

# HOW LOW CAN YOU GO? next generation immunoassay systems and the revival of protein biomarkers

Proteins are the machinery that drive most biological processes; however, measuring their concentrations at low levels has been historically impeded by a lack of appropriately sensitive technology. Advances in immunoassay technology, such as single molecule counting, have provided orders of magnitude of increased sensitivity. These novel technologies are enabling new discoveries and a clearer understanding of previously intractable biology. Here we discuss how single molecule counting technology can brighten the future of translational research, drug discovery and personalised medicine.

**T**here is a great paradox facing drug developers: get the most innovative and effective new drugs from bench to bedside as quickly as possible while meeting stringent regulatory demands that demonstrate robust safety and efficacy. This leaves quite a dilemma. Pressure to drive innovative drug candidates through the pharmaceutical pipeline into clinical trials leads to a high level of attrition, where even the most promising drug candidates can fail. This keeps promising therapies from needy patients and leaves pharma footing the bill. The key to solving this puzzle is to effectively rule-in or rule-out drug candidates early in the pipeline, before they enter into costly late phase clinical trials.

## **Biomarkers – the beacon of translational biology**

One can view classic biochemistry as the original drug discovery machine, beginning with discovery and purification of insulin from pancreatic islet cells in dogs. The medicinal application of this

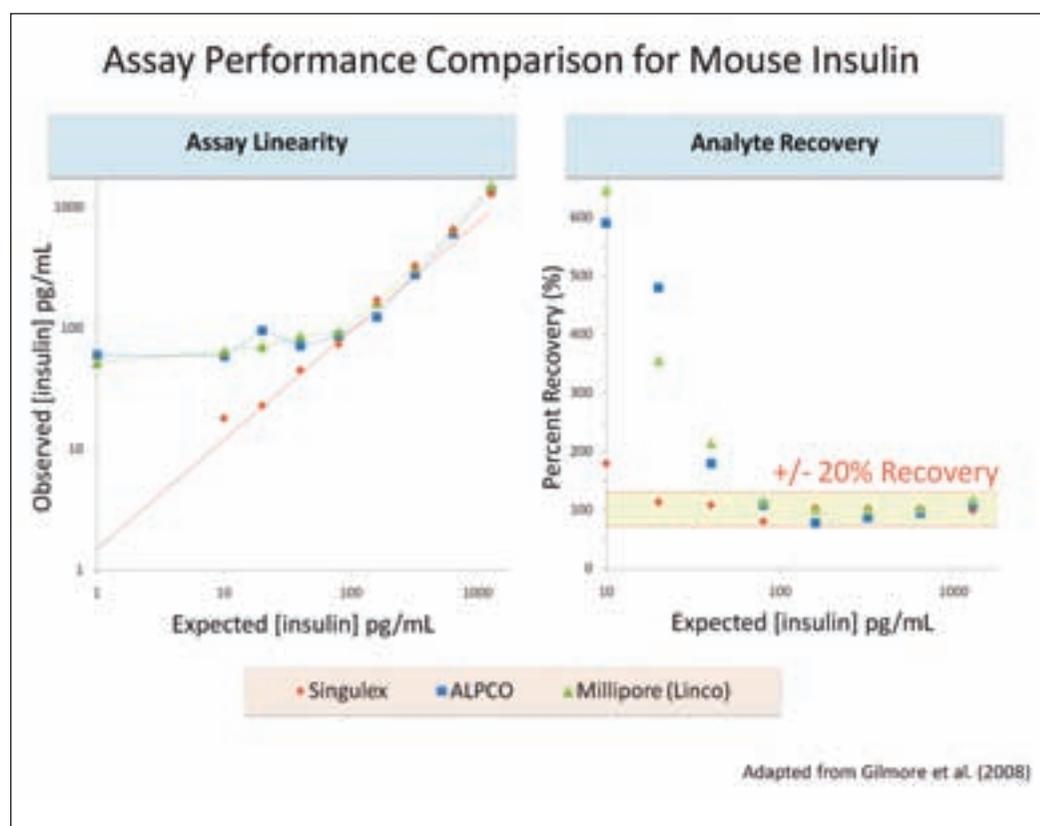
discovery led to a Nobel Prize in 1923 and paved the way for the first pharmaceutical giant: Eli Lilly & Sons. The biology around protein analysis remained the mainstay of big pharma until the molecular revolution of the 90s led into the genomics era. The difficulties of specific and sensitive protein detection helped set the stage for the molecular revolution, where genomics-based approaches have taken centre stage. However, this does not change the central dogma of biology, that our pathophysiology is ultimately determined at the protein level. Therefore we must continue to develop advanced protein detection systems, even for protein biomarkers with previously intractable biology.

The strength of the biomarker movement has been its promise to provide a standardised metric for evaluating new therapies across disease states. In this way biomarkers have become inseparable from the drug development process, and may combine physiological and molecular biomarkers for disease. However, molecular and protein biomarkers (ie

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**Figure 1**  
Comparison of mouse insulin  
assay performance



cTnI, insulin) have a strong advantage over physiological biomarkers (ie electrocardiogram, BMI) because they are more quantitative and less subjective. A desired approach is to use protein biomarkers for the targeted disease in early phase I/II studies to understand if a candidate drug exerts a dose-dependent pharmacodynamic (PD) effect on biomarkers that are either in the disease pathway or that are a direct target of the drug. If a drug candidate does not impact the concentration of a relevant biomarker in a dose dependent fashion, this will halt development of the compound. The earlier a problem drug can be screened out the better, saving the expense of costly late phase clinical trials. However, in many cases technology to do this effectively has been a limiting factor, especially for measuring protein biomarkers in readily available specimens such as blood plasma or serum.

Systems biology ushers in a new paradigm, and shifts attitudes from scepticism towards early adoption and implementation of cutting edge technology. We have seen the fields of genomics and bioinformatics grow under this strategy, which can now be applied towards validation of these discoveries at the protein level. In order for this to work requires the successful deployment of several approaches: biomarker discovery, target valida-

tion, assay development, instrumentation and clinical trial management. There has been an attempt to unify these approaches under the umbrella of translational research, which has become a hot buzz word even though few people seem to agree on how it is defined or executed. One thing most researchers agree on is that this integrative approach is the future of pharmaceutical development, as evidenced by investments by both the NIH and the European Commission towards funding translational research centres and consortiums<sup>1,2</sup>. The point of confusion usually concerns where and how researchers fit into the very broad 'bench-to-bedside' model. Diagnostics is unique in that it can play a supporting role across the entire bench-to-bedside spectrum, since there is something to be measured every step of the way.

### The disease continuum – a full spectrum of disease states

In a general sense, current diagnostic guidelines based on quantification of protein-based biomarkers suffer from three distinct challenges. First, assay sensitivity is often what determines the lower limit of the threshold and the diagnostic cut-off value. Second, the threshold system only reveals the tip of the iceberg when it comes

to disease states. As in cTnI, a potential gold mine of information resides beneath the 99th percentile threshold value for a clinically relevant biomarker. This valuable information, quantifiable by single molecule counting (SMC) technology in particular, could be used to diagnose, stratify, determine risk, or aid in disease prevention. Third, development of a disease state is not an on/off proposition. Rather, there is a continuum of disease development which makes disease onset and diagnosis difficult to mark. When does a patient cross the line to a full-fledged disease state? The fuzzy line marking disease onset is a common cause of diagnostic failures.

An excellent example of prolonged disease onset leading towards diagnostic failure is Alzheimer's Disease (AD). Right now, the only definitive diagnosis of AD is a post-mortem examination of brain tissue for amyloid plaques and neurofibrillary tangles. Currently, clinical diagnosis of this disease is diagnosed by ruling out other probable causes for the symptoms of AD, which are usually only apparent after significant disease progression has occurred. Thus there is no reliable way at this time to clinically define when this complex disease officially starts, and an FDA approved molecular diagnostic for AD is desperately needed.

The need for an early diagnosis for AD is especially important as new therapies for AD are developed, which provide better patient outcomes when administered early in the course of the disease. Currently, there is no FDA approved protein biomarker for clinical diagnosis of Alzheimer's disease, though there are some hopeful candidates: beta-amyloid and tau proteins.

However, further clinical validation of this new diagnostic information will be necessary to define the disease state and set the guidelines for early diagnosis and onset of AD.

In this way, protein biomarker programmes in AD and other disease areas can benefit immensely from the application of novel immunoassay technologies with ultra-sensitive detection.

### **The solution – integrating novel technology into next generation immunoassay systems**

**Fluoresphere immunoassays (Luminex) – xMAP** technology integrates flow cytometry, microspheres, lasers, digital signal processing and traditional chemistry to feature a flexible immunoassay system that can be configured to perform a wide variety of bioassays. Luminex uses colour-coded microspheres, which can be combined into 100 distinct sets. Each bead set can be coated

with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. Within the Luminex compact analyser, lasers excite the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. In this way, xMAP technology allows multiplexing of up to 100 unique assays within a single sample, both rapidly and precisely. However, multiplexing can come at the expense of assay sensitivity and this technology generally does not provide sensitive measurements beyond what has been historically obtained with 96-well enzymatic assays (eg ELISA).

**Gold nanoparticle probes (Nanosphere) –** Nanoparticles can be used as the solid phase for protein capture by attaching specific capture antibodies for specific analytes. Detection is achieved by attaching bio-barcode nucleic acid probes, which are specifically amplified and quantified. Sequence tagged barcodes are then correlated with specific analyte capture. Multiplexing is accomplished by changing the bio-barcode tags on a set of gold microparticles, providing a method for differential readout. However, the amplification step and multiplexing can increase background and can impact assay precision.

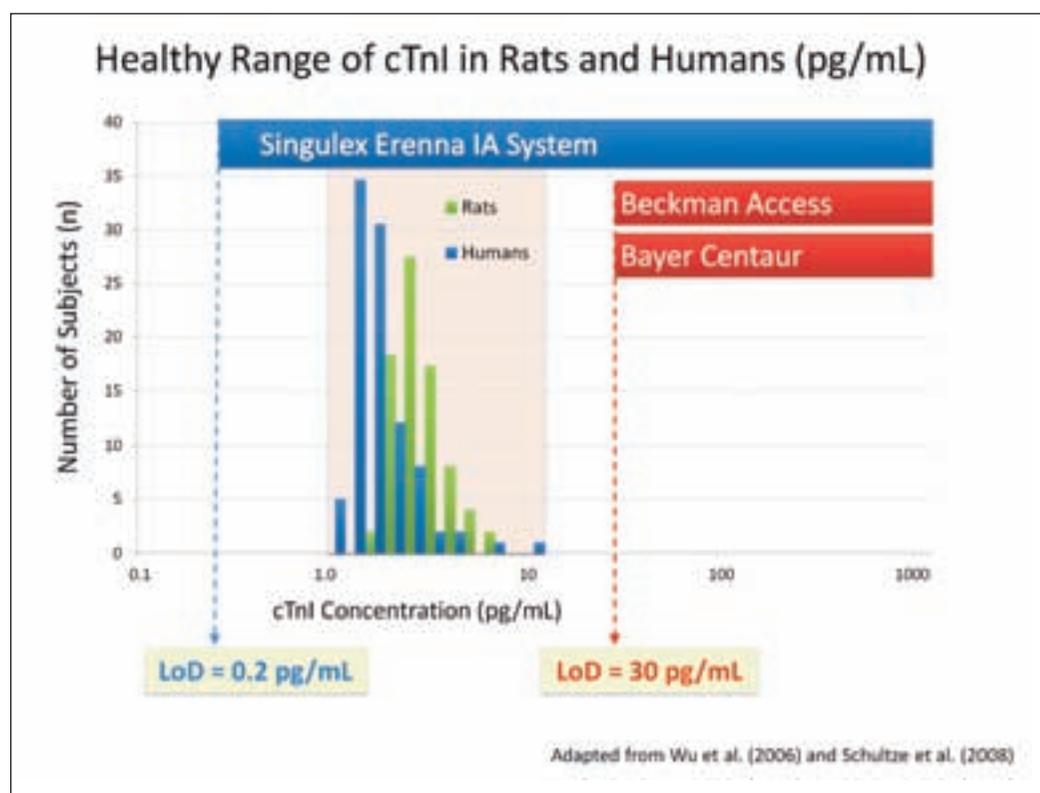
**Single Molecule Counting (SMC)=based systems (Singulex) –** Singulex has developed a proprietary immunoassay technology, which leads the next generation of molecular diagnostic technologies capable of quantifying biomarkers at the sub-picogram level. The proprietary Singulex immunoassay technology utilises paramagnetic microparticles (MPs) as the solid phase for immune-capture and detection of analyte from a complex biological sample, providing enhanced specificity, sensitivity and precision by 1-3 logs over existing plate-based methods. The incorporation of SMC technology into next generation immunoassays, like the Singulex Erenna System, is already having an impact by quantifying proteins at sub-picogram levels<sup>3,4</sup>.

### **The impact of next generation immunoassay systems**

Each of these new immunoassay systems adds value by increasing sensitivity to unprecedented levels of detection, some to below the femtomolar range. The benefit of this new limit of diagnostic sensitivity is the availability of a new tool kit for solving intractable problems in the biology of disease and to embark upon clinical investigations which were

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**Figure 2**  
Range of cTnI in humans and rats compared to assay LoD



previously considered untenable. There are several strategic applications of these new technologies towards key issues facing drug development.

### Making new biological discoveries

Fundamental to drug discovery is the understanding and manipulation of how biological pathways are altered during disease. These fundamental questions are tackled during preclinical stages of discovery and development, often times in cell culture or small animal model systems for which sensitivity and precision of measurements is an issue. For example, there are several genetic mouse models for diabetes, however accurate insulin measurements in fasted female mice are notoriously difficult. Recently the Singulex proprietary immunoassay has been shown to make accurate quantifications from only 5 $\mu$ L mouse plasma samples, allowing long-term monitoring of insulin over time<sup>5</sup>. In this model, the accuracy of the Erenna System has been shown to provide superior precision over currently available commercial assays (Figure 1). Long-term monitoring is also an issue for studies of cardiotoxicity studies in small animals, and the same immunoassay system has shown the ability to accurately quantify cTnI levels from 15 $\mu$ L samples of healthy rat serum<sup>6</sup>. Interestingly, the healthy range of cTnI levels in rats is consistent with meas-

urements in other animals, including humans<sup>7</sup> and is far below the LoD of other currently available commercial assays (Figure 2). Lastly, another factor important during the discovery phase is the ability to quantify multiple targets, which also benefits from small volume assays due to increased sensitivity. For example, a recent diagnostic test for diabetes was released that calculates risk based upon the quantification of a panel of biomarkers from samples as small as 1.3  $\mu$ L<sup>8</sup>.

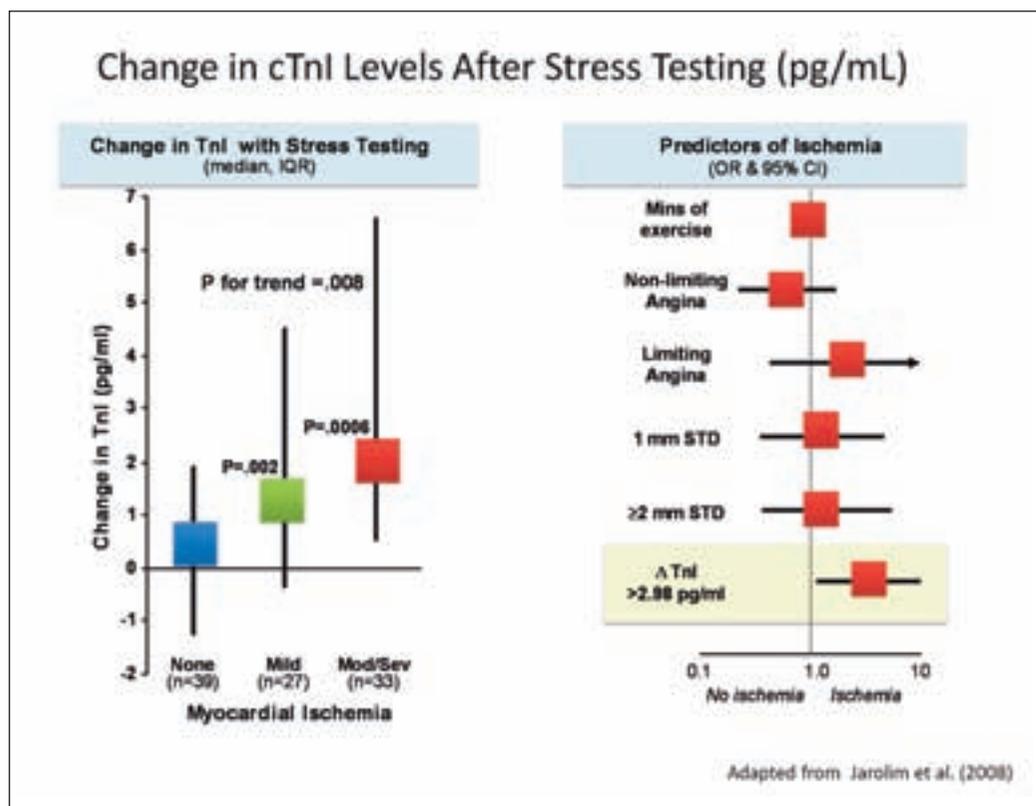
### Early disease detection

The ultimate diagnostic challenge is to detect the onset of disease as early as possible. In terms of patient risk, earlier diagnoses are correlated with better patient outcomes, assuming that efficacious therapeutic modalities can be employed. The development of clinically relevant molecular biomarkers has already had a huge impact on diagnosis of disease, however a continuing problem is that current guidelines rely on the establishment of cut-off values and thresholds for making a diagnosis. These requisite guidelines were set because technologies at that time could not provide the requisite sensitivity. Now we can measure the complete spectrum from healthy to disease state and hence need to think differently. For example, cardiac troponin-I (cTnI) is considered the gold standard for

diagnosis of acute myocardial infarction (AMI), and recent guidelines recommended using a diagnostic cut-point set at a cTnI concentration where the assay imprecision exceeds 10%CV. These cut-points are far removed from the reference range of healthy volunteers and hence small increases in cTnI in the population or within individuals cannot be measured. The Singulex immunoassay technology offers a solution by achieving the sensitivity and precision with which to quantify cTnI to levels below those found in healthy patients<sup>3,4</sup>, allowing accurate investigation into the clinical relevance of values below the threshold compared to other leading immunoassays (Figure 2). Additionally, quantifying acute changes in analyte may provide diagnostic information in the form of biomarker velocity. For example, cTnI velocity before and after stress testing has been shown to be a promising indicator to detect transient myocardial ischaemia<sup>9</sup>, diagnostic information which can be used for risk stratification and prediction of adverse cardiac events (Figure 3). Similarly, this concept is currently used for prostate cancer management, whereby a rapid increase in PSA levels after surgical intervention is associated with unfavourable outcomes. This new diagnostic information may allow earlier disease detection and therapeutic intervention, ultimately saving lives.

#### Targeting baseline levels in healthy states

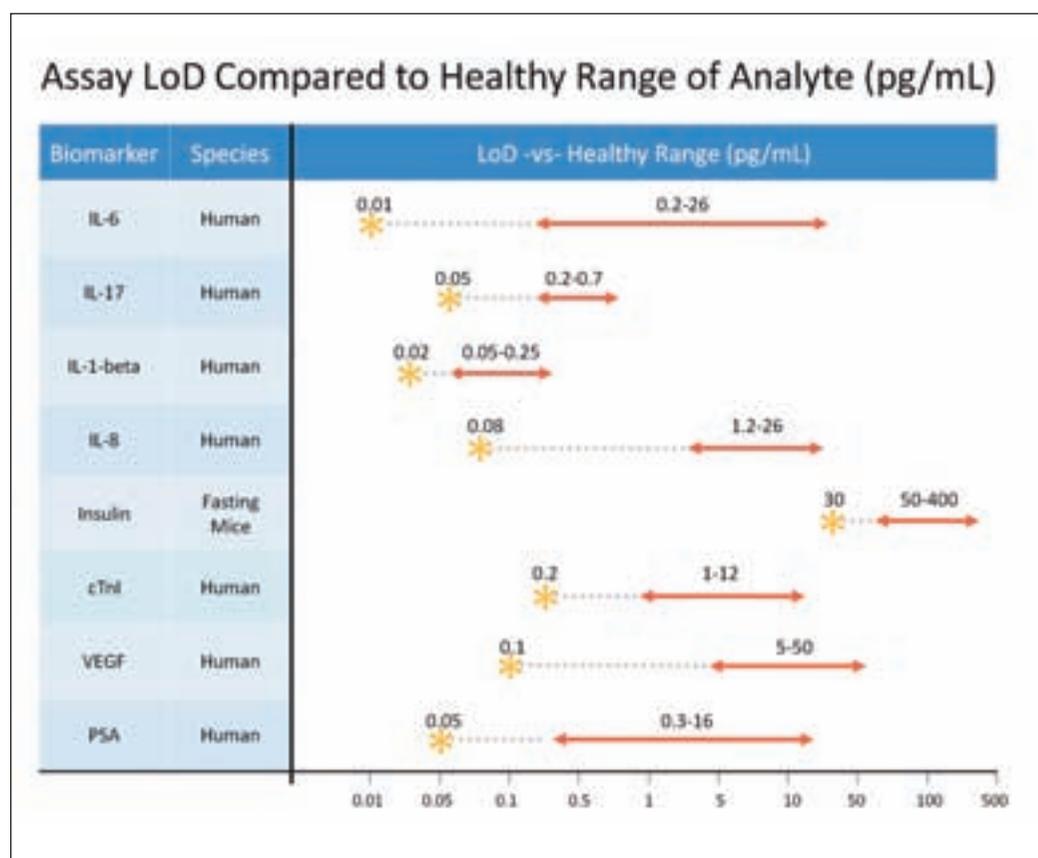
The issue still remains once 'official' guidelines are set, what diagnostic information resides below the threshold? Are we throwing away data that could have an impact on diagnosis, basic research and therapeutic development? The diagnostic goal should not be to merely capture the tip of the iceberg, but to gather all of the relevant data below the threshold – ultimately including baseline measurements of biomarkers in healthy subjects. After all, if you measure the sick and dying, then you will know when patients are sick and dying. If you measure the healthy, then you will know when patients are not healthy – which is a much better proposition. Development criteria for diagnostic sensitivity of immunoassay systems should be redefined in terms of quantifying biomarkers in easily collected, routine samples like serum from healthy individuals. By integrating SMC technology into immunoassay systems, the next generation of molecular diagnostics will have a large impact on translational research by the routine establishment of biomarker baseline profiles from healthy subjects. For example, recently a panel of pro-inflammatory cytokines was quantified in serum samples from normal healthy blood donors using the Erenna System (Singulex), a new IA system based on SMC technology<sup>10</sup>. As more ultra-sensitive



**Figure 3**  
Use of cTnI for detecting cardiac risk and stratification

## Immunoassays

**Figure 4**  
Erenna immunoassay system  
LoD compared to the  
measured range of analyte  
quantified in healthy human  
subjects



immunoassay systems are used for this type of research, healthy reference ranges can be determined that define baseline levels of clinically important biomarkers (Figure 4). This brings immediate benefit to current research and development programmes, and brings future benefits in clinical settings to improve patient outcomes.

### Better efficacy and safety

Evidence-based medicine is pushing pharmaceutical developers to prove that the clinical benefit outweighs risk of administering a drug. Next generation IA systems can immediately benefit pharmaceutical developers by increasing the sensitivity of assays for biomarkers used as surrogate endpoints and safety biomarkers. SMC technology strengthens this application of biomarkers by quantifying a reference range of baseline biomarker levels in healthy subjects, providing a necessary comparison for determining the efficacy and safety of new therapies. For showing drug efficacy, the return of a companion diagnostic biomarker to levels at or below those in healthy subjects builds a strong case for clinical benefit. During drug safety studies, biomarkers indicative of adverse events can be measured with SMC systems to be sure

they are not substantially elevated above healthy levels. Also, with SMC technology a panel of safety biomarkers can be detected in small animal model systems, allowing safety studies to be integrated very early in the product pipeline. A good example is the use of IA systems based on SMC to quantify cTnI in small volume samples from rats, dogs and monkeys<sup>7</sup> allowing for pre-clinical time-lapse studies of cardiotoxicity.

### Early rule in/rule out decisions

Shifting drug efficacy and safety studies earlier into the product pipeline allows rule-in/rule-out decisions to be made earlier in the development cycle to avoid costly late-phase attrition and market failures. SMC-based systems enhance this process by providing ultra-sensitive detection of biomarkers with high precision, providing a larger set of discreet data points with which to make quality decisions. SMC systems can be used to test very small volumes – as low as 1-5ul of serum<sup>5</sup> – without compromising assay sensitivity. This enables greater sampling from individual small animals, time-course study design, capability to test a panel of biomarkers in each sample, and better discrimination between samples. The ability to

conduct timed trials through serial testing allows for the measurement of biomarker velocity. The use of small volumes allows for efficient sample acquisition and conservation, enabling the use of valuable bio-repository samples (including plasma, CSF, interstitial fluids, sputum, etc) in order to determine the clinical relevance of a biomarker. All of these benefit the researcher by expanding opportunities for robust early phase clinical study design. In a similar fashion, SMC technology can be applied to reposition drugs at market, improve allocation of development resources, and decrease time to market, all of which ultimately bring benefit to the patient.

#### Enabling personalised medicine

We are in a post-omics era, and we are now seeing regular demonstrations of the clinical relevance of genomic and proteomic data. We know that individuals with the same disease can have unique risks, presentations and responses to therapy. How can new diagnostic technology add value to translational researchers in the post-omics era? One way SMC technology is bringing value to researchers is by enabling the translation of biomarker discovery into the determination of risk for disease development. For example, SMC technology has been used recently to discover and validate a panel of protein biomarkers constituting a new diagnostic assay, the PreDx Diabetes Risk Test, which predicts a patient's risk of developing Type 2 Diabetes within five years<sup>8</sup>. This new diagnostic assay quantifies a risk score from the measurement of a panel of relevant biomarkers identified through the discovery process. From studies like these, we continue to learn that different patients may have different pre-dispositions for disease, different disease pathologies, and require different therapies. With SMC technology, biomarker panels can be utilised to pre-screen patient cohorts for contra-indications, to administer targeted therapies for different disease presentations or patient profiles, or to avoid contra-indications for administering drugs to subsets of patients. In this way SMC diagnostic systems will evolve personalised medicine into more than just a buzz word, and into an approach which is implemented at the bedside.

#### Summary

SMC-based diagnostic technologies are continuing to increase the sensitivity of molecular diagnostic platforms, benefiting a broad spectrum of translational researchers. These new diagnostic systems should strive to meet the changing needs of trans-

lational researchers by playing a supporting role throughout the entire bench to bedside spectrum. This expanded role for diagnostics will require continued clinical evaluation of SMC-based diagnostic systems with the flexibility to provide a broad range of clinical utility. Looking to the future of molecular medicine, this kind of partnership will be invaluable. **DDW**

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