

## **Appendix D: Chemical Identification**

**ANALYTICAL RESULTS**

To Paul Tenning, Angela Lim

Copy Oliver Hasselwander

From Henrik Max Jensen

Re. Equivalence testing of 2'-FL and DiFL using <sup>1</sup>H NMR spectroscopyObjective

To state the identity of 2'-FL and DiFL and to compare the materials from DuPont/Inbiose E. coli production with human milk reference material by use of <sup>1</sup>H NMR Spectroscopy.

Samples

Sample name	Batch ID	Comments	Analytical ID
<b>2'-FL</b> 2'-Fucosyllactose	B60265, Kit 13	E. coli Fermentation	T8977-24 NMR Spectrum ID: T8977_20170516 21
<b>2'-FL Human derived</b> 2'-Fucosyllactose	35/8 Lot no. 187-131 <i>IsoSep AB</i>	Isolated from Human Breast Milk	T8977-21 NMR Spectrum ID: T8977_20170428 7
<b>DiFL</b> Lactodifucotetraose Fuc(α1-2)Gal(β1-4)[Fuc(α1-3)]Glc	DiFL Solution 13g/L, 03-04-2017	E. coli Fermentation	T8977-20 NMR Spectrum ID: T8977_20170516 31
<b>DiFL</b> Lactodifucotetraose Fuc(α1-2)Gal(β1-4)[Fuc(α1-3)]Glc	DiFL, dried Kantvik 22.5.2017	E. coli Fermentation	T8977-26 NMR Spectrum ID: T8977_20170516 50
<b>DiFL Human derived</b> Lactodifucotetraose Fuc(α1-2)Gal(β1-4)[Fuc(α1-3)]Glc	45/2 Lot no. 187-291 <i>IsoSep AB*</i>	Isolated from Human Breast Milk	T8977-23 NMR Spectrum ID: T8977_20170428 8

Table 1: Samples used for the equivalence report. \*) Source of reference standards: *IsoSep AB*, Dalkärrsvägen 11, SE-146 36 Tullinge, Sweden

Method

Samples T8977-20, T8977-24 and T8977-26 are isolated and purified from E. coli fermented 2'-FL production. Samples T8977-21 and T8977-23 are pure reference materials isolated from Human Breast Milk.

Samples (1-2mg) are dissolved in 200μL of deuterium water (D2O) and 160μL hereof is placed in a 3mm NMR tube (TSP is used as internal chemical shift reference at 0.0ppm). <sup>1</sup>H NMR spectra were recorded using the standard zg30 pulse-sequence (number of scans = 16) using either a 600MHz or 800MHz Bruker Advance NMR Spectrometer equipped with a TXI/TCI cryoprobe.

Results

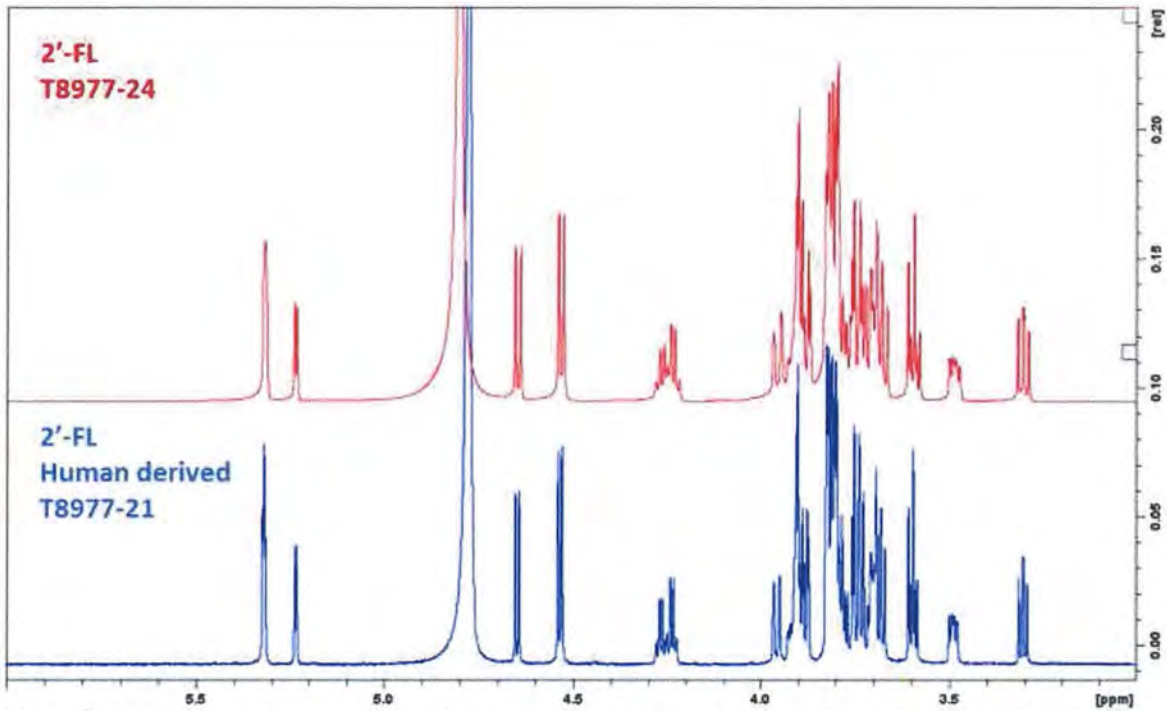


Fig. 1.  $^1\text{H}$  NMR Spectral comparison. Zoomed region (3-6ppm). The red spectrum is 2'-FL from *E. coli* fermentation and the lower blue spectrum is reference material of human origin.

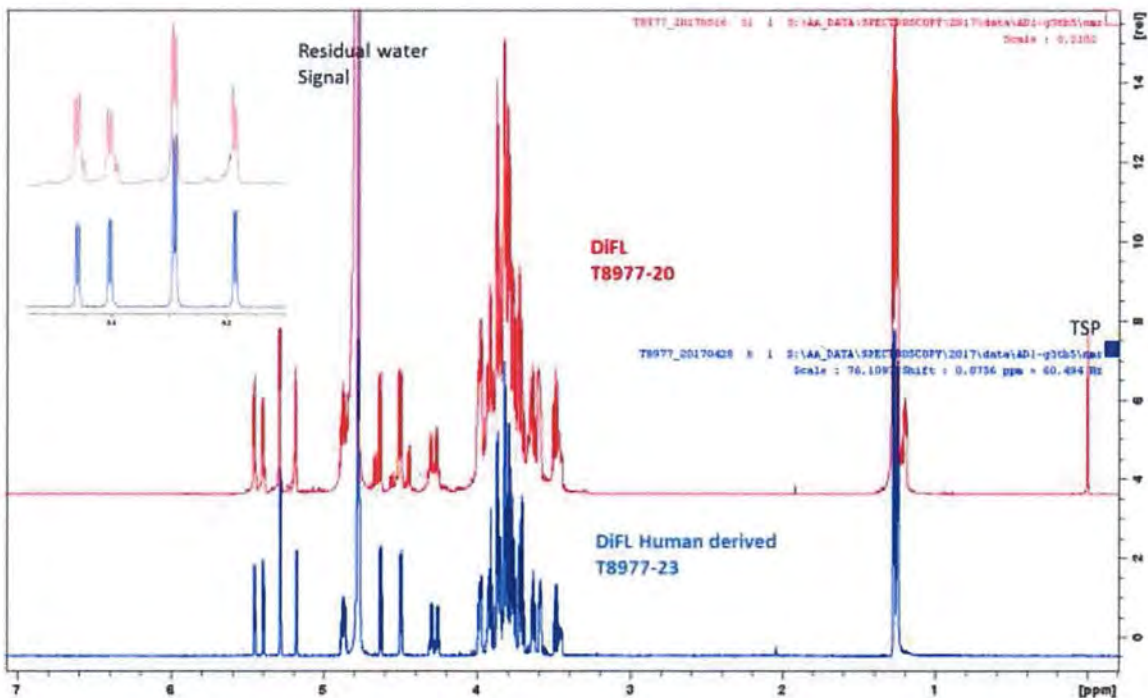


Fig. 2.  $^1\text{H}$  NMR Spectral comparison. Zoomed region (0-7ppm). The red spectrum is DiFL isolated from *E. coli* fermentation and the lower blue spectrum is reference material of human origin. The insert is a zoom of the four  $\alpha$ -anomeric protons of all four hexoses.

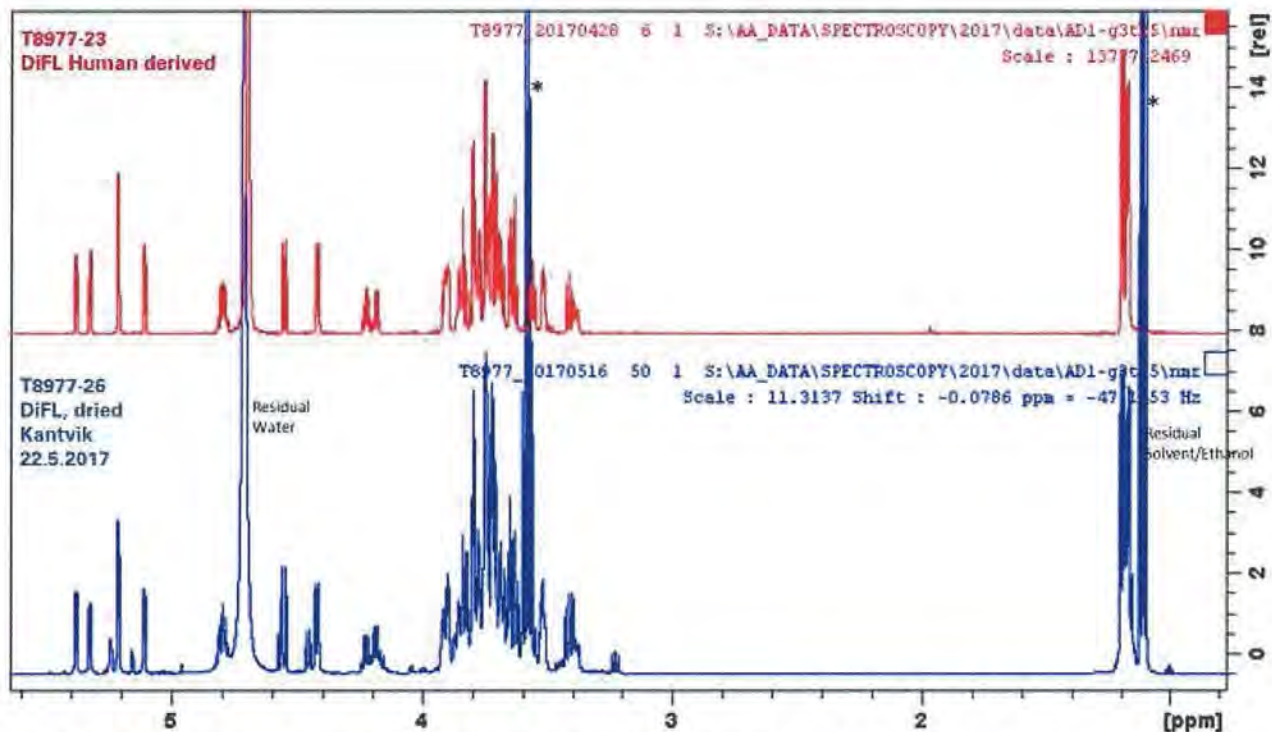


Fig. 3.  $^1\text{H}$  NMR Spectral comparison. Zoomed region (0.9-5.6ppm). The red spectrum (T8977-23) is reference material DiFL Human origin and the lower blue spectrum (T8977-26) is isolated and purified from *E. coli* fermentation. The \*) indicates observed residual solvent: Ethanol in T8977-26.

### Conclusion

All major well-resolved signals in the  $^1\text{H}$  NMR spectra of 2'-FL (Fig. 1) and DiFL (Fig.2, Fig. 3) are identical between materials isolated from the production and reference materials. The equivalence between materials isolated from production samples 2'-FL (T8977-24) and DiFL (T8977-20, T8977-26) to Human derived reference materials (T8977-21 and -23) are demonstrated in Fig. 1, 2 and 3.

The recorded  $^1\text{H}$  NMR spectra of the samples are identical to spectral data reported in the literature (van Leeuwen 2014).

Sign.

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Literature reference:

Sander S van Leeuwen, Ruud J W Schoemaker, Gerrit J Gerwig, Ellen J M van Leusen-van Kan, Lubbert Dijkhuizen, Johannes P Kamerling.

Rapid milk group classification by <sup>1</sup>H NMR analysis of Le and H epitopes in human milk oligosaccharide donor samples. *Glycobiology* vol. 24 no. 8 pp. 728–739, 2014

Technical Report

**Identification of carbohydrate constituents in 2'-Fucosyllactose samples by High Performance Anion Exchange Chromatography – Mass Spectrometry (HPAEC-MS).**

Report date: 29 May 2017

Technical analysts: Bogdan Szostek, Keith Prickett, Siiri Viikari and Henrik Max Jensen

## Summary.

This report summarizes the data supporting identification of carbohydrate constituents of 2'-fucosyllactose (2'-FL) powder samples. Batches of 2'-FL powders were analyzed by High-Performance Anion Exchange Chromatography – Pulsed Amperometric Detection-Mass Spectrometry (HPAEC-PAD-MS) using a PA100 column for separation of carbohydrate analytes. The mass spectrometric data: elemental formula of a carbohydrate derived from the accurate mass measurement and product ion spectra of carbohydrate standards were used to confirm the identify of carbohydrate peaks observed in the chromatograms of 2'-FL samples. The mass spectrometric data provided additional evidence and validation for carbohydrate identity in addition to a simple retention time match that is typically obtained from HPAEC-PAD analysis. Two of the carbohydrate peaks observed in the 2'-FL samples were not matched with any of available carbohydrate standards. Hence, these two peaks were isolated from the 2'-FL samples and subjected to NMR structure elucidation. Tentative structure assignments were proposed.

## Conclusions.

HPAEC-PAD analysis using a PA100 column separation is used to provide the quantitation of constituent carbohydrates, expressed a peak area percent, and to test the specification for different batches of the 2'-FL product. Table 1 shows such data for the F13/3 batch. As the specification analysis is done on a different system than the HPAEC-PAD-MS work done for this report, even though the same method is used: column and gradient conditions, there is a slight difference in the retention times of the carbohydrates from these two different system. Table 1 captures these retention time differences. The report refers to the retention times obtained on the HPAEC-PAD-MS system.

Table 2 captures the conclusions about the identity of carbohydrate analytes derived from the HPAEC-PAD-MS analysis and NMR analysis of two fractions isolated from the 2'-FL sample.

Table 1. Retention time equivalence for the F13/3 batch peaks analyzed on two different HPAEC-PAD systems.

Name	DuPont Specification	Batch F13/3 percent	Retention time (min), as in chromatogram in Figure 1A  (specification analysis system)	Retention time (min), as in chromatogram in Figure 1  (HPAEC-PAD-MS system)
2'-Fucosyllactose	Min. 82% (Area)	82.20	7.18	7.3
Tentatively identified as 2'-Fucosyllactulose		1.10	7.69	7.8
Lactose	Max. 8% (Area)	6.54	6.46	6.6
3-Fucosyllactose	-	<0.1	4.41	5.1
Difucosyllactose	Max. 7% (Area)	6.69	5.24	5.3
Fucosylgalactose		0.95	4.76	5.1
Glucose/galactose		<0.1	4.07	4.5
Fucose		<0.1	2.51	3.0
Sorbitol (galactitol)		1.02	2.08	2.5
Mannitol		<0.1	2.31	ND
Trihexose (tentatively identified as galactogluco-oligosaccharide)		1.22	11.38	13.3
Other carbohydrates (without 2FL, lactose, difucosyllactose)	Max. 6% (Area)	4.57	Not applicable	Not applicable



**Table 2.** Summary of evidence obtained from HPAEC-MS analysis of 2'-FL, batch F13/1 sample and NMR analysis of fractions for the 7.8 min and 13.3 min peaks.

Retention time (min)	Component Assignment	Evidence
2.5	Sorbitol/galactitol	The elemental formula of $C_6H_{14}O_6$ was determined for the 2.5 min peak. Product ion spectra and retention times of both sorbitol and galactitol match that of the 2.5 min peak in the 2'-FL sample. Product ion spectra of galactitol and sorbitol are almost identical. No differentiation between galactitol and sorbitol can be made based on the product ion spectra. Therefore, the 2.5 min peak may contain both sorbitol and galactitol.
3.0	Fucose	Retention time of the 3.0 min matched the retention time of fucose standard. No MS data was obtained because of low abundance of this peak.
4.5	Glucose/ galactose	Retention time of the 4.5 min peak matched the retention time of glucose and galactose standard. No MS data was obtained because of low abundance of this peak.
5.1	3-FL/ fucosylgalactose	Two carbohydrates were found to be associated with the peak at 5.1 min. The elemental formula of $C_{12}H_{22}O_{10}$ and $C_{18}H_{32}O_{15}$ were determined for these carbohydrates. Retention time and the product ion spectrum of Fucosylgalactose standard matched that of the $C_{12}H_{22}O_{10}$ carbohydrate peak. The other carbohydrate with elemental formula of $C_{18}H_{32}O_{15}$ was identified as 3-FL by matching the retention time and the product ion spectrum of 3-FL standard.
5.3	Difucosyllactose	The elemental formula of $C_{24}H_{42}O_{19}$ was determined for the 5.3 min peak. Product ion spectrum and the retention time of difucosyllactose standard matched that of the 5.3 min peak. The peak at 5.3 min was identified as difucosyllactose.
6.6	Lactose	The elemental formula of $C_{12}H_{22}O_{11}$ was determined for the 6.6 min peak. Retention time and the product ion spectrum of lactose standard matched that of the 6.6 min peak. Peak at the 6.6 min was identified as lactose.

7.3	2'-FL	The elemental formula of $C_{18}H_{32}O_{15}$ was determined for the 7.3 min peak. Product ion spectrum and the retention time of 2'-FL standard match that of the 7.3 min peak. Peak at the 7.3 min is identified as 2'-fucosyllactose.
7.8	2'-FL isomer (tentatively identified as 2'-fucosyllactulose)	Thorough analysis of MS data in the region of the 2'-FL peak where the 7.8 min shoulder peak was expected lead to a conclusion that the peak at 7.8 min represents an isobaric isomer of 2'-FL with elemental composition of $C_{18}H_{32}O_{15}$ . In addition, it was shown that the 7.8 min peak can be generated by exposure of 2'-FL to basic conditions (100 mM NaOH). It has been reported in literature that under such conditions the 2'-fucosyllactose converts to 2'-fucosyllactulose. Additional evidence from NMR analysis of isolated fraction of the 7.8 min peak points to the 2'-fucosyllactulose (Ref 1). Therefore, peak at 7.8 min is tentatively identified as 2'-fucosyllactulose. Standard of 2'-fucosyllactulose is not commercially available.
13.3	Tri-hexose (tentatively identified as galacto-gluco-oligosaccharide)	The elemental formula of $C_{18}H_{32}O_{16}$ was determined for the 13.3 min carbohydrate peak. The elemental composition and fragmentation pattern observed in the product ion spectrum of 13.3 min peak lead to a conclusion that the 13.3 min peak is a trihexose. It was postulated that the trihexose may be either 6'- or 3'-galactosyllactose. Product ion spectra and retention time of both the 6'- and 3'-galactosyllactose standards are different than that of the 13.3 min peak. Hence, the trihexose is not either 6'- or 3'-galactosyllactose. NMR analysis of isolated fraction of peak at 13.3 min lead to partial, tentative assignment for the 13.3 min peak as a galacto-gluco-oligosaccharide (Ref 1)

Reference 1. Technical report by Henrik Max Jensen entitled "Impurity profiling of 2'-FL fractions using  $^1H$  NMR spectroscopy."

## Results of constituent carbohydrates identification in three batches of 2'-FL powders.

Three batches of 2'-FL powder, namely F13/1, F13/2a, F13/3 were analyzed by HPAEC-PAD-MS. Figure 1 shows the resulting chromatograms obtained for injection of 0.02 mg/mL aqueous solutions of the 2'-FL samples. The retention times of integrated peaks in Figure 1 are the same as these listed in Table 2. This analysis is done with the amount of sample injected on column that provides optimal chromatographic resolution and detection for the purity analysis and calculation of area percent composition. However, it does not result in sufficient intensity of signal for the MS detection, especially for the lower abundance carbohydrates, for confident identification of carbohydrates using product ion spectra comparison. Therefore, much larger on-column sample loading was required, injections of 2 mg/mL aqueous solution were made. Figure 2 and 3 show resulting HPAEC-PAD chromatograms that were obtained at two different dates. It is apparent that these chromatograms are obtained under column overload conditions, which affected not only the peak shape but also the retention time of the analytes. As the separation is done under the same chromatographic conditions the retention order of carbohydrates is not affected, allowing mapping the peaks observed in the purity analysis (Figure 1 or 1A) to these shown in Figure 2 and 3. The MS data presented in the following figures is derived from either injection from Figure 2 or Figure 3. The MS-detected peaks are delayed vs. the PAD-detected peaks because of the MS detector being downstream of the PAD detector, and separated by the dead volume of the suppressor and the corresponding tubing. This report shows only MS data for the F13/1 batch. All three batches were analyzed by HPAEC-PAD/MS. The MS data and the conclusions presented here for the F13/1 batch are the same for the other two batches.

The MS data that supports the identification of peaks listed in Table 2 are presented in the following format. A figure that shows extracted ion chromatogram for a given  $m/z$ , the MS spectrum that corresponds to the peak observed for the particular  $m/z$ , and the elemental formula determined based on the accurate mass of the  $m/z$  ion. As the carbohydrates are ionized here by  $\text{Li}^+$  ion attachment to a neutral sugar molecule (M), the elemental formula of a carbohydrate is simply determined by subtraction of one Li from the elemental formula of the  $m/z$  ion.

Product ion spectra (MS/MS spectra) are obtained by isolation of a particular  $m/z$  ion and fragmentation of it at defined collisional energy conditions to yield the fragment ions. Isobaric carbohydrates (the same mass, elemental composition) can be distinguished by their product ion spectra. The structural features of a complex carbohydrate such as linkage or anomeric configuration or the identify of monomeric sugars cannot be determined from the product ion spectrum, but product ion spectra can be used for matching with the spectra of carbohydrate standards. Product ion spectra match vs. the standard provides additional evidence towards confirming the identity of a carbohydrate in addition to the retention time and MW/elemental composition match. A figure that shows that type of data is presented for each peak observed in the F13/1 batch of 2'-FL powder.

**Peak at 2.5 min (Table 2):**

The m/z 189 ion was found to be associated with the peak at 2.5 min. The elemental formula of  $C_6H_{14}O_6$  was determined for the 2.5 min peak carbohydrate (Figure 4). Product ion spectra and retention times of both sorbitol and galactitol match that of the 2.5 min peak in the 2'-FL batch F13/1 sample (Figure 5). Product ion spectra of galactitol and sorbitol are almost identical. No differentiation between galactitol and sorbitol can be made based on the product ion spectra. Therefore, the 2.5 min peak may contain both sorbitol and galactitol.

**Peak at 5.1 min (Table 2):**

The m/z 333 and m/z 495 ions were found to be associated with the peak at 5.1 min. The elemental formula of  $C_{12}H_{22}O_{10}$  was determined for the m/z 333 ion carbohydrate (Figure 6). Retention time and the product ion spectrum of Fucosylgalactose standard match that of the m/z 333 carbohydrate detected in the 2'-FL batch F13/1 sample (Figure 7). The peak at 5.1 min is identified as fucosylgalactose. The 5.1 min peak contains another carbohydrate with the m/z 495 ion and elemental formula of  $C_{18}H_{32}O_{15}$ . This represents an isomer of 2'-FL and was identified as 3-FL based on the retention time and the product ion spectra match with that of 3-FL standard (Figure 14).

**Peak at 5.3 min (Table 2):**

The m/z 641 ion was found to be associated with the peak at 5.3 min. The elemental formula of  $C_{24}H_{42}O_{19}$  was determined for the 5.3 min peak carbohydrate (Figure 8). Product ion spectrum and the retention time of difucosyllactose standard match that of the 5.3 min peak in the 2'-FL batch F13/1 sample (Figure 9). Peak at the 5.3 min is identified as difucosyllactose.

**Peak at 6.6 min (Table 2):**

The m/z 349 ion was found to be associated with the peak at 6.6 min. The elemental formula of  $C_{12}H_{22}O_{11}$  was determined for the 6.6 min peak carbohydrate (Figure 10). Product ion spectrum of lactose standard match that of the 6.6 min peak in the 2'-FL batch F13/1 sample (Figure 11). Peak at the 6.6 min is identified as lactose. There is some discrepancy in the retention time of lactose standard and the 6.6 min peak in the 2'-FL sample. The shift in the retention time may be caused by sample loading done for HPAEC-MS runs, its proximity to the major component of 2'-FL. However, perfect match of the retention time of lactose standard and all analytical runs with smaller amount of sample injected as in Figure 1 was observed for the 6.6 min peak.

**Peak at 7.3 min (Table 2):**

The m/z 495 ion was found to be associated with the peak at 7.3 min. The elemental formula of  $C_{18}H_{32}O_{15}$  was determined for the 7.3 min peak carbohydrate (Figure 12). Product ion spectrum and the retention time of 2'-FL standard match that of the 7.3 min peak in the 2'-FL batch F13/1 sample (Figure 13). Peak at the 7.3 min is identified as 2'-fucosyllactose. M/z 511 ion was also observed co-eluting with

m/z 495. Elemental formula calculated for the accurate mass of the m/z 511 ion is  $C_{18}H_{32}O_{15}Na$ , which is interpreted as formation of sodium adduct of 2'-FL.

#### **Peak at 7.8 min (Table 2):**

Thorough analysis of MS data in the region of the 2'-FL peak where the 7.8 min shoulder peak was expected lead to the conclusion that the peak at 7.8 min represents an isobaric isomer of 2'-FL with elemental composition of  $C_{18}H_{32}O_{15}$ . Analysis of the product ion spectra from different regions of the 2'-FL peak revealed that the relative intensity of selected fragment ions changes across the 2'-FL. This is an indication of another isobaric carbohydrate overlapping with the 2'-FL peak. This is illustrated in Figure 15 where the plot of the intensity of m/z 331 and m/z 477 fragment ions shows the outline of two chromatographic peaks. In addition, the product ion spectra taken at two different regions of the 2'-FL peak differ in the relative intensity of fragment ion peaks, confirming presence of two different species present in that peak. Data plotted in Figure 15 are for the F09 batch, which exhibited higher intensity of the shoulder peak. Similar data was obtained for the F13/1 batch, but the data was less clear as the intensity of the shoulder peak was smaller.

It was postulated that the shoulder peak (2'-FL isomer) results from conversion of 2'-FL under basic conditions. 2'-FL was placed in water and 100 mM sodium hydroxide solution and the solutions were analyzed by HPAEC-PAD/MS at time of 0, 9, 17.5, and 21 hours from preparation. Figure 16 shows HPAEC-PAD chromatograms obtained at different times of the experiment. The HPAEC-PAD and the MS data (not shown in this report) clearly show formation of the 2'-FL isomer from 2'-FL under basic conditions.

#### **Peak at 13.3 min (Table 2):**

The m/z 511 ion was found to be associated with the peak at 13.3 min. The elemental formula of  $C_{18}H_{32}O_{16}$  was determined for the 13.3 min carbohydrate peak (Figure 17). The elemental composition and the fragmentation pattern for the m/z 511 ion (Figure 18) lead to a conclusion that the 13.3 min peak is a tri-hexose. It was postulated that the unknown tri-hexose may be either 6'- or 3'-galactosyllactose. The product ion spectra of both the 6'- and 3'-galactosyllactose are different than that of the 13.3 min peak. In addition, the retention time of the standards has not matched that of the 13.3 min peak. The MS data shows that the unknown tri-hexose is not 6'- or 3'-fucosyllactose.

### **Carbohydrate standards:**

2'-Fucosyllactose (2'-FL), CAS Registry Number: 41263-94-9, Carbosynth Limited

3-Fucosyllactose (3-FL), CAS Registry Number: 41312-47-4, Carbosynth Limited

Difucosyllactose (DiFL), CAS Registry Number: 20768-11-0, Carbosynth Limited

Fucosylgalactose (Blood Group H disaccharide), CAS Registry Number: 24656-24-4, Carbosynth Limited

Lactose, CAS Registry Number: 63-42-3, Sigma-Aldrich

Sorbitol, CAS Registry Number: 50-70-4, Sigma-Aldrich

Galactitol, CAS Registry Number: 608-66-2, Sigma-Aldrich

3'-Galactosyllactose, CAS Registry Number: 32694-82-9, Carbosynth Limited

6'-Galactosyllactose, CAS Registry Number: 32582-31-0, Carbosynth Limited

## Experimental conditions:

Carbohydrate HPAEC-PAD/MS analysis was performed on a Dionex ICS-5000+ instrument. Columns were Dionex CarboPac PA-100 (250 x 2 mm) with CarboPac PA-100 guard column (50 x 2 mm). The column was run at a flowrate of 0.2 mL/min and a column temperature at 35°C. Mass spectrometric analysis was performed on a Thermo Fisher Scientific LTQ Orbitrap Velos mass spectrometer interfaced with the ICS-5000+ chromatograph. The MS ionization of the carbohydrates was done by use of electrospray probe operating in the positive ion mode. The mobile phase exiting the PAD detector was introduced to a AERS 500, 2 mm suppressor to suppress the mobile phase and hence enable electrospray ionization. Lithium chloride solution in methanol was added to the suppressed mobile via a T-connection before the electrospray probe to enable formation of lithium adducts of carbohydrates. Hence, the carbohydrates were detected as (M+Li)<sup>+</sup> adducts.

Eluent composition:

Channel	Eluent
A	H <sub>2</sub> O
B	0.1 M NaOH
C	0.1 M NaOH/0.3 M NaOAc
D	0.1 M NaOH/1 M NaOAc

Gradient:

Time (Min)	A (%)	B (%)	C (%)	D (%)
0.0	0	99.6	0.4	0
20.0	0	99.6	0.4	0
21.0	0	75.0	25.0	0
30.0	0	75.0	25.0	0
31.0	0	99.6	0.4	0
55.0	Stop Run			

**Figures:**

**Figure 1.** HPAEC-PAD chromatograms of three batches of 2'-FL powder. Five microliter injection of 0.02 mg/mL 2'-FL powder aqueous solution.

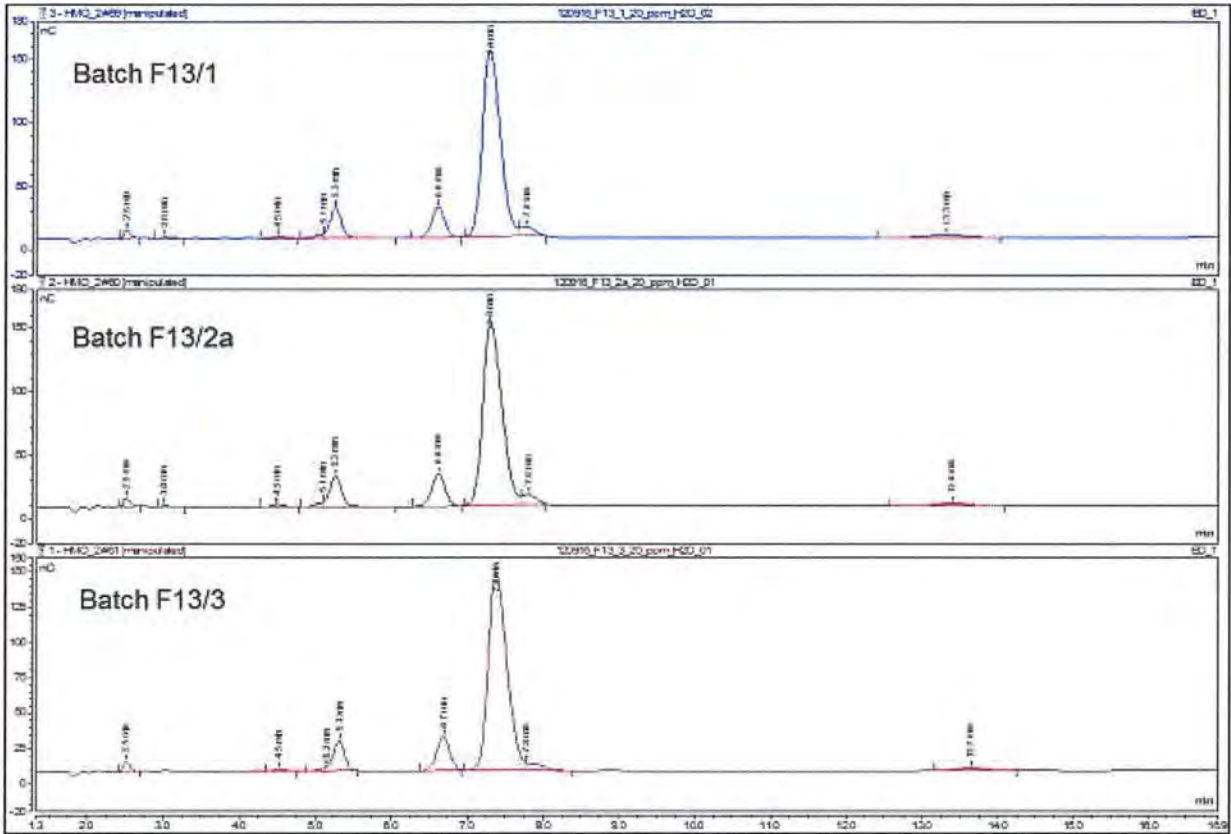




Figure 1A. HPAEC-PAD chromatogram of F13/3 batch. Relative peak area % and retention times reported in Table 1 are derived from this chromatogram.

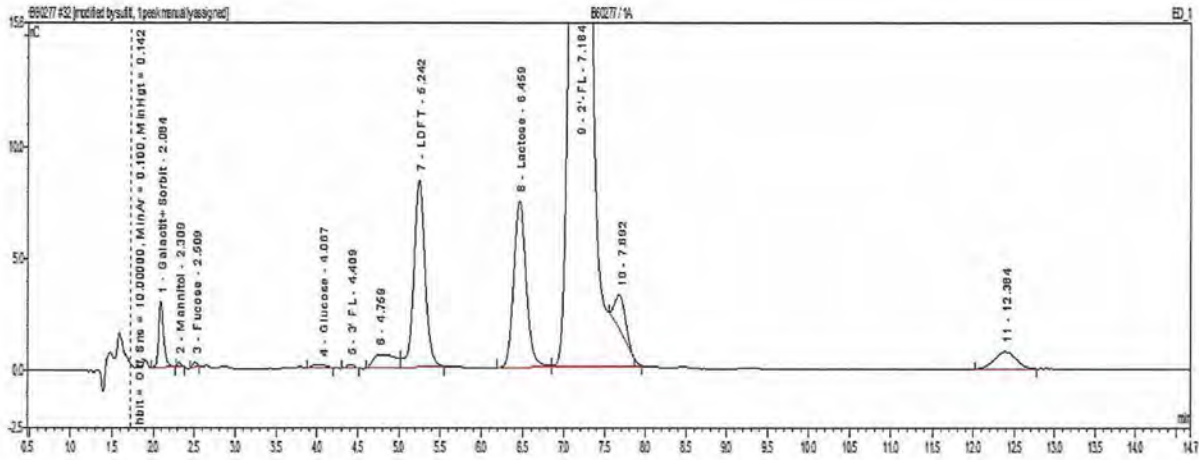
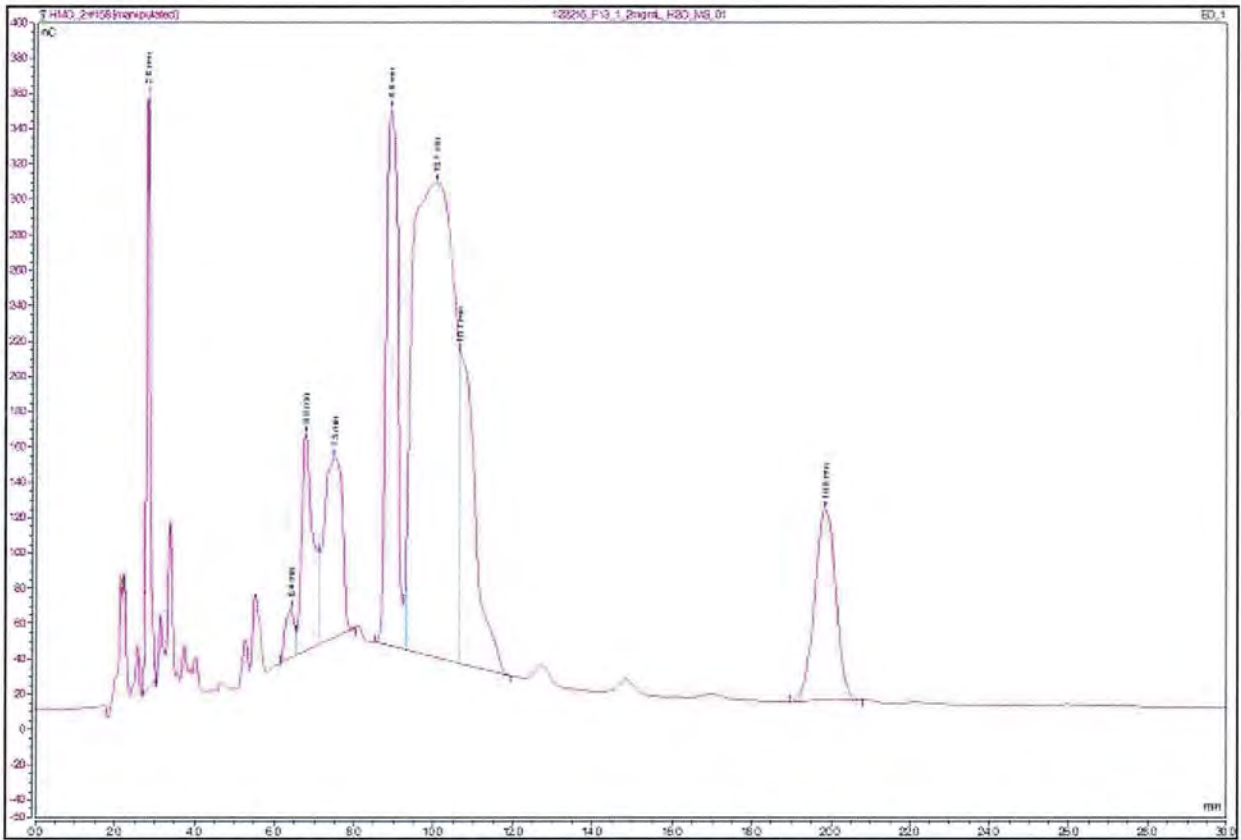
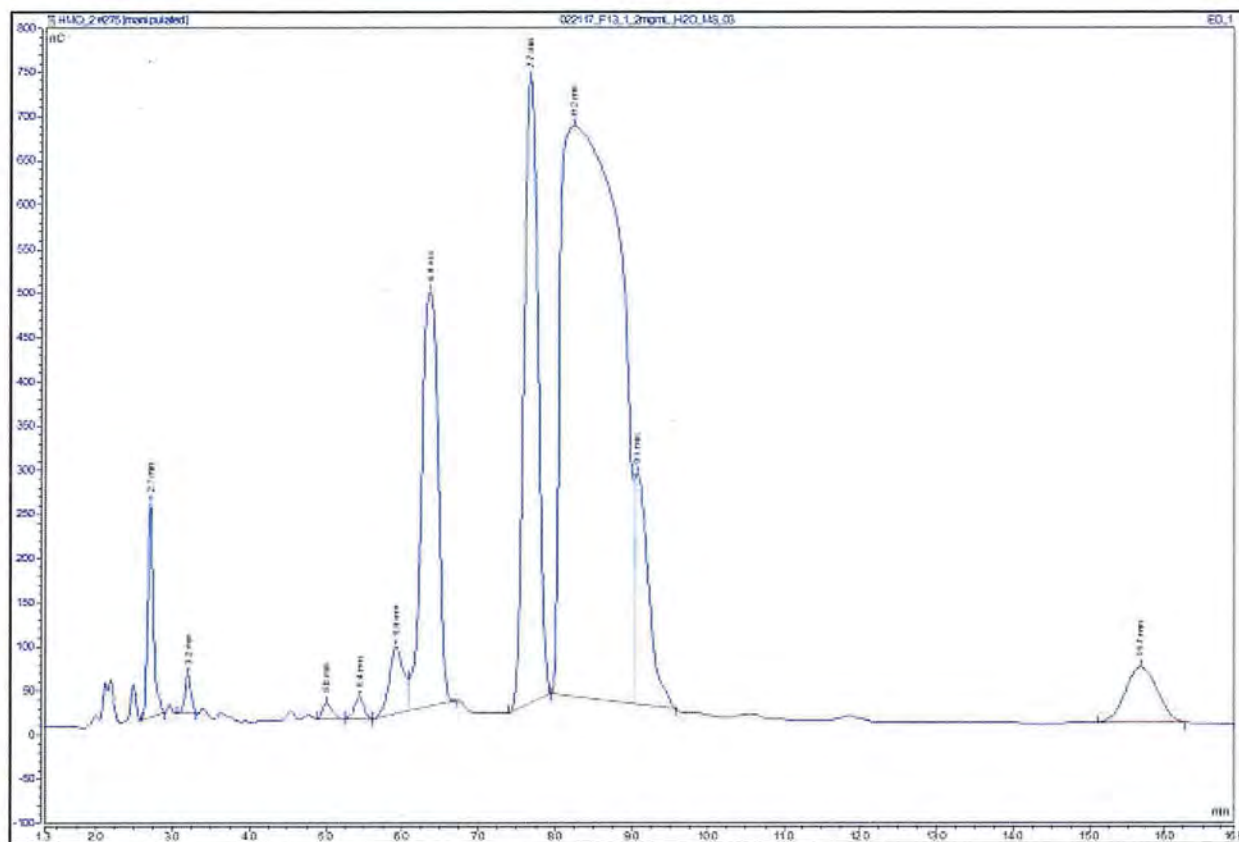


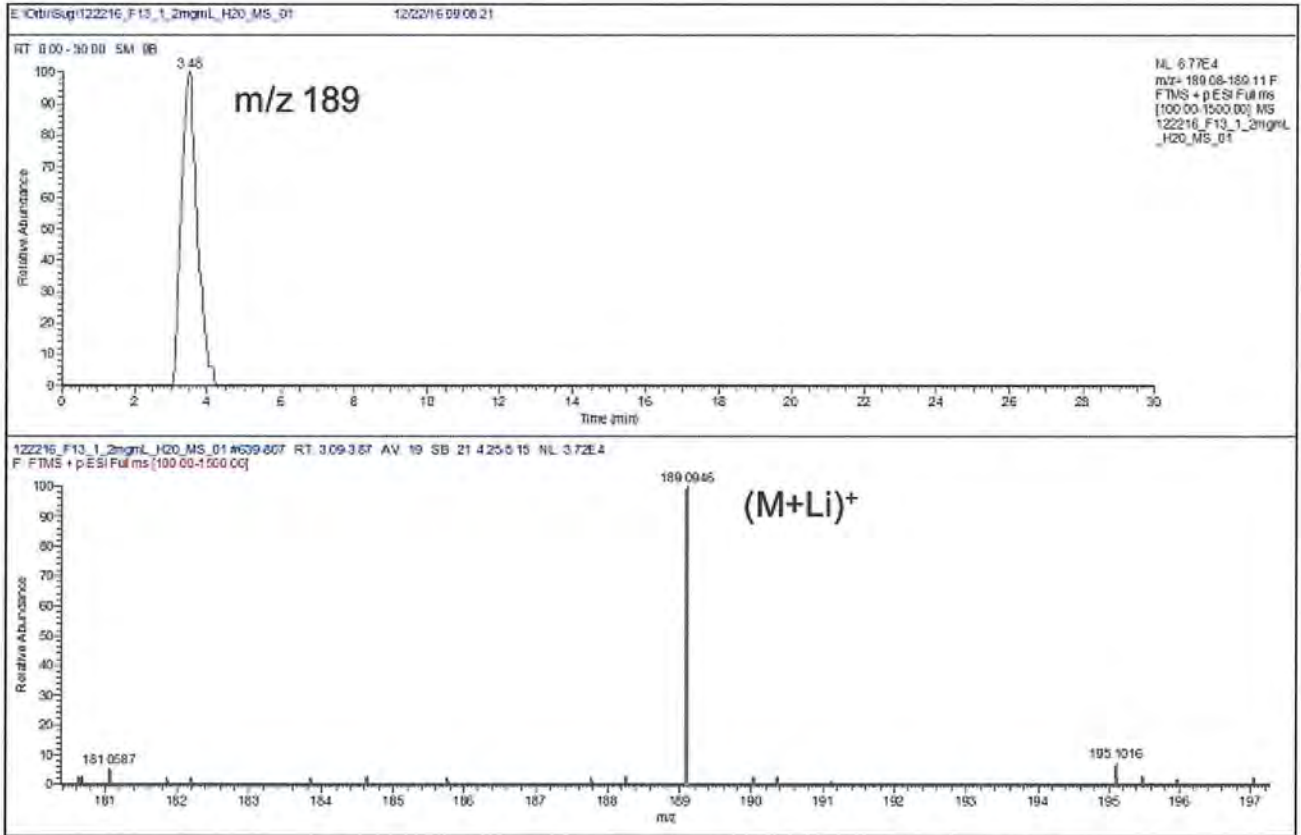
Figure 2. HPAEC-PAD chromatogram of 2'-FL powder, batch 13/1. Five microliter injection of 2 mg/mL 2'-FL powder aqueous solution.



**Figure 3.** HPAEC-PAD chromatogram of 2'-FL powder, batch 13/1. Five microliter injection of 2 mg/mL 2'-FL powder aqueous solution.

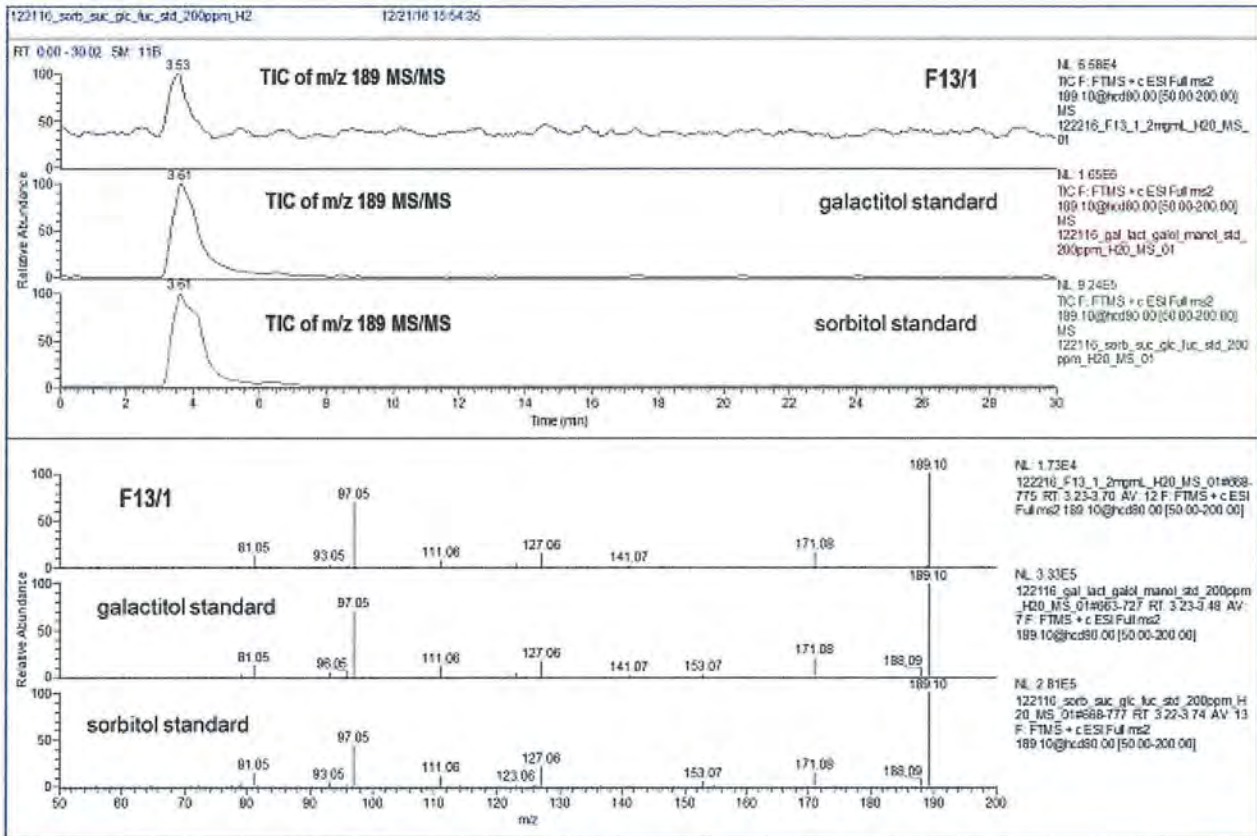


**Figure 4.** HPAEC-MS extracted ion chromatogram of m/z 189 for the 2'-FL powder, batch 13/1 sample. MS spectrum of 3.48 min peak and elemental formula calculated for the m/z 189 ion. Elemental formula of carbohydrate corresponding to m/z 189 ion is C<sub>6</sub>H<sub>14</sub>O<sub>6</sub>.

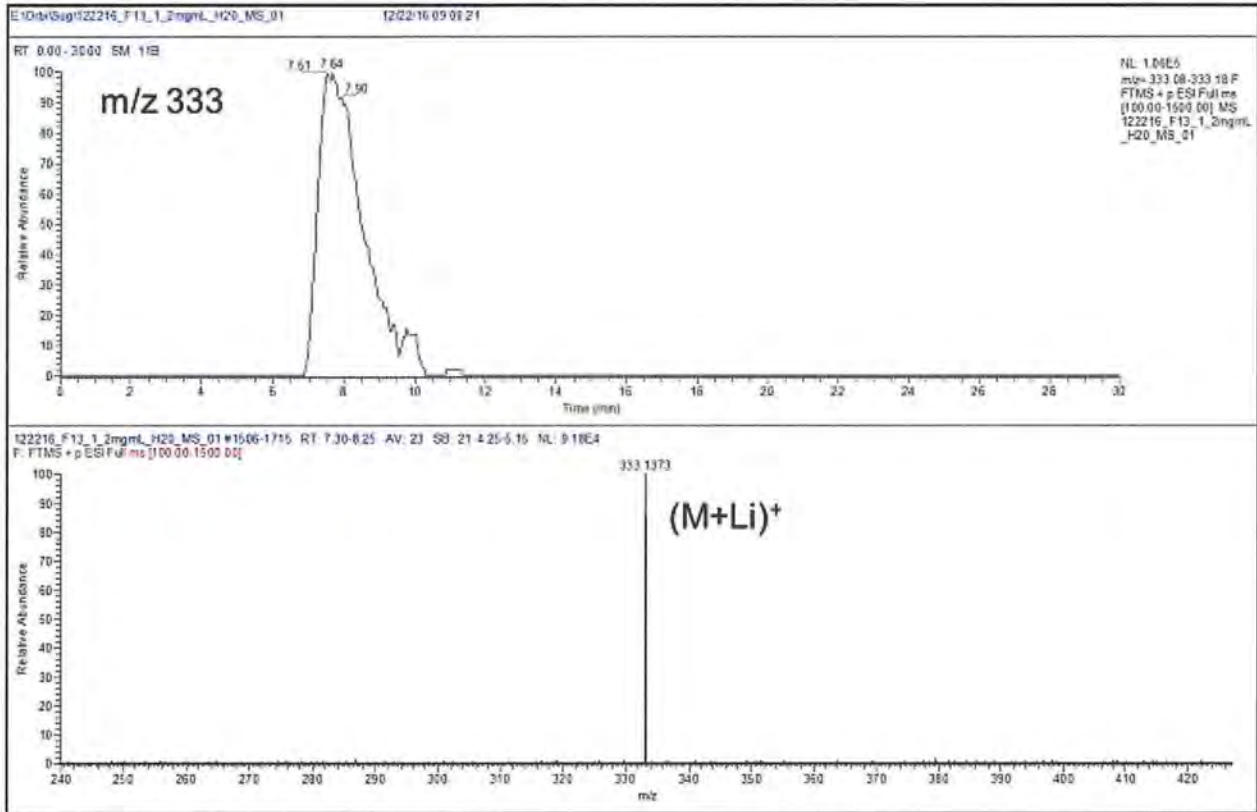


m/z	Theo. Mass	Delta (mmu)	RDB equiv.	Composition
189.0946	189.0945	0.10	-0.5	C6 H14 O6 Li

**Figure 5.** HPAEC-MS chromatograms representing TIC of m/z 189 product ion spectra for the 2'-FL powder, batch 13/1 sample, galactitol, and sorbitol standards. Product ion spectra of the m/z 189 peak of F13/1 sample, and galactitol, sorbitol standards.

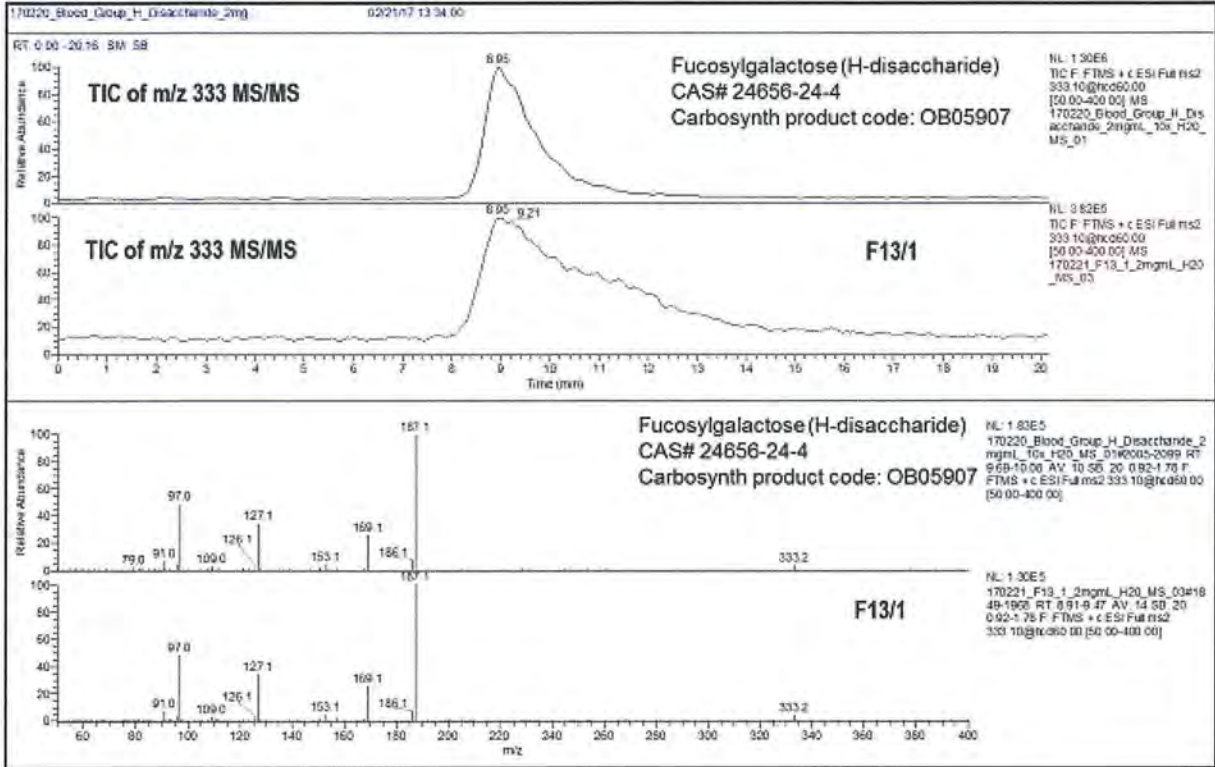


**Figure 6.** HPAEC-MS extracted ion chromatogram of m/z 333 for the 2'-FL powder, batch 13/1 sample. MS spectrum of 7.6 min peak and elemental formula calculated for the m/z 333 ion. Elemental formula of carbohydrate corresponding to m/z 333 ion is C<sub>12</sub>H<sub>22</sub>O<sub>10</sub>.

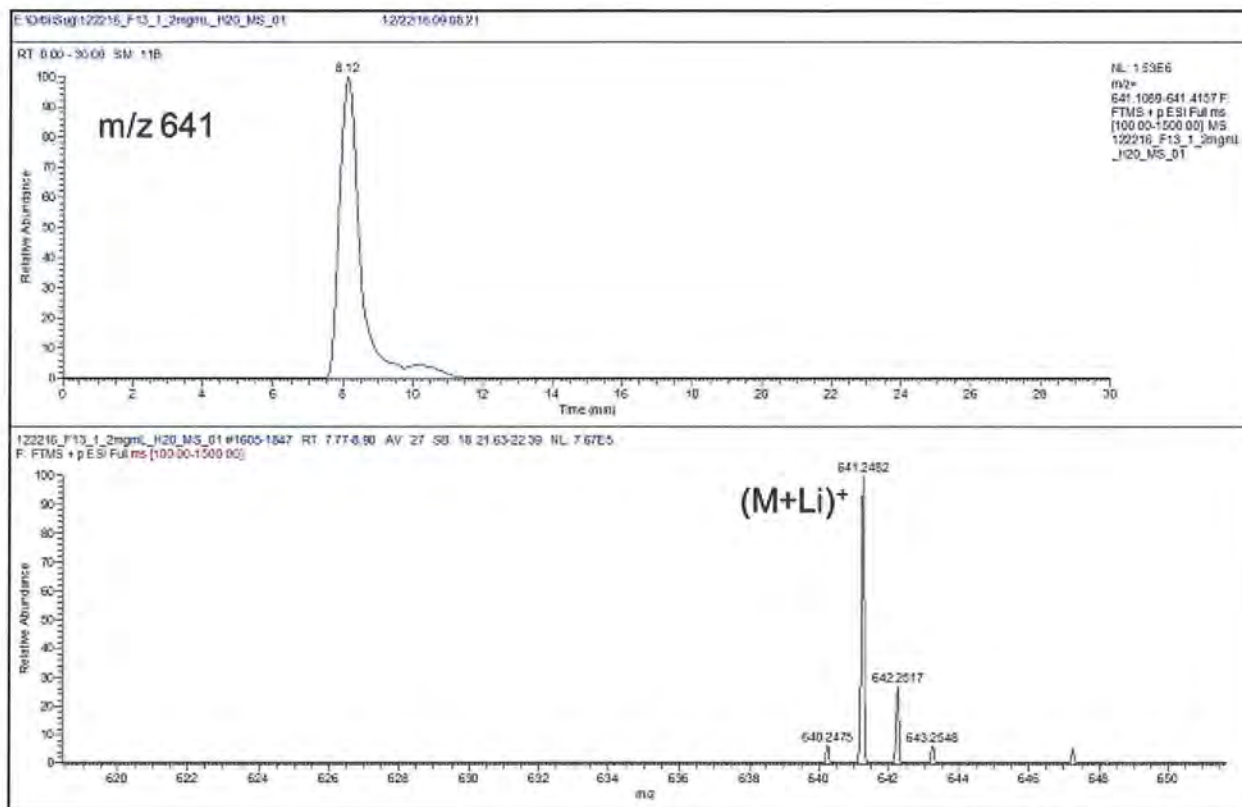


m/z	Theo. Mass	Delta (mmu)	RDB equiv.	Composition
333.1371	333.1368	0.37	1.5	C <sub>12</sub> H <sub>22</sub> O <sub>10</sub> Li

**Figure 7.** HPAEC-MS chromatograms representing TIC of m/z 333 product ion spectra for the 2'-FL powder, batch 13/1 sample and fucosylgalactose standard. Product ion spectra of the m/z 333 peak of F13/1 sample and fucosylgalactose standard.



**Figure 8.** HPAEC-MS extracted ion chromatogram of m/z 641 for the 2'-FL powder, batch 13/1 sample. MS spectrum of 8.12 min peak and elemental formula calculated for the m/z 641 ion. Elemental formula of carbohydrate corresponding to m/z 641 ion is  $C_{24}H_{42}O_{19}$ .



m/z	Theo. Mass	Delta (mmu)	RDB equiv.	Composition
641.2482	641.2475	0.69	3.5	C <sub>24</sub> H <sub>42</sub> O <sub>19</sub> Li



Figure 9. HPAEC-MS chromatograms representing TIC of m/z 641 product ion spectra for the 2'-FL powder, batch 13/1 sample and difucosyllactose standard. Product ion spectra of the m/z 641 peak of F13/1 sample and difucosyllactose standard.

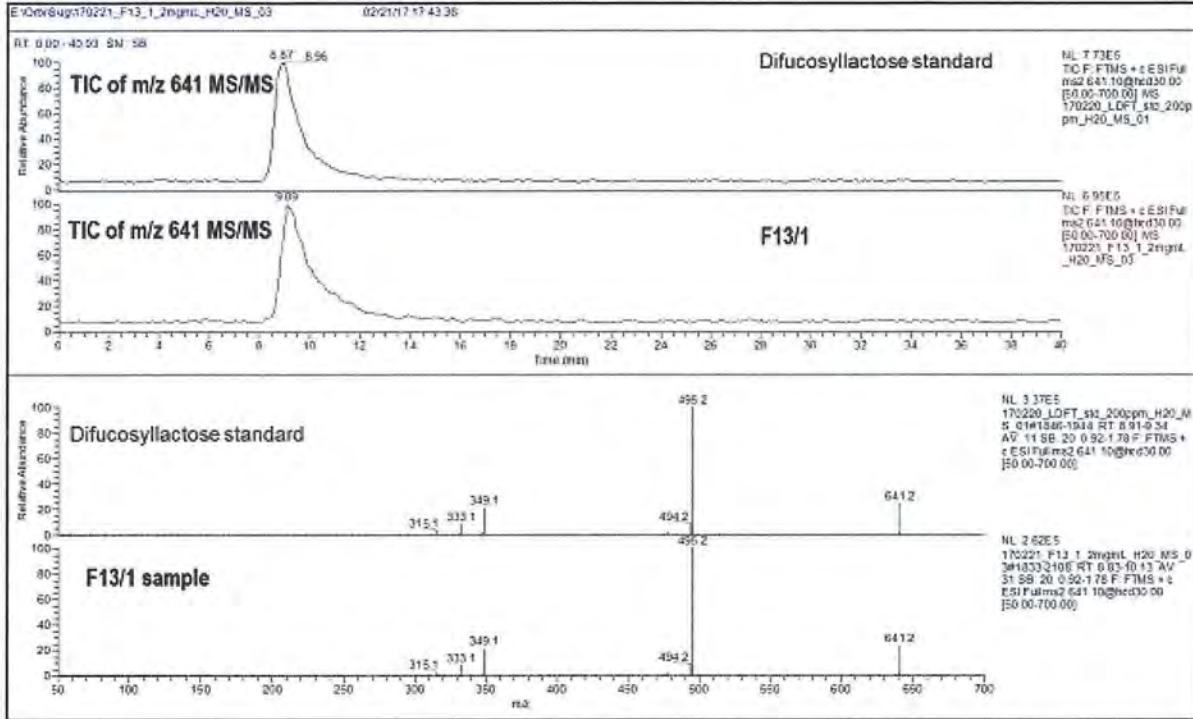
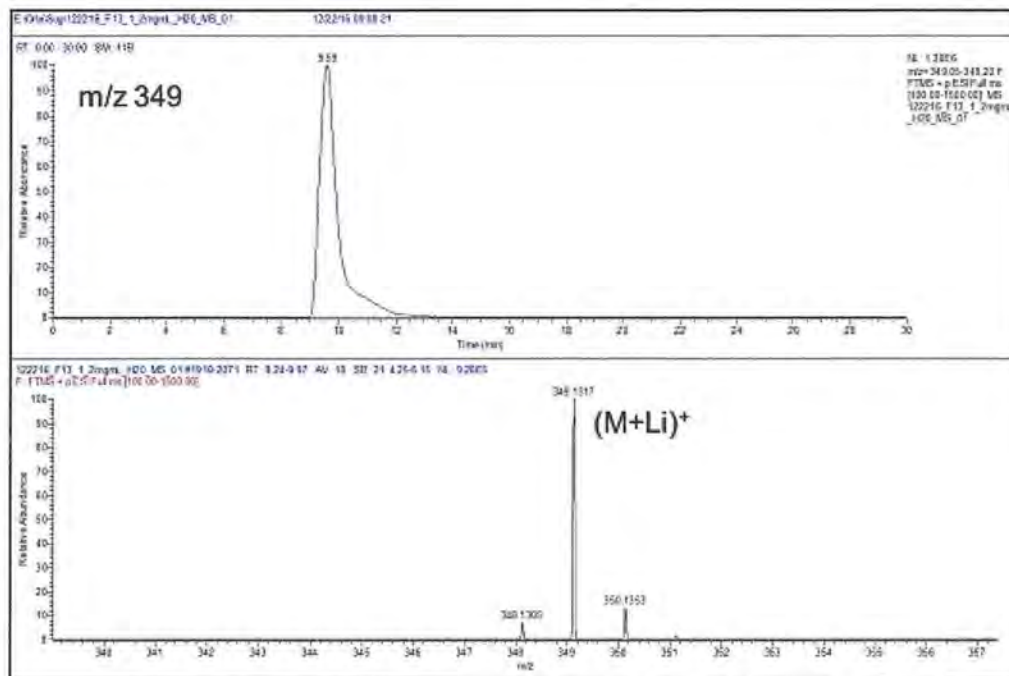
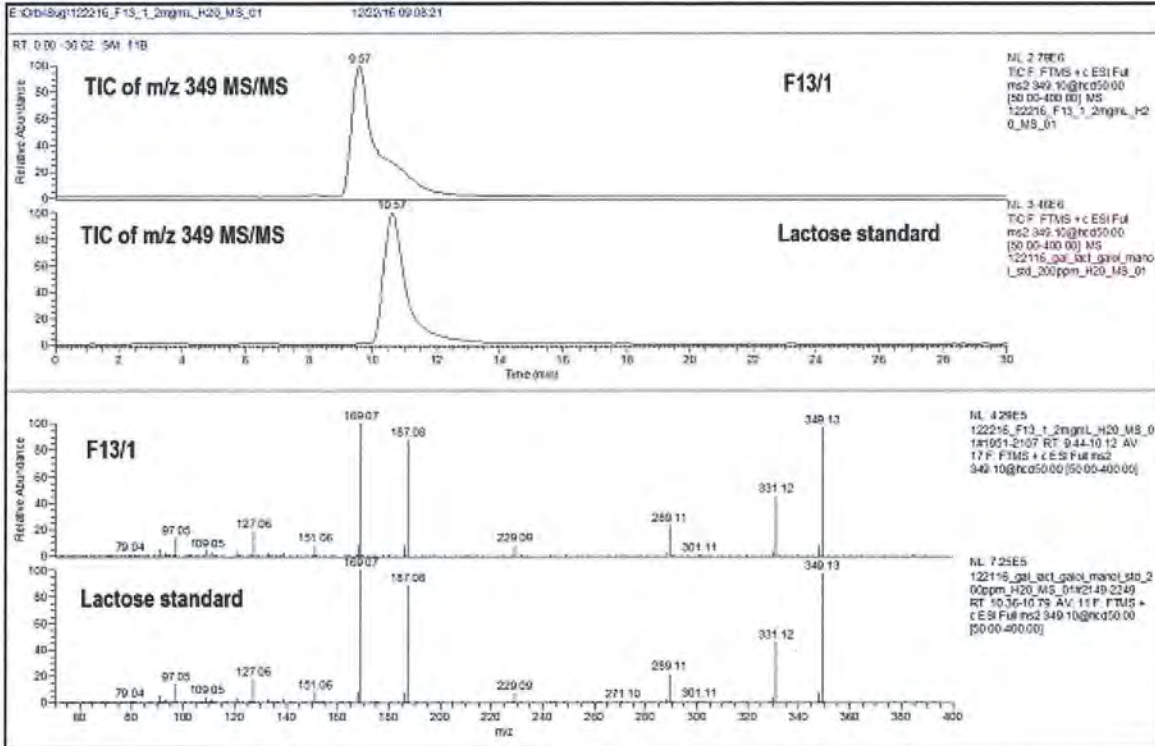


Figure 10. HPAEC-MS extracted ion chromatogram of m/z 349 for the 2'-FL powder, batch 13/1 sample. MS spectrum of 9.6 min peak and elemental formula calculated for the m/z 349 ion. Elemental formula of carbohydrate corresponding to m/z 349 ion is C<sub>12</sub>H<sub>22</sub>O<sub>11</sub>.

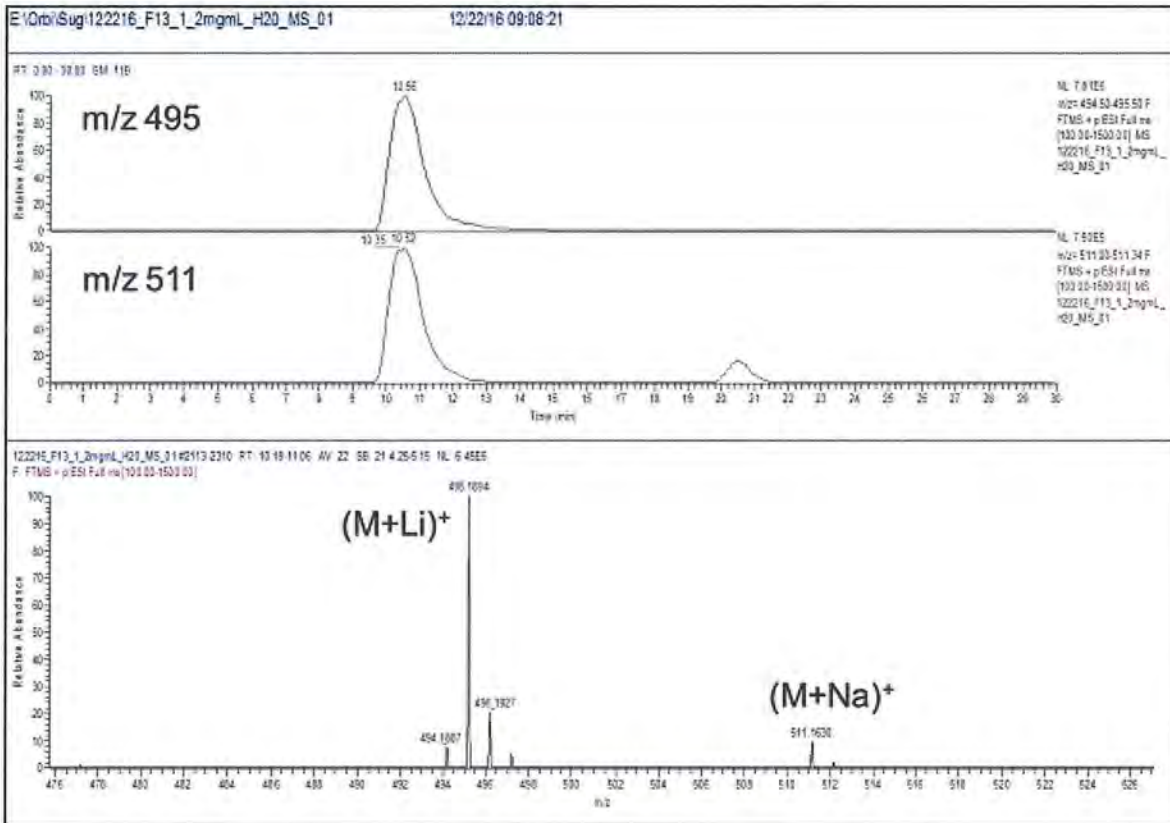


m/z	Theo. Mass	Delta (mmu)	RDB equiv.	Composition
349.1317	349.1317	0.02	1.5	C <sub>12</sub> H <sub>22</sub> O <sub>11</sub> Li

**Figure 11.** HPAEC-MS chromatograms representing TIC of m/z 349 product ion spectra for the 2'-FL powder, batch 13/1 sample and lactose standard. Product ion spectra of the m/z 349 peak of F13/1 sample and lactose standard.



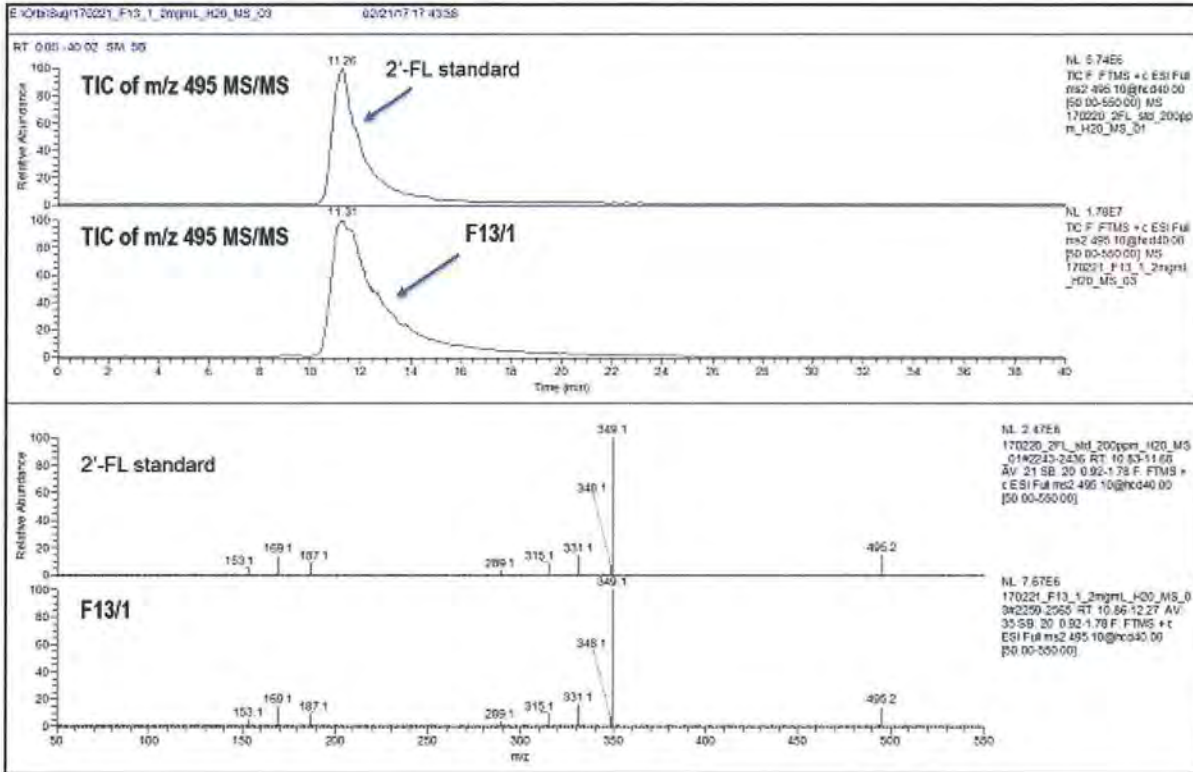
**Figure 12.** HPAEC-MS extracted ion chromatogram of m/z 495 and m/z 511 for the 2'-FL powder, batch 13/1 sample. MS spectrum of 10.5 min peak and elemental formula calculated for the m/z 495 ion and m/z 511 ion eluting at 10.5 min. Elemental formula of carbohydrate corresponding to m/z 495 ion is  $C_{18}H_{32}O_{15}$ .



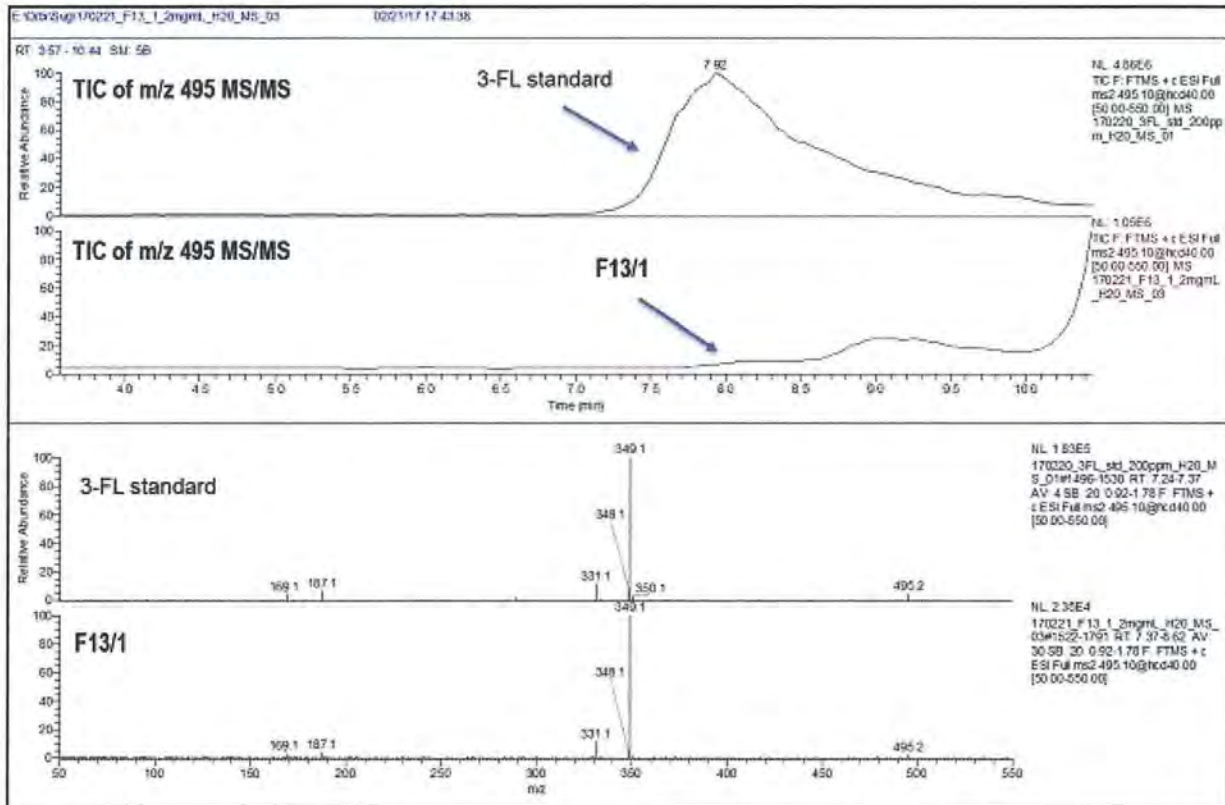
m/z	Theo. Mass	Delta (mmu)	RDB equiv.	Composition
495.1896	495.1896	0.01	2.5	C <sub>18</sub> H <sub>32</sub> O <sub>15</sub> Li

m/z	Theo. Mass	Delta (mmu)	RDB equiv.	Composition
511.1632	511.1633	-0.12	2.5	C <sub>18</sub> H <sub>32</sub> O <sub>15</sub> Na

**Figure 13.** HPAEC-MS chromatograms representing TIC of m/z 495 product ion spectra for the 2'-FL powder, batch 13/1 sample and 2'-FL standard. Product ion spectra of the m/z 495 peak of F13/1 sample and 2'-FL standard.



**Figure 14.** HPAEC-MS chromatograms representing TIC of m/z 495 product ion spectra for the 2'-FL powder, batch 13/1 sample and 2-FL standard. Product ion spectra of the m/z 495 peak of F13/1 sample and 3-FL standard.



**Figure 15.** HPAEC-MS chromatograms representing TIC of m/z 495 product ion spectra for the 2'-FL powder, batch F09 sample and extracted ion chromatograms the m/z 331 and m/z 477 fragment ions. Product ion spectra form different regions of the m/z 495 peak.

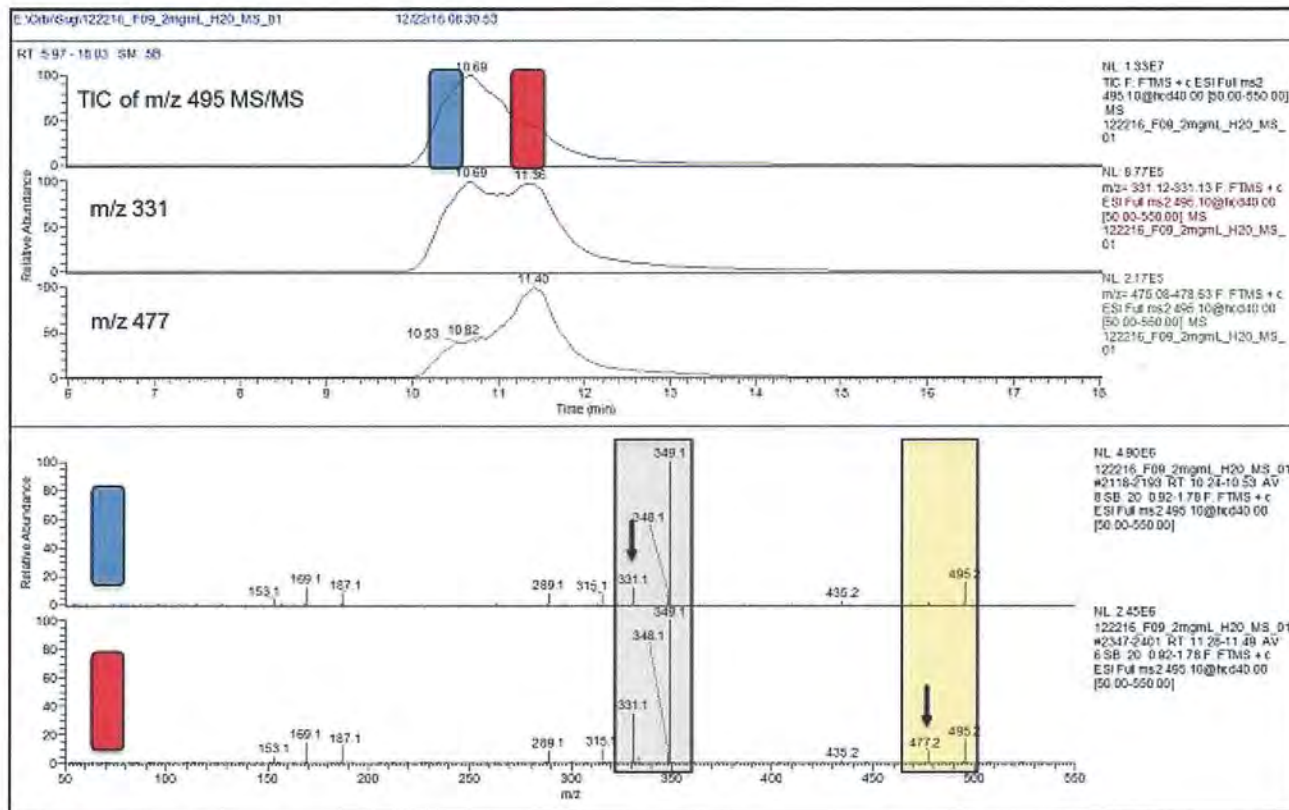
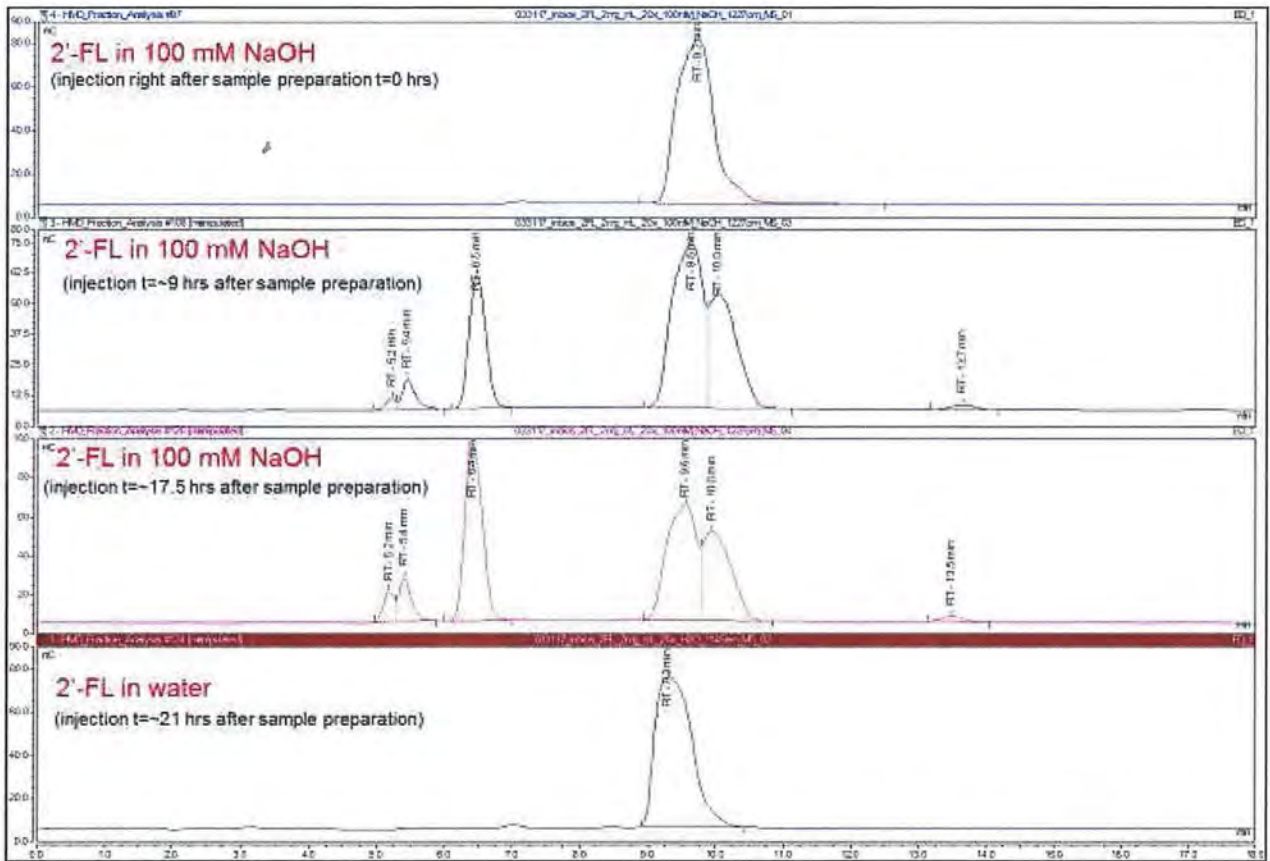
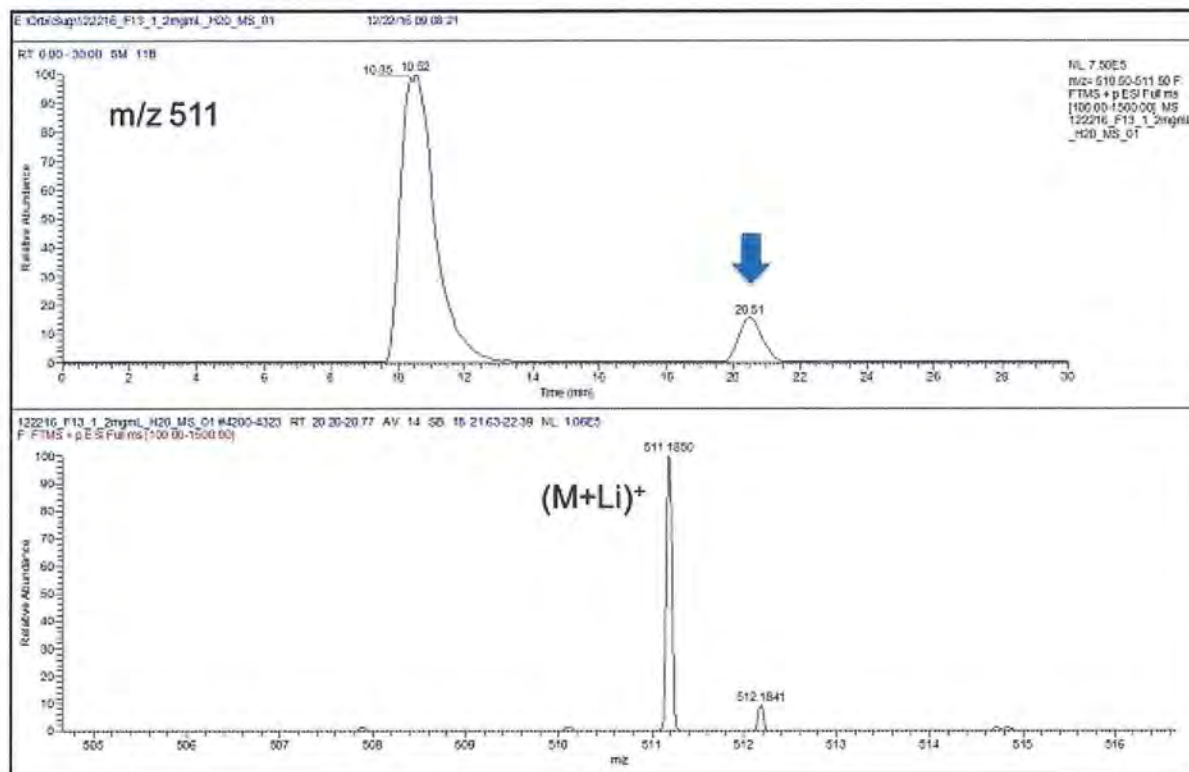


Figure 16. HPAEC-PAD chromatograms showing formation of the 2'-FL isomer peak under basic conditions (100 mM sodium hydroxide aqueous solution).



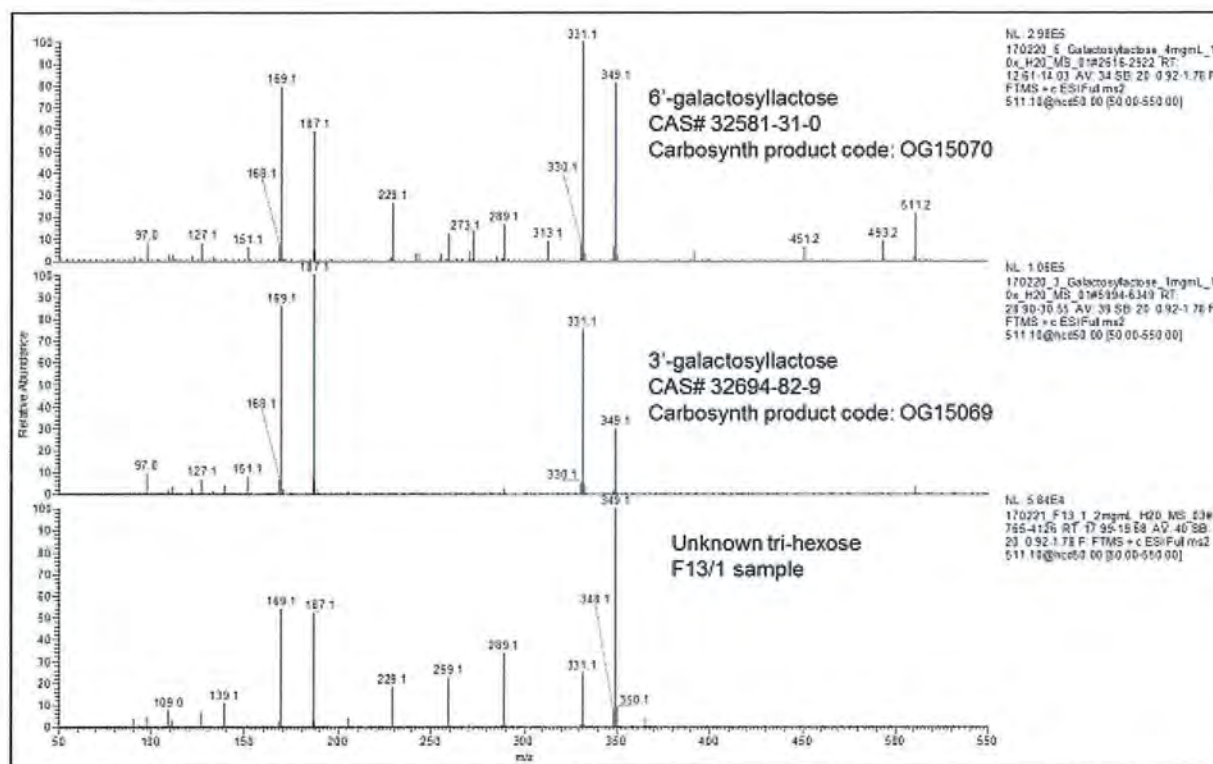


**Figure 17.** HPAEC-MS extracted ion chromatogram of m/z 511 for the 2'-FL powder, batch 13/1 sample. MS spectrum of 20.5 min peak and elemental formula calculated for the m/z 511 ion. Elemental formula of carbohydrate corresponding to m/z 511 ion is  $C_{18}H_{32}O_{16}$ .



m/z	Theo. Mass	Delta (mmu)	RDB equiv.	Composition
511.1850	511.1845	0.52	2.5	C <sub>18</sub> H <sub>32</sub> O <sub>16</sub> Li

**Figure 18.** Product ion spectra of 6'- and 3'-galactosyllactose and the m/z 511 tri-hexose detected in the 2'-FL powder, batch F13/1 sample.



## Appendix E: Affirmations

STATEMENT

Probiotics/Cultures & Food Protection

<b>Supplier</b>	DuPont Nutrition & Health
<b>Product</b>	2'Fucosyllactose
<b>Produced in</b>	Europe
<b>Rev. no.</b>	1
<b>Date</b>	30 June 2017

**DuPont Nutrition & Health 2'Fucosyllactose Food Grade Statement**

DuPont Nutrition & Health is committed to providing innovative products that meet or exceed the expectations of our customers. This includes commitment to manufacture products safely and sustainably, and to meet applicable regulatory and statutory requirements through implementation of our quality and food safety management processes.


DuPont Nutrition & Health manufacturing sites have Management Systems in place that have been certified according to FSSC or other GFSI standards. These include Standard Operation Procedures to ensure and document that the materials used in commercial production are of a suitable grade to be used in food.

Furthermore, the raw material suppliers must be certified according to ISO standards. The evaluation of suppliers is based on food safety standards which include supplier questionnaires and assessments which are covered by supplier audits.

For the production of DuPont Nutrition & Health 2'Fucosyllactose, raw materials are qualified and selected based on EU food and infant food regulations; as such, are in conformance with food grade requirements and suitable for use in food manufacturing.

The 2'Fucosyllactose will be manufactured under conditions suitable for human consumption, in accordance to EU food law requirements and EU regulations on hygiene for foodstuffs as well as Food GMPs.

Best regards,



Dagmar Pettke  
Quality & Food Safety EMEA  
Probiotics / Cultures & Food Protection  
DuPont Nutrition & Health

STATEMENT

Probiotics/Cultures & Food Protection

<b>Supplier</b>	DuPont Nutrition & Health
<b>Product</b>	2'Fucosyllactose
<b>Produced in</b>	Europe
<b>Rev. no.</b>	1
<b>Date</b>	17 July 2017


**DuPont Nutrition & Health 2'Fucosyllactose - GMO Statement**

2'Fucosyllactose does not contain or consist of GMOs, as defined in Regulation (EC) 1829/2003 on genetically modified food and feed, and Regulation (EC) 1830/2003 on the traceability and labelling of genetically modified food and feed products produced from GMOs.

2'Fucosyllactose is produced with a genetically engineered E. coli K-12 production strain. The microorganisms used for producing 2'Fucosyllactose are not present in the final product.

Apart from the E. coli K-12 production strain, no other genetically modified ingredients or genetic modification technology was used in the production of the 2FL.

Best regards,



Dagmar Pettke  
Quality & Food Safety EMEA  
Probiotics / Cultures & Food Protection  
DuPont Nutrition & Health

## Appendix F: Test for Residual Bacterial DNA

Confidential Information

Confidential Business Information



Date: April 27, 2017			
To: Oliver Hasselwander, Angela Lim, Paul Tenning			
cc: Jeff Pucci, Siiri Viikari			
Analysis Performed By: Katie Scarbinsky			
Responsible Manager: JoElla Heil			
Manager Signature: signature available upon request			
File	PIR (New Product Initiation Request)	<i>Escherichia coli</i>	GICC03482

## EXECUTIVE SUMMARY

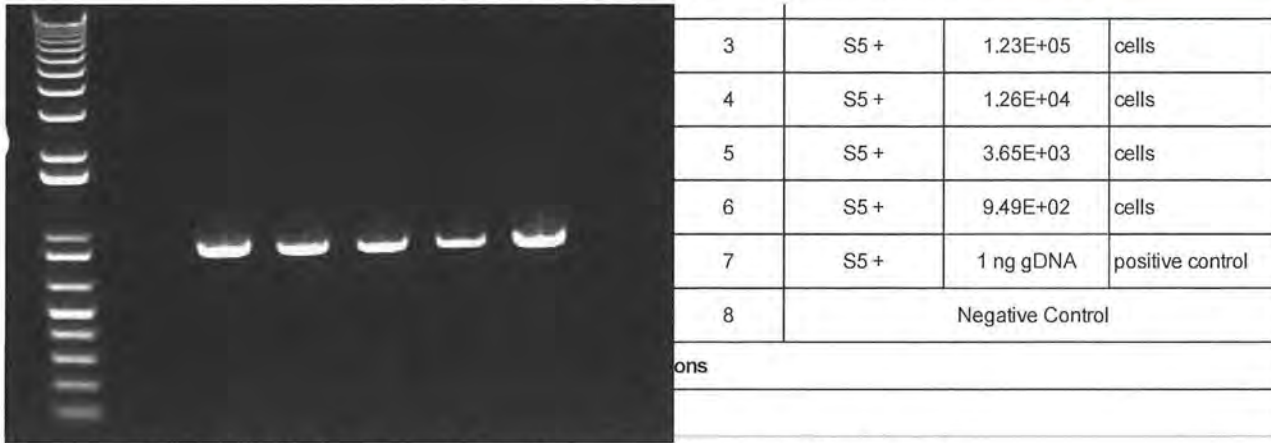
<ul style="list-style-type: none"> <li>The effectiveness of the cell lysis step was demonstrated. The band intensity decreased proportionately with the amount of cells added to the sample.</li> </ul>
<ul style="list-style-type: none"> <li>The limit of detection of rDNA for the assay was demonstrated by adding decreasing amounts of gDNA until DNA amplicon extinction was reached. The limit of detection for rDNA lies between 0.04885 ng/μl and 0.1953 ng/μl for the SP primers (amplifying chromosomal DNA), and 0.00075 ng/μl and 0.00305 ng/μl for the FT primers (amplifying plasmid DNA).</li> </ul>
<ul style="list-style-type: none"> <li>5 lots of Flow-Mo / 2' Fucosyl Lactose spray dried sample was tested using two primer sets: one amplifying a gene from the plasmid, and a second set for a gene on the chromosomal DNA. Spray dried samples were reconstituted in a 1:10 dilution of water, and then 1:5 in cell lysis buffer.</li> </ul>
<ul style="list-style-type: none"> <li>Based on the conditions cited in this test, it can be stated that no rDNA was detected in this sample using the FT primers (amplifying plasmid DNA). No rDNA was detected using the SP primers (amplifying chromosomal DNA) for this sample.</li> </ul>

## SAMPLE INFORMATION

Product:	Flow-Mo / 2' Fucosyl Lactose			Number of Samples
GICC#:	GICC03482			5
#	Batch	Sample Type	Fermentation Site	Spray Dried Site
S1	F13/3	Spray Dried	Inbiose	Brabrand
S2	F21	Spray Dried	Inbiose	Brabrand
S3	F22	Spray Dried	Inbiose	Brabrand
S4	F23	Spray Dried	Inbiose	Brabrand
S5	F25	Spray Dried	Inbiose	Brabrand

## RESULTS

1A - Sample with Intact Cells									
#	Primers	Batch Number						Sample Dilution	
S5	Fucosyltransferase on the Plasmid	F25						1:50	
Gel Results									
1	2	3	4	5	6	7	8	Lane	Description
								1	Invitrogen E-gel 1 kb Plus DNA Ladder
								2	Sample 5 (S5)
								A-95	



- Bands were clearly detected in lanes 3, 4, 5 and 6 when intact cells were spiked into the sample.
- The positive control spike (1 ng gDNA) was detected in lane 7.
- No rDNA was detected in the negative control, as seen by the lack of bands in lane 8.
- **The decreasing band intensity demonstrates that the cell lysis step is effective.**

Detailed method description can be found at end of report under Description of Assay Methods.

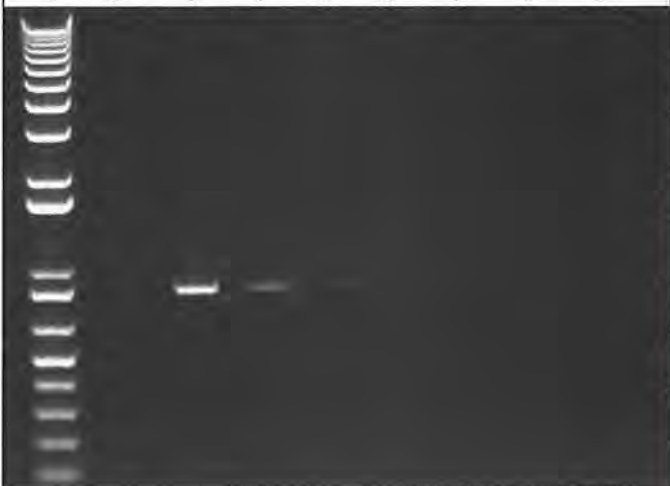
**RESULTS**

1B - Sample with Intact Cells									
#	Primers	Batch Number		Sample Dilution					
S5	Sucrose Phosphorylase on the Chromosome	F25		1:50					
Gel Results									
1	2	3	4	5	6	7	8	Lane	Description
								1	Invitrogen E-gel 1 kb Plus DNA Ladder
								2	Sample 5 (S5)
								3	S5 + 1.23E+05 cells
								4	S5 + 1.26E+04 cells
								5	S5 + 3.65E+03 cells
								6	S5 + 9.49E+02 cells
								7	S5 + 1 ng gDNA positive control
								8	Negative Control
Observations									
• No rDNA was detected in lot F25 alone (lane 2).									
• Bands were clearly detected in lanes 3, 4, 5 and 6 when intact cells were spiked into the sample.									
• The positive control spike (1 ng gDNA) was detected in lane 7.									
• No rDNA was detected in the negative control, as seen by the lack of bands in lane 8.									
• <b>The decreasing band intensity demonstrates that the cell lysis step is effective.</b>									

Detailed method description can be found at end of report under Description of Assay Methods.



**RESULTS**

2A - Limit of Detection												
#	Primers				Batch Number					Sample Dilution		
S5	Fucosyltransferase on the Plasmid				F25					1:50		
Gel Results												
1	2	3	4	5	6	7	8	9	Lane	Description		
									1	Invitrogen E-gel 1 kb Plus DNA Ladder		
									2	Sample 5 (S5)		
									3	S5 +	0.003906	ng gDNA
									4	S5 +	0.000977	ng gDNA
									5	S5 +	0.000244	ng gDNA
									6	S5 +	0.000061	ng gDNA
									7	S5 +	0.000015	ng gDNA
									8	S5 +	0.000004	ng gDNA
									9	Negative Control		
Observations												
<ul style="list-style-type: none"> <li>No rDNA was detected in lot F25 alone (lane 2); bands for spiked gDNA were clearly detected in lanes 3 and 4. A faint band for spiked gDNA was detected in lanes 5 and 6. No band was seen in lane 7 or 8.</li> <li>No rDNA was detected in the negative control, as seen by the lack of bands in lane 9.</li> <li>It can be established that the limit of detection lies between 0.000015 ng and 0.000061 ng.</li> <li>Accounting for sample dilution, the limit of rDNA detection in our samples is between 0.00075 ng/µl and 0.00305 ng/µl.</li> </ul>												

Detailed method description can be found at end of report under Description of Assay Methods.

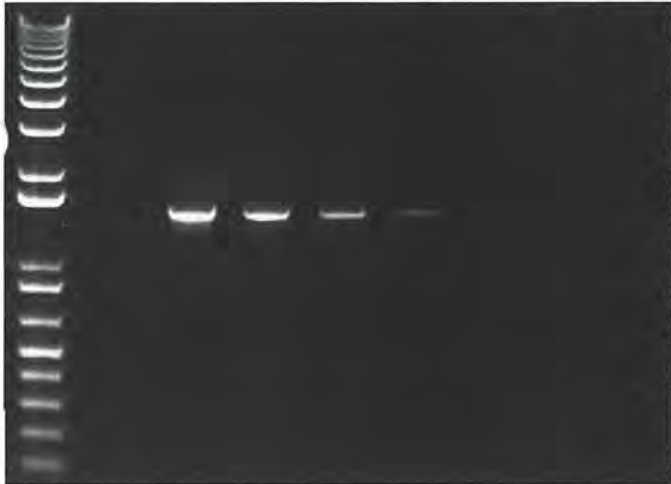
**RESULTS**

2B - Limit of Detection												
#	Primers				Batch Number					Sample Dilution		
S5	Sucrose Phosphorylase on the Chromosome				F25					1:50		
Gel Results												
1	2	3	4	5	6	7	8	9	Lane	Description		
									1	Invitrogen E-gel 1 kb Plus DNA Ladder		
									2	Sample 5 (S5)		
									3	S5 +	1.000000	ng gDNA
									4	S5 +	0.250000	ng gDNA
									5	S5 +	0.062500	ng gDNA
									6	S5 +	0.015625	ng gDNA

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5/4/2017

GICC03482\_2017\_Registration\_Flow-Mo\_Brabrand\_Inbiose\_UPDATED.xlsx



7	S5 +	0.003906	ng gDNA
8	S5 +	0.000977	ng gDNA
9	Negative Control		

ons

gDNA were clearly detected in lanes 3, 4 and 5. A faint band for in lane 8.

ck of bands in lane 9.

000977 ng and 0.003906 ng.

our samples is between 0.04885 ng/μl and 0.1953 ng/μl.

on of Assay Methods.

**RESULTS**

3A - Samples Tested in Triplicate							
#	Primers	Batch Number	Sample Dilution				
S1	Fucosyltransferase on the Plasmid	F13/3	1:50				
Gel Results							
1	2	3	4	5	6	Lane	Description
						1	Invitrogen E-gel 1 kb Plus DNA Ladder
						2	S1 replicate 1
						3	S1 replicate 2
						4	S1 replicate 3
						5	S1+ 1 ng gDNA
						6	Negative Control
Observations							
<ul style="list-style-type: none"> <li>No rDNA was detected in Batch F13/3; there were no bands detected in lanes 2, 3, and 4 using primers amplifying the Fucosyltransferase gene on the plasmid.</li> <li>The positive control spike of 1 ng gDNA was detected in lane 5.</li> <li>No rDNA was detected in the negative control, as seen by the lack of bands in lane 6.</li> </ul>							

Detailed method description can be found at end of report under Description of Assay Methods.

**RESULTS**

3B - Samples Tested in Triplicate							
#	Primers	Batch Number	Sample Dilution				
S1	Sucrose Phosphorylase on the Chromosome	F13/3	1:50				
Gel Results							
1	2	3	4	5	6	Lane	Description
						1	Invitrogen E-gel 1 kb Plus DNA Ladder

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2	S1	replicate 1
3	S1	replicate 2
4	S1	replicate 3
5	S1+	1 ng gDNA
6	Negative Control	

- Observations**
- No rDNA was detected in Batch F13/3; there were no bands detected in lanes 2, 3, and 4 using primers amplifying the Sucrose Phosphorylase gene on the chromosome.
  - The positive control spike of 1 ng gDNA was detected in lane 5.
  - No rDNA was detected in the negative control, as seen by the lack of bands in lane 6.

Detailed method description can be found at end of report under Description of Assay Methods.

**RESULTS**

3C - Samples Tested in Triplicate			
#	Primers	Batch Number	Sample Dilution
S2	Fucosyltransferase on the Plasmid	F21	1:50

Gel Results								
1	2	3	4	5	6	Lane	Description	
						1	Invitrogen E-gel 1 kb Plus DNA Ladder	
						2	S2	replicate 1
						3	S2	replicate 2
						4	S2	replicate 3
						5	S2+	1 ng gDNA
						6	Negative Control	

- Observations**
- No rDNA was detected in Batch F21; there were no bands detected in lanes 2, 3, and 4 using primers amplifying the Fucosyltransferase gene on the plasmid.
  - The positive control spike of 1 ng gDNA was detected in lane 5.
  - No rDNA was detected in the negative control, as seen by the lack of bands in lane 6.

Detailed method description can be found at end of report under Description of Assay Methods.

**RESULTS**

3D - Samples Tested in Triplicate			
#	Primers	Batch Number	Sample Dilution
S2	Sucrose Phosphorylase on the Chromosome	F21	1:50

A-99

Gel Results								
1	2	3	4	5	6	Lane	Description	
						1	Invitrogen E-gel 1 kb Plus DNA Ladder	
						2	S2	replicate 1
						3	S2	replicate 2
						4	S2	replicate 3
						5	S2+	1 ng gDNA
						6	Negative Control	
Observations								
<ul style="list-style-type: none"> <li>No rDNA was detected in Batch F21; there were no bands detected in lanes 2, 3, and 4 using primers amplifying the Sucrose Phosphorylase gene on the chromosome.</li> <li>The positive control spike of 1 ng gDNA was detected in lane 5.</li> <li>No rDNA was detected in the negative control, as seen by the lack of bands in lane 6.</li> </ul>								

Detailed method description can be found at end of report under Description of Assay Methods.

**RESULTS**

3E - Samples Tested in Triplicate												
#	Primers	Batch Number	Sample Dilution									
S3	Fucosyltransferase on the Plasmid	F22	1:50									
S4	Fucosyltransferase on the Plasmid	F23	1:50									
Gel Results												
1	2	3	4	5	6	7	8	9	10	Lane	Description	
										1	Invitrogen E-gel 1 kb Plus DNA Ladder	
										2	S3	replicate 1
										3	S3	replicate 2
										4	S3	replicate 3
										5	S3+	1 ng gDNA
										6	S4	replicate 1
										7	S4	replicate 2
										8	S4	replicate 3
										9	S4+	1 ng gDNA
										10	Negative Control	
Observations												
<ul style="list-style-type: none"> <li>No rDNA was detected in Batch F22; there were no bands detected in lanes 2, 3, and 4 using primers amplifying the Fucosyltransferase gene on the plasmid.</li> </ul>												

<ul style="list-style-type: none"> <li>No rDNA was detected in Batch F23; there were no bands detected in lanes 6, 7, and 8 using primers amplifying the Fucosyltransferase gene on the plasmid.</li> </ul>
<ul style="list-style-type: none"> <li>The positive control spike of 1 ng gDNA was detected in lanes 5 and 9.</li> </ul>
<ul style="list-style-type: none"> <li>No rDNA was detected in the negative control, as seen by the lack of bands in lane 10.</li> </ul>

Detailed method description can be found at end of report under Description of Assay Methods.

**RESULTS**

3F- Samples Tested in Triplicate			
#	Primers	Batch Number	Sample Dilution
S3	Sucrose Phosphorylase on the Chromosome	F22	1:50
S4	Sucrose Phosphorylase on the Chromosome	F23	1:50

Gel Results													
1	2	3	4	5	6	7	8	9	10	Lane	Description		
											1	Invitrogen E-gel 1 kb Plus DNA Ladder	
											2	S3	replicate 1
											3	S3	replicate 2
											4	S3	replicate 3
											5	S3+	1 ng gDNA
											6	S4	replicate 1
											7	S4	replicate 2
											8	S4	replicate 3
											9	S4+	1 ng gDNA
											10	Negative Control	

Observations	
<ul style="list-style-type: none"> <li>No rDNA was detected in Batch F22; there were no bands detected in lanes 2, 3, and 4 using primers amplifying the Sucrose Phosphorylase gene on the chromosome.</li> </ul>	
<ul style="list-style-type: none"> <li>No rDNA was detected in Batch F23; there were no bands detected in lanes 6, 7, and 8 using primers amplifying the Sucrose Phosphorylase gene on the chromosome.</li> </ul>	
<ul style="list-style-type: none"> <li>The positive control spike of 1 ng gDNA was detected in lanes 5 and 9.</li> </ul>	
<ul style="list-style-type: none"> <li>No rDNA was detected in the negative control, as seen by the lack of bands in lane 10.</li> </ul>	

Detailed method description can be found at end of report under Description of Assay Methods.

**RESULTS**

3G - Samples Tested in Triplicate			
#	Primers	Batch Number	Sample Dilution
S5	Fucosyltransferase on the Plasmid	F25	1:50

A-101

Gel Results								
1	2	3	4	5	6	Lane	Description	
						1	Invitrogen E-gel 1 kb Plus DNA Ladder	
						2	S5	replicate 1
						3	S5	replicate 2
						4	S5	replicate 3
						5	S5+	1 ng gDNA
						6	Negative Control	
Observations								
<ul style="list-style-type: none"> <li>No rDNA was detected in Batch F25; there were no bands detected in lanes 2, 3, and 4 using primers amplifying the Fucosyltransferase gene on the plasmid.</li> <li>The positive control spike of 1 ng gDNA was detected in lane 5.</li> <li>No rDNA was detected in the negative control, as seen by the lack of bands in lane 6.</li> </ul>								

Detailed method description can be found at end of report under Description of Assay Methods.

**RESULTS**

3H - Samples Tested in Triplicate								
#	Primers	Batch Number	Sample Dilution					
S5	Sucrose Phosphorylase on the Chromosome	F25	1:50					
Gel Results								
1	2	3	4	5	6	Lane	Description	
						1	Invitrogen E-gel 1 kb Plus DNA Ladder	
						2	S5	replicate 1
						3	S5	replicate 2
						4	S5	replicate 3
						5	S5+	1 ng gDNA
						6	Negative Control	
Observations								
<ul style="list-style-type: none"> <li>No rDNA was detected in Batch F25; there were no bands detected in lanes 2, 3, and 4 using primers amplifying the Sucrose Phosphorylase gene on the chromosome.</li> <li>The positive control spike of 1 ng gDNA was detected in lane 5.</li> <li>No rDNA was detected in the negative control, as seen by the lack of bands in lane 6.</li> </ul>								

Detailed method description can be found at end of report under Description of Assay Methods.

Description of Assay Methods	
PCR Conditions	1.1 PCR is carried out using the GE Healthcare "puReTaq Ready to Go PCR Beads"

1.2 PCR Amplification:		
<b>Gene Amplified</b>	Fucosyltransferase on the Plasmid	
<b>Primer</b>	<b>Sequence</b>	<b>Concentration</b>
<u>FTfor</u>	<u>GCAATCAGATGTTTCAGTATG</u>	0.4 µM
<u>FTrev</u>	<u>AGGCATTATATTCTGGCTTTTC</u>	0.4 µM
<b>Gene Amplified</b>	Sucrose Phosphorylase on the Chromosome	
<b>Primer</b>	<b>Sequence</b>	<b>Concentration</b>
<u>SPfor</u>	<u>AGGTGCAGCTCATCACTTAC</u>	0.4 µM
<u>SPrev</u>	<u>ATTGGCGATCAGATCATCCG</u>	0.4 µM
1.3 1 µL of prepared sample and/ or control is added to 24 µL primer solution w/ PCR Beads		
1.4 PCR reactions are mixed and run in a PCR machine with a cycle program optimized for this product sample type		
1.5 Gel electrophoresis is performed on resulting PCR products		
1.5.1 Gel used: Invitrogen E-gel Agarose (GP)		
1.5.2 Molecular Weight Standard used: Invitrogen E-gel 1 kb Plus DNA Ladder		
1.6 Amplified DNA is visualized by ethidium bromide		
<b>Sample Analysis</b>	2.1 DNA is extracted / released from any residual host organisms by incubating the samples in a lysis buffer at 95°C for 15 minutes, then incubating ≤1 hour at room temperature	
	2.2 Extracted samples are added to the PCR reaction as described (step 1.3)	
	2.3 When possible, three independent lots are analyzed in triplicate	
<b>Control Validation</b>	<b>DNA extraction (cell lysis)</b>	
	3.1 Host cells are added to the experimental sample at multiple concentrations prior to DNA extraction (step 2.1).	
	3.2 Lysis and DNA extraction are confirmed by the presence of a dose-dependent change in PCR product intensity	
	<b>DNA Controls</b>	
	3.3 Sample Positive Control: gDNA is added to the experimental sample prior to DNA extraction (step 2.1)	
3.4 gDNA Extinction: gDNA in serial dilutions is added to the experimental sample prior to DNA extraction (step 2.1)		

Limit of Detection Calculation	
$LOD \text{ for sample rDNA concentration } \left( \frac{ng}{\mu L} \right) = PCR \text{ reaction gDNA spike (ng)} \times \text{Sample dilution } (\mu L^{-1})$	
Assay Requirements Based on EFSA Guidelines	
<b>Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. EFSA Panel on Genetically Modified Organisms:</b>	
<i>EFSA Journal 2011;9(6):2193 Section 2.2.3 Information on the possible presence of recombinant DNA is required in products belonging to Categories 1, 2 and 3. If recombinant DNA corresponding to full-length coding sequences is found, the likelihood of gene transfer must be assessed (See Section B.4.). All the methods should be documented in detail.</i>	
1	All DNA present in the product should be extracted. Therefore, a cell lysis step must be included in the protocol to extract DNA from products belonging to Categories 2 or 3. Special attention should be given to the detection of DNA present in microorganisms that are resistant to cell lysis, like those capable of forming spores. To verify the efficacy of the lysis step, intact cells of the GMM must be added in different dilutions before DNA extraction as a positive control.
2	Control DNA should be added to the sample in different dilutions until DNA extinction before commencing the DNA extraction process, in order to check the limit of detection of recombinant DNA in the sample.
3	The presence of DNA should be assessed using a PCR-based method. The reliability, efficacy and sensitivity of the DNA detection

	method should be documented. Positive and negative controls must be included to ensure functional PCR and to exclude PCR inhibition. As control DNA, total DNA of the GMM must be used. Should PCR inhibition be encountered when testing the product, samples taken before formulation may be used.
4	At least one functional gene has to be targeted. Because DNA degradation can be sequence-dependent, all functional genes, if of concern (e.g. antimicrobial resistance genes, virulence genes, genes encoding toxic compounds), inserted into the GMM should be targeted specifically. The PCR should span the full length of the coding sequences but should not exceed it.
5	At least three independent batches of product preparations should be sampled, each analyzed in triplicate. A proper sampling method should be chosen and documented (see point 2.2.1).



## Appendix G: Summary of Animal Studies

## APPENDIX G: TOXICOLOGY AND NON-TOXICOLOGICAL STUDIES ON 2FL

### In vitro Toxicology Studies

#### **Bacterial Reverse Mutation Test (Ames Test)**

Coulet et al. (2014) investigated the mutagenic potential of 2FL in a bacterial reverse mutation test using the plate incorporation and pre-incubation methods. Five *Salmonella typhimurium* strains (TA98, TA100, TA1535, TA1537, and TA102) were exposed to 52, 164, 512, 1600, and 5000 µg/plate of 2FL (Glycom/AS) in the presence or absence of metabolic activation (rat liver S9 fraction mix). The negative control in this study was water and the positive controls were 2-nitrofluorene, sodium azide, 9-aminoacridine, *t*-butylhydroperoxide, 2-aminoanthracene. There was no evidence of cytotoxicity or precipitation in the study. Strain TA 100 showed statistically significant increases in the number of revertants in the absence of metabolic activation at doses of 164 and 5000 µg/plate and strain TA102 showed significant increases in the number of revertants at 52 and 164 µg/plate; however, there was no dose-response relationship observed and the increases in number of revertants were not greater than two-fold. The authors concluded that 2FL was not mutagenic at doses of up to 5000 µg/mL.

Glycom A/S (2016) described the results of an unpublished bacterial reverse mutation assay that examined the mutagenicity of Glycom's 2FL (97.6% purity) in *Salmonella typhimurium* strains TA98, TA 100, TA1535, and TA1537 and in *Escherichia coli* strain WP2uvrA in the presence and absence of metabolic activation (S9) (Verspeek-Rip, 2015). The study adhered to the OECD principles of GLP and followed OECD Test guideline No 471. In both the plate incorporation method and the preincubation methods, 5000 µg/plate did not elicit cytotoxicity or a significant increase in the number of revertant colonies. The authors concluded that doses of up to 5000 µg/plate of Glycom's 2FL were not mutagenic in the presence or absence of metabolic activation.

Jennewein Biotechnologie (2015) conducted a *Salmonella* mutation assay in which the mutagenicity of up to 5000 µg/plate of Jennewein's 2FL was tested in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 in the presence and absence of metabolic activation using the plate incorporation and preincubation methods. No cytotoxicity or elevated numbers of revertant colonies were observed; however, the positive controls showed a significant increase in the number of revertant colonies. The authors concluded that that Jennewein's 2FL was not cytotoxic or mutagenic.

#### **In vitro Mammalian Cell Gene Mutation Assay in Mouse Lymphoma L5178Y Cells**

In the mouse lymphoma assay, cultured L51784 *tk*<sup>+/+</sup> mouse lymphoma cells were treated with doses of up to 5000 µg/mL of chemically synthesized 2FL (Glycom/AS) for four hours in the presence or absence of metabolic activation (Coulet et al., 2014). The positive controls were methylmethanesulfonate (MMS) and cyclophosphamide (CP). No biologically relevant increases in the frequency of mutations, cytotoxicity, or precipitation were observed at any dose of 2FL.

#### **Mammalian Micronucleus Test**

Verbaan (2015a) conducted an in vitro micronucleus test in which human peripheral blood lymphocytes were exposed to doses of up to 2000 µg/mL of chemically synthesized 2FL in the presence and absence of metabolic activation (S9) (unpublished study). The authors reported no significant increase in the

number of micronucleated peripheral human lymphocytes at dose of up to 2000 µg/mL. This study was reviewed by (EFSA Panel on Dietetic Products, 2015).

GRN 650 described an unpublished in vitro micronucleus test in which human peripheral blood lymphocytes were exposed to doses of up to 2000 µg/mL 2FL (Verbaan, 2015b). In the first study, exposure to 2FL produced from fermentation (97.6% purity) was for 3 h with a 27h harvest time in the presence or absence of metabolic activation and in the second experiment exposure lasted for 24 hours and the harvest time was at 24 hours. . No precipitation or cytotoxicity was observed and there were no statistically or biologically significantly increased concentrations numbers of mononucleated or binucleated cells. Exposure to 2FL was for 24 h with a 24h harvest time and occurred in the absence of metabolic activation. The study reported no clastogenicity or aneugenicity of 2FL at doses of up to 2000 µg/mL.

#### **In vivo Mammalian Micronucleus Test**

The ability of Jennewein's 2FL to damage chromosomes or the mitotic apparatus was investigated in an in vivo micronucleus assay (Jennewein Biotechnologie, 2015). A preliminary acute oral toxicity study was conducted by administering, via oral gavage, doses of 500, 1000, or 2,000 mg/kg bw via oral gavage in one animal per sex and dose. No systemic toxicity was observed with these doses of therefore, the same doses were used in the main study.

A single dose of 500, 1000, or 2000 mg/kg bw of Jennewein's 2FL were given to 5 (CrI:CD(SD)) rats per sex per dose group via oral gavage in the main study. The negative control was the vehicle (0.8% aqueous hydroxypropylmethylcellulose) and the positive control was cyclophosphamide. The rats were killed at 24 and 48 hours after dosing and bone marrow smears were prepared. There was no evidence of systemic toxicity in the rats at any dose and there was no significant increase in the number of micronucleated polychromatic erythrocytes at any dose administered.

### **Animal Toxicology**

#### **Subacute Toxicity Studies**

In GRN 571, Jennewein Biotechnologie described a dietary toxicity study in which ten female (CrI:CD(SD)) rats were given a control diet or the same diet supplemented with 10% Jennewein 2FL (Jennewein Biotechnologie, 2015). No deaths of animals or changes in appearance or behavior were noted and the food consumption and body weight were described as being comparable for both diet groups.

Coulet et al. (2014) conducted a 14-day tolerability dose-range finding study in 2FL in Wistar IGS:CrI:WI Han rats. Juvenile rats were administered via gavage 0, 2000, 5000, or 7500 mg/kg bw/day of 2FL (5 females and 5 males per dose group) in a dose volume of 10 mL/kg bw/day from PND 7 through PND 20 (weaning). The rats were assessed twice daily for general health, mortality, and morbidity. The authors stated that the 2000 mg/kg bw/day dose was well-tolerated. Liquid or yellowish liquid feces were observed in most rats in the 7500 mg/kg bw/day FOS group, and in the 5000 and 7500 mg/kg bw/day 2FL dose groups from days one to three and nine to eleven. This occurred with erythema in the urogenital region. There were short term reductions in body weight gain from days 0 to 3 in the FOS group and the 7500 mg/kg bw/day dose group compared with controls. The mean body weight gain was similar for all groups at the end of the study with the exception on female rats in the 7500 mg/kg bw/day dose group

and the FOS group which continued to show a slight reduction in body weight gain than the control group. Two females from the 7500 mg/kg bw/day dose group were found dead. One was unable to be necropsied and no cause of death was determined for the other. Two macroscopic findings were observed: a herniation between the right and left median liver lobes in one female in the 5000 mg/kg bw/day dose group and a smaller than normal testis in a male in the 2000 mg/kg bw/day dose group were considered to be incidental and were not attributed to 2FL. Based on the results of this study, the authors used a maximum dose lower than the 7500 mg/kg bw/day dose group in the 90-day study.

Hanlon and Thorsrud (2014) conducted a 3-week oral toxicity study in which, starting at lactation day 2, neonatal pigs were administered a liquid diet (Purina Pronurse® milk replacement formula) containing 0, 200, 500 or 2000 mg/L of 2'-FL (Jennewein), prepared via a fermentation process corresponded to 29.37, 72.22 and 291.74 mg/kg/day, respectively for males and 29.30, 74.31, and 298.99 mg/kg/day, respectively for females. The numbers of male piglets per dose group was 6, 8, 7, and 6 and the number of females per dose group was 6, 4, 5 and 6, respectively. None of the animals died during the course of the study, there were no clinical signs providing evidence of toxicity, and there was no effect on body weight or food consumption. There were no adverse effects on hematological or coagulation parameters or on urinalysis measurements. Some significant differences were observed in clinical chemistry measurements such as a reduction in gamma glutamyltransferase activity in males on day 7 but not day 21 and males in the 2000 mg/L dose group had a significant increase in alanine aminotransferase on days 7 and day 21. In addition, microscopic findings included mild to moderate inflammation within the keratinized portion of the squamous epithelium in the non-glandular part of the stomach for one female in the 500 and 2000 mg/kg bw/day dose groups. These effects were observed in the absence of macroscopic findings. No macroscopic or microscopic findings were attributed to 2FL. The authors described 2FL as being well tolerated at the doses administered and having no treatment related effects on growth and development.

### **Subchronic Toxicity Study**

Coulet et al. (2014) investigated the subchronic oral toxicity of 0, 2000, 5000, and 6000 mg/kg bw/day of 2FL or 6000 mg/kg bw/day FOS (n=10 rats/sex/dose group) when administered to juvenile rats for 90 consecutive days in a dose volume of 10 mL/kg bw/day. An additional 5/rats/sex/dose group for the control, FOS, and 6000 mg 2FL/kg bw/day dose group were treated for 90 days and then were allowed to recover for 28 days before being killed. One rat of each sex in the 6000 mg/kg bw/day 2FL dose group and two males in the FOS group died during the primary study. One female in the FOS group died during the recovery period. These deaths were considered to be unrelated to the treatments because there were no histopathological correlates. All animals in the 5000 and 5000 mg/kg bw/day 2FL dose groups and in the FOS group experienced diarrhea typically associated with erythema in the urogenital region for the FOS and the 6000 mg/kg bw/day dose groups. Hyper-salivation was observed in 50% of animals in the 5000 mg/kg bw/day dose group and for all animals in the 6000 mg/kg bw/day dose group and the FOS group. The initial body weight gain was lower. Female rats in the 5000 and 6000 mg/kg bw/day 2FL and the FOS dose groups showed minimal cortical tubular epithelial cytoplasmic vacuolation in the kidney at the conclusion of the dosing period and was related to reduced kidney weight in the 6000 mg/kg bw/day dose group and the increase in serum creatinine in the 5000 and 6000 mg/kg bw/day dose groups. This effect also occurred in control rats at the end of the recovery period, did not occur in males, was not dose related, and was not associated with associated clinical pathology effect of histopathological correlates. As a result, this effect was considered to be "non-adverse, of unclear origin, and unrelated to

treatment". There were some instances of significant increase in hematological parameters; however, the values remained within the range for historical controls, did not show a dose-response relationship, typically occurred in one sex, and also occurred in the FOS reference group or showed no clinical pathology or histopathological correlates. The NOAEL for this study was considered by the authors to be 5000 mg/kg bw/day.

This study was later reviewed by (EFSA Panel on Dietetic Products, 2015) and they determined a NOAEL of 2000 mg/kg bw/day, based on the reduction in the relative kidney weight and two unexplained deaths of females in the 6000 mg/kg bw/day 2FL dose group, and significant differences between groups in hematological parameters and clinical blood measurement in the 5000 and 6000 mg/kg bw/day dose groups.

The Coulet et al. (2014) 90-day subchronic toxicity study was also reviewed by the Expert Panel for GRN 650 (Glycom A/S, 2016). Glycom described the reduction in red blood cell counts as being slight, and observed that this effect did not occur in both sexes, and there were no corroborative histopathological or gross pathological effects observed. In addition, although there was a dose response relationship with reductions in AST levels, male and female rats that were treated with FOS (positive controls) also showed reductions for males and females. There were no additional clinical chemistry, hematological, or histopathological correlates. In GRN 650, Glycom concluded that the NOAEL of 5000 mg/kg bw/day that was established by Coulet et al. (2014) was correct.

GRN 571 described a 90-day dietary toxicity study in which male and female rats were fed a control diet or the me diet to which a diet containing 10% of Jennewien's 2FL (n=10 rats/sex/dose group) (Jennewein Biotechnologie, 2015). There was no difference between groups in body weight or body weight gain and food consumption was described as being comparable. There was no reduction in food consumption during the course of the study. There was a reduction in the intake of 2FL from 11.54 g/kg/day to 5.25 g/kg/day in male rats (mean=7.66±2.21 g/kg/day) and from 12.07 g/kg/day to 5.78 g/kg/day (mean=8.72 ±1.9g/kg/day) in female rats. Between days 9 and 69 of the study, 7 males and 4 females that were fed the diet containing 2FL had pale stools. This was attributed to undigested 2FL in the stool and was not thought to be adverse. On male that was fed 2FL had soft stool for 15 days starting on day 14, but this was not considered to adverse. The authors did not observe adverse clinical signs, behavior, hematology, clinical chemistry, or urinalysis and no adverse ophthalmological effect were observed. There were no significant differences in organ weight or gross pathological findings. The authors concluded that Jennewein's 2FL was safe for consumption at doses of 7.66 g/kg/day in male rats and 8.72 g/kg bw/day in female rats and the NOAEL was the dose tested.

Penard (2015) conducted an adapted 90-day toxicity study in neonatal Wistar [CrI:WI(Han)rats] starting at age 7 days as described in GRN 650 (Glycom A/S, 2016). The authors administered doses of 2000, 4000, and 5000 mg/kg bw/day of 2FL produced by fermentation (Glycom A/S, purity of 97.6%) or 5000 mg/kg bw/day of FOS (reference group). The study adhered to OECD principles. Additional animals were assigned to recovery groups and were treated for 90 days with 0 (control), 2FL, or FOS (5 males and 5 females) and the animals were killed after 28 days. In additions dams with reconstituted litters of a minimum of 5 male and 5 female pups were housed in plastic cages. Until the animals were weaned on postnatal day 21 at which time they were housed 5 pup per sex per dose group per cage. The rats were fed a standard diet. There no mortalities that were attributed to 2FL during the study. The feces of most rats that were fed FOS were liquid as was the case for rats in the mid and high dose 2FL groups.

Soiled urogenital regions were observed for rats in the mid and high dose 2FL groups. Also observed were hypersalivation, abnormal foraging, and/or pedaling in animals in the animals that were given 4000, and 5000 mg/kg bw/day of 2FL starting on day 35, but these effects were not observed during the recovery period. There were no test-article-related effects on body weight, body weight gain, or food consumption. No results that were considered to be of toxicological relevance were observed in tibia length, reflex, and physical development, time to sexual maturation, learning capacity, memory, motor activity, exploratory behavior, or general movement were observed. No ophthalmological effects were observed. There were some minor differences in some hematological measurement; however, these effects were not considered to be toxicologically significant as they were either small or remained within the historical control data range, and were no longer present during the recovery period. There were no differences related to treatment with 2FL with respect to uranalysis, organ weights, or macroscopic weights, histological analyses for animals in the 2FL and control and reference groups. The NOAEL for this study was determined to be 5000 mg/kg bw/day, the highest dose administered.

#### **Non-toxicological Studies on 2FL**

In order to study the synaptic plasticity and learning capabilities in rodents, Vazquez et al. (2015) fed adult male C57BL/6 mice diets containing 0.312% 2FL (Inalco Pharmaceuticals) for 12 weeks or adult male Sprague Dawley rats diet containing 0.625% 2FL for 5 weeks. This latter dose provided approximately 350 mg/kg bw/day of 2FL. The investigators reported that the addition of 2FL to the diet resulted in no difference in daily food intake or body weight between control and 2FL treatment groups. Treatment with 2FL was associated with better performance than the control in learning and memory tasks as evidenced by improvements in input/output curves, and long-term potentiation responses (LTP) at the hippocampal CA3-CA1 synapse, better performance in motor and cognitive tests in the IntelliCage, better performance in the Skinner box task, and enhanced expressions of brain functional markers.

In a follow up study, Vazquez et al. (2016) investigated whether the reported effects of 2FL on LTP and learning and memory occurred because of the integrity of the molecule and whether the observed effect of 2FL in the previous study occurred via the gut-brain axis, in particular, via the vagus nerve. The authors reported that these effects were elicited by 2FL and not by L-fucose and that bilateral subdiaphragmatic vagotomy resulted in inhibition of the effects. The authors inferred that the gut brain axis may be involved in the cognitive benefits associated with supplementation with 2FL.

Cilieborg et al. (2016) studied the effects of a 2FL-enriched formula on the immature intestine during the immediate postnatal period. Thirty-three piglets from two sows (Large White × Danish Landrace) were delivered by Caesarian section and were passively immunized by providing sow's serum via the umbilical catheter for 24 hr after which the piglets were fed parenteral nutrition supplemented with minimal enteral nutrition for two days. The piglets were given an oral inoculation of maternal fecal bacteria ( $2.5 \times 10^4$  colony forming units) on day 1 and were given full enteral feeding on days three through five. The animals were fed either a control formula (n=17) or 2FL formula (n=16) containing freeze-dried 2'-fucosyllactose (Glycom) at 5g/L. Limited effects on bacterial colonization, mucosal fucosylation, mucosal structure, digestive function and NEC sensitivity were reported within the first 5 d after preterm birth.

Cilieborg et al. (2017) investigated whether 2FL competitively inhibits pathogen adhesion and prevents diarrhea in a highly sensitive caesarian-section delivered newborn pigs. Both the porcine pathogen *E. coli* F18 and the F-18 negative control strain ATCC 25922 adhere to PS1c1 cells from jejunal epithelium of an adult pig; however, 5 g/L of 2FL inhibited adhesion of *E. coli* F18, but not ATCC 25922 to PS1c1 cells. Another study in this report investigated the tolerance of pigs to 2FL. Thirty newborn pigs were given plasma from their mothers to provide passive immunization and were also given parenteral nutrition. Initial gut colonization was standardized by giving an oral fecal suspension from the sows. Pigs were either inoculated or not inoculated with enterotoxigenic *Escherichia coli* F18 and fed one of four diets supplemented with 2FL at doses of 0 (control, n = 8), 2 g/L (n = 7), 5 g/L (n = 8), or 10 g/L (n = 7). There was no difference in weights of pigs at any time point. All rats in treatment groups developed diarrhea and there were no differences in mean fecal score, intestinal permeability, dry weight mucosal proportions in the proximal and distal small intestine. There was no difference in blood gas treatment but there was a reduction in hematocrit in the 2 g/L dose group compared with the 10 g/L dose group. To identify the optimal *E. coli* inoculation dose, 31 newborn pigs at term were treated each day by gavage with 1ml of culture medium (CON, n = 8) or one of three doses of F18,  $1 \times 10^7$  CFU/d (n = 9),  $2 \times 10^8$  CFU/d (n = 7) or  $8 \times 10^9$  CFU/d (n = 7). There was a significant difference in incidence of diarrhea in the high dose and the control groups and a trend for median dose vs control and the high dose vs the low dose. They reported that diarrhea still occurred in the presence of 2FL but F18 and 2FL pigs had more severe diarrhea and diarrhea started earlier than in control animals. In an intervention study in 25 pigs that had passive immunization and initial gut colonization and were inoculated with daily with  $7.5 \times 10^{10}$  F18 (n = 9), one group was given the same dose of *E. coli* F18 plus 10 g/L 2FL in the milk replacer as described in the tolerance study above (n = 8) and one control group received only milk replacer (Control, n = 8). The authors reported that overall 2FL was tolerated well.

Castillo-Courtade et al. (2015) investigated the effect of treatment of mice with 2FL or 3'-sialyllactose on allergy induced by a by oral ovalbumin (OVA) challenge in sensitized mice. The administration of 1 mg of 2FL to 8-to 9-month-old male Balb/mice for 15 days resulted in reduced symptoms of food allergy by the induction of IL-10(+) T regulatory cells and the indirect stabilization of mast cells.

Weichert et al. (2013) reported that 2FL synthesized by whole cell biocatalysis inhibited adhesion of *Campylobacter jejuni*, enteropathogenic *Escherichia coli*, *Salmonella enterica* serovar *fyris*, and *Pseudomonas aeruginosa* to the intestinal human Caco-2 cells. In addition, 3FL inhibited adhesion of enteropathogenic *E. coli* 29%, *P. aeruginosa* 26% in Caco-2 cells. Both 2FL and 3FL inhibited attachment of *P. aeruginosa* to the human respiratory epithelia cell line A549.

He et al. (2016) reported that 2FL inhibited the binding and invasion of enteropathogenic *E. coli* and the release of IL-8. The addition of 2FL to the diet protected against weight loss induced by aggregating invasive *E. coli* pathogens and reduced inflammation.

Oliveros et al. (2016) investigated whether treatment of rats with 2FL during the lactation period had an effect on cognitive abilities of rats. Rat pups were assigned to be treated with 2FL or water (n=12rats/treatment group) during the lactation period. The rats underwent classic behavioral testing at age 4 to 6 weeks and at age 1 year. The authors reported that rats in the two treatment groups showed similar behaviors at age 4 to 6 weeks with the exception of an apparent slight improvement in

performance in the Morris Water Maze for the 2FL group. At age one year, rats in the 2FL treatment group showed better performance in the Novel Object Recognition and Y maze paradigms and more intense and longer long-term potentiation (young and adult rats) than rats in the control group.

Good et al. (2016) investigated the effect of treatment with 2FL on protection against necrotizing enterocolitis (NEC), which is a significant cause of illness and death in premature infants. They reported that treatment of 7- to 10-day old mice with 0.25 mg/g bw of 2FL once/day (5 mg/mL of formula) reduced the severity of NEC compared with control formula, prevented the inhibition of mesenteric perfusion seen in newborn mice, restored intestinal perfusion in neonatal mice by maintaining the expression of endothelial nitric oxide synthase and showed that 2FL regulated eNOS gene expression and function.

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## Appendix H: Expert Panel Consensus Statement and Qualifications

## EXPERT PANEL CONSENSUS STATEMENT

### Introduction

DuPont Nutrition and Health (“DuPont”) convened a panel of independent scientists (the “Expert Panel”), qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, to conduct a critical and comprehensive evaluation of the available pertinent data and information on 2'-*O*-fucosyllactose (2FL) and to determine whether the proposed uses in food would be Generally Recognized as Safe (GRAS) based on scientific procedures. The Expert Panel consisted of the following qualified experts: Joseph Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine); Edward L. Carmines, Ph.D. (Carmines Consulting LLC); and Roger A. Clemens, Dr.PH, FIFT, CFS, FASN, FACN, CNS, FIAFST, Adjunct Professor, Pharmacology and Pharmaceutical Sciences, University of Southern California. Kara Lewis, Ph.D. (Independent Consultant) and Michael C. Falk, Ph.D. (LSRO Solutions, LLC) served as technical advisors to the Expert Panel.

The Expert Panel independently and collectively critically evaluated a comprehensive package of scientific information and data compiled from the literature. The information was presented in a dossier produced by LSRO Solutions LLC (“Comprehensive GRAS Assessment of 2'-*O*-fucosyllactose In Term Infant Formulas, Toddler Formulas, and Foods Targeted to Toddlers”; November 17, 2017). The Expert Panel evaluated other information deemed appropriate or necessary. To the best of our knowledge, this determination is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the uses of this ingredient in food.

### Summary and Basis for GRAS

2FL, a trisaccharide consisting of galactose, glucose and fucose, is the most abundant oligosaccharide of human breast milk. The 2FL content of breast milk varies according to geographic location, lactational stage, ethnicity, Lewis-blood group status, and secretor status. Although concentrations of 2FL in breast milk as high as 8.4 g/L have been reported, studies investigating the mean content of 2FL in breast milk have reported values clustered around 2.4 g/L.

Three previous GRAS notifications (GRN546 (2014), GRN571 (2015), and GRN650 (2016)) were submitted to the U.S. FDA regarding the use of 2FL as an ingredient in infant formulas, toddler formulas and foods, and conventional foods and beverages (Glycom A/S, 2014, 2016; Jennewein Biotechnologie, 2015). In GRN 546 the proposed use was as an ingredient in non-exempt infant formula at a maximum of 2.4 g/L and in baked good and mixes, beverages and beverage bases, coffee and tea, dairy product analogs, infant and toddler foods, grain products and pastas, milk (whole and skim), milk products, processed fruits and fruit juices, processed vegetables and vegetable juices, and sugar substitutes at maximum levels ranging from 0.084 to 2.4 g/serving (Glycom A/S, 2014). In GRN 571, the maximum proposed use as an ingredient in non-exempt, milk based term infant formulas and in toddler formulas was at up to 2 g/L of reconstituted formula (Jennewein Biotechnologie, 2015). In GRN 650, the proposed uses as an ingredient in nonexempt infant formulas for term infants at a maximum use level of 2.4 grams per liter (g/L) of reconstituted formula; and, in beverages and beverage bases, dairy product analogs, infant and toddler foods including follow-on formulas, grain products and pastas, milk and milk products, and processed fruits and fruit juices at use levels ranging from 0.084 to 2.04 g/serving (Glycom A/S, 2016). These notifications all received letters of no objection from the U.S. FDA (U.S. FDA, 2014, 2015, 2016).

The Food Safety Authority of Ireland (FSAI) evaluated the use of 2FL in foods for infants, all users of infant formula and foods specifically designed for young children, and as a dietary supplement at levels of up to 3 g/day. The FSAI did not identify any safety concerns under the proposed conditions of use. The European Food Safety Authority (EFSA) also reviewed the safety of 2FL as a novel food ingredient and found it safe for infants (in combination the Lacto-N-neotetraose), for follow-on and young child formulas, and when added to other foods for children and adults at levels up to 3 g/person/day.

DuPont's 2FL is produced by a fermentative process using an *E.coli* MG1655 (K12) host with engineered modifications via classical genetics and is 100% stable after at least 50 generations. The strain was modified to produce 2FL by the integration into the chromosome of four genes derived from other bacteria and the deletion from the chromosome of various genes involved in carbohydrate and intermediary metabolism. The modified strain contains no known lytic phages or conjugation plasmids and no residual trace of the helper plasmids or antibiotic markers. The modified strain is inactivated during the manufacturing process and no living or dead organisms were reported in the final 2FL product.

The production process utilizes standard, well-documented fermentation techniques under current Good Manufacturing Practice (GMP) and/or Global Food Safety Initiative certifications and meeting the US manufacturing requirements consistent with Food Safety Modernization Act (FSMA) rules and/or the foreign supplier verification requirements. Dupont's 2FL is chemically identical to reference standards as confirmed by high pressure anion exchange chromatography, mass spectroscopy, and <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectroscopy.

Based on analytical data on multiple batches of DuPont's 2FL, the Expert Panel concluded that the manufacturing process is reproducible and reliable producing DuPont's 2FL that meets specifications for appearance, chemical identity, carbohydrate profile, proximates, and contaminants (heavy metals, endotoxin, microbial, and any known adverse microbial metabolites). Dupont's 2FL is also substantially equivalent to the 2FL products produced by other manufacturers and approved as GRAS by the FDA. The 2FL content of DuPont's 2FL product is at least 82% by weight, slightly lower than the previously approved GRAS 2 FL products. The associated carbohydrate by-products are equivalent to the other products and all are common components of breast milk and/or infant formula.

DuPont's 2FL is stable for 26 weeks under accelerated stability testing conditions (40 °C, 75% Relative Humidity). Levels of 2FL and related carbohydrates and microbial contaminants were unchanged during testing. Although the moisture content rose significantly during testing, this did not result in any microbial contamination. This rise was attributed to inadequate packaging. DuPont Nutrition and Health intends to change to less moisture permeable packaging to prevent moisture accretion in the future. The method of analysis for moisture determination was changed during the course of the stability testing due to methodological limitations. DuPont will ensure that any future packaging material be compliant with infant formula regulations and that future stability testing be conducted with a uniform analytical technique.

DuPont intends to market 2FL as an ingredient in non-exempt term infant formula, toddler formula, and conventional foods and beverages targeted at toddlers. 2FL will be added to infant and toddler formulas at 2.4 g/L, to infant and toddler foods at 12.0 g/kg, and to toddler drinks at 1.2 g/L. These levels of 2FL are commensurate with those in human breast milk and are equivalent to the levels proposed in the previously FDA-approved GRAS notifications, adjusted for the 2FL content of DuPont's 2FL. DuPont's 2FL is an alternative to the 2FL products already in commercial use and is not expected to add to the overall daily intake of 2FL from formula and food.

2FL has been evaluated by *in vitro* and *in vivo* genotoxicity studies, subacute oral toxicity studies in rats and pigs, and subchronic toxicity studies in rats. 2FL was reported as non-mutagenic and non-clastogenic in all

studies conducted. A No Observed Adverse Effect Level (NOAEL) of 5000 mg/kg bw/day was reported by Coulet et al. (2014) in a 90-day subchronic, gavage study in male and female Wistar (CrI:W19(Han)) rats. EFSA evaluated the same study and reported a NOAEL of 2000 mg/kg bw/day based on decreased relative kidney weight in the high dose female group, two unexplained deaths, and hematological clinical effects in the high and mid dose groups (EFSA Panel of Dietetic Products, 2015). The observations upon which the EFSA panel based their findings were disputed by Glycom in GRN 650 (Glycom A/S, 2016). Glycom A/S (2016) concluded that the slight reductions in red blood cell counts were not associated with histopathological or gross pathological correlates and aspartate aminotransferase reductions were comparable to control values and thus, were not considered adverse. On these bases Glycom defended their NOAEL of 5000 mg/kg bw/day (Glycom A/S, 2016). In another 90-day, subchronic, gavage study in male and female Wistar rats (CrI:W19(Han)), Penard (2015) reported a NOAEL of 5000 mg/kg bw/day, the highest dose tested for Glycom's 2FL. Jennewein Biotechnologie (2015) reported another 90-day, subchronic study in male and female CrI:CD(SD) rats. In this study, rats were fed a diet containing 10% 2FL. The authors reported NOAELs of 7660 mg/kg bw/day and 8720 mg/kg bw/day for females and males, respectively (Jennewein Biotechnologie, 2015). On the basis of these findings, the Expert Panel for the Evaluation of Dupont's 2FL concludes that a NOAEL of 7660 mg/kg bw/day is an appropriate basis for a determination of safety.

In two clinical trials during which infants received up to 1 g/L of 2FL in the presence of galactoligosaccharides (Marriage et al., 2015) for up to 6 months or 1 g/L of 2FL in the presence or absence of lacto-N-neotetraose for up to 4 months showed that 2FL was safe and well-tolerated and supported age-appropriate growth (Puccio et al., 2017). The authors concluded that 2FL posed "no safety concerns" (Marriage et al., 2015) and was "safe and well-tolerated" (Puccio et al., 2017).

In one clinical trial in 100 healthy adults, 2FL dissolved in water was consumed at doses of 5, 10 or 20 g/day for two weeks (Elison et al., 2016). At the highest dose, subjects reported mild and reversible adverse effects including flatulence, bloating, and constipation. The authors concluded, "all subjects tolerated the investigational products throughout the trial period" thus confirming "the safety of [2FL]".

Based on the substantial equivalence of 2FL to marketed 2FL products whose safety has already been established, the intended use levels commensurate with levels present in human breast milk, multiple repeat-dose toxicology and other toxicology studies on various manufactured forms of 2FL, and the history of safe use in human clinical trials, the Expert Panel concludes that 2FL is safe for use in term infant and toddler formulas, and foods intended for toddlers when used at the proposed by Dupont.

#### Common Knowledge Elements of GRAS

The first common knowledge element for a GRAS determination is that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing published, peer-reviewed scientific journals for the safety assessment. The animal studies and human clinical studies on which this GRAS determination has been based have been published in the peer-reviewed scientific literature.

The second common knowledge element required for a GRAS determination is consensus among qualified scientists that the safety of the proposed uses of the substance has been demonstrated. The Expert Panel agrees there is adequate data in the scientific literature to conclude that 2FL is a common component of breast milk, that 2FL has been reviewed and approved as a food sources for humans by the U.S. FDA and other international regulatory agencies, and that the weight of the available evidence demonstrates that the proposed uses are safe.

Conclusion

We, the undersigned members of the Expert Panel, have individually and collectively critically evaluated the materials summarized above on the safety of DuPont's 2FL and 2FL and other information deemed appropriate and unanimously conclude that DuPont's 2FL, manufactured as described in the dossier and consistent with cGMP, and meeting appropriate food grade specifications, is Generally Recognized As Safe (GRAS) based on scientific procedures for use as an ingredient in term infant formulas and toddler formulas at 2.4 g/L and in various foods targeted to toddlers at levels specified in the accompanying dossier.

It is our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusions.

(b) (6)

November 29, 2017

Joseph F. Borzelleca, Ph.D.  
Virginia Commonwealth University

Date

(b) (6)

November 29, 2017

Edward L. Carmines, Ph.D.  
Carmines Consulting, LLC

Date

(b) (6)

November 29, 2017

Roger A. Clemens, Dr.PH  
University of Southern California

Date

(b) (6)

November 29, 2017

Michael C. Falk, Ph.D.  
LSRO Solutions LLC  
Advisor to the Expert Panel

Date

(b) (6)

November 29, 2017

Kara D. Lewis, Ph.D.  
Consultant  
Advisor to the Expert Panel

Date

## References

Coulet, M., Phothirath, P., Allais, L., Schilter, B., 2014. Pre-clinical safety evaluation of the synthetic human milk, nature-identical, oligosaccharide 2'-O-Fucosyllactose (2'FL). *Regul Toxicol Pharmacol* 68, 59-69.

EFSA Panel of Dietetic Products, N.a.A., 2015. Scientific opinion on the safety of 2'-O-fucosyllactose as a novel food ingredient pursuant to Regulation (EC) No 259/97. *EFSA Journal* 13.

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Glycom A/S, 2014. GRAS exemption claim for 2'-O-fucosyllactose (2'-FL): GRN000546. U.S. FDA, GRAS Notice Inventory.

Glycom A/S, 2016. GRAS exemption claim for 2'-O-fucosyllactose (2'-FL) produced by fermentation: GRN000650. U.S. FDA, GRAS Notice Inventory.

Jennewein Biotechnologie, G., 2015. GRAS Exemption claim for use of 2'fucosyllactose (2'-FL) in term infant and toddler formulas:GRN000571. U.S. FDA, GRAS Notice Inventory.

Marriage, B.J., Buck, R.H., Goehring, K.C., Oliver, J.S., Williams, J.A., 2015. Infants Fed a Lower Calorie Formula With 2'FL Show Growth and 2'FL Uptake Like Breast-Fed Infants. *J Pediatr Gastroenterol Nutr* 61, 649-658.

Penard, L., 2015. 2'-FL – 13-Week Oral (Gavage) Juvenile Toxicity Study in the Rat Followed by a 4-Week Treatment-Free Period. Confidential. (Study Number AB20757; Sponsor Reference Number GSN037). Prepared by DD 's-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom A/S.

Puccio, G., Alliet, P., Cajozzo, C., Janssens, E., Corsello, G., Sprenger, N., Wernimont, S., Egli, D., Gosoniu, L., Steenhout, P., 2017. Effects of Infant Formula With Human Milk Oligosaccharides on Growth and Morbidity: A Randomized Multicenter Trial. *J Pediatr Gastroenterol Nutr* 64, 624-631.

U.S. FDA, 2014. Agency response letter GRAS Notice No. GRN 000546, in: C.O.o.F.A. Safety (Ed.). U.S. FDA, GRAS Notice Inventory.

U.S. FDA, 2015. Agency response letter to GRAS Notice No. GRN 000571, in: C.O.o.F.A. Safety (Ed.). U.S. FDA, GRAS Notice Inventory.

U.S. FDA, 2016. Agency response letter to GRAS Notice No. GRN 000650, in: C.O.o.F.A. Safety (Ed.). U.S. FDA, GRAS Notice Inventory.

**Bonnette, Richard**

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**Subject:** FW: [EXTERNAL] RE: Revised submission for 2'-O-fucosyllactose - question regarding appendix marked "confidential"

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**From:** Lim, Angela [mailto:Angela.Lim@dupont.com]  
**Sent:** Wednesday, December 13, 2017 2:31 PM  
**To:** Bonnette, Richard <Richard.Bonnette@fda.hhs.gov>  
**Subject:** Re: [EXTERNAL] RE: Revised submission for 2'-O-fucosyllactose - question regarding appendix marked "confidential"

Dear Mr Bonnette,

Thank you for your inquiry. I would like to confirm that "Appendix F: Test for Residual Bacterial DNA" (pages A-94 through A-105) is releasable under FOIA. The confidential watermark you noted is an editorial oversight.

Thank you for your assistance with this notification.

Best regards,

Angela Lim  
Sr. Manager, Regulatory Affairs  
DuPont Nutrition & Health

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**From:** Bonnette, Richard  
**Sent:** Tuesday, December 12, 2017 1:35 PM  
**To:** [angela.lim@dupont.com](mailto:angela.lim@dupont.com)  
**Subject:** Revised submission for 2'-O-fucosyllactose - question regarding appendix marked "confidential"

Dear Ms. Lim,

We received the revised submission for 2'-O-fucosyllactose dated December 8, 2017. After reading through the submission we had just one point to clarify. In part 1 (page 7), you note that the submission does not contain information that is exempt from disclosure (other than the minor items redacted for personal privacy). I wanted to confirm that "Appendix F: Test for Residual Bacterial DNA" (pages A-94 through A-105) is releasable under FOIA though it's labelled "Confidential Business Information." Was this confidential watermark an oversight? If you agree that this information should not be considered confidential, please let me know by responding to this email. I'll then add your email response to the administrative record (the email will be posted to our website along with the submission) and we'll be able to move forward with filing the submission as a GRAS notice. If you do indeed intend for Appendix F to be CBI, you will need to submit a new version of the submission that complies with 170.225(c)(8), and 170.250(d) & (e).

Regards,  
Richard

Richard E. Bonnette, M.S.  
Center for Food Safety and Applied Nutrition



Office of Food Additive Safety  
U.S. Food and Drug Administration  
Tel: 240-402-1235  
[richard.bonnette@fda.hhs.gov](mailto:richard.bonnette@fda.hhs.gov)



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DuPont Nutrition & Health  
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200 Powder Mill Road  
Wilmington, DE 19803  
Tel: +1 (302) 695-6786

December 8, 2017

Paulette Gaynor, PhD  
GRAS Notification Program  
Office of Food Additive Safety (HFS-200)  
Center for Food Safety and Applied Nutrition  
Food and Drug Administration  
5001 Campus Drive  
College Park, MD 20740-3835



**Re: GRAS Notification for 2'-O-Fucosyllactose (2FL)**

Dear Dr Gaynor:

On behalf of DuPont Nutrition & Health (DuPont) and in accordance to 21 CFR 170 Subpart E Generally Recognized as Safe (GRAS) Notice, I am submitting one paper copy of the comprehensive GRAS assessment that was conducted on DuPont's 2'-O-Fucosyllactose (2FL) for use in term infant formula, toddler formulas and foods targeted to toddlers. This GRAS notification comprises DuPont's claim of exclusion of DuPont's 2'-O-Fucosyllactose (2FL) from the requirements of pre-market approval on the basis of a GRAS conclusion by scientific procedures, the supporting data and information and the independent GRAS Expert Panel Consensus statement.

Thank you for reviewing DuPont's GRAS notice.

Sincerely,

(b) (6)

Angela Lim  
Sr Manager, Regulatory Affairs  
DuPont Nutrition & Health

**From:** [Lim, Angela](#)  
**To:** [Morissette, Rachel](#)  
**Subject:** RE: GRN 000749 questions to be addressed  
**Date:** Tuesday, February 13, 2018 1:43:15 PM  
**Attachments:** [image007.png](#)

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Dear Ms Morissette,

I'd like to confirm receipt of the list of questions to be addressed for GRN 000749. I will work on compiling DuPont's responses and sending them back promptly.

Thank you.

Best regards, Angela

Angela Lim  
Sr Manager, Regulatory Affairs  
DuPont Nutrition & Health

DuPont Experimental Station 320/221  
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Tel: + 1 (302) 695-6786 (Office)  
Email: [Angela.Lim@DuPont.com](mailto:Angela.Lim@DuPont.com)

---

**From:** Morissette, Rachel [mailto:Rachel.Morissette@fda.hhs.gov]  
**Sent:** Tuesday, February 13, 2018 11:57 AM  
**To:** Lim, Angela <Angela.Lim@dupont.com>  
**Subject:** [EXTERNAL] GRN 000749 questions to be addressed

Dear Ms. Lim,

Please see attached a list of questions to be addressed for GRN 000749. Please send your responses within 10 business days in an email or separate document. Please do not send a revised notice. Let me know if you have any questions.

Best regards,

**Rachel Morissette, Ph.D.**  
*Consumer Safety Officer*

Center for Food Safety and Applied Nutrition  
Office of Food Additive Safety  
U.S. Food and Drug Administration  
[rachel.morissette@fda.hhs.gov](mailto:rachel.morissette@fda.hhs.gov)



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[http://www.DuPont.com/corp/email\\_disclaimer.html](http://www.DuPont.com/corp/email_disclaimer.html)

February 26, 2018

Dear Dr Morissette,

Please find below DuPont's responses to the questions received on February 13, 2018 regarding GRAS Notice GRN 000749 for 2'-fucosyllactose (2'-FL).

**Question (1):**

On page 22 of the notice, DuPont states that dietary exposures to 2'-FL were estimated using the most recent NHANES data (i.e., 2011-2014). However, we note that Table 9 (page 23 of the notice) includes citations for the 2009-2010 and 2011-2012 NHANES, but does not include data from the more recent 2013-2014 NHANES. Please clarify which NHANES was used to calculate the dietary exposures.

**Response:**

The sentence *"Where applicable, the most recent National Health and Nutrition Examination Surveys (NHANES 2011-2014) have been utilized to estimate the mean and 90th percentile daily intake of 2FL among the U.S. populations"* is an editorial error from a previous version and should be deleted.

The intake data in Table 9 was based upon the 2009-2010 and the 2011-2012 NHANES data. DuPont was not able to find an analysis of the 2013-2014 NHANES data for infants and toddlers in the literature.

**Question (2):**

On page 21 of the notice under the heading "10. Allergens", the first sentence states:

*"None of the genes introduced into the production strain were secreted proteins (sucrose phosphorylase, fructokinase, sucrose permease, and fucosyltransferase)."*

We suggest rewording this sentence as follows:

*"None of the genes introduced into the production strain encode secreted proteins (sucrose phosphorylase, fructokinase, sucrose permease, and fucosyltransferase)."*

**Response:**

DuPont agrees with the suggested rewording. The genes introduced into the production strain encode for sucrose phosphorylase, fructokinase, sucrose permease, and fucosyltransferase; proteins are not secreted.

**Question (3):**

On page 40 of the notice, DuPont states:

*"Based on a NOAEL of 7660 mg/kg bw/day determined by subchronic oral toxicity studies and applying a 100-fold uncertainty safety factor, an ADI of 77 mg/kg bw/day was determined to be safe."*

The NOAEL used for the ADI determination is from an unpublished subchronic study in adult rats. Additionally, the EDI is greater than the ADI. Please discuss how a safety conclusion for the intended use of 2'-FL can be reached without an ADI determination and relying only on data and information that are

publicly available in support of a general recognition of safety. Please do not send a revised ADI calculation.

**Response:**

The inclusion of the ADI discussion in the dossier was to reflect the totality of issues, favorable and potentially unfavorable, reviewed by the Expert Panel. In their deliberations, the Expert Panel noted the existence of an unpublished, sub-chronic study, the ADI derived from the resulting NOAEL and that the EDI for the intended use exceeded the ADI. They did not consider this study to be pivotal.

The conclusion that DuPont's 2'-FL is safe for its intended use is based upon published studies discussing the molecular structure of 2'-FL, the 2'-FL content in human milk and clinical studies with 2'-FL.

The 2'-FL manufactured by DuPont is chemically and structurally identical to the 2'-FL present in human milk and to the GRAS notified 2'-FL products (GRN546 (2014), GRN571 (2015), and GRN650 (2016)).

Studies investigating 2'-FL content in human milk report values clustering around 2.4 g/L. Several studies report 2'-FL concentrations of approximately 7 g/L in mature breast milk and 8.4 g/L in colostrum from mothers with the 2'-FL secretor phenotype. Therefore, consumption of 2'-FL at these levels by infants can be concluded as safe.

The Davies et al (1994) study cited in GRN650 (2016), reported a mean consumption of 890.6 ml/day in 6-week old infants with a mean body weight of 4.7 kg. Therefore, the mean intake of 2'-FL from human milk can be expressed as 2.14 g/person/day or 455 mg/kg bw/day. For infants with mothers having the 2'-FL secretor phenotype, 2'-FL intake levels may exceed 6.23 g/person/day or 1326 mg/kg bw/day.

Addition of DuPont's 2'-FL to term infant and toddler formula at a maximum of 2.4 g/L will result in a mean EDI of 2.02 g/person/day and a 90<sup>th</sup> percentile EDI of 2.91 g/person/day by infants of 0-6 months, the age group with the highest intake. The mean EDI for this application is consistent with the mean 2'-FL intake resulting from consumption of human milk while the 90<sup>th</sup> percentile EDI falls well within the background exposure to 2'-FL from human milk of 2'-FL secretor mothers.

DuPont's 2'-FL will also be added to baby & toddler foods and toddler drinks at a maximum of 12.0 g/kg and 1.2 g/L respectively. 2'-FL intakes by infants of 7-12 months are highest for these combined food and beverage categories; the mean EDI is 4.63 g/person/day and the 90<sup>th</sup> percentile is 8.36 g/person/day. Healthy infants in this age group can be expected to have an average body weight of 8.1 kg or greater.<sup>1</sup> Thus, the mean EDI and 90<sup>th</sup> percentile may be expressed as 572 mg/kg bw/day and 1032 mg/kg bw/day. These values are within the background exposure to 2'-FL from consumption of human milk from 2'-FL secretor mothers. Furthermore, the derived EDI are based upon the conservative premise that all foods and drinks within this category will be supplemented with 2'-FL at the maximum concentration. In practice, not all foods and drinks within this category will be supplemented with 2'-FL and those that are, may be supplemented lower levels.

Moreover, DuPont's 2'-FL will be an alternative to the 2'-FL products already in commercial use and is intended to be used at equivalent levels in infants and toddler formulas, baby & toddler foods and/or toddler drinks; applications where 2'-FL is already permitted. Therefore, DuPont's 2'-FL is not expected to add to the overall daily intake of 2'-FL, which has previously been determined as safe. As such, DuPont's 2'-FL can likewise be considered as safe for the intended use.

Lastly, the clinical studies confirm that formula containing 1g/L 2'-FL is well tolerated and no safety concerns were reported, thereby demonstrating a history of safe use in human clinical trials.

---

<sup>1</sup> CDC Clinical growth charts [https://www.cdc.gov/growthcharts/html\\_charts/wtageinf.htm](https://www.cdc.gov/growthcharts/html_charts/wtageinf.htm)

**Question (4):**

On page 41 of the notice, DuPont states:

*“The key evidence in this determination has been published in a peer review journal. Various other safety assessment, risk assessments, animal and human studies have all been published in peer reviewed journals or made publicly available on government websites.”*

GRAS conclusions or unpublished data and information contained in GRAS notices (i.e. GRN 000571) available only through government websites, such as the GRAS Notice Inventory, are not considered “peer-reviewed” and can only be used as corroborative evidence. The peer-reviewed primary literature references contained within a notice may be incorporated into a new notice with adequate discussion. Given the number of unpublished studies mentioned and discussed in this notice, as well as the use of an unpublished study for the ADI determination, it is unclear if the general recognition of safety requirement has been met in this notice. Please clearly identify which studies were pivotal studies and which studies were corroborative studies for DuPont’s GRAS conclusion.

**Response:**

The pivotal published studies upon which DuPont and the Expert Panel arrived at a conclusion of GRAS status are as follows:

- Structural analysis of 2'-FL
  - van Leeuwen, S. et al (2014) Rapid milk group classification by 1H NMR analysis of Le and H epitopes in human milk oligosaccharide donor samples. *Glycobiology* 24(8), 728-739.
- 2'-FL content in human milk
  - McGuire, M.K. et al (2017) What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. *Am J Clin Nutr* 105, 1086-1100.
  - Musumeci, M. et al (2006) Oligosaccharides in colostrum of Italian and Burkinabe women. *J Pediatr Gastroenterol Nutr* 43, 372-378.
  - Gabrielli, O. et al (2011) Preterm milk oligosaccharides during the first month of lactation. *Pediatrics* 128, e1520-1531.
  - Galeotti, F. et al (2012) On-line high-performance liquid chromatography-fluorescence detection-electrospray ionization-mass spectrometry profiling of human milk oligosaccharides derivatized with 2-aminoacridone. *Anal Biochem* 430, 97-104.
  - Castanys-Munoz, E. et al (2013) 2'-fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. *Nutr Rev* 71, 773-789.
  - Erney, R.M. et al (2000) Variability of human milk neutral oligosaccharides in a diverse population. *J Pediatr Gastroenterol Nutr* 30, 181-192.
  - Thurl, S. et al (1996) Quantification of individual oligosaccharide compounds from human milk using high-pH anion-exchange chromatography. *Anal Biochem* 235, 202-206.
  - Sprenger, N. et al (2017) Longitudinal change of selected human milk oligosaccharides and association to infants' growth, an observatory, single center, longitudinal cohort study. *PLoS ONE* 12(2): e0171814.
- Clinical studies with 2'-FL
  - Puccio, G. et al (2017) Effects of Infant Formula with Human Milk Oligosaccharides on Growth and Morbidity: A Randomized Multicenter Trial. *J Pediatr Gastroenterol Nutr* 64, 624-631.
  - Marriage, B.J. et al (2015) Infants Fed a Lower Calorie Formula with 2'FL Show Growth and 2'FL Uptake Like Breast-Fed Infants. *J Pediatr Gastroenterol Nutr* 61, 649-658.

- Davis, P.S.W. et al (1994) Adjusting milk intake for body size in early infancy.

The following studies and safety evaluations were considered corroborative.

- Subchronic and subacute oral toxicity studies:
  - Coulet, M. et al (2014) Pre-clinical safety evaluation of the synthetic human milk, nature-identical, oligosaccharide 2'-O-Fucosyllactose (2'-FL). Regul Toxicol Pharmacol 68, 59-69.
  - Hanlon, P.R. & Thorsrud, B.A. (2014) A 3-week pre-clinical study of 2'-fucosyllactose in farm piglets. Food Chem Toxicol 74, 343-348.
  - Penard, L. (2015) 2'-FL – 13-Week Oral (Gavage) Juvenile Toxicity Study in the Rat Followed by a 4-Week Treatment-Free Period. Confidential. (Study Number AB20757; Sponsor Reference Number GSN037). Prepared by DD 's-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom A/S.
- Clinical studies
  - Elison, E. et al (2016) Oral supplementation of healthy adults with 2'-O-fucosyllactose and lacto-N-neotetraose is well tolerated and shifts the intestinal microbiota. The British journal of nutrition 116, 1356-1368.
- Safety evaluations
  - Glycom A/S (2014) GRAS exemption claim for 2'-O-fucosyllactose (2'-FL): GRN000546. U.S. FDA, GRAS Notice Inventory.
  - Glycom A/S (2016) GRAS exemption claim for 2'-O-fucosyllactose (2'-FL) produced by fermentation: GRN000650. U.S. FDA, GRAS Notice Inventory.
  - Jennewein Biotechnologie, G. (2015) GRAS Exemption claim for use of 2'-fucosyllactose (2'-FL) in term infant and toddler formulas: GRN000571. U.S. FDA, GRAS Notice Inventory.
  - EFSA Panel of Dietetic Products (2015) Scientific opinion on the safety of 2'-O-fucosyllactose as a novel food ingredient pursuant to Regulation (EC) No 259/97. EFSA Journal 13.

**Question (5):**

On page 6 of the notice, DuPont states:

*“The intended effect is as a nutrient necessary for the body’s nutritional and metabolic processes, serving as a prebiotic for commensal gut bacteria which metabolize prebiotics into short-chain fatty acids used for energy by colonocytes, and to stimulate sodium and water absorption....”*

Given that the majority of the existing infant formulas on the market do not contain 2'-FL and that breastmilk by non-secreting mothers contains little or no 2'-FL, this would suggest that 2'-FL does not serve a necessary function for infants’ “nutritional and metabolic processes.” Please clarify what is meant by this statement.

**Response:**

Upon review of FDA’s comments, DuPont notes that the cited statement on the intended effect is poorly written. We were trying to communicate that

- the presence of 2'-FL can aid in infant/toddler gut microbiota development by serving as a prebiotic/nutrient for commensal gut bacteria.
- an individual’s commensal gut bacteria play a supporting role in the body’s nutritional and metabolic processes.



To confirm, the intended use of DuPont's 2'-FL is as a food ingredient in term infant and toddler formulas, baby & toddler foods and toddler drinks at the levels listed in Table 1.

**Question (6):**

On page 9 of the notice, DuPont states:

*"All ingredients/process aids used in the fermentation phases are food or FCC grade and/or are permitted for direct addition to foods as GRAS ingredients and/or food additives."*

Please provide a citation for the specific FCC reference in this statement.

**Response:**

DuPont requires our suppliers who claim that their products are FCC grade to conform to the compendial monographs of the most current edition. The 10<sup>th</sup> edition is the most current edition.

**Question (7):**

The Sprenger et al. (2016) reference on page 39 could not be found in the notice. We presume that DuPont is referring to:

*Sprenger et al. (2017) "Longitudinal change of selected human milk oligosaccharides and association to infants' growth, an observatory, single center, longitudinal cohort study." PLoS ONE 12(2): e0171814*

Please confirm if this is the intended reference.

**Response:**

Yes, the intended reference is the Sprenger et al. 2017 noted above; the publication date was incorrectly listed as 2016.

**Question (8):**

The Smilowitz et al. (2017) reference on page 39 could not be found in the notice. We presume that DuPont is referring to:

*Smilowitz et al. (2017) "Tolerability and safety of the intake of bovine milk oligosaccharides extracted from cheese whey in healthy human adults." J. Nutr. Sci. 6: e6*

If this is not the correct citation, please provide the correct citation. If this is the citation, please clarify:

- 1) how a study with bovine milk oligosaccharides in general relates specifically to the safety of 2'-FL and
- 2) how a study done with healthy human adults is relevant to safety in infants.

**Response:**

DuPont agrees that the referenced study with bovine milk oligosaccharides has no relevance in the safety discussion on 2'-FL use in infant and toddler formulas and foods. The referenced paragraph is an editorial error from a previous version; it should have been deleted.

**Question (9):**

On the last page of the Expert Panel statement above the signatures (page A-118), the panel members state the following:

*“We, the undersigned members of the Expert Panel, have individually and collectively critically evaluated the materials summarized above on the safety of DuPont’s 2FL and 2FL and other information deemed appropriate and unanimously conclude that DuPont’s 2FL, manufactured as described in the dossier and consistent with cGMP, and meeting appropriate food grade specifications, is Generally Recognized As Safe (GRAS) based on scientific procedures for use as an ingredient in term infant formulas and toddler formulas at 2.4 g/L and in various foods targeted to toddlers at levels specified in the accompanying dossier.”*

Please clarify the nature of the “other information deemed appropriate” that was provided to the Expert Panel and whether this information was used in the panel’s conclusion of general recognition of safety. Please clarify if this other information was available to FDA and is publicly available.

**Response:**

All the information provided to the Expert Panel is contained in the dossier and in the literature cited within the dossier. No other information was used by the Expert Panel in its conclusion of general recognition of safety. The Expert Panel’s conclusion of general recognition of safety was entirely supported by publicly available information.

The phrase “other information deemed appropriate” is a carryover from a previous version and had been intended communicate the comprehensiveness of the Expert Panel review of available data and the thoroughness of their evaluation.

I hope the above responses are fully responsive to the questions and clarifications requested. If further questions arise or additional clarification is needed, I can be reached via the contact information included in the notification. Thank you for your reviewing our notification.

Sincerely,

Angela Lim  
Sr Manager, Regulatory Affairs  
DuPont Nutrition & Health

**From:** [Lim, Angela](#)  
**To:** [Morissette, Rachel](#)  
**Subject:** RE: GRN 000749 questions to be addressed  
**Date:** Monday, February 26, 2018 1:54:43 PM  
**Attachments:** [image007.png](#)  
[2-13-18 GRN749 Questions for Notifier - Response \(Feb 26, 2018\).pdf](#)

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Dear Dr Morissette,

Please find attached DuPont's response to the questions raised and the clarifications requested for GRN 000749.

Thank you for assisting with this notification.

Best regards, Angela

Angela Lim  
Sr Manager, Regulatory Affairs  
DuPont Nutrition & Health

DuPont Experimental Station 320/221  
200 Powder Mill Road, Wilmington, DE 19803

Tel: + 1 (302) 695-6786 (Office)  
Email: [Angela.Lim@DuPont.com](mailto:Angela.Lim@DuPont.com)

---

**From:** Morissette, Rachel [mailto:Rachel.Morissette@fda.hhs.gov]  
**Sent:** Tuesday, February 13, 2018 11:57 AM  
**To:** Lim, Angela <Angela.Lim@dupont.com>  
**Subject:** [EXTERNAL] GRN 000749 questions to be addressed

Dear Ms. Lim,

Please see attached a list of questions to be addressed for GRN 000749. Please send your responses within 10 business days in an email or separate document. Please do not send a revised notice. Let me know if you have any questions.

Best regards,

**Rachel Morissette, Ph.D.**  
*Consumer Safety Officer*

Center for Food Safety and Applied Nutrition

Office of Food Additive Safety  
U.S. Food and Drug Administration  
[rachel.morissette@fda.hhs.gov](mailto:rachel.morissette@fda.hhs.gov)



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February 26, 2018

Dear Dr Morissette,

Please find below DuPont's responses to the questions received on February 13, 2018 regarding GRAS Notice GRN 000749 for 2'-fucosyllactose (2'-FL).

**Question (1):**

On page 22 of the notice, DuPont states that dietary exposures to 2'-FL were estimated using the most recent NHANES data (i.e., 2011-2014). However, we note that Table 9 (page 23 of the notice) includes citations for the 2009-2010 and 2011-2012 NHANES, but does not include data from the more recent 2013-2014 NHANES. Please clarify which NHANES was used to calculate the dietary exposures.

**Response:**

The sentence *"Where applicable, the most recent National Health and Nutrition Examination Surveys (NHANES 2011-2014) have been utilized to estimate the mean and 90th percentile daily intake of 2FL among the U.S. populations"* is an editorial error from a previous version and should be deleted.

The intake data in Table 9 was based upon the 2009-2010 and the 2011-2012 NHANES data. DuPont was not able to find an analysis of the 2013-2014 NHANES data for infants and toddlers in the literature.

**Question (2):**

On page 21 of the notice under the heading "10. Allergens", the first sentence states:

*"None of the genes introduced into the production strain were secreted proteins (sucrose phosphorylase, fructokinase, sucrose permease, and fucosyltransferase)."*

We suggest rewording this sentence as follows:

*"None of the genes introduced into the production strain encode secreted proteins (sucrose phosphorylase, fructokinase, sucrose permease, and fucosyltransferase)."*

**Response:**

DuPont agrees with the suggested rewording. The genes introduced into the production strain encode for sucrose phosphorylase, fructokinase, sucrose permease, and fucosyltransferase; proteins are not secreted.

**Question (3):**

On page 40 of the notice, DuPont states:

*"Based on a NOAEL of 7660 mg/kg bw/day determined by subchronic oral toxicity studies and applying a 100-fold uncertainty safety factor, an ADI of 77 mg/kg bw/day was determined to be safe."*

The NOAEL used for the ADI determination is from an unpublished subchronic study in adult rats. Additionally, the EDI is greater than the ADI. Please discuss how a safety conclusion for the intended use of 2'-FL can be reached without an ADI determination and relying only on data and information that are

publicly available in support of a general recognition of safety. Please do not send a revised ADI calculation.

**Response:**

The inclusion of the ADI discussion in the dossier was to reflect the totality of issues, favorable and potentially unfavorable, reviewed by the Expert Panel. In their deliberations, the Expert Panel noted the existence of an unpublished, sub-chronic study, the ADI derived from the resulting NOAEL and that the EDI for the intended use exceeded the ADI. They did not consider this study to be pivotal.

The conclusion that DuPont's 2'-FL is safe for its intended use is based upon published studies discussing the molecular structure of 2'-FL, the 2'-FL content in human milk and clinical studies with 2'-FL.

The 2'-FL manufactured by DuPont is chemically and structurally identical to the 2'-FL present in human milk and to the GRAS notified 2'-FL products (GRN546 (2014), GRN571 (2015), and GRN650 (2016)).

Studies investigating 2'-FL content in human milk report values clustering around 2.4 g/L. Several studies report 2'-FL concentrations of approximately 7 g/L in mature breast milk and 8.4 g/L in colostrum from mothers with the 2'-FL secretor phenotype. Therefore, consumption of 2'-FL at these levels by infants can be concluded as safe.

The Davies et al (1994) study cited in GRN650 (2016), reported a mean consumption of 890.6 ml/day in 6-week old infants with a mean body weight of 4.7 kg. Therefore, the mean intake of 2'-FL from human milk can be expressed as 2.14 g/person/day or 455 mg/kg bw/day. For infants with mothers having the 2'-FL secretor phenotype, 2'-FL intake levels may exceed 6.23 g/person/day or 1326 mg/kg bw/day.

Addition of DuPont's 2'-FL to term infant and toddler formula at a maximum of 2.4 g/L will result in a mean EDI of 2.02 g/person/day and a 90<sup>th</sup> percentile EDI of 2.91 g/person/day by infants of 0-6 months, the age group with the highest intake. The mean EDI for this application is consistent with the mean 2'-FL intake resulting from consumption of human milk while the 90<sup>th</sup> percentile EDI falls well within the background exposure to 2'-FL from human milk of 2'-FL secretor mothers.

DuPont's 2'-FL will also be added to baby & toddler foods and toddler drinks at a maximum of 12.0 g/kg and 1.2 g/L respectively. 2'-FL intakes by infants of 7-12 months are highest for these combined food and beverage categories; the mean EDI is 4.63 g/person/day and the 90<sup>th</sup> percentile is 8.36 g/person/day. Healthy infants in this age group can be expected to have an average body weight of 8.1 kg or greater.<sup>1</sup> Thus, the mean EDI and 90<sup>th</sup> percentile may be expressed as 572 mg/kg bw/day and 1032 mg/kg bw/day. These values are within the background exposure to 2'-FL from consumption of human milk from 2'-FL secretor mothers. Furthermore, the derived EDI are based upon the conservative premise that all foods and drinks within this category will be supplemented with 2'-FL at the maximum concentration. In practice, not all foods and drinks within this category will be supplemented with 2'-FL and those that are, may be supplemented lower levels.

Moreover, DuPont's 2'-FL will be an alternative to the 2'-FL products already in commercial use and is intended to be used at equivalent levels in infants and toddler formulas, baby & toddler foods and/or toddler drinks; applications where 2'-FL is already permitted. Therefore, DuPont's 2'-FL is not expected to add to the overall daily intake of 2'-FL, which has previously been determined as safe. As such, DuPont's 2'-FL can likewise be considered as safe for the intended use.

Lastly, the clinical studies confirm that formula containing 1g/L 2'-FL is well tolerated and no safety concerns were reported, thereby demonstrating a history of safe use in human clinical trials.

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<sup>1</sup> CDC Clinical growth charts [https://www.cdc.gov/growthcharts/html\\_charts/wtageinf.htm](https://www.cdc.gov/growthcharts/html_charts/wtageinf.htm)

**Question (4):**

On page 41 of the notice, DuPont states:

*“The key evidence in this determination has been published in a peer review journal. Various other safety assessment, risk assessments, animal and human studies have all been published in peer reviewed journals or made publicly available on government websites.”*

GRAS conclusions or unpublished data and information contained in GRAS notices (i.e. GRN 000571) available only through government websites, such as the GRAS Notice Inventory, are not considered “peer-reviewed” and can only be used as corroborative evidence. The peer-reviewed primary literature references contained within a notice may be incorporated into a new notice with adequate discussion. Given the number of unpublished studies mentioned and discussed in this notice, as well as the use of an unpublished study for the ADI determination, it is unclear if the general recognition of safety requirement has been met in this notice. Please clearly identify which studies were pivotal studies and which studies were corroborative studies for DuPont’s GRAS conclusion.

**Response:**

The pivotal published studies upon which DuPont and the Expert Panel arrived at a conclusion of GRAS status are as follows:

- Structural analysis of 2'-FL
  - van Leeuwen, S. et al (2014) Rapid milk group classification by 1H NMR analysis of Le and H epitopes in human milk oligosaccharide donor samples. *Glycobiology* 24(8), 728-739.
- 2'-FL content in human milk
  - McGuire, M.K. et al (2017) What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. *Am J Clin Nutr* 105, 1086-1100.
  - Musumeci, M. et al (2006) Oligosaccharides in colostrum of Italian and Burkinabe women. *J Pediatr Gastroenterol Nutr* 43, 372-378.
  - Gabrielli, O. et al (2011) Preterm milk oligosaccharides during the first month of lactation. *Pediatrics* 128, e1520-1531.
  - Galeotti, F. et al (2012) On-line high-performance liquid chromatography-fluorescence detection-electrospray ionization-mass spectrometry profiling of human milk oligosaccharides derivatized with 2-aminoacridone. *Anal Biochem* 430, 97-104.
  - Castanys-Munoz, E. et al (2013) 2'-fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. *Nutr Rev* 71, 773-789.
  - Erney, R.M. et al (2000) Variability of human milk neutral oligosaccharides in a diverse population. *J Pediatr Gastroenterol Nutr* 30, 181-192.
  - Thurl, S. et al (1996) Quantification of individual oligosaccharide compounds from human milk using high-pH anion-exchange chromatography. *Anal Biochem* 235, 202- 206.
  - Sprenger, N. et al (2017) Longitudinal change of selected human milk oligosaccharides and association to infants' growth, an observatory, single center, longitudinal cohort study. *PLoS ONE* 12(2): e0171814.
- Clinical studies with 2'-FL
  - Puccio, G. et al (2017) Effects of Infant Formula with Human Milk Oligosaccharides on Growth and Morbidity: A Randomized Multicenter Trial. *J Pediatr Gastroenterol Nutr* 64, 624-631.
  - Marriage, B.J. et al (2015) Infants Fed a Lower Calorie Formula with 2'FL Show Growth and 2'FL Uptake Like Breast-Fed Infants. *J Pediatr Gastroenterol Nutr* 61, 649-658.

- Davis, P.S.W. et al (1994) Adjusting milk intake for body size in early infancy.

The following studies and safety evaluations were considered corroborative.

- Subchronic and subacute oral toxicity studies:
  - Coulet, M. et al (2014) Pre-clinical safety evaluation of the synthetic human milk, nature-identical, oligosaccharide 2'-O-Fucosyllactose (2'-FL). Regul Toxicol Pharmacol 68, 59-69.
  - Hanlon, P.R. & Thorsrud, B.A. (2014) A 3-week pre-clinical study of 2'-fucosyllactose in farm piglets. Food Chem Toxicol 74, 343-348.
  - Penard, L. (2015) 2'-FL – 13-Week Oral (Gavage) Juvenile Toxicity Study in the Rat Followed by a 4-Week Treatment-Free Period. Confidential. (Study Number AB20757; Sponsor Reference Number GSN037). Prepared by DD 's-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark, Glycom A/S.
- Clinical studies
  - Elison, E. et al (2016) Oral supplementation of healthy adults with 2'-O-fucosyllactose and lacto-N-neotetraose is well tolerated and shifts the intestinal microbiota. The British journal of nutrition 116, 1356-1368.
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  - Jennewein Biotechnologie, G. (2015) GRAS Exemption claim for use of 2'-fucosyllactose (2'-FL) in term infant and toddler formulas: GRN000571. U.S. FDA, GRAS Notice Inventory.
  - EFSA Panel of Dietetic Products (2015) Scientific opinion on the safety of 2'-O-fucosyllactose as a novel food ingredient pursuant to Regulation (EC) No 259/97. EFSA Journal 13.

**Question (5):**

On page 6 of the notice, DuPont states:

*“The intended effect is as a nutrient necessary for the body’s nutritional and metabolic processes, serving as a prebiotic for commensal gut bacteria which metabolize prebiotics into short-chain fatty acids used for energy by colonocytes, and to stimulate sodium and water absorption....”*

Given that the majority of the existing infant formulas on the market do not contain 2'-FL and that breastmilk by non-secreting mothers contains little or no 2'-FL, this would suggest that 2'-FL does not serve a necessary function for infants’ “nutritional and metabolic processes.” Please clarify what is meant by this statement.

**Response:**

Upon review of FDA’s comments, DuPont notes that the cited statement on the intended effect is poorly written. We were trying to communicate that

- the presence of 2'-FL can aid in infant/toddler gut microbiota development by serving as a prebiotic/nutrient for commensal gut bacteria.
- an individual’s commensal gut bacteria play a supporting role in the body’s nutritional and metabolic processes.



To confirm, the intended use of DuPont's 2'-FL is as a food ingredient in term infant and toddler formulas, baby & toddler foods and toddler drinks at the levels listed in Table 1.

**Question (6):**

On page 9 of the notice, DuPont states:

*"All ingredients/process aids used in the fermentation phases are food or FCC grade and/or are permitted for direct addition to foods as GRAS ingredients and/or food additives."*

Please provide a citation for the specific FCC reference in this statement.

**Response:**

DuPont requires our suppliers who claim that their products are FCC grade to conform to the compendial monographs of the most current edition. The 10<sup>th</sup> edition is the most current edition.

**Question (7):**

The Sprenger et al. (2016) reference on page 39 could not be found in the notice. We presume that DuPont is referring to:

*Sprenger et al. (2017) "Longitudinal change of selected human milk oligosaccharides and association to infants' growth, an observatory, single center, longitudinal cohort study." PLoS ONE 12(2): e0171814*

Please confirm if this is the intended reference.

**Response:**

Yes, the intended reference is the Sprenger et al. 2017 noted above; the publication date was incorrectly listed as 2016.

**Question (8):**

The Smilowitz et al. (2017) reference on page 39 could not be found in the notice. We presume that DuPont is referring to:

*Smilowitz et al. (2017) "Tolerability and safety of the intake of bovine milk oligosaccharides extracted from cheese whey in healthy human adults." J. Nutr. Sci. 6: e6*

If this is not the correct citation, please provide the correct citation. If this is the citation, please clarify:

- 1) how a study with bovine milk oligosaccharides in general relates specifically to the safety of 2'-FL and
- 2) how a study done with healthy human adults is relevant to safety in infants.

**Response:**

DuPont agrees that the referenced study with bovine milk oligosaccharides has no relevance in the safety discussion on 2'-FL use in infant and toddler formulas and foods. The referenced paragraph is an editorial error from a previous version; it should have been deleted.

**Question (9):**

On the last page of the Expert Panel statement above the signatures (page A-118), the panel members state the following:

*“We, the undersigned members of the Expert Panel, have individually and collectively critically evaluated the materials summarized above on the safety of DuPont’s 2FL and 2FL and other information deemed appropriate and unanimously conclude that DuPont’s 2FL, manufactured as described in the dossier and consistent with cGMP, and meeting appropriate food grade specifications, is Generally Recognized As Safe (GRAS) based on scientific procedures for use as an ingredient in term infant formulas and toddler formulas at 2.4 g/L and in various foods targeted to toddlers at levels specified in the accompanying dossier.”*

Please clarify the nature of the “other information deemed appropriate” that was provided to the Expert Panel and whether this information was used in the panel’s conclusion of general recognition of safety. Please clarify if this other information was available to FDA and is publicly available.

**Response:**

All the information provided to the Expert Panel is contained in the dossier and in the literature cited within the dossier. No other information was used by the Expert Panel in its conclusion of general recognition of safety. The Expert Panel’s conclusion of general recognition of safety was entirely supported by publicly available information.

The phrase “other information deemed appropriate” is a carryover from a previous version and had been intended communicate the comprehensiveness of the Expert Panel review of available data and the thoroughness of their evaluation.

I hope the above responses are fully responsive to the questions and clarifications requested. If further questions arise or additional clarification is needed, I can be reached via the contact information included in the notification. Thank you for your reviewing our notification.

Sincerely,

Angela Lim  
Sr Manager, Regulatory Affairs  
DuPont Nutrition & Health

**From:** [Lim, Angela](#)  
**To:** [Morissette, Rachel](#)  
**Subject:** RE: question on lactose source for GRN 000749  
**Date:** Wednesday, March 14, 2018 4:13:31 PM  
**Attachments:** [image013.png](#)  
[image019.png](#)

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Thanks for this information, Rachel.

Best regards, Angela

Angela Lim  
Sr Manager, Regulatory Affairs  
DuPont Nutrition & Health

DuPont Experimental Station 320/221  
200 Powder Mill Road, Wilmington, DE 19803

Tel: + 1 (302) 695-6786 (Office)  
Email: [Angela.Lim@DuPont.com](mailto:Angela.Lim@DuPont.com)

---

**From:** Morissette, Rachel [mailto:[Rachel.Morissette@fda.hhs.gov](mailto:Rachel.Morissette@fda.hhs.gov)]  
**Sent:** Wednesday, March 14, 2018 3:10 PM  
**To:** Lim, Angela <[Angela.Lim@dupont.com](mailto:Angela.Lim@dupont.com)>  
**Subject:** [EXTERNAL] RE: question on lactose source for GRN 000749

No, we just needed to have that information for the record since it didn't appear in the notice anywhere.

Thanks,

*Rachel*

---

**Rachel Morissette, Ph.D.**

*Consumer Safety Officer*

Center for Food Safety and Applied Nutrition  
Office of Food Additive Safety  
U.S. Food and Drug Administration  
[rachel.morissette@fda.hhs.gov](mailto:rachel.morissette@fda.hhs.gov)



**From:** Lim, Angela [<mailto:Angela.Lim@dupont.com>]  
**Sent:** Wednesday, March 14, 2018 3:09 PM  
**To:** Morissette, Rachel <[Rachel.Morissette@fda.hhs.gov](mailto:Rachel.Morissette@fda.hhs.gov)>  
**Subject:** RE: question on lactose source for GRN 000749

Hi Rachel,

Yes, the lactose used in production of DuPont's 2'-FL in GRN 000749 is from cow's milk.

Is there a concern? Thanks.

Best regards, Angela

Angela Lim  
Sr Manager, Regulatory Affairs  
DuPont Nutrition & Health

DuPont Experimental Station 320/221  
200 Powder Mill Road, Wilmington, DE 19803

Tel: + 1 (302) 695-6786 (Office)  
Email: [Angela.Lim@DuPont.com](mailto:Angela.Lim@DuPont.com)

---

**From:** Morissette, Rachel [<mailto:Rachel.Morissette@fda.hhs.gov>]  
**Sent:** Wednesday, March 14, 2018 2:54 PM  
**To:** Lim, Angela <[Angela.Lim@dupont.com](mailto:Angela.Lim@dupont.com)>  
**Subject:** [EXTERNAL] question on lactose source for GRN 000749

Hi Angela,

Can you please confirm if the source of lactose used in the production of DuPont's 2'-FL in GRN 000749 is from cow's milk?

Thanks,

*Rachel*

---

**Rachel Morissette, Ph.D.**

*Consumer Safety Officer*

Center for Food Safety and Applied Nutrition  
Office of Food Additive Safety  
U.S. Food and Drug Administration  
[rachel.morissette@fda.hhs.gov](mailto:rachel.morissette@fda.hhs.gov)



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