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CASE STUDY SMART Digest ImmunoAffinity (IA) Kit

Simplifying ImmunoAffinity Capture Workflow

Rapid, Sensitive, LC-SRM Quantitative Analysis of Proteins in Plasma

As pharmaceuticals grow more efficacious, reporting regulations grow more rigorous. With more low abundance proteins shown to be key biomarkers the need for high sensitivity assays that are easy to perform and amenable to high-throughput workflows gain importance. For protein biomarkers in particular, effective, rapid digestion and detection strategies are becoming increasingly critical to analytical workflows. Current methods can be laborious, time consuming and often hard to reproduce.

The Thermo Scientific™ SMART Digest™ ImmunoAffinity (IA) Kits enable a simple combined workflow of protein enrichment by immunoaffinity capture (IAC) and high temperature proteolytic digestion in a single, easy-to-use reagent.

Introduction

PharmaCadence Analytical Services is a professional analytical services organization for pharmaceutical and life sciences with expertize in LC-MS services. PharmaCadence specialize in high quality quantitative Liquid Chromatography Mass Spectrometry (LC-MS) bioanalytical methods for research and early development. This includes services in biomarker quantification such as protein and peptide quantitative analysis by Liquid Chromatography Single Reaction Monitoring (LC-SRM).

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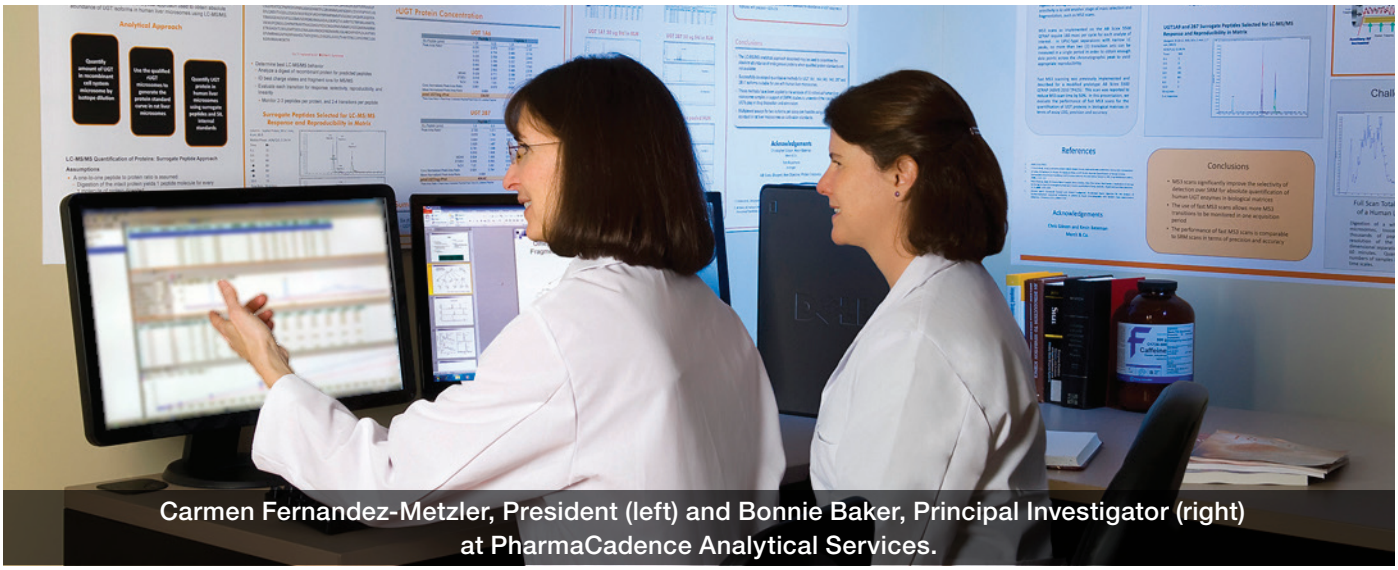
Rapid and Easy Protein Enrichment and Digestion

Dr. Fernández-Metzler, President, and Dr. King, Laboratory Director, at PharmaCadence are leading projects to deliver quantitative information on biological therapeutics and low level biomarkers. The increased efficacy and low abundance of these compounds is driving the analytical community to deal with extremely low levels of analyte in complex biological matrices.

The most sensitive LC-MS protein quantitation and biomarker analytical procedures utilize an immunoaffinity enrichment step to concentrate the target analyte and reduce matrix background. The captured target protein is then digested to yield surrogate peptides amenable to sensitive detection by LC-SRM. The IAC LC-SRM workflow is ideal for maximizing the signal to noise from a minimum amount of sample while providing the selectivity and sensitivity needed to achieve the lowest possible limits of quantification.

Dr. Fernández-Metzler and Dr. King identified the affinity capture and subsequent protein digestion as being a time consuming, multistep process which causes a significant bottleneck in a standardized IAC LC-SRM workflow. The multiple steps and the additive imprecision were found to affect the quality of their analytical results. In order to overcome these challenges the team at PharmaCadence implemented SMART Digest ImmunoAffinity kits that allow protein enrichment and rapid proteolytic cleavage to be performed in a single, easy to use reactor in less than 4 hours. This has significantly improved their throughput, reduced assay complexity and maintained or improved the overall quality of the results.



Carmen Fernandez-Metzler, President (left) and Bonnie Baker, Principal Investigator (right) at PharmaCadence Analytical Services.

"Coupling thermally stable trypsin and co-immobilized streptavidin allows for a simplified workflow with unprecedented speed and sensitivity from raw sample to purified digest..."

—Carmen Fernandez-Metzler, President

The Method

The team at PharmaCadence used the SMART Digest IA Streptavadin kit sample preparation protocol to prepare a circulating biomarker protein targeted for LC-MS analysis.

The SMART Digest IA kit combines immunoaffinity capture and digestion of the protein into a single well. This is achieved by immobilizing the streptavidin and heat-stable trypsin onto the same bead (Figure 1). Using this procedure immunoaffinity capture was achieved in 2 hours. The biomarker is captured onto the magnetic bead, washed and solvent exchanged to the SMART Digest buffer, followed by a 1 hour digestion. The immobilized trypsin being activated by elevation to high-temperature (70 ºC) and addition of the pre-prepared SMART Digest buffer supplied with the kit.

The results obtained with this novel approach were compared to a conventional immunoaffinity capture procedure. This was achieved by using a high capacity streptavidin gel to capture the circulating biomarker in plasma, followed by tryptic digestion of the enriched protein.

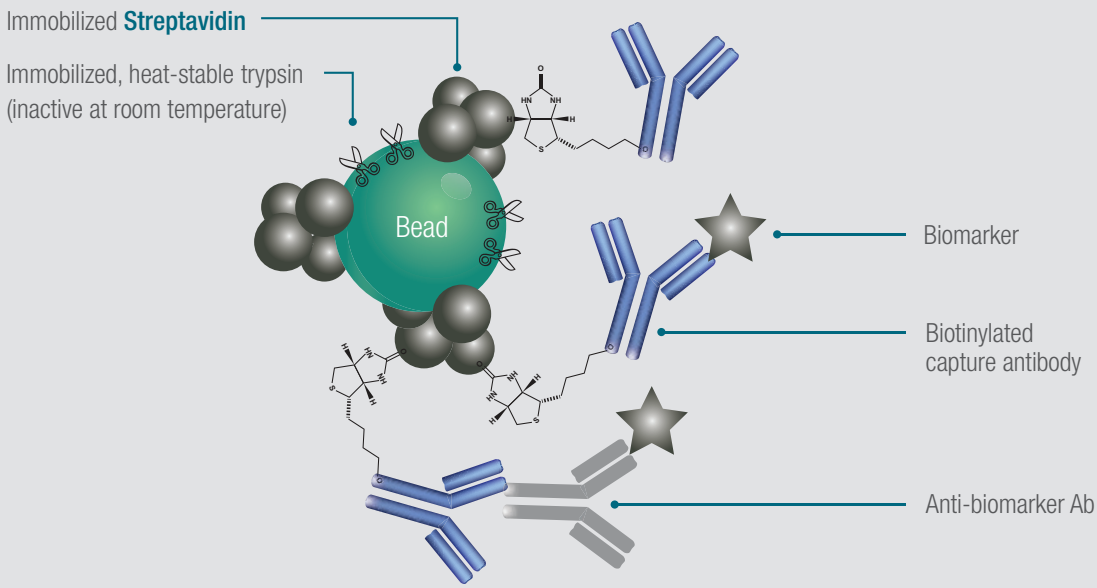


Figure 1. SMART Digest IA heat-stable, immobilized enzyme design combined with immunoaffinity capture.

Table 1. Materials.

Material	Function	Supplier
SMART Digest IA, Streptavidin magnetic bead kit (P/N 60110-104)	Protein enrichment and digestion	Thermo Fisher Scientific
High capacity Streptavidin Agarose	Protein enrichment	Thermo Fisher Scientific (Pierce™)
Trypsin	In-solution digestion	Promega Trypsin Gold
Human plasma	Biological matrix	BioreclamationIVT

Table 2. LC-MS.

UHPLC Conditions		
Column	C18, 300 Å, 3 µm, 1 × 50 mm	
Mobile Phase	A: water containing 0.1% formic acid	
	B: acetonitrile containing 0.1% formic acid	
Gradient	Time (min)	%B
	0	5
	0.2	5
	0.5	5
	4.5	20
	5.5	45
	6.5	95
	7.5	95
	8	5
	10	5
Flow Rate	50 µL/min	
Detection	SRM on a trap Mass Spectrometer	
Ionization	Electrospray	

Table 3. Preparation of Standard Curves and Quality Controls.

Recombinant protein standard curve and QCs were prepared in 4% BSA and diluted in human plasma.
Calibration curve from 125 to 500 ng/mL.
QCs in replicate.
Recovery was calculated by spiking a known level of protein into a blank sample before digestion.
Calibration curve read back concentrations and back calculated QC values were determined using the peak area ratios of target peptide and the corresponding stable isotope labeled (SIL) -peptide internal standard.

Workflow Time Reduction

The co-immobilized streptavidin and heat-activated temperature stable trypsin technology provided by the SMART Digest IA kit enabled Dr. Fernández-Metzler and Dr. King to dramatically accelerate their workflow (Figure 2) with the added benefit of achieving very high sensitivities with excellent reproducibility. In the example shown the workflow was reduced from 21 hours to 3–4 hours, with an increase in sensitivity of up to 3 times.

The ability to perform digestion on the same resin as the immunoaffinity removed the need to perform a lengthy subsequent protein digestion. This dramatically reduces the amount of manual handling required and thus eliminates potential sources for error and sample loss. This directly contributes to the improved recovery and repeatability observed with the new approach. Additionally, the thermally stable trypsin allowed them to digest their samples in only 1 hour, significantly faster than the traditional over-night protocol they had employed before. Coupling these two advancements allowed the PharmaCadence team to routinely increase the speed, sensitivity and precision of their workflow.

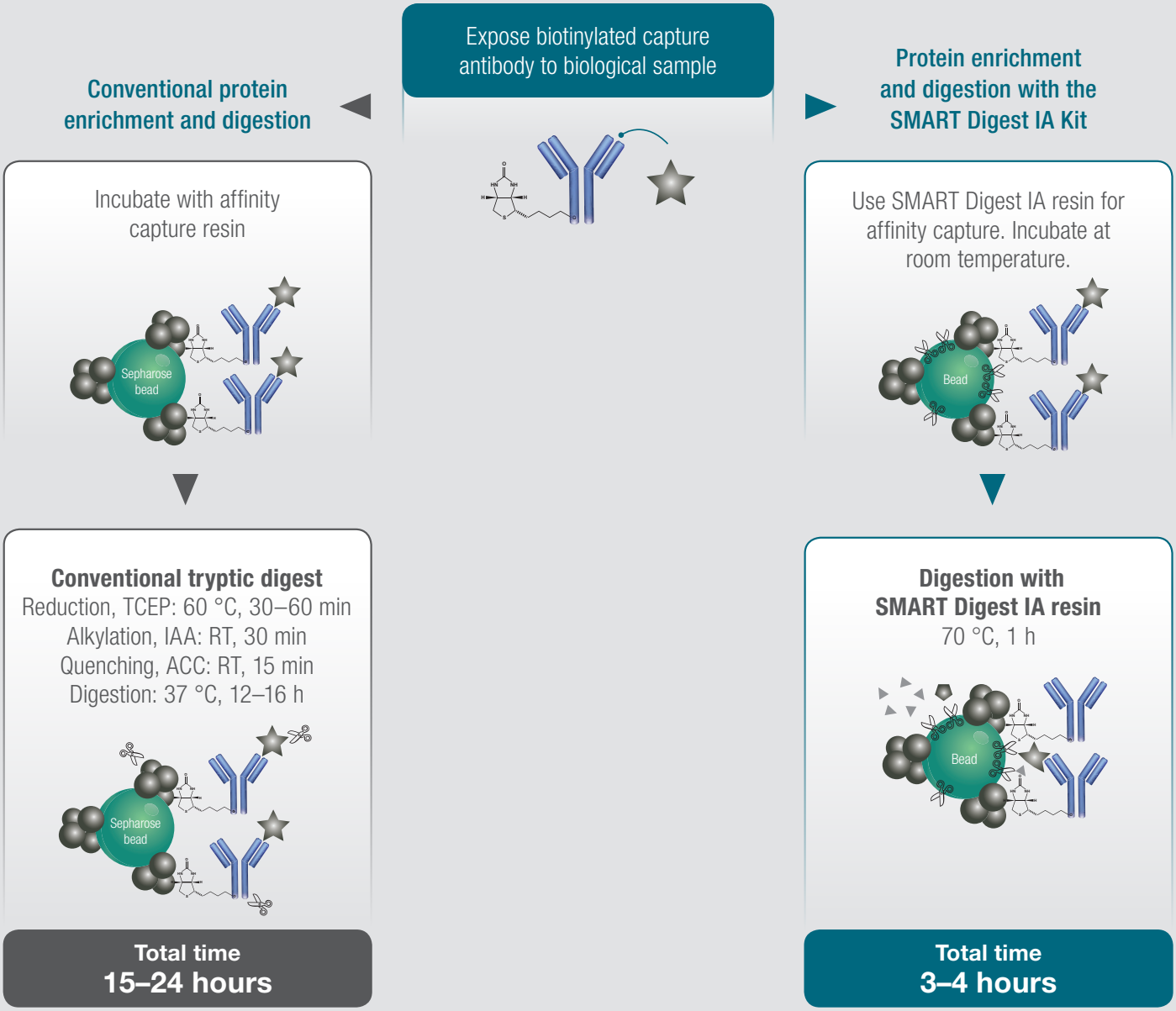


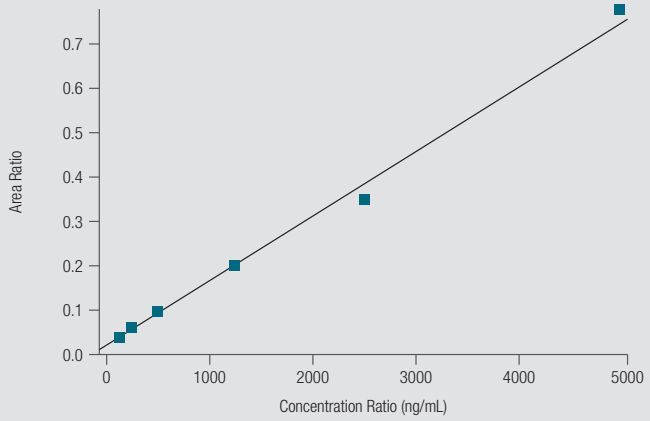
Figure 2. SMART Digest IA kit analytical approach compared to a conventional enrichment and digestion approach.

IAC LC-SRM Platform Technology

A critical part of protein analysis in pharmaceuticals, food, agriculture, and many other industries is developing detection methods with good sensitivities that can be used as a standard platform. The capture protocols previously available to PharmaCadence often involve a large number of pipetting and washing steps that are best performed by an experienced and skilled analyst. The ability to purify protein from plasma and perform a complete digestion in less than 4 hours with the SMART Digest IA kits dramatically simplifies the overall IAC method. The simplified method not only takes less time and skill to perform but consistently yields excellent precision and LLOQ (Figure 3 and 5, Table 4). The general utility of the streptavidin-biotin binding and the rapid protein digestion afforded by the SMART Digest IA kits improves upon what the team could previously achieve (Figure 4 and 5, Table 5). They greatly enhanced efficiency making the SMART Digest IA kit an ideal platform technology for the IAC LC-SRM.

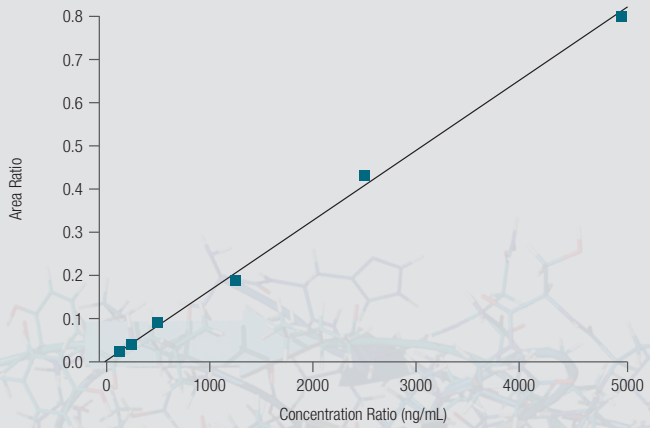
"The general utility, high reliability and ease of use make the SMART Digest IA kits an ideal platform technology for IAC LC-SRM methods..."

—Bonnie Baker, Principal Investigator



Standard Curve (n = 1)			Quality Controls (n = 4)		
Actual Conc (ng/mL)	Accuracy (%)	Calc Value (ng/mL)	Actual Conc (ng/mL)	CV (%)	Accuracy (%)
125	93	116.5			
250	107	266.3	250	11.5	90.2
500	106	531.1			
1250	100	1247	1250	7.4	99.1
2500	90	2251			
5000	104	5212			

Figure 3. SMART Digest IA streptavidin kit. Human plasma calibration curve.



Standard Curve (n = 1)			Quality Controls (n = 4)		
Actual Conc (ng/mL)	Accuracy (%)	Calc Value (ng/mL)	Actual Conc (ng/mL)	CV (%)	Accuracy (%)
125	105	131			
250	90	225	250	14.5	111.2
500	109	544			
1250	92	1149	1250	4.1	104.8
2500	106	2654			
5000	99	4922			

Figure 4. Conventional streptavidin IAC workflow. Human plasma calibration curve.

Table 4. SMART Digest IA streptavidin kit. Biomarker recovery from human plasma.

Recovery with SMART Digest IA kit	
500 ng/mL spike	7330 (cps)
Recovery	64%

Table 5. Conventional streptavidin IAC. Biomarker recovery from human plasma.

Recovery with conventional approach	
500 ng/mL spike	2778 (cps)
Recovery	35%

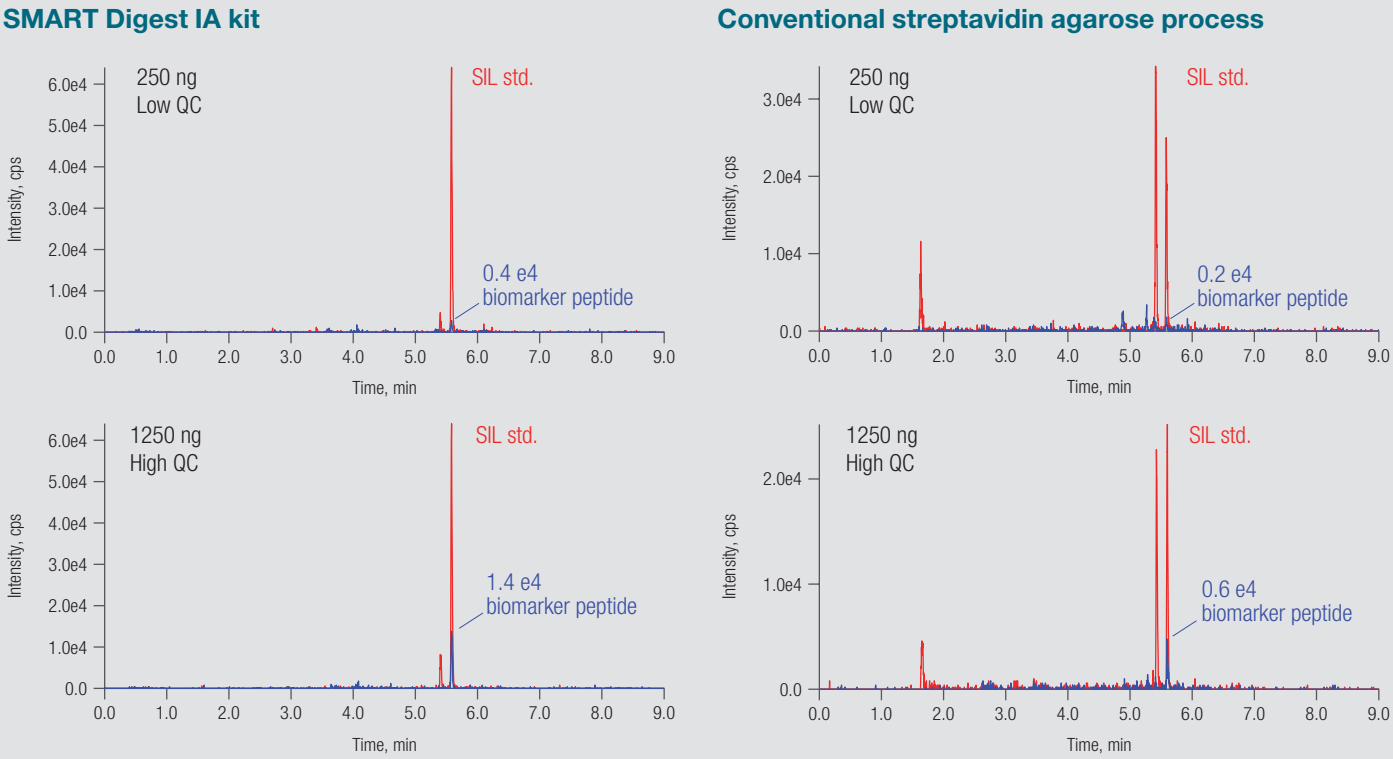


Figure 5. MS chromatographic response at low and high QC levels for sample prepared with SMART Digest IA kit, compared to a conventional protein enrichment and digestion approach. This shows improved method recovery with samples prepared with the SMART Digest IA kit.

Conclusions

The PharmaCadence team have experienced that as pharmaceuticals grow more efficacious, reporting regulations on food and agriculture grow more rigorous. Also, as more low abundance proteins are shown to be key biomarkers the need for simple, rapid and robust methods for IAC LC-SRM are required for success.

Dr. Fernández-Metzler, Dr. King and their team found that the co-immobilized streptavidin and heat-activated temperature stable trypsin in the SMART Digest ImmunoAffinity kits is capable of significantly improving efficiency while delivering high quality results to allow them to keep pace with the increasing application demands.

"Coupling thermally stable trypsin and co-immobilized streptavidin allows for a generic protein quantification workflow with performance equivalent to traditional methods that can be performed routinely in a fraction of the time typically required..."

—Rick King, Laboratory Director

Find out more at **www.thermofisher.com/SMARTdigest**

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