

# **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 1.3 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 ( $\delta_H$ =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<sub>H</sub> ppm 5.30 (1H), reducing 4-α-Glc 5.22 (0.4H), 4-β-Glc, δ<sub>H</sub> ppm 4.62 (doublet, 0.6H), and doublet for 2-β-Gal, δ<sub>H</sub> ppm 4.51 (J<sub>1, 2</sub>=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, δ<sub>H.C</sub> ppm

	Residue	Nuclei	1	2	3	4	5	6	NOE, HMBC
A	4-a-Glc	ı,H	5.22 94.5	3.58 74.1	3.80 74.3	3.71 78.6	3.88 73.1	3.88/3.79 62.8	C1-A4
В	4–β-Glc	ъс пос	4.62 98.6	3.29 76.7	3.58 77.0	3.70 78.6	3.47 78.1	3.77/3.93 63.0	C1-84
c	2-β-Gal	1H 13C	4.51 101.0	3.66 77.2	3.84 74.0	3.87 78.0	3.68 78.0	3.79/3.73 63.8	84-C1, D1-C2
D	α-Fuc->2	1H 13C	5.30 101.9	3.79 70.9	3.78 72.3	3.80 74.3	4.23 69.7	1.22	C2-D1

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

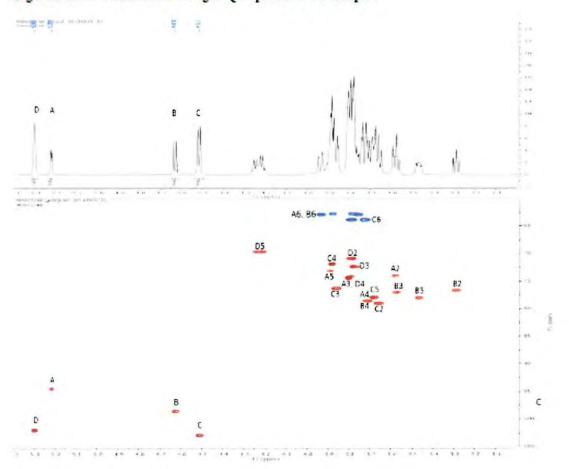


Fig 2 2'-Fucosyllactose

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

9/29/17

# Appendix 1.2 University of Groningen



Purity determination of 2'-FL samples by 1D 1H 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<sub>2</sub>O with intermediate lyophilisation. One-dimensional <sup>1</sup>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<sub>2</sub>O. <sup>1</sup>H are expressed in ppm in reference to internal acetone (8<sup>1</sup>H 2.225). 1D 600-MHz <sup>1</sup>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 'H NMR spectrum (Figure 1). The intensity of the anomeric peak specific for 2'-FL at  $\delta$  5.31 was compared with the intensity of anomeric peaks representing LDFT and 3-FL at  $\delta$  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, area, where  $\delta$  1.16-1.19 ppm corresponds with Fuc-( $\alpha$ 1- $\alpha$ 3)- in 3-FL and  $\delta$  1.23-1.26 corresponds with Fuc-( $\alpha$ 1- $\alpha$ 2)- plus Fuc-( $\alpha$ 1- $\alpha$ 3)- in LDFT. Due to overlap with the strong signal for Fuc-( $\alpha$ 1- $\alpha$ 2)- in 2'-FL ( $\delta$ 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 '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 'H NMR integrations. This product is the most pure 2'-FL preparation analysed so far.

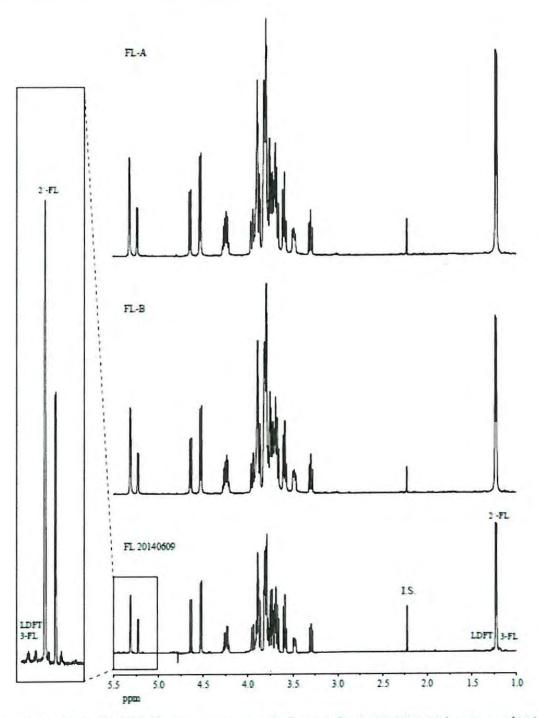


Figure 1. 600 MHz 1D  $^{1}$ H NMR spectra representing the four samples. For FL 20140609 (previous analysis) the anomeric region is expanded into the inset, showing the peaks used for integration in determining 2  $^{\circ}$ -FL purity. LS. is acetone internal standard ( $\delta$  2.225 ppm).

# 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

Notherlands

Monitor. Jan Bastiaans

Test Facility: Spectral Service AG

Emil-Hoffmann Straße 33

D 50996 Köln Germany

Date: 30 January 2017

Emil-Hoffmann-Straße 33 D-50996 Köln (Germany)

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# Spectral Service AG

# Study Report

FFD63190

#### 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 Fems

Tab. 2 Chemicals actually used as reference and calibration items

No	Substance name	stance name Distributor			
14	Tetramethylsilane (TMS, for NMR calibration)	Sigma-Aldrich Chemie Gnb/l 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 <sub>6</sub> (DMSO-d <sub>6</sub> ).	Euriso-top, Saarbrücken D)	D010H
Degree of deuteration 99.8%		

#### 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

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#### 4 METHODS

#### 4.1.1 NMR Spectroscopy

A 1H-NMR spectrum was recorded to characterise the test item. Approx. 10 mg of the test item have been dissolved in 1 ml D<sub>2</sub>O.

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<sub>2</sub>O 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 
$$MOLis [mMol] = \frac{IWs [mg] * Cis [\%]}{MWis [g/mol] * 100}$$

Equation 2  $MOLis [mMol] = \frac{Im * NAis * MOLis [mMol]}{Is * NAis}$ 

Equation 3  $Cis [\%] = \frac{MWis [mg] * Cis [\%]}{IWis [mMol] * MOLis [mMol] * 100}$ 
 $Im * NAis * MOLis [mMol] = \frac{MWis [g/mol] * MOLis [mMol] * 100}{IWis [mg]}$ 

Tab. 4 Declaration of variables

	test item (T1)	internal standard (IS)
molecular weight [g/mol]	MWti	MWis
initial weight [mg]	IW <sub>TI</sub>	We
content (%-by weight)	Cti	Cıs
Mol [mMol]	MOLTI	MOL <sub>15</sub>
integral	In	113
number of atoms*)	NATO	NAs

<sup>&</sup>quot;) alom refers to NMR active nucleus missianed (e.g. 1H 13C, 19F, 19P)

For determination of the water content 0.7 ml of DMSO-ds 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.

Spectral Service AG

Study Report

FFD63190

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<sub>190</sub> [%] = (IN

(INT 100 (ft) / NA 100) \* Ch

(INT n / NA n)

Equation 2

Ciao [%] =

MOL H20 \* MW H20

MW tr

Tab. 5 Declaration of variables

	test item (TI)	H <sub>2</sub> O
molecular weight [g/Mol]	MWTI	MWHZO
Mol-% [%]		MOLH20
content (%-by weight)	Cu	
integral	INTn	INTego
number of atoms *)	NATI	NAHZO
content water [% w/w]		CH20

<sup>&</sup>quot;) atom refers to NMR active nucleus measured (e.g. <sup>1</sup>H, <sup>13</sup>C, <sup>19</sup>F, <sup>19</sup>P)

Spectral Service AG

Study Report

FFD63190

#### 5 RESULTS AND DISCUSSION

Signal chemical shifts and multiplicity 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 $_5$ ) and 3.33 ppm (singlet of water).

Tab. 6 Calculation of cortent

		Exce	l-Version	16.0	Version 02.01		valid from:	6/15/2015
T	Integral	Initial weight [mg]	integral	initial weight [mg]		mMol	Content [mg]	Content [%]
Test	11	T	15	15	15	Ti	11	11
NS.A	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
	Molecula	r weight TI	488.44				Average	95.3
	Number	of atoms TI-1	3	Number of atoms	TI2	3	Std -dev.	13
	Number	of atoms IS-1	1	Number of atoms	IS-2	1	%RSD	13
teciniting	Molecula	r weight IS-1	122 13	Molacular weight	IS-2	122 13	Balance	30PE-R42
	Committee for the Committee of the Commi		99.9	Content [%] IS-2		99.9	Metter-Toledo XPE-205DR/M	

Comment Initial weight is higher from required MeMiligh of 10 mg

Tab. 7 Calculation of water content

INT H <sub>2</sub> O (pure solvent)	154.3	INT n	23.4
INT H <sub>3</sub> O (solvent + TI)	215.1	NA n	1
INT H <sub>2</sub> O (TI)	60.8	INT n / NA n	23.4
Content TI (% by weight)	95.3	MOL HOU	123 8
MW T	504.4	C 100	4.4
MW HOD	18.0		

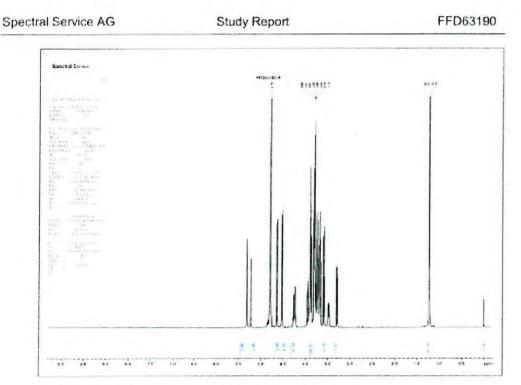


Fig. 1 'H-NMR spectrum of test item FFD63190 in D<sub>2</sub>O

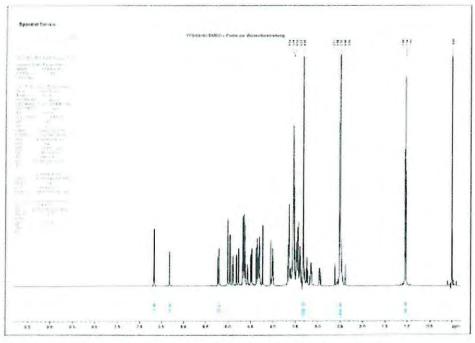


Fig. 2 1H-NMR spectrum of test item FFD63190 in DMSO-d<sub>6</sub>, water content

Spectral Service AG Study Report FFD63190

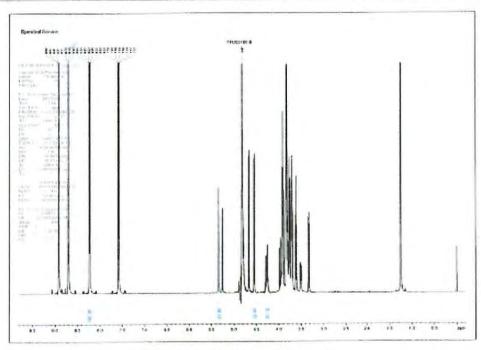


Fig 3 ¹H-NMR spectrum of test item FFD63190 in D₂O, quant. B

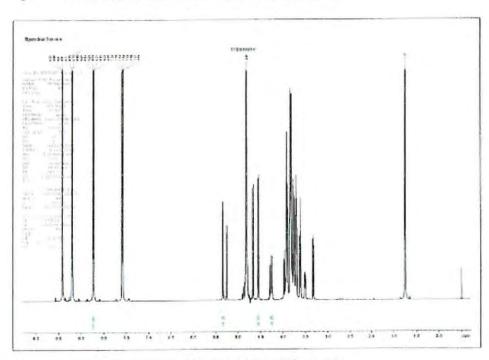


Fig. 4 H-NMR spectrum of test item FFD63190 in D<sub>2</sub>O, quant C

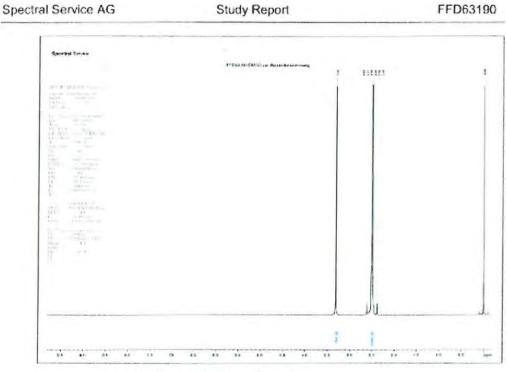


Fig. 5 'H-NMR spectrum of DMSO-de, water content

Spectral Service AG Study Report FFD63190

#### 6 SUMMARY

The proposed structure of the test item was confirmed by ¹H-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:

Or Bernd Diehl,

Chemist

Co-worker.

Andreas Beyer.

Biological technical assistant

Karin Seitz

Tallin Com.

Chemist

Quality assurance: Dr. Minam 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	$\alpha$ -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: E41-101V10



ROQUETTE® DEXTROSE MONOR	HYDRATE M	PAGE 1/
DEFINITION :		
DEXTROSE MONOHYDRATE. Purified	and crystalliza	d r-glucese.
containing one molecule of water	er of crystalliss	
CAS no : 77938-63-7		Productio dintribution da
EINECS : 200 075 1		<b>U</b> UNIVAR
SPECIFICATIONS :		4 in Coldena, 18 - 22 h3 AVI and 5 - 39 for 45 (27) 1 - 92 (0) 45 2 4 months above com-
· PHYSICO-CHEMICAL VALUES		C-00,3449/041
APPRARANCE	MCE	Crystalline powder,
AFFERRANCE	100.0	white and edourless
TARTE		Sweet.
LOSS ON DEVING	MCL.	9.1 % max
DENTROSE (D-GENICONE)	PAC 11	99 5 9 min.
SPECIFIC ROTATION	MCP	53.6 - 53.2 dagreen
PH IN SOLUTION	MC.T.	4 - 6
SULPHATED ASH	MCL	0.1 % max.
RESISTIVITY	MCL	100 kOhn on min.
PARTICLE SIZE	MCL	
- RESIDUE ON 500 MIC.		10 % max.
* MICROBIOLOGICAL VALUES		
- TOTAL COUNT	NMC	1000/g max.
- YEASTS	NNC	10/g task .
HOULDS	NISCI	10/g max.
- B.COLI	WARC	Absent in 10 g
- SALMONELLAE	MMC	Absent in 25 g
TYPICAL VALUES :		
ENERGY VALUE		
calculated, on 100 g commercial	l product	1547 kJ (164 kcal)
NCL, NMC : ROQUBITE Methods		
QUALITY ASSURANCE / RUMAN FOOD		government 25, 2014

CARTALE SOCIALE NIT. WHIS IN \$165,000. CONTRET RECAIR TABILITATION IN 0016 1980005. BEG. MF N 242/27/775 INBUSTALE D. TORTONIA. C. C. LA A ALESSANDERIA N. 73302.

State of the last

GRAS ASSOCIATES, LLC



**EPECIPICATIONS** 

Ref: \$41-101W10

#### ROQUETTE" DEXTROSE MONOHYDRATE M

PMR 2/2

COMMENTE :

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

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

#### COMPOSMITY :

- CODEX STAN 212 1999
- EU directive 2801/111/80 (GJBC L. 10 dd 12/01/02).
- Current FOOD CHEWICKLE CODEX.
- DS Code of federal regulations 21 CFR & 168.111.

STORAGE I

Standard packaging : bulk road tenker.

25 kg paper bags + polyothylen free film
1000 kg FIBC

Minimum durability date of the packaged product: Nanufacturing date + 12 conths.

Enelf life: Namufacturing date 4 5 years.

DOCUMENTO (b) (6) CTATO

DAIA 9-14-2014

MCI. NHC | FORESTTE Mathods

QUALITY ASSURANCE / HUNGAY FOOD

NOVEMBER 25, 3014

NOVEMBER 2415 Spiriture Assure con Socio Unico Regarda Friend SA (FANNOS)

STER UGAIT DEFENDAÇE STABILIPATION (1904) CASSAND SENICIA (AL) - NA SERVAÇAÇE (1904)

THEFOND: 6143 774 THE THEX TIDIAL ROCKET T, THERAY: 6143 677 795
CANDAE SOCIALE MY VERS 4-2 TAX COD CODICE RICKER BARTIN VAIN, COTA 1980065 - REG. IMP. N 245/27/275
TREPLANT DIRECTORA - C.C.L.A.A ARESSANDRIA N. 72302

3

# Certificate of analysis

#### Appendix 2.2 α-Lactose Monohydrate

Patherin Capito S.p.A. Strada Statale Appla 46/48 #104 L Copum (Caserta) ITALY:



#### Product

Product code Order no. Customer no. Batch no. Date of production Refest date.

#### Description

Typical analysis

# Sensorial

: Lactopure®

Refined Crystals 502309 2226137

Capua Bioservices : 707304

09 08-2015

: 09-08-2018

: High quality lactose

: Lactose monohydrate 99.6%, protein 0.1%, inmetals 0.1%, free moisture 0.1%

: White crystalline powder, odnorless, slightly sweet

#### Intended use

# Infant formula, dry blending application

Interiora data	2 mant formula, bry blending application				
Chemical / physical:	Specification	Results	Method of analysis		
Sulphated ash	max 0.15%	< 0.1 %	NFN GB10 (modified)		
Free maisture	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 + -	4	FC-method equivalent to ADPI 916 / ISO 5739 / IDF 107		
pH (23%, 20°C)	3.0 - 2.0	3.6	FC-method using NEN 3775		
Particle size:					
> 250 micron	max. 30%	7%	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/q	4/0	FC-method equivalent to ISO 4833		
Enterobacteriaceae	absent in 10 x 10 g		FC-method, BWP 18h 37°C, SD, VRBG 18-24h 37°C		
F roli	abcont in 10 m	abrent	EC mathed LMV 3Eh Cal 10 34h		

max 500 cfu/g	4/g absent	FC-method equivalent to ISO 4833 FC-method, BWP 18h 37°C, SD,
	addent	VRBG 18-24h 37°C
absent in 10 n	absent	FC-method, LMX 25h, Coli ID 24h
max 10 du / g	< 1/0	FC-method equivalent to ISO 6611
max 10 cfu / o	<1/9	FC-method equivalent to ISO 6611
max. 50 cfu/g	= 10/g	FC-method equivalent to ISO 7932
absent in 5 x 1 g	absent	FC-method, G&C 42h 37°C, PCR
max, 10 cfu/g	< 1/9	FC-method, using I)FM 27 (1995)
		185 200 Weenk
absent in 60 x 25 g	absent	FC-method equivalent to ISO 6579
absent in 30 x 10 g	absent	FC-method equivalent to ISO/TS 22964
	absent in 10 x 10 g max 10 du / g max 10 du / g max 10 du / g max 50 du/g absent in 5 x 1 g mex, 10 du/g absent in 60 x 25 g	absent in 10 x 10 y absent  absent in 10 g absent  max 10 cfu / g < 1 / g  max 10 cfu / g < 1 / g  max 50 cfu / g < 10 / g  absent in 5 x 1 g absent

Borculo, 08-09-2016



Manager Parist augmented (14,6 Parist Parist Milli) American 10 (14) (14) (15) (16) (16) (16) 10 (14) (16) (17) (17) (17) 10 (15) (17) (17) (17) 10 (15) (17) (17) (17)

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Entsteld Lampto Devin APAL E-19-12, Lood 12, Clock Building E-tan Spore, Inc. 2, June 1971 Herbit Stating Base Schenge Devil See Bulleyea Bu

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#### FrieslandCampina Domo

Stationsplein 4
3818 LE Ameristoort
The Netherlands
P.O. Box 1551
3800 BN Ameristoort
The Netherlands
1 +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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FriedlandCampine there 8.V.: ErisalandCampine Domo is a tradesame of FriedlandCampine Domo 8.V. (registration number 3116-917), with this happiness office in Amendian, The settlemads. FreshindCampine 5.V. is a subsidiary of buyel FriedlandCampine 6.V. The persons some and common respectively safe and delivery of fuery freedlandCampine 6.V. that there been registered at the Chamber of Commence under registration number 310531944, are applicable to all transactions and understooding residently there from





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P.O. Box 1551
3800 BN Ameriscont
The Netherlands
t +31 (0)33 7133 333
f +31 (0)33 7133 334

www.frieslandcampina.com

# 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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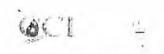
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Priodized/Designe Scene 8.4.; Friedand/Lampine Domo is a tradectamic of Friedand/Lampine Scimo 8.4. (Inglishmon number 2115497), with this registered office in Amendian, The embeddenics. Friedand/Lampine Scimo 8.4. is suitedary of Royal Friedand/Lampine Scimo 8.4. is suitedary of Royal Friedand/Lampine 8.4. The persons terms and conditions of purchase respectively sate and estimate delivery of Royal Friedand/Lampine 8.4. that have been registered at the Chamber of Commerce-Landon registration number 31053544, are applicable to 88 transactions and unidenticating resoluting them from:



#### Appendix 2.3 **Phosphoric Acid**



# Certificate of Analysis

MIOCEGTRON DZ

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

RATCH N		GRADE		RE-TEST DATE
11777/16		FCG		OC1 - 2019
RESULTS		SPECS		
Positive		Positivo		PACKING
Complies		Limpid Colouriess Lio	und	1860 Kg
25.1	*	30.0 - 40.0	%	1000
* 2	ppm	3	ppm max	
• 9	ppm	3	ppm max	
- 10	ppni	10	ppm max	MANUFACTURING
- 5	ppm	3	ppm max	DATE OCT - 2016
	Positive Compiles 35.1   3   10	Positive Compace 25-1 N 2 ppm 10 ppm 10 ppm	### PCG    Positive	### PCG    Positive

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- Issued by QC

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

Amenta not Sistema Caulifa IIM 150 1601:2018
Ademia con Sistema di Gestione Ambienti (IM 150 14101:2004
Adienda con Sistema di Gestione Ambienti (IM 150 14101:2004
Adienda con Baisema (650 per legistedenti de Sastrato Cossestion EFRC) 2012
Siste 16009: Viz Totama (1401-2005) 1601 Vescha (100)
Siste prodetti Costano (1401-2005) 1601 Vescha (100)
Siste prodetti con Caulifa (1401-2005) 1601 Vescha (100)
Siste prodetti con Caulifa (1401-2005) 1601 Vescha (100)

9/29/17

# Appendix 2.4

# **Ammonium Hydroxide**



Certificate of Analysis

# Certificato di Analisi

Ammonium Solution

Predotto

SOLUZIONE AMMONIACALE

Data Analisi 06.05.2016

Targa mezzo AB 18509 Analista RATINI

Parametro analizzato	Valore	Unità di Misura		
TITOLO IN AMMONIACA Titer Amounta IDENTIFICAZIONE Ident Jicotion	30,68 POSITIVA	% PESO 1/ Neight		
RESIDUO NON VOLATILE sone latte reich	6	PPM		
METALLIPESANTI Heavy Melds	<5	PPM		
SOSTANZE OSSIDABILI Oxilisalie Salshaus	COMPLIES			
APPARENZA Appearance	COMPLIES			

30,68 90 NH4 solution in equivalent to 20,49 % NH3 solution

YARA ITALIA S.p.A.

Pagina 1 di 1



Yara Italia S p.A.

Sade Legale, Uffixi Amministrativi e Direzione Commerciale Vis B. Crespi, 57 –20159 MILANO Telefono: 02 75415287 – Telefax: 02 75416228 Registro implese Milanole C.F., 01974300921 P. MA: 11843280154 - C.C.I.A.A. n. 1383867 Cep. Soc. Euro 130.000.000;00 t/x

# Appendix 2.5 di-Ammonium Hydrogen Phosphate



# Certificate of Analysis

M1QC007Rev.02

# di-AMMONIUM HYDROGEN PHOSPHATE COD. 06650 C

	BATCH No	GRADE		EXP.DATE
	Typical	FCG		After 5 years
TEST	RESULTS	SPECS		
IDENTIFICATION		Positive		PACIONG
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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Clean Consult International S.p.A.

Azienda con Sistema Cuatità UNI ISO 9001:2008
Azienda con Sistema di Gestione Ambiente UNI ISO 16001:2004
Azienda con Sistema GRF per ingredienti del Settore Cosmistico EPICI 2012
Sede legate via Toscana, 146 - 28655 - Lodi Veccho (LD)
Sede produttiva e statistimento via Patula, 6486 - 80030 - Castello di Cistema (Na)

# Appendix 2.6 Monopotassium Phosphate



Certificate of Analysis

# POTASSIUM PHOSPHATE MONOBASIC

8ATCH N: 11972/16		GHADE PHARMA		EXP.DATE NOV - 2021
RESULTS		SPECS		
Positive		Pusitive		PACKING
Comples		White or almost what crysteline cowder, or colorations covered		25 Kg
Comules		Freely solutions water, pratically		
Company				
100.3	46	98 C 100 E	4	
4.3		4.2 - 4.5		MANUFACTURING
= 1.D	*	10	N may	DATE
prosper test		to pass best	in livery	NOV -2016
* 2000	ppm	2000	com may	1104 - 2010
< 200	ppm	200		
* 300	ppm	300		
+.2	(ifter:	2	2000 000	
*5	ppen	5		
5 10	gagatti	19	2.32	
< 10	tition	19		
≠ 0.1	FPITI	0.1	to max	
< 10	ppm	10		
	## SULTS  Positive Complete Complete Complete Complete Complete 100.3 4.3	Positive Complete Complete Complete Complete Commans 100.3 % 4.3 % 7.5.0 % Positive 2000 ppm 4.200 ppm	### PMARMA    RESULTS   SPECS	### PHARMA    RESULTS

#### REMARKS:

APPROVED by
Mass me Napolitane
Quality Manager
Crean Consult International S.p.A.

As larde con Sistama Quellik Usli 200 (0031)2660 Adirects con Sistama (Fi Gastinna Ambanis III) 190 (1408) 2004 Anisand et al. Sistama (SAP) per Reprimburit to Sistam Connectic Effici 2013 Santi Rayde va Toossay, 1465 (2005), Lard Vector (III) (1005) Santi Rayde va Sistama (SAP) va Toossay, (Arth. 1800) (2004) Cases in the Connectic Plant

<sup>-</sup> PHARMA / Ph Eur ane USF - NF Current Edition

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#### Appendix 2.7 L-Tryptophan



# 元 锡 品 海 凱 基 戳 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 MANE:1-色氨酸 1,-TRYPTOPHAN

(h) JES BATCH NO: 1160903

执行标准 STANDARD: FCC8

致量 QUANTITY: 75KGS

生产日期 PRODUCTION DATE: 2016-09-14 报告日期 REPORT DATE: 2016-09-22 有效日期 EXPIRY DATE: 2017-09-13

CAS NO. :73-22-3

储存条件 STORAGE COMPITION: CODE AND DRY PLACE AWAY FROM DIRECT SUNLIGHT(<201)

Tests	标准规定 Linits	公司 See results
TEAK Description	White crystals or crystalline puwder	conforms.
監別(紅角) Identification(IR)	concarding with the reference spectrum	conforms
18 fd Reserv	98. 67-101. 9%	89 174
比較度 Specific constinu(a)。" [a]。"	-30, 0° ~ -33, 0°, -28, 7° ~ -32, 7°	-31.0" -31.3°
tn Lead(1%)	ं प्रीमृत्याः )	_ < days
子從失重 toss on drying	≤0.30%	0.153
秋野鉄道 Residue on Ignitium	€0.10%	-0.01%
说: 水區燈及追答合FCUS li methician: Phased test accordin 18.		<b>*</b>

# Appendix 2.8 Magnesium Sulfate Heptahydrate



# Certificate of Analysis

M1QCC0/Rev.02

# MAGNESIUM SULFATE heptahydrate cop. 18450 c

	BATCH Nr		GRADE		EXP.DATE
	11919/16		PHARMA		NOV - 2021
TEST	RESULTS		SPECS		
BENTIFICATION	Positive		Postive		, PACKING
APPEARANCE	Complies		Whyte or abrust white, costables powder or billiant, colouriess cycles		25 kg
SOLUDILITY	Complete		Freety soluble in water, very soluble in builing water, protectly insoluble a ethanol (96%).		
APPEARANCE of SOLUTION	Complies		Client and Colourloss		
ASSAY (as MgSO <sub>4</sub> after ignition)	99.9		99.0 - 100.5	%	
LOSS on IGNITION	50.7		45.0 - 52.0	5	MANUFACTURING
LOSS or DRYING	*2	%	2	14 (2191	DATE
pH (sol. 5 %)	6.2		50-97		NOV 2016
ACIDITY or ALGALIN'TY	passes test		to pass tost		1107 2010
CHLORIDES (CI)	< 140	ppm	140	ppm max	
IRON (Fe)	< 20	ppm	20	pom max	
HEAVY METALS (FD)	< 10	gratern	10	ppm max	
ARSENIC (As)	< 2	ppm	2	pom max	
SELENIUM (Se)	< 30	ppm	30	рот тих	

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Massimo Napofitano
Quality Managar
Clean Consult toternational S.p.A

Attentin von Bistleres Guette URI ISO DKO1 (244) Astendo con Bistleres di Garcijona Ambrarda URI ISO 14791 (20)4 Adiando con Bistleres GBIP per ingredient del Settiere Commelica (2710) (20)5 Soda escrite va Tomatana, Alle (1865) Led Vencifica (Co.) Soda (productiva) o statid reactio, et al finduce 54055 (1811)) Condreto di Cultura (Ne)

# Appendix 2.9

Citric Acid

# **Product Specification**

# Jungbunzlauer

# Citric Acid Monohydrate

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

in ether

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

PROMANA CHIMICI S.P.A.
PRODOTTORING CHIMICI S.P.A.
CODICE (1)(1000 200) (6)
APPROVAZIONE R.D.

We herewith confirm that this product meets the requirements of the latest edition of the European Pharmacopoela (PP). Eur.), the United States Pharmacopoela (USP), the Food Chemical Codes (PCC) 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 SO: EN

# Appendix 2.10 Iron (II) Sulfate Heptahydrate



# Certificate of Analysis

M1QC807Rev.01

# FERROUS SULFATE HEPTAHYDRATE COD. 13120 C

	BATCHN		GRADE		RETEST DATE
	12295/14		PHARMA		DEC - 2016
TEST	RESULTS		SPECS		
DENTIFICATION	Postvo		Positive		PACKING
APPEARANCE	Complies		Light green, crystatine powder or blush-green crystals, efflorescent in air		25 Kg
SOLUBILITY	Comples		Free soluble in water, very soluble in boiling water, pratically insoluble in etherol 96%.		
ASSAY (as FeSO <sub>4</sub> ,7H <sub>2</sub> O)	90.5	%	99.5 - 104.5	94	
ARSENIC	< 3	ppm	3	pon mas	
LEAD	< 10	ppm	10	ppm max	MANUFACTURING
MERCURY	• 3	ppm	3	ppm max	DATE DEC - 2014

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Aziende sen Bistems Queltà UNI IBO 2001:2006 Aziende sen Sistema di Caslicta Ambente UNI IBO 14031:2004 Sada lugala: en Inscana, 148: 78655 - Lost Veccha (LO) Sada stratutiva e stabilimento via Poccia, 64/66 - 80030 - Cancelo di Gazarra (Na)

# Appendix 2.11 Manganese Chloride Tetrahydrate

# SIGMA-ALDRICH

and oldered com

3050 Spruce Street, Seire Louis, MO 63103, USA Weiselle: www.signassidrich.com Ernst USA: technery@sist.com Outside USA: eufechsery@sist.com

# **Product Specification**

Product Name:

Manganeseilli chlonde tetrahydrate - meers USP testing specifications

Product Number:

M8054

CAS Number: MDL 13446-34-9 MFCD00149792 Ct2Mn + 4H2O

Formula: Formula Weight:

197.91 g/mol

TEST

## Specification

MnClo · 4HpO

Identification
pH
Loss on Drying
Insoluble matter
Sulfate
Substances not ppt by
arctronium sulfide (as sulfate)
Iron (Fe)
Zinc
Heavy Metal
Assay
Dry Basis
Residual Solvents USP 467

35 - 60 360 - 365 % < 0005 % < 0005 % < 02 %

≤ 5 ppm
Pass
≤ 5 ppm
98.0 - 101.0 %

Meets Requirements

Specification. PRD.1.206.18000038469

Recommended Retest Period

2 Years

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 evaluable 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.

# 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 <sub>3</sub> )	≤ 0.01	%	
Sulphate (SO <sub>4</sub> )	≤ 0.005	%	
Ca (Calcium)	≤ 0.005	%	
Cu (Copper)	≤ 0.0005	%	
Fe (Iron)	≤ 0.001	%	
(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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### Appendix 2.13 Cupric Sulfate Pentahydrate



Certificate of Analysis

M1QC007Rev.02

# CUPRIC SULFATE pentahydrate COD. 21330 C

	BATCH No TYPICAL	GRADE FCG		EXP.DATE 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 PRECIPI by HYDROGEN SULFIDE	TATED	0.3	% max	MANUFACTURING DATE

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Azienda con Bistema di Gestione Ambiente Uni ISO 14001:2004
Azienda con Bistema GMP per ingredienti del Settore Cosmetico EPICI 2012
Sede legale val Toscana, 1465 - 28855 - Lod Vecchio (LO)
Sede produtiva e stabilimento via Padula, 6486 - 80030 - Castello di Cistema (Na)

# Appendix 2.14 Boric Acid



### Certificate of Analysis

M1QC007Rev.02

# BORIC ACID

	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	passos test		to pass test		
COMPLETENESS of SOLUTION	passes tost		to pass les!		
ASSAY	100.0	36	99.5 - 100.5	3%	
LOSS on DRYING	< 5000	ppm	5000	kew sudd	MANUFACTURING
HEAVY METALS (Pb)	< 20	ppm	20	ррт тах	DATE JAN - 2016

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Clean Consult International S.p.A.

Azienda con Statema Gustità UNI ISO 9101.2008
Azienda con Statema di Gestiono Ambiente UNI ISO 14051 2004
Azienda con Statema GRP per improblenti del Bellono Cosmolico EFFCI 2012
Foota Ingula: via Teccasa, 1489—20156 - Lod Vectios (LO)
Code produtiva e statistima rino van Parkia, CAPSI - 80050 - Cesteiro di Costoma (No)

# 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		Postve		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
CADMIUM (Cd)		2	ppm max	DATE
LEAD (Pb)		4	ppm max	
MERCURY (Hg)		5	ppm max	
SELENIUM (Se)		30	ppm max	

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Azienda con Sistema Gualità UNI ISO 9001-2008 Azienda con Sistema di Gastione Ambiente UNI ISO 14001-2504 Azienda con Sistema GMP use improblenti del Settore Cosmetico EFICI 2012 Sede legani via Toscarra, 14/8 - 26555 - Lod Vecchio (LO)

### Appendix 2.16 Sodium Hydroxide



# Certificate of Analysis

M1QC007Rev.02

# SODIUM HYDROXIDE 25%

Cod 22111

11805/16		GRADE FCG / DSM		RE-TEST DATE OCT - 2019
RESULTS		SPECS		
Postive Complies		Positive Limpid Colourless Lie	uid	PACKING 1200 Kg
24 8	16.	24 5 - 25 5	4.	C-1
< 2.0	94	2.0	% mar	
< 0.1	ppm	0.1	ppm max	
	Postive Compiles 24 8 < 2.0	Postive Comptins 24 8 8 < 20 92	Positive Positive Compiles & \$24.5 - 25.5 < 2.0	Positive Compiles Empid Colombias Liquid 24 8 24 5 - 25 5 % < 2.0 % max

MANUFACTURING DATE OCT - 2016

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Accords con Sistema Grafillo UNI ISO 9961-2505
Accords con Sistema of Coptime Ambiento UNI ISO 14001-2004
Accords con Sistema GMP per Impedient del Solicer Committe Effect 2912
Secontegral via Tectrana 1445 - 28761 - 200 Wester d.C.)
Serie produtiva e staliditatema via Fodula 1445 - 82(0) - Castedo di Chilena (Na)

# Appendix 2.17 Sodium Molybdate Dihydrate



Certificate of Analysis

MIGCOUTREV.01

# SODIUM MOLYBDATE dihydrate

COD 22160 C

	BATCH N		GRADE		RE TEST DATE
	10103/16		PHARMA		JAN - 2021
					4.
TEST	RESULTS		SPECS		
IDENTIFICATION	Positive		Fastive		PACKING
APPEARANCE	Congress.		Vitale or almost write pos-	ster	2 0000
SOLUBLITY	Complies		Freely soluble in water		
APPEARANCE SOLUTION	Comples		Clear and colourless		
ASSAY	59.7	*	96.0 - 100.5	%.	
CHLORIDES (CI)	× 50	ppm	56	Com mass	MANUFACTURING
PHOSPHATES (PO.)	< 200	gepren	200	ppm max	DATE
AMIONUM (NH))	< 10	ppm	10	pom mes	JAN - 2016
HEAVY METALS (Fb)	× 10	\$14afr1	10	ppm max	
LOSS on DRYING	14,5	- CV	14 0 16 0	%	

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Quelity Manager
Glean Consult International S.p.A.

Ariende con Beleine Gestine BRISC 9001-2002 Ariende con Beleine di Gestines Arithere UN ISO 14001-2400 Brish espec va Footbill, 1419 - 20055 Led Visales (ICH Brish Crest/Anne state-franch via Padala 5400-48003) - Candala d' Chierca (Un)

### 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

Harperinkskamp 5 BORCULO 7271 AR

Am : FRESLAND CAMPINA

NORBERT DENTRESSANGLE

### NORIT ACTIVATED CARBON CERTIFICATE OF ANALYSIS

021394048536SO / 1 Customer P.O.Number

morning Plant

4501721755 Klazienaveen

NORIT PN 2

Customer Grade

2.880.00 EG

untry Shipped Corne Name

PA'O FERRYMASTERS ROZE

Vehicle ID

BT-SZ-69 3923896

Let Number

12 Feb 2016

Pack Date 10Nov2015

PHYSICAL AND CHEMICAL PROPERTIES							
Property Description	Unit	9.d	Specification Me	Indonedual Value	Specification bits s		
Moisture (as packed)	mass-%	NSTM 3.08		2	10		
Ash content	mass-%	NSTM 3.02		10			
Molasses number (EUR)		NSTM 2.19		350	390		
рН		NSTM 3.09	6.0	6.5	7.2		

02139400091922 Dec 15

02139400091811 Nov 15

021393954477 15 Sep 15

0213939544764 Aug 15

The data above was obtained from tests on sample; taken during the time of production and or packaging of this product using Nortt Standard Test Methods (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.

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

### PalletNa/Container ID

Seal No:

Signature: Mr. F. de Graaf Lab Manager



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.

SP	ECIFICATIONS	
Molasses number (EUR)	max. 390	LA.
pH	min. 6.0	-
pH	max. 7.2	4,
Moisture (as packed)	max, 10	mass-%

GENERAL	CHARACTERISTICS	
lodine number	850	
Molasses number (EUR)	350	
Methylene blue adsorption	15	g/100 g
Total surface area (B.E.T.)	950	m <sup>2</sup> /g
Apparent density (tamped)	470	kg/m
Particle size D <sub>io</sub>	3	μ <del>m</del>
Particle size D <sub>so</sub>	20	μm
Particle size D <sub>so</sub>	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



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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 prebletic 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 charistals need to be of HPLC- or analytical quality and highly purified water for HPLC (MES-Q or equivalent).

· 50% Sodiom hydroxide (carbonate free)

Baker 3727

- 2"-Fucosy: Lactose

FriedandCampina standard

- Hakum

- Micro Riters: 0.2 pm, GHP Acrodus: 13 (Pall) or equal
- HPLC Vists 2 mL with split septem suitable for the ICS used
- Carbopac PAI column (4\*250 mm) (Dinnex p/n. 35391)
- Carbonat PAY guard column (4°50 mm) (Dienex p/n. 43096)

# 6. Equipment

- Analytical Balance, accuracy 0.1 mg
- Belance, accuracy 0.01 gram
- Vortex mixer
- · Contribute
- Otheror
- Bris moter
- Thermo Edemino HPAEC economent ICS-5000 or equal, equipped with:
  - Pump (ICS-5000+ OP)
  - Thermostatic autocomplex (ICS-5000 AS-AP)
  - Detector / Chromatography module (ICS-5000+ DC)
    - EU detector PAD, with electorid carbohydrate waveform for Ag/AgCl refelectrode
    - An 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 erienmeyer 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-500) 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 Lectose 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;

\$2\$3199	Standard 1	Standard 2	Standard 3	Standard 4
I'-Fuccey	1.25	2.50	5.00	10.00
Lactosa				



### 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

Sample tray temp.

Column temp.

Flow

: 30°C, : 1.0 ml/minute

Injection volume

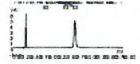
: 30 µL (with a 25 µl injection loop)

Detector

: PAD with an AU-electrode, Data Collection Rate=2 Hz.

### Gradient conditions:

Tild (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)
Fucuse	0.33
Galactose + Glucose	0.52
Fructose	0.61
3-Fucosyl Lactose	0.64
di-Fucosyi 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

9/29/17

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)

# Accuray based on reference sample with 100% purity. The reference sample was used as a 100% pure 2-fucces/lactose standard.

Determine the difference with student T-test.

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

Dale	Sample ID	product	purity 1	%.	difference
			NutriGontroi	Helerence	NG/reference
5-12-2016	PMRS01-1	2-hicosyl·lactose	100,7	100,0	0,7
5-12-2016	PMRS01/2	2-tucosyl·lactose	100,9	100/0	0,9
5-12-2016	PMRS01-3	2-hicosyl-lactosa	8,008	100,0	0,8
5 12 2016	PMRS01-4	2 tucosyl lactose	100,8	100,0	8,0
5-12-2016	PMR801-5	2-hicosyl·lactose	100,5	100,0	0,5
5-12-2016	PMRS01=8	2 hicosyl·laciose	100,1	100,0	0,1
5-12-2016	FMRS01-7	2 hicosyl-lactose	8,69	100,0	-0,2
5-12-2016	PMRS0148	2 hicosyl lactose	100,4	100,0	0,4
5-12-2016	PMRS01-9	2-hicosyl-factose	99,7	100,0	-0,3
5-12-2016	FWRS01-10	2 hicosyl factose	99,9	100,0	-0,1
7-12-2016	PWRS01-11	2-tucosyl-lactose	99,7	100,0	-0,3
7-12-2016	FMRS01-12	2 hicosyl·lactose	100,1	100/0	0,1
15-12-2016	PMRS01-18	2 hicosyl·lactose	100,5	100,0	0,5
15-12-2016	PMRS01-14	2 hicosyl-lactose	t00,1	100,0	0,1
15-12-2016	PMRS01-15	2-hicosyl-lactose	99,3	100/0	-0,8

	bet suwbearheid	95%
	reamber (n)	15
	average diff.	0,10
	eldev(r+1)	0,47
stder(n-1)	/aq root(n) =	0,122
	-0,07 =< y <= (	0.45

confidence interval:

There is no significant blas Conclusion:

Validationreport:

Quality manual:

Method:

Determination of 2-fucosyl-tactons purity

and the transfer of

Date:

27-1-2017

version: 1

author(s): nuthorhor: (Sanker) Anales Product developper

analysis method:

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

### To determine:

- BOOLINGLY
- · opposite de la composite de l
- within-laboratory reproducibility
- madelinement lincortainty
- selectivity & specificity

### postello.

### Accuracy

There is no analytical 2-functories countries available. The reference comple supplied by the customer was used as a 100% pure exciption elevators.

### Bearing the Sty

The retailve standard deviation under repostability (PSDr) conditions is 0,4%.

Approximately
The relative standard deviation under within-tab reproducibility (RSD<sub>R</sub>) conditions is 0.4%.

### Robbin annaucound monthly insued of

The relative transpurpment uncertainty is 1%

### Salvettay & Specially

Selectivity:
The salactivity of the method is based on chromatographic separation of components in time on an enton-exchange cotumn.

### Specificity.

The specificity of the method is bissed on the use of the pulsed amperometric detector which seems only to For example, hydroxyl groups in certahydrates are existed on a working electrode surface and the resulting current is executed existrable components.

### Conchiden

The HPLO-PAD method for the determination of the purity of 2-succeyt-tecture is fit for purpose.

Surrey

2/6

# Accuracy based on reference sample with 100% purity. The reference sample was used as a 100% pure 2-hecosy-bacters standard. model 1.2

Determine the difference with student Y-best.

hypothesis: The wine p must be equal to "0".

Dain Sample D	Sample D		proy			
			hebiContel	Patanage	NCAsterance	
5-12-3010	PMP\$01-1	2-tacony/inclose	100,7	100.0	0.7	
5-12-2016	PMR\$01-0	2-fuscept-backson	200.9	100,0	0.0	
5-12-2016	PLESSI-S	2-huneyl-last up	100,6	100,0	44	
5-12-2010	PMRS01-4	2 Aucosyl-Locknop	\$00.jb	100,0	mirraconnii/Q.#	
5-12-2016	FMS01-6	2-hucosyl-laidsne	100,5	100,0	0,5	
\$-12-2010	PURSON-4	2-hasoyl-lectore	1.00.1	100.0	34	
5-12-2016	PMR501-7	2 history/factors	動点	100.0	62	
5-12-2016	FMF801-8	2-haconyl-hadaso	100,4	100,0	0,4	
5-12-2015	PMR\$01-0	2 ductory) doctors	49,7	0.00¢	403	
5-12-2016	PMP801-10	2 turny lineare	10.3	100.0	-0,3	
	PMR501-11	2-Auccoyl-textons	100.7	700.0	- QS	
7-12-2016	PMP801-12	2-Accorditactions	100.1	400.0	4.1	
15-12-2016	PMREDI-13	2-tucosyl-lastane	1005	100.0	8.3	
NAME OF TAXABLE PARTY.	PMRSD1-14	2-Automyl-England	100.1	100.0	0.1	
15-12-2010	PMPISO1-15	2 turnyl terions	191,3	100.0		

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Charles Sandra March	
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model 2.1

Repeatability

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2-fucción finalismo telefrio apiec.	PMRS10-2	6-12-2016	.98,5	66,1	<b>SEC</b>	0,0000
2-fuccent-lectors within epic.	FMRS10-0	\$-12-XD18	99,7	66.6	99.2	0.00013
Africa A lackum with spec.	PMP6104	6-12-BH6	98.8	\$8.9	88.0	0.000
9-tyconyl-inchaso within spec.	PMR2104	<b>卷-12-2010</b>	50,6	50,0	98,8	0,0000
2-futnosyl-historia COS	FMFS09-1	7-12-2016	86.6	89.8	89.7	0,00000
2-futcomit-incition COS	PMRS09-2	7-12-2016	<b>高温</b>	00.2	10.1	0.0000
2-Nucosyl-Isotose OOS	PMRS00-8	7-12-2016	20,1	20,5	80,8	0,0000
2-fucosyl-bottom OOS	FMR809-4	7-12-2016	80,8	40.6		0.0000
2-fucción lactore OOS	PMRS00-5	7-12-2016	85.4	85,3	86,4	0,00000
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model 2.2

# Reproducibility

erantela.

pure 2-fucusyl-business

Best ROD (%) as

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argumented on Andi:

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

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rreni					<b></b>	
2-hacocyl-lackons within spec.	PM810-1	Nick/Kevin	984	68,6	96.0	0,0000
	PMR3102	HICKINGVIII	98,8	68.8	98.0	0.0000
2-facos/l-betos with soot.	PMP310-3	Nick/Kevia	96.2	193,4	<b>03</b>	0,0000
Managharkan with spor.	FMR8104	New Yearts	02.0	98,6	98.00	0,0000
2-happeyl-lacture within spec.	PMPS10-6	NieldKents	8.8	<b>第2.7</b>	30.0	0,0000
2 Auconyl-factors COS	PARSON	Nick Kovin	60,7	40.6	00.7	0.0000
2-fuzzose/-bardossa COS	PMRSORQ	Palacia/Acadim	\$10.5	60,t	60,1	0,0000
2-fuccest-factors OCS	PMRS09-6	Nick/Kovin	80,00	80,7	<b>(1)</b> , (2)	0,0000
2-hannost-factoria OOS	PLPS00-4	Nick/Kovin	製多	A0,0	<b>20.7</b>	0,0000
2-fuccey/-incione GOS	PMRSODS	NickAcevin	80,A	60,5	00,0	0,0001
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# model 2.3 Measurement Uncertainty (expanded)

The reseasurement encertainty is build up from a varieties deviation (RSD<sub>N</sub>) and a systematic deviation (rescircum relative bias compared to the as true assumed value from a relative biastoratory test or a professory test or a reference material)

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

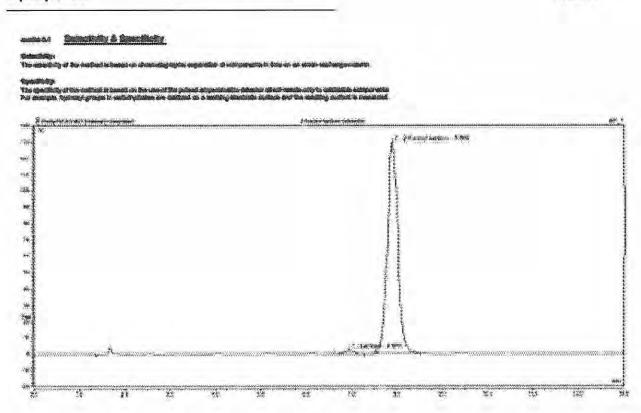
$$M(\%) = 2 \times \sqrt{(RSD_R)^2 + (D)^2}$$

M - minibe inscrimental productly (%)

RED m wastellan coefficient for reproductably

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

D (%)=	J 0.0
RED, (N) -	0.4 %
M (%) = relative measurement ancestabily =	



### Kritiekewaarden voor de Cochran maximumvariantietosts, kritisch nivesu 5 %

0.006 0.067 0.841 0.781 0,727 0,550 0,638 0,570 0.541 0,515 6,482 0,471 0,452 0,434 0,418 0,459 0,989 0,377 0.954 0,248 0,325 0,310 0,000 0,300 0,280 0.273 0,267 0,200 0.266 0.251 0.246 0,242 0.237 0.172 0,100

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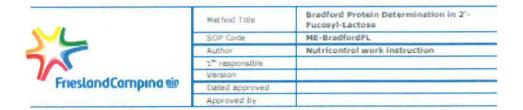


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27.7	0,90	1,09
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	0.04	1,00
	0.95	1,05
	0.96	1,04

Tabel ut W.J. Dison on F. Massey "Introduction to statistical analysis"; Mc Graw-Hill Book Company, Inc. New York 1967-II

# Appendix 3.3 Bradford Protein Determination in 2'-FL



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7.2 Liquid sample preparation Error! Bookmark not de	fined.
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### 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

- Bovine Serum Albumin

UV-Cuvette semi-micro
 5 mL Polystyrene Round-Bottom Tube

VWR; E530-1L

Sigma; A9418 Brand; 7591 50

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.

FrieslandCampina Innovation Centre Brenland 20, 6708 WH Wageningen

Page 2 of 4



# 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 NaCI-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

9/29/17

# 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)

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.

### Repestability (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.

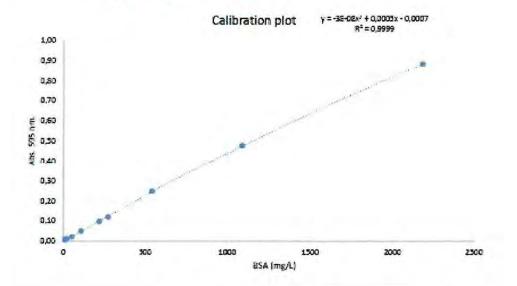
The LOQ of this method is 0,01% (m/m)

### Accuracy & calibration

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

The correlation coefficient (R2) is higher when a second-order polynomial equation is used.

Co	one (mg/L) AUFS	5 595 nm		
	2181,00	0,882		
	1090,50	0,479	equation	on coefficients
	545,25	0,248	a	-3,00E-08
	272,63	0,120	D	0,0005
	218,10	0,098	c	-0,0007
	109,05	0,051		
	54,53	0,023		
	21,81	0,013		
LOQ	10,91	0.007		



			Go	al Seek val	LIIB .	Ī					BSA
		Sample weight	calculated X	measured	Y	addition o	f		Recovery		% (m/m)
		mg in 10 ml	mg BSA/L	<b>AUFS 595</b>	nm	BSA (ml)	BSA (mo	i/L)			
2-fucosyl-lactose reference	PMRSOI	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	96	0,58
2-Jucosyl-lactose reference	PMRSOT	1098	1093,9	0,511	0,510	+1,0 ml	1090,5	mg/L	100,3	%	1,00
2-fucosyl-factose OOS	PMRS09	984	<10	-0,003							<0,01
2-fucosyl-lactose OOS	PMRS09	962	<10	0,001							<0,01
2-fucosyl-tactose OOS	PMRS09	879	543,1	0,262	0,262	+0,5 mi	545,26	mg/L	99,6	%	0,62
2-fucosyl-lactosa OOS	FMRS09	636	1022,1	0,479	0,479	+1,0 mi	1090,5	mg/L	93,7	%	1,61
2-fucosyl-lactose within spec.	PMRS10	996	<10	-0,002							<0,01
2-fucceyl-factose within spec.	PMRS10	1044	<10	0,001			-	-		1 1	<0,01
2-lucxeyi-landuse within spac.	PMRS10	964	558, 1	0,269	0,269	+0,5 m	545,25	ushy.	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	96	1,07

everage 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 ana	analysis 1a	analysis 1b	average	(X <sub>1</sub> -X <sub>2</sub> ) <sup>2</sup> X <sub>200</sub> <sup>2</sup>		
product	sample ID	date	% (m/m)	% (m/m)	% (m/m)	
2-fucesyl-tectose with 1,0 ml BSA	PMRS01	25-1-2017	0,95	0,94	0,95	0,00013
2 hucosyl-lactose with 0,5 ml BSA	PMRS01	25-1-2017	0,48	0,48	0,48	0,00000
2 fucosyl tectose 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-fucesyl-tectose with 1,0 ml BSA	PMRS09	25-1-2017	0,98	1,02	1,00	0,00190
2-fucosyl-tectose with 0,5 ml BSA	PMRS09	25-1-2017	0,46	0,48	0,47	0,00309
2-fucesyl-lactose with 0,2 mi BSA	PMRS09	25-1-2017	0,21	0,20	0,21	0,00029
2-fucesyl-lactose with 0,1 mi BSA	PMRS09	25-1-2017	0,11	0,10	0,11	0,00235
2-hucosyl-tactose with 1,0 ml BSA	PMRS10	25-1-2017	1,00	1,03	1,01	0,00104
2 fucceyl-tectose with 0,5 ml BSA	PMRS10	25-1-2017	0,49	0,49	0,49	0,00006
2-fucesyl-lactose with 0,2 ml BSA	PMRS10	25-1-2017	0,21	0,20	0,20	0,00007
2-fucosyl-tactose with 0,1 mi BSA	PMRS10	25-1-2017	0,11	0,11	0,11	0,00219
2000 20						
and date and he to and in the						0.04500

raw data can be found in sheet "raw data"

Σ 0,01539

number of duplo's (k) must be a 8

cochran-test	0,201	1
cochran-table value (kgn-1)	0,541	
number of duplo's (k)	12	
RSD,	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,	complies with limit	
product .	oduct sample ID date % (m/m)	% (m/m)	% (m/m)	% (m/m)	(%)		

# model 2.2 Reproducibility

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

Ilmit 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 duple analysis			analysis 1a	analysis 2a	average	(X1-X2)2 X2002
product	sample ID	date:	% (m/m)	% (m/m)	% (m/m)	
2 huopsyl-lactose with 1,0 ml BSA	PMRS01	25/26-1-2017	0,95	0,92	0,9	0,00078
2-fucesyl-lactions with 0,5 mil BSA	PMRS01	25/25-1-2017	0,48	0,49	0,5	0,00077
2-fuodeyl-botose with 0,2 ml BSA	PMRS01	25/25-1-2017	0,20	0,19	0,2	0,00070
2-fuonsyl-lactose with 0,1 ml BSA	PMRS01	25/26-1-2017	0,10	0,11	0,1	0,01271
2-tuopsyl-lactors with 1,0 ml BSA	PMRS09	25/26-1-2017	0,98	0,97	1,0	0,00012
2-tuopsyl-tectors with 0,5 ml BSA	PMRS09	25/26-1-2017	0,46	0,50	0,5	0,00616
2 fuonsyl-lactone with 0,2 ml BSA	PMRS09	25/26-1-2017	0,21	0,21	0,2	0,00107
2-fuopsyl-lactors with 0,1 ml BSA	PMR\$09	25/26-1-2017	0,11	0,11	0,1	0,00027
2-fuopsyl-lactose with 1,0 ml BSA	PMRS10	25/26-1-2017	1,00	0,94	1,0	0,00388
2-fuoncyl-lactors with 0,5 ml BSA	PMRS10	25/26-1-2017	0,49	0,46	0,5	0,00345
2-hucosyl-lactose with 0,2 ml BSA	PMRS10	25/25-1-2017	0,21	0,21	0,2	0,00007
2-fuonsyl-kactose with 0,1 ml BSA	PMRS10	25/26-1-2017	0,11	0,11	0,1	0,00414
raw data can be found in she	et "raw data"	1			Σ	0,03411

| 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<sub>R</sub> | 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 <sub>R</sub>	complies with limit
product	sample ID	analist 1/2	2 % (m/m)	% (m/m)	% (m/m)	(%)	
		-					

# model 2.3 Measurement Uncertainty (expanded)

The measurement uncertainty is build up from a variable deviation (RSD<sub>R</sub>) 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 (%)

	metrix 2-fucosyl-lactose	
$D\left(\%\right)=$	0.5	%
RSD <sub>R</sub> (%) =	3,8	%
M (%) = relative measurement uncertainty =	8	%

# 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 %

### 0,999 0,967 0,906 0,841 0.781 0,727 0,680 0,638 0,602 0,570 0,541 0,515 0,492 0,471 16 0,452 0,434 0,418 0,403 0,389 0,377 0,365 0,354 0,343 0,334 0,325 0,316 0,308 0,300 0,293 0,206 0,280 0,273 0,267 0,262 0,256 0,251 0,246 0,242 0,237 0,172 120 0,100

author:	H. Dahimana
Company:	Nutricontrol, Netherlands

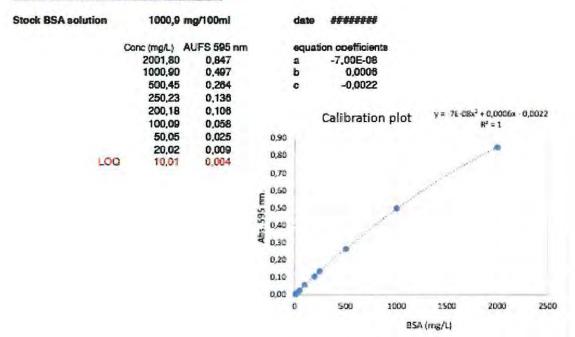


### Kritieke waarde P voor toesen precisiekenmerken

R	parameter (		
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 1150 0,90 1,09 200 0,92 1,08 300 0,93 1,07 400 0,94 1,06 500 0,95 1,05	1		
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 1100 0,88 1,12 1100 0,88 1,12 1100 0,88 1,12 1100 0,88 1,12 1100 0,99 1,09 200 0,99 1,08 300 0,99 1,09 200 0,99 1,08 300 0,99 1,09	k	P	Q
3			
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 0,68 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 0,88 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	2	0.23	
5	3	0,34	-
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 1150 0,90 1,09 200 0,92 1,08 300 0,93 1,07 400 0,94 1,06 500 0,95 1,05	4	0,42	1
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 1100 0.88 1,12 1100 0.88 1,12 1100 0.99 1,09 200 0.99 1,08 300 0.99 1,08 300 0.99 1,09 200 0.99 1,08 300 0.99 1,09	5	0,48	-
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 1,27 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 1,12 100 0,88 1,12 100 0,88 1,12 100 0,88 1,12 100 0,99 1,09 200 0,92 1,08 300 0,93 1,07 400 0,94 1,06 500 0,95 1,05	6	0,52	
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	7		1,42
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 1,12 100 0,88 1,12 100 0,88 1,12 100 0,88 1,12 100 0,88 1,12 100 0,88 1,12 100 0,88 1,12 100 0,88 1,12 100 0,88 1,12 100 0,99 1,08 300 0,93 1,07 400 0,94 1,06 500 0,95 1,05	8		1,39
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 1,12 100 0,88 1,12 100 0,88 1,12 100 0,88 1,12 100 0,88 1,12 100 0,88 1,12 100 0,88 1,12 100 0,88 1,12 100 0,88 1,12 100 0,99 1,08 300 0,93 1,07 400 0,94 1,06 500 0,95 1,05	9	0,61	1,37
11	10	0,63	1.35
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 1,12 100 0,88 1,12 100 0,88 1,12 100 0,90 1,09 200 0,92 1,08 300 0,93 1,07 400 0,94 1,06 500 0,95 1,05	11	0,64	1,34
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 1,12 100 0,88 1,12 100 0,88 1,12 100 0,90 1,09 200 0,92 1,08 300 0,93 1,07 400 0,94 1,06 500 0,95 1,05	12		1,32
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 1,12 100 0,88 1,12 100 0,88 1,12 100 0,90 1,09 200 0,92 1,08 300 0,93 1,07 400 0,94 1,06 500 0,95 1,05	177.77		1,31
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			1,30
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		0.70	1,29
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	16		1,28
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 0.74 1,05	17		1,27
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			1,27
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		0.73	1,26
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	20	0,74	1,25
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	25		1,23
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	30		1,21
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	35		1,19
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	40		1,18
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	45		1,17
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			1,16
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		0,85	1,15
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			1,14
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		0,87	1,13
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		0.00	1,12
200 0,92 1,08 300 0,93 1,07 400 0,94 1,06 500 0,95 1,05			
300 0,93 1,07 400 0,94 1,08 500 0,95 1,05			1,09
400 0,94 1,06 500 0,95 1,05	200		1,08
500 0,95 1,05	300		
1000 0 98 1 04			
1,01	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

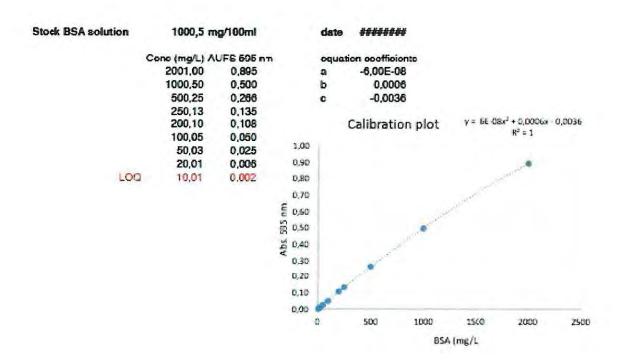


					calculated	measured Y		BSA	BSA *
analysis 1a		weight (mg)	AUFS	mg BSA/L	AUFS 595 nm		% (m/m)	% (m/m)	
24 lactose with 1,0 ml BSA	PMRS01	25-1-2017	1010,7	0,505	950,7	0,505	0,505	0,94	0,95
24-factose with 0,5 ml BSA	PMRS01	25-1-2017	1008,9	0,270	480,5	0,27	0,270	0,48	0,48
2-f-Inctone with 0,2 ml BSA	FMRS01	25-1-2017	1036,6	0,115	200,0	0,115	0,115	0,19	0,20
2-1-lactose with 0,1 ml BSA	FMRS01	25-1-2017	1092,6	0,056	98,1	0,056	0,056	0,09	0,10
2-f-Inchose with 1,0 ml BSA	PMRS09	25-1-2017	1050,4	0,517	976,5	0,517	0,517	0,93	0,98
2-1-factors 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-Inchose 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-Inctose with 0,1 ml BSA	PMR\$09	25-1-2017	1003,7	0,062	108,7	0,062	0,082	0,11	0,11
24-lactone with 1,0 ml BSA	PMRS10	25-1-2017	1007,6	0,527	998,2	0,527	0,527	0,99	1,00
2-1-factors 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-factors with 0,2 ml BSA	PMR\$10	25-1-2017	1048,8	0,118	205,2	0,118	0,118	0,20	0,21
2-f-isotoso with 0,1 ml BSA	PMRS10	25-1-2017	1113,0	0,085	114,3	0,065	0,065	0,10	0,11
		-							

Goal Seek Excel

					Goal Seek Excel				
					calculated	measured	Υ	BSA	BSA *
analysis 1b			weight (mg)	AUFS	mg BSA/L AUFS 59		nm	% (m/m)	% (m/m)
24-lactose with 1,0 ml BSA	PMRS01	25-1-2017	1015,6	0,500	940,0	0,500	0,500	0,93	0,94
2-1-lactose with 0,5 ml BSA	PMRS01	25-1-2017	1005,2	0,270	480,5	0,270	0,270	0,48	0,48
24-inctose 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-lectose with 0,1 ml BSA	PMRS01	25-1-2017	1023,4	0,058	101,8	0.058	0,058	0,10	0,10
24-lactose with 1,0 ml BSA	PMRS09	25-1-2017	1054,3	0,537	1020,0	0,537	0,537	0,96	1,02
24-lectose with 0,5 ml BSA	PMRS09	25-1-2017	1026,6	0,272	484,2	0,272	0,272	0,47	0,48
24-inctose with 0,2 ml BSA	PMRS09	25-1-2017	1110,2	0,117	203,5	0,117	0,117	0,18	0,20
2-1-tectose with 0,1 ml BSA	PMRS09	25-1-2017	1041,8	0,059	103,6	0,059	0,059	0,10	0,10
24-factose with 1,0 ml BSA	PMRS10	25-1-2017	1105,0	0,542	1030,9	0,542	0,542	0,93	1,03
24-lactose with 0,5 ml BSA	PMRS10	25-1-2017	1016,9	0,273	486,1	0,273	0,273	0,48	0,49
24-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	0,082	109,0	0,062	0,062	0,10	0,11
			+						

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



					Go	al Seek valu	18	C	
			255		calculated	measured	Υ	BSA	BSA *
analysis 2a			weight (mg)	AUFS	mg BSA/L	AUFS 595 nm		% (m/m)	% (m/m)
24-lactose with 1,0 ml BSA	PMRS01	26-1-2017	1058,9	0,500	924,5	0,500	0,500	0,87	0,92
24 lactorse with 0,5 ml BSA	PMRS01	26-1-2017	1173,7	0,278	494,0	0,278	0,278	0,42	0,49
24-lactose with 0,2 ml BSA	PMRS01	26-1-2017	1117,9	0,111	194,8	0,111	0,111	0,17	0,19
24-lactose with 0,1 ml BSA	PMRS01	26-1-2017	1095,4	0,061	109,8	0,061	0,062	0,10	0,11
24-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-lactorse with 0,2 ml BSA	PMRS09	25-1-2017	1057,1	0,122	213,9	0,122	0,122	0,20	0,21
24 lactose with 0,1 mil BSA	PMRS09	26-1-2017	1103,5	0,062	110,5	0,062	0,062	0,10	0.11
24 lactose with 1,0 ml BSA	PMRS10	26-1-2017	1070,1	0,507	937,8	0,507	0,506	0,88	0,94
24 lactose with 0,5 ml BSA	PMRS10	25-1-2017	1087,5	0,261	461,9	0,261	0,261	0,42	0,46
24 larsose 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-lectose with 0,1 ml BSA	PMRS10	26-1-2017	1091,4	0,060	107,1	0,060	0,060	0,10	0,11

BSA1: the sample weight is corrected to exactly 1000 mg to be able to compare results for calucation of repeatability and reproducibility.

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

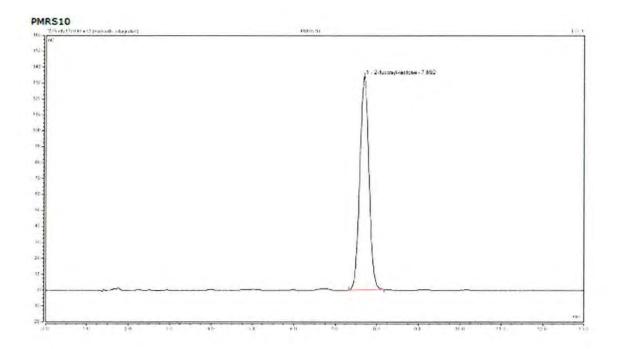
### Appendix Chromatograms of HPAEC 2'-fucosyllactose method

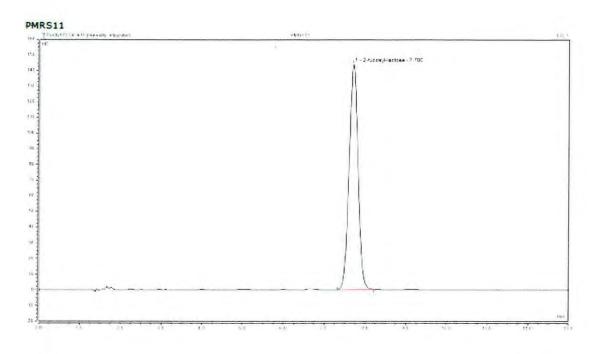
Isocratic HPAEC of the 2'-fucosyllactose end product (ME-AV042FL Isocratic HPAEC)

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

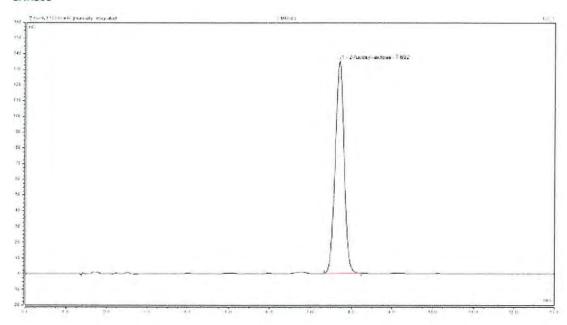
Identification and quantification of 2'-fucosyllactose is done with a standard, PMRS01, of which the 2'-fucosyllactose is identified and quantified with qNMR (see report Spectral Services, Köln, Germany)

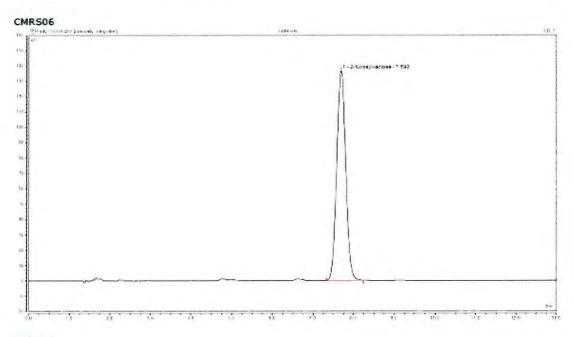
### Plots Chromatogram of registration batches, isocratic HPAEC method



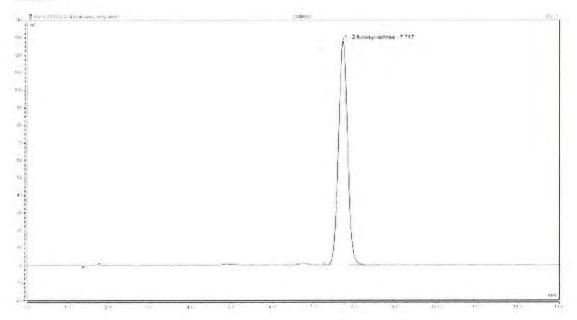


### CMRS03





### CMRS07



9/29/17

# 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

# Certificate of analysis

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



Balle	11 11	4.0	71	No.	
Date	100	Par.	100	4	-

## Description

### Pressal analysis

### Sensorial:

: Z-focosylactose

: PMRS10 - 78 18 7916

26.10.2016

Human milk obgosacchische

2.-Fucusyllactors: 93%, marsture 4%, success 1%, and factors: 1%, phonor: 41%, fucusy 41%.

Write immergeneous powder, neutral to stightly sweet, so of these

		SEC KILL LINESCO.	
Chemical/physical	Specification	Result	Hethod of analysis
Total mosture	max 5%	3.83%	150 260 (modified). Kari Fescher
2' Eurosyllarione	1994 98%	94.2%	FC methial using HPAFT PAD
Lactore	mer 4%	17.15/41	FC method using HPAEC PAD
Ako-Lactore	max 2%	0.5%	H. method using MPALC PAD
Cd45 12000	mer 2%	0.1%	FL midfied many MPALL PAD
Galactose	max 2%	< 0.1%;	FC method using HPAEC PAD
FIEDS	max 2%	0.2%	PC inettest using APALC PAD
Profess	mex. 0.01%.	-0.01%	Syladford
Sulphated ash	man 0.2%	H 112%	MEN 6810 (modified)
Hente	max. 1 maku	- 0.1 marks	ISO 14673-2/IDE 189-2
fit tata	mera. 3/0 mg/log	n 2 markg	150 14673 2780F 189-2
Scorefied particles	resien ebra A	A	PC method equivalent to
			ADM 916/05/0 5739/05/ 107
ph (turn)	1.0 7.5	4.11	FC method using NEW 3775
Albertenami	max. 4 8 reg/kg	<ul> <li>2 mg/kg</li> </ul>	FC method using ISO 17294
Armenai	max. 0.1 mg/kg	no bid impraig	FC method mens 150 17294
C.o. b votatto	max 0.01 mg/kg	c0 005 mg/kg	FC method using 150 17294
Mescury	ных, 0.05 выдел	< 0.001 mg/kg	FC method using 190 17294
Lennyt	max. 0.05 reg/kg	<0.02 mg/kg	FC method using 15th 17294
Aflatorin M1	mex 0.2 pg/kg	<0.01 µg/log	ISO (4501/IDF 171
Nicrobiological	Specification	Result	Method of analysis

CALCULATION CALLETY CALL
Aerobic mesophilic count
Entercharterwise
E coli
Years.
Mosside
Presumptive Backs ceres
Staphylococcus aureus
Substate recluring electricis
spores
i beneratien periranjere.
Samonella
Cronobacter sop
tralationers
GMO detection

Specification	Result
man, 3000 da/o	330 111/0
absent in 14 g	accent in 10
absorbed in 11 gr	eceptin 10
max. In their	< 1 chi/g
max. 10 chills	- 1 cluic
max. 198 cti/q	10 000
absent in 1.1	absent in Lg
max. 30 kfulg	) churg
absent to Ly	absent in Lg
alment in 25 g	abasis in 250
absent in 75 g	alisani in 25 i
may 10 Pulmo	0.88 EU/mg
migative	megative

	Method of analysis	
	FC method equivalent to ISD 4813	
E	FC method, 69W 18h 37"E, 5D, 98BG 18-24h 37"E	
	FC method, LMX 25h, Coll ID 24h	
	FC-method equivalent to ISO 661.1	
	FC (redhod equivalent to 250 6611	
	FC-method equivalent to ISO 2912	
	15/0 6888 3, G&C 42h 37*	
	FC method using 1999 27 (1995) 185-286 Wrenk	
	FC method, RPM 20h 46°C, confirmation	
	FC method contralent to ISO 6579	
	FC in without equivalent to ISO/TS 22964	
	Eur. Ph. 2.6-14 and USF <85>	

QPCR.

Wagening Bast Function: R&D Manager

District Compress (Novel 1986)
Additional on the Novel 1986 of Additional on the Novel 1880 of the America and P.O. Son Will 1880 of the America and Time Riverbands (S. 1981), P. 1981 (A. 1981), P. 1981

for earth, ergin Paris, Arenda EFS Toleron Rod Sett N/2 Palartie NEDWO (NA Local Allerton / Settle Ned Allerton Ned Frieddisch, andere a Descentification of the Section Co. Control Residence of Paris Section Co. Control Residence of Paris September 1992 of the Section Control Residence of Technologies (Control Residence of Technologies (Con

Physical Control of the Fig. 1984 of the Special Society of London Control of the Control of Contro

Certificate of analysis

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



Product Batch member Date of production Bond Beitre

Description Lypical analysis

Sensorial:

2" Focusyllaction PHRSII 40 11 2016 30 11 2019

Numer milk disposacchende

2' Furosyllactose 93%, musture 9%, lactose 1%, aido lactura 1 %, glacese «1%, ficuse «1%

White homogeneous powder, nestro to slightly sweet, no off firem

Result

93.2%

0.5%

41. b) %

0.1%

**20.1%** 

-0.01%

soften Los

Edgen 0.1

<0.7 mg/kg

< 0.01 mg/kg

c0 005 mg/kg

- II Off 3 mg/kg

=0.02 mg/kg

n.ave

0.2%

Chemical/physical Total mosture 2 Pucosyllactose Lactors Alo-Lactose Carrier GARBITORSO FIN Ires Products Suiphatest ash PARTIE Scorefied particles

DH (10%.) Ahmenum Arsenic Carbresian MOT DUTY Listair Alluticeen Mil

Microbiological

E. coli

VESSES.

Noulds

**Sporos** 

Samonella

Enstatason

Faret

Cronobacter spp.

GMO detection

Enterobacteriaceae

Acrober mesophier count

Presumptive Bacifus careus.

Substitle reducing closbridge

Staphylococcus aureus

Diestrichum perfringens

max The (Fig. 2%) Italy 2h trus 2% mus 25 max 0.01% max 0.7% man I marky mas 50 mg/kg more dies #

Specificat on

max 5%

min. 90%

3.8 7.5 max. 4.8 mg/kg max. U.1 mg/kg max 0.01 mg/kg max. If 65 madeu max, 0.65 reg/kg mas 0.2 µg/kg

almost to be-

<0.01 µg/kg Specification Result max. 1000 July - 1000 tru/a absent in 10-9 Street in 10 a absent in 10 g Atisent in 10 g mark 10 chara C) CHIVE max 10 cfufo. · LOTO/G mas 100 cf s/g e l'eferts Atment in 1 g max 30 cfuto - Lidwig Absent in 1 g

absent mile atment in 25 q Absent in 25 o Absent in 25 a about a 25 o may 10 EU-m) 0.54 EWing nespetive Megative

Method of analysis

150 750 (modified), Kirl Discher FC medical using MPAFC PAD FC method using HPAEC PAD PC method princ HPAEC PAD PC. Hedrical using HPAFC PAD FC-method using HPAEC PAO ht method using HPAEC PAD Bradford NEN 6810 (modified) 150 14673 2/lbf 189-2 150 14673 2/IBF 189 2 FC method equivalent to

ADPI 915/150 1739/30F 107 PC method using NEM 1775 PC method using ISO 17294 Hi melliod using ISD 17794 FC method using ISO 17294 FE method using 150 17294 fil method using this 17294 150-14501/JDF 171

Method of analysis

FC method equivalent to ISO 4833 FC method, BPV 18h 37°C, 50, VRBG 18-24h 37°C FC method, LMI 25h, Col ID 24h FC method equivalent to ISO 6611 FC Preffind employeet to 1523 66 (1 FC-method equivalent to ISO 7932

15O 6888 3, G8C 42h 37\*

FC mothed using DFM 27 (1995) 185-200 Weens

FC method, RPN 20th 46°C, confirmation FE method equivalent to ISO 6579 FC-method equivalent to ISO/TS 22964 Eur. Ph. 2.6.14 and USP < 85>

OPER

Wage

tricrited paper (non-1964)
to margin 4, 300 (1964)
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to tricrite (1971) 300 (1964)
to reference
to referenc

Tradicial argume (tomo revious 81% Particion Roset Name NAZ Particion no reg 87%)2

Aprile no reg 87%(2)

Frankerth without Danie Med. § 18-12 I famility (1992 highling) § New Square France John 1997 Inches part France Joyn. Malagna.

# Certificate of analysis

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



Product Satch number Owte of production Best Before

Description

Typical analysis

Chemical/physical

Sensorial:

Total mountains

Alfor Lieuteray

Sulphated ash

Scorched particles

Lactose

tales of

Parameter.

Protein

Dit tenter

hitrate.

net / 1/PM 1

Aluminam

Cadminaria

Affatoen Ma.

Microbiological

Enterolog fet at par

Aerobic mesophisc count

Presumptive Bacillus cereus

Sulphile rectating clostrida

Staphylococcus aureus

Chestralium per immpere.

Mercury

Local

E. cchi

Vangerin

Minute

Samonella

firmlestenoute

Cronobacter sop

Louisin

Galactose

2" Funday/surface

. 2" factos/dactose | CMRS03 | 05-01-2017 | 05-01-2020

Hamilto milk tilkgroses chemile

2 Furceylactose 93%, moisture 4%, lactose 1%, alto-lactose 1 %, glacose +1%, lucisie <1%

White becognition punder, certified to Highly sweet, on all these

0.1%

- 0.1%

<0.01%

+0.7 mg/kg

CL3 regittio

-0.2 mg/kg

<0.010 mg/kg

ell 1005 mg/kg

G 92%

1.64

0.0%

mos 2% mos 2% mos 2% mos 2% mos 0.0% mos 0.2%

3.0 - 7.5 max - 4.8 ma/kg max - 0.1 mg/kg max - 0.01 mg/kg max - 0.05 mg/kg max - 0.05 mg/kg

mas. 0.2 mater

Specification

absent in lite.

absent to long

max 10 ch/q

max 10 ch/g

mas 100 curo

absent in 10

max. 3000 share

mace. Sti ang/lag

man, disc A

-0.003 mg/kg -0.02 mg/kg -0.01 pg/kg -0.00 cla/g -0.00 cla/g -0.00 cla/g -0.00 cla/g -0.00 cla/g -0.00 cla/g

Alment in 1 u

max 20 ctu/g <1 ctu/g

attent in 1 g Absent in 1 p

attent in 25 g Absent in 25 g

attent in 25 g Absent in 25 g

attent in 25 g Absent in 25 g

max 10 ftPmg invaline invaline

Method of analysis ISD 760 (modified), ICerl fischer FC method using HPASC FAD FC method using HPASC FAD

FC method using HPAEC PAD FC method using HPAEC PAD FC method using HPAEC PAD FC method using HPAEC PAD

Bradford RED 58 (0 (modified)

ISG 14673 2/IDF 189-2 ISG 14673 2/IDF 189-2 IC method equivalent to ADM 916/ISD 5739/IDF 107 IC method using NEN 3775 IT method using ISO 17294 IC method using ISO 17294 ISO 1450/IOF 171

Method of analysis

Pt. method equivalent to 450-4633. FC-roichad, BPW 18h-37°C, SD, VSBG 11h-24h-37°C FC-method, CMX 25h, Coli ID 24h FC-method equivalent to 150-6611.

FC method equivalent to ISO 6611 FC method equivalent to ISO 6611 FC method equivalent to ISO 7932 ISO 6685-1, GSC 426-375

FC method using UFM 27 (1995) 185-200 Weersk

EC method, RFM 200 46°C, confirmation FC method equivalent to ISO 6579 FC method equivalent to ISO/TS 22964 Eor. Ph. 2.6.14 and USP < BS>

GPCH

(b) (6)

Wisall

Dan Bay

Function: Riskl Manager

Fundand corporation of the bition outsident of BEBS of Americans FO has that 1980 bit toronishmen The his particular for 1970(\$1) 70 44 his tor 1970(\$1) 71 44 his Emergent angles Done America Et 1, Promise State ATT Paramies St DMG 1554 State of July ANA Teach of July ANA Teach of July ANA Teach of July ANA Teach of July ANA Friedman Complete Depay of ac F. 15-12 Leave D. Committation F. 15-12 Leave D. Committation From Square Two J. Index Tails the Stational Sequence to support for a bill 178 475 DOO

 $F(\theta,\phi)$  with several divina of an exhibit into diving the least state of an appear constraints of the second several state of the second sec

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



Product Batch number Date of production Best Before

### Description

Typical analysis

Sensorial:

Cadmium

Allatoxin MI

Microbiological

Enterobacteriaceae

Aerobic mesophilic count

Presumptive Bacillus cereus

Sulphite reducing clostridia

Staphylococcus aureus

Ckstridium perfringers

Mercury

Lead

E. coli

Yeasts

Moulds

spores

Salmonella

Endotoxin

Cronobacter spp.

GMO-detection

- 2"-Fucosyllactose
- CMRS06

Result

3.59°W

92.7%

D.B%

0.2%

0.1%

0.1%

0.1%

< 0.1%

< 0.01%

< 0.1 mg/kg

1.6 mg/kg

- 24-03-2017 24-03-2020
- Human milk digosacchanise
- 2 Fucosyllactose 93%, moisture 4%, lactose 1%, allo lactose 1 %, glucose <1%, focose <1%
- : White homogeneous powder, neutral to slightly sweet, no off flavor

# Certificate of analysis

Chemical/physical	Specification
Total moisture	max 5%
2' Furosyflactose	man. 90%
Lactose	max 3%
Allo Lactose	max. 2%
Glucase	max 2%
Galactose	max 2%
Fucuse	max 2%
Protein	max. 0.01%
Sulphated ash	max. 0.2%
Nitnte	max. 1 mg/kg
Bitrate	max 50 mc/kg
Scorched particles	max disc A
pH (10%)	3.0 7.5
Aluminum	max. 4.8 mg/kg
Arsenic	max. 0.1 mg/kg

	7.5 4.8 ms/kg
	0.1 mg/kg
max.	0.01 mg/kg
max.	0.05 mg/kg
max.	0.05 mg/kg
max.	0.2 µ0.kg

max 3000 stole

absent in 10 3

absent in 10 a

max 10 cfu/g

max. 10 chi/g

max. 100 cfurg

absent in 1 a

max, 30 cfu/g

absent in 1 g

absent in 25 g

absent in 25 g

max. 10 EU/mg

negative

4 03
<0.2 mg/kg
<0.01 mg/kg
<0.00% mg/kg
< 0.006 mg/kg
<0.02 mg/kg
< 0.01 pu/kg
Result
< 1000 cfu/g
Absent in 10g
Absent in 10 g
<1 cfu/g
<1 ctu/g

<1000 cfu/g
Absent in 10g
Absent in 10 g
<1 cfu/g
<1 ctu/g
<1 rfu/g
Absent in 1 g
<1 clu/g
Absent in 1 g
Absent in 25 g
Absent in 25 g
< 0.1 EU/mg
Negative

Method of analysis 150-760 (modified). Karl Fischer FC method using HPAEC-PAD EC method using HPAEC-PAD FC method using HPAEC PAD FC method using HPAEC PAD FC-method using HPAEC PAD FC-method using HPAEC PAD Bradford NEN 6810 (modified) 150 14673-2/ICF 189-2 ISO 14673 2/ICF 189-2 H; method equivalent to ADPI 916/ISO 5739/IDF 107 FC-method using NEN 3775 FC-method using ISO 17294 FC method using ISO 17294 FC method using ISO 17294 R1 method using ISO 17294 FC-method using (50 17294) ISO 14501/IDF 171

Method of analysis FC-method equivalent to ISO 4833 TC method, BPW 18h 37°C, SD, VRBG 18 24h 37°C FC-method, LMX 25h, Coli ID 24h FC-method equivalent to 150 6611 FC-method equivalent to ISO 6611 FC method equivalent to ISO 7932 150 6888 3, G&C 42h 37\* FC-method using IJFM 27 (1995) 185-200 Weenk

FC-method, RPM 20h 46°C, confirmation FC-method equivalent to ISO 6579 FC-method equivalent to ISO/TS 22964 Eur. Ph. 2.6.14 and USP <85> **GPCR** 

Wageninggr, 10-04-2017

(b) (6)

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Foresteell, Ampère Danne, I Fili A. Sentiorigips (4: 38 III (1) Ameryline) F. F. Box 1551 (1807) Br. Ameryline) The Eintherland: Tel + 11 10 (1) 277 (1) [1] Tax + 33 (0011) 113 (1) 1.

erestandicampina Dunic AFAL F. PE. V. Level M. Crest Building 3 him Space No. 2, place 1971 66 IBB Petalog Bayl Manager Carol Found

Certificate of analysi

### Appendix 5.5

## Certificate of Analysis 2'-FL Lot CMRS07



Product Batch number Date of production

Best Before Description

Typical analysis

Sensorial:

2' Fucosyllactose CMRS07

09 05 2017 09 05 2020

: Homan milk oboosarchande

: 2' Ficosyllactose 93%, moisture 4%, lactose 1%, allo lactose 1 %, glurose «1%, fucose «1%

White homogeneous powder, neutral to slightly sweet,

Result

93.8%

0.5%

1.0%

<0.1%

-0H 196.

=0.01%

< 0.1 mg/kg

a a marka

0.08%

5 117

B. 2%.

Chemical/physical Total moisture 2'-Fucosyllactose Lactors Alle Lactose Glucies Galactose Fuerse Protein

Sulphated ash Mitrite. Mitrate Scorched particles pH (10%) Aleminum Arsento Cadmuos

Mercury Lead Aflatoxin M1 Microbiological

Aerobic mesophilic count Enterobacteriaceae E coli Yeasts. Moulds Presumptive Bacillus cereus Staphylococcus aureus Sulphite reducing clostridia **SPORES** Clostridium perfringens Salmonella Cronobacter spp. Endotoxin

GMO detection

min. 90% max. 3% max. 2% max. 2% max. 2% max. 2% max 0.01% max 0.2%

Specification

max Lingday max 50 mg/kg max disc / 30 - 7.5 max 4.8 mulion max 0.1 me/kg max 0.01 mg/kg

max 0.05 mg/kg max 0.05 rog/kg max. 0.2 jig/kg Specification max 3000 sfu/g

absent in 10 g absent in 10 q. max 10 alulu max. 10 chilo max. 100 ct.//o absent in 1 ] max 30 chillip

absent in 1 q absent in 25 q ebsent in 25 g max. 10 EUmig negative

< 0.4 mg/kg <0.020 ma/kg -0.010 maylos <0.011 mg/kg <0.07 mg/hg <0.01 pg/kg

<1000 cfu/g Absent in 10 g Absent in 10 g el chija < 1 cfu/q 20 cfu/u Absent in Lg = i chuyq

Absent in 1 g Absent in 25 g Absent in 25 g <0.1 f t/mg Negative

Method of analysis ISO 760 (modified), Karl Fischer

FC method using HPAEC PAD FC method using HPAFC PAD FC method using HPAEC PAD FC method using HPAEC PAD FC method using HPAEC PAD FC method using HPAEC-PAD

Bradford NEN 6810 (modified) ISO 14673 2/IDF 189-2 ISO 14673 2/IDF 189 2 FC-method equivalent to ADPI 916/ISO 5739/IDF 107

FC-method using NEN 3775 FC method using ISO 17294 HC method using ISO 17294 HC method using ISO 17294 FC method using ISO 17294 FC method using ISO 17294 150 14501/IDF 171

Method of analysis

FC-method equivalent to ISO 4833 FC method, 8PW 18h 37°C, SD, V8BG 18 74h 37°C FC method, LMX 25h, Coli ID 24h

FC method equivalent to ISO 6611 FC method equivalent to ISO 6611 FC method equivalent to ISO 7932 150 6888 3, 680 42h 375

FC-method using UFM 27 (1995) 185-200 Weenk

FC-method, RPM 20h 46°C, confirmation FC method equivalent to ISO 6529 FC method equivalent to ISO/75 22964 Fur. Ph 2 6.14 and USP <85>

OPCR

Wage Jan B

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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	(HO)	(*) months	ted months	t=12 months	t=24 treatities	t+36 months	ted munito	t=t munth	
Parameters tested									
Chemistry (%ar/w)									
Fotal maisture (Karl Fisher)	33%	2.7%	4.0%				4.3%	3.7%	
Ash	0.11%	*0.01	0.01				0.03%	0.02%	
271	96.3%	54.3%	54.8%				95.0%	94.2%	
Locion	0.6%	0.6%	1.3%				2,1%	2.1%	
Allo-loctore	0.1%	1.1%	1.6%				1.2%	1.6%	
Ghease	0.1%	0.1%	0.3%				0.1%	DAN	
Microbiology									
Mesophilic aerobic aell count	*10 cfu/g	< 2D offully	< 20 chair				< 10 cfu/g	<10 dujg	
Enterobacterioceae/ 10 g	Negative	Negulibre	Negation				Negative	Negative	
Salmanella/25 g	Negative	Negative	Negative				Negative	Negative	
Cronabacter spp/ 25 g	Negative	Negative	Negative				Negative	<b>Along at thes</b>	
Semortal	NOCEMBER 1								
smell .	Premier fed A	A bit serent	A bit secont				A bit sweet	A bit sweet	
apjero/ance	White Homogra- nous fire powder	White Homoger- nouti fine powder	White Homoger- rous Fire powder		u mana angawa daka ngant di sala angawa ngaka ng		White Homoge- nous fire pawder	White Harmage- reset fire gaveder	

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 6<sup>th</sup> 2017 t=24 months: December 6<sup>th</sup> 2018 t-36 months: December 6 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

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Appendix 11 Bacterial Reverse Mutation Test with 2'-Fucosyllactose



### STUDY REPORT

### V20805/05

Bacterial reverse mutation test with 2'-fucosyllactose

IA 30 March 2017

Arma(s) M.J.M. van den Wijngaard

Sposson Friesland Campina Innovation

Bronland 20

6708WH Wageningen The Netherlands

Temperature (formerly 093.26005/02.41)

TRISKSLIGH BYTTHY CODE 20805/05

SICOSOGRATATA CAREF

GUIDHUNG OECD 471

STATES Final

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The rights and obligations of contracting parties are subject to either the General Terms and Conditions for Commissions to Triskelion, or the relevant agreement concluded between the contracting parties.

Should any doubt arise from the publication of the Triskelion report in an electronic form, the authorized printed version shall be considered authentic.

2017 Triskelion

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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)<sup>1</sup>. 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

(b) (6)	
	30 March 2017
F.A.A. van Acker, PhD	Date

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

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# **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		

(b) (6)

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<sup>+</sup> and trp<sup>+</sup> 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.

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### 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: danne.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 40

3704 HE Zeist The Netherlands

Phone: +31 88 866 2800

1.3 Responsible Personnel

Study director: F.A.A. van Acker, PhD Phone: +31 88 856 26 18

E-mail: frederique.vanacker@triskelion.nl

1.4 Time schedule

The test was conducted between 06 and 10 October 2016.

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### 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

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# 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.

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### 4 Materials and methods

### 4.1 Characterization of test substance

Test material name 1 : 2'-fucosyllactose

Chemical name <sup>1</sup> : 2'-FL

Batch number <sup>1</sup> : MRS02

CAS number <sup>1</sup> : 41263-94-9

Appearance <sup>1</sup> : white powder

Purity <sup>1</sup> : 94 %

Molecular formula <sup>1</sup> : C<sub>1e</sub>H<sub>32</sub>O<sub>15</sub>

Molecular weight <sup>1</sup> : 488.99 g/mol

Storage conditions 1 : 2-10°C, protected from light

 Date of receipt
 : 19 July 2016

 Expiry date <sup>1</sup>
 : 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 (NaN3) from Aldrich, Brussels, Belgium.

<sup>1</sup> Characteristics provided by the sponsor

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### 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 <sup>1</sup>					
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 <sup>+</sup>	uvrA*	-R			

<sup>1</sup> 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<sub>2</sub> 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

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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  $\mu$ m 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  $\mu$ g/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 5. typhimunium 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.

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### 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.

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### 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<sup>†</sup> (S. typhimurium) and trp<sup>†</sup> (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.

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### 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.

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### 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.

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# Appendix 1 - Results of the bacterial reverse mutation test

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

		TA 15	35	TA 1537		TA 98	3	TA 100		E. Coli	
		-59	+59	-59	+59	-59	+59	-59	+59	-59	+59
n µg/plate		34	23	16	11	31	44	144	182	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	181	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	80	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
558 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	18	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	65
	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
		35	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	1889	B41	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	1905	927	2722	483	576
	StDev	31	20	1269	29	97	39	82	192	69	38

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Mean StDev

Average number of revertants per plate Standard deviation

59

Pos. Control

Liver homogenate from rats treated with aroclor Positive control; see text for actual concentrations of reference mutagens

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### Annex 1 - GLP compliance monitoring unit statement



### **ENDORSEMENT OF COMPLIANCE**

WITH THE OECD PRINCIPLES OF GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Manitoring 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 Triskeion BV Utrachtsawag 48, 3701 HE Zelst PO Box 844, 3703 AV Zelst

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.



Heelth Care Impectorate of the Ministry of Health, Wefare and Spot Sectepanese 1, 202 - AZ Usecto P.C. Box 2500, 3500 GR Usech, The Netherlands

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# 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	Additi	Additional mutations <sup>1</sup>					
		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	141		
<sup>1</sup> rfa uvr	: - = sensitive to crystal vi		ak sensiti	ve to crystal	violet			
R-factor	: - = sensitive to ampicillir	n; + = resista	ent to amp	picillin				
His	: - = requires histidine							
Тгр	: - = 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: 247-365
- 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.

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# 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

# MOLTOX

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# 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.

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# 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	ļ.,	-	•	+	4		-
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)

<sup>+</sup> mutagenic (positive)

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#### 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.

	Mean ± SD number of revertants per plate (number of assays)						
Strain	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)	$30 \pm 7 (4)$			
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 59-mix	•			0			
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 UVIA	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)	

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# Annex 5 - continued

#### Historical positive controls

Overview historical positive control.

Data from assays conducted between 2011 and September 2016.

		Mutation Rati	o <sup>1</sup>			
Strain	Compound <sup>2</sup>	mean ± standard deviation; rang (number of assays)				
without S9-m	nix					
TA 1535	NaN₃, 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 <sub>3</sub> , 1 μg/plate	6 ± 1	3-9	(45)		
WP2 uvrA	ENU, 100 µg/plate	11 ± 3	5-18	(40)		
with S9-mix	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 ug/plate	9 ± 4	5-24	(41)		

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

ENU = N-nitroso N-ethylurea

2-AA = 2-aminoanthracene

9-AA = 9-aminoacridine

BP = benzo(a)pyrene

2-NF = 2-nitrofluorene

NaN<sub>3</sub> = natrium azide

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# Annex 6 - Certificate of analysis

A mala all some statements of production of production control of the control of		r Wichel Ft  AJ (50-clasments product)  MESS2  07 07 26 (6  an 70 cm Book (FBD)			
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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

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B. Usta, BSc

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Friesland Campina Innovation

Bronland 20

6708WH Wageningen The Netherlands

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The rights and obligations of contracting parties are subject to either the General Terms and Conditions for Commissions to Triskelion, or the relevant agreement concluded between the contracting parties.

Should any doubt arise from the publication of the Triskelion report in an electronic form, the authorized printed version shall be considered authentic.

2017 Triskelion

Study director

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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<sup>1</sup>. 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.

(b) (6)	
	OY April 2017
	Date

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

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# **Quality Assurance Statement**

 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

TRISKELION B.V.
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# **Abbreviations**

GLP Good Laboratory Practice

OECD Organisation for Economic Co-operation and Development

QA Quality Assurance QAU Quality Assurance Unit TRISKELION B.V.
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## 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.

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# 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

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

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# 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.

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# 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.

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#### 4 Materials and methods

# 4.1 Characterization of the test substance

Test material name 1 : 2'-fucosyllactose

Chemical name <sup>1</sup> : 2'-FL

Batch number <sup>1</sup> : MRS02

CAS number <sup>1</sup> : 41263-94-9

Appearance <sup>1</sup> : white powder

Purity <sup>1</sup> : 94%

Molecular formula <sup>4</sup> : C<sub>18</sub>H<sub>32</sub>O<sub>15</sub>

Molecular weight <sup>4</sup> : 488.99 g/mol

Storage conditions 1 : 2-10°C, protected from light

Expiry date <sup>1</sup> : 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 : 33047
CAS Reg. Number : 6055-19-2
Molecular formula : C<sub>7</sub>H<sub>15</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>2</sub>P.H<sub>2</sub>O
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

<sup>1</sup> Characteristics provided by the sponsor

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Aneugenic positive control:

Name : Vinblastine sulphate

Appearance : a white powder

 Batch number
 : 35366

 CAS Reg. Number
 : 143-67-9

 Molecular formula
 : C46H58N4O9

 Molecular weight
 : 810.974 g/mol

Purity : 100%
Storage conditions : 2-10 °C
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, 56°C) fetal calf serum (20% v/v), penicillin (100 U/ml medium), streptomycin (100  $\mu$ g/ml medium) and phytohemagglutinin (2.4  $\mu$ g/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.

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#### 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  $\mu$ g/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 blood in 4.5 ml culture medium, were incubated for 48 hours at ca. 37°C in humidified air containing ca. 5% CO<sub>2</sub>. 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 concentrations 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

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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<sub>2</sub> 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  $\mu$ l 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  $\mu$ g/ml and used as positive control (final concentration 0.0125  $\mu$ g/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<sub>2</sub> 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.

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#### 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 = <u>no. of mononucleates + 2 x no. of binucleates + 3 x no. of multinucleates</u>
(total number of cells)

Replication index (%) = 100 x <u>CBPI T (mean) - 1</u>
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.

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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.

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#### 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  $\mu$ g/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).

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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).

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# 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.

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# 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.

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## 8 References

- OECD guideline 487 for the testing of chemicals: In Vitro Mammalian Cell Micronucleus Test (MNvit); adopted 29 July2016.
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# **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	

<sup>&</sup>quot;) 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)

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#### Appendix 2: Tables of results

Table 2.1 Test 1≯Pulse treatment method with metabolic activation

Treatm / recovery time (h)	Dose level (µg/m1)	ana	ell stage alysis/50 O-BN-M	00	BN (%)	CBPI	(mean)	RI (%)	% Cytotox. (100- RI)	Selected for MN analysis (+/-)	MNBN/ 1000BN	MNBN/ 2000 BN (%)	Statistics (p-value)
	NC	243	255	2	51.00	1.52	1.52	100	0	+	7	14	
		250	243	7	48.60	1.51			1		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	1.45					6	(0.65)	
4/20	500	259	238	3	47.60	1.49	1.47	90	10	+	4	13	n.s.
(+59)		281	217	2	43.40	1.44					9	(0.65)	
	250	253	242	5	48.40	1.50	1.49	95	5	7			-
		262	237	1	47.40	1.48							
	125	270	227	3	45.40	1.47	1.47	91	9	- 2	4		-
		269	228	3	45.60	1.47							
	62.5	271	223	6	44.60	1.47	1.47	92	8	- 14 N		1.0	10=6
		265	232	3	45.40	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		1 2/2			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

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# Appendix 2 - continued

Table 2.2 Test 1▶Pulse treatment method without metabolic activation

Treatm / recovery time (h)	Dose level (µg/ml)	an	ell stag alysis/5 IO-BN-M	00	BN (%)	CBPI	CBPI (mean)	RI (%)	% Cytotox. (100-RI)	Selected for MN analysis (+/-)	MNBN/ 1000BN	MNBN/ 2000 BN (%)	Statistics (p-value)
	NC	245	249	6	49.80	1.52	1.52	100	0	+	В	17	-
		246	246	8	49.20	1.52					9	(0.85)	
	2000	246	245	9	49.00	1.53	1.54	103	3 0	+ 1	8	15	n.s.
		230	265	5	53.00	1.55					7	(0.75)	
	1000 500	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)	
4/20		237	253	10	50.60	1.55			0	+	9	17	n.s.
(-59)		220	278	2	55.60	1.56			11111		8	(0.85)	
	250	225	272	3	54.40	1.56	1.57	108	0	-		4.	-
		219	275	6	55.00	1.57							
	125	251	242	7	48.40	1.51	1.50	96	4	-		. 1	1
1		259	234	7	46.80	1.50					122-1		
	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)! 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
 <sup>1)</sup> Chi-square test (one-sided)

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#### Appendix 2 - continued

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

Treatm / recovery time (h)	Dose level (µg/ml)	an	Cell stag alysis/5 IO-BN-M	00	BN (%)	CBPI	CBPI (mean)	RI (%)	% Cytotox. (100-RI)	Selected for MN analysis (+/-)	MNBN/ 1000BN	MNBN/ 2000BN (%)	Statistics (p-value)
	NC	298	191	11	38.2	1.426	1.396	100	0	+	В	17	
		330	157	13	31.4	1.366					9	(0.85)	
24h	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.
(-59)		349	137	14	27.4	1.330				1 = 1	В	(0.90)	
	750	299	188	13	37.6	1.428	1.396	100	0				- 1
		325	168	7	33.6	1.354					70-0		
	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	457	43	0	8.6	1.086	1.073	18	8 82		56	127	<0.0001
	0.0125	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

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#### Annexes

#### Annex 1: GLP Compliance Monitoring Unit Statement



# **ENDORSEMENT OF COMPLIANCE**

WITH THE OCCUPRINCIPLES OF GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 2004 SPEC the conformity with the Off CD Principles of GLP was assessed on 29 September 6 Outster and 9 December 2015 at

TNO Instance by Unscrewing 45 3704 NO Zerol PO Bus 644 2700 NV Zerol

It is nervisely coefficient that the affore-mentioned test tackly is currently operating in complience with the OECD Principles of Good Laboratory Practice in the following press of expense: Toxicity multigenoisty analytical and crinical stremmers safety phaemacology isnetics, metabolism and in vitro studies.



Healt Care Inspections of the Ministry of Health Andrew and Special Geography - The Call Charles P.O. Son ZARO SIGO OF Minister The Natherlands

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#### Annex 2: The quality certificate of 59

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

# MOLTOX

#### POST MITOCHONDRIAL SUPERNATANT (89) QUALITY CONTROL & PRODUCTION CERTIFICATE

Part Number Influenties LOT NO. 1 2 14 PART NO 1 1 (9) VOLVIE 1 2 11 A 10L BI SEPR - 1 2 VIA C 1 STORAGE 41 at beins 40 C PREP (mid-o Shi<sup>2</sup> ENPHO medan 1 200 (NR) ING AGENT warm (Na (Riemann Klair) 50 (a kara Astroclassics SPECIES SE SPECIES SE STRAIN: SESSIBLE LINE SE SEN ALIE AGE 1 CHERK WHIGHT 12 (1988) TISS I Lace CLEAN PRESCRIPTION IN THE CLEAN CONTINUES. Service Park behavior Accord F3.28 Interested | Asker for ethiosystematic Polent (SOO) pentrol pentr BIOLNAMA

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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.
- The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.
- The two nuclei in a binucleated cell should be approximately equal size, staining pattern and staining intensity.
- 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
- 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.
- 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:

- 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.
- 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.
- Micronuclei may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
- 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.

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#### 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 + 59	35	*	0.70 ± 0.18	0.40 - 1.20
4 / 20 - 59	35	*	0.68 ± 0.19	0.20 - 1.15
(24 /-) - 59	4	1% DMSO	0.87 ± 0.18	0.65 - 1.15
(24 /-) - 59	3	2% DMSO	1.16 ± 0.30	0.90 - 1.65

<sup>\*</sup>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 ± 5.D.)	Range (%)
4 / 20 + 59	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

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# Annex 5: Certificate of Analysis

Product : Vivinal FL
Product code NA (Severapmental product)
Retensumber MRSO2
Date of production (S2-C7-2018
Concact person 3an-William Books (RAD)

Description : Fluman milk objects change

Typical analysis

2 Orv matter 97%, moisture 1%, 2\*-Fucosyllactose 94%, tectore 1%, glucose 1%, fucose 1%

Chemical/physical:	Specification	Besults	Method of acalesis
Total moisture  7 Fucosyllecture 3-Fucosyllecture 3-Fucosyllecture Fucosyllecture Lacture Guiose Froteir Sulphoted ash Nertis Nitrate pH (10%)	max 5% men 90% max 2% max 2% max 2% max 2% max 0 01% max 0 2% max 50 mg/kg 1.0 7.5	5% 54% 51% 61% 61% 61% 60025% 601 203 303	ISO 760 (moddled), Karl Fischer FC-method using HPAEC PAD FC-method using NEN 3775
Microbiological:			
Acrosic mesobilic count Enterobetteriaceae E. coli Yeaxta Moultat Presumptive Bastilius census Salphite reducing dostriole Costradium perfungess Salmoneila Considerater spa.	absent in \$ 5	<1000 <1 <1 <1 <1 <1 <1 <1 <1 neg neg neg	FC-method equivalent to 130 4933 FC method, 99W 16h 27°C, 50, VRSG 18-24h 37°C, FC method, LMX 25h, Cos ID 24h FC-method equivalent to 150 6611 FC-method equivalent to 150 6615 FC method equivalent to 150 7932 FC method equivalent to 150 7932 FC method using DPM 27 (1995) 185-700 Weens FC-method, GSC 42h 37°C, PCR FC method equivalent to 150 65°C, confirmation FC method equivalent to 150 65°C, for the 150 for 1

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