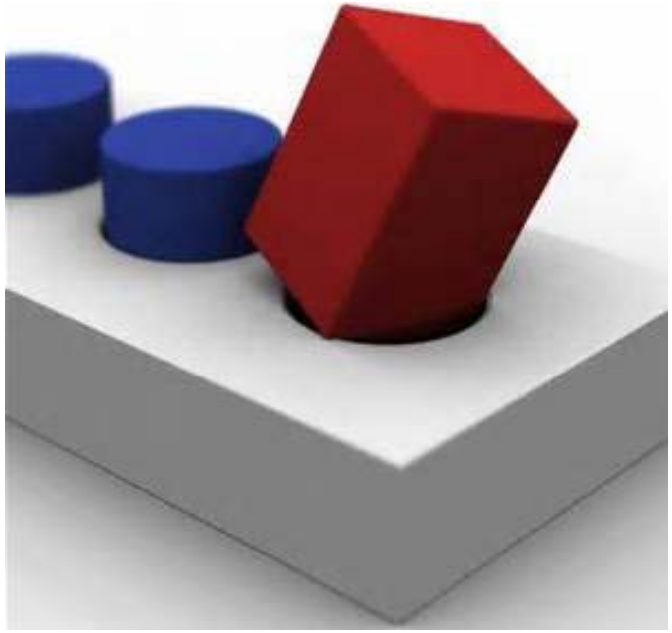
Sample volume – does it need to restrict your biomarker strategy?

Richard Hughes Ph.D, Senior Scientist, Drug Development Services

EBF Open Meeting, Barcelona, Nov 2015.

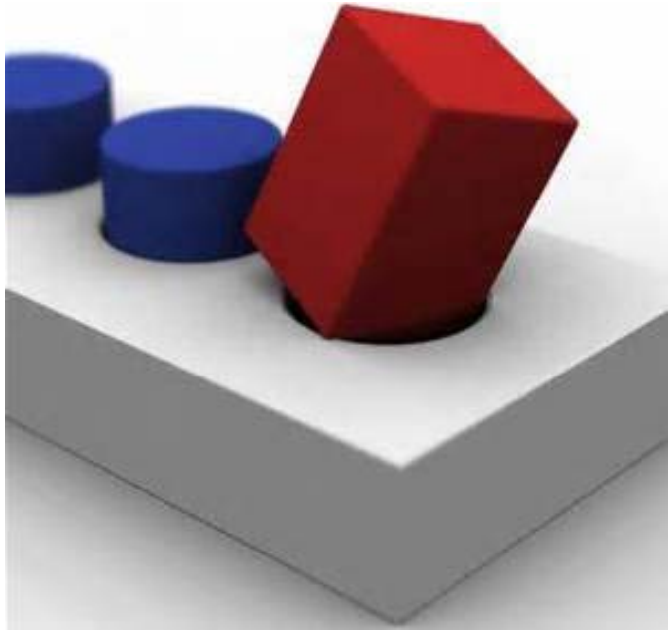
Introduction

- Biomarker analysis often dictates the need to detect low abundance proteins from all manner of unusual and difficult to work with matrices.
- This has evolved from the requirement for datasets to be valued, supportive, and of physiological relevance.



Introduction

- Biomarker analysis often dictates the need to detect low abundance proteins from all manner of unusual and difficult to work with matrices.
- This has evolved from the requirement for datasets to be valued, supportive, and of physiological relevance.
- Non-standardised sampling techniques can often harvest unusual matrices inconsistently, leading to sample volumes which are insufficient for meaningful analysis.
- Consideration must be paid to the goals of the analysis upfront. Measurement of multiple biomarkers means the choice of appropriate technology requires a balance between good precision, sensitivity, through-put, reagent availability, multiplexing, and cost.



Introduction

- In this presentation, we discuss a variety of options for designing effective biomarker strategies for working with ‘difficult’ samples.
- The benefits of certain assay types and platforms
- Efficiency savings for fit-for-purpose analysis
- The benefits of tailoring the analysis to maximise information from volume-limiting samples

Working with ‘difficult’ samples

Challenges/options with small sample volumes

- Move to 384- well immunoassays.

  Not really an option for off-the-shelf reagents or many commonly used platforms

  While assay volumes are greatly reduced, well miniaturization has its limitations, in particular when dealing with non-standard sample matrices. Pipette inaccuracy, adhesion and reduced wash efficacy can lead to increased between-well imprecision as wells become smaller.

- Multiplexing vs singleplex

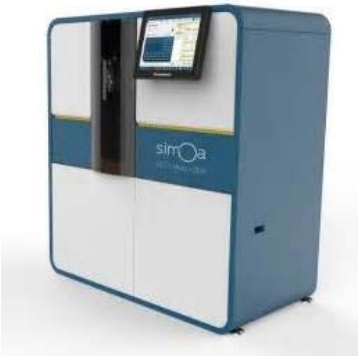
  Evolving guidelines for regulatory assay validation
  Fit for purpose method qualification
  Compromises on analyte compatibility, assay conditions, and/or acceptance criteria
  Potential for cross-talk between analytes.

Single-plex biomarker platforms

Platform	Sample volume (µL)
ELISA	150 – 450
Singulex	100 - 250
Siemens Immulite	120 - 300
Randox Daytona	30 – 50
Gyrolab	10
Optimiser	15

Multiplex biomarker platforms

Platform	Sample volume (µL)
Randox Evidence	300
Aushon	250
Quanterix Simoa	200
Imperacer	100
MSD	50 -100
CBA	50 - 100
Luminex	50 - 100



Benefits of certain assays and platforms

REVIEW

For reprint orders, please contact reprints@future-science.com

Miniaturized immunoassays: moving beyond the microplate

After more than 40 years, immunoassays are still the backbone of protein biomarker analysis in clini

REVIEW

For reprint orders, please contact reprints@future-science.com

Analytical assay platforms for soluble target engagement biomarkers: old favorites and emerging technologies

Quantification of soluble biomarkers is critical for assessing target engagement in both preclinical and
Since the advent of the commonly used ELISA, there has been an array of immunoassay platforms
goal, and many researchers will have a favorite. This review will compare and contrast immunoass
used by many pharmaceutical and CRO groups and highlight emerging assay platforms that may deliver
assays. Several case studies will be used throughout as the changing immunoassay landscape is discu

Preclinical and clinical development of a drug molecule is a lengthy process that requires well-designed studies with carefully chosen and defined outputs. The assessment of target engagement and modulation is a critical part in this process, especially during Phase I studies. Development of characterized and validated PD

be quantified, which is of particular importance for novel, first-in-class mechanisms. In addition, the optimal duration of impacting the target and subsequent repeat administration regimens can be determined. Such information can contribute to interspecies **allometric scaling**, whereby data from preclinical species can be used to inform

Joanne Goodman* & Balaji Agoram
Medimmune, Cambridge, CB21 6GH, UK
*Author for correspondence:
Tel.: +44 122 389 8241
E-mail: goodmanj@medimmune.com

The AAPS Journal, Vol. 16, No. 6, November 2014 (© 2014)
DOI: 10.1208/s12248-014-9660-1

Mini-Review

Theme: Emerging Technologies in the BioAnalytical Space Applied to Large Molecule Determinations
Guest Editors: Johanna Mora and Binodh DeSilva

Next Generation Ligand Binding Assays—Review of Emerging Technologies' Capabilities to Enhance Throughput and Multiplexing

Johanna Mora,[1] Allison Given Chunyk,[2] Mark Dysinger,[3] Shobha Purushothama,[4] Claude Ricks,[5] Karolina Österlund,[6] and Valerie Theobald[7,8]

Received 2 June 2014; accepted 22 August 2014; published online 6 September 2014

Abstract. The purpose of this manuscript is to provide a summary of the evaluation done by the Throughput and Multiplexing subteam on five emerging technologies: Single molecule array (Simoa™), Optimiser™, CyTOF® (Mass cytometry), SQIDLite™, and iLite™. Most of the information is presented with a minimum amount of published data and much is based on discussions with users and the vendor, to help provide the reader with an unbiased assessment of where the subteam sees each technology fitting best in the bioanalysis of large molecules. The evaluation focuses on technologies with advantages in throughput and multiplexing, but is wide enough to capture their strengths in other areas. While all platforms may be suited to support bioanalysis in the discovery space, because of their emergent nature, only Optimiser and SQIDLite are currently ready to be used in the regulated space. With the exception of Optimiser, each instrument/technology requires an up-front investment from the bioanalytical lab that will need justification during capital budget discussions. Ultimately, the platform choice should be driven by the quality of data, project needs, and the intended use of the data generated. In a time- and resource-constrained environment, it is not possible to evaluate all emergent technologies available in the market; we hope that this review gives the reader some of the information needed to decide which technology he/she may want to consider evaluating to support their drug development program in comparison to the options they already have in their hands.

KEY WORDS: emerging; high throughput; ligand binding assay; multiplexing; technology.

The AAPS Journal, Vol. 16, No. 6, November 2014

Bioanalysis (2013) 5(23), 2919–2931

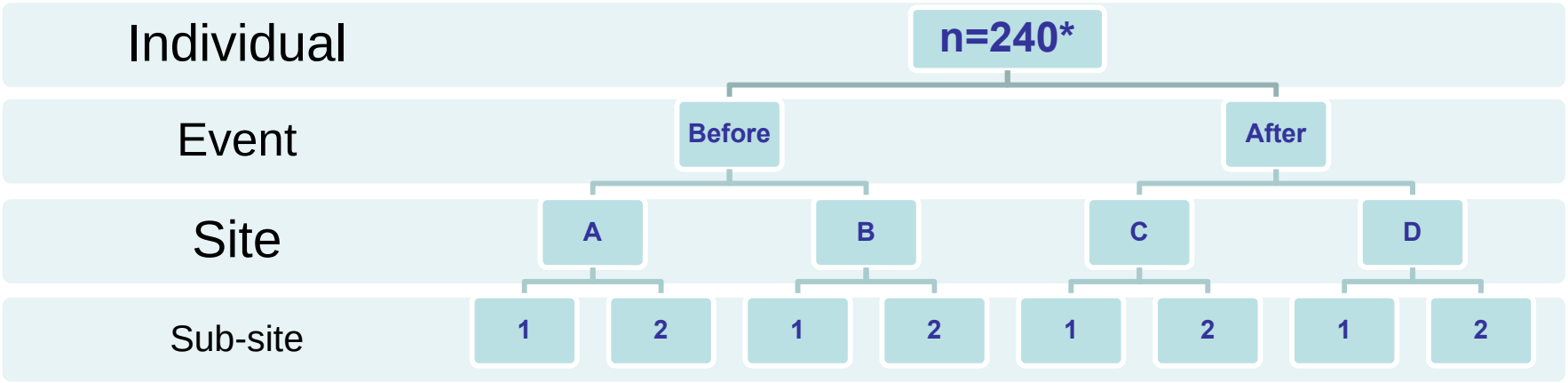
“Into New Territories” with next generation biomarker platforms

Platform			
Biosims Dynaxi	“Analysis can be undertaken with sample volumes down to 10 µL: plasma, urine, CSF, supernatants..”		
Ayoxxa Lunaris	“down to 3 µL”		
Genalyte Maverick	“5 to 10 µL”		

Case Study – Biomarker panel analysis from low/variable volume matrix

- A company with an interest in developing biomarker sampling techniques from low abundance and tricky to harvest biological fluids
- Duplicate biological samples were collected before and after an event from two sites from each individual

- 8 samples per individual



*** Only 176 suitable for analysis**

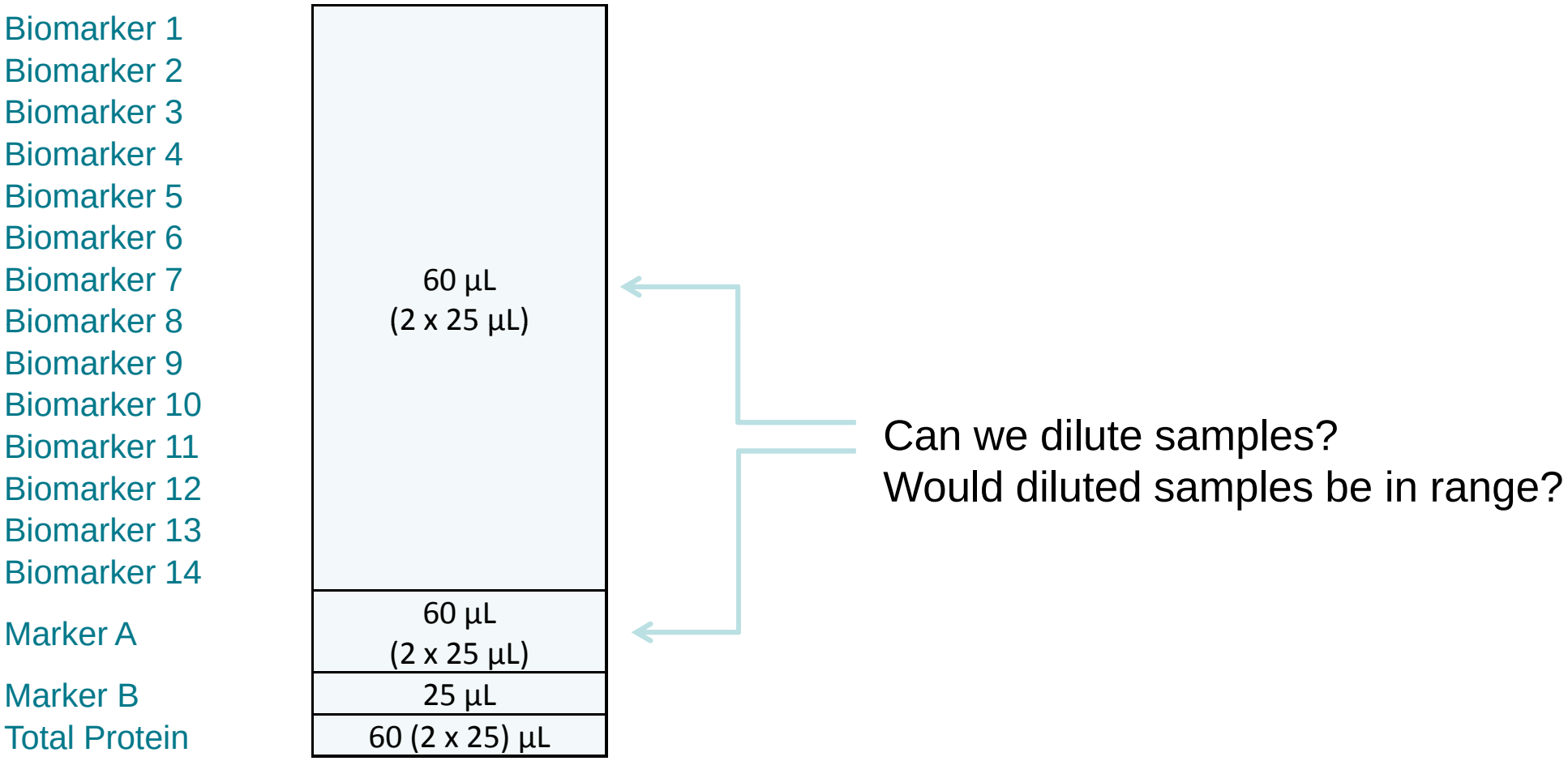
Case Study – Biomarker panel analysis from low/variable volume matrix

- Requested Markers

Biomarker 1 Biomarker 2 Biomarker 3 Biomarker 4 Biomarker 5 Biomarker 6 Biomarker 7 Biomarker 8 Biomarker 9 Biomarker 10 Biomarker 11 Biomarker 12 Biomarker 13 Biomarker 14	HCYTOMAG-60K | MILLIPLEX MAP Human Biomarker Magnetic Bead Panel - Immunology Multiplex Assay	60 µL (2 x 25 µL)
Marker A	HAGP1MAG-12K | MILLIPLEX MAP Human Magnetic Bead Panel - Cancer Multiplex Assay	60 µL (2 x 25 µL)
Marker B	Aviva Systems Biology competitive ELISA	25 µL
Total Protein	BCA assay	60 (2 x 25) µL

Case Study – Biomarker panel analysis from low/variable volume matrix

- Where can we make efficiency savings?



Case Study – Biomarker panel analysis from low/variable volume matrix

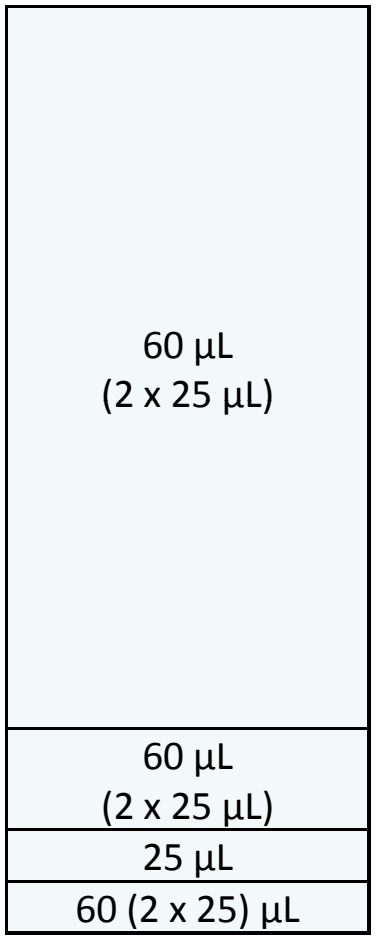
- Where can we make efficiency savings?

Biomarker 1
Biomarker 2
Biomarker 3
Biomarker 4
Biomarker 5
Biomarker 6
Biomarker 7
Biomarker 8
Biomarker 9
Biomarker 10
Biomarker 11
Biomarker 12
Biomarker 13
Biomarker 14

Marker A

Marker B

Total Protein



Can we dilute samples?
Would diluted samples be in range?

Can this be reduced
without compromising the LLoQ?

How do we make the most out of the samples available?

	Vol Requirement (µL)
Nothing	60
Protein plus Marker B	60 - 75
Protein plus Plex	75 - 120
Protein plus Plex plus Marker B	120 - 150
All analytes	> 0.150

It is important to have a clear indication of what volumes are available prior to designing a flexible strategy to analyse them

Before								After							
Sample n	subject n		site	weight (g)	Sum L	Sum R	Sum All	Sample n	subject n		site	weight	Sum L	Sum R	Sum All
001	001	A	1	0.0102	0.0303	0.0493	0.0796	157	001	C	1	0.0021	0.0855	0.0274	0.1129
002	001	A	2	0.0201				158	001	C	2	0.0834			
003	001	B	1	0.0015				159	001	D	1	0.0175			
004	001	B	2	0.0478				160	001	D	2	0.0099			
013	004	A	1	0.001	0.0471	0.0479	0.0950	173	004	C	1	0.0003	0.0655	0.0025	0.068
014	004	A	2	0.0461				174	004	C	2	0.0652			
015	004	B	1	0.0004				175	004	D	1	0.0025			
016	004	B	2	0.0475				176	004	D	2	-			
								161	005	C	1	0.0156	0.0418	0.0796	0.1214
								162	005	C	2	0.0262			
								163	005	D	1	0.0299			
								164	005	D	2	0.0497			
								165	007	C	1	0.0067	0.0171	0.1211	0.1382
								166	007	C	2	0.0104			
								167	007	D	1	0.0776			
								168	007	D	2	0.0435			
381	08	A	1	0.0365	0.0900	0.0638	0.1538								
382	08	A	2	0.0535											
383	08	B	1	0.0480											
384	08	B	2	0.0158											
177	009	A	1	0.0147	0.2537		0.2537								
178	009	A	2	0.239											
033	014	A	1	0.0005	0.0089	0.0546	0.0635	189	014	C	1	0.0043	0.0503	0.0844	0.1347
034	014	A	2	0.0084				190	014	C	2	0.0460			
035	014	B	1	0.0327				191	014	D	1	0.0482			
036	014	B	2	0.0219				192	014	D	2	0.0362			
037	019	A	1	0.0336	0.053	0.0665	0.1195								
038	019	A	2	0.0194											
039	019	B	1	0.0395											
040	019	B	2	0.027											
041	021	A	1	0.0003	0.044	0.0398	0.0838	197	021	C	1	0.0005	0.0048	0.1236	0.1284
042	021	A	2	0.0437				198	021	C	2	0.0043			
043	021	B	1	0.0006				199	021	D	1	0.0008			
044	021	B	2	0.0392				200	021	D	2	0.1228			
045	022	A	1	0.3269	0.6371	0.6702	1.3073	201	022	C	1	0.0254	0.2911	0.3179	0.6090
046	022	A	2	0.3102				202	022	C	2	0.2657			
047	022	B	1	0.3435				203	022	D	1	0.1536			
048	022	B	2	0.3267				204	022	D	2	0.1643			

How do we make the most out of the samples available?

	Vol Requirement (µL)
Nothing	60
Protein plus Marker B	60 - 75
Protein plus Plex	75 - 120
Protein plus Plex plus Marker B	120 - 150
All analytes	> 0.150

How many samples have

1. Enough before and after volume in both Site/sub-site samples for all methods? ~ 4
2. Sufficient *for both* before and after volume if Site/sub-site samples are pooled? ~ 28
3. Sufficient *in either* before and after volume if Site/sub-site samples are pooled? 17 before /20 after

How do we make the most out of the samples available?

	Vol Requirement (µL)
Nothing	60
Protein plus Marker B	60 - 75
Protein plus Plex	75 - 120
Protein plus Plex plus Marker B	120 - 150
All analytes	> 0.150

How many samples have

1. Enough before and after volume in both Site/sub-site samples for all methods? ~ 4
2. Sufficient *for both* before and after volume if Site/sub-site samples are pooled? ~ 28
3. Sufficient *in either* before and after volume if Site/sub-site samples are pooled? 17 before /20 after

4. Assay establishment work on 3 samples from point 1 above had to take place before a strategy to analyse the remaining samples could be implemented
   - Can the samples be diluted?
   - Can the samples be pooled?
   - Should the analytes be prioritised?

Magpix 14-plex

	Ind x		Biomarker 1	Biomarker 2	Biomarker 3	Biomarker 4	Biomarker 5	Biomarker 6	Biomarker 7	Biomarker 8	Biomarker 9	Biomarker 10	Biomarker 11	Biomarker 12	Biomarker 13	Biomarker 14
Before (pg/mL)	A1	S045	8.81	BLQ	10.67	BLQ	4.12	229	BLQ	BLQ	BLQ	BLQ	BLQ	9.73	BLQ	13.0
	A2	S046	9.98	BLQ	17.11	31.16	131	1248	5.09	BLQ	BLQ	53.6	BLQ	288	BLQ	20.5
	B1	S047	3.72	BLQ	5.22	BLQ	1.63	190	BLQ	BLQ	BLQ	BLQ	BLQ	7.19	BLQ	BLQ
	B2	S048	5.92	BLQ	11.7	22.0	104	1175	3.06	BLQ	BLQ	16.4	BLQ	77.2	BLQ	5.82
-																
After (pg/mL)	C1	S201	2.39	BLQ	BLQ	12.2	39.0	1015	2.15	BLQ	BLQ	9.27	BLQ	62.0	BLQ	BLQ
	C2	S202	9.43	BLQ	11.9	35.7	678	2243	27.4	BLQ	BLQ	13.5	BLQ	98.0	BLQ	18.7
	D1	S203	6.79	BLQ	5.22	BLQ	0.50	203	BLQ	BLQ	BLQ	BLQ	BLQ	13.3	BLQ	10.9
	D2	S204	3.72	BLQ	8.80	34.7	746	1251	23.6	BLQ	BLQ	19.5	BLQ	101	BLQ	BLQ

Magpix 14-plex – normalisation

			Protein (µg/mL)	Biomarker 6 (pg/mL)	pg Biomarker 6/µg protein
Ind x Before	A	S045	70.1	229	3.3
		S046	245	1248	5.1
	B	S047	31.3	190	6.1
		S048	232	1175	5.1
Ind y Before	A	S673	290	1631	5.6
		S674	276	2296	8.3
	B	S675	183	1569	8.6
		S676	214	1210	5.7
Ind z Before	A	S429	89	2168	24.4
		S430	130	2603	20.0
	B	S431	68.2	2925	42.9
		S432	89.6	1155	12.9

Differences in biomarker concentrations were not so significant after normalisation suggesting maybe that pooling site A and site B samples was probably the best option to maximise volume but one which compromised generating intra-site data.

“ISR” ? ISR-lite??

Useful to identify a sample from its initial analysis which has enough volume to provide a measure of reproducibility for all subsequent runs

	Biomarker 5	Biomarker 6	Biomarker 12
Run 1	44.8	727	19.9
	44.6	737	20.0
Run 2	46.9	498	26.4
	46.6	510	26.8
Run 3	40.5	487	25.1
	40.7	476	26.1
Run 4	40.4	539	27.3
	54.7	571	28.4
Run 5	40.6	583	28.3
	37.8	579	25.5
%CV	**11**	**16**	**12**

Data shown from three representative in range markers

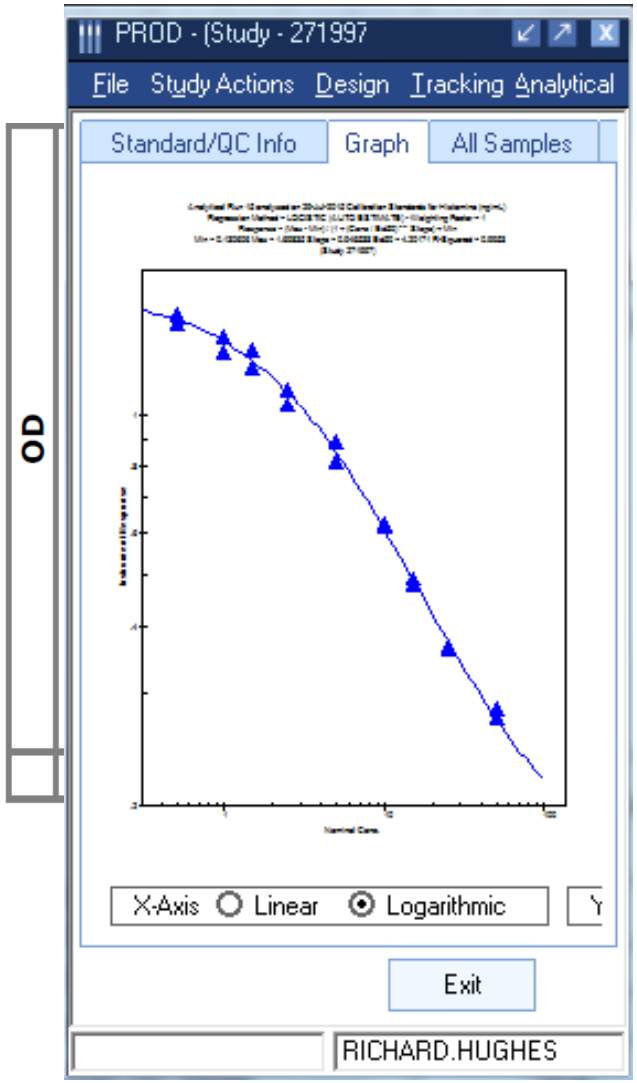
Marker A - Magpix

Ind x	Before				After			
	A1	A2	B1	B2	C1	C2	D1	D2
	S045	S046	S047	S048	S201	S202	S203	S204
Rep 1	1.93	5.27	1.93	2.88	2.64	< 2.7	2.24	2.88
Rep 2	1.93	4.82	2.38	2.88	INS	< 2.7	2.09	2.88

Ind y	Before				After			
	A1	A2	B1	B2	C1	C2	D1	D2
	S421	S422	S423	S424	S673	S674	S675	S676
Rep 1	< 2.7	INS	0.82	1.93	< 2.7	< 2.7	< 2.7	0.98
Rep 2	INS	INS	0.82	2.51	< 2.7	< 2.7	< 2.7	1.12

Ind z	Before				After			
	A1	A2	B1	B2	C1	C2	D1	D2
	S429	S430	S431	S432	S697	S698	S699	S700
Rep 1	3.90	5.69	3.42	4.58	1.58	2.51	2.76	1.93
Rep 2	3.80	5.90	3.21	4.58	1.76	1.76	2.24	2.24

Marker B – Competitive ELISA kit



5 non-zero standards in solution
Addition of extra standard curve points to make the assay more robust between 0.5 – 50 ng/mL

Kit supplies 2 QCs in water (?) which do not adequately span the range

A high and low spike was prepared in surrogate matrix, and the concentration of two pooled endogenous samples were established for QC material

	QC1 (top Std spiked into surrogate matrix	Pooled sample 1 QC2	Pooled sample 2 QC3	QC4 (top Std spiked into surrogate matrix
Mean Concentration Found (ng/mL)	0.897	1.26	5.65	20.2
Inter-run SD	0.216	0.283	0.29	2.67
Inter-run %CV	24.1	22.5	5.1	13.2
Inter-run %Bias	-10.3	-2.3	-4.2	1
n	22	12	12	22

Marker B – Competitive ELISA kit

- Normal Range

Individual x	Before				After			
	A1	A2	B1	B2	C1	C2	D1	D2
	S045	S046	S047	S048	S201	S202	S203	S204
Mean concentration (ng/mL)	2.54	7.32	1.36	8.53	3.57	13.7	1.40	8.80
%CV	23.5	11.4	4.8	8.4	15.7	8.4	24.4	1.4
Individual y	Before				After			
	A1	A2	B1	B2	C1	C2	D1	D2
	S421	S422	S423	S424	S673	S674	S675	S676
Mean concentration (ng/mL)	5.98	3.71	1.58	0.968	3.19	2.61	0.645	BLQ
%CV	2.9	2.1	19.9	18.2	11.3	13.5	10.7	NA

- Parallelism (pool made from 4 samples)

		Mean (ng/mL)	x dil factor	RE to undiluted
Pool 1	6.03	5.50	5.50	NA
	4.97			
[Pool 1] 2	3.19	2.95	5.89	7
	2.70			
[Pool 1] 4	1.45	1.68	6.72	22
	1.91			
[Pool 1] 8	0.937	0.960	7.68	40
	0.982			

Marker B – Competitive ELISA kit

- Normal Range

Individual x	Before				After			
	A1	A2	B1	B2	C1	C2	D1	D2
	S045	S046	S047	S048	S201	S202	S203	S204
Mean concentration (ng/mL)	2.54	7.32	1.36	8.53	3.57	13.7	1.40	8.80
%CV	23.5	11.4	4.8	8.4	15.7	8.4	24.4	1.4
Individual y	Before				After			
	A1	A2	B1	B2	C1	C2	D1	D2
	S421	S422	S423	S424	S673	S674	S675	S676
Mean concentration (ng/mL)	5.98	3.71	1.58	0.968	3.19	2.61	0.645	BLQ
%CV	2.9	2.1	19.9	18.2	11.3	13.5	10.7	NA

- Parallelism (pool made from 4 samples)

- Spike Recovery

	EQC (L) +Spike 1	EQC (L) +Spike 6	EQC (L) +Spike 10
ng/mL	0.975	1.74	10.2
	0.961	2.00	10.3
Mean	0.968	1.87	10.3
SD	0	0.2	0.1
%CV	1.0	9.8	0.7
%RE	-6	-8	-7

Marker B – Competitive ELISA kit

- Sample acylation – an opportunity to stretch the sample

Sample preparation and acylation

1. Pipette **25 µL** of **standards**, **controls** and **plasma samples**, into the respective wells of the **Reaction Plate.**
2. Add **25 µL** of **Acylation Buffer** to all wells.
3. Add **25 µL** of **Acylation Solution** to all wells.
4. Incubate for 45 min at **RT** (20 - 25 °C) on a **shaker** (approx. 600 rpm).
5. Add **100 µL** of **water** to all wells.
6. Incubate for **15 min** at **RT** (20 - 25 °C) on a **shaker** (approx. 600 rpm).
7. Take **25 µL of the prepared standards, controls and samples** for the **ELISA**

- After acylation 25 µL of sample is diluted into a total volume of 175 µL, enough for repeat analysis should it be needed

Marker B – Competitive ELISA kit

- Sample acylation – an opportunity to stretch the sample

Sample preparation and acylation

1. Pipette **25 µL** of **standards**, **controls** and **plasma samples**, into the respective wells of the **Reaction Plate.**
2. Add **25 µL** of **Acylation Buffer** to all wells.
3. Add **25 µL** of **Acylation Solution** to all wells.
4. Incubate for 45 min at **RT** (20 - 25 °C) on a **shaker** (approx. 600 rpm).
5. Add **100 µL** of **water** to all wells.
6. Incubate for **15 min** at **RT** (20 - 25 °C) on a **shaker** (approx. 600 rpm).
7. Take **25 µL of the prepared standards, controls and samples** for the **ELISA**
8. PUT REMAINING IN FREEZER

- After acylation 25 µL of sample is diluted into a total volume of 175 µL, enough for repeat analysis should it be needed

S041 (ng/mL)	14.4
S041	13.9
frozen sample S041	13.9
frozen sample S041	13.2

S044 (ng/mL)	1.07
S044	1.17
frozen sample S044	1.24
frozen sample S044	1.21

Total Protein Assay

- Must have assay for normalisation
- BCA offers better precision than Bradford and the sample matrix did not deliver reproducible data from spectrophotometry
- Normally performed using 25 µL of sample + 200 µL reagent per well to provide adequate sensitivity
- Volumes reduced by 2.5 (10 µL) and sensitivity remained reproducible at 31.3 µg/mL with good precision

Total Protein Assay

- Normal Range of samples

ID		µg/mL
S429	Before A1	89.0
S430	Before A2	130
S431	Before B1	68.2
S432	Before B2	89.6
S967	After C1	110
S698	After C2	181
S699	After D1	76.9
S700	After D2	BLQ

- Parallelism of pooled matrix told us that samples could be analysed diluted if volumes were short

	Pool	Pool/4	Pool/8	Pool/16
µg/mL	1540	367	161	66.5
	1600	378	160	72.7
Mean	1570	372.5	160.5	69.6
SD	42.4	7.8	0.7	4.4
%CV	2.7	2.1	0.4	6.3
RE	--	-5	-18	-29

Precision very reproducible could even consider singlicate analysis if needs be

How do we make the most out of the samples available?

Priorities for Sample Analysis

1. Total Protein – reduce assay volumes, singlicate if required
2. 14 plex – Pool and (if required) dilute samples
3. Marker B ELISA – Pool and dilute two fold, freeze after acylation
4. Marker A – not taken forward into analysis due to analyte concentrations

Total sample spend of < 100 µL, increasing the number of potentially analysable samples

Sample volume – does it need to restrict your biomarker strategy?

An effective biomarker strategy from a inconsistent or low volume matrix depends on choosing the right platform and the right assay.

Advance planning, organised sample collection, and pragmatic analyte choices facilitate efficient bioanalysis

Assessing sample pooling, dilution, or miniaturising has its benefits but also its compromises (e.g. sensitivity) and is not a wholesale solution.

A few years ago almost certainly yes, but as we move into new horizons technology solutions are providing us with the tools to generate multiplex data from only a few microlitres. Reagent availability on these remains behind industry standard platforms. Data from CRO and pharma over the next few years will be key to seeing which emerge.

Sample volume – does it need to restrict your biomarker strategy?

Richard Hughes Ph.D, Senior Scientist, Drug Development Services

EBF Open Meeting, Barcelona, Nov 2015.

The Sponsor Company

John Allinson

Science
for a safer world