genomeweb ProteoMonitor
The Global Newsweekly of Proteomics Technology

Choice of Trypsin Key to Reproducibility, Accuracy in Targeted Proteomic Assays

May 23, 2014
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NEW YORK (GenomeWeb News) – As mass spec-based proteomics continues its progress towards the clinic, issues of assay accuracy and reproducibility have moved to the fore.

This was apparent, for instance, at this year's Mass Spectrometry Applications to the Clinical Laboratory annual meeting where a number of presenters focused on such questions.

Among the various parts of the mass spec workflow, the reproducibility of trypsin digestion has emerged as one of the most significant issues. And within this process, many researchers are concerned with the question of properly accounting for the differential decay of peptides during digestion.

Mass spec workflows for targeted protein quantitation typically use stable isotope labeled peptides as internal standards. However, researchers have found that decay of these peptide standards and their corresponding native peptides during the digestion process may lead to bias.

Most notably, a 2012 study led by North Carolina State University researcher David Muddiman found that this phenomenon led to greater than 30-fold differences in protein measurements in an investigation of quantitative mass spec assays to 24 enzymes linked to the formation of lignin in the woody plant, *Populus trichocarpa*.

Detailed in a paper in *Molecular & Cellular Proteomics*, the study looked at how assay results varied depending on whether the researchers added their SIL peptides before, during, or after trypsin digestion. Due to each peptide having differential rates of production and decay, the quantity arrived at in each case depended on where in the workflow the SIL standards were added.

"We realized that these peptides were decaying over the course of [the 16-hour] digestion, and the problem was they were being produced and decaying at different rates," Philip Loziuk, a graduate student in Muddiman's lab, told *ProteoMonitor*. "So you had some peptides that were stable and others that decayed at a fast rate."

The researchers developed an equation for estimating the amount of bias introduced for each peptide by these differential rates of production and decay. Using this equation they found that nearly half the assays they looked at in their study exhibited quantitative errors greater than 10 percent, with several cases exhibiting greater than 100 percent error.

Compounding the issue, the authors noted, was the fact that, with regard to timing the addition of SIL standards, "no specific procedure has been agreed upon, or demonstrated to be best."

Seeking to establish such a procedure, they determined through the study that standards are best added to a sample at the same time as trypsin. They also proposed that when choosing proteotypic peptides for protein quantitation researchers should take into account peptide stability in order to minimize bias due to decay during digestion.

Loziuk noted, however, that in a follow-up study published in 2013 in the *Journal of Proteome Research*, Muddiman's lab found that optimization of the digestion process eliminated the peptide decay observed in the *MCP* study along with the associated bias.

Among their findings was that use of modified trypsin – which is more specific for tryptic cleavage sites and retains optimal activity under more basic conditions and at higher temperatures compared to the unmodified enzyme – is key to avoiding the problem of peptide decay.

In fact, Loziuk said, their results suggested that use of unmodified trypsin "was almost totally the reason for the peptide decay" observed in the *MCP* study. Unmodified trypsin is subject to autolysis, which leads to the chymotrypsin-like activity that causes peptide decay during digestion.

On the other hand, "modified trypsin has much less non-specific activity, so when you look at the digest curve, you get very little to no decay when you use [it] in a targeted proteomics experiment," he said.

Modified trypsin is significantly more expensive than unmodified trypsin, however, which is what led Muddiman and his team to use it for their *Populus trichocarpa*assays, Loziuk said. "We were doing these large-scale studies where we were trying to process a lot of samples, and we switched from modified trypsin to unmodified to save on costs."

Since determining that unmodified trypsin was the cause of the problematic peptide decay, the lab has largely gone back to using modified versions of the enzyme, Loziuk noted. However, he said, many researchers still use unmodified trypsin.

Unmodified trypsin is often treated with a chymotrypsin inhibitor to eliminate the chymotryptic activity behind the peptide decay, but, Loziuk said, this isn't actually very effective at getting rid of such activity.

Several different types of modifications are used to eliminate trypsin's autolytic activity, such as dimethylation or acetylation of the enzyme's lysine residues. Trypsin immobilization – linking the enzyme to some sort of polymer – is another commonly used modification approach.

In addition to preventing autolysis, immobilization can also significantly speed up the digestion process, notes John Griffiths, senior mass spectrometrist at the Cancer Research UK Manchester Institute.

Griffiths this year began using Perfinity Biosciences' Flash Digest immobilized trypsin kit after being approached by the company with beta versions of the product.

"What we were interested in was the speed with which the digestion could take place and the reproducibility of the digest," he told *ProteoMonitor*, noting that he and his colleagues had seen improvements along both lines with the new kit.

In terms of assay time, the immobilized enzyme allows them to go from raw sample to mass spec analysis in three hours, compared to more than 20 hours using their conventional workflow. Analyzing the reproducibility of the method, Griffiths said he found that from a cell lysate they could quantify roughly 1,500 proteins from replicate digests with coefficients of variation of 15 percent or below, compared to around 750 proteins with their old process.

Griffiths noted that he is primarily interested in global proteomics experiments, as opposed to targeted mass spec, but, he said, he expects that the observed improvements would hold for multiple-reaction monitoring-style assays, as well.

The method "is something that we plan to adopt full time as our workflow," he said. "We're able to give people their data so much faster, and there are no drawbacks that we've come across so far."