High-Throughput Comparative Proteome Analysis Using a Quantitative Cysteinyl-Peptide Enrichment Technology (QCET)

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1. Principle

QCET involves several sequential steps (Figure 1). First, protein samples from two cell states are prepared, then separately digested by trypsin under identical conditions. The tryptic peptides from each sample are labeled with either ^{16}O or ^{18}O by immobilized-trypsin-catalyzed oxygen exchange (Figure 2), in either regular or ^{18}O-enriched water. Next, the differentially labeled peptide samples are combined and cysteinyl-peptides are selectively captured using a thiol-specific affinity resin by forming a disulfide bond between the cysteinyl-peptide and the resin (Figure 3). Following stringent washing, bound cysteinyl-peptides are released from the resin by incubating with DTT. Finally, the enriched cysteinyl-peptides are analyzed, identified, and quantified using the accurate mass and time (AMT) tag approach.

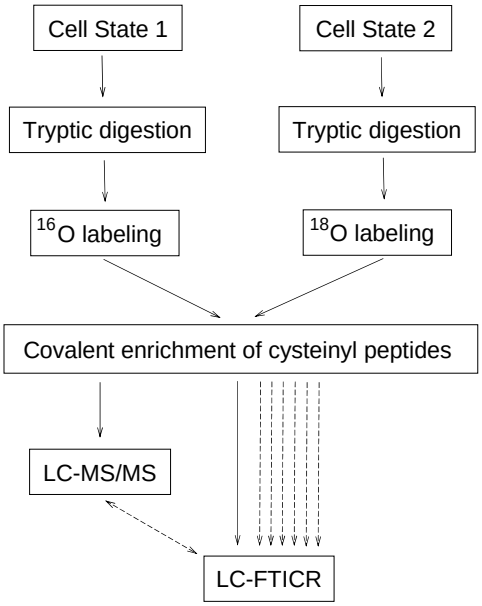

Figure 1. Strategy for quantification of differential protein expression using QCET.

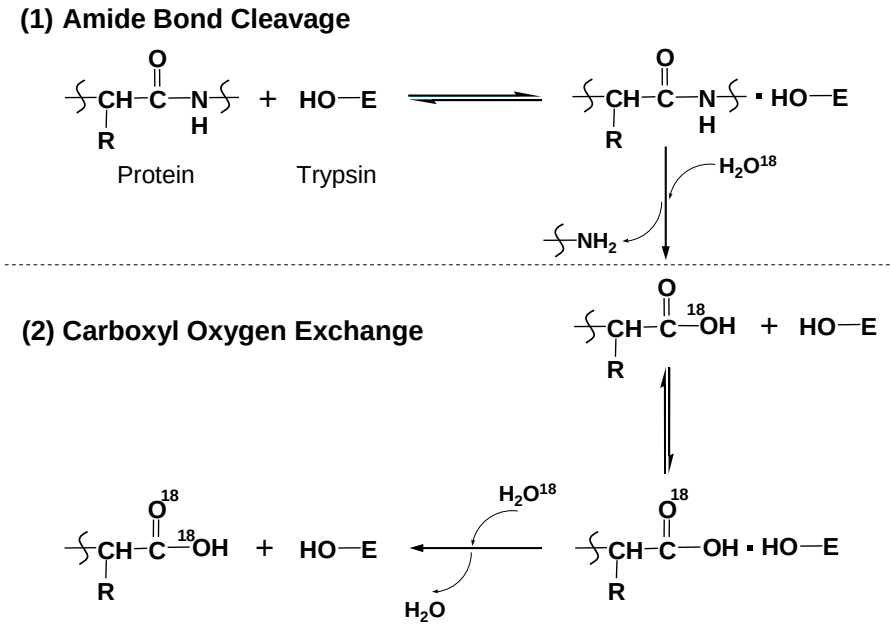

Figure 2. Principle of post-digestion ^{18}O labeling.

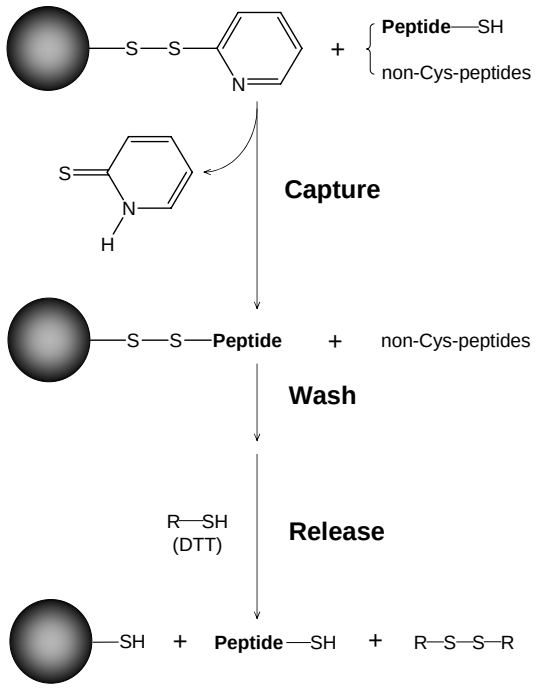

Figure 3. Principle of cysteinyl-peptide enrichment using Thiopropyl Sepharose 6B.

2. Protocols

- **Tryptic digestion.** Proteins from cell lysate were subjected to tryptic digestion after disulfide bonds in the denatured proteins were reduced (50 mM Tris buffer, pH 8.2, 8 M urea, 10 mM DTT) for 1 hour at 37 °C. The reduced protein mixture was diluted 10 times using 20 mM Tris buffer, pH 8.2, and Sequencing Grade Modified Trypsin (porcine) (Promega) was added at a trypsin:protein ratio of 1:50 and incubated at 37 °C for 3 hours. Residual trypsin activity was quenched by boiling the digest for 10 minutes then immediately incubating on ice for 10 minutes. The tryptic digest was loaded on a 1-mL SPE C18 column (Supelco) and washed with 4 mL of 0.1% TFA/5% acetonitrile. Peptides were eluted from the SPE column with 1 mL of 0.1% TFA/80% acetonitrile and dried under reduced pressure.

- **Trypsin-catalyzed ^{18}O labeling.** To dissolve the dried peptides, 20 μL of acetonitrile was first added to the dried digest, followed by the addition of 100 μL of 50 mM NH_4HCO_3 in either ^{18}O-enriched water (95%, Isotec) or regular ^{16}O water. Then, 1 μL of 1 M $CaCl_2$ and 5 μL of immobilized trypsin (Applied Biosystems) were added to the digests and the samples were mixed continuously for 24 hours at 30 °C. Supernatants were collected by centrifuging the samples for 5 minutes at 15,000×g. The immobilized trypsin beads were further washed 3 times using 60% methanol. The corresponding supernatants of ^{16}O and ^{18}O-labeled samples were pooled and combined, then dried under vacuum.

- **Cysteinyl-peptide enrichment by Thiopropyl Sepharose 6B.** Unless otherwise noted, all solutions used in this step were degassed to prevent oxidation of the thiol content. The tryptic digest from 100 μg protein was reduced with 5 mM DTT in 20 μL of 50 mM Tris buffer, pH 7.5, 1 mM EDTA (coupling buffer) for 30 minutes at 37 °C, after which the sample was diluted to 100 μL by adding coupling buffer. 100 μL of Thiopropyl Sepharose 6B thiol-affinity resin (Amersham Biosciences) were prepared from dried powder per the manufacturer's instruction. Briefly, the dried powder was rehydrated in water for 15 minutes and washed by 50 bed volumes of water, followed by 50 bed volumes of coupling buffer in a Handee Mini-Spin column (Pierce). The reduced peptide sample was then incubated with the resin for 1 hour at RT with gentle mixing and the unbound portion was removed from the resin by spinning the column at low speed. The resin was washed in the spin column sequentially with 0.5 mL×5 of 50 mM Tris buffer, pH 8.0, 1 mM EDTA (washing buffer), 2 M NaCl, 80% acetonitrile/0.1% TFA solution, and washing buffer. To release the captured cysteinyl-peptides, 100 μL of 20 mM DTT freshly prepared in washing buffer was added to the resin and incubated for 30 minutes at RT. The resin was further incubated twice with 100 μL of 20 mM DTT for 10 minutes, after which 100 μl of 80% acetonitrile was added to wash the resin. The pH of the combined eluates was adjusted to 8.0 and the sample was alkylated with 80 mM iodoacetamide for 30 minutes at RT in the dark. The sample was then cleaned up by SPE C18 column and lyophilized.

- **SCX fractionation and LC-MS/MS analysis** (use *unlabeled* cys-peptides for building the AMT database).

- **LC-FTICR analysis** (use the *labeled* cys-peptides for quantitation).

Reference:

[1] Liu T, WJ Qian, EF Strittmatter, DG Camp, GA Anderson, BD Thrall, and RD Smith. 2004. "High-Throughput Comparative Proteome Analysis Using a Quantitative Cysteinyl-Peptide Enrichment Technology." *Analytical Chemistry* 76:5345-5353.