Analysis of left-censored multiplex immunoassay data: A unified approach

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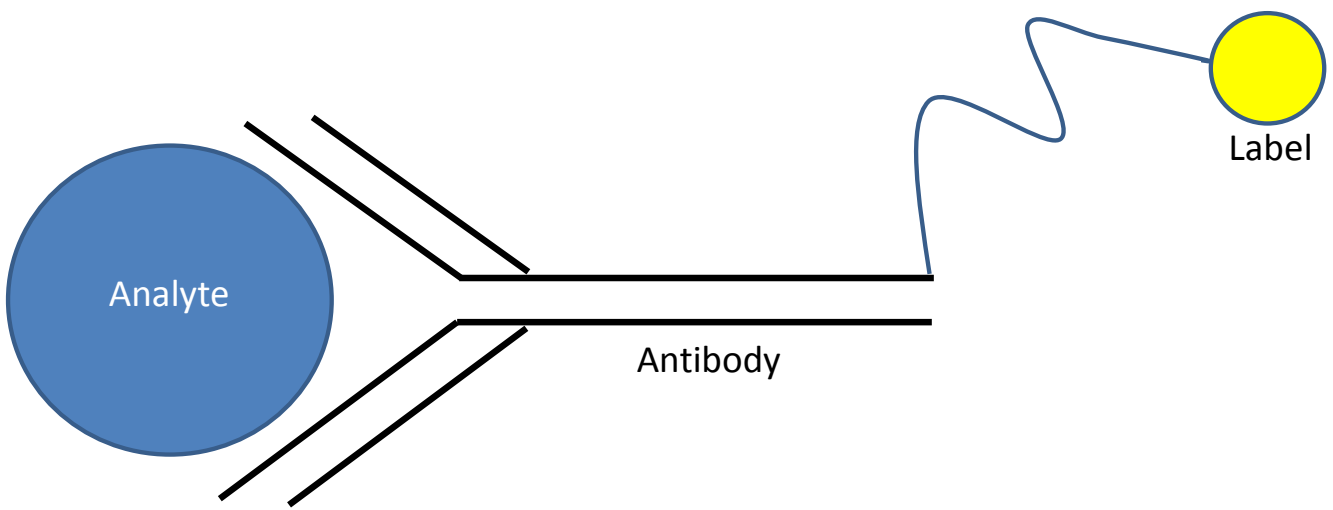
Talk Outline

An immunoassay is ...

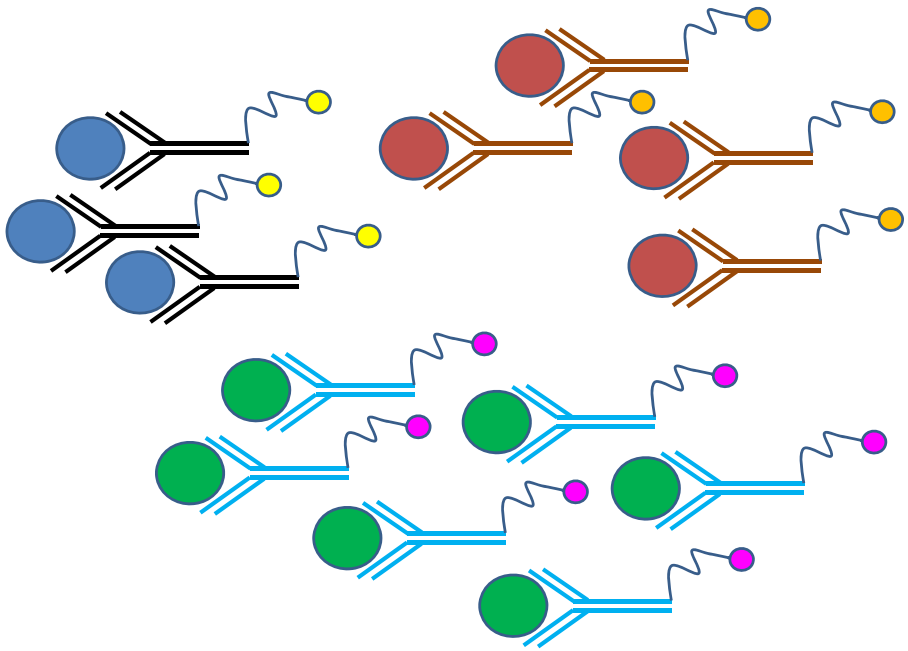
- A chemical test used to measure the concentration of a molecule in solution
- Target molecule - often a protein - called an *analyte*
- Analyte is “captured” by an analyte-specific antibody
- Measureable “label” (e.g. intensity of fluorescent signal) facilitates quantitation



Conventional uses

- Detect presence (absence) of a protein
  - Pregnancy test
  - Steroid use
  - Goal: *"Is analyte X detectable in this subject?"*
- Measure the concentration of protein
  - Blood panel
  - Viral load (HIV+ patients)
  - Goal: *"What is the concentration of analyte X for this subject?"*
- Emphasis is on analyte measurement for an individual

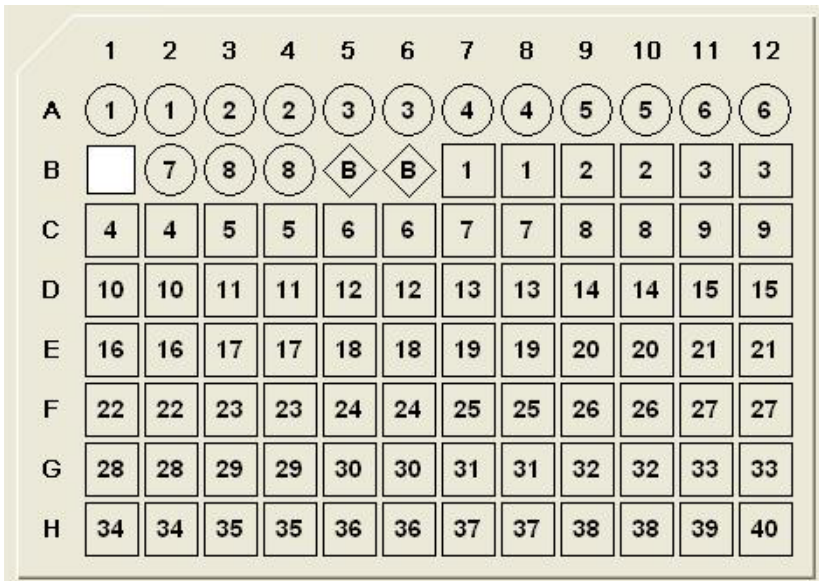
Multiplex immunoassay



- Simultaneous quantitation of a panel of analytes
- Commonly used as biomarker discovery tool
- Emphasis on group-level comparisons
- Goal
  - *"Is analyte X associated with disease status?"*
  - *"Does analyte X predict disease status?"*

Multiplex immunoassay

- All analytes accommodated on a single 96-well plate
- k-plex, $k = 5 - 30$ (typically)
- Multiple plates used to facilitate large sample sizes
- Each well detects all k analytes



Standards – serially diluted known analyte concentrations

Blanks – no analyte concentration

Patient samples – unknown analyte concentration

Workflow

For every analyte on each plate

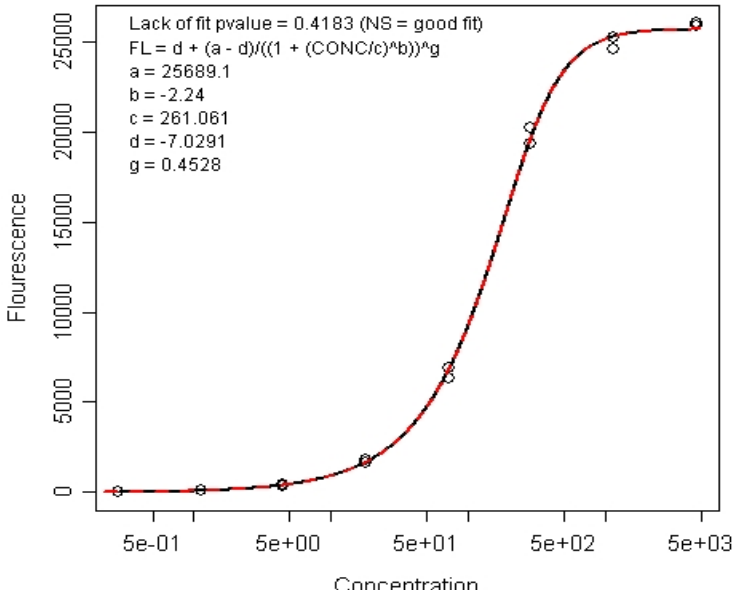
1. Place $8 - 10$ duplicate standard concentrations
2. Place duplicate blanks
3. Use remaining wells for duplicate patient samples (serum, urine, saliva)
4. Measure signal intensity (*response*)
5. Calculate average response for blanks
6. "Correct" standard and patient responses by subtracting the average blank response
7. Fit a standard curve to paired known standard concentrations and background corrected (BgC) responses
8. Average the patient BgC responses
9. Back-fit average BgC patient response values to obtain estimates of analyte concentrations
10. Conduct group-level inference

Standard curve

5-Parameter Logistic Model

$$f(x_{ijk}|\beta_{jk}) = d_{jk} + \frac{a_{jk} - d_{jk}}{\left[1 + \left(\frac{x_{ikj}}{c_{jk}}\right)^{b_{jk}}\right]^{g_{jk}}}$$

where x_{ijk} is the ith known concentration for analyte j plate k and $\beta_{jk} = (a_{jk}, b_{jk}, c_{jk}, d_{jk}, g_{jk})'$.

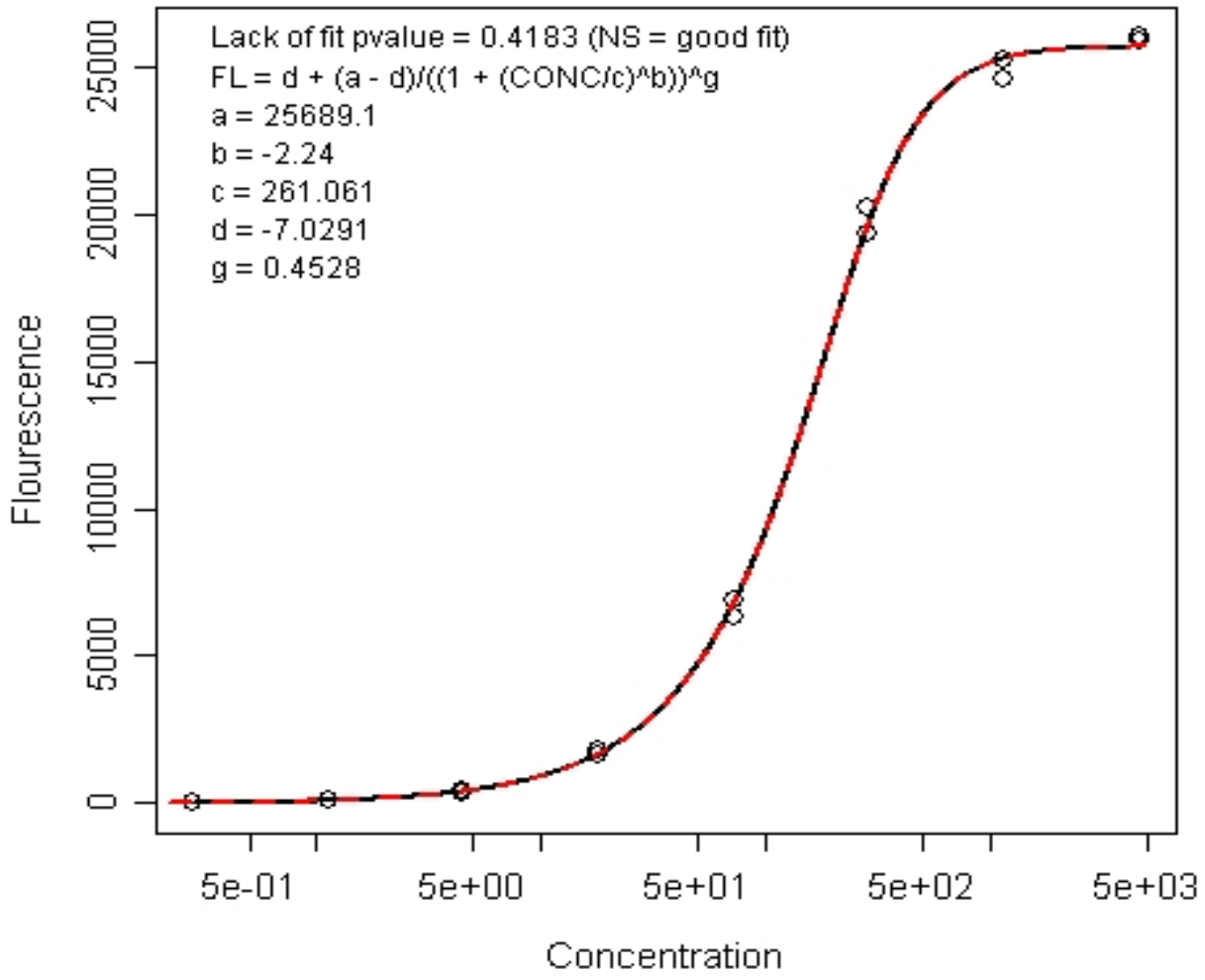


a_{jk} - upper asymptote
d_{jk} - lower asymptote
b_{jk} - transition rate between asymptotes
c_{jk} - concentration at inflection point
g_{jk} - asymmetry parameter

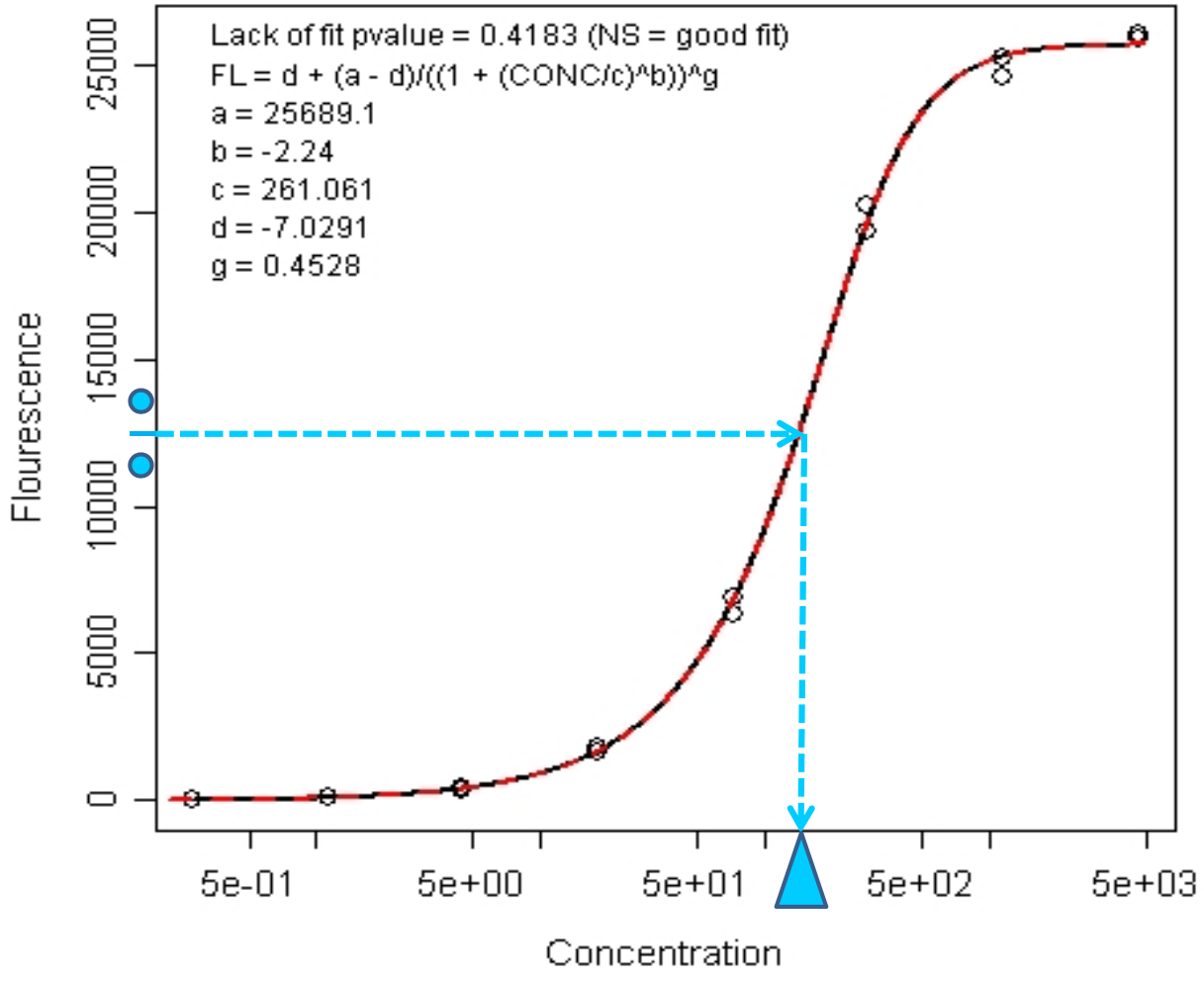
Fitting the 5-PL standard curve

- $y^*_{ijk\ell}$ = observed BgC response corresponding to ℓth replicate of standard concentration x_{ijk}
- $y^*_{ijk\ell} \sim \text{Normal}(f(x_{ijk}|\beta_{jk}), \text{Var}(y^*_{ijk\ell}))$
- Variance systematically related to mean
  - Noise in signal detectors is proportional to response magnitude
  - Kinetics associated with antibody binding
  - Power of the mean variance function
  - $\text{Var}(y^*_{ijk\ell}) = \sigma^2_{jk} f(x_{ijk}|\beta_{jk})^{2\theta}$
- Curve fitting achieved using generalized least squares (GLS)
- GLS = weighted least squares with weights estimated by $\text{Var}(y^*_{ijk\ell})^{-1}$

Standard curve



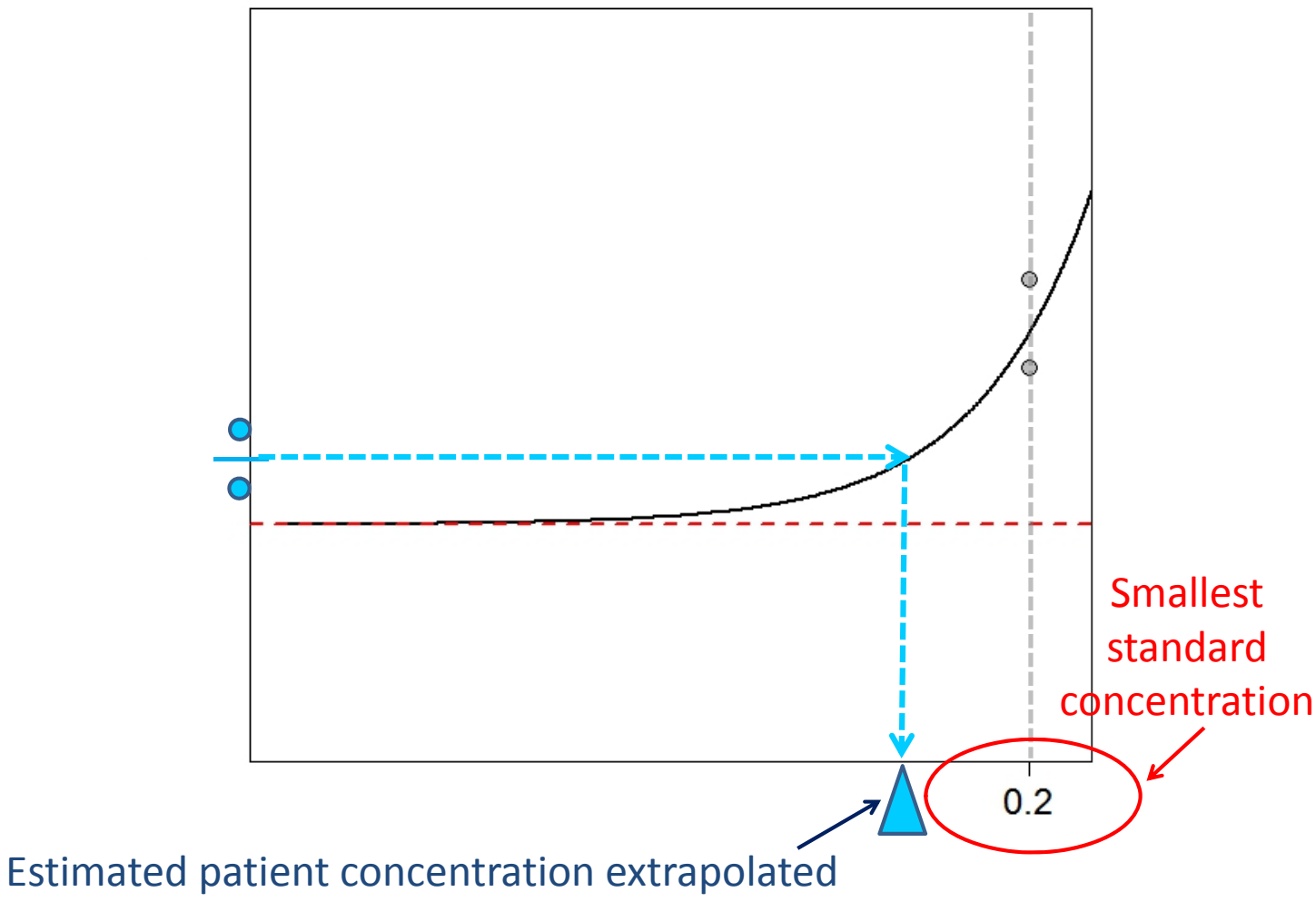
Patient analyte concentration estimation



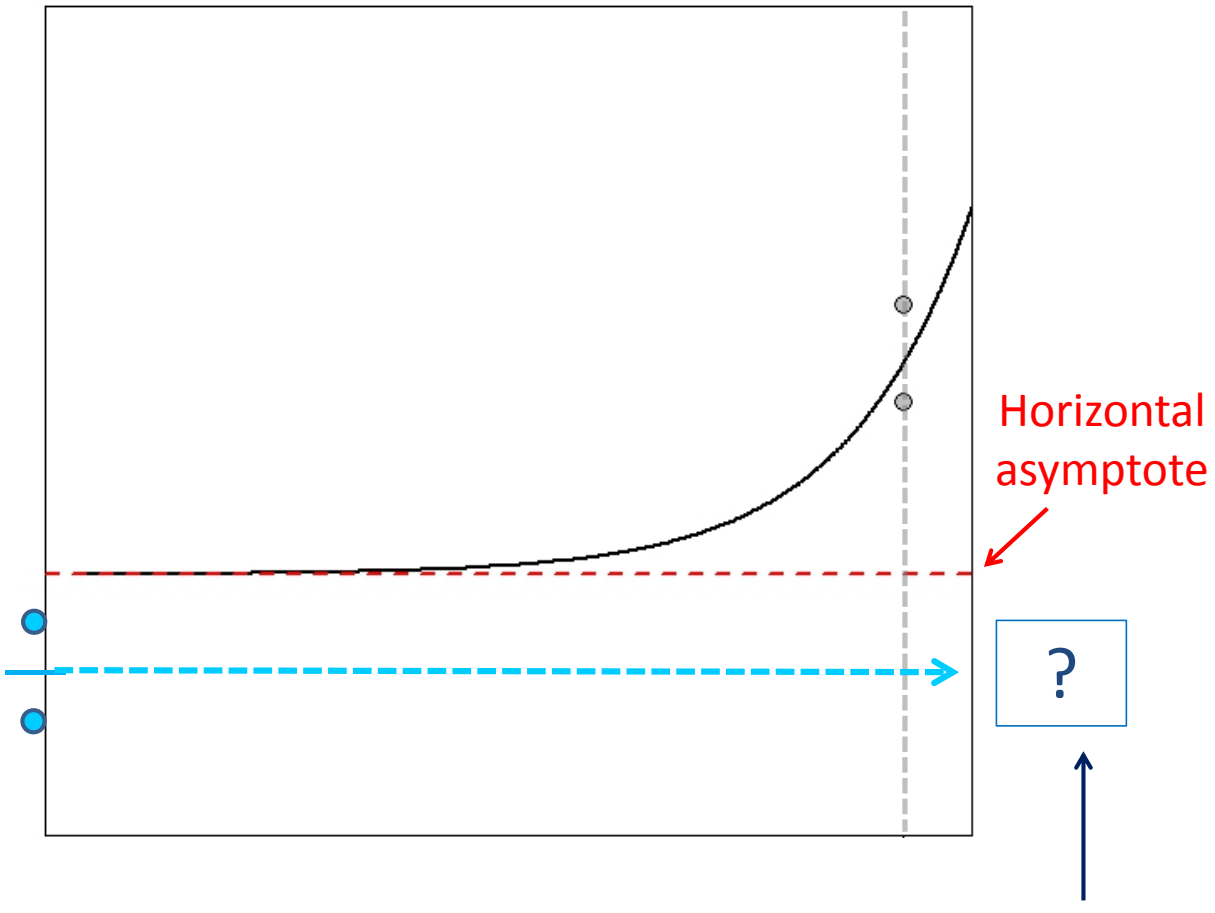
Sounds straightforward until you look at the data

Well	IL-1b	IL-2	IL-4	IL-5
B7,B8	*0.28	23.41	0.42	1.74
B9,B10	*0.61	51.61	1.07	3.94
B11,B12	OOR <	5.58	OOR <	OOR <
C1,C2	1.04	58.38	1.18	5.59
C3,C4	*0.79	63.17	1.66	5.69
C5,C6	*0.27	25.23	0.38	2.32
C7,C8	OOR <	8.46	OOR <	*0.45
C9,C10	*0.25	47.58	0.96	3.87
C11,C12	OOR <	OOR <	OOR <	OOR <
D1,D2	*0.19	27.14	0.52	2.17
D3,D4	OOR <	22.98	0.6	1.95
D5,D6	*0.48	46.6	0.95	4.44
D7,D8	10.07	8.21	OOR <	5.16
D9,D10	*0.71	50.53	1.24	3.8

Starred concentrations



OOR<



Back-fit fails – concentration can't be estimated

What causes this problem?

In biomarker discovery context

- Analytes' expected concentrations uncertain
- Results in poorly tuned dose-response curves
- Pervasive problem - percent of analytes with extrapolated or out-of-range concentrations can range from 0% - >90%
- Occurs less frequently at high end of curve

How can we analyze the data and address the investigator's research hypotheses?

Simple "solution"?

Well	IL-1b	IL-2	IL-4	IL-5
B7,B8	0	23.41	0.42	1.74
B9,B10	0	51.61	1.07	3.94
B11,B12	0	5.58	0	0
C1,C2	1.04	58.38	1.18	5.59
C3,C4	0	63.17	1.66	5.69
C5,C6	0	25.23	0.38	2.32
C7,C8	0	8.46	0	0
C9,C10	0	47.58	0.96	3.87
C11,C12	0	0	0	0
D1,D2	0	27.14	0.52	2.17
D3,D4	0	22.98	0.6	1.95
D5,D6	0	46.6	0.95	4.44
D7,D8	10.07	8.21	0	5.16
D9,D10	0	50.53	1.24	3.8

Simple “solution”?

Well	IL-1b	IL-2	IL-4	IL-5
B7,B8	0	23.41	0.42	1.74
B9,B10	0	51.61	1.07	3.94
B11,B12	0	5.58	0	0
C1,C2	1.04	58.38	1.18	5.59
C3				
C5				
C7				
C9				
C1				
D1,D2	0	27.14	0.52	2.17
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D5,D6	0	46.6	0.95	4.44
D7,D8	10.07	8.21	0	5.16
D9,D10	0	50.53	1.24	3.8

- Induces bias
- Underestimates variability
- Inflates type I error

Other ideas?

- Use established methods for analysis of left-censored data
  - ML with left-censored values contributing 1 - CDF to likelihood
  - Harrell's C-index - AUC equivalent for censored time-to-event data
- *Where to censor?*
- Conventional approach - censor at LOD or LOQ
- No established assay detection/quantitation limits
- Would need to be determined for each analyte and plate
- Previous efforts resulted in high rates of censored data - not an agreeable solution to the scientific investigator
- Shouldn't we account for uncertainty in curve and background estimation?

A "unified" approach

Incorporate background estimation, standard curve fitting, and patient analyte concentration estimation into a single Bayesian hierarchical model

1. *Background*

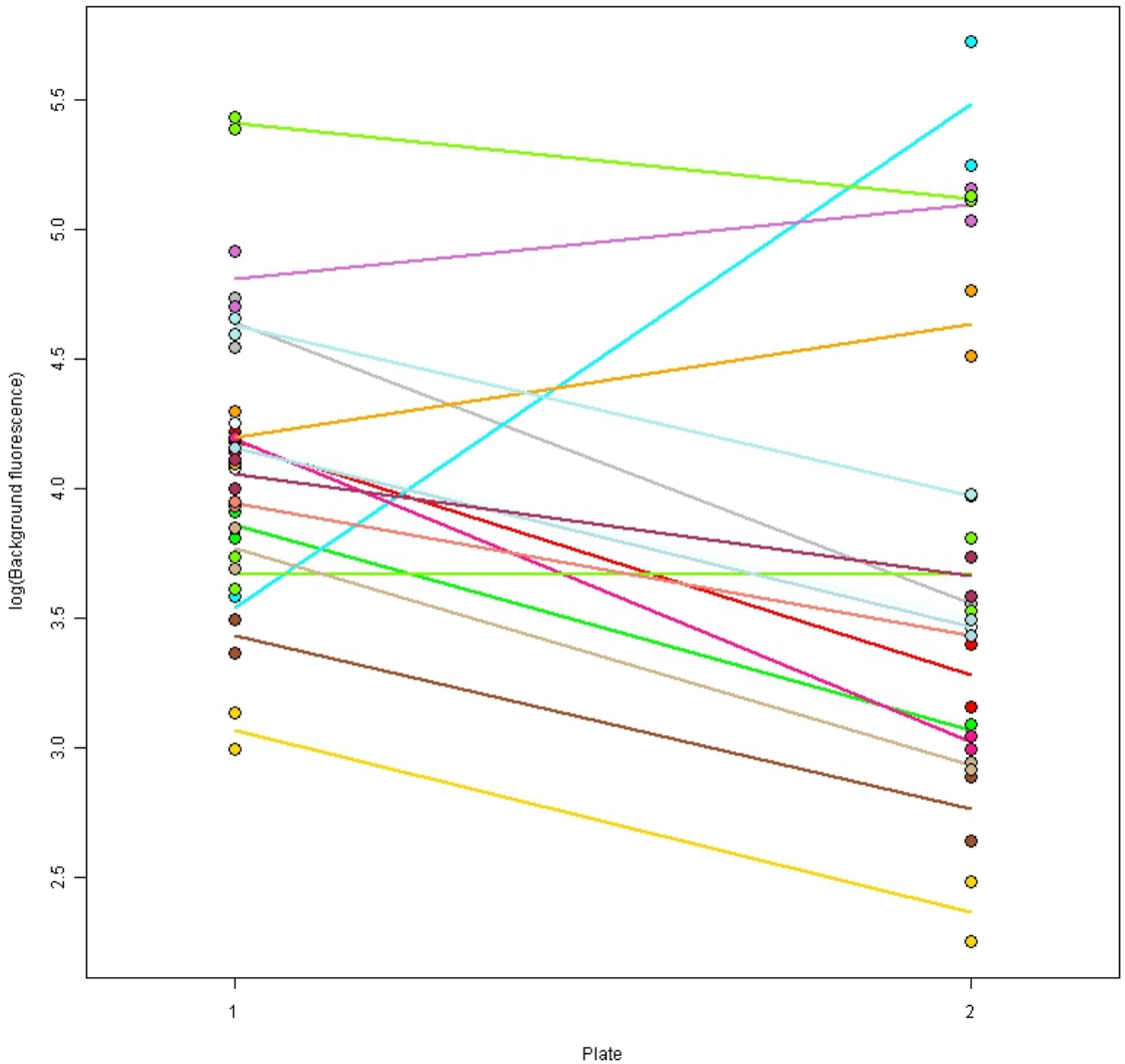
   $\delta_{jk} =$ observed background for analyte j, plate k

   $$\log(\delta_{jk}) \sim \text{Normal}(\mu_{jk}, \varphi)$$

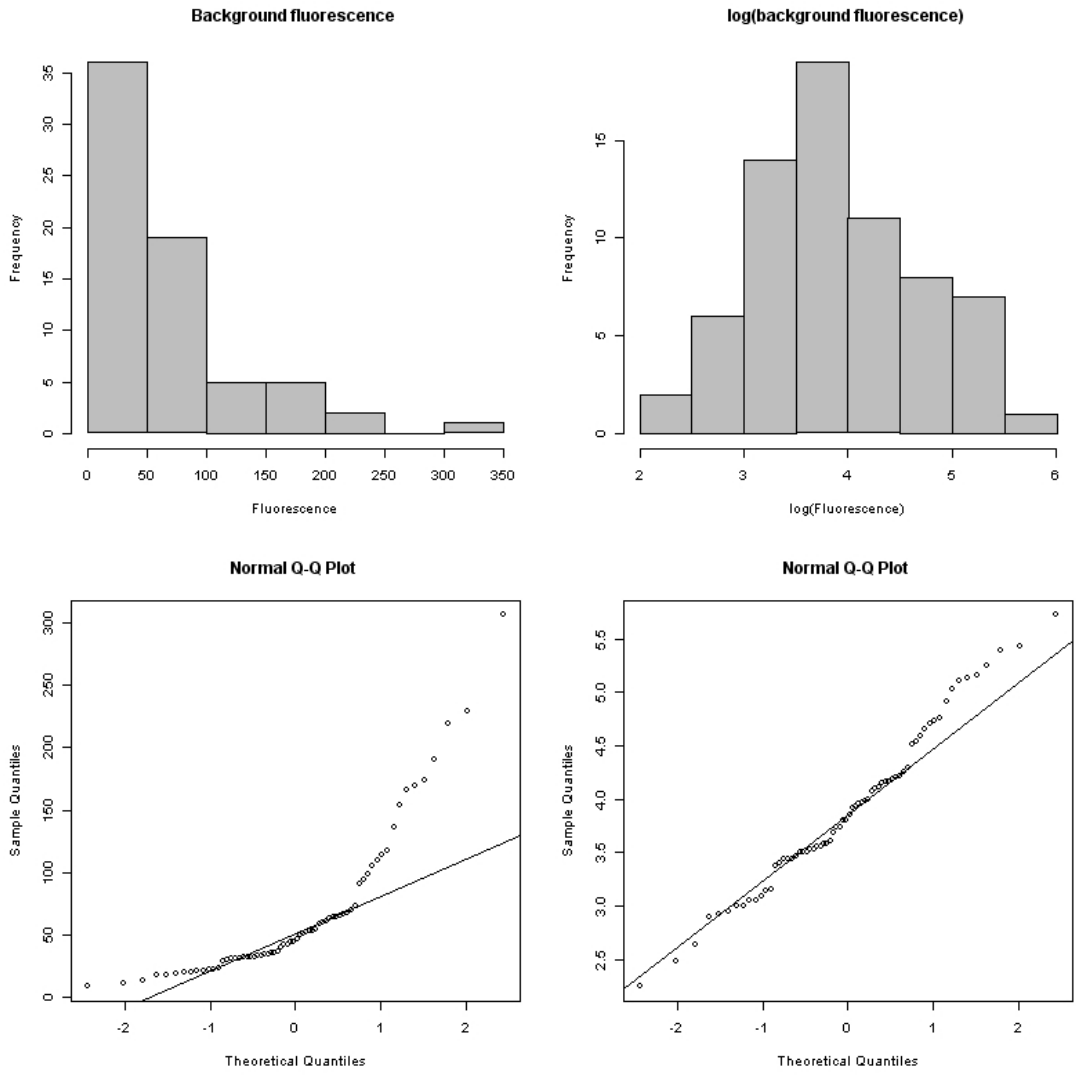
   with

   $$E(\delta_{jk}) = e^{\mu_{jk}}$$
   $$\text{Var}(\delta_{jk}) = \lambda_{jk} = e^{2\mu_{jk}} e^{\varphi}(e^{\varphi} - 1)$$

Background mean



Background distribution



Standard curve

2. *Standard curve*

$y_{ijk\ell}$ = observed (not BgC) response corresponding to the ℓth replicate of x_{ijk}, the ith known concentration for analyte j, plate k

$$y_{ijk\ell}|\mu_{jk} \sim \text{Normal}(f(x_{ijk}|\boldsymbol{\beta}_{jk}) + e^{\mu_{jk}}, \tau_{ijk})$$

where

$$\tau_{ijk} = \sigma^2_{jk} f(x_{ijk}|\boldsymbol{\beta}_{jk})^{2\theta}$$

Patient data

3. *Patient analyte concentrations*

$\nu_{hj} =$ patient h's true (latent) concentration of analyte j

$$\log(\nu_{hj}) \sim \text{Normal}(\gamma_{0j} + \gamma_{1j} D_h, \eta_j)$$

where

$$D_h = \begin{cases} 1, & \text{patient } h \text{ is disease positive} \\ 0, & \text{patient } h \text{ is disease negative.} \end{cases}$$

$e^{\gamma_{1j}} =$ fold-change comparing Ds+ to Ds- patients

Patient data

4. *Patient response values*

$z_{hjk\ell}$ = observed (not BgC) response corresponding to the ℓth replicate of patient h's true concentration for analyte j, plate k

$$z_{hjk\ell}|\mu_{jk} \sim \text{Normal}(f(\nu_{hj}|\beta_{jk}) + e^{\mu_{jk}}, \xi_{hjk})$$

where

$$\xi_{hjk} = \sigma_{jk}^2 f(\nu_{hj}|\beta_{jk})^{2\theta} \cdot 1(\nu_{hj} \geq x_{1hj}) + \lambda_{jk} \cdot 1(\nu_{hj} < x_{1hj})$$

Simulation study - experimental design

- Three plates
- Ten analytes per plate (“A” - “J”)
- Ten duplicate standards for each analyte
- Two blanks per plate
- Duplicate samples for 15 disease and 15 control subjects per plate (total sample size = 45 disease and 45 control)

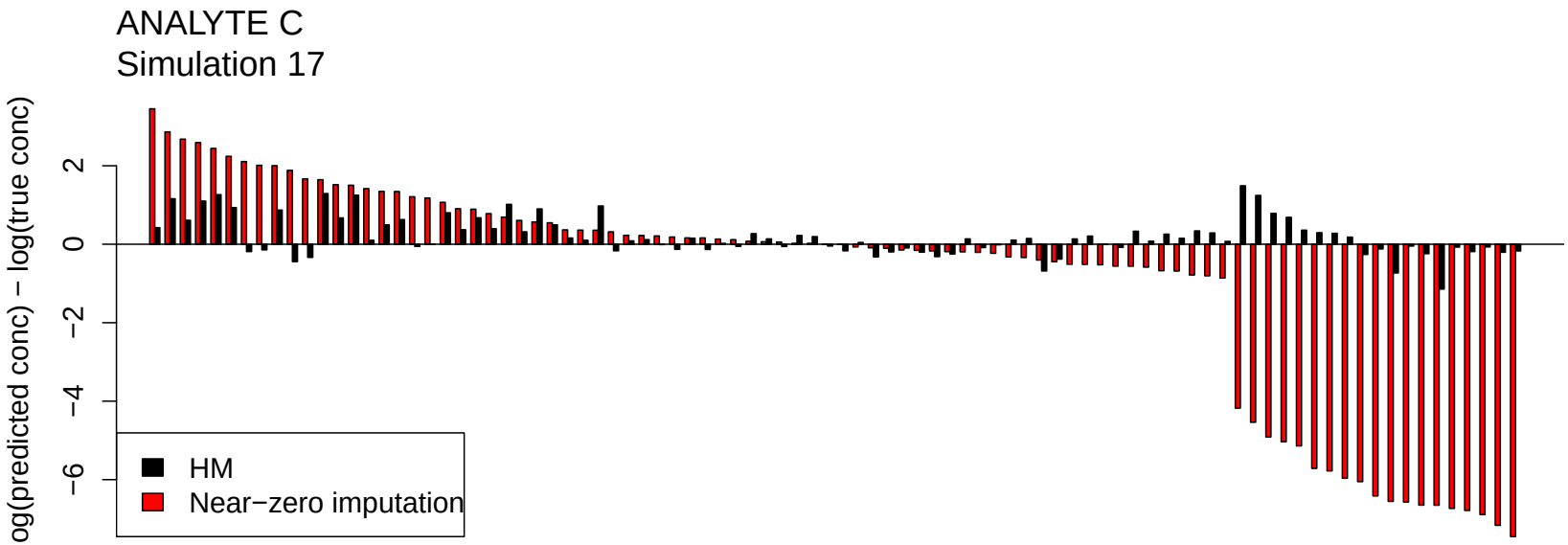
Simulation study - data description

- Prob($\nu_{hj} < x_{1jk}|$control) $= 0.2 \forall j, k$
- $e^{\gamma_{1j}} = 1, 1.5, 2, 4$, and 6, respectively, for analytes A/F, B/G, C/H, D/I, and E/J
- For control subjects, SD(log conc) = 1 for all analytes
- For disease subjects, SD(log conc) = 1 for analytes A - E
- For disease subjects, SD(log conc) = 1.25 for analytes F - J

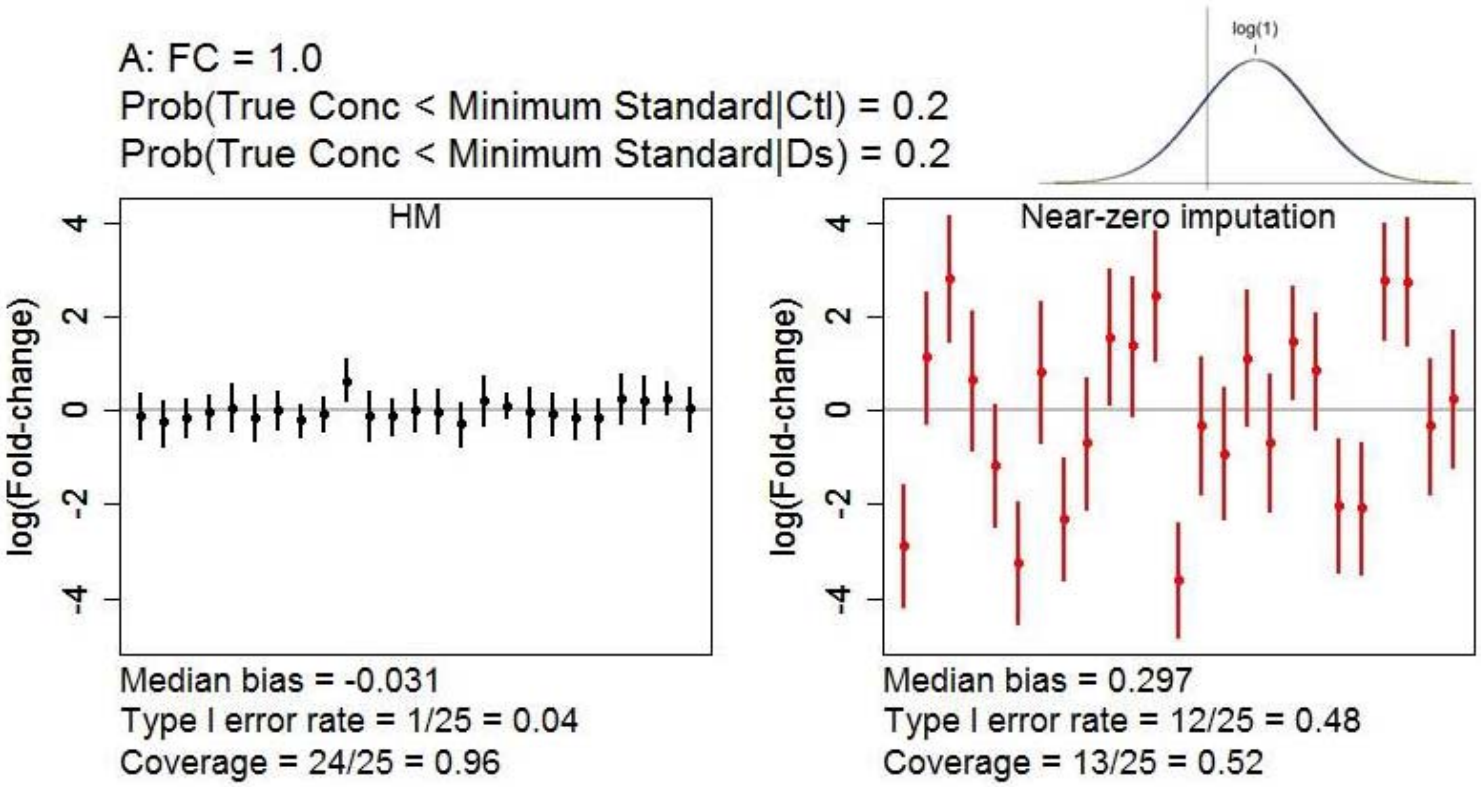
Simulation study - inference

- 25 independent simulations in WinBUGS
- Burn-in = 50K iterations with posterior inference chain length of 10K
- Constructed FC and AUC from posterior estimates of patient analyte concentrations
- Compared to standard workflow
  - "Near-zero" imputation for low extrapolated or out-of-range concentrations
  - Linear regression for FC estimation and inference
    $H_0: \text{FC} = 1$ vs $H_1: \text{FC} \neq 1$
  - Empirical estimate of AUC with inference based on corresponding 95% CI
    $H_0: \text{AUC} = 0.5$ vs $H_1: \text{AUC} > 0.5$
- Type I error, power, coverage, bias, MSE to results using standard workflow

Results

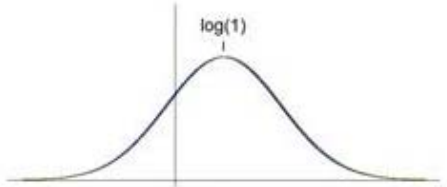

ANALYTE C
Simulation 17
log(predicted conc) − log(true conc)
2
0
−2
−4
−6
HM
Near−zero imputation

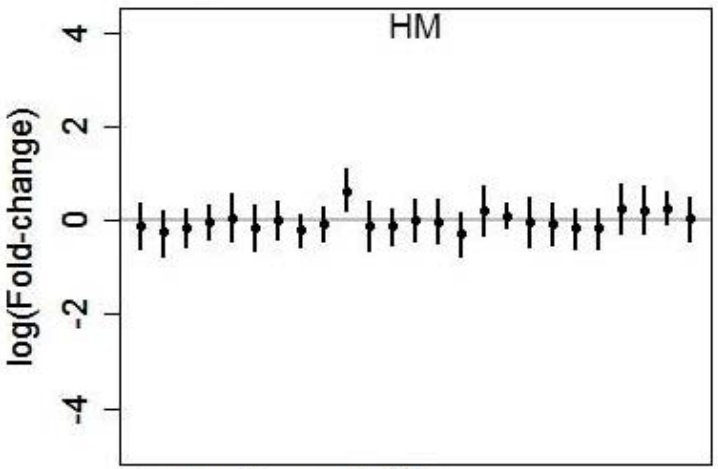
Fold change results



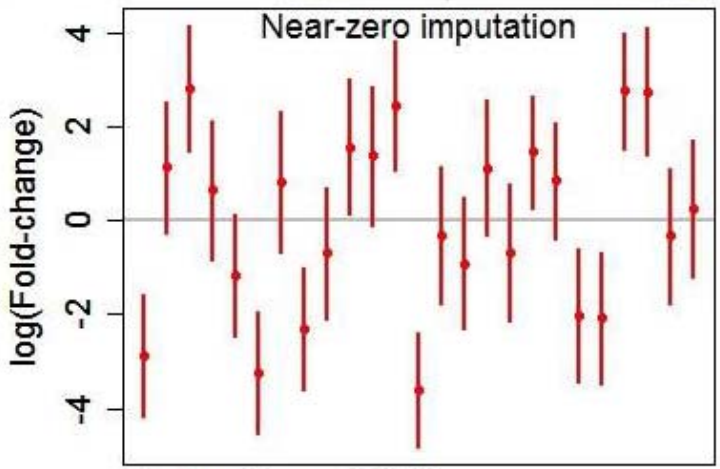
Fold change results

A: FC = 1.0
Prob(True Conc < Minimum Standard|Ctl) = 0.2
Prob(True Conc < Minimum Standard|Ds) = 0.2





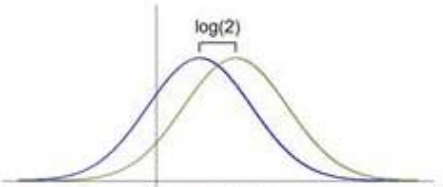
Median bias = -0.031
Type I error rate = 1/25 = 0.04
Coverage = 24/25 = 0.96

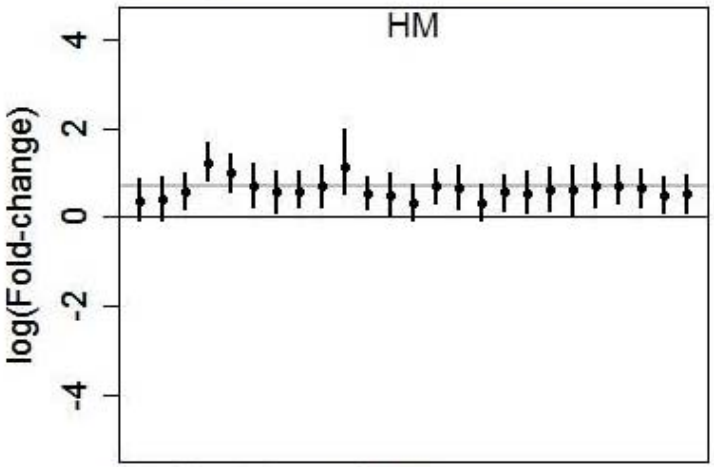


Median bias = 0.297
Type I error rate = 12/25 = 0.48
Coverage = 13/25 = 0.52

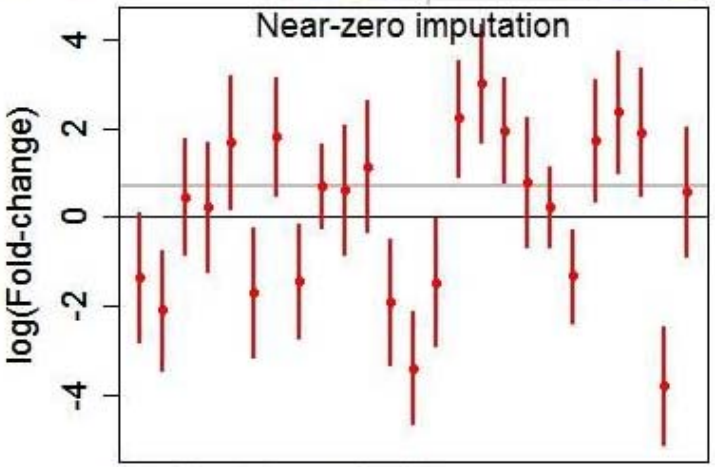
Fold change results

C: FC = 2
Prob(True Conc < Minimum Standard|Ctl) = 0.2
Prob(True Conc < Minimum Standard|Ds) = 0.06





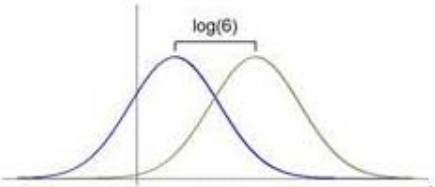
Median bias = -0.215
Power = 21/25 = 0.84
Coverage = 24/25 = 0.96

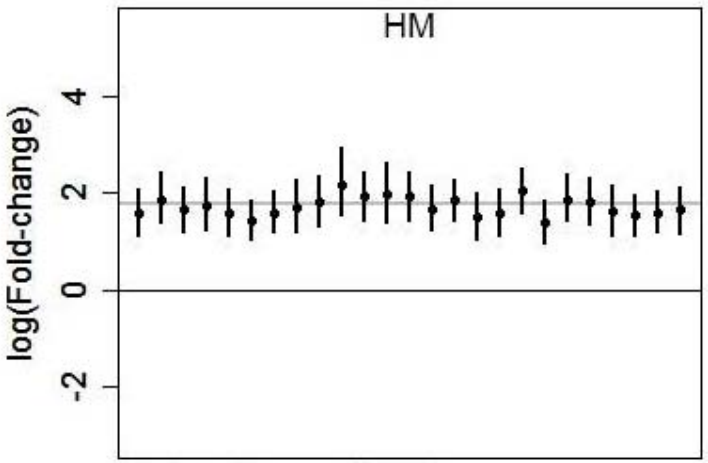


Median bias = -0.248
Power = 16/25 = 0.64
Coverage = 12/25 = 0.48

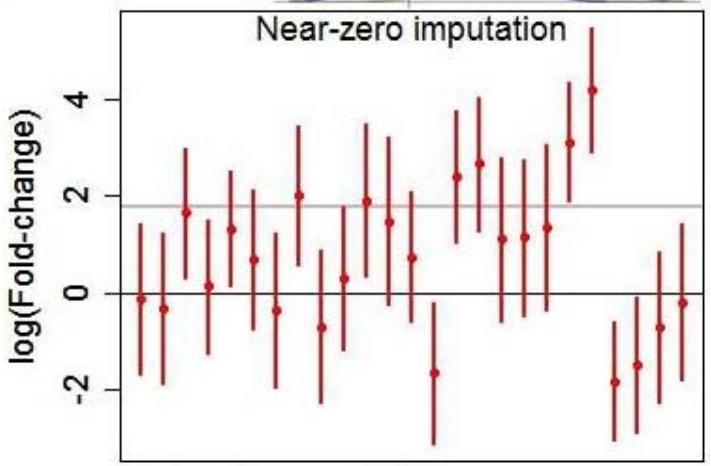
Fold change results

E: FC = 6
Prob(True Conc < Minimum Standard|Ctl) = 0.2
Prob(True Conc < Minimum Standard|Ds) = 0.004





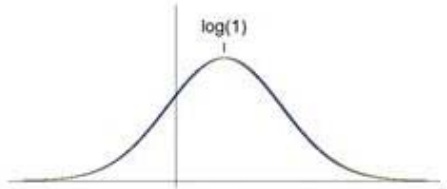
Median bias = -0.693
Power = 25/25 = 1
Coverage = 25/25 = 1

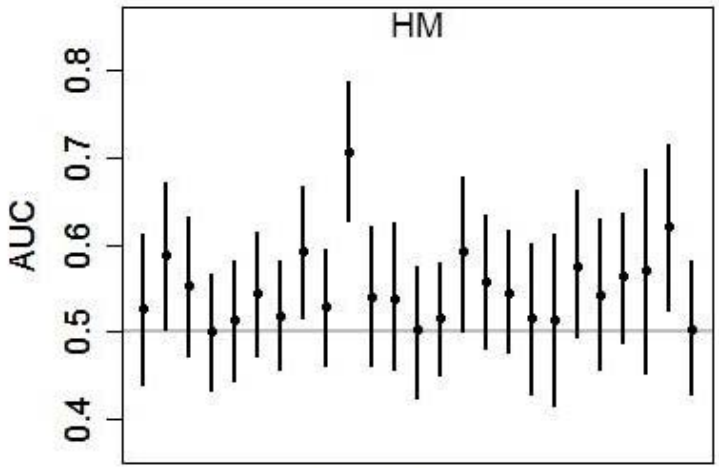


Median bias = -1.908
Power = 11/25 = 0.44
Coverage = 16/25 = 0.64

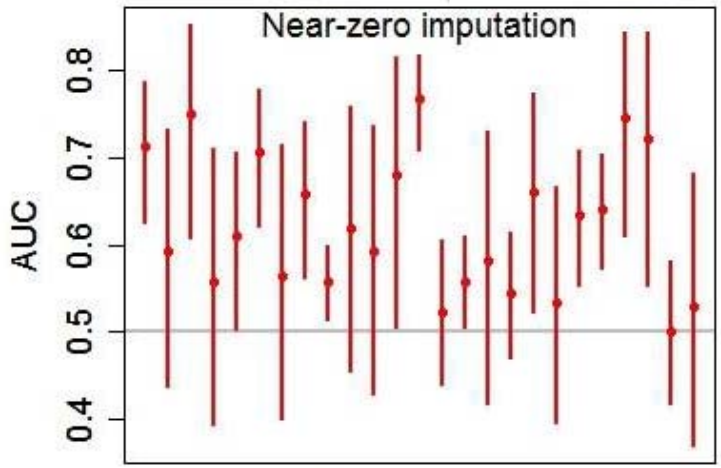
AUC results

A: AUC = 0.5
Prob(True Conc < Minimum Standard|Ctl) = 0.2
Prob(True Conc < Minimum Standard|Ds) = 0.2



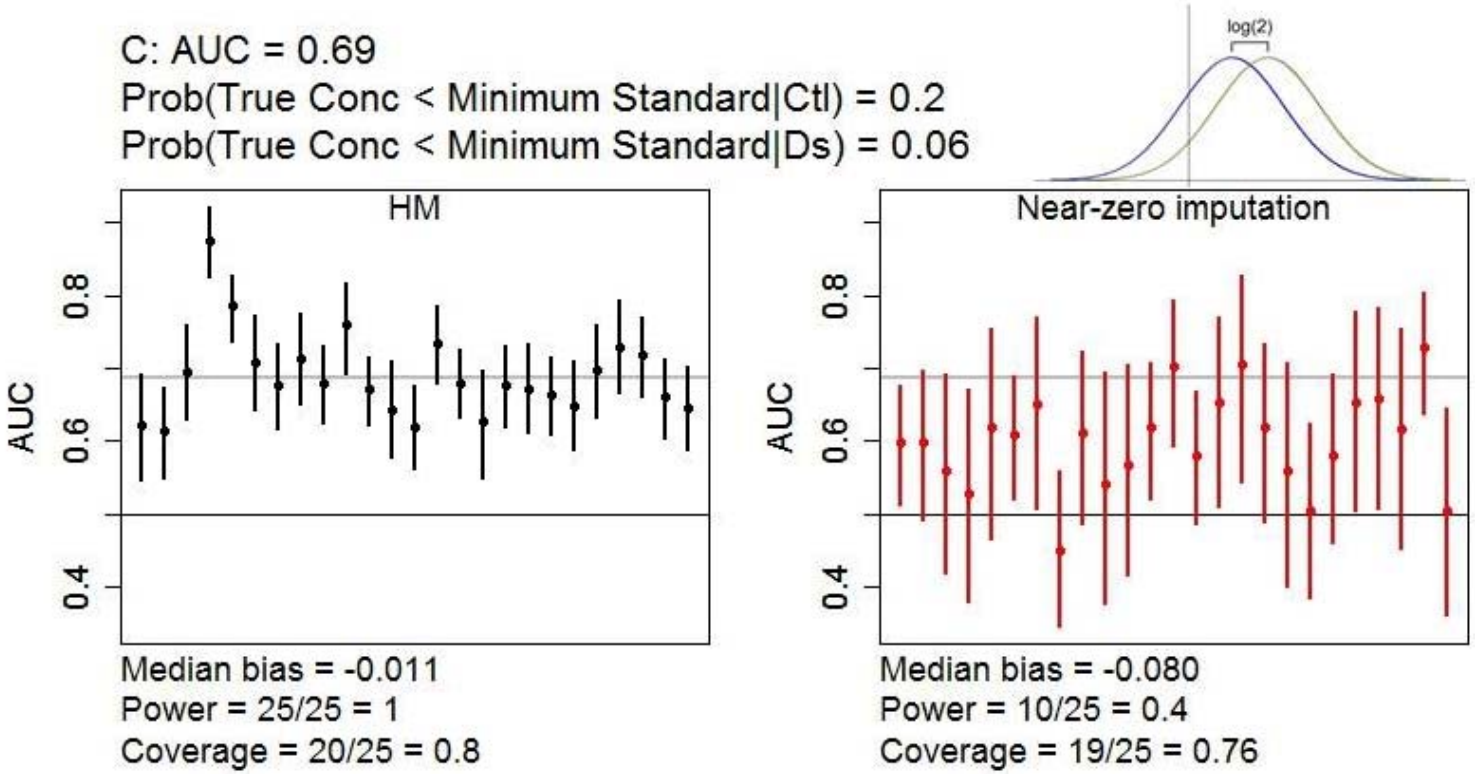


Median bias = 0.043
Type I error rate = 5/25 = 0.2
Coverage = 20/25 = 0.8

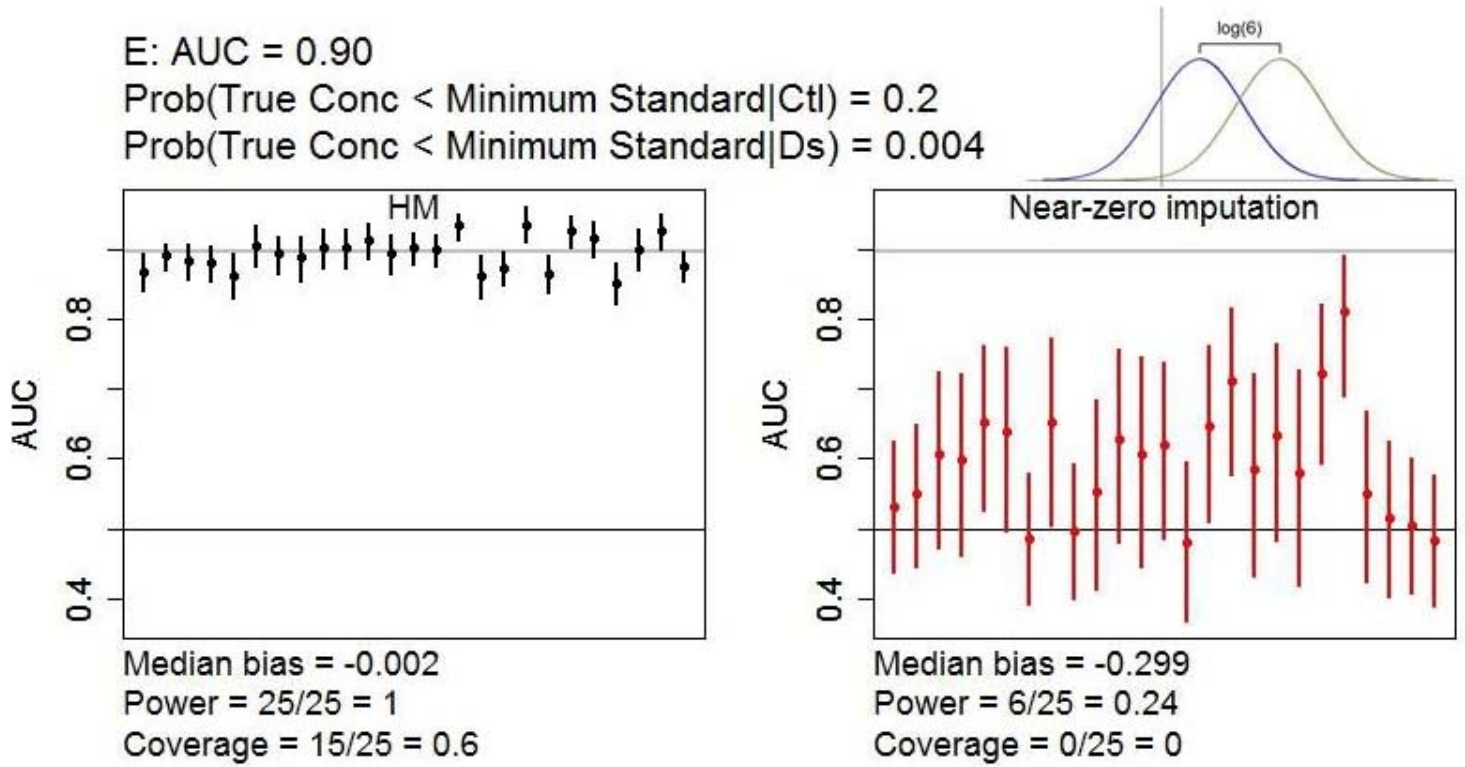


Median bias = 0.109
Type I error rate = 14/25 = 0.56
Coverage = 11/25 = 0.44

AUC results


C: AUC = 0.69
Prob(True Conc < Minimum Standard|Ctl) = 0.2
Prob(True Conc < Minimum Standard|Ds) = 0.06
log(2)
HM
AUC
0.4
0.6
0.8
Median bias = -0.011
Power = 25/25 = 1
Coverage = 20/25 = 0.8
Near-zero imputation
AUC
0.4
0.6
0.8
Median bias = -0.080
Power = 10/25 = 0.4
Coverage = 19/25 = 0.76

AUC results


E: AUC = 0.90
Prob(True Conc < Minimum Standard|Ctl) = 0.2
Prob(True Conc < Minimum Standard|Ds) = 0.004
log(6)
HM
AUC
0.4
0.6
0.8
Median bias = -0.002
Power = 25/25 = 1
Coverage = 15/25 = 0.6
Near-zero imputation
AUC
0.4
0.6
0.8
Median bias = -0.299
Power = 6/25 = 0.24
Coverage = 0/25 = 0

Head and neck cancer application

Hypothesis: HPV16 infection affects tumor immunity, resulting in differences between patients with HPV-associated and non-associated head and neck cancer tumors.

- Serum samples from 15 HPV+, 18 HPV-, and 19 control patients
- Multiplex analysis of 9 cytokines and chemokines
- Two plates
- Eight standards per analyte and plate
- Two background wells per analyte per plate
- All patient samples measured in duplicate
- Percent of estimated concentrations below minimum standard (based on standard workflow) ranges from 0% to 41%
- FC and AUC estimated using hierarchical model

HNCa results

IL6 was 3.4-times higher in HPV- samples relative to control (FC = 3.4, 95% CI = 1.6 to 7.9).

Cytokines significantly predictive of HPV-associated and non-associated HNCa, based on AUC posterior inference. AUC (95% CI)			
Analyte	**HPV-:Ctl**	**HPV-:HPV+**	**HPV+:Ctl**
GCSF	**0.70 (0.56, 0.83)**	0.54 (0.39, 0.68)	**0.66 (0.51, 0.79)**
IL4	0.58 (0.49, 0.67)	**0.64 (0.54, 0.73)**	0.45 (0.33, 0.56)
IL6	**0.79 (0.75, 0.83)**	**0.67 (0.63, 0.71)**	**0.65 (0.59, 0.70)**
IL12	**0.61 (0.54, 0.68)**	**0.60 (0.53, 0.69)**	0.51 (0.41, 0.60)
MCP1	**0.66 (0.61, 0.70)**	**0.59 (0.54, 0.64)**	0.54 (0.48, 0.60)
MIP1b	0.52 (0.48, 0.56)	0.46 (0.42, 0.51)	**0.55 (0.51, 0.60)**
TNFa	**0.62 (0.55, 0.68)**	0.58 (0.50, 0.66)	0.55 (0.47, 0.62)

Summary

- What we've learned so far
  - If goal is biomarker discovery, near-zero imputation is a bad idea
  - FC estimation and inference has good accuracy
  - AUC estimation and inference seems slightly conservative
- What's left
  - Additional simulations
  - Compare to censored data approaches

Future directions

- Multivariate distribution for analyte concentrations
- Design considerations - number of background replicates, number of plates (sample size)
- Flexible dose-response curve fitting - e.g. splines
- Hopefully the next grant!

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