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Development and Validation of Methods for Detection of Aflatoxin-Lysine Adduct in Dried Blood Spot Samples

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Aflatoxins

- A group of potent mycotoxins produced mainly by *Aspergillus flavus* and *A. parasiticus;*
- Widespread food contaminants, especially for corn & corn products, peanuts & other groundnuts, and rice;
- Human aflatoxicosis and hepatocellular carcinoma;
- Immunosuppressors;
- Anti-nutritional agents;
- Inhibition of children growth and development.

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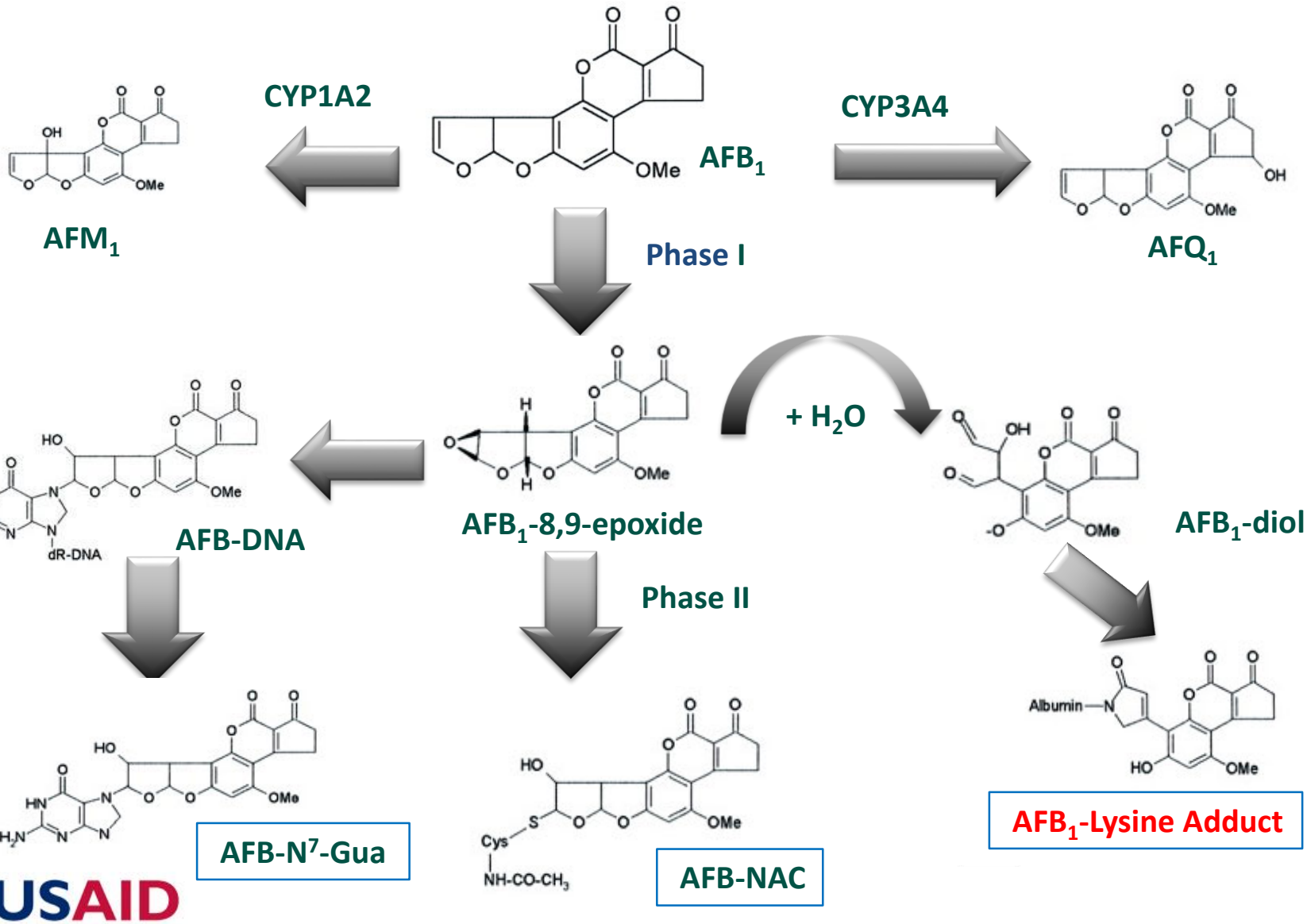
Metabolic Pathways of AFB_1



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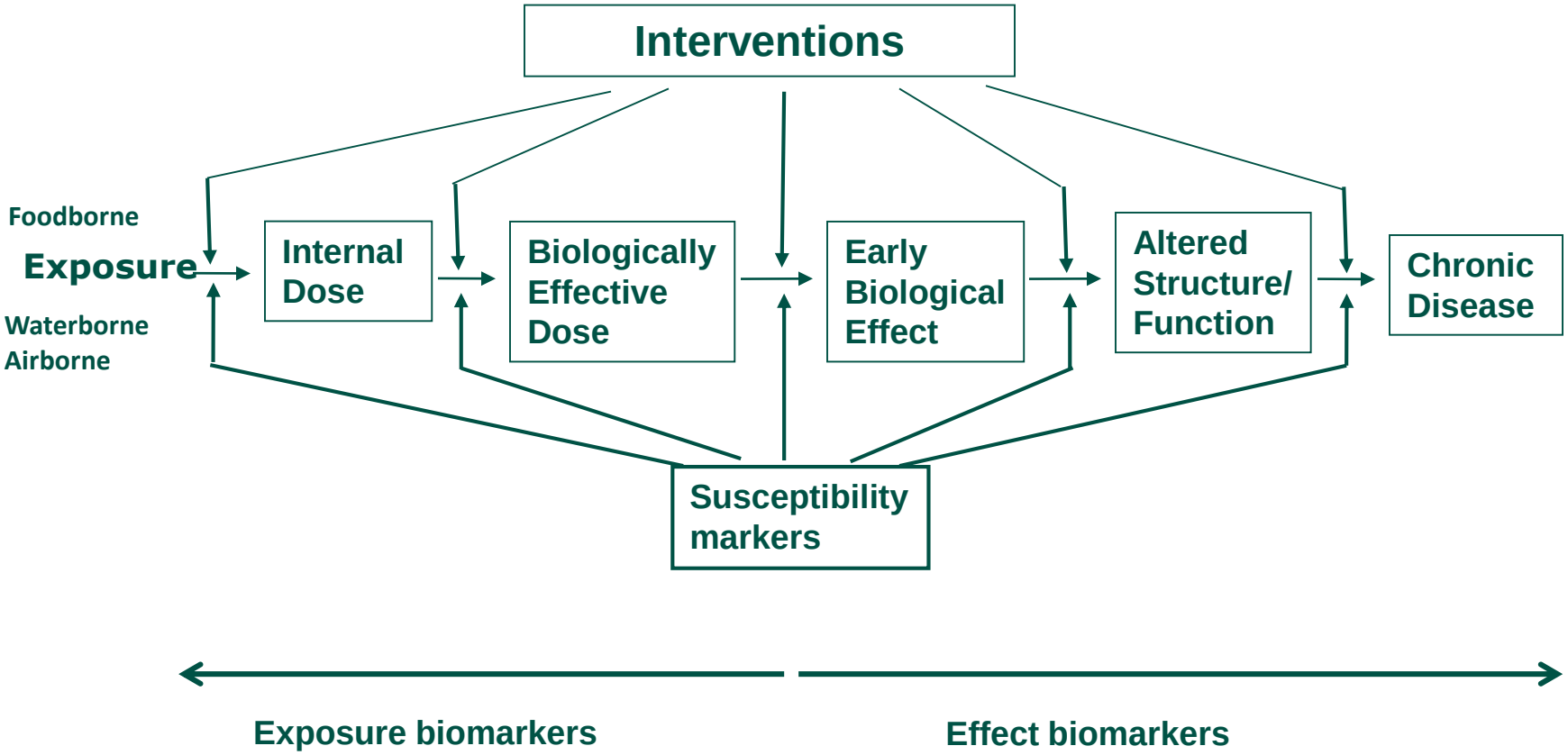
Biomarkers



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Original Goals

- To establish and validate methods for measuring major aflatoxin biomarkers in human dried blood spot (DBS) samples;
- To support needs of aflatoxin exposure assessment in USAID supported Peanut and Mycotoxin Innovation Laboratory (PMIL) and Nutrition Innovation Laboratory (NIL) research projects.

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Working Hypothesis

- Levels of AFB_1-lysine adduct in human blood or DBS samples are correlated to dietary aflatoxin exposure and will be a reliable effective biological response indicator for aflatoxin-linked adverse health effects in high-risk human populations.

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Background Information

- DBS sampling technique was first developed to screen newborn babies for the genetic metabolic disorder, phenylketonuria.
- DBS has been applied to nutritional supplement studies and pharmacokinetics' studies during new drug development.
- DBS has been used for various "omics" studies.
- DBS has been proposed to use for HIV and HCV research and various environmental exposure studies.

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Advantages of DBS Technique

- Less invasive;
- Uses smaller blood volumes;
- Utilizes simple storage methods;
- Minimizes shipping expenses;
- Offers convenient sampling;
- Reduces risk of blood-borne pathogens such as HIV, etc.

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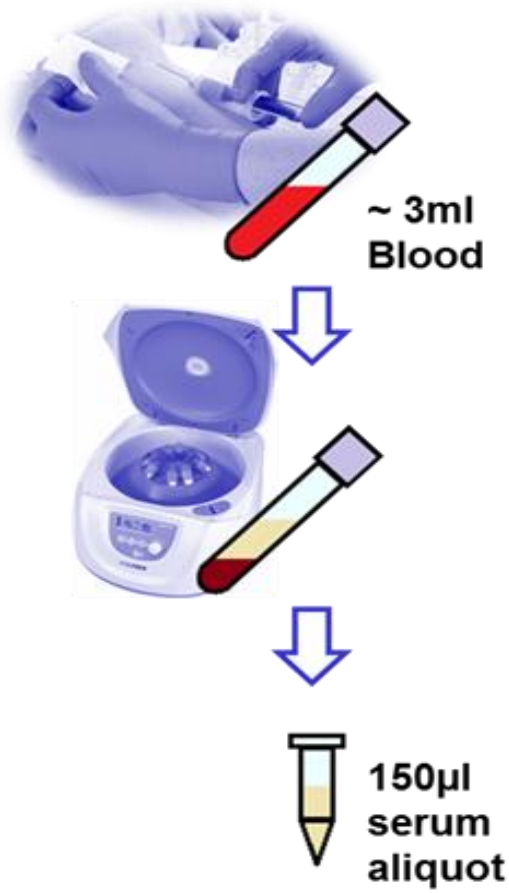
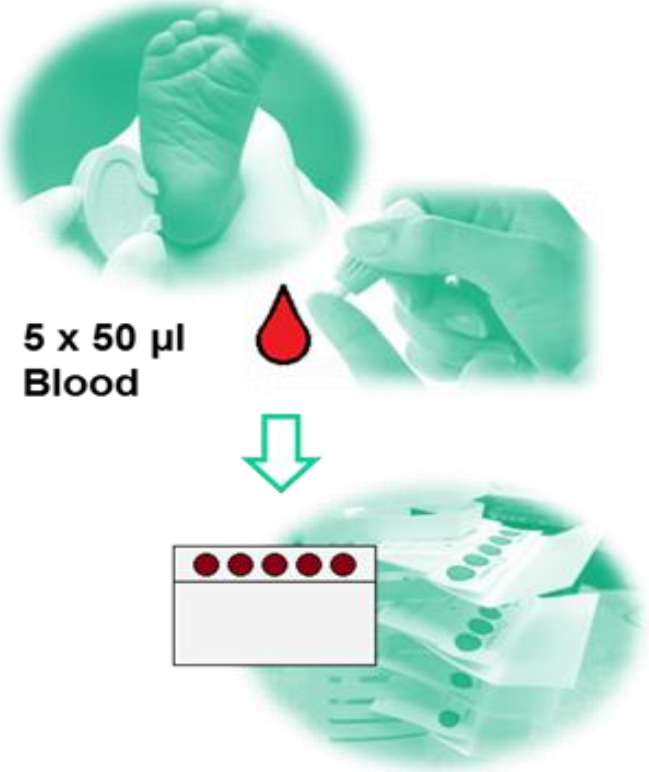
Regular methods



Dried Blood Spots



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Challenges

- Hold enough mycotoxin or test target?
- Sensitivity?
- Specificity?
- Accuracy?
- Platform analysis for large quantity of samples?
- Acceptation?

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Phase 1 Objectives

- To compare capacity of DBS cards from different commercial sources for holding the whole blood, serum/plasma, and to optimize the elution procedure for recovery of all materials in DBS cards.
- To establish methods to measure concentrations of total proteins and albumin in diluted micro-volume eluting solutions and to optimize conditions of enzyme digestion to release aflatoxin-bound lysine adduct from the albumin.

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Phase 1 Objectives (continued)

- To develop method for concentration and purification of aflatoxin-lysine adduct in digests for instrument analysis;
- To determine analytical chemistry parameters, such as accuracy, precision, sensitivity (limit of detection), reproducibility, and recovery for the method.

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Commercial DBS Card Comparison

Untreated Cards

- Ahlstrom 226
- Munktell TFN
- GE Whatman DMPK C - 31ETF base paper
- GE Whatman 903

Treated Cards

- GE Whatman DMPK A (FTA) – 31ETF base paper
  4 additives, for 'protection' of genetic material
- GE Whatman DMPK B (FTA Elute) – 903 base paper
  1 additive, will denature proteins

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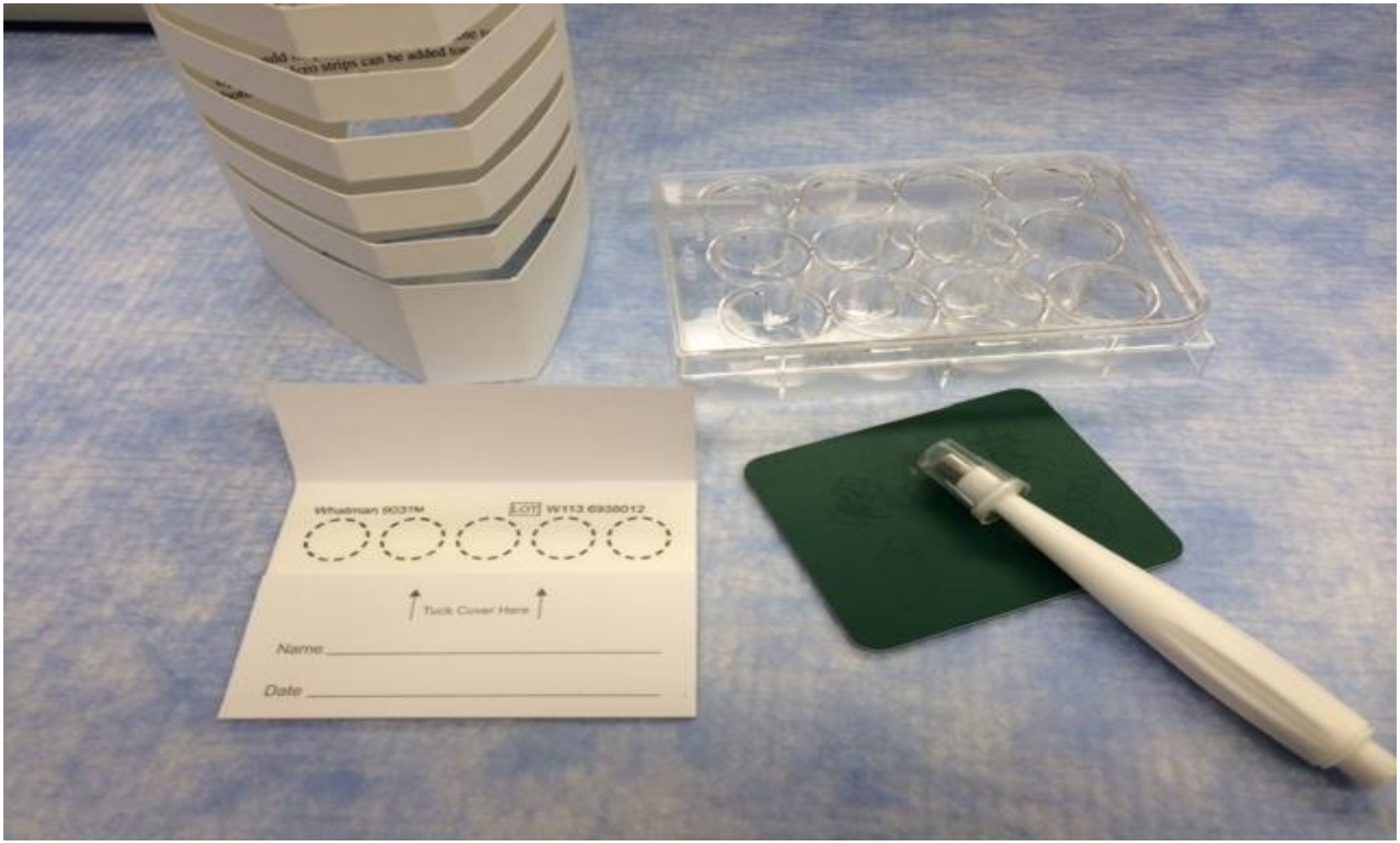
Main Supplies



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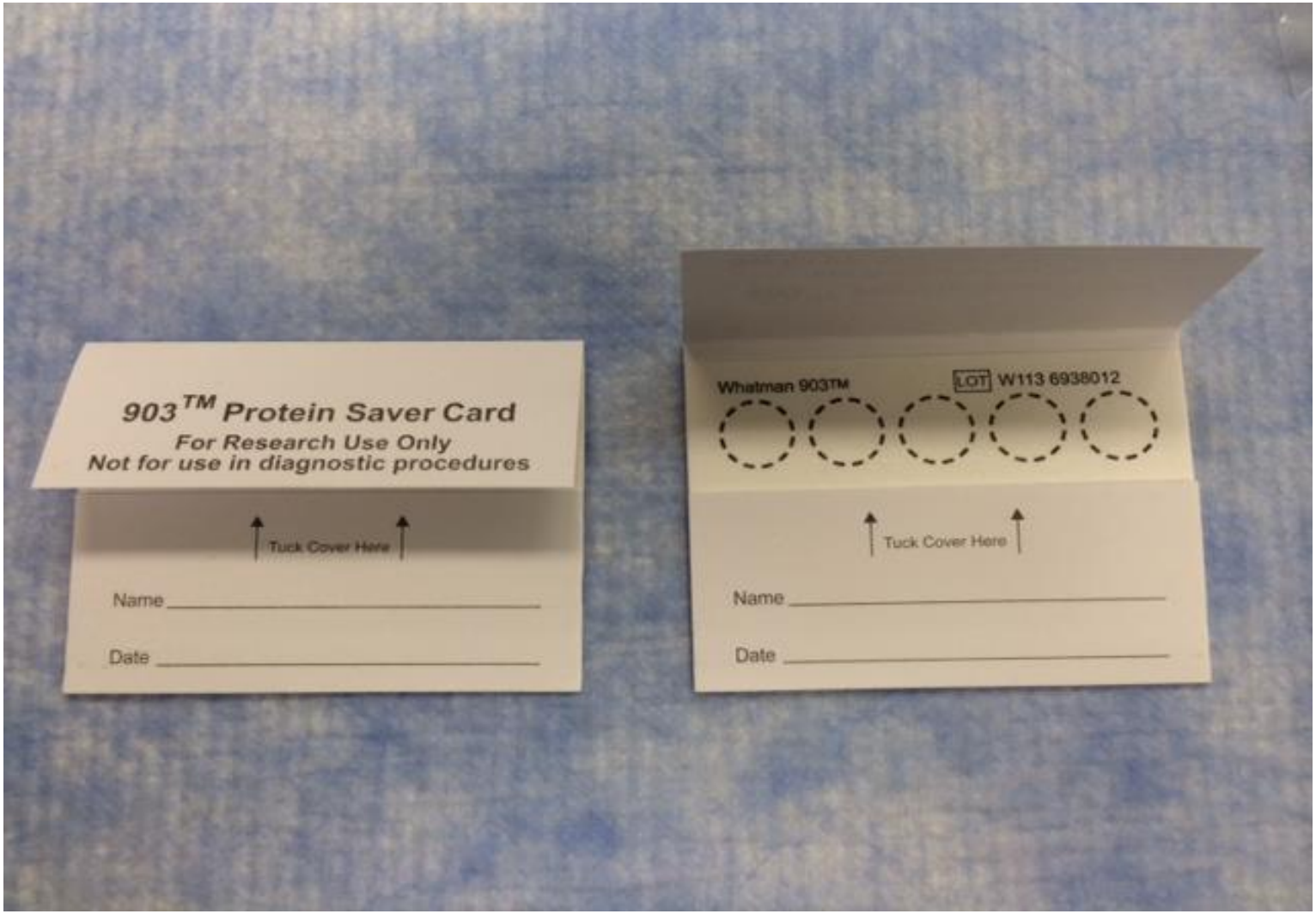
903™ Protein Saver Card
For Research Use Only
Not for use in diagnostic procedures
Tuck Cover Here
Name
Date
Whatman 903™
LOT W113 6938012
Tuck Cover Here
Name
Date


Whatman 903™
LOT W112 6932812
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Name 1
Date 5-20-13
Whatman 903™
LOT W112 6932812
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Name 3
Date 5-20-13

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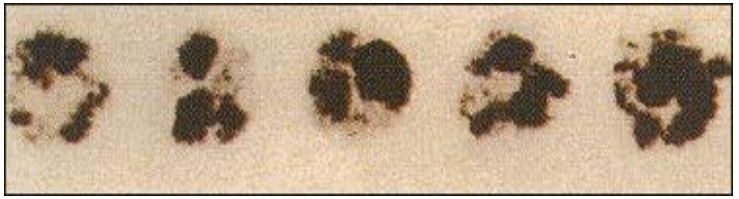
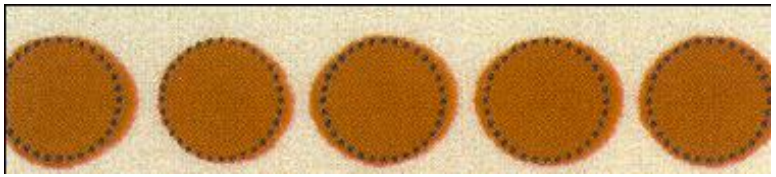
Unsatisfactory Specimens

Supersaturated

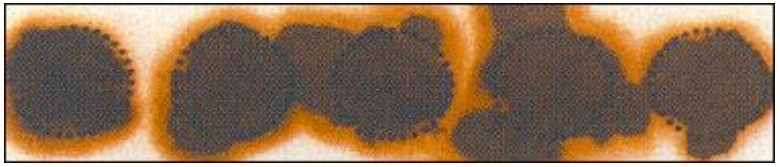


Quantity Insufficient for Testing



Specimen Not Dried Before Mailing

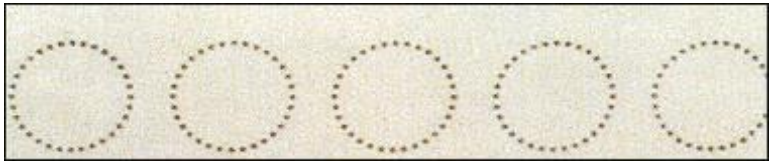
Scratched or Abraded



Serum Rings

Diluted, Discolored, or Contaminated

Clotted or Layered



No Blood

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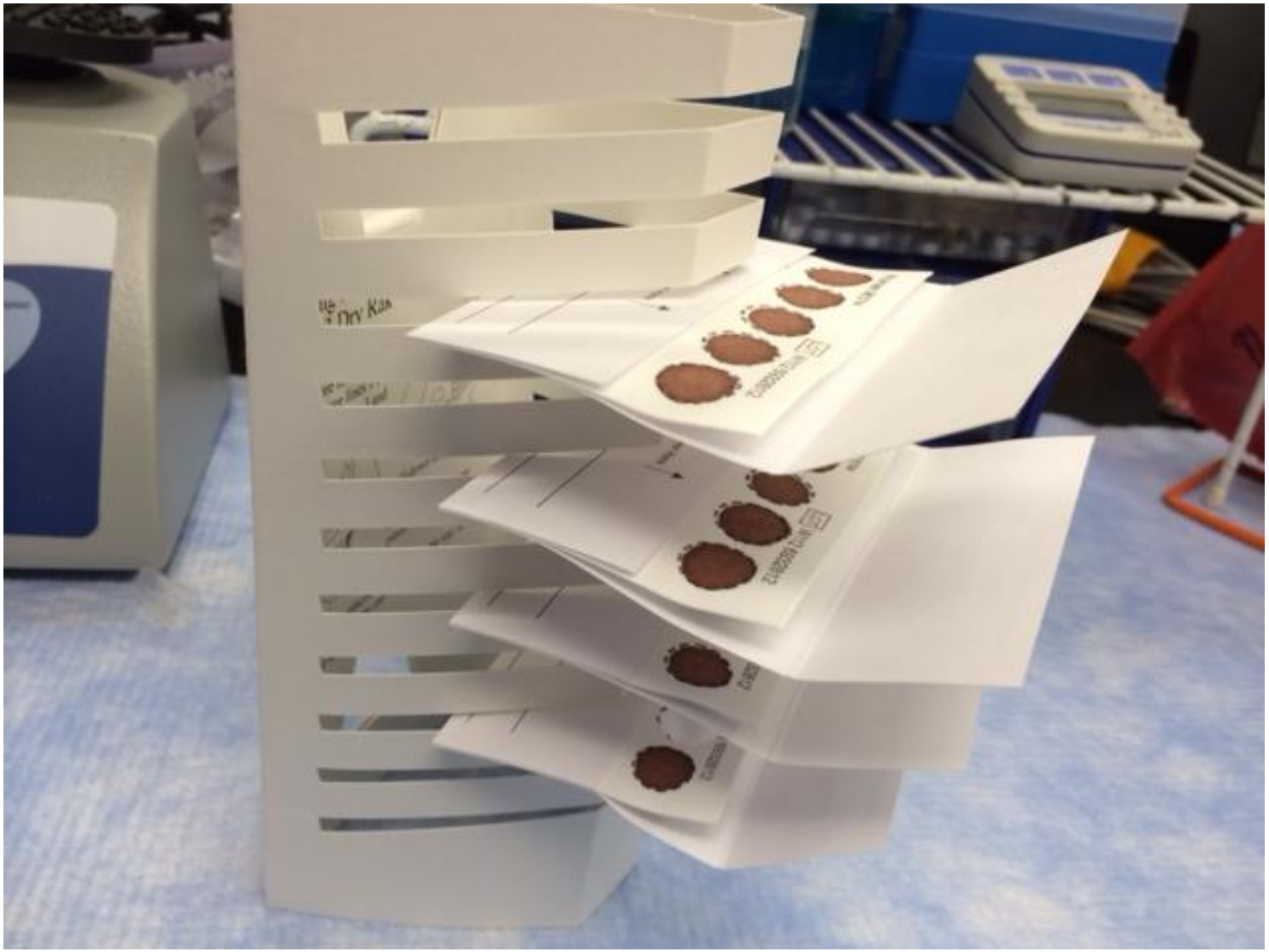

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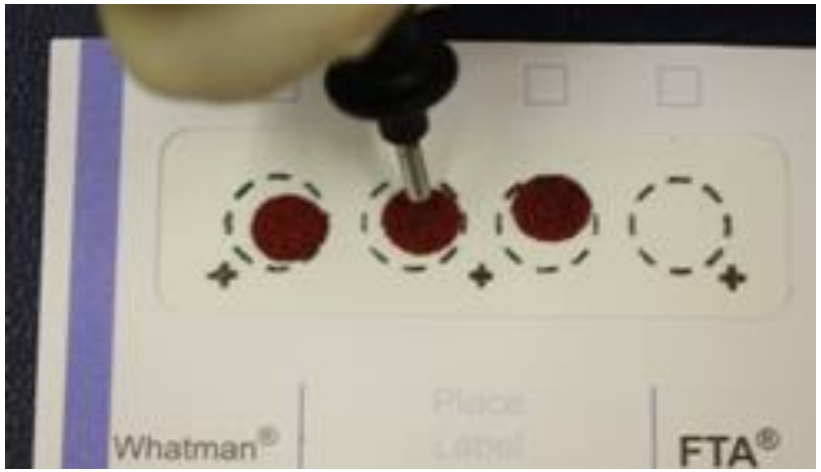
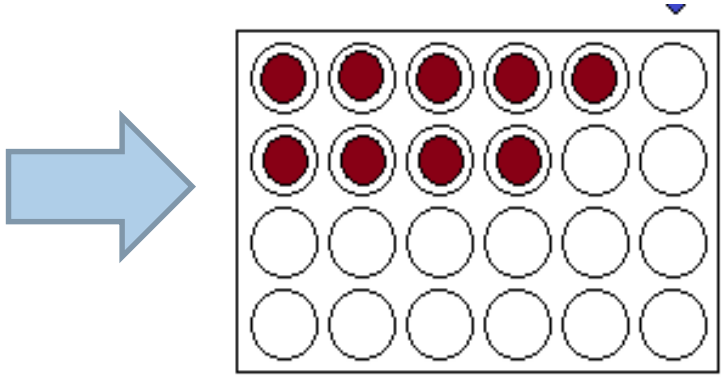
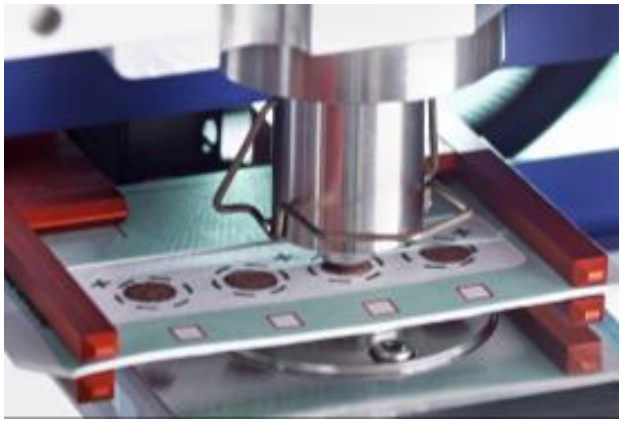




Manual



Selection of DBS Extraction System

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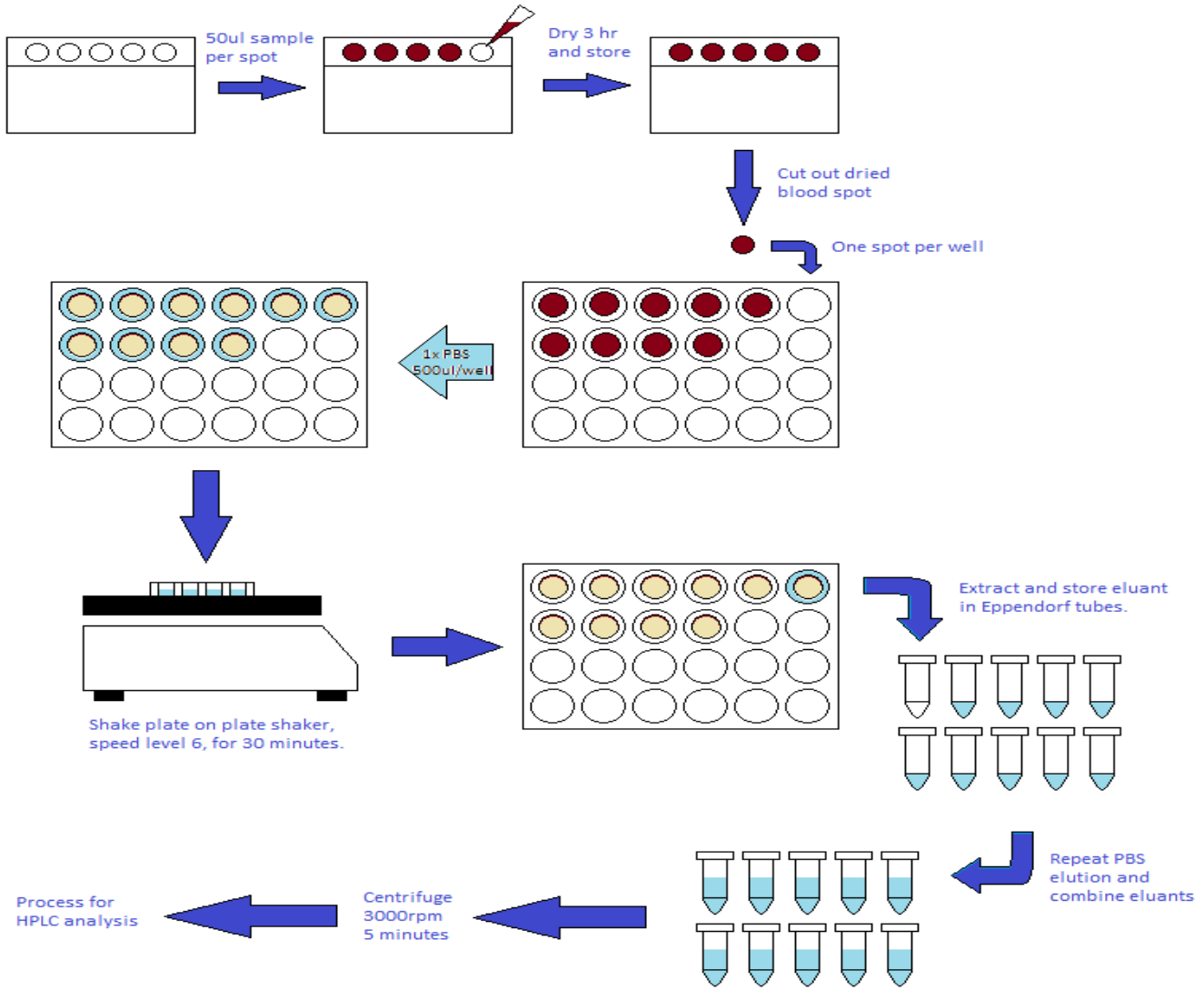
Lab Procedure



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Method Developed

- Sample collection : ≤ 50 µl blood in DBS card
- Washing Solution: Triton-100-PBS and rebuild the sample solution;
- Determination of albumin and total protein concentrations;
- Pronase digestion, solid-phase concentration and purification;
- HPLC-fluorescent detection, and MS confirmation;
- Limit of detection: 20 fg/mg albumin;
- Recovery: averaged 75% for various spiked concentrations.

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Elution & Washing Efficiency

Total Protein (µg)

	Serum	Dilute to 1.5ml	Serum Spot Eluent			
			Wash 1	Wash 2	Wash 3	Sum
20µl	1365.61	1409.63	1416.97	117.10	-56.75	1534.07
40µl	2731.23	2644.29	2206.00	322.65	-8.86	2528.66
60µl	4096.84	4131.43	2964.46	490.41	4.55	3459.42

Triplicate experiments

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Elution & Washing Efficiency

Total Albumin (µg)						
	Serum	Dilute to 1.5ml	Serum Spot Eluant			
			Wash 1	Wash 2	Wash 3	Sum
20ul	884.13	410.00	649.17	-11.67	-77.50	649.17
40ul	1768.27	1211.25	1606.67	77.50	-64.58	1684.17
60ul	2652.40	2205.00	2575.83	163.75	-49.17	2739.58

Triplicate experiments

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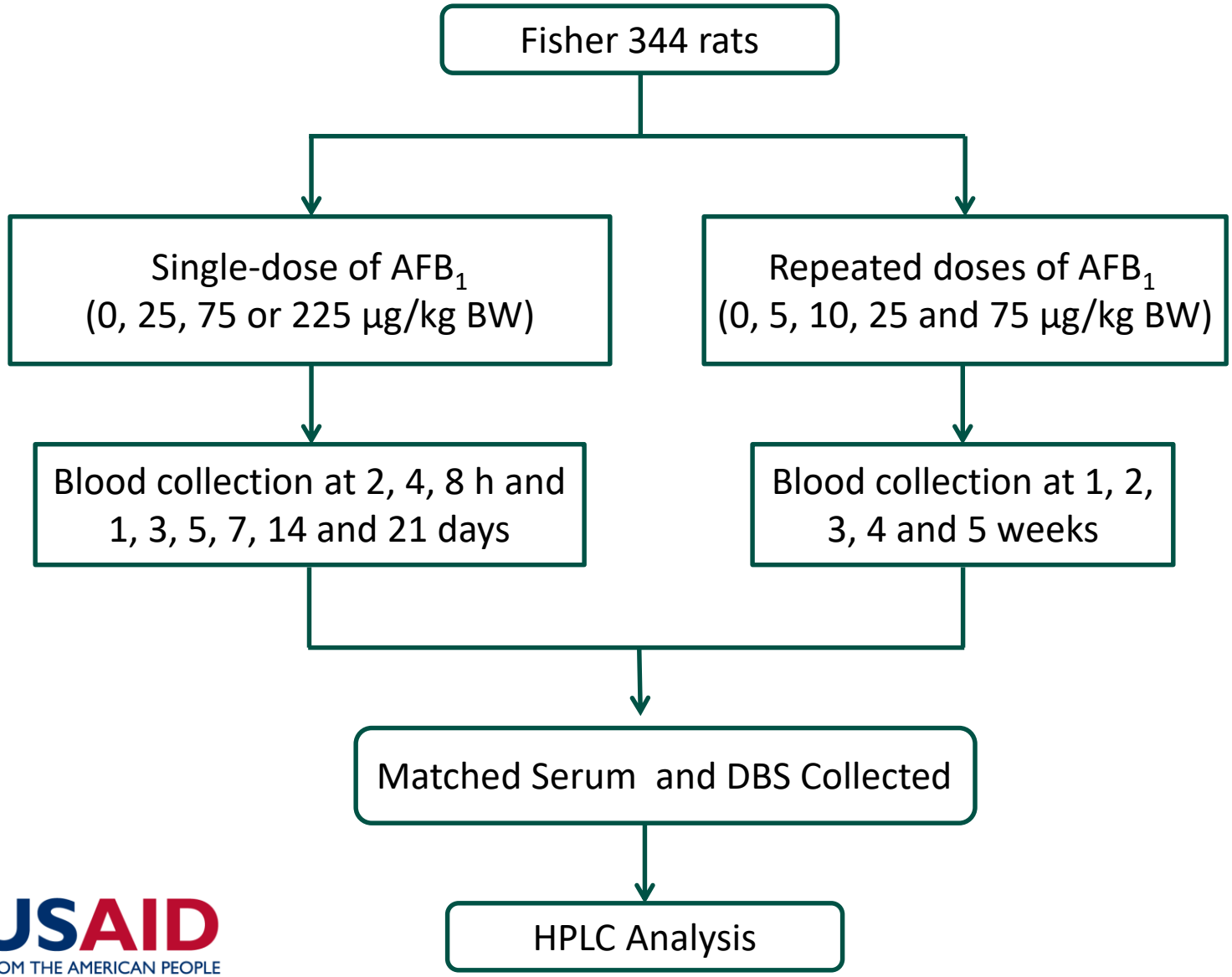
Phase 2A: Animal Validation Studies



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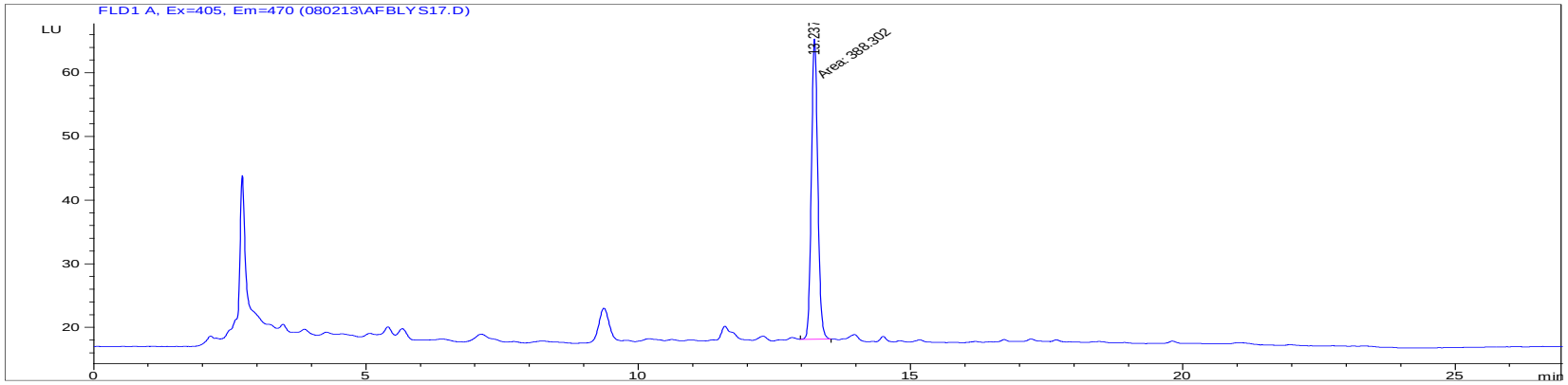
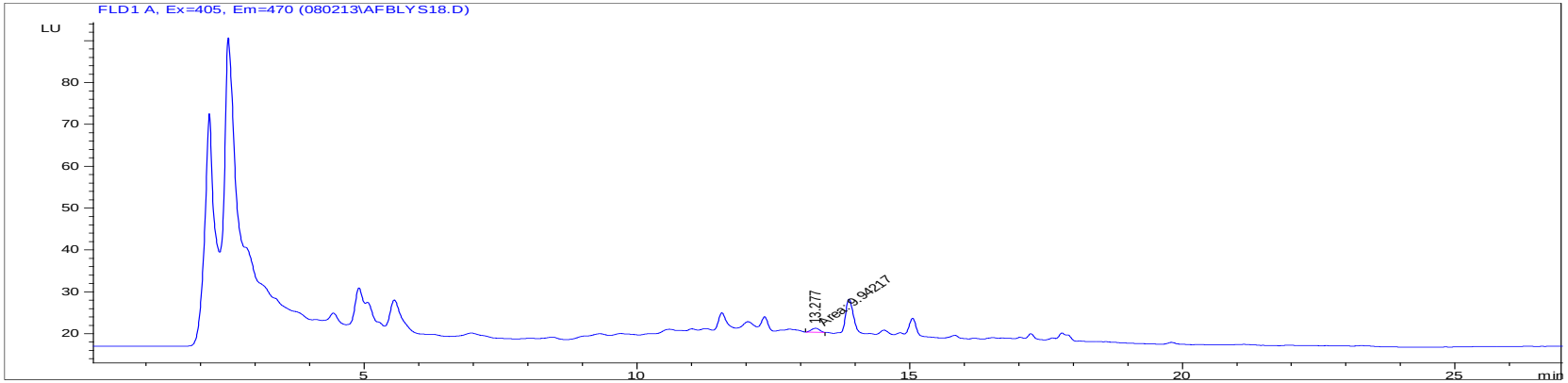
DBS samples from AFB_1-dosed animal blood

Whole blood DBS sample from high dose treated animals



Whole blood DBS sample from low dose treated animals



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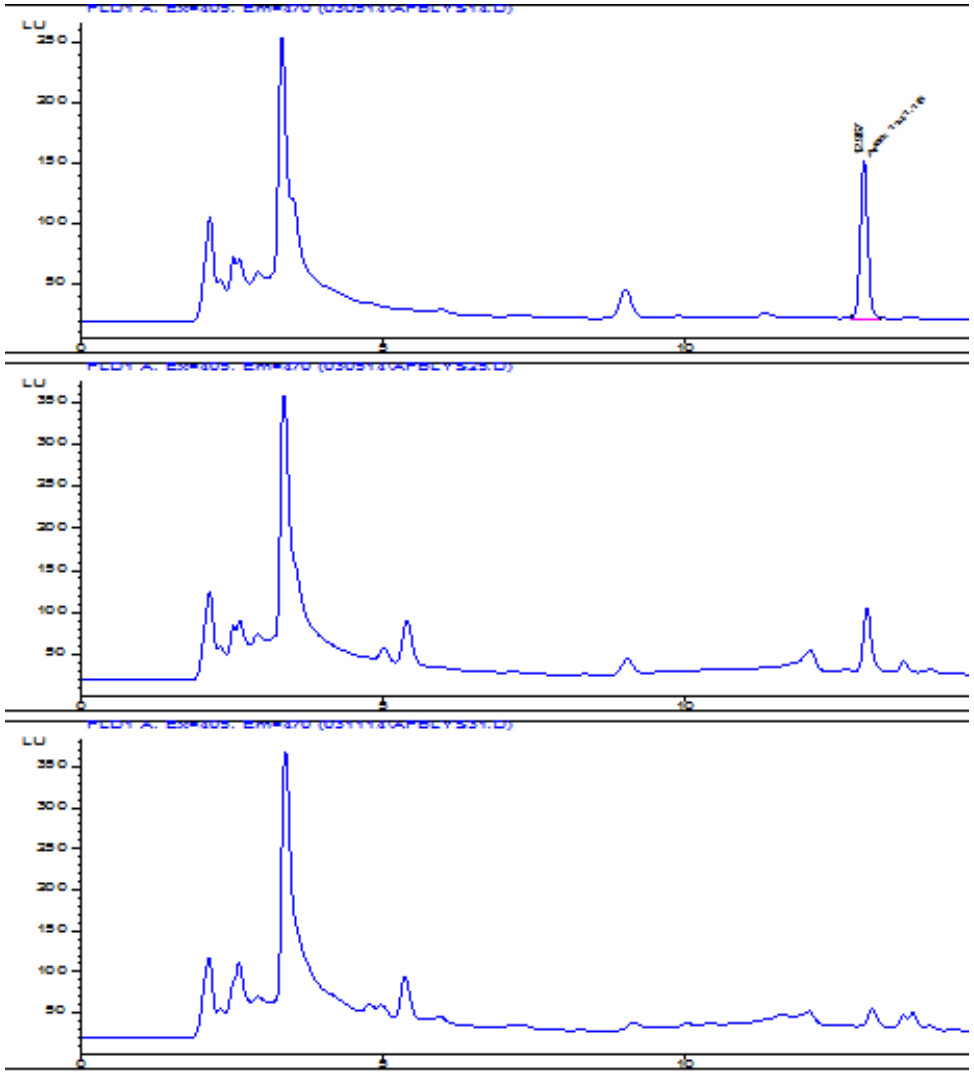
HPLC chromatograph of AFB_1-treated rat DBS



Single dose of 225 µg/kg AFB1

Single dose of 75 µg/kg AFB1

Single dose of 25 µg/kg AFB1

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Single dose

	2h	24hr	3d	5d	7d
Control	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.01
25µg/kg	16.48±2.58	5.62±0.42	5.90±1.02	2.83±0.16	1.34±0.16
75µg/kg	54.8±0.53	12.77±1.68	15.11±2.49	8.71±2.03	5.19±0.79
225µg/kg	143.98±20.45	96.19±10.67	80.72±5.80	66.63±16.91	36.18±7.57

N=6

Repeated Dose

Dose (µg/kg)	AFB-Lys (ng/mg alb.)				
	week 1	week 2	week 3	week 4	week 5
0	0.02±0.00	0.02±0.00	0.03±0.01	0.02±0.01	0.02±0.00
5	0.17±0.01	0.27±0.01	0.30±0.03	0.38±0.02	0.41±0.03
10	0.51±0.02	0.76±0.06	0.90±0.06	0.98±0.03	1.04±0.07
25	1.44±0.11	2.01±0.13	2.09±0.08	2.56±0.16	2.79±0.16
75	2.02±0.13	2.76±0.16	2.76±0.28	2.64±0.11	2.50±0.13

N=6

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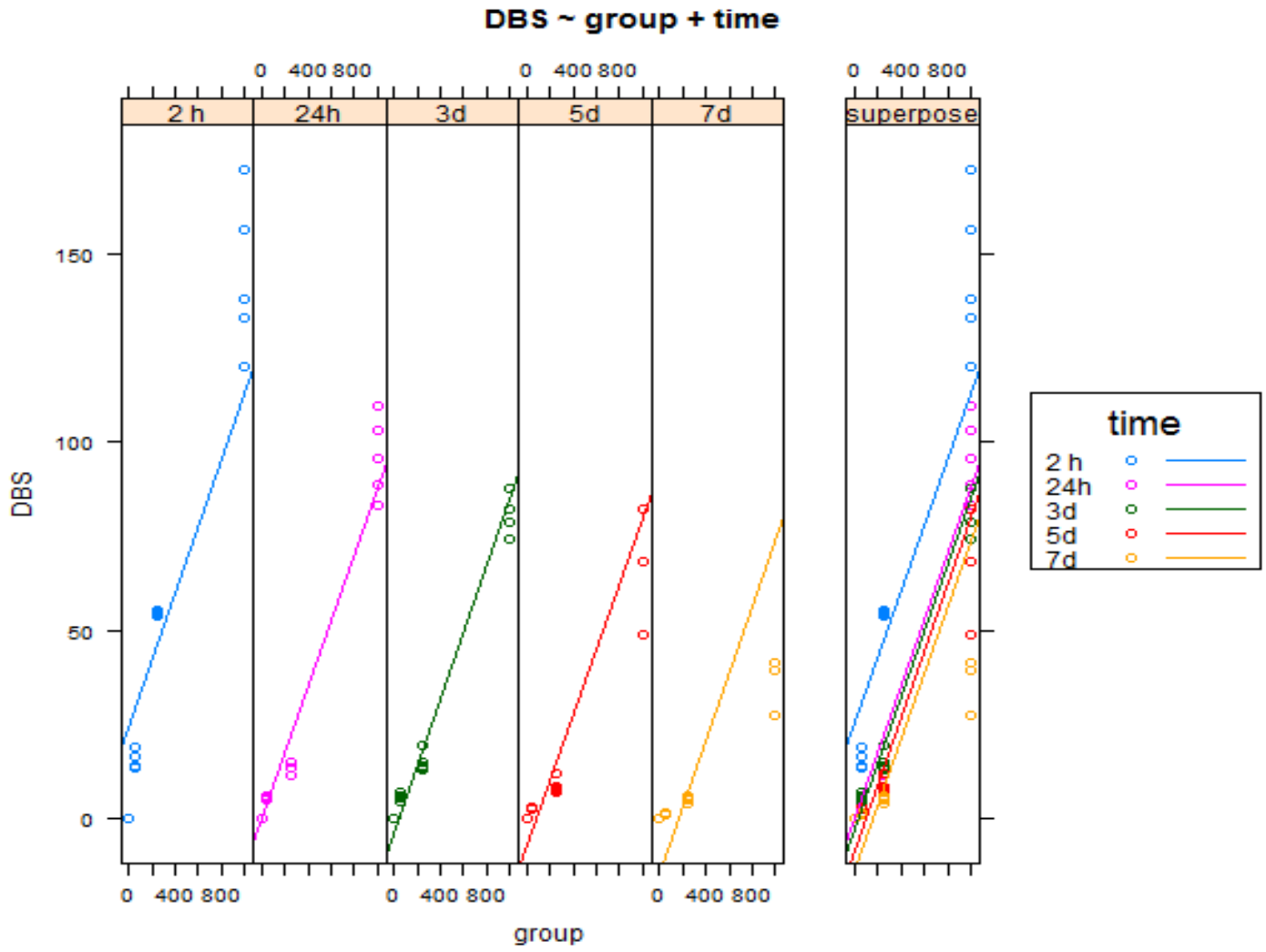
Single Dose


DBS ~ group + time
2 h
24h
3d
5d
7d
superpose
DBS
group
0
50
100
150
0
400
800
time
2 h
24h
3d
5d
7d

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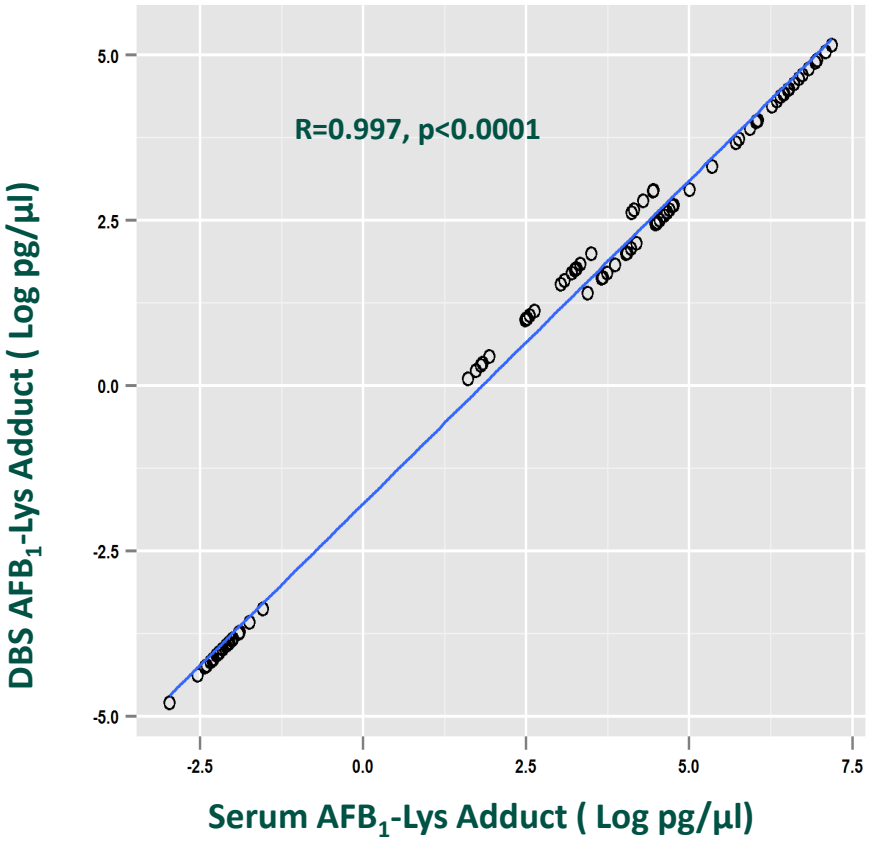
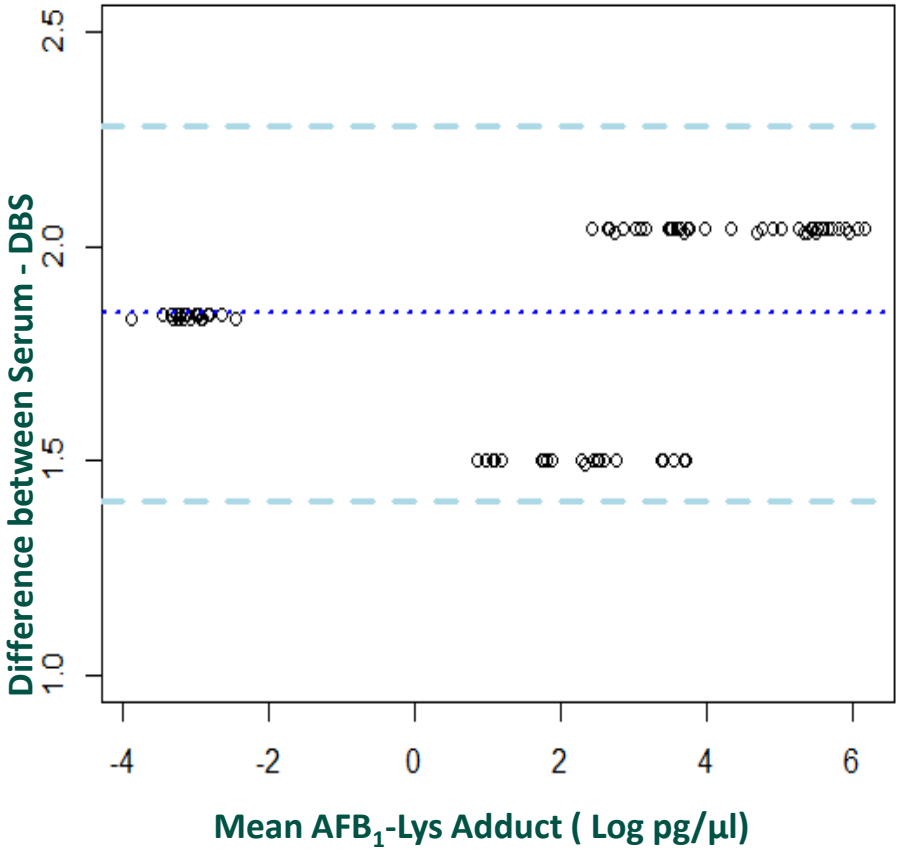
Single Dose





Scatter plots (left) and Bland–Altman plots (right) for paired serum and DBS specimens measured by HPLC . In scatter plots, solid line = linear regression. In Bland–Altman plots, center line indicates mean difference between serum and DBS measures; upper and lower lines indicate the 95% confidence interval.

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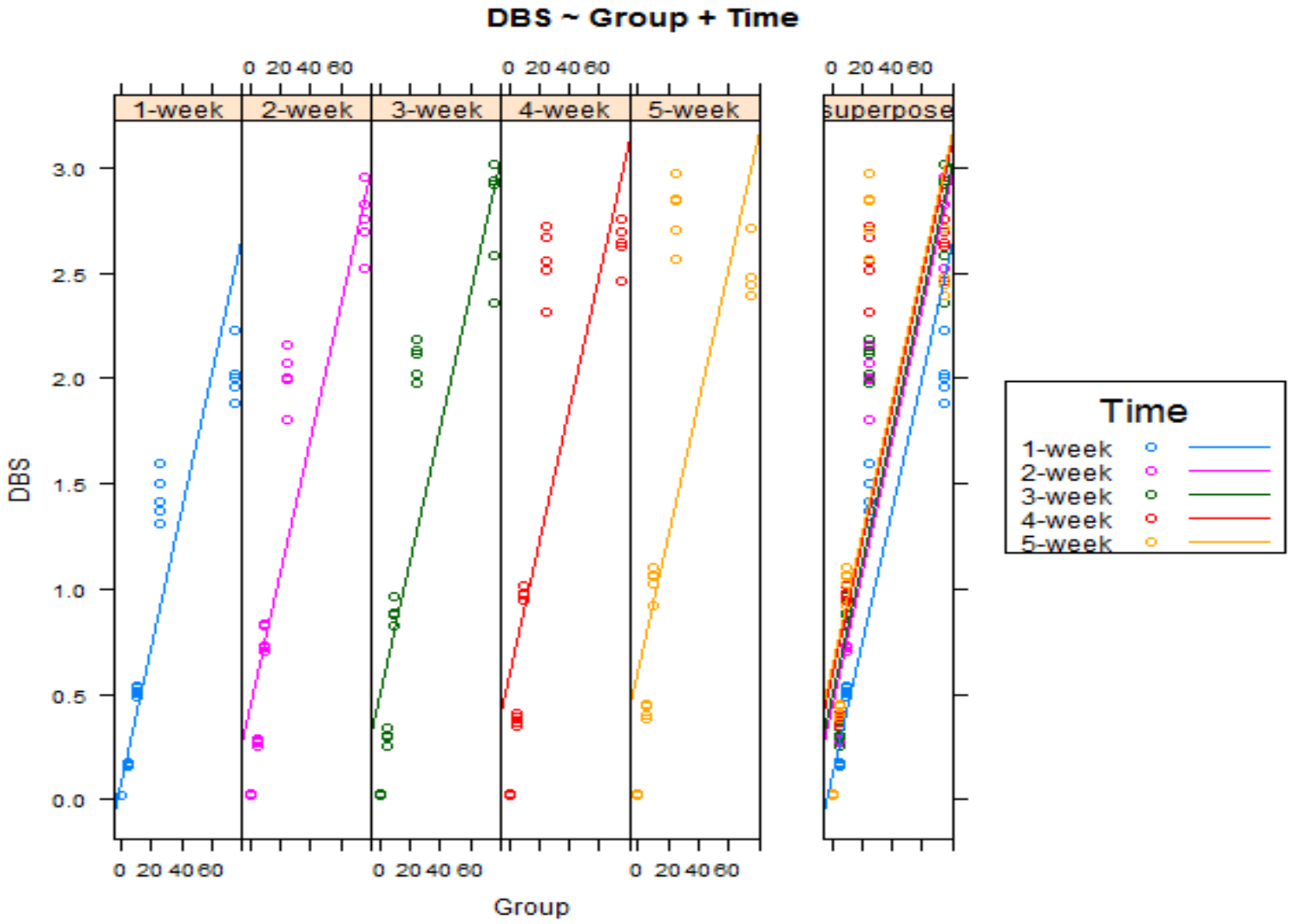
Repeated Doses



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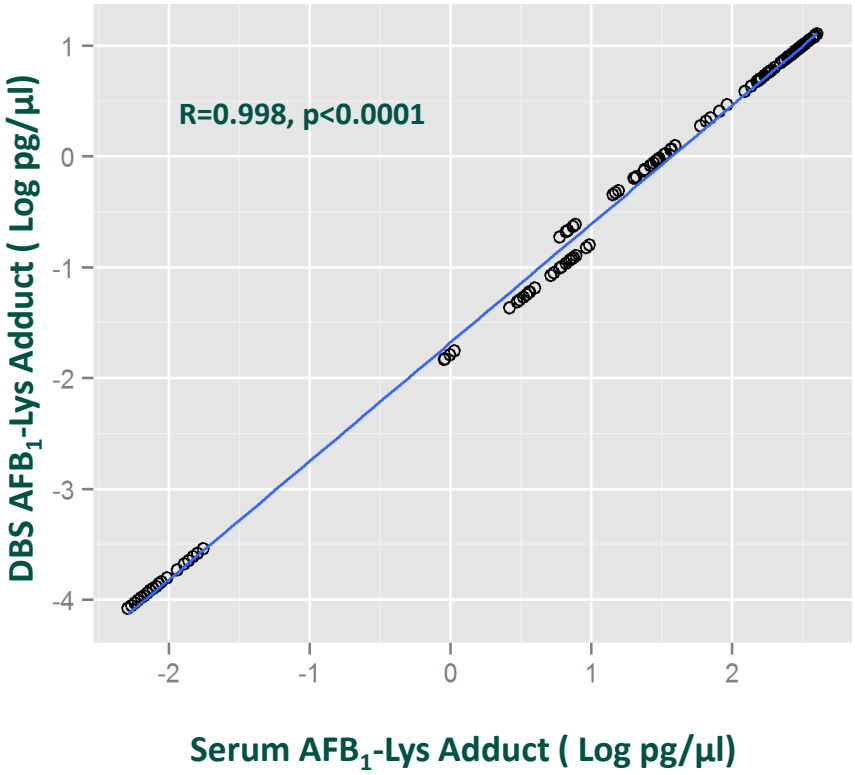
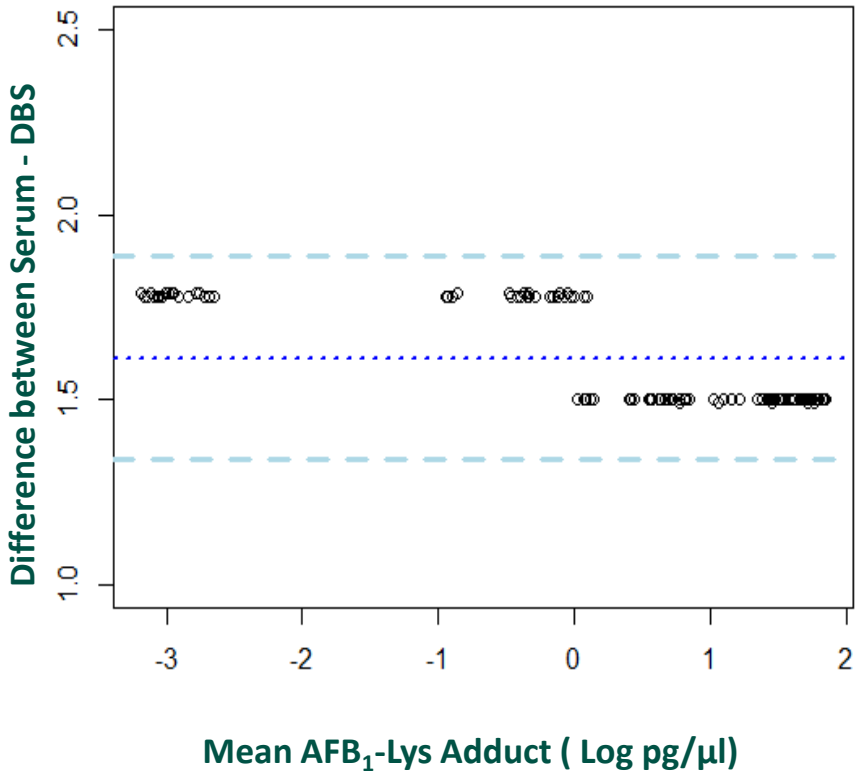
Repeated Dose





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Phase 2B. Human Validation Study

Dietary aflatoxin exposure	Low	Middle	High
Participant numbers	12	12	12
Detection rate (%)	50 (6/12)	58.3 (7/12)	100 (12/12)
Median level of AFB-lysine adduct (pg/mg albumin)	3.92	12.18	136.26
Range of AFB-lysine adduct (pg/mg albumin)	0-4.78	0-24.64	61.49-992.42

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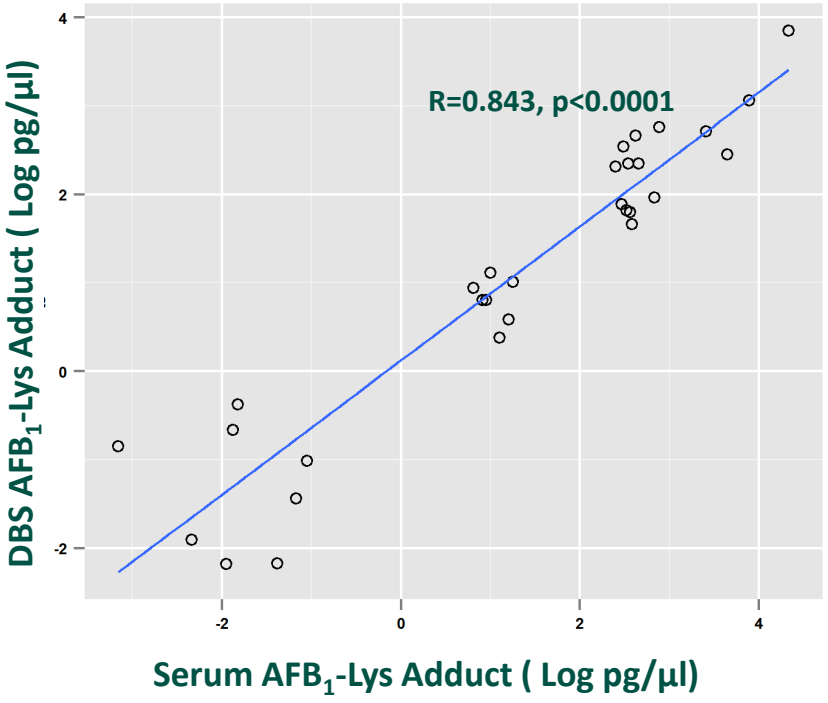
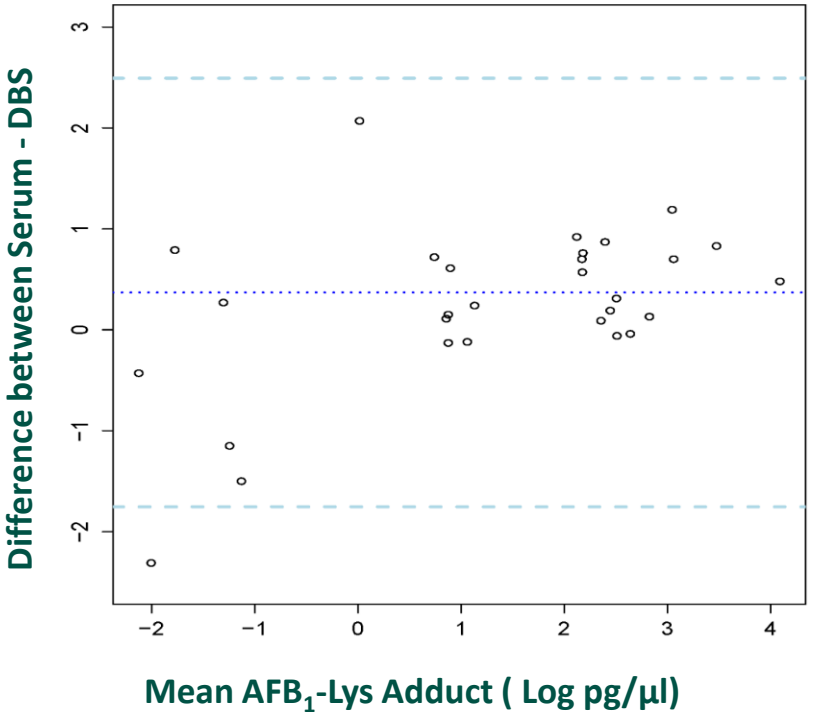
Human Validation Study





Scatter plots (left) and Bland–Altman plots (right) for paired human serum and DBS specimens. In scatter plots, solid line = linear regression. In Bland–Altman plots, center line indicates mean difference between serum and DBS measures; upper and lower lines indicate the 95% confidence interval.

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Phase 3. Application Study

Use of DBS samples collected from USAID NIL conducted Aflatoxin Birth Cohort Study in Nepal to assess mother/children aflatoxin exposure and its correlation with adverse growth/development effects.

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AFB-Lys Adduct Levels in Five Batches of DBS Samples from Nepal Birth Cohort Study

Parameter	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
AFB-Lys Adduct (pg/mg albumin)					
Number	171	128	320	222	222
Detection rate (%)	98.83	96.88	100	100	100
Median	1.65	1.21	2.99	8.64	15.68
Geometric Mean	4.24	1.77	2.92	8.94	14.71
95% CI	3.92 – 4.57	1.51 – 2.04	2.76 - 3.07	8.49 - 9.42	13.88- 15.88
Minimal	0.40	0.20	0.43	3.51	2.92
Maximal	147.32	14.10	75.31	40.25	44.85

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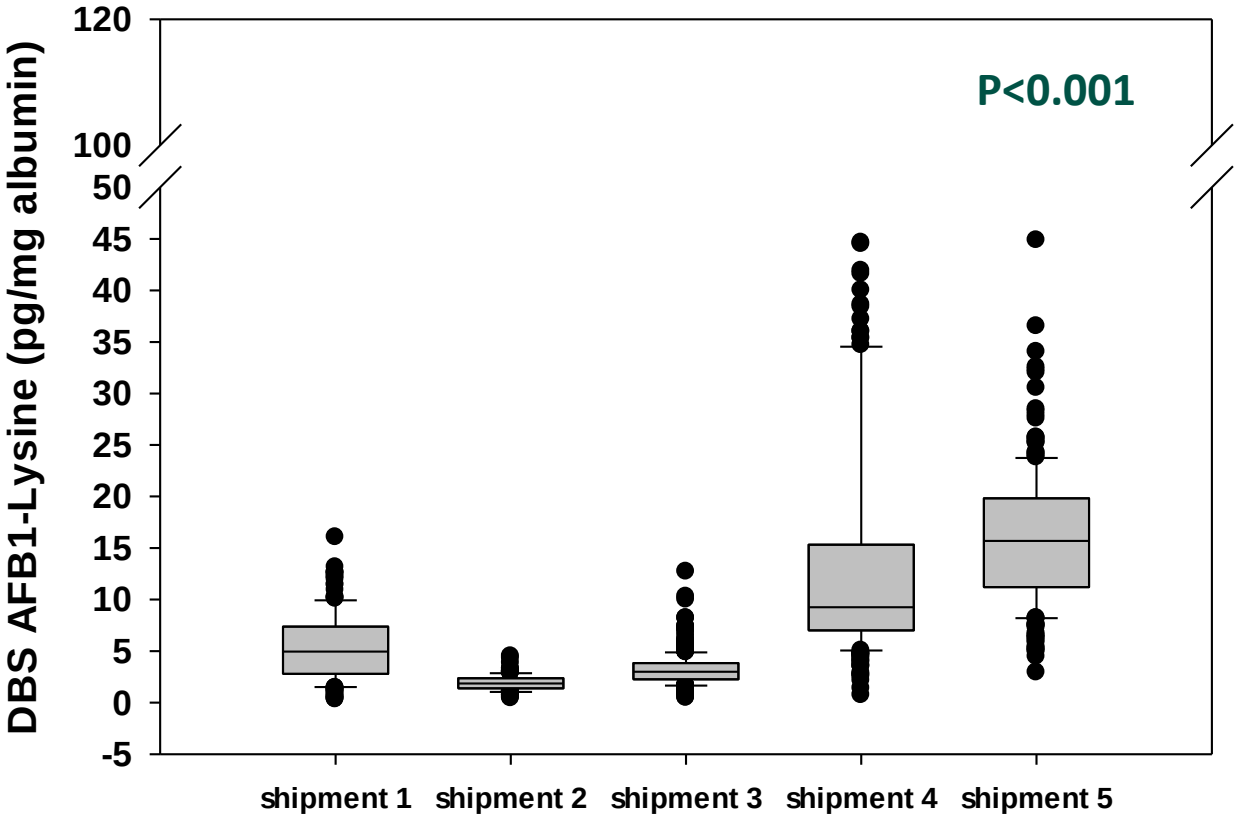
Distribution of AFB-Lys adduct Levels in DBS Samples of Nepal Birth Cohort



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Outcomes and Impacts

- Highly innovative and significant;
- Meet urgent needs;
- Fill the research gaps;
- Generate data for understanding the relationship between biological response and aflatoxin exposure.
- Significantly beneficial for the health-effect assessment of children as a result of long-term exposure to aflatoxins in developing world.

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- Collaborators from NIL and PMIL Focused Countries
  - Nepal site
  - Uganda site

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