under the conditions of the tests. Further tests were performed *in vitro* (two micronucleus tests, one mammalian cell gene mutation test) and *in vivo* (one micronucleus test) with negative results. 2'-FL was shown not to be mutagenic in prokaryotic and eukaryotic cells in published and generally available studies, a conclusion that applies also to the notifier's 2'-FL.

Oral toxicity (6.3.2)

2'-FL from four different sources have been examined in 90-day repeated oral toxicity studies. No treatment related toxicity was observed in any of the four (published and unpublished) studies, the no observed adverse effect levels was in each study the highest administrated dose (between 5,000 and 8,720 mg/kg per day). 2'-FL is not toxic when administered to rats at high levels up to 8,720 mg/kg per day, a conclusion that applies also to the notifier's 2'-FL.

In one study administration of 2'-FL in a milk replacement formula to neonatal piglets, from birth to age 3 weeks, at concentrations of up to 2,000 mg 2'-FL per L per day was well tolerated, a finding that applies also to the notifier's 2'-FL.

Metabolism and clinical studies (6.6)

A significant number of studies in new-borns, infants, and adults investigated the biological effects of adding 2'-FL to the diet. In case of infants of breast-feeding age this included comparison of administration of 2'-FL-fortified formula with human breast-milk containing naturally 2'-FL. No adverse effects were noted, added 2'-FL was generally well tolerated, being in infants comparable to naturally fed 2'-FL. This conclusion for 2'-fucosyllactose from different sources applies also to the notifier's 2'-FL.

Other data (6.4, 6.5)

2'-FL was shown to affect positively several parameters in developmental animal models.

Conclusion

We have reviewed the publicly available safety and clinical studies and conclude that they support the safety of the proposed uses of the notifier's 2'-FL for infants, toddlers and adults. This conclusion is supported by corroborative studies discussed by previous GRAS notices on 2'-FL. In addition, the estimates (see Part 5) that infants consuming breast milk may receive 600 mg 2'-FL per kg bw per day or more affirms the safety of the proposed use in infant formula where the highest exposure estimated is in the consumer-only population group of infants aged 0 to 5 months (mean of 315 mg per kg bw per day, 90th percentile, 532 mg per kg bw per day, respectively). In addition, we conclude that the proposed use in foods would be well tolerated and safe.

6.8 Statement in accordance with 21 CFR 170.250 (c) (2)

We, that is our experts specialized in chemistry, molecular biology, toxicology, and human nutrition, have reviewed the available data and information and we are not aware of any data and information that are, or may appear to be, inconsistent with our conclusion of GRAS status for 2'-fucosyllactose.

7 Data and information used in the notice

As stated in Part 6, the data and information used in this GRAS notice are listed separately according to those documents which are generally available and those which are not. Documents that are generally available are listed below in Section 7.1 using standard bibliographic citations.

Those documents that are not generally available are listed in Section 7.2 using the respective names of the Appendices.

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Annexes

Annex I

NMR Spectroscopy

Qualitätsmanagement und -kontrolle EN

BASF We create chemistry

Elektronische Rohdaten

Auftragsnummer: 18P00477

NMR-Spektroskopie / Labor NMR / Box LQ

Probenbezeichnung:	2-Fucosyl Lactose 012644-L 06 Turmaustrag AM-08-12-B1 H.st.05.06.2018
Bestimmung:	Quantitative NMR, min. 94.0% (+quantitation of impurities)
EDV-System:	ScanFreigabeVirtuell Version: 1.0.3.0

Erstellt von: Fr. Birgit Faath

Erstellt am: 02.07.2018

Saum, Stephan
G-ENH/MT F31

BASF
We create chemistry

Auftrag : 18P00477.1 3393200037
Probe : 2-Fucosyl Lactose 012644-L 06 Turmaustrag AM-08-12-B1 H.st.05.06.2018

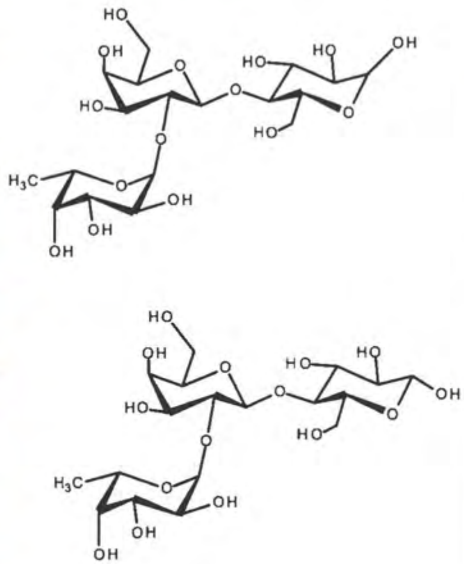
Instrument : AV401 2018Yjn18055

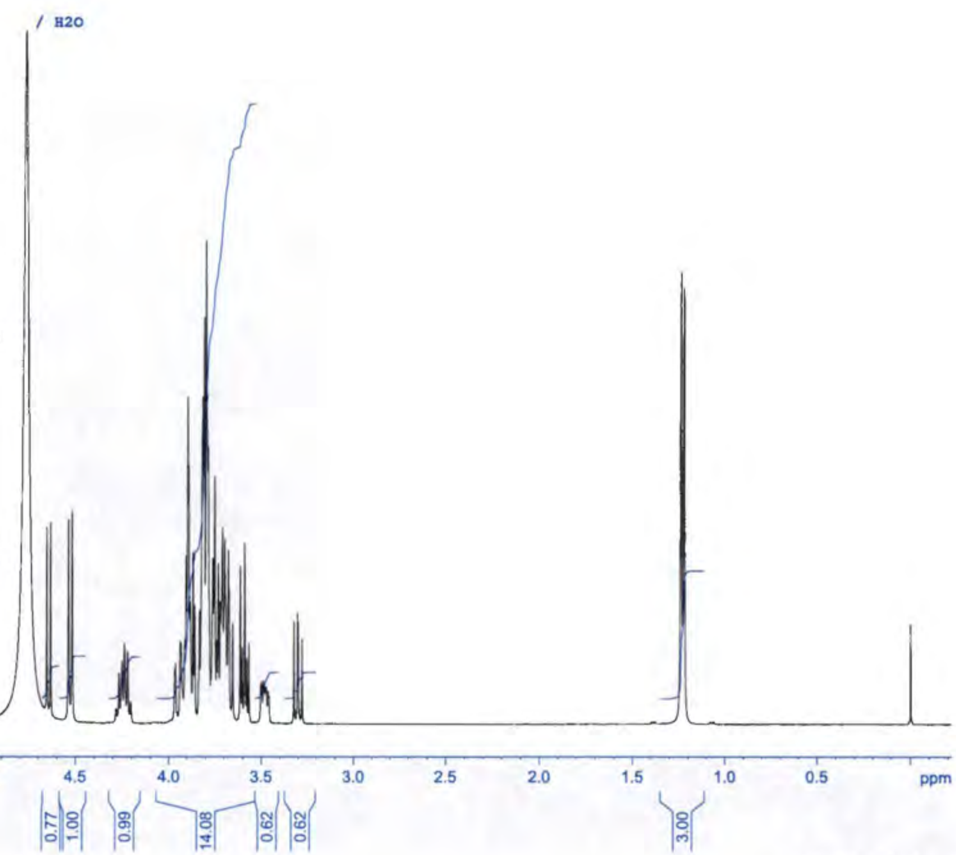
overview

```
Current Data Parameters
NAME          18P00477.1
EXPNO                 10
PROCNO                 1

F2 - Acquisition Parameters
Date_           20180619
Time               17.22
INSTRUM          AV401_a
PROBHD    5 mm PATBO BB-
PULPROG             zg30
TD                 65536
SOLVENT              D2O
NS                    16
DS                     2
SWH            8802.817 Hz
FIDRES         0.134320 Hz
AQ            3.7224448 sec
RG                  50.8
DW                56.800 usec
DE                 15.66 usec
TE                 298.2 K
D1            1.00000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1         400.3320017 MHz
NUC1                  1H
P1                 13.50 usec
PLW1         32.00000000 W

F2 - Processing parameters
SI                 65536
SF           400.3299680 MHz
WDW                   EM
SSB        0
LB                  0.10 Hz
GB         0
PC                  1.00
SR                -32.00 Hz
```

edited by: B.Faath
Phone : +49-621-6045338

isomeric mixture





Freigabe Daten: 02.07.2018 / eU ID: 2027235 / Fr. Birgit Faath

Saum, Stephan
G-ENH/MT F31

BASF
We create chemistry

Auftrag : 18P00477.1 3393200037
Probe : 2-Fucosyl Lactose 012644-L 06 Turmaustrag AM-08-12-B1 H.st.05.06.2018

Instrument : AV401 2018Yjn18055

```
Current Data Parameters
NAME          18P00477.1
EXPNO                 10
PROCNO                 1

F2 - Acquisition Parameters
Date_          20180619
Time              17.22
INSTRUM        AV401_a
PROBHD   5 mm PATBO BB-
PULPROG            zg30
TD                65536
SOLVENT             D2O
NS                   16
DS                    2
SWH            8802.817 Hz
FIDRES         0.134320 Hz
AQ            3.7224448 sec
RG                 50.8
DW               56.800 usec
DE                15.66 usec
TE                298.2 K
D1           1.00000000 sec
TD0                   1

======== CHANNEL f1 ========
SFO1        400.3320017 MHz
NUC1                 1H
P1                13.50 usec
PLW1        32.00000000 W

F2 - Processing parameters
SI                65536
SF          400.3299680 MHz
WDW                  EM
SSB      0
LB                 0.10 Hz
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PC                 1.00
SR               -32.00 Hz
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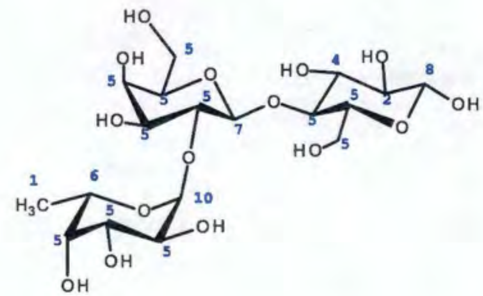
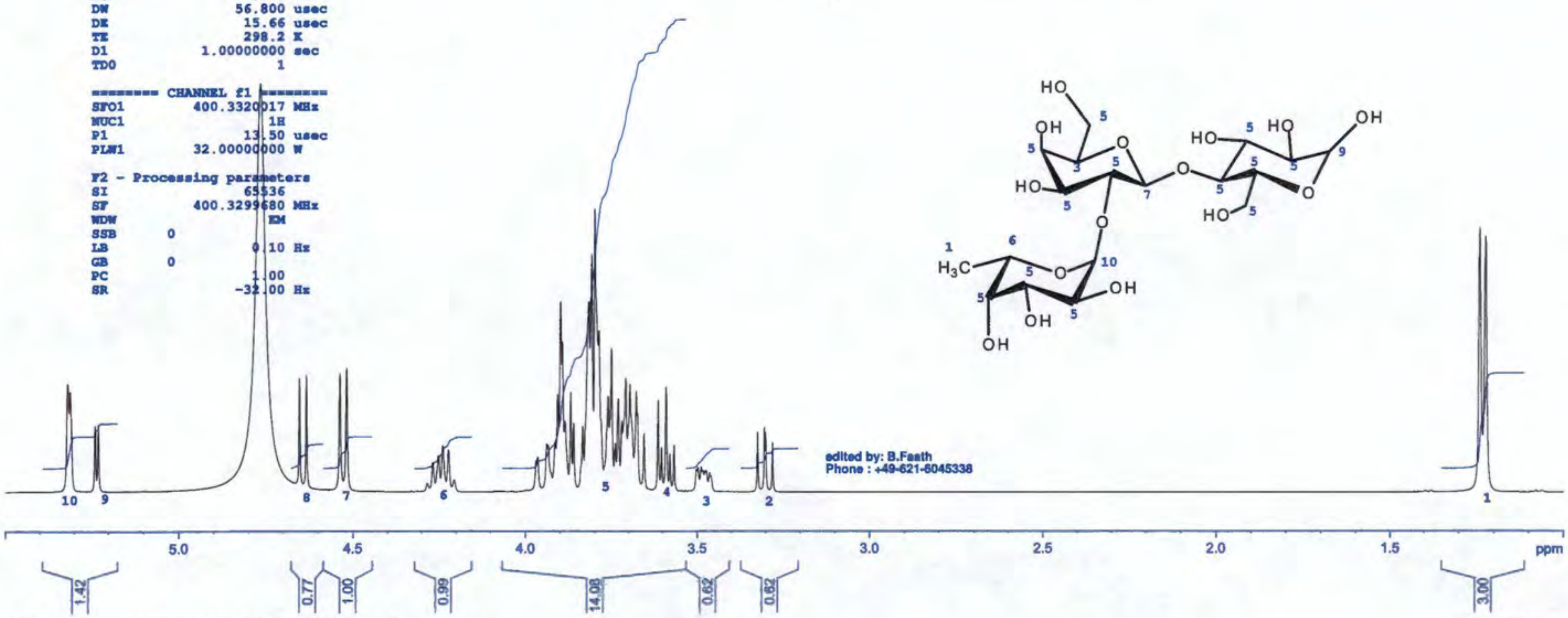
enlargement





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A-4

2-Fucosyllactose

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25.03.2019

Saum, Stephan
G-ENH/MT F31

Auftrag : 18P00477.1 3393200037
Probe : 2-Fucosyl Lactose 012644-L 06 Turmaustrag AM-08-12-B1 H.st.05.06.2018

Instrument : AV401 2018Yjn18055

Current Data Parameters
NAME 18P00477.1
EXPNO 11
PROCNO 1

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Time 18.14
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PULPROG zgpg30
TD 131072
SOLVENT D2O
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DS 2
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FIDRES 0.183399 Hz
AQ 2.7262976 sec
RG 203
DW 20.800 usec
DE 15.00 usec
TE 298.2 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1

======== CHANNEL f1 ========
SFO1 100.6731253 MHz
NUC1 13C
P1 9.00 usec
PLW1 62.00000000 W

======== CHANNEL f2 ========
SFO2 400.3332026 MHz
NUC2 1H
CPDPRG[2 waltz16
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PLW2 32.00000000 W
PLW12 0.76880002 W
PLW13 0.62273002 W

F2 - Processing parameters
SI 65536
SF 100.6629688 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.00
SR -90.15 Hz

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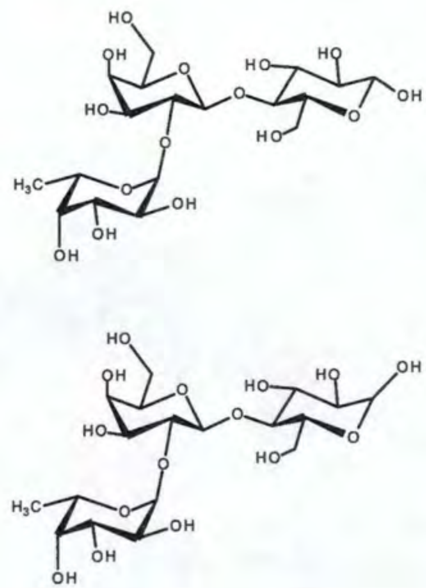
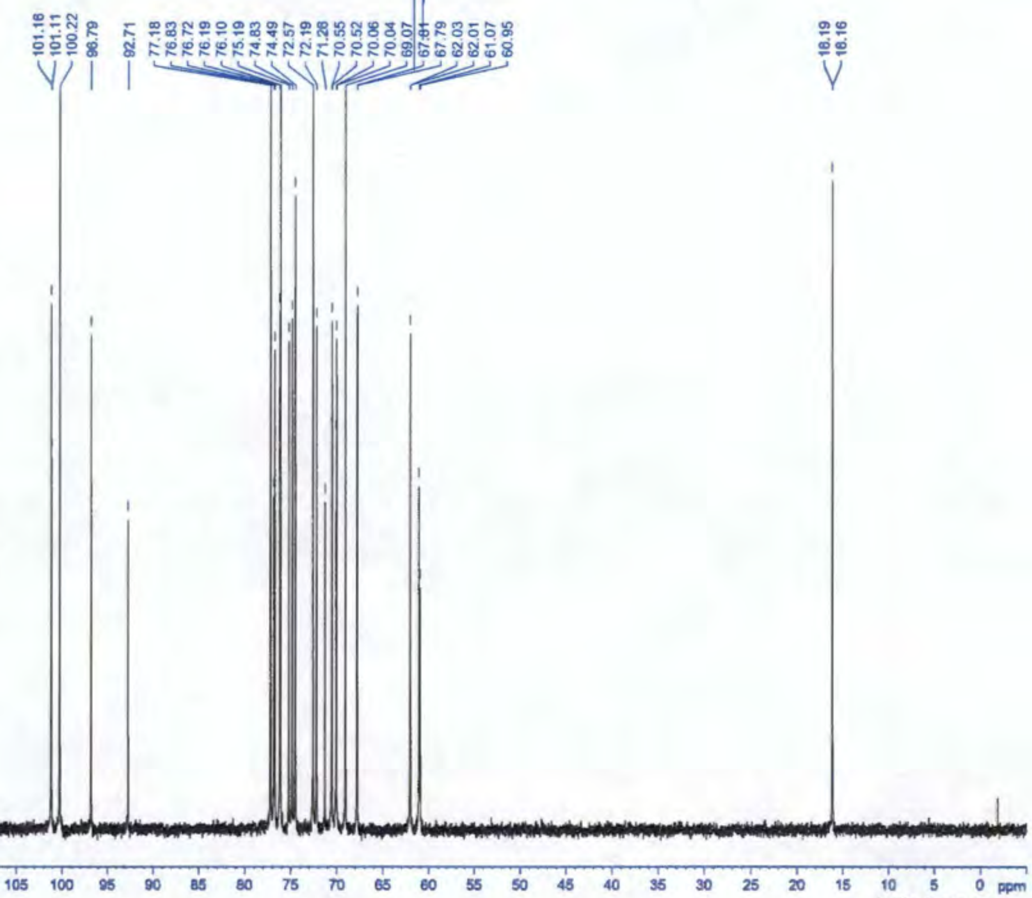
Phone : +49-621-6045338

overview

isomeric mixture



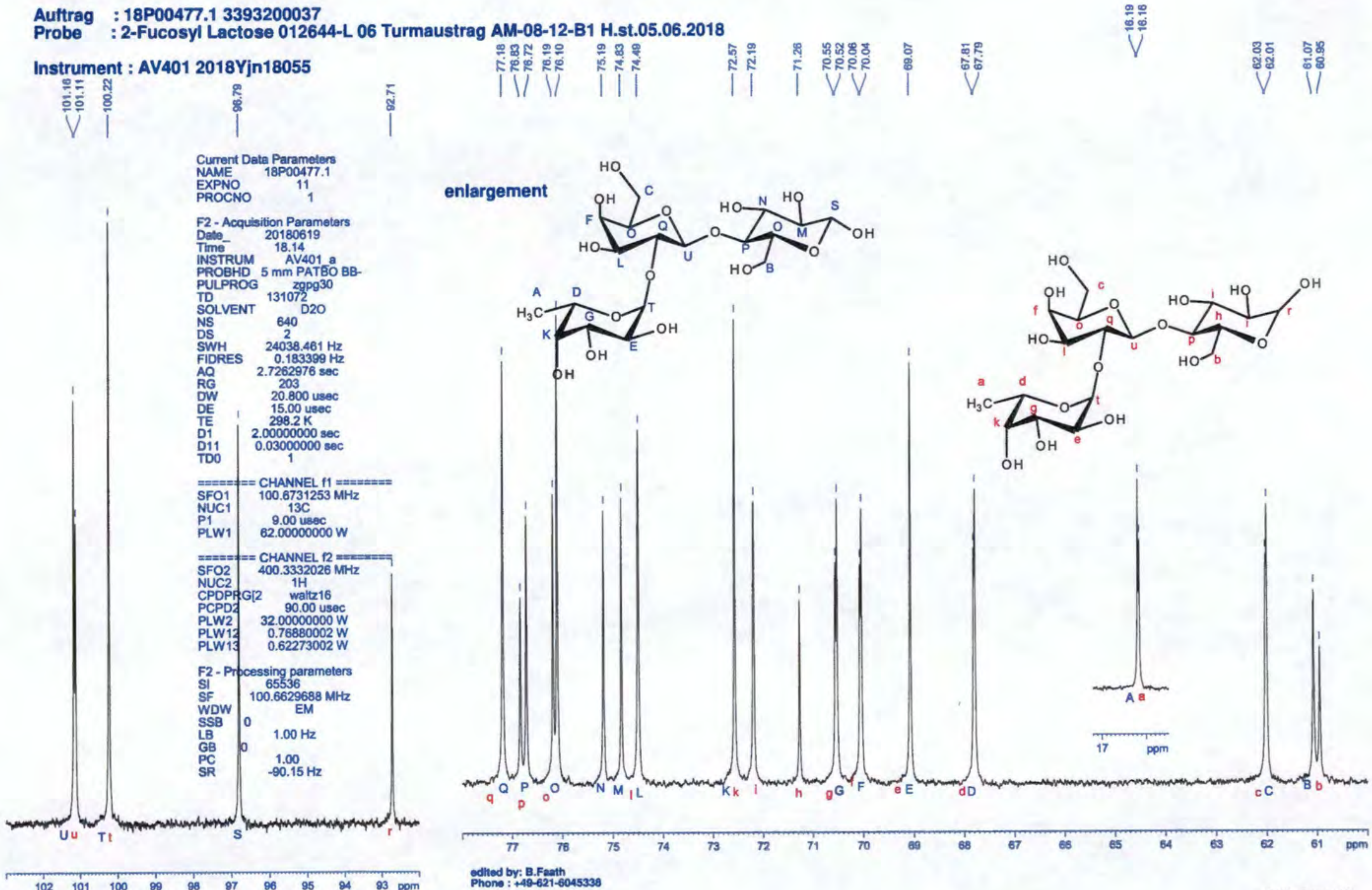


Saum, Stephan
G-ENH/MT F31

BASF
We create chemistry

Auftrag : 18P00477.1 3393200037
Probe : 2-Fucosyl Lactose 012644-L 06 Turmaustrag AM-08-12-B1 H.st.05.06.2018

Instrument : AV401 2018Yjn18055



edited by: B.Faath
Phone : +49-621-6045338

Freigabe Daten: 02.07.2018 / eU ID: 2027235 / Fr. Birgit Faath

Saum, Stephan
G-ENH/MT F31

Auftrag : 18P00477.1 3393200037
Probe : 2-Fucosyl Lactose 012644-L 06 Turmaustrag AM-08-12-B1 H.st.05.06.2018

Instrument : AV401 2018Yjn18055

BASF
We create chemistry

Current Data Parameters
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EXPNO 13
PROCNO 1

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NS 8
DS 16
SWH 8802.817 Hz
FIDRES 2.149125 Hz
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RG 203
DW 56.800 usec
DE 6.50 usec
TE 298.4 K
CNST2 144.0000000
CNST17 -0.5000000
D0 0.00000300 sec
D1 1.00000000 sec
D4 0.00173611 sec
D11 0.03000000 sec
D16 0.00020000 sec
D21 0.00345000 sec
D24 0.00089000 sec
IN0 0.00002480 sec

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NUC1 1H
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P2 25.88 usec
P28 1000.00 usec
PLW1 32.00000000 W

======== CHANNEL f2 ========
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NUC2 13C
CPDPRG[2 garp

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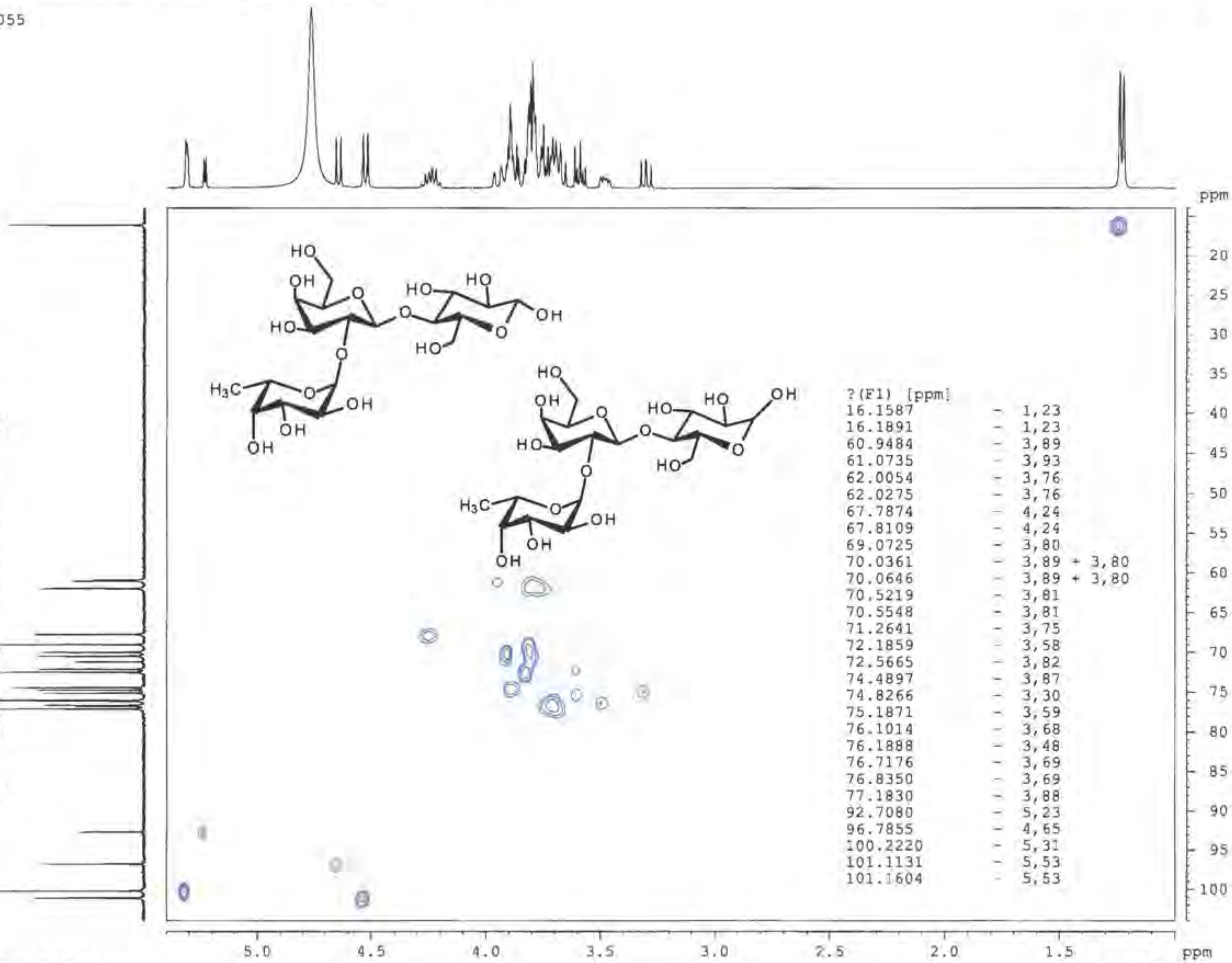

A-7

2'-Fucosyllactose

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25.03.2019

Saum, Stephan
G-ENH/MT F31

Auftrag : 18P00477.1 3393200037
Probe : 2-Fucosyl Lactose 012644-L 06 Turmaustrag AM-08-12-B1 H.st.05.06.2018

Instrument : AV401 2018Yjn18055

BASF We create chemistry

Current Data Parameters
NAME 18P00477.1
EXPNO 14
PROCNO 1

F2 - Acquisition Parameters
Date_ 20180619
Time 19.09
INSTRUM AV401_a
PROBHD 5 mm PATBO BB-
PULPROG hmbcgpndqf
TD 4096
SOLVENT D2O
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DS 16
SWH 8802.817 Hz
FIDRES 2.149125 Hz
AQ 0.2326528 sec
RG 203
DW 56.800 usec
DE 6.50 usec
TE 298.2 K
CNST13 8.0000000
D0 0.00000300 sec
D1 1.00000000 sec
D6 0.06250000 sec
D16 0.00020000 sec
IN0 0.00002240 sec

======== CHANNEL f1 ========
SFO1 400.3319016 MHz
NUC1 1H
P1 12.90 usec
P2 25.80 usec
PLW1 32.00000000 W

======== CHANNEL f2 ========
SFO2 100.6731253 MHz
NUC2 13C
P3 9.00 usec
PLW2 62.00000000 W

====== GRADIENT CHANNEL =====
GPNAM[1] SMSQ10.100
GPNAM[2] SMSQ10.100

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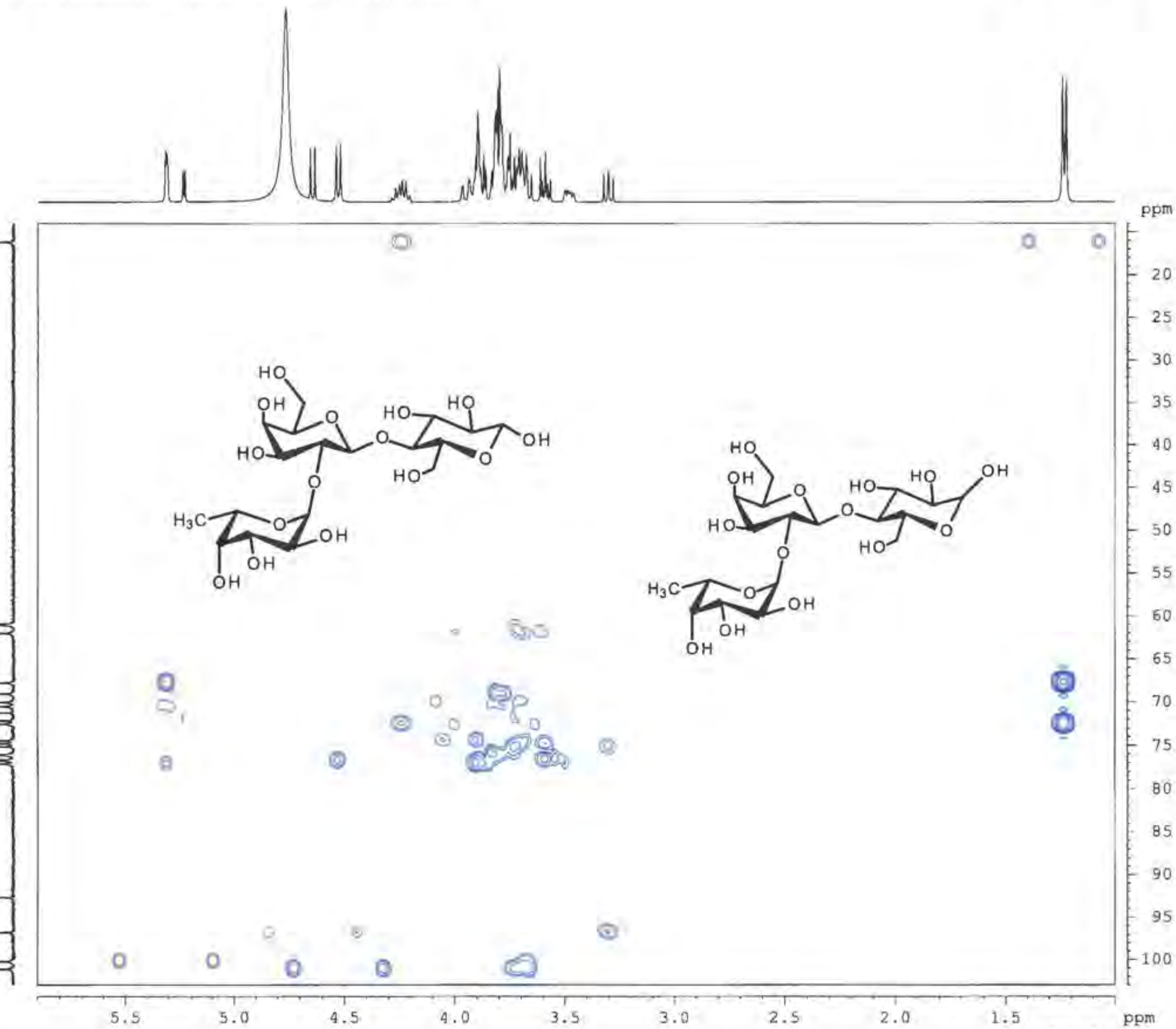

Saum, Stephan
G-ENH/MT F31

Auftrag : 18P00477.1 3393200037
Probe : 2-Fucosyl Lactose 012644-L 06 Turmaustrag AM-08-12-B1 H.st.05.06.2018

Instrument : AV401 2018Yjn21004

D2O + MS 1.w.

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PROCNO                 1

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PULPROG             zg30
TD                131072
SOLVENT              D2O
NS                     8
DS                     2
SWH          8802.817 Hz
FIDRES       0.067160 Hz
AQ          7.4448895 sec
RG                   128
DW            56.800 usec
DE            15.66 usec
TE               298.2 K
D1       35.00000000 sec
TD0                    1

======== CHANNEL f1 ========
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NUC1                  1H
P1            13.50 usec
PLW1      32.00000000 W

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SI                 65536
SF        400.3299655 MHz
WDW                   EM
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LB               0.10 Hz
GB       0
PC                  1.00
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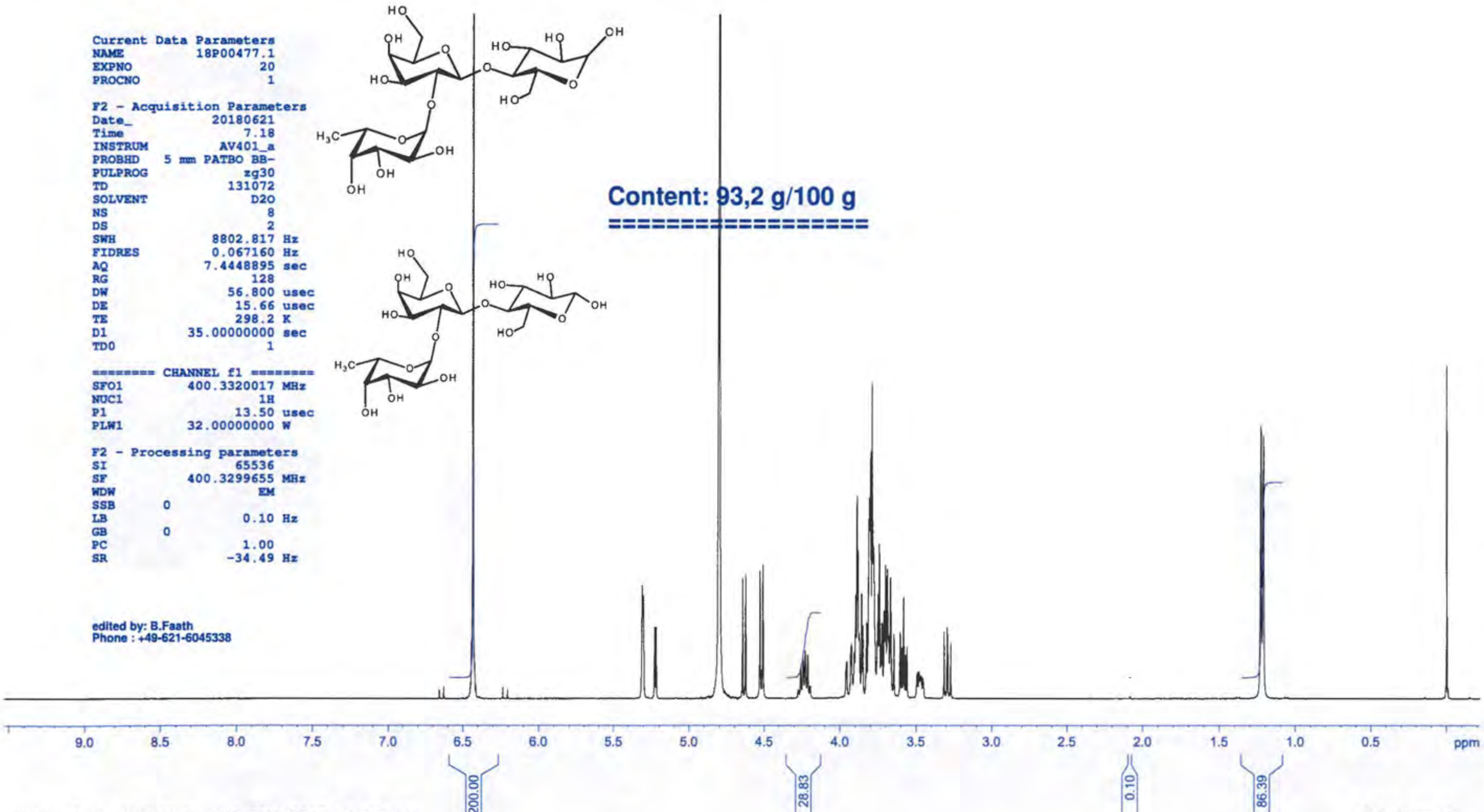
Phone : +49-621-6045338



BASF We create chemistry

2-Fucosyllactose

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25.03.2018

A-9

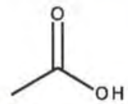
Saum, Stephan
G-ENH/MT F31

BASF
We create chemistry

Auftrag : 18P00477.1 3393200037
Probe : 2-Fucosyl Lactose 012644-L 06 Turmaustrag AM-08-12-B1 H.st.05.06.2018

Instrument : AV401 2018Yjn21004

D2O + MS 2.w.



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SOLVENT              D2O
NS                     8
DS                     2
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FIDRES          0.067160 Hz
AQ             7.4448895 sec
RG                   114
DW                56.800 usec
DE                 15.66 usec
TE                 298.2 K
D1           35.00000000 sec
TD0                    1

======== CHANNEL f1 ========
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P1                 13.50 usec
PLW1         32.00000000 W

F2 - Processing parameters
SI                 65536
SF           400.3299646 MHz
WDW                   EM
SSB       0
LB                  0.10 Hz
GB        0
PC                  1.00
SR                -35.42 Hz
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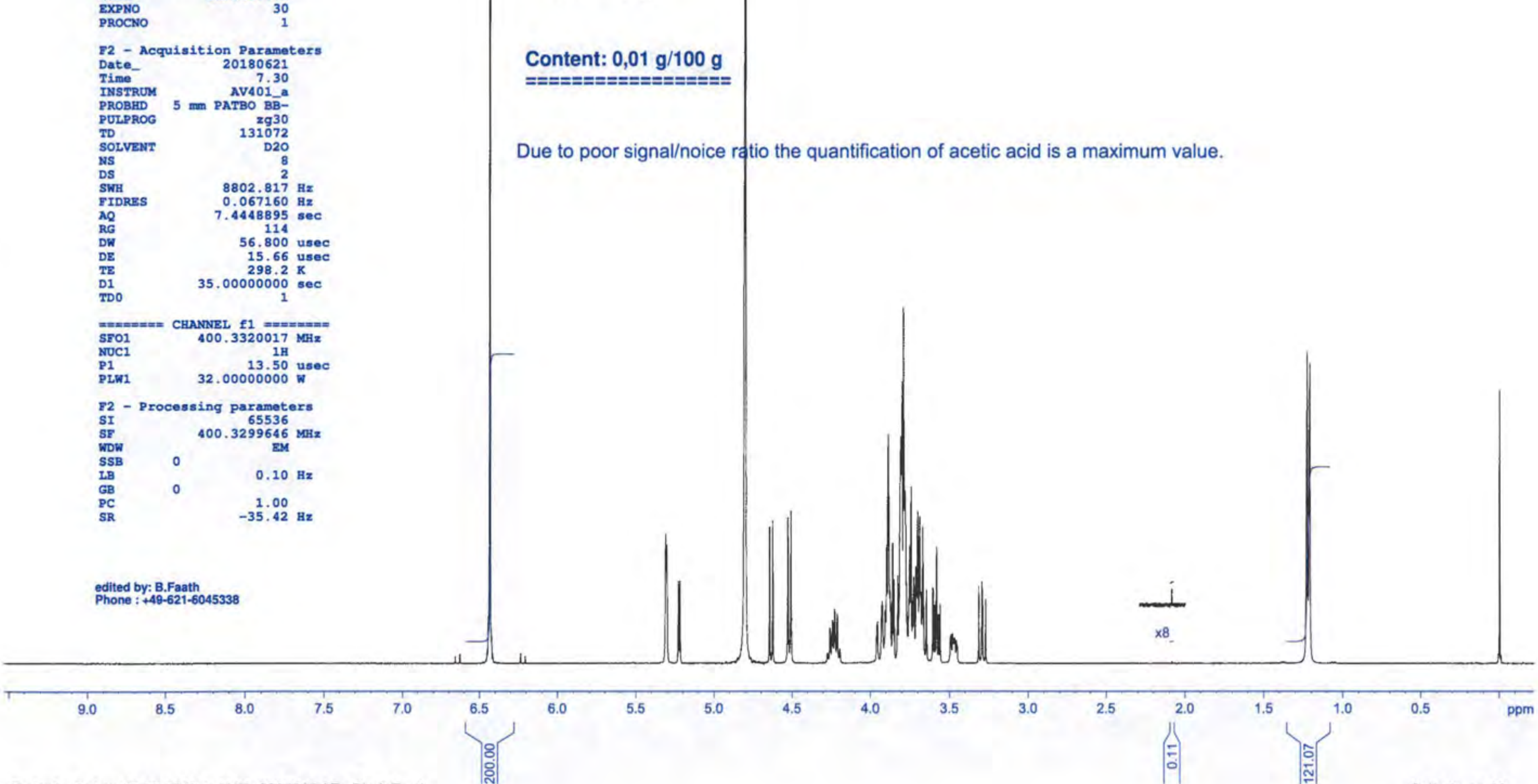
edited by: B.Faath
Phone : +49-621-6045338

Content: 0,01 g/100 g

Due to poor signal/noice ratio the quantification of acetic acid is a maximum value.



A-10

GRAS Notice / BASF SE

25.03.2019

Saum, Stephan
G-ENH/MT F31

BASF
We create chemistry

2-Fucosyllactose

Auftrag : 18P00477.1 3393200037
Probe : 2-Fucosyl Lactose 012644-L 06 Turmaustrag AM-08-12-B1 H.st.05.06.2018

Instrument : AV401 2018Yjn21004

D2O + MS 3.w.

A-11

Current Data Parameters

NAME	18P00477.1
EXPNO	40
PROCNO	1

F2 - Acquisition Parameters

Date_	20180621
Time	7.42
INSTRUM	AV401_a
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PULPROG	zg30
TD	131072
SOLVENT	D2O
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DS	2
SWH	8802.817 Hz
FIDRES	0.067160 Hz
AQ	7.4448895 sec
RG	128
DW	56.800 usec
DE	15.66 usec
TE	298.2 K
D1	35.00000000 sec
TD0	1

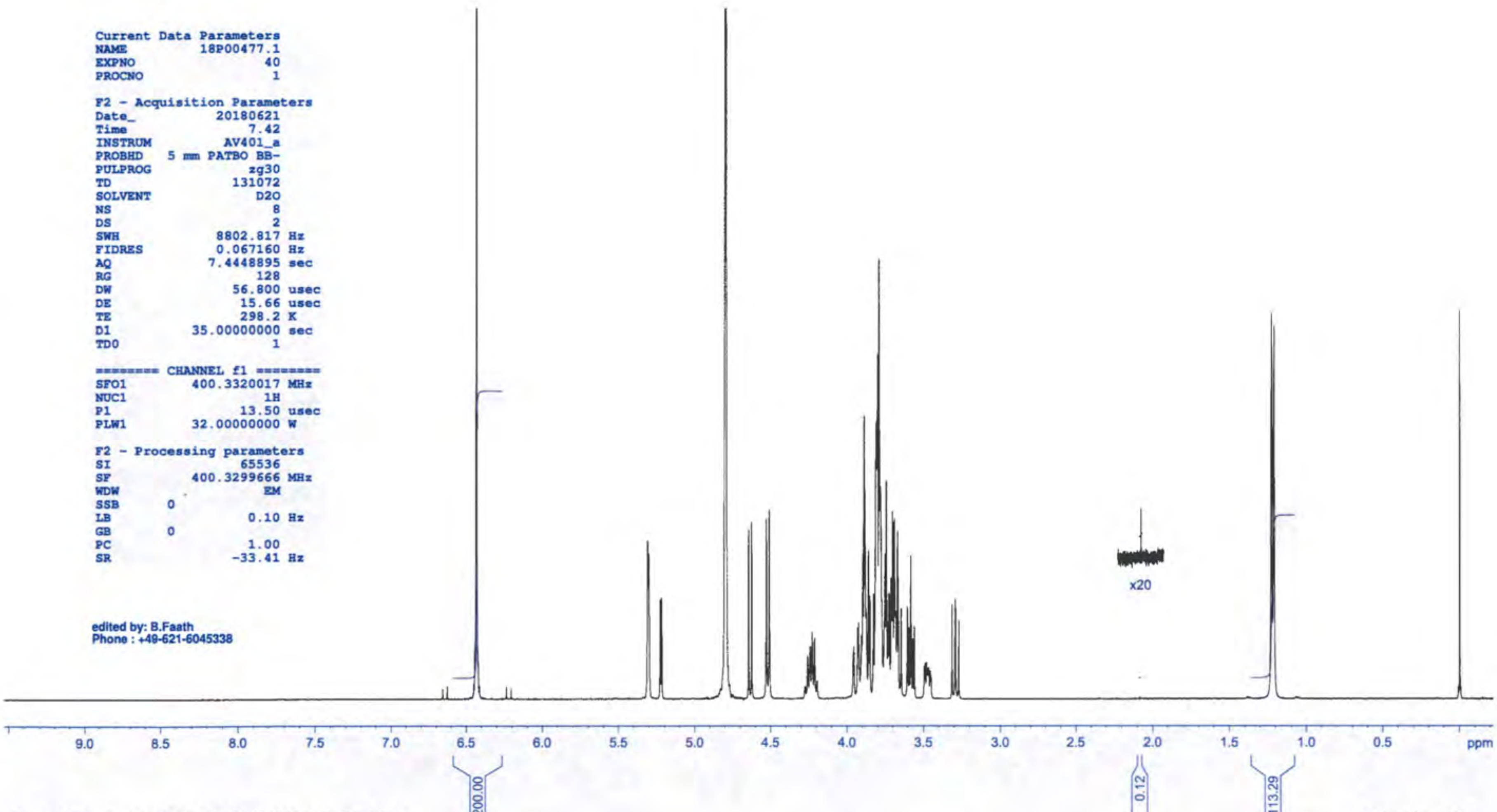
======== CHANNEL f1 ========

SFO1	400.3320017 MHz
NUC1	1H
P1	13.50 usec
PLW1	32.00000000 W

F2 - Processing parameters

SI	65536
SF	400.3299666 MHz
WDW	EM
SSB	0
LB	0.10 Hz
GB	0
PC	1.00
SR	-33.41 Hz

edited by: B.Faath
Phone : +49-621-6045338



GRAS Notice / BASF SE

25.03.2019

Annex II

Analytical certificates

BASF SE, 68623 Lampertheim, Deutschland

To whom it may concern

18/10/2018
Technical Marketing
Human Nutrition
Dr. Stephan Saum
Tel.: +65 6393-5233
stephan.saum@basf.com

The following table summarizes analytical data of 2'-Fucosyllactose batch No. 012644-L 01 produced by BASF.

Parameter	Specification	012545-L	Method
Assay			
Assay, HPLC	mind. 94%	96.7	BASF-HPLC method
Identification			
Appearance, visual	powder or agglomerates	complies	MSZ ISO 6658:2007
Color, visual	white to off-white powder	complies	MSZ ISO 6658:2007
Identification	Rt standard +/- 3%	complies	BASF-HPLC method
Related substances			
D-Lactose	≤ 3.0 %	0.7	BASF-HPLC method
L-Fucose	≤ 2.0 %	< 0.5	BASF-HPLC method
2'-Difucosyl-D-Lactose	≤ 2.0 %	< 0.5	BASF-HPLC method
2'-Fucosyl-D-Lactulose	≤ 2.0%	< 0.5	BASF-HPLC method
Characters			
pH (20°C, 5% solution)	3.2 - 7.5	3.5	Ph. Eur. 2.2.3
Sulfated Ash	≤ 1.5 %	< 0.05	Ph. Eur. 6.7 04/2010:20414
Acetic acid, enzym. (as free acid and/or sodium acetate)	≤ 1.0%	0.75	Megazyme K-ACETRM 07/12
Water, Karl-Fischer	≤ 9.0 % (weight)	5.4	Karl-Fischer (Ph. Eur. 2.5.12)
Heavy Metals / Contaminants			
Pb	≤ 0.05 mg/kg	< 0.05	ICP-MS
Cd	≤ 0.05 mg/kg	< 0.05	ICP-MS
Hg	≤ 0.05 mg/kg	< 0.05	ICP-MS
As	≤ 0.1 mg/kg	< 0.05	ICP-MS
Endotoxin	≤ 10 EU/mg	< 0.1	Limulus amebocyte lysate kinetic chromogenic assay described in the European Pharmaceopoeia
Residual Protein (Bradford)	≤ 0.01 %		modified Bradford Assay
Microbiology			
Total microbial aerobic count	<500 CFU/g	< 10	MSZ-EN-ISO 4833-1:2014
Yeasts and Molds	<100 CFU/g	< 10	MSZ-ISO 7954:1999
Enterobacteria & other Gram-neg	absent in 10 g	complies	ISO 21528-1:2004, MSZ ISO 21528-2:2007
Cronobacter sakazakii	absent in 10 g	complies	ISO-TS 22964:2006
Salmonella	absent in 25 g	complies	MSZ-EN-ISO 6579:2006
Listeria monocytogenes	absent in 25 g	complies	MSZ-EN-ISO 11290-1:1996/ A1:2005, MSZ-EN-ISO 11290-1:1998

BASF SE, 68623 Lampertheim, Deutschland

To whom it may concern

18/10/2018
Technical Marketing
Human Nutrition
Dr. Stephan Saum
Tel.: +65 6393-5233
stephan.saum@basf.com

The following table summarizes analytical data of 2'-Fucosyllactose batch No. 012644-L 04 produced by BASF.

Parameter	Specification	012545-L	Method
Assay			
Assay, HPLC	mind. 94%	98.4	BASF-HPLC method
Identification			
Appearance, visual	powder or agglomerates	complies	MSZ ISO 6658:2007
Color, visual	white to off-white powder	complies	MSZ ISO 6658:2007
Identification	Rt standard +/- 3%	complies	BASF-HPLC method
Related substances			
D-Lactose	≤ 3.0 %	0.8	BASF-HPLC method
L-Fucose	≤ 2.0 %	< 0.5	BASF-HPLC method
2'-Difucosyl-D-Lactose	≤ 2.0 %	< 0.5	BASF-HPLC method
2'-Fucosyl-D-Lactulose	≤ 2.0%	0.7	BASF-HPLC method
Characters			
pH (20°C, 5% solution)	3.2 - 7.5	3.5	Ph. Eur. 2.2.3
Sulfated Ash	≤ 1.5 %	< 0.05	Ph. Eur. 6.7 04/2010:20414
Acetic acid, enzym. (as free acid and/or sodium acetate)	≤ 1.0%	0.68	Megazyme K-ACETRM 07/12
Water, Karl-Fischer	≤ 9.0 % (weight)	4.7	Karl-Fischer (Ph. Eur. 2.5.12)
Heavy Metals / Contaminants			
Pb	≤ 0.05 mg/kg	< 0.05	ICP-MS
Cd	≤ 0.05 mg/kg	< 0.05	ICP-MS
Hg	≤ 0.05 mg/kg	< 0.05	ICP-MS
As	≤ 0.5 mg/kg	< 0.05	ICP-MS
Endotoxin	≤ 10 EU/mg	< 0.1	Limulus amebocyte lysate kinetic chromogenic assay described in the European Pharmaceopoeia
Residual Protein (Bradford)	≤ 0.01 %	< 0.01	modified Bradford Assay
Microbiology			
Total microbial aerobic count	<500 CFU/g	< 10	MSZ-EN-ISO 4833-1:2014
Yeasts and Molds	<100 CFU/g	< 10	MSZ-ISO 7954:1999
Enterobacteria & other Gram-neg	absent in 10 g	complies	ISO 21528-1:2004, MSZ ISO 21528-2:2007
Cronobacter sakazakii	absent in 10 g	complies	ISO-TS 22964:2006
Salmonella	absent in 25 g	complies	MSZ-EN-ISO 6579:2006
Listeria monocytogenes	absent in 25 g	complies	MSZ-EN-ISO 11290-1:1996/ A1:2005, MSZ-EN-ISO 11290-1:1998

BASF SE, 68623 Lampertheim, Deutschland

To whom it may concern

18/10/2018
Technical Marketing
Human Nutrition
Dr. Stephan Saum
Tel.: +65 6393-5233
stephan.saum@basf.com

The following table summarizes analytical data of 2'-Fucosyllactose batch No. 012644-L 05 produced by BASF.

Parameter	Specification	012545-L	Method
Assay			
Assay, HPLC	mind. 94%	99.3	BASF-HPLC method
Identification			
Appearance, visual	powder or agglomerates	complies	MSZ ISO 6658:2007
Color, visual	white to off-white powder	complies	MSZ ISO 6658:2007
Identification	Rt standard +/- 3%	complies	BASF-HPLC method
Related substances			
D-Lactose	≤ 3.0 %	0.5	BASF-HPLC method
L-Fucose	≤ 2.0 %	< 0.5	BASF-HPLC method
2'-Difucosyl-D-Lactose	≤ 2.0 %	< 0.5	BASF-HPLC method
2'-Fucosyl-D-Lactulose	≤ 2.0%	0.7	BASF-HPLC method
Characters			
pH (20°C, 5% solution)	3.2 - 7.5	3.5	Ph. Eur. 2.2.3
Sulfated Ash	≤ 1.5 %	< 0.05	Ph. Eur. 6.7 04/2010:20414
Acetic acid, enzym. (as free acid and/or sodium acetate)	≤ 1.0%	0.68	Megazyme K-ACETRM 07/12
Water, Karl-Fischer	≤ 9.0 % (weight)	4.7	Karl-Fischer (Ph. Eur. 2.5.12)
Heavy Metals / Contaminants			
Pb	≤ 0.05 mg/kg	< 0.05	ICP-MS
Cd	≤ 0.05 mg/kg	< 0.05	ICP-MS
Hg	≤ 0.05 mg/kg	< 0.05	ICP-MS
As	≤ 0.1 mg/kg	< 0.05	ICP-MS
Endotoxin	≤ 10 EU/mg	< 0.1	Limulus amebocyte lysate kinetic chromogenic assay described in the European Pharmaceopoeia
Residual Protein (Bradford)	≤ 0.01 %	< 0.01	modified Bradford Assay
Microbiology			
Total microbial aerobic count	<500 CFU/g	< 10	MSZ-EN-ISO 4833-1:2014
Yeasts and Molds	<100 CFU/g	< 10	MSZ-ISO 7954:1999
Enterobacteria & other Gram-neg	absent in 10 g	complies	ISO 21528-1:2004, MSZ ISO 21528-2:2007
Cronobacter sakazakii	absent in 10 g	complies	ISO-TS 22964:2006
Salmonella	absent in 25 g	complies	MSZ-EN-ISO 6579:2006
Listeria monocytogenes	absent in 25 g	complies	MSZ-EN-ISO 11290-1:1996/ A1:2005, MSZ-EN-ISO 11290-1:1998

BASF SE, 68623 Lampertheim, Deutschland

To whom it may concern

18/10/2018
Technical Marketing
Human Nutrition
Dr. Stephan Saum
Tel.: +65 6393-5233
stephan.saum@basf.com

The following table summarizes analytical data of 2'-Fucosyllactose batch No. 012644-L 02 produced by BASF.

Parameter	Specification	012545-L	Method
Assay			
Assay, HPLC	mind. 94%	96.9	BASF-HPLC method
Identification			
Appearance, visual	powder or agglomerates	complies	MSZ ISO 6658:2007
Color, visual	white to off-white powder	complies	MSZ ISO 6658:2007
Identification	Rt standard +/- 3%	complies	BASF-HPLC method
Related substances			
D-Lactose	≤ 3.0 %	< 0.5	BASF-HPLC method
L-Fucose	≤ 2.0 %	< 0.5	BASF-HPLC method
2'-Difucosyl-D-Lactose	≤ 2.0 %	< 0.5	BASF-HPLC method
2'-Fucosyl-D-Lactulose	≤ 2.0%	< 0.5	BASF-HPLC method
Characters			
pH (20°C, 5% solution)	3.2 - 7.5	6.5	Ph. Eur. 2.2.3
Sulfated Ash	≤ 1.5 %	< 0.05	Ph. Eur. 6.7 04/2010:20414
Acetic acid, enzym. (as free acid and/or sodium acetate)	≤ 1.0%	<0.1	Megazyme K-ACETRM 07/12
Water, Karl-Fischer	≤ 9.0 % (weight)	5.3	Karl-Fischer (Ph. Eur. 2.5.12)
Heavy Metals / Contaminants			
Pb	≤ 0.05 mg/kg	< 0.05	ICP-MS
Cd	≤ 0.05 mg/kg	< 0.05	ICP-MS
Hg	≤ 0.05 mg/kg	< 0.05	ICP-MS
As	≤ 0.1 mg/kg	< 0.05	ICP-MS
Endotoxin	≤ 10 EU/mg	< 0.1	Limulus amebocyte lysate kinetic chromogenic assay described in the European Pharmaceopoeia
Residual Protein (Bradford)	≤ 0.01 %	< 0.01	modified Bradford Assay
Microbiology			
Total microbial aerobic count	<500 CFU/g	< 10	MSZ-EN-ISO 4833-1:2014
Yeasts and Molds	<100 CFU/g	< 10	MSZ-ISO 7954:1999
Enterobacteria & other Gram-neg	absent in 10 g	complies	ISO 21528-1:2004, MSZ ISO 21528-2:2007
Cronobacter sakazakii	absent in 10 g	complies	ISO-TS 22964:2006
Salmonella	absent in 25 g	complies	MSZ-EN-ISO 6579:2006
Listeria monocytogenes	absent in 25 g	complies	MSZ-EN-ISO 11290-1:1996/ A1:2005, MSZ-EN-ISO 11290-1:1998

BASF SE, 68623 Lampertheim, Deutschland

To whom it may concern

18/10/2018
Technical Marketing
Human Nutrition
Dr. Stephan Saum
Tel.: +65 6393-5233
stephan.saum@basf.com

The following table summarizes analytical data of 2'-Fucosyllactose batch No. 012644-L 06 produced by BASF.

Parameter	Specification	012545-L	Method
Assay			
Assay, HPLC	mind. 94%	100.7	BASF-HPLC method
Identification			
Appearance, visual	powder or agglomerates	complies	MSZ ISO 6658:2007
Color, visual	white to off-white powder	complies	MSZ ISO 6658:2007
Identification	Rt standard +/- 3%	complies	BASF-HPLC method
Related substances			
D-Lactose	≤ 3.0 %	< 0.3	BASF-HPLC method
L-Fucose	≤ 2.0 %	< 0.3	BASF-HPLC method
2'-Difucosyl-D-Lactose	≤ 2.0 %	< 0.3	BASF-HPLC method
2'-Fucosyl-D-Lactulose	≤ 2.0%	< 0.3	BASF-HPLC method
Characters			
pH (20°C, 5% solution)	3.2 - 7.5	6.4	Ph. Eur. 2.2.3
Sulfated Ash	≤ 1.5 %	< 0.05	Ph. Eur. 6.7 04/2010:20414
Acetic acid, enzym. (as free acid and/or sodium acetate)	≤ 1.0%	<0.1	Megazyme K-ACETRM 07/12
Water, Karl-Fischer	≤ 9.0 % (weight)	5.29	Karl-Fischer (Ph. Eur. 2.5.12)
Heavy Metals / Contaminants			
Pb	≤ 0.05 mg/kg	< 0.05	ICP-MS
Cd	≤ 0.05 mg/kg	< 0.01	ICP-MS
Hg	≤ 0.05 mg/kg	< 0.05	ICP-MS
As	≤ 0.1 mg/kg	< 0.05	ICP-MS
Endotoxin	≤ 10 EU/mg	< 0.1	Limulus amebocyte lysate kinetic chromogenic assay described in the European Pharmaceopoeia
Residual Protein (Bradford)	≤ 0.01 %	< 0.01	modified Bradford Assay
Microbiology			
Total microbial aerobic count	<500 CFU/g	< 100	MSZ-EN-ISO 4833-1:2014
Yeasts and Molds	<100 CFU/g	< 100	MSZ-ISO 7954:1999
Enterobacteria & other Gram-neg	absent in 10 g	complies	ISO 21528-1:2004, MSZ ISO 21528-2:2007
Cronobacter sakazakii	absent in 10 g	complies	ISO-TS 22964:2006
Salmonella	absent in 25 g	complies	MSZ-EN-ISO 6579:2006
Listeria monocytogenes	absent in 25 g	complies	MSZ-EN-ISO 11290-1:1996/ A1:2005, MSZ-EN-ISO 11290-1:1998

BASF SE, 68623 Lampertheim, Deutschland

To whom it may concern

18/10/2018
Technical Marketing
Human Nutrition
Dr. Stephan Saum
Tel.: +65 6393-5233
stephan.saum@basf.com

The following table summarizes analytical data of 2'-Fucosyllactose batch No. 012644-L 10 produced by BASF.

Parameter	Specification	012545-L	Method
Assay			
Assay, HPLC	mind. 94%	100.1	BASF-HPLC method
Identification			
Appearance, visual	powder or agglomerates	complies	MSZ ISO 6658:2007
Color, visual	white to off-white powder	complies	MSZ ISO 6658:2007
Identification	Rt standard +/- 3%	complies	BASF-HPLC method
Related substances			
D-Lactose	≤ 3.0 %	0.5	BASF-HPLC method
L-Fucose	≤ 2.0 %	< 0.3	BASF-HPLC method
2'-Difucosyl-D-Lactose	≤ 2.0 %	< 0.3	BASF-HPLC method
2'-Fucosyl-D-Lactulose	≤ 2.0%	0.4	BASF-HPLC method
Characters			
pH (20°C, 5% solution)	3.2 - 7.5	6.8	Ph. Eur. 2.2.3
Sulfated Ash	≤ 1.5 %	< 0.05	Ph. Eur. 6.7 04/2010:20414
Acetic acid, enzym. (as free acid and/or sodium acetate)	≤ 1.0%	< 0.1	Megazyme K-ACETRM 07/12
Water, Karl-Fischer	≤ 9.0 % (weight)	5.48	Karl-Fischer (Ph. Eur. 2.5.12)
Heavy Metals / Contaminants			
Pb	≤ 0.05 mg/kg		ICP-MS
Cd	≤ 0.05 mg/kg		ICP-MS
Hg	≤ 0.05 mg/kg		ICP-MS
As	≤ 0.1 mg/kg		ICP-MS
Endotoxin	≤ 10 EU/mg	< 0.1	Limulus amebocyte lysate kinetic chromogenic assay described in the European Pharmaceopoeia
Residual Protein (Bradford)	≤ 0.01 %	< 0.01	modified Bradford Assay
Microbiology			
Total microbial aerobic count	<500 CFU/g	< 10	MSZ-EN-ISO 4833-1:2014
Yeasts and Molds	<100 CFU/g	< 10	MSZ-ISO 7954:1999
Enterobacteria & other Gram-neg	absent in 10 g	complies	ISO 21528-1:2004, MSZ ISO 21528-2:2007
Cronobacter sakazakii	absent in 10 g	complies	ISO-TS 22964:2006
Salmonella	absent in 25 g	complies	MSZ-EN-ISO 6579:2006
Listeria monocytogenes	absent in 25 g	complies	MSZ-EN-ISO 11290-1:1996/ A1:2005, MSZ-EN-ISO 11290-1:1998

Annex III

Analysis of the fucT2 Gene by PCR Assay

BASF SE
Dr. Birgit Hoff
RBW/D - A030
Carl-Bosch-Strasse 38
67056 Ludwigshafen

Freiburg, 2017-12-13

Certificate No.: 1991 – 01

EFG Order No.: 1991 EFG 5DE0203 2222 V02 Sample received: 2017-11-07

Samples

Sample description:	powder
Sample labelling:	2'-O-Fucosyl-D-Lactose
Your Sample Lot:	012644-L01
Amount of sample:	50 g
Sample Condition:	No remarks

Test **Analysis for the Presence of the Full-Length Sequence of the fucT2 Gene of a Recombinant *Escherichia coli* Production Strain by Means of a Qualitative Gel-Based PCR Assay**

Subsample analyzed: 3 x 200 mg per batch

Sample	Batch-No.	EFG Code	Start of Analysis	End of Analysis	Result fucT2-PCR	Spike recovery
2'-O-Fucosyl-D-Lactose	**012644-L01**	GO-4154	04.12.2017	05.12.2017	**Negative**	**yes**

Comment: In case of a negative result no band was observed as detected by UV-illumination after gel-electrophoresis. It is possible that the sample may contain residual production strain DNA in quantities below the method-specific LOD (limit of detection). In case of a positive result the amount of residual production strain DNA is equal to or greater than the method-specific LOD. Residual DNA quantification is not possible by this test.

Dr. Nicole Appel, Head of Special Testing Services

The results exclusively refer to the actually analyzed portion of the sample delivered and therefore they do not have to be representative of the product from which the sample was taken.

DAkkS Deutsche Akkreditierungsstelle D-PL-17111-01-00

Freiburg, 2017-12-13

BASF SE
Dr. Birgit Hoff
RBW/D - A030
Carl-Bosch-Strasse 38
67056 Ludwigshafen

Certificate No.: 1991 – 04

EFG Order No.: 1991 EFG 5DE0203 2222 V02 Sample received: 2017-12-06

Samples

Sample description:	powder
Sample labelling:	2'-O-Fucosyl-D-Lactose
Your Sample Lot:	012644-L04
Amount of sample:	50 g
Sample Condition:	No remarks

Test **Analysis for the Presence of the Full-Length Sequence of the fucT2 Gene of a Recombinant *Escherichia coli* Production Strain by Means of a Qualitative Gel-Based PCR Assay**

Subsample analyzed: 3 x 200 mg per batch

Sample	Batch-No.	EFG Code	Start of Analysis	End of Analysis	Result fucT2-PCR	Spike recovery
2'-O-Fucosyl-D-Lactose	**012644-L04**	GO-4178	08.12.2017	12.12.2017	**Negative**	**yes**

Comment: In case of a negative result no band was observed as detected by UV-illumination after gel-electrophoresis. It is possible that the sample may contain residual production strain DNA in quantities below the method-specific LOD (limit of detection). In case of a positive result the amount of residual production strain DNA is equal to or greater than the method-specific LOD. Residual DNA quantification is not possible by this test.

Dr. Nicole Appel, Head of Special Testing Services

The results exclusively refer to the actually analyzed portion of the sample delivered and therefore they do not have to be representative of the product from which the sample was taken.

DAkkS Deutsche Akkreditierungsstelle D-PL-17111-01-00

BASF SE
Dr. Birgit Hoff
RBW/D - A030
Carl-Bosch-Strasse 38
67056 Ludwigshafen

Freiburg, 2017-12-13

Certificate No.: 1991 – 05

EFG Order No.: 1991 EFG 5DE0203 2222 V02 Sample received: 2017-12-06

Samples

Sample description:	powder
Sample labelling:	2'-O-Fucosyl-D-Lactose
Your Sample Lot:	012644-L05
Amount of sample:	50 g
Sample Condition:	No remarks

Test **Analysis for the Presence of the Full-Length Sequence of the fucT2 Gene of a Recombinant *Escherichia coli* Production Strain by Means of a Qualitative Gel-Based PCR Assay**

Subsample analyzed: 3 x 200 mg per batch

Sample	Batch-No.	EFG Code	Start of Analysis	End of Analysis	Result fucT2-PCR	Spike recovery
2'-O-Fucosyl-D-Lactose	**012644-L05**	GO-4180	08.12.2017	12.12.2017	**Negative**	**yes**

Comment: In case of a negative result no band was observed as detected by UV-illumination after gel-electrophoresis. It is possible that the sample may contain residual production strain DNA in quantities below the method-specific LOD (limit of detection). In case of a positive result the amount of residual production strain DNA is equal to or greater than the method-specific LOD. Residual DNA quantification is not possible by this test.

Dr. Nicole Appel, Head of Special Testing Services

The results exclusively refer to the actually analyzed portion of the sample delivered and therefore they do not have to be representative of the product from which the sample was taken.

DAkkS Deutsche Akkreditierungsstelle D-PL-17111-01-00

BASF SE
Dr. Birgit Hoff
RBW/D - A030
Carl-Bosch-Strasse 38
67056 Ludwigshafen

Freiburg, 2017-12-13

Certificate No.: 1991 – 02

EFG Order No.: 1991 EFG 5DE0203 2222 V02 Sample received: 2017-11-17

Samples

Sample description: powder
Sample labelling: 2'-O-Fucosyl-D-Lactose
Your Sample Lot: 012644-L02
Amount of sample: 50 g
Sample Condition: No remarks

Test **Analysis for the Presence of the Full-Length Sequence of the fucT2 Gene of a Recombinant *Escherichia coli* Production Strain by Means of a Qualitative Gel-Based PCR Assay**

Subsample analyzed: 3 x 200 mg per batch

Sample	Batch-No.	EFG Code	Start of Analysis	End of Analysis	Result fucT2-PCR	Spike recovery
2'-O-Fucosyl-D-Lactose	**012644-L02**	GO-4176	04.12.2017	05.12.2017	**Negative**	**yes**

Comment: In case of a negative result no band was observed as detected by UV-illumination after gel-electrophoresis. It is possible that the sample may contain residual production strain DNA in quantities below the method-specific LOD (limit of detection). In case of a positive result the amount of residual production strain DNA is equal to or greater than the method-specific LOD. Residual DNA quantification is not possible by this test.

Dr. Nicole Appel, Head of Special Testing Services

The results exclusively refer to the actually analyzed portion of the sample delivered and therefore they do not have to be representative of the product from which the sample was taken.

DAkkS Deutsche Akkreditierungsstelle D-PL-17111-01-00

Freiburg, 2018-07-30

BASF SE
Dr. Anne-Catrin Letzel
RBW/D - A30
67056 Ludwigshafen

Certificate No.: 2009-4401-01

EFG Order No.: 2009 EFG 5DE0203 2222 V01 Sample received: 2018-06-19

Samples

Sample description:	powder
Sample labelling:	2-Fucosyl Lactose
Your Sample Lot:	012644-L06
Amount of sample:	50 g
Sample Condition:	No remarks

Test **Analysis for the Presence of the Full-Length Sequence of the fucT2 Gene of a Recombinant *Escherichia coli* Production Strain by Means of a Qualitative Gel-Based PCR Assay**

Subsample analyzed: 3 x 200 mg per batch

Sample	Batch-No.	EFG Code	Start of Analysis	End of Analysis	Result fucT2-PCR	Spike recovery
2-Fucosyl Lactose	**012644-L06**	GO-4401	23.07.2018	30.07.2018	**Negative**	**Yes**

Comment: In case of a negative result no band was observed as detected by UV-illumination after gel-electrophoresis. It is possible that the sample may contain residual production strain DNA in quantities below the method-specific LOD (limit of detection). In case of a positive result the amount of residual production strain DNA is equal to or greater than the method-specific LOD. Residual DNA quantification is not possible by this test.

Petra Richl, Head of Method Development / Special Testing

The results exclusively refer to the actually analyzed portion of the sample delivered and therefore they do not have to be representative of the product from which the sample was taken.

DAkkS Deutsche Akkreditierungsstelle D-PL-17111-01-00

1/1

Freiburg, 2018-07-30

BASF SE
Dr. Anne-Catrin Letzel
RBW/D - A30
67056 Ludwigshafen

Certificate No.: 2009-4403-01

EFG Order No.: 2009 EFG 5DE0203 2222 V01 Sample received: 2018-07-09

Samples

Sample description:	powder
Sample labelling:	2-Fucosyl Lactose
Your Sample Lot:	012644-L10
Amount of sample:	50 g
Sample Condition:	No remarks

Test **Analysis for the Presence of the Full-Length Sequence of the fucT2 Gene of a Recombinant *Escherichia coli* Production Strain by Means of a Qualitative Gel-Based PCR Assay**

Subsample analyzed: 3 x 200 mg per batch

Sample	Batch-No.	EFG Code	Start of Analysis	End of Analysis	Result fucT2-PCR	Spike recovery
2-Fucosyl Lactose	**012644-L10**	GO-4403	23.07.2018	30.07.2018	**Negative**	**Yes**

Comment: In case of a negative result no band was observed as detected by UV-illumination after gel-electrophoresis. It is possible that the sample may contain residual production strain DNA in quantities below the method-specific LOD (limit of detection). In case of a positive result the amount of residual production strain DNA is equal to or greater than the method-specific LOD. Residual DNA quantification is not possible by this test.

Petra Richl, Head of Method Development / Special Testing

The results exclusively refer to the actually analyzed portion of the sample delivered and therefore they do not have to be representative of the product from which the sample was taken.

DAkkS Deutsche Akkreditierungsstelle D-PL-17111-01-00

Annex IV

Stability data

Newtrition

Stability data

Version 1, 05.2018

2'-Fucosyllactose

PRD no. XXXXXX
Manufacturing site: BASF SE, Ludwigshafen, Germany

Stability study (no.): 17H00088

Storage conditions:
Defined climatic conditions: 40°C/75% RH

Analytical method:

2'-Fucosyllactose assay:	HPLC (internal method; dried substance)
Related substance (D-Lactose):	HPLC (internal method; w/w-%)

Packaging:
The stability test is conducted on product packaged in water-resistant aluminum foil bags, similar to the primary packaging material used for storage and distribution of sales product.

Test attributes:
The stability study includes testing of those attributes that are susceptible to change during storage and are likely to influence the quality, safety and/or efficacy.
Assay (total amount of 2'-Fucosyllactose) and related substance (D-Lactose) are the most important attributes that are susceptible to change during storage.

Accelerated stability study:
2'-Fucosyllactose stored at 40°C ± 2°C/ 75% RH ± 5% RH

Analytical method	Batch	Start of the study	Spec.	0 m	3 m	6 m
Assay (HPLC) [%] (iDM)	012545-L	08/2017	Min. 94.0	**97.3**	**99.0**	**98.3**
D-Lactose (HPLC) [%]	012545-L	08/2017	Max. 3.0	**<0.5**	**0.6**	**0.7**

Conclusion

2'-Fucosyllactose is stable over the time course of at least 6 months at 40°C (75% relative humidity) when it is stored in the original unopened bag. According to common understanding these storage conditions are equivalent to a storage time of 24 months at 25°C. Therefore, based on the above-mentioned results a shelf-life of at least 24 months at 25°C is expected.

Annex V

Documentation of deposition of strain LU20297 at DSMZ

FOR SAFE DEPOSIT PURPOSES ONLY!
Not to be used for scientific publications (e.g. description of type strains) or patent purposes!

DSMZ

LEIBNIZ-INSTITUT DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH
Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures
Inhoffenstr. 7 B
D-38124 Braunschweig
GERMANY

ACCESSION FORM for SAFE DEPOSIT OF bacteria, archaea, fungi for completion by the depositor	*To be completed by the Depositary Authority:* DSMZ ACCESSION NUMBER: DATE CULTURE RECEIVED:

BACTERIA/ARCHAEA/FUNGI[1]

I. IDENTIFICATION OF THE MICROORGANISM[1]

Identification reference: LU20297 Taxonomic designation: E coli K12 JM109 derivative	The culture to be deposited is: (X) a pure culture ( ) a mixture of microorganisms (not more than two components)

II. CONDITIONS FOR CULTIVATION ( )[2]

Medium: pH Luria Broth	before sterilisation: Sterilisation 20 min at 121 °C pH after sterilisation: 7 Oxygen relationship: (X) aerobic (X) microaerophilic ( ) obligate anaerobic Specific gaseous requirements: none Incubation temperature: 37 °C Incubation time: 24h-48h Short term storage at: 4 °C Interval of transfer: 7d

1 The DSMZ only accepts for deposit microorganisms which belong to risk group 1 or 2 according to EU Council Directive 2000/54 on the protection of workers from risks related to exposure to biological agents at work and can be classified as S1 or S2 organisms according to the German Law Regulating Genetic Engineering or Class 1 or 2 according to Directive 2009/41/EC of the European Parliament and of the council on the contained use of genetically modified micro-organisms respectively.

2 Mark with a cross if additional information is given on an attached sheet.

FOR SAFE DEPOSIT PURPOSES ONLY!
- not for patent purposes or scientific publications -

DSMZ ACCESSION FORM safe deposit of bacteria/archaea/fungi (first page) 07/2016

FOR SAFE DEPOSIT PURPOSES ONLY!
***Not to be used* for scientific publications (e.g. description of type strains) or patent purposes!**

III. CONDITIONS FOR LONG TERM STORAGE	( )[2]
growth overnight in Luria broth. Addition of 10% glycerol and freezing in dry ice methanol mixture, Storage at -8	

IV. CONDITIONS FOR TESTING VIABILITY	( )[2]
plating/streaking of frozen cells on LB Agar, incubation at 37°C for 24h-48h	

V. COMPONENTS OF MIXED CULTURES (WHEN APPLICABLE)	( )[2]
Description of components (not more than two components): Method(s) for checking presence of components:	

VI. PROPERTIES DANGEROUS TO HEALTH OR ENVIRONMENT	( )[2]
RISK GROUP of the microorganism[1]: (x) risk group 1 ( ) risk group 2 CLASSIFICATION in case the microorganism is genetically engineered[1]: (x) Class 1/S1 ( ) Class 2/S2 THE STRAIN HAS TO BE HANDLED UNDER LABORATORY CONTAINMENT LEVEL[1]: (x) L1 ( ) L2 IS THIS STRAIN DANGEROUS TO HEALTH OR THE ENVIRONMENT ? ( ) YES (x) NO If yes, please specify: (x) the undersigned is not aware of such properties	

1 The DSMZ only accepts for deposit microorganisms which belong to risk group 1 or 2 according to EU Council Directive 2000/54 on the protection of workers from risks related to exposure to biological agents at work and can be classified as S1 or S2 organisms according to the German Law Regulating Genetic Engineering or Class 1 or 2 according to Directive 2009/41/EC of the European Parliament and of the council on the contained use of genetically modified micro-organisms respectively.

2 Mark with a cross if additional information is given on an attached sheet

FOR SAFE DEPOSIT PURPOSES ONLY!
- not for patent purposes or scientific publications -

DSMZ ACCESSION FORM safe deposit of bacteria/archaea/fungi (second page) 07/2016

FOR SAFE DEPOSIT PURPOSES ONLY!

***Not to be used** for scientific publications (e.g. description of type strains) or patent purposes!*

VII. IF THE MICROORGANISM IS GENETICALLY MANIPULATED ( )[2]

Please absolutely give complete answers!

*1. DATA CONCERNING THE **HOST ORGANISM***

designation: JM109 derivative of E. coli K12

risk group[1]: (x) risk group 1 ( ) risk group 2

sensitivities: none
resistances: streptomycin resistance (natural rpsL* allele)
auxotrophies: thiamine

special properties:
(e.g. restriction/modification system, general genetic recombination) recA deficient strain

*2. DATA CONCERNING THE **DONOR ORGANISM***

designation: Helicobacter pylori

risk group[1]: ( ) risk group 1 (X) risk group 2 ( ) risk group 3

description of the **cloned DNA fragment**:

cloned information: fucosyltransferase fucT2

size of the cloned DNA (in bp): 903bp
( ) complete genome ( ) cDNA
( ) subgenomic ( ) subgenic (X) synthetic

potential risk of the cloned DNA:

(X) no potential risk ( ) pathogenic ( ) tumorigenic
( ) toxigenic ( ) allergenic

*3. DATA CONCERNING THE **VECTOR***

designation:

derivative of:

host specificity:

resistances:

plasmid size (in kb): without insert: with insert:

promoters:

additional reading frames:

own infectiosity:	( ) yes	( ) no
mobilisable plasmid:	( ) yes	( ) no
own transfer system:	( ) yes	( ) no
transfer by endogenous viruses:	( ) yes	( ) no

*4. DATA CONCERNING THE **GENETICALLY MANIPULATED ORGANISM***

special properties: production of 2 Fucosyl-Lactose
(e.g. production of ...; use as ...-vector etc.)

foreign DNA: (X) chromosomally integrated ( ) episomal

potential risk: ( ) pathogenic ( ) tumorigenic
( ) toxigenic ( ) allergenic
( ) no potential risk
please indicate why:

According to the regulations of the German Law Regulating Genetic Engineering the DSMZ can only accept genetically manipulated, potentially pathogenic organisms for deposition when a copy of the permit issued bythe competent authority (or by an equivalent national biological safety commission) for work on the organisms accompanies the deposition form.

[2] Mark with a cross if additional information is given on an attached sheet.

DSMZ ACCESSION FORM safe deposit of bacteria/archaea/fungi (third page) 07/2016

FOR SAFE DEPOSIT PURPOSES ONLY!
- not for patent purposes or scientific publications -

FOR SAFE DEPOSIT PURPOSES ONLY!

***Not to be used* for scientific publications (e.g. description of type strains) or patent purposes!**

VIII. SCIENTIFIC DESCRIPTION ( )[8]

JM109 a synthetic gene cluster containing the fucT2 gene from Helicobacter and the genes gmd, wcaG, manC, manB from E coli integrated into the fucIK locus

IX. ADDITIONAL DATA ( )[8]

X. DEPOSITOR[9]

The microorganism described above is sent for long term maintenance (safe deposit). It is understood that cultures of the strain remain the property of the depositor. They will not be included into the DSMZ catalogue of strains and not supplied to third parties without the written authorization of the depositor. This kind of deposit is not suitable for patent purposes and it will not be accepted for scientific publications. *Type strains* must be deposited in the public collection of the DSMZ (accession form).

Institution/ legal entity:
BASF SE, RBW

Name of signing person(s) (typewritten):
Carsten Sieden
Oskar Zelder

The signing person(s) deposit(s):
LU20297

(X) on behalf of the legal entity
( ) as private depositor(s)

Address:
BASF SE
RBW/D-A30
67056 Ludwigshafen

Signature (b) (6) [redacted] (Zelder) (Sieden)

Phone: 06216042305

Fax:

E-Mail: stammsammlung-a30@basf.com

Date: 05.10.2017

[8] Mark with a cross if additional information is given on an attached sheet.

[9] This Deposition Form is thecontract between the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and the depositor. Therefore it must be signed bythe depositor. In case of a legal entitythe signatures of two representatives, officially nominated by this entity, are recommended. Indication of the e-mail address helps to accelerate communication.

This deposition form must be signed by the depositor.

FOR SAFE DEPOSIT PURPOSES ONLY!
- not for patent purposes or scientific publications -

DSMZ ACCESSION FORM safe deposit of bacteria/archaea/fungi (fourth and last page) 07/2016

Annex VI

Alignment of LU20297_planned and LU20297_sequenced with consensus sequence

MUSCLE Alignment of LU20297_planned and LU20297_sequenced with consensus sequence (middle)

Identities = 6849/6849 (100%), Positives = 6849/6849 (100%), Gaps = 0/6849 (0%)

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LU20297_planned        1 TCCTGGCCGCCTACGCATTAATCGCTATGGCACTGTGCCTGATCTCAGCCTTCGCTGGCG   60
                         TCCTGGCCGCCTACGCATTAATCGCTATGGCACTGTGCCTGATCTCAGCCTTCGCTGGCG
LU20297_sequenced      1 TCCTGGCCGCCTACGCATTAATCGCTATGGCACTGTGCCTGATCTCAGCCTTCGCTGGCG   60

LU20297_planned       61 GTCATGTGGGCTTAATAGCCCTGACTTTATGCAGCGCCTTTATGTCGATTCAGTACCCAA  120
                         GTCATGTGGGCTTAATAGCCCTGACTTTATGCAGCGCCTTTATGTCGATTCAGTACCCAA
LU20297_sequenced     61 GTCATGTGGGCTTAATAGCCCTGACTTTATGCAGCGCCTTTATGTCGATTCAGTACCCAA  120

LU20297_planned      121 CAATCTTCTCGCTGGGCATTAAGAATCTCGGCCAGGACACCAAATATGGTTCGTCCTTCA  180
                         CAATCTTCTCGCTGGGCATTAAGAATCTCGGCCAGGACACCAAATATGGTTCGTCCTTCA
LU20297_sequenced    121 CAATCTTCTCGCTGGGCATTAAGAATCTCGGCCAGGACACCAAATATGGTTCGTCCTTCA  180

LU20297_planned      181 TCGTTATGACCATTATTGGCGGCGGTATTGTCACTCCGGTCATGGGTTTTGTCAGTGACG  240
                         TCGTTATGACCATTATTGGCGGCGGTATTGTCACTCCGGTCATGGGTTTTGTCAGTGACG
LU20297_sequenced    181 TCGTTATGACCATTATTGGCGGCGGTATTGTCACTCCGGTCATGGGTTTTGTCAGTGACG  240

LU20297_planned      241 CGGCGGGCAACATCCCCACTGCTGAACTGATCCCCGCACTCTGCTTCGCGGTCATCTTTA  300
                         CGGCGGGCAACATCCCCACTGCTGAACTGATCCCCGCACTCTGCTTCGCGGTCATCTTTA
LU20297_sequenced    241 CGGCGGGCAACATCCCCACTGCTGAACTGATCCCCGCACTCTGCTTCGCGGTCATCTTTA  300

LU20297_planned      301 TCTTTGCCCGTTTCCGTTCTCAAACGGCAACTAACTGAACATATTTTCCGAATAAAGTGG  360
                         TCTTTGCCCGTTTCCGTTCTCAAACGGCAACTAACTGAACATATTTTCCGAATAAAGTGG
LU20297_sequenced    301 TCTTTGCCCGTTTCCGTTCTCAAACGGCAACTAACTGAACATATTTTCCGAATAAAGTGG  360

LU20297_planned      361 AGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATT  420
                         AGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATT
LU20297_sequenced    361 AGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATT  420

LU20297_planned      421 TCACACAGGAAACAGAATTCATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCCTT  480
                         TCACACAGGAAACAGAATTCATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCCTT
LU20297_sequenced    421 TCACACAGGAAACAGAATTCATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCCTT  480

LU20297_planned      481 TAAAGTTGTGCAGATTTGTGGTGGTTTAGGTAATCAGATGTTTCAATACGCTTTTGCAAA  540
                         TAAAGTTGTGCAGATTTGTGGTGGTTTAGGTAATCAGATGTTTCAATACGCTTTTGCAAA
LU20297_sequenced    481 TAAAGTTGTGCAGATTTGTGGTGGTTTAGGTAATCAGATGTTTCAATACGCTTTTGCAAA  540

LU20297_planned      541 GTCCCTGCAGAAGCACCTGAACACGCCGGTCCTGCTGGATACCACGTCGTTCGATTGGAG  600
                         GTCCCTGCAGAAGCACCTGAACACGCCGGTCCTGCTGGATACCACGTCGTTCGATTGGAG
LU20297_sequenced    541 GTCCCTGCAGAAGCACCTGAACACGCCGGTCCTGCTGGATACCACGTCGTTCGATTGGAG  600

LU20297_planned      601 CAATCGTAAAATGCAACTGGAACTGTTCCCGATTGATTTGCCGTATGCGAACGCAAAAGA  660
                         CAATCGTAAAATGCAACTGGAACTGTTCCCGATTGATTTGCCGTATGCGAACGCAAAAGA
LU20297_sequenced    601 CAATCGTAAAATGCAACTGGAACTGTTCCCGATTGATTTGCCGTATGCGAACGCAAAAGA  660

LU20297_planned      661 AATTGCGATTGCCAAAATGCAGCACCTCCCGAAACTGGTTCGCGATGCGCTGAAGTACAT  720
                         AATTGCGATTGCCAAAATGCAGCACCTCCCGAAACTGGTTCGCGATGCGCTGAAGTACAT
LU20297_sequenced    661 AATTGCGATTGCCAAAATGCAGCACCTCCCGAAACTGGTTCGCGATGCGCTGAAGTACAT  720

LU20297_planned      721 CGGCTTCGACCGCGTCAGCCAAGAAATCGTTTTCGAGTATGAGCCGAAGCTGCTGAAGCC  780
                         CGGCTTCGACCGCGTCAGCCAAGAAATCGTTTTCGAGTATGAGCCGAAGCTGCTGAAGCC
LU20297_sequenced    721 CGGCTTCGACCGCGTCAGCCAAGAAATCGTTTTCGAGTATGAGCCGAAGCTGCTGAAGCC  780

LU20297_planned      781 GAGCCGTCTGACCTATTTCTTTGGCTACTTTCAGGATCCGCGTTACTTCGACGCAATTTC  840
                         GAGCCGTCTGACCTATTTCTTTGGCTACTTTCAGGATCCGCGTTACTTCGACGCAATTTC
LU20297_sequenced    781 GAGCCGTCTGACCTATTTCTTTGGCTACTTTCAGGATCCGCGTTACTTCGACGCAATTTC  840

LU20297_planned      841 CAGCCTGATTAAACAAACCTTCACTCTGCCACCACCTCCGGAGAATAACAAGAACAATAA  900
                         CAGCCTGATTAAACAAACCTTCACTCTGCCACCACCTCCGGAGAATAACAAGAACAATAA
LU20297_sequenced    841 CAGCCTGATTAAACAAACCTTCACTCTGCCACCACCTCCGGAGAATAACAAGAACAATAA  900

LU20297_planned      901 CAAAAAAGAGGAAGAGTACCAGCGCAAGTTGAGCCTGATCTTGGCGGCGAAGAACAGCGT  960
                         CAAAAAAGAGGAAGAGTACCAGCGCAAGTTGAGCCTGATCTTGGCGGCGAAGAACAGCGT
LU20297_sequenced    901 CAAAAAAGAGGAAGAGTACCAGCGCAAGTTGAGCCTGATCTTGGCGGCGAAGAACAGCGT  960

LU20297_planned      961 TTTTGTGCATATCCGCCGTGGTGACTACGTCGGTATTGGTTGCCAACTGGGCATCGACTA 1020
                         TTTTGTGCATATCCGCCGTGGTGACTACGTCGGTATTGGTTGCCAACTGGGCATCGACTA
LU20297_sequenced    961 TTTTGTGCATATCCGCCGTGGTGACTACGTCGGTATTGGTTGCCAACTGGGCATCGACTA 1020

LU20297_planned     1021 TCAAAAAAAAGCGTTGGAGTACATGGCGAAGCGTGTCCCGAATATGGAACTGTTTGTGTT 1080
                         TCAAAAAAAAGCGTTGGAGTACATGGCGAAGCGTGTCCCGAATATGGAACTGTTTGTGTT
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LU20297_sequenced   1021 TCAAAAAAAAGCGTTGGAGTACATGGCGAAGCGTGTCCCGAATATGGAACTGTTTGTGTT 1080

LU20297_planned     1081 TTGCGAAGATCTGAAGTTCACGCAGAATTTGGACCTGGGTTACCCGTTCACCGATATGAC 1140
                         TTGCGAAGATCTGAAGTTCACGCAGAATTTGGACCTGGGTTACCCGTTCACCGATATGAC
LU20297_sequenced   1081 TTGCGAAGATCTGAAGTTCACGCAGAATTTGGACCTGGGTTACCCGTTCACCGATATGAC 1140

LU20297_planned     1141 CACGCGTGACAAAGAAGAAGAGGCCTATTGGGACATGCTGCTGATGCAAAGCTGTAAACA 1200
                         CACGCGTGACAAAGAAGAAGAGGCCTATTGGGACATGCTGCTGATGCAAAGCTGTAAACA
LU20297_sequenced   1141 CACGCGTGACAAAGAAGAAGAGGCCTATTGGGACATGCTGCTGATGCAAAGCTGTAAACA 1200

LU20297_planned     1201 CGGCATCATCGCGAACAGCACCTACTCTTGGTGGGCGGCATATCTGATGGAGAACCCGGA 1260
                         CGGCATCATCGCGAACAGCACCTACTCTTGGTGGGCGGCATATCTGATGGAGAACCCGGA
LU20297_sequenced   1201 CGGCATCATCGCGAACAGCACCTACTCTTGGTGGGCGGCATATCTGATGGAGAACCCGGA 1260

LU20297_planned     1261 GAAAATCATTATCGGCCCGAAACATTGGCTGTTTGGTCACGAGAATATTCTTTGCAAGGA 1320
                         GAAAATCATTATCGGCCCGAAACATTGGCTGTTTGGTCACGAGAATATTCTTTGCAAGGA
LU20297_sequenced   1261 GAAAATCATTATCGGCCCGAAACATTGGCTGTTTGGTCACGAGAATATTCTTTGCAAGGA 1320

LU20297_planned     1321 ATGGGTGAAAATCGAGAGCCACTTCGAGGTGAAAAGCCAGAAGTACAACGCTTAACTCGA 1380
                         ATGGGTGAAAATCGAGAGCCACTTCGAGGTGAAAAGCCAGAAGTACAACGCTTAACTCGA
LU20297_sequenced   1321 ATGGGTGAAAATCGAGAGCCACTTCGAGGTGAAAAGCCAGAAGTACAACGCTTAACTCGA 1380

LU20297_planned     1381 GACAGAGGAATAATACATGTCAAAAGTCGCTCTCATCACCGGTGTAACCGGACAAGACGG 1440
                         GACAGAGGAATAATACATGTCAAAAGTCGCTCTCATCACCGGTGTAACCGGACAAGACGG
LU20297_sequenced   1381 GACAGAGGAATAATACATGTCAAAAGTCGCTCTCATCACCGGTGTAACCGGACAAGACGG 1440

LU20297_planned     1441 TTCTTACCTGGCAGAGTTTCTGCTGGAAAAAGGTTACGAGGTGCATGGTATTAAGCGTCG 1500
                         TTCTTACCTGGCAGAGTTTCTGCTGGAAAAAGGTTACGAGGTGCATGGTATTAAGCGTCG
LU20297_sequenced   1441 TTCTTACCTGGCAGAGTTTCTGCTGGAAAAAGGTTACGAGGTGCATGGTATTAAGCGTCG 1500

LU20297_planned     1501 CGCATCGTCATTCAACACCGAGCGCGTGGATCACATTTATCAGGATCCGCACACCTGCAA 1560
                         CGCATCGTCATTCAACACCGAGCGCGTGGATCACATTTATCAGGATCCGCACACCTGCAA
LU20297_sequenced   1501 CGCATCGTCATTCAACACCGAGCGCGTGGATCACATTTATCAGGATCCGCACACCTGCAA 1560

LU20297_planned     1561 CCCGAAATTCCATCTGCATTATGGCGACCTGAGTGATACCTCTAACCTGACGCGCATTTT 1620
                         CCCGAAATTCCATCTGCATTATGGCGACCTGAGTGATACCTCTAACCTGACGCGCATTTT
LU20297_sequenced   1561 CCCGAAATTCCATCTGCATTATGGCGACCTGAGTGATACCTCTAACCTGACGCGCATTTT 1620

LU20297_planned     1621 GCGTGAAGTACAGCCGGATGAAGTGTACAACCTGGGCGCAATGAGCCACGTTGCGGTCTC 1680
                         GCGTGAAGTACAGCCGGATGAAGTGTACAACCTGGGCGCAATGAGCCACGTTGCGGTCTC
LU20297_sequenced   1621 GCGTGAAGTACAGCCGGATGAAGTGTACAACCTGGGCGCAATGAGCCACGTTGCGGTCTC 1680

LU20297_planned     1681 TTTTGAGTCACCAGAATATACCGCTGACGTCGACGCGATGGGTACGCTGCGCCTGCTGGA 1740
                         TTTTGAGTCACCAGAATATACCGCTGACGTCGACGCGATGGGTACGCTGCGCCTGCTGGA
LU20297_sequenced   1681 TTTTGAGTCACCAGAATATACCGCTGACGTCGACGCGATGGGTACGCTGCGCCTGCTGGA 1740

LU20297_planned     1741 GGCGATCCGCTTCCTCGGTCTGGAAAAGAAAACTCGTTTCTATCAGGCTTCCACCTCTGA 1800
                         GGCGATCCGCTTCCTCGGTCTGGAAAAGAAAACTCGTTTCTATCAGGCTTCCACCTCTGA
LU20297_sequenced   1741 GGCGATCCGCTTCCTCGGTCTGGAAAAGAAAACTCGTTTCTATCAGGCTTCCACCTCTGA 1800

LU20297_planned     1801 ACTGTATGGTCTGGTGCAGGAAATTCCGCAGAAAGAGACCACGCCGTTCTACCCGCGATC 1860
                         ACTGTATGGTCTGGTGCAGGAAATTCCGCAGAAAGAGACCACGCCGTTCTACCCGCGATC
LU20297_sequenced   1801 ACTGTATGGTCTGGTGCAGGAAATTCCGCAGAAAGAGACCACGCCGTTCTACCCGCGATC 1860

LU20297_planned     1861 TCCGTATGCGGTCGCCAAACTGTACGCCTACTGGATCACCGTTAACTACCGTGAATCCTA 1920
                         TCCGTATGCGGTCGCCAAACTGTACGCCTACTGGATCACCGTTAACTACCGTGAATCCTA
LU20297_sequenced   1861 TCCGTATGCGGTCGCCAAACTGTACGCCTACTGGATCACCGTTAACTACCGTGAATCCTA 1920

LU20297_planned     1921 CGGCATGTACGCCTGTAACGGAATTCTCTTCAACCATGAATCCCCGCGCCGCGGCGAAAC 1980
                         CGGCATGTACGCCTGTAACGGAATTCTCTTCAACCATGAATCCCCGCGCCGCGGCGAAAC
LU20297_sequenced   1921 CGGCATGTACGCCTGTAACGGAATTCTCTTCAACCATGAATCCCCGCGCCGCGGCGAAAC 1980

LU20297_planned     1981 CTTCGTTACCCGCAAAATCACCCGCGCAATCGCCAACATCGCCCAGGGGCTGGAGTCGTG 2040
                         CTTCGTTACCCGCAAAATCACCCGCGCAATCGCCAACATCGCCCAGGGGCTGGAGTCGTG
LU20297_sequenced   1981 CTTCGTTACCCGCAAAATCACCCGCGCAATCGCCAACATCGCCCAGGGGCTGGAGTCGTG 2040

LU20297_planned     2041 CCTGTACCTCGGCAATATGGATTCCCTGCGTGACTGGGGCCACGCCAAAGACTACGTAAA 2100
                         CCTGTACCTCGGCAATATGGATTCCCTGCGTGACTGGGGCCACGCCAAAGACTACGTAAA
LU20297_sequenced   2041 CCTGTACCTCGGCAATATGGATTCCCTGCGTGACTGGGGCCACGCCAAAGACTACGTAAA 2100

LU20297_planned     2101 AATGCAGTGGATGATGCTGCAGCAGGAACAGCCGGAAGATTTCGTTATCGCGACCGGCGT 2160
                         AATGCAGTGGATGATGCTGCAGCAGGAACAGCCGGAAGATTTCGTTATCGCGACCGGCGT
LU20297_sequenced   2101 AATGCAGTGGATGATGCTGCAGCAGGAACAGCCGGAAGATTTCGTTATCGCGACCGGCGT 2160

LU20297_planned     2161 TCAGTACTCCGTGCGTCAGTTCGTGGAAATGGCGGCAGCACAGCTGGGCATCAAACTGCG 2220
                         TCAGTACTCCGTGCGTCAGTTCGTGGAAATGGCGGCAGCACAGCTGGGCATCAAACTGCG
LU20297_sequenced   2161 TCAGTACTCCGTGCGTCAGTTCGTGGAAATGGCGGCAGCACAGCTGGGCATCAAACTGCG 2220
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LU20297_planned     2221 CTTTGAAGGCACGGGCGTTGAAGAGAAGGGCATTGTGGTTTCCGTCACCGGGCATGACGC 2280
                         CTTTGAAGGCACGGGCGTTGAAGAGAAGGGCATTGTGGTTTCCGTCACCGGGCATGACGC
LU20297_sequenced   2221 CTTTGAAGGCACGGGCGTTGAAGAGAAGGGCATTGTGGTTTCCGTCACCGGGCATGACGC 2280

LU20297_planned     2281 GCCGGGCGTTAAACCGGGTGATGTGATTATCGCTGTTGACCCGCGTTACTTCCGTCCGGC 2340
                         GCCGGGCGTTAAACCGGGTGATGTGATTATCGCTGTTGACCCGCGTTACTTCCGTCCGGC
LU20297_sequenced   2281 GCCGGGCGTTAAACCGGGTGATGTGATTATCGCTGTTGACCCGCGTTACTTCCGTCCGGC 2340

LU20297_planned     2341 TGAAGTTGAAACGCTGCTCGGCGACCCGACCAAAGCGCACGAAAAACTGGGCTGGAAACC 2400
                         TGAAGTTGAAACGCTGCTCGGCGACCCGACCAAAGCGCACGAAAAACTGGGCTGGAAACC
LU20297_sequenced   2341 TGAAGTTGAAACGCTGCTCGGCGACCCGACCAAAGCGCACGAAAAACTGGGCTGGAAACC 2400

LU20297_planned     2401 GGAAATCACCCTCAGAGAGATGGTGTCTGAAATGGTGGCTAATGACCTCGAAGCGGCGAA 2460
                         GGAAATCACCCTCAGAGAGATGGTGTCTGAAATGGTGGCTAATGACCTCGAAGCGGCGAA
LU20297_sequenced   2401 GGAAATCACCCTCAGAGAGATGGTGTCTGAAATGGTGGCTAATGACCTCGAAGCGGCGAA 2460

LU20297_planned     2461 AAAACACTCTCTGCTGAAATCTCACGGCTACGACGTGGCGATCGCGCTGGAGTCATAAGC 2520
                         AAAACACTCTCTGCTGAAATCTCACGGCTACGACGTGGCGATCGCGCTGGAGTCATAAGC
LU20297_sequenced   2461 AAAACACTCTCTGCTGAAATCTCACGGCTACGACGTGGCGATCGCGCTGGAGTCATAAGC 2520

LU20297_planned     2521 ATGAGTAAACAACGAGTTTTTATTGCTGGTCATCGCGGGATGGTCGGTTCCGCCATCAGG 2580
                         ATGAGTAAACAACGAGTTTTTATTGCTGGTCATCGCGGGATGGTCGGTTCCGCCATCAGG
LU20297_sequenced   2521 ATGAGTAAACAACGAGTTTTTATTGCTGGTCATCGCGGGATGGTCGGTTCCGCCATCAGG 2580

LU20297_planned     2581 CGGCAGCTCGAACAGCGCGGTGATGTGGAACTGGTATTACGCACCCGCGACGAGCTGAAC 2640
                         CGGCAGCTCGAACAGCGCGGTGATGTGGAACTGGTATTACGCACCCGCGACGAGCTGAAC
LU20297_sequenced   2581 CGGCAGCTCGAACAGCGCGGTGATGTGGAACTGGTATTACGCACCCGCGACGAGCTGAAC 2640

LU20297_planned     2641 CTGCTGGACAGCCGCGCCGTGCATGATTTCTTTGCCAGCGAACGTATTGACCAGGTCTAT 2700
                         CTGCTGGACAGCCGCGCCGTGCATGATTTCTTTGCCAGCGAACGTATTGACCAGGTCTAT
LU20297_sequenced   2641 CTGCTGGACAGCCGCGCCGTGCATGATTTCTTTGCCAGCGAACGTATTGACCAGGTCTAT 2700

LU20297_planned     2701 CTGGCGGCGGCGAAAGTGGGCGGCATTGTTGCCAACAACACCTATCCGGCGGATTTCATC 2760
                         CTGGCGGCGGCGAAAGTGGGCGGCATTGTTGCCAACAACACCTATCCGGCGGATTTCATC
LU20297_sequenced   2701 CTGGCGGCGGCGAAAGTGGGCGGCATTGTTGCCAACAACACCTATCCGGCGGATTTCATC 2760

LU20297_planned     2761 TACCAGAACATGATGATTGAGAGCAACATCATTCACGCCGCGCATCAGAACGACGTGAAC 2820
                         TACCAGAACATGATGATTGAGAGCAACATCATTCACGCCGCGCATCAGAACGACGTGAAC
LU20297_sequenced   2761 TACCAGAACATGATGATTGAGAGCAACATCATTCACGCCGCGCATCAGAACGACGTGAAC 2820

LU20297_planned     2821 AAACTGCTGTTTCTCGGATCGTCCTGCATCTACCCGAAACTGGCAAAACAGCCGATGGCA 2880
                         AAACTGCTGTTTCTCGGATCGTCCTGCATCTACCCGAAACTGGCAAAACAGCCGATGGCA
LU20297_sequenced   2821 AAACTGCTGTTTCTCGGATCGTCCTGCATCTACCCGAAACTGGCAAAACAGCCGATGGCA 2880

LU20297_planned     2881 GAAAGCGAGTTGTTGCAGGGCACGCTGGAGCCGACTAACGAGCCTTATGCTATTGCCAAA 2940
                         GAAAGCGAGTTGTTGCAGGGCACGCTGGAGCCGACTAACGAGCCTTATGCTATTGCCAAA
LU20297_sequenced   2881 GAAAGCGAGTTGTTGCAGGGCACGCTGGAGCCGACTAACGAGCCTTATGCTATTGCCAAA 2940

LU20297_planned     2941 ATCGCCGGGATCAAACTGTGCGAATCATACAACCGCCAGTACGGACGCGATTACCGCTCA 3000
                         ATCGCCGGGATCAAACTGTGCGAATCATACAACCGCCAGTACGGACGCGATTACCGCTCA
LU20297_sequenced   2941 ATCGCCGGGATCAAACTGTGCGAATCATACAACCGCCAGTACGGACGCGATTACCGCTCA 3000

LU20297_planned     3001 GTCATGCCGACCAACCTGTACGGGCCACACGACAACTTCCACCCGAGTAATTCGCATGTG 3060
                         GTCATGCCGACCAACCTGTACGGGCCACACGACAACTTCCACCCGAGTAATTCGCATGTG
LU20297_sequenced   3001 GTCATGCCGACCAACCTGTACGGGCCACACGACAACTTCCACCCGAGTAATTCGCATGTG 3060

LU20297_planned     3061 ATCCCAGCATTGCTGCGTCGCTTCCACGAGGCGACGGCACAGAATGCGCCGGACGTGGTG 3120
                         ATCCCAGCATTGCTGCGTCGCTTCCACGAGGCGACGGCACAGAATGCGCCGGACGTGGTG
LU20297_sequenced   3061 ATCCCAGCATTGCTGCGTCGCTTCCACGAGGCGACGGCACAGAATGCGCCGGACGTGGTG 3120

LU20297_planned     3121 GTATGGGGCAGCGGTACACCGATGCGCGAATTTCTGCACGTCGATGATATGGCGGCGGCG 3180
                         GTATGGGGCAGCGGTACACCGATGCGCGAATTTCTGCACGTCGATGATATGGCGGCGGCG
LU20297_sequenced   3121 GTATGGGGCAGCGGTACACCGATGCGCGAATTTCTGCACGTCGATGATATGGCGGCGGCG 3180

LU20297_planned     3181 AGCATTCATGTCATGGAGCTGGCGCATGAAGTCTGGCTGGAGAACACCCAGCCGATGTTG 3240
                         AGCATTCATGTCATGGAGCTGGCGCATGAAGTCTGGCTGGAGAACACCCAGCCGATGTTG
LU20297_sequenced   3181 AGCATTCATGTCATGGAGCTGGCGCATGAAGTCTGGCTGGAGAACACCCAGCCGATGTTG 3240

LU20297_planned     3241 TCGCACATTAACGTCGGCACGGGCGTTGACTGCACTATCCGCGAGCTGGCGCAAACCATC 3300
                         TCGCACATTAACGTCGGCACGGGCGTTGACTGCACTATCCGCGAGCTGGCGCAAACCATC
LU20297_sequenced   3241 TCGCACATTAACGTCGGCACGGGCGTTGACTGCACTATCCGCGAGCTGGCGCAAACCATC 3300

LU20297_planned     3301 GCCAAAGTGGTGGGTTACAAAGGCCGGGTGGTTTTTGATGCCAGCAAACCGGATGGCACG 3360
                         GCCAAAGTGGTGGGTTACAAAGGCCGGGTGGTTTTTGATGCCAGCAAACCGGATGGCACG
LU20297_sequenced   3301 GCCAAAGTGGTGGGTTACAAAGGCCGGGTGGTTTTTGATGCCAGCAAACCGGATGGCACG 3360

LU20297_planned     3361 CCGCGCAAACTGCTGGATGTGACGCGCCTGCATCAGCTTGGCTGGTATCACGAAATCTCA 3420
                         CCGCGCAAACTGCTGGATGTGACGCGCCTGCATCAGCTTGGCTGGTATCACGAAATCTCA
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LU20297_sequenced   3361 CCGCGCAAACTGCTGGATGTGACGCGCCTGCATCAGCTTGGCTGGTATCACGAAATCTCA 3420

LU20297_planned     3421 CTGGAAGCGGGGCTTGCCAGCACTTACCAGTGGTTCCTTGAGAATCAAGACCGCTTTCGG 3480
                         CTGGAAGCGGGGCTTGCCAGCACTTACCAGTGGTTCCTTGAGAATCAAGACCGCTTTCGG
LU20297_sequenced   3421 CTGGAAGCGGGGCTTGCCAGCACTTACCAGTGGTTCCTTGAGAATCAAGACCGCTTTCGG 3480

LU20297_planned     3481 GGGTAACCTGCAGGAAGAGGAGAAATTAACTATGGCTCAATCTAAATTGTATCCTGTTGT 3540
                         GGGTAACCTGCAGGAAGAGGAGAAATTAACTATGGCTCAATCTAAATTGTATCCTGTTGT
LU20297_sequenced   3481 GGGTAACCTGCAGGAAGAGGAGAAATTAACTATGGCTCAATCTAAATTGTATCCTGTTGT 3540

LU20297_planned     3541 GATGGCTGGCGGTTCTGGTTCACGTTTGTGGCCACTGAGCCGTGTGCTGTATCCGAAACA 3600
                         GATGGCTGGCGGTTCTGGTTCACGTTTGTGGCCACTGAGCCGTGTGCTGTATCCGAAACA
LU20297_sequenced   3541 GATGGCTGGCGGTTCTGGTTCACGTTTGTGGCCACTGAGCCGTGTGCTGTATCCGAAACA 3600

LU20297_planned     3601 ATTCCTGTGTCTGAAAGGTGATCTGACCATGCTGCAAACCACCATTTGCCGCCTGAACGG 3660
                         ATTCCTGTGTCTGAAAGGTGATCTGACCATGCTGCAAACCACCATTTGCCGCCTGAACGG
LU20297_sequenced   3601 ATTCCTGTGTCTGAAAGGTGATCTGACCATGCTGCAAACCACCATTTGCCGCCTGAACGG 3660

LU20297_planned     3661 CGTCGAATGCGAGAGCCCGGTCGTTATCTGCAATGAACAACACCGCTTCATCGTCGCAGA 3720
                         CGTCGAATGCGAGAGCCCGGTCGTTATCTGCAATGAACAACACCGCTTCATCGTCGCAGA
LU20297_sequenced   3661 CGTCGAATGCGAGAGCCCGGTCGTTATCTGCAATGAACAACACCGCTTCATCGTCGCAGA 3720

LU20297_planned     3721 ACAGCTGCGTCAGCTGAACAAACTGACGGAGAACATTATTCTGGAGCCTGCGGGTCGTAA 3780
                         ACAGCTGCGTCAGCTGAACAAACTGACGGAGAACATTATTCTGGAGCCTGCGGGTCGTAA
LU20297_sequenced   3721 ACAGCTGCGTCAGCTGAACAAACTGACGGAGAACATTATTCTGGAGCCTGCGGGTCGTAA 3780

LU20297_planned     3781 CACCGCACCAGCAATTGCACTGGCTGCATTGGCAGCGAAGCGTCATAGCCCGGAATCCGA 3840
                         CACCGCACCAGCAATTGCACTGGCTGCATTGGCAGCGAAGCGTCATAGCCCGGAATCCGA
LU20297_sequenced   3781 CACCGCACCAGCAATTGCACTGGCTGCATTGGCAGCGAAGCGTCATAGCCCGGAATCCGA 3840

LU20297_planned     3841 CCCGCTGATGCTGGTGCTTGCGGCCGACCACGTGATCGCCGACGAGGACGCATTTCGTGC 3900
                         CCCGCTGATGCTGGTGCTTGCGGCCGACCACGTGATCGCCGACGAGGACGCATTTCGTGC
LU20297_sequenced   3841 CCCGCTGATGCTGGTGCTTGCGGCCGACCACGTGATCGCCGACGAGGACGCATTTCGTGC 3900

LU20297_planned     3901 CGCCGTTCGTAACGCTATGCCATACGCAGAGGCGGGCAAACTGGTTACGTTCGGTATCGT 3960
                         CGCCGTTCGTAACGCTATGCCATACGCAGAGGCGGGCAAACTGGTTACGTTCGGTATCGT
LU20297_sequenced   3901 CGCCGTTCGTAACGCTATGCCATACGCAGAGGCGGGCAAACTGGTTACGTTCGGTATCGT 3960

LU20297_planned     3961 TCCGGATCTGCCGGAAACCGGCTATGGCTACATTCGTCGTGGCGAGGTTTCTGCGGGTGA 4020
                         TCCGGATCTGCCGGAAACCGGCTATGGCTACATTCGTCGTGGCGAGGTTTCTGCGGGTGA
LU20297_sequenced   3961 TCCGGATCTGCCGGAAACCGGCTATGGCTACATTCGTCGTGGCGAGGTTTCTGCGGGTGA 4020

LU20297_planned     4021 GCAAGATATGGTTGCGTTTGAGGTTGCTCAGTTCGTGGAAAAACCGAACTTGGAAACCGC 4080
                         GCAAGATATGGTTGCGTTTGAGGTTGCTCAGTTCGTGGAAAAACCGAACTTGGAAACCGC
LU20297_sequenced   4021 GCAAGATATGGTTGCGTTTGAGGTTGCTCAGTTCGTGGAAAAACCGAACTTGGAAACCGC 4080

LU20297_planned     4081 GCAGGCCTATGTCGCGTCGGGTGAGTATTACTGGAATAGCGGTATGTTTCTGTTTCGTGC 4140
                         GCAGGCCTATGTCGCGTCGGGTGAGTATTACTGGAATAGCGGTATGTTTCTGTTTCGTGC
LU20297_sequenced   4081 GCAGGCCTATGTCGCGTCGGGTGAGTATTACTGGAATAGCGGTATGTTTCTGTTTCGTGC 4140

LU20297_planned     4141 TGGTCGCTACCTGGAGGAGTTGAAGAAATACCGTCCGGATATCCTGGACGCGTGTGAGAA 4200
                         TGGTCGCTACCTGGAGGAGTTGAAGAAATACCGTCCGGATATCCTGGACGCGTGTGAGAA
LU20297_sequenced   4141 TGGTCGCTACCTGGAGGAGTTGAAGAAATACCGTCCGGATATCCTGGACGCGTGTGAGAA 4200

LU20297_planned     4201 AGCCATGTCCGCGGTGGATCCGGACTTAAACTTTATTCGCGTGGACGAGGAAGCGTTCTT 4260
                         AGCCATGTCCGCGGTGGATCCGGACTTAAACTTTATTCGCGTGGACGAGGAAGCGTTCTT
LU20297_sequenced   4201 AGCCATGTCCGCGGTGGATCCGGACTTAAACTTTATTCGCGTGGACGAGGAAGCGTTCTT 4260

LU20297_planned     4261 GGCGTGCCCGGAAGAGAGCGTCGACTACGCAGTGATGGAACGTACTGCGGATGCGGTTGT 4320
                         GGCGTGCCCGGAAGAGAGCGTCGACTACGCAGTGATGGAACGTACTGCGGATGCGGTTGT
LU20297_sequenced   4261 GGCGTGCCCGGAAGAGAGCGTCGACTACGCAGTGATGGAACGTACTGCGGATGCGGTTGT 4320

LU20297_planned     4321 GGTGCCGATGGATGCAGGCTGGAGCGATGTCGGTTCGTGGAGCAGCCTGTGGGAGATTAG 4380
                         GGTGCCGATGGATGCAGGCTGGAGCGATGTCGGTTCGTGGAGCAGCCTGTGGGAGATTAG
LU20297_sequenced   4321 GGTGCCGATGGATGCAGGCTGGAGCGATGTCGGTTCGTGGAGCAGCCTGTGGGAGATTAG 4380

LU20297_planned     4381 CGCACACACGGCCGAGGGTAATGTTTGTCACGGCGATGTGATCAACCACAAGACCGAGAA 4440
                         CGCACACACGGCCGAGGGTAATGTTTGTCACGGCGATGTGATCAACCACAAGACCGAGAA
LU20297_sequenced   4381 CGCACACACGGCCGAGGGTAATGTTTGTCACGGCGATGTGATCAACCACAAGACCGAGAA 4440

LU20297_planned     4441 TAGCTACGTCTATGCGGAGAGCGGTTTGGTTACGACCGTGGGCGTCAAAGACCTGGTCGT 4500
                         TAGCTACGTCTATGCGGAGAGCGGTTTGGTTACGACCGTGGGCGTCAAAGACCTGGTCGT
LU20297_sequenced   4441 TAGCTACGTCTATGCGGAGAGCGGTTTGGTTACGACCGTGGGCGTCAAAGACCTGGTCGT 4500

LU20297_planned     4501 TGTTCAGACCAAAGACGCGGTCCTGATCGCAGATCGTAATGCGGTCCAGGATGTTAAGAA 4560
                         TGTTCAGACCAAAGACGCGGTCCTGATCGCAGATCGTAATGCGGTCCAGGATGTTAAGAA
LU20297_sequenced   4501 TGTTCAGACCAAAGACGCGGTCCTGATCGCAGATCGTAATGCGGTCCAGGATGTTAAGAA 4560
```

```
LU20297_planned      4561 AGTTGTTGAACAGATTAAGGCCGATGGCCGCCATGAACACCGTGTCCATCGCGAAGTTTA 4620
                          AGTTGTTGAACAGATTAAGGCCGATGGCCGCCATGAACACCGTGTCCATCGCGAAGTTTA
LU20297_sequenced    4561 AGTTGTTGAACAGATTAAGGCCGATGGCCGCCATGAACACCGTGTCCATCGCGAAGTTTA 4620

LU20297_planned      4621 CCGTCCGTGGGGTAAGTACGACAGCATCGACGCGGGTGACAGATACCAAGTCAAGCGTAT 4680
                          CCGTCCGTGGGGTAAGTACGACAGCATCGACGCGGGTGACAGATACCAAGTCAAGCGTAT
LU20297_sequenced    4621 CCGTCCGTGGGGTAAGTACGACAGCATCGACGCGGGTGACAGATACCAAGTCAAGCGTAT 4680

LU20297_planned      4681 TACCGTCAAGCCTGGCGAAGGCCTGAGCGTGCAGATGCACCACCATCGCGCGGAGCATTG 4740
                          TACCGTCAAGCCTGGCGAAGGCCTGAGCGTGCAGATGCACCACCATCGCGCGGAGCATTG
LU20297_sequenced    4681 TACCGTCAAGCCTGGCGAAGGCCTGAGCGTGCAGATGCACCACCATCGCGCGGAGCATTG 4740

LU20297_planned      4741 GGTAGTTGTGGCGGGTACGGCCAAAGTGACTATTGATGGTGACATCAAGTTGCTGGGCGA 4800
                          GGTAGTTGTGGCGGGTACGGCCAAAGTGACTATTGATGGTGACATCAAGTTGCTGGGCGA
LU20297_sequenced    4741 GGTAGTTGTGGCGGGTACGGCCAAAGTGACTATTGATGGTGACATCAAGTTGCTGGGCGA 4800

LU20297_planned      4801 GAATGAAAGCATCTATATCCCGCTGGGTGCAACGCACTGCCTGGAAAACCCGGGCAAAAT 4860
                          GAATGAAAGCATCTATATCCCGCTGGGTGCAACGCACTGCCTGGAAAACCCGGGCAAAAT
LU20297_sequenced    4801 GAATGAAAGCATCTATATCCCGCTGGGTGCAACGCACTGCCTGGAAAACCCGGGCAAAAT 4860

LU20297_planned      4861 TCCGCTGGACCTGATTGAAGTTCGTTCCGGCTCCTACCTGGAAGAAGATGATGTCGTTCG 4920
                          TCCGCTGGACCTGATTGAAGTTCGTTCCGGCTCCTACCTGGAAGAAGATGATGTCGTTCG
LU20297_sequenced    4861 TCCGCTGGACCTGATTGAAGTTCGTTCCGGCTCCTACCTGGAAGAAGATGATGTCGTTCG 4920

LU20297_planned      4921 TTTCGCGGACCGTTATGGTCGCGTCTAATCGATAAGAGGAGAAATTAACTATGGCAGCCA 4980
                          TTTCGCGGACCGTTATGGTCGCGTCTAATCGATAAGAGGAGAAATTAACTATGGCAGCCA
LU20297_sequenced    4921 TTTCGCGGACCGTTATGGTCGCGTCTAATCGATAAGAGGAGAAATTAACTATGGCAGCCA 4980

LU20297_planned      4981 GCGTACGCGCGAACTATTGTCTGAAAAAGGGTAATGACATGAAGAAACTGACGTGCTTCA 5040
                          GCGTACGCGCGAACTATTGTCTGAAAAAGGGTAATGACATGAAGAAACTGACGTGCTTCA
LU20297_sequenced    4981 GCGTACGCGCGAACTATTGTCTGAAAAAGGGTAATGACATGAAGAAACTGACGTGCTTCA 5040

LU20297_planned      5041 AAGCGTACGACATCCGTGGTAAATTGGGTGAAGAACTGAATGAAGATATTGCCTGGCGCA 5100
                          AAGCGTACGACATCCGTGGTAAATTGGGTGAAGAACTGAATGAAGATATTGCCTGGCGCA
LU20297_sequenced    5041 AAGCGTACGACATCCGTGGTAAATTGGGTGAAGAACTGAATGAAGATATTGCCTGGCGCA 5100

LU20297_planned      5101 TTGGTCGTGCGTATGGTGAGTTCCTGAAACCGAAAACGATCGTTCTGGGTGGTGACGTCC 5160
                          TTGGTCGTGCGTATGGTGAGTTCCTGAAACCGAAAACGATCGTTCTGGGTGGTGACGTCC
LU20297_sequenced    5101 TTGGTCGTGCGTATGGTGAGTTCCTGAAACCGAAAACGATCGTTCTGGGTGGTGACGTCC 5160

LU20297_planned      5161 GTCTGACCAGCGAAACCCTGAAGCTGGCGTTGGCGAAGGGTCTGCAAGATGCGGGCGTCG 5220
                          GTCTGACCAGCGAAACCCTGAAGCTGGCGTTGGCGAAGGGTCTGCAAGATGCGGGCGTCG
LU20297_sequenced    5161 GTCTGACCAGCGAAACCCTGAAGCTGGCGTTGGCGAAGGGTCTGCAAGATGCGGGCGTCG 5220

LU20297_planned      5221 ATGTTCTGGATATCGGCATGTCTGGCACCGAAGAAATCTATTTTGCAACCTTCCACCTGG 5280
                          ATGTTCTGGATATCGGCATGTCTGGCACCGAAGAAATCTATTTTGCAACCTTCCACCTGG
LU20297_sequenced    5221 ATGTTCTGGATATCGGCATGTCTGGCACCGAAGAAATCTATTTTGCAACCTTCCACCTGG 5280

LU20297_planned      5281 GCGTGGATGGTGGCATTGAAGTCACCGCCTCCCATAATCCGATGGACTACAACGGCATGA 5340
                          GCGTGGATGGTGGCATTGAAGTCACCGCCTCCCATAATCCGATGGACTACAACGGCATGA
LU20297_sequenced    5281 GCGTGGATGGTGGCATTGAAGTCACCGCCTCCCATAATCCGATGGACTACAACGGCATGA 5340

LU20297_planned      5341 AACTGGTGCGTGAGGGTGCGCGTCCGATTAGCGGTGATACCGGTCTGCGTGACGTGCAAC 5400
                          AACTGGTGCGTGAGGGTGCGCGTCCGATTAGCGGTGATACCGGTCTGCGTGACGTGCAAC
LU20297_sequenced    5341 AACTGGTGCGTGAGGGTGCGCGTCCGATTAGCGGTGATACCGGTCTGCGTGACGTGCAAC 5400

LU20297_planned      5401 GTCTGGCTGAGGCGAACGATTTTCCGCCTGTGGACGAAACCAAGCGTGGCCGCTACCAAC 5460
                          GTCTGGCTGAGGCGAACGATTTTCCGCCTGTGGACGAAACCAAGCGTGGCCGCTACCAAC
LU20297_sequenced    5401 GTCTGGCTGAGGCGAACGATTTTCCGCCTGTGGACGAAACCAAGCGTGGCCGCTACCAAC 5460

LU20297_planned      5461 AGATTAACTTGCGCGATGCGTACGTGGATCACCTGTTCGGTTACATCAATGTCAAGAACC 5520
                          AGATTAACTTGCGCGATGCGTACGTGGATCACCTGTTCGGTTACATCAATGTCAAGAACC
LU20297_sequenced    5461 AGATTAACTTGCGCGATGCGTACGTGGATCACCTGTTCGGTTACATCAATGTCAAGAACC 5520

LU20297_planned      5521 TGACCCCGCTGAAGCTGGTTATCAATAGCGGTAATGGTGCAGCTGGCCCAGTGGTCGATG 5580
                          TGACCCCGCTGAAGCTGGTTATCAATAGCGGTAATGGTGCAGCTGGCCCAGTGGTCGATG
LU20297_sequenced    5521 TGACCCCGCTGAAGCTGGTTATCAATAGCGGTAATGGTGCAGCTGGCCCAGTGGTCGATG 5580

LU20297_planned      5581 CGATTGAGGCGCGCTTTAAGGCTCTGGGTGCACCGGTCGAGCTGATCAAAGTTCACAACA 5640
                          CGATTGAGGCGCGCTTTAAGGCTCTGGGTGCACCGGTCGAGCTGATCAAAGTTCACAACA
LU20297_sequenced    5581 CGATTGAGGCGCGCTTTAAGGCTCTGGGTGCACCGGTCGAGCTGATCAAAGTTCACAACA 5640

LU20297_planned      5641 CGCCGGACGGTAACTTTCCGAACGGTATCCCAAATCCGCTGCTGCCGGAATGTCGTGACG 5700
                          CGCCGGACGGTAACTTTCCGAACGGTATCCCAAATCCGCTGCTGCCGGAATGTCGTGACG
LU20297_sequenced    5641 CGCCGGACGGTAACTTTCCGAACGGTATCCCAAATCCGCTGCTGCCGGAATGTCGTGACG 5700

LU20297_planned      5701 ACACCCGCAATGCAGTGATCAAGCATGGCGCGGATATGGGCATTGCGTTCGACGGTGACT 5760
                          ACACCCGCAATGCAGTGATCAAGCATGGCGCGGATATGGGCATTGCGTTCGACGGTGACT
```

```
LU20297_sequenced   5701 ACACCCGCAATGCAGTGATCAAGCATGGCGCGGATATGGGCATTGCGTTCGACGGTGACT 5760

LU20297_planned     5761 TTGACCGTTGTTTCTTGTTTGATGAGAAAGGCCAATTCATTGAGGGTTACTACATCGTGG 5820
                         TTGACCGTTGTTTCTTGTTTGATGAGAAAGGCCAATTCATTGAGGGTTACTACATCGTGG
LU20297_sequenced   5761 TTGACCGTTGTTTCTTGTTTGATGAGAAAGGCCAATTCATTGAGGGTTACTACATCGTGG 5820

LU20297_planned     5821 GTTTGCTGGCGGAAGCATTTCTGGAGAAAAACCCGGGTGCCAAGATTATTCACGACCCGC 5880
                         GTTTGCTGGCGGAAGCATTTCTGGAGAAAAACCCGGGTGCCAAGATTATTCACGACCCGC
LU20297_sequenced   5821 GTTTGCTGGCGGAAGCATTTCTGGAGAAAAACCCGGGTGCCAAGATTATTCACGACCCGC 5880

LU20297_planned     5881 GTCTGAGCTGGAACACCGTCGATGTTGTGACGGCTGCTGGCGGTACTCCGGTTATGTCTA 5940
                         GTCTGAGCTGGAACACCGTCGATGTTGTGACGGCTGCTGGCGGTACTCCGGTTATGTCTA
LU20297_sequenced   5881 GTCTGAGCTGGAACACCGTCGATGTTGTGACGGCTGCTGGCGGTACTCCGGTTATGTCTA 5940

LU20297_planned     5941 AGACGGGCCACGCATTCATTAAAGAGCGCATGCGCAAAGAAGATGCGATTTATGGTGGCG 6000
                         AGACGGGCCACGCATTCATTAAAGAGCGCATGCGCAAAGAAGATGCGATTTATGGTGGCG
LU20297_sequenced   5941 AGACGGGCCACGCATTCATTAAAGAGCGCATGCGCAAAGAAGATGCGATTTATGGTGGCG 6000

LU20297_planned     6001 AGATGTCCGCGCATCATTACTTCCGTGATTTCGCCTATTGCGACTCCGGCATGATCCCGT 6060
                         AGATGTCCGCGCATCATTACTTCCGTGATTTCGCCTATTGCGACTCCGGCATGATCCCGT
LU20297_sequenced   6001 AGATGTCCGCGCATCATTACTTCCGTGATTTCGCCTATTGCGACTCCGGCATGATCCCGT 6060

LU20297_planned     6061 GGCTGCTGGTTGCGGAACTGGTCTGCCTGAAAGACAAAACCCTCGGCGAGCTGGTTAGAG 6120
                         GGCTGCTGGTTGCGGAACTGGTCTGCCTGAAAGACAAAACCCTCGGCGAGCTGGTTAGAG
LU20297_sequenced   6061 GGCTGCTGGTTGCGGAACTGGTCTGCCTGAAAGACAAAACCCTCGGCGAGCTGGTTAGAG 6120

LU20297_planned     6121 ATCGCATGGCCGCATTTCCTGCGAGCGGTGAGATCAATTCGAAGTTGGCGCAGCCGGTTG 6180
                         ATCGCATGGCCGCATTTCCTGCGAGCGGTGAGATCAATTCGAAGTTGGCGCAGCCGGTTG
LU20297_sequenced   6121 ATCGCATGGCCGCATTTCCTGCGAGCGGTGAGATCAATTCGAAGTTGGCGCAGCCGGTTG 6180

LU20297_planned     6181 AGGCCATTAACCGTGTGGAGCAGCACTTCAGCCGTGAAGCCTTGGCTGTCGACCGTACCG 6240
                         AGGCCATTAACCGTGTGGAGCAGCACTTCAGCCGTGAAGCCTTGGCTGTCGACCGTACCG
LU20297_sequenced   6181 AGGCCATTAACCGTGTGGAGCAGCACTTCAGCCGTGAAGCCTTGGCTGTCGACCGTACCG 6240

LU20297_planned     6241 ACGGTATCAGCATGACCTTTGCAGACTGGCGCTTCAACTTACGTACCAGCAATACGGAAC 6300
                         ACGGTATCAGCATGACCTTTGCAGACTGGCGCTTCAACTTACGTACCAGCAATACGGAAC
LU20297_sequenced   6241 ACGGTATCAGCATGACCTTTGCAGACTGGCGCTTCAACTTACGTACCAGCAATACGGAAC 6300

LU20297_planned     6301 CGGTCGTTCGTCTGAACGTTGAGAGCCGTGGCGATGTGCCGCTGATGGAAGCGCGCACTC 6360
                         CGGTCGTTCGTCTGAACGTTGAGAGCCGTGGCGATGTGCCGCTGATGGAAGCGCGCACTC
LU20297_sequenced   6301 CGGTCGTTCGTCTGAACGTTGAGAGCCGTGGCGATGTGCCGCTGATGGAAGCGCGCACTC 6360

LU20297_planned     6361 GCACTCTGTTGACGCTGCTGAATGAGTAATGAACTAGTGCCAAATAAAACGAAAGGCTCA 6420
                         GCACTCTGTTGACGCTGCTGAATGAGTAATGAACTAGTGCCAAATAAAACGAAAGGCTCA
LU20297_sequenced   6361 GCACTCTGTTGACGCTGCTGAATGAGTAATGAACTAGTGCCAAATAAAACGAAAGGCTCA 6420

LU20297_planned     6421 GTCGGAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAG 6480
                         GTCGGAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAG
LU20297_sequenced   6421 GTCGGAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAG 6480

LU20297_planned     6481 GACAAATTTAATTAAGTACTGCTTAATACCACGCGGGGGCATTTCTATCGCGCGGCGCTG 6540
                         GACAAATTTAATTAAGTACTGCTTAATACCACGCGGGGGCATTTCTATCGCGCGGCGCTG
LU20297_sequenced   6481 GACAAATTTAATTAAGTACTGCTTAATACCACGCGGGGGCATTTCTATCGCGCGGCGCTG 6540

LU20297_planned     6541 GAAGGGTTAACTGCGCAATTACAGCGCAATCTACAGATGCTGGAAAAAATCGGGCACTTT 6600
                         GAAGGGTTAACTGCGCAATTACAGCGCAATCTACAGATGCTGGAAAAAATCGGGCACTTT
LU20297_sequenced   6541 GAAGGGTTAACTGCGCAATTACAGCGCAATCTACAGATGCTGGAAAAAATCGGGCACTTT 6600

LU20297_planned     6601 AAGGCCTCTGAATTATTGTTAGTCGGTGGAGGAAGTCGCAACACATTGTGGAATCAGATT 6660
                         AAGGCCTCTGAATTATTGTTAGTCGGTGGAGGAAGTCGCAACACATTGTGGAATCAGATT
LU20297_sequenced   6601 AAGGCCTCTGAATTATTGTTAGTCGGTGGAGGAAGTCGCAACACATTGTGGAATCAGATT 6660

LU20297_planned     6661 AAAGCCAATATGCTTGATATTCCGGTAAAAGTTCTCGACGACGCCGAAACGACCGTCGCA 6720
                         AAAGCCAATATGCTTGATATTCCGGTAAAAGTTCTCGACGACGCCGAAACGACCGTCGCA
LU20297_sequenced   6661 AAAGCCAATATGCTTGATATTCCGGTAAAAGTTCTCGACGACGCCGAAACGACCGTCGCA 6720

LU20297_planned     6721 GGAGCTGCGCTGTTCGGTTGGTATGGCGTAGGGGAATTTAACAGCCCGGAAGAAGCCCGC 6780
                         GGAGCTGCGCTGTTCGGTTGGTATGGCGTAGGGGAATTTAACAGCCCGGAAGAAGCCCGC
LU20297_sequenced   6721 GGAGCTGCGCTGTTCGGTTGGTATGGCGTAGGGGAATTTAACAGCCCGGAAGAAGCCCGC 6780

LU20297_planned     6781 GCACAGATTCATTATCAGTACCGTTATTTCTACCCGCAAACTGAACCTGAATTTATAGAG 6840
                         GCACAGATTCATTATCAGTACCGTTATTTCTACCCGCAAACTGAACCTGAATTTATAGAG
LU20297_sequenced   6781 GCACAGATTCATTATCAGTACCGTTATTTCTACCCGCAAACTGAACCTGAATTTATAGAG 6840

LU20297_planned     6841 GAAGTGTGA 6849
                         GAAGTGTGA
LU20297_sequenced   6841 GAAGTGTGA 6849
```

Annex VII

Sequence similarity of phage lambda genes *gam*, *beta*, *alpha* and strain LU20297

Similarity & Homology ▾ DNA Analysis ▾ Protein Analysis ▾ Databases ▾ Patent Tools ▾

```
BLASTN 2.2.26 [Sep-21-2011]


Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs", Nucleic Acids Res. 25:3389-3402.

Query=
         (1882 letters)

Database:
/mmprojects/tokovebt/genomes/Escherichia_coli_N8_2/Escherichia_coli_N8
_2_contigs.fa
           2 sequences; 4,656,925 total letters

Searching..................................................done

 ***** No hits found ******



  Database: /mmprojects/tokovebt/genomes/Escherichia_coli_N8_2/Escheri
  chia_coli_N8_2_contigs.fa
    Posted date:  Sep 20, 2017 11:19 AM
  Number of letters in database: 4,656,925
  Number of sequences in database:  2

Lambda     K      H
    1.37    0.711     1.31

Gapped
Lambda     K      H
    1.37    0.711     1.31


Matrix: blastn matrix:1 -3
Gap Penalties: Existence: 5, Extension: 2
Number of Sequences: 2
Number of Hits to DB: 98,143
Number of sequences better than 1.0e-01: 0
Number of HSP's gapped: 0
Number of HSP's successfully gapped: 0
Length of query: 1882
Length of database: 4,656,925
Length adjustment: 17
Effective length of query: 1865
Effective length of database: 4,656,891
Effective search space: 8685101715
Effective search space used: 8685101715
X1: 11 (21.8 bits)
X2: 15 (29.7 bits)
X3: 50 (99.1 bits)
S1: 19 (38.2 bits)
S2: 19 (38.2 bits)
```

Annex C1: Sequence search for the phage lambda gam beta alpha genes in LU20297 using the blast algorithm shows the absence of those genes.

```
Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs", Nucleic Acids Res. 25:3389-3402.

Query=
         (4592 letters)

Database:
/mmprojects/tokovebt/genomes/Escherichia_coli_W8_2/Escherichia_coli_W8
_2_contigs.fa
           2 sequences; 4,656,925 total letters

Searching..................................................done



                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

1:W8-2_1 ()                                                         48   7e-05
2:W8-2_2 ()                                                         40   0.018


>1:W8-2_1 ()
          Length = 4425750

 Score = 48.1 bits (24), Expect = 7e-05
 Identities = 24/24 (100%)
 Strand = Plus / Minus


Query: 22     gctgttttggcggatgagagaaga 45
               ||||||||||||||||||||||||
Sbjct: 682586 gctgttttggcggatgagagaaga 682563
```

extract aligned sequence to VNTI   adding 1000 up and downstream sequence

open Blast Hit in genome browser

```
 Score = 48.1 bits (24), Expect = 7e-05
 Identities = 24/24 (100%)
 Strand = Plus / Plus


Query: 22      gctgttttggcggatgagagaaga 45
                ||||||||||||||||||||||||
Sbjct: 1432630 gctgttttggcggatgagagaaga 1432653
```

extract aligned sequence to VNTI   adding 1000 up and downstream sequence

open Blast Hit in genome browser

```
 Score = 48.1 bits (24), Expect = 7e-05
 Identities = 24/24 (100%)
 Strand = Plus / Plus


Query: 22      gctgttttggcggatgagagaaga 45
                ||||||||||||||||||||||||
Sbjct: 2128615 gctgttttggcggatgagagaaga 2128638
```

Annex C2: Sequence search for the the bases bp 4195 to bp 8786 (total of 4592bp) of the Red/ET plasmid in LU20297 using the blast algorithm shows the absence of those genes.

```
BLASTN 2.2.26 [Sep-21-2011]


Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs",  Nucleic Acids Res. 25:3389-3402.

Query=
         (660 letters)

Database:
/mmprojects/tokovebt/genomes/Escherichia_coli_N8_2/Escherichia_coli_N8
_2_contigs.fa
           2 sequences; 4,656,925 total letters

Searching..................................................done

 ***** No hits found ******


  Database: /mmprojects/tokovebt/genomes/Escherichia_coli_N8_2/Escheri
  chia_coli_N8_2_contigs.fa
    Posted date:  Sep 20, 2017 11:19 AM
  Number of letters in database: 4,656,925
  Number of sequences in database:  2

Lambda     K      H
    1.37    0.711     1.31

Gapped
Lambda     K      H
    1.37    0.711     1.31


Matrix: blastn matrix:1 -3
Gap Penalties: Existence: 5, Extension: 2
Number of Sequences: 2
Number of Hits to DB: 35,320
Number of sequences better than 1.0e-01: 0
Number of HSP's gapped: 0
Number of HSP's successfully gapped: 0
Length of query: 660
Length of database: 4,656,925
Length adjustment: 16
Effective length of query: 644
Effective length of database: 4,656,893
Effective search space: 2999039092
Effective search space used: 2999039092
X1: 11 (21.8 bits)
X2: 15 (29.7 bits)
X3: 50 (99.1 bits)
S1: 18 (36.2 bits)
S2: 18 (36.2 bits)
```

Annex C3: Sequence search for the chloramphenicol resistance gene in LU20297 using the blast algorithm shows the absence of the *cmR* gene.

```
BLASTN 2.2.26 [Sep-21-2011]


Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs",  Nucleic Acids Res. 25:3389-3402.

Query=
         (375 letters)

Database:
/mmprojects/tokovebt/genomes/Escherichia_coli_N8_2/Escherichia_coli_N8
_2_contigs.fa
           2 sequences; 4,656,925 total letters

Searching..................................................done

 ***** No hits found ******


  Database: /mmprojects/tokovebt/genomes/Escherichia_coli_N8_2/Escheri
  chia_coli_N8_2_contigs.fa
    Posted date:  Sep 20, 2017 11:19 AM
  Number of letters in database: 4,656,925
  Number of sequences in database:  2

Lambda     K      H
    1.37    0.711     1.31

Gapped
Lambda     K      H
    1.37    0.711     1.31


Matrix: blastn matrix:1 -3
Gap Penalties: Existence: 5, Extension: 2
Number of Sequences: 2
Number of Hits to DB: 14,950
Number of sequences better than 1.0e-01: 0
Number of HSP's gapped: 0
Number of HSP's successfully gapped: 0
Length of query: 375
Length of database: 4,656,925
Length adjustment: 15
Effective length of query: 360
Effective length of database: 4,656,895
Effective search space: 1676482200
Effective search space used: 1676482200
X1: 11 (21.8 bits)
X2: 15 (29.7 bits)
X3: 50 (99.1 bits)
S1: 17 (34.2 bits)
S2: 17 (34.2 bits)
```

Annex C4: Sequence search for the zeocin resistance gene in LU20297 using the blast algorithm shows the absence of the *zeoR* gene.

Ms. Ellen Anderson
Consumer Safety Officer
Division of Food Ingredients
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

August 12, 2019

Re: GRAS Notice No. GRN 000852

Dear Ms. Anderson,

This is in reference to your letter dated July 31, 2019 asking for clarification on 5 questions that have been raised as a part of your evaluation of GRN 000852 for 2”-Fucosyllactose (2’-FL).

1. *The notice does not state whether a search of published scientific literature was conducted to identify any information that might contradict with your conclusion that 2’-FL is GRAS under the intended use. If a literature search was conducted, please describe how the search was conducted, including the time frame of the search and any pertinent results.*

   Response:

   Two literature searches have been conducted by BASF to identify relevant information for assessing the safety of BASF’s 2’-fucosyllactose:

   The first search on any toxicological data of 2’-fucosyllactose was conducted using the STN service (http://www.stn-international.de/stn_home.html). STN is an online database service that provides access to published research, journal literature, patents, structures, sequences, properties, and other data. The database covers worldwide literature from all areas of chemistry, biochemistry, chemical engineering, and related sciences. The following databases covered by STN were searched for literature regarding toxicological substance information: BIOSIS, HCAPLUS, REGISTRY, EMBASE and TOXCENTER. The CAS number for 2-Fucosyllactose was used to collect additional substance identifiers in the file REGISTRY. All relevant identifiers were used to select toxicological significant information in the other files. The period of the research covered all references in the databases up to October 2018.

   In addition to the STN search, further national and international substance databases (e.g. EPA IRIS, US NTP, RTECS) were searched with the aim to identify information on toxicological properties or harmful effects in humans or animals. We have considered only regulatory relevant studies, but not any explorative studies.

   While preparing the GRAS notice a second literature search was conducted in October 2018 to identify recent literature on the safety of 2’-fucosyllactose which have not been considered in other GRAS notifications on 2’-fucosyllactose before. As a reference point BASF used GRN 735 for which FDA issued a “no-questions” letter on April 6, 2018. GRN 735 had been submitted in

September 2017, so it was decided to restrict the literature search to studies published in 2017 and 2018. This search was performed in the Primo Central Index (using the search interface provided by the ETH Zurich library). The term used was “Fucosyllactose” which resulted in approx. 540 titles which were screened whether they contained relevant safety information. Likewise, Google Scholar was searched with a time limitation to 2017 and later (the first fifty hits were assessed). From these two searches 5 new relevant studies have been identified: GIBSON (2017), MOUKARZEL (2017), E. J. REVERRI (2018), VAN BERLO (2018), VANDENPLAS (2018).

None of the 5 identified new publications contradict with our conclusion that BASF’s 2’-FL is GRAS under the intended use.

2. *Please indicate the protein source (e.g., milk-based, soy-based, whey-based, etc.) of the infant formula to which 2’-FL is intended to be added.*

Response:

2’-FL is intended to be used in infant formula for full term infants. Adding 2’-FL to infant formula aims at narrowing the nutritional gap between human milk and formula, independent of the protein source of that formula. Thus, 2’-FL is intended to be added to milk-based, whey-based and soy-based infant formula.

3. *In Table 14 on page 32 of the notice, it states 2’-FL is intended for use in “ready-to-drink formula or formula prepared from powder.” Is 2’-FL intended to be used in concentrated infant formulas as well? We interpret “ready-to-drink” to mean that no reconstitution is required, which is different from concentrated infant formula.*

Response:

BASF’s 2‘-FL can also be used for concentrated infant formula. The concentration of 2‘-FL /100 kcal of the ready-to-drink formula will remain unchanged. The 2’-FL concentration in 100 ml of the concentrated formula would be correspondently higher, reflecting the reconstitution with water which is necessary before feeding the formula to an infant.

Examples of products based on concentrated infant formula are:

- Enfamil PREMIUM Infant, infant formula, prepared from liquid concentrate, made with water, NFS (food code: 11710632)
- Enfamil PREMIUM Infant, infant formula, prepared from liquid concentrate, made with tap water (Food Code 11710633),
- Enfamil PREMIUM Infant, infant formula, prepared from liquid concentrate, made with plain bottled water (Food Code 11710634)
- Gerber Good Start Gentle Plus, infant formula, prepared from liquid concentrate, made with water, NFS (Food Code 11710912).

Hence, we would like to amend Table 14 of our notification as follows:

Table 14 Intended food categories and use levels for 2'-FL

Proposed Food Category (21 CFR 170.3)	Food Uses	Maximum 2'-FL use level (g / serving)	RAAC [a]) (g or mL)	Maximum 2'-FL use levels (g / 100 g)
Beverages and Beverage Bases	Energy drinks	0.28	360	0.08
	Fitness water and thirst quenchers, sports and isotonic drinks	0.28	360	0.08
Breakfast cereals	Ready-to-eat breakfast cereals for adults and children	1.2	15 (puffed) 40 (high-fiber) 60 (biscuit-types)	8.0 3.0 2.0
	Hot cereals for adults and children	1.2	40 (dry) ~ 250 (prepared)	0.48 (as consumed)
Dairy Product Analogs	Milk substitutes such as soy milk and imitation milks	0.28	240	0.12
Frozen Dairy Desserts and Mixes	Frozen desserts including ice creams* and frozen yogurts, frozen novelties	1.2	~ 70	1.7
Gelatins, Puddings and Fillings	Dairy-based puddings, custards and mousses	1.2	~ 70	1.7
	Fruit pie filling	1.2	85	1.41
	"Fruit prep" such as fruit filling in bars, cookies, yogurt and cakes	1.2	~40	3.0
Grain Products and Pastas	Bars, including snack bars, meal-replacement bars and breakfast bars	0.48	40	1.20
Jams and Jellies, Commercial	Jellies and jams, fruit preserves*, and fruit butters	1.2	~20	6.0
Milk, Whole and Skim	All *acidophilus* or fortified milks, non-fat and low-fat milk fluids, including fluid milk and reconstituted milk powder*	0.28	240	0.12
Milk Products	Flavored milks, including chocolate milk, coffee drinks, cocoa, smoothies (dairy and fruit-based), other fruit and dairy combinations, yogurt drinks, and fermented milk drinks including kefir **	0.28	240	0.12
	Milk-based meal replacement beverages or diet beverages**	0.28	240	0.12
	Yogurt*,**	1.2	225	0.53

Proposed Food Category (21 CFR 170.3)	Food Uses	Maximum 2'-FL use level (g / serving)	RAAC a) (g or mL)	Maximum 2'-FL use levels (g / 100 g)
	Formula intended for pregnant women ("mum" formulas, -9 to 0 months)	1.2	200 b)	0.6
Processed Fruits and Fruit Juices	Fruit drinks, including vitamin- and mineral-fortified products	0.28	240	0.12
	Fruit juices*	0.28	240	0.12
Sweet Sauces, Toppings and Syrups	Syrups used to flavor milk beverages	0.28	40	0.70
Other Categories				
Non-Exempt Infant and Follow-On Formula	Infant formula (0-6 months), including ready to drink formula, concentrated formula requiring reconstitution with water and powder requiring reconstitution with water	0.24	100 b)	0.24 (0.40 g / 100 kcal) c
	Follow-on formula (6 to 12 months), including ready to drink formula or formula prepared from powder	0.24	100 b)	0.24 (0.40 g / 100 kcal) c
Baby Foods	Meal replacement products such as Pediasure	0.24	120 b)	0.2
	Growing-up (toddler) milks (12-36 months)	0.24	120 b)	0.2
	Ready-to-eat, ready-to-serve hot cereals	1.2	15 (dry) 110 (ready-to-serve)	1.09 (as consumed)
	Yogurt and juice beverages identified as "baby" drinks	1.2	120	1.0
	Desserts including fruit desserts, cobblers, yogurt/fruit combinations ("junior type" desserts)	1.2	110	1.09
	Baby crackers, pretzels, cookies, and snack items	0.4	7	5.7

4. *In Table 10 on page 23 of the notice, lactose is listed as a component in the growth media used in the production of 2'-FL. Please indicate the source of the lactose.*

<u>Response:</u>

For production of the batches as mentioned in this notification, lactose from cow's milk (food grade) is used. Specification parameters of the lactose used are in the attached file.

Lactose

5. *In Table 12 on page 27 of the notice, there is a parameter listed for residual protein with a specification of less than or equal to 0.01% in the 2'-FL ingredient. Please explain the origin of this residual protein.*

   Response:

   2'-fucosyllactose is produced by fermentation followed by a downstream process that aims at effectively removing the biomass of the used microorganism. The biomass consists especially of cell-walls incl. proteins. Neither the raw materials in the fermentation (growth media components) nor the processing aids used in the purification process contain any protein source as indicated in Tables 10 and 11 of our notification. Thus, the only relevant protein source in the production process is the fermentation organism.

   However, the protein limit of NMT 0.01% is a quality control parameter for an effective downstream processing resulting in an absence of the microorganism and its constituents in the final product. As described in the notice (see 2.3.2 Isolation and purification) the 2'-FL containing liquid phase is filtered by a cross-flow filtration process with an ultrafiltration membrane to remove large molecules (e.g. protein, DNA and lipopolysaccharides). Hence, our manufacturing process results in a product in which no residual protein is detected. The limit of 0.01% which is described in our product specification (Table 12) represents the limit of detection (LoD) of the modified Bradford Assay.

   It should be noted that this threshold of 0.01% is also specified in the other 2'-FL GRAS notifications, which have received the "no questions" letters from FDA, and using the same production host (*E. coli* K12) (please see the notification of Glycom GRN 650, Glycosyn/Friesland GRN 735 and Dupont GRN 749). Moreover, the LoD of 0.01% has also been recognized by the European Food Safety Authority (EFSA) in their latest scientific opinion regarding "Safety of 2'-fucosyllactose/ difucosyllactose mixture as a novel food pursuant to Regulation (EU) 2015/2283" (doi: 10.2903/j.efsa.2019.5717). Cross references to other applications/notifications using the same production host can therefore be made.

We hope the responses to your questions are satisfactory. We are looking forward to your completed evaluation. If you have any further questions or need clarification, please reach out to me or my colleague claudia.callies-kluepfel@basf.com.

Sincerely,

(b) (6)

Haresh. P. Madeka, PhD
Sr. Regulatory & External Affairs Manager