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# Final Study Plan



Combined chronic toxicity and carcinogenicity study in rats fed  
GM maize NK603

Multi-Site Study Plan

**G-TwYST**  
**GMP Two Year Safety Testing**  
**632165 A/2015/GLP**

9 June 2015

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Prof. Dr. Pablo Steinberg

	Combined chronic toxicity and carcinogenicity study in rats fed GM maize NK603 according to OECD Test Guideline 453 and EFSA Considerations on the applicability of OECD TG 453 to whole food/feed testing	 Reg. No. 061/G-036 Version: ..... Page No.: 2/38
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### Acknowledgment and Disclaimer

The authors of this document thank all project partners for their valuable contributions and comments on draft versions of this document.

This document expresses the view of the G-TwYST consortium, and does not reflect an official opinion of the European Commission. Responsibility for the information and views expressed therein lies entirely with the authors.



In the whole document, the acronym “**G-TwYST**” has been used to refer to the project.

“GMP Two Year Safety Testing” (G-TwYST) is a Collaborative Project of the Seventh Framework Programme of the European Community for Research, Technological Development and Demonstration Activities.

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SZU, Limbová 14, 833 03 Bratislava 37, Slovak Republic,		Testing Laboratories Center, Laboratory of Toxicology
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## Combined chronic toxicity and carcinogenicity study in rats fed GM maize NK603

### Multi-Site Study Plan




Study No: 632165 A/2015/GLP

**Sponsor:** EU Project G-TwYST

**Sponsor's representative:** Prof. Dr. Pablo Steinberg

**Test Facility:** Slovak Medical University  
Testing Laboratories Center  
Laboratory of Toxicology  
Limbová 14,  
83303 Bratislava  
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## 1. SIGNATURES OF APPROVAL OF THE MULTI-SITE STUDY PLAN



<b>Study Director:</b>	<b>Name</b> Dagmar Zeljenková	<b>Date</b>	<b>Signature</b>
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<b>Test Facility Management:</b>	<b>Name</b> Martin Gajdoš	<b>Date</b>	<b>Signature</b>
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<b>Sponsor's Representative:</b>	<b>Name</b> Pablo Steinberg	<b>Date</b>	<b>Signature</b>
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**Confirmation of Study plan in accordance with GLP**  
 This study plan meets the requirements for GLP compliance

<b>Head of QAU</b>	<b>Name</b> Eva Němcová	<b>Date</b>	<b>Signature</b>
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## 2. NATIONAL REGULATIONS, GUIDELINES AND STANDARDS

### 2.1. Good Laboratory Practice

The study will be conducted in accordance with the OECD Principles of Good Laboratory Practice, as revised in 1997 (ENV/MC/CHEM(98)17), and the EU Commission Directive 2004/10/EC (adopted on the 11<sup>th</sup> of February 2004; Official Journal No L 50/44).

The Center of Testing Laboratories (Laboratory of Toxicology, Laboratory of Immunotoxicology - including the laboratory of haematology - and the experimental animal housing rooms) of the Slovak Medical University has received a statement of GLP compliance from the Slovak National Accreditation Service (certificate No. G-036). The National GLP Compliance Programme in the Slovak Republic is based on Act No. 67/2010 Coll. and in compliance with Government Decree No. 320/2010 Coll. The Laboratory of Clinical and Experimental Biochemistry of the Slovak Medical University is not GLP compliant, but holds an accreditation certificate (ISO 17025) from the Slovak National Accreditation Service (certificate No. M-013), is subject to the National Quality Control Programme for Clinical Biology and is controlled by the Quality Assurance Unit (QAU) of the Slovak Medical University. All procedures performed by the above-mentioned laboratories are described in standard operating procedures approved by the QAU.

Each Principal Investigator at the histology processing test site and the histopathology examination test site will be responsible for compliance with their national GLP regulations, for any work performed at their test site and for data provided to the test facility for inclusion in the report. Any phase report or data provided by the principal investigator should include a statement of GLP compliance signed by them and a quality assurance statement signed by the test site quality assurance.



These principles are compatible with Good Laboratory Practice regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA), and Japan (MHLW, MAFF and METI).

Test site 3, the biostatistics study phase, will not be claiming GLP compliance for this phase of the study. This test site does not hold a national certificate of GLP compliance, however the expertise of the Principal Investigator (Contributing Scientist) was considered by the Sponsor to be necessary for the study.

### 2.2. Other Guidelines

The study design is based on the procedures indicated by the following internationally accepted guidelines and recommendations:

- The OECD Test Guideline 453 for Testing of Chemicals; "Combined Chronic Toxicity/Carcinogenicity Studies" (adopted on the 7<sup>th</sup> of September 2009)

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- The EFSA Consideration on the applicability of OECD TG 453 to whole food/feed testing (EFSA Journal 2013; 11(7):3347).

### 2.3. Animal Welfare

The study will be conducted in accordance with EU Directive 2010/63/EU of the European Parliament and the Council of 22<sup>nd</sup> September 2010 on the protection of animals used for scientific purposes. This study has been approved by the Veterinary State Administration, Slovak Republic (Statna veterinarna a potravinova sprava Slovenskej republiky). Animal care will be in compliance with SOPs of the Department of Toxicology, Slovak Medical University Bratislava and with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

The criteria described in the OECD Guidance Document on the recognition, assessment and use of clinical signs as humane endpoints for experimental animals used in safety evaluation (ENV/JM/MONO[2000]7) such as changes in external physical appearance and clinical signs (described in Annex 3 of the above-mentioned OECD Guidance Document) will be taken into account to determine when an animal is in a moribund condition, is expected to become moribund or experiences pain and distress, and should therefore be euthanised.



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### 3. GENERAL INFORMATION

#### 3.1. Multi-Site Study Details

**Test Sites:**

**Study Phase:**

**Histology Processing**

**Test Site 1:**

Institute of Pathology  
 University of Veterinary Medicine Hannover  
 Bischofsholer Damm 15  
 30173 Hannover  
 Germany

**Principal Investigator:**

Prof. Dr. Wolfgang Baumgärtner  
 wolfgang.baumgaertner@tiho-hannover.de

**Test Site Quality Assurance:**

Dr. Ilona Fleischhauer  
 Fraunhofer Institut für Toxikologie und Experimentelle  
 Medizin  
 Leitung Qualitätssicherung  
 Nikolai-Fuchs-Str. 1  
 30625 Hannover, Germany  
 ilona.fleischhauer@item.fraunhofer.de

**Study Phase:**

**Histopathology**

**Test Site 2:**




Roger Alison Ltd.,  
 Caerfyrddin Fach,  
 Cilcennin,  
 Lampeter,  
 SA48 8RN  
 United Kingdom

**Principal Investigator:**

Roger Alison, BVSc., MRCVS, DipIQCVP  
 roger@rogeralison.com

**Test Site Quality Assurance:**

Clare Alison, BSc., MSc., PhD., MRQA,  
 Roger Alison Ltd.  
 clare@clarealison.com

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**Study Phase:**

**Biostatistics**

**Test Site 3:**

Stichting Dienst Landbouwkundig Onderzoek (DLO)  
 Wageningen University and Research Centre  
 Droevendaalsesteeg 1  
 6708 PB Wageningen  
 The Netherlands

**Principal Investigator:**

Dr. Hilko van der Voet  
 hilko.vandervoet@wur.nl



**3.2. Additional Responsibilities**

Toxicology: Dagmar Zeljenková, VMD, PhD  
 Clinical Chemistry: Prof. Spustova Viera, M.D., Ph.D.  
 Haematology: Jana Tulinská, M.D., Ph.D.  
 Ophthalmology: Prof. Andrej Černák, M.D., Dr.Sc.  
 Necropsy: Katarina Ambrušová, VMD  
 Lead Quality Assurance: Eva Němcová, Mgr.  
 Ethics Committee: Ludmila Novotná, Dr.  
 Histopathological Peer Reviewer: To be added by amendment

**3.3. Distribution List**

The original signed study plan will be retained in the study file, to be archived at the completion of the study. Copies of the final study plan along with any amendments will be distributed to all relevant staff via supervisors/department heads specified as follows:

Sponsor: pablo.steinberg@tiho-hannover.de  
 Study Director: dagmar.zeljenkova@szu.sk  
 Clinical Chemistry: viera.spustova@szu.sk  
 Haematology: jana.tulinska@szu.sk  
 Ophthalmology: andrej.cernak@pe.unb.sk  
 Necropsy : katarina.amrusova@szu.sk  
 Lead Quality Assurance: eva.nemcova@szu.sk  
 All Principal Investigators: See multi site study details

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All Test Site QA: See multi site study details

### 3.4. Study Plan Amendments and Deviations

Any intended change to the study plan will result in an amendment to study plan approved by the study director and also signed by test facility management and the Sponsor. Amendments will be distributed to all recipients of the study plan.

Deviations (unplanned changes) from the study plan will be documented and acknowledged by the study director. Each principal investigator will document deviations from the study plan affecting their study phase, acknowledge and report them to the study director.

### 3.5. Quality Assurance

Lead quality assurance will audit and inspect study-related procedures and will report any audit and inspection results in writing to the study director and test facility management. This includes review of the study plan and any amendments, inspection of specific critical phases of the study and audit of the final report. Details of inspections will be included within the Quality Assurance Statement issued with the final report.

Test site quality assurance will audit and inspect study-related work conducted at their test site according with their SOPs and will report any audit and inspection results in writing to the principal investigator, test site management, study director, test facility management and lead quality assurance. Details of inspections will be included within the test site Quality Assurance Statement.

### 3.6. Reporting



A GLP compliant report will be presented. This will include the reporting requirements as described in OECD Test Guideline 453 and will be written in the English language. A draft report will be sent to the Sponsor for review and comments before issue of the final report. The pre-QA draft report and the post-QA draft report will be issued before the final report.

The report will be prepared by the study director based on the raw data / phase reports received from the responsible principal investigator/contributing scientist; the phase reports received from the principal investigator/contributing scientist will be included in the appendices of the report.

### 3.7. Archiving

The following documents will be archived under code number 632165A/2015/GLP at the Registry of accredited laboratories and laboratories with GLP certificate of SZU until the year 2026:

- the study plan and any amendments
- correspondence between the Study Director and test sites

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- QA reports of audits/inspections
- all raw data (paper and electronic)
- all original documents/primary documentation (including chain of custody records)
- samples of the test items
- copy of the histology processing records (original at the Department of Pathology, University of Veterinary Medicine Hannover, Germany)
- the original histopathology phase report
- reports from contributing scientists




Further details of documents to be retained are included in the Appendix, Attachment 3. No data will be discarded without the Sponsor's written consent.

Paraffin blocks will be archived at the Department of Pathology, University of Veterinary Medicine Hannover, Hannover Germany, while the tissue slides will be archived at Roger Alison Ltd., Lampeter, United Kingdom.

### 3.8. Proposed Time Schedule




Test feeds arrive:	June-July 2015
Arrival of animals:	July - August 2015
Starting of the treatment:	
- males	August 2015 - day 1-5 and 8-12 (in groups)
- females	September 2015 - day 15-19 and 22-26 (in groups)
Necropsy of the animals (1 year):	day 366 of each group
Histological processing:	1 month after day 392
Histopathology evaluation:	2 months after day 392
Draft Report to Sponsor:	3 months after day 392
Necropsy of the animals (2 years):	day 731 of each group
Histological processing:	1 month after day 760
Histopathology evaluation:	4 months after day 760
Draft report to sponsor:	6 months after day 760

See Appendix, Attachment 1, for a more detailed proposed time frame.

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#### 4. OBJECTIVE

The purpose of this oral toxicity study is to assess the effects of GM maize NK603 when fed to rats for a period of 1 year and 2 years. This study is being conducted in association with two 90-day feeding studies (designed according to the OECD Test Guideline 408) as part of a bigger project. This study will provide a comparative assessment of the results of shorter term subchronic toxicity studies versus an extended chronic toxicity/ carcinogenicity study.

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## 5. TEST AND CONTROL ITEMS

### 5.1. Test Item



GM maize: Variety containing the NK603 event expressing the glyphosate tolerant 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). Variety to be chosen after the analyses of harvests from different locations.

The test item is referred to as NK603 maize hereafter. Untreated NK603 maize as well as NK603 maize treated with the glyphosate-containing herbicide Roundup will be used

Records including test item and reference item characterisation, batch number, purity, composition/concentrations, date of receipt, expiry date, storage conditions, quantities received and used will be maintained within the study file.

### 5.2. Control Item

Near-isogenic non-GM crop: Variety to be chosen after the analyses of harvests from different locations.

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## 6. TEST SYSTEM

### 6.1. Species and strain

Rat Wistar Rcc Han/Specific Pathogen Free (SPF)

### 6.2. Source

Harlan Italy, reg. No 2-2914 - 15-06-1994

### 6.3. Approximate weight and age



Upon arrival, all rats will be 5 weeks old, the male animals will weigh between 120 and 140g and the female animals between 100 and 120 g. The animals will be 6-7 weeks old at the start of the study. Ideally, they should be born within 1-5 days of each other and be of uniform weight ( $\pm$  20% of the mean).

### 6.4. Identification

Each rat will be marked by a code (tattoo) on the tail base or marked with a chip on the neck in accordance with SOP ŠPP/TOX/V002 to identify the animals individually. Each cage will be marked with a colored cage card.

### 6.5. Justification for the selection and number of animals

The animal species (*Rattus norvegicus* ssp. *alba*) and strain (Wistar Rcc Han) is recognized by international guidelines as a recommended test system for chronic toxicity/carcinogenicity studies. Females will be nulliparous and non-pregnant. The number of animals used in this study is planned to be 20 males and 20 females per dose group for the 1-year study; 50 males and 50 females for the 2-years study in each of the five dose groups, a total of 700 animals, as recommended by the OECD Test Guideline 453 (2009) and EFSA (2013). A prospective power analysis will be performed to critically assess proposed sample sizes and meaningful effect sizes. Ten additional male and female rats not assigned to the study will be used as sentinels in the carcinogenicity study, which will be held in the same rooms as the rest of the animals in this study. Two animals of the same gender will be placed in one cage, and cages will be considered as experimental units. Male rats will be numbered 1 to 360 (this includes 10 sentinels) while female rats will be numbered 501 to 860 (also including 10 sentinels).

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## 7. MATERIALS AND METHODS

### General Remark

Details of the materials and methods that are not specified in the subsequent sections of this study plan are contained in the appropriate standard operating procedures (list of SOPs is attached)

### 7.1. Test item preparation - Diet formulation

The test item will be supplied to the test facility as a pre-prepared complete pelleted diet. The diet formulation will be done so as to produce separate diet compositions according to the dose group requirements. The test diets will be provided as single batches (containing portions of diets packed in separate vacuum, gamma-irradiated packs). Specific details of this process and the analyses performed will be included in the accompanying „Chronic toxicity/carcinogenicity feeding trail - Supplementary Information“ and records/data will be retained at the Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants (Quedlinburg, Germany; staff member involved: Ralf Wilhelm).

### 7.2. Storage conditions

The pelleted test diets will be stored in a closed storage room (cool and dry, controlled temperature and humidity) by the test facility. The temperature and humidity of the room will be recorded and the records will be kept.

### 7.3. Water



The rats will be supplied water *ad libitum* during the acclimatisation and study periods. Tap water with a special filter to eliminate microorganisms will be used. The bottles containing this water will be autoclaved before use. The microbiological and chemical quality of the water from the local mains will be monitored quarterly by the Waterworks Bratislava. The test facility will receive a corresponding quality certificate.

### 7.4. Animal housing

All animals will be housed in rooms B2-308, 309, 310, 311 and 312 of the Specific Pathogen Free (SPF) experimental animal house equipped with a pressurized climatic system at the Department of Toxicology of the Slovak Medical University. The temperature and relative humidity in the animal room will be recorded every 30 minutes and every week the computer readout for the past week will be evaluated. Mean temperature will be maintained at  $22 \pm 3^{\circ}\text{C}$  and relative humidity at 30-70%. The animals will be subjected to a 12-hour light/12-hour dark cycle.

Rats will be housed in Tecniplast cages Type 2145 F from Tecniplast Italy. The cages have a high-density polypropylene body, measuring 480 x 265 x 210 mm - floor area 940 cm<sup>2</sup>.



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The animals will be provided with environmental enrichment items: wooden chew blocks and a plastic tunnel or suitable alternatives. Certificates of analysis for the environmental enrichment items will be provided by the supplier. These enrichment items are considered not to contain any contaminants that could be expected to affect the study in any way.

We will use sterilized animal bedding (sawdust, JRS Lignocel®) from Germany. It will be stored in the clean, dry and cold store room on the first floor in the animal facility.

The cages will be cleaned twice a week outside of the animal room. Animals will first be transferred to a clean cage. The cages will then be emptied and cleaned with water and detergent. After cleaning they will be dried and thereafter immersed in disinfectant. The cages will then be brought into the animal house and placed in an additional Tecniplast disinfectant solution. Then the cages will be placed in the SPF unit on a drying rack before use.

The cage racks will be cleaned in the SPF rooms every week manually with water and detergent.

Feed containers and any other containers or equipment being used in the SPF rooms will be cleaned in the same way as the cages are cleaned.

Bottles will be exchanged and cleaned twice a week according to SOP ŠPP/SPF/V005. They will be cleaned in a special automatic washing machine set aside for the bottles in this study. The cleaning solution will include detergent followed by a disinfectant.



## 7.5. Experimental Design

### Animal receipt and acclimatisation

All animals will be purchased from Harlan and will only be a few days apart in age. Therefore, we will have the required number of test animals of uniform weight and age, and house them all under identical conditions

Upon arrival, the animals will be placed in cages, 4 per cage. 48 hours after arrival, the animals will be weighed and kept in cages for the next 9 days prior to the start of the study to allow for acclimatisation to the laboratory conditions. These are identical to those defined for the feeding trial. During this period of time the health status of the animals will be monitored twice a day (see the section Periodical Health Status Observations below for a full description of the health status evaluation) according to SOP ŠPP/TOX/V003.

One day before the start of treatment, all animals will be housed in 5 separate rooms (2 for males, 3 for females) under standard SPF conditions. To verify the health condition of the rats, a detailed examination of all animals will be carried out on study day 1, prior to the start of the treatment (see the section Periodical Health Status Observations for a full description).

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## Randomization




The experiment will use four racks (numbered Ch1-Ch4) for the chronic toxicity study where each rack contains 5 rows of 5 cages. Ten racks (numbered Ca1-Ca10) will be used for the carcinogenicity study and these racks will also contain 5 rows of 5 cages. Each cage houses two rats. The five dose groups are randomized within rows. This implies that the design is a randomized complete block design in which rows constitute the blocks. Male rats will be housed in racks Ch1-2 and in racks Ca1-5, while female rats will be housed in racks Ch3-4 and Ca6-10.

The experiment starts in week 1 with 1 block for the chronic study and 3 blocks for the carcinogenicity study on Monday (total 4 blocks, 20 cages, 40 male animals). This is repeated on the subsequent days in week 1. Racks will be filled from top to bottom, for the chronic and the carcinogenicity study separately. In week 2 the scheme is continued with on each day 1 block for the chronic study and 2 blocks for the carcinogenicity study (total 3 blocks, 15 cages, 30 male animals per day). The scheme of weeks 1-2 is repeated in weeks 3-4 with female rats.

To keep the units within blocks as homogeneous as possible, the 10 heaviest male rats (based on the weights on day 9 = day of the randomisation) will be housed in the first block of the chronic study, the next 10 heaviest male rats will be housed in the first block of the carcinogenicity study, etc. Within each block the allocation of the 10 rats to their cages will be random. The same scheme will be employed for the female rats. Because we are starting with the heaviest animals this has the additional advantage that, because not all rats can be housed on the same day, the true starting weight of the rats in the experiment will be as similar as possible.

Tables with cage numbers and the random diet assignment (as specified in the table below) will be prepared by the local statisticians. We will use the Random Number Generators (RNG) of SPSS software to randomly allocate the 10 rats to the five cages within each block, separately for male and female animals. The male rats will be numbered 1 to 360 (this includes 10 sentinel rats) while the female rats will be numbered 501 to 860 (also including 10 sentinels). Ten random male and ten random female rats will be selected, irrespective of their weights, as sentinels.

All observations will be done on a block by block basis. For instance, at the end of the chronic feeding trial experiment the cages are handled block by block in the same order as at the start of the experiment. This design ensures that possible differences between starting and ending days, and also possible differences between racks and between the vertical position of cages within racks, are confounded with blocks, implying that the analysis accounts for such differences. Table 1 give the random allocation of the five dose groups named A-E for each rack in the chronic study, while Table 2 gives the allocation for the carcinogenicity study. Sentinel animals in the carcinogenicity study, which will be held in the same rooms as the rest of the animals in this study, are not included in the experimental design described below.

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**Table 1.** Randomised order of the 5 dose groups named A-E for each row in the chronic toxicity testing phase. The dose group codes A-E are randomised by the feed supplier over the five dose groups in the study.

StartWeek	Sex	BlockNr	Row	StartDay	Rack Ch1 Male				
Week 1	Male	Block 1	Row 1	Monday	E	D	B	C	A
Week 1	Male	Block 2	Row 2	Tuesday	C	E	D	B	A
Week 1	Male	Block 3	Row 3	Wednesday	B	D	E	C	A
Week 1	Male	Block 4	Row 4	Thursday	A	D	E	C	B
Week 1	Male	Block 5	Row 5	Friday	B	A	C	E	D

StartWeek	Sex	BlockNr	Row	StartDay	Rack Ch2 Male				
Week 2	Male	Block 6	Row 1	Monday	B	C	A	D	E
Week 2	Male	Block 7	Row 2	Tuesday	D	B	A	C	E
Week 2	Male	Block 8	Row 3	Wednesday	C	A	B	D	E
Week 2	Male	Block 9	Row 4	Thursday	E	A	B	C	D
Week 2	Male	Block 10	Row 5	Friday	B	E	A	D	C

StartWeek	Sex	BlockNr	Row	StartDay	Rack Ch3 Female				
Week 3	Female	Block 11	Row 1	Monday	D	E	C	B	A
Week 3	Female	Block 12	Row 2	Tuesday	B	D	E	C	A
Week 3	Female	Block 13	Row 3	Wednesday	D	C	A	E	B
Week 3	Female	Block 14	Row 4	Thursday	E	A	D	B	C
Week 3	Female	Block 15	Row 5	Friday	A	D	E	B	C

StartWeek	Sex	BlockNr	Row	StartDay	Rack Ch4 Female				
Week 4	Female	Block 16	Row 1	Monday	E	C	B	D	A
Week 4	Female	Block 17	Row 2	Tuesday	C	B	D	E	A
Week 4	Female	Block 18	Row 3	Wednesday	A	C	B	D	E
Week 4	Female	Block 19	Row 4	Thursday	E	B	C	A	D
Week 4	Female	Block 20	Row 5	Friday	E	B	C	A	D

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**Table 2.** Randomised order of the 5 dose groups named A-E for each row in the carcinogenicity testing phase. The dose group codes A-E are randomised by the feed supplier over the five dose groups in the study.

StartWeek	Sex	BlockNr	Row	StartDay	Rack Ca1 Male				
Week 1	Male	Block 1	Row 1	Monday	D	E	A	B	C
Week 1	Male	Block 2	Row 2	Monday	B	C	E	D	A
Week 1	Male	Block 3	Row 3	Monday	C	E	B	D	A
Week 1	Male	Block 4	Row 4	Tuesday	D	B	C	A	E
Week 1	Male	Block 5	Row 5	Tuesday	A	E	D	C	B

StartWeek	Sex	BlockNr	Row	StartDay	Rack Ca2 Male				
Week 1	Male	Block 6	Row 1	Tuesday	E	A	D	C	B
Week 1	Male	Block 7	Row 2	Wednesday	C	E	D	B	A
Week 1	Male	Block 8	Row 3	Wednesday	D	A	E	C	B
Week 1	Male	Block 9	Row 4	Wednesday	D	C	A	B	E
Week 1	Male	Block 10	Row 5	Thursday	E	C	D	A	B

StartWeek	Sex	BlockNr	Row	StartDay	Rack Ca3 Male				
Week 1	Male	Block 11	Row 1	Thursday	A	C	B	D	E
Week 1	Male	Block 12	Row 2	Thursday	D	B	C	A	E
Week 1	Male	Block 13	Row 3	Friday	B	D	E	A	C
Week 1	Male	Block 14	Row 4	Friday	D	A	C	B	E
Week 1	Male	Block 15	Row 5	Friday	E	C	D	B	A

StartWeek	Sex	BlockNr	Row	StartDay	Rack Ca4 Male				
Week 2	Male	Block 16	Row 1	Monday	D	B	C	A	E
Week 2	Male	Block 17	Row 2	Monday	C	B	D	A	E
Week 2	Male	Block 18	Row 3	Tuesday	C	E	B	A	D
Week 2	Male	Block 19	Row 4	Tuesday	E	C	A	B	D
Week 2	Male	Block 20	Row 5	Wednesday	B	A	D	C	E

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StartWeek	Sex	BlockNr	Row	StartDay	Rack Ca5 Male				
Week 2	Male	Block 21	Row 1	Wednesday	D	A	B	E	C
Week 2	Male	Block 22	Row 2	Thursday	C	D	B	E	A
Week 2	Male	Block 23	Row 3	Thursday	A	D	C	E	B
Week 2	Male	Block 24	Row 4	Friday	E	C	A	B	D
Week 2	Male	Block 25	Row 5	Friday	C	D	E	A	B

StartWeek	Sex	BlockNr	Row	StartDay	Rack Ca6 Female				
Week 3	Female	Block 26	Row 1	Monday	D	C	B	A	E
Week 3	Female	Block 27	Row 2	Monday	B	E	C	D	A
Week 3	Female	Block 28	Row 3	Monday	D	E	B	A	C
Week 3	Female	Block 29	Row 4	Tuesday	C	D	E	A	B
Week 3	Female	Block 30	Row 5	Tuesday	C	E	D	B	A

StartWeek	Sex	BlockNr	Row	StartDay	Rack Ca7 Female				
Week 3	Female	Block 31	Row 1	Tuesday	E	B	D	C	A
Week 3	Female	Block 32	Row 2	Wednesday	A	B	C	E	D
Week 3	Female	Block 33	Row 3	Wednesday	C	B	D	E	A
Week 3	Female	Block 34	Row 4	Wednesday	C	B	A	D	E
Week 3	Female	Block 35	Row 5	Thursday	D	E	A	C	B

StartWeek	Sex	BlockNr	Row	StartDay	Rack Ca8 Female				
Week 3	Female	Block 36	Row 1	Thursday	E	C	D	A	B
Week 3	Female	Block 37	Row 2	Thursday	C	A	D	B	E
Week 3	Female	Block 38	Row 3	Friday	C	D	A	E	B
Week 3	Female	Block 39	Row 4	Friday	E	C	D	B	A
Week 3	Female	Block 40	Row 5	Friday	C	D	E	B	A

StartWeek	Sex	BlockNr	Row	StartDay	Rack Ca9 Female				
Week 4	Female	Block 41	Row 1	Monday	A	C	B	E	D
Week 4	Female	Block 42	Row 2	Monday	B	D	C	E	A
Week 4	Female	Block 43	Row 3	Tuesday	D	B	A	E	C
Week 4	Female	Block 44	Row 4	Tuesday	D	E	A	C	B
Week 4	Female	Block 45	Row 5	Wednesday	D	E	A	C	B

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StartWeek	Sex	BlockNr	Row	StartDay	Rack Ca10 Female				
Week 4	Female	Block 46	Row 1	Wednesday	E	C	B	A	D
Week 4	Female	Block 47	Row 2	Thursday	C	B	D	A	E
Week 4	Female	Block 48	Row 3	Thursday	E	B	C	A	D
Week 4	Female	Block 49	Row 4	Friday	E	A	B	D	C
Week 4	Female	Block 50	Row 5	Friday	A	D	C	B	E




On a regular basis (once per week) cages within each row of cages will be rotated from top to bottom. Racks will be rotated clockwise every two weeks within the original room configuration.

Skeleton analyses of variances with the appropriate degrees of freedom are given below (Tables 3 and 4), both for analyses with all cages including both sexes as well for an analysis for a single sex. The analysis for both studies shows that sex is confounded with starting week.

**NK603 Chronic toxicity testing phase (5 dose groups, 100 cages, 200 rats)**

**Table 3.** A skeleton analysis of variances with the appropriate degrees of freedom

ANOVA for both sexes		ANOVA for a single sex	
Source of variation	d.f.	Source of variation	d.f.
StartWeek stratum sex	1	block stratum	9
StartWeek.block stratum	18	block.cage stratum	
StartWeek.block.cage stratum		dosegroup	4
dosegroup	4	Residual	36
sex.dosegroup	4	Total	49
Residual	72		
Total	99		

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### NK603 Carcinogenicity testing phase (5 dose groups, 250 cages, 500 rats)

**Table 4.** A skeleton analysis of variances with the appropriate degrees of freedom

ANOVA for both sexes		ANOVA for a single sex	
Source of variation	d.f.	Source of variation	d.f.
StartWeek stratum		block stratum	24
sex	1	block.cage stratum	
StartWeek.block stratum	48	dosegroup	4
StartWeek.block.cage stratum		Residual	96
dosegroup	4	Total	124
sex.dosegroup	4		
Residual	192		
Total	249		



### Route of administration

The route of administration will be the oral route as this route is the most appropriate for the safety assessment of foods. The test item will be incorporated into the diet, since this is the way humans could be exposed to the test item. Attention will be paid that there will be no nutritional imbalances as a result of dietary incorporation of the test item.

### General experimental design with NK603 maize, start August - September 2015

**Table 5.** Experimental design

Group	Isogenic maize (% of diet)	NK603 only (% of diet)	NK603 + Roundup (% of diet)	No of animals			
				Chronic toxicity		Carcinogenicity	
				No. of Males	No. of Females	No. of Males	No. of Females
1	33	0	0	20	20	50	50
2	22	11	0	20	20	50	50
3	0	33	0	20	20	50	50
4	22	0	11	20	20	50	50
5	0	0	33	20	20	50	50
Sentinels <sup>1</sup>						10	10
Total animals				100	100	260	260

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<sup>1</sup> Sentinels will be fed the standard rat diet Teklad Global Diet<sup>®</sup>.

The different diets will be randomised and labelled A-E by the supply company. The codes will only be given to Ralf Wilhelm (JKI). Feed containers and scoops will be colour-coded. However, animal house staff will be “blind” with respect to the identity of the diets.

Roger Alison, the study histopathologist, will receive the codes immediately before starting the histopathological evaluation, i.e. the histopathological evaluation of the tissues will not be performed blind.

Blood and urine collection, haematology, clinical chemistry and urine analyses as well as body weight, feed consumption and organ weight measurements will be performed block by block, from cages in the order of the randomisation scheme given in Table 1 and 2. This minimises sampling variation between dose groups within blocks.

## 7.6. Periodical Health Status Observations

### **Morbidity, mortality**

Normally observations are done twice a day. However, in case of moribund animals, we will isolate them in the quarantine area to prevent cannibalism and will carefully observe them at least 4 times daily. Selection criteria are made explicit in SOP ŠPP/TOX/V004. If a study animal dies, we will subject it to necropsy as soon as possible after death. The criteria described in the OECD Guidance Document on the recognition, assessment and use of clinical signs as humane endpoints for experimental animals used in safety evaluation (ENV/JM/MONO[2000]7) such as changes in external physical appearance and clinical signs (described in Annex 3 of the above-mentioned OECD Guidance Document) will be taken into account to determine when an animal is in a moribund condition, is expected to become moribund or experiences pain and distress, and should therefore be euthanized. In such a case animals will be anaesthetized with ketamine/xylazine (SOP ŠPP/TOX/V005) and thereafter immediately necropsied.

### **Clinical signs**



#### ***Cage side observations / uncovered cage***

Rats will be inspected twice daily for evidence of reaction to treatment or illness, which includes the following signs: changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions as well as activity level and change in behavior in accordance with SOP ŠPP/TOX/V003.

#### ***Detailed physical examination and functional assessment***

Rats will be examined outside the cage once weekly. Any deviations from normal will be recorded in terms of nature and severity, date and time of onset, duration and progress of the observed response. Signs noted will include changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity such as lacrimation, piloerection, pupil size, and unusual respiratory patterns as well as activity level and change in behavior.



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Changes in gait, posture and response to handling as well as the occurrence of clonic or tonic movements or bizarre behavior (self-mutilation, walking backwards) will also be recorded. The outcome of this examination will be recorded for each animal in accordance with the SOP ŠPP/TOX/V003 (Origin of score system: Ország A. et al. [1985] Veterinárna ortopédia a rontgenológia, Bratislava: Príroda, 243 p.). The animals will also be assessed for gait disturbances using the *Accuplacer* treadmill equipment.

#### ***Ophthalmologic examination***

The eyes of all animals will be examined prior to the administration of the test feeds and at the end of the study. Pupillar dilation and ophthalmologic examination of both eyes will be performed by an experienced ophthalmologist in the conscious rat during gentle manual restraint by a technician. In a first step, the eyes and the peribulbar structures will be macroscopically examined. Thereafter, direct ophthalmoscopy will be performed using an ophthalmoscope. Ophthalmoscopic findings will be recorded on data sheets and transcribed into the computer system for compilation and analysis.

#### ***Body weight***

Each animal will be weighed at the following times: 1) 48 hours after arrival and on the day of randomization, 2) on the first day of feeding, 3) weekly during the first 13 weeks, 4) monthly thereafter, 5) at the end of the study, 6) in the event of an early death or sacrifice *in extremis*.



#### ***Feed consumption and feed efficiency***

Feed will be supplied *ad libitum*. Measurement of feed consumption, reported as the total amount of feed consumed by two animals in one cage, and food efficiency will be made once weekly for the first 13 weeks and once monthly thereafter. At the beginning of each feed consumption measurement, full feeders with stainless steel lids will be weighed and placed in each cage. The feeders will be weighed again on the day of the feeder change-out, the difference in weight being an estimate of the total amount consumed by two rats in one cage. Feed spillage will be documented and the amount will be noted and subtracted.

### **7.7. Procedures For Sample Collection**

Samples will be collected according to the OECD Test Guideline 453 for the following analyses: haematology, clinical chemistry, urinalysis and histopathology. Samples collected will include blood, urine and tissues/organs. Blood samples will be divided for the haematology and clinical chemistry analyses. Tissues/organs will be removed and evaluated histopathologically.

In addition, at the end of the first and second year 500 µl plasma/rat and 2-3 ml urine/rat from 16 rats per experimental group and at the end of the second year liver and kidney samples from 16 rats per experimental group will be sent by SZU to the French consortium GMO90+, which will analyze the expression of a number of biomarkers of effects in the above-mentioned samples.

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### Urine and blood collection and processing in months 3, 6 and 12 as well as 7 days before necropsy in month 24

Sufficient personnel will be available:

Urine will be collected by person No. 1

Urine processing and transport to the Laboratory of Clinical and Experimental Biochemistry (urinalysis) - person No. 2

Blood taking from the tail vein - person No. 3

Blood processing, dividing of samples - person No. 4

Blood transport to the Laboratory of Immunotoxicology (haematology) - person No. 5

Blood transport to the Laboratory of Clinical and Experimental Biochemistry (clinical chemistry) - person No. 6

### Blood and tissue collection and processing at the day of necropsy in month 24

This will be done in accordance with SOP ŠPP/TOX/V005. Sufficient personnel will be available:

Animals will be anaesthetized by person No. 1

Blood taking from the abdominal aorta (for GMO90+) - person No. 2

Blood processing and transport to the Laboratory of Clinical and Experimental Biochemistry (for GMO90+) - person No. 3

Animal transport to the autopsy room on the same floor - person No. 4

Necropsy of the thorax part of the body - person No. 5

Necropsy of the abdominal part of the body - person No. 6

Necropsy of the genital organs - person No. 7

Removal and weighing of tissues and organs in line with OECD guideline 453 - person No. 8

Decapitation and necropsy of the head including brain - person No. 9

All organs will be stored in formalin or Bouin's solutions for the histological examination - person No. 10



Details will be documented by subsequent amendment.

### Haematology

At months 3, 6 and 12 and 7 days before necropsy in month 24, blood samples from the tail vein will be taken from all animals for haematological examination after 16 hours fasting. EDTA will be used as anticoagulant. Blood samples will be stored at room temperature (17-25°C), maximally up to 4 hours, until measurement. Haematological analysis will be performed in accordance with SOP ŠPP/IMU/M002 by using a Sysmex K-4500 automated haematology analyzer (Sysmex, Kobe, Japan).

Parameters to be determined are:

- erythrocyte count (RBC)
- haematocrit (HT)
- haemoglobin (Hb)

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- leukocyte count (WBC)
- differential leukocyte count (lymphocytes, neutrophils, eosinophils, basophils and monocytes)
- platelet count (PLT)
- mean corpuscular volume (MCV)
- mean corpuscular haemoglobin (MCH)
- mean corpuscular haemoglobin concentration (MCHC)

Differential leukocyte counts will be performed by using a light microscope. Blood smears will be subjected to panoptic staining by using May-Grunwald and Giemsa-Romanowski dyes. The percentage of lymphocytes, neutrophils, eosinophils, basophils and monocytes will be determined by examining 200 cells.

#### Clinical chemistry

At months 3, 6 and 12 and 7 days before necropsy in month 24, blood samples from the tail vein will be taken from all animals for blood chemistry examination after 16 hours fasting. Samples will be analysed using an Ortho Clinical Vitros® 250 Chemistry System (Ortho-Clinical Diagnostics, Raritan, NJ, USA). Methodologies include colorimetric, potentiometric and rate tests using multi-layered Vitros Slides in accordance with SOP ŠPP/LEKB/M001. Blood samples will be stored at room temperature (17-25°C) for a maximum of 4 hours until measurement. Parameters to be determined will be:

- total protein
- albumin
- aspartate aminotransferase
- alanine aminotransferase
- alkaline phosphatase
- creatinine
- glucose
- urea
- total cholesterol
- triglycerides
- gamma-glutamyl transpeptidase
- Na
- K
- Cl
- Ca
- P

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### Urinalysis

At months 3, 6, and 12 of the study and during the last week of the study urine analyses will be performed. Urine will be collected from 10 individual rats per gender and group in metabolic cages (one rat per metabolic cage) under the same conditions for 16 hours; during this time period the animals will continue to receive the corresponding diets. The total volume of urine excreted during the 16-hour period will be measured and recorded at the end of the 16-hour collection period, and the animals will be brought back from the metabolic cages to their respective conventional cages. Each collected urine sample will be identified by a unique code.

Parameters to be determined will be:

- appearance
- volume
- osmolality
- pH
- total protein
- glucose
- occult blood
- ketone
- urobilinogen



## 7.8. Necropsy and Histopathology

### Gross necropsy

A complete necropsy will be performed on all animals at the end of the study (after 1 year for the chronic phase and after 2 years for the carcinogenicity phase). Organs/tissues will be examined macroscopically for any deviations from normal (in accordance with ŠPP/TOX/V005). A supervising toxicopathologist will be present during the scheduled necropsy. The results will be manually recorded and subsequently transferred and saved in the computer system.

The wet weight of the following organs of animals sacrificed after 1 year will be recorded:

- adrenal glands (paired)
- brain
- epididymides (paired)
- heart
- kidneys
- liver
- ovaries (paired)
- spleen
- testes (paired)
- thyroid (weighed post-fixation)
- parathyroid (weighed post-fixation)
- uterus



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As described in the OECD Test Guideline 453, organ weights of animals sacrificed after 2 years will not be recorded, since geriatric changes and the development of tumours will confound the usefulness of organ weight data.

Organs and tissues for histopathological examination will be formalin-fixed (neutrally buffered 10% formalin). Details will be added later by amendment.

As described in the OECD Test Guideline 453, the following tissues of animals sacrificed after 1 year and of animals sacrificed after 2 years will be subjected to a histopathological examination after fixation:

- all gross lesions
- adrenal glands (left and right, recorded separately)
- aorta
- brain (representative regions including cerebrum, cerebellum, medulla/pons and pituitary)
- caecum
- cervix
- colon
- epididymides
- eyes (including retina)
- femur (femoro-tibial joint)
- gut-associated lymphoid tissue (GALT)
- Harderian gland
- heart
- ileum
- jejunum
- kidneys
- lacrimal gland (exorbital)
- liver
- lymph nodes: mandibular and mesenteric
- oesophagus
- ovaries
- pancreas
- parathyroid
- peripheral nerve (sciatic) preferably in close proximity to the muscle
- pituitary
- prostate
- rectum
- salivary glands
- seminal vesicles and coagulating gland
- skeletal muscle

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- skin with mammary gland area
- spinal cord (cervical, mid-thoracic and lumbar regions)
- spleen
- sternum with bone marrow
- stomach
- testes (left and right, recorded separately)
- thymus
- thyroid
- tongue
- trachea and lungs inflated with fixative and then immersed in formalin
- urinary bladder
- uterus
- vagina
- additional tissues may need to be investigated based on clinical or any other findings



Trimming will be done by the Department of Toxicology at SZU, who will ship the tissue samples to the histology processing test site at the Institute of Pathology at the University of Veterinary Medicine Hannover immediately after the tissue samples have been fixed. Tissue samples of animals that have to be prematurely necropsied (because of their moribund condition) will also be shipped immediately after having fixed the samples.

### Histology processing

The trimmed tissue specimens will be transported in labelled cassettes in 10% buffered formalin to the histology processing test site at the Department of Pathology, University of Veterinary Medicine, Hannover, Germany. They will be stored in neutral buffered 10% formalin at room temperature until they are further processed. The whole processing will take place under GLP compliant conditions in the Department of Pathology (building 02-29, rooms 216 and 217), and all procedures will be performed by trained technicians.

Briefly, trimmed tissue samples within the cassettes will be checked and recorded and a confirmatory dispatch note will be sent back to SZU. Then, specimens will automatically be embedded in paraffin wax according to a standardized protocol. Paraffin blocks will be made manually. Each block will be cut until the whole tissue specimen is visible on its surface. Then, a 3-5µm thick section will be taken, straightened on a warm water bath and mounted on a glass slide. The glass slides will be labelled according to the labelling on the respective cassette and paraffin block. Slides will be stained with haematoxylin and eosin according to a standardized protocol. The slides will then be covered with a cover glass, dried, and stored at room temperature until shipped. The slides will then be packed in a shatter-proof manner and shipped to the histopathology examination test site Roger Alison Ltd. by DHL or a comparable courier.

The paraffin blocks will be stored at the Department of Pathology, University of Veterinary Medicine, Hannover, Germany, temporarily. Final archiving will take place at SZU.



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### Histopathology

The above-mentioned tissue specimens of all animals in the control group (dose group 1) and the two high dose groups (groups 3 and 5) will be examined. If test item-related morphologic changes are detected in organs of any high-dose animal, then these target tissues of all animals in the low-dose groups will also be analyzed. Furthermore, all tissue specimens from animals having died or having had to be sacrificed before the actual end of the feeding trial as well as all tissues showing macroscopic abnormalities will be examined microscopically.

A signed histopathology phase report will be provided by the principal investigator for inclusion in the main report as an appendix. A peer review of findings will be performed and the peer review statement will be attached as a separate appendix within the final report.

All histological slide samples will be returned to the test facility for archiving.

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## 8. DATA EVALUATION AND STATISTICAL ANALYSIS

Evaluation of the data and screening for any obvious errors and outliers will be performed by the local statistical team at the test facility, SZU. Outliers will be checked against the original paper records. Outliers which are not due to transcription or other obvious types of error will be retained, but noted. The statistical analysis will be performed by the Biostatistics test site, the Biometris group of partner DLO. Analyses will be performed with and without the outliers. If the conclusion depends on the presence of one or more outliers, then this will require further investigation on a case-by-case basis. If an outlier makes no difference to the conclusions, it will be retained.



The statistical analysis will be performed according to a pre-established protocol. Cages will be the experimental units. Summary statistics will be tabulated. Weight and food consumption data will be plotted over time. Data of males and females will be analysed together unless there is a prior biological argument to analyse males and females separately. Conclusions for males and females will also be reported separately if a significant interaction between treatment and sex is found in the joint analysis.

The statistical analysis will present the results as differences between the treated group and the control group on an appropriate scale with a 95% confidence interval, and compare these results with a zero difference (difference test) and pre-specified limits of concern (equivalence test). The precise statistical methods may vary depending on the nature of the data. For many quantitative endpoints an ANOVA type analysis with fixed factors group and sex will be appropriate. The results of the statistical analyses will be presented in tabular and graphical form.

The data used within the statistical analyses will be made publically available on the G-TwYST web site.

For histopathology data, statistical analyses may be performed at the discretion of the Study Pathologist if required and full details of statistical tests employed will be included in the histopathology phase report.



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