# Development of a Condensed Workflow to Simultaneously Characterize Post Translational Modifications and Quantitate Host Cell Protein Impurities During the Development of a Protein Therapeutic

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PELICAN™
*P. fluorescens* expression technology
A Ligand® Technology

## 1. ABSTRACT

The Pelican Expression Technology™ platform, is a *Pseudomonas fluorescens*-based protein expression system, which is a robust and scalable platform for recombinant protein production. It is especially well-suited for complex therapeutic protein production including antibody derivatives, vaccine antigens, therapeutic enzymes, human cytokines, and growth factors. Therapeutic proteins are large molecules comprised of long polypeptide chains that are used as drugs to treat many diseases. During development, product post-translational modifications (PTMs) of amino acid side-chains are monitored through peptide mapping analysis. Also monitored are the quantity and identity of host cell proteins (HCP). Two different workflows are used to monitor PTMs and to identify and quantitate residual HCPs from *P. fluorescens*, the bacterial host for Ligand's Pelican Expression Technology™ platform.

## 2. BACKGROUND

During development of a therapeutic protein, product quality attributes are monitored throughout the purification process using peptide mapping analysis. These include:

- Deamidation
- Isomerization
- Oxidation
- Pyroglutamate formation

Also monitored are the amount and identity of host cell proteins (HCP) at different steps of the purification process. These two different workflows are used to monitor chemical degradation and to identify and quantitate residual HCPs.

## 3. OBJECTIVE

Our initial analysis methods to discover and quantitate PTMs as well as HCPs were developed and used separately during the development of this drug product candidate. Over time we developed more tailored orbitrap acquisition methods that were able to combine multiple workflows into a single workflow and generate the same information that was being obtained using the separate analysis techniques. We developed an analysis method that enabled us to observe trace HCP peptides for *P. fluorescens* in the presence of large amounts of drug substance peptides and as a result allows for rapid acquisition of both product and process related impurities with minimal sample requirements, expedited data analysis, and simplified sample preparation workflows.

## 4. METHODOLOGY

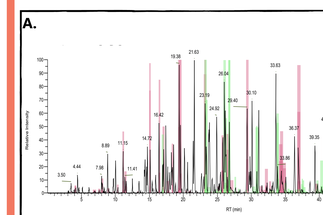
### Individual Workflows

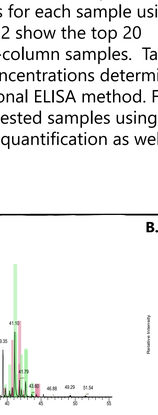
**Peptide Mapping:**
Each sample was denatured and reduced with 4M guanidine, 50 mM DTT and 50 mM Tris-Cl pH 7.5 with heating at 40°C for 60 min. Next the samples were buffer exchanged into 10 mM Tris-Cl pH 7.5, 2 mM $CaCl_2$ and trypsin was added to a final ratio of 1:20 and incubated at 40°C for 2 hours. The reaction was quenched with formic acid and analyzed by LCMS, with PTM identifications assigned using orbitrap data (Method B) and PTM quantifications determined using Q-TOF data.

**HCP Analysis:**
Samples at 2 mg/ml or above had Tris-Cl pH 7.5 added to a concentration of 50 mM and known amounts of standard proteins followed by trypsin added at a ratio of 1:100 and incubated overnight at 35°C. The following morning the samples were heated at 95°C for 5 minutes, followed by centrifugation at 11,000 RPM for 10 minutes. The supernatant was removed and analyzed by orbitrap LCMS (Method B). Quantitation in ppm was based on the intensity of known proteins spiked into the sample.

### Combined Workflow

The peptide mapping method used in the induvial workflows was used exactly as written, except known amounts of standard proteins were spiked into the sample prior to digestion and three injections were made for each sample using three individually optimized orbitrap methods described in Figure 1.



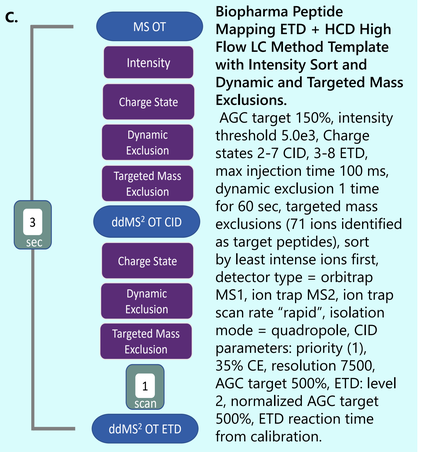




**Figure-1 A, B, C:** Thermo Scientific Orbitrap Fusion Method Editor settings with critical parameter description.

## 5. RESULTS SUMMARY

Target protein samples from a purification step were analyzed for HCP content and PTMs using combined and separate workflows. Figure 2 shows PTM values for each sample using the different workflows. Tables 1 and 2 show the top 20 abundant HCPs for the pre- and post-column samples. Table 3 shows a comparison between HCP concentrations determined using each MS workflow and a traditional ELISA method. Figure 3 shows the elution profile for the digested samples using the separate workflows for PTM and HCP quantification as well as the combined workflow for PTMs.

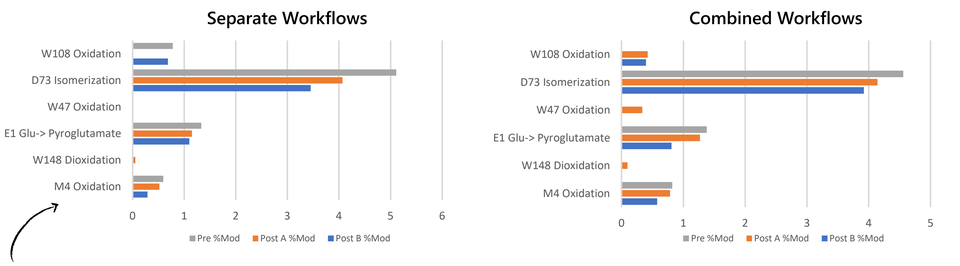



**Figure 2.** Post translational modification values quantitated using the original default acquisition method and after the method was changed to enable combined PTM and HCP analysis. A comparison of the values resulted in a difference of 0.3% or less for all modification values.




**Figure 3.** The elution profiles for the digested target protein (pre-column A; post-column B) using separate workflows to quantitate PTMs (top), combined workflows to quantitate PTMs (middle), and after precipitation to remove the target protein using the HCP specific workflow (bottom). Subunit A peptides are highlighted in red, while subunit B peptides are colored green. Sequence coverage when the target protein was precipitated was between 44% and 55% for subunits A and B for all samples. Sequence coverage for the combined and PTM specific methods was identical for both methods with 100% coverage for subunit A and 92.3% for subunit B. The m/z peaks corresponding to peptides that were included in the dynamic exclusion tables continued to be identified by the processing software due to lower abundant m/z peaks present.

| Protein Name | Col_Load Separate Workflows (%) | Col_Load Combined Workflows(%) |
|---|---|---|
| HCP 4 | 0.35 | 0.28 |
| HCP 5 | 0.62 | 0.16 |
| HCP 6 | 0.13 | 0.03 |
| HCP 20 | 0.12 | 0.05 |
| HCP 7 | 0.66 | 0.36 |
| HCP 8 | 0.26 | 0.23 |
| HCP 2 | 23.39 | 20.01 |
| HCP 9 | 1.75 | 1.38 |
| HCP 10 | 6.43 | 5.72 |
| HCP 11 | 0.62 | 0.38 |
| HCP 12 | 0.44 | 0.21 |
| HCP 13 | 2.23 | ND |
| HCP 3 | 3.14 | 2.98 |
| HCP 1 | 38.92 | 39.74 |
| HCP 14 | 0.76 | 0.59 |
| HCP 15 | 0.77 | 0.83 |
| HCP 16 | 2.12 | 0.03 |
| HCP 17 | 1.87 | 1.46 |
| HCP 18 | 0.41 | 0.05 |
| HCP 19 | 0.87 | 1.03 |
| 19 Low Abundance HCPs | ~15% | ~15% |
| Total Number Observed | 47 | 46 |
| Concentration (ppm) | 5870 | 8640 |

**Table 1.** Residual HCP identification information and percent of total protein for the pre-column sample. 47 HCPs were observed for the load using the separate workflow method while 46 were identified using the combined method. The table shows the top twenty HCPs by percent, with the remaining HCPs making up ~15% for the load.

| Protein Name | Col_FT Separate Workflows (%) | Col_FT Combined Workflows (%) |
|---|---|---|
| HCP 6 | 0.94 | 0.96 |
| HCP 7 | 0.48 | 0.22 |
| HCP 4 | 0.38 | 0.48 |
| HCP 5 | 0.34 | 0.12 |
| HCP 9 | 1.08 | 1.08 |
| HCP 20 | 0.74 | 0.68 |
| HCP 8 | 1.92 | 0.88 |
| HCP 2 | 28.36 | 30.58 |
| HCP 11 | 0.74 | 0.86 |
| HCP 12 | 0.48 | 0.5 |
| HCP 3 | 1.58 | 0.78 |
| HCP 1 | 42.88 | 43.58 |
| HCP 10 | 5.48 | 6.98 |
| HCP 13 | 0.46 | 0.94 |
| HCP 14 | 3.92 | 0.24 |
| HCP 15 | 0.26 | 0.2 |
| HCP 16 | 2.62 | 0.02 |
| HCP 17 | 2.04 | 0.56 |
| HCP 18 | 1.38 | 0.14 |
| HCP 19 | 0.78 | 0.5 |
| 18 Low Abundance HCPs | ~5% | ~5% |
| Total Number Observed | 36 | 35 |
| Concentration (ppm) | 798 | 758 |

**Table 2.** Residual HCP identification information and percent of total protein for the post column sample. 36 HCPs were observed for the eluate using the separate workflow method and 35 were identified for the combined method. The table shows the top twenty HCPs by percent, with the remaining HCPs making up ~5% for the elution.

| Sample | ELISA (ppm) | Seperatre WF (ppm) | Combined WF (ppm) |
|---|---|---|---|
| Pre-Col | 6761 | 5870 | 8640 |
| Post A | 838 | 798 | 758 |
| Post B | 878 | 1107 | 519 |

**Table 3.** A comparison between HCP concentrations determined with each MS workflow and with a traditional ELISA method.

## 6. CONCLUSION

Mass spectrometry analysis was successful in determining *P. fluorescens* HCP content by comparing the total intensity of all observed HCP proteins against the intensities of known non-bacterial proteins spiked into the sample.
The results observed for the separate workflows were almost identical to the results observed for the combined workflows for the identity and amount of PTMs as well as the identity and number of HCPs present in each sample.

To validate our HCP concentration results, we compared our values to one obtained by a traditional ELISA method and for samples with HCP content below 1000 ppm the values were similar. The MS derived values appeared to diverge from the ELISA derived values as the absolute amount of HCP present increases in value (greater than 1000 ppm). The ability to combine process and product related impurity assessment in a single MS experiment has provided significant opportunity to expedite analysis, improve sample throughput and provide deeper characterization during process development of protein therapeutics.

## REFERENCES & ACKNOWLEDGEMENTS

Retallack, Diane M., Hongfan Jin, and Lawrence Chew. "Reliable protein production in a Pseudomonas fluorescens expression system." Protein expression and purification 81, no. 2 (2012): 157-165.

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