



**SHRIRAM INSTITUTE FOR INDUSTRIAL RESEARCH**

**Confidential**

**PROJECT No.** : TOX-346B  
**PRODUCT** : Bt COTTONSEEDS  
**STUDY** : PRIMARY SKIN IRRITATION TEST IN RABBITS  
**REPORT No.** : 000041135  
**DATE** : 22.032007

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**PRIMARY SKIN IRRITATION TEST IN RABBITS**

**WITH**

**Bt COTTONSEEDS**

**Report for:**

**METAHELIX LIFE SCIENCES PRIVATE LIMITED**  
PLOT NO.3, KIADB 4<sup>th</sup> 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**  
(A Unit of Shriram Scientific & Industrial Research Foundation)

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**QUALITY ASSURANCE STATEMENT**

This is to certify that the work described in the study report entitled 'Primary Skin Irritation Study' with Bt Cottonseeds on rabbits 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.

<u>Phases of study</u>	<u>Dates of Inspection</u>	<u>Dates of Reporting</u>
Protocol	10.11.2006	10.11.2006
Conduct	16.11.2006	16.11.2006
Records	29.11.2006	29.11.2006
Report	22.03.2007	22.03.2007

*Binu 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 'Primary Skin Irritation Test on Rabbits' 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.

*Ahilla*  
STUDY DIRECTOR

*P. Sreeni*  
SCIENTIST PATHOLOGY

*M. Aggarwal*  
HEAD, DEPT. OF TOXICOLOGY

Approved for issue

*A. K. Gupta*  
DEPUTY DIRECTOR  
(MANAGEMENT)



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**SCIENTIFIC PERSONNEL INVOLVED IN THE STUDY**

**Dr. RAJUL SAXENA, M.V.Sc.**  
**(Scientist Pathology)**

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

**Mr. MANOJ KUMAR, M.Sc.**  
**(Sr. Analyst)**

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



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## SUMMARY

In the current study, 0.5 gm of the test samples i.e. Non-Bt Cottonseeds (Sample –I) and Bt Cottonseeds (Sample –II), supplied by Methelix Life Sciences Private Limited was applied to the skin of albino rabbits. After 4 hours i.e. the exposure period, the degree of irritation was read and scored at 1, 24, 48, 72 hours, 7 and 14 days after the patch removal.

The primary skin irritation index was calculated and came as 0.00 after patch removal. Hence, it was concluded that the test substance Bt Cottonseeds (Sample –II) was '**non-irritant**' to the skin of rabbits when compared with the Non-Bt Cottonseeds (Sample-I) and the control group.



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## **INTRODUCTION**

Primary skin irritation is the production of reversible inflammatory changes in the skin following the application of a test substance as it involves the interaction of chemicals with the sensory receptors in the skin at the site of application.

On the basis, of this study, the possible hazard likely to arise from exposure of the skin to the test substance can be assessed.



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## **OBJECTIVE**

To evaluate local irritant effects to rabbit skin following a single application of the test samples.

## **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 : Bt COTTONSEEDS & NON-Bt  
COTTONSEEDS (SAMPLE I & II)

SPONSOR : METAHELIX LIFE SCIENCES  
PRIVATE LIMITED

MATERIAL DESCRIPTION : YELLOWISH BROWN COLOURED  
POWDER

PACKED IN : BROWN COLOURED PAPER  
CARTONS

DATE OF COMMENCEMENT : 16.11.2006  
OF STUDY

DATE OF COMPLETION : 29.11.2006  
OF STUDY



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## **EXPERIMENTAL DESIGN**

### **ANIMALS**

In the present study three young adult rabbits of the New Zealand White Strain were taken per sample / per group.



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## HUSBANDRY

All animals were housed in metal cages fitted with perforated floors. Water and standard rabbit feed were given *ad libitum*.

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

The light conditions were controlled to give 12 hours artificial light (8 a.m. - 8 p.m. ) each day.



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## **ACCLIMATIZATION**

A minimum of 7 days acclimatization was allowed before the commencement of the study.



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## **IDENTIFICATION OF ANIMALS**

Each rabbit cage was attached with a tag marked with the animal number, the test and the product name.



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## EXPERIMENTAL PROCEDURE

### SKIN PREPARATION

Twenty-four hours before the test (dose application), hair on the back and flanks of each rabbit were closely clipped exposing (approximately 6 cm<sup>2</sup> area) of skin.

**DOSE : 0.5 gm / animal**



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### **APPLICATION PROCEDURE**

0.5 gm sample of 'Non-Bt Cottonseeds (Sample –I) in powdered form was evenly applied to a small area (approximately 6 cm square) of the closely clipped skin of each of three rabbits. The site of application was covered with a cotton gauze patch, which was kept in contact with skin by means of semi-occlusive dressing. 0.5 gm sample of powdered 'Bt Cottonseeds (Sample –II) was similarly treated with each of another three rabbits.

At the termination of 4 hours exposure period, the bandages / gauze were removed and treatment sites were cleaned with wet gauze to remove any residual test substance.



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## OBSERVATIONS

Skin reaction at the site of application was subjectively assessed and scored once daily at 1, 24, 48, 72 hours, 7 and 14 days after patch removal (post test observation period) according.

The reaction at the site of application was assessed and scored according to the following numerical system :

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SKIN REACTION	SCORE
<b>(A ) ERYTHEMA AND ESCHAR FORMATION</b>	
- No erythema	0
- Very slight erythema ( barely perceptible )	1
- Well defined erythema	2
- Moderate to severe erythema	3
- Severe erythema ( beet redness to eschar formation )	4

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**SKIN REACTION**

**SCORE**

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**( B ) EDEMA FORMATION**

- |  |   |
|--|---|
| - No edema   | 0 |
| - Very slight edema ( barely perceptible )                                       | 1 |
| - Slight edema ( edges of area well raised )                                     | 2 |
| - Moderate edema ( raised approx.1mm )   | 3 |
| - Severe edema ( raised more than 1mm and extending<br>beyond area of exposure ) | 4 |
-



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### EVALUATION OF PRIMARY SKIN IRRITATION INDEX

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EVALUATIONS	SCORE
Non Irritant	0.0
Negligible Irritant	0.1- 0.4
Slight Irritant	0.41-1.9
Moderate Irritant	2.0 - 4.9
Severe Irritant	5.0 - 8.0

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**EVALUATION OF REACTIONS ( Draize's Method )**  
**TABLE-I**  
**CONTROL GROUP**

	Rabbit Numbers			Average	Combined Index
	1M	2M	3M		
<b>1 Hrs.</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>24 Hrs.</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>48 Hrs.</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>72 Hrs.</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>7 Days</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>14 Days</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	



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**EVALUATION OF REACTIONS ( Draize's Method )**  
**TABLE-II**  
**NON-Bt COTTONSEEDS (Sample-I)**

	Rabbit Numbers			Average	Combined Index
	1M	2M	3M		
<b>1 Hrs.</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>24 Hrs.</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>48 Hrs.</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>72 Hrs.</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>7 Days</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>14 Days</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	

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**EVALUATION OF REACTIONS ( Draize's Method )**  
**TABLE-III**  
**Bt COTTONSEEDS (Sample-II)**

	Rabbit Numbers			Average	Combined Index
	1M	2M	3M		
<b>1 Hrs.</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>24 Hrs.</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>48 Hrs.</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>72 Hrs.</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>7 Days</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	
<b>14 Days</b>					
Erythema Score	0	0	0	0.00	0.00
Edema Score	0	0	0	0.00	

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## RESULT

The given sample of Bt Cottonseeds (Sample –II) was found to be '**non-irritant**' to the skin of rabbits when compared with the sample of Non-Bt Cottonseeds (Sample –I).

The sample has been tested as per 'DBT, Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant parts' for non clinical laboratory studies.



# SHRIRAM INSTITUTE FOR INDUSTRIAL RESEARCH

(A unit of Shriram Scientific and Industrial Research Foundation)

An ISO - 9001:2000 Certified Institute

## TEST CERTIFICATE

000041135

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

J.O.No. TOX/346 B  
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 Primary skin irritation test on rabbits.

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 4<sup>th</sup> Phase, Bommasandra,  
Bangalore-560 099, India.

### TEST RESULTS

**Primary skin irritation test on Rabbits**

The given sample of 'Bt Cottonseeds (Sample-II)' was found to be 'Non-irritant' to the skin of rabbits when compared with the sample of Non- Bt Cottonseeds (Sample-I).

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

*M. Aggarwal*  
AUTHORISED SIGNATORY  
(EMPLOYEE CODE: 6006)

19, University Road, Delhi - 110007.

E-Mail: [qad@shriraminstitute.org](mailto: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 11<sup>th</sup> September 2006.

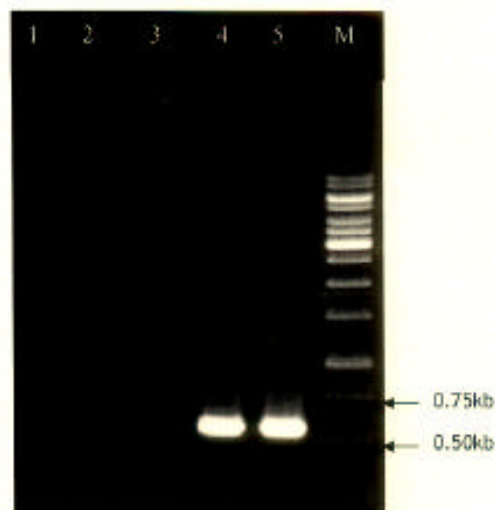
1. Confirmation of transgenic nature by PCR specific to the transgene
2. Confirmation of presence of Cry1C protein and its quantitation by ELISA

#### 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. H<sub>2</sub>O 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.



### 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

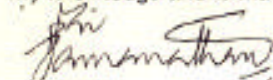
Sl no	Entry ID	A <sub>450</sub>	Cry1C concentration (µg/g on fresh wt)
1	Blank	0.09	NA
2	1 ppb standard	0.3	0.92
3	5 ppb standard	1.44	5.2
4	10 ppb standard	2.21	9.93
5	Nontransgenic	0.092	NA
6	Transgenic	2.9	13.08

### 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