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PROGRAMME

Final Study Plan

90-Day subchronic toxicity study 1 in rats fed GM maize NK 603

Multi-Site Study Plan

G-TwYST

GMP Two Year Safety Testing

632165 B/2016/GLP

9 June 2015

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Prof. Dr. Pablo Steinberg

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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Project website: www.g-twyst.eu

	<p>90-Day subchronic toxicity study in rats fed GM maize NK603 based on OECD Test Guideline 408, EFSA Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed (2011) and EFSA explanatory statement complementing the above-mentioned EFSA Guidance (2014)</p>	<div style="border: 1px solid blue; padding: 5px; text-align: center;">  Reg. No. 061/G-036 </div> <p>Version: Page No.: 3/34</p>
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90-Day subchronic toxicity study 1 in rats fed GM maize NK 603

Multi-Site Study Plan

Study No: 632165 B/2016/GLP

Sponsor: EU Project G-TwYST

Sponsor's representative: Prof. Dr. Pablo Steinberg

Test Facility: Slovak Medical University
Testing Laboratories Center
Laboratory of Toxicology
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1. Signatures of Approval of the Multi-Site Study Plan:

Study Director:	Name Dagmar Zeljenková	Date	Signature
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Test Facility Management:	Name Martin Gajdoš	Date	Signature
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Sponsor's Representative:	Name Pablo Steinberg	Date	Signature
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Confirmation of Study plan in accordance with GLP

This study plan meets the requirements for GLP compliance

Head of QAU	Name Eva Němcová	Date	Signature
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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 11th 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 408 for Testing of Chemicals; "Repeated Dose 90-Day Oral Toxicity Study in Rodents" (adopted on the 21st of September 1998)





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- The EFSA Guidance on repeated-dose 90-day oral toxicity studies on whole food/feed in rodents (EFSA Scientific Opinion, 2011).
- The EFSA Explanatory statement for the applicability of the Guidance of the EFSA Scientific Committee on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed for GMO risk assessment (EFSA Statement, 2014).

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 22nd 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:

Department 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, DipIPECVP
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

Deputy Study Director:	Jana Tulinská, MD, PhD
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
Deputy Study Director:	jana.tulinska@szu.sk
Clinical Chemistry:	viera.spustova@szu.sk
Haematology:	jana.tulinska@szu.sk
Ophthalmology:	andrej.cernak@pe.unb.sk
Necropsy :	katarina.ambrusova@szu.sk
Lead Quality Assurance:	eva.nemcova@szu.sk

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All Principal Investigators: See multi-site study details

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 408 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 632165 B/2016/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:	January 2016
Arrival of animals:	January-February 2016
Starting of the treatment:	
- males	February - day 1
- females	February - day 14
Necropsy of the animals:	successively, start day 91
Histological processing:	1 month after day 91
Histopathology evaluation:	2 months after day 91
Draft report to sponsor:	3 months after day 91



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

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.

	<p>90-Day subchronic toxicity study in rats fed GM maize NK603 based on OECD Test Guideline 408, EFSA Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed (2011) and EFSA explanatory statement complementing the above-mentioned EFSA Guidance (2014)</p>	<div style="border: 1px solid blue; padding: 5px;">  <p>Reg. No. 061/G-036</p> </div> <p>Version: Page No.: 13/34</p>
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4. OBJECTIVE

The purpose of this oral toxicity study is to assess the effects of GM maize NK 603 when fed to rats for a period of 90 days at an incorporation rate of 11 and 33% in the feed. This 90-day study is being conducted in association with a long-term feeding study designed according to the OECD Test Guideline 453 as part of a bigger project. These studies will provide a comparative assessment of the results of a subchronic toxicity study 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-enoylpyruvylshikimate-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 140 g and the female animals between 100 and 120 g. The animals will be 6 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 subchronic toxicity studies. Females will be nulliparous and non-pregnant. The number of animals used in this study is planned to be 16 males and 16 females in each of the five dose groups, a total of 160 animals, as recommended by the OECD Test Guideline 408 (1998). A prospective power analysis will be performed to critically assess proposed sample sizes and meaningful effect sizes. Six additional male and female rats not assigned to the study will be used as sentinels, 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 86 (this includes 6 sentinels) while female rats will be numbered 101 to 186 (also including 6 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.

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 „90-day feeding trial Study plan - 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 N° B 2/317 and 318 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 cm².

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

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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 second floor in the animal facility. One lot of sawdust bedding will be purchased and used for the entire study.

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 4 - 6 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 2 separate rooms (1 for males, 1 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).

Randomization

The experiment will use four racks where each rack contains 4 rows of 5 cages. Each cage houses two rats. The five dose groups are randomized within each row. This implies that the design is

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a randomized complete block design in which the rows constitute the blocks. Male rats will be housed in racks 1-2 while female rats will be housed in racks 3-4.

The experiment starts in week 1 with two male blocks on Monday (10 cages, 20 male animals), two male blocks on Tuesday, two male blocks on Wednesday and finally two male blocks on Thursday. Racks will be filled from top to bottom. This is repeated in week 3 with female rats.

To keep the units within blocks as homogeneous as possible, the 10 heaviest male rats (based on the weights 48 hrs after arrival) will be housed in the first block, the next 10 heaviest male rats will be housed in block 2, 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 86 (this includes 6 sentinel rats) while the female rats will be numbered 101 to 186 (also including 6 sentinels). Six random male and six 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 90-days 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 gives the random allocation of the five dose groups named A-E for each rack. Sentinel animals, 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.

Table 1. Randomised order of the 5 dose groups named A-E for each row in the 90-day 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 1 Male				
Week 1	Male	Block 1	Row 1	Monday	C	B	D	E	A
Week 1	Male	Block 2	Row 2	Monday	A	E	C	D	B
Week 1	Male	Block 3	Row 3	Tuesday	A	C	B	E	D
Week 1	Male	Block 4	Row 4	Tuesday	E	C	B	A	D

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

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StartWeek	Sex	BlockNr	Row	StartDay	Rack 3 Female				
Week 3	Female	Block 9	Row 1	Monday	B	A	C	D	E
Week 3	Female	Block 10	Row 2	Monday	C	E	D	B	A
Week 3	Female	Block 11	Row 3	Tuesday	A	C	D	B	E
Week 3	Female	Block 12	Row 4	Tuesday	E	D	A	C	B

StartWeek	Sex	BlockNr	Row	StartDay	Rack 4 Female				
Week 3	Female	Block 13	Row 1	Wednesday	C	A	D	B	E
Week 3	Female	Block 14	Row 2	Wednesday	E	B	C	A	D
Week 3	Female	Block 15	Row 3	Thursday	C	A	D	B	E
Week 3	Female	Block 16	Row 4	Thursday	D	C	E	A	B

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.



A skeleton analysis of variances with the appropriate degrees of freedom is given below (Table 2), both for an analysis with all 80 cages including both sexes as well for an analysis for a single sex.

Table 2. A skeleton analysis of variance 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	7
sex	1	block.cage stratum	
startweek.block stratum	14	dosegroup	4
startweek.block.cage stratum		Residual	28
dosegroup	4	Total	39
sex.dosegroup	4		
Residual	56		
Total	79		

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.

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General experimental design with NK603 maize, start February 2016

Table 3. Experimental design



Group	Isogenic maize (% of diet)	NK603 only (% of diet)	NK603 + Roundup (% of diet)	No. of Males	No. of Females
1	33	0	0	16	16
2	22	11	0	16	16
3	0	33	0	16	16
4	22	0	11	16	16
5	0	0	33	16	16
Sentinels ¹				6	6
Total animals				86	86

¹ Sentinels will be fed the standard rat diet Teklad Global Diet®.

The different diets will be randomized 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, immunology, endocrinology 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 randomization scheme given in Table 1. This minimizes sampling variation between dose groups within blocks.

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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.

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.

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Body weight

Each animal will be weighed at the following times: 1) 48 hours after arrival and on the day of the randomisation, 2) on the first day of feeding, 3) weekly during the study period, 4) at the end of the study, 5) 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 per week, and feed efficiency will be made once weekly for 90 days. 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 (once weekly), 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.

7.8. Procedures For Sample Collection

Samples will be collected for the following analyses: haematology, clinical chemistry, immunology, endocrinology, urinalysis and histopathology. Samples collected will include blood, urine, vaginal smears and tissues/organs. Blood samples will be divided for the haematology and clinical chemistry analyses. Tissues/organs will be removed and evaluated histopathologically.

Urine and blood collection and processing

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

Tissue collection and processing at the day of necropsy

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

Animals will be anaesthetized by person No. 1

Animal transport to the autopsy room on the same floor - person No. 2

Necropsy of the thorax part of the body - person No. 3



Necropsy of the abdominal part of the body - person No. 4

Necropsy of the genital organs - person No. 5

Removal and weighing of tissues and organs in line with OECD guideline 408 - person No. 6

Decapitation and necropsy of the head including brain - person No. 7

All organs will be stored in formalin or Bouin's solutions for the histological examination - person No. 8

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Details will be documented by subsequent amendment.

Haematology

At the end of the study (before sacrifice), 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)
- 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 the end of the study (before sacrifice), blood samples from the tail vein will be taken from all anaesthetized 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
- urea
- glucose

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- total cholesterol
- triglycerides
- Na
- K
- Cl
- Ca
- P
- 17 β -estradiol
- testosterone

Immunology

If the spleens show no macroscopic alterations at necropsy, the spleen of all animals will be cut in two halves, and one half will be transferred into plastic tubes with sterile RPMI medium containing heparin. The spleen samples will be transported to Lab. No. B2-222 at SZU and used to determine the proliferative activity of lymphocytes in accordance with SOP ŠPP/IMU/M006 and the *in vitro* production of cytokines following SOP ŠPP/IMU/M009.



The proliferative activity of lymphocytes will be examined using a liquid scintillation technique. Spleen cells will be cultured in the absence of a proliferative stimulus (to determine spontaneous proliferation) or in the presence of a specific antigen (feed extract) or mitogens (concanavalin A, phytohaemagglutinin, pokeweed mitogen). The degree of proliferation will be quantified by determining the incorporation of ³H-labelled thymidine into DNA.

The ***in vitro* production of cytokines** will be analysed after incubating the spleen cells with specific antigens (feed extract) and/or a mitogen (concanavalin A). Cytokine release (Th₁/Th₂ response) will be measured in culture supernatants by using commercially available assays (ELISA or Luminex® kits).

A **phenotypic analysis of leukocytes** based on a flow cytometric method according to SOP: ŠPP/IMU/M007 will be performed. Samples of spleen, two mesenteric lymph nodes and one half of the thymus will be collected from all animals (if no macroscopic alterations at necropsy are observed), placed in PBS buffer or RPMI medium and transported to the Lab. No. B2-222 at SZU. The cells obtained from the above-mentioned tissue samples will be incubated with commercially available monoclonal anti CD3, CD4, CD8, CD45R and CD161 antibodies (to identify rat T-cells, T-helper cells, T-cytotoxic cells, B-cells and NK-cells, respectively) labelled with fluorescent dyes, and then a flow cytometry analysis will be performed.

Heparinised whole blood of animals of all groups will be incubated with fluorescein-labelled *Staphylococcus aureus* bacteria and dihydroethidine at 37°C, and thereafter the **phagocytic activity and the oxidative burst of leukocytes** will be analyzed by flow cytometry according to SOP ŠPP/IMU/M008.

In order to assess the CP4 EPSPS allergenicity/immunogenicity and the effect of the genetic modification on the intrinsic allergenicity/immunogenicity of maize, once the groups have been unblinded the **total and anti-maize/CP4 EPSPS specific antibody levels** (i.e. IgG, IgM and IgE) will be

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determined in the plasma of animals in the control and in the 33% GM maize (+/- Roundup)-fed groups.

Endocrinology



Vaginal smears will be obtained daily between 08.00-09.00 a.m. during 10 days prior to necropsy and on the day of necropsy. The smears will be spread on slides, fixed with methanol, stained with Giemsa-Romanowski reagent and examined under a low-power light microscope. The stage of the estrous cycle will be determined based on the presence of leukocytes (metestrus, diestrus), nucleated epithelial cells (proestrus), and cornified epithelial cells (estrus). A constant 4- or 5-day vaginal estrous cycle is regarded as a regular estrous cycle. An extended estrus is defined as exhibiting cornified cells with no leukocytes for 3 or more days and an extended diestrus is defined as the presence of leukocytes for 4 or more days. The number of regular and irregular cycles, percentage of female rats having constant and aberrant cycles and the duration of the estrus cycle stages will be recorded for all female animals in all experimental groups. Once the groups have been unblinded, 17 β -estradiol, triiodothyronine (T3) and thyroxine (T4) levels will be determined in the serum samples of female animals in the control and in the 33% GM maize (+/- Roundup)-fed groups, while testosterone, T3 and T4 will be determined in the serum samples of male animals in the control and in the 33% GM maize (+/- Roundup)-fed groups.

Urinalysis

During the last week of the study urine analyses will be performed. Urine will be collected from each individual rat 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

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7.9. Necropsy and Histopathology

Gross necropsy

A complete necropsy will be performed on all animals at the end of the study. The weight of organs will be recorded in line with OECD Test Guideline 408 and EFSA (2014), and 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 will be recorded:

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

Organs and tissues for histopathological examination will be formalin-fixed (neutrally buffered 10% formalin). Testes, epididymides and eyes will be preserved in Davidson's solution and subsequently transferred to 10% neutral buffered formalin after the initial fixation is achieved.

As described in the OECD Test Guideline 408, the following tissues will be subjected to a histopathological examination after fixation:

- all gross lesions
- adrenal glands
- aorta
- brain (representative regions including cerebrum, cerebellum, medulla/pons and pituitary)
- caecum
- cervix
- colon
- epididymides
- eyes (including retina)
- duodenum
- femur (femoro-tibial joint)
- gut-associated lymphoid tissue (GALT)



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- heart
- ileum
- jejunum
- kidneys
- 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
- skin with mammary gland area
- spinal cord (cervical, mid-thoracic and lumbar regions)
- spleen
- sternum with bone marrow
- stomach
- testes
- 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 at ambient temperature 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.

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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. 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.

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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	90-Day subchronic toxicity study in rats fed GM maize NK603 based on OECD Test Guideline 408, EFSA Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed (2011) and EFSA explanatory statement complementing the above-mentioned EFSA Guidance (2014)	 Reg. No. 061/G-036

APPENDIX

Attachment 1



Proposed time schedule

				Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7
	day - 7	day 0	day 1			day 90				
Quarantine	7 days									
Randomization		males, day 0; females, day 0+14*								
Ophthalmology	day -5/6				week 11					
Application males			day 1-4 start			day 90 end				
Application females			day 14 - 17 start			day 90 + 14 end				
Weighing of the feed		every 7 days	every 7 days	every 7 days	every 7 days	XX				
Weighing of animals		every 7 days	every 7 days	every 7 days	every 7 days	day of necropsy				
General clinical observations		every day	every day twice	every day twice	every day twice					
Detailed clinical observations		every 7 days	every 7 days	every 7 days	every 7 days	day of necropsy				
Sensory reactivity		every 7 days	every 7 days	every 7 days	every 7 days	XX				
Hematology, males+females						days 83-87 (blood from the tail vein)				
Clinical chemistry, males+females						days 83-87 (blood from the tail vein)				
Urinalysis, males+females						days 83-87				
Gross necropsy, males						successively, start day 91	blood from the aorta at necropsy			

 	90-Day subchronic toxicity study in rats fed GM maize NK603 based on OECD Test Guideline 408, EFSA Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed (2011) and EFSA explanatory statement complementing the above-mentioned EFSA Guidance (2014)	 Reg. No. 061/G-036

				Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7
	day - 7	day 0	day 1			day 90				
Gross necropsy, females						successively, start day 91 + 14	blood from the aorta at necropsy			
Tissue processing							X			
Histopathology								X	X	
Draft report										X

* arrival of females 14 days later

SZU, Limbová 14, 833 03 Bratislava 37, Slovak Republic,		Testing Laboratories Center, Laboratory of Toxicology
	90-Day subchronic toxicity study in rats fed GM maize NK603 based on OECD Test Guideline 408, EFSA Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed (2011) and EFSA explanatory statement complementing the above-mentioned EFSA Guidance (2014)	 Reg. No. 061/G-036
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Attachment 2: List of Materials and Equipment

Laboratory of Toxicology, SZU

- Electronic balance Kern ABJ 220-4M, No. WB 0850106, range: 0.01-220g, precision: 0.0000g, Kern & Sohn GmbH, Germany, room No. B2-326
- Electronic balance Chyo JL-180 No. 306227, range: max.180 g, room No. B2-326
- Personal computers, office

Experimental animal rooms:

- Temperature and humidity detector, G-RECO TH, rooms No. B 2/317 and 318
- Personal computers, office
- Data backup system - 1 external hard drive and the eXplorer system established by JKI
- Electronic balance Sartorius BP 1200, No. 70707472, range: 0-1000 g, Sartorius AG, Germany, the operating room of experimental animal rooms
- Pressure air conditioning system VENTO, No. RMK 01.2, REMAK LTD., Czech Republic, experimental animal rooms on the 3th floor at SZU
- Personal computers, office
- Type of animal cages in TECNIPLAST cages Type 2145 F from the Tecniplast Company, Italy. The cages have a high density polypropylene body, measuring 480 x 265 x 210 mm - floor area 940 cm²
- Ophthalmoscope Welch Allyn
- Apparatus for neurobehavioural testing: Accupacer treadmill

Laboratory of Immunotoxicology, SZU

- Haematological analyzer Sysmex K-4500, SYSMEX TOA Medical Electronics Co. LTD, Japan, No. VČ F-1466, room B2-212.
- Personal computers, office

Laboratory of Clinical and Experimental Biochemistry, SZU

- Analyzer Vitros 250, Ortho-Clinical Diagnostics, No. 219037234, USA, room B-048
- Personal computers, office, software for processing of the data
- Windows XP, program Office 2003
- Windows 2007, program Office 2010
- Software SPPS version 16.0.

Materials:



- Syringes, needles, tubes, tubes microvette, tips, gloves, gauze, racks, cages, paper, cartridge

Department of Pathology, University of Veterinary Medicine Hannover

- Embedding apparatus, embedding solutions, paraffin wax, microtome blades, glass slides, cover glasses, staining solutions, packing materials, paraffin block storage cabinets

Equipment for Histopathology, Roger Alison Ltd.

- PathData software, Olympus microscope, personal computers, slide storage cabinets

	<p>90-Day subchronic toxicity study in rats fed GM maize NK603 based on OECD Test Guideline 408, EFSA Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed (2011) and EFSA explanatory statement complementing the above-mentioned EFSA Guidance (2014)</p>	<div style="border: 1px solid blue; padding: 5px; text-align: center;">  Reg. No. 061/G-036 </div> <p>Version: Page No.: 34/34</p>
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Attachment 3

List of records to be maintained for this study includes:

- animal receipt records and quarantine records
- randomization records
- serology reports
- feed log and analysis reports
- water analysis reports
- moribundity/mortality checks
- rack/cage rotation records
- Temperature/relative humidity/light intensity and cycle checks
- dose analysis data
- dose preparation and accountability records
- dose administration records
- necropsy and histopathological findings
- pathology specimens as specified
- copy of the histology processing records (original at the Department of Pathology, University of Veterinary Medicine Hannover, Germany)