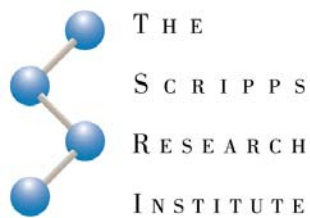
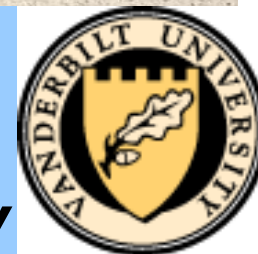


Biomarker Quantification by Compensated Interferometry: Lowering the LOQ to Improve Case-Control Separation for IPNs



THE
SCRIPPS
RESEARCH
INSTITUTE

Professor Darryl J. Bornhop
Vanderbilt Department of Chemistry
Vanderbilt Institute for Chemical Biology



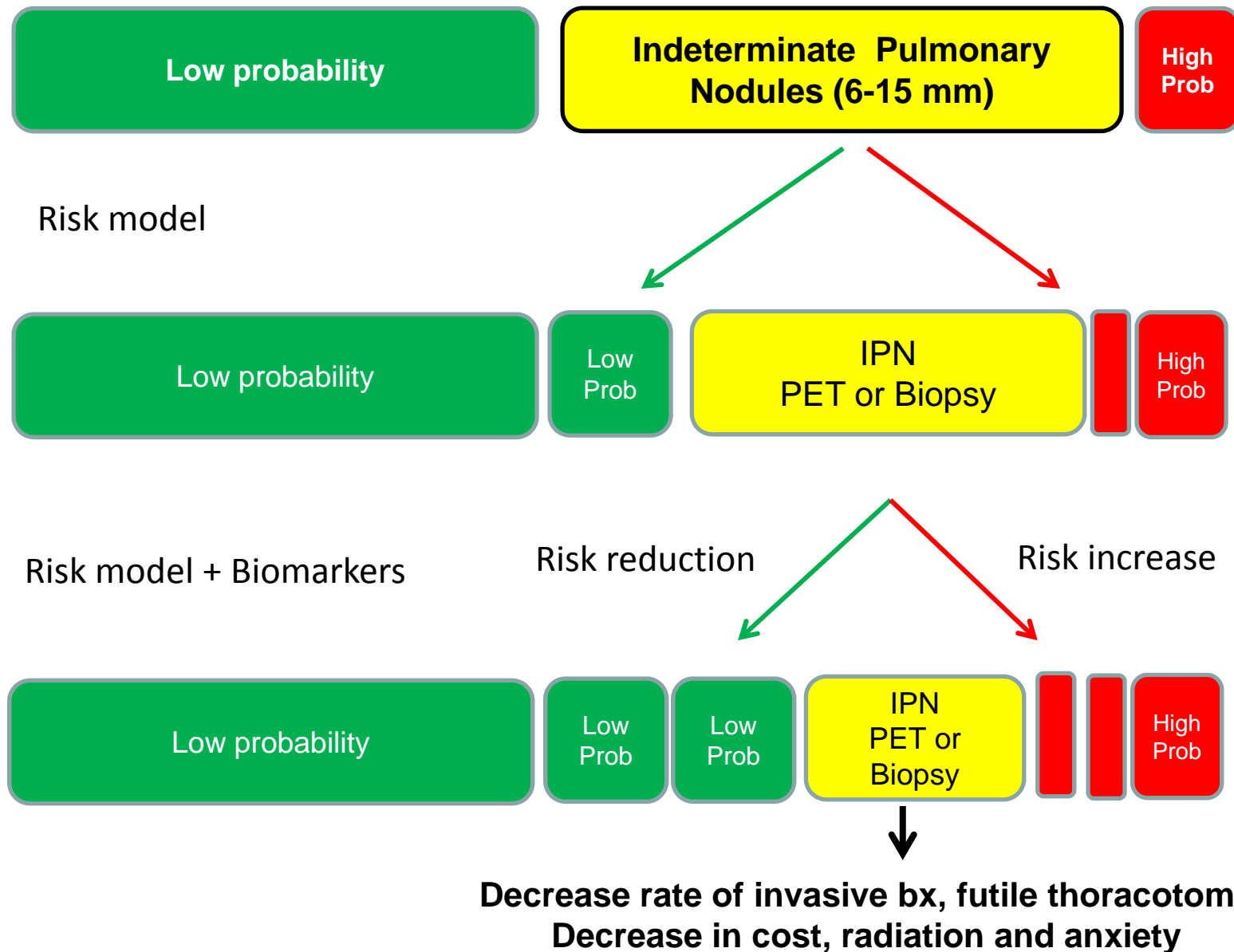
Today's Lineup

- Background
 - *Cyfra-21.1 in IPNs and Lung Cancer*
- FreeSRF Assay and Interferometric Sensing
- Improving S/N while Speeding Assay
- Translational Engineering
- CYFRA 21-1 Biomarker Quantification
- Results from Patient Cohort

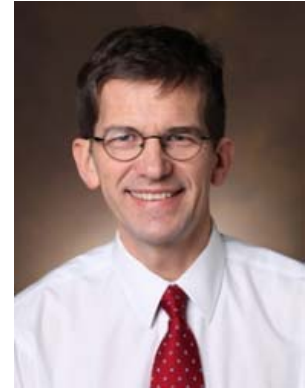
Early Detection Desperately Needed for Lung Cancer

- 5-Year Survival Still Poor at **16%**
- > 70% of Patients Diagnosed at Advanced Stage and *Incurable*.
- Current screening methods are time consuming, expensive and insensitive.
- Rapid, simple **sensitive** diagnostic assay key to improving clinical outcomes and reducing costs

Biomarkers For Clinical Management



CRP and hsCRP

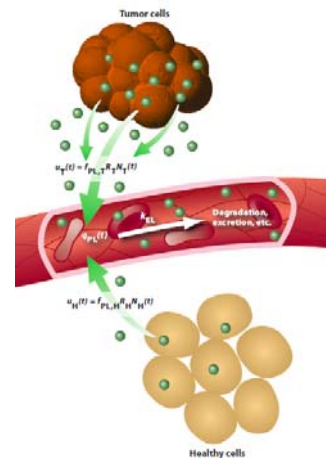
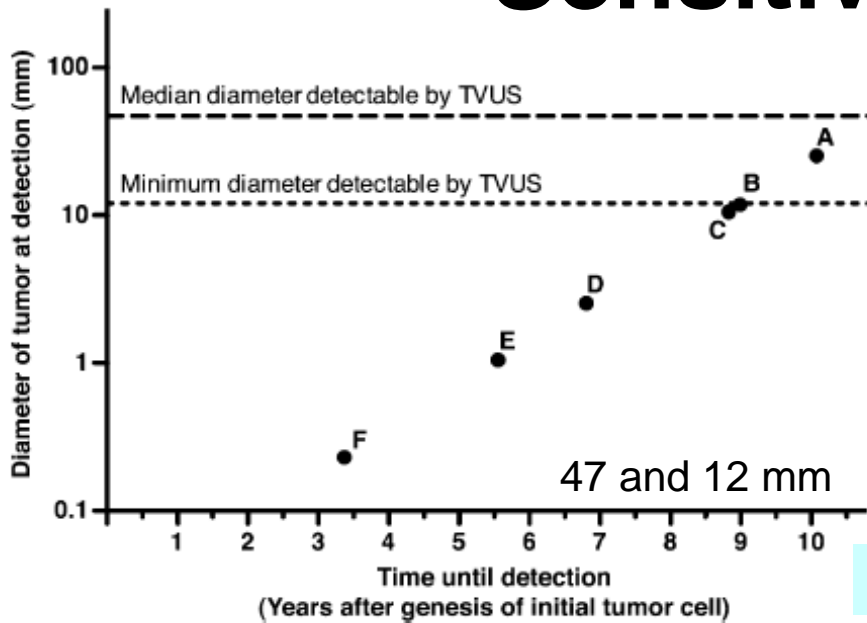


- Historically, CRP was just a general inflammation marker.
- Now CRP is marker of Cardiovascular risk due to the 30-fold improvement in sensitivity afforded by the hsCRP assay.

Rifai, Clin Chem 2001
Ridker NEJM 2001

- Can we repurpose existing (cancer) biomarkers and improve our risk assessment of disease?
- Can lowering the CYFRA-21.1 LOQ expand the dividing line between case and control.

Sensitivity Matters



- A: ELISA
- B: 100% vasc permeability
- C: not shed by healthy cells
- D: shedding is 1000x nl
- E: not shed in nl and improved 1,000 x assay sensitivity from baseline
- F: not shed in nl and improved 100,000 assay sensitivity from baseline

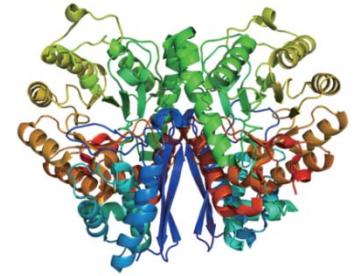
*Free-solution Assay Quantifies MAdCAM in Colon Tissue**

- Probe Volume: $\approx 4\text{nL}$ (nanoliter)
- At D.L. of 45fM (femtomolar)
 - $(50 \times 10^{-15} \text{ moles/L}) \times (4 \times 10^{-9} \text{ L}) = 200 \times 10^{-24} \text{ moles}$
 - $(200 \times 10^{-24} \text{ moles}) \times (6.02 \times 10^{23} \text{ molecules/mole})$

=120 molecules

*BJP, 70-81(2017)
DOI:10.1111/bph.13654.

CYFRA 21-1



- Fragment of Cytokeratin 19.
 - CK19 epitope is a polypeptide likely released following cell death (Sheard et al, 2002).
- Serum fragments are readily detected using anti-CYFRA 21-1 antibody (Pujol et al, 1993).
- Patients with nonmalignant disease showed almost no elevation of serum CYFRA 21-1
 - *Except* in cases of cirrhosis, renal failure, or infectious lung disease.
- CYFRA 21-1 is reasonably stable in serum
 - 48 h room temperatures and 14 days at 4 °C.

CYFRA 21.1 has a history of being used as a Cancer Biomarker

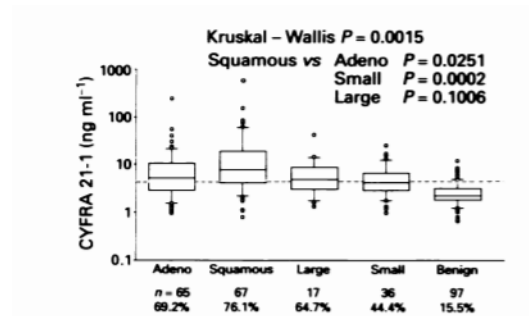
Table 2. - CYFRA 21-1

	>3.2 ng·ml ⁻¹	
	n	%
SCC*	95/158	60***
ACL	32/85	38
LCLC	7/23	30
SCLC	2/14	14
All LC**	136/280	49

SCC: squamous cell carcinoma; SCLC: small cell lung carcinoma; LC: lung carcinoma (variance among four histological types, $p=0.002$; **: -squared test).

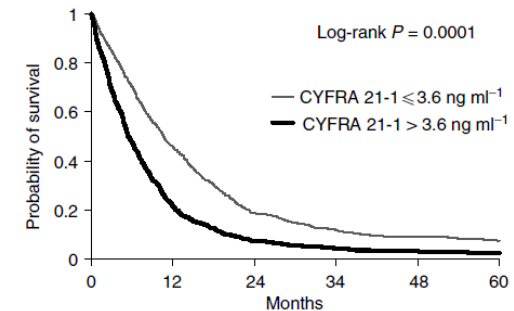
3.2 ng/mL

Rapellino ERJ 1995



3.5 ng/mL

Takada, BJC 1995



3.6 ng/mL

Pujol, BJC 2004

Recently CYFRA21-1 was shown to be important in predicting metastasis and poor prognosis in lung cancer” (Zhang et al. BMC Cancer (2017) 17:96)

Several reports have focused on using CYFRA 21-1 as part of predictive panels based on learning algorithms.

Numerous assay kits exist to quantify CYFRA 21.1

The Free-solution Assay Approach

Mix-and-Read Free-solution Assays and Interferometry:
“ELISA on steroids”.



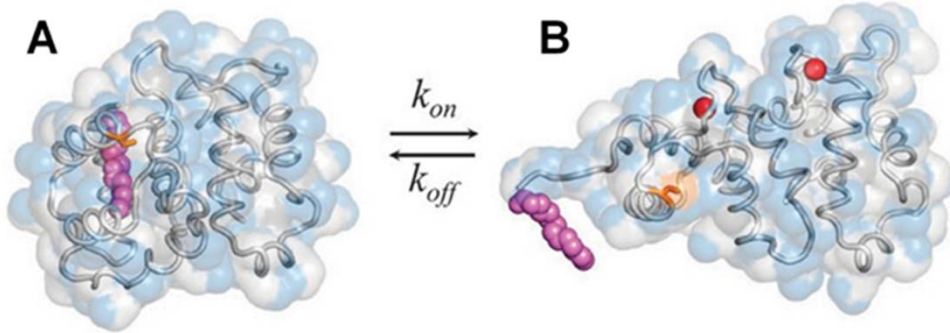
Advantages of our New Assay Method

Speed, Reduced Cost, High Sensitivity, Wide Applicability, and Complex Matrix Compatibility

The Economist, Print ed. June 2, 2011 “New light on proteins.
Biomedicine: Using lasers to study proteins on the outer membranes of cells.”

The Free-Solution Signal

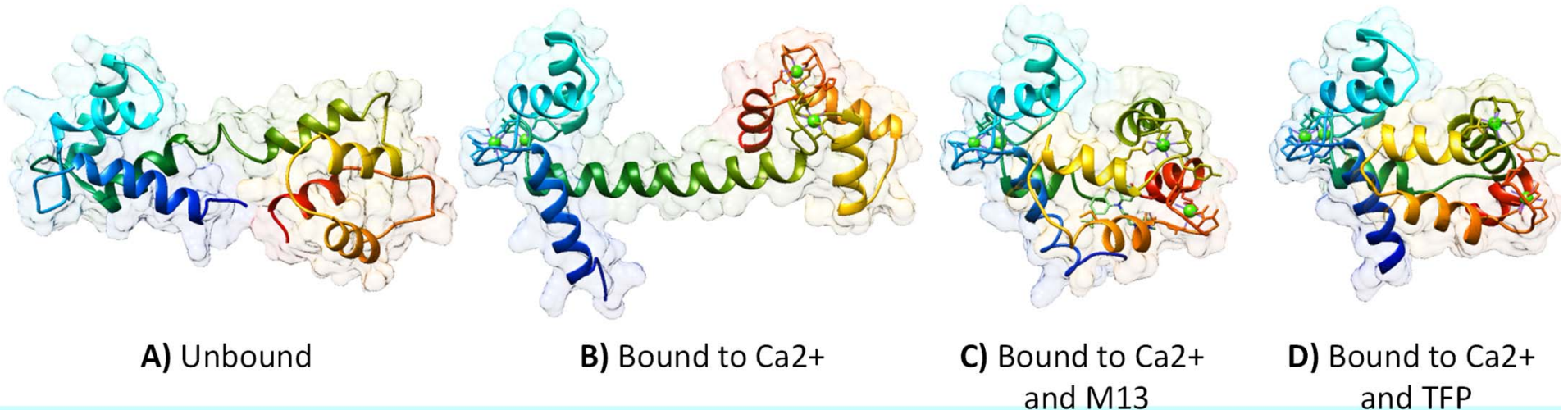
NMR structures of apo and Ca²⁺-mRecoverin



- Solvent Accessible Surface Area (SASA)
- Radius of Gyration (R_{gyr})

Δ in conformation and hydration $\sim \Delta RI$

Assay is robust, reproducible and sensitive.
Predictative model recently published (PNAS)



Bornhop, D.J., Michael N. Kammer, A. Kussrow, R. A. Flowers, and J. Meiler. "Origin and Prediction of Free-solution Interaction Studies Performed Label-free." *PNAS USA* 113.12 (2016)

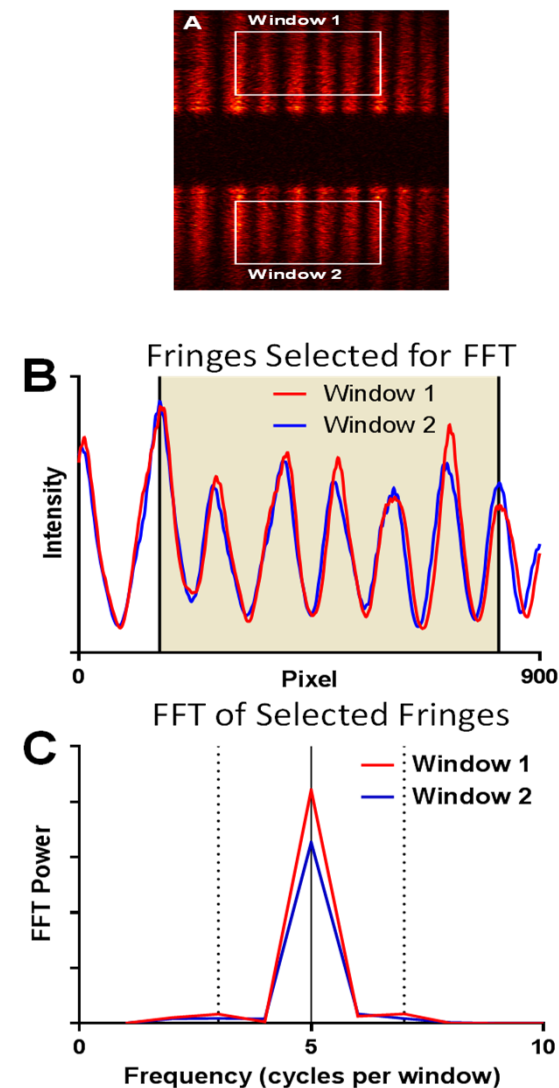
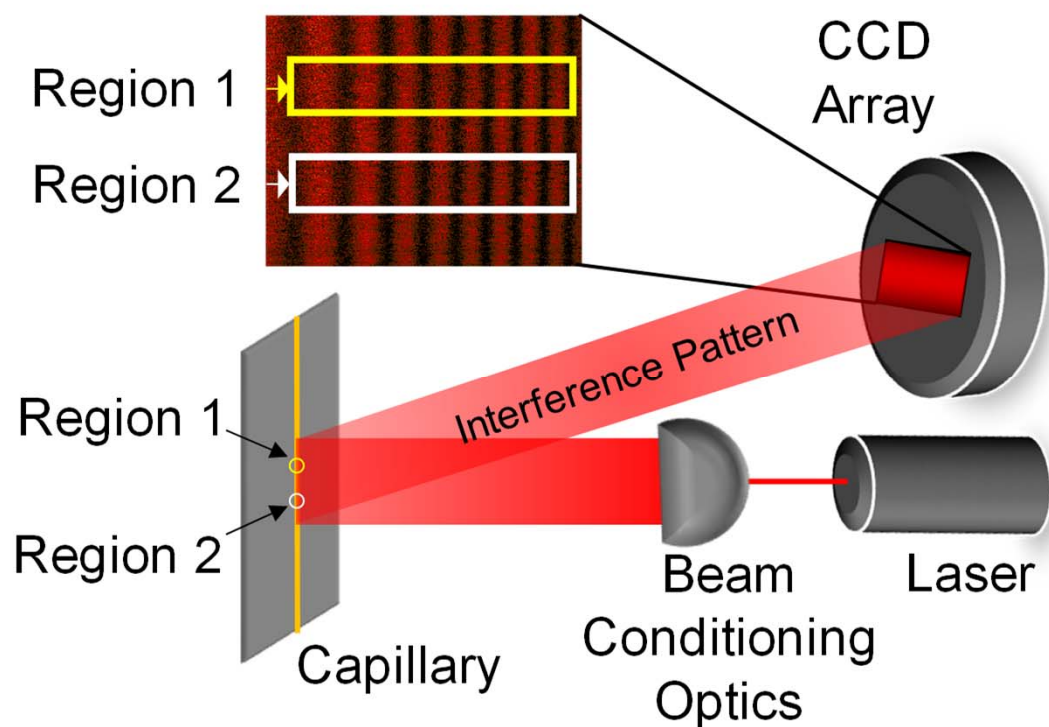
Path to Improved Assay Performance and Throughput

- **New Label-Free Reader**
 - Compensated Interferometer
 - Inexpensive, Simple Optical Train
 - Temperature Insensitive!
 - Improved S/N, Capillary flow cell
- **Free-solution Assay using Droplet Generation**
- **Mix-and-Read**
 - No immobilization or derivitization
 - Small sample volumes (nL- μ L)
 - Automated sample introduction



NOW WHAT?

The Reader: Compensated Interferometer (CI)

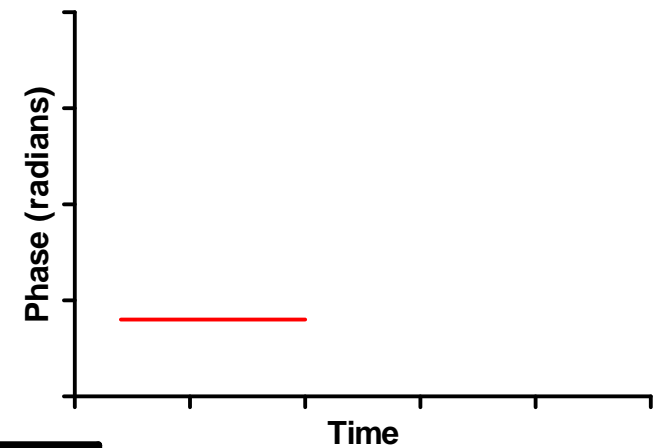
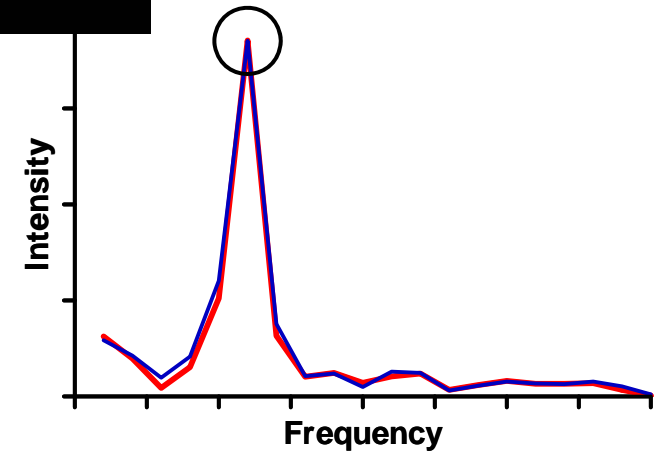
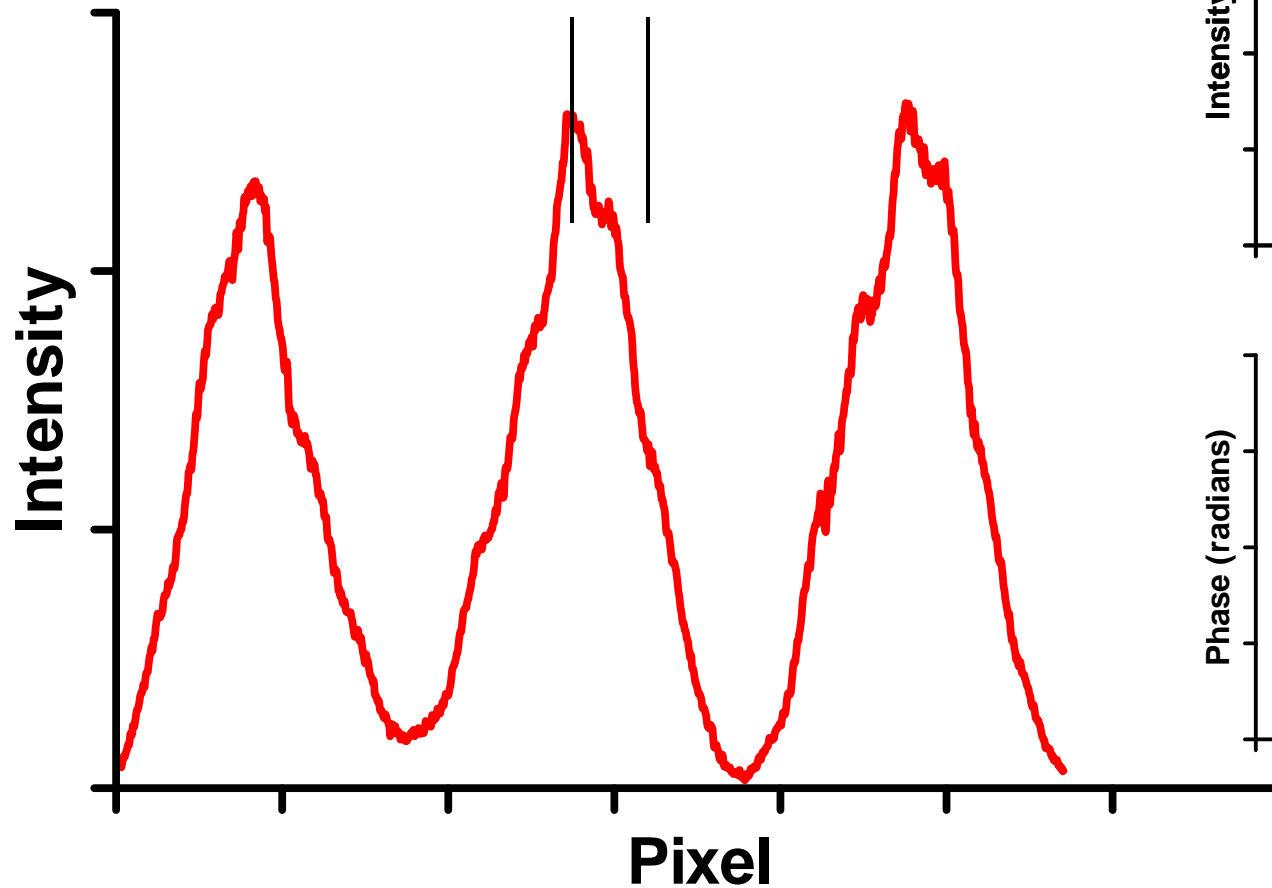
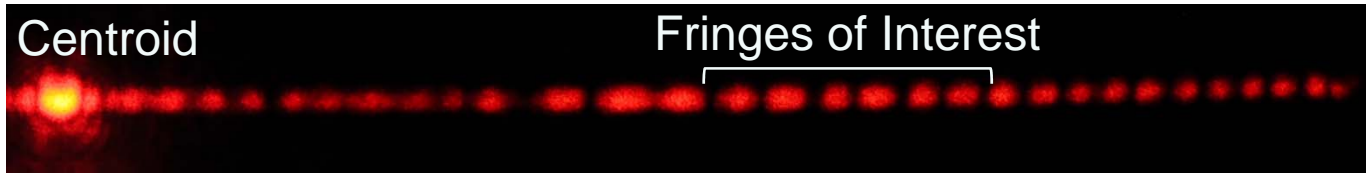


Compensation: 1.1×10^{-8} RIU baseline
Largest undetectable Temp. Change = 8° C

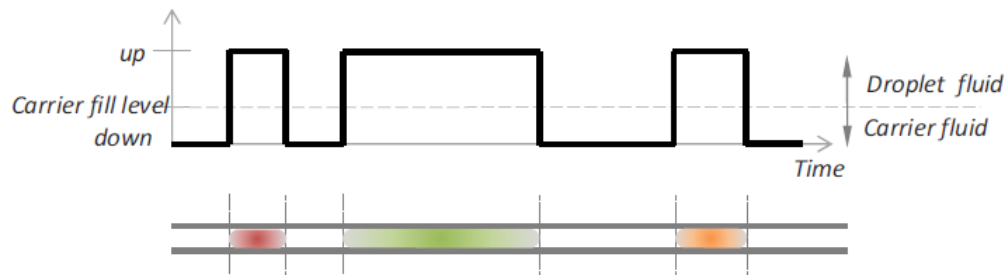
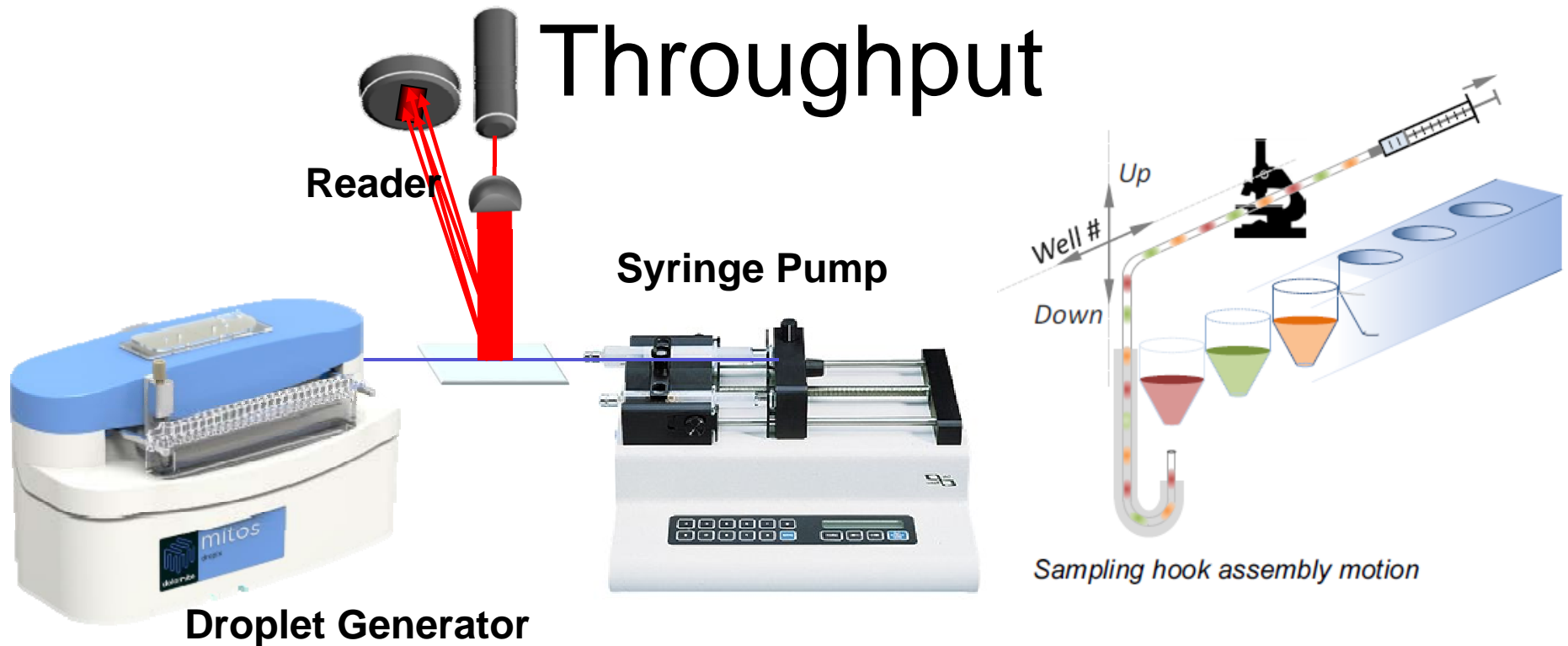
Kammer, et.al., *Anal. Chem.*, Revised (Feb. 2017)
US and PCT patents granted and pending.

Measurement of the Fringe Shift

Fringe Pattern:



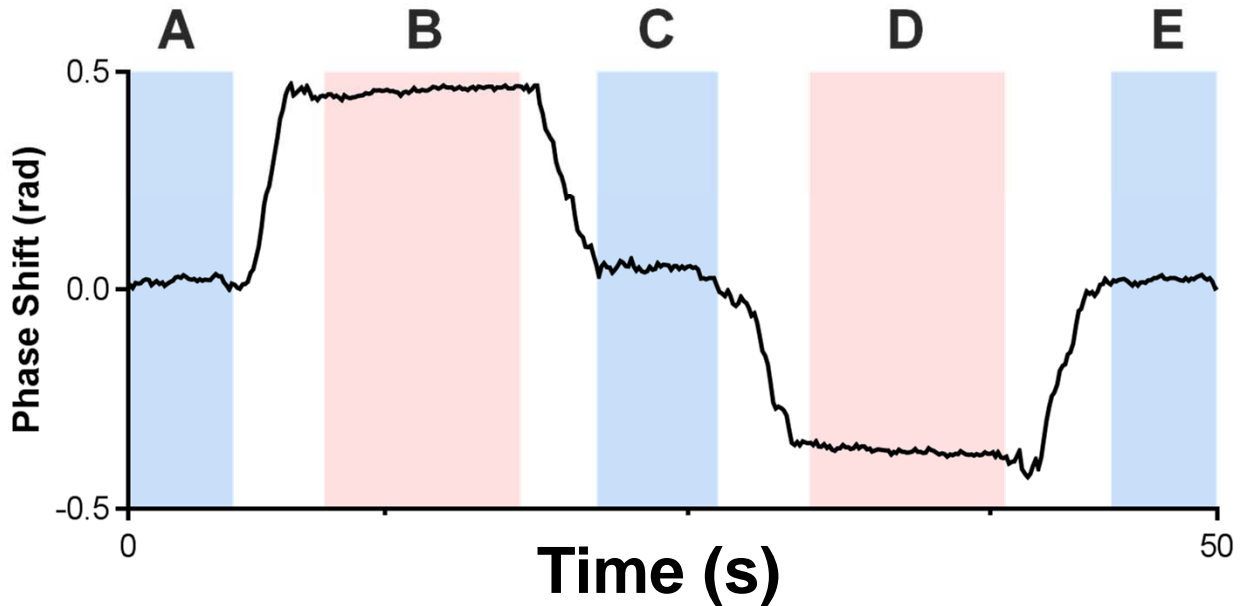
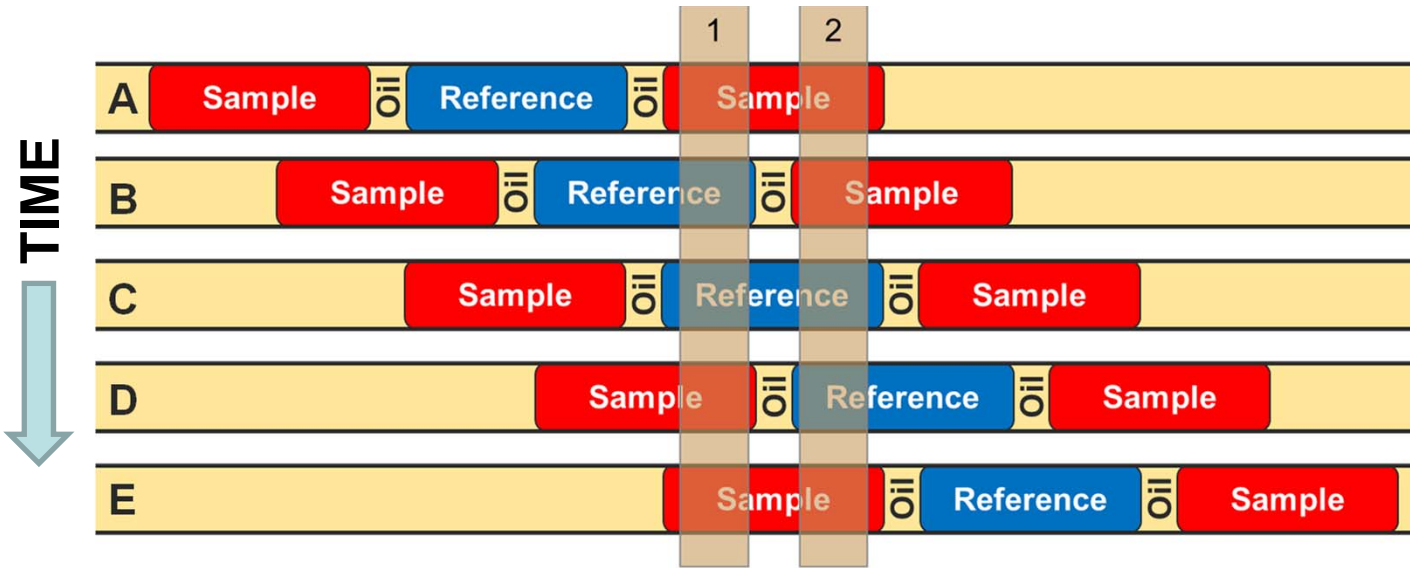
Droplet Generation for Higher Throughput



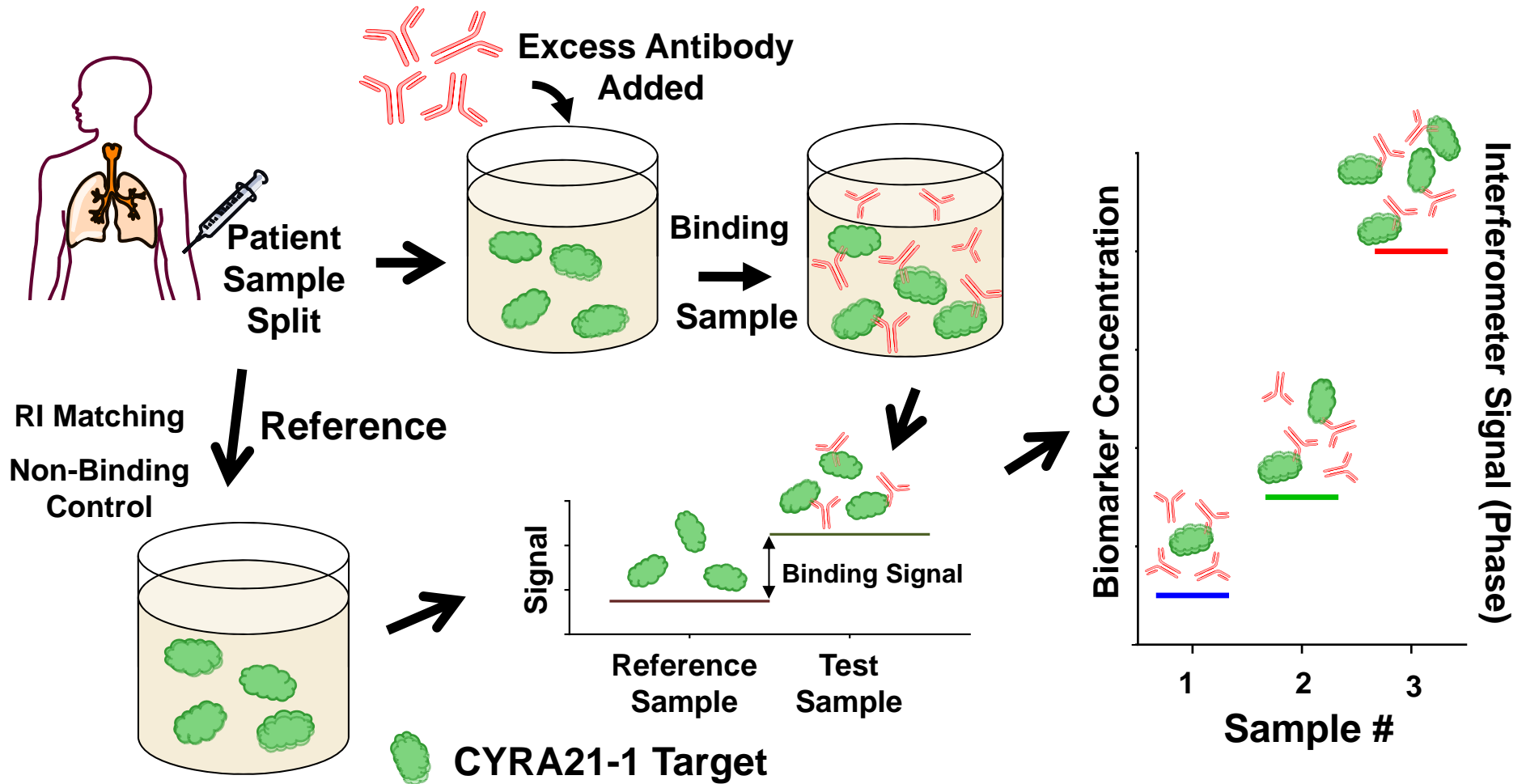
'Up' and 'down' Motion of the sampling nozzle controls droplet size and carrier spacing.

Signal Extraction in HT-CI

Detection Windows

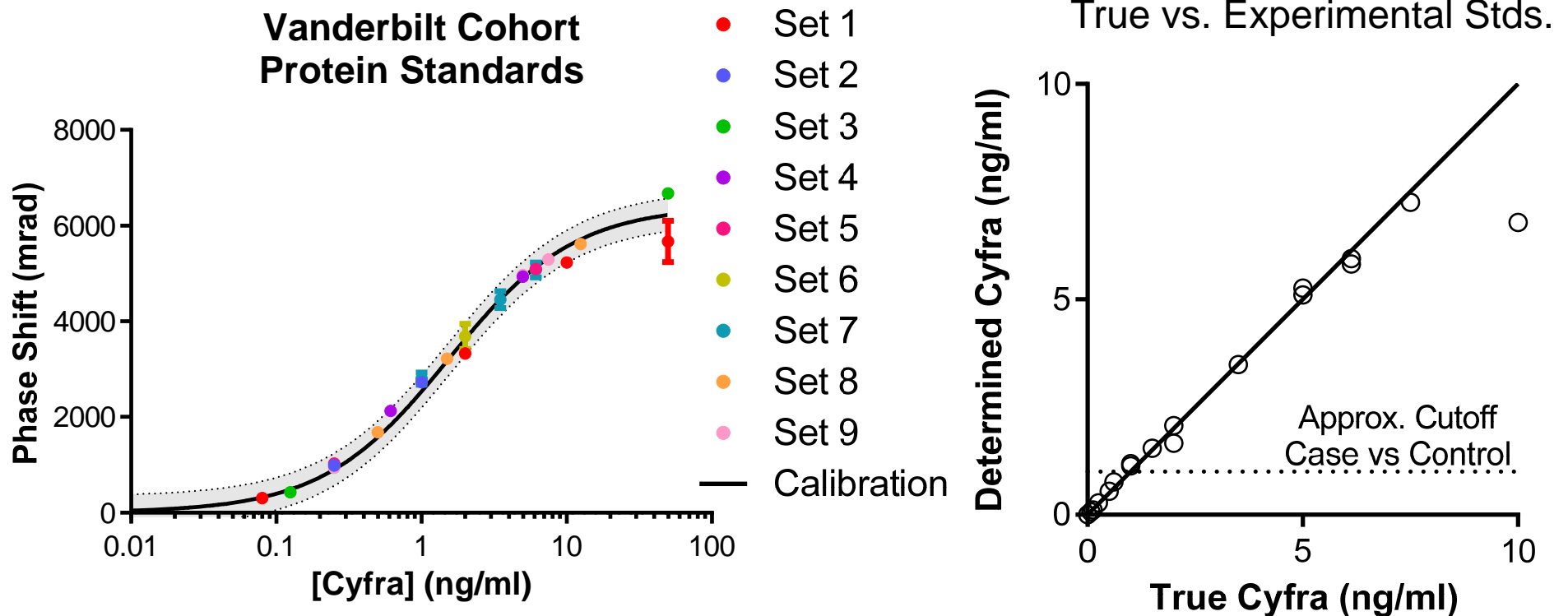


Mix-and-Read Free-Solution Assay



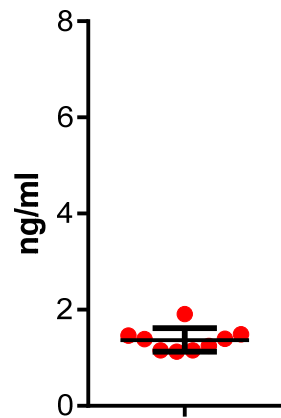
- Determine if probe produces quantifiable signal.
- Calibration done using spiked serum samples.
- Non-specific binding tested by titrating antibody with serum.

Daily Standards of Spiked Serum Ensure Accurate CYRFA Quantification



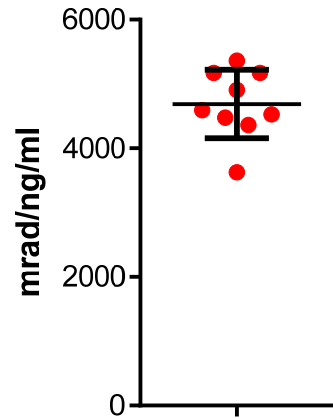
- Black line = Pooled calibration curve
- Grey area = 95% confidence interval
- Colored dots = Protein standards run on each day
- **Minimum of 6 replicates at each concentration**

Vandy Cohort Analytical Figures of Merit



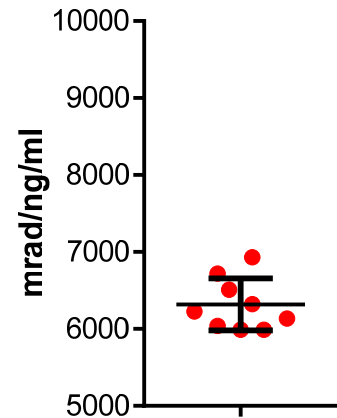
K_D

1.37 ± 0.24
C.V. 17.8%



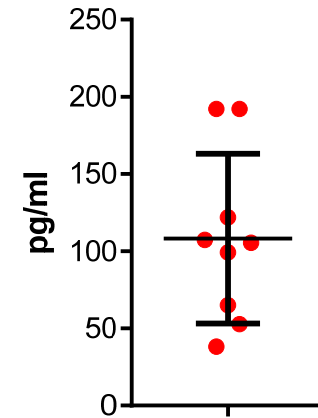
Slope

4689 ± 533
C.V. 11.3%



B_{max}

6318 ± 337
C.V. 5.3%



LOQ

108 ± 55
C.V. 50.8%

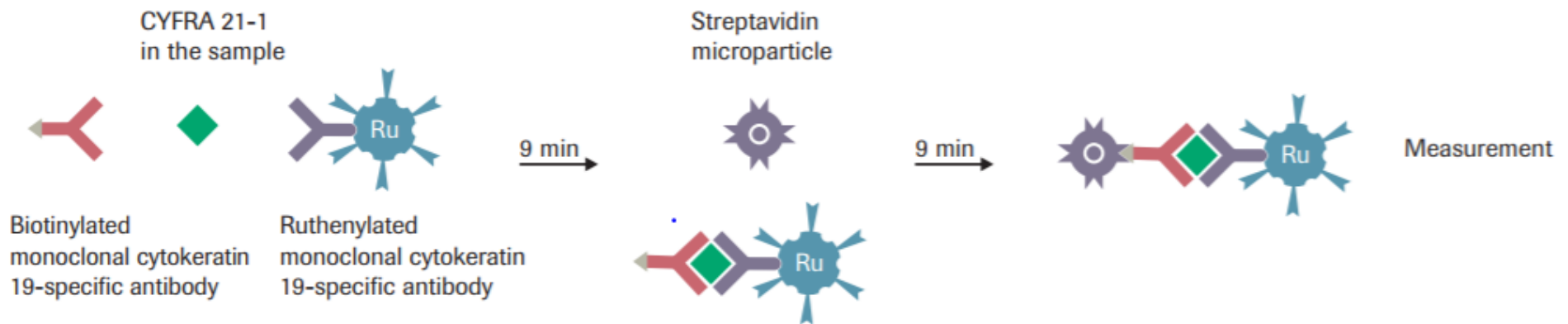
Probe = Cyfra 21-1(CK19) Monoclonal Antibody

Made from Mouse

https://www.mybiosource.com/prods/Antibody/Monoclonal/Cyfra211/datasheet.php?products_id=850246

Electrochemiluminescence Immunoassay (ECLIA) (ELISA)

Test principle: one-step sandwich assay



The Elecsys CYFRA 21-1 assay is based on two specific monoclonal antibodies (KS 19.1 and BM 19.21) against fragments of cytokeratin 19 having a molecular weight of approx. 30 kD.⁹



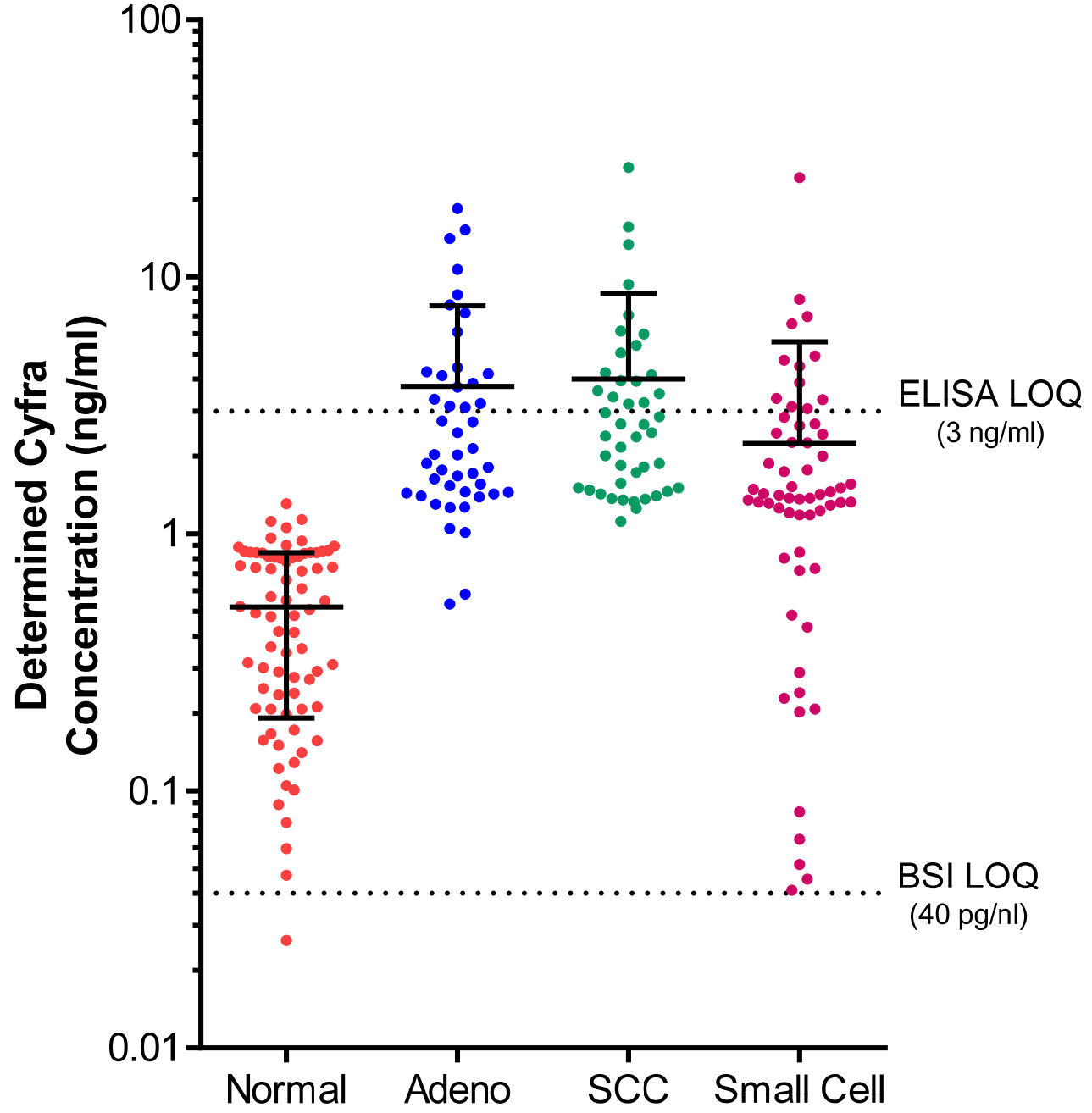
- absorbance using capture
- Direct vs Sandwiched
- Pros: Cost, Throughput, Accessibility
- Cons: Mileage may vary, probes must be developed

Patients Characteristics

Assembled cohorts	Preliminary data VUMC n=225			
	ADC (%)	SCC (%)	SCLC (%)	No Cancer (%)
Patients	N=45	N=44	N=61	N=75
Age ± SD	65.2 ± 8.0	65.8 ± 7.8	63.9 ± 8.9	59.2 ± 12.7
Gender				
Male	26 (58)	29 (66)	36 (59)	40 (53)
Female	19 (42)	15 (34)	25 (41)	35 (47)
Nodule Size (cm) ± SD	2.7 ± 1.7	2.7 ± 2.0	3.6 ± 2.6	2.5 ± 1.6
Smoking Status				
Never Smoker	0 (0)	0 (0)	3 (5)	1 (1)
Ex-Smoker	26 (58)	23 (52)	18 (30)	38 (51)
Current Smoker	19 (42)	21 (48)	40 (65)	36 (48)
Pack Years ± SD	50.1 ± 31.3	53.9 ± 23.5	63.7 ± 32.8	41.1 ± 30.3
Cancer Path Stages				
IA-IB	33 (73)	34 (77)	0 (0)	0 (0)
IIA-IIIA	12 (27)	10 (23)	0 (0)	0 (0)
IIIB-IV	0 (0)	0 (0)	0 (0)	0 (0)
Limited	0 (0)	0 (0)	33 (54)	0 (0)
Extensive	0 (0)	0 (0)	28 (46)	0 (0)
Cancer histologies				
Adenocarcinoma	45			
Squamous carcinoma		44		
Large cell carcinoma				
Small cell carcinoma			61	
Other malignant				
Benign Histologies				
TB				6 (8)
Fungal infection				7 (9)
Bacterial infection				10 (13)
Inflammation				45 (60)
Fibrosis				3 (7)
Hamartoma				2 (3)
COPD				

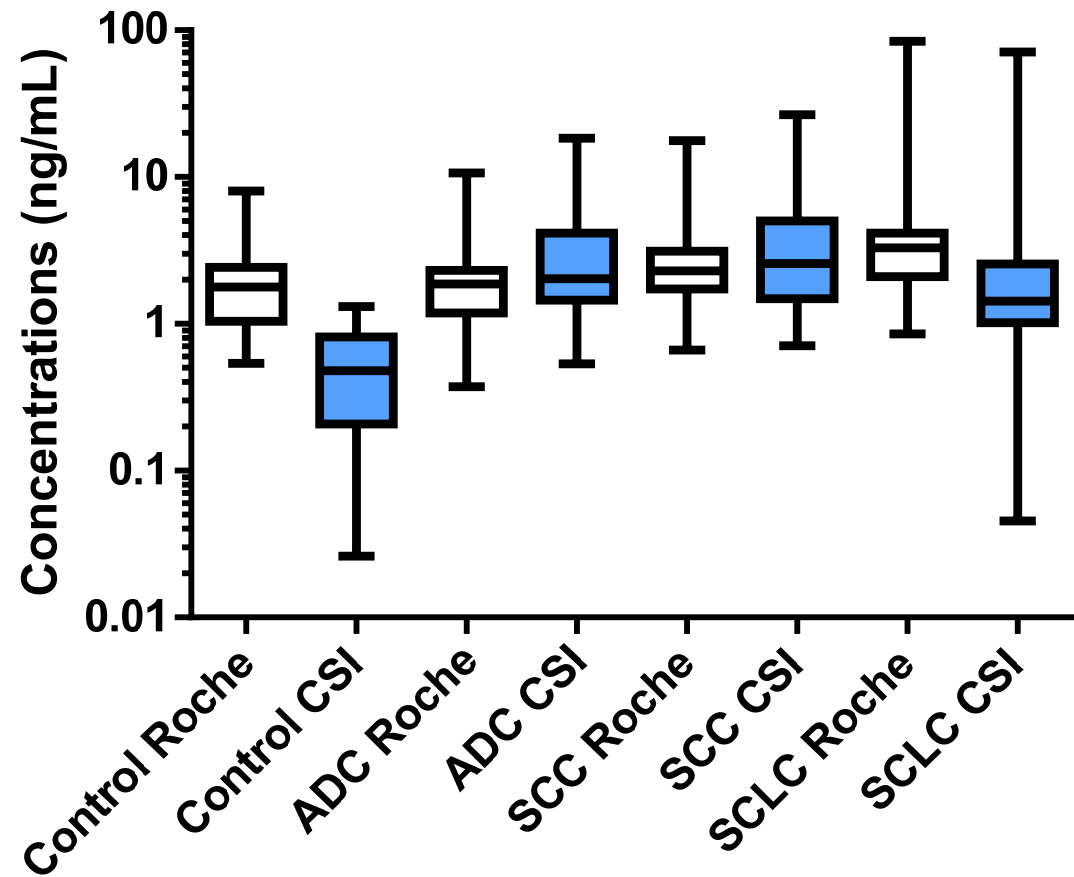
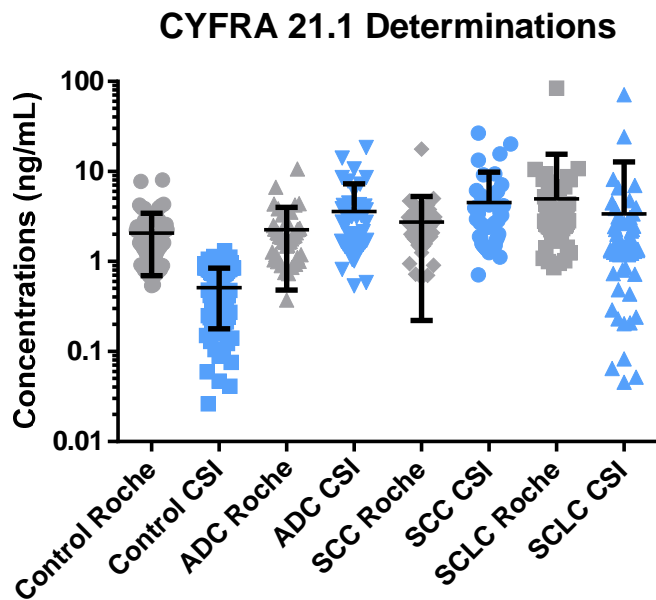


Results from Vanderbilt 225 Patient Cohort

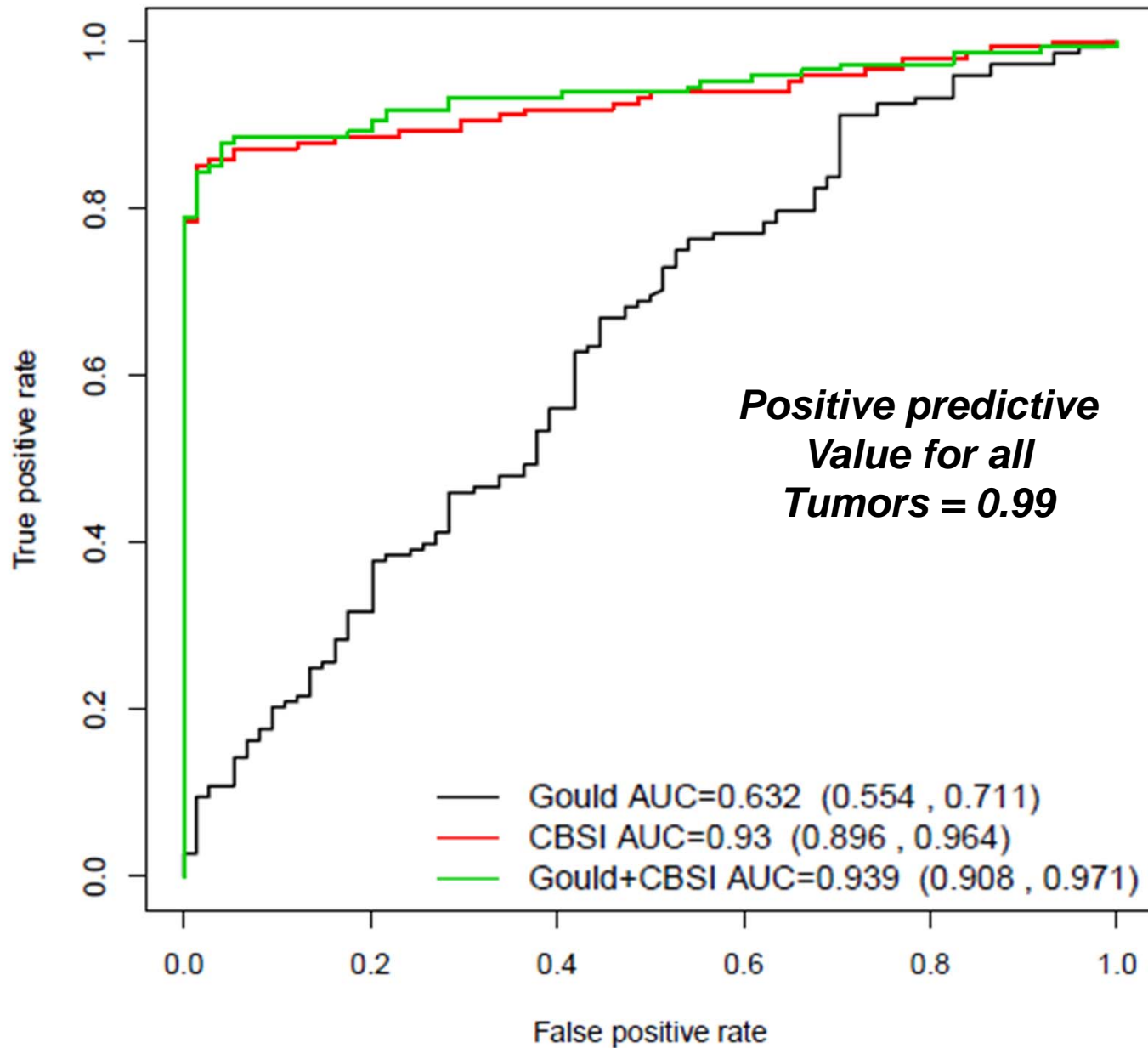


Bake Off with Roche Cobas ELC Assay

CYFRA 21.1 Determinations



ROC Curve for Cyfra-21.1



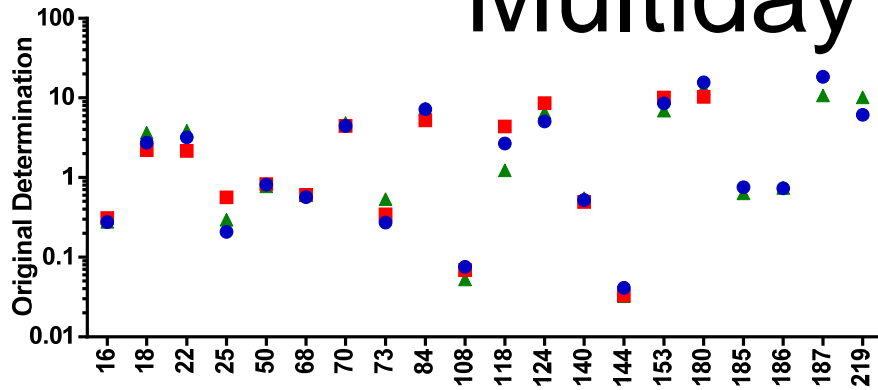


Preliminary Results

CYFRA 21.1-CBSI	All tumors	Adenocarcinoma	Squamous Ca	Small cell Lung Ca
AUC	0.924 (0.889, 0.96)	0.972 (0.943, 1)	0.99 (0.973, 1)	0.841 (0.763, 0.92)
cutoff	1.19	1.01	1.12	1.19
Sensitivity	0.85	0.93	0.98	0.75
Specificity	0.99	0.95	0.97	0.99
PPV	0.99	0.91	0.96	0.98
NPV	0.77	0.96	0.99	0.83
<u>DLR.Positive</u>	<u>64</u>	<u>17.5</u>	<u>36.6</u>	<u>56.6</u>
DLR.Negative	0.15	0.07	0.02	0.25
FP	1	4	2	1
FN	22	3	1	15
Optimal Criterion	0.84	0.88	0.95	0.74

Multiday Validation

10 cases & 10 controls selected by Pierre's group to rerun on two more days, each day undergoing another freeze thaw cycle.



● Original Determination
■ +1 Freeze Thaw Cycle
▲ +2 Freeze Thaw Cycle

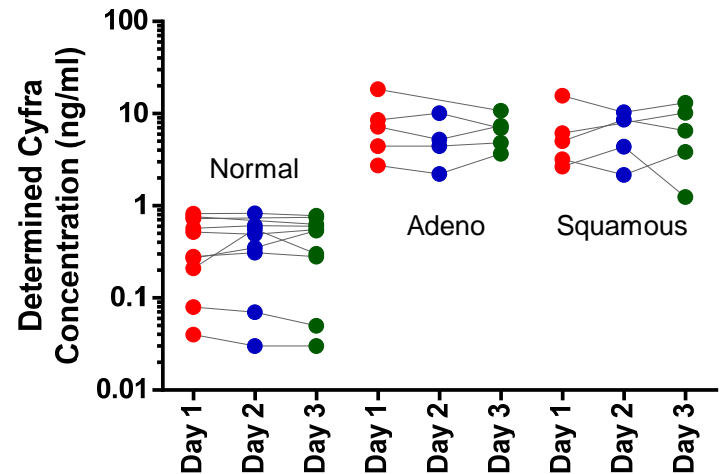
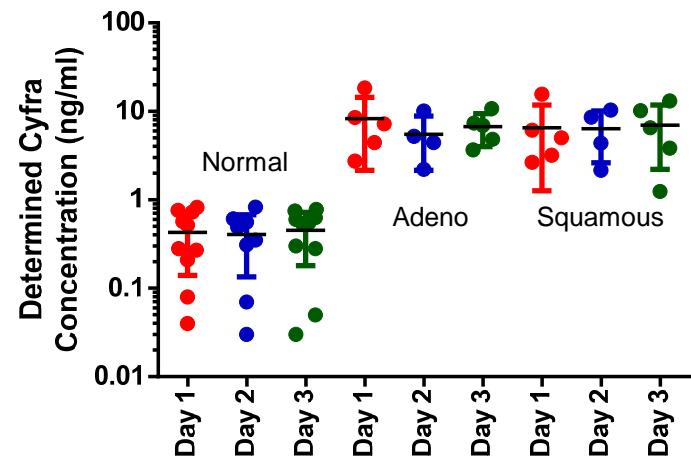
Day 1	Day 2	Day 3	C.V.	Average
0.28	0.31	0.28	6.4%	0.29
2.75	2.21	3.66	25.6%	2.85
3.20	2.16	3.86	28.0%	3.07
0.21	0.56	0.30	52.0%	0.357
0.82	0.83	0.78	3.1%	0.81
0.57	0.61	0.60	3.1%	0.59
4.45	4.45	4.85	5.1%	4.58
0.27	0.35	0.54	35.5%	0.38
7.23	5.24	7.41	18.2%	6.62
0.08	0.07	0.05	18.1%	0.067
2.67	4.39	1.24	57.1%	2.76**
5.05	8.60	6.53	26.5%	6.73
0.52	0.49	0.55	5.6%	0.52
0.04	0.03	0.03	13.6%	0.03
8.52	10.14	6.95	18.7%	8.53
15.65	10.33	13.17	20.4%	13.05
0.76	*	0.63	12.2%	0.69
0.73	*	0.75	1.2%	0.74
18.47	*	10.77	37.2%	14.62
6.15	*	10.22	35.1%	8.18
Average C.V.			21.1%	

* Poor droplet performance

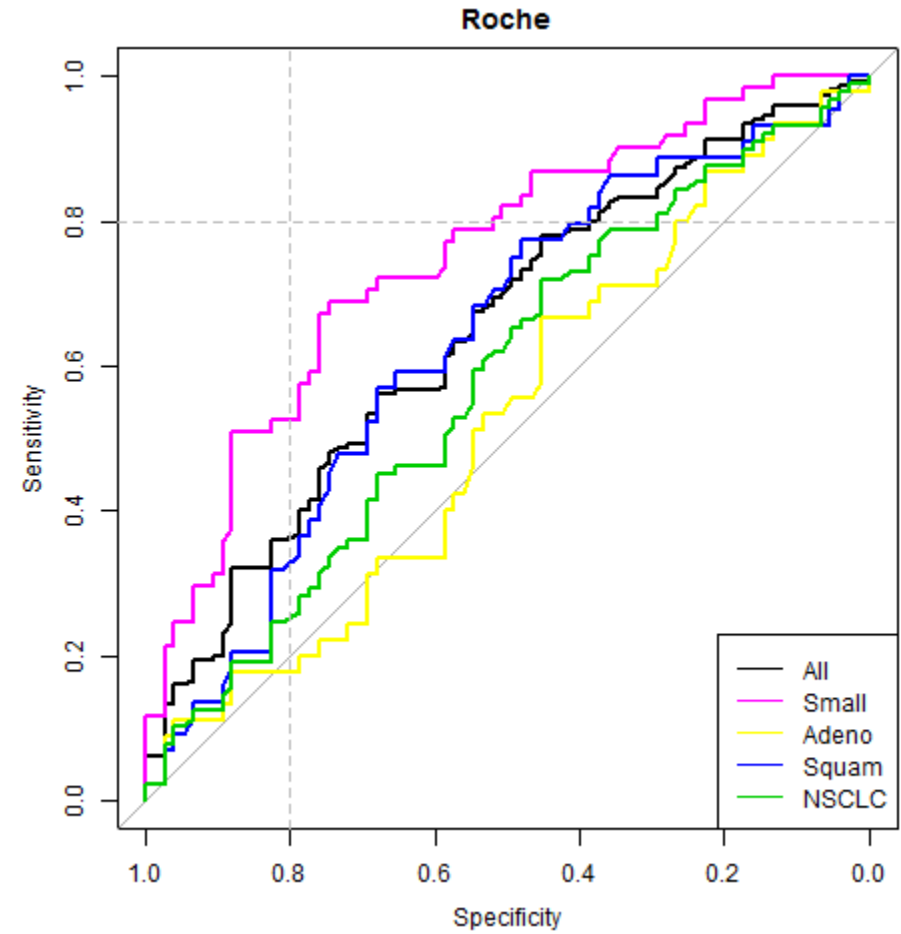
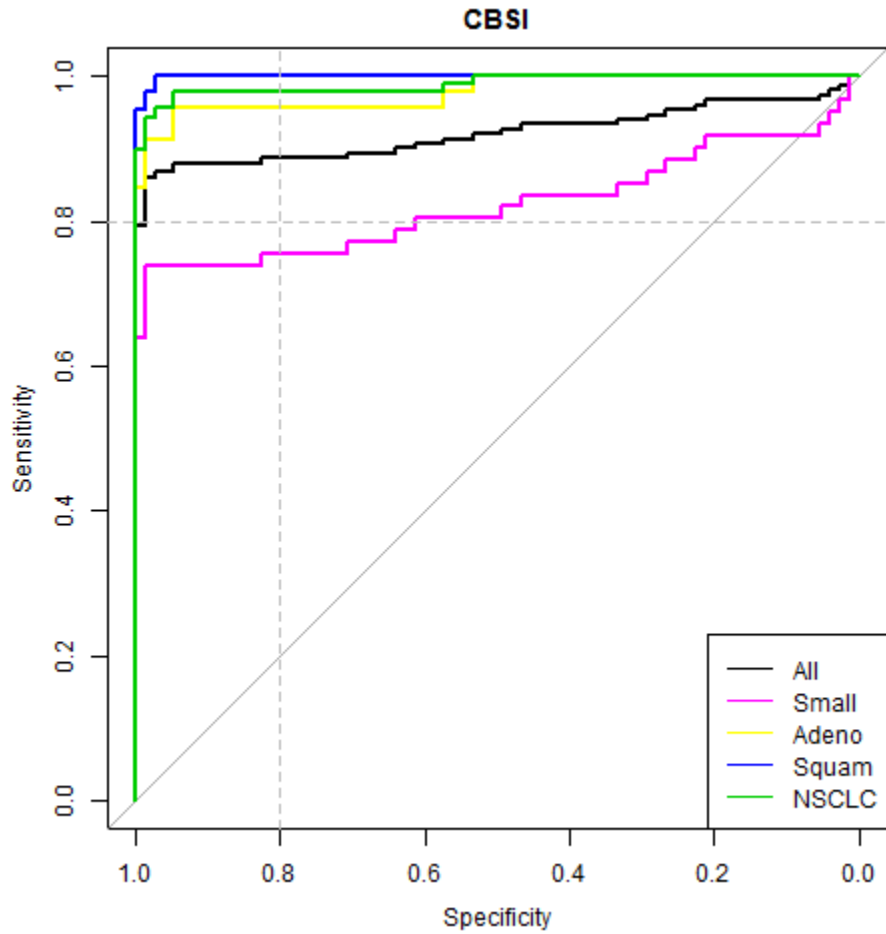
**Still a case

Large C.V.s seen at ends of Calibration curve.

Day 3 = 3rd freeze thaw cycle



Reanalysis By Fred Hutch



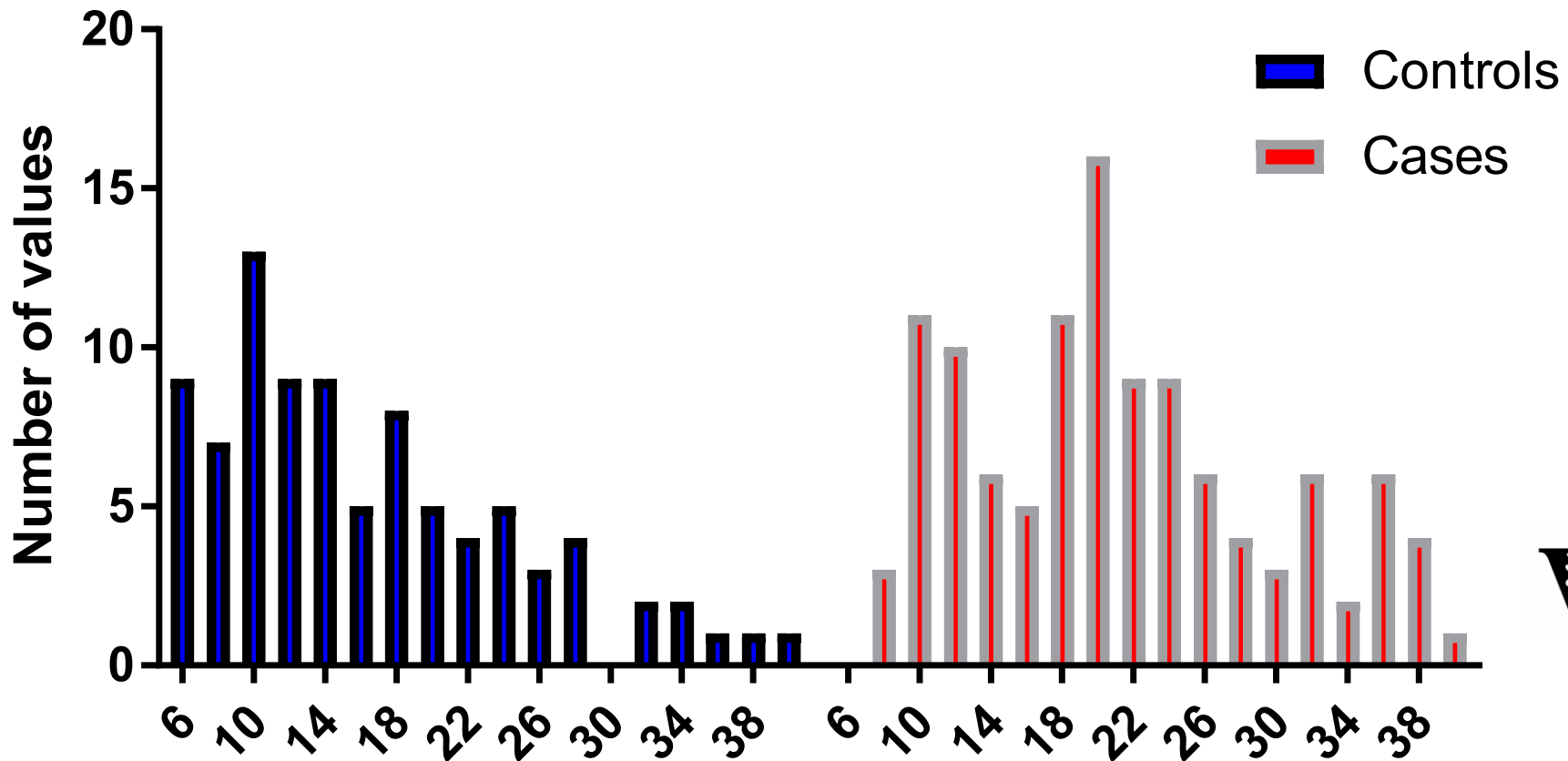
Independent Analysis of CYFRA 21-1 for Vanderbilt Cohort - Fred Hutch

- Results consistent with those presented in the application to the EDRN.
- The discrimination improvement of CBSI over Roche is quite impressive. (Figure 2, Tables 3 and 4)
- The discrimination by CBSI between controls and SCLC cases is not as good as for NSCLC cases
- The opposite is observed when measuring CYFRA 21-1 by the Roche approach.
- Cohort has a somewhat higher percentage of males, are slightly older, and have greater smoking intensity (pack-years) than the controls; cases and controls appear well-balanced with respect to nodule size. (Table 1)

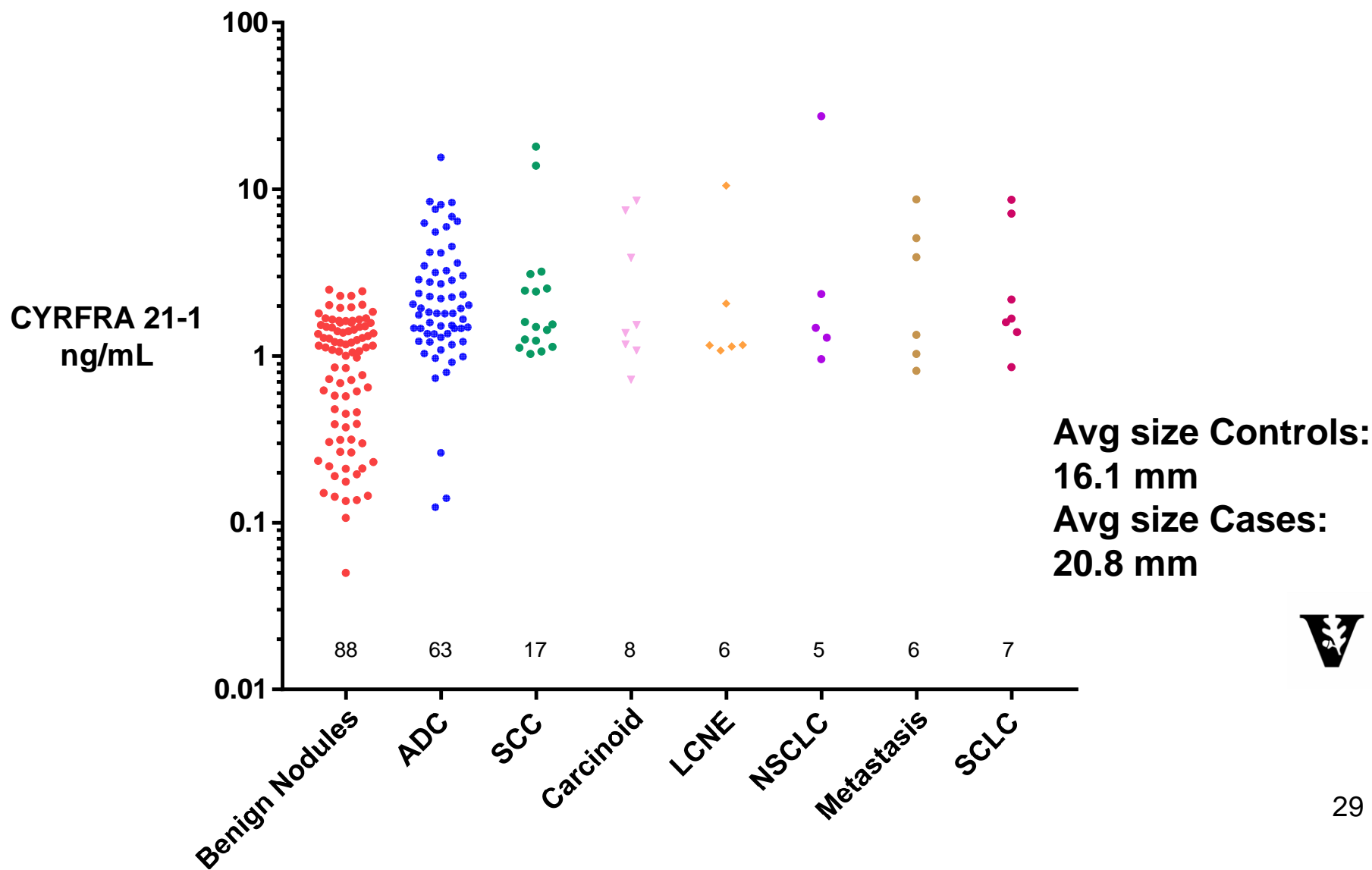
In Hutch DMCC analysis of Vanderbilt data, we took a conservative approach to evaluating the biomarker as a test and used a threshold that gave 80% specificity in the data set. (89% sensitivity at specificity=80%.)

Validation Cohort II : 200 prospective IPNs from VUMC/VA

Histogram of Nodule size



Validation Cohort II: 200 prospective IPNs from VUMC/VA



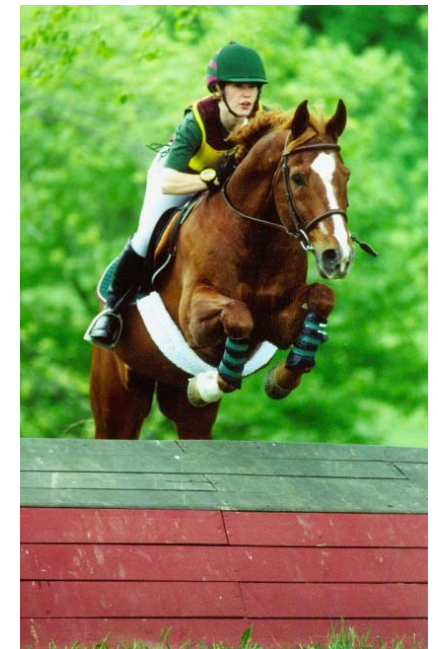
Toward Clinical Translation

- LOQ improvement predicted with the implementation of a automated sample preparation (robot).
- Automated signal extraction software
 - Written and being tested.
- Enhanced S/N based on FreeSRF
 - Enhanced structural / hydration changes
 - Aptamer, Second antibody, etc.
- General instrumentation refinements.

Collaborators

Vanderbilt: Dr. Pierre Massion
and Megan Hoeksema,
Profs. Jens Meiler,
Rick Haselton, and Heidi Chen
Lehigh: Prof. Robert Flowers
Fred Hutch: Dr. Tracey Marsh

Lab Peeps



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- *Tom Labrecque Foundation*



Vanderbilt Institute of Chemical Biology

