

Development of Recombinant Human Cardiac Troponin ITC-Complex Candidate Certified Reference Materials

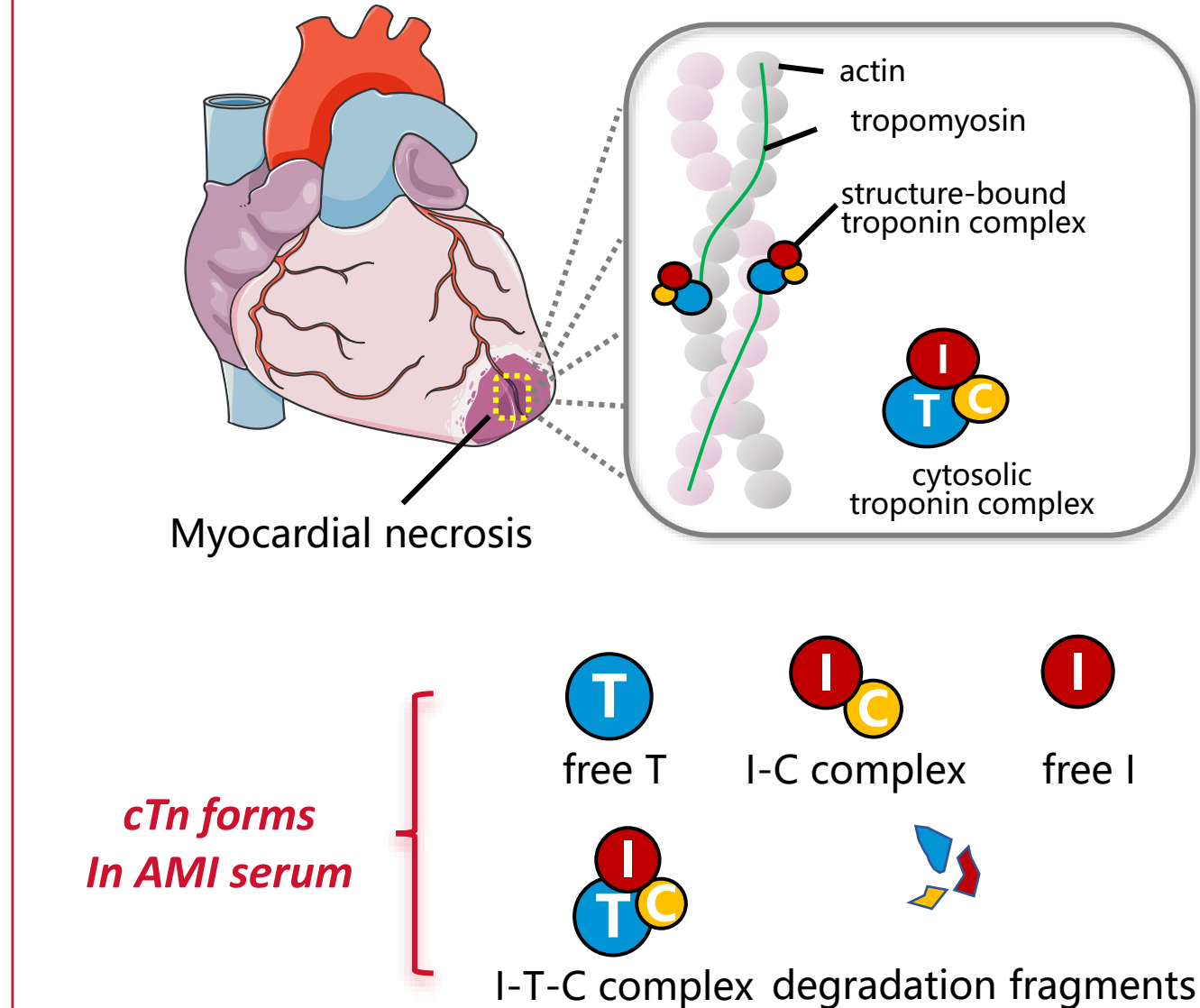
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Introduction

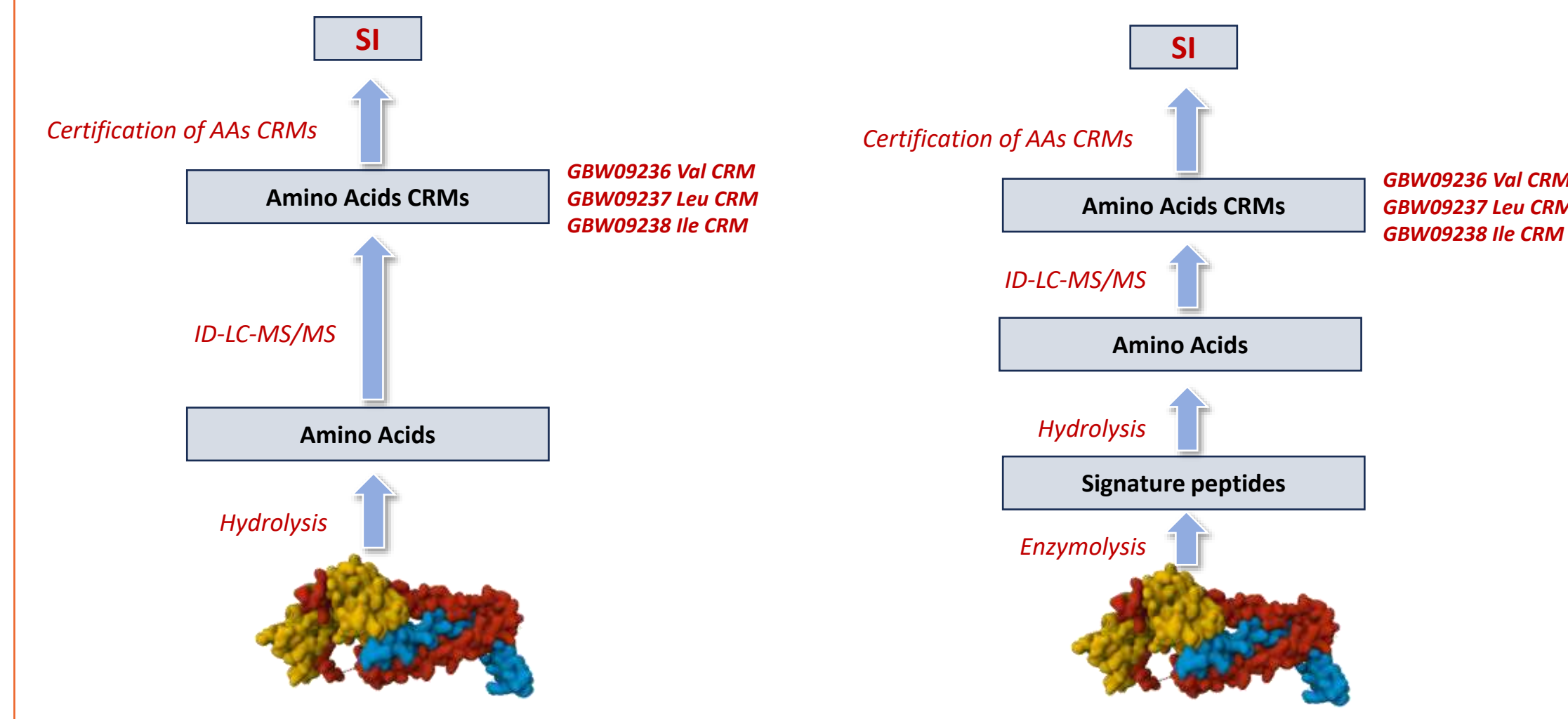
As one of the top ten causes of death worldwide, cardiovascular disease poses a serious threat to public health. Cardiac troponin (cTn) has been classified as an I-A recommendation for the diagnosis of Acute Myocardial Infarction (AMI) in relevant international guidelines, and its subunit, cTnI is regarded as the "gold standard" for the diagnosis of AMI. The major components of cTnI in serum samples from AMI patients exist as a ternary cTnI-cTnT-TnC complex (ITC) and a binary cTnI-TnC complex (IC). The complex existence of cTnI in AMI samples and its unstable characteristics have prevented the standardization of cTnI being realized.



Methods

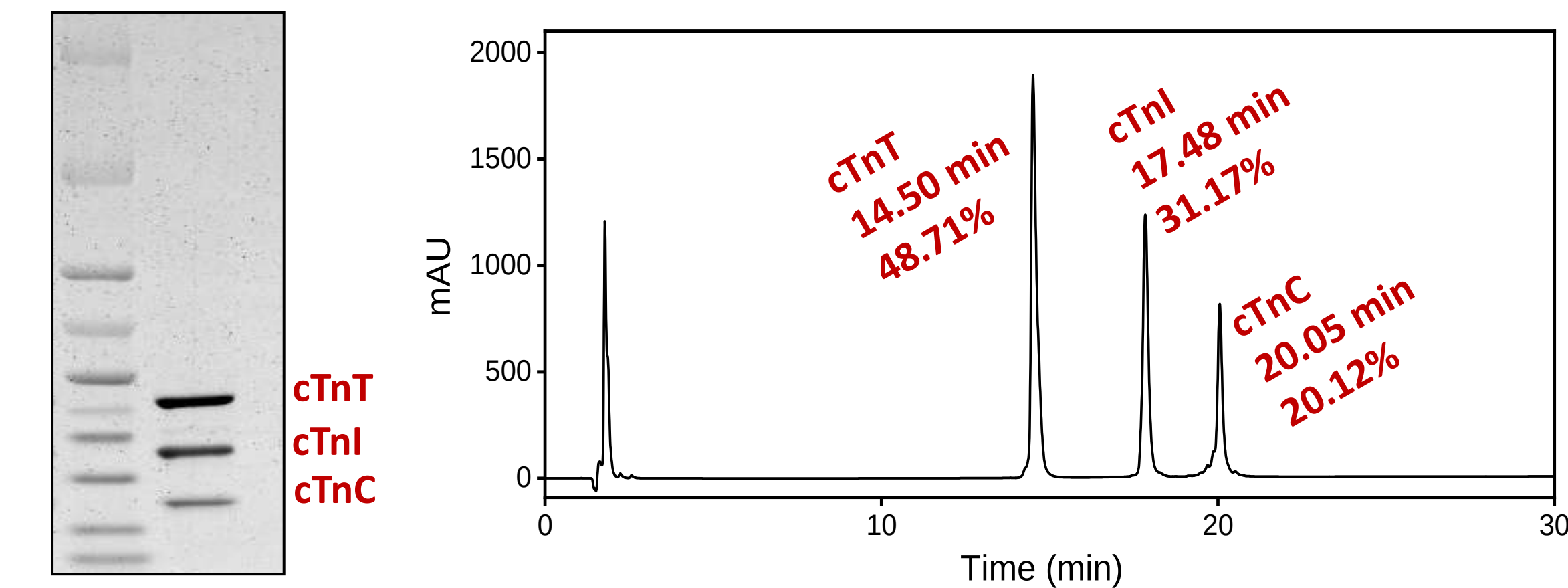
The cTn solution was quantified by both amino acids analysis – isotope dilution mass spectrometry (AAA-IDMS) and signature peptide – isotope dilution mass spectrometry (SP-IDMS).

- **AAA-IDMS:** cTn CRM solution and mixed isotope-labeled amino acids (Leucine, isoleucine, valine) were hydrolyzed using 6 M HCl at 110°C for 36 h. And using ID-LC-MS/MS to detect Amino Acids. The quantification result can be traced to Amino Acids CRMs, which notes GBW09236 valine CRM, GBW09237 leucine CRM and GBW09238 Isoleucine CRM. And then be traced to SI unit.
- **SP-IDMS:** We developed the Enzymatic Hydrolysis IDMS to quantify cTn CRMs. cTn CRM solution and mixed isotope-labeled signature peptides were hydrolyzed using trypsin at 37°C for 16 h. And using ID-LC-MS/MS to detect peptides. The quantification result can be traced to Amino Acids CRMs, which notes GBW09236 valine CRM, GBW09237 leucine CRM and GBW09238 Isoleucine CRM. And then be traced to SI unit.

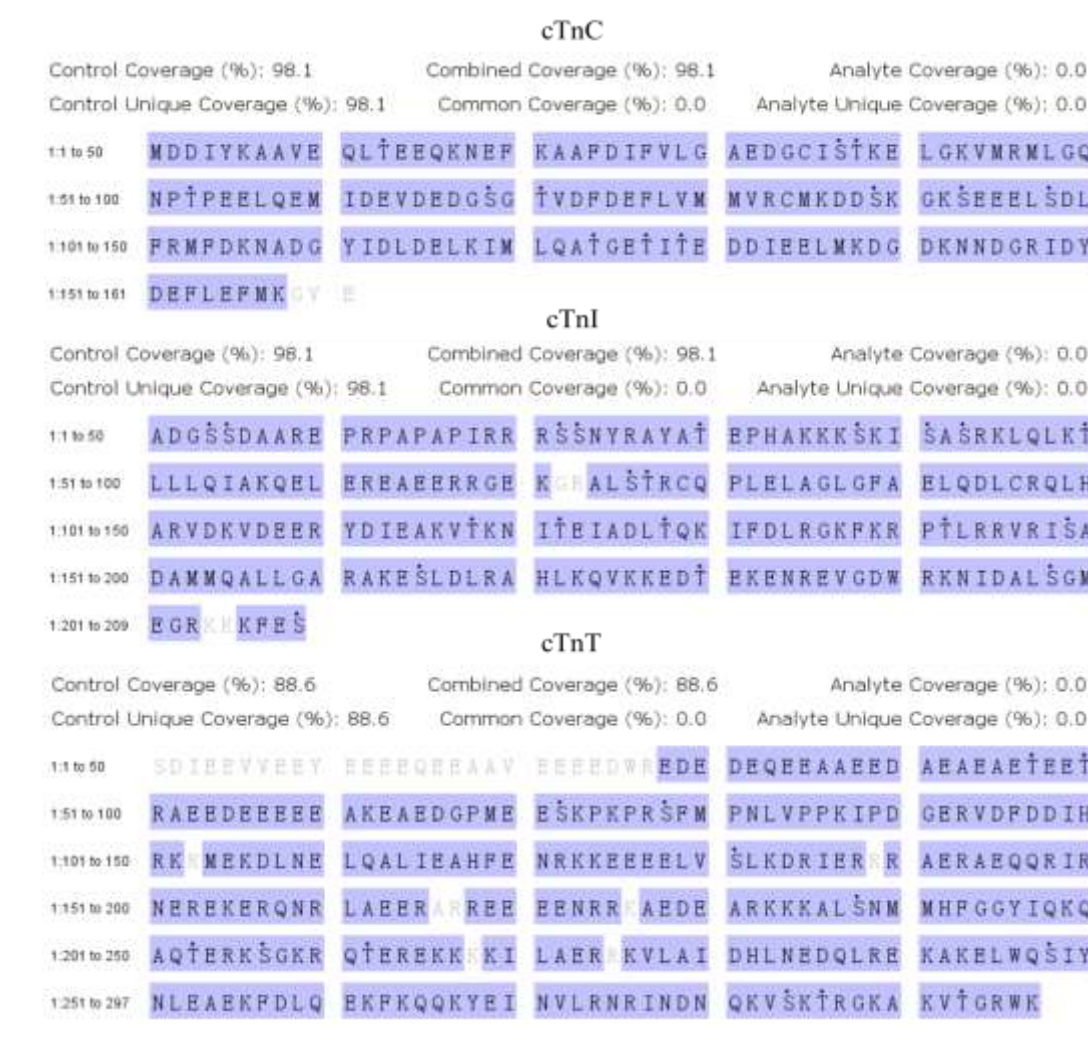


Characterization of cTn CRMs

➢ High purity by SDS-PAGE and HPLC, and it shows three protein subunits



➢ >98% peptide mass fingerprint matching ratio



- cTnT: 34456 Da
- cTnI: 23875 Da
- cTnC: 18403 Da

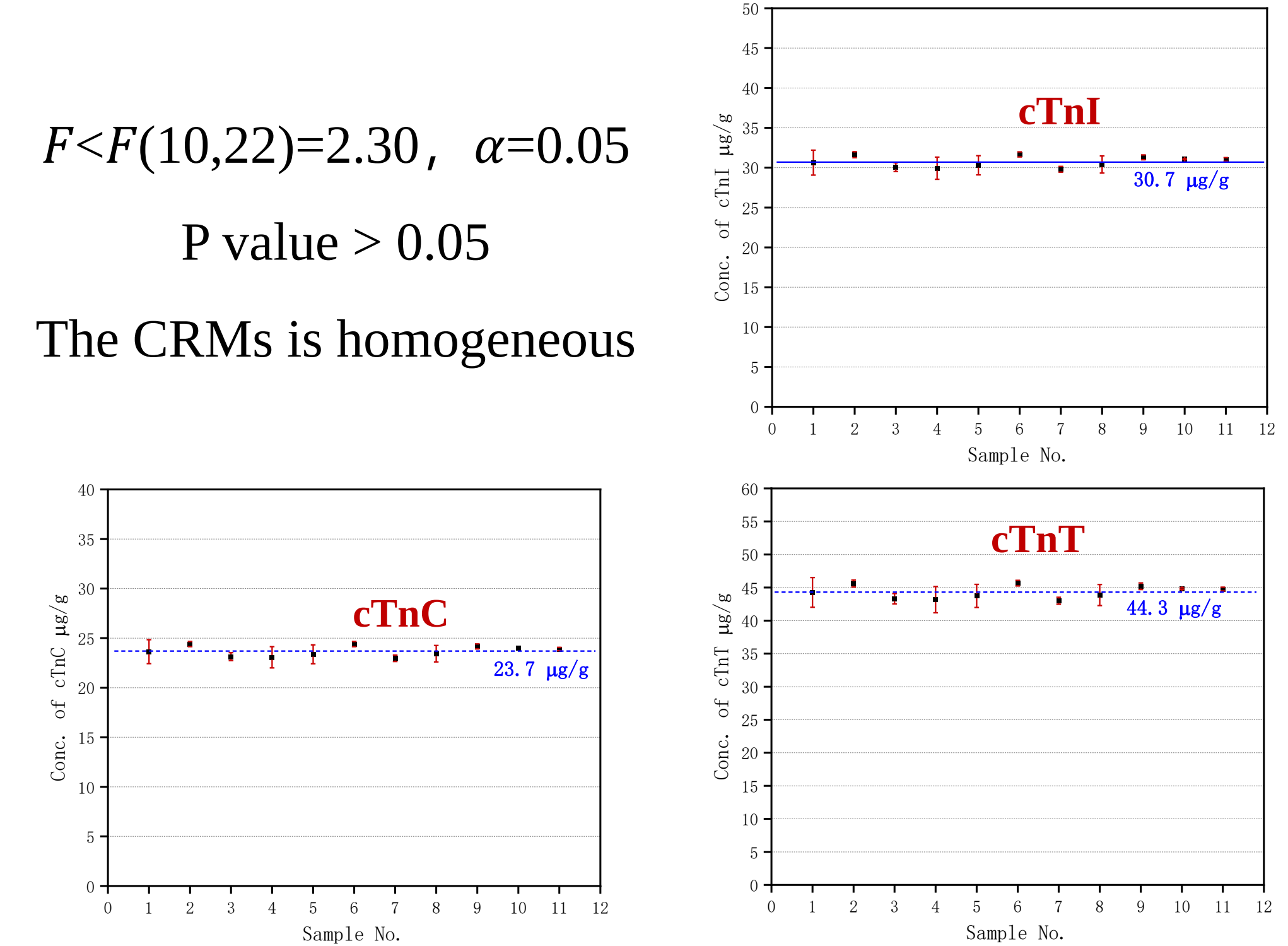
Homogeneity and Stability

➢ Estimate the homogeneity of candidate CRMs by AAA-IDMS. The result shows the CRMs are homogeneous.

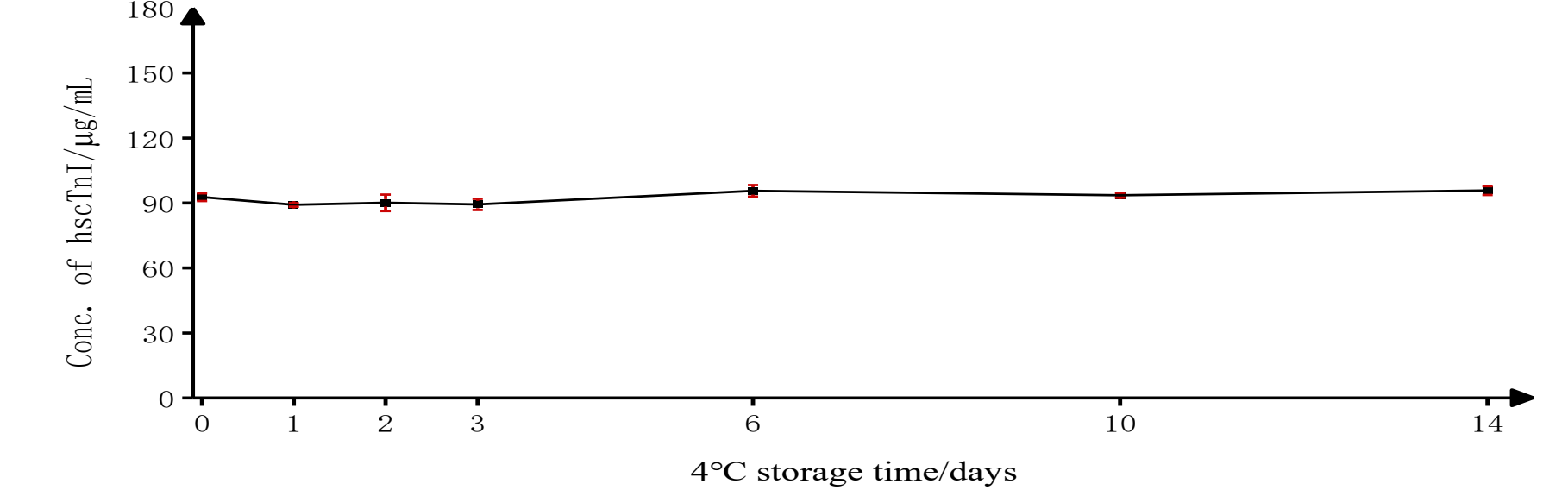
$$F < F(10, 22) = 2.30, \alpha = 0.05$$

P value > 0.05

The CRMs are homogeneous



➢ Estimate the short-time stability of candidate CRMs by CLIA. The result shows the CRMs are stable for 14 days at 4°C.

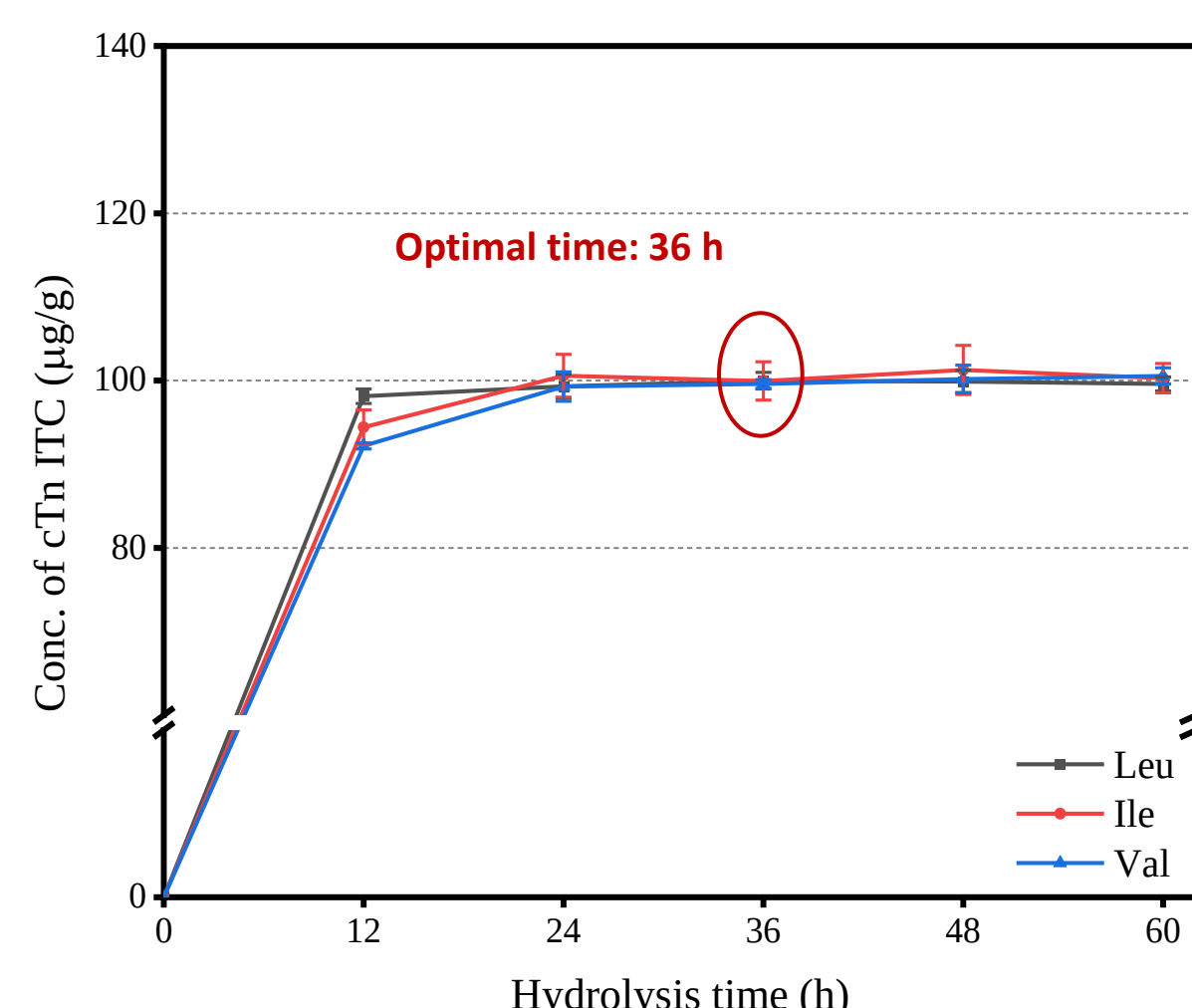
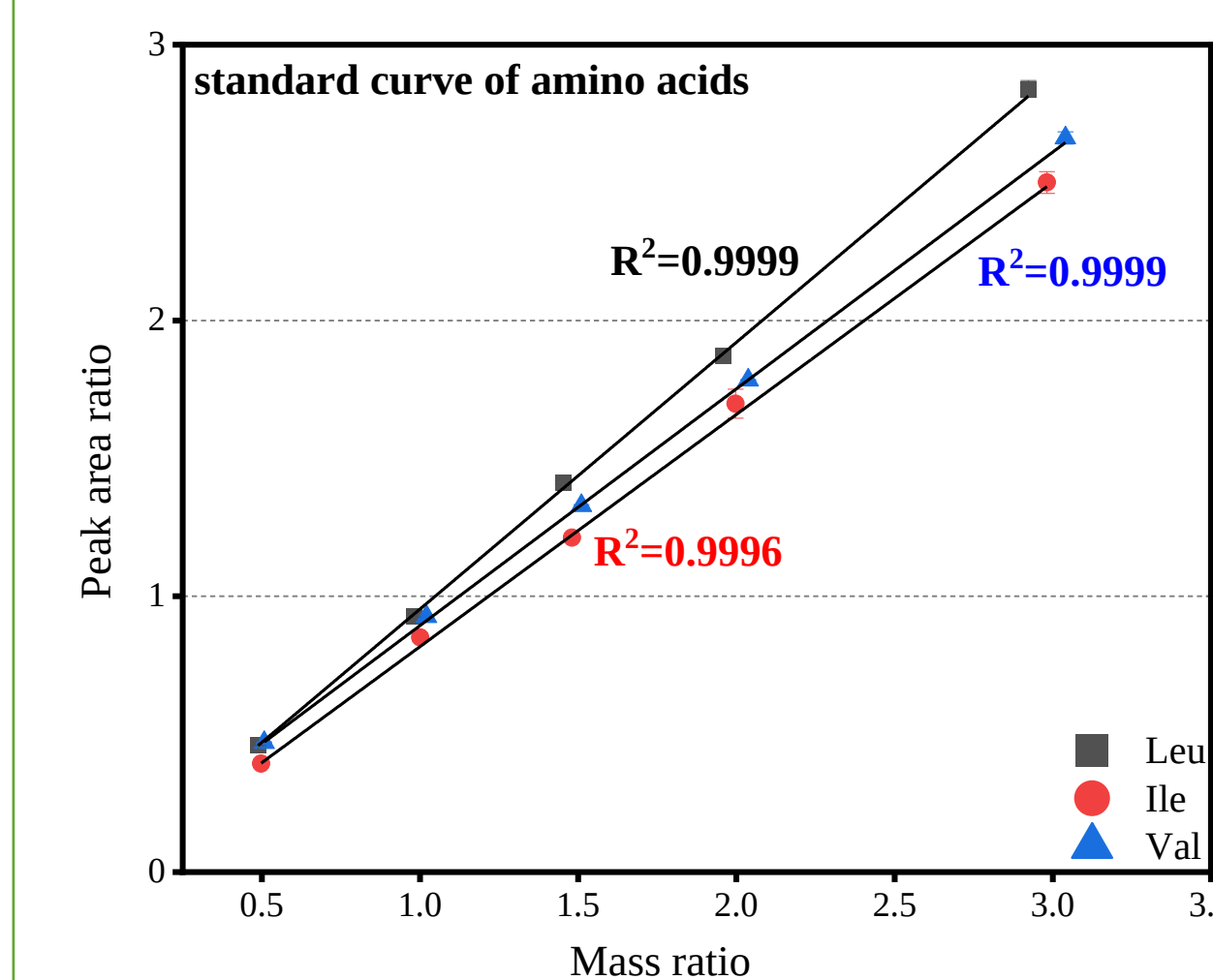


AAA-IDMS quantitative results

The standard curves of three amino acids showed good linear relationship. We optimized the hydrolysis time to 36 hours. The averaged peak area ratios of each amino acid were calculated to quantify the cTn concentration by bracketing calibrations. Concentration of each amino acid was calculated as the left formula, and the concentration of cTn was obtained from the average of three amino acids. Because the molar ratio of three subunits is 1:1:1, concentration of each unit equals to concentration of cTn complex multiplied by the ratio of subunit Molecular weight to cTn complex molecular weight. The cTnI mass concentration obtained from AAA-IDMS was 31.4 µg/g. The cTnT mass concentration obtained from AAA-IDMS was 45.1 µg/g. The cTnC mass concentration obtained from AAA-IDMS was 24.1 µg/g.

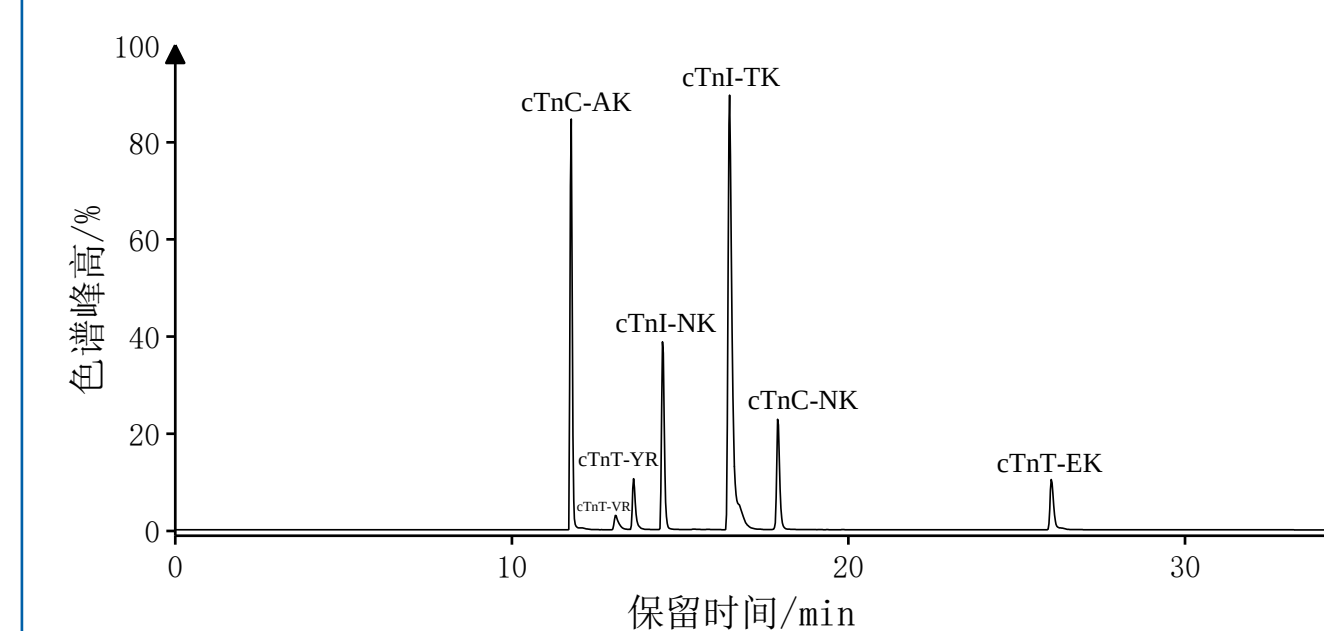
$$C_{AAs} = \left[\frac{\left(\frac{A_s}{A'_s} - \frac{A_l}{A'_l} \right) \cdot \left(\frac{c_h}{c'_h} - \frac{c_l}{c'_l} \right)}{\frac{A_h}{A'_h} - \frac{A_l}{A'_l}} + \frac{c_l}{c'_l} \right] \times \frac{m'_s}{M_s}$$

$$C_{cTn} = (c_{Leu} + c_{Ile} + c_{Val})/3$$

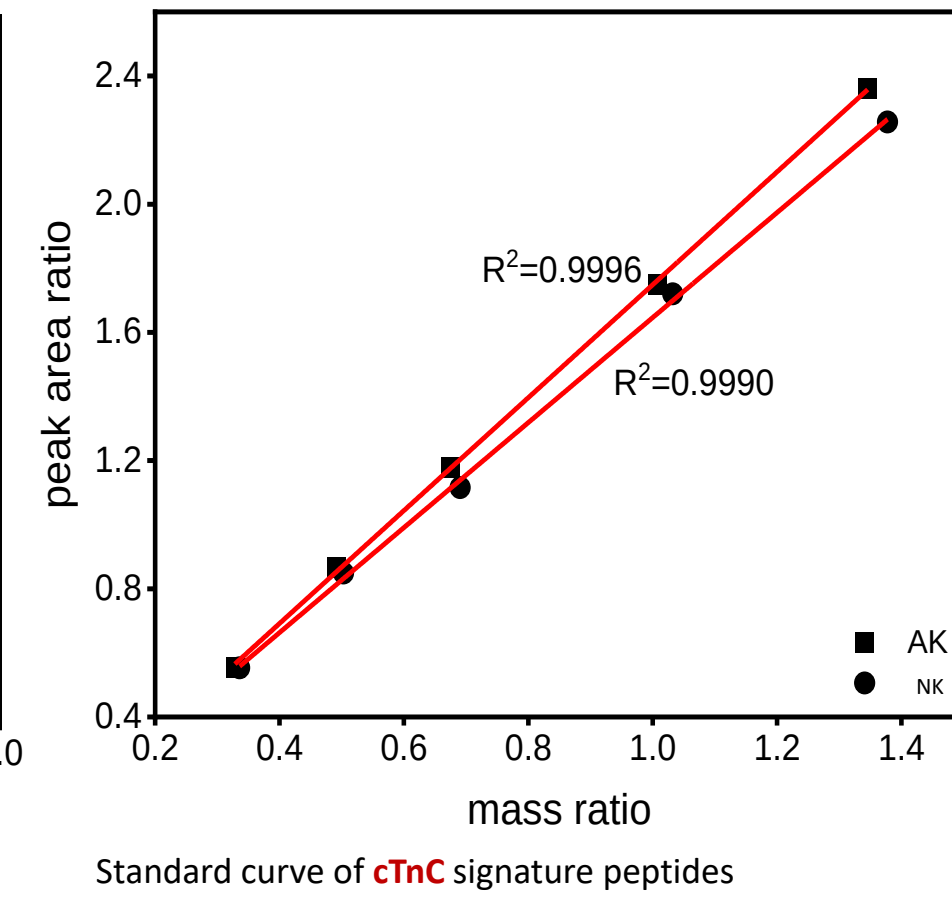
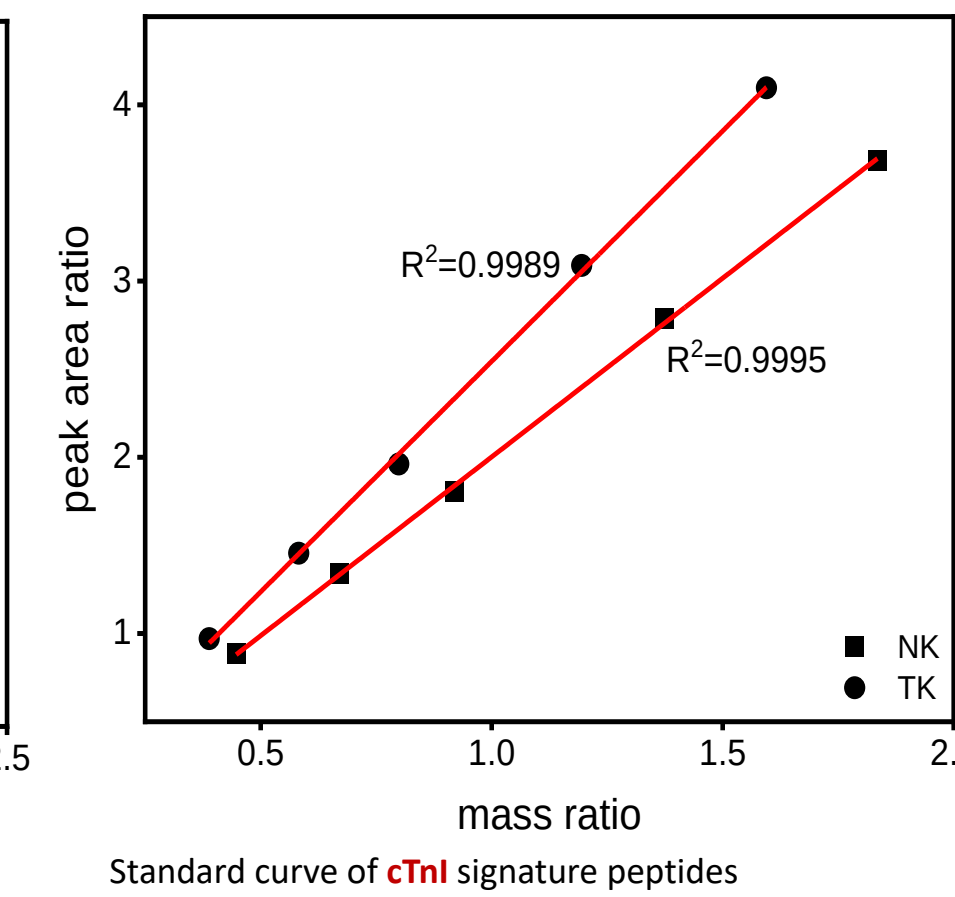
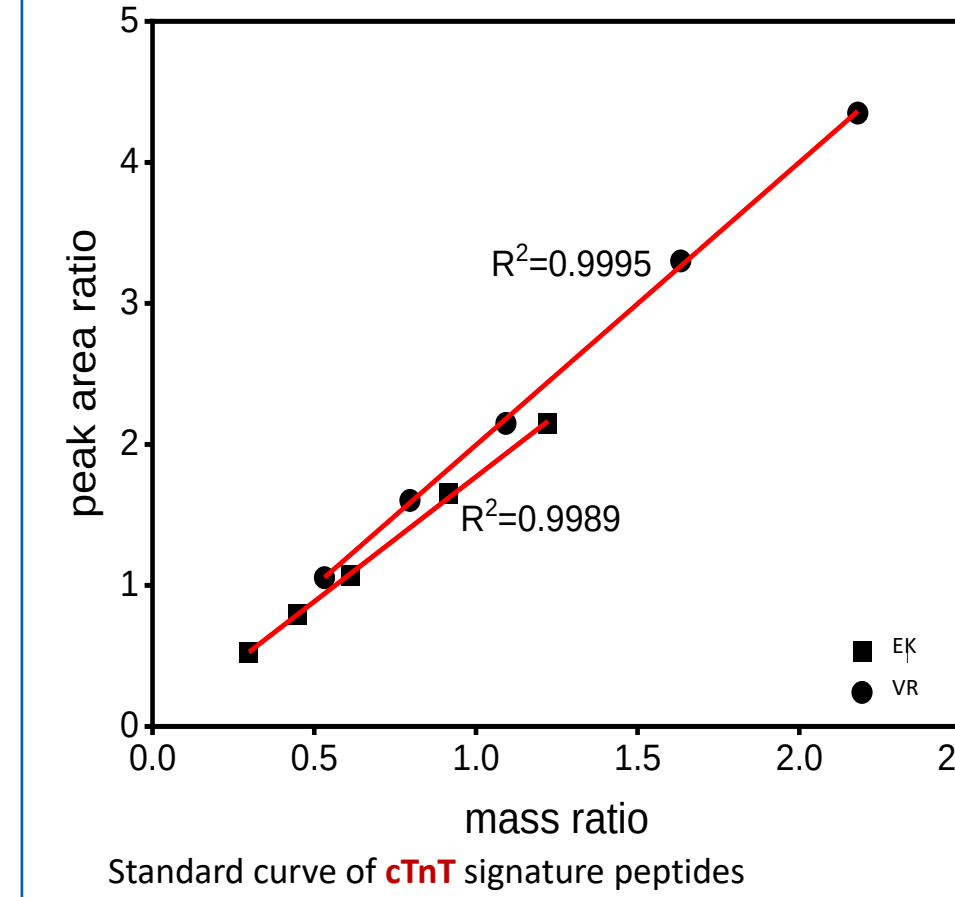


SP-IDMS quantitative results

The cTn digested solution is detected by Agilent 6410 LC-MS/MS. As shown in the chromatogram, six peptides in the cTn digested solution were all well separated with each other. The standard curve of each peptide shows good linear relationship. The averaged peak area ratios of each peptide were calculated to quantify the cTn concentration by bracketing calibrations. Concentration of each SP was calculated as the formula, and the concentration of each cTn subunit was obtained from the average of peptides. The cTnI mass concentration obtained from AAA-IDMS was 29.0 µg/g. The cTnT mass concentration obtained from AAA-IDMS was 42.0 µg/g. The cTnC mass concentration obtained from AAA-IDMS was 20.4 µg/g.



$$C_{pep} = \left[\frac{\left(\frac{A_s}{A'_s} - \frac{A_l}{A'_l} \right) \cdot \left(\frac{c_h}{c'_h} - \frac{c_l}{c'_l} \right)}{\frac{A_h}{A'_h} - \frac{A_l}{A'_l}} + \frac{c_l}{c'_l} \right] \times \frac{m'_s}{M_s}$$



Conclusion

In this study, we selected the recombinant cTn ITC complex expressed by E. coli as the raw material for CRM. This complex has an unchanged tertiary structure, high cTnI stability, and the same form as in AMI samples. The cTn candidate CRM was successfully quantified through amino acid analysis coupled with isotope-dilution mass spectrometry (AAA-IDMS) and signature peptide-IDMS (SP-IDMS). The results can be traced to the International System of Units (SI) via absolutely quantified amino acids and peptides. After optimizing the conditions of protein hydrolysis, amino acids and signature peptides were detected by LC-MS/MS. The cTnI mass concentration obtained from the average of AAA-IDMS and SP-IDMS was (30.2 ± 2.3) µg/g (k = 2). The cTnT mass concentration obtained from the average of AAA-IDMS and SP-IDMS was (43.6 ± 3.5) µg/g (k = 2). The cTnC mass concentration obtained from the average of AAA-IDMS and SP-IDMS was (22.3 ± 1.4) µg/g (k = 2). The consistency between the two methods was successfully validated, ensuring absolute quantitative accuracy.

This study establishes the foundation for the development of high-order reference materials for the clinical detection of troponin, which can enhance the accuracy, reliability, and consistency of clinical troponin test results.

References

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