Directly testing the linearity assumption for assay validation

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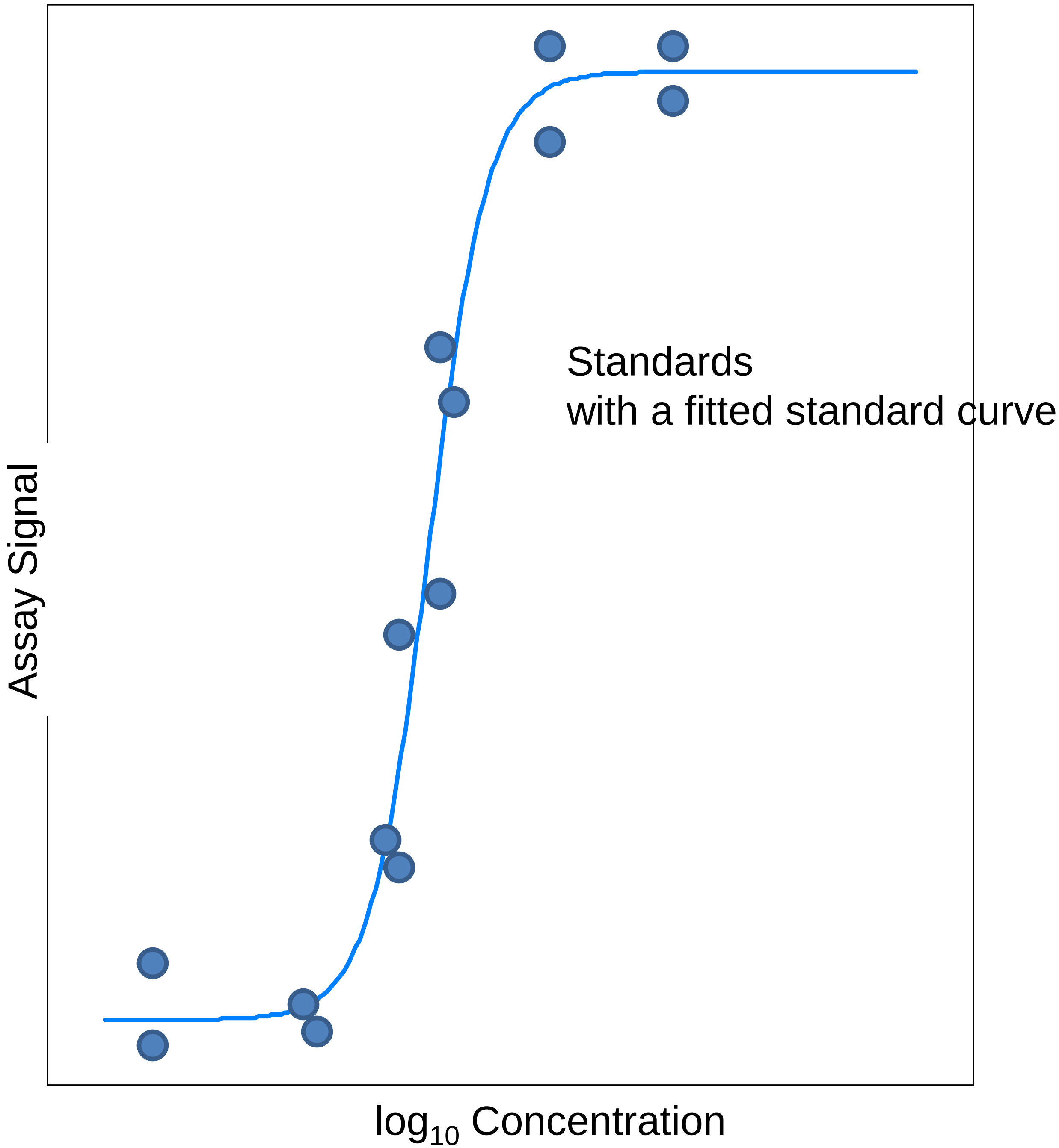
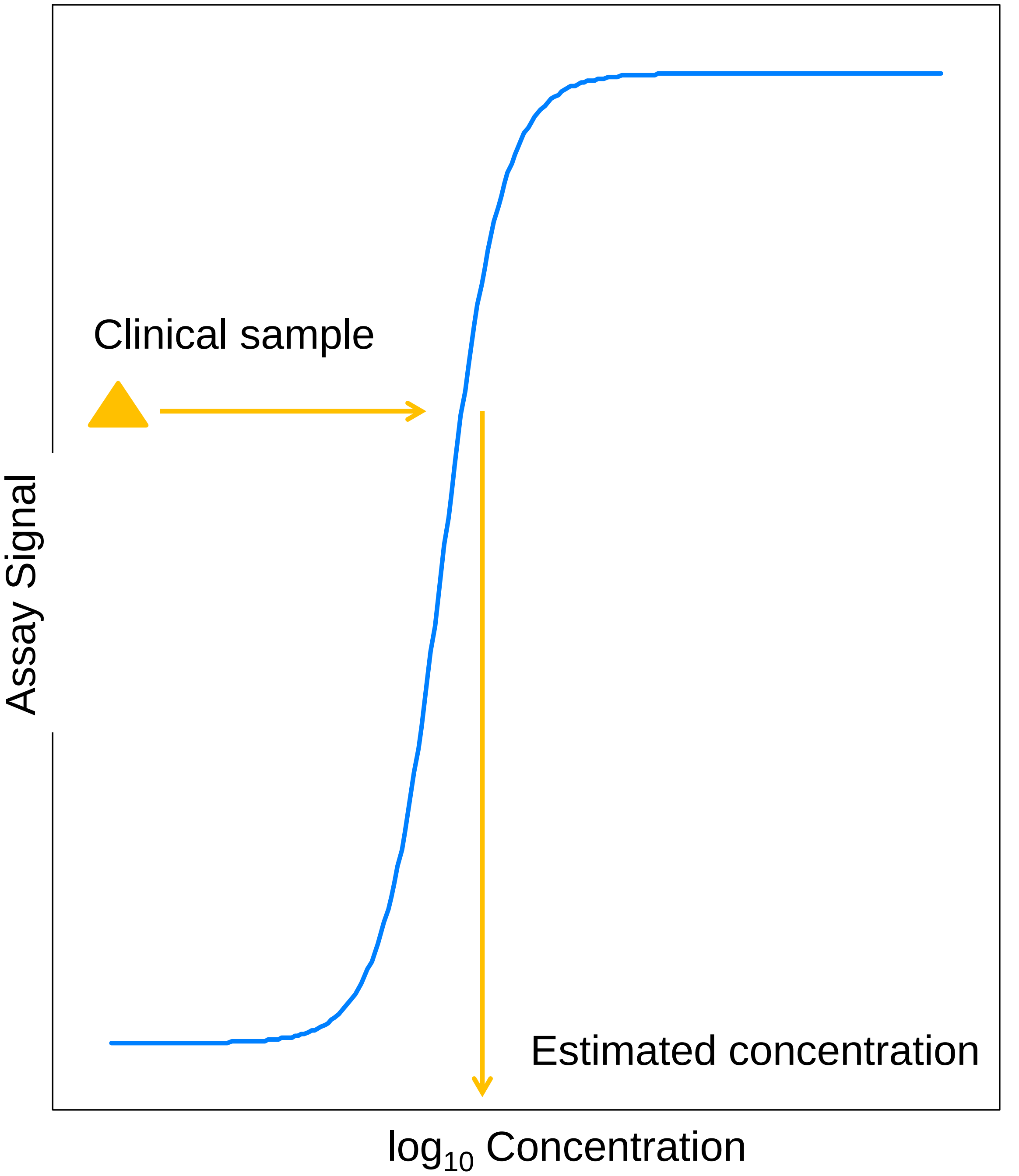
Purpose

- Illustrate a novel method to test for linearity in an analytical assay.

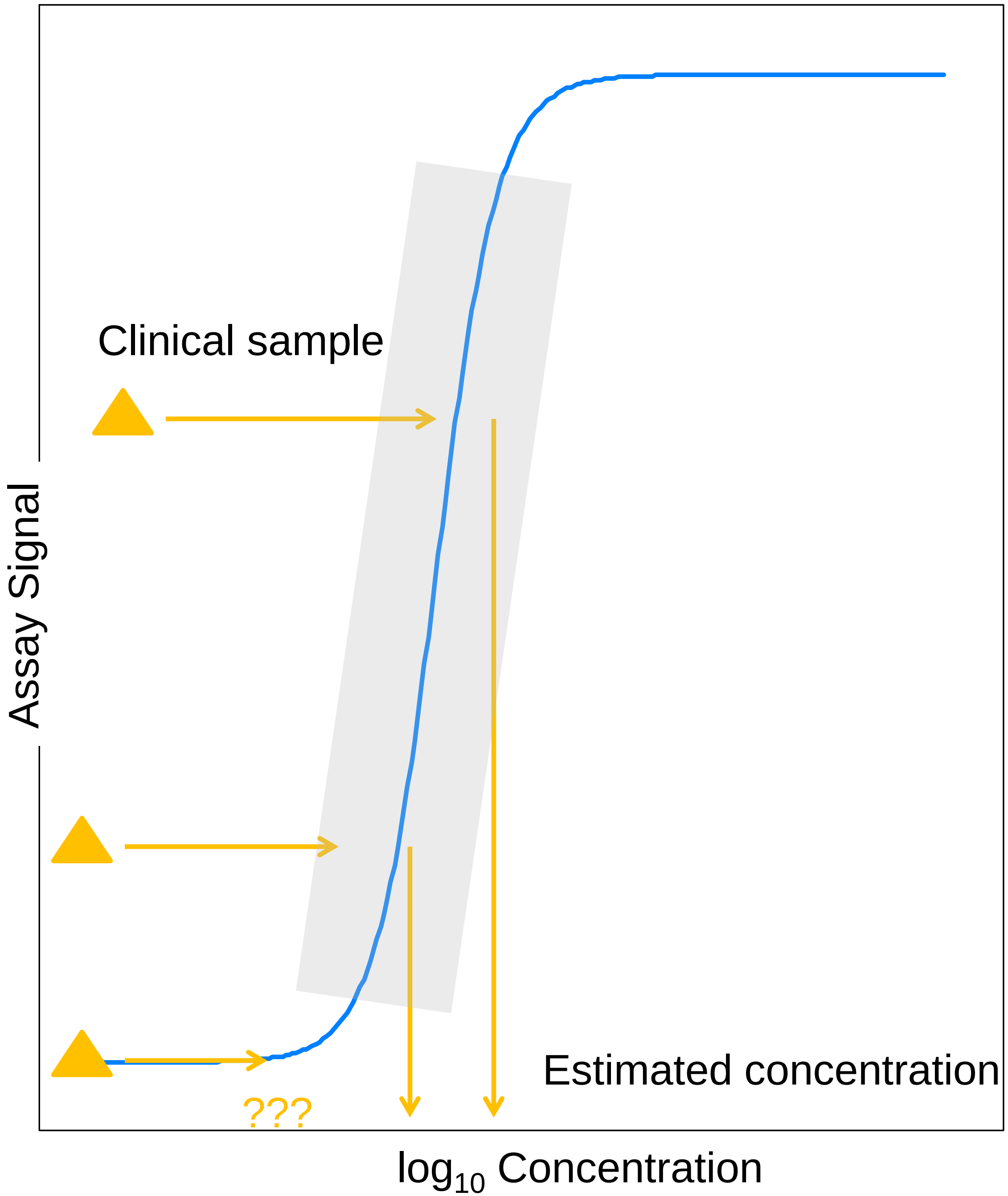
Analytical assay – standard curve

- We wish to measure the concentration of an analyte (e.g., a protein) in clinical sample.
- Standards = known concentrations of an analyte.
- To estimate the concentration, we create a standard curve.


Standards
with a fitted standard curve
Assay Signal
$\log_{10}$ Concentration


Clinical sample
Estimated concentration
Assay Signal
$\log_{10}$ Concentration

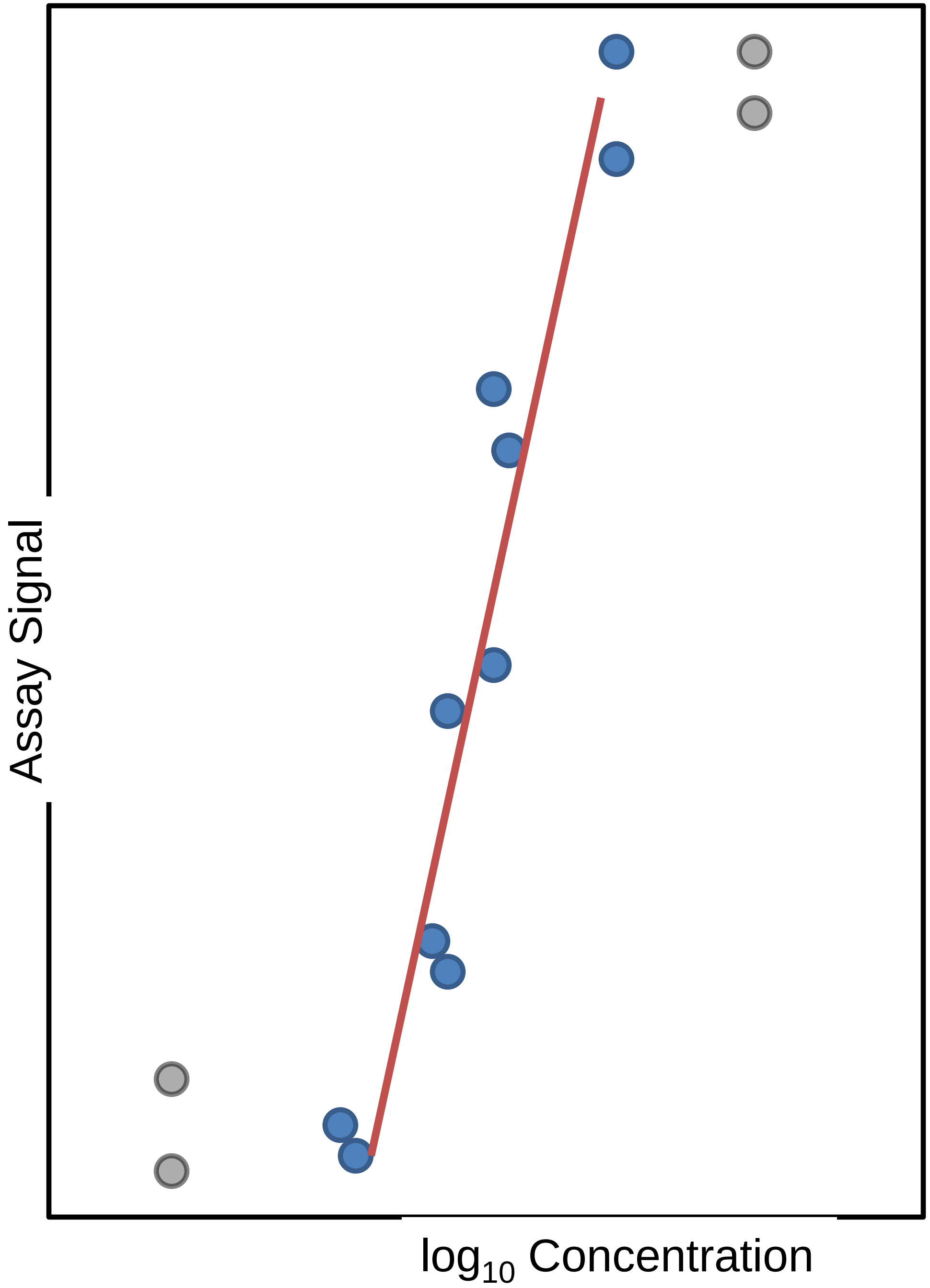
To many, the only interest lies in the linear portion of the curve.



ICH Q2(R1) guideline

- http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf

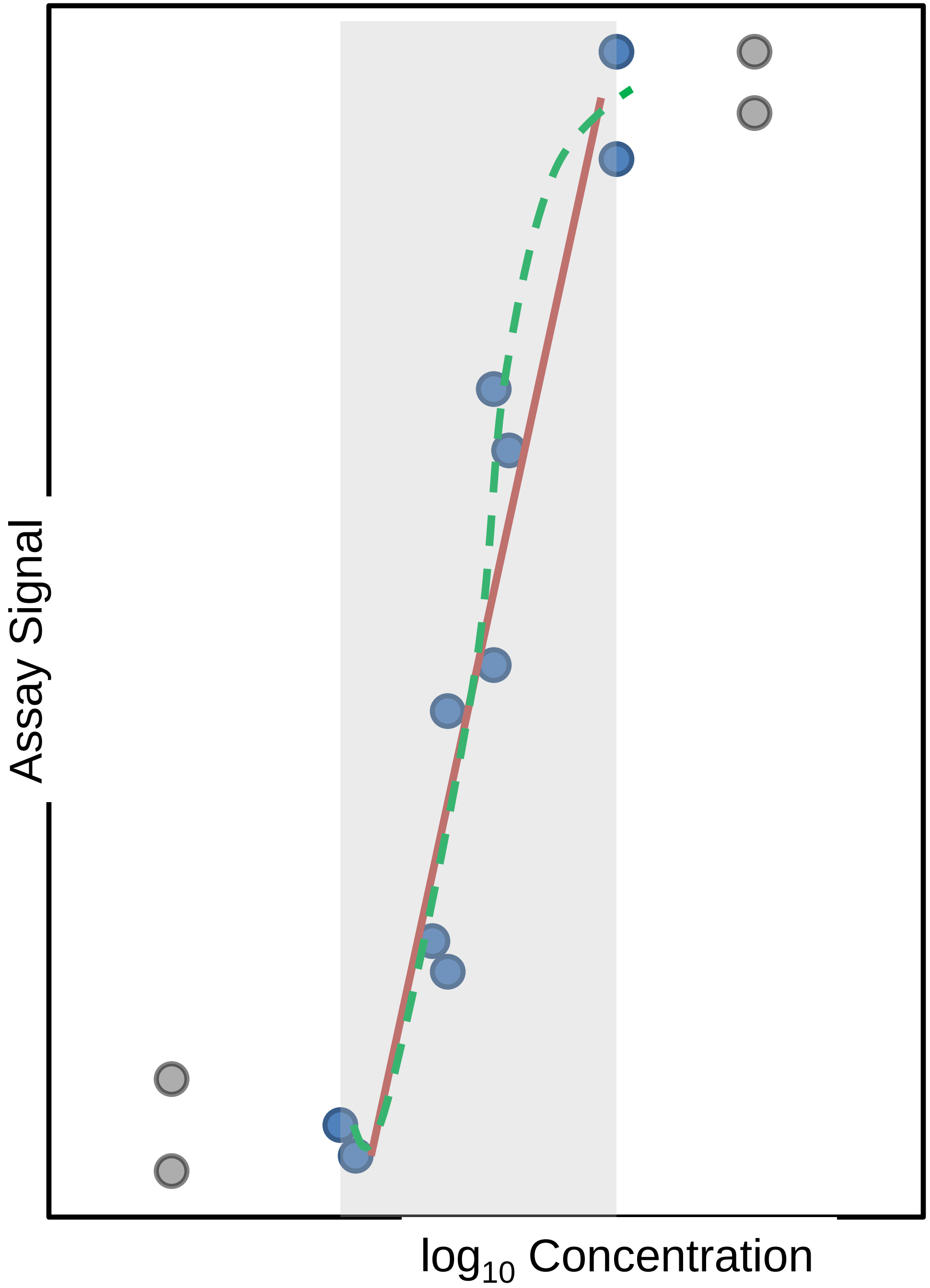
- Evaluate linearity by visual inspection



Assay Signal
log$_{10}$ Concentration

The EP6-A guidelines

- Clinical and Laboratory Standards Institute
  - http://www.clsi.org/source/orders/free/ep6-a.pdf
- Compare straight-line to higher-order polynomial curve fits
  - Recommendation: Test higher-order coefficients.


Assay Signal
$\log_{10}$ Concentration

Notation

$$E[\ Y_{ij} \mid X_i\ ] = a_1 + b_1 X_i \qquad = g_1(X_i)$$

$$E[\ Y_{ij} \mid X_i\ ] = a_2 + b_2 X_i + c_2 X_i^2 \qquad = g_2(X_i)$$

$$E[\ Y_{ij} \mid X_i\ ] = a_3 + b_3 X_i + c_3 X_i^2 + d_3 X_i^3 \qquad = g_3(X_i)$$

- Assume IID normally distributed errors with equal variance.

$i = 1, 2, \ldots, L$ concentrations

$j = 1, 2, \ldots, n_i$ replicates

Orthogonal polynomials

- The orthogonal polynomial of degree *k* is of the form

$$\sum_{r=0}^{k} \theta_r f_r(x),$$

θ_r = (k+1) constants (to be estimated)

$f_r(x)$ = (k+1) orthogonal polynomials

x = concentration

See: Robson, 1959

Orthogonal polynomials properties

$$\sum_{i=1}^{L} n_i f_r(X_i) = 0$$

$$\sum_{i=1}^{L} n_i f_r(X_i) f_s(X_i) = 0$$

$$\sum_{i=1}^{L} n_i f_r^2(X_i) = 1$$ ortho-normal property

See: Robson, 1959

OLS: orthogonal polynomials

$$E\lfloor \boldsymbol{Y} \rfloor = \boldsymbol{F}\boldsymbol{\theta} \qquad Var[\boldsymbol{Y}] = \sigma^2 \boldsymbol{I}_N,$$ N=total sample size

$$\boldsymbol{Y} = \begin{pmatrix} Y_{11} \\ Y_{12} \\ \vdots \\ Y_{1n_1} \\ \vdots \\ Y_{L1} \\ Y_{L2} \\ \vdots \\ Y_{Ln_L} \end{pmatrix}, \qquad \boldsymbol{F} = \begin{pmatrix} f_0(X_1) & f_1(X_1) & \cdots & f_k(X_1) \\ \vdots & \vdots & & \vdots \\ f_0(X_{Ln_L}) & f_1(X_{Ln_L}) & & f_k(X_{Ln_L}) \end{pmatrix}, \qquad \boldsymbol{\theta} = \begin{pmatrix} \theta_0 \\ \theta_1 \\ \vdots \\ \theta_k \end{pmatrix}$$

$$\hat{\boldsymbol{\theta}} = \boldsymbol{F}^T \boldsymbol{Y}$$ and $$Var[\hat{\boldsymbol{\theta}}] = \sigma^2 \boldsymbol{I}_{k+1}.$$

$$\widehat{\boldsymbol{\theta}}[\text{cubic}] = \begin{pmatrix} f_0(X_1) & f_0(X_2) & \cdots & f_0(X_{Ln_L}) \\ f_1(X_1) & f_1(X_2) & \cdots & f_1(X_{Ln_L}) \\ f_2(X_1) & f_2(X_2) & \cdots & f_2(X_{Ln_L}) \\ f_3(X_1) & f_3(X_2) & \cdots & f_3(X_{Ln_L}) \end{pmatrix} \quad \mathbf{Y}$$

$$\mathbf{F}^{\mathbf{T}}$$

$$\widehat{\boldsymbol{\theta}}[\text{linear}] = \begin{pmatrix} f_0(X_1) & f_0(X_2) & \cdots & f_0(X_{Ln_L}) \\ f_1(X_1) & f_1(X_2) & \cdots & f_1(X_{Ln_L}) \end{pmatrix} \quad \mathbf{Y}$$

Intercept and slope estimates are same for both!

Literature

- Krouwer and Schlain (1993)
  - Assume linearity, except at last concentration
  - Ha: μ_{max} - $(a_1 + b_1 X_{max}) \neq 0$

Assumes linearity?

- EP6-A
  - Ha: One or both of c_3, $d_3 \neq 0$

Wrong power profile

  - Ha: $\left| g_k\left(X_i\right) - g_1\left(X_i\right) \right| < \delta$ for all i = 1, 2, …, L

Tested without use of inference

- Kroll et al. (2000) $\sum_{i=1}^{L} \left\{ g_k\left(X_i\right) - g_1\left(X_i\right) \right\}^2 / X_i$
  - Composite statistic, ADL
  - Ha: ADL > λ

Wrong power profile
Difficult to choose λ

More literature

Choice of concentrations?

- Hsieh and Liu (2008)
  - Ha: I-U tests $\left|g_k\left(X_i\right)-g_1\left(X_i\right)\right|<\delta$ for all *i* = 1, 2, ..., *L*

Difficult to choose ψ
Generally $\psi \ll \delta^2$!

- Hsieh, Hsiao, and Liu (2009)
  - Composite statistic, $SSDL = L^{-1}\sum_{i=1}^{L}\left\{g_k\left(X_i\right)-g_1\left(X_i\right)\right\}^2.$
  - Ha: SSDL < ψ
  - Generalized pivotal quantity (GPQ) method

Our proposed hypothesis

- Ha: $\left|g_k(x) - g_1(x)\right| < \delta$ for all $x \in [x_L, x_U]$

- Similar to I-U testing, but instead of individual concentrations, performed across a range of interesting concentrations.

- Bayesian(or GPQ) methods.
  - Linear models
  - Test is function of linear contrast

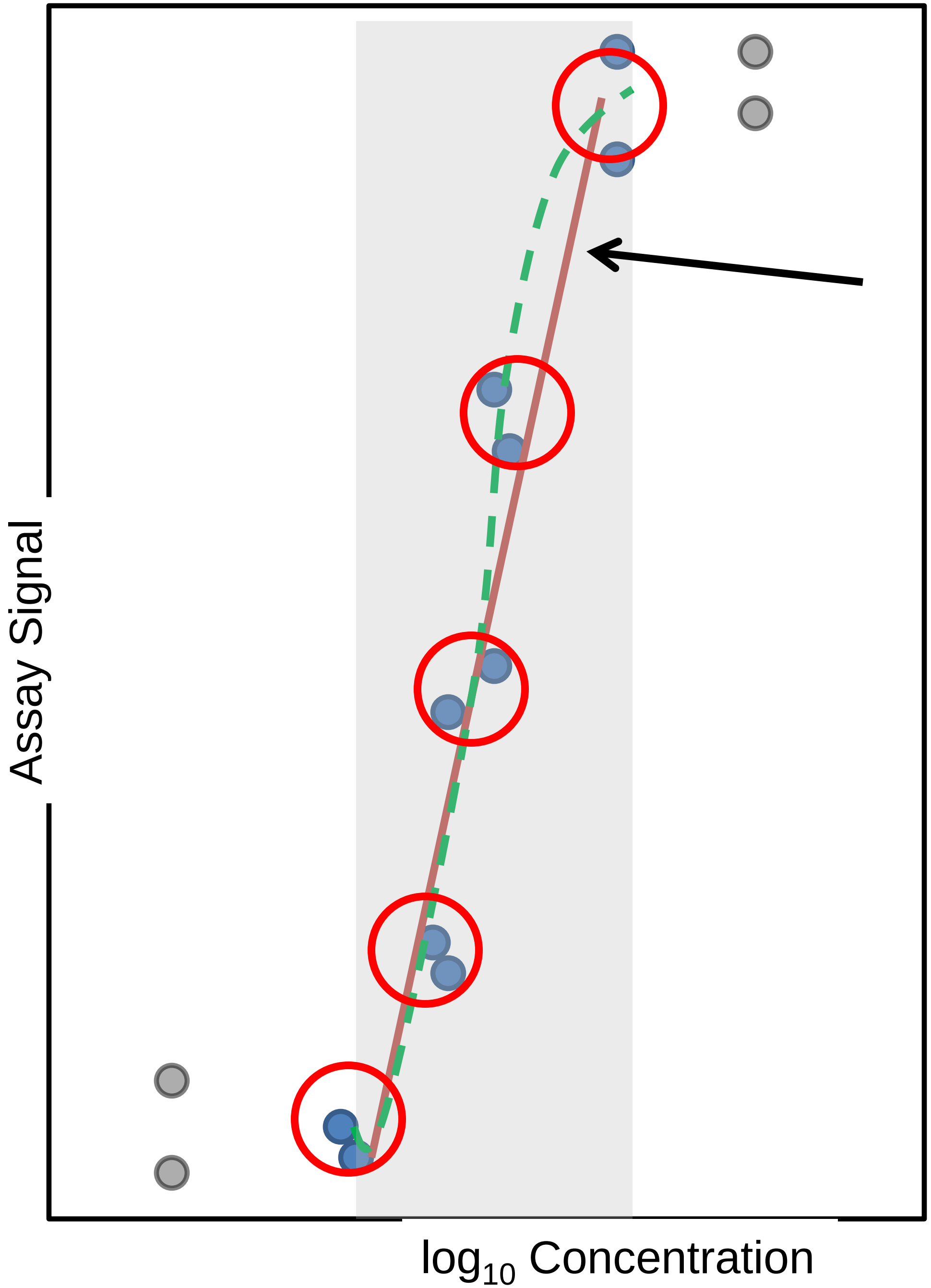
Our proposed test statistic

$$\left|g_k(x) - g_1(x)\right| < \delta \quad \text{for all} \quad x \in [x_L, x_U]$$

$$\Rightarrow \quad \max_{x \in [x_L, x_U]} \left|\sum_2^k \theta_r f_r(x)\right| < \delta$$

$$p(\delta, x_L, x_U) = Pr\left\{ \max_{x \in [x_L, x_U]} \left|\sum_{r=2}^k \theta_r f_r(x)\right| < \delta \,\middle|\, data \right\}$$

- Accept linearity if $p > p_0$ (e.g., $p > 0.9$).


Assay Signal
log10 Concentration

How to: with Jeffrey's prior (or GPQ)

- Given **Y** (responses) and **F** (orth poly design matrix). Assume a kth-degree polynomial.

- Let $\hat{\theta}$ and $\hat{\sigma}$ be OLS estimates
- Error degrees of freedom = N-(k+1)

- Generate two random variables

$Z \sim N_{k+1}(\mathbf{0}, \mathbf{I})$

$U \sim \chi^2(N\text{-}k\text{-}1)$

$$\theta_{Bayes} = \hat{\theta} - \hat{\sigma} \frac{Z}{\sqrt{U / (N-k-1)}}$$

Generate "B" of these

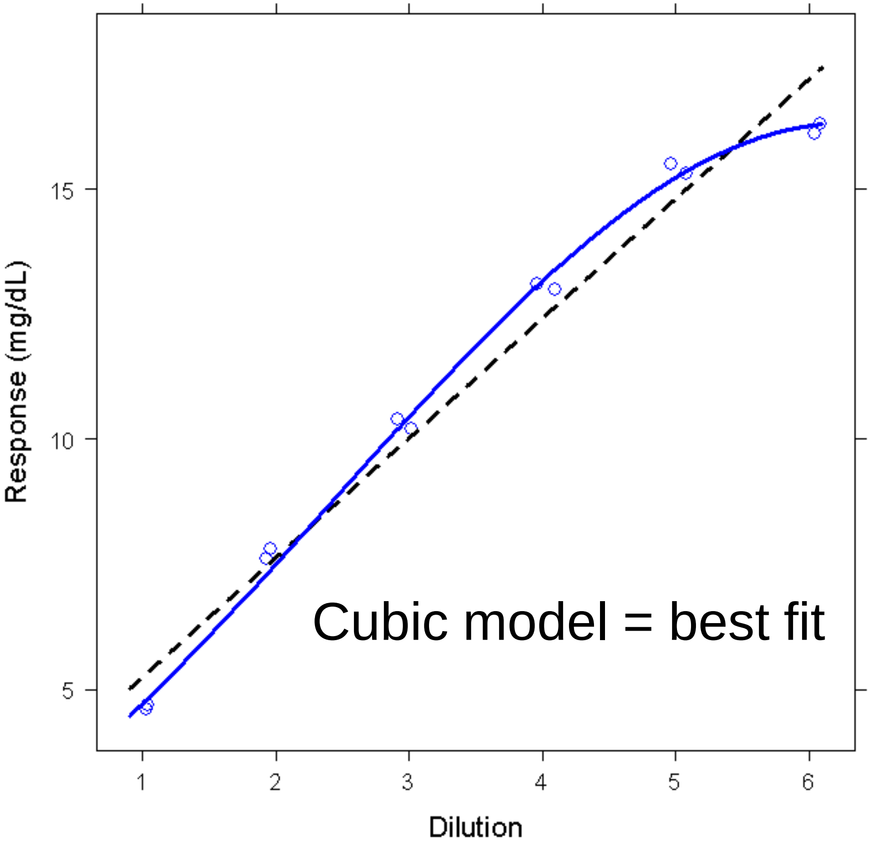
To estimate $p(\delta, x_L, x_U)$,
count the proportion of times:

$$\max_{x \in [x_L, x_U]} \left| \sum_{2}^{k} \theta_{Bayes,r} f_r(x) \right| < \delta$$

Calcium Assay example

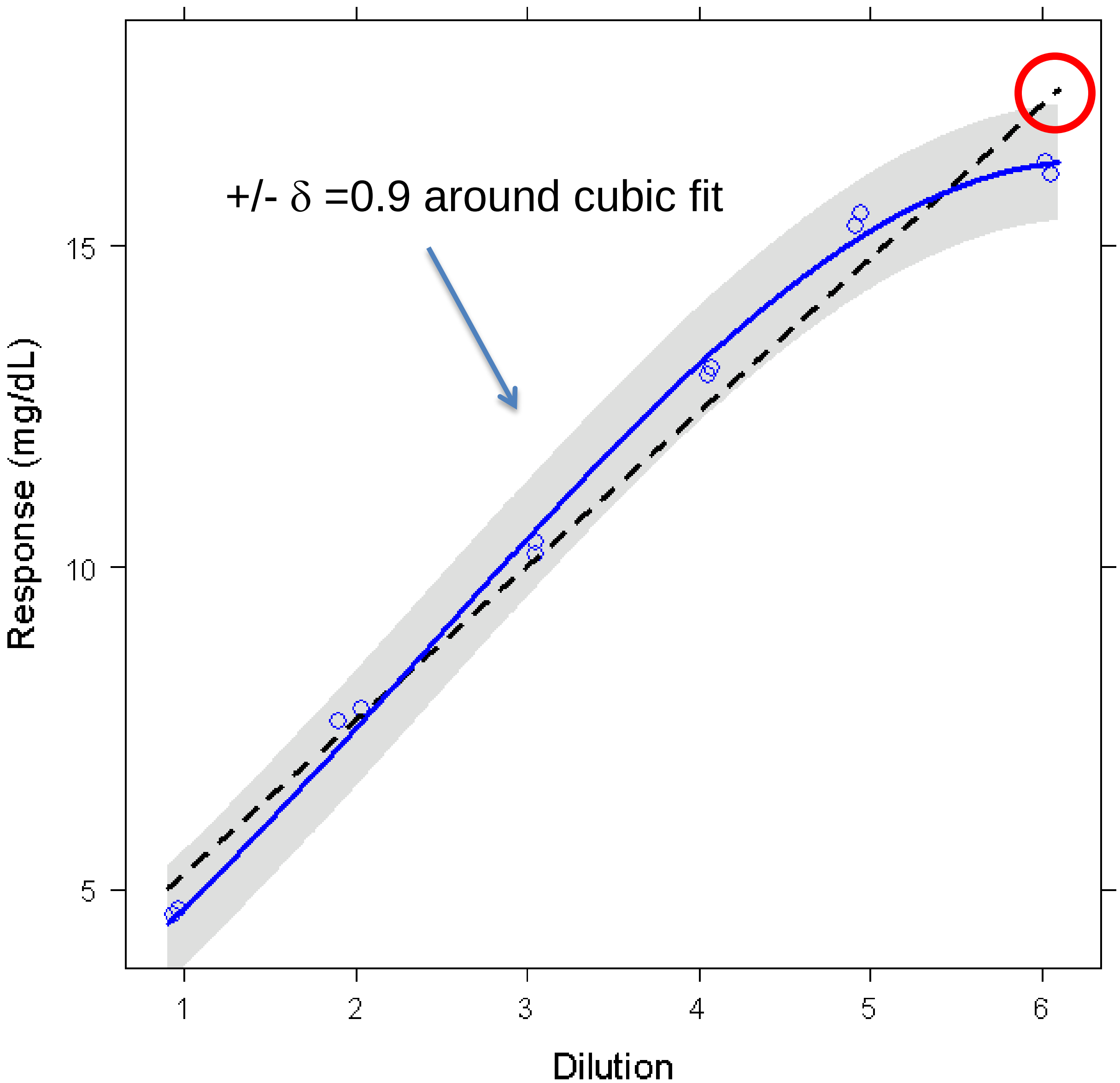
- From NCCLS EP6-A, Appendix C, ex. 2

Dilution	Replicate 1	Replicate 2
1	4.7	4.6
2	7.8	7.6
3	10.4	10.2
4	13	13.1
5	15.5	15.3
6	16.3	16.1



Compare cubic to linear from Dilution =1 to Dilution = 6: **δ= 0.9** for testing.

NCCLS EP6-A: δ=0.2 mg/dL



Dilution	**Mean difference: Cubic – Linear**
1	-0.53
2	-0.13
3	0.42
4	0.74
5	0.42
6	***-0.93***

Clear failure at Dilution = 6

- Hsieh and Liu I-U test p-value = 0.61
  - $\left|g_3(X_i) - g_1(X_i)\right| < \delta$ for all *i* = 1, 2, ..., *6*
  - Linear fit not adequate

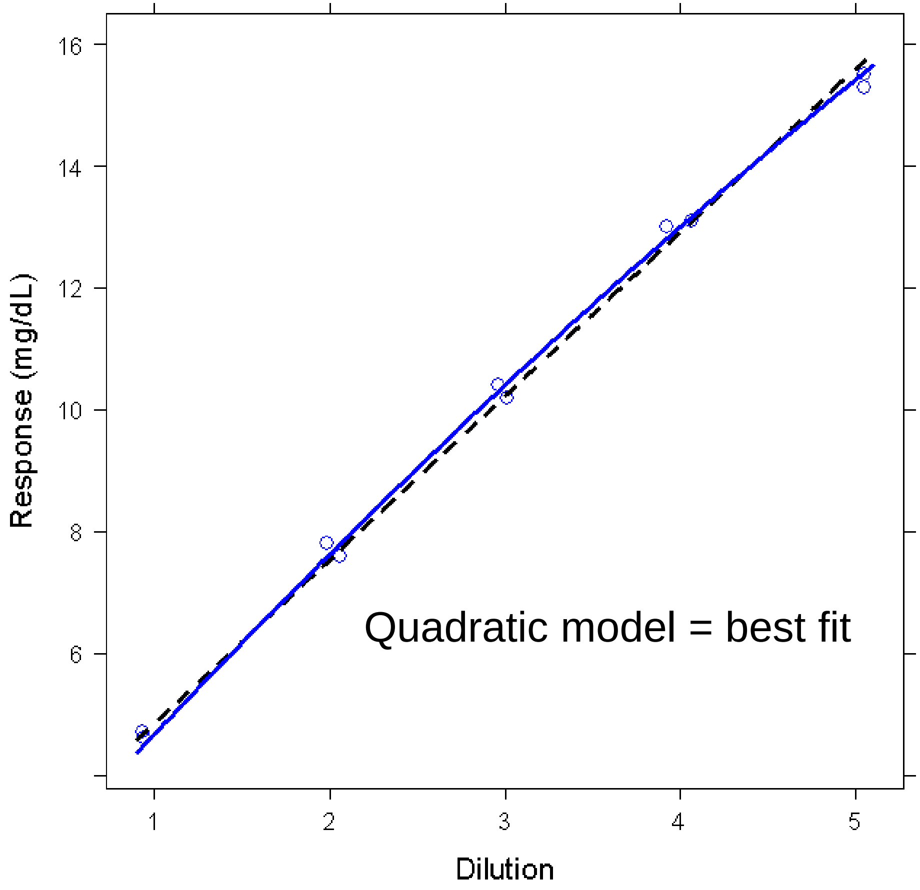
- $p(\delta, x_L, x_U) = 0.39$
  - $\left|g_3(x) - g_1(x)\right| < \delta$ for all $1 \le x \le 6$
  - Linear fit not adequate

- Probability $SSDL = \left(\frac{1}{6}\right)\sum_{i=1}^{6}\left\{g_3(X_i) - g_1(X_i)\right\}^2 < \delta^2$ > 0.99
  - *Linear fit is equivalent to cubic fit*

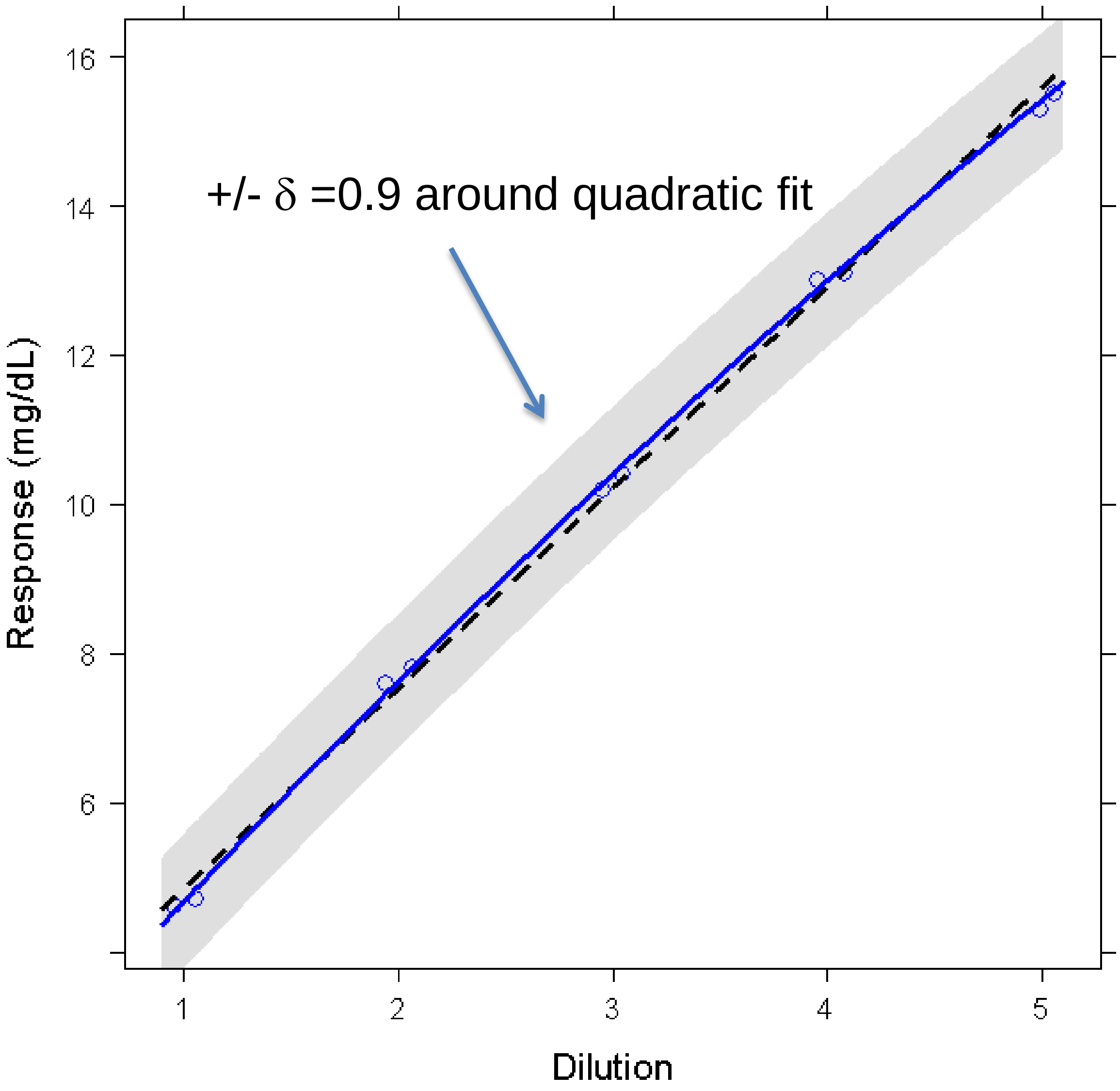
Try again without last dilution

- From NCCLS EP6-A, Appendix C, ex. 2

Dilution	Replicate 1	Replicate 2
1	4.7	4.6
2	7.8	7.6
3	10.4	10.2
4	13	13.1
5	15.5	15.3
6		



Compare quadratic to linear from Dilution =1 to Dilution = 5 with **δ= 0.9** for testing.



Dilution	**Mean difference: Quad – Linear**
1	-0.18
2	0.09
3	0.18
4	0.09
5	-0.18
6	

Linear and quadratic fits equivalent

- Hsieh and Liu I-U test p-value < 0.01
- $p(\delta, x_L, x_U) > 0.99$
- SSDL probability > 0.99
- *Linear fit is equivalent to quadratic fit for dilutions between 1 and 5.*

Simulation

Quadratic vs. Linear Simulation

- $g_2(x) = 10 + (1-40\phi)x + \phi x^2, \quad 1 \leq x \leq 40$

- $\phi = 0 =$ linear: $g_2(x) = 10 + x$
- $\phi = 0.04 =$ large quadratic component.

- Y ~ N($g_2(x)$, σ=3). $10 \leq \mathrm{g}_2(\mathrm{x}) \leq 50$

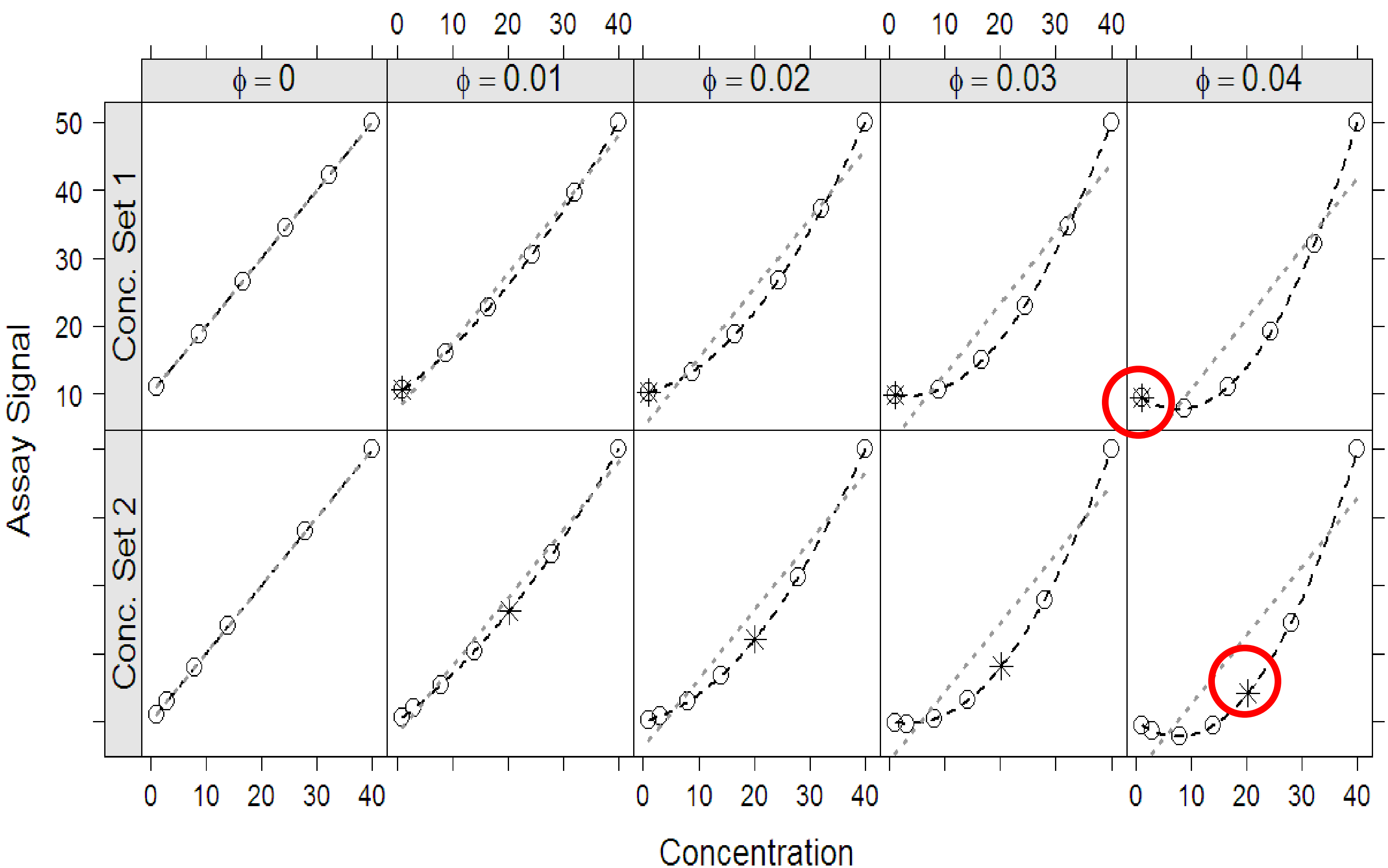
- 6 concentrations with two replicates each
- Testing limit: $\delta = 6.1$

Two sets of concentrations

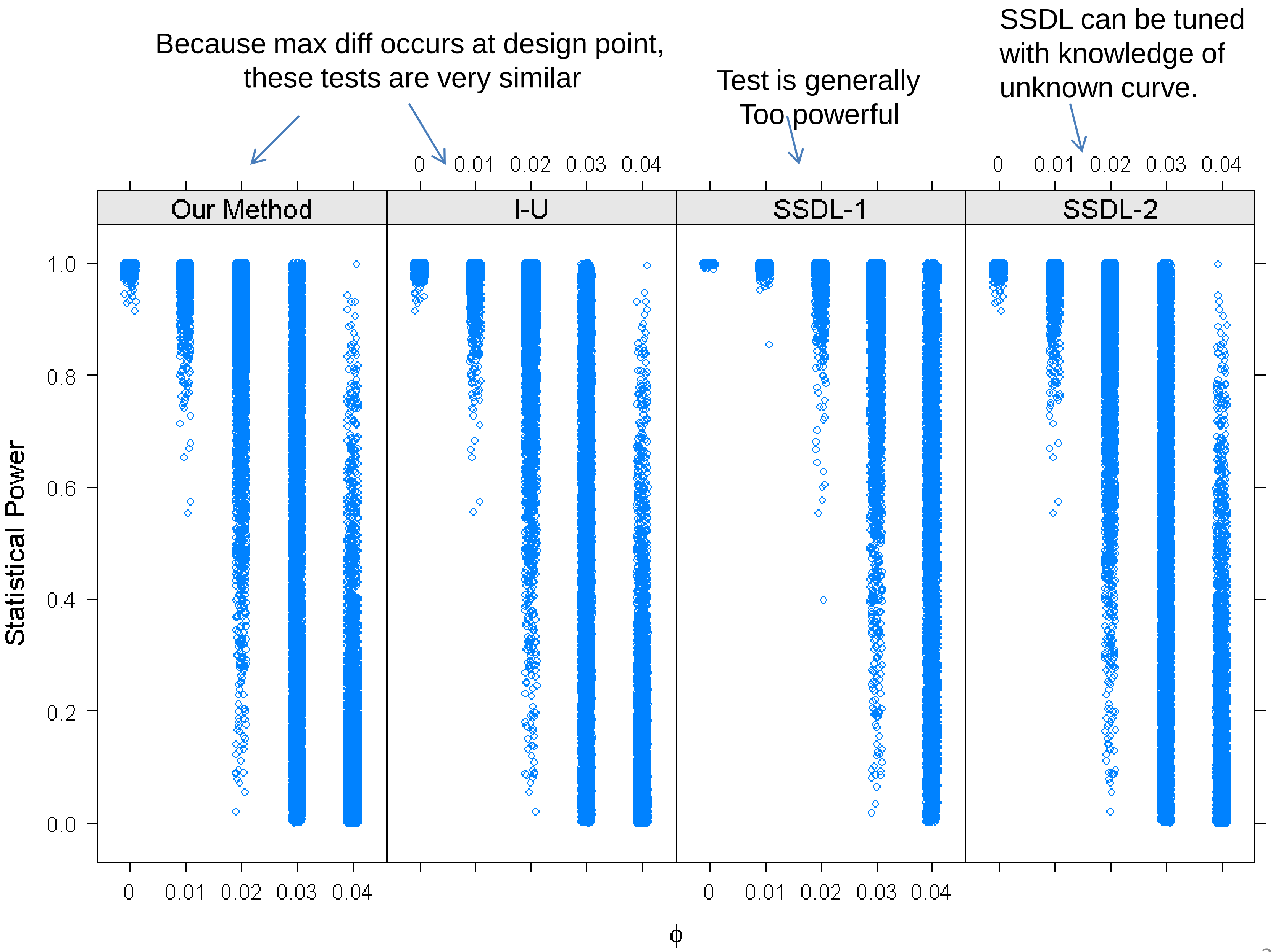
Set 1: Maximum deviation occurs at $x = 1$

Set 2: Maximum deviation occurs between points

This case is H0/H1 border



Maximum difference occurs at $x = 1$

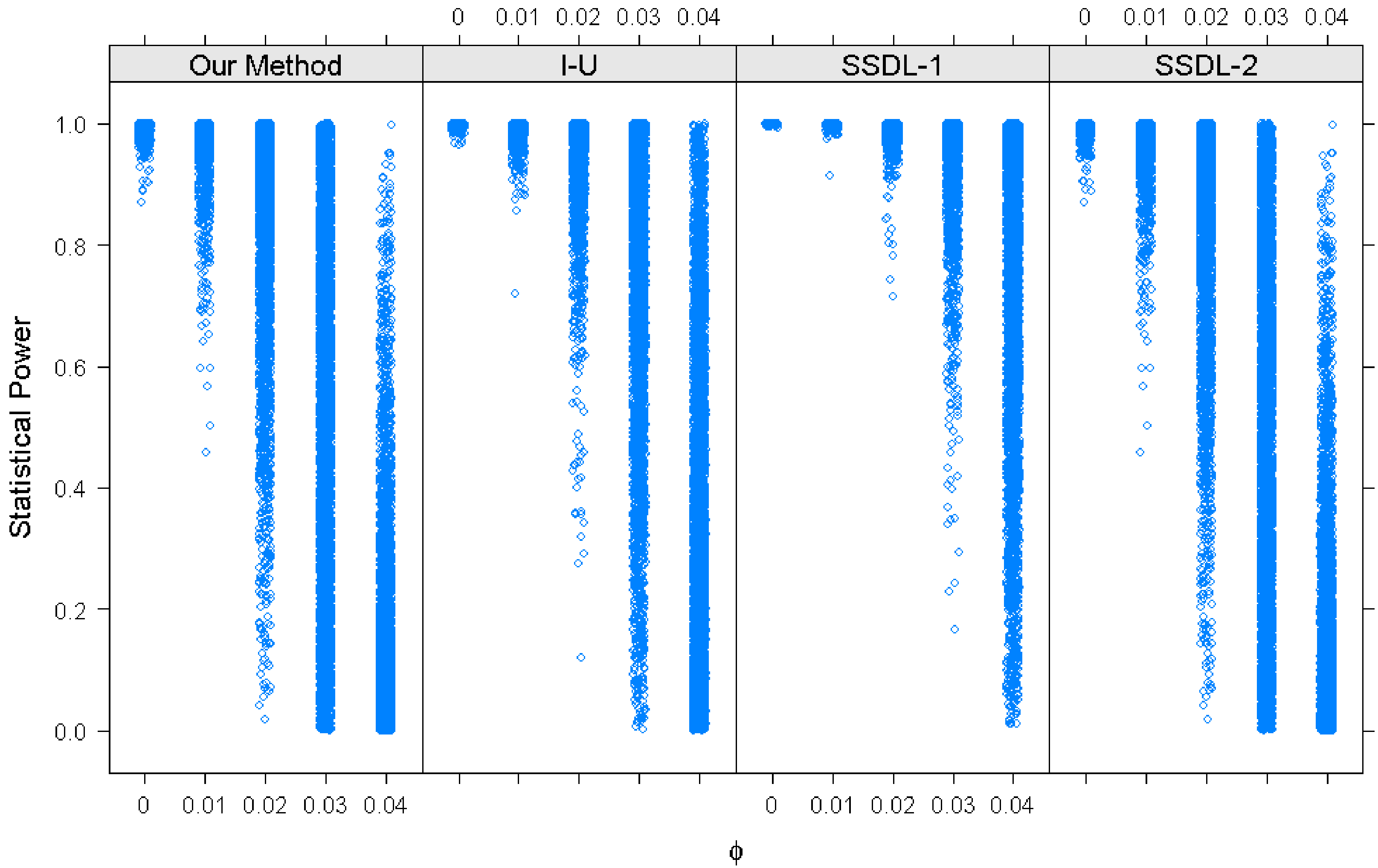


Maximum difference occurs between points

Our method retains similar power profile

Tests are generally Too powerful

SSDL can be tuned with knowledge of unknown curve.



Inverting the test

- Consider the true mean response $g_k(x)$ and the reduced model $g_1(x)=a+bx$.

- Let $z_k(x) = \{ g_k(x) - a \}/b$
  - This is polynomial Y-value back-calculated with best-fitting straight line.

- How close is $z_k(x)$ to x ?

A few hypotheses to consider

- $| z_k(x) - x | < \delta$ for all $x_L \le x \le x_U$
- $| 100\%\{z_k(x) - x\}/x | < \delta$
- $| \log\{z_k(x)\} - \log(x) | < \delta$

Many others!

- We can compute conditional probability

$$\Pr\{ |\ \log\{z_k(x)\} - \log(x)\ | < \delta \ \ |\ \text{data}\ \}$$

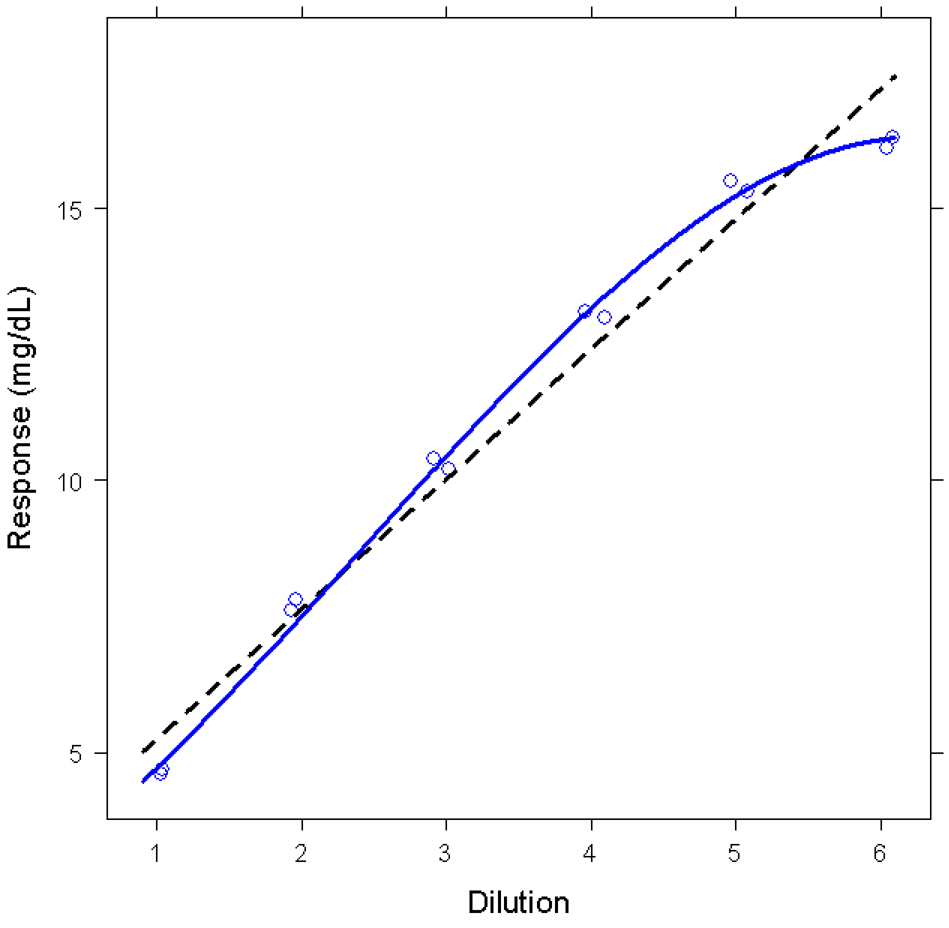
Or

- Find δ such that

$$\Pr\{ |\ \log\{z_k(x)\} - \log(x)\ | < \delta \ \ |\ \text{data}\ \} = 0.95$$

$$\Pr\{|\log\{z_3(x)\} - \log(x)| < 0.15 \mid \text{data}\} = 0.95$$

for all $1 \le x \le 6$

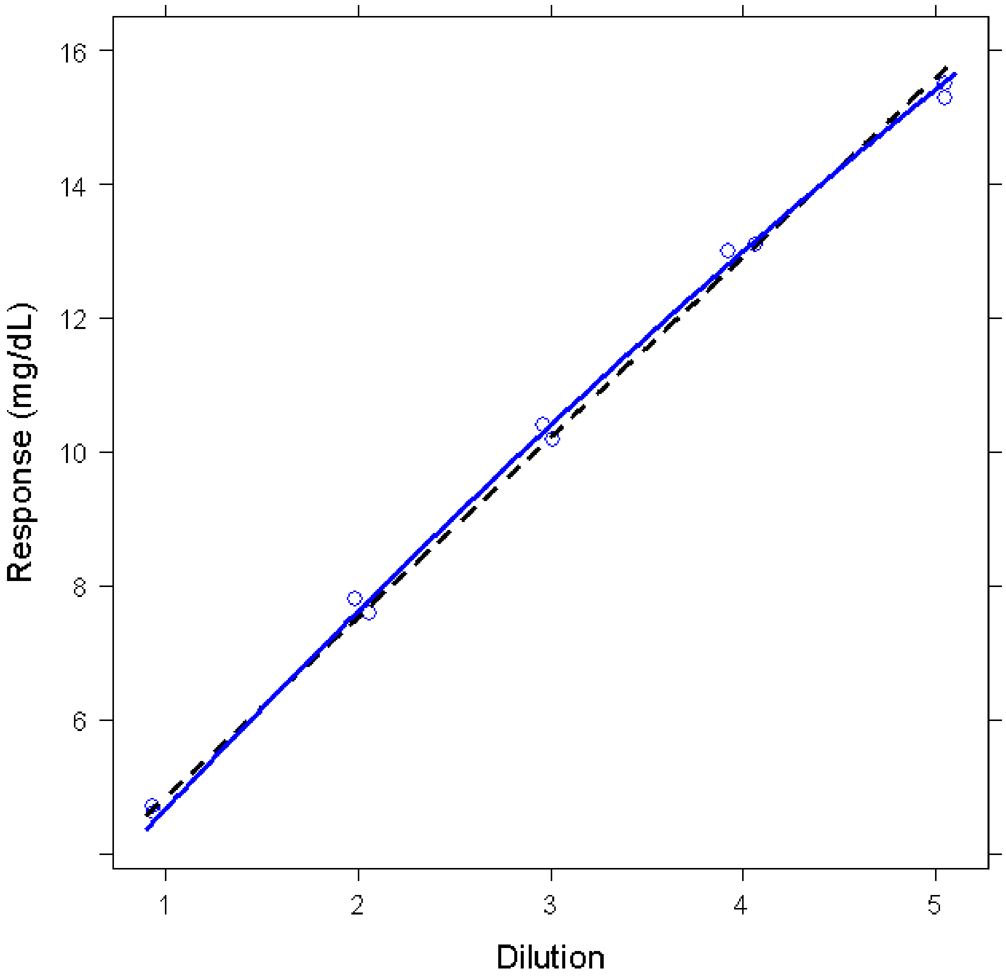


Back-calculated values are within 0.15 log10 units of true value.

Back-calculated values are within $100\%(10^{0.15}-1)$ = **40%** of true value.

$$\Pr\{|\log\{z_2(x)\} - \log(x)| < 0.05 \mid \text{data}\} = 0.95$$

$$\text{for all } 1 \le x \le 5$$



Back-calculated values are within 0.05 log10 units of true value.

Back-calculated values are within $100\%(10^{0.05}-1)$ = **12%** of true value.

Extra bits

- When k=2 (quadratic vs. linear), the proposed test statistic is central T distributed.
- When $k = 2$, by altering the testing limits, the I-U, SSDL, and proposed test methods can be made equal.
- From simulations, test size for the proposed test statistic appears to be $\leq \alpha$, depending on the experimental design.

Summary

- Test method extends idea of NCCLS EP6-A by computing probability that best-fit curve is equivalent to a linear fit.
- Testing performed across a range of concentrations and *not* at experimental design points.

References: Guidelines

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Thank you!

Questions?