



**SHRIRAM INSTITUTE FOR INDUSTRIAL RESEARCH**

**Confidential**

**PROJECT No.** : TOX-346D  
**PRODUCT** : Bt COTTONSEEDS  
**STUDY** : IRRITATION TO MUCOUS MEMBRANE IN RABBITS  
**REPORT No.** : 000041136  
**DATE** : 22.03.2007

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**IRRITATION TO MUCOUS MEMBRANE 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 'Irritation to Mucous Membrane in Rabbits' 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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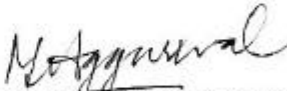
**STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE**

We, the undersigned take overall responsibility to conduct the work described in the study entitled 'Irritation to Mucous Membrane in 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.

  
STUDY DIRECTOR

  
SCIENTIST PATHOLOGY

  
HEAD, DEPT. OF TOXICOLOGY

Approved for issue

  
DEPUTY 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)**

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

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



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## SUMMARY

In the current study, 0.1gm 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 as such to the vaginal mucous membrane of the female 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.

The irritation index was calculated and it came out as 0.00. Hence, under these conditions it was concluded that the test sample i.e. Bt Cottonseeds (Sample –II), was '**non irritant**' to the mucous membrane of rabbits when compared with the Non-Bt Cottonseeds (Sample-I) and the control group.



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## **INTRODUCTION**

In the present study localized inflammatory response, on the mucous membrane of the rabbits is evaluated by a single application of the test substance as it involves the interaction of chemicals with the sensory receptors in the skin.

This study serves to indicate the existence of possible hazards likely to arise from exposure on the mucous membrane to the test substance.



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## **OBJECTIVE**

To determine the degree of irritation produced by the test substances i.e. Non-Bt cotton seeds (Sample-I) and Bt cotton seeds (Sample-II), on the mucous membrane following a single application.

## **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**  
**(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**

Note: For characterization details of test samples, see Annexure-I provided by the sponsor



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## **EXPERIMENTAL DESIGN**

### **ANIMALS**

In the present study three female, young adult (nulliparous, non-pregnant) rabbits of the New Zealand White strain per group were taken.



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## HUSBANDRY

All the animals were housed individually in metal cages 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**

The exact quantity of the given test samples i.e. Non Bt Cottonseeds (Sample-I\_ in powdered form was applied carefully to the vaginal mucous membrane of each of the three female rabbits.

Similarly powdered Bt Cottonseeds (Sample -II) was applied carefully to the vaginal mucous membrane of each of the three another female rabbits

**DOSE : 0.1 gm / animal**



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## **OBSERVATIONS**

The mucous membrane of the rabbits were examined for any discharge and inflammatory reaction at 1, 24, 48, 72 hours, 7 and 14 days after dose application.



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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</b>            |       |
| No erythema                    | 0     |
| Very slight erythema           | 1     |
| Well defined erythema          | 2     |
| Moderate to severe erythema    | 3     |
| Severe erythema (Beet redness) | 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 (moderate swelling of vulvar lips )                                   | 3 |
| Severe edema (excessive swelling of vulvar lips with<br>eversion of vaginal mucosa ) | 4 |

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**EVALUATION OF IRRITATION INDEX**

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| <b>EVALUATION</b>   | <b>SCORE</b> |
|---------------------|--------------|
| Non Irritant        | 0.00         |
| Negligible Irritant | 0.00-0.40    |
| Slight Irritant     | 0.41-1.99    |
| Moderate Irritant   | 2.00-4.99    |
| Severe Irritant     | 5.00-8.00    |

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**EVALUATION OF REACTIONS**  
**TABLE-I**  
**CONTROL GROUP**

---

|                | RABBIT NUMBERS |    |    |         |                |
|----------------|----------------|----|----|---------|----------------|
|                | 1F             | 2F | 3F | AVERAGE | COMBINED INDEX |
| <b>1HOURS</b>  |                |    |    |         |                |
| Erythema Score | 0              | 0  | 0  | 0       | 0.00           |
| Edema Score    | 0              | 0  | 0  | 0       |                |
| <b>24HOURS</b> |                |    |    |         |                |
| Erythema Score | 0              | 0  | 0  | 0       | 0.00           |
| Edema Score    | 0              | 0  | 0  | 0       |                |
| <b>48HOURS</b> |                |    |    |         |                |
| Erythema Score | 0              | 0  | 0  | 0       | 0.00           |
| Edema Score    | 0              | 0  | 0  | 0       |                |
| <b>72HOURS</b> |                |    |    |         |                |
| Erythema Score | 0              | 0  | 0  | 0       | 0.00           |
| Edema Score    | 0              | 0  | 0  | 0       |                |
| <b>7 DAYS</b>  |                |    |    |         |                |
| Erythema Score | 0              | 0  | 0  | 0       | 0.00           |
| Edema Score    | 0              | 0  | 0  | 0       |                |
| <b>14 DAYS</b> |                |    |    |         |                |
| Erythema Score | 0              | 0  | 0  | 0       | 0.00           |
| Edema Score    | 0              | 0  | 0  | 0       |                |

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**EVALUATION OF REACTIONS**  
**TABLE-II**  
**NON-Bt COTTONSEEDS (Sample-I)**

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|                | <b>RABBIT NUMBERS</b> |           |           |                |                       |
|----------------|-----------------------|-----------|-----------|----------------|-----------------------|
|                | <b>1F</b>             | <b>2F</b> | <b>3F</b> | <b>AVERAGE</b> | <b>COMBINED INDEX</b> |
| <b>1HOURS</b>  |                       |           |           |                |                       |
| Erythema Score | 0                     | 0         | 0         | 0              | 0.00                  |
| Edema Score    | 0                     | 0         | 0         | 0              |                       |
| <b>24HOURS</b> |                       |           |           |                |                       |
| Erythema Score | 0                     | 0         | 0         | 0              | 0.00                  |
| Edema Score    | 0                     | 0         | 0         | 0              |                       |
| <b>48HOURS</b> |                       |           |           |                |                       |
| Erythema Score | 0                     | 0         | 0         | 0              | 0.00                  |
| Edema Score    | 0                     | 0         | 0         | 0              |                       |
| <b>72HOURS</b> |                       |           |           |                |                       |
| Erythema Score | 0                     | 0         | 0         | 0              | 0.00                  |
| Edema Score    | 0                     | 0         | 0         | 0              |                       |
| <b>7 DAYS</b>  |                       |           |           |                |                       |
| Erythema Score | 0                     | 0         | 0         | 0              | 0.00                  |
| Edema Score    | 0                     | 0         | 0         | 0              |                       |
| <b>14 DAYS</b> |                       |           |           |                |                       |
| Erythema Score | 0                     | 0         | 0         | 0              | 0.00                  |
| Edema Score    | 0                     | 0         | 0         | 0              |                       |

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**EVALUATION OF REACTIONS**  
**TABLE-III**  
**Bt COTTONSEEDS (Sample-II)**

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|                | RABBIT NUMBERS |    |    |         |                |
|----------------|----------------|----|----|---------|----------------|
|                | 1F             | 2F | 3F | AVERAGE | COMBINED INDEX |
| <b>1HOURS</b>  |                |    |    |         |                |
| Erythema Score | 0              | 0  | 0  | 0       | 0.00           |
| Edema Score    | 0              | 0  | 0  | 0       |                |
| <b>24HOURS</b> |                |    |    |         |                |
| Erythema Score | 0              | 0  | 0  | 0       | 0.00           |
| Edema Score    | 0              | 0  | 0  | 0       |                |
| <b>48HOURS</b> |                |    |    |         |                |
| Erythema Score | 0              | 0  | 0  | 0       | 0.00           |
| Edema Score    | 0              | 0  | 0  | 0       |                |
| <b>72HOURS</b> |                |    |    |         |                |
| Erythema Score | 0              | 0  | 0  | 0       | 0.00           |
| Edema Score    | 0              | 0  | 0  | 0       |                |
| <b>7 DAYS</b>  |                |    |    |         |                |
| Erythema Score | 0              | 0  | 0  | 0       | 0.00           |
| Edema Score    | 0              | 0  | 0  | 0       |                |
| <b>14 DAYS</b> |                |    |    |         |                |
| Erythema Score | 0              | 0  | 0  | 0       | 0.00           |
| Edema Score    | 0              | 0  | 0  | 0       |                |

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## RESULT

The given sample of Bt cottonseeds (Sample –II) was found to be '**non-irritant**' to the vaginal mucous membrane of the rabbits when compared to the Non-Bt cottonseeds (Sample-I).

The compound 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

000041136

**Issued to :**

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

J.O.No. TOX/346 D  
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 Irritation to mucous membrane test in 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

**Irritation to mucous membrane test in Rabbits**

The given sample of Bt cottonseeds (Sample -II) was found to be 'Non-irritant' to the vaginal mucous membrane of the rabbits when compared to the Non-Bt cottonseeds (Sample-I) and the control group of animals.

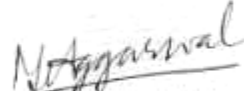
The sample has been conducted as per DBT, Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant parts.

(Annexure enclosed)

\*\*\*\*

DOR : 06-11-2006

DOC : 22-03-2007

  
AUTHORISED SIGNATORY  
(EMPLOYEE CODE 6000)

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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.

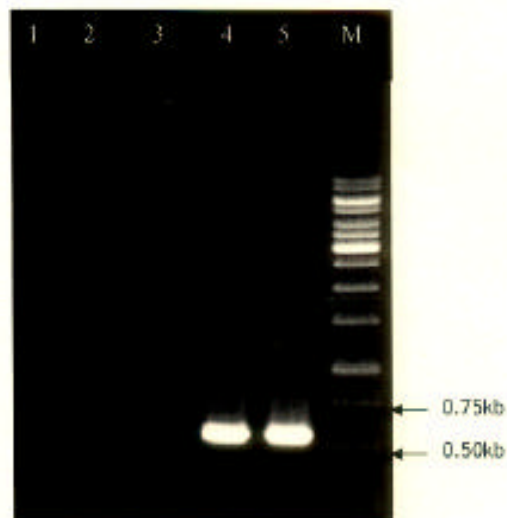
1. Confirmation of transgenic nature by PCR specific to the transgene
2. Confirmation of presence of Cry1C protein and its quantitation by ELISA

#### 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

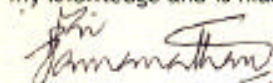
| Sl no | Entry ID        | A <sub>450</sub> | Cry1C concentration (µg/g on fresh wt) |
|-------|-----------------|------------------|--|
| 1     | Blank           | 0.09             | NA                                     |
| 2     | 1 ppb standard  | 0.3              | 0.92                                   |
| 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*