GRAS Notification

of

Purified 2'-Fucosyllactose (2'-FL)

Food Usage Conditions for General Recognition of Safety

on behalf of

Glycosyn, LLC
Woburn, MA

and

FrieslandCampina Domo B.V.
Amersfoort, The Netherlands

Volume 2 of 2

9/29/17

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Appendix 1 NMR Analytical Reports

Appendix 1.1 Complex Carbohydrate Research Center

Appendix 1.2 University of Groningen

Appendix 1.3 Spectral Service (Cologne)

Appendix 1.1 Complex Carbohydrate Research Center

COMPLEX CARBOHYDRATE RESEARCH CENTER

ANALYTICAL SERVICE REPORT

Date: 10/8/14

Investigator: **John McCoy**
Glycosyn Inc.
196 Boston Avenue Suite 1200
Medford MA 02155
(Email: dmajumdar@glycosyninc.com Debatosh Majumdari)

Subject: NMR-spectroscopic analysis of 1 sample

Sample Synthetic Oligosaccharide

CCRC Code: **JM092314B**

Analyst: Radnaa Naran

Cost:

Methods:

Please note:

Should any of these data be used in a publication, please include the following statement in the acknowledgment: "This research was supported in part by the National Institutes of Health (NIH)-funded Research Resource for Integrated Glycotechnology (NIH grant no. 5P41GM10339024) to Parastoo Azadi at the Complex Carbohydrate Research Center.

NMR Spectroscopy

The sample was deuterium exchanged 3 times by lyophilization in D_2O, then re-dissolved in 0.5 mL D_2O and placed in a 5-mm NMR tube. 1-D proton and 2-D gCOSY, TOCSY, gHSQC, gHMBC, ROESY spectra were obtained on Varian Inova 600 MHz spectrometer at 25°C using standard Varian pulse sequences. Proton chemical shifts were measured relative to water peak (δ_H=4.78 at 25°C). Carbon chemical shifts were referenced using the absolute chemical shift scale in Mnova.

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Results

In anomeric region (Fig 1: Presat and gHSQC spectra), 4 signals were detected: terminal α-Fuc, δ_H ppm 5.30 (1H), reducing 4-α-Glc 5.22 (0.4H), 4-β-Glc, δ_H ppm 4.62 (doublet, 0.6H), and doublet for 2-β-Gal, δ_H ppm 4.51 ($J_{1,2}$=7.45Hz; 1H).

Proton and carbon chemical shifts for each glycosyl residue were assigned by interpretation of 1-D Proton and 2-D gCOSY, zTOCSY, gHSQC, and gHMBC spectra (Table 1, Fig 1), and inter-residue linkages were confirmed from ROESY and HMBC correlations. The trisaccharide structure was identified as 2'-Fucosyllactose shown in Fig 2.

Table 1 NMR- Chemical shift assignments for 2'-Fucosyllactose, $\delta_{H/C}$ ppm

	Residue	Nuclei	1	2	3	4	5	6	NOE, HMBC
A	4-α-Glc	^1H	5.22	3.58	3.80	3.71	3.88	3.88/3.79	C1-A4
		^{13}C	*94.5*	*74.1*	*74.3*	*78.6*	*73.1*	*62.8*	
B	4-β-Glc	^1H	4.62	3.29	3.58	3.70	3.47	3.77/3.93	C1-B4
		^{13}C	*98.6*	*76.7*	*77.0*	*78.6*	*78.1*	*63.0*	
C	2-β-Gal	^1H	4.51	3.66	3.84	3.87	3.68	3.79/3.73	B4-C1, D1-C2
		^{13}C	*101.0*	*77.2*	*74.0*	*78.0*	*78.0*	*63.8*	
D	α-Fuc->2	^1H	5.30	3.79	3.78	3.80	4.23	1.22	C2-D1
		^{13}C	*101.9*	*70.9*	*72.3*	*74.3*	*69.7*	*17.9*	

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Fig 1 Partial 1-D Presat and 2-D gHSQC spectra of the sample

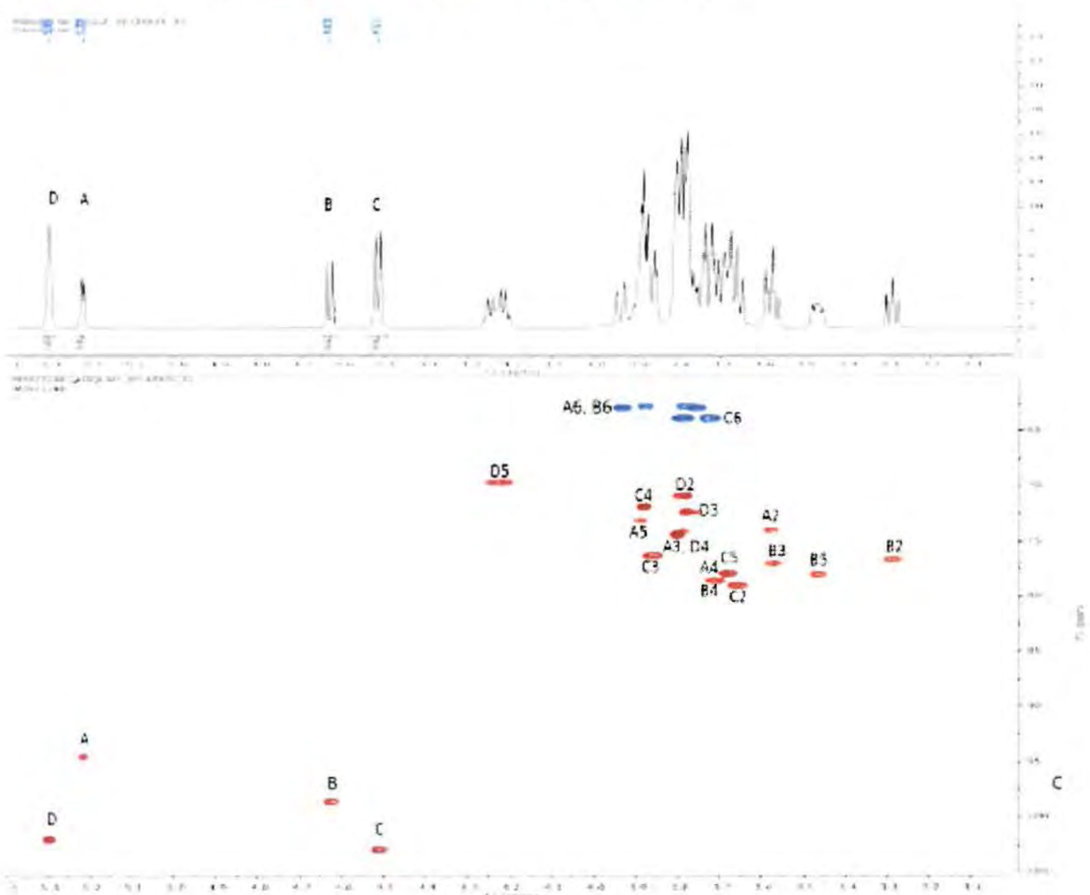

Fig 2 2'-Fucosyllactose

2-β-Gal
4-α,β-Glc
α-Fuc

If you have any further questions please contact Dr. Parastoo Azadi at 706-583-0629 or azadi@ccrc.uga.edu

Appendix 1.2 University of Groningen

Purity determination of 2'-FL samples by 1D ^{1}H NMR spectroscopy

Sander van Leeuwen, PhD
Microbial Physiology
University of Groningen

Nijenborgh 7
9747 AG Groningen

Methods

Samples of 3-6 mg were exchanged twice with 500 µL 99.9 atom% D_2O with intermediate lyophilisation. One-dimensional ^{1}H NMR spectra were recorded at a probe temperature of 298K on a Varian Inova 600 spectrometer (NMR Department, University of Groningen). Samples were finally dissolved in 650 µL D_2O. ^{1}H are expressed in ppm in reference to internal acetone (δ^{1}H 2.225). 1D 600-MHz ^{1}H NMR spectra were recorded in triplicate with 5000 Hz spectral width at 16k complex data points, using a WET1D pulse to suppress the HOD signal. Spectra were processed using MestReNova 5.3 (Mestrelabs Research SL, Santiago de Compostella, Spain), using Whittaker Smoother baseline correction.

Results

All samples showed a highly similar 1D ^{1}H NMR spectrum (Figure 1). The intensity of the anomeric peak specific for 2'-FL at δ 5.31 was compared with the intensity of anomeric peaks representing LDFT and 3-FL at δ 5.45 and 5.40 ppm were used to determine the purity of the samples. The levels of LDFT and 3-FL were estimated from the Fuc CH_3 area, where δ 1.16-1.19 ppm corresponds with Fuc-(α1→3)- in 3-FL and δ 1.23-1.26 corresponds with Fuc-(α1→2)- plus Fuc-(α1→3)- in LDFT. Due to overlap with the strong signal for Fuc-(α1→2)- in 2'-FL (δ 1.20-1.22 ppm) the estimation of LDFT and 3-FL has to be taken as a rough indication. One representative spectrum for each sample is shown (Figure 1). Each of three spectra were integrated twice and averages of a total of 6 integrations per sample are shown (Table 1).

Table 1. Percentages of 2'-FL and LDFT and 3-FL for each sample, based on 1D ^{1}H NMR integrations. FL-A and FL-B are the samples of the new batch, the four others were taken from the previous report.

	2'-FL (%)	LDFT (%)	3-FL (%)
FL-20140609	95.6 (+/- 0.52)	1.2	3.2
FL-20140610	94.1 (+/- 0.47)	1.6	4.3
FL-20140611	94.2 (+/- 0.59)	1.2	4.6
FL-20140612	95.2 (+/- 0.41)	1.6	3.2
2FL-A	98.4 (+/- 0.54)	0.9	0.7
2FL-B	98.2 (+/- 0.34)	1.0	0.9

Conclusions

The 2'-FL in batches A and B have comparable purity as determined by 1D ^{1}H NMR integrations. This product is the most pure 2'-FL preparation analysed so far.

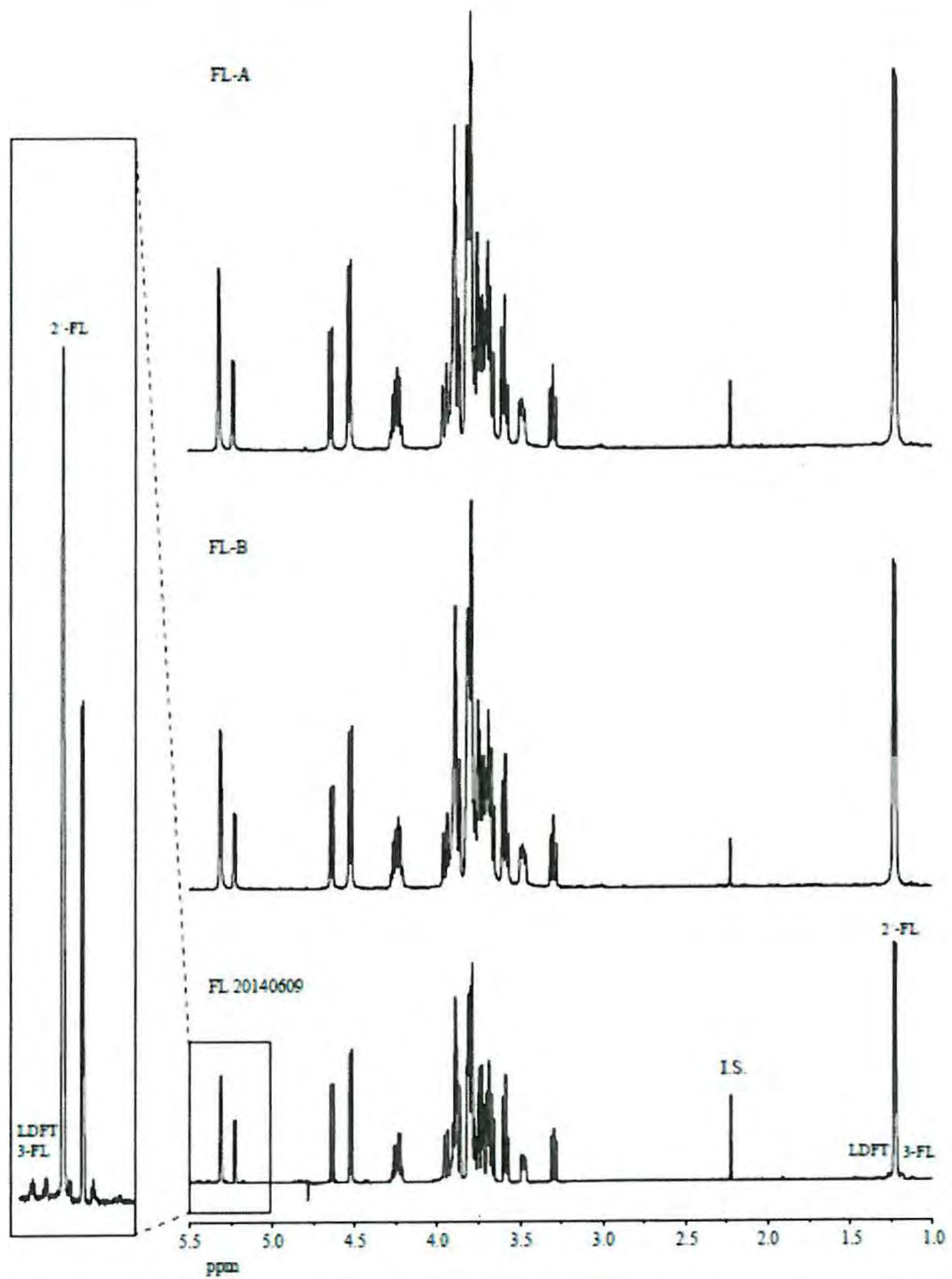

Figure 1. 600 MHz 1D ¹H NMR spectra representing the four samples. For FL 20140609 (previous analysis) the anomeric region is expanded into the inset, showing the peaks used for integration in determining 2'-FL purity. I.S. is acetone internal standard (δ 2.225 ppm).

Appendix 1.3 Spectral Service (Cologne)

STUDY REPORT FFD63190

- 2-fucosyllactose powder -

- Quantification and Characterisation, Water Content -

Sponsor: FrieslandCampina Innovation Centre
Bronland 20
NL-6708WH Wageningen
Netherlands

Monitor: Jan Bastiaans

Test Facility: Spectral Service AG
Emil-Hoffmann Straße 33
D-50996 Köln
Germany

Date: 30 January 2017

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1 OBJECTIVES

The purpose of analysis is to determine identity and content of the test item and water using spectroscopic methods.

2 TEST ITEM

Tab. 1 Test item data

Sample name	Lot no.	Spectral Service code	Arrival
2-fucosyllactose powder	PMRS01	FFD63190-1	19.01.2017

3 MATERIALS

3.1 Reference and Calibration Items

Tab. 2 Chemicals actually used as reference and calibration items

No.	Substance name	Distributor	Order no.
14	Tetramethylsilane (TMS, for NMR calibration)	Sigma-Aldrich Chemie GmbH Steinheim (D)	T2,400-7
9	Nicotinic acid amid (NSA)	Sigma-Aldrich, Buchs (CH)	72340

3.2 Chemicals and Materials

Tab. 3 Chemicals and materials used in the study

Substance name	Distributor	Order no.
Deuteriumoxide, Degree of deuteration 99.9%	Euriso-top, Saarbrücken D)	D214H
Dimethylsulfoxide-d_6 (DMSO-d_6), Degree of deuteration 99.8%	Euriso-top, Saarbrücken D)	D010H

3.3 Instruments

NMR spectrometer Avance III 600 (Bruker, Karlsruhe, D), magnetic flux density 14.1 Tesla
BBO cryo probe (5 mm CPBBO BB-), automated sample changer Bruker B-ACS 120
Computer Intel Xeon E5 8-Core 3.7 GHz under MS Windows 7, Bruker TopSpin 3.2
Standard operation procedure SAA-GMR028-05

Micro balance Mettler-Toledo XPE 26 (Greifensee, CH)
Balance printer Mettler Toledo LC-P45 (Greifensee, CH)
Standard operation procedure SAA-GMR047-02

4 METHODS

4.1.1 NMR Spectroscopy

A ^{1}H-NMR spectrum was recorded to characterise the test item. Approx. 10 mg of the test item have been dissolved in 1 ml D_2O.
The actually used NMR parameters appear on the spectrum plot.

For quantification, according to standard operation procedure SAA-MET001-03 appropriate amounts of the test item and of internal standard have been exactly weighed (s. Table 'Calculation of content', Chapter 6 - NMR Spectroscopy), dissolved in 1 ml D_2O and measured. Integrated signals of the test item and of the internal standard nicotinic acid amide, (NSA) have been used for calculation.

The ratio of integrals per atom corresponds to the molar ratio of the compared substances. For calculation software Microsoft Excel 9.0 was used.

Calculation:

Equation 1
$$MOL_{IS}\,[mMol] = \frac{IW_{IS}\,[mg] \cdot C_{IS}\,[\%]}{MW_{IS}\,[g/mol] \cdot 100}$$

Equation 2
$$MOL_{TI}\,[mMol] = \frac{I_{TI} \cdot NA_{IS} \cdot MOL_{IS}\,[mMol]}{I_{IS} \cdot NA_{TI}}$$

Equation 3
$$C_{TI}\,[\%\text{-by weight}] = \frac{MW_{TI}\,[g/mol] \cdot MOL_{TI}\,[mMol] \cdot 100}{IW_{TI}\,[mg]}$$

Tab. 4 Declaration of variables

	test item (TI)	internal standard (IS)
molecular weight [g/mol]	MW_{TI}	MW_{IS}
initial weight [mg]	IW_{TI}	IW_{IS}
content [%-by weight]	C_{TI}	C_{IS}
Mol [mMol]	MOL_{TI}	MOL_{IS}
integral	I_{TI}	I_{IS}
number of atoms*)	NA_{TI}	NA_{IS}

*) atom refers to NMR active nucleus measured (e.g. ^{1}H, ^{13}C, ^{19}F, ^{31}P)

For determination of the water content 0.7 ml of DMSO-d_6 were filled in a NMR tube and measured. The water signal (zero value) was normalised using the solvent signals. Approx. 10 mg of the test item was added to the solvent and measured again. The corrected water signal was compared with the signals of the test item.

The ratio of integrals per atom corresponds to the molar ratio of the compared substances. For calculation software Microsoft Excel 14.0 was used.

Calculation:

Equation 1

$$MOL_{H2O}\ [\%] = \frac{(INT_{H2O(TI)} / NA_{H2O}) * C_{TI}}{(INT_{TI} / NA_{TI})}$$

Equation 2

$$C_{H2O}\ [\%] = \frac{MOL_{H2O} * MW_{H2O}}{MW_{TI}}$$

Tab. 5 Declaration of variables

	test item (TI)	H_2O
molecular weight [g/Mol]	MW_{TI}	MW_{H2O}
Mol-% [%]		MOL_{H2O}
content [%-by weight]	C_{TI}	
integral	INT_{TI}	INT_{H2O}
number of atoms *)	NA_{TI}	NA_{H2O}
content water [% w/w]		C_{H2O}

*) atom refers to NMR active nucleus measured (e.g. ^{1}H, ^{13}C, ^{19}F, ^{31}P)

5 RESULTS AND DISCUSSION

Signal chemical shifts and mutiplicity are in accordance with the given structure of 2'FL. Fructose is not detected; however, some smaller amounts of carbohydrate are visible and may disturb the integration slightly. In summary, the total amount of 2'FL and water content corresponds to 100 % within the limits of the method.

Signals not caused by the test item: 0.0 ppm (singlet of TMS); 2.50 ppm (multiplet of DMSO-d_5 in DMSO-d_6) and 3.33 ppm (singlet of water).

Tab. 6 Calculation of content

Excel-Version 16.0					Version 02.01		valid from:	6/15/2015
Test	Integral TI	Initial weight [mg] TI	Integral IS	Initial weight [mg] IS	mMol IS	mMol TI	Content [mg] TI	Content [%] TI
NSA	69.7	10.40	100.0	10.57	0.0865	0.0201	9.8172	94.4
NSA	72.7	10.28	100.0	10.20	0.0834	0.0202	9.8821	96.2
	Molecular weight TI		486.44				Average	95.3
	Number of atoms TI-1		3	Number of atoms TI-2		3	Std.-dev.	1.3
	Number of atoms IS-1		1	Number of atoms IS-2		1	%RSD	1.3
Recording	Molecular weight IS-1		122.13	Molecular weight IS-2		122.13	Balance	
	Content [%] IS-1		99.9	Content [%] IS-2		99.9	Mettler-Toledo XPE205DR/M	

Comment: initial weight is higher than required MinWeigh of 10 mg

Tab. 7 Calculation of water content

INT H_2O (pure solvent)	154.3		INT_{TI}	23.4
INT H_2O (solvent + TI)	215.1		NA_{TI}	1
INT H_2O (TI)	60.8		INT_{TI} / NA_{TI}	23.4
Content TI [%-by weight]	95.3		MOL_{H2O}	123.8
MW_{TI}	504.4		**C_{H2O}**	**4.4**
MW_{H2O}	18.0			

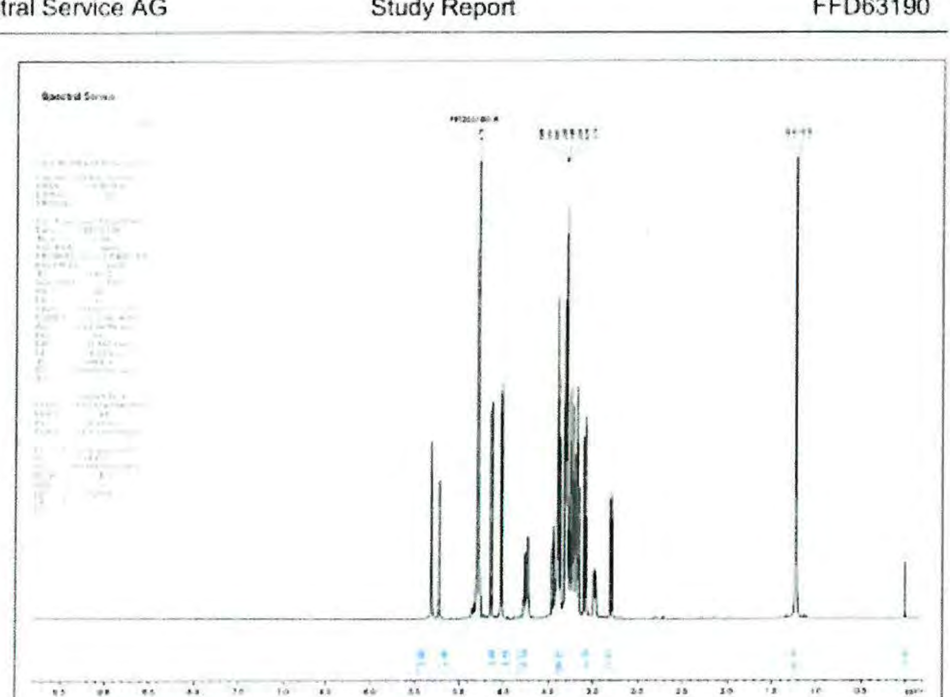

Fig. 1 ^{1}H-NMR spectrum of test item FFD63190 in D_2O

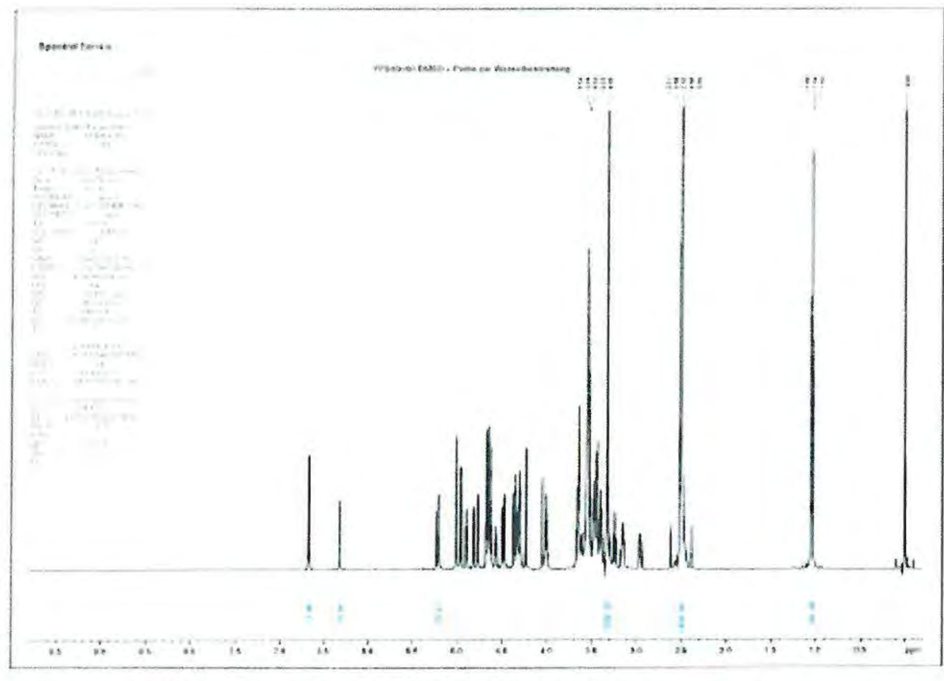

Fig. 2 ^{1}H-NMR spectrum of test item FFD63190 in DMSO-d_6, water content

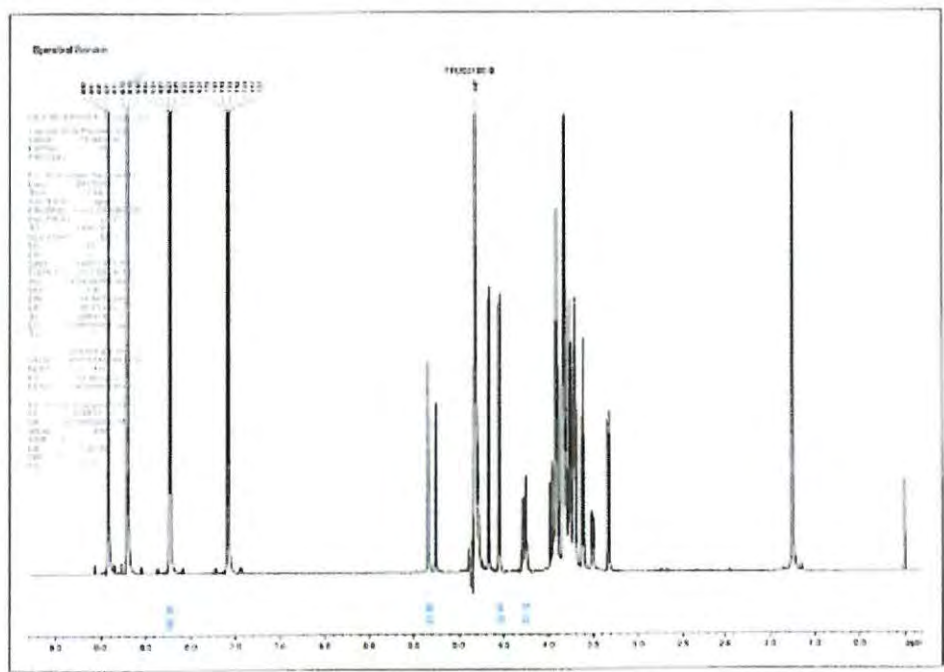

Fig 3 ^{1}H-NMR spectrum of test item FFD63190 in D_2O, quant. B

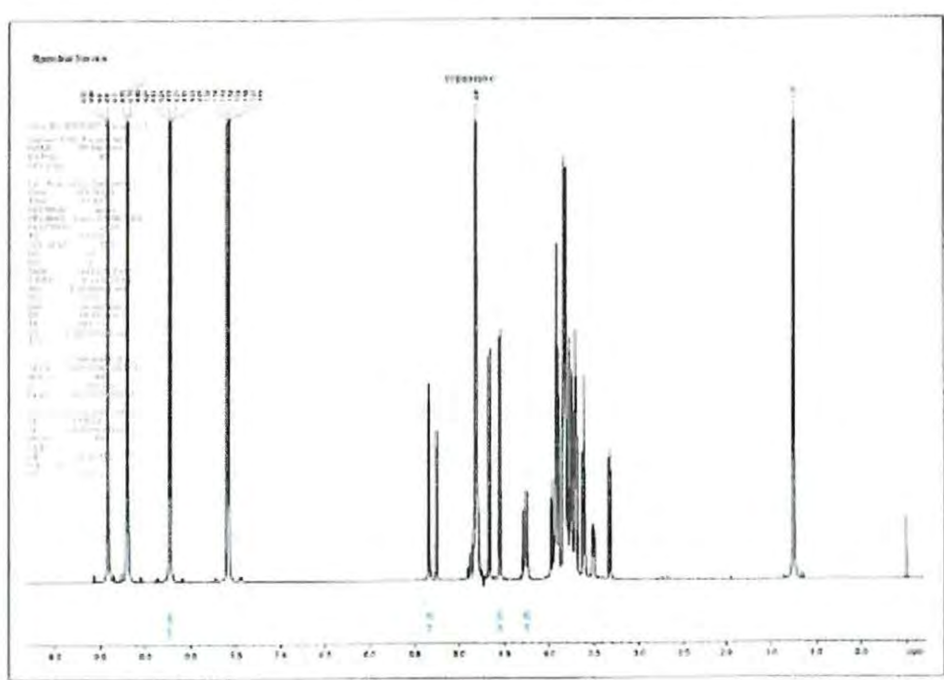

Fig 4 ^{1}H-NMR spectrum of test item FFD63190 in D_2O, quant C

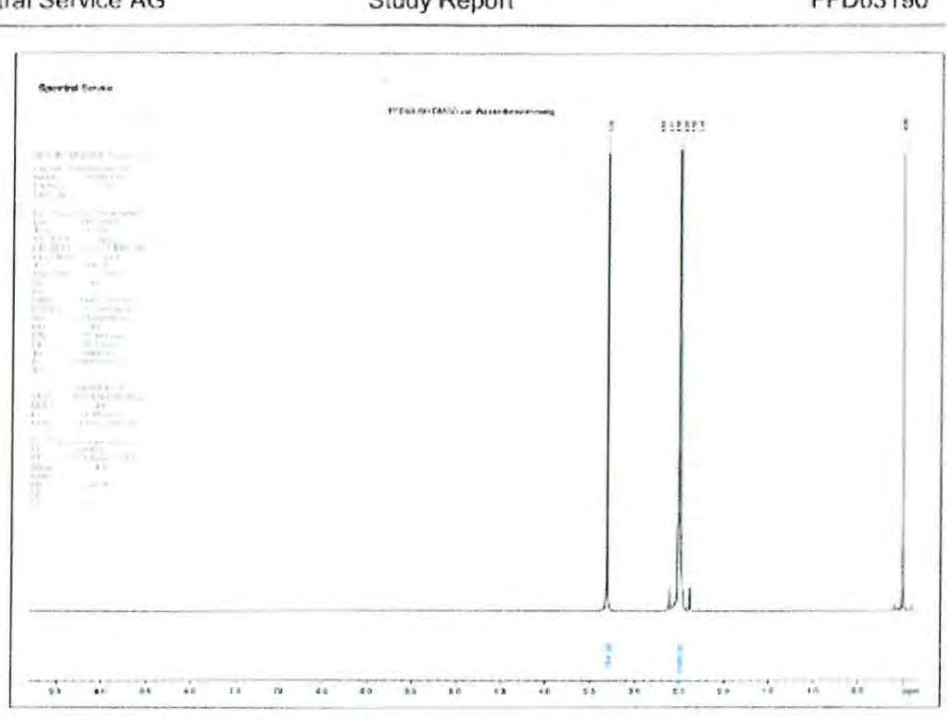

Fig. 5 ¹H-NMR spectrum of DMSO-d_6, water content

6 SUMMARY

The proposed structure of the test item was confirmed by ^{1}H-NMR-Spectroscopy. The average content was determined to 95 3 weight-%, the water amount to 4.5 %.

The result refers exclusively to test item analysed by Spectral Service AG. Because no specification was given to the Spectral Service AG for this analysis order, the assessment of the plausibility of this result is the responsibility of the customer.

7 PERSONNEL

Study director:	Dr Bernd Diehl,	Chemist
Co-worker:	Andreas Beyer,	Biological technical assistant
	Karin Seitz,	Chemist
Quality assurance:	Dr Miriam Sobieray,	1. State exam

All are staff members of the test facility.

8 CONFIRMATION OF THE STUDY REPORT

Page 1 of the original report is printed on Spectral Service business paper; the report is authorised by original signature and company's stamp. Use or publication in parts is not authorised on principle and is not allowed to be connected with the company's name or a signature of a staff member. Misuse will be prosecuted.

Appendix 2 Specifications and Certificates of Analysis for Raw Materials and Production Processing Aids

Appendix 2.1	**Glucose Monohydrate**
Appendix 2.2	**α-Lactose Monohydrate**
Appendix 2.3	**Phosphoric Acid**
Appendix 2.4	**Ammonium Hydroxide**
Appendix 2.5	**di-Ammonium Hydrogen Phosphate**
Appendix 2.6	**Monopotassium Phosphate**
Appendix 2.7	**L-Tryptophan**
Appendix 2.8	**Magnesium Sulfate Heptahydrate**
Appendix 2.9	**Citric Acid**
Appendix 2.10	**Iron (II) Sulfate Heptahydrate**
Appendix 2.11	**Manganese Chloride Tetrahydrate**
Appendix 2.12	**Cobalt (II) Chloride Hexahydrate**
Appendix 2.13	**Cupric Sulfate Pentahydrate**
Appendix 2.14	**Boric Acid**
Appendix 2.15	**Zinc Sulfate Heptahydrate**
Appendix 2.16	**Sodium Hydroxide**
Appendix 2.17	**Sodium Molybdate Dihydrate**
Appendix 2.18	**Activated Carbon**

Appendix 2.1 Glucose Monohydrate

SPECIFICATIONS Ref: E41-101010

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ROQUETTE® DEXTROSE MONOHYDRATE M

DEFINITION :

DEXTROSE MONOHYDRATE. Purified and crystallized D-glucose, containing one molecule of water of crystallisation.

Prodotto distribuito da
UNIVAR®

CAS n° : 77938-63-7
EINECS : 200-075-1

SPECIFICATIONS :

• PHYSICO-CHEMICAL VALUES

APPEARANCE	MCL	Crystalline powder, white and odourless
TASTE		Sweet.
LOSS ON DRYING	MCL	9.1 % max
DEXTROSE (D-GLUCOSE)		99.5 % min.
SPECIFIC ROTATION	MCL	52.6 - 53.2 degrees
pH IN SOLUTION	MCL	4 - 6
SULPHATED ASH	MCL	0.1 % max.
RESISTIVITY	MCL	100 kOhm.cm min.
PARTICLE SIZE	MCL	
- RESIDUE ON 500 MIC.		10 % max.

• MICROBIOLOGICAL VALUES

- TOTAL COUNT	NMC	1000/g max.
- YEASTS	NMC	10/g max.
MOULDS	NMC	10/g max.
- E.COLI	NMC	Absent in 10 g
- SALMONELLAE	NMC	Absent in 25 g

TYPICAL VALUES :

ENERGY VALUE calculated, on 100 g commercial product	1547 kJ (364 kcal)

MCL,NMC : ROQUETTE Methods

QUALITY ASSURANCE / HUMAN FOOD November 25, 2014

ROQUETTE ITALIA - Società per Azioni con Socio Unico - Roquette Frères SA (FRANCIA)
SEDE LEGALE, DIREZIONE E STABILIMENTO 15063 CASSANO SPINOLA (AL) - VIA SERRAVALLE, 26
TELEFONO 0143 774 1 r.a., TELEX: 210161 ROQUET 1, TELEFAX: 0143 477 295
CAPITALE SOCIALE INT. VERS. € 5.165.000 - CODICE FISCALE PARTITA IVA N. 00216980065 - REG. IMP. N 242/27/275
TRIBUNALE DI TORTONA - C.C.I.A.A. ALESSANDRIA N. 73302

SPECIFICATIONS

Ref: R41-101R10

PAGE 2/2

ROQUETTE® DEXTROSE MONOHYDRATE M

COMMENTS :

Due to its fine particle size, this product is liable to become compacted.

Store at room temperature, in a dry place, and in its unopened original packing.

CONFORMITY :

- CODEX STAN - 212 - 1999
- EU directive 2001/111/EC (OJEC L. 10 dd 12/01/02).
- Current FOOD CHEMICALS CODEX.
- US Code of federal regulations - 21 CFR § 168.111.

STORAGE :

Standard packaging : bulk road tanker,
25 kg paper bags + polyethylen free film
1000 kg FIBC

Minimum durability date of the packaged product: Manufacturing date + 12 months.

Shelf life: Manufacturing date + 5 years.

UNIVAR S.p.A.
DOCUMENTO (b) (6) CATO
FIRMA ....
DATA ...... 9-12-2014....

MCL.MMC : ROQUETTE Methods

QUALITY ASSURANCE / HUMAN FOOD November 25, 2014

ROQUETTE ITALIA - Società per Azioni con Socio Unico - Roquette Frères SA (FRANCIA)
SEDE LEGALE, DIREZIONE E STABILIMENTO: 15063 CASSANO SPINOLA (AL) - VIA SERRAVALLE, 26
TELEFONO: 0143 774 1 r.a. TELEX 210161 ROQUET I, TELEFAX: 0143 477 275
CAPITALE SOCIALE INT. VERS. € 5.165.000 - CODICE FISCALE PARTITA IVA N. 00161980055 - REG. IMP. N 245/27/275
TRIBUNALE DI TORTONA - C.C.I.A.A. ALESSANDRIA N. 73302

Appendix 2.2 α-Lactose Monohydrate

Patheon Capua S.p.A.
Strada Statale Appia 46/48
81043 Capua (Caserta)
ITALY

Certificate of analysis

Product	: **Lactopure®** Refined Crystals
Product code	: 502309
Order no.	: 2226137
Customer no.	: Capua Bioservices
Batch no.	: 707304
Date of production	: 09-08-2015
Retest date	: 09-08-2018
Description	: High quality lactose
Typical analysis	: Lactose monohydrate 99.6%, protein 0.1%, minerals 0.1%, free moisture 0.1%
Sensorial	: White crystalline powder, odourless, slightly sweet
Intended use	: **Infant formula, dry blending application**

Chemical / physical:	**Specification**	**Results**	**Method of analysis**
Sulphated ash	max. 0.15%	< 0.1 %	NEN 6810 (modified)
Free moisture	max. 0.2 %	< 0.1 %	FC-method, 2h 80°C
Total moisture	max. 5.2 %	5.1 %	ISO 760 (modified), Karl Fischer
Scorched particles	max. value + +	+	FC-method equivalent to ADPI 916 / ISO 5739 / IDF 107
pH (23%, 20°C)	3.0 - 7.0	3.6	FC-method using NEN 3775

Particle size:

> 250 micron	max. 30%	2 %	FC-method Laser diffraction, Sympatec
> 150 micron	18 - 64 %	44 %	FC-method Laser diffraction, Sympatec
> 75 micron	65 - 90 %	77 %	FC-method Laser diffraction, Sympatec

Microbiological:

Aerobic mesophilic count	max. 500 cfu/g	4 / g	FC-method equivalent to ISO 4833
Enterobacteriaceae	absent in 10 x 10 g	absent	FC-method, BWP 18h 37°C, SD, VRBG 18-24h 37°C
E. coli	absent in 10 g	absent	FC-method, LMX 25h, Col: ID 24h
Yeasts	max. 10 cfu / g	< 1 / g	FC-method equivalent to ISO 6611
Moulds	max. 10 cfu / g	< 1 / g	FC-method equivalent to ISO 6611
Presumtive Bacillus cereus	max. 50 cfu/g	< 10 /g	FC-method equivalent to ISO 7932
Staphylococcus aureus	absent in 5 x 1 g	absent	FC-method, G&C 42h 37°C, PCR
Sulphite reducing clostridia spores	max. 10 cfu/g	< 1 / g	FC-method, using IDFM 27 (1995) 185-200 Weenk
Salmonella	absent in 60 x 25 g	absent	FC-method equivalent to ISO 6579
Cronobacter spp.	absent in 30 x 10 g	absent	FC-method equivalent to ISO/TS 22964

Borculo, 08-09-2016

(b) (6)

Manager QC

FrieslandCampina Domo

Stationsplein 4
3818 LE Amersfoort
The Netherlands

P.O. Box 1551
3800 BN Amersfoort
The Netherlands

t +31 (0)33 7133 333
f +31 (0)33 7133 334

www.frieslandcampina.com
www.domo.nl

TO WHOM IT MAY CONCERN

We, FrieslandCampina Domo, herewith declare that our Lactopure product range is suitable for human consumption.

On behalf of FrieslandCampina Domo,

(b) (6)

FrieslandCampina Domo

Hendri de Geest
QA Sales Officer
Amersfoort, 19 January 2017

This document is valid until one year after publication date.

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FrieslandCampina Domo

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3818 LE Amersfoort
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P.O. Box 1551
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The Netherlands

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www.frieslandcampina.com
www.domo.nl

TO WHOM IT MAY CONCERN

We, FrieslandCampina Domo, herewith declare that our Lactopure is manufactured, packaged and labelled according to the relevant EU regulations for food and food ingredients, and/or FAO/ WHO Codex Alimentarius, when relevant. For Lactopure the Codex Alimentarius tenth edition applies

On behalf of FrieslandCampina Domo,

(b) (6)

FrieslandCampina Domo

Hendri de Geest
QA Sales Officer
Amersfoort, 01 February 2017

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Appendix 2.3 Phosphoric Acid

Certificate of Analysis

M1OC607Rev 02

ortho-PHOSPHORIC ACID 35 %
(PHOSPHORIC ACID)
COD. 02480 C

BATCH Nr	GRADE	RE-TEST DATE
11777/16	FCG	OCT - 2019

TEST	RESULTS		SPECS	
IDENTIFICATION	Positive		Positive	
APPEARANCE	Complies		Limpid Colourless Liquid	
ASSAY	35.1	%	30.0 - 40.0	%
ARSENIC	< 3	ppm	3	ppm max
CADMIUM	< 3	ppm	3	ppm max
FLUORIDE	< 10	ppm	10	ppm max
LEAD	< 3	ppm	3	ppm max

PACKING
1000 Kg

MANUFACTURING DATE
OCT - 2016

REMARKS:

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APPROVED by
Massimo Napolitano
Quality Manager
Clean Consult International S.p.A

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Azienda con Sistema di Gestione Ambiente UNI ISO 14001:2004
Azienda con Sistema GMP per Ingredienti del Settore Cosmetico EFfCI 2012
Sede legale: via Toscana, 14/B - 20855 - Lodi Vecchio (LO)
Sede produzione e stabilimento: via Padula, 64/66 - 80030 - Castello di Cisterna (Na)

Appendix 2.4 Ammonium Hydroxide

Certificate of Analysis

Certificato di Analisi

Ammonium Solution

Prodotto SOLUZIONE AMMONIACALE

Data Analisi 06.05.2016

Targa mezzo AB 18509

Analista RATINI

Parametro analizzato	Valore	Unità di Misura
TITOLO IN AMMONIACA Titer Ammonia (NH_4)	30,68	% PESO % Weight
IDENTIFICAZIONE Identification	POSITIVA	
RESIDUO NON VOLATILE non-volatile residue	6	PPM
METALLI PESANTI Heavy Metals	<5	PPM
SOSTANZE OSSIDABILI Oxidisable Substances	COMPLIES	
APPARENZA Appearance	COMPLIES	

30,68 % NH_4 solution is equivalent to 28,49 % NH_3 solution

YARA ITALIA S.p.A.

Pagina 1 di 1

Yara Italia S p.A.

Sede Legale, Uffici Amministrativi e Direzione Commerciale
Via B. Crespi, 57 – 20159 MILANO
Telefono: 02 75415287 – Telefax: 02 75416228

Registro Imprese Milano e C.F.: 01974300921
P. IVA: 11843280154 - C.C.I.A.A. n. 1383867
Cap. Soc. Euro 130.000.000,00 i.v.

Appendix 2.5 di-Ammonium Hydrogen Phosphate

Certificate of Analysis
M1QC007Rev.02

di-AMMONIUM HYDROGEN PHOSPHATE
COD. 06650 C

	BATCH Nr	GRADE		EXP.DATE
	Typical	FCG		After 5 years

TEST	RESULTS	SPECS		
IDENTIFICATION		Positive		PACKING
APPEARANCE		Whyte crystals		
APPEARANCE of SOLUTION		Clear and Colourless		
ASSAY		96.0 - 102.0	%	
pH (sol. 1%)		7.6 - 8.2		
ARSENIC (As)		3	ppm max	
FLUORIDE (F)		10	ppm max	MANUFACTURING
LEAD (Pb)		4	ppm max	DATE

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Sede produttiva e stabilimento: via Padula, 64/66 - 80030 - Castello di Cisterna (Na)

Appendix 2.6 Monopotassium Phosphate

Certificate of Analysis

M1QC007Rev.02

POTASSIUM PHOSPHATE MONOBASIC
COD. 20710 C

BATCH Nr	GRADE	EXP.DATE
11922/16	PHARMA	NOV - 2021

TEST	RESULTS		SPECS	
IDENTIFICATION	Positive		Positive	
APPEARANCE	Complies		White or almost white, crystalline powder or colourless crystals	
SOLUBILITY	Complies		Freely soluble in water, praticaly insoluble in ethanol 96%	
APPEARANCE of SOLUTION	Complies		Clear and colouness	
ASSAY (dried substance)	100.3	%	98.0 - 100.5	%
pH (sol. 5%) 20°C	4.3		4.2 - 4.5	
LOSS on DRYING	< 1.0	%	1.0	% max
REDUCING SUBSTANCES	passes test		to pass test	
INSOLUBLE SUBSTANCES	< 2000	ppm	2000	ppm max
CHLORIDES (Cl)	< 200	ppm	200	ppm max
SULPHATES (SO_4)	< 300	ppm	300	ppm max
ARSENIC (As)	< 2	ppm	2	ppm max
LEAD (Pb)	< 5	ppm	5	ppm max
HEAVY METALS (Pb)	< 10	ppm	10	ppm max
IRON (Fe)	< 10	ppm	10	ppm max
SODIUM (Na)	< 0.1	ppm	0.1	% max
LIMIT of FLUORIDE	< 10	ppm	10	ppm max

PACKING
25 Kg

MANUFACTURING DATE
NOV - 2016

REMARKS:

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Quality Manager
Crean Consult International S.p.A.

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Azienda con Sistema di Gestione Ambiente UNI ISO 14001:2004
Azienda con Sistema GMP per Ingredienti del Settore Cosmetico EFfCI 2013
Sede legale: via Toscana, 14/B - 20852 - Lodi Vecchio (LO)
Sede produttiva e stabilimento: via Padula, 64/66 - 80030 - Castello di Cisterna (NA)

Appendix 2.7 L-Tryptophan

无锡晶海氨基酸股份有限公司
WUXI JINGHAI AMINO ACID CO.,LTD
214199 GANGXIA WUXI CHINA TEL:86-510-88760012/88761785 FAX 86-510-88760012

成品检验报告单
CERTIFICATE OF ANALYSIS

品名 PRODUCT NAME: L-色氨酸 L-TRYPTOPHAN
生产日期 PRODUCTION DATE: 2016-09-14
出厂批号 BATCH NO: 1160903
报告日期 REPORT DATE: 2016-09-22
执行标准 STANDARD: FCC8
有效日期 EXPIRY DATE: 2017-09-13
数量 QUANTITY: 75KGS
CAS NO.: 73-22-3
储存条件 STORAGE CONDITION: COOL AND DRY PLACE AWAY FROM DIRECT SUNLIGHT (≤20°)

检验项目 Tests	标准规定 Limits	检测结果 Test results
性状 Description	White crystals or crystalline powder	conforms
鉴别(红外) Identification (IR)	concordant with the reference spectrum	conforms
含量 Assay	98.5~101.5%	99.0%
比旋度 Specific rotation[α]D [α]D	-30.0°~-33.0° -29.7°~-32.7°	-31.0° -31.3°
铅 Lead(Pb)	≤3ppm	<3ppm
干燥失重 Loss on drying	≤0.30%	0.15%
炽灼残渣 Residue on Ignition	≤0.10%	0.01%

结 论: 本品检验符合FCC8 指标规定。
Conclusion: Passed test according to the Standard of FCC8.

化验员(Inspector): 复核人(Checker): 质检主任(QC Manager): 质量受权人(Qualified Person):

(b) (6)

REC-009-FCC8-A0

Appendix 2.8 Magnesium Sulfate Heptahydrate

Certificate of Analysis

M1QC00/Rev.02

MAGNESIUM SULFATE heptahydrate

COD. 18400 C

BATCH Nr	GRADE	EXP.DATE
11919/16	PHARMA	NOV - 2021

TEST	RESULTS		SPECS	
IDENTIFICATION	Positive		Positive	
APPEARANCE	Complies		White or almost white, crystalline powder or brilliant, colourless crystals	
SOLUBILITY	Complies		Freely soluble in water, very soluble in boiling water, praticaly insoluble in ethanol (96%)	
APPEARANCE of SOLUTION	Complies		Clear and Colourless	
ASSAY (as $MgSO_4$ after ignition)	99.9		99.0 - 100.5	%
LOSS on IGNITION	50.7		48.0 - 52.0	%
LOSS on DRYING	< 2	%	2	% max
pH (sol. 5 %)	6.2		5.0 - 9.2	
ACIDITY or ALCALINITY	passes test		to pass test	
CHLORIDES (Cl)	< 140	ppm	140	ppm max
IRON (Fe)	< 20	ppm	20	ppm max
HEAVY METALS (Pb)	< 10	ppm	10	ppm max
ARSENIC (As)	< 2	ppm	2	ppm max
SELENIUM (Se)	< 30	ppm	30	ppm max

PACKING
25 kg

MANUFACTURING DATE
NOV 2016

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Sede legale: via Toscana, [illegible] · 26866 · Lodi Vecchio (LO)
Sede produttiva e stabilimento: via Padula [illegible] · 80130 · Castello di Cisterna (NA)

Appendix 2.9 Citric Acid

Product Specification

Jungbunzlauer

Citric Acid Monohydrate

Food & Pharmaceutical Grade (conforms to Ph. Eur. / USP / FCC / EC)

Product name	Citric acid monohydrate	$C_6H_8O_7 \cdot H_2O$
EC No.	201-069-1	
CAS No.	5949-29-1	
E-No.	E 330	
Characteristics	Colourless crystals or a white, crystalline powder; effloresces in dry air, very soluble in water, freely soluble in ethanol (96%), sparingly soluble in ether	

Odour	typical, practically odourless
Identification	conforms
Appearance of solution	clear and colourless
Clarity of solution (USP)	conforms
Colour of solution (USP)	conforms
Readily carbonisable substances (Ph. Eur. / EC / JP)	conforms
Readily carbonisable substances (USP / FCC)	conforms
Oxalic acid / oxalate	< 100 mg/kg
Sulphate	< 100 mg/kg
Heavy metals	< 5 mg/kg
Arsenic	< 1 mg/kg
Lead	< 0.5 mg/kg
Mercury	< 0.5 mg/kg
Calcium	< 30 mg/kg
Iron	< 3 mg/kg
Chloride	< 5 mg/kg
Residue on ignition (USP / FCC)	< 0.05 %
Sulphated ash	< 0.05 %
Water	7.5 - 8.8 %
Assay	99.7 - 100.3 %

ROMANA CHIMICI S.p.A.
PRODOTTO [illegible]
CODICE [illegible]
APPROVAZIONE R.D. (b) (6)

We herewith confirm that this product meets the requirements of the latest edition of the European Pharmacopoeia (Ph. Eur.), the United States Pharmacopeia (USP), the Food Chemical Codex (FCC) and of Commission Directive 2008/84/EC. All analytical methods are in accordance with the latest requirements of the Ph. Eur., the USP, the FCC or equivalent methods. Test methods are available on request.

Version 06.09, supersedes 02.09 1/1 CAM_S01_EN

Appendix 2.10 Iron (II) Sulfate Heptahydrate

Certificate of Analysis

M1QC007Rev.01

FERROUS SULFATE HEPTAHYDRATE
COD. 13120 C

BATCH Nr	GRADE	RETEST DATE
12295/14	PHARMA	DEC - 2016

TEST	RESULTS		SPECS		
IDENTIFICATION	Positive		Positive		**PACKING** 25 Kg
APPEARANCE	Complies		Light green, crystalline powder or bluish-green crystals, efflorescent in air		
SOLUBILITY	Complies		Free soluble in water, very soluble in boiling water, pratically insoluble in ethanol 96%		
ASSAY (as $FeSO_4 \cdot 7H_2O$)	90.5	%	99.5 - 104.5	%	
ARSENIC	< 3	ppm	3	ppm max	
LEAD	< 10	ppm	10	ppm max	**MANUFACTURING DATE** DEC - 2014
MERCURY	< 3	ppm	3	ppm max	

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Quality Manager
Clean Consult International S.p.A.

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Azienda con Sistema di Gestione Ambiente UNI ISO 14001:2004
Sede legale: via Toscana, 14/B - 26855 - Lodi Vecchio (LO)
Sede produttiva e stabilimento: via Pezzulo, 64/66 - 80030 - Castello di Cisterna (Na)

Appendix 2.11 Manganese Chloride Tetrahydrate

SIGMA-ALDRICH

sigma-aldrich.com

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

Product Specification

Product Name:
Manganese(II) chloride tetrahydrate - meets USP testing specifications

Product Number:	**M8054**	$MnCl_2 \cdot 4H_2O$
CAS Number:	13446-34-9	
MDL:	MFCD00149792	
Formula:	Cl2Mn · 4H2O	
Formula Weight:	197.91 g/mol	

TEST	Specification
Identification	Pass
pH	3.5 - 6.0
Loss on Drying	36.0 - 38.5 %
Insoluble matter	≤ 0.005 %
Sulfate	≤ 0.005 %
Substances not ppt. by ammonium sulfide (as sulfate)	≤ 0.2 %
Iron (Fe)	≤ 5 ppm
Zinc	Pass
Heavy Metal	≤ 5 ppm
Assay Dry Basis	98.0 - 101.0 %
Residual Solvents USP 467	Meets Requirements
Recommended Retest Period 2 Years	----------------

Specification: PRD.1.ZQ6.10000030489

Sigma-Aldrich warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

1 of 1

Appendix 2.12 Cobalt (II) Chloride Hexahydrate

Specification

1.02539.0100 Cobalt(II) chloride hexahydrate for analysis EMSURE® ACS,Reag. Ph Eur

	Specification	
Assay (complexometric)	99.0 - 102.0	%
Insoluble matter	≤ 0.010	%
Nitrate (NO_3)	≤ 0.01	%
Sulphate (SO_4)	≤ 0.005	%
Ca (Calcium)	≤ 0.005	%
Cu (Copper)	≤ 0.0005	%
Fe (Iron)	≤ 0.001	%
K (Potassium)	≤ 0.005	%
Mg (Magnesium)	≤ 0.002	%
Mn (Manganese)	≤ 0.001	%
Na (Sodium)	≤ 0.01	%
Ni (Nickel)	≤ 0.005	%
Pb (Lead)	≤ 0.0005	%
Zn (Zinc)	≤ 0.002	%

Dr. Andreas Lang
Responsible laboratory manager quality control

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Merck KGaA, Frankfurter Straße 250, 64293 Darmstadt (Germany): +49 6151 72-0
EMD Millipore Corporation - a subsidiary of Merck KGaA, Darmstadt, Germany
290 Concord Road, Billerica, MA 01821, USA, Phone: (978) 715-4321
SALSA **Version 494427** /000000000000// Date: 12.10.2016

Appendix 2.13 Cupric Sulfate Pentahydrate

Certificate of Analysis

M1QC007Rev.02

CUPRIC SULFATE pentahydrate
COD. 21330 C

	BATCH Nr	**GRADE**		**EXP.DATE**
	TYPICAL	**FCG**		**After 5 years**
TEST	**RESULTS**	**SPECS**		
IDENTIFICATION		Positive		**PACKING**
APPEARANCE		Blue crystals		
ASSAY		98.0 - 102.0	%	
IRON (Fe)		100	ppm max	
LEAD (Pb)		4	ppm max	
SUBSTANCES NOT PRECIPITATED by HYDROGEN SULFIDE		0.3	% max	**MANUFACTURING DATE**

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Sede legale: via Toscana, 14/B - 26855 - Lodi Vecchio (LO)
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Appendix 2.14 Boric Acid

Certificate of Analysis
M1QC007Rev.02

BORIC ACID
COD. 00580 C

BATCH Nr	GRADE	EXP.DATE
10100/16	PHARMA	JAN - 2021

TEST	RESULTS		SPECS		
IDENTIFICATION	Positive		Positive		**PACKING**
APPEARANCE	Complies		White, or almost white crystalline powder		**1000 g**
SOLUBILITY in ALCOHOL	passes test		to pass test		
COMPLETENESS of SOLUTION	passes test		to pass test		
ASSAY	100.0	%	99.5 - 100.5	%	
LOSS on DRYING	< 5000	ppm	5000	ppm max	**MANUFACTURING DATE**
HEAVY METALS (Pb)	< 20	ppm	20	ppm max	**JAN - 2016**

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Sede legale: via Toscana, 14/B - 26856 - Lodi Vecchio (LO)
Sede produttiva e stabilimento: via Padula, 64/56 - 80030 - Castello di Cisterna (Na)

Appendix 2.15 Zinc Sulfate Heptahydrate

Certificate of Analysis

M1QC007Rev.02

ZINC SULFATE HEPTAHYDRATE
COD. 23760 C

	BATCH Nr	GRADE		EXP.DATE
	Typical	FCG		After 5 years
TEST	**RESULTS**	**SPECS**		
IDENTIFICATION		Positive		**PACKING**
CHARACTERISTICS		White Crystals		
APPEARANCE of SOLUTION		Clear and Colourless		
ASSAY		99.0 - 108.7	%	
ACIDITY		to pass test		
ALKALIES and ALKALINE EARTHS		5000	ppm max	**MANUFACTURING DATE**
CADMIUM (Cd)		2	ppm max	
LEAD (Pb)		4	ppm max	
MERCURY (Hg)		5	ppm max	
SELENIUM (Se)		30	ppm max	

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Appendix 2.16 Sodium Hydroxide

Certificate of Analysis

M1QC007Rev.02

SODIUM HYDROXIDE 25%
Cod. 22111

	BATCH Nr		GRADE		RE-TEST DATE
	11805/16		FCG / DSM		OCT - 2019
TEST	**RESULTS**		**SPECS**		
IDENTIFICATION	Positive		Positive		**PACKING**
APPEARANCE	Complies		Limpid Colourless Liquid		1200 Kg
ASSAY (NaOH)	24.8	%	24.5 - 25.5	%	
CARBONATES (Na_2CO_3)	< 2.0	%	2.0	% max	
MERCURY (Hg)	< 0.1	ppm	0.1	ppm max	

MANUFACTURING DATE
OCT - 2016

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Sede legale: via Toscana 14/B - 20851 - Lodi Vecchio (LO)
Sede produttiva e stabilimento: via Padule 64/66 - 82030 - Castello di Cisterna (Na)

Appendix 2.17 Sodium Molybdate Dihydrate

Certificate of Analysis

M1QC007Rev.01

SODIUM MOLYBDATE dihydrate

COD 2216[illegible]

	BATCH Nr		GRADE		RE-TEST DATE
	10103/16		PHARMA		JAN - 2021
TEST	**RESULTS**		**SPECS**		
IDENTIFICATION	Positive		Positive		**PACKING**
APPEARANCE	Complies		White or almost white powder		1000 g
SOLUBILITY	Complies		Freely soluble in water		
APPEARANCE SOLUTION	Complies		Clear and colourless		
ASSAY	99.7	%	98.0 - 100.5	%	
CHLORIDES (Cl)	< 50	ppm	50	ppm max	**MANUFACTURING**
PHOSPHATES (PO_4)	< 200	ppm	200	ppm max	DATE
AMMONIUM (NH_4)	< 10	ppm	10	ppm max	JAN - 2016
HEAVY METALS (Pb)	< 10	ppm	10	ppm max	
LOSS on DRYING	14.5	%	14.0 - 16.0	%	

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Azienda con Sistema di Gestione Ambientale UNI ISO 14001:[illegible]
[illegible]
[illegible]

Appendix 2.18 Activated Carbon

Cabot Norit Nederland B.V.

Astronaut 34
3824 MJ Amersfoort
P.O.Box 105
3800 AC Amersfoort, THE NETHERLANDS

+31 33 464 8911

NORIT ACTIVATED CARBON CERTIFICATE OF ANALYSIS

Sales Order Number	021394048536SO / 1
Customer P.O.Number	4501721755
Manufacturing Plant	Klazienaveen
Grade	**NORIT PN 2**
Customer Grade	
Quantity Shipped	2,880.00 KG
Carrier Name	P&O FERRYMASTERS ROZE
Vehicle ID	BT-SZ-69
Lot Number	3923896
Shipping Date	12 Feb 2016
Pack Date	10Nov2015

NORBERT DENTRESSANGLE
Harpenkskamp 5
BORCULO 7271 AR
Attn : FRIESLAND CAMPINA

PHYSICAL AND CHEMICAL PROPERTIES

Property Description	Unit	Ref	Specification Min	Individual Value	Specification Max
Moisture (as packed)	mass-%	NSTM 3.08		2	10
Ash content	mass-%	NSTM 3.02		10	
Molasses number (EUR)		NSTM 2.19		350	390
pH		NSTM 3.09	6.0	6.5	7.2

021394000919 22 Dec 15   021394000918 11 Nov 15   021393954477 15 Sep 15   021393954476 4 Aug 15

The data above was obtained from tests on samples taken during the time of production and/or packaging of this product using Norit Standard Test Method (NSTM). We do not guarantee the same results will be obtained by others in other laboratories and we disclaim liability resulting from the use of the contents of this report.

Date of Manufacture:
This has been replaced with a "Pack Date" which represents the "Date of Manufacture".

PalletNo/Container ID

Seal No:

Signature: Mr. F. de Graaf Lab Manager

NORIT ACTIVATED CARBON

25 May 2011

Food & Beverage / PN2

NORIT® PN 2

Powdered Activated Carbon

WHY CABOT

Cabot Norit Activated Carbon is a premier activated carbon manufacturer respected for experienced people, diverse products and strong customer relationships. Cabot's history of innovation, product performance, technical expertise and customer focus ensure that you receive the right products and solutions for your specific purification needs.

Norit PN 2 is suitable for decolourisation and purification of food products at which the use of a carbon with a neutral reaction is required. Norit PN 2 is an established grade for liquid sugar treatment. It is widely used in the soft drink industry where the highest standards regarding final colour, brightness and the sensory (organoleptic) character of sugar syrups must be met.
Norit PN 2 is a neutralized steam activated carbon with a high adsorptive capacity, dedicated for removal of small colour bodies and undesired taste and odour compounds.

Norit PN 2 meets the requirements of the latest version of the U.S. Food Chemicals Codex. It is produced under the scope of a Quality Management System which complies with the requirements of CDX HACCP. The corresponding Certificate of Registration is available upon request.

SPECIFICATIONS

Molasses number (EUR)	max. 390	-
pH	min. 6.0	-
pH	max. 7.2	-
Moisture (as packed)	max. 10	mass-%

GENERAL CHARACTERISTICS

Iodine number	850	-
Molasses number (EUR)	350	-
Methylene blue adsorption	15	g/100 g
Total surface area (B.E.T.)	950	m^2/g
Apparent density (tamped)	470	kg/m^3
Particle size D_{10}	3	µm
Particle size D_{50}	20	µm
Particle size D_{90}	140	µm
Ash content	12	mass-%
Moisture (as packed)	3	mass-%
Filtration time	25	min

Appendix 3 Analytical Methodology for Purified 2'-Fucosyllactose (2'-FL) Analysis

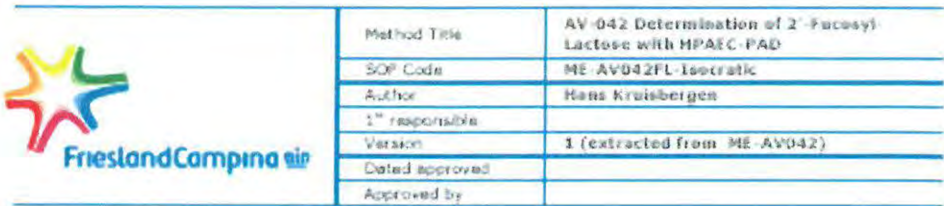
Appendix 3.1 **Determination of 2'-FL by Isocratic HPAEC-PAD**

Appendix 3.2 **Validation Report for Determination of 2'-FL by Isocratic HPAEC-PAD**

Appendix 3.3 **Bradford Protein Determination in 2'-FL**

Appendix 3.4 **Validation Report for Bradford Protein Determination in 2'-FL**

Appendix 3.1 Determination of 2'-FL by Isocratic HPAEC-PAD



Method Title	AV-042 Determination of 2'-Fucosyl-Lactose with HPAEC-PAD
SOP Code	ME-AV042FL-Isocratic
Author	Hans Kruisbergen
1st responsible	
Version	1 (extracted from ME-AV042)
Dated approved	
Approved by	

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1. Safety

HPLC equipment is expensive and delicate. Use of this equipment is allowed for trained technicians who have permission to use the equipment. Before using any equipment familiarize yourself with the standard area operating procedure and check whiteboard for any messages concerning the area.

1.1 Hazards/Risks

Use of concentrated Sodium Hydroxide.

1.2 Personal safety

Use of labcoat and safety glasses are mandatory for the area.

Use gloves by preparing solvents of Sodium Hydroxide from concentrated stocks.

1.3 Waste

Vials can be disposed in special vial containers located in lab A3.48. The waste stream consisting of low concentrations of Sodium Hydroxide, lead to a special drain close to the equipment and is no risk to the environment.

2. Scope/background

This method is used to measure the 2'-Fucosyl Lactose content in 2'-Fucosyl Lactose related products and is especially for users who want to know the 2'-Fucosyl Lactose content more accurate. This method has a relative error of 5%.

3. Objective

2'-Fucosyl Lactose a Human Milk Oligosaccharide (HMO) and is known for its prebiotic effect. 2'-Fucosyl Lactose is an ingredient that can be added to different food applications.
Peptides and proteins will disturb the detector and have to be removed before analyzing on HPLC.

4. Version information

First version.

5. Materials

Unless stated otherwise chemicals need to be of HPLC- or analytical quality and highly purified water for HPLC (Milli-Q or equivalent).

- 50% Sodium hydroxide (carbonate free) — Baker 3727
- 2'-Fucosyl Lactose — FrieslandCampina standard
- Helium
- Micro filters: 0.2 µm, GHP Acrodisc 13 (Pall) or equal
- HPLC vials 2 mL with split septum suitable for the ICS used
- Carbopac PA1 column (4*250 mm) (Dionex p/n. 35391)
- Carbopac PA1 guard column (4*50 mm) (Dionex p/n. 43096)

6. Equipment

- Analytical Balance, accuracy 0.1 mg
- Balance, accuracy 0.01 gram
- Vortex mixer
- Centrifuge
- Dilutor
- Brix meter
- Thermo Scientific HPAEC equipment ICS-5000 or equal, equipped with:
  - Pump (ICS-5000+ DP)
  - Thermostatic autosampler (ICS-5000 AS-AP)
  - Detector / Chromatography module (ICS-5000+ DC)
    - ED detector PAD, with standard carbohydrate waveform for Ag/AgCl ref-electrode
    - Au electrode for carbohydrates

7. Reagents

Eluens A (200 mM NaOH carbonate free)
Weigh 984.0 g Milli-Q (degas the Milli-Q when used for DX-600) into a 2 L erlenmeyer add 16.0 g 50% NaOH. Stir a short time on a magnetic stirrer and pore the liquid quickly in the bottle in the Solvent Organizer and put under helium.

Eluens B
Not applicable.

Eluens C
Not applicable.

Eluens D (Water)
Fill a solvent bottle with Milli-Q (degas the Milli-Q when used for DX-600) and place it in the Solvent Organizer and put under helium.

8. Procedure

8.1 Sample preparation

- Dissolve samples in water and dilute the samples with Milli-Q to concentration 1-10 ppm of the amount of 2'-Fucosyl Lactose that has to be analyzed.

8.3 Calibration/Standards

- Prepare a calibration curve of 2'-Fucosyl Lactose with a concentration of 1.25 - 2.5 - 5 and 10 ppm.
- Weigh accurate 100-120 mg 2'-Fucosyl Lactose into a graduated flask of 100 ml. Dissolve and fill up till 100 ml with Milli-Q.
- Dilute the right amount with a diluter into a 100 ml graduated flask to get the following concentrations in Milli-Q:

ppm	Standard 1	Standard 2	Standard 3	Standard 4
2'-Fucosyl Lactose	1.25	2.50	5.00	10.00

8.4 Operating procedure

HPLC conditions:

Analytical column : CarboPac PA1: 250 x 4 mm anion-exchange (Dionex)
Pre-column : CarboPac PA1: 50 x 4 mm (Dionex)
Max.column pressure : 4000 PSI
Sample tray temp. : 10°C
Column temp. : 30°C,
Flow : 1.0 ml/minute
Injection volume : 30 µL (with a 25 µl injection loop)
Detector : PAD with an AU-electrode, Data Collection Rate=2 Hz.

Gradient conditions:

Tijd (min)	Eluens A (%)	Eluens B (%)	Eluens C (%)	Eluens D (%)
0	75			25
15	75			25

Typical chromatogram:Typical retentiontime 2'-Fucosyl Lactose approximately 8.4 min.

Other components:

Compound	Relative Retention Time (RRT)
Fucose	0.33
Galactose + Glucose	0.52
Fructose	0.61
3-Fucosyl Lactose	0.64
di-Fucosyl Lactose	0.77
allo-Lactose	0.87
Lactose	0.90
2'-Fucosyl Lactose	1.00

8.5 Data analysis

Calculate the concentration of 2'-Fucosyl Lactose in the samples based on the calibration curves. Check if the areas of the carbohydrates are within the limits of the calibration curve. Correct for the dilution of the sample to obtain the concentration of the carbohydrate (ppm).

8.6 Trouble shooting

See manuals or contact supplier

9. Relating documents and literature

Related SOP's:
ME-AV042, ME-AV044, ME-AV045 and ME-AV049.

Related MSDS:
50% NaOH.

Related AST numbers
This method can be used on the following equipment:
AST 07894 : DX600
AST 04458 : ICS3000
AST 04457 : ICS5000

Appendix 3.2 Validation Report for Determination of 2'-FL by Isocratic HPAEC-PAD

Validationreport: **Determination of 2-fucosyl-lactose purity**

Date: **27-1-2017** **version: 1**

Veghel

H. Dahlmans (b) (6)

model 1.2 **Accuray based on reference sample with 100% purity.**

The reference sample was used as a 100% pure 2-fucosyl-lactose standard.

Determine the difference with student T-test.

hypothesis: The value μ must be equal to "0".

Date	Sample ID	product	purity %		difference
			NutriControl	Reference	NC/reference
5-12-2016	FMRS01-1	2-fucosyl-lactose	100,7	100,0	0,7
5-12-2016	FMRS01-2	2-fucosyl-lactose	100,9	100,0	0,9
5-12-2016	FMRS01-3	2-fucosyl-lactose	100,8	100,0	0,8
5-12-2016	FMRS01-4	2-fucosyl-lactose	100,8	100,0	0,8
5-12-2016	FMRS01-5	2-fucosyl-lactose	100,5	100,0	0,5
5-12-2016	FMRS01-6	2-fucosyl-lactose	100,1	100,0	0,1
5-12-2016	FMRS01-7	2-fucosyl-lactose	99,8	100,0	-0,2
5-12-2016	FMRS01-8	2-fucosyl-lactose	100,4	100,0	0,4
5-12-2016	FMRS01-9	2-fucosyl-lactose	99,7	100,0	-0,3
5-12-2016	FMRS01-10	2-fucosyl-lactose	99,9	100,0	-0,1
7-12-2016	FMRS01-11	2-fucosyl-lactose	99,7	100,0	-0,3
7-12-2016	FMRS01-12	2-fucosyl-lactose	100,1	100,0	0,1
15-12-2016	FMRS01-13	2-fucosyl-lactose	100,5	100,0	0,5
15-12-2016	FMRS01-14	2-fucosyl-lactose	100,1	100,0	0,1
15-12-2016	FMRS01-15	2-fucosyl-lactose	99,3	100,0	-0,8

betrouwbaarheid	95%
number (n)	15
average diff.	0,19
stdev(n-1)	0,47
stdev(n-1) / sq root(n) =	0,122

confidence interval: -0,07 =< μ <= 0,45

Conclusion: There is no significant bias

Validationreport: **Quality manual:**

Method: **Determination of 2-fucosyl-lactose purity**

Date: **27-1-2017** **version: 1**

author(s): (Senior) Analist
authoriser: Product developper

analysis method: high pressure liquid chromatography with pulsed amperometric detection (HPLC-PAD).

To determine:
- accuracy
- repeatability
- within-laboratory reproducibility
- measurement uncertainty
- selectivity & specificity

results
Accuracy
There is no analytical 2-fucosyl-lactose standard available. The reference sample supplied by the customer was used as a 100% pure analytical standard.

Repeatability
The relative standard deviation under repeatability (RSDr) conditions is 0,4%.

Reproducibility
The relative standard deviation under within-lab reproducibility (RSD_R) conditions is 0,4%.

Relative measurement uncertainty (expanded)
The relative measurement uncertainty is 1%

Selectivity & Specificity
Selectivity:
The selectivity of the method is based on chromatographic separation of components in time on an anion-exchange column.

Specificity:
The specificity of the method is based on the use of the pulsed amperometric detector which reacts only to
For example, hydroxyl groups in carbohydrates are oxidized on a working electrode surface and the resulting current is measured oxidizable components.

Conclusion:
The HPLC-PAD method for the determination of the purity of 2-fucosyl-lactose is fit for purpose.

model 1.2 **Accuray based on reference sample with 100% purity.**

The reference sample was used as a 100% pure 2-fucosyl-lactose standard.

Determine the difference with student T-test.

hypothesis: The value μ must be equal to "0".

Date	Sample ID	product	purity %		difference
			NutriControl	Reference	NC-reference
5-12-2016	PMRSD1-1	2-fucosyl-lactose	100,7	100,0	0,7
5-12-2016	PMRSD1-2	2-fucosyl-lactose	100,9	100,0	0,9
5-12-2016	PMRSD1-3	2-fucosyl-lactose	100,5	100,0	0,5
5-12-2016	PMRSD1-4	2-fucosyl-lactose	100,6	100,0	0,6
5-12-2016	PMRSD1-5	2-fucosyl-lactose	100,5	100,0	0,5
5-12-2016	PMRSD1-6	2-fucosyl-lactose	100,1	100,0	0,1
5-12-2016	PMRSD1-7	2-fucosyl-lactose	99,8	100,0	-0,2
5-12-2016	PMRSD1-8	2-fucosyl-lactose	100,4	100,0	0,4
5-12-2016	PMRSD1-9	2-fucosyl-lactose	99,7	100,0	-0,3
5-12-2016	PMRSD1-10	2-fucosyl-lactose	99,9	100,0	-0,1
7-12-2016	PMRSD1-11	2-fucosyl-lactose	99,7	100,0	-0,3
7-12-2016	PMRSD1-12	2-fucosyl-lactose	100,1	100,0	0,1
15-12-2016	PMRSD1-13	2-fucosyl-lactose	100,5	100,0	0,5
15-12-2016	PMRSD1-14	2-fucosyl-lactose	100,1	100,0	0,1
15-12-2016	PMRSD1-15	2-fucosyl-lactose	99,3	100,0	-0,6

betrouwbaarheid.	95%
number (n)	15
average diff	0,10
stdev(n-1)	0,47
stdev(n-1) / sq.root(n) =	0,122

confidence interval: -0,07 =< μ =< 0,45

Conclusion: There is no significant bias

model 2.1

Repeatability

matrix pure 2-fucosyl-lactose

limit RSD$_R$ (%) = 1,3 estimated limit

argumentation limit: The limit is based on the value of the Horwitz equation multiplied by 2/3.

Repeatability from duplo analysis			analysis 1	analysis 2	average	$\frac{(X_1-X_2)^2}{X_{avg}^2}$
product	sample ID	date	%	%	%	
2-fucosyl-lactose within spec.	FMRS10-1	6-12-2016	[illegible]	[illegible]	[illegible]	0,00002
2-fucosyl-lactose within spec.	FMRS10-2	6-12-2016	[illegible]	[illegible]	[illegible]	0,00004
2-fucosyl-lactose within spec.	FMRS10-3	6-12-2016	[illegible]	[illegible]	99,2	0,00012
2-fucosyl-lactose within spec.	FMRS10-4	6-12-2016	[illegible]	[illegible]	[illegible]	[illegible]
2-fucosyl-lactose within spec.	FMRS10-5	6-12-2016	[illegible]	[illegible]	[illegible]	0,00001
2-fucosyl-lactose OOS	FMRS09-1	7-12-2016	[illegible]	[illegible]	[illegible]	0,00000
2-fucosyl-lactose OOS	FMRS09-2	7-12-2016	[illegible]	[illegible]	[illegible]	0,00001
2-fucosyl-lactose OOS	FMRS09-3	7-12-2016	[illegible]	[illegible]	[illegible]	0,00004
2-fucosyl-lactose OOS	FMRS09-4	7-12-2016	[illegible]	[illegible]	[illegible]	0,00001
2-fucosyl-lactose OOS	FMRS09-5	7-12-2016	[illegible]	[illegible]	[illegible]	0,00000
					Σ	[illegible]

number of duplo's (k) must be ≥ [illegible]

cochran-test	0,474	
cochran-table value (k,n-1)	0,602	
number of duplo's (k)	10	
RSD$_r$	0,4	%
factor = 1 (k)	1,00	

conclusion: the repeatability variation coefficient complies with the limit

The relative measurement uncertainty is 1%.

Re-validation of repeatability from duplo analysis			analysis 1	analysis 2	average	RSD$_r$	complies with limit
product	sample number	date	%	%	%	(%)	

model 2.2

Reproducibility

matrix: pure 2-fucosyl-lactose

limit RSD_R (%) = 2,0 — estimated limit

argumentation limit: The limit is based on the limit for reproducibility calculated by the Horwitz Equation

Reproducibility from duplo analysis			analysis 1	analysis 2	average	$\frac{(X_1-X_2)^2}{X_{avg}^2}$
product	sample ID	analist 1/2	%	%	%	
2-fucosyl-lactose within spec.	PMRS10-1	Nick/Kevin	99,4	98,6	99,0	0,00007
2-fucosyl-lactose within spec.	PMRS10-2	Nick/Kevin	98,8	98,8	98,8	0,00000
2-fucosyl-lactose within spec.	PMRS10-3	Nick/Kevin	99,2	99,4	99,3	0,00000
2-fucosyl-lactose within spec.	PMRS10-4	Nick/Kevin	98,9	98,6	98,8	0,00001
2-fucosyl-lactose within spec.	PMRS10-5	Nick/Kevin	98,5	98,7	98,6	0,00000
2-fucosyl-lactose OOS	PMRS09-1	Nick/Kevin	89,7	89,6	89,7	0,00000
2-fucosyl-lactose OOS	PMRS09-2	Nick/Kevin	90,1	90,1	90,1	0,00000
2-fucosyl-lactose OOS	PMRS09-3	Nick/Kevin	89,6	89,7	89,6	0,00000
2-fucosyl-lactose OOS	PMRS09-4	Nick/Kevin	89,5	89,9	89,7	0,00002
2-fucosyl-lactose OOS	PMRS09-5	Nick/Kevin	89,4	90,5	90,0	0,00015
					Σ	0,00025

number of duplo's (k) must be ≥ 8

cochran-test	0,565
cochran-table value (k,n-1)	0,602
number of duplo's (k)	10
RSD_R	0,4 %
factor = 1 (k)	1,00

conclusion: the reproducibility variation coefficient complies with the limit

Re-validation of reproducibility from duplo analysis			analysis 1	analysis 2	average	RSD_R	complies with limit
product	sample number	date	%	%	%	(%)	

model 2.2 Reproducibility

model 2.3

Measurement Uncertainty (expanded)

The measurement uncertainty is build up from a variable deviation (RSD_R) and a systematic deviation (maximum relative bias compared to the as true assumed value from a reliable interlaboratory test or a proficiency test or a reference material)

The relative measurement uncertainty (M) can be estimated by the following equation (95% confidence);

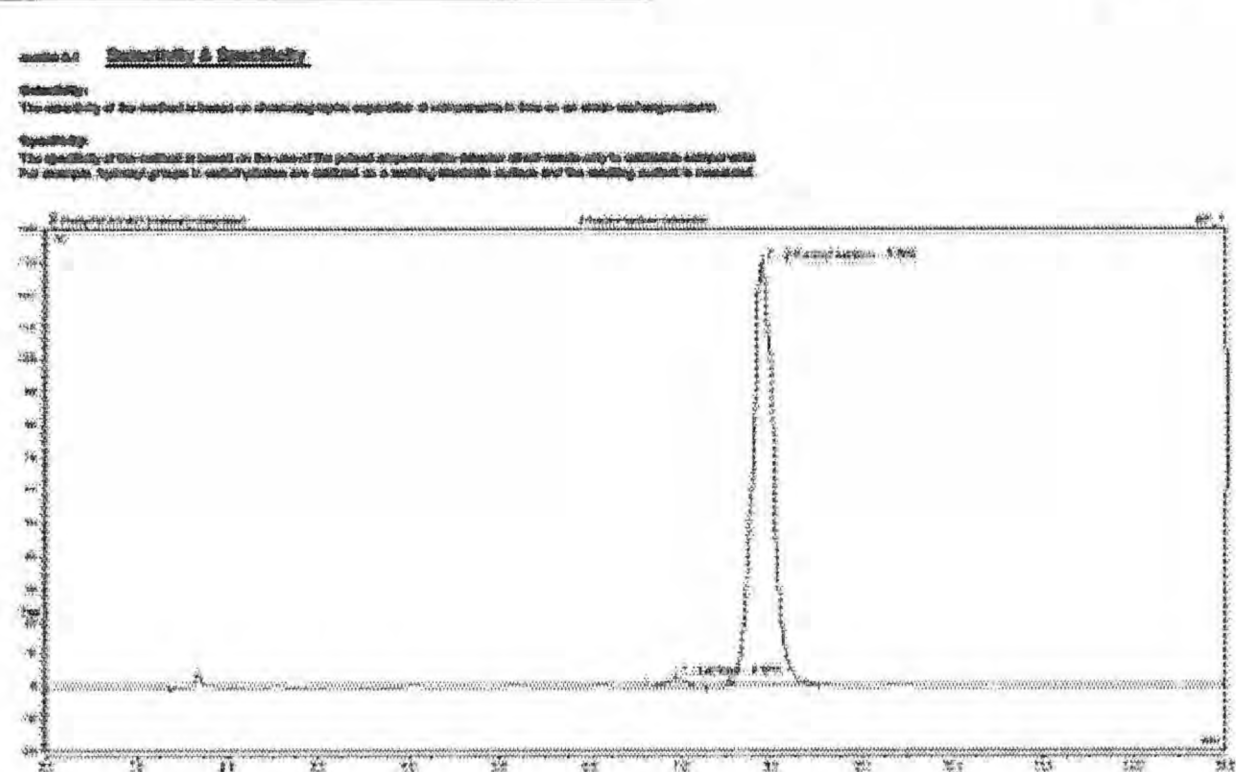
$$M(\%) = 2 \times \sqrt{(RSD_R)^2 + (D)^2}$$

M = relative measurement uncertainty (%)

RSD_R = variation coefficient for reproducibility

D = relative bias (%) compared to supplied reference material (model 1.1)

	matrix 2-fucosyl-lactose	
D (%) =	0.0	%
RSD_R (%) =	0.4	%
M (%) = relative measurement uncertainty =	1	%



Kritiekewaarden voor de Cochran maximumvariantietoets, kritisch niveau 5 %

k \ n-1	1
2	0,999
3	0,967
4	0,906
5	0,841
6	0,781
7	0,727
8	0,680
9	0,638
10	0,602
11	0,570
12	0,541
13	0,515
14	0,492
15	0,471
16	0,452
17	0,434
18	0,418
19	0,403
20	0,389
21	0,377
[illegible]	0,365
[illegible]	0,354
[illegible]	0,343
[illegible]	0,334
[illegible]	0,325
[illegible]	0,316
[illegible]	0,308
[illegible]	0,300
[illegible]	0,293
[illegible]	0,286
[illegible]	0,280
[illegible]	0,273
[illegible]	0,267
[illegible]	0,262
[illegible]	0,256
[illegible]	0,251
[illegible]	0,246
[illegible]	0,242
[illegible]	0,237
[illegible]	0,172
[illegible]	0,100
[illegible]	0

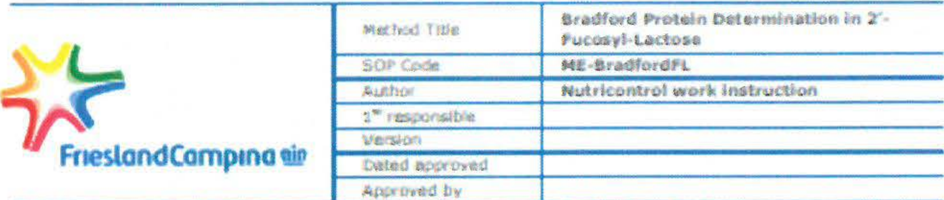
author:	[illegible]
Company:	[illegible]

Kritieke waarde P voor toetsen precisiekenmerken

k	P	Q
	0,08	—
2	0,23	—
3	0,34	—
4	0,42	—
5	0,48	—
6	0,52	—
7	0,56	1,42
8	0,58	1,39
9	0,61	1,37
10	0,63	1,35
11	0,64	1,34
12	0,66	1,32
13	0,67	1,31
14	0,69	1,30
15	0,70	1,29
16	0,71	1,28
17	0,71	1,27
18	0,72	1,27
19	0,73	1,26
20	0,74	1,25
[illegible]	0,76	1,23
[illegible]	0,79	1,21
[illegible]	0,80	1,19
[illegible]	0,81	1,18
[illegible]	0,82	1,17
[illegible]	0,83	1,16
[illegible]	0,85	1,15
[illegible]	0,86	1,14
[illegible]	0,87	1,13
[illegible]	0,88	1,12
[illegible]	0,88	1,12
[illegible]	0,90	1,09
[illegible]	0,92	1,08
[illegible]	0,93	1,07
[illegible]	0,94	1,06
[illegible]	0,95	1,05
[illegible]	0,96	1,04

Tabel uit W.J. Dixon en F. Massey "Introduction to statistical analysis"; Mc Graw-Hill Book Company , Inc. New York 1957-II

Appendix 3.3 Bradford Protein Determination in 2'-FL



FrieslandCampina		
	Method Title	Bradford Protein Determination in 2'-Fucosyl-Lactose
	SOP Code	ME-BradfordFL
	Author	Nutricontrol work instruction
	1st responsible	
	Version	
	Dated approved	
	Approved by	

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7.2 Liquid sample preparation ......Error! Bookmark not defined.
7.3 Calibration/Standards 3
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8. Relating documents and literature 4

1. Safety

1.1 Hazards/Risks

Use of Bradford reagent.

1.2 Personal safety

Use of labcoat and safety glasses are mandatory for the area.

Wear gloves by using Bradford reagent.

1.3 Waste

Bradford reagent can be disposed in containers located in lab A3.48.

2. Scope/background

This method is used to measure the protein content in 2'-Fucosyl Lactose.

3. Objective

2'-Fucosyl Lactose a Human Milk Oligosaccharide (HMO) and is known for its prebiotic effect. 2'-Fucosyl Lactose is an ingredient that can be added to different food applications.
Since 2'-Fucosyl Lactose is produced by bio fermentation the method is used to demonstrate that less than 100 ppm protein is present in the end product.

4. Version information

First version.

5. Materials

- Bradford reagent VWR; E530-1L
- Bovine Serum Albumin Sigma; A9418
- UV-Cuvette semi-micro Brand; 7591 50
- 5 mL Polystyrene Round-Bottom Tube Falcon; 352054
- 0.15 mol/L NaCl – solution: weigh 8.77 gram NaCl in a volumetric flaks of 1000 ml, add demi-water and fill up to 1000 ml and mix.

6. Equipment

- Spectrophotometer Molecular Devices; SpectraMax Plus 384, o.e.

7. Procedure

7.1 Solid sample preparation

- Prepare a sample solution in NaCl-solution by weight (weigh approx. 1 gram , accuracy 0.1 mg) in a tube of 50 ml and fill up to 10 ml and mix.

7.2 Calibration/Standards

- Prepare BSA-standard solution (approx. 400 mg/L): weigh, accuracy 0,1 mg, 0,4 gram BSA in a 100 ml beaker. Dissolve in approx. 30 ml NaCl-solution. Bring the volume quantitatively over in a volumetric flask of 1000 ml. Fill up to 1000 ml and mix.
- Dilute the BSA-standard solution with a diluter 40-20-10-5-2-0 times in 50 ml tubes. Use 0.15 mol/L NaCl-solution as the dilution medium.

7.3 Colour reaction and absorption measurement

- Add with a pipet 5 ml Bradford reagent in a tubes of 12 ml
- Add with a pipet 0.5 ml sample solution and mix with a Vortex – mixer.
- Do the same with the calibration-solution standards and use NaCl-solution as blank.
- Let the reagent react for at least 10 minutes before further handling

7.4 Operating procedure

Spectrophotometer conditions:

- Set the wavelength at 595 nm.
- Fill a semi micro cuvette with water and push the reference button.
- Measure a blank sample, mix gently before transfer to cuvette, measure directly after transfer.
- Measure all the samples and standards, mix gently before transfer to cuvette, measure directly after transfer.

7.5 Data analysis

Subtract the absorption of the blank from all measurements. Calculate the concentration of protein in the samples based on the calibration curve. Check if the responses are within the limits of the calibration curve. Correct for the dilution of the sample to obtain the concentration of the protein (ppm).

7.6 Trouble shooting

See manuals.

8. Relating documents and literature

Bradford, M.M. (1976), "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", Analytical Biochemistry 72 (1-2), 248-254.DOI:10.1016/0003-2697(76)90527-3

Appendix 3.4 Validation Report for Bradford Protein Determination in 2'-FL

Validationreport: **Determination of Protein (Bradford method)**

Date: **27-1-2017** **version: 1**

Veghel

H. Dahlmans (b) (6)

Validationreport:		**Quality manual:**	
Method:	**Determination of Protein (Bradford method)**		NutriControl
Date:	**27-1-2017**	**version: 1**	

author(s): (Senior) Analist
authorisor: Product developper

analysis method: The Bradford method is a colorimetric protein assay, based on an absorbance shift of the dye Coomassie Brilliant Blue G-250

To determine:
- accuracy & calibration
- repeatability
- within-laboratory reproducibility
- measurement uncertainty
- selectivity & specificity

results

Accuracy & calibration

The accuracy of the protein assay was determined by addition of BSA to a 2-fucosyl-lactose sample supplied by the customer.
Bovine Serum Albumine (BSA) was used as the protein source.
A stock solution of BSA was diluted and measured a 595 nm with a spectrofotometer.
The concentration of BSA was plotted against the measured absorption at 595 nm.
A second-order polynomial equation was used to calculate the concentration of protein in the samples with added BSA.
The average recovery of 99,5% was then calculated.

Repeatability (RSDr)

The limit for RSDr is set at 2/3 of the RSDR.
The relative standard deviation under repeatability (RSDr) conditions is 2,5%.

Reproducibility (RSDR)

The limit for RSDR is based on the limit for reproducibility calculated by the Horwitz Equation at a concentration of 0,1%.
The relative standard deviation under within-lab reproducibility (RSDR) conditions is 3,8%.

Relative measurement uncertainty (expanded)

The relative measurement uncertainty is 8%

Selectivity & specificity

The Bradford protein assay is used to measure the concentration of total protein in a sample. The principle of this assay is that the binding of protein molecules to Coomassie dye under acidic conditions results in a color change from brown to blue. This method actually measures the presence of the basic amino acid residues, arginine, lysine and histidine, which contributes to formation of the protein-dye complex.

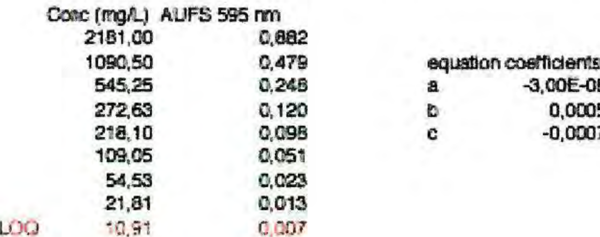
Conclusion:

The Bradford method for the determination of protein in 2-fucosyl-lactose is fit for purpose.
The LOQ of this method is 0,01% (m/m)

Accuracy & calibration

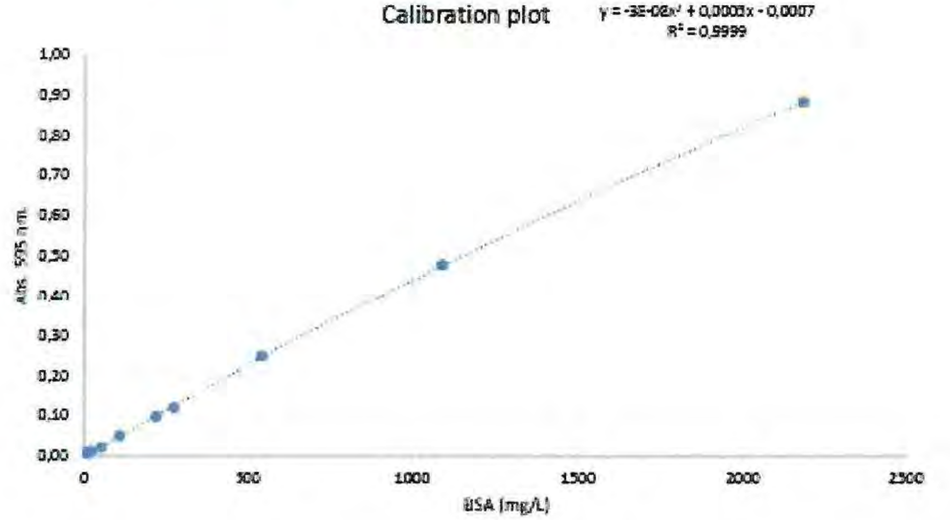
Stock BSA solution **1090,5 mg/100ml** **date** **10-1-2017**

The correlation coefficient (R^2) is higher when a second-order polynomial equation is used.



	Conc (mg/L)	AUFS 595 nm
	2181,00	0,882
	1090,50	0,479
	545,25	0,246
	272,63	0,120
	218,10	0,098
	109,05	0,051
	54,53	0,023
	21,81	0,013
LOQ	10,91	0,007

equation coefficients	
a	-3,00E-08
b	0,0005
c	-0,0007



		Sample weight mg in 10 ml	Goal Seek value calculated X mg BSA/L	measured Y AUFS 595 nm		addition of BSA (ml)	BSA (mg/L)		Recovery		BSA % (m/m)
2-fucosyl-lactose reference	PMRS01	1038	<10	0,003							<0,01
2-fucosyl-lactose reference	PMRS01	986	<10	0,001							<0,01
2-fucosyl-lactose reference	PMRS01	1007	560,3	0,27	0,270	+0,5 ml	545,25	mg/L	102,8	%	0,56
2-fucosyl-lactose reference	PMRS01	1098	1093,9	0,511	0,510	+1,0 ml	1090,5	mg/L	100,3	%	1,00
2-fucosyl-lactose OOS	PMRS09	984	<10	-0,003							<0,01
2-fucosyl-lactose OOS	PMRS09	982	<10	0,001							<0,01
2-fucosyl-lactose OOS	PMRS09	879	543,1	0,262	0,262	+0,5 ml	545,25	mg/L	99,6	%	0,62
2-fucosyl-lactose OOS	PMRS09	636	1022,1	0,479	0,479	+1,0 ml	1090,5	mg/L	93,7	%	1,61
2-fucosyl-lactose within spec.	PMRS10	996	<10	-0,002							<0,01
2-fucosyl-lactose within spec.	PMRS10	1044	<10	0,001							<0,01
2-fucosyl-lactose within spec.	PMRS10	994	558,1	0,269	0,269	+0,5 ml	545,25	mg/L	102,4	%	0,56
2-fucosyl-lactose within spec.	PMRS10	1006	1073,6	0,502	0,502	+1,0 ml	1090,5	mg/L	98,5	%	1,07

average recovery: **99,5 %**

average recovery loss: **0,5 %**

model 2.1

Repeatability

matrix	pure 2-fucosyl-lactose
limit RSD_R *(%) =*	3,8 — estimated limit
argumentation limit:	The limit is based on the value of the Horwitz equation multiplied by 2/3.

Repeatability from duplo analysis			analysis 1a	analysis 1b	average	$\frac{(X_1-X_2)^2}{X_{ave}^2}$
product	sample ID	date	% (m/m)	% (m/m)	% (m/m)	
2-fucosyl-lactose with 1,0 ml BSA	PMRS01	25-1-2017	0,95	0,94	0,95	0,00013
2-fucosyl-lactose with 0,5 ml BSA	PMRS01	25-1-2017	0,48	0,48	0,48	0,00000
2-fucosyl-lactose with 0,2 ml BSA	PMRS01	25-1-2017	0,20	0,19	0,19	0,00290
2-fucosyl-lactose with 0,1 ml BSA	PMRS01	25-1-2017	0,10	0,10	0,10	0,00137
2-fucosyl-lactose with 1,0 ml BSA	PMRS09	25-1-2017	0,98	1,02	1,00	0,00190
2-fucosyl-lactose with 0,5 ml BSA	PMRS09	25-1-2017	0,46	0,48	0,47	0,00309
2-fucosyl-lactose with 0,2 ml BSA	PMRS09	25-1-2017	0,21	0,20	0,21	0,00029
2-fucosyl-lactose with 0,1 ml BSA	PMRS09	25-1-2017	0,11	0,10	0,11	0,00235
2-fucosyl-lactose with 1,0 ml BSA	PMRS10	25-1-2017	1,00	1,03	1,01	0,00104
2-fucosyl-lactose with 0,5 ml BSA	PMRS10	25-1-2017	0,49	0,49	0,49	0,00006
2-fucosyl-lactose with 0,2 ml BSA	PMRS10	25-1-2017	0,21	0,20	0,20	0,00007
2-fucosyl-lactose with 0,1 ml BSA	PMRS10	25-1-2017	0,11	0,11	0,11	0,00219
raw data can be found in sheet "raw data"					Σ	0,01539

number of duplo's (k) must be ≥ 6

cochran-test	0,201
cochran-table value (k;n-1)	0,541
number of duplo's (k)	12
RSD_r	2,5 %
factor = 1 (k)	1,00

conclusion: the repeatability variation coefficient complies with the limit

Re-validation of repeatability from duplo analysis			analysis 1	analysis 2	average	RSD_r	complies with limit
product	sample ID	date	% (m/m)	% (m/m)	% (m/m)	(%)	

model 2.2

Reproducibility

matrix 2-fucosyl-lactose and BSA standard, analysed on 25th and 26th january 2017

limit RSD_R *(%) =* | 5,7 | estimated limit |

argumentation limit: The limit is based on the limit for reproducibility calculated by the Horwitz Equation at a concentration of 0,1%.

Reproducibility from duplo analysis			analysis 1a	analysis 2a	average	$\frac{(X_1-X_2)^2}{X_{ave}^2}$
product	sample ID	date:	% (m/m)	% (m/m)	% (m/m)	
2-fucosyl-lactose with 1,0 ml BSA	PMRS01	25/26-1-2017	0,95	0,92	0,9	0,00078
2-fucosyl-lactose with 0,5 ml BSA	PMRS01	25/26-1-2017	0,48	0,49	0,5	0,00077
2-fucosyl-lactose with 0,2 ml BSA	PMRS01	25/26-1-2017	0,20	0,19	0,2	0,00070
2-fucosyl-lactose with 0,1 ml BSA	PMRS01	25/26-1-2017	0,10	0,11	0,1	0,01271
2-fucosyl-lactose with 1,0 ml BSA	PMRS09	25/26-1-2017	0,98	0,97	1,0	0,00012
2-fucosyl-lactose with 0,5 ml BSA	PMRS09	25/26-1-2017	0,46	0,50	0,5	0,00616
2-fucosyl-lactose with 0,2 ml BSA	PMRS09	25/26-1-2017	0,21	0,21	0,2	0,00107
2-fucosyl-lactose with 0,1 ml BSA	PMRS09	25/26-1-2017	0,11	0,11	0,1	0,00027
2-fucosyl-lactose with 1,0 ml BSA	PMRS10	25/26-1-2017	1,00	0,94	1,0	0,00389
2-fucosyl-lactose with 0,5 ml BSA	PMRS10	25/26-1-2017	0,49	0,46	0,5	0,00345
2-fucosyl-lactose with 0,2 ml BSA	PMRS10	25/26-1-2017	0,21	0,21	0,2	0,00007
2-fucosyl-lactose with 0,1 ml BSA	PMRS10	25/26-1-2017	0,11	0,11	0,1	0,00414
					Σ	0,03411

raw data can be found in sheet "raw data"

number of duplo's (k) must be ≥ 6

cochran-test	0,372	
cochran-table value (k,n-1)	0,541	
number of duplo's (k)	12	
RSD_R	3,8	%
factor = 1 (k)	1,00	

conclusion: the reproducibility variation coefficient complies with the limit

Re-validation of reproducibility from duplo analysis			analysis 1	analysis 2	average	RSD_R	complies with limit
product	sample ID	analist 1/2	% (m/m)	% (m/m)	% (m/m)	(%)	

model 2.3 **Measurement Uncertainty (expanded)**

The measurement uncertainty is build up from a variable deviation (RSD_R) and a systematic deviation (maximum relative bias compared to the as true assumed value from a reliable interlaboratoy test or a proficiency test or a reference material)

The relative measurement uncertainty (M) can be estimated by the following equation (95% confidence);

$$M(\%) = 2 \times \sqrt{(RSD_R)^2 + (D)^2}$$

M = relative measurement uncertainty (%)
RSD_R = variation coefficient for reproducibility
D = relative bias (%) compared to supplied reference material (model 1.1) or recovery loss (%)

	matrix 2-fucosyl-lactose	
D (%) =	0,5	%
RSD_R (%) =	3,8	%
M (%) = relative measurement uncertainty =	8	%

model 2.3 Meas. uncertainty 6/10

model 5.1 Selectivity and Specificity

The Bradford protein assay is used to measure the concentration of total protein in a sample. The principle of this assay is that the binding of protein molecules to Coomassie dye under acidic conditions results in a color change from brown to blue. This method actually measures the presence of the basic amino acid residues, arginine, lysine and histidine, which contributes to formation of the protein-dye complex.

Kritiekewaarden voor de Cochran maximumvariantietoets, kritisch niveau 5 %

k \ n-1	1
2	0,999
3	0,967
4	0,906
5	0,841
6	0,781
7	0,727
8	0,680
9	0,638
10	0,602
11	0,570
12	0,541
13	0,515
14	0,492
15	0,471
16	0,452
17	0,434
18	0,418
19	0,403
20	0,389
21	0,377
22	0,365
23	0,354
24	0,343
25	0,334
26	0,325
27	0,316
28	0,308
29	0,300
30	0,293
31	0,206
32	0,280
33	0,273
34	0,267
35	0,262
36	0,256
37	0,251
38	0,246
39	0,242
40	0,237
60	0,172
120	0,100
∞	0

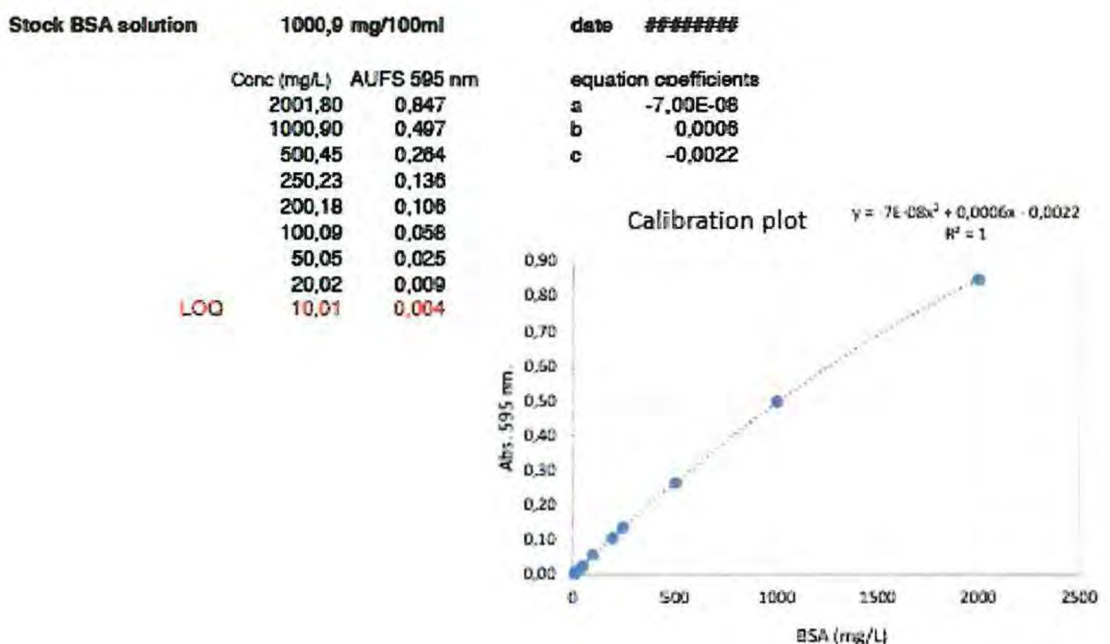
author:	H. Dahlmans
Company:	Nutricontrol, Netherlands

Kritieke waarde P voor toesen precisiekenmerken

k	P	Q
	0,06	--
2	0,23	--
3	0,34	--
4	0,42	--
5	0,48	--
6	0,52	--
7	0,56	1,42
8	0,58	1,39
9	0,61	1,37
10	0,63	1,35
11	0,64	1,34
12	0,66	1,32
13	0,67	1,31
14	0,69	1,30
15	0,70	1,29
16	0,71	1,28
17	0,71	1,27
18	0,72	1,27
19	0,73	1,26
20	0,74	1,25
25	0,76	1,23
30	0,79	1,21
35	0,80	1,19
40	0,81	1,18
45	0,82	1,17
50	0,83	1,16
60	0,85	1,15
70	0,86	1,14
80	0,87	1,13
90	0,00	1,12
100	0,88	1,12
150	0,90	1,09
200	0,92	1,08
300	0,93	1,07
400	0,94	1,06
500	0,95	1,05
1000	0,96	1,04

Tabel uit W.J. Dixon en F. Massey "introduction to statistical analysis"; Mc Graw-Hill Book Company , Inc. New York 1957-II

Raw data Repeatability and Reproducibility



Stock BSA solution 1000,9 mg/100ml

date ########

	Conc (mg/L)	AUFS 595 nm
	2001,80	0,847
	1000,90	0,497
	500,45	0,264
	250,23	0,136
	200,18	0,106
	100,09	0,056
	50,05	0,025
	20,02	0,009
LOQ	10,01	0,004

equation coefficients

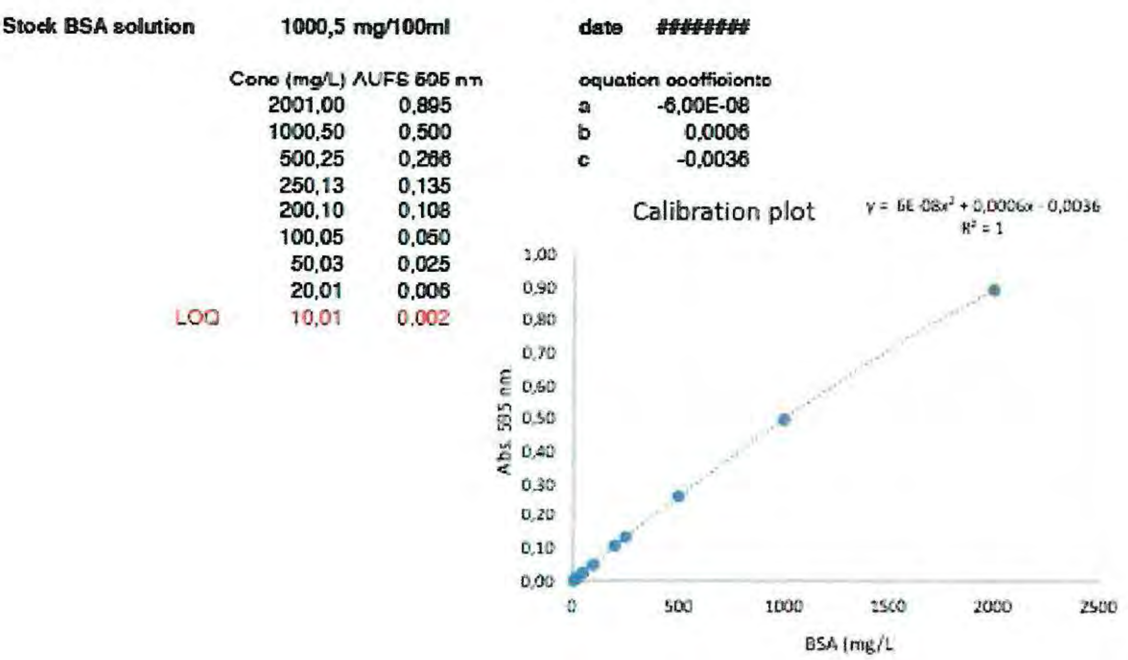
a	-7,00E-08
b	0,0006
c	-0,0022

analysis 1a			weight (mg)	AUFS	Goal Seek Excel: calculated mg BSA/L	Goal Seek Excel: measured Y AUFS 595 nm		BSA % (m/m)	BSA * % (m/m)
2-f-lactose with 1,0 ml BSA	PMRS01	25-1-2017	1010,7	0,505	950,7	0,505	0,505	0,94	0,95
2-f-lactose with 0,5 ml BSA	PMRS01	25-1-2017	1008,9	0,270	480,5	0,27	0,270	0,48	0,48
2-f-lactose with 0,2 ml BSA	PMRS01	25-1-2017	1036,6	0,115	200,0	0,115	0,115	0,19	0,20
2-f-lactose with 0,1 ml BSA	PMRS01	25-1-2017	1092,6	0,056	98,1	0,056	0,056	0,09	0,10
2-f-lactose with 1,0 ml BSA	PMRS09	25-1-2017	1050,4	0,517	976,5	0,517	0,517	0,93	0,98
2-f-lactose with 0,5 ml BSA	PMRS09	25-1-2017	1013,6	0,258	458,0	0,258	0,258	0,45	0,46
2-f-lactose with 0,2 ml BSA	PMRS09	25-1-2017	1017,9	0,119	207,0	0,119	0,119	0,20	0,21
2-f-lactose with 0,1 ml BSA	PMRS09	25-1-2017	1003,7	0,062	106,7	0,062	0,062	0,11	0,11
2-f-lactose with 1,0 ml BSA	PMRS10	25-1-2017	1007,6	0,527	998,2	0,527	0,527	0,99	1,00
2-f-lactose with 0,5 ml BSA	PMRS10	25-1-2017	1052,6	0,275	489,9	0,275	0,275	0,47	0,49
2-f-lactose with 0,2 ml BSA	PMRS10	25-1-2017	1048,8	0,118	205,2	0,118	0,118	0,20	0,21
2-f-lactose with 0,1 ml BSA	PMRS10	25-1-2017	1113,0	0,065	114,3	0,065	0,065	0,10	0,11

analysis 1b			weight (mg)	AUFS	Goal Seek Excel: calculated mg BSA/L	Goal Seek Excel: measured Y AUFS 595 nm		BSA % (m/m)	BSA * % (m/m)
2-f-lactose with 1,0 ml BSA	PMRS01	25-1-2017	1015,6	0,500	940,0	0,500	0,500	0,93	0,94
2-f-lactose with 0,5 ml BSA	PMRS01	25-1-2017	1005,2	0,270	480,5	0,270	0,270	0,48	0,48
2-f-lactose with 0,2 ml BSA	PMRS01	25-1-2017	1041,2	0,109	189,5	0,109	0,109	0,18	0,19
2-f-lactose with 0,1 ml BSA	PMRS01	25-1-2017	1023,4	0,058	101,8	0,058	0,058	0,10	0,10
2-f-lactose with 1,0 ml BSA	PMRS09	25-1-2017	1064,3	0,537	1020,0	0,537	0,537	0,96	1,02
2-f-lactose with 0,5 ml BSA	PMRS09	25-1-2017	1026,6	0,272	484,2	0,272	0,272	0,47	0,48
2-f-lactose with 0,2 ml BSA	PMRS09	25-1-2017	1110,2	0,117	203,5	0,117	0,117	0,18	0,20
2-f-lactose with 0,1 ml BSA	PMRS09	25-1-2017	1041,8	0,059	103,6	0,059	0,059	0,10	0,10
2-f-lactose with 1,0 ml BSA	PMRS10	25-1-2017	1105,0	0,542	1030,9	0,542	0,542	0,93	1,03
2-f-lactose with 0,5 ml BSA	PMRS10	25-1-2017	1016,9	0,273	486,1	0,273	0,273	0,48	0,49
2-f-lactose with 0,2 ml BSA	PMRS10	25-1-2017	1018,0	0,117	203,5	0,117	0,117	0,20	0,20
2-f-lactose with 0,1 ml BSA	PMRS10	25-1-2017	1081,8	0,062	109,0	0,062	0,062	0,10	0,11

BSA * : the sample weight is corrected to exactly 1000 mg to be able to compare results for calucaltion of repeatability and reproducibility. raw data

9/10



Stock BSA solution 1000,5 mg/100ml

date ########

	Conc (mg/L)	AUFS 595 nm
	2001,00	0,895
	1000,50	0,500
	500,25	0,266
	250,13	0,135
	200,10	0,108
	100,05	0,050
	50,03	0,025
	20,01	0,006
LOQ	10,01	0,002

equation coefficients

a	-6,00E-08
b	0,0006
c	-0,0036

analysis 2a			weight (mg)	AUFS	Goal Seek value: calculated mg BSA/L	Goal Seek value: measured Y AUFS 595 nm		BSA % (m/m)	BSA * % (m/m)
2-f-lactose with 1,0 ml BSA	PMRS01	26-1-2017	1056,9	0,500	924,5	0,500	0,500	0,87	0,92
2-f-lactose with 0,5 ml BSA	PMRS01	26-1-2017	1173,7	0,278	494,0	0,278	0,278	0,42	0,49
2-f-lactose with 0,2 ml BSA	PMRS01	26-1-2017	1117,9	0,111	194,8	0,111	0,111	0,17	0,19
2-f-lactose with 0,1 ml BSA	PMRS01	26-1-2017	1095,4	0,061	109,8	0,061	0,062	0,10	0,11
2-f-lactose with 1,0 ml BSA	PMRS09	26-1-2017	1044,0	0,520	965,7	0,520	0,520	0,93	0,97
2-f-lactose with 0,5 ml BSA	PMRS09	26-1-2017	1173,0	0,279	495,4	0,279	0,279	0,42	0,50
2-f-lactose with 0,2 ml BSA	PMRS09	26-1-2017	1057,1	0,122	213,9	0,122	0,122	0,20	0,21
2-f-lactose with 0,1 ml BSA	PMRS09	26-1-2017	1103,5	0,062	110,5	0,062	0,062	0,10	0,11
2-f-lactose with 1,0 ml BSA	PMRS10	26-1-2017	1070,1	0,507	937,8	0,507	0,506	0,88	0,94
2-f-lactose with 0,5 ml BSA	PMRS10	26-1-2017	1087,5	0,261	461,9	0,261	0,261	0,42	0,46
2-f-lactose with 0,2 ml BSA	PMRS10	26-1-2017	1177,4	0,118	206,9	0,118	0,118	0,18	0,21
2-f-lactose with 0,1 ml BSA	PMRS10	26-1-2017	1091,4	0,060	107,1	0,060	0,060	0,10	0,11

BSA * : the sample weight is corrected to exactly 1000 mg to be able to compare results for calucation of repeatability and reproducibility.

raw data

Appendix 4 Representative Chromatograms for Multiple Production Bathes of Purified 2'-Fucosyllactose (2'-FL)

Appendix Chromatograms of HPAEC 2'-fucosyllactose method

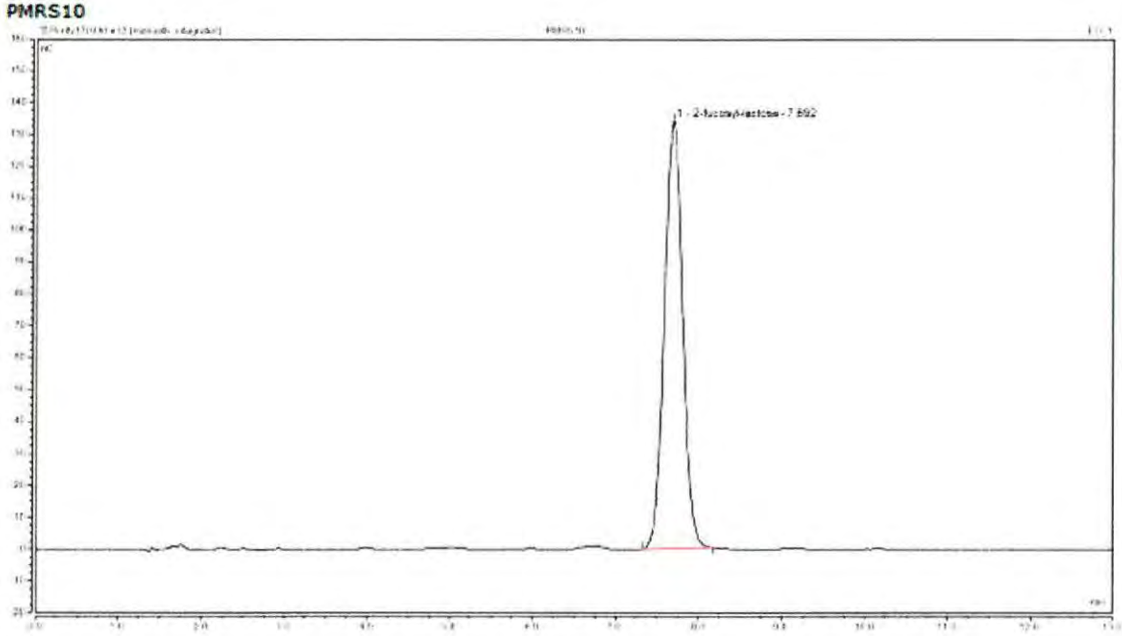
Isocratic HPAEC of the 2'-fucosyllactose end product (ME-AV042FL Isocratic HPAEC)

In this document the Chromatograms of the registration batches are presented, registration batches PMRS10, PMRS 11, CMRS03, CMRS06 and CMRS07.

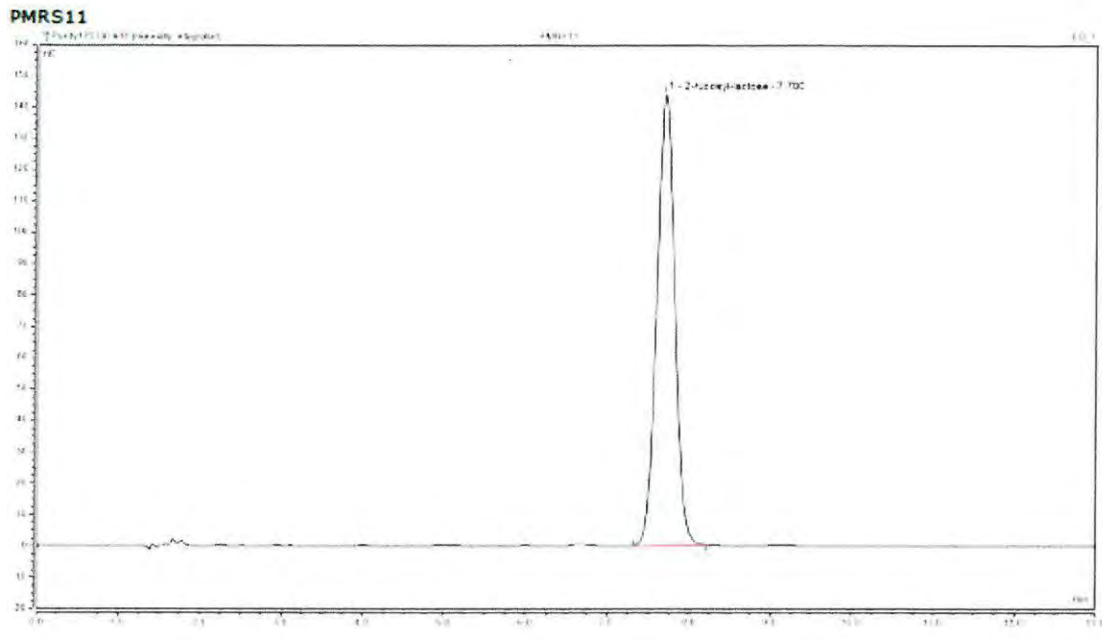
Identification and quantification of 2'-fucosyllactose is done with a standard, PMRS01, of which the 2'-fucosyllactose is identified and quantified with qNMR (see report Spectral Services, Köln, Germany)

Plots Chromatogram of registration batches, isocratic HPAEC method

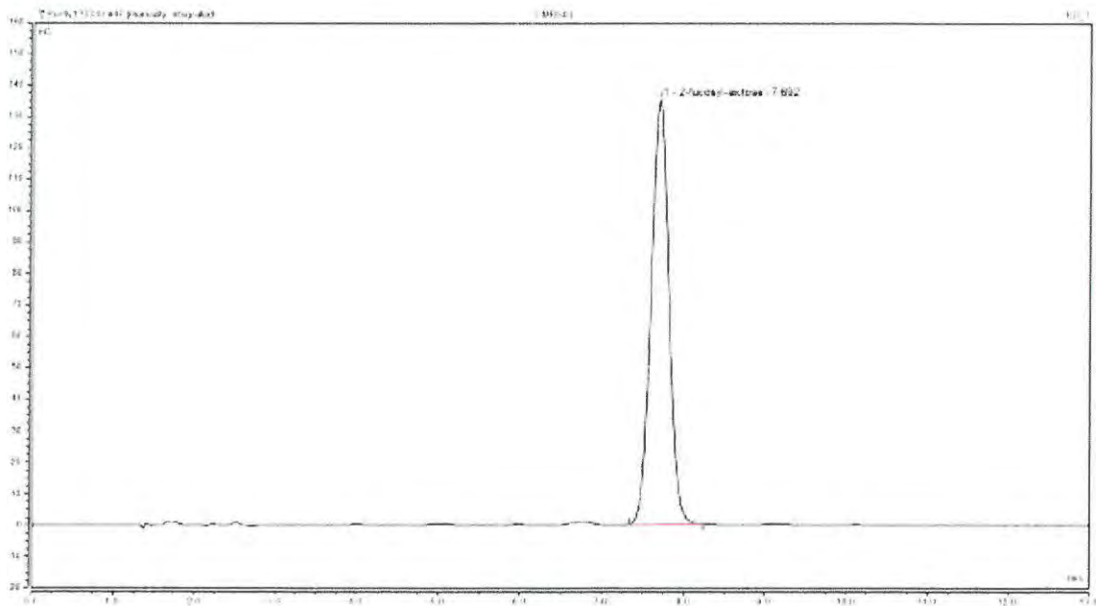
PMRS10



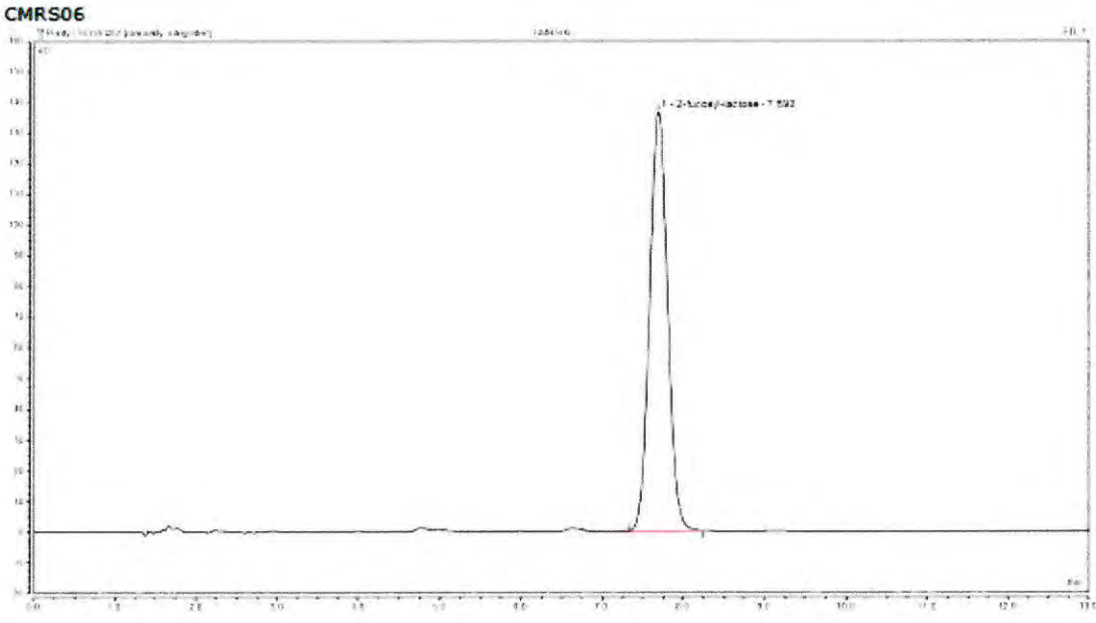
PMRS11



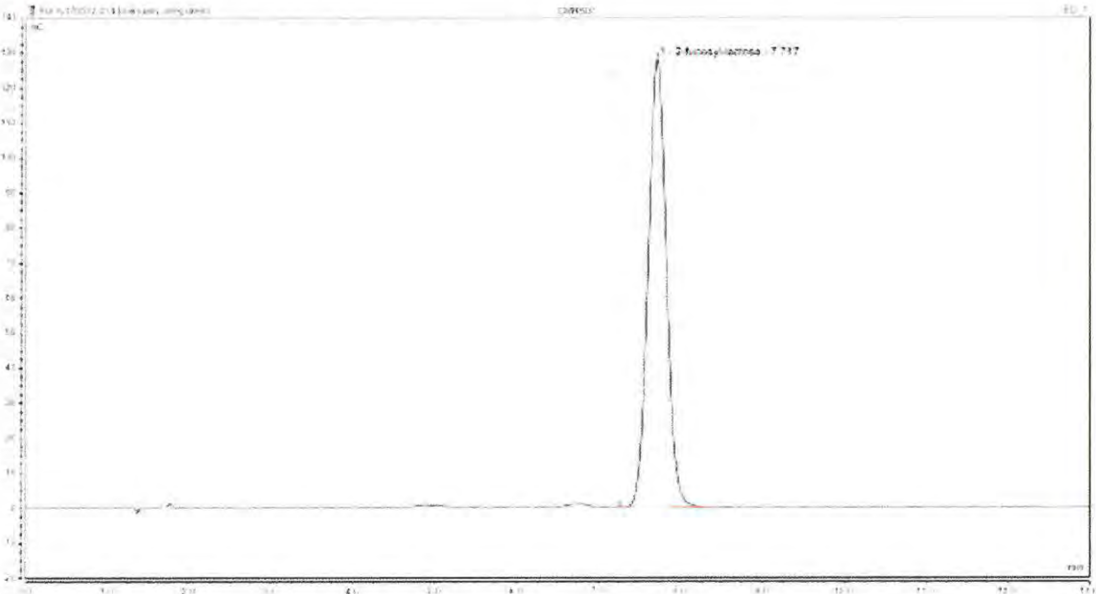
CMRS03



CMRS06



CMRS07



Appendix 5 Certificates of Analysis for Multiple Production Batches of Purified 2'-Fucosyllatose (2'-FL)

Appendix 5.1 **Certificate of Analysis 2'-FL Lot PMRS10**

Appendix 5.2 **Certificate of Analysis 2'-FL Lot PMRS11**

Appendix 5.3 **Certificate of Analysis 2'-FL Lot CMRS03**

Appendix 5.4 **Certificate of Analysis 2'-FL Lot CMRS06**

Appendix 5.5 **Certificate of Analysis 2'-FL Lot CMRS07**

Appendix 5.1 Certificate of Analysis 2'-FL Lot PMRS10

Certificate of analysis

Product : 2'-Fucosyllactose
Batch number : PMRS10
Date of production : 28.10.2016
Best Before : 28.10.2019

Description : Human milk oligosaccharide

Typical analysis : 2'-Fucosyllactose 93%, moisture 4%, lactose 1%, allo lactose 1 %, glucose <1%, fucose <1%

Sensorial: White homogeneous powder, neutral to slightly sweet, no off flavor

Chemical/physical	Specification	Result	Method of analysis
Total moisture	max. 5%	3.83%	ISO 760 (modified), Karl Fischer
2'-Fucosyllactose	min. 90%	94.2%	FC method using HPAEC-PAD
Lactose	max. 4%	0.6%	FC method using HPAEC-PAD
Allo-Lactose	max. 2%	0.5%	FC method using HPAEC-PAD
Glucose	max. 2%	0.1%	FC method using HPAEC-PAD
Galactose	max. 2%	<0.1%	FC method using HPAEC-PAD
Fucose	max. 2%	0.2%	FC method using HPAEC-PAD
Protein	max. 0.01%	<0.01%	Bradford
Sulphated ash	max. 0.2%	0.02%	NEN 6810 (modified)
Nitrite	max. 1 mg/kg	<0.1 mg/kg	ISO 14673-2/IDF 189-2
Nitrate	max. 50 mg/kg	0.7 mg/kg	ISO 14673-2/IDF 189-2
Scorched particles	max. disc A	A	FC method equivalent to ADPI 916/ISO 5739/IDF 107
pH (10%)	4.0 - 7.5	4.1	FC method using NEN 3775
Aluminum	max. 4.8 mg/kg	<0.2 mg/kg	FC method using ISO 17294
Arsenic	max. 0.1 mg/kg	<0.010 mg/kg	FC method using ISO 17294
Cadmium	max. 0.01 mg/kg	<0.005 mg/kg	FC method using ISO 17294
Mercury	max. 0.05 mg/kg	<0.001 mg/kg	FC method using ISO 17294
Lead	max. 0.05 mg/kg	<0.02 mg/kg	FC method using ISO 17294
Aflatoxin M1	max. 0.2 µg/kg	<0.01 µg/kg	ISO 14501/IDF 171

Microbiological	Specification	Result	Method of analysis
Aerobic mesophilic count	max. 3000 cfu/g	300 cfu/g	FC method equivalent to ISO 4833
Enterobacteriaceae	absent in 10 g	absent in 10 g	FC method, BPW 18h 37°C, SD, VRBG 18-24h 37°C
E. coli	absent in 10 g	absent in 10 g	FC method, LMX 25h, Coli ID 24h
Yeasts	max. 10 cfu/g	<1 cfu/g	FC method equivalent to ISO 6611
Moulds	max. 10 cfu/g	<1 cfu/g	FC method equivalent to ISO 6611
Presumptive Bacillus cereus	max. 100 cfu/g	10 cfu/g	FC method equivalent to ISO 7932
Staphylococcus aureus	absent in 1 g	absent in 1 g	ISO 6888-3, G&C 42h 37°
Sulphite reducing clostridia spores	max. 30 cfu/g	1 cfu/g	FC method using DIN 27 (1995) 18h-28h Weenk
Clostridium perfringens	absent in 1 g	absent in 1 g	FC method, RPM 20h 46°C, confirmation
Salmonella	absent in 25 g	absent in 25 g	FC method equivalent to ISO 6579
Cronobacter spp	absent in 25 g	absent in 25 g	FC method equivalent to ISO/TS 22964
Endotoxins	max. 10 EU/mg	0.88 EU/mg	Eur. Ph. 2.6.14 and USP <85>
GMO detection	negative	negative	qPCR

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Function: R&D Manager

Appendix 5.2 Certificate of Analysis 2'-FL Lot PMRS11

Certificate of analysis

Product	2'-Fucosyllactose
Batch number	PMRS11
Date of production	30-11-2016
Best Before	30-11-2019

Description: Human milk oligosaccharide

Typical analysis: 2'-Fucosyllactose 93%, moisture 4%, lactose 1%, allo-lactose 1%, glucose < 1%, fucose < 1%

Sensorial: White homogeneous powder, neutral to slightly sweet, no off flavor

Chemical/physical	Specification	Result	Method of analysis
Total moisture	max. 5%	1.33%	ISO 760 (modified), Karl Fischer
2'-Fucosyllactose	min. 90%	93.2%	FC method using HPAEC-PAD
Lactose	max. 3%	0.5%	FC method using HPAEC-PAD
Allo-Lactose	max. 2%	0.6%	FC method using HPAEC-PAD
Glucose	max. 2%	0.1%	FC method using HPAEC-PAD
Galactose	max. 2%	<0.1%	FC method using HPAEC-PAD
Fucose	max. 2%	0.2%	FC method using HPAEC-PAD
Protein	max. 0.01%	<0.01%	Bradford
Sulphated ash	max. 0.2%	0.01%	NEN 6810 (modified)
Nitrite	max. 1 mg/kg	<0.1 mg/kg	ISO 14673-2/IDF 189-2
Nitrate	max. 50 mg/kg	1.0 mg/kg	ISO 14673-2/IDF 189-2
Scorched particles	max. disc A	A	FC method equivalent to ADPI 916/ISO 5739/IDF 107
pH (10%)	3.0 - 7.5	4.0	FC method using NEN 3775
Aluminum	max. 4.8 mg/kg	<0.2 mg/kg	FC method using ISO 17294
Arsenic	max. 0.1 mg/kg	<0.01 mg/kg	FC method using ISO 17294
Cadmium	max. 0.01 mg/kg	<0.005 mg/kg	FC method using ISO 17294
Mercury	max. 0.05 mg/kg	<0.003 mg/kg	FC method using ISO 17294
Lead	max. 0.05 mg/kg	<0.02 mg/kg	FC method using ISO 17294
Aflatoxin M1	max. 0.2 µg/kg	<0.01 µg/kg	ISO 14501/IDF 171

Microbiological	Specification	Result	Method of analysis
Aerobic mesophilic count	max. 3000 cfu/g	<1000 cfu/g	FC method equivalent to ISO 4833
Enterobacteriaceae	absent in 10 g	Absent in 10 g	FC method, BPW 18h 37°C, SO, VRBG 18-24h 37°C
E. coli	absent in 10 g	Absent in 10 g	FC method, LMI 25h, Coli ID 24h
Yeasts	max. 10 cfu/g	<1 cfu/g	FC method equivalent to ISO 6611
Moulds	max. 10 cfu/g	<1 cfu/g	FC method equivalent to ISO 6611
Presumptive Bacillus cereus	max. 100 cfu/g	<1 cfu/g	FC method equivalent to ISO 7932
Staphylococcus aureus	absent in 1 g	Absent in 1 g	ISO 6888-3, GSC 42h 37°
Sulphite reducing clostridia spores	max. 30 cfu/g	<1 cfu/g	FC method using DFM 27 (1995) 185-200 Weenk
Clostridium perfringens	absent in 1 g	Absent in 1 g	FC method, RPM 20h 46°C, confirmation
Salmonella	absent in 25 g	Absent in 25 g	FC method equivalent to ISO 6579
Cronobacter spp.	absent in 25 g	Absent in 25 g	FC method equivalent to ISO/TS 22964
Endotoxin	max. 10 EU/mg	0.54 EU/mg	Eur. Ph. 2.6.14 and USP <85>
GMO detection	negative	Negative	qPCR

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Appendix 5.3 Certificate of Analysis 2'-FL Lot CMRS03

Certificate of analysis

Product	: 2' Fucosyllactose
Batch number	: CMRS03
Date of production	: 05 01 2017
Best Before	: 05 01 2020

Description : Human milk oligosaccharide

Typical analysis : 2'-Fucosyllactose 93%, moisture 4%, lactose 1%, allo-lactose 1 %, glucose < 1%, fucose <1%

Sensorial: : White homogeneous powder, neutral to slightly sweet, no off flavor

Chemical/physical	Specification	Result	Method of analysis
Total moisture	max. 5%	4.1%	ISO 760 (modified), Karl Fischer
2' Fucosyllactose	min. 90%	93.7%	FC method using HPAEC PAD
Lactose	max. 3%	1.1%	FC method using HPAEC PAD
Allo Lactose	max. 2%	0.6%	FC method using HPAEC PAD
Glucose	max. 2%	0.1%	FC method using HPAEC PAD
Galactose	max. 2%	<0.1%	FC method using HPAEC PAD
Fucose	max. 2%	0.4%	FC method using HPAEC PAD
Protein	max. 0.01%	<0.01%	Bradford
Sulphated ash	max. 0.2%	0.02%	NEN 6810 (modified)
Nitrite	max. 1 mg/kg	<0.1 mg/kg	ISO 14673 2/IDF 189-2
Nitrate	max. 50 mg/kg	0.3 mg/kg	ISO 14673 2/IDF 189 2
Scorched particles	max. disc A	A	FC method equivalent to ADPI 916/ISO 5739/IDF 107
pH (10%)	3.0 - 7.5	4.64	FC-method using NEN 3775
Aluminum	max. 4.8 mg/kg	<0.2 mg/kg	FC-method using ISO 17294
Arsenic	max. 0.1 mg/kg	<0.010 mg/kg	FC method using ISO 17294
Cadmium	max. 0.01 mg/kg	<0.005 mg/kg	FC-method using ISO 17294
Mercury	max. 0.05 mg/kg	<0.003 mg/kg	FC method using ISO 17294
Lead	max. 0.05 mg/kg	<0.02 mg/kg	FC method using ISO 17294
Aflatoxin M1	max. 0.2 µg/kg	<0.01 µg/kg	ISO 14501/IDF 171

Microbiological	Specification	Result	Method of analysis
Aerobic mesophilic count	max. 3000 cfu/g	<1000 cfu/g	FC method equivalent to ISO 4833
Enterobacteriaceae	absent in 10 g	Absent in 10 g	FC-method, BPW 18h 37°C, 50, VRBG 18-24h 37°C
E. coli	absent in 10 g	Absent in 10 g	FC-method, LMX 25h, Coli ID 24h
Yeasts	max. 10 cfu/g	<1 cfu/g	FC method equivalent to ISO 6611
Moulds	max. 10 cfu/g	<1 cfu/g	FC method equivalent to ISO 6611
Presumptive Bacillus cereus	max. 100 cfu/g	10 cfu/g	FC method equivalent to ISO 7932
Staphylococcus aureus	absent in 1 g	Absent in 1 g	ISO 6888 1, GBC 42h 37°
Sulphite reducing clostridia spores	max. 30 cfu/g	<1 cfu/g	FC method using IIFM 27 (1995) 185 200 Weenk
Clostridium perfringens	absent in 1 g	Absent in 1 g	FC method, RPM 20h 46°C, confirmation
Salmonella	absent in 25 g	Absent in 25 g	FC method equivalent to ISO 6579
Cronobacter spp	absent in 25 g	Absent in 25 g	FC method equivalent to ISO/TS 22964
Endotoxin	max. 10 EU/mg	0.21 EU/mg	Eur. Ph. 2.6.14 and USP <85>
GMO detection	negative	negative	qPCR

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Function: R&D Manager

Appendix 5.4 Certificate of Analysis 2'-FL Lot CMRS06

Certificate of analysis

Product	: 2'-Fucosyllactose
Batch number	: CMRS06
Date of production	: 24-03-2017
Best Before	: 24-03-2020
Description	: Human milk oligosaccharide
Typical analysis	: 2'-Fucosyllactose 93%, moisture 4%, lactose 1%, allo lactose 1 %, glucose <1%, fucose <1%
Sensorial:	: White homogeneous powder, neutral to slightly sweet, no off flavor

Chemical/physical	**Specification**	**Result**	**Method of analysis**
Total moisture	max. 5%	3.59%	ISO 760 (modified), Karl Fischer
2'-Fucosyllactose	min. 90%	92.7%	FC method using HPAEC-PAD
Lactose	max. 3%	0.8%	FC method using HPAEC-PAD
Allo Lactose	max. 2%	0.2%	FC method using HPAEC-PAD
Glucose	max. 2%	0.1%	FC method using HPAEC-PAD
Galactose	max. 2%	<0.1%	FC-method using HPAEC-PAD
Fucose	max. 2%	0.1%	FC-method using HPAEC-PAD
Protein	max. 0.01%	<0.01%	Bradford
Sulphated ash	max. 0.2%	0.1%	NEN 6810 (modified)
Nitrite	max. 1 mg/kg	<0.1 mg/kg	ISO 14673-2/IDF 189-2
Nitrate	max. 50 mg/kg	1.6 mg/kg	ISO 14673-2/IDF 189-2
Scorched particles	max. disc A	A	FC method equivalent to ADPI 916/ISO 5739/IDF 107
pH (10%)	3.0 - 7.5	4.03	FC-method using NEN 3775
Aluminum	max. 4.8 mg/kg	<0.2 mg/kg	FC-method using ISO 17294
Arsenic	max. 0.1 mg/kg	<0.01 mg/kg	FC method using ISO 17294
Cadmium	max. 0.01 mg/kg	<0.005 mg/kg	FC-method using ISO 17294
Mercury	max. 0.05 mg/kg	<0.006 mg/kg	FC method using ISO 17294
Lead	max. 0.05 mg/kg	<0.02 mg/kg	FC-method using ISO 17294
Aflatoxin M1	max. 0.2 µg/kg	<0.01 µg/kg	ISO 14501/IDF 171

Microbiological	**Specification**	**Result**	**Method of analysis**
Aerobic mesophilic count	max. 3000 cfu/g	<1000 cfu/g	FC-method equivalent to ISO 4833
Enterobacteriaceae	absent in 10 g	Absent in 10g	FC-method, BPW 18h 37°C, SD, VRBG 18-24h 37°C
E. coli	absent in 10 g	Absent in 10 g	FC-method, LMX 25h, Coli ID 24h
Yeasts	max. 10 cfu/g	<1 cfu/g	FC-method equivalent to ISO 6611
Moulds	max. 10 cfu/g	<1 cfu/g	FC-method equivalent to ISO 6611
Presumptive Bacillus cereus	max. 100 cfu/g	<1 cfu/g	FC method equivalent to ISO 7932
Staphylococcus aureus	absent in 1 g	Absent in 1 g	ISO 6888-3, G&C 42h 37°
Sulphite reducing clostridia spores	max. 30 cfu/g	<1 cfu/g	FC-method using IJFM 27 (1995) 185-200 Weenk
Clostridium perfringens	absent in 1 g	Absent in 1 g	FC-method, RPM 20h 46°C, confirmation
Salmonella	absent in 25 g	Absent in 25 g	FC-method equivalent to ISO 6579
Cronobacter spp.	absent in 25 g	Absent in 25 g	FC-method equivalent to ISO/TS 22964
Endotoxin	max. 10 EU/mg	<0.1 EU/mg	Eur. Ph. 2.6.14 and USP <85>
GMO-detection	negative	Negative	qPCR

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Appendix 5.5 Certificate of Analysis 2'-FL Lot CMRS07

Certificate of analysis

Product	: 2'-Fucosyllactose
Batch number	: CMRS07
Date of production	: 09-05-2017
Best Before	: 09-05-2020
Description	: Human milk oligosaccharide
Typical analysis	: 2'-Fucosyllactose 93%, moisture 4%, lactose 1%, allo-lactose 1 %, glucose <1%, fucose <1%
Sensorial:	White homogeneous powder, neutral to slightly sweet, no off flavor

Chemical/physical	**Specification**	**Result**	**Method of analysis**
Total moisture	max. 5%	3.6%	ISO 760 (modified), Karl Fischer
2'-Fucosyllactose	min. 90%	93.8%	FC method using HPAEC-PAD
Lactose	max. 3%	0.5%	FC method using HPAEC-PAD
Allo-Lactose	max. 2%	1.0%	FC method using HPAEC-PAD
Glucose	max. 2%	<0.1%	FC method using HPAEC-PAD
Galactose	max. 2%	<0.1%	FC method using HPAEC-PAD
Fucose	max. 2%	0.2%	FC method using HPAEC-PAD
Protein	max. 0.01%	<0.01%	Bradford
Sulphated ash	max. 0.2%	0.08%	NEN 6810 (modified)
Nitrite	max. 1 mg/kg	<0.1 mg/kg	ISO 14673-2/IDF 189-2
Nitrate	max. 50 mg/kg	0.9 mg/kg	ISO 14673-2/IDF 189-2
Scorched particles	max. disc A	A	FC-method equivalent to ADPI 916/ISO 5739/IDF 107
pH (10%)	3.0 - 7.5	5.07	FC-method using NEN 3775
Aluminum	max. 4.8 mg/kg	<0.4 mg/kg	FC method using ISO 17294
Arsenic	max. 0.1 mg/kg	<0.020 mg/kg	FC method using ISO 17294
Cadmium	max. 0.01 mg/kg	<0.010 mg/kg	FC method using ISO 17294
Mercury	max. 0.05 mg/kg	<0.011 mg/kg	FC-method using ISO 17294
Lead	max. 0.05 mg/kg	<0.02 mg/kg	FC method using ISO 17294
Aflatoxin M1	max. 0.2 µg/kg	<0.01 µg/kg	ISO 14501/IDF 171

Microbiological	**Specification**	**Result**	**Method of analysis**
Aerobic mesophilic count	max. 3000 cfu/g	<1000 cfu/g	FC-method equivalent to ISO 4833
Enterobacteriaceae	absent in 10 g	Absent in 10 g	FC-method, BPW 18h 37°C, SD, VRBG 18-24h 37°C
E. coli	absent in 10 g	Absent in 10 g	FC method, LMX 25h, Coli ID 24h
Yeasts	max. 10 cfu/g	<1 cfu/g	FC method equivalent to ISO 6611
Moulds	max. 10 cfu/g	<1 cfu/g	FC-method equivalent to ISO 6611
Presumptive Bacillus cereus	max. 100 cfu/g	20 cfu/g	FC method equivalent to ISO 7932
Staphylococcus aureus	absent in 1 g	Absent in 1 g	ISO 6888-3, GBC 42h 37°
Sulphite reducing clostridia spores	max. 30 cfu/g	<1 cfu/g	FC-method using UFM 27 (1995) 185-200 Weenk
Clostridium perfringens	absent in 1 g	Absent in 1 g	FC-method, RPM 20h 46°C, confirmation
Salmonella	absent in 25 g	Absent in 25 g	FC method equivalent to ISO 6579
Cronobacter spp	absent in 25 g	Absent in 25 g	FC method equivalent to ISO/TS 22964
Endotoxin	max. 10 EU/mg	<0.1 EU/mg	Eur. Ph. 2.6.14 and USP <85>
GMO detection	negative	Negative	qPCR

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Appendix 6 Evaluation of 2'-FL for Absence of Genes of the *E. coli* Production Strain by qPCR

Please refer to the Appendix 6 report, provided as a separate file.

Appendix 6 qPCR 2'-fucosyllactose.pdf

Appendix 7 Stability Testing Report for Purified 2'-Fucosyllatose (2'-FL)

Status report stability test 2-'FL

The stability of the 2' FL produced by fermentation is currently being assessed over a period of 36 months, in total. 2' FL samples are stored under 2 storage conditions:

1) Normal storage conditions: 25°C and 60% humidity
2) Accelerated storage conditions: 40°C and 75% humidity

The samples are analyzed on chemical composition and microbiological composition on different time points. An overview of the stability test, including the timings and parameters that are assessed, is shown in table 1.

Table 1. *Stability study on 2' Fucosyllactose (2' FL) under normal (25°C and 60% humidity) and accelerated (40°C and 75% humidity) storage conditions.*

storage condition	25°C and 60% humidity						40°C and 75% humidity	
timing	t=0	t=3 months	t=6 months	t=12 months	t=24 months	t=36 months	t=3 months	t=6 months
Parameters tested								
Chemistry (%w/w)								
Total moisture (Karl Fisher)	3.3%	3.7%	4.0%				4.2%	3.7%
Ash	0.11%	<0.01	0.01				0.03%	0.02%
2'FL	96.3%	93.3%	94.8%				95.0%	94.2%
Lactose	0.6%	0.6%	1.3%				1.1%	2.1%
Allo-lactose	0.1%	1.1%	1.8%				1.2%	1.6%
Glucose	0.1%	0.1%	0.3%				0.2%	0.4%
Microbiology								
Mesophilic aerobic cell count	<10 cfu/g	<10 cfu/g	<10 cfu/g				<10 cfu/g	<10 cfu/g
Enterobacteriaceae/ 10 g	Negative	Negative	Negative				Negative	Negative
Salmonella/ 25 g	Negative	Negative	Negative				Negative	Negative
Cronobacter spp/ 25 g	Negative	Negative	Negative				Negative	Negative
Sensorial								
smell	A bit sweet	A bit sweet	A bit sweet				A bit sweet	A bit sweet
appearance	White Homoge-nous fine powder	White Homoge-nous fine powder	White Homoge-nous fine powder				White Homoge-nous fine powder	White Homoge-nous fine powder

The first measurements, at t=0, t=3months, and t=6months, have been performed. The results are shown in table 1. The stability test is currently ongoing; the remaining measurements still need to be performed, according to the schedule:

t=12 months: December 6th 2017
t=24 months: December 6th 2018

t=36 months: December 6th 2019
An update of this status report of the stability test will be made available with the additional data, once the analyses have been performed.

The methodologies used to assess the parameters, as shown in table 1, are the same methodologies used to analyze the composition of the 2'FL. These methodologies are described elsewhere in the dossier (Appendix ...).

Appendix 8 Estimated Daily Intake Levels of Purified 2'-Fucosyllactose (2'-FL)

Please refer to the Appendix 8 report, provided as a separate file.

Appendix 8 U.S. Intakes Report 2'-FL.pdf

Appendix 9 14-Day Oral (Diet) Dose-Range Finding Study in Male Rats with 2'-Fucosyllactose

Please refer to the Appendix 9 report, provided as a separate file.

Appendix 9 Final study report 14-day DRF 2'-FL toxicology test.pdf

Appendix 10 Sub-Chronic (13-week) Oral Toxicity Study with 2'-Fucosyllactose in Rats

Please refer to the Appendix 10 report, provided as a separate file.

Appendix 10 Sub-Chronic (13-week) Oral Toxicity Study.pdf

Appendix 11 Bacterial Reverse Mutation Test with 2'-Fucosyllactose

STUDY REPORT

V20805/05

Bacterial reverse mutation test with 2'-fucosyllactose

DATE	**30 March 2017**
AUTHOR(S)	**M.J.M. van den Wijngaard**
SPONSOR	**Friesland Campina Innovation** **Bronland 20** **6708WH Wageningen** **The Netherlands**
TRISKELION PROJECT NUMBER	**P10197 (formerly 093.26005/02.41)**
TRISKELION STUDY CODE	**20805/05**
SPONSOR STUDY CODE	**--**
GUIDELINE	**OECD 471**
STATUS	**Final**
NUMBER OF PAGES	**26**

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Statement of GLP compliance

I, the undersigned, hereby declare that this report constitutes a complete and accurate representation of the study and its results.

All study activities performed by Triskelion B.V. were carried out in compliance with the current OECD Principles of Good Laboratory Practice (GLP)[1]. The OECD principles of Good Laboratory Practice are accepted by Regulatory Authorities throughout the European Community, USA and Japan. Chemical analysis for the verification of test substance identity and properties was not performed in this study.

Study director

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F.A.A. van Acker, PhD

30 March 2017
Date

[1] The most recent endorsement of compliance of the test facility with these principles is attached to the report as Annex 1

Quality Assurance Statement

I, the undersigned, hereby declare that this report provides an accurate record of the procedures employed and the results obtained in this study; all audits were study-based and were reported to the study director and management on the dates indicated.

Phase	Start date of audit	Date of audit report
Authorised study plan	1 February 2016	1 February 2016
Authorised study plan amendment 1	21 March 2016	21 March 2016
Authorised study plan amendment 2	24 June 2016	24 June 2016
Authorised study plan amendment 3	19 July 2016	19 July 2016
Authorised study plan amendment 4	14 September 2016	14 September 2016
Authorised study plan amendment 5	11 November 2016	11 November 2016
Authorised study plan amendment 6	22 November 2016	28 November 2016
Authorised study plan amendment 7	21 February 2017	21 February 2017
Test substance dissolution	5 February 2016	5 February 2016
Preparation of dosing solutions	5 February 2016	5 February 2016
Pilot exp. Fungal and bacterial contamination Amendment 5	11 November 2016	11 November 2016
Counting revertants	5 December 2016	5 December 2016
Draft report and study file	23 February 2017	23 February 2017
Final report	30 March 2017	30 March 2017

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M.L.A. de Kuijper – van Buurt
Quality Assurance auditor

Date: 30 Mar 2017

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Summary

The test substance, 2'-fucosyllactose was examined for its possible mutagenic activity in the bacterial reverse mutation test using the histidine-requiring *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100 and the tryptophan-requiring *Escherichia coli* strain WP2 *uvrA*, in the absence and presence of a liver fraction of Aroclor 1254-induced rats for metabolic activation (S9-mix).

A single test was performed. All strains, both in the absence and presence of S9-mix, were treated with five concentrations of the test substance. A stock solution of the test substance of 50 mg/ml in PBS was prepared; this resulted in a clear colorless solution. Negative controls (solvent) and positive controls were run simultaneously with the test substance.

The mean numbers of his^+ and trp^+ revertant colonies of the negative controls used were within the acceptable range in all strains. The positive controls gave the expected increase in the mean numbers of revertant colonies. Therefore, the test was considered valid.

No toxicity was observed in any strain, this was evidenced by an absence of a clearing of the background lawn of bacterial growth compared to the negative controls, no decrease in the mean number of revertants was observed and pinpoint colonies did not occur.

The test substance did not induce a more than 2-fold and/or dose related increase in the mean number of revertant colonies compared to the background spontaneous reversion rate observed with the negative control with strains TA 1535, TA 1537, TA 98, TA 100 and WP2 *uvrA*, in both the absence and presence of S9-mix.

It is concluded that the results obtained in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100, and in the *Escherichia coli* strain WP2 *uvrA*, in both the absence and presence of the S9-mix, indicate that the test substance **2'-fucosyllactose is not mutagenic** under the conditions used in this study.

1 General

1.1 Study Sponsor

Sponsor:	Friesland Campina Innovation Bronland 20 6708WH Wageningen The Netherlands
Monitor:	D. Delsing, PhD
Phone:	+31 6 5359 8111
E-mail:	dianne.delsing@frieslandcampina.com

1.2 Test facility

Triskelion B.V.	www.triskelion.nl
Postal address:	P.O. Box 844 3700 AV Zeist The Netherlands
Location:	Utrechtseweg 48 3704 HE Zeist The Netherlands
Phone:	+31 88 866 2800

1.3 Responsible Personnel

Study director:	F.A.A. van Acker, PhD
Phone:	+31 88 866 26 18
E-mail:	frederique.vanacker@triskelion.nl

1.4 Time schedule

The test was conducted between 06 and 10 October 2016.

2 Introduction

2.1 Objective

The objective of this study was to provide data on the possible mutagenic activity of 2'-fucosyllactose, in four selected strains of *Salmonella typhimurium*, TA 1535, TA 1537, TA 98 and TA 100, and in the *Escherichia coli* mutant WP2 *uvrA*, in both the absence and presence of a metabolic activation system (S9-mix).

2.2 Applicable guidelines

This study was conducted in accordance with the following guideline:

OECD guideline no. 471, Genetic Toxicology: Bacterial Reverse Mutation Test, adopted 21 July 1997

3 Study plan and deviations

3.1 Study plan

The study was conducted according to study plan P20805/05 entitled 'Bacterial reverse mutation test with 2'-fucosyllactose'. The study plan was approved by the study director on 27 September 2016.

3.2 Deviations

No deviations from the study plan occurred.

4 Materials and methods

4.1 Characterization of test substance

Test material name [1]	: 2'-fucosyllactose
Chemical name [1]	: 2'-FL
Batch number [1]	: MRS02
CAS number [1]	: 41263-94-9
Appearance [1]	: white powder
Purity [1]	: 94 %
Molecular formula[1]	: $C_{18}H_{32}O_{15}$
Molecular weight[1]	: 488.99 g/mol
Storage conditions [1]	: 2-10°C, protected from light
Date of receipt	: 19 July 2016
Expiry date [1]	: 15 July 2018
Supplier	: sponsor
Triskelion ref. no.	: 160161

The Certificate of Analysis of the batch of the test substance used for the study is included as Annex 6.

4.2 Other chemicals

Nicotinamide adenine dinucleotide phosphate, disodium salt (NADP) was obtained from Roche Diagnostics, Woerden, The Netherlands; Minimal glucose agar plates from Biotrading, Mijdrecht, The Netherlands; Biotine, L-histidine and L-Tryptophan from Merck KGaA, Darmstadt, Germany; D-glucose-6-phosphate, disodium salt (G-6-P), 9-aminoacridine (9-AA), N-ethyl-N-nitrosourea (ENU), dimethylsulphoxide (DMSO), Benzo(a)pyrene (B[a]P) from Sigma Chemical Company, St. Louis, USA; S9 from Trinova Biochem, Giessen, Germany and 2-nitrofluorene (2-NF), 2-amino-anthracene (2-AA) and sodium azide (NaN_3) from Aldrich, Brussels, Belgium.

[1] Characteristics provided by the sponsor

4.3 Characterization of the test system

The *Salmonella typhimurium* strains and the *Escherichia coli* WP2 *uvrA* strain were purchased from Trinova Biochem (Giessen, Germany) and were originally from Moltox Molecular Toxicology Incorporated (Boone, USA).

The genotype of the *Salmonella typhimurium* and *Escherichia coli* strains are given below:

		Additional mutations[1]		
Strain	Amino acid mutation	LPS	UV-repair	R-factor
TA 98	his D3052	rfa^-	$uvrB^-$	+R
TA 100	his G46	rfa^-	$uvrB^-$	+R
TA 1535	his G46	rfa^-	$uvrB^-$	-R
TA 1537	his C3076	rfa^-	$uvrB^-$	-R
WP2 *uvrA*	trp	rfa^+	$uvrA^-$	-R

[1] rfa: this mutation causes partial loss of the lipopolysaccharide (LPS) barrier that coats the surface of the bacteria; it increases the permeability to large molecules, e.g. crystal violet

uvrB/A: these mutations comprise deletions of a gene coding for the DNA excision repair system, which results in greatly increased sensitivity in detecting many mutagens including UV radiation

R-factor: the R-factor strains contain the plasmid pKM 101, which increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA-repair system normally present in *S. typhimurium*, it carries an ampicillin resistance gene

Frozen stocks of each strain were checked for histidine (his) or tryptophan (trp) requirement and for sensitivity to ampicillin, crystal violet and UV radiation. The results for the stocks used in this study are presented in Annex 2.

The S9 liver homogenate was purchased from Trinova Biochem (Giessen, Germany) and was originally from Moltox Molecular Toxicology Incorporated (Boone, USA). On the day of use, aliquots of S9 liver homogenate were thawed and mixed with a NADPH generating system. The final concentrations of the various ingredients in the S9-mix were: $MgCl_2$ 8 mM; KCl 33 mM; G-6-P 5 mM; NADP 4 mM; sodium phosphate 100 mM (pH 7.4), NaCl 46 mM, and S9 10 %. The S9-mix was kept on ice until use.

4.4 Experimental procedures

4.4.1 Dose levels of the test substance and reference substances

The plate-incorporation method was applied and the histidine-requiring *Salmonella typhimurium* mutants TA 1535, TA 1537, TA 98 and TA 100 and the tryptophan-requiring *Escherichia coli* mutant WP2 *uvrA* strains were used. The assay has been described in detail by Ames et al. (1975) and by Maron and Ames (1983). A preliminary test to assess the toxicity of

the test substance was not performed. Therefore, the toxicity test was incorporated in the mutagenicity assay.

PBS was used as the solvent for the test substance. Just before use, a solution of 50 mg/ml was prepared, based on the purity of the test substance (94 %). The stock solution was sterilized by passage through a 0.45 µm filter. The stock solution resulted in a clear colorless solution both before and after filter sterilization. Serial 3-fold dilutions of the test substance were prepared in the solvent; five concentrations were tested in all strains, ranging from 62 to 5000 µg/plate, both in the absence and presence of S9-mix.

The actual concentrations of the test substance in the test solutions were not determined. Therefore, the concentrations quoted in this report are nominal concentrations.

Negative controls (PBS) and positive controls were run simultaneously with the test substance in all experiments.

The reference mutagens used as positive controls were as follows:

Strain	in the absence of the S9-mix	in the presence of the S9-mix
TA 1535	sodium azide: 1.0 µg/plate	2-aminoanthracene: 2.0 µg/plate
TA 1537	9-aminoacridine: 80 µg/plate	benzo(a)pyrene: 4.0 µg/plate
TA 98	2-nitrofluorene: 2.0 µg/plate	2-aminoanthracene: 2.0 µg/plate
TA 100	sodium azide: 1.0 µg/plate	2-aminoanthracene: 2.0 µg/plate
WP2 *uvrA*	N-ethyl-N-nitrosourea: 100 µg/plate	2-aminoanthracene: 80 µg/plate

4.4.2 Mutation analysis

Fresh bacterial cultures were prepared by inoculation of nutrient broth with a thawed aliquot of the stock culture and subsequent incubation for 10-16 hours at ca. 37°C while shaking. Briefly, the mutagenicity assay was carried out as follows: to 2 ml molten top agar (containing 0.6 % agar, 0.5 % NaCl and 0.05 mM L-histidine.HCl and 0.05 mM biotin for the *S. typhimurium* strains or 0.05 mM tryptophane for the *E. coli* WP2 *uvrA* strain), was added subsequently: 0.1 ml of a fully grown culture of the appropriate strain, 0.1 ml of the test substance or of the negative control or of the positive control substance solution, and 0.5 ml S9-mix for the experiments with metabolic activation or 0.5 ml sodium phosphate 100 mM (pH 7.4) for the experiments without metabolic activation. The ingredients were thoroughly mixed and the mix was immediately poured onto minimal glucose agar plates (1.5 % agar in Vogel and Bonner medium E with 2 % glucose).
All determinations were made in triplicate. The plates were incubated for 48-72 hours at ca. 37°C. Subsequently, the his^+ or trp^+ revertants were counted.

4.5 Analysis of test results

The mutagenicity study was considered valid if the mean colony counts of the negative control values of the strains were within acceptable ranges, if the results of the positive controls met the criteria for a positive response (all as presented in Annexes 4 and 5), if no more than 5 % of the plates was lost through contamination or other unforeseen events and if at least three doses were non-toxic.

Toxicity was defined as a reduction (by at least 50 %) in the number of revertant colonies and/or a clearing of the background lawn of bacterial growth as compared to the negative control and/or the occurrence of pinpoint colonies.

A test substance was considered to be positive in the bacterial gene mutation test if the mean number of revertant colonies on the test plates was increased in a dose-related manner or if a two-fold and/or greater increase was observed compared to the negative control plates. A clear positive response would not need to be verified. Marginally or weakly positive results should be verified by additional testing.

A test substance was considered to be negative in the bacterial gene mutation test if it showed neither a dose-related increase in the mean number of revertant colonies nor a reproducible positive response at any of the concentrations tested.

Positive results from the bacterial reverse mutation test indicate that a test substance induces point mutations by base pair substitutions or frameshifts in the genome of either *Salmonella typhimurium* and/or *Escherichia coli*. Negative results indicate that, under the test conditions used, the test substance is not mutagenic in the tested strains.
Although most studies give clearly positive or negative results, in rare cases the data set may preclude making a definite judgement about the mutagenic potential of the test substance. Results may remain equivocal in this case.

Both numerical significance and biological relevance were considered together in the evaluation. No statistical analysis was performed.

Omission of a second test under these conditions is acceptable as a single test does not, or hardly ever results in false negative conclusions (Triskelion historical data in Annex 5 and Kirkland and Dean, 1994).

Historical data on the bacterial reverse mutation tests, including data on positive and negative controls, are presented in Annex 5.

5 Results and discussion

The results of the bacterial reverse mutation test are shown in Table 1 (Appendix 1).

A single test was performed. A stock solution of the test substance of 50 mg/ml in PBS was prepared, based on the purity (94 %), this resulted in a clear colorless solution. All strains, both in the absence and presence of S9-mix, were treated with five concentrations of the test substance, ranging from 62 to 5000 µg/plate. Negative controls (solvent) and positive controls were run simultaneously with the test substance

The mean numbers of his^+ (*S. typhimurium*) and trp^+ (*E. coli*) revertant colonies of the negative controls used were within the acceptable range in all strains, and the positive controls gave the expected increase in the mean numbers of revertant colonies. Therefore, the test was considered valid.

No toxicity was observed in any strain, this was evidenced by an absence of a clearing of the background lawn of bacterial growth compared to the negative controls, no decrease in the mean number of revertants was observed and pinpoint colonies did not occur.

In the test with strains TA 1535, TA 1537, TA 98, TA 100 and WP2 *uvrA*, in both the absence and presence of S9-mix, the test substance did not induce a more than 2-fold and/or dose related increase in the mean number of revertant colonies compared to the background spontaneous reversion rate observed with the negative control.

6 Conclusion

It is concluded that the results obtained in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100, and in the *Escherichia coli* strain WP2 *uvrA*, in both the absence and presence of the S9-mix, indicate that the test substance **2'-fucosyllactose** is **not mutagenic** under the conditions used in this study.

7 Documentation and retention of records

The following study specific materials will be archived for 5 years:

- Raw data (or true copies if unstable)
- Correspondence
- All other information related to the study

The following study specific materials will be archived for 15 years

- Original study plan and final report, and any amendments thereof

General raw data will be retained for at least 25 years, after which they may be destroyed without further notice. These may include, but are not necessarily limited to:

- Facility-based documents
- Calibration and quality control data
- General registrations potentially used for more than one study

Remaining test substance will be retained for at least one month and then returned to the sponsor.

At the end of the archiving period, the sponsor will be asked whether the study plan, final report, amendments, raw data and correspondence should be discarded, retained for an additional period, or transferred to the archives of the sponsor.

All materials will be retained in the archives of TNO, Utrechtseweg 48, 3704 HE Zeist, The Netherlands. The archiving period for starts on the cover date of the final report.

Appendix 1 – Results of the bacterial reverse mutation test

Table 1: Number of revertants counted in the bacterial reverse mutation test

	TA 1535		TA 1537		TA 98		TA 100		E. Coli	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0 µg/plate	34	23	16	11	31	44	144	162	53	52
	20	24	5	16	35	53	154	179	57	49
	30	27	14	17	41	51	164	167	44	66
Mean	28	25	12	15	36	49	154	169	51	56
StDev	7	2	6	3	5	5	10	9	7	9
62 µg/plate	23	23	17	22	46	38	154	161	57	55
	32	16	11	27	29	51	158	185	55	49
	30	26	19	24	23	52	132	210	69	62
Mean	28	22	16	24	33	47	148	185	60	55
StDev	5	5	4	3	12	8	14	25	8	7
185 µg/plate	18	23	22	18	40	45	145	187	53	55
	30	25	13	19	44	55	159	190	53	56
	27	25	11	16	36	62	137	187	53	55
Mean	25	24	15	18	40	54	147	188	53	55
StDev	6	1	6	2	4	9	11	2	0	1
556 µg/plate	29	34	17	17	35	40	179	180	58	69
	27	24	16	23	32	51	146	201	55	57
	29	20	14	16	28	57	155	192	62	72
Mean	28	26	16	19	32	49	160	191	58	66
StDev	1	7	2	4	4	9	17	11	4	8
1667 µg/plate	35	20	13	29	36	51	173	171	38	69
	23	27	15	23	30	61	155	181	72	61
	17	20	9	20	29	53	169	142	71	67
Mean	25	22	12	24	32	55	166	165	60	66
StDev	9	4	3	5	4	5	9	20	19	4
5000 µg/plate	32	29	12	26	42	38	180	202	72	69
	20	24	18	24	33	55	188	175	56	83
	38	27	17	16	33	43	153	204	73	52
Mean	30	27	16	22	36	45	174	194	67	68
StDev	9	3	3	5	5	9	18	16	10	16
Positive Control	832	268	2290	180	2086	1869	841	2562	430	589
	891	307	4394	234	2149	1946	1004	2668	457	605
	843	277	2112	226	1959	1904	937	2935	561	533
Mean	855	284	2932	213	2065	1906	927	2722	483	576
StDev	31	20	1269	29	97	39	82	192	69	38

Mean	Average number of revertants per plate
StDev	Standard deviation
S9	Liver homogenate from rats treated with aroclor
Pos. Control	Positive control; see text for actual concentrations of reference mutagens

Annex 1 – GLP compliance monitoring unit statement

ENDORSEMENT OF COMPLIANCE

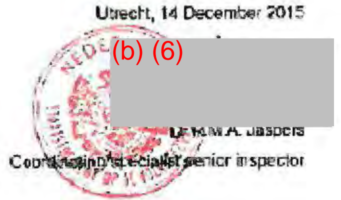
WITH THE OECD PRINCIPLES OF GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 2004/9/EC the conformity with the OECD Principles of GLP was assessed on 29 September-6 October and 9 December 2015 at

TNO Triskelion BV
Utrechtseweg 48, 3704 HE Zeist
PO Box 844, 3700 AV Zeist

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Toxicity, mutagenicity, analytical and clinical chemistry, safety pharmacology, kinetics, metabolism and in-vitro studies.

Utrecht, 14 December 2015



Dr. R.M.A. Jaspers
Coordinating specialist senior inspector

Health Care Inspectorate of the Ministry of Health, Welfare and Sport
Stadsplateau 1, 3521 AZ Utrecht
P.O. Box 2680, 3500 GR Utrecht, The Netherlands

Annex 2 – Characteristics of Salmonella typhimurium and Escherichia coli strains

Frozen stocks of each strain are yearly checked for histidine or tryptophan requirement and for sensitivity to ampicillin, crystal violet and UV radiation at the date of freezing. The results for the stocks used in the present assays are:

Strain	Stock date	Additional mutations[1]				
		rfa	uvr	R-factor	his	trp
TA 1535	06 August 2015	-	-	-	-	NT
TA 1537	15 January 2016	-	-	-	-	NT
TA 98	15 January 2016	-	-	+	-	NT
TA 100	15 January 2016	-	-	+	-	NT
WP2 *uvrA*	15 January 2016	±	-	-	NT	-

[1]rfa	: - = sensitive to crystal violet; ± = weak sensitive to crystal violet
uvr	: - = sensitive to UV radiation
R-factor	: - = sensitive to ampicillin; + = resistant to ampicillin
His	: - = requires histidine
Trp	: - = requires tryptophan
NT	: not tested

References

- Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for detecting carcinogens and mutagens with the *Salmonella* mammalian microsome mutagenicity test. Mutation Res. 31: 347-365.
- Maron, D.M. and B.N. Ames (1983) Revised methods for the *Salmonella* mutagenicity test. Mutation Res. 113: 173-215. + ERRATUM, Mutation Res. 113: 533.

Annex 3 – Quality control and production certificate of Aroclor 1254-induced rat liver homogenate

The batch of S9 was obtained from Trinova Biochem (Giessen, Germany) and was originally from Moltox Molecular Toxicology Incorporated (Boone, USA). The quality certificate was provided by the supplier.

TRiNOVA Biochem GmbH

MOLTOX

POST MITOCHONDRIAL SUPERNATANT (S9)
QUALITY CONTROL & PRODUCTION CERTIFICATE

Animal Information
SPECIES:
STRAIN:
SEX:
AGE:
WEIGHT:
TISSUE: Liver

Part Number Information
LOT NO.:
PART NO.:
VOLUME:
BUFFER:
STORAGE:

PREP:
EXPIRY:
INDUCING AGENT:

REFERENCE:

For Research Purposes Only

BIOCHEMISTRY
- PROTEIN:

Annex 4 – Acceptance ranges for negative and positive control data

Acceptable ranges for negative control data, plate incorporation method

Strain	revertant colonies per plate (with and without S9-mix): negative control, acceptable range
TA 1535	10 - 75
TA 1537	4 - 40
TA 98	20 - 95
TA 100	100 - 230
WP2 *uvrA*	47 - 98

Acceptable ranges for positive control data, plate incorporation method

Strain	in the absence of the S9-mix	Minimum Mutation Ratio	in the presence of the S9-mix	Minimum Mutation Ratio
TA 1535	sodium azide: 1.0 µg/plate	5	2-aminoanthracene: 2.0 µg/plate	5
TA 1537	9-aminoacridine: 80.0 µg/plate	10	Benzo(a)pyrene: 4.0 µg/plate	3
TA 98	2-nitrofluorene: 2.0 µg/plate	5	2-aminoanthracene: 2.0 µg/plate	3
TA 100	sodium azide: 1.0 µg/plate	3	2-aminoanthracene: 2.0 µg/plate	3
WP 2 *uvrA*	N-ethyl-N-nitrosourea: 100 µg/plate	3	2-aminoanthracene: 80 µg/plate	3

Mutation Ratio: number of induced revertants/number of control revertants.

Annex 5 – Historical data of bacterial reverse mutation test

False negative responses

Reproducibility between first and second assay with respect to predicting overall absence of mutagenicity. Data from studies until September 2016.

Mutagenicity: overall judgement	-			+	+		-
Mutagenicity: judgement First / second assay	- / -	- / +	+ / -	- / +	+ / +	+/-	+/- (second assay according to 'treat and plate')
Number of studies	151	3	22	0	18	0	14

- non-mutagenic (negative)

+ mutagenic (positive)

Annex 5 – continued

Historical solvent controls

Demonstration of the absence of mutagenic effects for several commonly used solvents. Data from assays conducted between 2011 and September 2016.

Strain	Mean ± SD number of revertants per plate (number of assays)			
	Methanol/ ethanol	water	DMSO	PBS
without S9-mix				
TA 1535	23 ± 10 (3)	26 ± 13 (10)	22 ± 7 (27)	20 ± 3 (4)
TA 1537	10 ± 1 (2)	11 ± 3 (11)	11 ± 3 (30)	10 ± 4 (4)
TA 98	23 ± 0 (2)	30 ± 4 (10)	27 ± 4 (25)	30 ± 7 (4)
TA 100	112 ± 7 (2)	144 ± 23 (11)	148 ± 34 (27)	133 ± 6 (4)
WP2 *uvrA*	56 ± 12 (2)	60 ± 8 (10)	58 ± 7 (23)	52 ± 5 (4)
with S9-mix				
TA 1535	30 ± 13 (2)	18 ± 5 (10)	20 ± 5 (26)	21 ± 4 (4)
TA 1537	9 ± 1 (2)	17 ± 4 (10)	15 ± 5 (30)	17 ± 6 (4)
TA 98	50 ± 2 (2)	49 ± 9 (10)	44 ± 7 (27)	50 ± 11 (4)
TA 100	139 ± 14 (2)	149 ± 20 (10)	147 ± 23 (28)	171 ± 14 (4)
WP2 *uvrA*	62 ± 4 (2)	71 ± 13 (10)	68 ± 7 (24)	74 ± 13 (4)

Historical negative control (solvent) data, all solvents together.
Data from assays conducted between 2011 and September 2016.

	Number of revertants per plate mean ± standard deviation; range; (number of assays)					
Strain	without S9-mix			with S9-mix		
TA 1535	23 ± 8	13-60	(44)	20 ± 6	7-39	(43)
TA 1537	11 ± 3	5-17	(48)	15 ± 5	6-24	(47)
TA 98	28 ± 5	18-36	(42)	46 ± 8	26-69	(44)
TA 100	144 ± 29	91-219	(45)	150 ± 22	109-192	(45)
WP2 *uvrA*	58 ± 8	47-78	(40)	69 ± 10	52-98	(41)

Annex 5 – continued

Historical positive controls

Overview historical positive control.

Data from assays conducted between 2011 and September 2016.

Strain	Compound[2]	Mutation Ratio[1] mean ± standard deviation; range (number of assays)		
without S9-mix				
TA 1535	NaN_3, 1 µg/plate	31 ± 03	8-61	(44)
TA 1537	9-AA, 80 µg/plate	207 ± 91	73-469	(48)
TA 98	2-NF, 2 µg/plate	42 ± 16	13-82	(42)
TA 100	NaN_3, 1 µg/plate	6 ± 1	3-9	(45)
WP2 *uvrA*	ENU, 100 µg/plate	11 ± 3	5-18	(40)
with S9-mix				
TA 1535	2-AA, 2 µg/plate	18 ± 7	8-43	(43)
TA 1537	BP, 4 µg/plate	19 ± 12	4-69	(47)
TA 98	2-AA, 2 µg/plate	30 ± 11	14-69	(44)
TA 100	2-AA, 2 µg/plate	15 ± 6	3-25	(45)
WP2 *uvrA*	2-AA, 80 µg/plate	9 ± 4	5-24	(41)

1 Mutation Ratio: number of induced revertants/number of control revertants

2 NaN_3 = natrium azide
ENU = N-nitroso N-ethylurea
2-AA = 2-aminoanthracene
9-AA = 9-aminoacridine
BP = benzo(a)pyrene
2-NF = 2-nitrofluorene

Annex 6 – Certificate of analysis

[illegible]	[illegible] FL
[illegible]	[illegible] (sovidsomentsi product)
[illegible]	[illegible]
Date of production	02-07-2016
Contact person	[illegible] (R&D)

Description — Human milk oligosaccharide

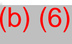
Typical analysis — Dry matter 97%, moisture 3%, 2'-Fucosyllactose 94%, lactose 1%, glucose 1%, fucose 1%

Chemical/ physical	Specification	Results	Method of analysis
Total moisture	max 5%	3%	ISO 760 (modified), Karl Fischer
2'-Fucosyllactose	min 90%	> 94 %	FC-method using HPAEC-PAD
3-Fucosyllactose	max 3%	< 1%	FC-method using HPAEC-PAD
Difucosyllactose	max 3%	< 1%	FC method using HPAEC-PAD
Fucose	max 2%	< 1%	FC method using HPAEC-PAD
Lactose	max 2%	< 1%	FC method using HPAEC-PAD
Glucose	max 2%	< 1%	FC method using HPAEC-PAD
Protein	max 0,01%	0,002%	Bradford
Sulphated ash	max 0,2%	0,06%	NEN 6810 (modified)
Nitrite	max 1 mg/kg	< 0,[illegible]	ISO 14673-3/IDF 189-2
Nitrate	max 50 mg/kg	0,2	ISO 14673-2/IDF 189-2
pH (10%)	[illegible]	[illegible]	FC method using NEN 3775

Microbiological			
Aerobic mesophilic count	max 500 cfu/g	< 100	FC method equivalent to ISO 4833
Enterobacteriaceae	absent in 1 g	< 1	FC method, BPW 18h 37°C, 50 VRBG 18-24h 37°C
E. coli	absent in 1 g	< 1	FC method, LMX 25h Col 10 24h
Yeasts	max 50 cfu/g	< 1	FC method equivalent to ISO 6611
Moulds	max 50 cfu/g	< 1	FC method equivalent to ISO 6611
Presumptive Bacillus cereus	max 100 cfu/g	< 1	FC method equivalent to ISO 7932
Staphylococcus aureus	absent in 1 g	< 1	FC method, GBC 42h 37°C, PCR
Sulphite reducing clostridia spores	max 30 cfu/g	< 1	FC method using DFM 27 (1995) 165-200 [illegible]
Clostridium perfringens	absent in 1 g	neg	FC method, RPM 20h 46°C, confirmation
Salmonella	absent in 1 g	neg	FC method equivalent to ISO 6579
Cronobacter spp.	absent in 1 g	neg	FC method equivalent to ISO/TS 22964

Wageningen, 15-07-2016

Jan-Willem Boots



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Appendix 12 *In vitro* Micronucleus Test with 2'-Fucosyllactose in Cultured Human Lymphocytes

STUDY REPORT

V20817/05

In vitro micronucleus test with 2'-fucosyllactose in cultured human lymphocytes

DATE	**4 April 2017**
AUTHOR(S)	**B. Usta, BSc**
SPONSOR	**Friesland Campina Innovation** **Bronland 20** **6708WH Wageningen** **The Netherlands**
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Statement of GLP compliance

I, the undersigned, hereby declare that this report constitutes a complete, true and accurate representation of the study and its results. All study activities performed by Triskelion were carried out in compliance with the current OECD Principles of Good Laboratory Practice[1]. The OECD principles of Good Laboratory Practice are accepted by Regulatory Authorities throughout the European Community, USA and Japan. Chemical analysis for the verification of the test substance identity and properties was not performed in this study.

Study director

(b) (6)

04 April 2017
Date

[1] *The most recent endorsement of compliance of the test facility with these principles is attached to the report as Annex 1.*

Quality Assurance Statement

I, the undersigned, hereby declare that this report provides an accurate record of the procedures employed and the results obtained in this study; all audits were study-based and were reported to the study director and management on the dates indicated.

Phase	Start date of audit	Date of audit report
Authorised study plan	4 October 2016	4 October 2016
Authorised study plan amendment 1	4 April 2017	4 April 2017
Cell washing	12 October 2016	12 October 2016
Cell harvesting	13 October 2016	13 October 2016
Slide preparation	24 October 2016	24 October 2016
Counting micronuclei	24 October 2016	24 October 2016
Preparation of dosing solutions	2 November 2016	2 November 2016
Draft report and study file	29 March 2017	29 March 2017
Final report	4 April 2017	4 April 2017

(b) (6)

M.T.A. Wolters Date : 4-4-2017
Quality Assurance auditor

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Abbreviations

GLP	Good Laboratory Practice
OECD	Organisation for Economic Co-operation and Development
QA	Quality Assurance
QAU	Quality Assurance Unit

Summary

The test substance, 2'-fucosyllactose was examined for its potential to induce micronuclei in cultured binucleated human lymphocytes, in both the absence and presence of a metabolic activation system (S9-mix). Two independent test were conducted for which blood was obtained from two different donors. Culture medium (RPMI1640) was used as a solvent for the test substance. The final concentrations of the test substance in the cultures ranged from 3.9 to 2000 µg/ml. In the both tests, the maximum final concentration in the culture medium was 2000 µg/ml, based on the purity of the test substance. Duplicate cultures were used in all experiments. Cytotoxicity was determined from the Cytokinesis-Block Proliferation Index (CBPI). In the first test, in the presence and absence of S9-mix, the treatment/recovery time was 4/20 hours (pulse treatment). In the second test, in the continuous treatment group the treatment/recovery time was 24/0 hours. Solvent control and positive controls were run in parallel.

In the performed experiments, the solvent control was within the range of historical data of the test facility. Treatment with the positive controls Cyclophosphamide and Vinblastine sulphate resulted in statistically significant increases in the numbers of binucleated cells containing micronuclei, when compared to the numbers observed in the cultures treated with the solvent control in both experiments. This demonstrates the validity of the study.

In the first experiment, in the pulse treatment groups both with and without S9-mix, the test substance did not show a clear cytotoxicity to the cells. In the pulse treatment group with S9-mix, at a concentration of 1000 µg/ml, the observed marginal cytotoxicity (13%) was considered to be not biologically relevant. Three dose levels (2000, 1000 and 500 µg/ml) of the test substance, together with the solvent control and positive control were analyzed for micronucleus induction in binucleated lymphocytes. In both pulse treatment groups, the test substance did not show a statistically significant increase in the number of binucleated cells containing micronuclei at any of the concentrations analyzed when compared to the concurrent solvent control cultures.

In the second experiment, in the continuous treatment group without S9-mix, no cytotoxicity was observed at the analyzed concentrations, except at a concentration of 1000 µg/ml. At this concentration the test substance was slightly cytotoxic to the cells. Due to the absence of a dose related cytotoxicity, the observed cytotoxicity of 18% was considered to be not biologically relevant. Three dose levels (2000, 1000 and 500 µg/ml), together with the solvent control and positive control were analyzed for micronucleus induction in binucleated lymphocytes. The test substance did not show a statistically significant increase in the number of binucleated cells containing micronuclei at any of the concentrations analyzed when compared to the concurrent solvent control.

From the results obtained in the *in vitro* micronucleus test it is concluded that, under the conditions used in this study, the test substance, 2'-fucosyllactose, was not clastogenic and/or aneugenic to cultured human lymphocytes.

1 General

1.1 Study Sponsor

Sponsor:	**Friesland Campina Innovation** **Bronland 20** **6708WH Wageningen** **The Netherlands**
Monitor:	**D. Delsing, PhD**
Phone:	**+31 6 5359 8111**
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1.2 Test facility

Triskelion B.V.	**www.triskelion.nl**
Postal address:	**P.O. Box 844** **3700 AV Zeist** **The Netherlands**
Location:	**Utrechtseweg 48** **3704 HE Zeist** **The Netherlands**
Phone:	**+31 88 866 2800**
Study director:	F.A.A. van Acker
Phone:	**+31 88 866 2618**
E-mail:	**frederique.vanacker@triskelion.nl**

1.3 Time schedule

Experimental start date: 6 October 2016
Experimental completion date: 10 November 2016

2 Introduction

2.1 Objective and background

The purpose of this study was to determine the potential of the test substance, 2'-fucosyllactose to induce micronuclei *in vitro* in binucleated human lymphocytes. The *in vitro* micronucleus test was used for the detection of chemicals that induce the formation of small membrane-bound DNA fragments in the form of micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes that were unable to migrate with the rest of the chromosomes during the anaphase of cell division. The assay thus has the potential to detect the activity of both clastogenic and aneugenic chemicals. The actin polymerisation inhibitor cytochalasin B, added during the target mitosis, allowed the identification of nuclei that have undergone one division as binucleates. At predetermined intervals after treatment, the cells were harvested, fixed and transferred onto microscopic slides. After staining, the slides were analyzed microscopically for the presence of micronuclei in binucleated cells.

2.2 Applicable guidelines

The study plan has been drafted in accordance with the following guideline:
OECD guideline 487 for the testing of chemicals: *In Vitro* Mammalian Cell Micronucleus Test (MNvit); adopted 29 July 2016.

3 Study plan and deviations

3.1 Study plan

The study was conducted according to study plan P20817/05 entitled: "In vitro micronucleus test with 2'-fucosyllactose in cultured human lymphocytes" and one amendment. The study plan was approved by the study director on 30 September 2016.

3.2 Deviations

No deviations from the study plan occurred.

4 Materials and methods

4.1 Characterization of the test substance

Test material name [1]	: 2'-fucosyllactose
Chemical name [1]	: 2'-FL
Batch number [1]	: MRS02
CAS number [1]	: 41263-94-9
Appearance [1]	: white powder
Purity [1]	: 94%
Molecular formula [1]	: $C_{18}H_{32}O_{15}$
Molecular weight [1]	: 488.99 g/mol
Storage conditions [1]	: 2-10°C, protected from light
Expiry date [1]	: 15 July 2018
Supplier	: sponsor
Triskelion ref. no.	: 160161

The Certificate of Analysis of the batch of the test substance used for this study was provided by the sponsor and is included as Annex 5.

4.2 Characterization of the positive control substances

Indirect acting clastogenic positive control:

Name	: Cyclophosphamide
Appearance	: white plaque
Batch number	: 3J047
CAS Reg. Number	: 6055-19-2
Molecular formula	: $C_7H_{15}Cl_2N_2O_2P.H_2O$
Molecular weight	: 279.10 g/mol
Purity	: 100%
Storage conditions	: ambient temperature (15-25°C)
Date received	: 18 March 2014
Expiry date	: 31 October 2016
Supplier	: Baxter B.V.
Triskelion dispense no.	: 1400A5

[1] Characteristics provided by the sponsor

Aneugenic positive control:

Name	: Vinblastine sulphate
Appearance	: a white powder
Batch number	: 35366
CAS Reg. Number	: 143-67-9
Molecular formula	: $C_{46}H_{58}N_4O_9$
Molecular weight	: 810.974 g/mol
Purity	: 100%
Storage conditions	: 2-10 ºC
Date received	: 11 July 2014
Expiry date	: 11 July 2019
Supplier	: Sigma-Aldrich
Triskelion GROS no.	: 104661

4.3 Tissue culture media and other chemicals

Fetal calf serum; RPMI 1640 medium (with HEPES and Glutamax) and penicillin-streptomycin were purchased from Life Technologies, Paisley, U.K.; nicotinamide-adenine dinucleotide phosphate disodium salt (NADP) from Roche Diagnostics, Almere, The Netherlands; glacial acetic acid from Merck-Darmstadt, Darmstadt, Germany; methanol from Biosolve, B.V., Valkenswaard, the Netherlands; dimethylsulfoxide (DMSO), D-glucose-6-phosphate disodium salt (G-6-P), Vinblastine sulphate, acridine-orange and Cytochalasin B from Sigma-Aldrich Chemie GmbH, Germany; phytohemagglutinin (PHA-L) from BioChrom AG, Germany; Cyclophosphamide from Baxter B.V., Utrecht, the Netherlands.

4.4 Characterisation of the test system

Blood samples were obtained by venapuncture from two young healthy, non-smoking individuals (37 and 28 years old) with no known recent exposures to genotoxic chemicals or radiation. The blood was collected in sterile, heparinized vacutainer tubes and gently mixed before use to prevent clotting. A different donor was used for the first and second test. The cultures were set up within 1 hour after withdrawal of the blood.

The medium for culturing the human peripheral blood lymphocytes consisted of RPMI 1640 medium (with HEPES and Glutamax), supplemented with heat-inactivated (30 min, 56ºC) fetal calf serum (20% v/v), penicillin (100 U/ml medium), streptomycin (100 µg/ml medium) and phytohemagglutinin (2.4 µg/ml).

4.5 Metabolic activation system

The S9-mix consisted of a liver homogenate fraction (S9) and cofactors as described by Ames et al. (1975) and Maron and Ames (1983). The S9 liver homogenate used in this study was purchased from Trinova Biochem (Giessen, Germany) and were originally from Moltox Molecular Toxicology Incorporated (Boone, USA). Annex 2 presents the quality of the used S9 batch. Immediately before use, S9-mix was prepared by mixing the thawed S9 with a NADPH-generating system. The final concentrations of the various ingredients in the S9-mix were: magnesium chloride 8 mM; potassium chloride 33 mM; G-6-P 5 mM; NADP 4 mM; sodium phosphate 100 mM (pH 7.4) and S9 40% (v/v). The final concentration of the S9 in the culture medium was 4% v/v.

4.6 Preliminary tests / measurements

A maximum stock concentration of 20 mg/ml was prepared in culture medium (RPMI1640) based on the purity of 94%. Serial dilutions of 10, 5, 2.5 and 1.25 mg/ml were prepared from the stock concentration in culture medium. Subsequently, 0.5 ml of the stock solution and serial dilutions were added to 4.5 ml culture medium without serum. The final concentrations of the test substance were: 2000, 1000, 500, 250 and 125 µg/ml. Shortly after preparation at ambient temperature changes with respect to the test substance were checked visually. In addition, pH and osmolality measurements were performed. The results are summarized in Appendix 1, Tables 1.1 and 1.2.

4.7 Dose levels in the experiments

In the first test, pulse treatment both with and without metabolic activation was conducted. Prior to dosing, a stock concentration of 20 mg/ml was prepared in culture medium based on the purity of 94%. The stock concentration was briefly mixed. Hereafter, serial stock dilutions (10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078 and 0.039 mg/ml) were prepared in culture medium from the stock concentration.

In the pulse treatment group both with and without metabolic activation the final concentrations of the test substance in culture medium were: 2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8 and 3.9 µg/ml.

In the second test, continuous treatment was conducted. Prior to dosing, a stock concentration of 20 mg/ml was prepared as described in the first test. The serial dilutions of the test substance were: 15, 10, 7.5, 5, 2.5, 1.25, 0.625, 0.313 and 0.156 mg/ml in culture medium from the stock concentration. The final concentrations of the test substance were: 2000, 1500, 1000, 750, 500, 250, 125, 62.5, 31.3 and 15.6 µg/ml.

The concentrations of the test substance were not determined analytically; they were therefore nominal concentrations.

4.8 Experimental procedures

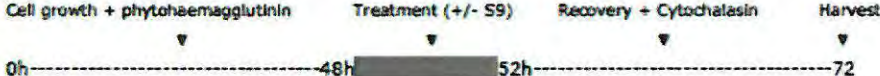
In the presence of phytohemagglutinine (PHA-L), aliquots of 0.5 ml of whole blooc in 4.5 ml culture medium, were incubated for 48 hours at *ca.* 37ºC in humidified air containing *ca.* 5% CO_2. The incubation was carried out in sterile (loosely) screw-capped centrifuge tubes. At approximately 48 hours after initiation of the cultures, the cells were harvested by low speed centrifugation and suspended in freshly prepared tissue culture medium without fetal calf serum and PHA-L. Subsequently, the cultures were exposed to different concentratons of the test substance, solvent or positive control as described in paragraphs 4.9 and 4.10. Duplicate cultures were used for each test group.

4.9 First test

A volume of 0.5 ml of the test substance concentrations, solvent control (RPMI1640 medium) or 50 µl positive control solution was added to the tissue culture medium in individual culture tubes. Cyclophosphamide, which requires metabolic activation in order to induce a clastogenic response, was dissolved in culture medium to a concentration of 2 mg/ml and used as positive control for the pulse treatment group in the presence of S9-mix (final concentration 20 µg/ml). This single positive control response is considered to demonstrate both the activity of the S9-mix and the response of the test system. To all cultures of the pulse treatment groups in the presence of the S9-mix, 0.5 ml of S9-mix (see paragraph 4.5) was added. To all

cultures of the pulse treatment groups in the absence of S9-mix, an additional 0.5 ml culture medium was used instead. The total volume in each culture was 5 ml. After the 4 hours treatment period, the culture medium was removed. The cells were washed twice with phosphate-buffered saline (pH 7.4) and subsequently supplied with 5 ml freshly prepared culture medium enriched with fetal calf serum (20%), PHA-L and cytochalasin B (6 µg/ml; final concentration). The cells were incubated for an additional 20 hours at *ca.* 37°C in humidified air containing ca. 5% CO_2 and harvested 72 hours after initiation of the cultures (second cell-cycle). A schematic overview of the pulse treatment groups are presented below.

Pulse treatment method (4 hours) with and without S9-mix:



4.10 Second test

In the continuous treatment group, a volume of 0.5 ml of the test substance concentrations, solvent control or 50 µl positive control solution was added to the tissue culture medium in individual culture tubes. Vinblastine Sulphate was dissolved in culture medium to a concentration of 1.25 µg/ml and used as positive control (final concentration 0.0125 µg/ml). The total volume in each culture was 5 ml. The cells were incubated for 24 hours at ca. 37°C in humidified air containing *ca.* 5% CO_2 and harvested 72 hours after initiation of the cultures (second cell-cycle). A schematic overview of the experiments is presented below.

Continuous treatment method (24 hours)

4.11 Harvesting and slide preparation

At the end of the total incubation period the cells in each culture were harvested and processed. The cells were harvested by low speed centrifugation, briefly treated with a hypotonic solution (0.075 M potassium chloride), fixed three times with a freshly prepared mixture of methanol and acetic acid, spread on clean slides and air dried. All procedures were performed at room temperature.

Three slides were prepared from each selected culture of the test substance, the solvent controls and positive controls. The slides were coded by a qualified person not involved in scoring the slides to enable "blind" scoring and thereafter stained with a fluorescence DNA-specific dye (acridin-orange) for analysis.

One slide per culture was analyzed for Cytokinesis-Block Proliferation Index (CBPI) and two slides were analyzed for micronucleus formation.

4.12 Microscopic analysis of the slides

Quantitative evaluation of cytotoxicity was performed using the CBPI. The CBPI indicates the average number of cell cycles per cell during the period of exposure to cytochalasin B. The CBPI was determined from at least 500 cells per slide (in total 1000 cells per dose level) and was used to calculate cell proliferation and to estimate the percentage of cytotoxicity by comparing values in the treated and negative control cultures.

The CBPI, the replication index (RI) and cytotoxicity were calculated as follows:

$$CBPI = \frac{\text{no. of mononucleates} + 2 \times \text{no. of binucleates} + 3 \times \text{no. of multinucleates}}{\text{(total number of cells)}}$$

$$\text{Replication index (\%)} = 100 \times \frac{CBPI^{T(mean)} - 1}{CBPI^{C(mean)} - 1}$$

Cytotoxicity (%) = 100-Replication index

$^{T (mean)}$ Mean of two cultures treated with the test substance
$^{C (mean)}$ Mean of two cultures treated with the negative control

The CBPI was calculated for treated (selected doses) and control cultures as a measure of cell cycle delay. If observed, the concurrent measures of cytotoxicity (cell density on the slides, signs of apoptosis or necrosis) were recorded for all treated and negative control cultures. Based on the evaluation of cytotoxicity, analysis of micronucleus formation was carried out at least on three analyzable concentrations of the test substance, together with the solvent and the positive control cultures. Where cytotoxicity occurred, the concentrations selected aimed to cover a range from that producing 55 ± 5% cytotoxicity, moderate and little or no cytotoxicity.
At least two thousand binucleated cells per concentration (1000 per culture) were examined for the presence of micronuclei. Criteria for scoring cytokinesis-blocked (binucleated) cells and micronuclei are presented in Annex 3 of this report.

4.13 Evaluation and interpretation of the results

The frequencies of micronuclei found in the cultures treated with the test substance and positive control cultures were compared with those of the concurrent solvent control using the Chi-square test (one-sided). The results were considered statistically significant when the p-value of the Chi-square test was less than 0.05.

The study was considered valid if the clastogenic and aneugenic positive controls gave a statistically significant increase in the number of binucleated cells containing micronuclei and if the solvent controls were within the historical data of the test facility.
The response was considered clearly positive if all of the following criteria are met:
- at least one of the test concentrations exhibits a statistically significant increase compared to the concurrent negative control.
- the increase is dose-related in at least in one experimental condition when evaluated with an appropriate trend test
- any of the results are outside the distribution of the historical solvent control data.

A response was considered clearly negative if all of the following criteria are met:
- none of the test concentrations exhibits a statistically significant increase compared to the concurrent negative control.
- there is no dose-related increase when evaluated with an appropriate trend test
- all results are inside the distribution of the historical negative control data.

A test result was considered equivocal if the response was neither positive or negative even after further investigation.

Statistical methods were used as an aid in evaluating the test results. Both biological relevance and statistical analysis were considered in evaluation of the response. Biological relevance was evaluated by comparison of the test results with the test facility's historical range of the solvent control.

5 Results and discussion

The potential clastogenic and/or aneugenic effect of the test substance, 2'-fucosyllactose, was investigated using the *in vitro* micronucleus test. The distribution of mononucleated, binucleated and multinucleated cells was assessed to calculate proliferation indices (CBPI) and percentage cytotoxicity. In both experiments, cells were treated with the test substance up to the maximum final concentration of 2 mg/ml, based on the purity, as requested by the OECD guideline 487. The selection of dose levels for micronucleus analysis was based on the cytotoxicity as determined by the CBPI index. Subsequently, the number of binucleated cells containing one or more micronuclei were analysed to assess the clastogenic and/or aneugenic potential of the test substance.

There were no aberrant findings observed during the performance of the first and the second experiment of the *in vitro* micronucleus test with respect to the test substance and culture medium. The results of the experiments are summarized in Appendix 2, Tables 2.1 - 2.3. Annex 4 presents the historical data of *in vitro* micronucleus tests in cultured human lymphocytes performed at the test facility.

5.1 Preliminary tests / measurements

In the solubility test, it was observed that culture medium (RPMI 1640) was a suitable vehicle for the test substance. The stock concentration of 20 mg/ml appeared to be a clear solution showing no discoloration when compared to the solvent (culture medium). The osmolality and pH values were determined shortly after preparation at ambient temperature. The obtained pH and osmolality results were within the normal values (Appendix 1, Tables 1.1 – 1.2). Based on the observations during the solubility test and measurements, it was decided to use 2000 µg/ml as the maximum final concentration in the culture medium for the pulse treatment groups, in both the absence and presence of S9-mix.

5.2 Micronuclei induction as a result of treatment with the solvent control and positive controls

In both experiments, the solvent control (culture medium) was within the range of historical data of the test facility. Treatment with the positive control substances Cyclophosphamide and Vinblastine sulphate resulted in statistically significant increases in the number of binucleated cells containing micronuclei, when compared to the numbers found in the concurrent solvent control cultures. This demonstrated the validity of the *in vitro* micronucleus test (Appendix 2, Tables 2.1-2.3).

5.3 Cytotoxicity observed in the first and second test

In the first experiment, in the pulse treatment group with S9-mix, no cytotoxicity was observed at the analyzed concentrations, except for the concentration of 1000 µg/ml. At this concentration the test substance was very slightly cytotoxic (13%) to the cells. Due to the absence of a dose related cytotoxicity, the observed cytotoxicity at this concentration was considered to be a chance finding and not biologically relevant. In the pulse treatment group without S9-mix, no cytotoxicity was observed at any of the concentrations analysed when compared to the concurrent solvent control cultures. The positive control substance Cyclophosphamide (20 µg/ml) showed 59% cytotoxicity (Appendix 2,Tables 2.1 - 2.2).

In the second experiment, in the continuous treatment group without S9-mix, no cytotoxicity was observed at the analyzed concentrations when compared to the concurrent solvent control cultures, except for the concentration of 1000 µg/ml. At this concentration the test substance was slightly cytotoxic (18%) to the cells. Due to the absence of a dose related cytotoxicity, the observed cytotoxicity at this concentration was considered to be a chance finding and not biologically relevant. The positive control substance Vinblastine sulphate (0.025 µg/ml) showed 82% cytotoxicity (Appendix 2, Table 2.3)

5.4 Micronuclei induction as a result of treatment with the test substance

In all treatment groups, three test substance concentrations (2000, 1000 and 500 µg/ml), together with the solvent control and positive control were analyzed for micronucleus induction in binucleated lymphocytes. In all treatment groups, the test substance did not show a statistically significant, dose-dependent increase in the number of binucleated cells containing micronuclei at any of the concentrations analyzed when compared to the concurrent solvent cultures (Appendix 2, Table 2.1 - 2.3). In addition, the number of binucleated cells containing micronuclei were within the historical data range of the test facility (Annex 4).

6 Conclusion

From the results obtained in the *in vitro* micronucleus test it is concluded that, under the conditions used in this study, the test substance 2'-fucosyllactose was not clastogenic and/or aneugenic to cultured human lymphocytes.

7 Documentation and retention of records, samples and specimens

The following study specific materials will be archived for 5 years:
- Raw data (or true copies if unstable)
- Microscopic slides

The following study specific materials will be archived for 15 years:
- Original study plan and final report, and any amendments thereof

General raw data will be retained for at least 25 years, after which they may be destroyed without further notice. These may include, but are not necessarily limited to:
- Facility-based documents
- Calibration and quality control data
- General registrations potentially used for more than one study

The sponsor will be asked whether the study plan, final report, amendments, raw data, including microscopic slides, and correspondence should be discarded, retained for an additional period, or transferred to the archives of the sponsor.

All materials will be retained in the archives of TNO, Utrechtseweg 48, 3704 HE Zeist, The Netherlands. The archiving period for starts on the cover date of the final report.

8 References

- OECD guideline 487 for the testing of chemicals: *In Vitro* Mammalian Cell Micronucleus Test (MNvit); adopted 29 July2016.
- Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for detecting carcinogens and mutagens with the *Salmonella* mammalian microsome mutagenicity test. Mutation Res. 31: 347-365.
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- Fenech, M. et al. (2003), HUMN project: intra- and inter-laboratory variation in the scoring of micronuclei and nucleoplasmic bridges in binucleated human lymphocytes Results of an international slide-scoring exercise by the HUMN project, *Mutation Research*, Vol 534, 25-64.
- Lorge, E. et al. (2006), SFTG International collaborative study on *in vitro* micronucleus test. General conditions and overall conclusions of the study, *Mutation Research*, Vol 607/1, pp. 13-36.

Appendices

Appendix 1: Observations during solubility test and measurements

Table 1.1: Changes with respect to the test substance in culture medium without serum.

Final concentrations (µg/ml)	Results shortly after preparation at room temperature*.
2000	Appeared to be a clear medium color solution.
1000	
500	
250	
125	

*) no entry: no aberrant findings

Table 1.2: Osmolality and pH measurements

Final concentrations (µg/ml) in culture medium	pH measurements	Osmolality measurements (mOsmol/kg)
NC	7.19	300
2000	7.21	308
1000	7.24	304
500	7.25	303
250	7.25	302
125	7.24	302

NC: negative control (culture medium RPMI1640)

Appendix 2: Tables of results

Table 2.1 Test 1▶Pulse treatment method with metabolic activation

Treatm / recovery time (h)	Dose level (µg/ml)	Cell stage analysis/500 (MO-BN-MU)			BN (%)	CBPI	CBPI (mean)	RI (%)	% Cytotox. (100-RI)	Selected for MN analysis (+/-)	MNBN/ 1000BN	MNBN/ 2000 BN (%)	Statistics[1] (p-value)
4/20 (+S9)	NC	243	255	2	51.00	1.52	1.52	100	0	+	7	14	-
		250	243	7	48.60	1.51					7	(0.70)	
	2000	253	244	3	48.80	1.50	1.48	92	8	+	8	17	n.s.
		278	217	5	43.40	1.45					9	(0.85)	
	1000	278	221	1	44.20	1.45	1.45	87	13	+	7	13	n.s.
		275	222	3	44.40	1.46					6	(0.65)	
	500	259	238	3	47.60	1.49	1.47	90	10	+	4	13	n.s.
		281	217	2	43.40	1.44					9	(0.65)	
	250	253	242	5	48.40	1.50	1.49	95	5	-	-	-	-
		262	237	1	47.40	1.48					-		
	125	270	227	3	45.40	1.47	1.47	91	9	-	-	-	-
		269	228	3	45.60	1.47					-		
	62.5	271	223	6	44.60	1.47	1.47	92	8	-	-	-	-
		265	232	3	46.40	1.48					-		
	CP20	397	102	1	20.40	1.21	1.21	41	59	+	25	51	<0.0001
		393	106	1	21.20	1.22					26	(2.55)	***

The fixed cells of dose levels (3.9 to 31.3 µg/m)l were stored without slide preparation.

Abbreviations:
Treatm: treatment time
Cytotox: cytotoxicity
MO: Mononucleated Cells
BN: Binucleated Cells
MU: Multinucleated Cells
CBPI: Cytokinesis-Block Proliferation Index
RI: Replication index
MN: Micronuclei
MNBN: Micronucleated Binucleated Cells
NC: negative control (culture medium RPMI1640),
CP: Cyclophosphamide
n.s: not significant compared to the concurrent control
- : not selected
[1] Chi-square test (one-sided); *** p≤0.0001

Appendix 2 – continued

Table 2.2 Test 1▶Pulse treatment method without metabolic activation

Treatm / recovery time (h)	Dose level (µg/ml)	Cell stage analysis/500 (MO-BN-MU)			BN (%)	CBPI	CBPI (mean)	RI (%)	% Cytotox. (100-RI)	Selected for MN analysis (+/-)	MNBN/ 1000BN	MNBN/ 2000 BN (%)	Statistics[1] (p-value)
4/20 (-S9)	NC	245	249	6	49.80	1.52	1.52	100	0	+	8	17	-
		246	246	8	49.20	1.52					9	(0.85)	
	2000	246	245	9	49.00	1.53	1.54	103	0	+	8	15	n.s.
		230	265	5	53.00	1.55					7	(0.75)	
	1000	236	259	5	51.80	1.54	1.52	100	0	+	8	15	n.s.
		258	229	13	45.80	1.51					7	(0.75)	
	500	237	253	10	50.60	1.55	1.56	106	0	+	9	17	n.s.
		220	278	2	55.60	1.56					8	(0.85)	
	250	225	272	3	54.40	1.56	1.57	108	0	-	-	-	-
		219	275	6	55.00	1.57					-		
	125	251	242	7	48.40	1.51	1.50	96	4	-	-	-	-
		259	234	7	46.80	1.50					-		
	62.5	227	267	6	53.40	1.56	1.58	111	0	-	-	-	-
		210	279	11	55.80	1.60					-		

The fixed cells of dose levels (3.9 and 31.3 µg/m)l were stored without slide preparation.

Abbreviations:
Treatm: treatment time
Cytotox: cytotoxicity
MO: Mononucleated Cells
BN: Binucleated Cells
MU: Multinucleated Cells
CBPI: Cytokinesis-Block Proliferation Index
RI: Replication index
MN: Micronuclei
MNBN: Micronucleated Binucleated Cells
NC: negative control (1% DMSO)
n.s: not significant compared to the concurrent control
- : not selected / determined
[1)] Chi-square test (one-sided)

Appendix 2 – continued

Table 2.3: Test 2▶Continuous treatment method without metabolic activation

Treatm / recovery time (h)	Dose level (µg/ml)	Cell stage analysis/500 (MO-BN-MU)			BN (%)	CBPI	CBPI (mean)	RI (%)	% Cytotox. (100-RI)	Selected for MN analysis (+/-)	MNBN/ 1000BN	MNBN/ 2000BN (%)	Statistics[1] (p-value)
24h (-S9)	NC	298	191	11	38.2	1.426	1.396	100	0	+	8	17	-
		330	157	13	31.4	1.366					9	(0.85)	
	2000	318	168	14	33.6	1.392	1.369	93	7	+	10	19	n.s.
		332	163	5	32.6	1.346					9	(0.95)	
	1500	321	162	17	32.4	1.392	1.405	102	0	-	-	-	-
		306	179	15	35.8	1.418					-		
	1000	351	138	11	27.6	1.320	1.325	82	18	+	10	18	n.s.
		349	137	14	27.4	1.330					8	(0.90)	
	750	299	188	13	37.6	1.428	1.396	100	0	-	-	-	-
		325	168	7	33.6	1.364					-		
	500	316	171	13	34.2	1.394	1.399	101	0	+	8	19	n.s.
		310	178	12	35.6	1.404					11	(0.95)	
	250	306	182	12	36.4	1.412	1.401	101	0	-	-	-	-
		314	177	9	35.4	1.390					-		
	VB 0.0125	457	43	0	8.6	1.086	1.073	18	82	+	56	127	<0.0001
		470	30	0	6.0	1.060					71	(6.35)	***

The fixed cells of dose level (15.6 to 125 µg/m)l were stored without slide preparation.

Abbreviations:
Treatm: treatment time
Cytotox: cytotoxicity
MO: Mononucleated Cells
BN: Binucleated Cells
MU: Multinucleated Cells
CBPI: Cytokinesis-Block Proliferation Index
RI: Replication index
MN: Micronuclei
MNBN: Micronucleated Binucleated Cells
NC: negative control (culture medium RPMI1640),
VB: Vinblastin sulphate
n.s: not significant compared to the concurrent control
- : not selected
[1] Chi-square test (one-sided); *** p≤0.0001

Annexes

Annex 1: GLP Compliance Monitoring Unit Statement

ENDORSEMENT OF COMPLIANCE

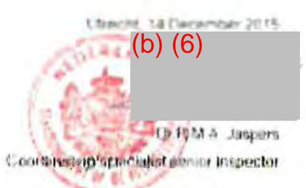
WITH THE OECD PRINCIPLES OF
GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 2004/9/EC the conformity with the OECD Principles of GLP was assessed on 29 September 6 October and 9 December 2015 at

TNO Triskelion BV
Utrechtseweg 48, 3704 HE Zeist
PO Box 844, 3700 AV Zeist

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Toxicity, mutagenicity, analytical and clinical chemistry, safety pharmacology, kinetics, metabolism and in-vitro studies.

Utrecht, 14 December 2015



Dr R.M.A. Jaspers
Coordinating specialist senior inspector

Health Care Inspectorate of the Ministry of Health, Welfare and Sport
[illegible]
P.O. Box 2680, 3500 GR Utrecht, The Netherlands

Annex 2: The quality certificate of S9

The batch of S9 used obtained from Trinova Biochem (Giessen. Germany) and were originally from Moltox Molecular Toxicology Incorporated (Boone. USA). The quality certificate was provided by the supplier.

TRiNOVA Biochem GmbH

MOLTOX

POST MITOCHONDRIAL SUPERNATANT (S9)
QUALITY CONTROL & PRODUCTION CERTIFICATE

Animal Information
SPECIES: [illegible]
STRAIN: [illegible]
SEX: Male
AGE: [illegible]
WEIGHT: [illegible]
TISSUE: Liver

Part Number Information
LOT NO.: [illegible]
PART NO.: [illegible]
VOLUME: [illegible]
BUFFER: [illegible]
STORAGE: [illegible]

PREP: [illegible]
EXPIRY: [illegible]
INDUCING AGENT: [illegible]

REFERENCE: [illegible] *Mutat Res* 113: 173 [illegible]

For Research Purposes Only

BIOCHEMISTRY
PROTEIN: [illegible]

[illegible] ACTIVITIES

[illegible]

BIOASSAY
TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS
[illegible]

PROMUTAGEN ACTIVATION
[illegible]

[illegible]

Approved [illegible]

MOLECULAR TOXICOLOGY, INC. [illegible]

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Germany

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fax +49 (0) 641 - 94300-22
info@trinova.de
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Annex 3: Criteria for analysis of cytokinesis-blocked cells and micronuclei

Criteria for scoring cytokinesis-blocked (binucleated) cells:

The cytokinesis-blocked cells that may be scored for micronuclei frequency should have the following characteristics:

1. The cells should be binucleated.
2. The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.
3. The two nuclei in a binucleated cell should be approximately equal size. staining pattern and staining intensity.
4. The two nuclei within a binucleated cell may be attached by a fine nucleoplasmic bridge which is no wider than one-fourth of the largest nuclear diameter
5. The two main nuclei in a binucleated cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable.
6. The cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

Criteria for scoring micronuclei (MN):

Micronuclei are morphologically identical to but smaller than nuclei. They have the following characteristics:

1. The diameter of micronuclei usually varies between 1/16 and 1/3 of the diameter of the main nuclei.
2. Micronuclei are round or oval in shape.
3. Micronuclei are nonrefractile and can therefore be readily distinguished from artefacts such as staining particles.
4. Micronuclei are not linked or connected to the main nuclei.
5. Micronuclei may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
6. Micronuclei usually have the same staining intensity as the main nuclei but occasionally staining is more intense.

References

- M. Fenech. W.P. Chang. M. Kirsch-Volders. N. Holland. S. Bonassi. E. Zeiger (2003) HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutation Research 534. 65-75.
- Michael Fenech (1993) The cytokinesis-block micronucleus technique: A detailed description of the method and its application to genotoxicity studies in human populations. Mutation Research 285. 65-75.

Annex 4: Historical data of the *in vitro* micronucleus tests

Historical solvent control data of *in vitro* micronucleus tests performed at the test facility with cultured human lymphocytes: summarized data 2008 - 2016.

1. Historical negative controls

Treatment time / Recovery time (h)	Number of tests (n)	Vehicle	% of binucleated cells containing micronuclei/2000 binucleated cells (Mean ± S.D.)	Range (%)
4 / 20 + S9	35	*	0.70 ± 0.18	0.40 – 1.20
4 / 20 - S9	35	*	0.68 ± 0.19	0.20 – 1.15
(24 /-) - S9	4	1% DMSO	0.87 ± 0.18	0.65 – 1.15
(24 /-) - S9	3	2% DMSO	1.16 ± 0.30	0.90 – 1.65

*All solvents: culture medium, 1% Ethanol and 1% DMSO

2. Historical positive control data of the indirect acting clastogen Cyclophosphamide (in the presence of metabolic activation)

Treatment time / Recovery time (h)	Number of tests (n)	Dose level (µg/ml)	% of binucleated cells containing micronuclei/2000 binucleated cells (mean ± S.D.)	Range (%)
4 / 20 + S9	35	20	3.56 ± 1.04	1.80 - 6.30

3. Historical positive control data of the aneugenic compound Vinblastine sulphate (in the absence of metabolic activation)

Treatment time / Recovery time (h)	Number of tests (n)	Dose level (µg/ml)	% of binucleated cells containing micronuclei/2000 binucleated cells (mean ± S.D.)	Range (%)
(24 /-) - S9	6	0.0125	4.86 ± 1.14	2.80 – 6.05

Annex 5: Certificate of Analysis

Product	: **Vivinal FL**
Product code	: NA (developmental product)
Batchnumber	: MRS02
Date of production	: 02-07-2016
Contact person	: Jan-Willem Boots (RAD)

Description : Human milk oligosaccharide

Typical analysis : Dry matter 97%, moisture 3%, 2'-Fucosyllactose 94%, lactose 1%, glucose 1%, fucose 1%

Chemical/ physical:	Specification	Results	Method of analysis
Total moisture	max 5%	3%	ISO 760 (modified), Karl Fischer
2'-Fucosyllactose	min 90%	>94%	FC-method using HPAEC-PAD
3-Fucosyllactose	max 3%	<1%	FC-method using HPAEC-PAD
Difucosyllactose	max 7%	<1%	FC-method using HPAEC-PAD
Fucose	max 2%	<1%	FC-method using HPAEC-PAD
Lactose	max 2%	<1%	FC-method using HPAEC-PAD
Glucose	max 2%	<1%	FC-method using HPAEC-PAD
Protein	max 0.01%	0.002%	Bradford
Sulphated ash	max 0.2%	0.06%	NEN 6810 (modified)
Nitrite	max 1 mg/kg	<0.1	ISO 14673-2/IDF 189-2
Nitrate	max 50 mg/kg	0.2	ISO 14673-2/IDF 189-2
pH (10%)	3.0 - 7.5	3.9	FC-method using NEN 3775

Microbiological:			
Aerobic mesophilic count	max 3000 cfu/g	<1000	FC-method equivalent to ISO 4833
Enterobacteriaceae	absent in 1 g	<1	FC-method, BPW 18h 37°C, SD, VRBG 18-24h 37°C
E. coli	absent in 1 g	<1	FC-method, LMX 25h, Coli ID 24h
Yeasts	max 30 cfu/g	<1	FC-method equivalent to ISO 6611
Moulds	max 30 cfu/g	<1	FC-method equivalent to ISO 6611
Presumptive Bacillus cereus	max 100 cfu/g	<1	FC-method equivalent to ISO 7932
Staphylococcus aureus	absent in 1 g	<1	FC-method, G&C 42h 37°C, PCR
Sulphite reducing clostridia spores	max 30 cfu/g	<1	FC-method using DFM 27 (1995) 185-200 Weenk
Clostridium perfringens	absent in 1 g	neg	FC-method, RPM 20h 45°C, confirmation
Salmonella	absent in 1 g	neg	FC-method equivalent to ISO 6579
Cronobacter spp.	absent in 1 g	neg	FC-method equivalent to ISO/TS 22964

Wageningen, 15-07-2016

Jan-Willem Boots

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PSB-en

02 AUG. 2016

END