Preliminary Evaluation and Comparison of Five Commercial Microcystin Enzyme-Linked Immunosorbent Assays to Liquid Chromatography Tandem Mass Spectrometry

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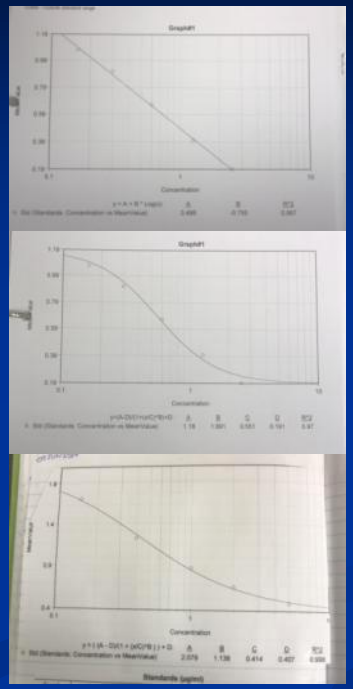
The Cyanotoxin Data Acquisition Process:

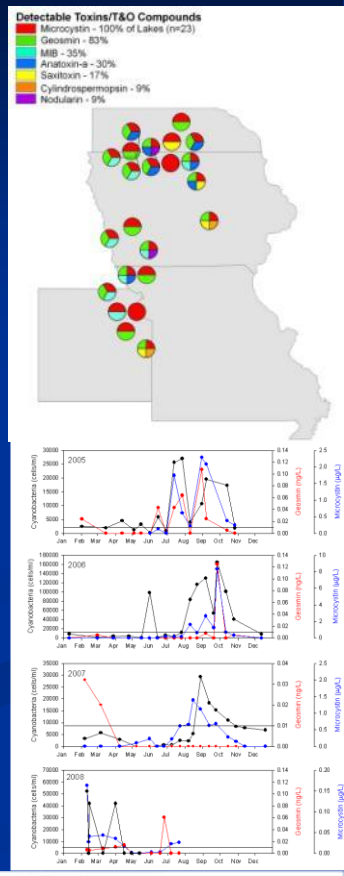
Sample Collection

Laboratory Processing

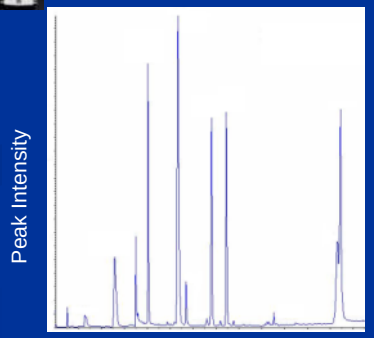
Analysis

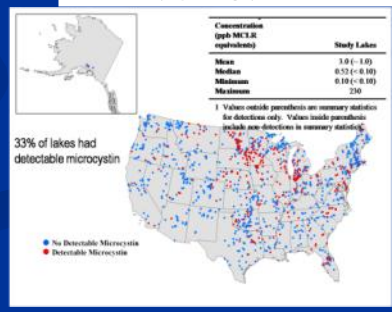


Data Reduction



Algal Toxin Analysis





Interpretation

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Experimental Design

- Hypothesis:
  - The lack of precision and accuracy in microcystin analyses are not a function of bad ELISA and LC/MS/MS technology.
- Samples: 31 surface samples from the Midwestern United States (bloom and non-bloom).
- Objectives:
  - 1. Evaluate and compare 5 commercially available microcystin ELISAs, independent of other sources of error
    - sample collection
    - sample splitting
    - laboratory processing (eg. cell lysis technique, filtration)
    - standards
    - analyst
    - data reduction
  - 2. Confirm by LC/MS/MS and compare to ELISA
    - Evaluate detection frequency, concentrations, and congeners

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Comparison of 5 Commercial Microcystin ELISAs

	ELISA Abbreviation				
Parameter	**Abraxis-DM**	**Abraxis-ADDA**	**Beacon**	**Envirologix**	**Strategic Diagnostics**
Manufacturer	Abraxis	Abraxis	Beacon	Envirologix	Strategic Diagnostics
Antibody Type	monoclonal	polyclonal	polyclonal	polyclonal	polyclonal
Binding Reagent Format	antibody in solution	immobilized antigen	immobilized antibody	immobilized antibody	immobilized antibody
Incubation steps	2	3	2	3	3
Incubation time (minutes)	90/20	90/30/20	30/30	30/30/30	30/30/30
Total Incubation time minutes)	110	150	60	90	90
Sample Size (uL)	100	50	50	20	100
Standard	MCLR [d]	MCLR [d]	MCLR [d]	MCLR [e]	MCLR surrogate [f]
Standards (ppb)	0.15, 0.40, 1.0, 2.0, 5.0	0.15, 0.40, 1.0, 2.0, 5.0	0.1, 0.3, 0.8, 2.0	0.16, 0.60, 2.5	0.1, 0.2, 0.4, 0.56, 0.8, 1.6
Positive Control Standard (µg/L)	0.75	0.75	1.0	No	No
Sensitivity (ppb) - MDL	0.10 (90 % B/Bo)	0.10	?	0.15 (81.3 % B/Bo)	0.10
Manufacturer Recommended Curve Fitting Functions [b]	FP, LL, PP, SL	FP, LL, PP, SL	FP, SL	SL	SL

a pre-incubation
b FP, four parameter; LL, log/logit, PP, point-to-point, SL, semi-log
c The material safety data sheet did not identify the substrate further.
d MCLR, microcystin-LR
e This assay used a microcystin-LR surrogate prior to XXXX
f MCLR surrogate,

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Microcystin ELISA Cross-Reactivity

No ELISA is MCLR specific!

- With over 80+ microcystin and 10+ nodularins, most cross-reactivities are unknown!

	Percent							
Microcystin Assays	**MCLA**	**MCLF**	**MCLR**	**MCRR**	**MCLW**	**MCLY**	**MCYR**	**NODR**
Monoclonal Assays								
Abraxis-DM	48	72	100	53	102	NA	64	76
Polyclonal Assays								
Abraxis-ADDA	125	108	100	91	114	NA	81	169
Beacon	5	NA	100	87	NA	NA	48	31
Envirologix	62	NA	100	54	NA	NA	35	69
Strategic Diagnostics	23	NA	100	97	NA	NA	82	66

- ELISA Response = Σ (Cross-Reactivity x Actual Congener Concentration) ,

- Example: Theoretical Concentration: 1 ppb MCLR + 1 ppb MCLA = 2 µg/L

Abraxis-DM	≈ 1.48 µg/L
Abraxis-ADDA	≈ 2.25 µg/L
Beacon	≈ 1.05 µg/L
Envirologix	≈ 1.62 µg/L
SDI	≈ 1.23 µg/L

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Cyanotoxin LC/MS/MS Analysis (MRL < 0.010 µg/L)

Anatoxin-a (ANAA)

Cylindrospermopsin (CYLS)
Deoxycylindrospermopsin (DCYL)

Domoic Acid (DMAC)

Lyngbyatoxin-a (LYGA)

Microcystin-LA (MCLA)
Microcystin-LF (MCLF)
Microcystin-LR (MCLR)
Microcystin-LW (MCLW)
Microcystin -LY (MCLY)
Microcystin-RR (MCRR)
Microcystin-YR (MCYR)

Nodularin-R (NODR)

Okadaic Acid (OKAC)

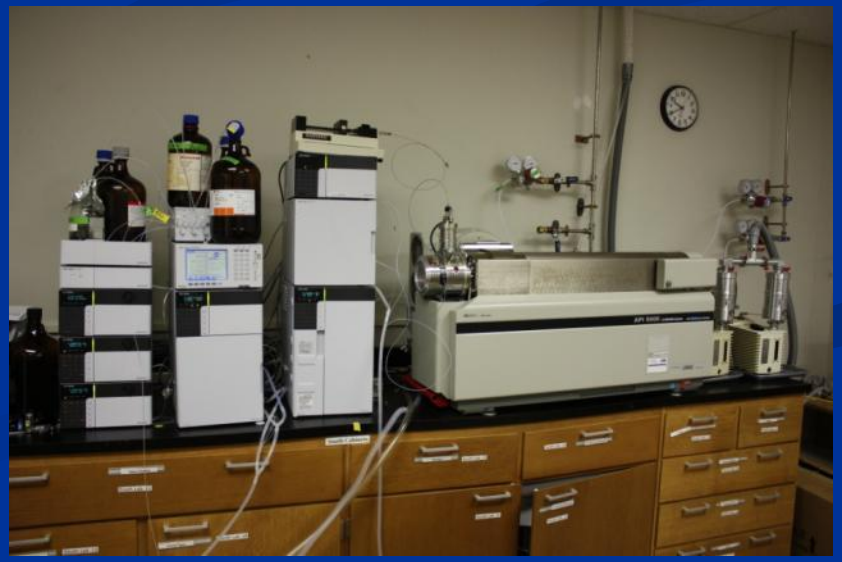
Shimadzu Prominence Liquid Chromatograph

- Direct Injection – 100 µL
- Active / Passive Needle Rinse: 0.1 % THF in MeOH
- Mobile Phase A: 0.1% Formic Acid (aq)
- Mobile Phase B: 0.1% Formic Acid (MeOH)
- Atlantis dC18 150 x 3 mm, 3 µ with guard cartridge

MDS Sciex/Applied Biosystems API 5000

- ES +/- Multiple Reaction Monitoring

Quantitation – Standard Addition at 1 µg/L



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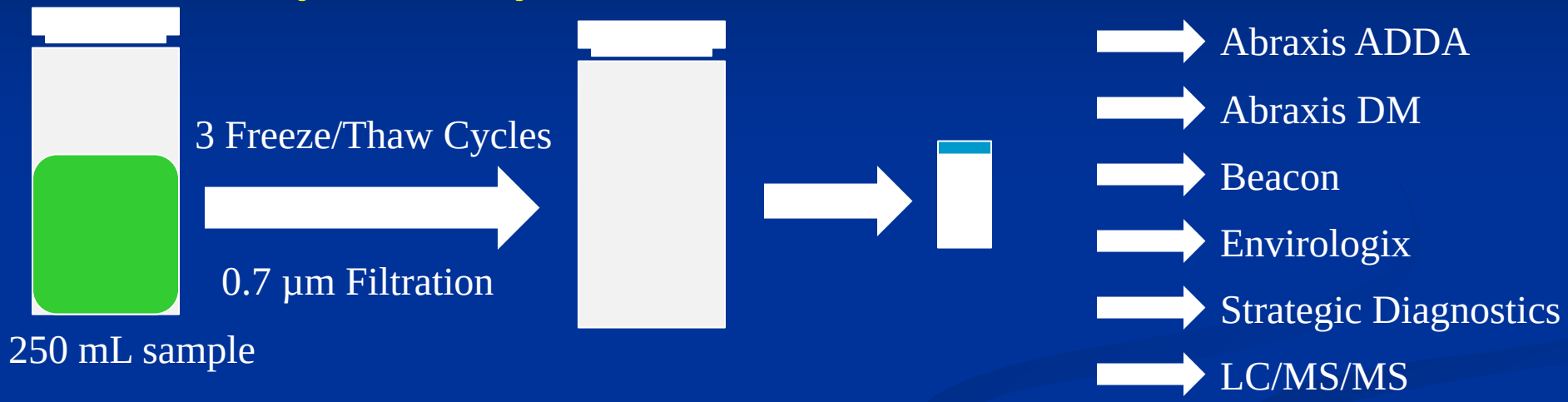
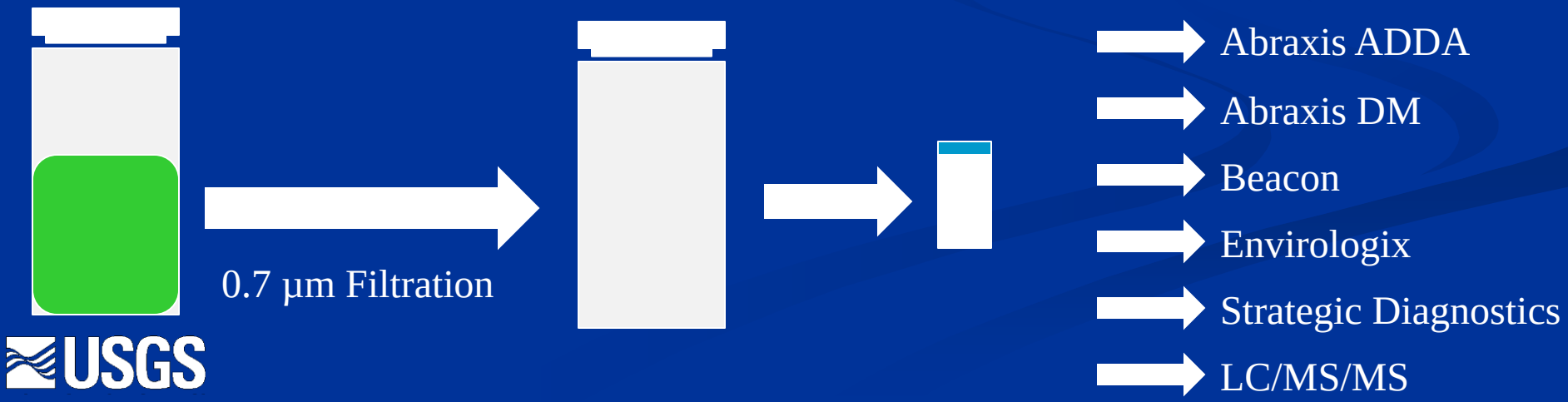
Study Design for Total and Dissolved Microcystin Analysis

Minimization of the Error Associated with Sample Collection and Laboratory Sample Processing.

Total Microcystin Analysis



Dissolved Microcystin Analysis



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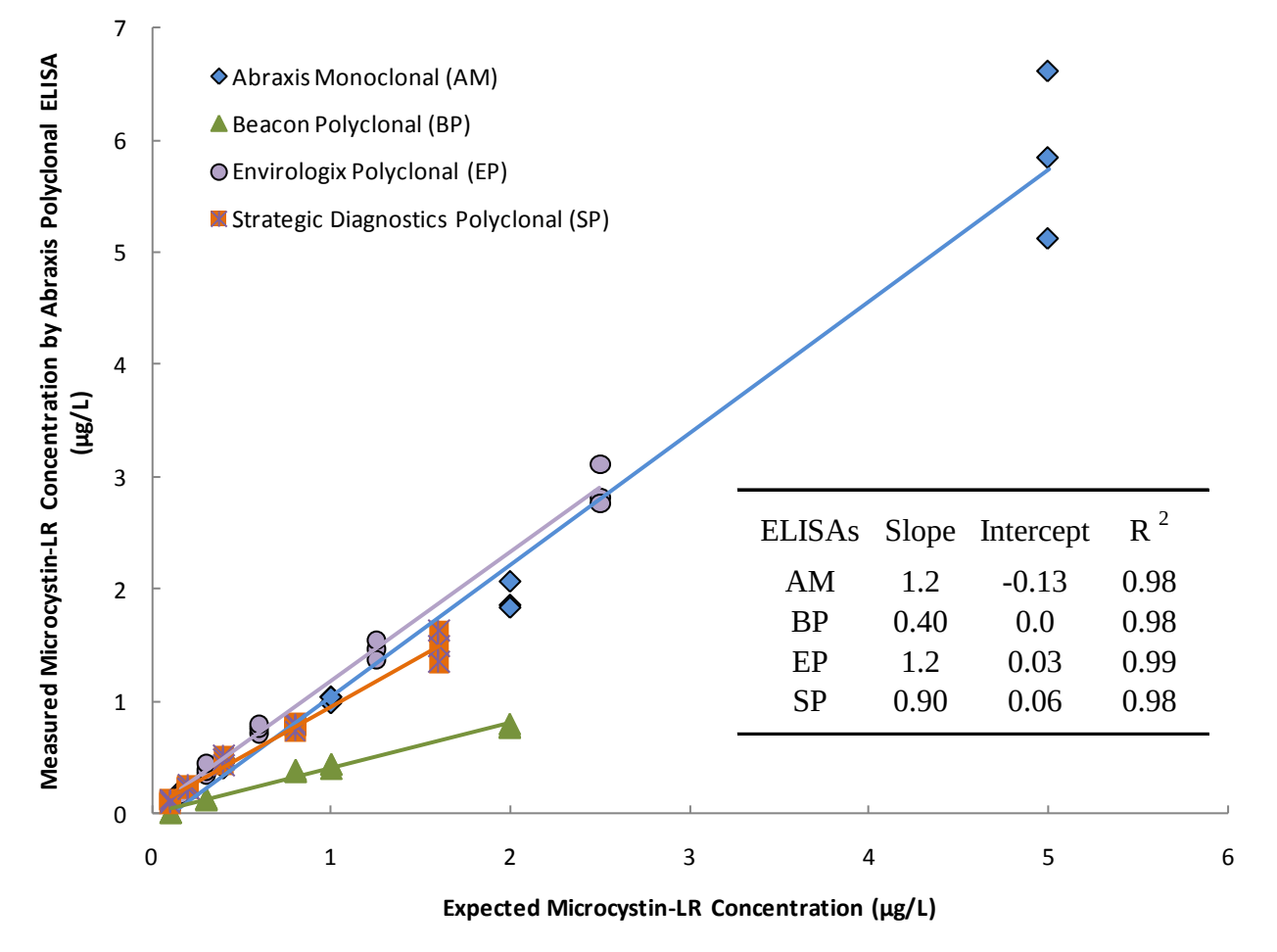
MCLR Standards Provided with the Abraxis-DM, Envirologix, and Strategic Diagnostics ELISAs showed good agreement when Measured by the Abraxis-ADDA ELISA (4-Parameter Curve Fit).



ELISAs	Slope	Intercept	R^2
AM	1.2	-0.13	0.98
BP	0.40	0.0	0.98
EP	1.2	0.03	0.99
SP	0.90	0.06	0.98

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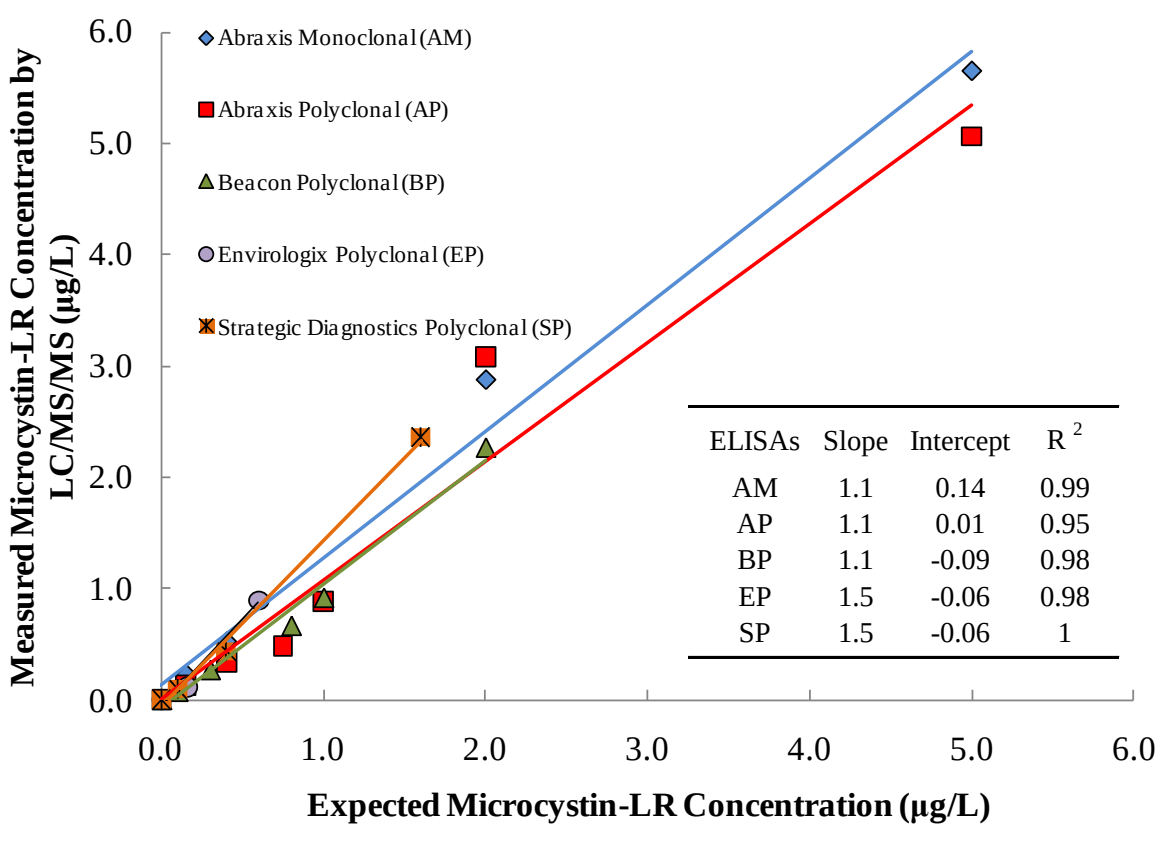
ELISA MCLR Calibration Standards were Microcystin-LR and gave a linear response by LC/MS/MS (Standard Addition).

- MCLR ELISA standards seemed to be pure MCLR
- Both the Envirologix and Strategic Diagnostics Assays had slopes of 1.5.



ELISAs	Slope	Intercept	R^2
AM	1.1	0.14	0.99
AP	1.1	0.01	0.95
BP	1.1	-0.09	0.98
EP	1.5	-0.06	0.98
SP	1.5	-0.06	1

Summary Statistics for LC/MS/MS Analyses of 62 Dissolved and Total Environmental Samples for Cyanotoxins

- **Microcystin-LF and Microcystin-LW, two of the more hydrophobic microcystins, did not frequently occur in the dissolved-phase compared to occurrence in the total-phase.**
- **Implication: Microcystin-LF and Microcystin-LW producers had not lysed or sorption may be an issue…**
- **Based on detection frequency, Microcystin-LR was the dominant congener in dissolved and total analyses, followed by Microcystin-RR.**
- **Based on concentration, Microcystin-RR was frequently equivalent or greater than Microcystin-LR concentrations in individual samples.**
- **<u>This finding is significant because Microcystin-RR is reported to have approximately 10 times less toxicity than Microcystin-LR!</u>**

	Anatoxin-a	Microcystin-LA	Microcystin-LF	Microcystin-LR	Microcystin-LW	Microcystin-LY	Microcystin-RR	Microcystin-YR	Nodularin-R
Dissolved (UnLysed, filtered) toxin samples									
Detections Frequency	19	13	10	74	6.5	10	42	6.5	3.2
Mean (µg/L)	0.53	0.081	0.12	0.87	0.37	0.28	3.1	0.62	0.012
Median (µg/L)	0.025	0.042	0.013	0.065	0.37	0.015	0.13	0.62	0.012
Minimum (µg/L)	0.014	0.010	0.012	0.011	0.010	0.010	0.034	0.040	0.012
Maximum (µg/L)	2.8	0.23	0.35	16	0.73	0.82	38	1.2	0.012
Total (Lysed) toxin samples									
Detections Frequency (%)	32	42	35	81	35	52	61	55	6
Mean (µg/L)	1.1	4.5	4.7	87	5.2	13	870	15.0	0.10
Median (µg/L)	0.080	0.20	0.04	1.0	0.067 [a]	0.10	0.75	0.13 [a]	0.10
Minimum (µg/L)	0.013	0.011	0.017	0.019	0.022	0.015	0.029	0.011	0.011
Maximum (µg/L)	10	54	51	2100	56	200	16000	240	0.19

a The median concentration being lower in concentration in the total toxin samples versus the dissolved toxin samples is due to the much greater detection frequency in the total samples.

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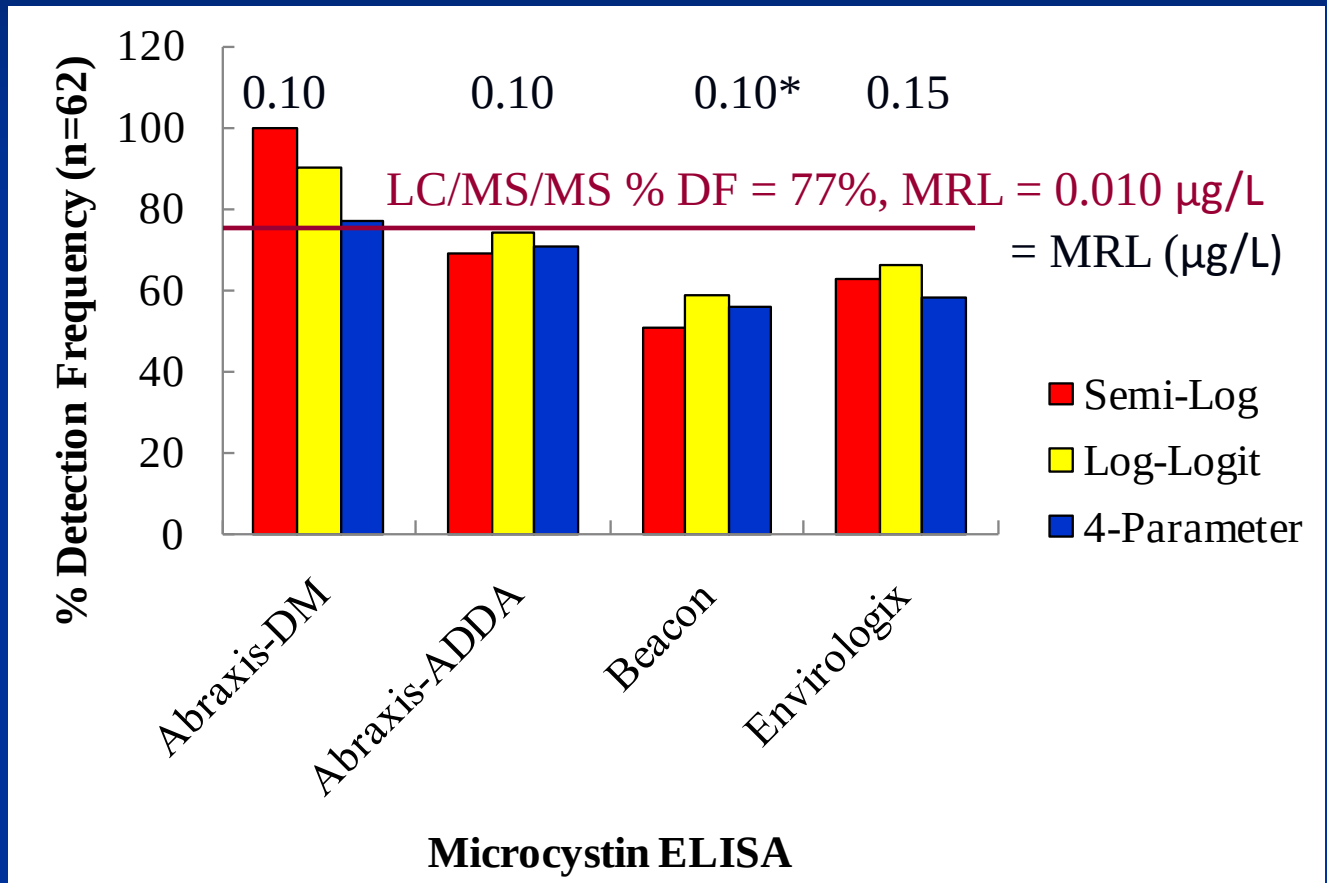
ELISA Calibration Curve Fitting Equations Can Impact Detection Frequency (DF) for Environmental Samples

- The Abraxis-DM ELISA exhibited the largest spread when comparing detection frequency between curve fits (77 to 100% - %RSD=13%).
- The Abraxis-ADDA ELISA appeared to be the least sensitive to curve fit based on detection frequency.
- The Abraxis-ADDA and Abraxis-DM ELISA (4-Parameter) appeared to agree most closely with LC/MS/MS given the 8 congeners measured and a ten times lower MRL.
- The Strategic Diagnostics assay did not meet QA/QC criteria for precision and accuracy for control standards and replicate analyses.



•Beacon does not have a manufacturer derived MRL. 0.10 used for Beacon MRL since low standard was 0.10 µg/L.

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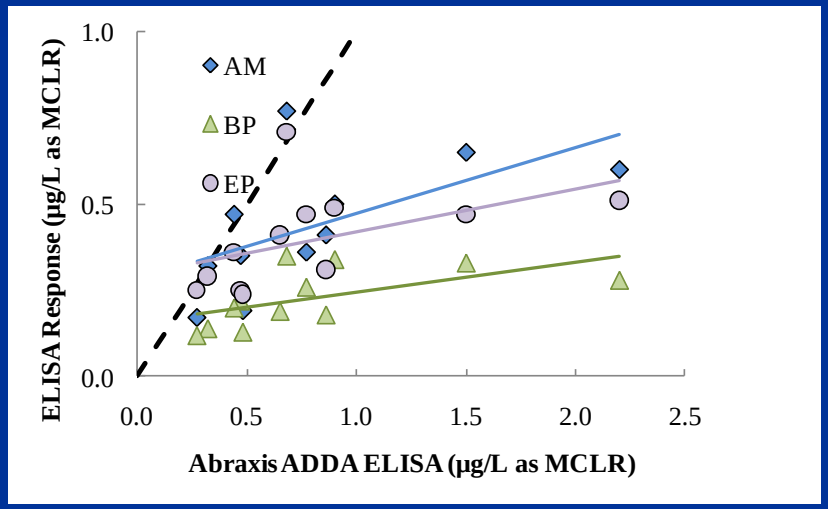
Microcystin ELISA Comparison
Correlations with Dissolved and Total Microcystin Detections

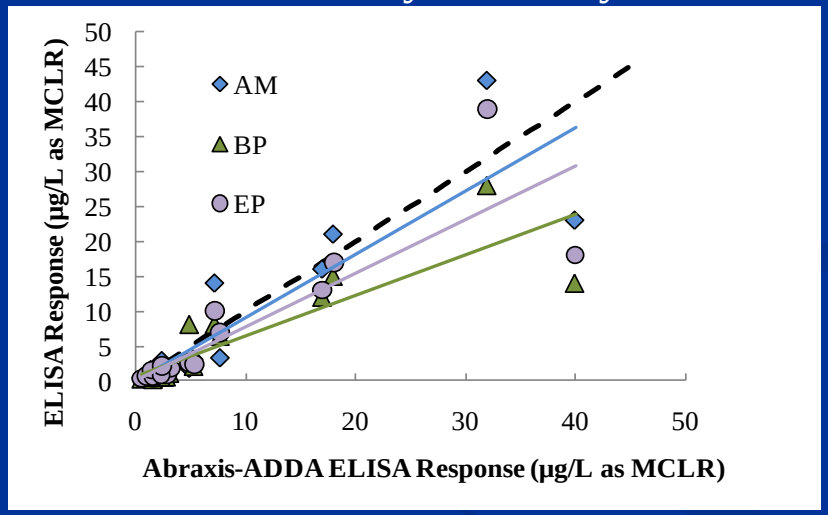
- It would appear that the Abraxis-ADDA ELISA does measure something not measured by other kits in many cases that is most noticeable in the dissolved-phase. Not sure what this is yet…
- The Beacon ELISA generally responded with lower values compared to the other ELISAs based on evaluation of regressed slopes **for this set of enviromental samples.**

Dissolved Microcystin Analyses



ELISA	Slope	Intercept	R^2
AM	0.19	0.28	0.35
BP	0.085	0.16	0.33
EP	0.12	0.30	0.23

Total Microcystin Analyses



ELISA	Slope	Intercept	R^2
AM	0.91	-0.072	0.79
BP	0.59	0.53	0.76
EP	0.77	0.051	0.77

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Abraxis-DM (AM), Beacon (BP), Envirologix (EP)

How Do We Compare ELISA and LC/MS/MS Results?

- ELISA and LC/MS/MS detect microcystins by completely different mechanisms.
- Therefore we shouldn't expect results from each technology to be comparable typically without some type of unit conversion or equivalence being introduced.
- Microcystin ELISA calibration curves and all results are based upon µg/L microcystin-LR equivalents.
- Equivalents have a definitive meaning in analytical chemistry that is generally discussed when anions and cations are being measured and converted to other units (eg. ammonia as N, etc.).
- ELISA MCLR equivalents also have cross-reactivity incorporated into the result for each respective ELISA.
- LC/MS/MS calibration and results are typically reported in units of µg/L of a particular compound.; each compound has its own standard curve (eg. Microcystin-RR is not reported in units of Microcystin-LR, but as Microcystin-RR).

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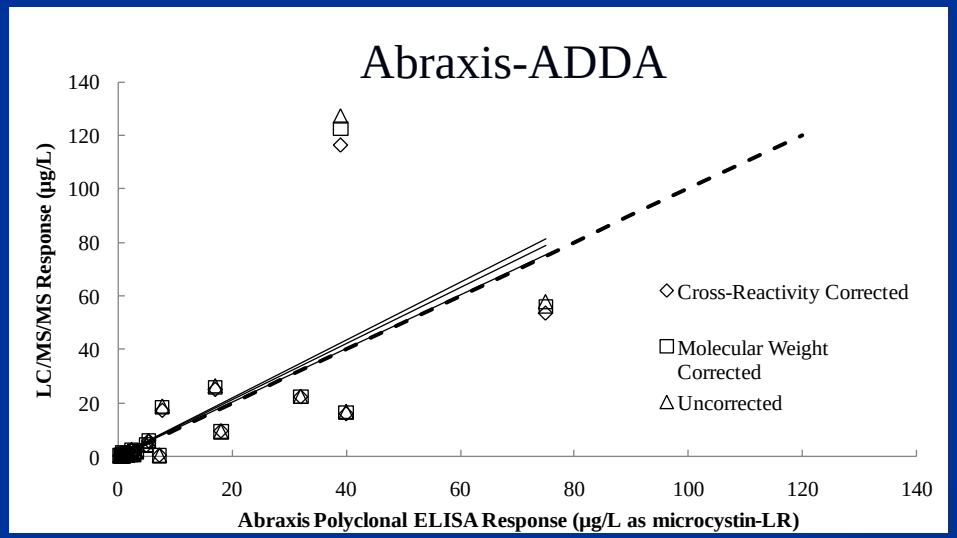
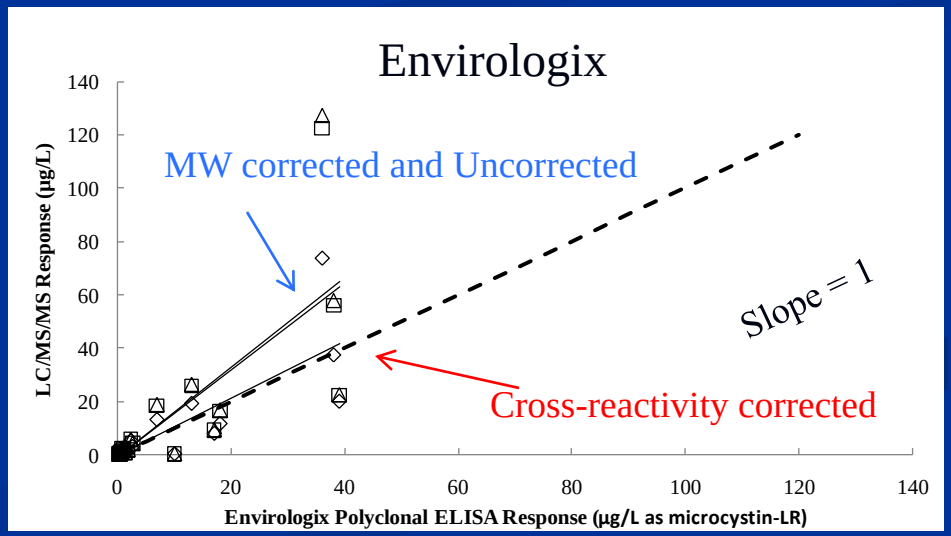
ELISA and LC/MS/MS Measure Different Characteristics:

Do units matter and can the results of both techniques be compared?

- Microcystin Concentration Comparisons:
  - 1. Uncorrected LC/MS/MS data ≠ ELISA… Exception may be the Abraxis-ADDA ELISA
  - 2. MW corrected LC/MS/MS data ≠ ELISA… Exception may be the Abraxis-ADDA ELISA
  - 3. Cross-reactivity corrected LC/MS/MS compares most favorably with all of the ELISAs

Sum Microcystin Concentration (LC/MS/MS) = Σ (Conc. MC $_{LC/MS/MS}$ x Cross-reactivity)





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	Slope [a]	Intercept [a]	R^2 [a]
Abraxis Monoclonal [b]			
Cross-Reactivity	0.95	0.061	0.61
Molecular Weight	1.4	-0.27	0.54
Uncorrected	1.5	-0.31	0.53
Abraxis Polyclonal [b]			
Cross-Reactivity	1.0	0.47	0.52
Molecular Weight	1.0	0.44	0.51
Uncorrected	1.1	0.41	0.51
Beacon Polyclonal [c]			
Cross-Reactivity	1.2	1.0	0.56
Molecular Weight	1.3	1.1	0.56
Uncorrected	1.4	1.1	0.55
Envirologix Polyclonal [c]			
Cross-Reactivity	1.1	-0.23	0.69
Molecular Weight	1.6	-0.79	0.61
Uncorrected	1.7	-0.85	0.60

a Slopes, intercepts, and correlation coefficients (R^2) were determined by linear regression using Excel v. 7.0.
b Cross-reactivity for microcystin-LY was not available and therefore was not incorporated into the calculation for the expected slope.
c Cross-reactivities for microcystin-LF, -LW, and -LY were not available and therefore was not incorporated into the calculation for the expected slope.

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Comparison of Results Between ELISA and LC/MS/MS: Units do Matter!!! Usually….

- All ELISAs have unique cross-reactivity patterns that are in relation to the calibration standard (microcystin-LR); ELISA measurements are proportional to the sum of the quantity of the cross-reactivity for each known congener multiplied by its actual concentration (not microcystin-LR equivalent).
- For direct comparison of chromatographic data to ELISA data we have to cross-reactivity correct the chromatographic data to get the data into similiar units.
- If matrix effects are impacting the LC/MS/MS data, cross-reactivity correction will not be sufficient for comparison. It is important to quantitate LC/MS/MS data using standard addition or isotope dilution if matrix effects are encountered.
- Generally, we found cross-reactivity correction of our chromatographic data greatly improved the correlation to ELISA data.
- The Abraxis-ADDA ELISA was the exception, since the cross-reactivity for each microcystin congener was fairly similar.
- Considerations and problems:
  - Cross-reactivity used for chromatographic data correction must be derived for each specific ELISAs' cross-reactivity pattern.
  - Not all ELISAs have the same number of cross-reactivities determined.
  - Standards may not be available in sufficient quantity and purity to determine cross-reactivities "in house".
  - This approach may not be practical in all cases and its usefulness will also be dependent on sample microcystin/nodularin composition.
  - Deviations greater than the error between measurement techniques may indicate a potentially toxic compound not measured by the chromatographic technique.

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Conclusions…
Most Things Depend on Sample Composition.

- Adequate precision is readily achievable with in +/- 20%, given error associated with sample collection, splitting, and laboratory processing are eliminated.
- ELISA accuracy is dependent on which microcystin / nodularin congeners are present in samples and how similar their cross-reactivities are with that of Microcystin-LR for a given ELISA.
- The Abraxis-ADDA can yield quite different results from other ELISAs, but surprisingly did not always yield the highest concentrations.
- The Beacon Assay typically yielded the lowest concentrations.
- Adequate comparisons can be made between a given ELISA and LC/MS/MS given:
  - Cross-reactivities are available for detected congeners
  - Cross-reactivity corrected chromatographic data is used
  - LC/MS/MS matrix effects do not complicate issues
  - Or an ELISA with approximately equal cross-reactivities is used
- Comparisons of results from studies using different methods should not be overinterpreted!

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Additional Information Available on the Web:

USGS KS Algal Toxin Research (http://ks.water.usgs.gov/Kansas/studies/qw/cyanobacteria)
Organic Geochemistry Research Laboratory (http://ks.water.usgs.gov/researchlab)
Toxic Substance Hydrology Program (http://toxics.usgs.gov/regional/emc)

USGS cyanotoxin sampling protocol: (http://pubs.usgs.gov/sir/2008/5038)
USGS OFR on Comparison of Two Cell Lysis Techniques: (http://pubs.usgs.gov/of/2008/1341/)

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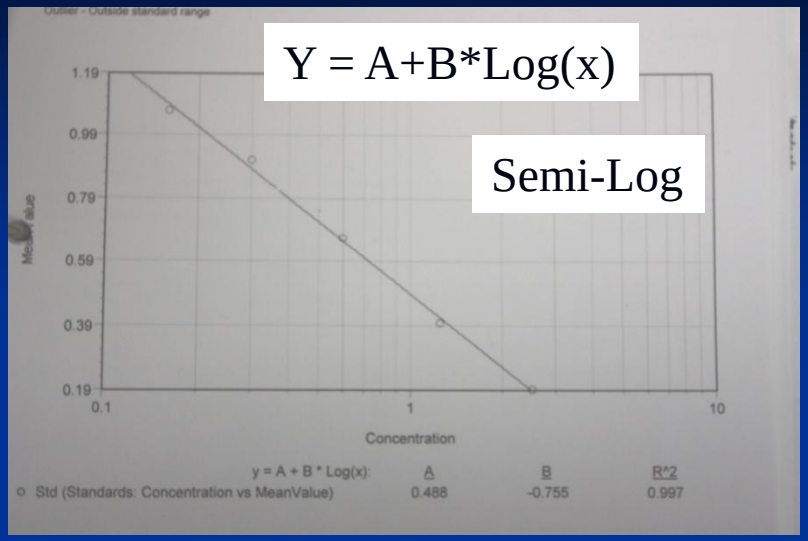
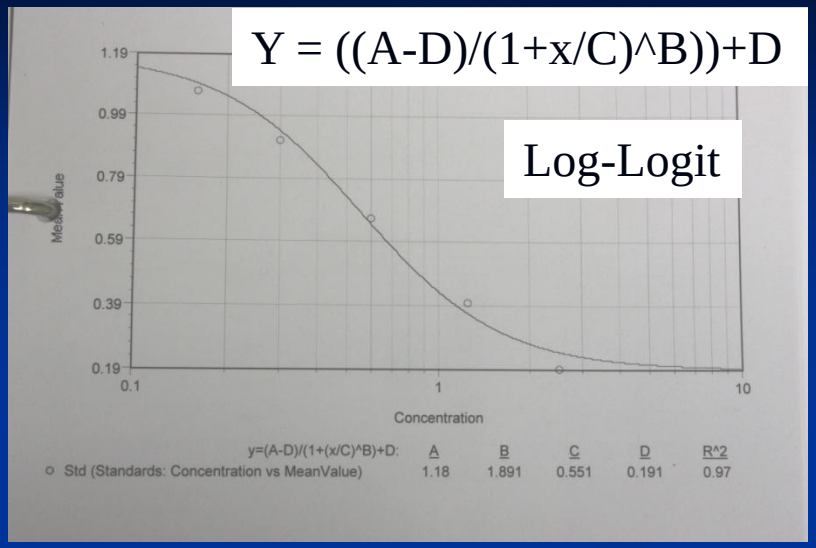
%RSD for all three ELISA fits for detection frequency

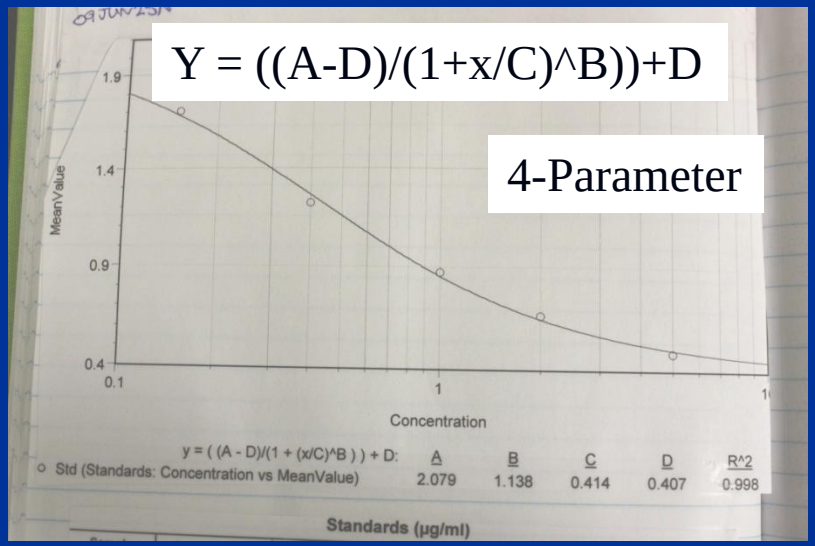
	Four-Parameter	Log-Logit	Semi-Log	LC/MS/MS	
Abraxis-DM					
Non-detects	14	6	0	14	
Detection Frequency (%)	77	90	100	77	12.9579
Average %RSD of Replicates	6.0	9.4	4.9	11	
Slope	--	1.1	0.84	--	
y-intercept	--	-0.19	0.50	--	
R^2	--	0.66	0.74	--	
Abraxis-ADDA					
Non-detects	18	16	19	--	
Detection Frequency (%)	71	74	69	--	3.52796
Average %RSD of Replicates	8.0	8.7	7.8	--	
Slope	--	1.0	1.2	--	
y-intercept	--	0.14	-0.054	--	
R^2	--	0.95	0.99	--	
Beacon					
Non-detects	27	25	30	--	
Detection Frequency (%)	56	59	51	--	7.30383
Average %RSD of Replicates	5.5	6.5	6.5	--	
Slope	--	0.60	0.70	--	
y-intercept	--	1.3	0.84	--	
R^2	--	0.80	0.88	--	
Envirologix					
Non-detects	26	21	23	--	
Detection Frequency (%)	58	66	63	--	6.48361
Average %RSD of Replicates	8.3	9.4	9.0	--	
Slope	--	0.75	0.83	--	
y-intercept	--	0.65	0.27	--	
R^2	--	0.86	0.87	--	

*** Of the 32 samples with detections evaluated by the Strategic Diagnostics assay, 6.3% (four-parameter) and 22% (log-logit and semi-log) of duplicate measures exceeded the 28.3% RSD precision criteria (± 20% of mean or expected value). Of samples exceeding the 28.3% RSD criteria for duplicates, mean, minimum, and maximum %RSD were 69, 55, and 83 % (four-parameter); 76, 30, and 130% (log-logit), and 79, 29, 130 % (semi-log).**

ELISA Curve Fits







- Semi-Log curve fits can be done on semi-log paper by hand and was commonly employed prior to computers.
- Log-Logit assumes that upper and lower limb have a slope of “0” and force shape of the curve.
- 4-parameter does assume shape of the curve, but uses all standards to define the shape of the curve through an iterative process until convergence.
- 4-parameter fits require at least five calibration levels to have sufficient degrees of freedom to solve unknowns in an equation.

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