



Subchronic toxicity and longitudinal metabolomics study



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Study Plan

Study No: 311957 – D / 14/ GLP

Draft Version

4 Dec 2013

Sponsor: EU Project GRACE

Sponsor's representative: Prof. Dr. Joachim Schiemann
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Head of the Institute for Biosafety in Plant Biotechnology
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joachim.schiemann@jki.bund.de

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Testing Laboratories Center
Laboratory of Toxicology
Limbová 14, 83303 Bratislava

Test Facility Representative: [REDACTED]
Slovak Medical University
Limbová 14, 83303 Bratislava
[REDACTED]

Study Director: Dagmar Zeljenková, MVD, PhD.
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Limbová 12, 83303 Bratislava
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[REDACTED]

**Test Site 1:
Histopathology
Test Site Principal Investigator:** [to be inserted depending on tender outcome]

**Test Site 2:
Diet analysis** RIKILT – Institute of Food Safety
Wageningen University and Research Center Campus
Building 123, Akkermaalsbos 2
NL-6708WB Wageningen

311957 – D / 14/ GLP



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Netherlands

Principal investigator: Dr G.A. Kleter
(gijs.kleter@wur.nl)

Test Site 3: Diet preparation

Mucedola s.r.l.

Formatiert: Schriftart: Nicht Fett, Englisch (Großbritannien), Nicht Hervorheben

Test Site 4: Maize production, diet and transcriptomic analysis

Center for Research in Agricultural Genomics (CRAG)
Campus UAB – CRAG building
Bellaterra, Cerdanyola del Vallès
08193 Barcelona
Spain

Principal investigator: Dr M. Pla de Sola Morales
(maria.pla@udg.edu)

Test Site 5: Diet analysis, and immunological and metabolomic analyses

Laboratoire d'Immuno-Allergie Alimentaire
Service de Pharmacologie et Immunologie (SPI)
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iBiTec-S
F-91191 Gif-Sur-Yvette cedex
France

Principal investigator: Dr Karine Adel-Patient
(Karine.Adel-patient@cea.fr)

SZU, Limbová 14, 833 03 Bratislava 37, Slovak Republic Testing Laboratories Center, Laboratory of Toxicology



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Approval of the Study plan

Study Director	Name	Date	Signature
	Dagmar Zeljenková, VMD, PhD.		

|



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**Test Facility
representative**

Name

Date

Signature

[Redacted Name]

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**Principal
Investigator**
Test Site 1

Name

[according to tender outcome]

Date

Signature

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**Principal
Investigator**
Test Site 2

Name
Dr Gijs A. Kleter

Date

Signature

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Principal Investigator
Test Site 3

Name

[Redacted Name]

Date

Signature

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**Principal
Investigator**
Test Site 4

Name
Dr Maria Pla de Sola Morales

Date

Signature

|

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**Principal
Investigator**
Test Site 5

Name

Date

Signature

Dr Karine Adel-Patient

← - - - - **Formatiert: Zentriert**

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Sponsor

Name

Date

Signature

Prof. Dr. Joachim Schiemann



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Confirmation of Study plan accordance with GLP

This study plan meets the requirements for GLP compliance

Head of QAU

Test Facility

Name

██████████

Date

Signature

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Head of QAU	Name	Date	Signature
Test Site 1	[according to tender outcome]		

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Confirmation of Study Plan accordance with ISO 17025

	Name	Date	Signature	Formatierte Tabelle
Head of QAU				
Test Site 2 Facility				Formatiert: Slowakisch (Slowakei)

SZU, Limbová 14, 833 03 Bratislava 37, Slovak Republic Testing Laboratories Center, Laboratory of Toxicology



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Head of OAU

Test Site 2

Name

██████████

Date

Signature

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Head of QAU

Name

Date

Signature

Test Site 3





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Regulatory Test Guidelines

The study will be carried out in accordance with OECD Test Guideline 408 for Testing of Chemicals, adopted September 21st, 1998 and the EFSA Guidance on repeated-dose 90-day oral toxicity studies on whole food/feed in rodents, EFSA Scientific Opinion, 2011.

Good Laboratory Practices

Animal trials (SZU, Slovakia):

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 of 11th February 2004 (Official Journal No L 50/44). 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 Toxicology of the Slovak Medical University and the Laboratory of Immunotoxicology have received a statement of GLP compliance from the Slovak National Accreditation Service (certificate No. G-036) [certificates of Test Site 1 will be included based on tender outcome]. The Laboratory of Clinical and Experimental Biochemistry of the Slovak Medical University holds an accreditation certificate (~~M-013~~ ISO EN 17025) from Slovak National Accreditation Service (certificate No. M-013) and 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 executed by the Laboratory of Toxicology, the Laboratory of Immunotoxicology and the Laboratory of Clinical and Experimental Biochemistry - [Test Site 1 will be included based on tender outcome] are described in standard operating procedures (SOP), approved by the QAU.

Formatiert: Englisch (USA)

Analysis of feed materials

Maize culture, harvesting and grain packaging was performed in experimental and commercial fields not subjected to specific GLP in 2012. This was supervised by CRAG-UdG. Sampling was performed according to EN ISO 24333:2009, based on "Cereals and cereal products".

Maize and diet samples collected at Mucedola srl. (Test Site 3, diet manufacturer) were sent to RIKILT (Test Site 2), where these samples were registered through the sample registration system based on information provided in the Sample Information Form ("MIF") to be prepared and submitted to RIKILT's Sample Room ("Monsterkamer"). These samples were assigned a Laboratory Information Management System (LIMS) number and divided into subsamples for dispatch towards the subcontractor Covance and the other Test Sites 2 and 4-5 for further analyses. Registration and processing of samples was done under the pertinent SOPs. The analyses of maize and diet samples for target constituents were carried out by various partners and a subcontractor, as follows:

- RIKILT (Test Site 2) analysed samples for mycotoxins, organic contaminants (dioxins, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), ~~and~~ nitrosamines, ~~and~~ It also tested samples for the presence of genetically modified organisms (GMOs). Analyses were carried out under ISO 17025:2005 on "General requirements for the competence of testing and calibration laboratories". Methods had been validated and accredited except for nitrosamines, which were carried out under a SOP. More specifically, the following SOPs apply: dioxins, A0565; PAHs, combined A0824 / A0834, PCBs (included in aforementioned SOPs); and GMOs, A1033 and A1132. The subcontractor Covance analysed maize and diets for key compounds according to the OECD consensus ~~document~~ document's recommendations (proximate composition, micronutrients including vitamins and minerals, fatty and amino acid profiles, anti-nutrients and other secondary metabolites) as well as heavy metals, pesticide residues, and nitrate.



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- Mucedola (Test Site 3) tested maize for the presence of mycotoxins, and maize and diets for microbiological quality and proximate composition, under ISO 17025. Manufacturing of custom feeds was done under Good Manufacturing Practice. Coding of diets and samples was carried out according to instructions received from the contractor.
- INRA (Test Site 5) tested selected samples of maize and diets for the presence of the newly expressed Cry1Ab protein (known to be present in the genetically modified MON810 maize).

Supplementary analyses of diet and animal tissues at Test Site 5

Analyses performed at INRA (Test Site 5) on the feed materials and animal tissues, plasma and urine will be done in respect of the quality reference system developed and used for research and experimentations at INRA in order to meet the objectives of INRA's quality policy, *i.e.* traceability of research activities and reliability of measurable results.

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 will be approved by the Veterinary State Administration, Slovak Republic (Statna veterinarna a potravinova sprava Slovenskej republiky) Ro-4372/12-221. Animal care will be in compliance with SOPs of the Department of Toxicology, Slovak Medical University Bratislava and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.



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1. OBJECTIVE

This study is a pilot study planned to determine the added scientific value of implementing additional metabolic endpoints in the experimental design of 90-day studies used for the risk assessment of GM food/feed using Monsanto and Pioneer maize MON810. Further, it aims to provide guidance for the use and improvement of existing and new assessment tools for GM food and feed safety evaluation. For that purpose, a longitudinal follow-up of every animal will be performed concerning their metabolic and immunological status and their respective variations. An untargeted metabolomic exploration will be performed in plasma, urine and tissues and will privilege extensive multivariate datamining to get the hidden statistical structuring of datasets summarized in statistical factorial maps. Structural identification of putative biomarkers involved in such a structuring of the metabolomic information will be tentatively performed.

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2. PROFESSIONAL AND SUPERVISORY STAFF

Test facility SZU:

CVs of all engaged scientists are deposited in the Department of Toxicology, Slovak Medical University.

Toxicology:

Dagmar Zeljenková, VMD, PhD.

SZU, Department of Toxicology, Limbová 14, 833 03 Bratislava 37, Slovak Republic

Veterinary and gross pathology:

[REDACTED]
SZU, Department of Toxicology, Limbová 14, 833 03 Bratislava 37, Slovak Republic

Clinical chemistry:

[REDACTED]
SZU, Laboratory of Clinical and Experimental Biochemistry, Limbová 14, 833 03 Bratislava 37, Slovak Republic

Haematology and Immunotoxicology:

[REDACTED]
SZU, Laboratory of Immunotoxicology
Limbová 14, 833 03 Bratislava 37, Slovak Republic

Ophthalmology:

[REDACTED]
SZU, University Hospital, Antolská 11, 85107 Bratislava, Slovak Republic

Lead Quality Assurance Manager:

[REDACTED], SZU QA Unit, Limbová 14, 833 03 Bratislava 37, Slovak Republic

Formatiert: Slowakisch (Slowakei)

Statistical Analysis:



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SZU, Department of Biophysics, Biostatistics and Informatics,
Limbová 14, 833 03 Bratislava 37, Slovak Republic

Ethics Committee:

[REDACTED]

Cancer Research Institute, Slovak Academy of Sciences, Vlárská 7, 83391 Bratislava, Slovak Republic

Test Site 1: [Histological laboratory has to be assigned according to tender outcome]

Histology preparation:

Histology evaluation

Quality assurance manager:

Histological peer review

Prof. VMD. František Jelínek, PhD, Dipl. ECVP

Veterinary Histopathological Laboratory

[REDACTED]

Test Site 2:

Diet analysis

Dr Esther J. Kok, Dr Gijs A. Kleter

Test Site 3:

Diet preparation

[REDACTED]

Test Site 4:

Maize production and handling, diet analysis

Dr Maria Pla

Test Site 5:

Diet analysis, and immunological and metabolomic analyses

Dr Karine Adel-Patient



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3. TEST FACILITIES

Test Facility:

Testing Laboratories Center
Laboratory of Toxicology,
Slovak Medical University
Limbová 14,
83303 Bratislava 37

Test Site 1: [Histological laboratory has to be assigned according to tender outcome]

Test Site 2:

RIKILT – Institute of Food Safety
Wageningen University and Research Center Campus
Building 123, Akkermaalsbos 2
NL-6708WB Wageningen
Netherlands

Test Site 3:

Mucedola s.r.l. (licensed by Harlan)
Via Galileo Galilei 4
20019 Settimo Milanese (MI)
Italy

Test Site 4:

Center for Research in Agricultural Genomics
Campus UAB - CRAG building
Bellaterra
Cerdanyola del Vallès
08193 Barcelona
Spain

Maize growing, harvesting and drying:
Estació Experimental Mas Badia
17134 La Tallada d'Empordà, Girona, Spain.

Test Site 5:

Laboratoire d'Immuno-Allergie Alimentaire
iBiTec-S, Service de Pharmacologie et Immunologie (SPI), Building 136
CEA de Saclay
F-91191 Gif-Sur-Yvette cedex
France



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4. TIME SCHEDULE

Test feeds arrival		Planned date: December, 2013
Arrival of animals		February 2014
Starting of the treatment	males	February 10 -14 , 2014 (2 animals/group/day)
	females	February 17-21, 2014 (2 animals/group/day)
Necropsy	males	May 12 – 16 , 2014 (2 animals/group/day)
	females	May 19 - 23, 2014 (2 animals/group/day)
Histology	Slides preparation	June 12 – July 30, 2014
	Histology evaluation	July 20 – September 30, 2014
Final report – draft to Sponsor:		November 30, 2014

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5. TEST AND CONTROL CROPS

- GM crop 1: Variety : DKC6667-YG (containing the MON810 event with insect-resistance trait based on expression of the newly expressed Cry1Ab protein)
- Non-GM near-isogenic crop 1: Variety: DKC6666
- GM crop 2: Variety : PR33D48 (containing the MON810 event with insect-resistance trait based on expression of the newly expressed Cry1Ab protein)
- Non-GM near-isogenic crop 2 Variety: PR32T16

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All crops: production during the 2012 season, all in a small area in the Empordà (NW of Catalonia, Spain) in the same conditions and according to the standard cultural practices in the region. No insecticides applied in any case. Herbicide and other treatments recorded. Monitoring of the date of sowing, flowering and harvesting, yield, grain humidity and relevant pathogen attacks, particularly corn borer incidence. Climatic data are available.

Crops were dried in a forced air oven at 60°C and sampled (EN_ISO_24333) to prepare about 90 or 100 kg (for GM and near-isogenic varieties, respectively) for preparation of the diets. Grains were packaged in autoclave plastic bags inside containers of 30-35 kg, each labeled with the full name of the variety and other details.

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Batches and batch numbers: Maize kernels were packed in bags of approximately 11 kg each. Three bags with a particular maize variety were packed into a container, containing approximately 35 kg of maize kernels. Batch numbers include the name of the variety plus a lot number affixed to it (see example).

Example of container label:

Producto / Product: MAIZE GRAIN

Variedad / Variety: PR32T16

Masa (Kg) / Mass weight (Kg): 10 Kg

Lote n° / Batch n°: PR32T16-1 (code is variable between varieties)

Proyecto o Contrato / Project or Contract: GRACE

Fecha realización / Date: 19/11/2012

Lugar realización / Location: Centre for Research in Agrigenomics (CRAG)

Persona contacto / Contact person: [REDACTED]

Muestra para / Sample for: Rat Feed Compound

Formatiert: Italienisch (Italien),
Hervorheben



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6. TEST SYSTEM

Species and strain

Rat Wistar Rcc Han /Specific Pathogen Free (SPF)

Source

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

Number of animals

65 male and 65 female rats will be ordered. Only 60 males and 60 females will be used for the study. Females will be nulliparous and non-pregnant. Animals not assigned to the study will be deemed as sentinels and will be used as veterinary control fed with Teklad diet as provided by the diet manufacturer.

Approximate weight and age

Upon arrival, the animals will weigh between 100-120 g ~~and~~. As the treatment of females will start with a time shift of 1 week compared to treatment of males, males will be 5 weeks old and females 4 weeks old upon arrival. The animals will be 6 weeks old at the start of the study and will weigh between 110-140 g. Ideally, they should be born within 1-5 days of each other and be of uniform weight ($\pm 20\%$ of the mean).

Identification

Within the frame of treatment groups, each rat will be marked by code (marked every 2 weeks with a permanent marker) on the tail base in accordance with SOP: ŠPP/TOX/V002 to identify the animal individually. Each cage will be marked with a colored cage card.

Justification for the selection and number of animals

This species (*Rattus norvegicus* ssp. *alba*) and strain (Wistar Rcc Han) of animal is generally recognized as appropriate for the conduct of subchronic toxicity studies. The Wistar rat is a widely used strain of rats for which significant control data are available. The toxicology laboratory of the Slovak Medical University has a record of the regular use of this strain of rats. Sample Group size was determined by a power analysis- for a two sample t-test for quantitative biomarkers such as haematology, clinical biochemistry and individual organ weights. With 10 animals per group (males and females combined) there will be a 90% power to detect a standardized effect size of 1.5 standard deviations and an 80% power to detect a standardized effect size of 1.3 standard deviations. - However, metabolomics generates a large number of biomarker observations which will need to be statistically analysed using multivariate methods. Currently it is not known how to determine sample size for this type of statistical analysis. For example, statistical power will depend to some extent on the number of measurements made on each individual animal. All that can be said at this stage is that these numbers seem to be a reasonable compromise which avoids using too many animals. For qualitative characters such as pathological lesions and tumours with a group size of 10 animals there is an 80% chance of detecting an incidence of 40% or more (8 animals) against a background of 5% (one animal) with a significance level of 0.05. Clearly these sample sizes are too small to be of much value in detecting qualitative effects.

Animal housing

All animals will be housed in rooms N° B 2/-31 and 4 of the Specific Pathogen Free (SPF) experimental animal house equipped with a pressure climatic system at the Department of Toxicology



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of the Slovak Medical University. The temperature and relative humidity in the animal room will be recorded every 20 minutes by the PMICRO-LCD-THSYS, Dallas Semiconductor system and every week the computer readout for the past week will be evaluated. Mean temperature will be maintained at $22 \pm 2^\circ\text{C}$ and relative humidity at 40-70%. The animals will be subjected to a 12-hour light/ 12-hour dark cycle.

Rats will be housed in TECNIPLAST cages ~~Typetype~~ 2145 F with an H-Temp™ (PSU) from the Tecniplast Company, Italy. The cages have a high density polypropylene body, measuring $480 \times 265 \times 210$ mm - floor area 940 cm^2 . Toys (plastic tubes) will be provided.

We will use sterilized bedding from JRS Lignocel®, hygienic animal bedding, sterilized sawdust from Charles River 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. The cages will be emptied and cleaned with water and detergent. After cleaning they will be dried and then 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 into 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 the same way that the cages are cleaned.

Bottles will be exchanged and cleaned daily. 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 disinfect.

Diet formulation, sampling and analysis

Diet formulation, sampling and shipping

- Maize harvested from the Catalonian production sites was shipped to the Italian production facility (Mucedola srl.) licensed by Harlan for the production of diets. Shipping was done with the monthly truck service offered by the facility. Grains were packaged in autoclave plastic bags inside containers of 30-35 kg, each labelled with the full name of the variety and other details.
- Milling of maize kernels was done by this facility, as was the formulation, *i.e.* mixing with other ingredients, using a customized pelletizing process ~~using~~utilizing a pasta press without the use of steam, which aids to prevent loss of heat-labile compounds.
- Formulation was carried out according to the diet composition recommended by the Harlan Company's nutritionist so as to achieve isoproteic and isocaloric diets with 11% and 33% transgenic variety and 22% and 33% for the near-isogenic maize inclusion levels. Hence, each diet contained 33% maize *in toto*. The composition included plant-based ingredients (hence no animal-derived ingredients), ~~such as wheat middlings, wheat, soybean meal, and soybean oil. No other maize-derived ingredients are incorporated besides the milled-maize prepared from the materials harvested for the GRACE project, the other ingredients being.~~ Samples for dispatch to the analytical laboratories for nutrition and contaminants were taken after milling and after pelletizing (before and after irradiation) according to instructions from the responsible GRACE scientist (company had been instructed to take multiple, *i.e.* at least five samples, at different spots from the batches prepared).



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- A complete battery of tests for different GMOs was performed on a sample of each variety at the RIKILT facilities (including a broad GMO screen and a quantitative event-specific PCR assay for MON810), while INRA tested for the presence of the Cry1Ab protein expressed in transgenic maize MON810.
- Diets were coded in a “double blind” fashion by the diet-producing company (Mucedola srl.). Samples of the diets were coded with different codes than the diets themselves. The coding scheme was shared with Gijs Kleter (the company’s contact within the GRACE consortium). It was kept confidential and therefore not distributed further among consortium members during the course of the previous animal experiments using the same feed materials, and analyses of samples derived from these experiments. The code of the analytical samples ~~was~~ will be broken after termination of the analytical activities for these previous experiments after analytical data had been recorded and stored. The code of the animal diets ~~provided to the animals~~ was partially broken (revealing which groups were high-dose GM and control groups ~~but without~~) and divulged towards the principal investigator in charge of the animal facility (~~Prof Dagmar~~ Zeljenkova) after the performance of the previous animal trials (Study No 311957 - A / 13/ GLP and 311957 - B / 13/ GLP) so as to allow focus of the ensuing histopathology on samples from groups fed these two diets. - The investigators and technical staff performing the histopathology had not been informed about the exact identity of the samples investigated, though, so as to still keep the experiment blind at that level. In a similar way, the codes are not ~~to~~ be revealed to them until the end of the current study under this study plan.

Analysis of maize material and diets (for each dosage group, diets will be provided in 3 follow-up batches by the diet manufacturer):

- Maize: The key parameters for the analysis of maize included:
 - Chemical composition
 - ☐ Macronutrients & ~~fibres~~ fibre (ADF, NDF, dietary),
 - ☐ Minerals,
 - ☐ Vitamins (A, B, C, E), zeaxanthin
 - ☐ Amino acid composition (including tryptophan),
 - ☐ Fatty acid composition,
 - ☐ Antinutrients (phytic acid, trypsin inhibitor),
 - ☐ Other secondary metabolites (~~fur~~ furfural, e.g., phenolics, sterols) and carbohydrates ~~(~~ e.g. raffinose, stachyose ~~).~~
 - GMOs (DNA ~~),~~)
 - Cry1Ab protein, ~~pesticide~~
 - Chemical contaminants:
 - Pesticide residues, ~~mycotoxins, heavy~~
 - Mycotoxins,
 - Heavy metals, ~~ether~~ and
 - ☐ Other contaminants (e.g. dioxins, PAHs, PCBs, nitrate, nitrosamine),
 - The key Diets: The analysis of diets was done on the pre-irradiation samples, while the post-irradiation samples of the same diets was stored as back-up for possible follow-up analyses if circumstances so require. The parameters of the analysis of the diets included:
 - Same parameters as for maize, plus:
 - Isoflavones
 - Lectins

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Besides these analyses of chemical composition and presence of GMOs, the maize and diets was also submitted to microbiological analysis. Both maize and diets (pre- and post-irradiation) was be



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analyzed for the presence of microorganisms, in particular bacteria (including coliforms, enterobacteriaceae, and specific bacteria) in all samples, plus other micro-organisms (including yeasts, moulds) in diets.

Storage conditions:

- Kernels and pellets were kept at ambient temperature and measures were taken to avoid build-up of moisture and fungal growth (e.g. transport of bags containing desiccant in closed boxes). The size of each bag is about 10 kg (autoclaved plastic bags). Every variety was considered as a single batch as it all was cultured, harvested and dried as a single batch. The size of the batches depended on the variety. Drying prevents grains from fungal infections, while gamma-irradiation of the diets was performed after milling and diet preparation.
- After receipt of the analytical samples, the receiving laboratories kept them under controlled cool, dry and confined conditions to ensure the stability of the sample.

Spare samples from the irradiated diets after receipt at the animal testing facility were taken and kept for later analysis in the refrigerator at 4°C (room B2-219).

Samples of diets were sent to the analytical laboratories contracted for the analysis of the composition (macronutrients, micronutrients, anti-nutrients, and other secondary metabolites) as well as for, the presence of genetically modified organisms (GMOs; element screen and event-specific test for MON810), as well as for mycotoxins, residues of pesticides and contaminants (e.g. dioxins, PAHs, PCBs, nitrate, nitrosamines, heavy metals), and pathogens.

Test diets were stored at -28 °C for 10 month in a freezer in room A 01 at SZU, Limbová 14, Bratislava, Slovak Republic. Before feeding the diets to the test animals, their quality will be controlled by sending them to the analytical laboratories contracted for the analysis of the composition. In order to check for the stability of the diets during storage, representative samples are to be analyzed for their content of fatty acids, amino acids, vitamins and Cry1Ab, and the outcomes compared to those obtained for the same diets before storage. During the study, storage of the test diet will be done in closed rooms (cool and dry, controlled temperature and humidity), at the Laboratory of Toxicology, SZU, Limbová 14, Bratislava, Slovak Republic

The test diets are provided as single batches (containing portions of diets packed in separate vacuum, gamma-irradiated packs).

Water

The rats will be supplied water *ad libitum* during the acclimation and study periods. We will use tap water with a special filter to eliminate microorganisms. The bottles containing this water will be autoclaved before use. The water from the local mains will be monitored for quality by testing for the microbiological and chemical quality by Waterworks Bratislava quarterly. We will receive a certificate of quality.



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7. EXPERIMENTAL DESIGN

Animal receipt and acclimation

All animals will be purchased from Harlan and will be only 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 3 ~~–5 days~~ **7 days for males and 10-14 days for females** prior to the start of the study to allow for acclimation to the laboratory conditions. These are identical to those defined for the experimental part of the study. During this period the animals will be monitored for their health status twice a day (see section 8. PERIODICAL HEALTH STATUS OBSERVATIONS below for a full description of the health status evaluation).

Randomisation

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 and will be randomized using completely randomised ~~designs (SOP: ŠPP/TOX/V001)~~ **design (SOP: ŠPP/TOX/V001)**. Experience shows that these young rats do not differ to any great extent in body weight. Should this be of concern at some future date, initial body weight could be taken into account by using an analysis of covariance. The multivariate statistical analysis will take account of gender as well as diet.

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Tables with cage numbers and the random diet assignment will be prepared by the local statisticians. The Random Number Generators (RNG) of SPSS software will be used for male and female animals, separately.

All animals will be numbered from 1 to 65 (males) and 66-130 (females). We will assign 2 animals into 1 cage, using RNG.

These animals will be excluded from next option and random choice will be repeated until animals 1-60 and 66-125 are randomly assigned to cages. Remaining 5 animals/gender will be used as sentinel group, 1 cage with 2 animals and 1 cage with 3 animals

Formatiert: Schriftart: Fett, Kursiv, Englisch (USA)

Formatiert: Links, Keine

2 animals will be placed in 1 cage. Animals will be randomly allocated to cages by dose group and gender. To minimise the chance of mistakes being made, cages of the same treatment groups will be clustered in vertically arranged groups, which will be rotated on a regular basis (once per week). Each vertical row of cages (within the same dose group) will be rotated from top to bottom. Racks will be rotated clockwise every two weeks within the original room configuration. **This is a reasonable compromise between the need to minimize possible mistakes and the need to ensure that each cage has, on average, the same environment within the animal house.**

Group allocation and dosing

Prior to the start of treatment on study day 1, a detailed examination of all animals will be carried out to verify their health condition (see section 8. PERIODICAL HEALTH STATUS OBSERVATIONS for a full description).

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 (maize) will be administered by incorporation into the diet since



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this mimics most human exposure to these foods. Attention will be paid that there will be no nutritional imbalance as a result of dietary incorporation of the test item.

Food will be supplied *ad libitum*. Feed consumption will be determined weekly for 90 days. At the beginning of each food consumption measurement, weighed full feeders with stainless steel lids will be placed in each cage. At feeder change-out (once weekly), the feeders will be weighed again, the difference in weight is an estimate of total amount consumed by the 2 occupants of the cage. **Food spillage will be documented and the amount will be noted and subtracted.** Feed consumption will be recorded, and will be reported as grams/animal/day. **Food conversion will be calculated (change in body weight in relation to food intake).**

Feed containers and scoops will also be colour coded. However, animal house staff will be “blind” with respect to the identity of the diets.

The different feeds will be coded and labelled by Mucedola company. The code was given only to Gijs Kleter. All others will be blinded to the feeds.

General experimental design with Monsanto and Pioneer MON810 maize, start February 2014

Group	% (w/w) of diet		Number of animals		Number of animals	
	GM	Near-Isogenic non-GM				
Unknown identity for the staff*	Referene diet	Othe ingredients	GM	Near-Isogenic non-GM		
x*	67	33	0	0	10	10
x*	67	11	22	0	10	10
x*	67	0	33	0	10	10
x*	67	0	0	33	10	10
x*	67	0	0	11	10	10
x*	67	0	0	0	10	10
Total					60	60

Gelöschte Zellen
 Formatierte Tabelle
 Gelöschte Zellen

Formatierte Tabelle

Group/ colour coding by Mucedola (example)	Diet No	Number of animals		Number of cages	
		Males	Females	Males	Females
1 light blue	1001	10	5	10	5
2 red	1002	10	5	10	5
3 green	1005	10	5	10	5
4 yellow	2006	10	5	10	5
5 white	2007	10	5	10	5
6 dark blue	2010	10	5	10	5
Sentinels beige	Teklad	5	2	5	2
Total		65	32	65	32

Formatierte Tabelle
 Eingefügte Zellen

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8. PERIODICAL HEALTH STATUS OBSERVATIONS

All observations will be made in a non randomized order.

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 observe them carefully at least 4 times daily. Selection criteria are made explicit in SOP: ŠPP TOX/V 004. If a study animal dies, we will subject it to necropsy as soon as possible after death. Any animal whose condition makes it unlikely that it will survive to the next observation period will be euthanized by ketamine/xylazine anaesthesia (SOP No.: ŠPP TOX/FS/004V/005) and immediately necropsied.

Clinical signs

Cage side observations / uncovered cage

Rats will be inspected twice daily for evidence of reaction to treatment or ill-health 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 behaviour in accordance with SOP: ŠPP TOX/V 003.

Detailed physical examination

Once weekly, rats will be examined out of cage. 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 behaviour.

Functional assessment

Towards the end of the exposure period changes in gait, posture and response to handling as well as the presence of clonic or tonic movements or bizarre behaviour (self-mutilation, walking backwards) will also be recorded. Sensory reactivity to stimuli of different modalities (e.g. auditory, visual and proprioceptive stimuli), will be recorded. The outcome of this examination will be recorded for each animal, in accordance with SOP: ŠPP /-TOX / V003 (Origin of score system: Ország A. et al. (1985): Veterinárnaortopédia a röntgenológia, Bratislava: Príroda, 243 s. (Veterinary orthopaedy and X-ray). The animals will also be assessed for gait disturbances using the AccuPacer treadmill equipment.

Ophthalmologic examination

Using an ophthalmoscope, we will examine the eyes of all animals prior to the administration of the test feeds and one week before the termination of the study. This will be done by the chief of ophthalmology who has expertise in this area.

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Pupillary dilation ophthalmologic examination of both eyes will be performed by an experienced ophthalmologist in the conscious rat during gently manual restraint by a technician. First, the eye and the peri-bulbar structures are macroscopically examined. Thereafter direct ophthalmocopy is performed using ophthalmoscope. Ophthalmoscopic findings are 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, 2) on the first day of feeding, 3) weekly during the study period, 4) at the termination of the study, 5) in the event of an

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early death or sacrifice *in extremis*. The General Linear Model (GLM) for Repeated Measures will be used for analysis of the body weight.

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9. PROCEDURES FOR SAMPLE COLLECTION

Sample collection during the feeding trial

Sample collection during the feeding trial will be done for ~~metabolomics~~metabolomic and ~~haematology~~haematologic analyses. Samples will be collected from all animals (n=120) and will include urine and blood (plasma). ~~Sample for metabolomics and serum for haematological analysis).~~
Timing of sample collection is detailed in attachment 1: A and B.

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Sample collection (days ~~1, 7, 28, 56~~29, 57 and 84):

Personal disposition for forced progress (12 animals per day)

- Urine will be collected by person No 1
- Urine processing and transport to the competent laboratory (Laboratory of Clinical and Experimental Biochemistry), dividing of samples: metabolomics samples will be immediately frozen in liquid nitrogen and then stored at -80°C - person No 2
- Blood taking from the tail vein by person No 3
- Blood processing, dividing of samples: metabolomics plasma samples will be immediately frozen in liquid nitrogen and then stored at -80°C - person No 4
- Blood transport to the competent laboratory: Laboratory of Immunotoxicology person No 5
- Clinical chemistry: Laboratory of Clinical and Experimental Biochemistry person No 6

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Urine

Urine will be collected from each individual feed deprived rat in metabolic cages ~~starting one~~on day ~~before the treatment period (i.e. D-1), and then on the 7th (D7), 28th (D28), 56th (D56)~~day 29 (D29), day 57 (D57) and 84th (D84) days. ~~day 92 (D92).~~ Urine will be collected under the same conditions in groups of 12 animals per day (6 test diets × 2 animals/test diet/gender) during 5 consecutive days, with a shift in time of 1 week between males and females. ~~D0 (see attachment 1B). D1~~ for each group will be assigned accordingly. For every collected group of animals, every test diet will be balanced for the number of animals submitted to urine collection.

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Animals will be kept in metabolic cages for 16 hours each day of urine collection, supplied with water but deprived from feed. Urine will be collected in a 20-ml vial containing 1 ml of a solution of 1% (i.e. initial concentration) of sodium azide (NaN₃) as preservative. The total volume of urine excreted during the 16-hour period will be measured. Urine will be homogenized before collecting two separate 1-ml aliquot samples in 3-ml polypropylene vials with cap ~~which will be~~ stored at -80°C.

At the end of every 16-hour collection period, blood will be collected from tail vein (see below) from the same animals before they are moved to their respective conventional cages.

Every sample collected at different time points will be identified by a unique code.

Data concerning the volumes of urine collected at different time points will be recorded. Observations related to a seemingly abnormal water consumption during the 16-hour urine collection in metabolic cages will be recorded.

Blood

Blood collections from tail vein will be performed the same days as the urine collection but after the urine collection (i.e. when the animals are removed from the metabolic cages and before they are put back to their respective cage). The timepoints for metabolomics/immunological blood sample collection are shifted according to the dosing time.

For each individual rat, blood (ca. 300-400 µL) will be collected in a heparinised vial which will be centrifuged at 3000 g at 4°C for 15 min. Plasma will be collected, dispatched in two separate 3-ml



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polypropylene vials (or equivalent vials) with a cap and stored at -80°C. Ca. 200 µl of plasma are required for the immunological and metabolomic analyses. For every animal, after collecting blood in one heparinised vial, samples will be kept not more than 15 min at 4°C before starting centrifugation to separate red cells from plasma and centrifuged plasma will be kept not more than 15 min at 4°C before preparation of aliquots and storage at -80°C.

Haematology

On day **8485**, blood samples from the tail vein will also be taken from all animals for haematological examination after 16 hours fasting. EDTA will be used as anticoagulant. Blood samples (ca. 200 µl) will be stored under room temperature (17-25°C) maximum up to 4 hours until measurement. Haematological analysis will be performed in accordance with SOP: ŠPP/IMU/M002 using Haematological analyzer Sysmex K-4500, SYSMEX TOA Medical Electronics Co. LTD, Japan. Parameters scheduled for examination are

- Erythrocyte Count (RBC)
- Haematocrit (HT)
- Haemoglobin (Hb)
- Mean Corpuscular Haemoglobin (MCH)
- Mean Corpuscular Haemoglobin Concentration (MCHC)
- Mean Cell Volume (MCV)
- Leukocyte Count (WBC)
- Differential Leukocyte Count
- Platelet Count (PLT)

Differential Leukocyte Count will be examined using light microscope. Blood smears will be stained by panoptic staining using May-Grunwald and Giemsa-Romanowski dyes. The percentage of lymphocytes, neutrophils, eosinophils, basophils and monocytes will be determined by examining of 200 cells.

Sample collection and tissue processing at the end of the feeding trial

Sample collection for the following analyses will be done: clinical chemistry and histopathology (according to OECD Guideline 408) as well as metabolomics and immunology. Samples collected will include blood, tissues and organs. Blood samples **will be taken from all the animals of the study and** will be divided for clinical chemistry, metabolomics and immunology analyses. Tissues and organs will be removed and evaluated by histology, metabolomics and immunology.

Sample collection and tissue processing:

Personal disposition for forced progress (12 animals per day will be necropsied):

- Animals will be **anaesthetized** by person No. 1
- **Blood taking** from the **abdominal vessel** will be done by person No. 2
- **Blood processing** and transport to the competent laboratory (Laboratory of Clinical and Experimental Biochemistry) – person No. 3
- **Animal transport** to the autopsy room on the same floor by person No. 4
- Removal and weighing of **selected organs** in line with OECD guideline 408 for the metabolomics study and their preparation – person No. 5
- ~~- Necropsy of **thorax part** body – person No. 6~~
- ~~- Necropsy of **abdominal part** body – person No. 7~~



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- ~~—Necropsy of **genital organs**—person No. 8~~
- ~~—Weighing of **organs** in line with OECD test guideline 408—person No. 9~~
- ~~—Decapitation and necropsy of the **head** including brain by person No. 10~~
- ~~—All organs for histopathology will be stored into formalin~~
- ~~—Omics samples- Samples for the metabolomics study~~ will be immediately frozen in liquid nitrogen and then stored at -80°C; this will be controlled by person No. ~~11~~6
- Necropsy of **thorax part** body - person No. 7
- Necropsy of **abdominal part** body - person No. 8
- Necropsy of **genital organs** - person No. 9
- Weighing of **organs** in line with OECD test guideline 408 - person No. 10
- Decapitation and necropsy of the **head** including brain by person No. 11
- All organs for histopathology will be stored into formalin
- Organs needed for immunological examination (~~part~~half of spleen, half of thymus, two mesenterial lymph nodes and bone marrow from left femur) will be stored at room temperature in RPMI media or PBS buffer and will be transported to the Laboratory of Immunotoxicology (lab. No. B2-222, SZU) within 1 hour - controlled by person No. 12.

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All steps are inspected by QA

Time schedule for the necropsy of animals

Males:

<i>Group/ colour coding by Mucedola (example)</i>	<i>Diet No</i>	<i>Monday, May 12, 2013</i>	<i>Tuesday, May 13, 2013</i>	<i>Wednesday, May 14, 2013</i>	<i>Thursday, May 15, 2013</i>	<i>Friday, May 16, 2013</i>
		<i>ID of animal</i>	<i>ID of animal</i>	<i>ID of animal</i>	<i>ID of animal</i>	<i>ID of animal</i>
1 light blue	<u>1001</u>	1-2	3-4	5-6	7-8	9-10
2 red	<u>1002</u>	11-12	13-14	15-16	17-18	19-20
3 green	<u>1005</u>	21-22	23-24	25-26	27-28	29-30
4 yellow	<u>2006</u>	31-32	33-34	35-36	37-38	39-40
5 white	<u>2007</u>	41-42	43-44	45-46	47-48	49-50
6 dark blue	<u>2010</u>	51-52	53-54	55-56	57-58	59-60

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Females:

<i>Group/ colour coding by Mucedola (example)</i>	<i>Diet No</i>	<i>Monday, May 19, 2013</i>	<i>Tuesday, May 20, 2013</i>	<i>Wednesday, May 21, 2013</i>	<i>Thursday, May 22, 2013</i>	<i>Friday, May 23, 2013</i>
		<i>ID of animal</i>	<i>ID of animal</i>	<i>ID of animal</i>	<i>ID of animal</i>	<i>ID of animal</i>
1 light blue	<u>1001</u>	101-102	103-104	105-106	107-108	109-110
2 red	<u>1002</u>	111-112	113-114	115-116	117-118	119-120
3 green	<u>1005</u>	121-122	123-124	125-126	127-128	129-130
4 yellow	<u>2006</u>	131-132	133-134	135-136	137-138	139-140
5 white	<u>2007</u>	141-142	143-144	145-146	147-148	149-150
6 dark blue	<u>2010</u>	151-152	153-154	155-156	157-158	159-160

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Blood sampling at sacrifice

At the end of the study and after 16 hours fasting, in anaesthesia before sacrifice, blood samples from the abdominal vessel will be taken from all animals for blood chemistry examination and metabolomic/immunological studies.

For every animal, blood samples will be taken under ketamine/xylazine anaesthesia. About 2 ml of blood will be collected in heparinized vials. 200 µl of whole blood will be pipetted into separate tubes for blood chemistry, 300 µl will be pipetted in laminar flow hood (lab. No.B2-222, SZU) into separate sterile tubes for phagocytic activity and respiratory burst of leukocytes.

Rest volume of blood collected in heparinised vials will be centrifuged at 3000g at 4°C for 15 min and all plasma collected. For every animal, after collecting blood in one heparinised vial, samples will be kept not more than 15 min at 4°C before starting centrifugation to separate red cells from plasma. Aliquots of plasma (ca. 500 µL) will be collected for the metabolomic and immunological analyses and kept at -80°C. Centrifuged plasma will be kept not more than 15 min at 4°C before preparation of aliquots and storage at -80°C. Plasma will be stored in 3-ml polypropylene vials with a cap or seemingly equivalent vials.

Clinical chemistry

Samples will be analysed using an Analyzer Vitros 250, Ortho-Clinical Diagnostics, No. 219037234, 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 will include:

- total protein (TP)
- albumin (ALB)
- aspartate aminotransferase (AST)
- alanine aminotransferase (ALT)
- alkaline phosphatase (ALP)
- creatinine (CREA)
- urea nitrogen
- fasting blood glucose
- total bilirubin (TBIL)
- total cholesterol
- triglycerides
- Na
- K
- Ca
- Cl
- P

Phagocytic activity and respiratory burst of leukocytes

Phagocytic activity and respiratory burst of leukocytes will be measured by flow cytometry - ŠPP/IMU/M008 in accordance with SOP: ŠPP/IMU/M008. Briefly, heparinised whole blood will be incubated with the fluorescein-labeled *Staphylococcus aureus* bacteria and dihydroethidine at 37°C, a negative control sample remains on ice. The investigation of phagocytosis and oxidative burst will be performed by flow cytometry.

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Humoral immunological studies of plasma

~~Plasma collected during and at the end of the 90 day trial will be tested for total and maize/Cry1Ab specific antibodies (i.e. IgG, IgM and IgE) using specific immunoassays developed and validated in Test Site 5.~~

← --- **Formatiert:** Kommentartext, Links

Metabolomic studies of plasma and urine samples for INRA (Test Site 5)

~~For metabolomic studies, plasma and urine samples will be extracted with organic solvents to discard insoluble macromolecules and non-polar analytes. The resulting hydrosoluble fractions will be fingerprinted by ultra-high resolution mass spectrometry to quantify the distribution of detectable analytes.~~

Plasma and urine samples will be sent on dry ice to INRA (Test Site 5) for further immunological ([see below](#)) and metabolomic analyses with the list of samples INRA must receive and their identification (e.g. n° of rats, type of sample, and day of collection). Each sample ~~must will~~ be clearly and unambiguously identified by the animal number/nature of the sample (plasma or urine).

~~For metabolomic studies, plasma samples will be extracted with organic solvent (methanol, 2 vol.) with Polytron disperser followed by centrifugation at 13000 g for 10 min and storage at -20°C for 16 hours to discard insoluble macromolecules and non-polar analytes. The resulting hydrosoluble fractions will be fingerprinted by ultra high resolution mass spectrometry (LTQ-orbitrap-MS, ThermoFisher Scientific, Villebon-Sur-Yvette, France) to quantify the distribution of detectable analytes in both negative and positive modes. Urine will be analyzed directly after dilution in water. Prior to processing of samples, a new codification of samples will be achieved and a randomization in selection of samples day after day will be performed. This randomized selection will be balanced to get one individual rat belonging to one of the different groups of animals in a same subset of samples processed at the same time. This procedure will be used to avoid any influence of a possible day-to-day variation in extraction on the mean fingerprint characterizing every group of treatment. Statistical structuring of the total metabolomic variance will be obtained by an extensive multivariate exploration of raw metabolomic data using R, SAS or Simca based statistical scripts or chemometric procedures available in the Test Site 5, and will be summarized on dedicated factorial maps. Putative biomarkers will be issued from the prior multivariate statistical exploration. Their identification will be achieved on a case-by-case basis by comparison of their mass spectra with those of authentic reference compounds, when available, and obtained in the same analytical conditions or by direct analyses by MSⁿ to describe fragments and then by confirmation of structural hypotheses thanks to available reference databases.~~

Humoral immunological studies of plasma

~~In order to assess the Cry1Ab allergenicity/immunogenicity, and the effect of the genetic modification on the intrinsic allergenicity/immunogenicity of maize, plasma collected during and at the end of the 90 day trial will be tested for total and maize/Cry1Ab specific antibodies (i.e. IgG, IgM and IgE) using specific immunoassays developed and validated in Test Site 5.~~

← --- **Formatiert:** Kommentartext, Links

10. PATHOLOGY

Gross necropsy

A complete necropsy will be performed on all animals at study termination on day ~~94~~92. The weight of organs will be recorded in line with OECD guideline 408 and organs/tissues will be examined macroscopically for any deviations from normal (in accordance with ~~ŠPP / TOX / V005~~ SOP: ŠPP /



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TOX / V005). A supervising toxicological pathologist will be present. All records are made manually and are subsequently transferred and saved in the computer system.

The wet-weight of the following organs will be recorded: brain, lungs; (weighed before inflation with the fixative), heart, liver, kidneys, spleen, adrenal glands, pancreas, testes, uterus, ovaries, epididymides, and thymus.

~~The Organs and tissues will be preserved in the fixative medium (neutral buffered 10% formalin) for histopathological examination are formalin-fixed (neutral buffered 10% formalin), except for gross lesions and parts eyes and male reproductive tissues , where Bouin 's solutions will be used.~~
Parts of specific organs will be snap frozen in liquid nitrogen or in PBS buffer or RPMI medium in order to allow for additional metabolomics and cellular immunology examinations.

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Tissue specimens include:

- all gross lesions
- brain (representative regions including cerebrum, cerebellum , medulla/pons and pituitary)
- spinal cord
- thyroid
- parathyroid
- thymus
- oesophagus
- aorta
- salivary glands
- stomach (forestomach and glandular stomach)
- small intestine
- pancreas
- large intestines (including Peyer's patches)
- liver
- kidneys (L, R)
- accessory sex organs
- adrenals
- spleen
- heart
- trachea and lungs (inflated with fixative and then immersed in formalin)
- gonads (testes, L, R; ovaries L, R)
- uterus
- female mammary glands
- prostate
- urinary bladder
- lymph nodes: submandibular and mesenteric
- peripheral nerve (sciatic or tibial) preferably in close proximity to the muscle
- section of bone marrow and/or a fresh bone marrow aspirate



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- skin from the back will be taken from the same area of each rat
- eyes (if changes were observed during ophthalmological examinations)
- additional tissues may need to be investigated based on clinical or any other findings. Also any organs/tissues that are likely to be considered as target organs based on the known toxicological properties of the test material should be preserved.

In the case of paired organs, e.g. kidney, adrenal, both organs will be singly weighed and preserved

Histopathology

Organs and tissues will be preserved in neutral buffered 10% formalin except for eyes and male reproductive tissues, where Bouin's solution will be used. Organs and tissues in formalin will be shipped to histological laboratory for histopathological evaluation in accordance with SOPs (has to be included according to tender outcome). Complete microscopic examination of the tissues listed above will be performed in accordance with the OECD TG 408 on all animals from the high dose and control group. Groups will be identified by Gijs Kleter, without informing SZU and the histological laboratory (has to be included according to tender outcome) about the identity of each group.

Cellular immunological studies

The procedure will be as follows:

After weighing, spleen will be cut on sterile Petri dish and ~~par~~half of the tissue will be put into plastic tubes with sterile RPMI media containing heparin. Spleen will be transported to (lab. No. B2-222, SZU). ~~Spleen will be -) and used for determination of the proliferation activity of lymphocytes (using liquid scintillation -) (in accordance with SOP: ŠPP/IMU/M006) and in vitro production of cytokines (TNF-alpha, IL-1beta; -ELISA -) following SOP: ŠPP/IMU/M009) after non-). Proliferation activity of lymphocytes will be examined using liquid scintillation. Spleen cells will be cultured either with no stimulus (for spontaneous proliferation) or with specific activation (antigen (extract from food) or mitogens: (concanavalin A, phytohemmagglutinin, pokeweed mitogen, anti-CD3)). The degree of proliferation will be quantified by determining the incorporation of ³H-labelled thymidine in the DNA. Cell fraction with incorporated ³H in the DNA will be determined by measuring filters in scintillation fluid. Radiation of ³H invokes the fluorescence effect measured by scintillation spectrophotometer. The degree of responsiveness will be measured as the counts per minute (cpm) for each variable.~~

Determination of In vitro production of cytokines (TNF-alpha, IL-1beta) will be preformed using ELISA method. Briefly, the spleen cells will be cultured with specific antigen (extract from food) and/or mitogen (concanavalin A). After incubation period, cytokine release (TNF-alpha, IL-1beta) will be measured in culture supernatants using commercially available ELISA sets.

Furthermore, ~~spleen, 2~~ mesenterial lymph nodes (optionally ~~par~~half of thymus and bone marrow from left femur) will be collected, placed into PBS buffer or RPMI media and transported to (lab. No. B2-222, SZU) and further used for Phenotypic analysis of leukocytes using flow cytometry according to the SOP: ŠPP/IMU/M007 Parameters M007. Parameters will include: CD3, CD4, CD8; (CD45R, CD161, DC optionally).

Phenotypic analysis of leucocytes

The phenotypic analysis of leukocytes using flow cytometry (ŠPP/IMU/M007) will include the following parameters: CD3, CD4, CD8; (CD45R, CD161, DC optionally).

Briefly, spleen, thymus and bone marrow cells will be washed using needle, mesenterial lymph nodes using homogenizer into medium with heparin or PBS. Cell suspensions will be standardized using light microscope. Commercially available monoclonal antibodies against rat T-cells, T-helper cells, T-

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cytotoxic cells, B-cells and NK-cells (CD3, CD4, CD8, CD45R, CD161, optionally DC) labelled with fluorescent dyes will be added to the cells. After incubation, rest of erythrocytes will be lysed and tubes will be analyzed using flow cytometry.

Metabolomic studies of tissue samples

The following tissues, i.e. the right lateral lobe of the liver and the third to the half higher part of the right kidney will be collected for metabolomic studies. The technical conditions of dissection and collection of these matrices are described in the following:

Liver:

After weighing, the right lateral lobe will be collected and immediately snap frozen in liquid nitrogen. Liver samples will be stored in 1.5 ml Eppendorf vials or seemingly equivalent containers and snap frozen in liquid nitrogen.

Kidney:

After weighing, the third to the half higher part of the right kidney will be cut and immediately snap-frozen in liquid nitrogen. Kidney samples will be stored in 1.5 ml Eppendorf vials snap and frozen in liquid nitrogen.

Organs will then be stored at -80°C until further processing and samples will be shipped on dry ice

Liver and kidney tissue samples will be sent on dry ice to INRA (Test Site 5) for further immunological and metabolomic analyses with the list of samples and their identification (e.g. n° of rats, type of sample, and day of collection) as well as the weight of the organs. Each sample must be clearly and unambiguously identified by the animal number/nature of the sample (e.g. liver, kidneys).

Aliquots are to be set aside so that these can be used in case of problems with transport of samples shipped from the animal testing facility to other test sites.

For metabolomic studies, liver and kidney samples will be extracted with ~~organic solvents~~ Polytron disperser in acetonitrile/water (50/50, 1 ml of solvent per 100 mg of tissue approximately) and then centrifuged at 13000 g for 10 min to discard insoluble macromolecules and non-polar analytes. ~~The~~ After dilution (50 to 100 fold) in the solvent used to introduce samples in mass spectrometer, the resulting hydrosoluble fractions will be fingerprinted by ultra high resolution mass spectrometry (liver and kidney extracts) or by nuclear magnetic resonance (liver extracts) to quantify the distribution of detectable analytes.

Statistical procedures used to mine raw data and analytical procedures used to identified putative biomarkers will be the same as those described above (p. 25) for metabolomic study of plasma and urine collected in all along the longitudinal follow-up of animals.

Storage of samples

Blood smears will be stored at the GLP archive (SZU, Test Facility). Supernatants from assays: *In vitro* production of cytokines will be stored in freezer -80°C (SZU, Test Facility). Plasma, urine and tissue samples will be stored in freezer -80°C at the SZU and delivered to the INRA (Test Site 5). Histopathology tissue samples will be stored at the GLP wet archive (SZU, Test Facility).



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11. DATA EVALUATION AND STATISTICAL ANALYSIS

The statistical analysis will be done by local statistical team, using any high-level statistical packages such as BMDP, SAS or R.

As a first step the data conventional data (haematology, clinical chemistry etc.) will be screened for any obvious errors and outliers. 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 of both the metabolomics and the conventional biomarker data will ~~then~~ be done 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.

Data Conventional data from males and females will be analysed separately ~~and together (ANOVA)~~ using the analysis of variance or a non-parametric method where necessary.

Summary statistics (e.g. "n", means, standard deviations and/or medians and quartiles, as appropriate), will be tabulated based on the cage means (as the cage is considered the experimental unit in this study). ~~A one-way analysis with planned or post hoc comparisons will be used to evaluate statistical significance of each outcome (trait). In some cases more detailed statistical analysis including correlations between characters or even a multivariate analysis may be needed, but this should be decided on a case-by-case basis. Methods of analysing longitudinal data such as growth and food consumption will be decided on a case-by-case basis.~~ Longitudinal data such as growth and food consumption will be analysed at monthly intervals. This analysis should not preclude additional methods of statistical analysis should they be considered useful.

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Tables of results (means, SDs and statistical significance; raw individual data) and the results of the metabolomics analysis will be prepared and in some cases additional statistical analyses and graphical methods may also be used.

The raw data will be made publically available on the GRACE web site.



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12. REFERENCES

- BMDP Statistics Software, Inc. (1990). BMDP Statistical Software Manual. W.J. Dixon, Chief Ed. 1990 rev. or later. University of California Press, Berkeley, CA, USA.
- European Committee for Standardization (2010) EN ISO 24333:2009 Cereals and cereal products – Sampling.
- European Committee for Standardization (2010) General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025:2005).
- EFSA Scientific Committee (2011) Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed. European Food Safety Authority (EFSA), Parma, Italy Journal 2011;9:2438.
- The European Parliament and the Council (2004) Directive 2004/10/EC. Official Journal of the European Union L 50: 44-45.
- The European Parliament and the Council (2010) Directive 2010/63/EU. Official Journal of the European Union L 276: 33-79.
- OECD (1998) Test No. 408 - Repeated Dose 90-day Oral Toxicity Study in Rodents. OECD Guidelines for the Testing of Chemicals, Section 4 Health Effects.
- OECD (1998) Principles of Good Laboratory Practice, as revised in 1997- ENV/MC/CHEM(98)17. Series on Principles of Good Laboratory Practice and Compliance Monitoring No. 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris.
- Slovak Republic, Act No 67/2010 on Conditions of Marketing of Chemical Substances and Chemical Mixtures and on amendment and supplement of other acts.
- Slovak Republic, Government Decree No. 320/2010 Coll.

13. ARCHIVING

Under the Code Number: 311957 - D / 14 / GLP

The following will be archived until the year 2023 at the SZU, the Registry of accredited laboratories and laboratories with GLP certificate:

- Study plan
- Correspondence
- Final report
- Reports of quality inspection
- All histological samples
- All original documents/Primary documentation

14. REPORTING

The final report will include the reporting requirements as described in OECD TG 408 method:

The final report will be written in English language. The sponsor can revise the draft report for 14 days from its date of issue. Then the final report will be finalized.



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The draft report will be made in two copies – one electronic copy for the sponsor and one paper copy for test facility. The study report will be made in four paper copies - two for the sponsor and two copies for the test facility and will include, but not limited to, the following:

- The name and address of the sponsor and the testing facility.
- The study schedule, the data of the start and the end of the study.
- The names of all personnel involved in the study, including the study director, other scientists and supervisory personnel.
- The item identification by code number. The appropriate properties of the item.
- The description of the test system, including species, strain, source, allocation, sex, age and method of identification.
- The description of the coded doses, dose regimen, route of administration and duration of the treatment period, the description of all methods used.
- Clinical signs and **relevant** raw data.
- The summary and description of all the toxic signs.
- Body weight data.
- Food consumption data.
- A description of all circumstances that may have affected the quality or integrity of the study.
- The authentication signed by study director.
- Test Facility Management Statement.
- The QAU Statement signed by **Lead** QA Manager.
- The copy of the Certificate of GLP.
- The storage locations of study plan, all raw data, specimens and the reports.

15. DISTRIBUTION

This study plan will be distributed as follows:

Issue No 1: Sponsor

Issue No 2: Study Director

Issue No 3: **Lead** QA Manager



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16. ATTACHMENTS

Attachment 1

A)

Subchronic toxicity and longitudinal metabolomics study

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Time schedule

	February 2014	March 2014	April 2014	May 2014	June 2014	July 2014	August 2014	September 2014
Quarantine	5-9 days males 12-16 days females (females are 1 week younger upon arrival)							
Randomisation	Males February 9 Females February 16							
Ophthalmology	February 7 Males, February 14 females			May 5 males, May 12 females				
Application Males -start	February 10-14 2014							
Application Females - start	February 17 --21 2014							
Weighing of the feed	Every 7 days	Every 7 days	Every 7 days	Every 7 days				
Weighing of animals	Every 7 days	Every 7 days	Every 7 days	Every 7 days				
General clinical observations	Everyday – Twice or more frequently	Everyday – Twice or more frequently	Everyday – Twice or more frequently	Everyday – Twice or more frequently				
Detailed clinical observations	Every 7 days	Every 7 days	Every 7 days	Every 7 days				
Sensory reactivity	Every 7 days	Every 7 days	Every 7 days	Every 7 days				
Hematology Males and females				Day <u>8485</u>				
Clinical Chemistry males females				Day <u>9492</u>				
Gross necropsy males				May 12 - 16, 2014				
Gross necropsy females				May 19 - 23, 2014				
Slides preparation					Start- June 2014			
Histology evaluation						Start- July 2014		
Immunology				Start May 12,	ELISA			September



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Final report to sponsor

2014

15, 2014
October
2014

B)

Sample collection during the study - blood and urine:

Day 7

Males:

<u>Group/ colour coding</u>	<u>Diet No</u>	<u>Monday, February 17, 2014</u>	<u>Tuesday, February 18, 2014</u>	<u>Wednesday, February 19, 2014</u>	<u>Thursday, February 20, 2014</u>	<u>Friday, February 21, 2014</u>
		<u>ID of animal</u>	<u>ID of animal</u>	<u>ID of animal</u>	<u>ID of animal</u>	<u>ID of animal</u>
1 light blue	1001	1-2	3-4	5-6	7-8	9-10
2 red	1002	11-12	13-14	15-16	17-18	19-20
3 green	1005	21-22	23-24	25-26	27-28	29-30
4 yellow	2006	31-32	33-34	35-36	37-38	39-40
5 white	2007	41-42	43-44	45-46	47-48	49-50
6 dark blue	2010	51-52	53-54	55-56	57-58	59-60

Females:

<u>Group/ colour coding</u>	<u>Diet No</u>	<u>Monday, February 24, 2014</u>	<u>Tuesday, February 25, 2014</u>	<u>Wednesday, February 26, 2014</u>	<u>Thursday, February 27, 2014</u>	<u>Friday, February 28 2014</u>
		<u>ID of animal</u>	<u>ID of animal</u>	<u>ID of animal</u>	<u>ID of animal</u>	<u>ID of animal</u>
1 light blue	1001	101-102	103-104	105-106	107-108	109-110
2 red	1002	111-112	113-114	115-116	117-118	119-120
3 green	1005	121-122	123-124	125-126	127-128	129-130
4 yellow	2006	131-132	133-134	135-136	137-138	139-140
5 white	2007	141-142	143-144	145-146	147-148	149-150
6 dark blue	2010	151-152	153-154	155-156	157-158	159-160



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After 1st month – Day 29

Males:

<u>Group/ colour coding</u>	<u>Diet No</u>	<u>Monday, March 10, 2014 ID of animal</u>	<u>Tuesday, March 11, 2014 ID of animal</u>	<u>Wednesday, March 12, 2014 ID of animal</u>	<u>Thursday, March 13, 2014 ID of animal</u>	<u>Friday, March 14, 2014 ID of animal</u>
1 light blue	1001	1-2	3-4	5-6	7-8	9-10
2 red	1002	11-12	13-14	15-16	17-18	19-20
3 green	1005	21-22	23-24	25-26	27-28	29-30
4 yellow	2006	31-32	33-34	35-36	37-38	39-40
5 white	2007	41-42	43-44	45-46	47-48	49-50
6 dark blue	2010	51-52	53-54	55-56	57-58	59-60

Females:

<u>Group/ colour coding</u>	<u>Diet No</u>	<u>Monday, March 17, 2014 ID of animal</u>	<u>Tuesday, March 18, 2014 ID of animal</u>	<u>Wednesday, March 19, 2014 ID of animal</u>	<u>Thursday, March 20, 2014 ID of animal</u>	<u>Friday, March 21, 2014 ID of animal</u>
1 light blue	1001	101-102	103-104	105-106	107-108	109-110
2 red	1002	111-112	113-114	115-116	117-118	119-120
3 green	1005	121-122	123-124	125-126	127-128	129-130
4 yellow	2006	131-132	133-134	135-136	137-138	139-140
5 white	2007	141-142	142-143	154-146	147-148	149-150
6 dark blue	2010	151-152	153-154	155-156	157-158	159-160

After 2nd month – Day 57

Males:

<u>Group/ colour coding</u>	<u>Diet No</u>	<u>Monday, April 7, 2014 ID of animal</u>	<u>Tuesday, April 8, 2014 ID of animal</u>	<u>Wednesday, April 9, 2014 ID of animal</u>	<u>Thursday, April 10, 2014 ID of animal</u>	<u>Friday, April 11, 2014 ID of animal</u>
1 light blue	1001	1-2	3-4	5-6	7-8	9-10
2 red	1002	11-12	13-14	15-16	17-18	19-20
3 green	1005	21-22	23-24	25-26	27-28	29-30
4 yellow	2006	31-32	33-34	35-36	37-38	39-40
5 white	2007	41-42	43-44	45-46	47-48	49-50
6 dark blue	2010	51-52	53-54	55-56	57-58	59-60



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Females:

<u>Group/ colour coding</u>	<u>Diet No</u>	<u>Monday, April 14, 2014</u> <u>ID of animal</u>	<u>Tuesday, April 15, 2014</u> <u>ID of animal</u>	<u>Wednesday, April 16, 2014</u> <u>ID of animal</u>	<u>Thursday, April 17, 2014</u> <u>ID of animal</u>	<u>Friday, April 18, 2014</u> <u>ID of animal</u>
1 light blue	1001	101-102	103-104	105-106	107-108	109-110
2 red	1002	111-112	113-114	115-116	117-118	119-120
3 green	1005	121-122	123-124	125-126	127-128	129-130
4 yellow	2006	131-132	133-134	135-136	137-138	138-140
5 white	2007	141-142	143-144	145-146	147-148	149-150
6 dark blue	2010	151-152	153-154	155-147	157-158	159-160

After 3rd month – Day 92

Males:

<u>Group/ colour coding</u>	<u>Diet No</u>	<u>Monday, May 12, 2014</u> <u>ID of animal</u>	<u>Tuesday, May 13, 2014</u> <u>ID of animal</u>	<u>Wednesday, May 14, 2014</u> <u>ID of animal</u>	<u>Thursday, May 15, 2014</u> <u>ID of animal</u>	<u>Friday, May 16, 2014</u> <u>ID of animal</u>
1 light blue	1001	1-2	3-4	5-6	7-8	9-10
2 red	1002	11-12	13-14	15-16	17-18	19-20
3 green	1005	21-22	23-24	25-26	27-28	29-30
4 yellow	2006	31-32	33-34	35-36	37-38	39-40
5 white	2007	41-42	43-44	45-46	47-48	49-50
6 dark blue	2010	51-52	53-54	55-56	57-58	59-60

Females:

<u>Group/ colour coding</u>	<u>Diet No</u>	<u>Monday, May 19, 2014</u> <u>ID of animal</u>	<u>Tuesday, May 20, 2014</u> <u>ID of animal</u>	<u>Wednesday, May 21, 2014</u> <u>ID of animal</u>	<u>Thursday, May 22, 2014</u> <u>ID of animal</u>	<u>Friday, May 23, 2014</u> <u>ID of animal</u>
1 light blue	1001	101-102	103-104	105-106	107-108	109-110
2 red	1002	111-112	113-114	115-116	117-118	119-120
3 green	1005	121-122	123-124	125-126	127-128	129-130
4 yellow	2006	131-132	133-134	135-136	137-138	139-140
5 white	2007	141-142	143-144	145-146	147-148	149-150
6 dark blue	2010	151-152	153-154	155-156	157-158	159-160



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Attachment 2

LIST OF MATERIAL AND EQUIPMENT

Equipment:

Laboratory of Toxicology:

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

Experimental animal rooms

- Temperature and humidity detector, PMICRO-LCD-THSYS, Dallas Semiconductor, rooms No. B2-609, B2-610.
- Personal computers, office
- Data backup system - 2 external hard drives and the eXplorer system established by JKI
- Electronic balance Sartorius BP 1200, No. 6080646, range: 0-1000g, 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.
- Type of animal cages in TECNIPLAST Filter top cages Type 2145 F with an H-Temp™ (PSU) durable filter cover 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

- Haematological analyzer Sysmex K-4500, SYSMEX TOA Medical Electronics Co. LTD, Japan, No. VČ F-1466, room B2-212.
- Laminar flow hood
- CO2 incubator
- Liquid scintillation spectrometer Microbeta 2, Canberra Packard
- Cell harvester
- Flow cytometer EpicsXL, Beckman Coulter
- Personal computers, office

Laboratory of Clinical and Experimental Biochemistry

- 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 SPSS version 16.0.

Material

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

SZU, Limbová 14, 833 03 Bratislava 37, Slovak Republic Testing Laboratories Center, Laboratory of Toxicology



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Equipments Histology [has to be inserted according to outcome of the tender]



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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
- Temperature/relative humidity checks
- Dose analysis data
- Dose preparation and accountability records
- Dose administration
- Necropsy and histopathological findings
- Pathology specimens as specified
- Histology processing records

Records – primary documentation -will be kept in room B 2 – 209

All records during the study will be kept in computer room B 2 – 221

External backup will be kept in room B2 – 210

Second external backup will be kept in room B – 358 (QA)

SZU, Limbová 14, 833 03 Bratislava 37, Slovak Republic Testing Laboratories Center, Laboratory of Toxicology



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Attachment 4 - 6 (available from SZU)

4. GLP CERTIFICATE SZU
5. GLP CERTIFICATE histopathological laboratory [to be inserted based on tender outcome]
6. ACCREDITATION CERTIFICATE Clinical and Experimental Biochemistry Laboratory

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Englisch (USA), Rechtschreibung und Grammatik nicht prüfen