SPOTLIGHT feature

Proteomics, Genomics & Microarrays

Rethinking oncological drug discovery with advances in analytical proteomics technology

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Despite advances in genomics, some aspects of cell behaviour can only be understood by exploring proteins. Cell signalling pathways, which commonly rely upon protein 'receptors' binding to signalling molecules to enable communication, allow a cell to interact with and respond to its environment appropriately, supporting healthy and normal growth, migration, division, and development and repair of tissue. These pathways can break down or become disrupted by various genetic or epigenetic alterations, leading to irregular cell function and the development of tumours and cancers.

To better understand the mechanisms behind cell health and disease, researchers aim to determine how cell signalling pathways are disrupted, and the points at which they are impeded along the sequential signalling pathway. Proteomics is a valuable tool here, as it can provide a level of information that other modalities cannot. By targeting specific proteins, proteomics directly measures the components of a cell rather than evaluating by inference based on RNA, DNA or other biomolecules. Quantitative proteomics advances these capabilities and identifies not only what is in a cell, but how much of it is present and how different proteins are interacting, providing a 'systems level' understanding of what is going on in a given cell or disease state.

While it generates invaluable insight, proteomics remains challenging due to the sheer complexity of the proteome. While the genome is made up of approximately 20,000 genes, the proteome comprises up to one million protein forms, some of which are present at low (and, therefore, hard-to-detect) abundances. The proteome is also highly dynamic and variable in nature; it responds to environmental influences and changes with age and individual characteristics, making sampling and interpretation difficult. Additionally, many laboratories have not yet fully embraced proteomics, considering it to be a somewhat specialised approach.

However, the drug discovery landscape is changing, with innovative analytical technology playing an increasingly vital role at this stage of the pipeline and removing traditional barriers to entry. Advanced proteomics methodologies offer more accessible, usable ways to characterise the complex molecular mechanisms involved in disease pathogenesis – without compromising on performance.

The promise of protein quantitation

Quantitative proteomics aims to paint a reliable, accurate picture of the proteome - of its protein concentrations, post-translational modifications, protein-protein interactions, and more - in order to understand the molecular drivers of disease. As the proteome is the main functional entity within a cell, proteomic analysis of cells in various disease states can identify potential biomarkers for use in novel biotherapeutics, and enable researchers to better understand the mechanisms underpinning diseases, such as cancer (which, in turn, could unlock faster diagnosis and treatment for patients). While oncological research is a notable example of the value of proteomics, quantitative proteomics holds promise in many other areas of study, from the development of wider biotherapeutics and personalised medicines to metabolomics, anti-doping, food and beverage testing, and more.

Traditionally, clinical diagnosis relies upon a mix of pathology, microscopy and genomic stratification – but this is insufficient for optimal understanding and diagnosis of health and disease given that many drugs act on proteins. With quantitative proteomics, diagnostic or prognostic protein biomarkers can be derived and used to monitor a specific set of targets, and map signalling networks and pathways. Such pathways are contextual in nature; measuring a specific node may return a specific set of conditions, but this gives just a snapshot of a particular pathway. For instance, a particularly active protein may appear to be in a certain state, but it may in fact be manipulated by a node further along the pathway. In this way, reaching a conclusion based on just one aspect of a sample's total suite of proteins may return false conclusions and, in turn, prompt incorrect clinical research decisions – or in a diagnostic setting, incorrect decisions regarding patient treatment. Additionally, it may be easy to measure the most visible proteins in a cell, but some signalling pathway components exist only in very low abundances. As with an iceberg, the 'tip' of higher-abundance proteins may be visible - but what if the important information lies beneath?

Despite its promise, quantitative proteomics requires advanced technology. A key issue relates to the aforementioned dynamism and variability of the proteome. This is far more pronounced than for the genome, and can enhance sampling bias. Sampling different parts of the same tumour results in differences, and methods of collection and storage become important considerations since, for example, post-resection freezing delays can impact the activation of signalling networks. In such cases, researchers are not seeing the real physiology of the disease, but the artefacts of collection and biobanking. Issues of sampling bias, or of changes occurring during collection and storage, do not arise for genomics samples as the DNA is stable. The comparative instability of the proteome, and associated sample variability, highlights the challenges inherent in oncology and disease proteomics.

Current antibody- and mass spectrometry-based approaches

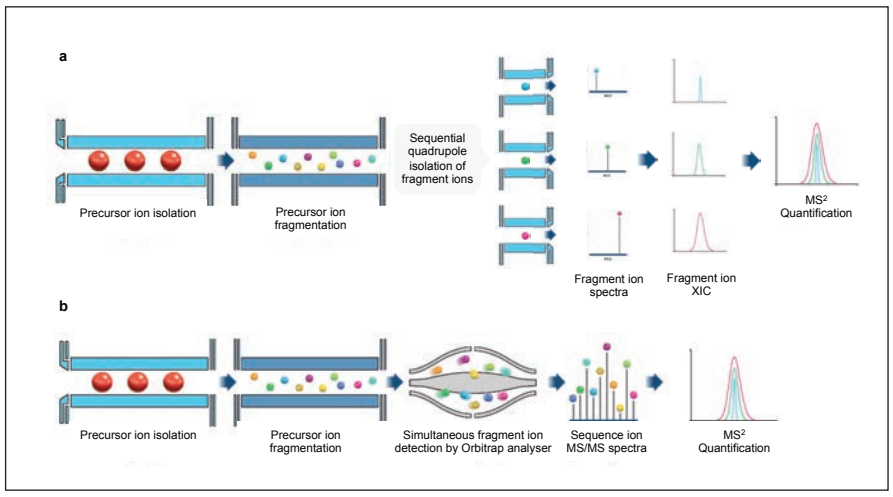
Currently, quantitative proteomics comprises a combination of discovery-based and targeted methods. Discovery methods aim to comprehensively survey a sample and identify all the components present, while targeted approaches instead seek to monitor and quantify selected targets of interest.

Many quantitative proteomics methodologies are of the former type – they look more broadly at the whole system – and are based on antibodies. Predominant antibody-based protein measurement methodologies include Luminex, tissue microarrays and western blotting. These techniques seek to determine which antigens within a sample are binding with specific and selective antibodies.

However, antibody-based methods are limited by the quality, availability and selectivity of the desired antibody, and can typically explore a maximum of several hundred or so targets. Mass spectrometry (MS), meanwhile, can explore thousands of targets without needing to verify the quality of an antibody, raising confidence in results. More targeted methods of quantitative proteomics use MS – specifically, liquid chromatography MS with 'selected reaction monitoring' triple quadrupole workflows (LC-MS; SRM). An example of such a workflow is one in which peptides representing proteins of interest are used to generate an 'assay' for their detection and quantification. For this, selected peptides are isolated and fragmented and characteristic fragment ions for that peptide are then sequentially isolated and detected. By tracing the properties of these precursor-product ion pairs, the target peptide – and, by extension, the target protein – can be quantified.

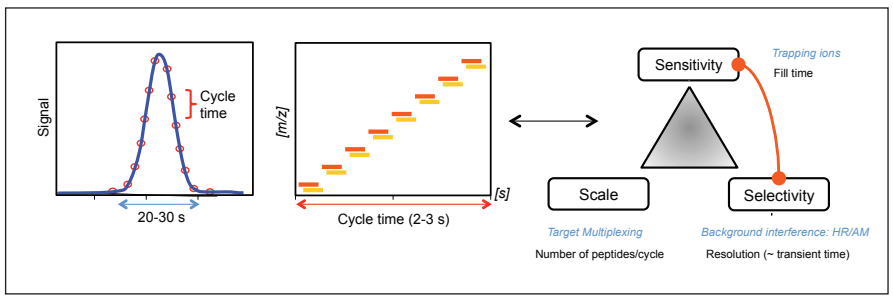
Quick, straightforward and cost-effective SRM approaches are limited by mass resolution and selectivity, especially in samples with complex matrixes such a biological material. However, such limitations can be overcome using high-resolution, accurate-mass MS (HRAM MS) and parallel-reaction monitoring workflows (PRM), such as those implemented by Thermo Scientific Orbitrap-based mass spectrometers. PRM isolates a target precursor, fragments it and then detects all resulting product ions simultaneously, allowing quantification of peptide abundance and comparison of results across multiple sample sets.

This brings greater selectivity and sensitivity alongside lower limits of detection, but remains limited by speed of acquisition, throughput and sometimes representation of the amount of target substance present (a property defined as peak area). Peak area is limited for PRM due to its slower acquisition, which decreases the chances of it achieving complete sampling of a peptide's elution profile.



SRM and PRM are conventional targeted LC-MS approaches. (a) In SRM assays the mass spectrometer is programmed to monitor for the presence of one or more precursor ions, collisionally fragment these ions, isolate, and detect the resulting fragment ions in sequential steps. The integration of extracted ion chromatograms (XIC) from the diagnostic fragment ions allows quantitation of the target. (b) PRM shares similarities with SRM, however, fragment ions are isolated and detected in parallel using high-resolution, accurate mass detectors such as Orbitrap mass analysers. This enables post-acquisition determination of the optimal fragment ions for quantification as well as higher measurement selectivity.

For both SRM and PRM, a major constraint lies in the relationship between target multiplexing (the number of analytes that can be measured reliably) in the desired cycle time and the amount of time devoted to each analyte within that timeframe. Cycle time is constrained by the properties of the LC setup, and so there is always a tradeoff between the highest performance (in terms of selectivity and sensitivity) and number of targets per analysis. Increasing the amount of time devoted to a single analyte brings improved sensitivity, for instance, but allows fewer analytes to be quantified. Conversely, large numbers of targets cannot be studied for as long, compromising data quality. Overall, LC-MS approaches either bring high-scale coverage with sub-optimal quantitation, or low-scale coverage with high quantitation, but not both.



Inter-dependencies between experiment scale, sensitivity, and selectivity. Chromatographic elution properties of the analyte and the desired sample rate (left) will ultimately determine the amount of time the mass spectrometer can spend taking measurements (centre). Within this fixed time, the instrument can be used to collect fewer measurements with high selectivity and sensitivity or alternatively, take more measurements but a reduced sensitivity (right).

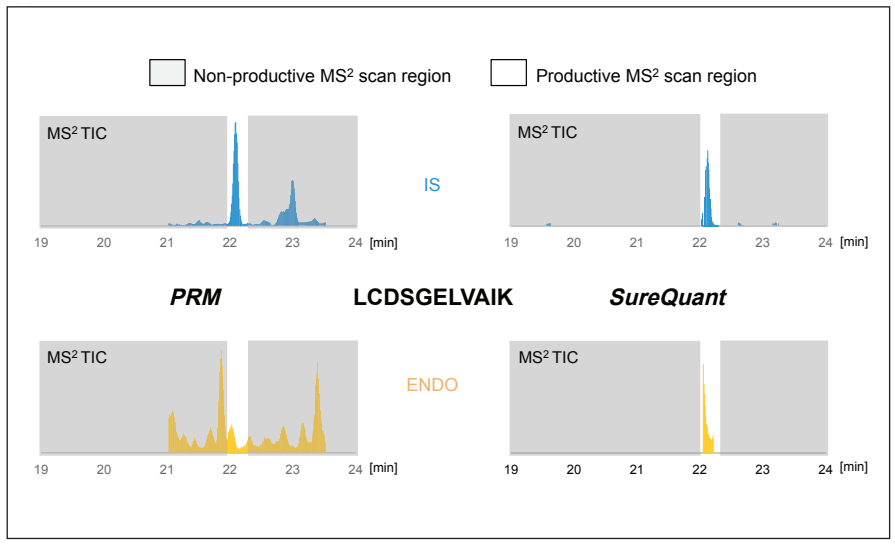
The way forward for proteomics researchers

The limitations of common LC-MS and antibody-based quantitative proteomics methodologies are far from abstract: they have real-world impacts on patients. They result in new therapeutic options either being delayed or unavailable, due to a limited understanding of how tumours and diseased cells signal, and how best to target them via treatment.

To overcome these hurdles, a new approach – internal standard triggered PRM (IS-PRM) – was developed that can dynamically guide targeted analysis in real time. This allows large numbers of targets to be reliably measured at high sensitivity and selectivity, protecting data quality and improving the efficiency of the analytical process. However, early iterations of IS-PRM require an advanced level of technical knowledge, with the user needing to utilise complicated programming interfaces and informatics tools to develop assays and prepare methods that achieve only a partial implementation. This has limited the uptake of IS-PRM by the proteomics community.

More recent developments remove this barrier. The Thermo Scientific SureQuant IS Targeted Quantitation method, for instance, is an evolution of the original IS-PRM approach. It presents the methodology in a usable, easily implementable way as a turnkey 'off the shelf' solution for any laboratory, including for use in biotherapeutics, plasma analysis, host-cell protein analysis, and to replace traditional biochemical analytical approaches such as ELISA.

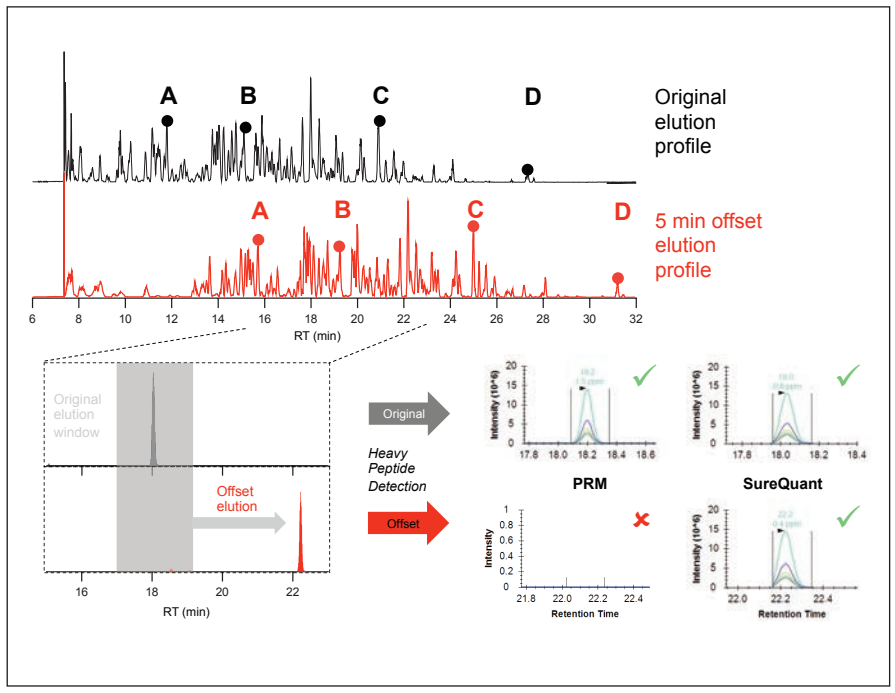
Targeted proteomics workflows have traditionally been challenging, especially for people new to the technology, as the amount of time and effort needed to develop, standardise and validate a targeted assay can be considerable. IS-PRM technologies, such as the SureQuant method, simplify this by bringing an intelligent approach to proteomics, which in turn maximises chances of successful detection and quantitation.



SureQuant intelligent detection of targets maximises instrument efficiency and productivity. The IS and endogenous detection of a representative peptide, LCDSGELVAIK, is shown from PRM and SureQuant acquisition. In the PRM experiment, many uninformative MS^2 scans are captured for the IS and endogenous target (grey region) during the 2.5 min monitoring window, and a smaller proportion of MS^2 scans are captured during the actual target elution time (white region). The dynamic nature of SureQuant acquisition minimizes unproductive scans allowing shorter duty cycles and higher productivity. Experiment details: 50 fmol IS spiked into 250 ng HeLa cell line digest. PRM MS^2 settings: 2.5 min RT window, 15000 resolution, 20 ms IT. SureQuant MS^2 settings: Watch mode 7500 resolution, 10 ms IT; Quant mode 60000 resolution, 116 ms IT.

Refined IS-PRM approaches such as SureQuant bring superior acquisition efficiencies of 80-90% (cf. 10-15% via other conventional targeted approaches), as acquisition parameters can be adjusted on-the-fly to maximise sensitivity and selectivity at the time-point when target is eluting. This productivity brings enhanced data quality and allows more targets to be quantified in the same amount of analysis time to increase target scale and throughput. Importantly, it enhances the chance of successful detection, as the use of internal standards intelligently guides measurement of the target of interest at precisely the right time.

The chances of missing a target measurement are, therefore, greatly minimised, resulting in more consistent, reliable and robust measurement and quantitation. Obtaining a more reliable and detailed dataset allows researchers to make fully informed decisions, and can help to meet compliance needs in highly regulated environments by enabling more precise quantification of the substances within a drug product.



SureQuant acquisition robustness overcomes chromatographic fluctuations. Targeted analysis of AKT-mTOR pathway proteins was performed by PRM and SureQuant acquisition using a standard gradient and an offset gradient which introduced a 5 minute artificial time delay to simulate LC retention time variations that can commonly occur (landmark peptides A-D are indicated for comparison). As an example, the observed heavy peptide LFDAPEAPLPSR (m/z 611.8526 ++) elution is shown at the original and offset retention times (bottom left). Notably, while PRM acquisition failed to capture the signal of the peptides with delayed elution, SureQuant acquisition maintained reliable measurement under these conditions.

Case study: Mapping signalling networks in colorectal tumours with SureQuant-based phosphoproteomics

Low-level cell signalling pathway modifications, such as tyrosine phosphorylation (pTyr) on proteins, play a key role in cell signalling, and have been found to be commonly dysregulated in cancer cells. Profiling the tumour pTyr proteome may, therefore, reveal insights central to the development of therapeutic oncology treatments. However, traditional MS-based methods do not offer adequate sensitivity, reproducibility and depth of coverage given that pTyr proteins account for less than 1% of cellular phosphorylation.

To overcome these challenges and enable research into phosphoproteomics, Professor Forest White's research group at the Massachusetts Institute of Technology (MIT), implemented SureQuant methodology, achieving reproducible and sensitive targeted quantitation as a result. White used this technique to leverage isotopically-labelled trigger peptides and reliably quantify several hundred commonly dysregulated pTyr targets in human colorectal tumour samples.

"SureQuant is uniquely positioned for phosphotyrosine tumour studies," said White, Professor of Biological Engineering at MIT. "Targeted approaches typically allow observation of dozens of targets at one time, but the SureQuant setups available instead allow thousands - and the number of pTyr events within the cell likely number in the same range, with at least 2,000 sites. We aim to expand our SureQuant work to develop a larger-scale model that can cover all functionally important pTyr sites, so we can monitor the phosphotyrosine signalling network."

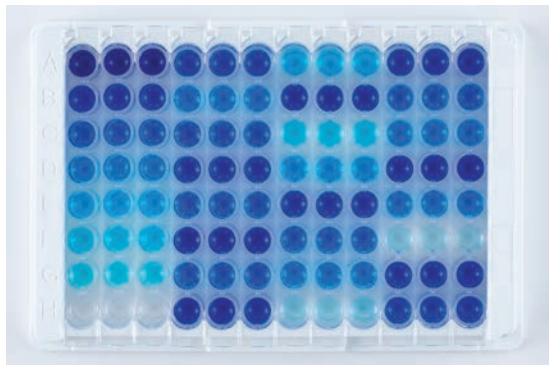
Value Prop	**SureQuant**	**PRM/SRM**	**PRM/SRM**
	Up to 500 pairs of H/L targets/30 min *Highest targeting efficiency *Complete quan profile every analysis *Maintain sensitivity *AND* target scale	Up to 100 pairs of H/L targets in 30 min (60K – 116ms) *Favouring sensitivity	Up to 500 pairs of H/L targets in 30 min (7.5K – 10ms) *Favouring target number
Quantification Performance (Precision/ Accuracy)	★★★★	★★★★	★★☆☆
Sensitivity (LLOQ)	★★★★	★★★★	★☆☆☆
Scale (# Targets)	★★★★	★☆☆☆	★★★★
Efficiency (Productive Scans)	★★★☆	★☆☆☆	★☆☆☆
Load-and-play (Minimal adjustment)	★★★☆	★☆☆☆	★☆☆☆

Summary of PRM/SRM and SureQuant capabilities. Traditional targeted approaches require tradeoffs between high-density targeted panels or high-sensitivity measurements, while SureQuant acquisition offers both large-scale profiling while still maintaining the highest quantification performance.

Conclusion

Quantitative proteomics methodologies are essential tools in the drug discovery toolkit. Discovery-based and targeted methods of quantitative proteomics are perceived as having opposing aims, with one type positioned to take a broader view and one a more detailed view. However, novel and refined methods of IS-PRM are capable of achieving large-scale target profiling with superior quantitative performance, filling a critical gap in protein quantification workflows. As well as enabling a deeper understanding of the molecular mechanisms behind cancers, such technology has great potential beyond oncological proteomics. The SureQuant method promises to play a crucial role in advancing our understanding of a wide range of complex diseases and signalling pathways, unlocking a wealth of knowledge about cell behaviour and health - and signposting potential routes to timely, effective treatments.

Pipetting Solutions for Improving ELISA Workflows



ELISAs (enzyme-linked immunosorbent assays) are popular plate-based methods to detect and quantify peptides, proteins, antibodies or hormones. However, they generally involve multiple pipetting steps that are time-consuming and tedious to perform, requiring consistent processing to ensure success. **Integra Biosciences** offers a range of liquid handling solutions to improve ELISA workflows, designed to make your lab life easier and your results more reproducible.

The VIAFLO 96/384 handheld electronic pipette can vastly improve ELISA workflows by enabling the transfer of samples and reagents into 96 or 384 wells simultaneously. All the steps needed to run the protocol can be saved as a custom program on the instrument, which then guides the user with easy to follow prompts. This streamlines the workflow and eliminates user variability. And for even higher throughput, it can quickly fill multiple plates using the Repeat Dispense mode.

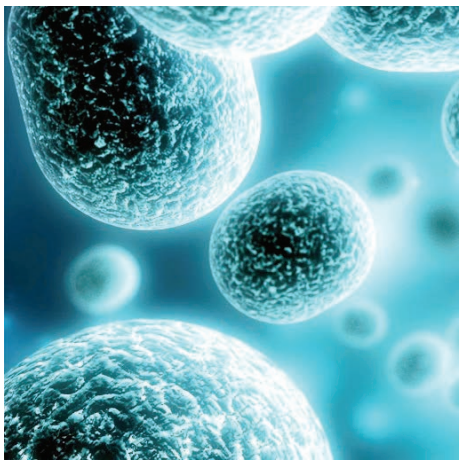
If you're ready for a more walk-away approach, the ASSIST PLUS pipetting robot can automate the ELISA process - just choose the VIALAB program and steps you need, place the appropriate labware on the deck, and let the robot do all the work. Pipetting settings and assay set-up options can easily be modified in VIALAB, making sure the system is always perfectly suited to your assay. Various sample and tube types, and multiple reagents can also be accommodated on the deck, and the choice of pipetting options is huge – you can mount any of Integra's 25 electronic pipettes onto the ASSIST PLUS.

These solutions are partnered with Integra's Low Retention GripTips, perfect for reducing the residual tip volumes when pipetting buffers that contain surfactants, such as Tween 20. This combination of flexibility and automated, optimised processing helps to guarantee the consistency and reproducibility of your ELISAs.

More information online: ilmt.co/PL/Epp0

For More Info, email: 51995pr@reply-direct.com

High-Throughput Screening using Luciferase Reporter Cell Lines



Amsbio reports on the positive customer feedback received for their One-Step Luciferase Assay kit recently introduced for quantitation of firefly luciferase activity in mammalian cell culture.

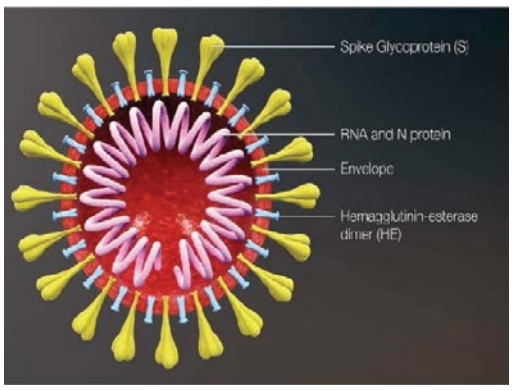
Dr Maja Petkovic, Custom Services Business Unit Manager at Amsbio said: "Initial uptake of this new kit has been strong particularly for high throughput screening applications. Our customers have appreciated that the kit offers not just high sensitivity but also the convenience of a homogeneous, single step protocol. In a recent study, researchers at Excellerate Bioscience were able to quantify agonist and antagonist in vitro pharmacological parameters in a luciferase reporter gene assay using our One-Step Luciferase assay system."

Luciferase is the general term given to a class of oxidative enzymes that catalyse reactions that give off bioluminescent light. Biological researchers can take advantage of this reaction and use it as a readout for processes where a promoter region from a gene of interest is placed immediately upstream of the coding sequence for luciferase.

The One-Step Luciferase Assay kit combines proprietary reagent buffer and reagent substrate components to form a working solution that contains all the necessary components for simple cell lysis and luciferase quantitation. Designed for flexibility - the kit works well with a variety of common media, delivers a signal output that is stable for more than two hours and does not require a luminometer with injectors.

For More Info, email: 52314pr@reply-direct.com

New Recombinant Eukaryotic SARS-CoV-2 S1-S2 Spike Proteins for Improved Diagnostic Testing of COVID19



Aalto Bio Reagents have announced the availability of its new range of recombinant SARS-CoV-2 spike (S) proteins for diagnostic test manufacturers, vaccine developers and researchers globally. Among all structural proteins of SARS-CoV-2, the S protein is the main antigenic component that is responsible for inducing host immune responses and neutralising antibodies. This makes it the principal focus of therapeutic and vaccine design for COVID-19.

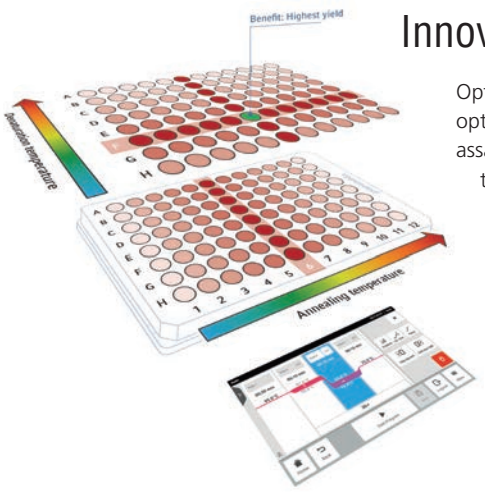
The S protein of SARS-CoV-2 is located on the surface of the viral particles and is comprised of two functional units, S1 and S2. The N-terminal S1 subunit is responsible for virus–receptor binding and the C-terminal S2 subunit mediates virus–cell membrane fusion. When used solely or in combination with the new insect-derived Nucleocapsid protein (N) (code BM 6424), the S1-S2 chimeric glycoprotein (code BM 6422) has demonstrated excellent reactivity in ELISA for detection of IgA, IgG and IgM antibodies against SARS-CoV-2.

"Patients continue to be screened for the virus by PCR on the frontline," said Philip Noone, CEO of Aalto Bio Reagents, "however there is an important need for serological tests as well to detect all those mild or even asymptomatic cases that may otherwise be missed. Having diagnostic tests that can work in different healthcare settings will be the critical next step in battling this virus and crucial to their development is the selection of high-quality, raw materials used to design them. Our new S1, S2 glycoproteins and S1-S2 Chimeric proteins, in addition to our E. coli-derived N protein launched in February, have been rapidly developed to meet this urgent need."

More information online: ilmt.co/PL/Gmz9

For More Info, email: 52292pr@reply-direct.com

Innovative 2D-Gradient for Enhanced PCR Optimisation



Optimising the annealing temperature to improve PCR results is a routine task, so why not optimise as far as you can? More and more findings indicate that optimisation of the denaturation temperature is worthwhile as well. A high denaturation temperature will harm the enzyme and other biomolecules in your assay. A low denaturation temperature may result in inefficient splitting of the complimentary DNA strands, as is often found with GC-rich templates or templates prone to form 'hairpin structures'.

Eppendorf's new 2D-Gradient allows optimisation of the annealing and the denaturation temperature in a single run - taking less time than ever before. This gives you the ideal set of temperatures for your PCR assay - quickly, conveniently, reliably.

Read the Application Note: ilmt.co/PL/Po2e

More information online: ilmt.co/PL/Koma

For More Info, email: 52318pr@reply-direct.com