NICEATM

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

ICCVAM

Interagency Coordinating Committee on the Validation of Alternative Methods

Allergic Contact Dermatitis (ACD) Case Studies

ICCVAM Workshop Series on Best Practices for Regulatory Safety Testing: Assessing the Potential for Chemically Induced Allergic Contact Dermatitis

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Case Study 1: Introduction

- You have submitted a protocol to the IACUC to use the LLNA to assess the ACD hazard potential of Chemical A
- The IACUC is pleased that you plan to use the LLNA rather than the guinea pig maximization test, the test your lab traditionally uses
- The IACUC further responds that you should consider performing the reduced LLNA (rLLNA) in order to reduce the number of animals used

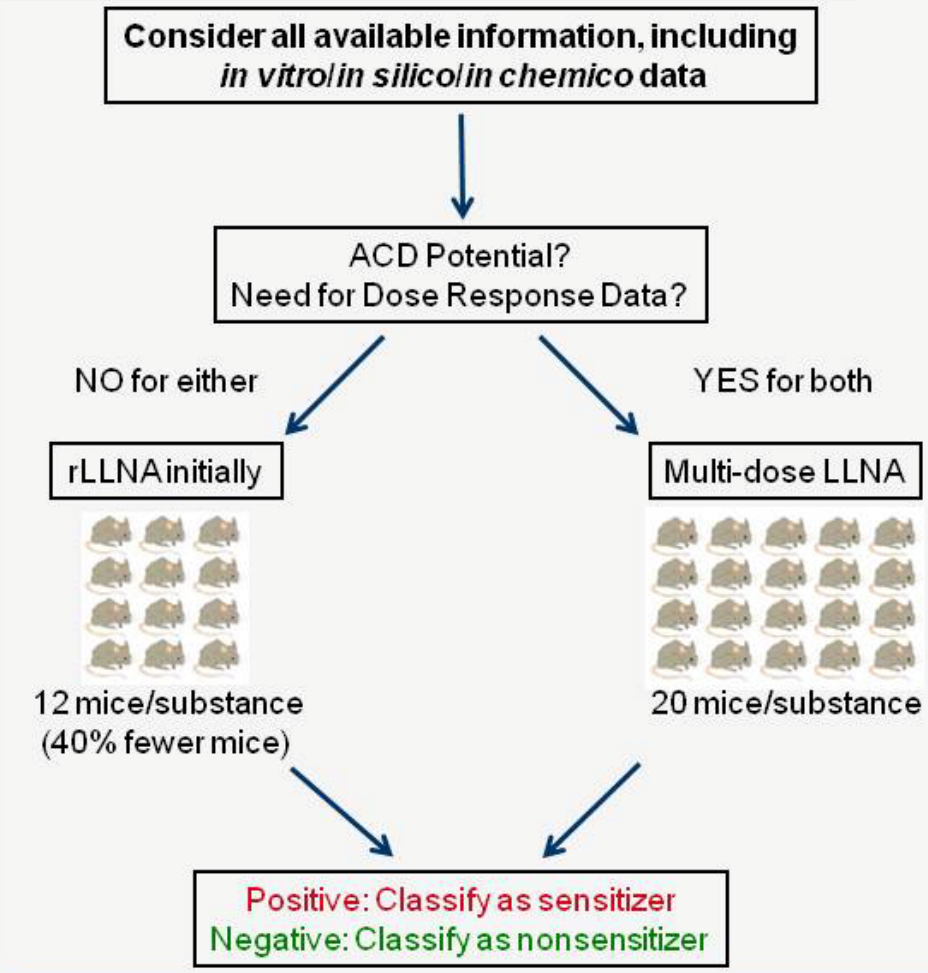
Case Study 1: Prior Chemical Information

- Information available for Chemical A
  - Molecular weight > 600
  - Log K_{ow} = 2.84
  - No structural alerts for skin sensitization
  - Structurally similar to Chemical B, which is a nonsensitizer
  - No other information is available
- Based on the information above, do you suspect that Chemical A may be a sensitizer or nonsensitizer?
  - Nonsensitizer based on its high molecular weight, similarity to Chemical B, and the lack of structural alerts for skin sensitization
  - Substances with molecular weights > 500 are less likely to be sensitizers due to limited penetration of the stratum corneum[1]
    - 70% (12/17) of substances in the NICEATM LLNA database with a molecular weight > 600 were nonsensitizers

[1]Bos JD, Meinardi MMHM. 2000. Exp Dermatol 9: 165-169.

Case Study 1: Decision Strategy for Using rLLNA

- You remember the following decision strategy and revise the protocol to perform the rLLNA



Case Study 1: IACUC Follow-up

- The IACUC was pleased with your revision to use the rLLNA and expeditiously approved the protocol
- Is there sufficient information to determine the dose for testing in the rLLNA or should a prescreen test be performed?
  - The dose tested must be the maximum concentration that does not produce overt systemic toxicity and/or excessive local skin irritation in the mouse
  - All existing toxicological information (i.e., acute toxicity and dermal irritation), structural information, and physicochemical information on Chemical A (and/or Chemical B, a structurally related substance) should be considered
  - A prescreen test must be performed because
    - There is no information on the doses that produce systemic toxicity or local skin irritation for Chemical A
    - The test and dose information for Chemical B is unavailable

Case Study 1: Dose Selection

- You perform a prescreen test using three doses with 2 mice/dose
  - The doses include the maximum soluble dose in acetone: olive oil (4:1) (AOO), 10%, with 5% and 2.5% as the lower doses
  - These doses are applied to the dorsum of the ears on days 1, 2, and 3
  - Body weights are measured on days 1 and 6
  - Ear thickness is measured on days 1, 3, and 6
  - Erythema of the dorsal surface of the ear is scored on days 1, 3, and 6
- No clinical signs of toxicity were observed at any dose; body weight, ear thickness, and ear erythema data are shown on the next slide

Case Study 1: Prescreen Test Data for Chemical A

Chemical A Dose	Animal	Change in Body Weight[a]	Change in Ear Thickness Day 3[b]	Change in Ear Thickness Day 6[b]	Erythema Score Day 1[c]	Erythema Score Day 3[c]	Erythema Score Day 6[c]
2.5%	1	+5.6%	10.7%	9.5%	0	0	0
	2	+4.9%	9.8%	10.2%	0	1	0
5%	3	+2.2%	16.9%	16.9%	0	2	1
	4	+3.7%	20.2%	18.9%	0	1	1
10%	5	-6.3%	26.2%	30.1%	0	3	2
	6	-7.1%	35.1%	33.4%	0	3	3

Erythema score: 0 = no erythema; 1 = very slight erythema (barely perceptible); 2 = well-defined erythema; 3 = moderate to severe erythema.

[a] Percent difference of Day 6 body weight compared to Day 1 body weight.

[b] Percent difference compared with Day 1 ear thickness (average of both ears).

[c] Average of both ears.

- What dose should be selected for testing Chemical A and why?
  - 5% should be tested because it is the highest dose that does not produce excessive local irritation (change in ear thickness <25% and erythema score < 3) and/or systemic toxicity (no clinical signs, body weight decrease <5%)

Case Study 1: rLLNA Test

- You test Chemical A at 5% in AOO using the rLLNA
- In addition to Chemical A and the vehicle control, what other substance should be concurrently tested and how many animals should be used to test it?
  - The positive control, 25% hexyl cinnamic aldehyde (HCA), should be tested using 4 animals as recommended by the ICCVAM protocol and by OECD Test Guideline 429
- How many animals should be used in the vehicle control group?
  - 4 animals should be treated only with AOO, the vehicle control
- How many animals should be used in the Chemical A test group?
  - 4 animals should be treated with 5% Chemical A in AOO
- What is the reduction in the number of animals using the rLLNA compared with the three-dose LLNA?
  - 8 fewer animals (40% [8/20]). The rLLNA uses 12 animals. The three-dose LLNA uses 20 animals

Case Study 1: rLLNA Data - 1

Group	Animal	DPM	Mean	SD	SEM	SI
Vehicle Control	1	175	300	143	71	
	2	225				
	3	300				
	4	500				
25% HCA[1]	5	1253	2531	946	473	
	6	2404				
	7	3080				
	8	3388				
5% Chemical A	9	350	488	155	77	
	10	400				
	11	500				
	12	700				

- Do any DPM values seem to be outliers?
- No statistical outliers were identified using Dixon's test

- Calculate the stimulation index (SI) values for each group
- SI = test substance mean dpm/vehicle control mean dpm

[1]Hexyl cinnamic aldehyde

Case Study 1: rLLNA Data - 2

Group	Animal	DPM	Mean	SD	SEM	SI
Vehicle Control	1	175	300	143	71	1.00
	2	225				
	3	300				
	4	500				
25% HCA[1]	5	1253	2531	946	473	8.44
	6	2404				
	7	3080				
	8	3388				
5% Chemical A	9	350	488	155	77	1.62
	10	400				
	11	500				
	12	700				

- Based on the positive control response, is the test acceptable?
  - Yes, the SI ≥ 3

- Student's *t*-test indicated that there was no significant difference between the control group and the 5% Chemical A group ($p = 0.1253$)

[1]Hexyl cinnamic aldehyde

Case Study 1: rLLNA Decision

- Would you classify Chemical A (SI = 1.62) as a sensitizer or nonsensitizer and why?
  - Chemical A is a nonsensitizer because the SI < 3

Case Study 1: Summary and Breakout Group Discussion

- This rLLNA case study demonstrated
  - The use of the rLLNA to test substances that are suspected to be nonsensitizers. Note that the rLLNA should also be used to test suspected sensitizers when dose-response information is not needed
  - Consideration and appropriate use of the rLLNA can decrease animal use by 40%
    - 80% of chemicals/products are **nonsensitizers** in standardized tests[1]
  - The use of a prescreen test to determine the dose to be tested in the rLLNA
- Some labs get more consistent results using 35% HCA as the positive control
- How can you be sure that you are accurately identifying mild/moderate sensitizers if the positive control response dips near the 3.0 threshold?
- The variability of the positive control response should be monitored and evaluated over time
- Because EPA, Australia, etc., require 5 animals per group, tests for multiple regulatory entities should use most conservative protocol
- The SI is usually expressed to one decimal point, so the cutoff should be expressed as SI < 3.0
  - A result of 2.95 would round to 3.0; this is technically a nonsensitizer but other factors such as dose response and solubility could result in consideration as a weak sensitizer
- How should medical devices be tested?
  - What, other than rLLNA results, can be used to support a negative result?

[1]Safford RJ. 2008. Reg Tox Pharmacol 51: 195-200.

Case Study 2: Introduction

- You have submitted a protocol to the IACUC to use the guinea pig maximization test to assess the ACD hazard potential of Chemical C
- The IACUC recommends the LLNA because it uses fewer animals and because positive responses do not produce pain and distress in the animals
- You respond to the IACUC that you cannot use the LLNA because your laboratory is not licensed to use radioactivity; you used this as a justification for the guinea pig test
- The IACUC notices that ICCVAM has recommended two nonradioactive LLNA methods, the LLNA: DA and the LLNA: BrdU-ELISA at http://iccvam.niehs.nih.gov/ and asks you to consider these

Case Study 2: Prior Chemical Information

- You have the following information about Chemical C
  - Molecular weight <200
  - log K_{ow} = -0.66
  - Soluble in water (up to 50%)
  - Only slightly soluble in AOO and *N,N*-dimethylformamide (DMF) and other organic solvents (up to 5%)
  - A structurally similar chemical, D, does not contain nickel and is not a potent ATP inhibitor or an ATP degrading enzyme
  - No other information is available
- Should you use the LLNA: DA or LLNA: BrdU-ELISA?
  - It depends on the substance to be tested and the equipment available. You decide to use the LLNA: DA because the substance does not contain nickel and it is not expected be a potent ATP inhibitor or an ATP degrading enzyme. A luminometer is readily available.

Case Study 2: Prior Chemical Information

- Is there sufficient information to determine the maximum dose for testing in the LLNA: DA or should a prescreen test be performed?
  - The maximum dose tested must be the maximum concentration that does not produce overt systemic toxicity or excessive local skin irritation in the mouse
  - All existing toxicological information (i.e., acute toxicity and dermal irritation), structural information, and physicochemical information on Chemical C (and/or a structurally related substance) should be considered
  - A prescreen test must be performed because there is no information on the doses of Chemical C that produce systemic toxicity or local skin irritation

Case Study 2: Prescreen Test

- What vehicle should be selected for prescreen testing?
  - 1% Pluronic® L92, a surfactant, or the equivalent, because Chemical C is very hydrophilic. Pluronic® L92 will allow Chemical C to adhere to the dorsum of the ear
- What concentrations should be selected for prescreen testing?
  - 50%, which is the maximum soluble concentration in water, and then 25% and 10%, from the recommended dose series
- For the prescreen study, four topical applications of each dose of Chemical C were given to each of two mice using the LLNA: DA treatment schedule (days 1, 2, 3, and 7)
- One hour prior to each application, the mice were pre-treated with 1% sodium lauryl sulfate applied to the dorsum of the ear

Case Study 2: Prescreen Results

- No clinical signs of toxicity were observed at any dose
  - Body weight, ear thickness, and ear erythema data are shown on the next slide

Case Study 2: Prescreen Data

Chemical C Dose	Animal	Change in Body Weight[a]	Change in Ear Thickness Day 3[b]	Change in Ear Thickness Day 7[b]	Change in Ear Thickness Day 8[b]	Erythema Score Day 1[c]	Erythema Score Day 3[c]	Erythema Score Day 7[c]	Erythema Score Day 8[c]
10%	1	+5.2%	9.8%	9.5%	9.5%	0	0	0	0
	2	+12.5%	10.7%	10.2%	10.3%	0	1	0	1
25%	3	+7.9%	20.2%	16.9%	17.5%	0	1	1	2
	4	+8.9%	16.9%	18.9%	19.4%	0	2	1	1
50%	5	+3.5%	25.2%	31.1%	32.1%	0	3	2	3
	6	+ 5.5%	34.1%	32.4%	33.7%	0	3	3	3

Erythema score: 0 = no erythema; 1 = very slight erythema (barely perceptible); 2 = well-defined erythema; 3 = moderate to severe erythema.

[a] Percent difference of Day 7 body weight compared to Day 1 body weight.

[b] Percent difference compared with Day 1 ear thickness (average of both ears).

[c] Average of both ears.

- What doses should be selected for LLNA: DA testing?
  - 25% as the maximum because it was the highest dose that did not produce excessive local irritation or systemic toxicity
    - Change in ear thickness <25%, erythema score < 3, and no systemic toxicity (no clinical signs, body weight decrease <5%)
  - 10% and 5% should be the lower doses (recommended by protocol)

Case Study 2: LLNA: DA Data

Group	Animal	Relative Luminescence Units[1]	Mean	SD	SEM	SI
Vehicle Control	1	15218	27188	10027	5014	1.00
	2	22764				
	3	33905				
	4	36866				
5% Chemical C	5	24319	36534	10199	5099	1.34
	6	32753				
	7	41322				
	8	47742				
10% Chemical C	9	20851	31201	10875	5438	1.15
	10	27887				
	11	29565				
	12	46499				
25% Chemical C	13	20734	30030	10456	5228	1.10
	14	21245				
	15	38401				
	16	39741				

- Do any values seem to be outliers?
  - No statistical outliers were identified using Dixon's test
- Calculate the SI values for each group

- SI = test substance mean RLU/vehicle control mean RLU

[1]Mean of two replicates

Case Study 2: LLNA: DA Decision

- For the positive control, 25% HCA, SI = 3.92
- Based on the positive control response, is the test acceptable?
  - Yes, the SI > 1.8, which is the criterion for potential skin sensitizers
- ANOVA of the the log-transformed relative luminescence units yielded F = 0.5666, p = 0.6475
- Based on a maximum SI of 1.34, would you classify Chemical C as a sensitizer or nonsensitizer and why?
  - Chemical C is a nonsensitizer because the maximum SI < 1.8. For the LLNA: DA, substances with SI ≥ 1.8 are potential skin sensitizers

Case Study 2: Summary and Breakout Group Discussion

- This case study provided an example of
  - Use of the LLNA: DA
  - Dose selection for the LLNA: DA using prescreen data
  - A test where the LLNA: DA SI < 1.8
- Experience with one or the other assay (i.e., LLNA: BrdU-ELISA) would be a factor in choosing the assay, as would whether the lab had validated the assay
- Pretreatment with 1% SLS a unique feature of the LLNA: DA used to increase sensitivity of the assay
  - Pluronic L92 is a surfactant also, would it do the same thing? Do you need SLS pretreatment if Pluronic L92 is used as the vehicle? This is an important practical point for further study.
- No “equivalent” of Pluronic L92 is known at this time; has SLS been tested using Pluronic L92 as a vehicle?
- Does the 1% SLS pretreatment impact aqueous/nonaqueous vehicle performance?
- If you had an outlier and excluded it, you’d have only 3 animals in that group
  - Would that be an acceptable test? The relevant regulatory agency should be consulted.
- If a substance is completely soluble in an organic vehicle (e.g., 10%) but makes a suspension in an aqueous vehicle at a higher concentration (e.g., 25%), which is the best solution for testing?

Case Study 3: Introduction

- You have submitted a protocol to the IACUC to use the Buehler test to assess the ACD hazard potential of Chemical E
- The IACUC recommends the LLNA because it uses fewer animals and because positive responses do not produce pain and distress in the animals
- You cannot use the LLNA because your laboratory is not licensed to use radioactivity, but you notice that ICCVAM has recommended two nonradioactive LLNA methods at http://iccvam.niehs.nih.gov/

Case Study 3: Prior Chemical Information

- You have only the following information on Chemical E
  - Molecular weight <170
  - log K_{ow} = 2.86
  - A structurally similar substance, Chemical F, is a sensitizer
  - The maximum concentration for testing Chemical E should be 25% in DMSO because it was the maximum soluble concentration that did not produce systemic toxicity or excessive local irritation
- Should you use the LLNA: DA or LLNA BrdU-ELISA?
  - It depends on the substance to be tested and the equipment available. You decide to use the LLNA: BrdU-ELISA because you are familiar with ELISA techniques and you have access to a microplate reader

Case Study 3: LLNA: BrdU-ELISA Data

Group	Animal	Absorbance[1]	Mean	SD	SEM	SI
Vehicle Control	1	0.119	0.243	0.221	0.110	1.00
	2	0.123				
	3	0.157				
	4	0.573				
5% Chemical E	5	0.171	0.227	0.048	0.024	0.93
	6	0.208				
	7	0.279				
	8	0.251				
10% Chemical E	9	0.089	0.200	0.102	0.051	0.82
	10	0.157				
	11	0.226				
	12	0.327				
25% Chemical E	13	0.197	0.296	0.122	0.061	1.22
	14	0.245				
	15	0.269				
	16	0.474				

[1]Mean of three replicates

- Calculate the SI values for each group
- SI = test substance mean abs/vehicle control mean abs

Case Study 3: Evaluation of LLNA: BrdU-ELISA Data

- For the positive control, 25% HCA, SI = 2.44
- Based on the positive control response, is the test acceptable?
  - Yes, the SI > 1.6, which is the criterion for potential skin sensitizers
- SI values were 0.93 at 5%, 0.82 at 10%, and 1.22 at 25%. Would you classify Chemical E as a sensitizer or nonsensitizer and why?
  - For the LLNA: BrdU-ELISA, substances with SI ≥ 1.6 are potential skin sensitizers. Based on these data, Chemical E appears to be a nonsensitizer because the maximum SI <1.6, however. . .

Case Study 3: Evaluation of Extreme Values

- After looking at the data your study director was surprised that Chemical E was negative because similar products, including Chemical F, were sensitizers
- The study director suggested that an outlier test be performed
  - Dixon's test indicated that the extreme value in the vehicle control group, 0.573, was an outlier at $p < 0.01$ among the 4 values in the vehicle control group
    - 0.573 was also an outlier at $p < 0.001$ among the 24 values in the historical vehicle control database for DMSO
  - The other two extreme values were not outliers
- You exclude the outlier and recalculate the SI values

Case Study 3: LLNA: BrdU-ELISA Data Without Outlier

Group	Animal	Absorbance[1]	Mean without Outlier	SD	SEM	SI without Outlier
Vehicle Control	1	0.119	0.133	0.021	0.012	1.00
	2	0.123				
	3	0.157				
	4	~~0.573~~				
5% Chemical E	5	0.171	0.227	0.048	0.024	1.71
	6	0.208				
	7	0.279				
	8	0.251				
10% Chemical E	9	0.089	0.200	0.102	0.051	1.50
	10	0.157				
	11	0.226				
	12	0.327				
25% Chemical E	13	0.197	0.296	0.122	0.061	2.22
	14	0.245				
	15	0.269				
	16	0.474				

- Calculate the SI values for each group
- SI = test substance mean abs/vehicle control mean abs

[1]Mean of three replicates

Case Study 3: LLNA: BrdU-ELISA Decision

- SI values with the outlier were 0.93 at 5%, 0.82 at 10%, and 1.22 at 25%
- SI values when excluding the outlier were 1.71 at 5%, 1.50 at 10%, and 2.22 at 25%
- Would you classify Chemical E as a sensitizer or nonsensitizer and why?
  - Chemical E is a sensitizer because the maximum SI = 2.22, which is >1.6. For the LLNA: BrdU-ELISA, substances with SI ≥ 1.6 are potential skin sensitizers

Case Study 3: Summary and Breakout Group Discussion

- This example shows that an outlier in the vehicle control group can produce erroneous results that may impact the classification of a substance using the LLNA: BrdU-ELISA
- In reviewing LLNA tests, NICEATM has also observed extreme low values in test substance groups that may produce false negative results
  - This emphasizes the need to collect individual animal data in order to identify outliers that could yield false negative results
- Structural similarities are of limited use when predicting sensitization potential
  - May be more useful when considering acute toxicity (i.e., when evaluating whether a prescreen is necessary)
- The absorbances in the historical vehicle control database should be evaluated
- A clear dose response is lacking, so the results are still questionable
- Other extreme values in the treatment groups may not be outliers, but may impact the evaluation as well (e.g., one high value in the high dose group)
- The number of cells or total protein applied to the wells isn't standardized and could be a source of variation
  - Consistency in processing the lymph nodes is very important

Case Study 4: Introduction and Prior Chemical Information

- You wish to assess the ACD hazard potential of another substance, Chemical G, using the LLNA: DA
- You have only the following information about Chemical G
  - Molecular weight = 100-150
  - log K_{ow} = 2.86
  - More soluble in AOO than DMF or other recommended organic solvents
  - Peptide reactivity is minimal
  - h-CLAT result is positive
- You use the LLNA: DA at a maximum concentration of 50%, the highest soluble concentration that did not produce excessive local irritation or systemic toxicity

Case Study 4: LLNA: DA Data

Group	Animal	Relative Luminescence Units[1]	Mean	SD	SEM	SI
Vehicle Control	1	15187	20576	5546	2773	1.00
	2	18744				
	3	20074				
	4	28298				
10% Chemical G	5	19026	25167	4299	2149	1.22
	6	25653				
	7	27127				
	8	28861				
25% Chemical G	9	27846	40921	10986	5448	1.99
	10	36281				
	11	47941				
	12	51618				
50% Chemical G	13	38134	49037	8244	4122	2.38
	14	47782				
	15	52938				
	16	57296				

- Do any values seem to be outliers?
  - No statistical outliers were identified using Dixon's test
- Calculate the SI values for each group
- SI = test substance mean RLU/vehicle control mean RLU

[1]Mean of two replicates

Case Study 4: Supporting Information and LLNA: DA Decision

- ANOVA of the log-transformed relative luminescence units: F = 12.61, p = 0.0005
- Dunnett's test
  - 10% - q = 1.322, p > 0.05
  - 25% - q = 4.210, p < 0.05
  - 50% - q = 5.4297, p < 0.05
- Additional data
  - Minimal peptide reactivity
  - Positive h-CLAT
- The maximum SI = 2.38. Would you classify Chemical G as a sensitizer or nonsensitizer and why?
  - Chemical G is a sensitizer because the maximum SI > 1.8

Case Study 4: LLNA: DA Interpretation

- 25% (3/12) of the nonsensitizers in the validation database were false positive with 1.8 < SI < 2.5. Do you have information that suggests the Chemical G results might be false positive?
  - Only the minimal peptide reactivity could possibly be used to suggest that the LLNA: DA result is false positive. 12% (6/52) of the sensitizers evaluated had minimal peptide reactivity[1]
  - The LLNA: DA result and h-CLAT result support a true positive result
  - Although SI = 2.38 is in the range where false positives may occur, the preponderance of the evidence supports the sensitizer classification

[1]Gerberick et al. 2007. Toxicol Sci 97: 417-427.

Case Study 4: Summary

- The purpose of this LLNA: DA example was to demonstrate how to interpret LLNA: DA results when the SI value is between 1.8 and 2.5, the range where false positive results may occur