

**NICEATM**  
National Toxicology Program  
Interagency Center for the Evaluation of  
Alternative Toxicological Methods

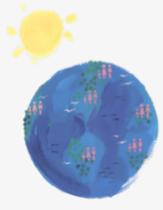
**ICCVAM**  
Interagency Coordinating Committee on  
the Validation of Alternative Methods

**Report on the Second Meeting of the  
Independent Scientific Peer Review  
Panel: Evaluation of the Updated  
Validation Status of New Versions and  
Applications of the Murine Local Lymph  
Node Assay**

**Introduction and Overview**

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Immunotoxicity Working Group

**June 26, 2009**  
SACATM Meeting  
Arlington, VA




**Alternative Method for Allergic Contact Dermatitis  
Testing: The Murine Local Lymph Node Assay (LLNA)**



- **LLNA (1998)**
  - First ICCVAM test method evaluation and independent peer review meeting
  - Avoids pain and distress
  - Valid substitute for traditional guinea pig tests
  - A reduction and refinement success
- **LLNA (2008)**
  - ICCVAM LLNA Performance Standards
  - rLLNA Test Method Evaluation Report (TMER)
  - Three Nonradioactive LLNA methods
  - LLNA Applicability domain
  - LLNA for potency

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**Legislation and Regulatory Protocol  
Requirements for Skin Sensitization**

Agency	Regulated Products	Legislation	Regulatory Protocol Requirements	Non-Governmental Standards
<b>United States</b>				
CPSC	Chemicals	FHSA	16 CFR 1500.3	OECD TG 429 OECD TG 406
EPA	Chemicals Pesticides	TSCA FIFRA	40 CFR 716, 721 OPPTS 870.2600	
OSHA	Workers	OSHA Act of 1970	NA	NA
<b>Europe</b>				
EU	Chemicals and mixtures	Council Directive 67/548/EEC Commission Directive 2001/155/EC Directive 2001/58/EC	Method B.6 in Council Directive 96/54/EC (Skin sensitization) <sup>1</sup> Method B.42 in Council Directive 2004/73/EC (Skin sensitization: Local Lymph Node Assay) <sup>2</sup>	ISO 10993-10 ECETOC Monograph No. 29 Skin Sensitization OECD TG 429 OECD TG 406

Abbreviations: CFR = Code of Federal Regulations; CPSC = U.S. Consumer Product Safety Commission; EC = European Commission; ECETOC = European Centre for Ecotoxicology and Toxicology of Chemicals; EEC = European Economic Community; EPA = U.S. Environmental Protection Agency; FDA = U.S. Federal Drug Administration; FHSA = Federal Hazardous Substances Act; FIFRA = Federal Insecticide, Fungicide and Rodenticide Act; EU = European Union; ISO = International Standards Organization; NA = not applicable; OECD = Organisation for Economic Co-operation and Development; OPPTS = Office of Prevention, Pesticides, and Toxic Substances; OSHA = U.S. Occupational Safety and Health Administration; TSCA = Toxic Substances Control Act.  
<sup>1</sup>Council Directives that contain the testing methods of Annex V to Council Directive 67/548/EEC.

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**Regulations and Guidelines for Skin  
Sensitization Testing**

Agency/Group	Regulation/Guideline	Test Requirements/Description
EPA	Health Effects Test Guidelines OPPTS 870.2600 Skin Sensitization (Mar 2003)	Provides guidelines for skin sensitization testing that harmonizes EPA OPPTS and OECD testing guidelines under TSCA and FIFRA. Includes LLNA and guinea pig test methods (Guinea Pig Maximization Test and Buehler Test).
ISO	ISO 10993-10 (2002)	Harmonized test methods described in <i>Biological Evaluation of Medical Devices: Tests for Irritation and Sensitization</i> . Includes LLNA and guinea pig test methods.
OECD	TG 406 (Jul 1992)	Test guidance provided in <i>Skin Sensitisation</i> . Includes guinea pig test methods (Guinea Pig Maximization Test and Buehler Test).
	TG 429 (Apr 2002)	<i>Skin Sensitisation: Local Lymph Node Assay</i>

Abbreviations: CDRH = Center for Devices and Radiological Health; ECETOC = European Centre for Ecotoxicology and Toxicology of Chemicals; EPA = U.S. Environmental Protection Agency; FDA-CDRH = U.S. Food and Drug Administration; FIFRA = Federal Insecticide, Fungicide and Rodenticide Act; ISO = International Standards Organization; OECD = Organisation for Economic Co-operation and Development; OPPTS = Office of Prevention, Pesticides, and Toxic Substances; OSHA = Occupational Safety and Health Administration; TG = Test Guideline.

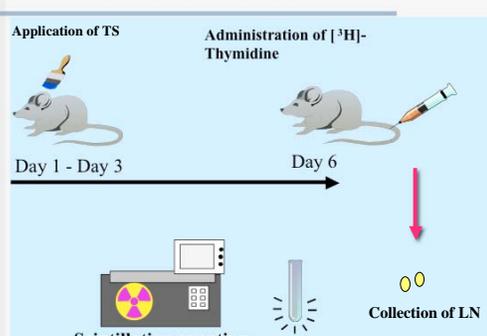
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**Overview of the Murine Local Lymph Node Assay (LLNA) Test Method Protocol**

- The purpose of the LLNA is to identify chemical sensitizers through quantification of lymphocyte proliferation
  - The LLNA uses a minimum of three dose levels
    - The highest dose level should be the maximum soluble concentration that does not cause systemic toxicity or excessive local irritation
- A Stimulation Index (SI) is calculated as the ratio of radioactivity incorporated into draining auricular lymph nodes cells of treated animals to that of vehicle control animals
  - $SI \geq 3$  is used to classify substances as skin sensitizers

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**LLNA Test Method Protocol**



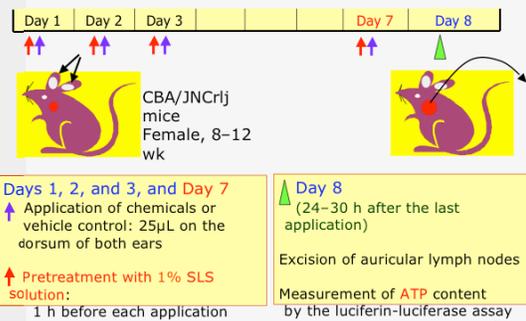
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## After the 2008 Peer Review Panel Meeting

- In 2008, the Peer Review Panel agreed with ICCVAM that more data were needed to evaluate the following:
  - Three modified versions of the LLNA not requiring radiolabeling
  - Application of the LLNA for pesticide formulations, other products, and substances tested in aqueous solutions
- Additional data were submitted to NICEATM
- The ICCVAM Immunotoxicity Working Group (IWG), working with NICEATM revised draft background review documents (BRDs), and ICCVAM updated the draft test method recommendations

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## 1. LLNA: DA Test Method Protocol (2009)



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## LLNA: DA Test Method Data

- The validation database in the 2009 revised draft BRD was updated from 2008 to include 15 additional substances
  - 44 substances with comparative data from the traditional LLNA
- Intralaboratory data analyzed (Idehara et al. 2008)
  - Two substances (isoeugenol and eugenol) were tested in the LLNA: DA at varying concentrations, in three different experiments, in order to assess intralaboratory reproducibility (previously included in 2008 draft BRD; individual animal data not available)
- Interlaboratory data recently evaluated
  - Two-phased interlaboratory validation study evaluated the reliability and relevance of the LLNA: DA (Omori et al. 2008)
    - First phase: 10 laboratories, 12 coded substances
    - Second phase: 7 different laboratories, 5 coded substances
    - Combined: 17 laboratories, 14 different coded substances

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## LLNA: DA Test Method Performance (Alternative Decision Criteria)

- Test method accuracy in 2009 revised draft BRD also evaluated two different decision criteria to classify sensitizers and nonsensitizers
  - SI  $\geq 2.5$  to classify substances as sensitizers
    - No false positive results compared to traditional LLNA
  - SI  $\leq 1.7$  to classify substances as nonsensitizers
    - No false negative results compared to traditional LLNA
  - There is a range of SI values (i.e.,  $1.7 < SI < 2.5$ ) for which the classification is not definitive (i.e., chance for false positives or false negatives)
    - 10 substances; 5 sensitizers and 5 nonsensitizers compared to traditional LLNA
  - Test method developers proposed using SI  $\geq 3.0$

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## LLNA: DA Interlaboratory Reproducibility (EC2.5 Values)

Substance	Laboratory										Mean EC2.5 (%)	% CV
	1	2	3	4	5	6	7	8	9	10		
DNCB	0.026	0.063	0.039	0.022	0.11	0.025	0.011	0.039	0.025	0.13	0.049	84
	(12.0 @ 0.3%)	(9.23 @ 0.3%)	(9.96 @ 0.3%)	(8.53 @ 0.3%)	(7.86 @ 0.3%)	(15.1 @ 0.3%)	(13.2 @ 0.3%)	(12.6 @ 0.3%)	(10.9 @ 0.3%)	(4.71 @ 0.3%)		
HCA	8.47	9.41	11.4	7.90	14.6	10.8	6.78	7.03	12.5	9.14	9.80	26
	(5.78 @ 25%)	(4.82 @ 25%)	(4.44 @ 25%)	(5.11 @ 25%)	(3.97 @ 25%)	(5.50 @ 25%)	(7.09 @ 25%)	(10.2 @ 25%)	(3.88 @ 25%)	(3.51 @ 25%)		

NOTE: Values in parentheses are highest SI values achieved. Shading indicates EC2.5 values that are outside of acceptable range recommended in ICCVAM LLNA Performance Standards (i.e., 5-20% for HCA and 0.025-0.1% for DNCB). Abbreviations: CV = coefficient of variation; DNCB = 2,4-dinitrochlorobenzene; HCA = hexyl cinnamic aldehyde.

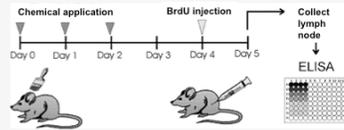
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## Draft ICCVAM Test Method Recommendations: Usefulness and Limitations

- The LLNA: DA can be used to identify substances as potential skin sensitizers and nonsensitizers, with specific defined limitations
  - Using a decision criterion of SI  $\geq 2.5$  to identify sensitizers results in no false positives (0/12)
  - Using a decision criterion of SI  $\leq 1.7$  to identify nonsensitizers results in no false negatives (0/32)
- Substances that produce SI  $\geq 1.7$  and  $\leq 2.5$  should be evaluated using an integrated decision strategy with all available and relevant information.
  - (such as, dose response information, QSAR information, statistical analyses of the differences between treated and vehicle control groups, peptide-binding activity, molecular weight, results from related chemicals, and other testing data)

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## 2. LLNA: BrdU-ELISA Test Method Protocol (2009)



- The LLNA: BrdU-ELISA protocol is the same as the traditional LLNA protocol except for the following items:
  - Lymph node cell proliferation is assessed by measuring the incorporation of BrdU into the cells using ELISA
  - BrdU is injected IP instead of <sup>3</sup>H-thymidine being injected IV as done in the traditional LLNA

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## LLNA: BrdU-ELISA Test Method Data

- The validation database in the 2009 revised draft BRD was updated from the 2008 draft BRD to include seven additional substances
  - 35 substances (31 with comparative traditional LLNA data)
  - All individual animal data are included in 2009 draft
- Intralaboratory reproducibility data for 8 substances tested (2-6 times) in one laboratory
  - Updated from 5 substances included in the 2008 draft BRD
- Interlaboratory data from the recently completed Japanese Society for Alternative Animal Experiments (JSAAE) validation study
  - 10 coded substances tested in three to seven laboratories
  - Not available for the 2008 draft BRD

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## LLNA: BrdU-ELISA Test Method Performance (Alternative Decision Criteria)

- Test method accuracy in 2009 revised draft BRD also evaluated two different decision criteria to identify sensitizers and nonsensitizers
  - SI  $\geq 2.0$  to identify substances as sensitizers
    - No false positive results compared to traditional LLNA
  - SI  $< 1.3$  to identify substances as nonsensitizers
    - No false negative results compared to traditional LLNA
  - There is a range of SI values (i.e.,  $1.3 \leq SI < 2.0$ ) for which a classification is not definitive (i.e., chance for false positives or false negatives)
    - 11 substances; 6 sensitizers and 5 nonsensitizers compared to traditional LLNA

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## LLNA: BrdU-ELISA Interlaboratory Reproducibility (EC2 Values)

Substance	Laboratory							Mean	% CV
	1	2	3	4	5	6	7		
DNCB	0.084 (4.3 @ 1%)	0.019 (8.37 @ 1%)	0.029 (5.89 @ 0.3%)	0.030 (5.50 @ 1%)	0.0025 (18.8 @ 0.3%)	0.025 (4.83 @ 0.3%)	0.053 (12.2 @ 1%)	0.035	76
HCA	16.2 (3.4 @ 50%)	— <sup>1</sup> (1.83 @ 50%)	24.0 (2.87 @ 50%)	9.36 (3.34 @ 50%)	4.07 (13.5 @ 50%)	13.0 <sup>2</sup> (3.27 @ 50%)	14.2 (3.84 @ 50%)	13.5	50

Note: Values in parentheses are highest SI values achieved. Shading shows EC2 values that are outside of the acceptable range from the ICCVAM LLNA Performance standards:  $5 \geq 20\%$  for HCA and  $0.025 \geq 0.1\%$  for DNCB. Abbreviations: CV = coefficient of variation; DNCB = 2,4-dinitrochlorobenzene; HCA = hexyl cinnamic aldehyde. <sup>1</sup>One test failed (positive control SI  $< 2$ ; vehicle control absorbance was unusually high), and this result is not included in the mean and CV. <sup>2</sup>Maximum SI = 1.27.

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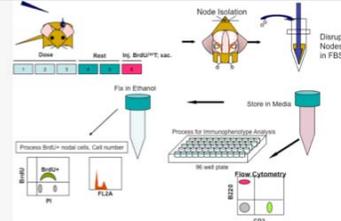
## Draft ICCVAM Test Method Recommendations: Usefulness and Limitations

- The LLNA: BrdU-ELISA can be used to identify substances as potential skin sensitizers and nonsensitizers, with specific defined limitations
  - Using a decision criterion of SI  $\geq 2.0$  to identify sensitizers results in no false positives (0/9)
  - Using a decision criterion of SI  $< 1.3$  to identify nonsensitizers results in no false negatives (0/22)
- Substances that produced  $1.3 \leq SI < 2.0$  should be evaluated using an integrated decision strategy with all available and relevant information
  - (such as, dose response information, QSAR information, statistical analyses of the differences between treated and vehicle control groups, peptide-binding activity, molecular weight, results from related chemicals, and other testing data)

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## 3. LLNA: BrdU-FC Test Method Protocol (2009)



- The LLNA: BrdU-FC protocol is the same as the traditional LLNA protocol except
  - Lymph node cell proliferation is assessed by measuring the incorporation of BrdU into the cells using flow cytometry
  - BrdU is injected IP instead of <sup>3</sup>H-thymidine being injected IV as done in the traditional LLNA
  - Optional endpoints for substances with SI  $\geq 3$ :
    - Irritation is assessed (mouse ear swelling)
    - The enhanced version of the test (LLNA: BrdU-FC) includes an optional immunophenotyping step (when mouse ear swelling  $> 25\%$ )

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### LLNA: BrdU-FC Test Method Data

- Initial data submitted by MB Research Labs from testing 45 substances (3 additional substances had no traditional LLNA data)
- New data to demonstrate intralaboratory reproducibility
  - 4 Tests of Hexyl cinnamic aldehyde in AOO
  - 4 Tests of 2,4-Dinitrochlorobenzene in AOO
- New data to indicate vehicle dependence of 2-Mercaptobenzothiazole (MBT) results

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### LLNA: BrdU-FC Test Method Performance

- The LLNA: BrdU-FC had a 93% accuracy and 97% sensitivity compared to the traditional LLNA (n = 45)
- The false positive and false negative rates were 12% and 3%, respectively

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### Intralaboratory Reproducibility - EC3 Results for HCA and DNCB Testing in the LLNA: BrdU-FC

Test Substance (Vehicle)	Test 1	Test 2	Test 3	Test 4	Acceptable Range <sup>1</sup>
HCA (AOO)	15%	16%	13%	8.4%	5-20%
DNCB (AOO)	0.06%	0.03%	0.05%	0.03%	0.025-0.10%

Abbreviations: AOO = Acetone:olive oil (4:1); DNCB = 2,4-Dinitrochlorobenzene; HCA = Hexyl cinnamic aldehyde; EC3 = Estimated Concentration to produce a Stimulation Index of 3.  
<sup>1</sup>ICCVAM LLNA Performance Standards

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### Draft ICCVAM Test Method Recommendations: Usefulness and Limitations

- Based on the available data, the LLNA: BrdU-FC appears useful for identifying substances as potential skin sensitizers or nonsensitizers
- However more information and data are needed before ICCVAM can make a recommendation on the LLNA: BrdU-FC
  - Original LLNA: BrdU-FC data needs to be obtained and a data quality audit conducted
  - Interlaboratory reproducibility should be evaluated in order to determine test method transferability

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### 4. Applicability Domain of the LLNA (2009)

- A comprehensive update of available data and information regarding the current usefulness and limitations of the LLNA for assessing the skin sensitizing potential of pesticide formulations and other products, substances tested in aqueous solutions, and metals
- Information in the revised draft addendum
  - Data from over 500 substances tested in LLNA (Jan. 2008)
  - Additional LLNA data added since Jan. 2008:
    - 52 pesticide formulations submitted by Dow AgroSciences (with corresponding guinea pig data)
    - 28 pesticide formulations submitted by DuPont Chemical Company
    - 12 natural complex substances submitted by RIFM (with corresponding human data)
    - 48 medical device eluates submitted by AppTec Laboratory Services
    - No new LLNA data for metals

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### Substances in the Updated Evaluation of the Applicability Domain for LLNA

- LLNA data for 104 pesticide formulations
  - 70 pesticide formulations with associated guinea pig data for the formulation, the active ingredient contained in the formulation, and/or a related substance
  - 22 pesticide formulations tested in both the LLNA and the guinea pig
- 6 textile dyes with both LLNA and guinea pig data
- 12 natural complex substances with LLNA and human data
- 24 substances tested in aqueous solutions in both the LLNA and the guinea pig

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## LLNA Testing of Pesticide Formulations

### Test Method Performance

- 22 pesticide formulations tested in both LLNA and guinea pig
  - The LLNA classifies more pesticide formulation as sensitizers (n = 13) than a guinea pig test (n = 3)
  - All formulations positive in the guinea pig are also positive in the LLNA

### Revised Draft ICCVAM Recommendations

- LLNA is more likely than a guinea pig test<sup>1</sup> to classify a pesticide formulation as a sensitizer
  - However, human data are not available for these pesticide formulations to confirm their human sensitization potential
  - These data indicate that the LLNA has utility for hazard classification of pesticide formulations, provided that the potential for possible overclassification is not a limitation

<sup>1</sup>18/22 Buehler test results, 1/22 guinea pig maximization test results, 3/22 unspecified guinea pig test results.

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## LLNA Testing of Dyes

### Test Method Performance

- Six textile dyes with both LLNA and guinea pig maximization test data; no human data
  - 50% (3/6) were sensitizers in the LLNA
  - 83% (5/6) were sensitizers in the guinea pig maximization test

### Revised Draft ICCVAM Recommendations

- More data are needed before a recommendation on the usefulness and limitations of the LLNA for testing these types of substances can be made
  - Due to the very limited number of dyes (n = 6) for which comparative reference data are available

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## LLNA Testing of Natural Complex Substances<sup>1</sup>

### Test Method Performance

- 75% (9/12) of these natural complex substances tested as sensitizers in the LLNA
- 33% (4/12) natural complex substances tested as sensitizers in the human maximization test
- Accuracy 42% (5/12)
  - LLNA overpredicts human 75% (6/8)
  - LLNA underpredicts human 25% (1/4)

### Revised Draft ICCVAM Recommendations

- The data suggest that the LLNA is protective against potential skin sensitization hazards
  - A definitive recommendation on the usefulness of the LLNA for testing natural complex substances cannot be made until a larger number of known human sensitizers that are natural complex substances have been tested in the LLNA

<sup>1</sup>"Natural complex substances" was a term recommended by the Panel. The revised draft Addendum refers to the same substances as "fragrance ingredients," and the draft ICCVAM recommendations referred to them as "essential oils."

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## LLNA Testing of Substances in Aqueous Solutions (1)

### Test Method Performance

- Based on 24 substances tested in aqueous solutions in both the LLNA and the guinea pig,<sup>1</sup> the LLNA classifies more substances as sensitizers (12) than the guinea pig test (4)
  - 10 substances for which LLNA and guinea pig results were discordant
    - Only one substance (neomycin sulfate) is negative in the LLNA and positive in the guinea pig; all other discordant substances are positive in the LLNA and negative in the guinea pig
  - Human data are available for one substance (neomycin sulfate) that is discordant between LLNA and guinea pig
    - This substance is also discordant between LLNA (negative) and human (positive)

<sup>1</sup>18/24 Buehler test results, 3/24 guinea pig maximization test results, 3/24 unspecified guinea pig test results

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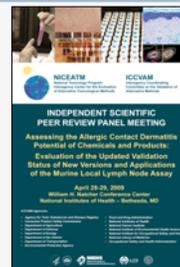
## LLNA Testing of Substances in Aqueous Solutions (2)

### Revised Draft ICCVAM Recommendations

- The available data suggest that the LLNA is more likely than a guinea pig test to classify a substance tested in an aqueous solution as a sensitizer
- Although the database analyzed was limited, the data indicate that the LLNA has utility for hazard classification of substances tested in aqueous solutions, provided that the potential for possible overclassification is not a limitation

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## ICCVAM LLNA Peer Review Panel Meeting



- Held April 28-29, 2009
  - William H. Natcher Conference Center
  - NIH, Bethesda, MD
- Expert Scientific Panel
  - 15 scientists
  - 6 countries
- Purpose: Evaluation of the updated validation status of new versions and applications of the Murine Local Lymph Node Assay (LLNA)

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## ICCVAM Charges to the Peer Review Panel

- Review the ICCVAM Revised Draft Background Review Documents (BRDs) for completeness, and identify any errors or omissions in the BRDs (*LLNA-DA; LLNA: BrdU-ELISA; LLNA: BrdU-FC*)
- Review the ICCVAM Revised Draft Applicability Domain Addendum for completeness, and identify any errors or omissions in the document
- Evaluate the information in the draft documents to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods (ICCVAM Submission Guidelines 2003) have been appropriately addressed
- Consider the ICCVAM revised draft test method recommendations for the following and comment on the extent to which they are supported by the information provided in the BRDs and Addendum:
  - Proposed test method use
  - Proposed recommended standardized protocols
  - Proposed test method performance standards
  - Proposed future studies