YAXINBIO

Scientific questions of peptide mapping analysis of protein / antibody

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Regulations

- August 1999 ICH Q6B Specifications: A.1.d.Peptide Map

A.1.d. Peptide map

Selective fragmentation of the product into discrete peptides is performed using suitable enzymes or chemicals, and the resulting peptide fragments are analyzed by high pressure liquid chromatography (HPLC) or other appropriate analytical procedures.

The peptide fragments should be identified to the extent possible using techniques such as amino acid compositional analysis, N-terminal sequencing, or mass spectrometry.

Peptide mapping of the drug substance or drug product using an appropriately validated procedure is a method that is frequently used to confirm desired product structure for lot release purposes.

Regulations

WHO 2013 rDNA Guidelines: A1.1.a) Peptide map

A1.1.a) Peptide map
Selective fragmentation of the product into discrete peptides is performed by using suitable enzymes or chemicals.
The resulting peptide fragments are analyzed by high-performance liquid chromatography (HPLC) or other appropriate analytical procedures.

The peptide fragments should be identified as far as possible using appropriate techniques such as mass spectrometry (MS) methods(e.g. Electrospray ionization MS, Matrix-assisted laser-desorption ionization time-of-flight MS).

The use of MS/MS coupling should also be considered as it could reveal more detailed sequence information about the analyzed peptide fragment.

If one fragmentation method does not deliver the complete amino acid sequence, the use of an orthogonal enzyme or chemical cleavage method can increase the sequence coverage.
The correct formation of the disulfide bridges may be characterized by the use of peptide mapping under reducing and non-reducing conditions.

The Peptide Mapping Analysis issues

Journal of Pharmaceutical and Biomedical Analysis

Volume 21, Issue 6, January 2000, Pages 1099–1128

J Pharm Biomed Anal. 2000 Jan;21(6):1099-128.

Validation of a peptide mapping method for a therapeutic monoclonal antibody: what could we possibly learn about a method we have run 100 times?

Bongers J[1], Cummings JJ, Ebert MB, Federici MM, Gledhill L, Gulati D, Hilliard GM, Jones BH, Lee KR, Mozdzanowski J, Naimoli M, Burman S.

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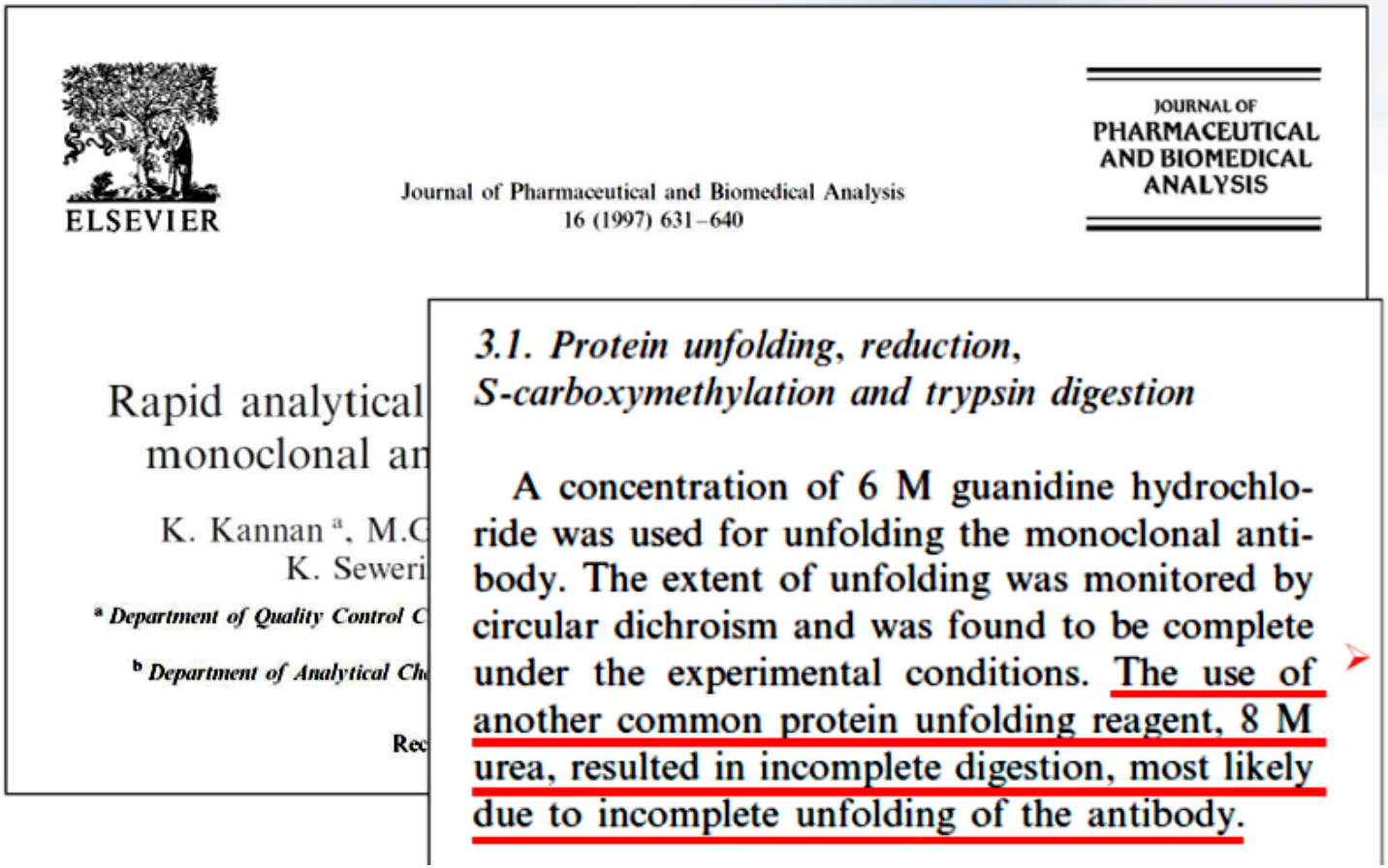
Abstract

Peptide mapping is a key analytical method for studying the primary structure of proteins. The sensitivity of the peptide map to even the smallest change in the covalent structure of the protein makes it a valuable 'finger-print' for identity testing and process monitoring. We recently conducted a full method validation study of an optimized reverse-phase high-performance liquid chromatography (RP-HPLC) tryptic map of a therapeutic anti-CD4 IgG1 monoclonal antibody. We have used this method routinely for over 1 year to support bioprocess development and test production lots for clinical trials. Herein we summarize the precision and ruggedness of the testing procedure and the main findings with respect to 'coverage of amino acid sequence' and limits-of-detection for various hypothetical structural variants. We also describe, in more detail, two unanticipated insights into the method gained from the validation study. The first of these is a potentially troublesome side-product arising during the reduction/alkylation step. Once the cause of this side-product was identified, it was easily prevented. We also report on subtle changes to the peptide map upon extended storage of the digest in the auto-sampler. These findings helped us to develop a 'robust' method for implementation in a quality control laboratory.

Selection Guanidine Hydrochloride vs. Urea



ELSEVIER

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JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Rapid analytical
monoclonal an

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K. Seweri

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[b] *Department of Analytical Ch*

Rec

3.1. Protein unfolding, reduction, S-carboxymethylation and trypsin digestion

A concentration of 6 M guanidine hydrochloride was used for unfolding the monoclonal antibody. The extent of unfolding was monitored by circular dichroism and was found to be complete under the experimental conditions. The use of another common protein unfolding reagent, 8 M urea, resulted in incomplete digestion, most likely due to incomplete unfolding of the antibody.

Preparation - Enzyme catalyzing system key points

Protease enzymatic catalyzing system buffer exchange method

BioProcess TECHNICAL

Solution-Phase Sample Preparation Approach for Peptide Mapping of Biological Therapeutics

Vajira Nanayakkara, William E. Werner, and Thomas Thorisult

Table 2: Summary of data for protein 1, comparing the PD10 column buffer exchange, dialysis, and ultrafiltration methods

	PD10	Dialysis	Ultrafiltration
Sequence coverage by AA residues (%)	75	80	94
H chain C — terminal Lys clipped	Yes	Yes	Yes
H chain Asn 34, 99, and 349 glycosylation	Weak signal	Weak signal	Yes
H chain N — terminal deamidation	Not found	Weak signal	Yes
Salt adduct ions	Yes	Yes	None or trace
Data quality and reproducibility	Poor	Poor	Excellent
Sample processing time per sample	36 hours	36 hours	12 hours

Preparation - Summary of key considerations

◆**Is denaturation of Antibody/protein adequate ?**
Whether non-folded state is consistently kept during enzymatic catalyzing,
is essential for peptide mapping analysis reproducibility.

◆**While focusing on the action and stability condition of the tools enzyme Trypsin, is another important prerequisite.**

Rituximab	*USP Medicine Compendium Final Authorized Version 1.0 (2012)*
Trastuzumab	*USP Medicine Compendium Final Authorized Version 1.0 (2013)*
Bevacizumab	*USP Medicine Compendium Final Authorized Version 1.0 (2014)*

USP—peptide mapping

Published on USP Medicines Compendium (http://www.usp.org/)
Rituximab, Final Authorized Version 1.0

<1055> Biotechnology-Derived Articles—Peptide Mapping
http://www.usp.org/sites/default/files/usp_pdf/EN/USPNF/peptideMapping.pdf

> 80% enzymatic digestion under reduced condition

B. Peptide Mapping

Use a chromatographic system.(Proceed as directed in Biotechnology Derived Articles-Peptide Mapping <1055>) Analyze the material to be tested by a chromatographic technique capable of resolving peptides generated from a Trypsin digest.

The digest is carried out under reducing conditions which provides NLT 80% digestion.

The test procedure used provides a minimum of 90% coverage of the protein sequence.

Standard solution: Digest and dilute a portion of USP Rituximab RS in an appropriate diluent.

Sample solution: Digest and dilute a quantity of Rituximab in an appropriate diluent to obtain a nominal concentration of Rituximab similar to that of the Standard solution.

Control solution: Digest and dilute a portion of an appropriate control (non-Rituximab monoclonal antibody) in an appropriate diluent to obtain a nominal concentration of the control that is similar to that of Standard solution.

[NOTE-The digests described in the Standard solution, Sample solution, and Control solution are conducted at the same time, using the same stock and concentration of reagents.]

USP—peptide mapping

IDENTIFICATION
A. Peptide Mapping
Solution A: 0.1% Trifluoroacetic acid in water
Solution B: 0.1% Trifluoroacetic acid in acetonitrile
Solution C: 0.5 M dithiothreitol in Water
Solution D: 0.5 M iodoacetamide in Water
Solution E: 0.25 M tris buffer in water. Adjust with dilute hydrochloric acid to a pH of 7.5.
Solution F: 6 M guanidine hydrochloride and 1 mM EDTA in Solution E (denaturing buffer)
Solution G: 0.1M tris buffer in water. Adjust with dilute hydrochloric acid to a pH of 7.8.
Solution H: 2 M urea in Solution G(digest buffer)
Solution I: 0.05 M acetic acid in water
Solution J: l mg/mL of trypsin in Solution I
Solution K: 10 mg/mL of USP Rituximab RS in water
Standard stock solution 1: Mix 100 µL of Solution K, 400 µL of Solution F, and 10 µL of Solution C, and incubate at 37° for 30 min. Add 24 µL of Solution D and incubate at room temperature for an additional 30 min in dark. Add 10 µL of Solution C and mix well.
Standard stock solution 2: Wash the PD-10 Sephadex G-25 column with 20 mL of water and equilibrate with 35-40 mL of Solution H.
Load Standard stock solution 1 on the column, and elute using Solution H in volumes of 700 µL each. Collect 6 independent fractions. Measure the absorbance of each fraction at 280 nm against Solution H. The fraction having an absorbance between 1.3 and 2.0 is used for digestion.
(NOTE--if the absorbance of the fraction is more than 2.0, dilute it using Solution H to get an absorbance of 2.0.
Standard solution: Mix well 50 µL of Standard stock solution 2 and 2 µL of Solution J and incubate for 18-20 h at room temperature.
Add 1 µL of trifluoroacetic acid and store the mixture at 4° .

Denaturing buffer

Digest buffer

Desalting column

RT enzymatic digestion 18-20 hours

USP—peptide mapping

Questions:

1) **Different buffer for denaturing (6M GuHCl) and for digest (2M urea)**
2) **Mode for buffer changing: G25 column**
3) **Digestion time: 18-20h in room temperature**

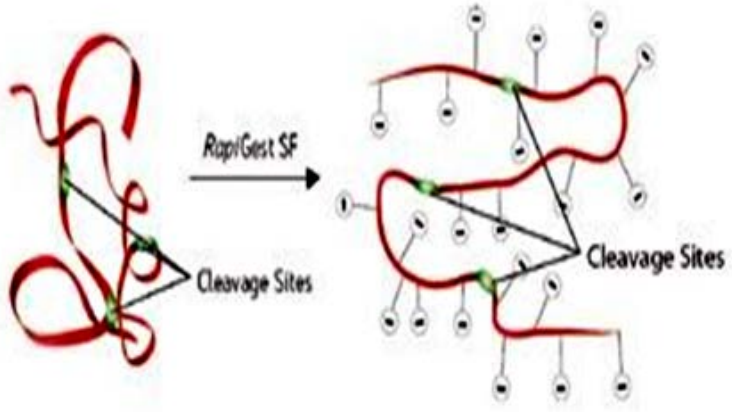
MS Identification and UV Release

1) MS identification: for example, if you have 100ug protein with 50ug digested，it is sufficient for finding all peptide segment and posttranslational modification。The resting of 50ug not cut is with no problem, that was generally seen for 4-5 hours of digestion from the articles by Waters and global pharmaceutical companies. Too long digestion may seen an increasing proportion of post-translational modifications, that is disadvantageous for identification.

2) UV release: not looking after the modification nor watching the coverage rate，but demands comparison of the UV spectra with that of the standard pharmacopoeia. This need to ensure that all 100ug protein is cut. So, we often see that in the pharmaceutical companies, as well as USP, the UV release monitoring are all digested overnight or even for 24 hours, and the digestions are performed after ultrafiltration or through the column solvent switching.

Sample pretreatment for peptide mapping

Rapigest SF surfactant from Waters



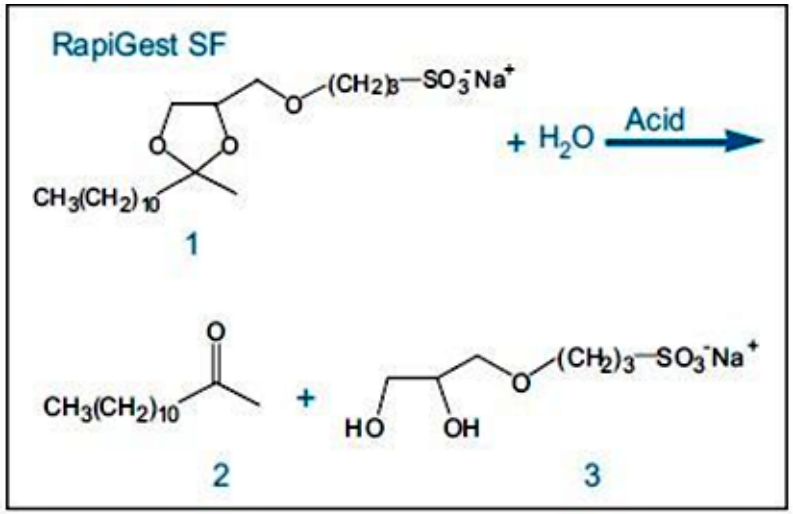


Figure 2.
RapiGest SF(1) decomposed to (2) and (3),T(1/2)=7.6min at pH=2

Similar as SDS, but more effective, and good activity for trypsin in Rapigest

Rapigest SF surfactant from Waters

Table 1. Activity detection of Trypsin, in the presence of selected denaturing agents

Trypsin solution (A)	Trypsin activity (B,%)	Trypsin solution (A)	Trypsin activity (B,%)
No additives	100	50% Methanol	31
0.1% RapiGest	100	50% Acetonitrile	92
0.5% RapiGest	87	1M Urea	97
0.1% SDS	20	2M Urea	83
0.5% SDS	1	0.5M Guanidine hydrochloride	21
0.1% RapiGest/ SDS	58	1M Guanidine hydrochloride	8

A: Add 0.5 ug Trypsin into 1mL 50mM Bicarbonate amine solution, Ph7.9, with 0.2mM BAEE.
B: Delta BAEE absorption value at 253nm(slope within 5 minutes)

Significance:

Find a condition under which the Trypsin maintains it's activity and target protein was dissolved and denatured.

Sample pretreatment for peptide mapping

Rapigest SF surfactant from Waters

Suggested procedure for in-solution digestions

1. Suspend lyophilized Rapigest SF powder in 1mL of 50mM NH4HCO3 to give 0.1%(w/v).
2. Suspend protein pellet in
3. Add DTT to 5mM
4. Heat the sample at 60℃ for 30 min
5. Cool the sample
6. Add IAA to 15mM and place the sample in dark 30min
7. Add enzyme for digestion (1:100 to 1:20, w/w)
8. Incubate at 37 ℃ for 1hr to overnight depending upon protein hydrophobicity

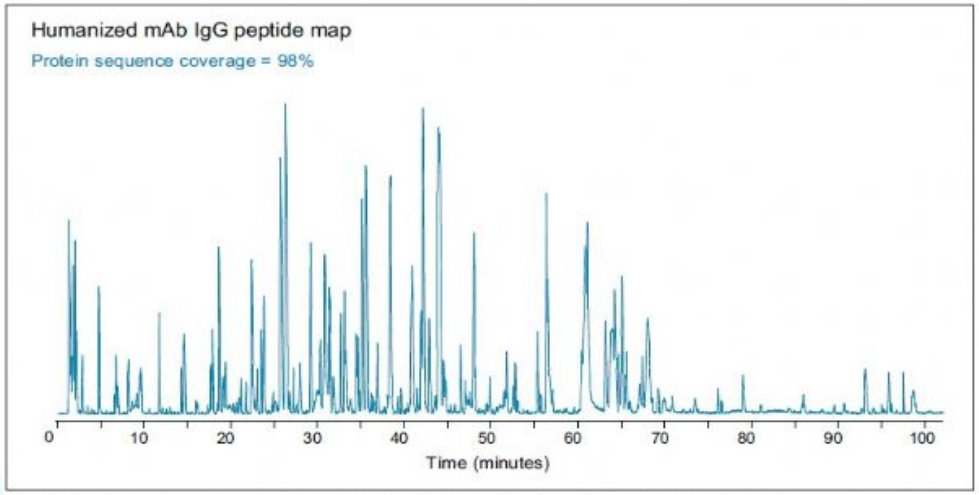


图4 胰蛋白酶消解人化单抗的LC/MS 分析。样品制备的复杂性显著降低。无需消化后清洁。总进样量为10 pmol 胰蛋白酶单抗。

Enzymes for peptide mapping

Sequencing grade Trypsin:

TPCK-Trypsin

Promega

Sigma

Recombinant Trypsin for peptide map

YaxinBio

Roche

TPCK-Trypsin

PRODUCT NAME	Comments	Content of chymotrypsin in Trypsin
USP-Trypsin	USP standard	<5%
Trypsin (sample)	Trypsin sample before TPCK treated	1.8%
TPCK-Trypsin	Trypsin after TPCK treated	0.56%

There is chymotrypsin activity in TPCK-treated trypsin.

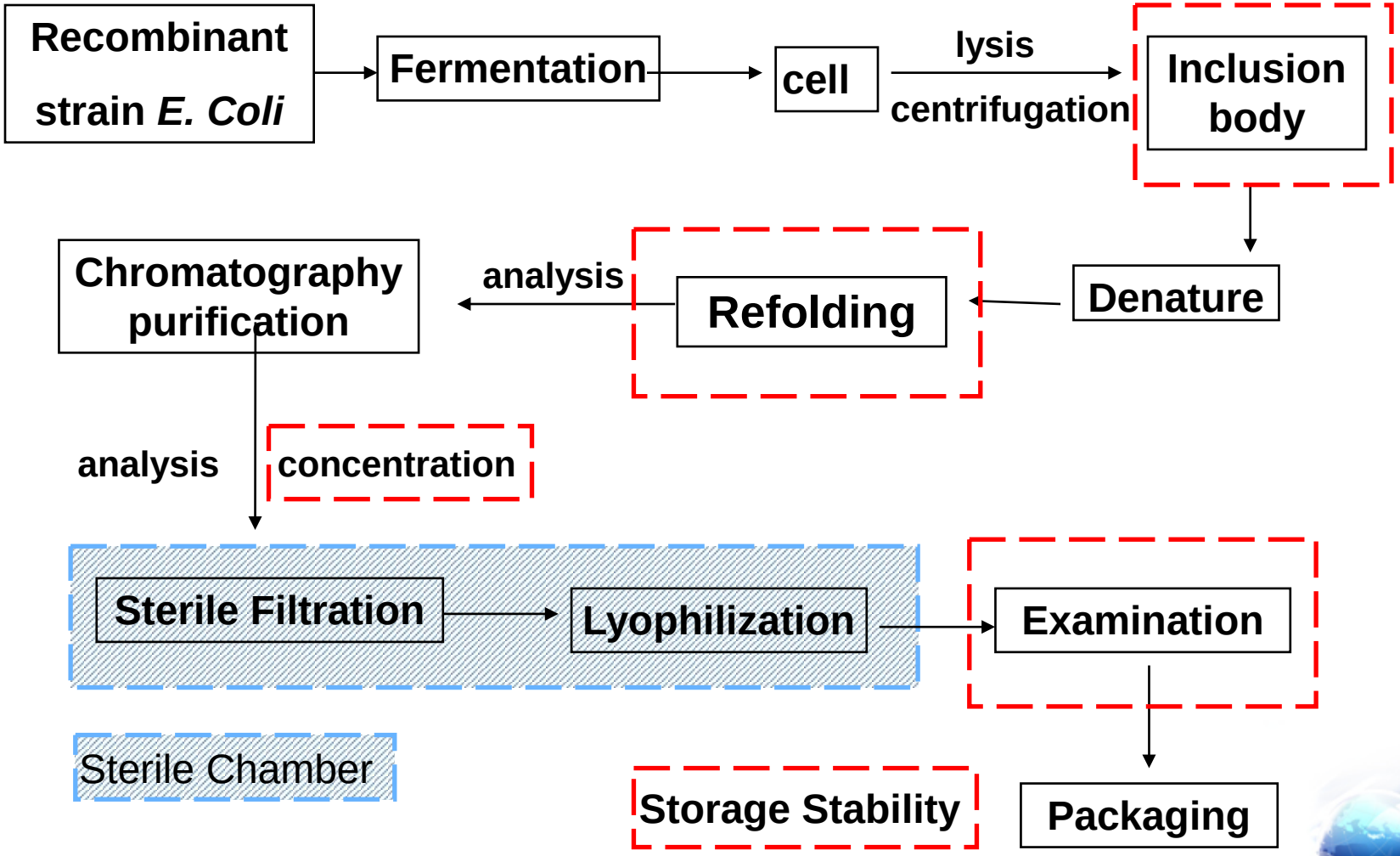
Recombinant Trypsin

No any other enzymes activity.

So, it is unnecessary for TPCK treated.

High specific activity

RECOMBINANT TRYPSIN



USP Recombinant Trypsin standard

BRIEFING

〈89〉 **Enzymes Used As Ancillary Material in Pharmaceutical Manufacturing.** This new general test chapter provides analytical procedures to aid in the assessment of quality for enzymes that are used in biopharmaceutical manufacturing. Examples include trypsin, collagenase, pepsin, and papain. This chapter does not discuss the applications of these enzymes but rather focuses on tests to assess the qualities as process materials. Further, the chapter does not provide ways to limit the enzymes in the final medicinal product. The first enzyme discussed in the chapter is recombinant trypsin. Other enzymes will be added in subsequent revisions of the chapter.

Trypsin purity analyzed by HPLC

The activity of recombinant trypsin is determined using a chromogenic peptide substrate carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate. The liquid chromatographic procedure in the test for *Purity* is based on analyses performed with the YMC-Pack ODS-A brand of L1 column. The retention time for the main peak is 12–17 min. A new Reference Standard, USP rTrypsin RS, is proposed for use during assessment of the system suitability for the proposed *Assay* and test for *Purity*.

(BIO: E. Chang.)

Correspondence Number—C126264

Comment deadline: November 30, 2013

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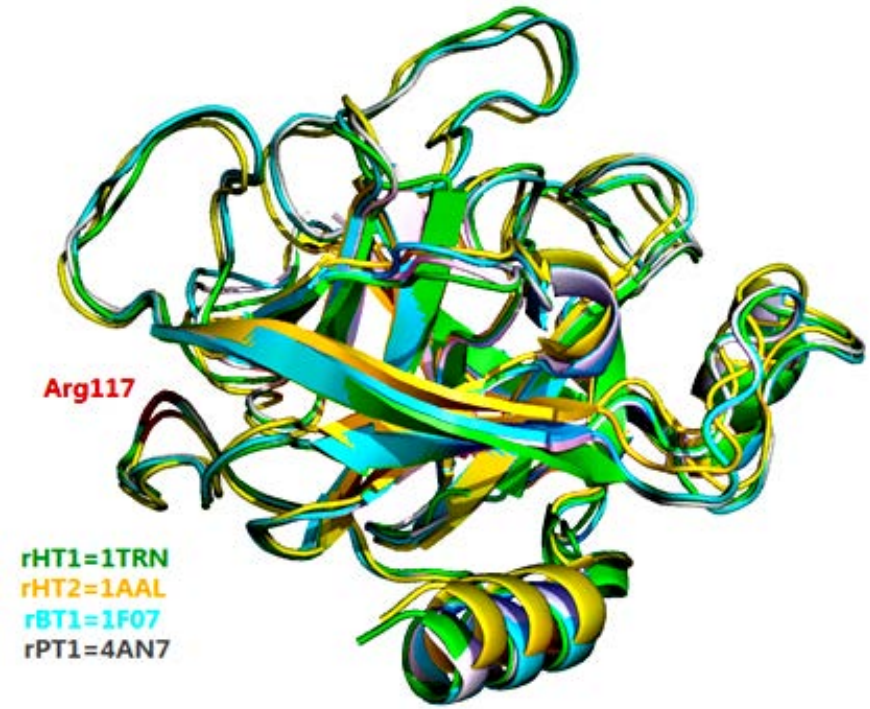
Comparison of Trypsin

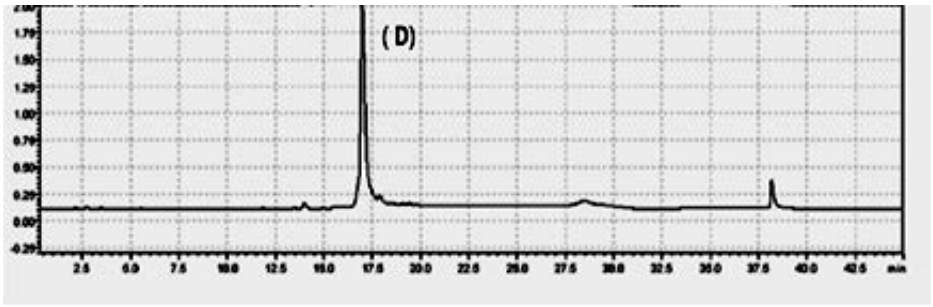
Test/Method	Specification 1:2500 in USP/CP	Specification 1:250
Appearance	White powder	Yellowish or brown
solubility	Soluble	Soluble overnight
Microbial limits	-	none
Staphylococcus aureus	Negative	none
Pseudomonas aeruginosa	Negative	none
Salmonella species	Negative	none
Loss on drying	<= 5.0%	none
Residue on ignition	NMT 2.5%	none
Limit of Chymotrypsin	NMT 5.0%	none
Activity	>= 2,500 USP units/mg	NLT 250 USP units/mg

Recombinant Trypsin in USP2014

Test/Method	Specification
Solubility	soluble
Bio-burden	NMT 100 CFU/ml
Specific Activity (USP u/mg pro)	NLT 3800
Purity (RP-HPLC)	NLT 70% β-trysin, NMT 20% α-trypsin

USP main standard differences of two Trypsins





Trypsin purity analyzed by HPLC

The retention time for the main peak for r-trypsin is 12-17min.

Retention time: >= 1.0min between two peaks of α-trypsin and β-trypsin.

>= 70% for the peak area of β-trypsin and <= 20% for the peak area of α-trypsin.

Stability of rTrypsin

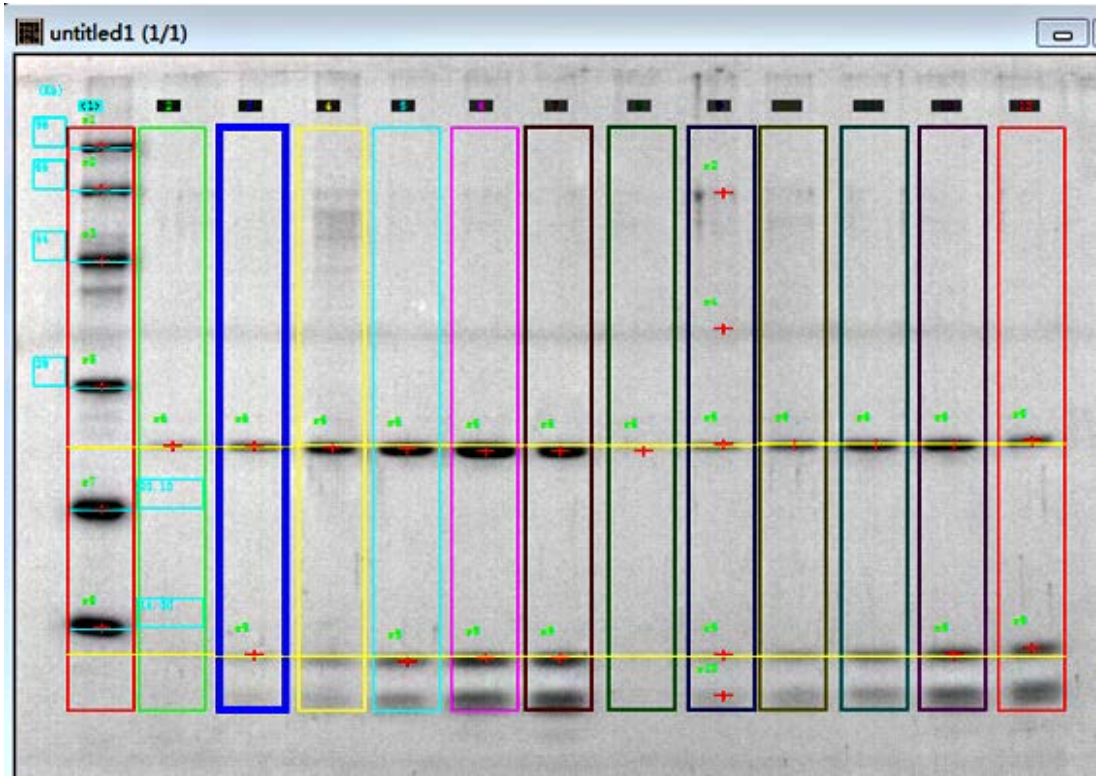
rTrypsin activity of different concentrations in 50mM NH_4HCO_3 pH7.8，37℃

ug/ml	10		20		50		100		200	
	U	100%	U	100%	U	100%	U	100%	U	100%
0h	110	100%	215	100%	600	100%	1140	100%	2300	100%
1h	115	105%	220	102%	575	96%	1170	103 %	2300	100%
2h	105	95%	240	112 %	540	90 %	1020	89%	2100	91 %
4h	120	109%	220	102 %	590	98 %	1160	102 %	1900	83 %
20h	75	68 %	135	63 %	290	48 %	440	39%	600	26%

Suggested used concentration：10-50ug/mL
For example: 1ug in 50uL

Suggested digestion time: 4h

Stability of rTrypsin



1：Marker
2：10ug/ml 10h 0.2ug
3：20ug/ml 10h 0.4ug
4：50ug/ml 10h 1ug
5：100ug/ml 10h 2ug
6：200ug/ml 10h 4ug
7：500ug/ml 10h 5ug
8：10ug/ml 20h 0.2ug
9：20ug/ml 20h 0.4ug
10：50ug/ml 20h 1ug
11：100ug/ml 20h 2ug
12：200ug/ml 20h 4ug
13：500ug/ml 20h 5ug

No self-degradation :
SDS-PAGE after incubated in 50mM NH_4HCO_3 ,pH7.8, 37℃,10h and 20h

Stability of rTrypsin

1mM Ca2+ Protection of different trypsin concentrations, in 37℃， 50mM NH_4HCO_3 pH7.8 中1mM

Time/hour	10ug/ml		20ug/ml		50ug/ml	
	0 mM Ca^{2+}	1mM Ca^{2+}	0 mM Ca^{2+}	1mM Ca^{2+}	0 mM Ca^{2+}	1mM Ca^{2+}
0h	100%	100%	100%	100%	100%	100%
1h	100%	100%	100%	100%	100%	100%
2h	100%	100%	100%	100%	100%	100%
4h	100%	100%	100%	100%	100%	100%
20h	68%	100%	63%	79%	49%	67%

Two groups paralleled, with or without 1mM Ca2 +, placed 4-5h at 37 ℃, both can maintain 100% activity.

1mM Ca2+ have certain protective effect for overnight insulation,

Such as: 20ug/ml, can avoid 16% loss of activity (increase from 63% to 79%); 50 ug/ml, can avoid 18% of the loss of activity (increase from 49% to 67%);10ug / ml has most obvious protective effect · for 24 hours of storage, 100% activity was maintained. Without 1mM Ca2 +, the activity was reduced by 32%.

YAXINBIO Stability of rTrypsin in GuHCL

Stability of Recombinant Trypsin at Different Concentrations in 2M, 1.5M Guanidine Hydrochloride, pH 8.0, at 37 ℃.

A：rTrypsin activity (%) in 50mM NH4HCO3 + 2M GuHCl pH8.0, 37℃

ug/ml	10	20	50	100	200
0h	100	100	100	100	100
2h	100	100	98	95	100
4h	100	100	91	1000	100
6h	100	100	89	96	86
22h	100	93	68	90	71

D：rTrypsin activity (%) in 50mM NH4HCO3 + 1.5M GuHCl pH8.0, 37℃

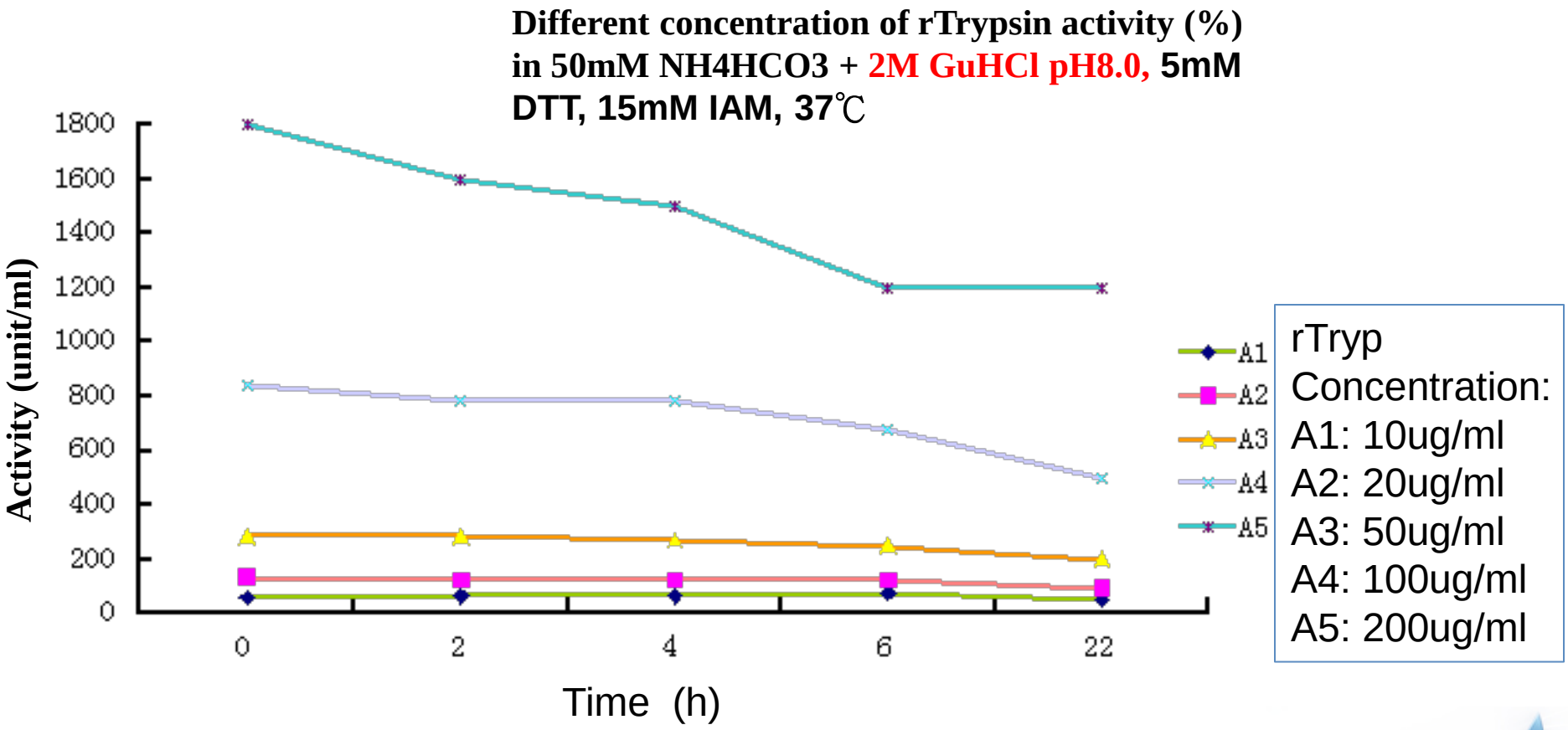
ug/ml	10	20	50	100	200
0h	100	100	100	100	100
2h	100	100	100	100	100
4h	100	100	100	100	100
6h	100	100	100	100	95
22h	100	100	96	100	95

Stable in 1.5M or 2M GuHCl, pH8.0, 37℃
No self-degradation, stable activity

Suggested process for pretreatment and trypsin digestion: 6M GuHCl in 50mM NH4HCO3 pH8.0 for denaturing and then dilute to 2M for trypsin digestion directly.
The digestion condition: 37 ℃, 4h-5h

YAXINBIO Stability of rTrypsin in GuHCL

Stability of Recombinant Trypsin at Different Concentrations
in 2M Guanidine Hydrochloride, pH 8.0, 5mM DTTT 15mM IAM, at 37 ℃.



Performance comparison test

Sequencing grade Trypsin Samples :

- **Promega modified Trypsin**
- **YaxinBio recombinant Trypsin**

- **Four aspects for peptide cleavage :**

1. Cut time and Peptides recovery rate

2. Missed cleavage peptides

3 Chymotrypsin cleavage peptides

4 Trypsin anto-lysis peptides

Performance comparison test

Sample：

- **Two monoclonal antibodies**

LC-MS:

- **UPLC-Xevo G2-S QTof system (Waters)**

Mobile phase：

- **A, 0.1FA% water**
- **B, 0.1FA% Acetonitrile**

Data processing：

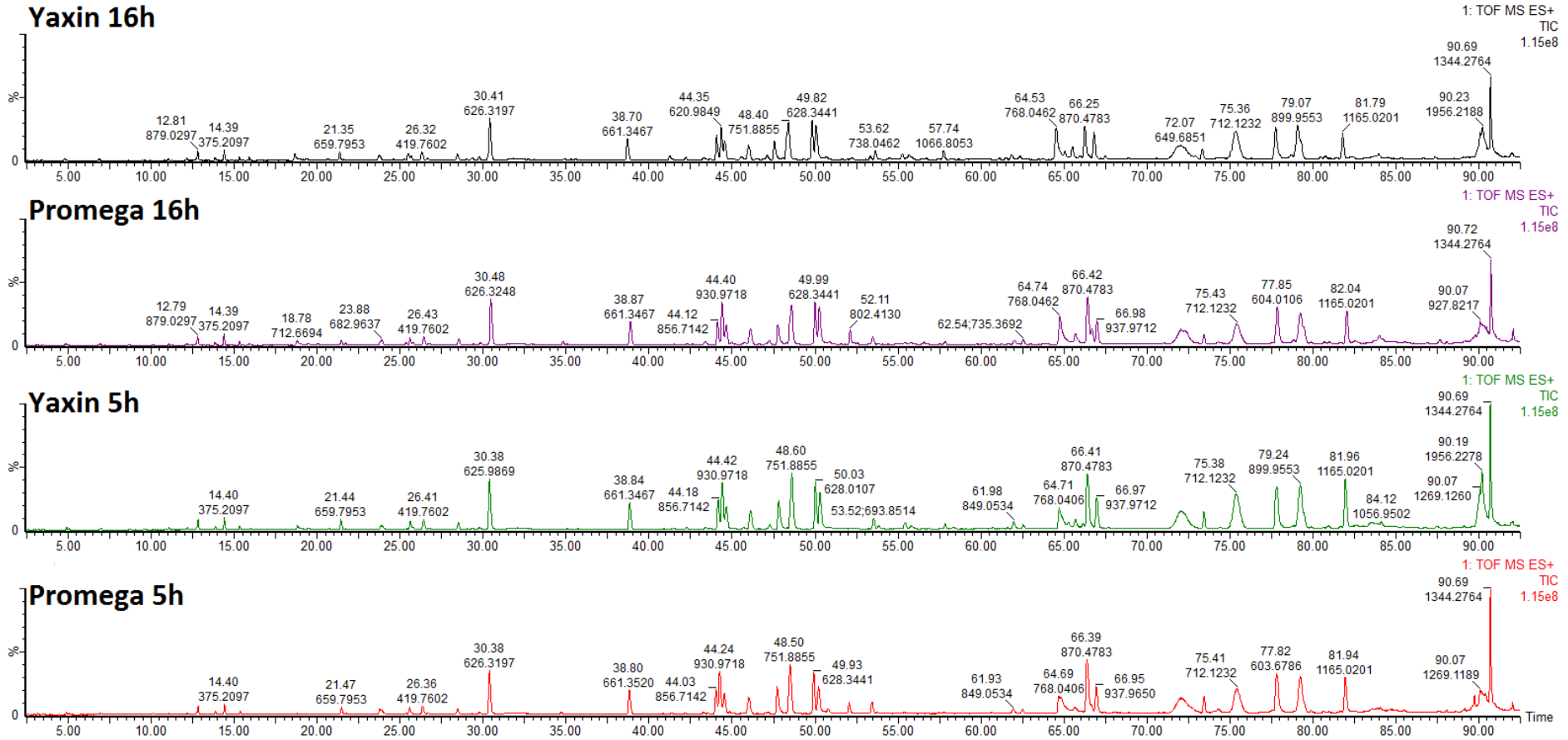
- **BiopharmaLynx (Waters)**

Performance comparison test

Sample Pretreatment Method :

- Reduced peptide mapping sample processing (HPLC/UPLC/MASS)
- The samples were diluted to 4-5g/L with ultrapure water, vortex for homogeneously mixed, dilute the sample to 2mg/ml with 6M Guanidine hydrochloride in 0.1 M/L Tris solution(Ph8.3) or 6M Guanidine hydrochloride in 0.1M Ammonium bicarbonate(AMBIC, pH about 8.0), and vortex for homogeneously mixed. (The final Guanidine hydrochloride concentration is 3-3.8M).
- Take 500μl diluted sample into the EP tube. Add 5ml 0.5MDTT solution, vortex mixing, bath 1.5-2.5h in 37 ℃ water bath. The DTT final concentration is 5mM).
- After water bath completed, add 13ul 0.5M IAM solution, the reaction was carried out with light avoided at room temperature for 45 minutes. (The final concentration of IAM is 13 mM)
- Put 400ul of the completely alkylated sample into to a 3KDa ultrafiltration centrifuge tube, and is centrifuged at 4℃, 12000rpm for 99 minutes.
- After centrifugation, add 150ul 0.1M Ammonium bicarbonate solution to the ultrafiltration tube and centrifuged at 12000rpm for 4 minutes at 4℃.
- After centrifugation, transfer the filtered fraction in a new outer tube and centrifuged at 4000 rpm for 3min at 4°C.
- Wash the membrane with 0.1M ammonium bicarbonate solution, wash twice each time with 180ul, each time the eluent is transferred to the outer tube.
- Gently blow and stir to mix homogeneously sample from the last step with a pipette gun, take 100ul into the EP tube, add 1ul 0.1M Calcium Chloride solution. (Calcium Chloride final concentration 1 mM)
- Then add 8ul Trypsin solution, vortex to mix for few seconds, and incubate the sample in water bath for enzyme digestion at 37 ℃ for 5, 15h (Trypsin : Protein = 1:50).
- Immediately after the digestion, the reaction was stopped by mixing 1%FA:Sample= 1:1 by volume.(FA final concentration 0.5%) pH<5.
- 10000rpm, low temperature centrifugation for 5min, take the supernatant for the following analysis of sample.

Performance comparison test



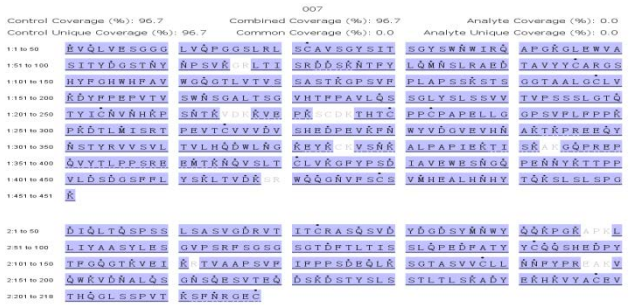
- **Almost the same chromatography profile (TIC)**

Performance comparison test

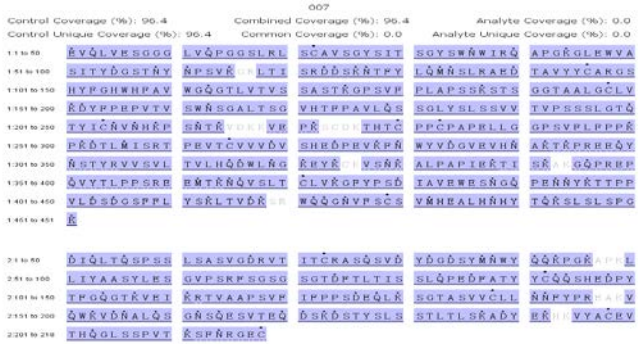
Peptide segment cover rate

Promega 5h：95.8%

Yaxin 5h：96.7%



Promega 16h：96.4%

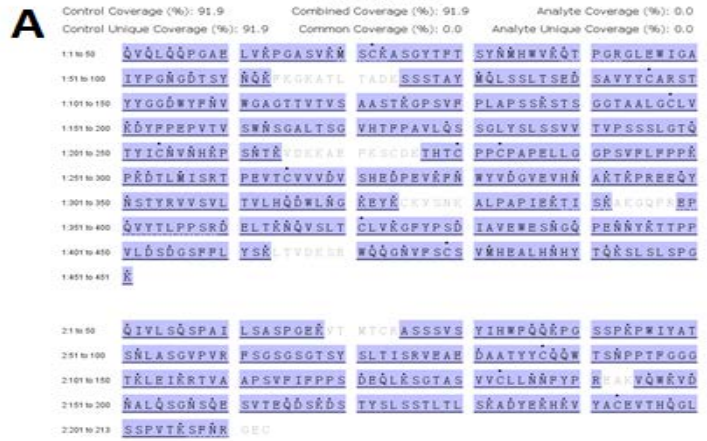


Yaxin 16h：97.3%

Performance comparison test

Figure 12 Peptide segment cover rate of the peptide mapping from Trypsin cleavage of 002 monoclonal antibody.

A: Promega-5h 91.9%



B: Yaxin-5h 94.9%

The peptide mapping of trypsin digestion from the Yaxin group get a higher coverage.

The peptide fragmentation information of individual peptide segments in Promega test group was not ideal and could not be identified.

Some peptide segments in the Yaxin group were not identified due to complete cleavage, such as the light-chain 15-16 peptide HKVYACEVTHQGLSSPVTK.

The HK peptide segment was too short and can only be indicated in the form of missed cleavage.

Performance comparison test

Enzyme cleavage and post-translational modification Identification

2:T010*	94.10%	93.60%	83.50%	83.60%	1:T024 B	3.10%	2.70%	1.40%	1.50%
2:T010*A	5.90%	6.40%	16.50%	16.40%	1:T021	97.70%	97.20%	90.80%	90.40%
1:T039*	96.60%	96.00%	89.50%	91.70%	1:T021A	1.50%	1.80%	7.70%	8.10%
1:T039*A	2.40%	3.10%	9.60%	7.40%	1:T021B	0.90%	1.10%	1.50%	1.50%
1:T039*B	1.00%	0.90%	0.90%	0.90%	1:T019	97.50%	97.60%	97.60%	97.60%
1:T035*	96.70%	96.90%	90.70%	87.70%	1:T019O	1.20%	1.20%	1.20%	1.30%
1:T035*A	/	/		9.30%	9.90%	1:T019B	1.30%	1.20%	1.20%	1.10%
1:T035*B	3.30%	3.10%	/		2.40%	1:T008	95.30%	93.50%	83.30%	78.70%
1:T034*	98.80%	98.60%	96.10%	95.70%	1:T008A	4.70%	6.50%	16.70%	21.30%
1:T034*A	1.20%	1.40%	3.90%	4.30%	1:T001	98.20%	98.30%	98.20%	98.10%
1:T024	84.40%	82.70%	69.30%	69.30%	1:T001C	1.80%	1.70%	1.80%	1.90%
1:T024 A	12.50%	14.60%	29.30%	29.20%					

Table 2: Comparison of major post-translational modification ratios (007 mAb)
1:T001 indicates the first peptide fragment of heavy chain cleavage by trypsin, “2:”indicates light chain.
* Indicates a fixed modification due to Iodoacetamide-modified Cysteine.
A: Deamidation, B:Deamidation intermediate, C: Pyroglutamate cyclization, O: oxidation.

11) Another function of the LC-MS assay is to determine and quantify post-translational modifications based on the mass secondary fragment results. Among them, post-translational modification, such as deamidation, oxidation, lysine excision, is a hotspot in the study of mAb quality.

2) Table 2 summarizes the peptide segments and ratios of the major post-translational modifications.
The results showed that，from the enzyme digestion peptide maps of the two types of Trypsin，the identified post-translational modification type and site are in consistent， and relevant post-translational modification ratio is basically the same.

3) It is worth noting that, because deamidation is a spontaneous chemical reaction in alkaline conditions, and Trypsin digestion pH is generally alkaline, so too long of digestion time will significantly lead to rise peptide deamidation ratio high.
In order to avoid this kind of post-translational modification brought about by the sample pretreatment , digestion time should not be too long.

Conclusion (I)Cut time—4-5h

AB and digestion time	Recovery rate	
	Promega-Trypsin	Yaxin-r-Trypsin
007 antibody - 5h	95.8	96.7
007 antibody -15h	96.4	97.3
002 antibody - 5h	91.9 (should less due to one identified as missed peptide)	94.9 (should high due to one HK too short to be identified)

It is worth noting that, because deamidation is a spontaneous chemical reaction in alkaline conditions, and Trypsin digestion pH is generally alkaline, so too long of digestion time will significantly lead to rise peptide deamidation ratio high.
In order to avoid this kind of post-translational modification brought about by the sample pretreatment, digestion time should not be too long.

Performance comparison test

Promega-Tryp & R-Tryp Comparison (1) ---Missed cleavage peptides

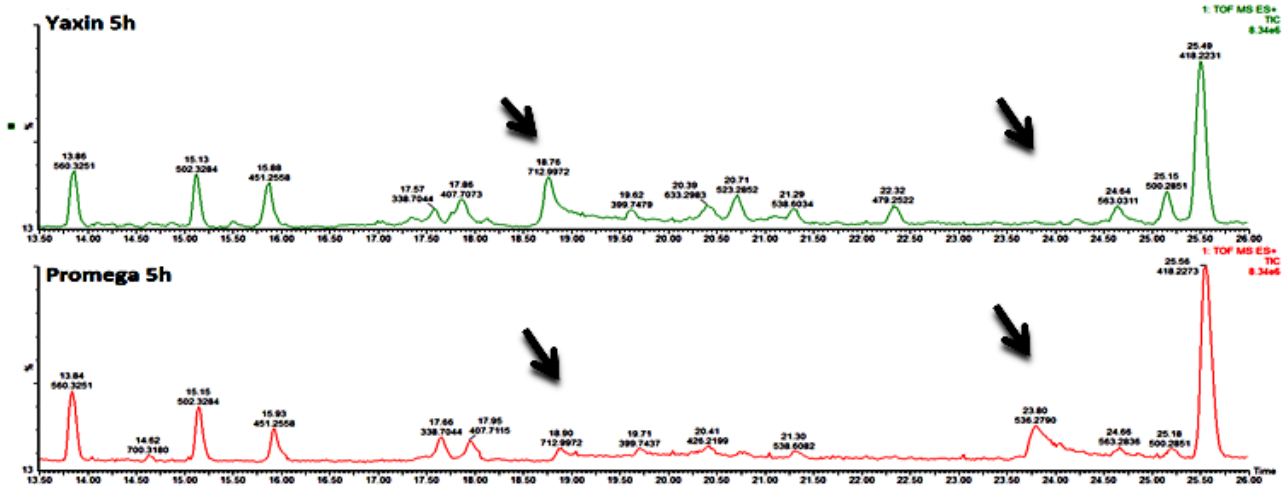


Figure 9, AB002 peptide map (13.5-26min)

(1) Missed cleavage segment too long :

18.9min peak is the 12th peptide fragment of normal light chain cleavage.

Yaxin group was able to cleave more light chain 12th peptide fragment,

while Promega group may be caused by missed cleavage leading to that some of the light chain 12th peptide is still connected to the previous or the next peptide segment, These two missed cleavage peptide segments are too long to be detected.

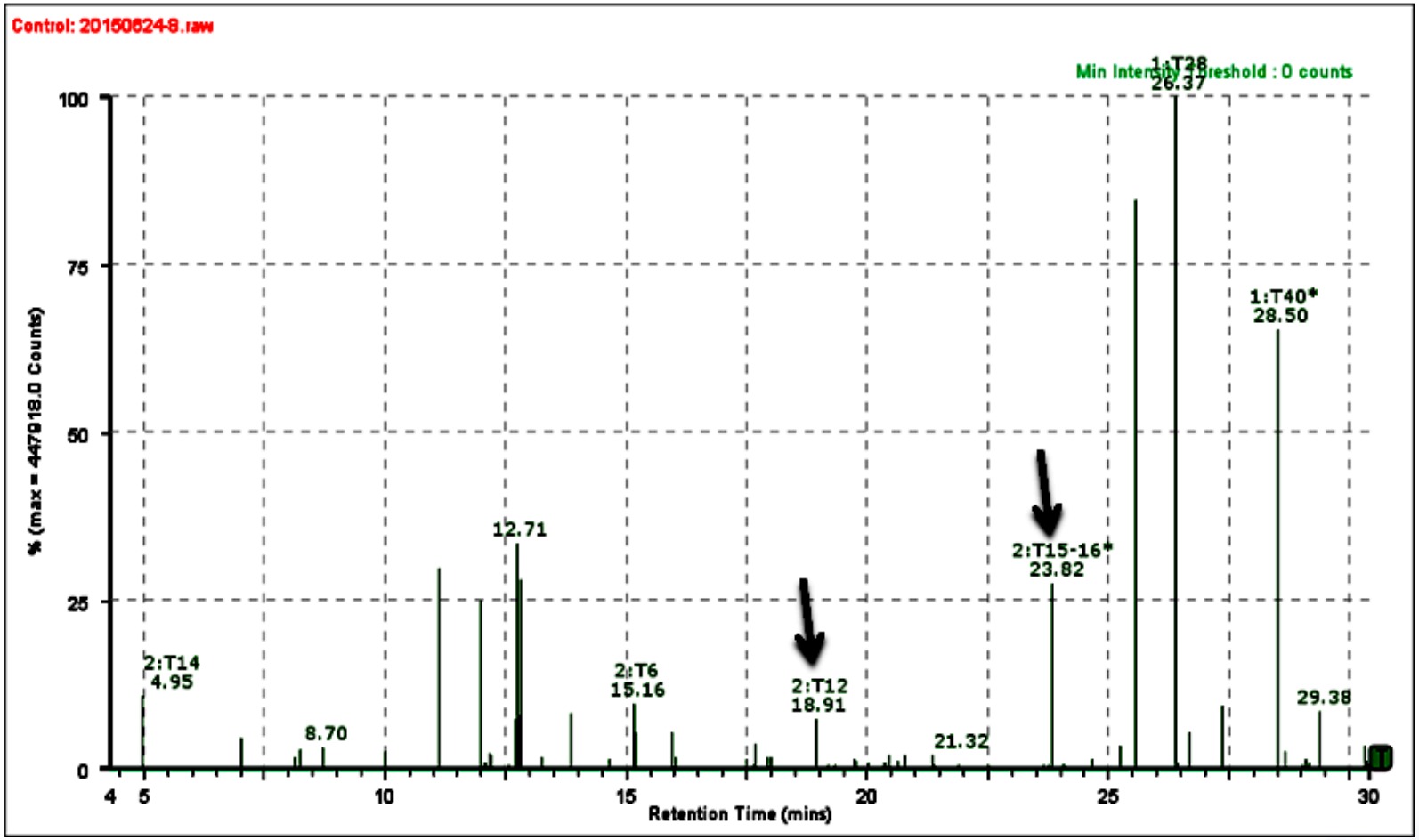
(2) Deduce by a missed cleavage fragment:

23.80min peak is a 15-16 peptide fragment for one missed cleavage point. HKVYACEVTHQGLSSPVTK, HK This peptide is too short and can not be identified normally , and the 23.80min peak is the form to indicate missed cleavage.

For the 15-16 segment, the Yaxin group had complete cleavage, while the Promega group had a certain amount of missed cleavage peptide segment.

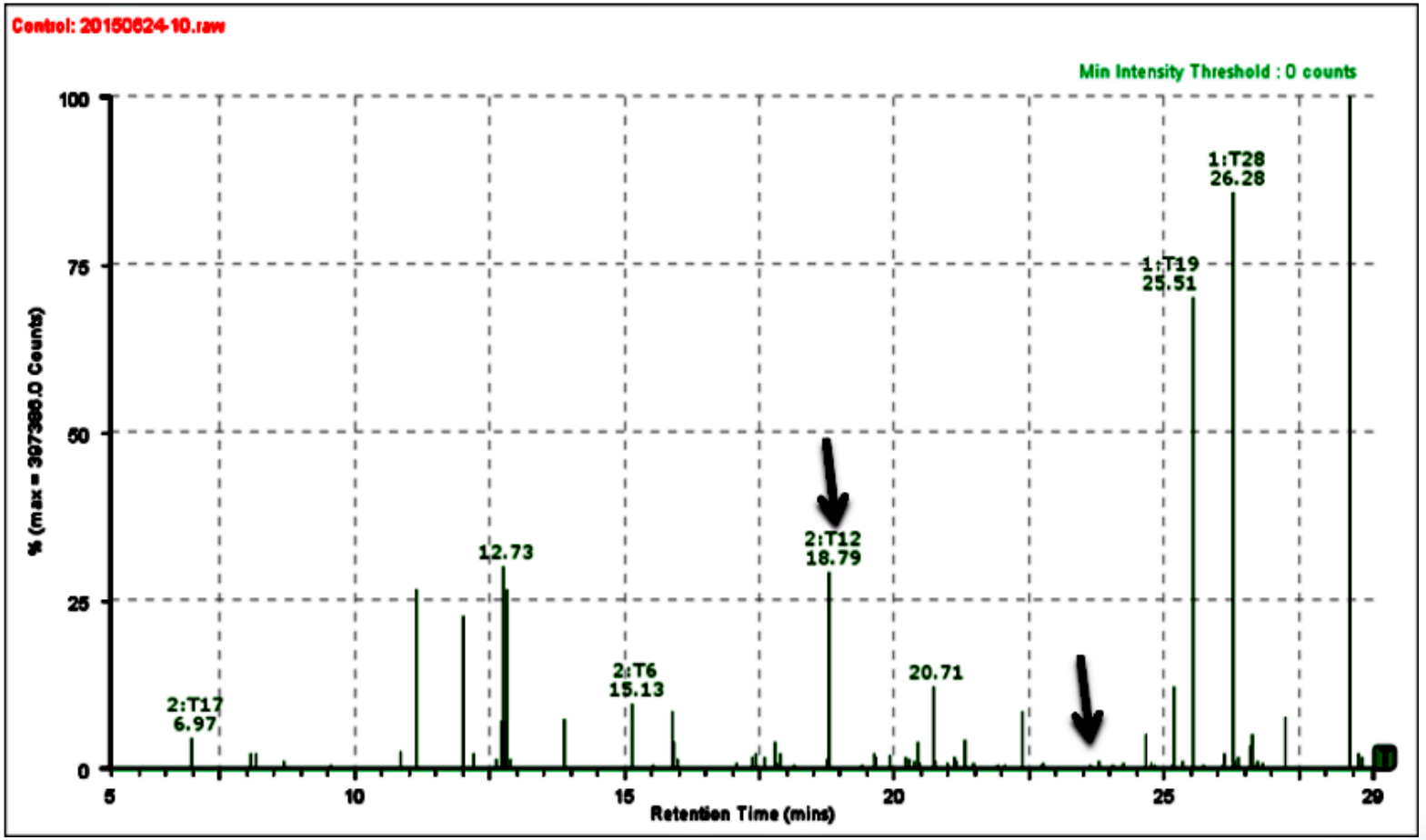
Performance comparison test

Figure10 Software processed 002 mAb Trypsin-digested peptide map (Promega 5h)



Performance comparison test

Figure11 Software processed 002 mAb Trypsin-digested peptide map (Yaxin 5h)



Performance comparison test

Promega-Tryp & R-Tryp Comparison (2) ---Missed cleavage peptides Conclusion

(1) For P15-16 peptide segment, Yaxin group was able to cleave completely, while Promega group had a certain amount of missed cleavage peptide segment.

Therefore, under the same enzymatic cleavage conditions, the enzyme digestion efficiency of Yaxin trypsin was higher and the digestion was completed within 5 hours.

(2) High coverage rate is got with Yaxin R-trypsin 94.9% to 91.9%.
While the fragmentation information of individual peptides in Promega group was not ideal and could not be identified.
Some peptide segment of Yaxin group was not identified because of complete cleavage, such as the 15-16 peptide segment of light chain HKVYACEVTHQGLSSPVTK. It is too short and can only be identified in contrast to missed cleavage.

Promega-Tryp & R-Tryp Comparison (3) ---Chymotrypsin cleavage

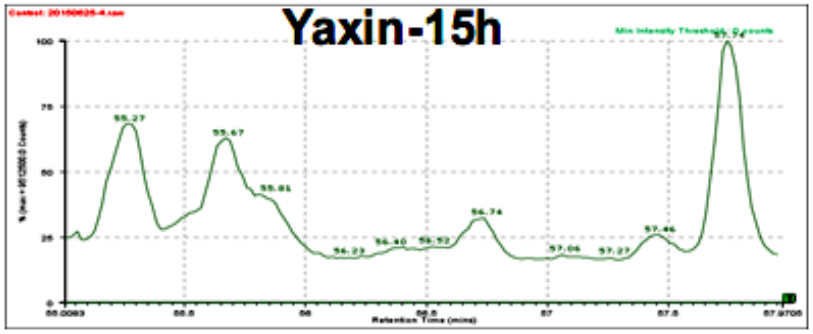
Table 1 Chymotrypsin cleavage peptides

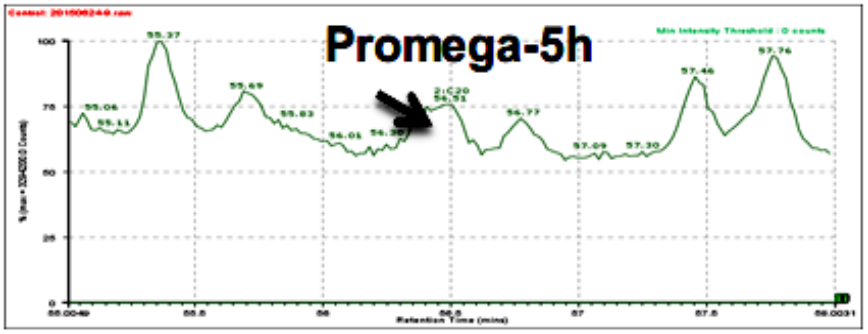
007 AB/signal strength	Promega-5h	Yaxin-5h	Promega-15h	Yaxin-15h
Heavy Chain C020	19002	None	90655	None
Heavy Chain C002	4407	None	23853	None
002AB/signal strength				
Heavy Chain C020	29613	None	/	/

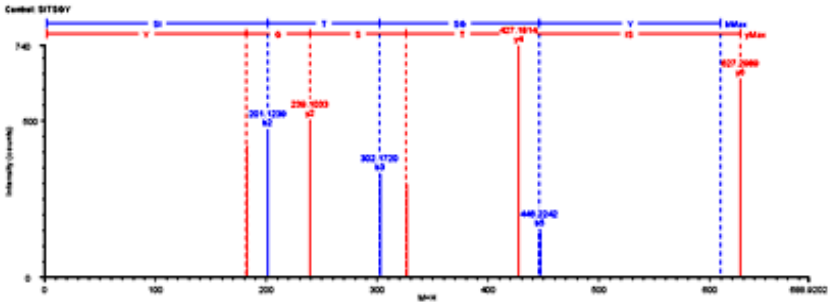
C020: the 20th peptide fragment of the aimed antibody by Chymotrypsin

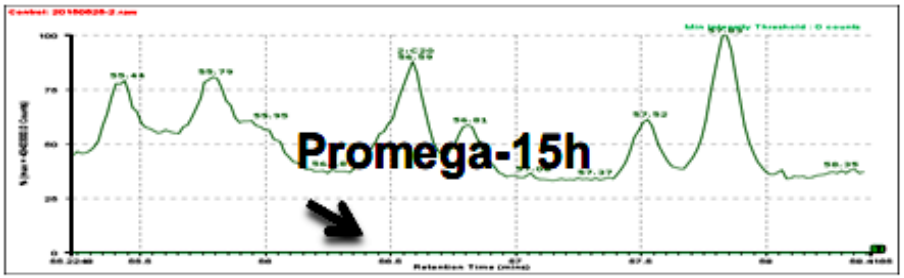
Through software search, we found that there exist chymotrypsin-specific cleavage peptide fragments in the Promega Trypsin-treated monoclonal antibody peptide map.
And the signal intensity increased with the reaction time.
The 007 McAb heavy chain C002 peaking at 18.2min with SLSSVVTVPSSSLGTQTY sequence, belongs to variable domain,
which is not found in 002 monoclonal antibody peptide map.
Heavy chain C020, peaking at 56.6min with SLSSVVTVPSSSLGTQTY sequence, belongs to constant region that can be found in the two monoclonal antibody peptide map.

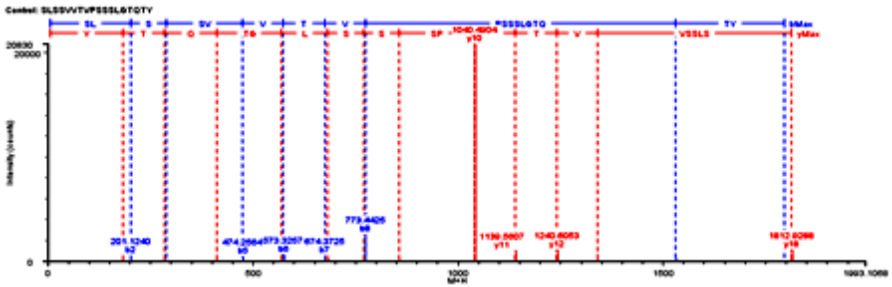
Promega-Tryp & R-Tryp Comparison (3) ---Chymotrypsin cleavage











Partial area map of C020 peptide segment in 007 mAb heavy chain

Secondary fragmentation map of C020 peptide segment in 007 mAb heavy chain

Table 2. Peptide auto-cleavage peptide segment(007 mAbs)

Peptide segment /Signal intensity	Promega-5h	Yaxin-5h	Promega-15h	Yaxin-15h
T007	30970	66064	46149	106509
T006	5887	41032	8934	71102
T005	9011	234266	8014	244695
T004	23456	180728	30468	435894

T007 represents the seventh peptide segment produced by trypsin auto-cleavage according to trypsin theoretical peptide mapping.

1) Both have four identical auto-cut fragments.
2) But it is clear that the auto-cleavage of Yaxin recombinant trypsin is more serious than that of Promega, especially the content of T005 and T004 peptide segment is much higher.

To Sum Up:

1) Two kinds of monoclonal antibody digestion peptide map results show that Yaxin Trypsin can achieve the effect of Promega Trypsin.

2) Ideal peptide segment coverage could be obtained by digestion of the two kinds of Trypsin for 5h.

3) The for 5h enzyme digestion efficiency of Yaxin Trypsin was higher than that of Promega Trypsin.

4) If you want to obtain 100% peptide segment coverage,
another protease digestion peptide map should be selected as a supplement.

Guanidine Hydrochloride denature and dilution then Enzyme cleavage

Pretreatment method:

After process for denature with Guanidine Hydrochloride and reductive alkylation, the protein solution was diluted with Ammonium bicarbonate solution (pH = 8) to 1M and 2M Guanidine Hydrochloride concentrations.
Subsequently, Trypsin was added at a ratio of 1:50 (enzyme: recombinant protein), and the reaction was carried out in a water bath at 37C. Digestion 5h and 16h, respectively. All the compared conditions including protein concentration, enzyme reaction temperature,
sample volume, etc. are consistent, only Trypsin different.

Guanidine Hydrochloride denature and dilution then Enzyme cleavage

Conditions:		Promega Modified Trypsin	YaxinBio-rTrypsin
1M Guanidine Hydrochloride	5h	90%	91.6%
	15h	91.6%	88.2%
2M Guanidine Hydrochloride	5h	79.8%	92.4%
	15h	90%	88.8%

In 1M Guanidine Hydrochloride and 5h enzyme cleavage,
all can reach the ideal coverage.
Yaxin group can find 32th peptide segment EPQVYTLPPSR of the heavy chain, yet Promega group did not, except the cleavage time was prolonged to 15h, indicating that Promega Trypsin takes longer to cut out the peptide segment. This consist with the results digestion of ultrafiltration without urease.

In 2M Guanidine Hydrochloride, the Yaxin group was observed decreased coverage rate along prolonged digestion time. This is because, such as, the heavy chain 17-18 peptide segment SCDKTHTCPPCPAPELL GGPSVFLFPPKPK, and the light chain 18-19 peptide segment SFNRGEC, is completely cleaved in prolonged cleavage time, producing heavy chain 17th peptide segment SCDK, light chain 18th peptide segment SFNR and 19 segment GEC.
These short peptides are often poorly retained on the column to be identified, resulting in reduced coverage.

For Promega group with prolonged cleavage time,
these peptide segments was still identified in missed cleavage forms,
indicating that this enzyme was unable to completely cleave the missed cleavage peptide segments.

Guanidine Hydrochloride denature and dilution then Enzyme cleavage

1）Compared with the ultrafiltration desalination experimental group, the coverage rate of peptide map shows that in the case of containing Guanidine Hydrochloride, The identified coverage rate of Trypsin digestion peptide map, (90% -91%), was lower than the ultrafiltration desalting group, (95% -96%).
Differences is analyzed and mainly because that some missed cleavage peptide segments and glycosylated peptide segment EEQYNSTYR was not identified in Guanidine Hydrochloride group.
It contain less missed cleavage peptide segments in normal circumstances.

2) Comparing the results, it was found that, despite of consistent protein amounts loaded, the mass spectrometry signal of Guanidine Hydrochloride group was almost 1 times lower. This suggests that the Guanidine Hydrochloride or some of the excipients or impurity in monoclonal antibody raw sample solution, may inhibit the overall mass spectrometry signal, resulting in no detection to some missed peptide segments that is intrinsically in low levels.
The identification method of Glycosylated peptide segments, in general, is first removal of sugar chains with glycosidase, so that it can be transformed into a common peptide identification.

Results Comparison : Guanidine Hydrochloride denature and dilution then Enzyme cleavage

To Sum Up:

1) In the solution containing Guanidine Hydrochloride,
the enzyme digestion efficiency of Yaxin recombinant Trypsin was higher than that of Promega.

2) In the presence of 1M Guanidine Hydrochloride,
Enzyme cleavages with both Trypsins for 5h all resulted in satisfactory peptide map coverage. In the 2M Guanidine Hydrochloride concentration,
Just 5h digestion with Yaxin Trypsin can get a better coverage of the peptide map, and Promega Trypsin requires longer digestion time.

3) Load with same protein sample content, the mass spectrometry signal of Quanidine Hydrochloride containing experimental group, is lower than the ultrafiltration desalination experimental group.

4) Due to lower mass spectrometry signal, many low content post-translational modified peptide segments are not detected in the Guanidine Hydrochloride-containing experimental groups.

C-Lysine certificate of AB

Enzyme: recombinant Carboxypeptidase B

About CPB Basic peak

CpB alkaline peak means that if the originator drug has acid peak 30, main peak 50, basic peak 20, while the sample has acid peak 30, main peak 45 and basic peak 25. And, after Cpb treatment, the originator drug remains unchanged, and the sample also became to 30,50,20, then it shows that the sample is only different from the originator drug on the alkaline peaks that is caused by 5% lysine variants.

This is acceptable on the declaration, and formal experiments have manifested no effect on the safety and effectiveness.
The European Union has generic drugs with this case, and have been approved.
So CpB in the application of antibody charge detection is necessary to use.

Acid Peak certificate of AB

Enzyme: V8
Aspartase, Glutamylase

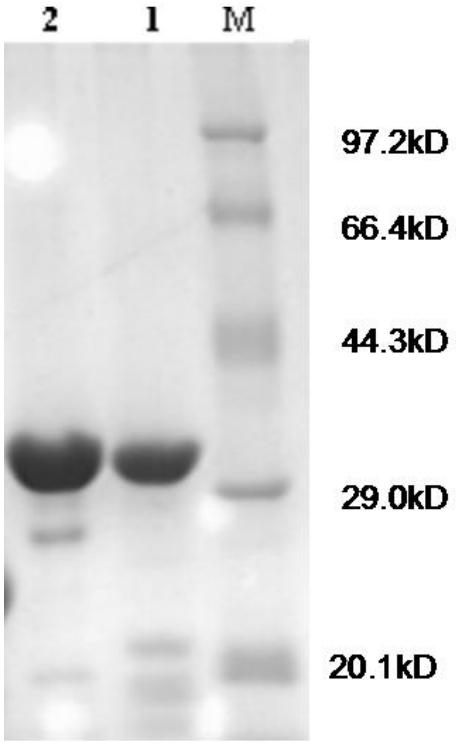
Antibody detection also requires identification of acidic peaks to control the degree of deamidation.
For antibody, the most important is the deamidation of Asn to form Asp.

Use the V8 enzyme.

V8 specifically cleaves the peptide bond formed by the C-terminus of Asp and Glu.

YAXINBIO C-Lysine certificate of AB

Enzyme: recombinant Carboxypeptidase B



1, Merck CPB
2, YaxinBio rCPB

ITEMS	SPECIFICATION
Source	Recombinant *E.Coli*
Purity	> 95%
Specific activity	>200 U/mg pro.
Trypsin content	<1ppm
Other enzymes	None
Form	Lyophilized powder
pI	5.4

STORAGE AND STABILITY

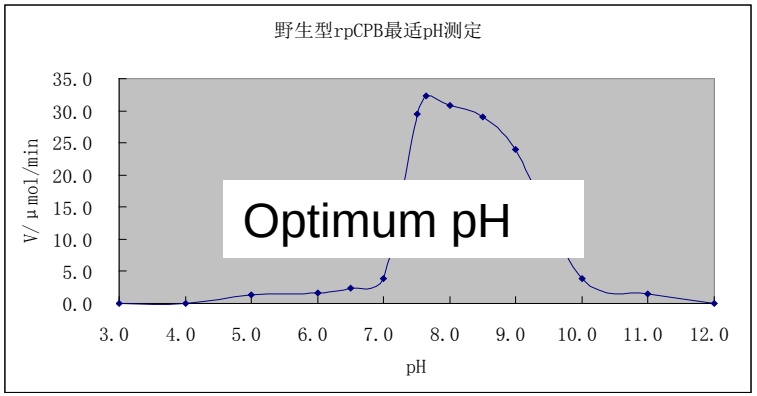
Recommend recombinant carboxypeptidase B lyophilized powder stored under 2-8℃ in sealed container. It is stable within 24 months.

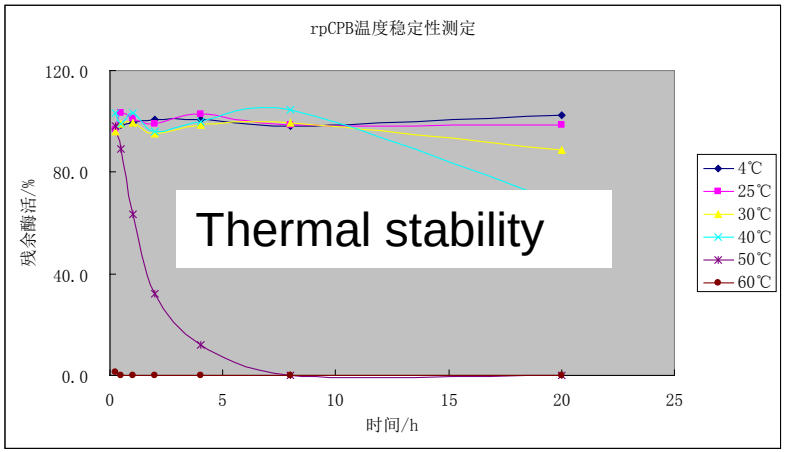
After dissolved, it should be stored under -20℃, It is stable within 24 months and no activity lose after 10 times repeated freezing and thawing.

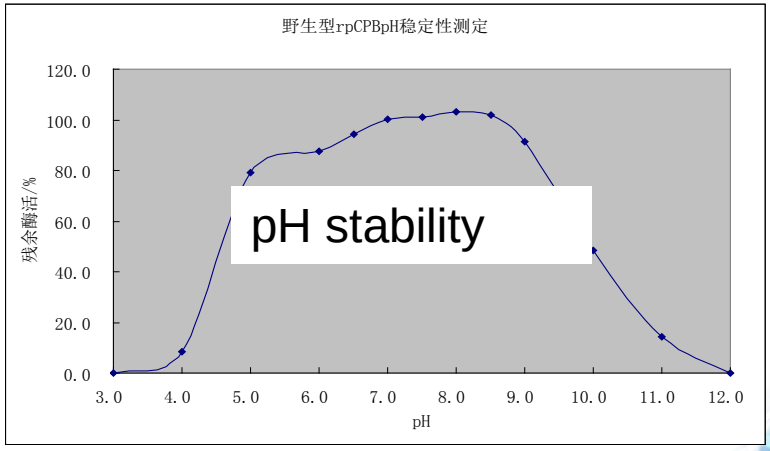
C-Lysine certificate of AB

Enzyme: recombinant Carboxypeptidase B

Suggested Methods:
Enzymolysis pH:7.5-9.0
Optimum pH:7.6
Analysis pH:6.0-9.0
Time enzymolysis:1h--3h
Enzymolysis Temperature:25℃-37 ℃



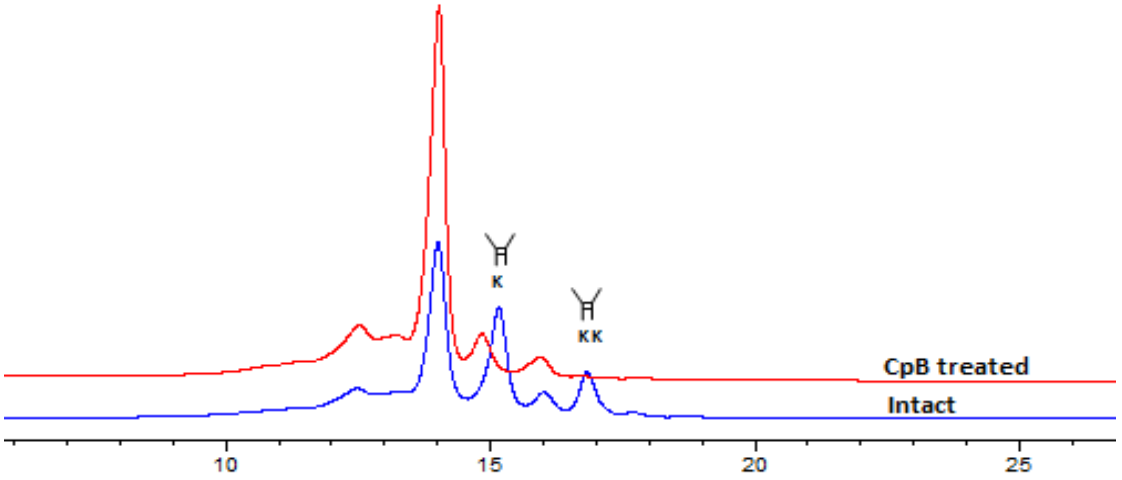




C-Lysine certificate of AB

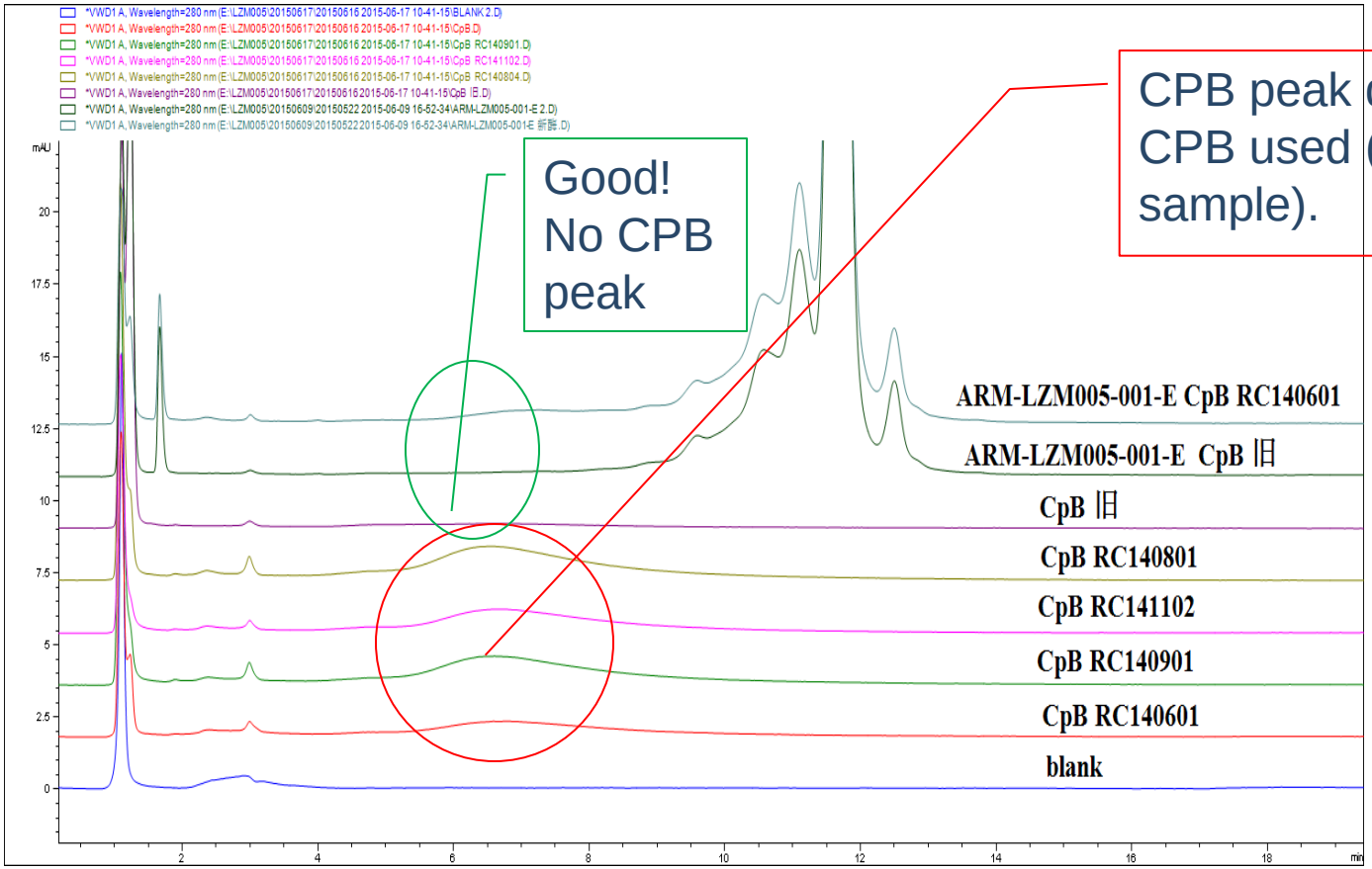
Example:

Carboxypeptidase B (RCPB): Yaxinbio RC01.
Dissolve Carboxypeptidase B in 1XPBS to 1g/L, and stored at-20° C.
The purified monoclonal antibody was ultra-filtered into phosphate buffer(pH = 7.5, 1-2 g/L), Add Carboxypeptidase B, according to the sample:enzyme = 100:1 ratio, react at 37 ℃ for 30 minutes, then direct injected for HPLC detection.



YAXINBIO C-Lysine certificate of AB

Enzyme: Carboxypeptidase B : Do not use too much



Suggested methods：
Ratio of AB to CPB： 20:1 to 100:1
Used concentration： 0.5ug-1ug in 50ul

YAXINBIO Sequencing grade r-chymotrypsin

Source	*E. Coli*
Purity	NLT 95% by HPLC analysis.
Physical form	Lyophilized powder
Specific activity	NLT 1500 USP units/mg pro.
No Contaminant activity	No carboxypeptidase A, or other proteases contaminant.

APPLICATION

Protein digests for peptide mapping applications or protein identification by peptide mass fingerprinting or MS/MS spectral matching. It is suitable for digestion reactions in-solution or in-gel.

RECOMMEND USAGE

To prepare 1-10mg/ml with 1mM HCl, used within 2 days, or stored below -20℃ after repacked

STORAGE AND STABILITY

Recommend recombinant Chymotrypsin lyophilized powder stored under 2-8℃ in sealed container. It is stable within 24 months. After dissolved, it should be stored under -20℃.

Main Products

Sequencing grade enzymes for protein analysis

Cat. No.	Product Name	Function	Charaters	Package
SRCPB0102	Recombinant Carboxypeptidase B (Sequencing grade)	---↓Lys/Arg/His	Specific Activity (unit/mg)	100μg
			NLT 200	1mg
SRT0202	Recombinant Trypsin (Sequencing grade)	---Lys/Arg↓---	Specific Activity (BAEE unit/mg)	100μg
			NLT 15000 BAEE unit	1mg
SRCT10	Recombinant Chymotrypsin (Sequencing grade)	---Tyr/Phe/Trp↓--- at a lower rate, at Leu and Met.	Specific Activity (unit/mg)	100μg
			NLT 1500	1mg
V813	V8 (Endoproteinase Glu-C) (Sequencing grade)	---Glu/Asp↓---	Activity (unit/mg)	50μg (1U)
			20	2mg

YAXINBIO Animal free questions in vaccine

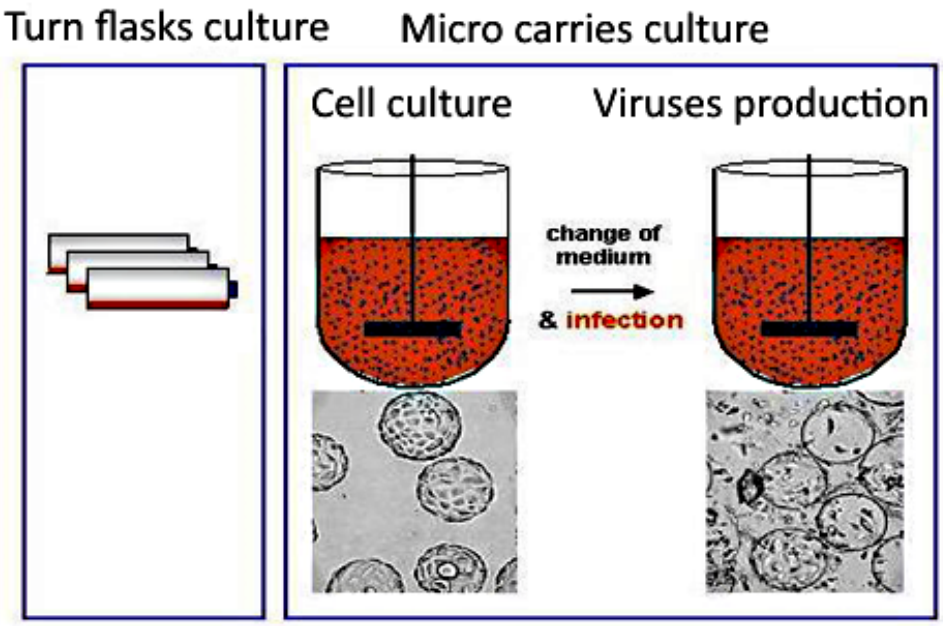
The first is : Non-serum media

The second is : recombinant Trypsin

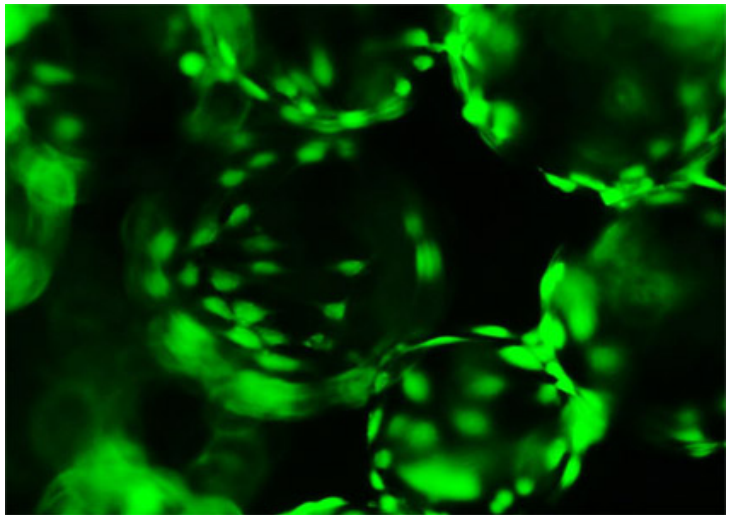
rTrypsin is to meet the needs of the cell culture and vaccine industry. With same performance to animal derived Trypsin, rTrypsin provides efficient dissociation of cells from surfaces and tissues while maintaining cell viability and integrity.

Additional question is : recombinant Trypsin inhibitor

Vaccine production



Micro carries culture



Vaccination, early training, stick a wall stage - harvest cell - culture

Trypsin is used during harvesting,
If propagate after harvesting, aprotinin is needed to inhibit Trypsin activity.

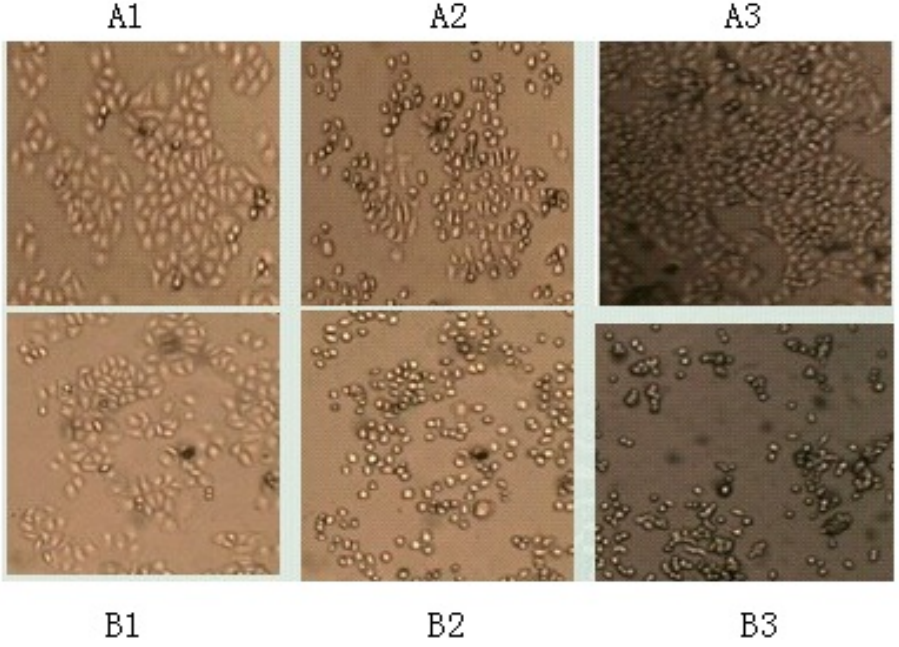
Trypsin history used in the vaccine production

◆ Trypsin (EC3.4.21.4) has been available for many years as native enzyme isolated from porcine and bovine pancreas.

- 1:250 Trypsin specially for cell dissociation
- 1:2500 Trypsin collected by CP, USP, EP and JP etc in 1970s.
- 1:3800 recombinant Trypsin issued by USP in 2014
- In 2015, CP2015 is preparing the standard for recombinant Trypsin.

RT used in cell culturing

Hela Cell **YaxinBio R-Trypsin**



A: rTryp1000 BAEE units/ml+0.01% EDTA

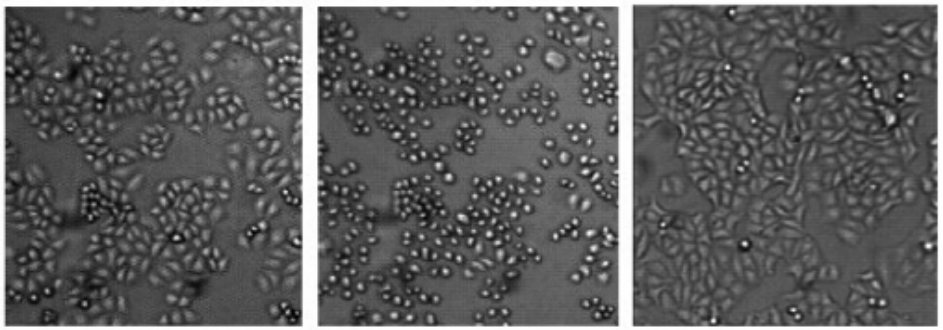
A1：before；A2：after 3min with rT；

A3：after 15h culturing

B: rTryp10000 BAEE units/ml+0.01% EDTA

B1：before；B2：after 30sec with rT；

B3：after 15h culturing



C: rTryp2000 BAEE units/ml+0.01% EDTA

C1：before；C2：after 2min with rT；

C3：after 24h culturing

RT used in cell culturing

The effects of Recombinant Trypsin components for cell detachment and its growth

Components	Time (min) for detachment	48 h Cell Growth condition
1000 BAEE units in PBS	7	+++
2000 BAEE units in PBS	6	+++
5000 BAEE units in PBS	4.5	+
10000 BAEE units in PBS	3	-
1000 BAEE units in PBS + 0.01% EDTA	3	+++++
2000 BAEE units in PBS + 0.01% EDTA	2	+++++
5000 BAEE units in PBS + 0.01% EDTA	1.8	+
10000 BAEE units in PBS + 0.01% EDTA	1.5	-

Recombinant Trypsin: YaxinBio
Cell Line: HeLa

Keys for Trypsin usage

1. The concentration of Trypsin
If shift from 1:250 trypsin, convert with activity, such as,
if 0.25% for 1:250 (250 USP unit/mg),
to 0.025 for 1:2500 (2500 USP unit/mg),
to 0.016 for 1:3800 (3800 USP unit/mg).

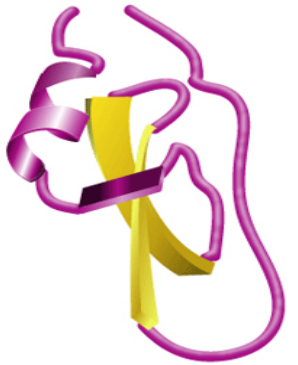
2. The time for Trypsin
If change FBS to non-serum medium, pay attention to the time, to stop the Trypsin with inhibitor.

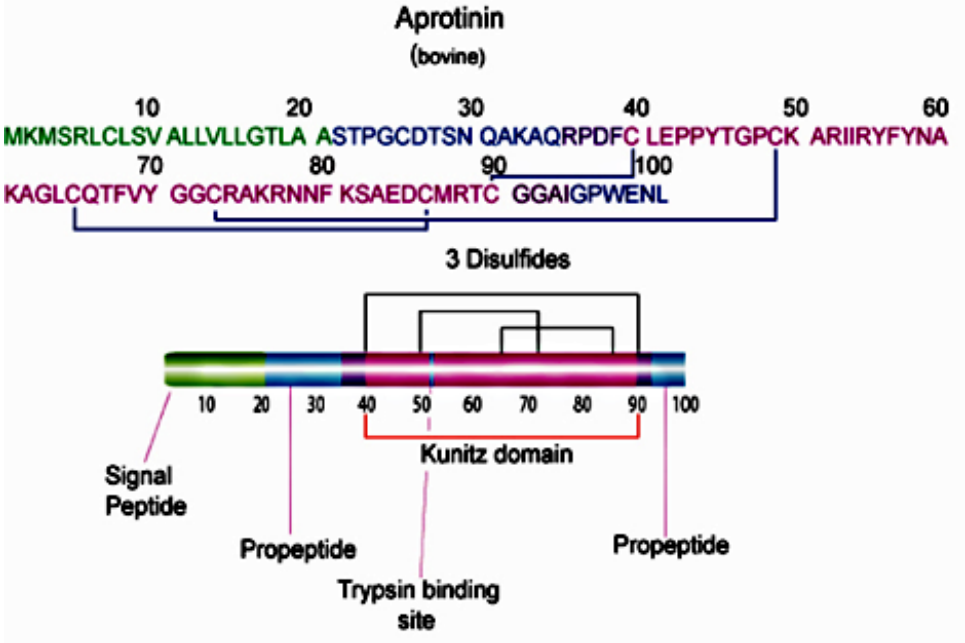
3. Different cell line owns different optimum concentration of Trypsin, find a optimum low effective concentration.

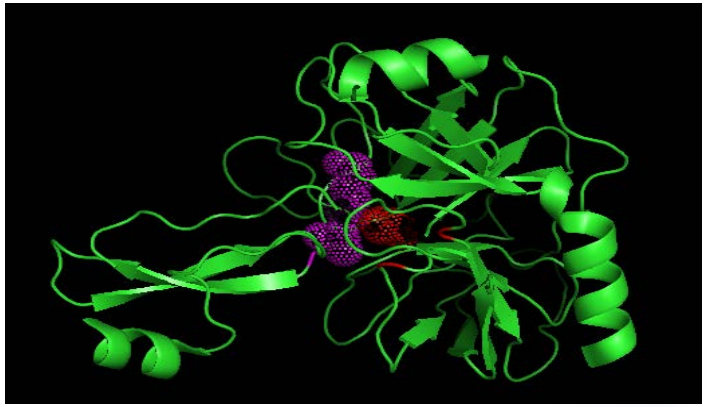
Trypsin Inhibitor

➢ Aprotinin/BPTI

While Aprotinin and bovine pancreatic Trypsin inhibitor (BPTI) are the same protein sequence, the term Aprotinin is typically used when describing the protein derived from bovine lung.





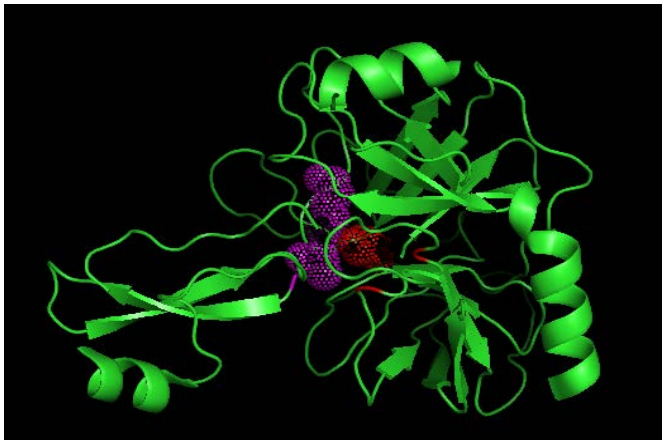


58 amino acid, 3 disulfides

Trypsin Inhibitor

- recombinant Aprotinin /BPTI --YaxinBio

Source	*E. Coli*
Purified by	HPLC
Physical form	Liquid in 0.1 M NaCl or White lyophilized powder
Specific activity	≥ 5 EPU/mg pro.
Purity	≥ 98% by SDS-PAGE
Contaminant activity	No any other protease contaminant.



Aprotinin-trypsin complex: mol:mol

UNIT DEFINITION

One trypsin inhibitor unit (EPU) will decrease the activity of 2 trypsin units by 50% where one trypsin unit will hydrolyze 1.0 µmole of N-benzoyl-L-arginineethyl ether (BAEE) per sec at pH 7.6 at 25 ℃.

A conversion factor for Aprotinin is: 1 EPU = 1 USP Aprotinin Unit = 1800 KIU.

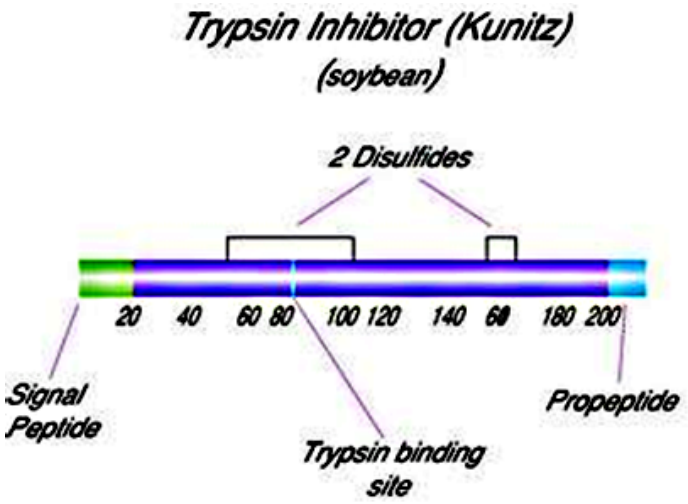
Usage: equal- mol trypsin (or 1/3 weight of trypsin)

store condition：2-8℃ or –20 ℃

Trypsin Inhibitor

- soybean trypsin inhibitor --- from TrypZean sigma

Soybean Trypsin inhibitor and other inhibitors work the same with TrypZean as they do with native Trypsins (on a weight-to-weight basis)



Activity: >7,000 BAEE units/mg
Usage suggestion: 0.25-0.5 mg/ml in PBS.
I**nhibit** equal- volume Trypsin
store condition：–5 ℃ -- –20 ℃

180 amino acid, 2 disulfides

Enterprise Vision

- Manufacturer of Animal Components Free recombinant proteins and protease.
- Supplier of recombinant protein and protease to global Biopharmaceutical company.
- Optimized customization service for biopharmaceutical customers

Main Products

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Products List

Cat. No.	Product Name	Function	Application	Charaters	Package
RCPB01	Recombinant Carboxypeptidase B	catalyzes lysine, arginine and histidine from C-terminal of polypeptides.	r-insulin production, antibody C-termial identification, sequencing, etc	Special Activity (unit/mg) NLT 170	10mg,100mg,1gr, or bulk.
RPT0201	Recombinant Trypsine (porcine)	endoproteinase, hydrolyzes polypeptides at the carboxyl side of lysine and arginine, comply with USP 2014	r-insulin production, biopharmaceutical process, cell culture	Special Activity (USPU/mg) NLT 3800	10mg,100mg,1gr, or bulk.
SRT0202	Recombinant Trypsine Sequence	endoproteinase, hydrolyzes polypeptides at the carboxyl side of lysine and arginine	peptide mapping, fingerprinting, and sequence analysis	Special Activity (USPU/mg) NLT 6000	20μg, 100μg, 1mg
RHT03	Recombinant Trypsine (human)	endoproteinase, hydrolyzes polypeptides at the carboxyl side of lysine and arginine, comply with USP 2014	biopharmaceutical process,cell culture,cell dissociation, human cell therapy, stem cell, etc.	Special Activity (USPU/mg) NLT 3800	10mg,100mg,1gr, or bulk.
RTS04	Recombinant Trypsine Solution	endoproteinase,hydrolyzes polypeptides at the carboxyl side of lysine and arginine, comply with USP 2014	cell culture,cell dissociation, biopharmaceutical process,etc.	Activity 2000 BAEE unit/ml	10ml,100ml,1L or bulk
RSPA05	Recombinant Protein A	binds to most human and mouse IgG subclasses. can be coupled to solid separation media to purify polyclonal or monoclonal IgG antibodies, can be coupled to a variety of molecules (such as fluorescent molecules, enzyme markers, biotin, colloidal gold and radioactive markers) used in antibody test in the process of Western-blot, ELISA or immunohistochemical tests, ect.		Purity NLT 95%	10mg,100mg,1gr, or bulk.
RLA06	Recombinant Lipase A	catalyzes the hydrolysis of fats and oils with excellent enantioselectivity.(1) Hydrolysis of trans-3-(4-methoxyphenyl) glycidic acid methyl ester [(±)-MPGM] (-)-MPGM.(2) Hydrolysis of (±)-naproxen methyl ester to produce (-)-naproxen. (3)Catalysis of ester substitution reaction, etc.		Activity 1000 unit/gr, 2000 unit/gr	1gr, 10gr, or bulk.
REK08	Enterokinase (EK)	cleaves lysine C-terminal preceded by four aspartic acids: Asp-Asp-Asp-Asp-Lys	delete extra N-terminal fusion protein to gain full recombinant protein	Activity (unit/μl) 10-50	100unit, 500unit, 1ku, or bulk.
RPK09	Recombinant proteinase K	endopeptidase. digest native proteins, thereby inactivating enzymes	used in the process of DNA extraction,etc.	Activity NLT 30	10mg,100mg,1gr, or bulk.
RCT10	Recombinant Chymotrypsine	endoproteinase, hydrolyzes polypeptides at the carboxyl side of aromatic amino acids: Tyr, Phe and Trp.	protein digests for peptide mapping or protein identification by peptide mass fingerprinting or MS/MS spectral matching.	Special Activity (unit/mg) NLT 1000	10mg,100mg or bulk.
V813	V8 (Endoproteinase Glu-C)	endoproteinase, cleaves peptide bonds C-terminal to Glu and Asp	insulin analysis, peptide mapping, fingerprinting, and sequence analysis	Activity (unit/mg) 20	1 unit, 50 μg, 2mg.

Main Products

- **Recombinant Trypsin - consistent with USP2014**

Series of products:

(1) Recombinant Trypsin (Human 1,PRSS I)
(2) Recombinant Trypsin (Human 2,PRSS II)
(3) Recombinant trypsin (Porcine, RPT）
(4) Recombinant trypsin (Bovine, RBT)
…..

Recombinant production, AOF(Animal-Free Origin),
completely improving the animal origin issues in vaccine production.
From an important way to avoid the spread of zoonoses.
Used in production of vaccine and recombinant protein,
immunotherapy and other fields.

Main Products

- **Manufacturer of AOF(Animal-Free Origin) recombinant enzymes for recombinant human insulin. the main products in this field:**

  (1). Recombinant Trypsin;

  (2). Recombinant Carboxypeptidase B;

  (3). V8 for Insulin Detection;

  (4). Recombinant Trypsin Inhibitor (Aprotinin)

  (5). Recombinant Endoproteinase Lys/Arg-Arg

  .....

Main Products

- **Other AOF(Animal-Free Origin) enzymes and proteins**

(1). Recombinant Enterokinase (large scale)

(2). Recombinant Protein A
(alkaline stable, for antibody purification)

(3). Recombinant human Chymotrypsin

…….

Independent intellectual property rights


证书号第1077877号

发明专利

发明名称：一种高稳定性的重组胰蛋白酶的

发明人：冯矗;赵致

专利号：ZL 2009 1 0055493.8

专利申请日：2009年07月28日

专利权人：上海雅心生物技术有限公司

授权公告日：2012年11月14日

局长 田力普

证书号第1077867号

发明专利

发明名称：一种高稳定性的重组羧肽酶B的

发明人：冯矗;赵致

专利号：ZL 2009 1 0055492.3

专利申请日：2009年07月28日

专利权人：上海雅心生物技术有限公司

授权公告日：2012年11月14日

局长 田力普

证书号第1077877号

发明专利

发明名称：一种高稳定性的重组胰蛋白酶的生产

发明人：冯矗;赵致

专利号：ZL 2009 1 0055493.8

专利申请日：2009年07月28日

专利权人：上海雅心生物技术有限公司

授权公告日：2012年11月14日

局长 田力普

证书号第1714571号

发明专利证书

发明名称：高稳定性的具抗体结合能力的重组蛋白A及其生产

发明人：李素霞;赵致;冯矗

专利号：ZL 2010 1 0614100.5

专利申请日：2010年12月30日

专利权人：上海雅心生物技术有限公司

授权公告日：2015年07月01日

局长 申长雨

2015年07月01日

Thank you!