John Allinson and **Steve Brooks**
Bioanalytical Systems, Ltd., Building 28, Stoneleigh-Deer Park, Stareton, Kenilworth, Warwickshire, UK CV8 2LQ

Biomarkers in Drug Development – A CRO Perspective

This article outlines the leading role contract service providers can play in development of biomarkers. It discusses the integration and considerations needed to bring the science and regulations into step so these exciting scientific developments can be used by the pharmaceutical industry to bring new medicines to market faster. A case study is presented to highlight the role a contract service provider can play to ensure that client and regulatory requirements are met.

Biomarker: A Definition

A characteristic that is objectively measured and evaluated as an indicator of:

- normal biologic processes,
- pathogenic processes, or
- pharmacologic response to a therapeutic intervention. (1)

The high costs incurred when drugs fail during clinical trials has prompted interest in biological indicators (biomarkers) associated with the progress of a disease and the effects of therapeutic interventions, together with indicators of drug-induced toxicity, for use in preclinical studies or earlier stages of the drug development process. Use of such data is not new to clinicians or toxicologists, since they have been used for decades in patient care and in safety assessment of new chemical entities. Some classic examples of biomarkers are blood glucose (for diabetes) and cholesterol (for cardiovascular risk), as well as liver, cardiac and renal function tests such as ALT, SGPT and CPK. Some others are listed in ***T1*** (2). What is new is the explosion in technology with proteomic and genomic techniques, automation, improvements in analytical sensitivity, and increased knowledge of biochemistry, physiology and cell and systems biology. These new technologies have sparked a tremendous and varied research effort into sensitive markers with the aim of predicting changes before manifestation of the disease or toxicity.

The techniques of genomics and proteomics have already been used to monitor multiple potential biomarkers simultaneously by examining gene and protein expression in diseased and healthy cells. For example, cDNA arrays have been used to detect changes in gene expression for hepato- (3) and nephrotoxicants (4), which were then correlated with clinical chemistry parameters and development of drug-induced lesions. Serum Proteomic Pattern Diagnostics have been used to identify proteomic patterns produced by anthracycline- and anthracenedione-induced cardiotoxicity (5).

The biomarkers listed in ***T1*** are somewhat misleading in that there is a well-defined correlation with the disease/therapeutic intervention, sometimes for a single biomarker. Typically, the correlation is not as clear cut and a panel of biomarkers is required. This complexity has inhibited acceptance of biomarkers as an integral part of drug development by both pharmaceutical companies and regulatory agencies. One consequence of the minimal acceptance by regulatory agencies is that biomarkers have only been used to eliminate potential leads due to, for example, toxicity issues, as opposed to being used to support IND and NDA applications. However, these issues are also being actively explored by the regulatory agencies, and increased utility of biomarkers will therefore require active and careful cooperation between regulatory agencies and pharmaceutical companies. Still, pharmaceutical companies are actively involved in biomarker research using pharmacodynamic (PD), safety, and efficacy biomarkers to facilitate go/no-go selections of lead compounds for development and, ideally, to act as surrogate endpoints and eventually used as a diagnostic tool (***F1***). As active participants in the drug development process, contract research organizations (CROs) must also be involved in the evolution and acceptance of biomarker assays at all levels of drug development.

The Role of CROs

Contract laboratory services supporting the pharmaceutical industry always face challenges in balancing the expressed needs of their clients with the requirements for compliance with relevant accreditation standards laid down by the regulatory authorities. Achieving this alignment requires contract laboratories to work with other stakeholders to enable the application of new processes that stay current with advances in scientific practice. It will be those service providers with the foresight, knowledge, and ability to keep abreast of the wider picture who will lead the way to achieving a confluence of innovative science with appropriate legislation. In the broad definition, biomarker determinations represent a branch of bioanalytical chemistry, just as clinical chemistry does. On the other hand, a narrower practical definition of bioanalytical chemistry relates specifically to determination of the drug substance itself and perhaps a metabolite or two. Bioanalytical CROs thus tend to use physical tools to achieve their objectives, tools such as chromatography and mass spectrometry. While these tools offer relatively high throughput and are useful for biomarker discovery and determination, it is

generally the case that protein biomarkers are studied with immunoassays that are unfamiliar to many bioanalytical groups. Immunoassays are clearly somewhat more ambiguous with respect to selectivity and linear range. They include biological selectivity elements which can be harder to control, and the validation criteria must be different. On the other hand, many immunoassays provide a selectivity and detection limit not approachable by physical methods. They also can provide for more parallel determinations using highly automated instruments of modest cost and are more familiar to clinicians and the diagnostics industry.

In addition to their different approaches, bioanalytical and central laboratories within large CROs are often deployed under different management structures and may be totally independent of each other from operational and study management standpoints. They, in fact, may be contracted by completely distinct and separate groups within a client organization. Therefore, one of the challenges with biomarkers is to ensure that the most appropriate method and analytical platform are used. To ensure this happens, the right mix of scientific staff and equipment within a single biomarkers group is needed.

Moreover, it is important that regulatory authorities accept biomarker data that is adequately supported both scientifically and clinically, even if it does not meet the usual "template" requirements. In this regard, the three areas of major conflict seem to be calibration, quality control, and physiological variability of these endogenous substances. It is not unusual to see methods validated in bioanalysis that would have no place in routine diagnostics. Equally, some methods in central laboratories may not be adequately validated to meet those parts of the guidelines that are relevant and required for regulatory submission of PD data. Fit-for-purpose evaluation (i.e., is the method suitable for the required concentration range) is often lacking or absent. For example, an excellent paper by Findlay et al. (6) identified many of the issues not addressed by the FDA Guidance for Industry for bioanalytical methods (7). Therefore, a balanced and sensible approach to these matters is needed. Recently, we have seen the move forward in acceptance of the issues discussed here and a real sense of collaboration between scientists active in this arena, a very welcome, if overdue, occurrence.

Biomarker Assay Validation

Method validation of biomarker assays should be considered a continuous and evolving process (***F2***). It will also be the case that biomarker assays used in research and discovery will not require the same degree of formal validation as those used in pre-clinical, GLP-regulated studies or clinical studies from first-in-man to Phase IV. In addition, some factors of the assays may not be able to be evaluated (and hence validated) until after sample analysis commences, due to the need for incurred samples containing significant concentrations of the biomarker of interest.

Case Study: Steroid Measurement

It is important to understand the limitations of the available methodologies and to ensure that the most appropriate one is used for the analysis. It is also important that the method will fulfil the requirements of both the client and any applicable regulatory body. Some laboratories ignore (or don't know of) the best method available and continue to use poorer methods that will please the regulators but may not fulfil the requirements of the study. Prior to using a method, a fit-for-purpose evaluation and justification of the method selection is critical.

In this case study, the sponsor requested analysis of a steroid in an animal species as a biomarker of preclinical efficacy. The measure of efficacy was a reduction in circulating levels of the steroid (by 10- to 20-fold), and the animal species used for the preclinical study typically has much lower circulating levels of the steroid than humans. Hence, this study required a method sensitive enough to detect changes in sub-clinical concentrations.

Measurement of this steroid in many CROs would typically be passed to the clinical chemistry/clinical pathology department for analysis by immunoassay, either in the form of an automated analyzer or by a manual kit-based method. Alternatively, since this is a preclinical study, it is possible the method could be measured using a bioanalytical approach such as LC/MS or GC/MS. The advantages and disadvantages of each of these approaches for this particular analysis will now be considered.

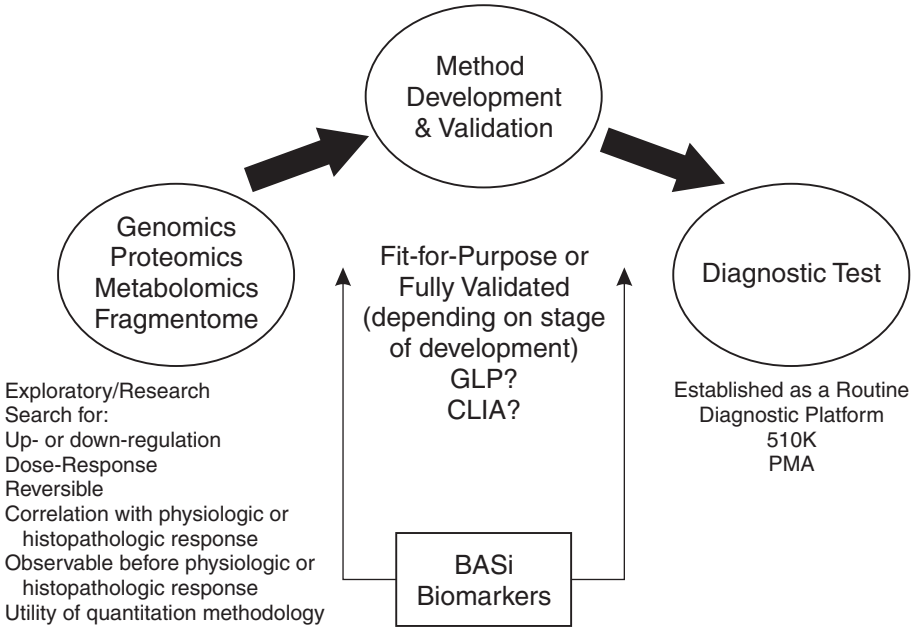
1. Clinical Chemistry / Clinical Pathology Approach

A clinical chemistry/clinical pathology department will generally focus on an analytical technique from a diagnostic, as opposed to a method, perspective. A clinical chemistry laboratory would normally consist of health care professionals trained in the clinical aspects of analytical measurement. Although the laboratory may understand the importance of the intended purpose of the study, they may not perform an extensive validation or fit-for-purpose assessment. Rather, methods would be validated/qualified to recognized criteria set down by such bodies as NCCLS (National Committee for Clinical Laboratory Standards) and CLIA (Clinical Laboratory Improvement Amendments). In particular, since the matrix in this case is animal serum, it is important for the laboratory to validate the matrix by performing experiments such as parallelism. It may also be important that the department understands regulatory requirements such as electronic records (CFR21 Part 11) and instrument qualification when selecting the most appropriate analytical platform.

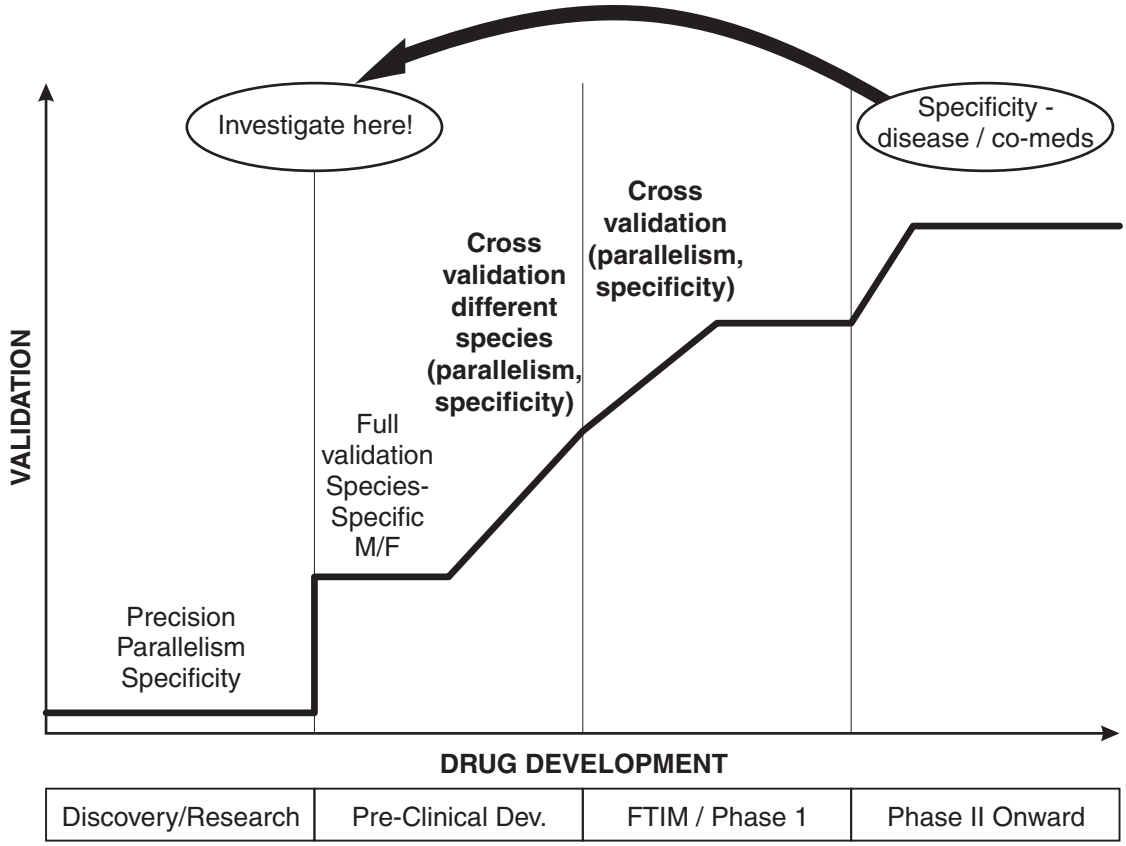
2. Bioanalytical Approach

A bioanalysis department will, in general, focus on an analytical technique from a method, as opposed to an application, perspective. A bioanalytical laboratory would normally consist of chemists trained in the development of analytical methodologies. Although the laboratory will perform an extensive validation of the assay, the preclinical (clinical) requirements of the study may not be taken into consideration. From a regulatory perspective, the majority of bioanalytical laboratories will be up to date with the latest regulatory requirements, including GLP, guidances, and

F1. Biomarkers, the transition from discovery to diagnostics.



F2. Parallel validation of biomarkers assays with drug development.



CFR 21 Part 11 compliance.

Steroid analysis using gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/ mass spectrometry (LC/MS) has some advantages over immunoassays. The main advantage is that of specificity, since it has been reported that commercial antibodies used in immunoassay kits may cross-react with other steroids. GC/MS is usually the preferred method, since it is most sensitive (refer to ***T2***) and specific, but unfortunately the sample preparation procedure is quite difficult and time-consuming. Normally a LC/MS assay would not have been sensitive enough to fulfil the requirements of this study, but more sensitive LC/MS methods have been reported recently (8).

3. BASi Biomarker Department Approach

The Biomarker Department at BASi combines the advantages of both the bioanalytical and clinical chemistry approaches. Both regulatory and clinical requirements are considered prior to the beginning of every study. The department has the analytical platforms and personnel required to develop both bioanalytical and immunoassay methods, and hence the most appropriate method is used for the assay. Sample volume, collection procedures, stability, and other issues related to endogenous molecules are all important and familiar to the Biomarker Department.

In this case study, although it is possible that the bioanalytical method would have fulfilled the requirements in terms of sensitivity, it was decided that the costs associated with this type of analysis would be too high. Moreover, the automation available for many immunoassays will often bring value in later phase development due to the ability to process large numbers of samples very easily when compared with GC/MS and LC/MS, since immunoassays often have very limited sample preparation or extraction processes. However, the existing immunoassay methods developed for clinical assays of this steroid were not fit-for-purpose, as they were not sensitive enough to measure sub-clinical concentrations.

Since the available methodologies would not meet the study requirements, a kit manufacturer was approached to discuss evaluation of an ultra-sensitive kit. This kit was an enzyme immunoassay method not currently available commercially. An agreement between the CRO and the diagnostic company was reached so the CRO would act as a "beta site" for the kit. The data generated in evaluation and validation of the kit would be sent to the diagnostic company to aid with their commercialization of the product. This new method proved to be suitable for the intended purpose of the study, and the method underwent a full validation procedure. ***T2*** shows how the method compares in sensitivity with the other available methods.

Although the LLOQ for the ultra-sensitive kit was slightly higher than that quoted for the LC/MS and GC/MS methods, it met the clinical requirements of the study (i.e., it is fit-for-purpose). Further, this immunoassay was four to ten times more sensitive than others available. It should also be noted that the limits for the other methods are quoted and not proven in our laboratory. Our experience has demonstrated that claims of performance are often difficult to meet in the laboratory. It is possible that the ultra-sensitive kit may be more than 40 times more sensitive from a practical perspective than some routine methods used in diagnostic laboratories. The data for the ultra-sensitive kit are those proven within our laboratory at validation. The method is fully automated. The cost of analysis is approximately 40 to 50% of methods that use GC/MS or LC/MS, and hence brings added value to the client. In addition, as a fully automated procedure, it is well placed for easy use in larger, later-phase studies (for large numbers of samples and short turnaround times). Unfortunately, as a beta test product, there have recently been some production issues to be addressed before scaling the method up

T1. Examples of biomarkers.

Disease	Biomarker
Type 1 and Type 2 diabetes	Glucose, fructosamine, and hemoglobin A1c. Retinal evaluations, nephropathy measures, peripheral neuropathy assessments
Hypertension	Angiotensin-I, angiotensin-II, plasma renin, aldosterone, ACE activity
Asthma, chronic obstructive pulmonary disease, rheumatoid arthritis	Cytokines, leukotrienes, chemokines
Hypertension	Blood pressure and heart rate measurements
Asthma, chronic obstructive pulmonary disease	Pulmonary function tests
Cardiovascular injury	cTroponin I and T
Liver damage	ALT, γGT

T2. Method comparison (example data).

Analytical Method	Analytical Range (ng/mL)	Lower Limit of Quantification (LLOQ) (ng/mL)	Lower Limit of Detection (LOD) (ng/mL)
Immunoassay Platform 1	0.5-16.0	0.5	0.2
Immunoassay Platform 2	0.2-18	0.2	ND
Immunoassay kit	0.5-25	0.5	0.05
GC/MS	0.025-12	0.025	ND
LC/MS	ND	0.01	ND
Ultra-sensitive kit	0.05-1	0.05	0.008

ND= data not available

for additional production use, but the approach is sound and the kit is expected to be a significant product.

This is a good example of how a multi-disciplinary staff working together with the client and commercial reagent manufacturers can ensure that *all* study implications, clinical and regulatory, are recognized and addressed. When combined with consideration for the bigger picture of drug development programs where such matters as cost and throughput of sample analysis are often important, this approach produces what we believe is a truly integrated and comprehensive biomarker assay service.

Senior author, ***John Allinson*** *(johna@basanalytics.co.uk), is a Fellow of the Institute of Biomedical Sciences (FIBMS) and, having spent 22 years in diagnostic laboratories in the British National Health Service, has experience in all major fields of clinical pathology. He has managed and developed specialist laboratory services for drug monitoring, trace metals, and immunoassays. During a tenure in a large CRO, he developed analytical services for a large central clinical laboratory conducting all phases of preclinical and clinical trials. This work necessitated the validation of more than 100 immunoassays on a variety of analytical platforms. John is now the Laboratory Director of BASi UK and has led the development of new analytical services including the biomarkers laboratory. John is a respected speaker at a number of conferences internationally, addressing the topic of biomarkers, regulatory issues, and the synergies with traditional bioanalytical laboratories. He is a member of the Biomarker Committee of the AAPS Ligand Binding Assay Bioanalytical Focus Group (LBABFG) whose aims are to produce recommendations for the industry for biomarker assays and their validation, soon to be published.*

Co-author, ***Steve Brooks*** *(sbrooks@bioanalytical.com) is Business Development Rep for BASi based in Wilmington, Delaware. Steve earned his MS degree in toxicology from the University of Arizona and worked in pharmaceutical R&D at ICI/Zeneca/AstraZeneca and other companies for close to 20 years with experience in safety assessment, drug metabolism and bioanalysis. He is chair-elect of the Delaware Valley Drug Metabolism Discussion Group and, as a hobby, is a member of the crew of the 17th century tall ship Kalmar Nyckel.*

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BIOMARKER CAPABILITIES

In addition to the assay list below, any commercial assay kit not included. Many assays may be validated for multi-species and multi-matrix.
Multiplexed assays available for some human and mouse assays.
Assay development and validation for molecules where suitable antibodies can be sourced.
Fully automated assays wherever possible.
Macromolecule PK method development, validation and sample analysis.
Hybridization assays.
Other analytical platforms used on study-specific basis where appropriate. Validation, true fit-for-purpose evaluation, clinical AND regulatory needs covered.

EQUIPMENT		TECHNIQUES AVAILABLE	
Upstate Luminex 100	DPC Immulite	*Enzymeimmunoassay (EIA)*	*Fluorescence Immunoassay*
Molecular Devices SPECTRA Max M2	Roche Cobas Mira Plus	*Chemiluminescence Immunoassay (CLIA)*	*Time-resolved Fluorescence (TRF)*
IL ACL Futura Coagulation System		*Scintillation Proximity Assays (SPA)*	*Immunogenicity Assays*
Dade-Behring BNProSpec Laser Nephelometer		*Fluorescence Resonance Energy Transfer (FRET)*	*Hybridization Assays*
Grifols Triturus 96-well Microplate Automated Analyzer		*Enzyme Linked Immunosorbent Assay (ELISA)*	

THERAPEUTIC AREA

NB - *many biomarkers have uses across multiple therapeutic areas. Where this is the case, this list has categorized these in the most likely therapeutic area of first choice.*

Adrenal/Pit	Allergy	Anemia	Bone	Cardiac	Diabetes	Specific Proteins	Inflammation	Reproductive
ACTH	Allergy Food Panel FP5E	EPO	25 Hydroxy Vitamin D	Angiotensin Conv. Enz.	C-Peptide	alpha 1-antitrypsin	CIC	Androstanedione
Cortisol	Allergy Screen AlaTOP	Ferritin	1 - 25 Di-Hydroxy Vit. D	Angiotensin I	Glucagon	ß2 Microglobulin	CRP	Androstenedione
11 Deoxycortisol	IgE Cat-specific	Folic Acid	Calcitonin	Angiotensin II	Insulin	ß-TP (ß-Trace Protein)	Cytokines (all)	Dehydrotestosterone
17 alpha Hydroxy	IGE Dog-specifc	Haptoglobin	N-Telopeptide	Apolipoprotein A I	Microalbumin	a 1-acid-glycoprotein	Hemopexin	DHEA-SO4
Progesterone	IgE Latex-specific	sTfR	Osteocalcin	Apolipoprotein A-II		Albumin	IL10	Free Testosterone
Aldosterone	IgE Mite-specific	Transferrin	PICP	Apolipoprotein B		alpha1-microglobulin	IL-1b	FSH
	IgE Total	Vitamin B12	PTH Intact	Apolipoprotein E		alpha2-macroglobulin	IL-2R	HCG
	ECP		PTHrP (Nichols)	Arginine Vasopressin		Ceruloplasmin	IL6	HCG Free Beta
			Pyridinium Crosslinks	Atrial Natriuretic Peptide		Ig/L-chain, K-Type	IL8	HCG Total
			Pyrilinks D	BNP		Ig/L-chain, L-Type	INTERLEUKINS (ALL)	LH
				CK-MB		IgA	LBP (Lipopolysaccharide BP)	Oestradiol
				CRP (High Sensitivity)		IgE	RF	Oestriol Unconjugated
				Endothelin 1		IgG	TNFa	Oestrone
				Homocysteine		IgG1		Progesterone
				Lp(a)		IgG2		Prolactin
				Myoglobin		IgG3		SHBG
				N' Atrial Natriuretic Pep.		IgG4		Testosterone Free
				N-terminal Pro-ANP		IgM		Testosterone Total
				Renin		Prealbumin		
				Troponin I		RBP		

Thyroid	Tumor Markers	Infectious	Coagulation	Growth	Renal Tox	Hepato-Tox	DOA/TDM	Miscellaneous
Anti-TG Ab	AFP	ADNase	Antithrombin III	HGH	Cystatin C	Alpha GST	Amphetamine	6 beta Hydroxy Cortisol
Anti-TPO Ab	CA 125(OM-MA)	Anti HBc-IgM	APTT	IGF1	Alpha GST	Collagen IV	Cannabinoids	C1
Free T3	CA 15-3 (BR-MA)	Anti HBs	Factor Assays	IGFBP3	Collagen IV	Pi GST	Carbamazepine	C4
Free T4	CA 19-9(GI-MA)	ASL	Fibrinogen		Pi GST		Cocaine metabalite	Cytokeratin 18
TBG	CEA	CMV IgG	Fibronectin		Urine Albumin		Digitoxin	MMP's
Thyroglobulin	HCG Free Beta	H. pylori-IgG	Plasminogen		Urine Alpha-1-microglobulin		Digoxin	Pancreatitis-Associated-Protein
Thyroid Uptake	NMP22	HbsAG	PT		Urine Beta-2-microglobulin		Metamphetamine	PAPP-A
Total T3	PSA	HbsAG confirmatory	Prothrombin Fragments		Urine IgG		Nicotine metabolite	TIMP's
Total T4	PSA 3rd generation	Herpes I & II IgG					Opiates	
TSH	PSA Free	Neopterin					Theophylline	
	TPS	Toxoplasma IgM					Valproic Acid	