



SHRIRAM INSTITUTE FOR INDUSTRIAL RESEARCH

Confidential

PROJECT No. : TOX-346E
PRODUCT : Bt COTTONSEEDS
STUDY : SKIN SENSITIZATION STUDY ON GUINES PIGS
REPORT No. : 000041137
DATE : 22.03.2007

SKIN SENSITIZATION STUDY ON GUINEA PIGS

WITH

Bt COTTONSEEDS

Report for:

METAHELIX LIFE SCIENCES PRIVATE LIMITED
PLOT NO.3, KIADB 4th PHASE, BOMMASANDRA,
BANGALORE-560 099, INDIA

Guidelines:

**‘DBT, Guidelines for Toxicity and Allergenicity Evaluation of
Transgenic Seeds, Plants and Plant Parts’**

Prepared by:

DEPARTMENT OF TOXICOLOGY
SHRIRAM INSTITUTE FOR INDUSTRIAL RESEARCH
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QUALITY ASSURANCE STATEMENT

This is to certify that the work described in the study report entitled 'Skin Sensitization Study' with Bt Cottonseeds on Guinea pigs has been audited and examined with respect to the study protocol and the Standard Operating Procedures in accordance to 'DBT, Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant parts' in compliance with Good laboratory Practices (G.L.P) for non clinical laboratory studies.

The report provides true and accurate record of results obtained.

The dates of inspections & dates on which findings were reported to the study director & SRI management are given below:

<u>Phases of study</u>	<u>Dates of Inspection</u>	<u>Dates of Reporting</u>
Protocol	07.12.2006	07.12.2006
Conduct	11.12.2006	11.12.2006
Records	17.01.2007	17.01.2007
Report	22.03.2007	22.03.2007

Rajni Bhat
Sr. SCIENTIST
QUALITY ASSURANCE



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STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE

We, the undersigned take overall responsibility to conduct the work described in the study entitled 'Skin Sensitization Study on Guinea pigs' with Bt Cottonseeds performed with respect to the study protocol and the Standard Operating Procedures in accordance to 'DBT, Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant parts' for non-clinical laboratory studies.

All the raw data, documentation, protocol and copy of final report are retained in the archives at Shriram Institute for Industrial Research, Delhi.

Chhita

STUDY DIRECTOR

Basera

SCIENTIST PATHOLOGY

Aggarwal

HEAD, DEPT. OF TOXICOLOGY

Approved for issue

[Signature]
 DEPT. DIRECTOR
 (MANAGEMENT)



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SCIENTIFIC PERSONNEL INVOLVED IN THE STUDY

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(Scientist Pathology)

Dr. ANIL KUMAR CHHILLAR, M.Sc., Ph.D.
(Research Associate)

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(Sr. Analyst)

Ms. ARPITA JAISWAL, M.Sc.
(Analyst)



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SUMMARY

Non-Bt Cottonseeds (Sample-I) was moistened with water and applied to the shaved skin of 10 young adult guinea pigs for 6 hours a day, three times a week for three weeks until 9 applications were made in the induction (sensitization) phase. Similarly, (Sample-II) was also applied to the guinea pigs and a control group of 5 guinea pigs was maintained which was not treated in the induction phase. After the last induction exposure the animals were left untreated for two weeks before conducting the challenge phase.

The day before the challenge, the hair from each guinea pig including 5 additional untreated animals (controls) was removed with a clipper from the mid back area and test substance was moistened and applied to the shaved area of the test and control guinea pigs. The test material was removed after 6 hours and the skin reaction at the site of application was assessed and scored immediately after challenge patch removal, and again after 24 hours and 48 hours.

Under the conditions of this study, the repeated application of “Bt-cotton seeds (Sample-II)” did not induce dermal sensitization (allergies) to the skin of any of the guinea pigs when compared to its corresponding “Non-Bt cotton seeds (Sample-I)” and the control group of animals.



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INTRODUCTION

This study was designed to determine the potential of the 'Non-Bt Cottonseeds (Sample-I) and Bt-Cottonseeds (Sample-II)' samples to elicit an immunological response through contact with the skin.



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OBJECTIVE

1. This test method is used to determine whether the test substance will elicit dermal sensitization in guinea pigs.
2. The rationale for this practice is based on the fact that the guinea pig has been shown to be the best animal model for human allergic contact dermatitis.

CHARACTERIZATION OF TEST AND CONTROL COTTONSEEDS

The test and control cottonseeds were characterized by the sponsor prior to their use in the study.



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TEST SUBSTANCE

PRODUCT NAME : NON-Bt COTTONSEEDS (SAMPLE I)
& Bt COTTONSEEDS (SAMPLE II)

SPONSOR : METAHELIX LIFE SCIENCES
PRIVATE LIMITED

MATERIAL DESCRIPTION : YELLOWISH BROWN COLOURED
POWDER

PACKED IN : BROWN COLOURED PAPER
CARTONS

DATE OF COMMENCEMENT : 11.12.2006
OF STUDY

DATE OF COMPLETION : 17.01.2007
OF STUDY



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EXPERIMENTAL DESIGN

Name of species : *Cavia porcellus*

No. of animals used per dose : 10 young adult healthy guinea pigs for the Bt-cotton seeds group, 10 for Non-Bt cottonseeds and 5 for negative control group were used.

Weight range : 300-500 gm

Acclimatization period : 7 Days

Route of administration : Dermal

ANIMAL GROUPS AND TEST APPLICATIONS

Group	No. of animals	<u>No. of applications</u>	
		Induction	Challenge
Control	5	0	1
(Non-Bt Cottonseeds)	10	9	1
Test (Bt Cotton seeds)	10	9	1



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HUSBANDRY

The animals were kept in group of 10 for test group and 5 for control group animals per pan with proper identification.

The room temperature was maintained at $22 \pm 3^{\circ}$ C with 30 - 70 % relative humidity.

The room was ventilated at the rate of approximately 15 air changes per hour.

Lighting was controlled to give 12 hours artificial light (8 a.m. - 8 p.m.) each day.

DIET

Water and standard pelleted feed (Amrut feeds Ltd.) was freely available to the experimental animals.



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IDENTIFICATION OF ANIMALS

Each cage was tagged having the details of animal group number, product name, dosage level, date of initiation and date of completion.

The animals were also marked with the help of marking ink.



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EXPERIMENTAL PROCEDURE (BUEHLER METHOD)

The dermal sensitization study comprised of two test phases -

1. Induction Phase
2. Challenge Phase

Sample preparation

The Non-Bt Cottonseeds (Sample-I) as well as Bt-Cottonseeds (Sample-II) were crushed and applied after moistening it with water.

Induction Phase

A day before the test, the hair was clipped from the mid back area of 20 guinea pigs designated as test animals. The sample was applied by moistening the crushed cottonseeds with water backed by 1 inch x 1 inch gauze patch was applied to the test area. The gauze patch was covered with non-reactive tape and wrapped with bandage. The test patches were removed after 6 hours and observations for the presence of erythema and oedema were recorded. The test article application procedure was repeated 3 times each week for 3 weeks; total 9 applications were made to the test area. After the last application of the induction phase these animals were left untreated for 2 weeks before conducting the challenge phase. A control group of 5 guinea pigs was also maintained which was not treated in the induction phase.



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2. Challenge Phase

The day before the challenge phase test, the hair of each guinea pig including 5 additional untreated animals (Control) was removed with a clipper from the mid back area.

The test substance (prepared by moistening the crushed cottonseeds with water) of Non-Bt cottonseeds (Sample I) as well as (Sample II) was applied respectively to the shaved area of the control and the test guinea pigs and tapped in place. The trunk of each animal was wrapped with bandage to maintain the test site. The test patches were removed after 6 hours.

Three observations for the presence of erythema and oedema were made-

1. Immediately after challenge patch removal.
2. After 24 hours.
3. After 48 hours.



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The following scoring criteria was used for evaluating the skin reaction:

SCORING CRITERIA FOR TEST

Reaction	Description	Score
Erythema	Erythema and eschar formation	
	- No erythema	0
	- Very slight erythema (Barely perceptible)	1
	- Well defined erythema (Pale red in colour)	2
	- Moderate to severe erythema (Red and area well defined)	3
	- Severe erythema (Beet redness to slight eschar formation)	4
Oedema	Oedema formation	
	- No oedema	0
	- Very slight oedema (Barely perceptible)	1
	- Slight oedema (Edges of area well defined by definite raising)	2
	- Moderate to severe oedema (Edges raised approx. 1mm)	3
	- Severe oedema (Raised more than 1mm and extending beyond area of exposure)	4



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RATING OF SENSITIZATION RESPONSE

Sensitized	Grades	Classification
0 to 8	I	No different than control
9 to 28	II	Mild
29 to 28	III	Moderate
65 to 80	IV	Strong
81 to 100	V	Extreme



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OBSERVATION

No noticeable dermal irritancy (erythema & oedema, Table - 1) was observed in any test and control guinea pigs during the induction phase and after the challenge phase.



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RESULT

Under the conditions of this study, the repeated application of “Bt-cotton seeds (Sample-II)” did not induce dermal sensitization (allergies) to the skin of any of the guinea pigs when compared to its corresponding “Non-Bt cotton seeds (Sample-I)” and the control group of animals.

The samples have been tested in accordance with Guidelines for toxicity and allergenicity, Evaluation of Transgenic seeds, plants and plant parts, Department of Biotechnology, Ministry of Science and Technology, Government of India for non-clinical laboratory studies.



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TABLE NO. 1
SUMMARY OF ERYTHEMA AND OEDEMA SCORE

Group	Induction Phase		Challenge Phase	
	Erythema	Oedema	Erythema	Oedema
control	-	-	0	0
Non-Bt cottonseeds	0	0	0	0
Bt cottonseeds	0	0	0	0



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TABLE NO. 2
INDUCTION PHASE
EVALUATION OF REACTION
NON-Bt COTTONSEEDS

Application No.	Skin Reaction	Guinea Pig Number										Average	Combined Index
		1	2	3	4	5	6	7	8	9	10		
1.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
2.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
3.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
4.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
5.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
6.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
7.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
8.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
9.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	



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TABLE NO. 3
INDUCTION PHASE
EVALUATION OF REACTION

Application No.	Skin Reaction	Guinea Pig Number										Average	Combined Index
		1	2	3	4	5	6	7	8	9	10		
1.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
2.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
3.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
4.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
5.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
6.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
7.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
8.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	
9.	Erythema	0	0	0	0	0	0	0	0	0	0	0	0.0
	Oedema	0	0	0	0	0	0	0	0	0	0	0	



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TABLE NO. 4
CHALLENGE PHASE
EVALUATION OF SKIN REACTION
NON-Bt COTONSEEDS

Skin Reaction	Time (hours)	Treated Animals (Non-Bt Cottonseeds)										Average Index	Combined Index	Untreated Animals (control)					Average Index	Combined Index
		1	2	3	4	5	6	7	8	9	10			1	2	3	4	5		
Erythema	After	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	
Oedema	6 hrs.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erythema	After	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	
Oedema	24 hrs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erythema	After	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	
Oedema	48 hrs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0



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TABLE NO. 5
CHALLENGE PHASE
EVALUATION OF SKIN REACTION
Bt COTONSEEDS

Skin Reaction	Time (hours)	Treated Animals (Bt Cottonseeds)										Average Index	Combined Index	Untreated Animals (control)					Average Index	Combined Index
		1	2	3	4	5	6	7	8	9	10			1	2	3	4	5		
Erythema	After	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	
Oedema	6 hrs.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erythema	After	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	
Oedema	24 hrs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erythema	After	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	
Oedema	48 hrs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0



SHRIRAM INSTITUTE FOR INDUSTRIAL RESEARCH

(A unit of Shriram Scientific and Industrial Research Foundation)

An ISO - 9001:2000 Certified Institute

TEST CERTIFICATE

000041137

Issued to :
METAHELIX LIFE SCIENCES PVT. LTD.
PLOT NO. 3, KIADB 4TH PHASE,
BOMMASANDRA
BANGALORE - 560099KARNATAKA

J.O.No. TOX/346 E
Reg.No. 4612570
Date 22-03-2007
GC-01 (REV-04)
Your Ref.No. --

Kind Attn: DR. M.J. VASUDEVA RAO , PRESIDENT

Sample Particulars :

One sample of "Bt Cottonseeds" was received for skin sensitization study on guinea pigs.

Date

Material Description : Non-Bt Cottonseeds (Sample-I)- Yellowish brown coloured powder
Bt Cottonseeds (Sample-II)- Yellowish brown coloured powder

Sponsor : Metahelix Life Sciences Private Limited
Plot no.3, KIADB 4th Phase, Bommasandra,
Bangalore-560 099, India.

TEST RESULTS

Skin Sensitization Study On Guinea pigs

Under the conditions of this study, the repeated application of "Bt-cotton seeds (Sample-II)" did not induce dermal sensitization (allergies) to the skin of any of the guinea pigs when compared to its corresponding "Non-Bt cotton seeds (Sample-I)" and the control group of animals.

The sample has been conducted as per DBT, Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant parts.

(Annexure enclosed)

DOR : 06-11-2006
DOC : 22-03-2007

AUTHORISED SIGNATORY
(EMPLOYEE CODE: 6008)

19, University Road, Delhi - 110007.
E-Mail: qad@shriraminstitute.org Website: <http://www.shriraminstitute.org>

Ph: 91-11-27667267, 27667983, 27667860
Fax: 91-11-27667676, 27667207

PCR & ELISA CONFIRMATION OF BIOSAFETY COTTONSEED MATERIAL

Objective: Quality Control of the cottonseed material from cry1C-9124 based intrahirsutum hybrids to be used for the biosafety studies; despatched on 11th September 2006.

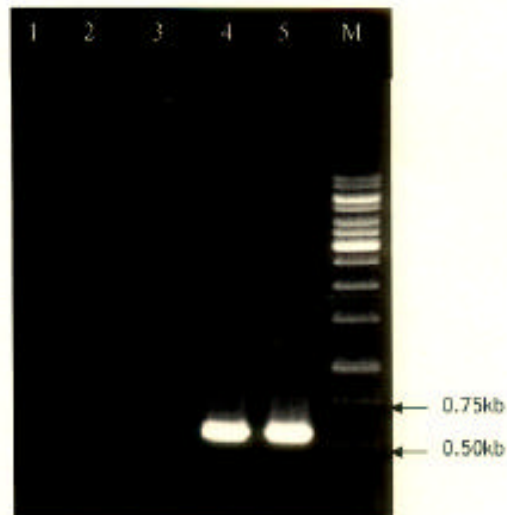
1. Confirmation of transgenic nature by PCR specific to the transgene
2. Confirmation of presence of Cry1C protein and its quantitation by ELISA

PCR confirmation

PCR was performed on Eppendorf Mastercycler Gradient machine with the following primers:

Upper: 5'-CCT CGC CAT TCT TCG TGA TTC C
Lower: 5'-GGT TGG CCT CCC TTC CGT AGA TA

1. H₂O CONTROL
2. -VE CONTROL (LEAF)
3. NON TRANSGENIC SEED DNA
4. TRANSGENIC SEED DNA
5. +VE CONTROL



EXPECTATION- 0.58 KB

Results and conclusion

As expected amplification from cry1C was observed in case of transgenic and positive control proving the presence of the gene. Water and negative controls were clear indicating the absence of gene.

Metahelix Life Sciences Private Limited

Plot No. 3, KIADB 4th Phase, Bommasandra, Bangalore 560 099, India.
Tel: +91-80-787 0236, 783 6086 Fax: +91-80-783 6084 www.meta-helix.com

ELISA confirmation

Quantitative ELISA for Cry1C protein was performed using the Quantiplate kit for Cry1C (Envirologix, USA; Catalog No. AP 007) according to the manufacturer's protocol

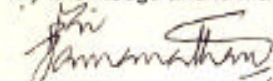
Sl no	Entry ID	A ₄₅₀	Cry1C concentration (µg/g on fresh wt)
1	Blank	0.09	NA
2	1 ppb standard	0.3	0.92
3	5 ppb standard	1.44	5.2
4	10 ppb standard	2.21	9.93
5	Nontransgenic	0.092	NA
6	Transgenic	2.9	13.08

Results

The absorbance value observed at 450nm for nontransgenic sample was nearly the same as blank and no colour development was observed in case of nontransgenic. Blue colour development was observed in case of transgenic samples indicating the presence of Cry1C protein.

Declaration

I hereby declare that the certificate of quality presented above is true to the best of my knowledge and is made on the basis of experiments carried out in our premises.



Val. Ramanathan

Head - Genomics