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Signal:noise? – The importance of the preanalytical phase in sample storage

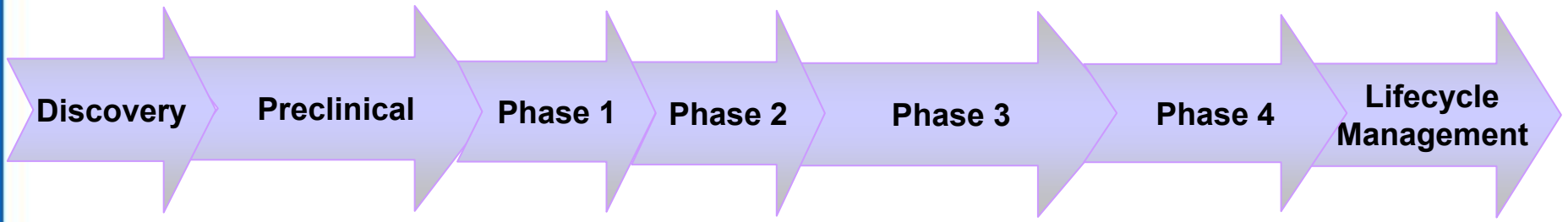
Scott D. Patterson, PhD
Executive Director, Medical Sciences

Outline

- Biomarkers in early drug development
- Biobank considerations
- Preanalytical issues
  - You wouldn't have to worry about a blood collection tube…would you?
  - Plasma analyte changes with freeze/thaw and centrifugation speed
  - Gene expression changes *ex vivo* and general handling of Paxgene tubes
- Conclusions

Drug Development: Traditional Approach vs. New Paradigm

Traditional Approach:



New Paradigm (FDA Critical Path Initiative):

MEDICAL SCIENCES
Early Development +
Biomarker Assay Technologies

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Why are we interested in biomarkers?

1. **Have we modulated the target?**
   - **Is it a proximal measure in the target tissue?**
2. **Can we use the data to help to guide dose ranging studies?**
   - **More likely from *in vivo* derived measurements than *ex vivo***
3. **Are there observable differences between individuals/patients?**
   - **Is there a chance the biomarker can stratify the population or even be used as a rapid measure of response?**

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Robust assays are required

- **Technical utility needs to be determined**
  - **i.e., can we conduct the assay in the manner desired?**
- **Clinical utility of the assay has to be evaluated (Clinical Performance)**
  - **How reliably test measures clinical condition**
  - **Will the derived information be relevant?**
- **Assay qualification (validation) is a critical component (Analytical Performance)**
  - **How reliably and correctly test measures analyte**
  - **Performance characteristics of the assay need to be determined**
  - **Over what time frame will the assay have to be performed?**
  - **Is the reagent vendor able to supply consistent product?**
  - **For many assays, unlikely full GLP is required, but must meet our quality standards**
  - **What preanalytical issues exist?**

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Molecular PD biomarkers currently used

CLINIC

- ✓ Cytokine/chemokine/other protein levels in blood/other fluids (including *ex vivo* stimulation) by MAP (multi-analyte profiling: beads/planar arrays)
- ✓ Signaling proteins in cell lysates by MAP (phospho & total)
- ✓ Signaling proteins in permeabilized cells by flow cytometry (Phosflow™)
- ✓ Second messengers in cell lysates
- ✓ Enzyme activity in tissue / fluids
- ✓ Cell surface proteins by flow cytometry
- ✓ Transcript levels in cell lysates
- ✓ Gene sequence and copy number in cell lysates
- ✓ Specific DNA levels in fluids
- ✓ Mass spectrometry based discovery

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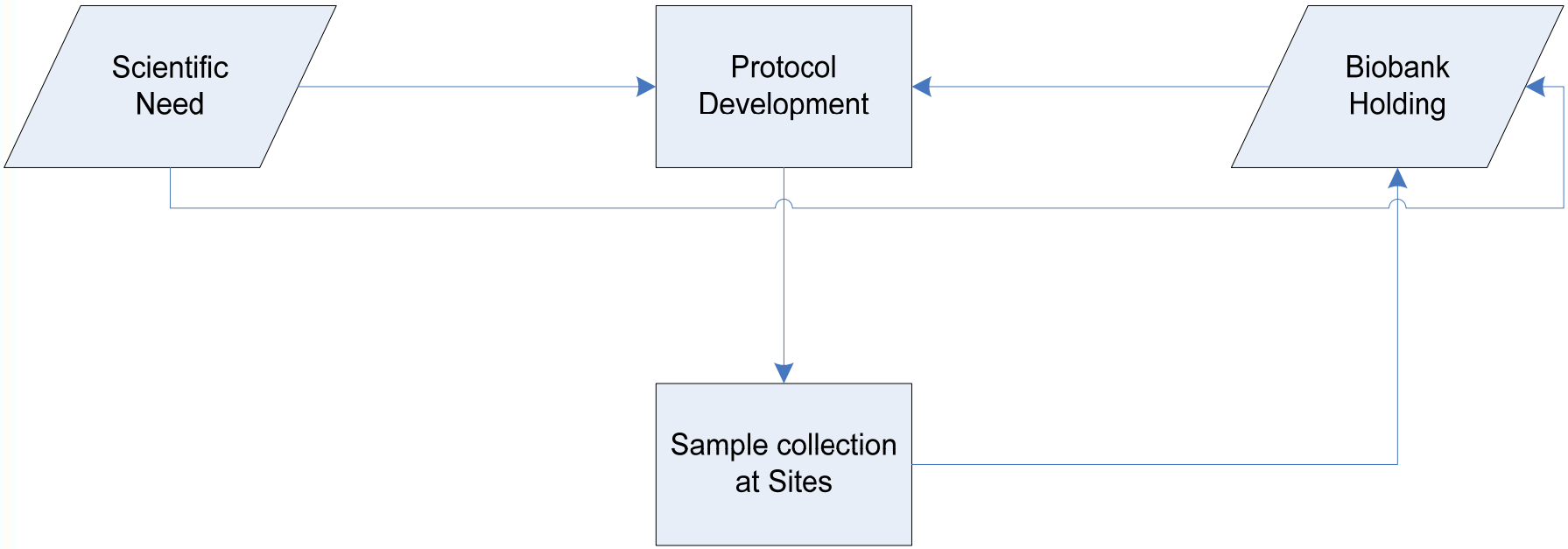
Biobank Process Overview



Drivers behind starting a biobank

Characteristics

- Desire to have access to well annotated clinical samples (often including outcome data) from a range of disease states

Uses

- Improving understanding of biological signature of various diseases
- Helping develop patient stratification markers
- Enabling earlier, better, and more informed decision in drug development via assessment of PD and safety biomarkers

Scale

- Samples from a number of selected clinical trials collected
- Costs vary by study (in some cases shipping can contribute >50%); turnover of samples can keep storage costs lower

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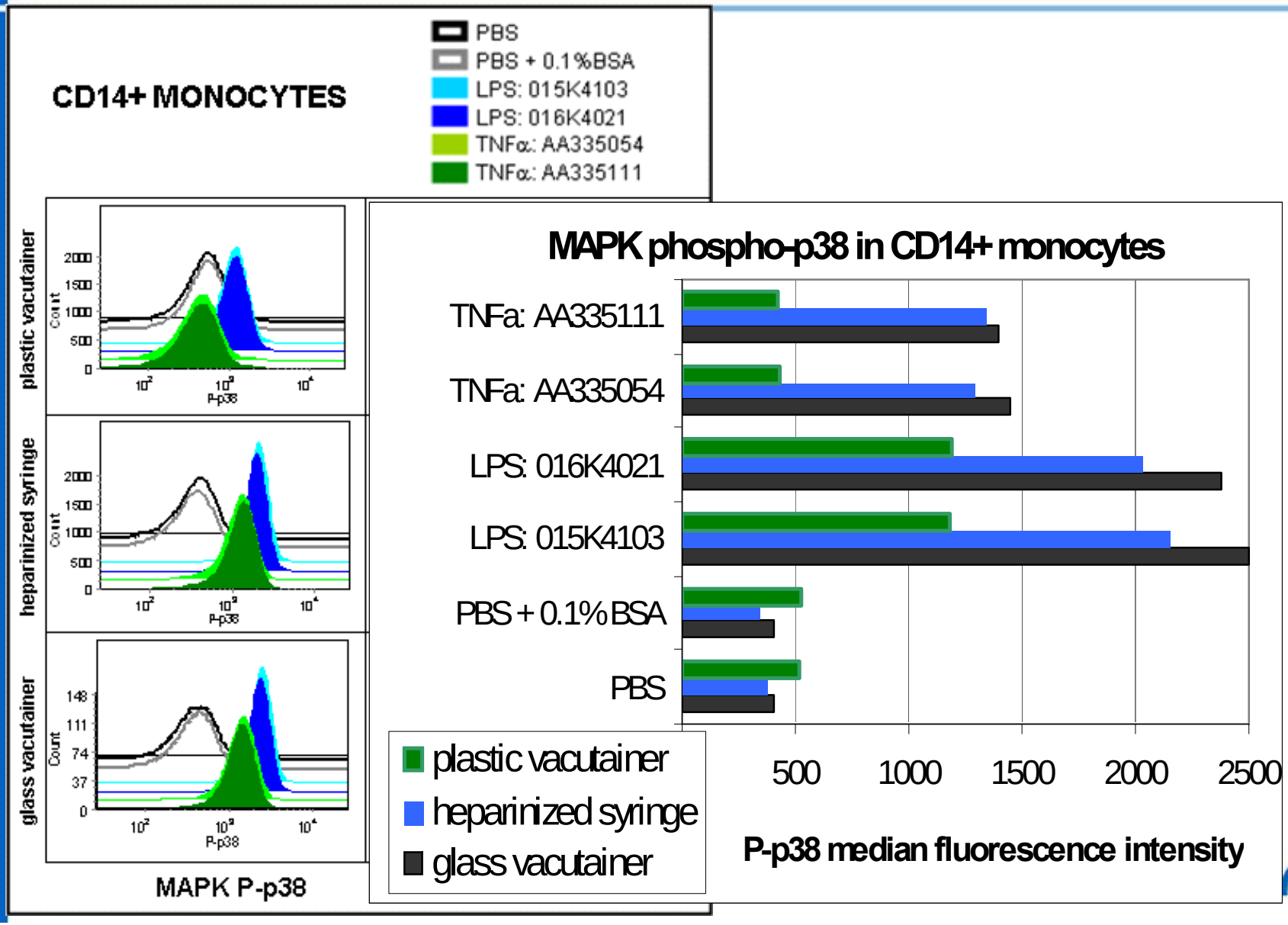
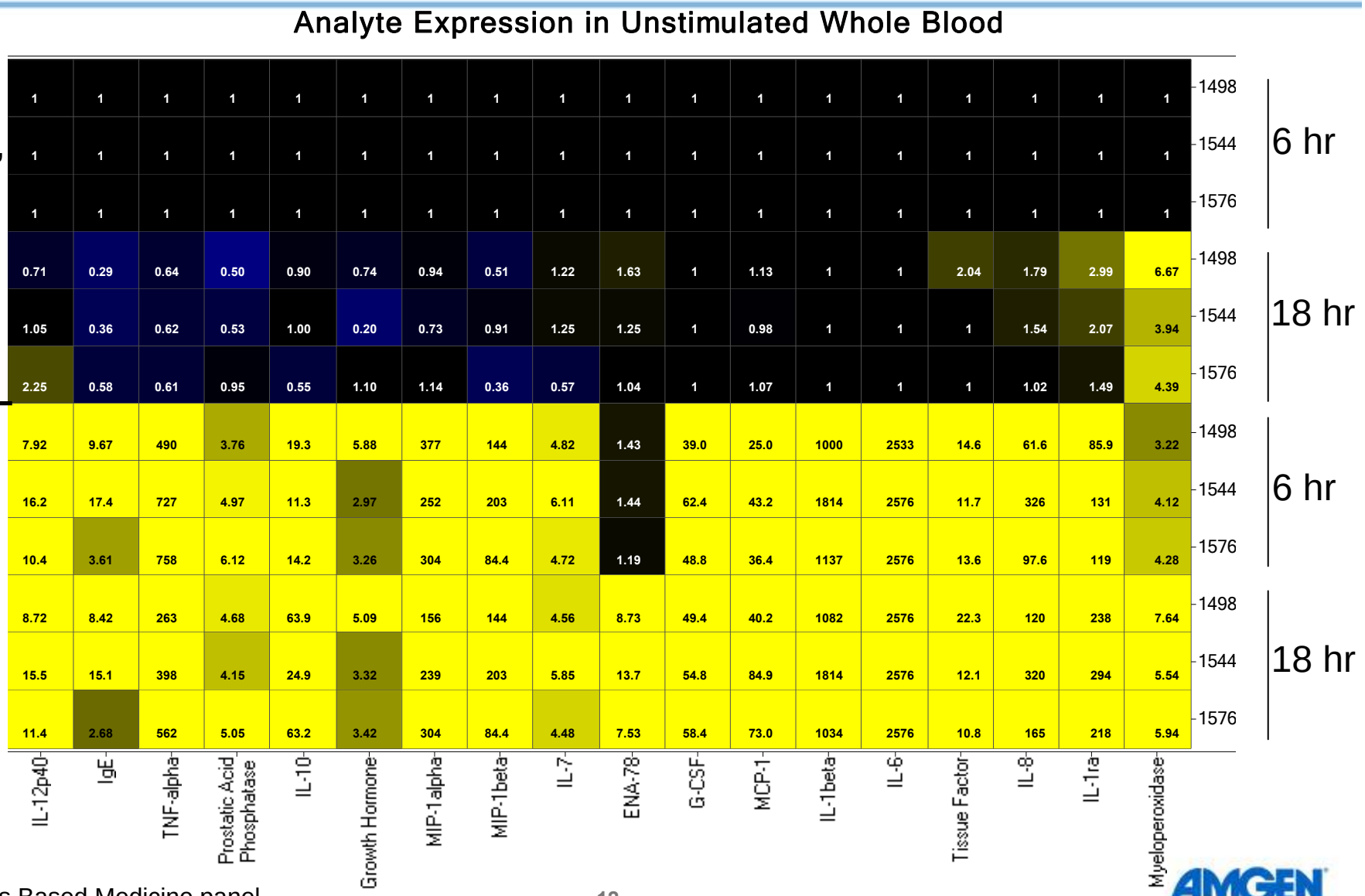
Endotoxin contaminated vacutainers tolerize against LPS & TNFα signaling in CD14 cells (p38)



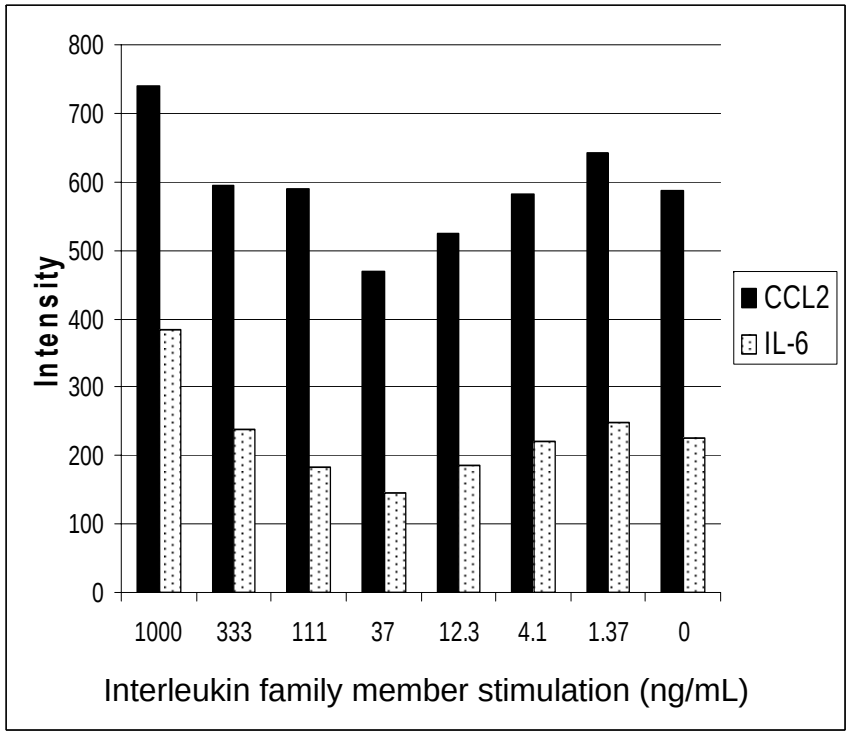
Proinflammatory cytokines are induced in plastic vacutainers containing trace endotoxin



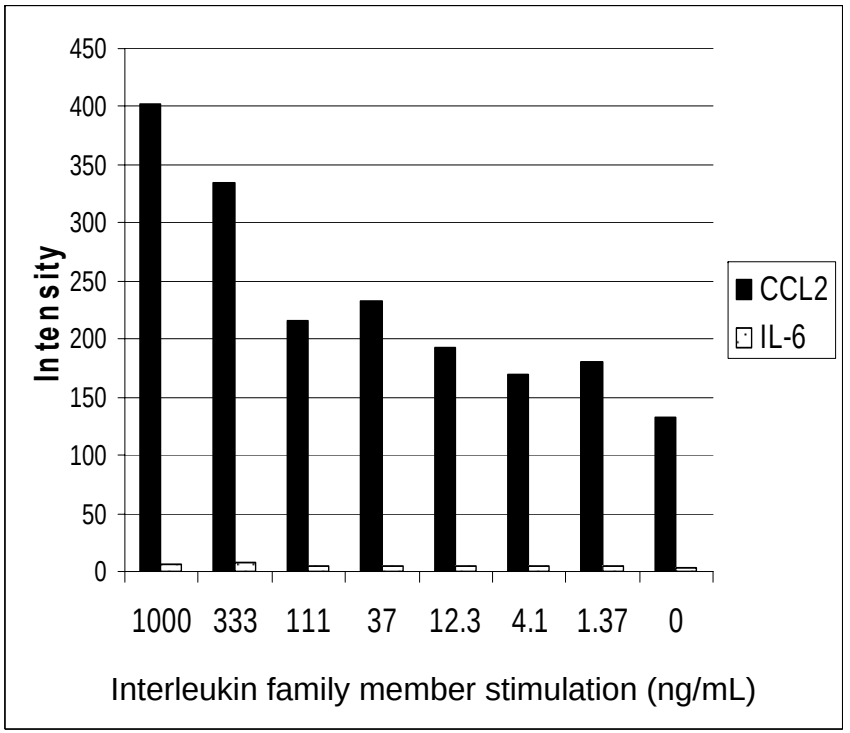
Rules Based Medicine panel

Baseline mRNA levels are ↑ in bDNA-based assays conducted on whole blood drawn in plastic tubes

A



B



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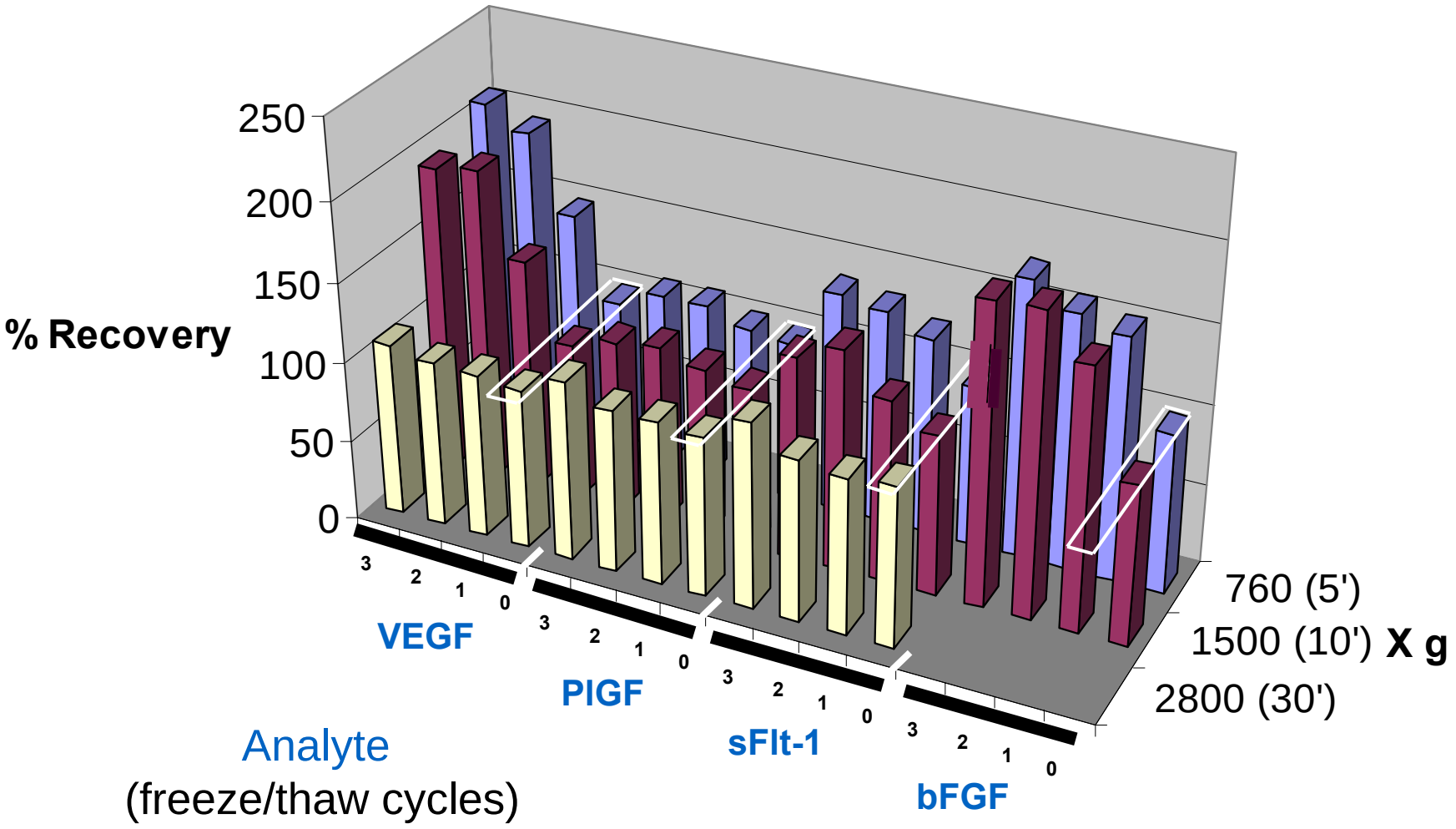
Effect of Centrifugation and Freeze/Thaw on Plasma Analytes



Russell et al (2008) *In:* Biomarker Methods in Drug Discovery and Development, F. Wang (ed), The Humana Press Inc, Totowa, in press.

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Don’t forget the biology!

Proteomics Clin. Appl. 2007, *1*, 1545–1558 DOI 10.1002/prca.200700141

RESEARCH ARTICLE

Cancer biomarker discovery *via* low molecular weight serum proteome profiling – Where is the tumor?

Michael T. Davis, Paul Auger, Chris Spahr and Scott D. Patterson

Department of Molecular Sciences, Amgen, Inc., One Amgen Center Dr., Thousand Oaks, CA, USA

Ernst Schering Res Found Workshop. 2007;(61):23-44.

2 Does the Serum Peptidome Reveal Hemostatic Dysregulation?

M.T. Davis, S.D. Patterson

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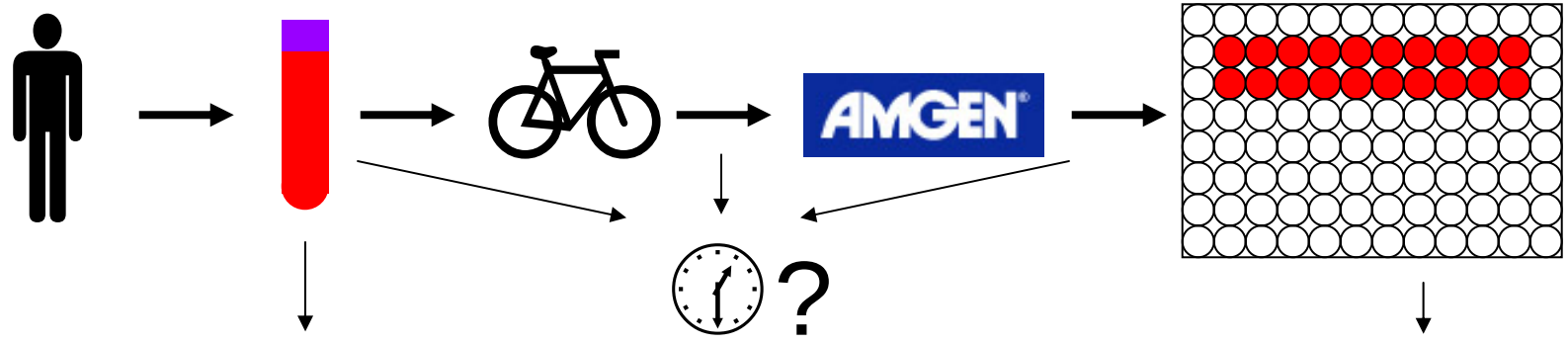
What Happens to the Gene Expression profile of Blood Samples Between the Clinic and Amgen?

Do various anticoagulants influence gene expression patterns?
Does the method of blood collection and extraction effect results?



<u>Anticoagulants Studied</u>
Sodium Heparin
K2 EDTA
Sodium Citrate

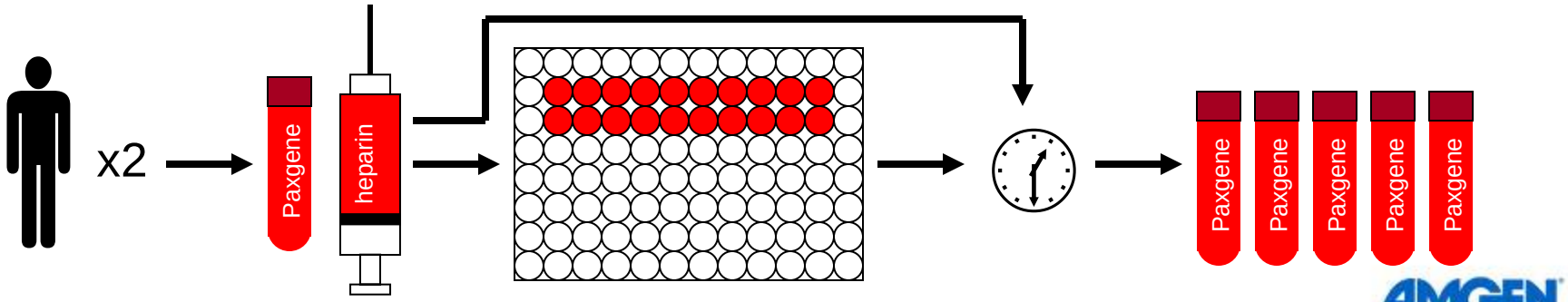
<u>Collection and Extraction Methods</u>
PreAnalytix Paxgene
Qiagen QiaAmp Whole Blood Kit

3. Assay Environment

- Whole blood was collected from two donors directly into Paxgene tubes and syringes containing heparin.
- Blood was then distributed to 96-well plates and kept at 37C.
- Remaining blood was kept in syringes at ambient temp.
- Blood was transferred to Paxgene tubes at the following times post-draw:

  30min – from ambient syringe
  1hr – from 37C plate
  3hr – from 37C plate
  6hr – from 37C plate and ambient syringe
  24hr – from 37C plate and ambient syringe

- Total RNA was extracted and subjected to QC and microarray analysis.



Both Assay Conditions Induce Inflammatory Gene Response, But they are Independent Sets

Up at Ambient in syringe (6 h)

Sequence Name	Sequence Description	QUERY_Fold Change	QUERY_P-value
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	100	7.75E-14
NR4A2	nuclear receptor subfamily 4, group A, member 2	78.02776	4.71E-37
AREG	amphiregulin (schwannoma-derived growth factor)	64.51153	4.34E-40
COX4I1	Sapiens, Similar to cytochrome c oxidase subunit IV isoform 1	49.98443	0.00019
RINZF	zinc finger protein RINZF	19.00078	8.22E-11
DTR	diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)	17.45212	1.00E-17
IL8	Homo sapiens interleukin 8 C-terminal variant (IL8) mRNA, complete cds.	13.90115	2.68E-22
CD69	CD69 antigen (p60, early T-cell activation antigen)	13.80183	0
EREG	epiregulin	12.63002	9.65E-14
JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	11.72579	6.04E-09
TCF8	transcription factor 8 (represses interleukin 2 expression)	10.90541	2.51E-39
BL34	BL34=B cell activation gene [human, mRNA, 1398 nt].	8.71506	2.53E-22
STK17B	serine/threonine kinase 17b (apoptosis-inducing)	8.00018	3.42E-41
SUI1	putative translation initiation factor	7.27559	1.59E-42
CD83	CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)	7.0555	7.86E-39
CDC42	cell division cycle 42 (GTP binding protein, 25kDa)	6.80316	7.64E-10
EGR3	early growth response 3	6.1772	1.55E-14
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	5.49497	0
TSSC3	tumor suppressing subtransferable candidate 3	4.3451	4.02E-06
HOXA5	homeo box A5	3.94295	0.00573
EGR1	early growth response 1	2.89132	3.75E-16

Up at 37C in TC plate (24 h)

Sequence Name	Sequence Description	QUERY_Fold Change	QUERY_P-value
SPON1	spondin 1, (f-spondin) extracellular matrix protein	19.29528	3.32E-06
EPHA4	EphA4	16.56425	0.04355
CCRL2	chemokine (C-C motif) receptor-like 2	11.89838	0.0051
KAB	KARP-1-binding protein	7.53622	0.00162
PCM1	pericentriolar material 1	7.44547	0.00633
CCL3	chemokine (C-C motif) ligand 3	7.02558	5.47E-44
UPB1	ureidopropionase, beta	5.50373	0.02926
EGR3	early growth response 3	5.47604	4.34E-10
CXCL2	chemokine (C-X-C motif) ligand 2	5.46494	1.44E-08
TNF	tumor necrosis factor (TNF superfamily, member 2)	4.73611	2.45E-08
IL1B	interleukin 1, beta	4.68472	2.88E-36
CXCR6	chemokine (C-X-C motif) receptor 6	4.38563	0.00029
CCL4	chemokine (C-C motif) ligand 4	4.18717	4.35E-33
KIAA0992	palladin	4.15859	4.77E-10
ZNF443	zinc finger protein 443	3.75138	0.00169
IL1B	interleukin 1, beta	3.56929	0
ICAM1	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	3.49135	3.31E-11
TNFAIP6	tumor necrosis factor, alpha-induced protein 6	3.40994	5.71E-35
NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	3.13851	5.11E-12
DMN	desmuslin	3.09306	0.00008
ADORA2A	adenosine A2a receptor	2.86423	2.26E-10
CREM	cAMP responsive element modulator	2.80096	0.00018
PLAU	plasminogen activator, urokinase	2.69374	0.00325
C3AR1	complement component 3a receptor 1	2.67405	1.48E-14
CD83	CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)	2.53232	1.88E-13
TNFAIP6	tumor necrosis factor, alpha-induced protein 6	2.3553	6.30E-24

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Summary of Expression Changes in Assay-Like Conditions

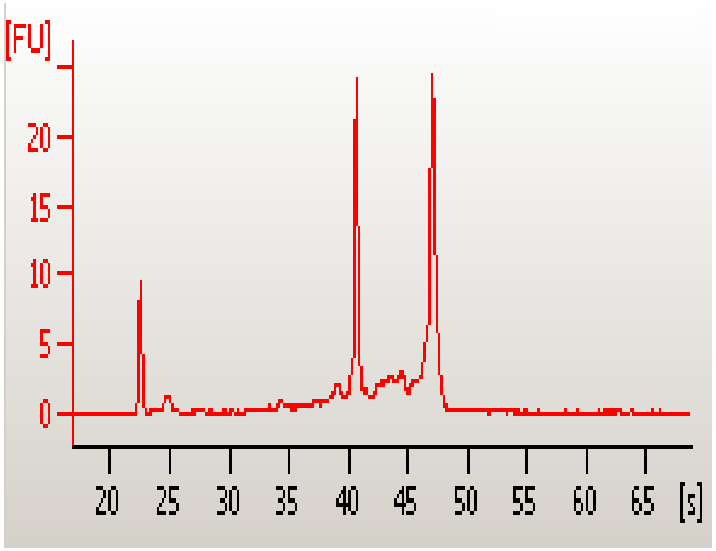
- RNA quality is excellent from all assay conditions
- RNA yields decrease with time in heparin and are lower overall at 37C
- There are different groups of genes upregulated by time at ambient temp in heparin syringe vs. time at 37C in TC plates

Recommendations for Paxgene handling

Failure to Mix PAXgene Tubes *May* Lead to RNA Degradation

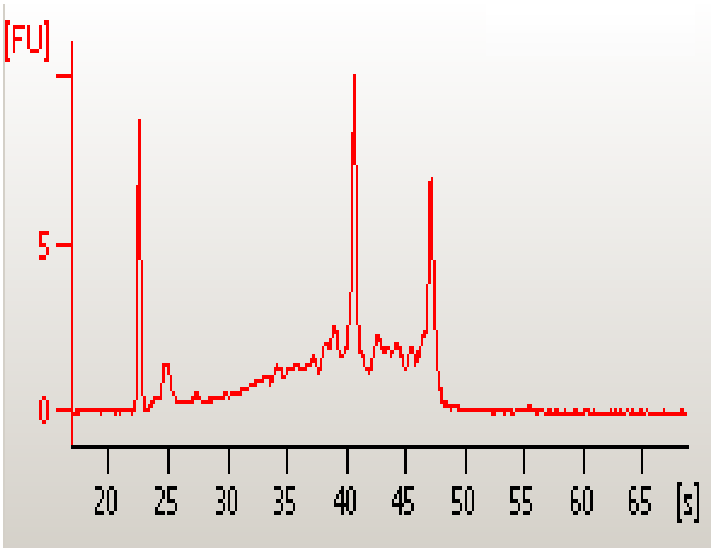
Tubes were collected from same donor and draw

Mixed



RIN = 8.9
28S/18S ratio = 1.7

Unmixed



RIN = 6.7
28S/18S ratio = 0.9

Microarray RNA QC cut-offs: RIN ≥7 or 28S/18S ratio ≥1.0

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Proper PAXgene Tube Handling at Clinical Sites

- Draw 2.5 ml of blood into each tube for optimal yield.
- Tubes need to be mixed immediately after draw! Failure to do so will result in:
  - Insoluble pellet in tube
  - Degraded RNA
  - Altered expression profile
- Consistent incubation time at room temperature is vital. Inconsistent times will result in:
  - Variable RNA yields
  - Altered expression profile

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Biomarker discovery in drug development

- **What do we desire?**
  - Greater number of accurate measures to provide a more complete picture of the PD effect
  - Ability to predict those patients who will benefit
- **What is limiting?**
  - The amount of sample and how frequently in can be collected
  - The type of clinical sample available
  - Multiplexing capability
  - Discovery in models is *not* limiting – but how relevant?
    - **Hence need human tissue**
- **How do we get there?**
  - Development/adaptation of technologies that generate robust data on clinically relevant material
  - What we *don't need* is overstated conclusion from small poorly controlled studies
  - **Well characterized and appropriately handled samples upon which to conduct the experiments**

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What kind of samples can be obtained?

- **Blood (plasma, serum, PBMCs, CTCs)**
  - ***Ex vivo* stimulations possible**
  - **Often used as a surrogate tissue**
  - ***Techniques:* ELISA, enzymatic assays, flow cytometry (cell surface, intracellular), transcript analysis**
- **Fine needle aspirates (repeat sampling possible sometimes)**
  - **Cells of interest can be enumerated and characteristics measured (few cells)**
  - **Preanalytics not yet well understood**
  - ***Techniques:* LSC, IHC**
- **Biopsies (repeat sampling difficult)**
  - **Skin, fat, muscle, tumor**
  - ***Techniques:* ELISA, LSC, enzymatic assays, transcript analysis**
- **Hair follicles**
  - **Potential for cell cycle related studies and some pathways of interest**
  - ***Techniques:* IHC, LSC**

METHODS THAT ALLOW ANALYSIS AT THE CELL LEVEL ARE *PREFERRED* IN HETEROGENOUS TISSUE SAMPLES

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Acknowledgements

- **Medical Sciences**
  - **Molecular Sciences & Computational Biology, Thousand Oaks and Seattle teams**
  - **Clinical Immunology – Cellular Immunology**
  - **Early Development**

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