
Characterization of biopharmaceuticals



October , 2011

Content



1. Introduction
2. Expression systems
3. Product characterization
4. Summary

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4. Summary

Biopharmaceuticals- current status



- ❖ Development of biosimilars
- ❖ Clear regulatory path in India
- ❖ EMEA guidance
- ❖ Likely guidance from US FDA
- ❖ Participation by many Indian companies



Protein characterization



- ❖ Know the product
- ❖ Consistency in batches
- ❖ Batch release
- ❖ Regulatory compliance
- ❖ Development of similar biologics



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Choice of expression system



- ❖ Bacteria

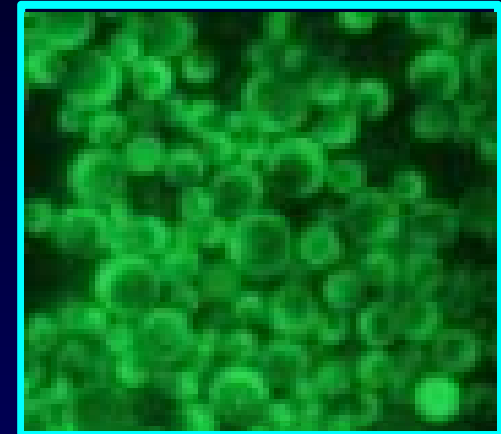
- ❖ E.coli



www.healthlifestylez.com

- ❖ Yeast

- ❖ Saccharomyces, Pichia



- ❖ Mammalian

- ❖ Chinese hamster ovary cells

sustainabledesignupdate.com



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Task tree for protein characterization



Protein

Determination of purity

HPLC analysis with
Diode array detector

SDS-PAGE

Isoelectric
focusing

Peptide mapping
Mass spectrometry

Chemical characterization

N-terminal
sequencing

Amino acid
analysis

Internal
Sequencing,
Mass spectrometry

Characterization of post-
Translational modifications

Conformational analysis

Circular dichroism

NMR spectroscopy

Fluorescence
spectroscopy

Colorimetry

Analysis of Quaternary structure

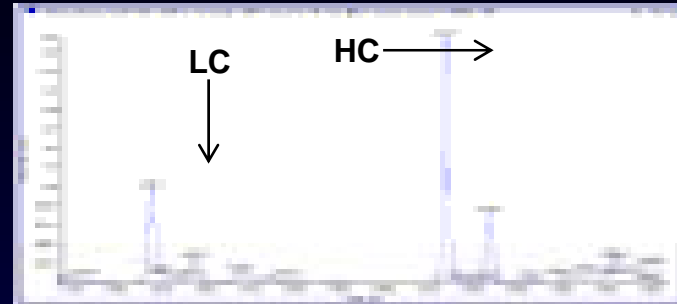
Gel filtration

Native gel electrophoresis

Analytical
Centrifugation

Biosimilar Antibody Sequence identification

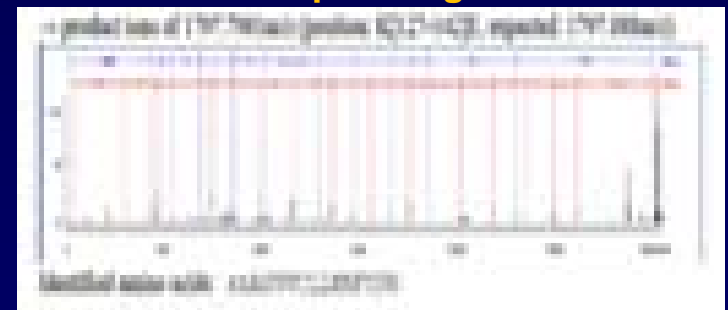
- ❖ Reliable primary sequence source critical
 - ❖ Patents, literature, publications
- ❖ If sequence information not available through any resource
 - ❖ De Novo Sequencing of Innovator product
 - ❖ Instrument and expertise intensive
 - ❖ Costly
 - ❖ Primary sequence analysis of Innovator product
 - ❖ N terminal sequencing of separated LC/HC
 - ❖ Double enzyme digests- LC -MS/MS, MALDI-TOF/TOF, MASCOT analysis
 - ❖ De Novo sequencing of peptides by MALDI-TOF/TOF



Mass analysis of Reduced and alkylated Fab By LC/MS



N-terminal sequencing of LC or HC



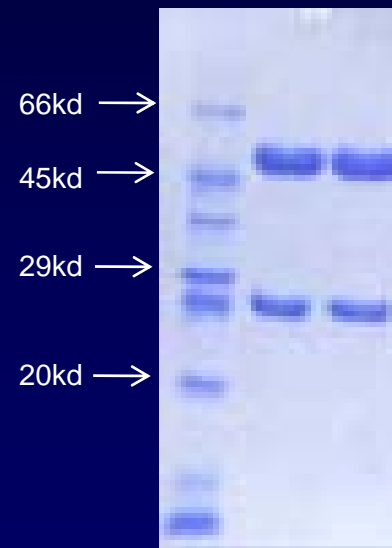
MALDI-TOF/TOF De Novo sequencing trace of enzymatic digests

Purity Analysis- SDS PAGE

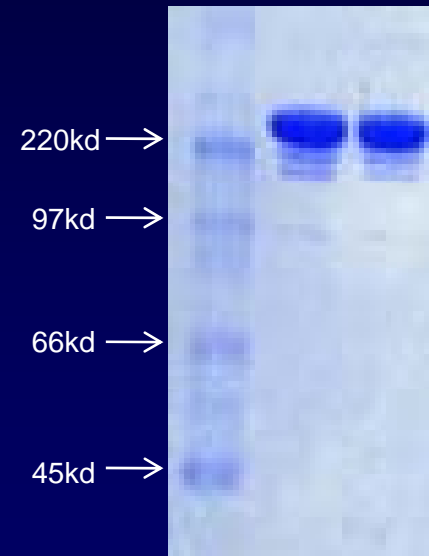


Adapted: PNAS, 2010, 107, 604-609

- ❖ Purity of bands
- ❖ Comparable mobility
- ❖ Presence of aggregates
- ❖ Pattern in Non-reducing gel
- ❖ Comparison of Main band intensity
- ❖ Relative abundance



SDS-PAGE Reducing



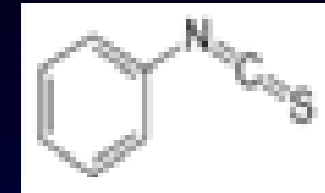
SDS-PAGE Non-reducing

N-terminal Sequencing



❖ Developed by Pehr Edman in 1950s

- ❖ N terminal AA derivatized with PITC and removed, rest chain intact
- ❖ Up to 10 residues



Phenyl isothiocyanate (PITC)

❖ Example

- ❖ Anti-IL2 receptor, Produced in NS0
 - ❖ Processing of HC signal sequence defective in innovator molecule
- ❖ Growth hormone (GH)
 - ❖ Biosimilar Showed Non-processivity of Methionine

Expected				Q	V	Q	L	V	Q	S
Observed	V	H	S	Q	V	Q	L	V	Q	S

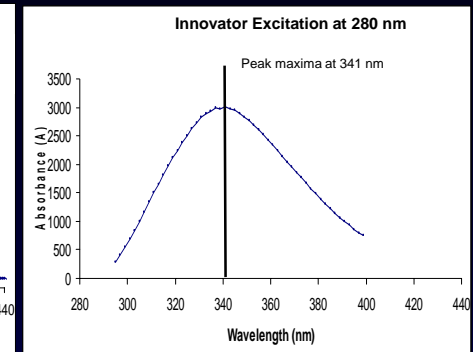
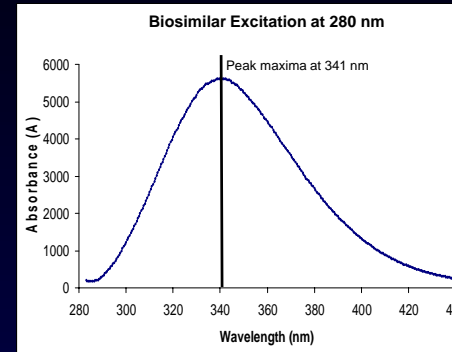
Expected		F	P	T	I	P	L	S	R	L
Observed	M	F	P	T	I	P	L	S	R	L

Secondary structure and folding



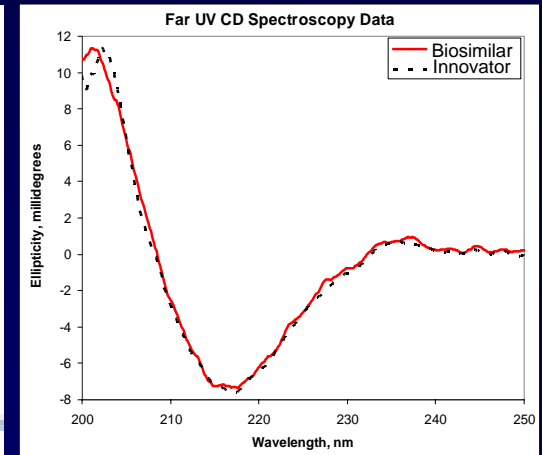
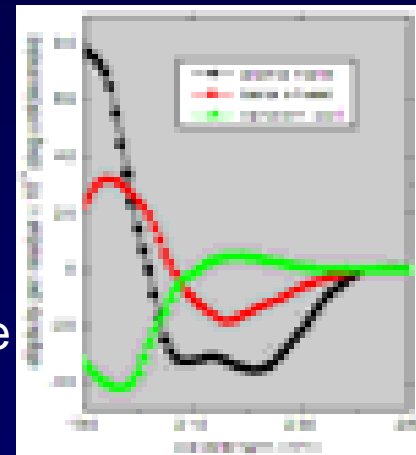
❖ Spectrofluorometric analysis

- ❖ F/Y/W fluorescence
- ❖ Improper folding Fluorescence profile changes
- ❖ Match Innovator vs. Biosimilar peak maxima



❖ Far UV CD spectroscopy

- ❖ 200-250 nm range
- ❖ Characteristic shape and magnitude
- ❖ Match Innovator vs. Biosimilar



Adapted: Poly L-Lysine CD far UV spectra analysis, Wikipedia

Characterization of Charge Heterogeneity



❖ Variations in acidic or basic species content

❖ Reasons

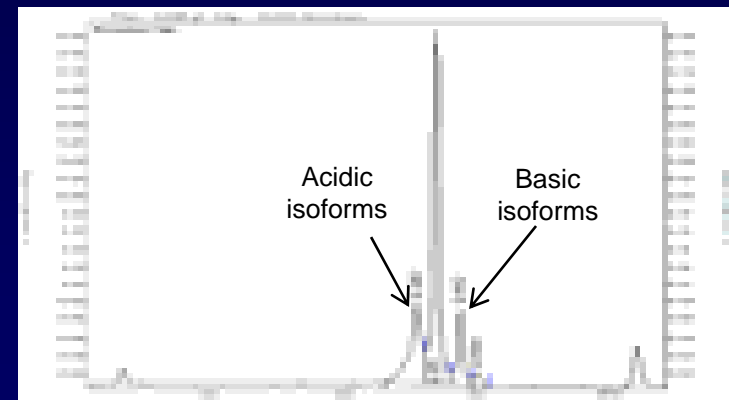
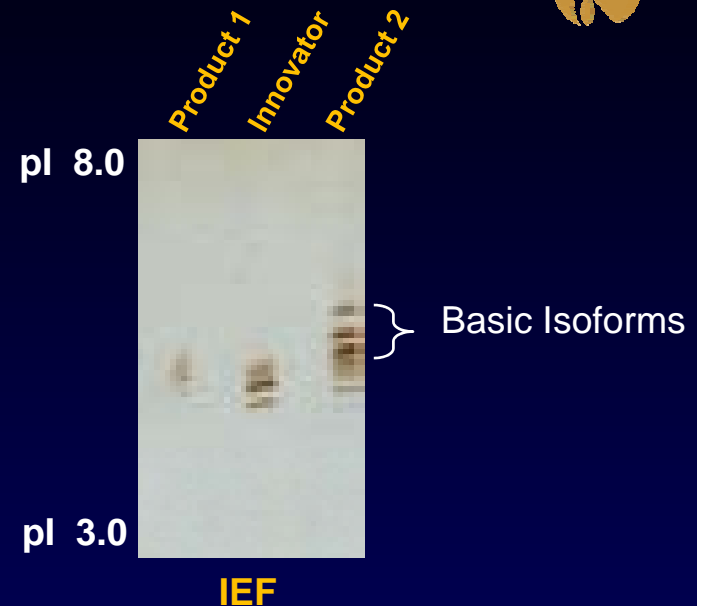
- ❖ Proteolytic degradation
- ❖ Deamidation
- ❖ Oxidation
- ❖ Differential glycosylation
- ❖ AA substitution/deletion

❖ Implications

- ❖ Product stability
- ❖ Product Quality

❖ Methods

- ❖ Isoelectric focusing (IEF)
- ❖ Capillary electrophoresis (CE)



Capillary electrophoresis

HPLC based characterization



❖ Size , charge based heterogeneity

❖ Recombinant Antibody A

- ❖ Initial clone HPLC (SEC) profile doesn't match
 - ❖ Strangely showed equivalent bioactivity
- ❖ Strategy changed, codon level changes done
- ❖ Perfect overlay with standard

❖ Product quality

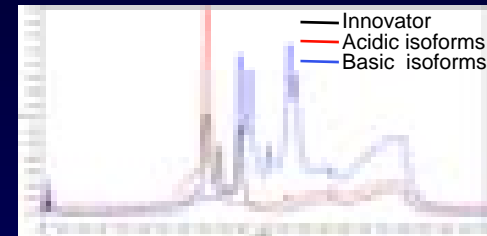
- ❖ Dimers and aggregates
- ❖ Impurity analysis

❖ Methods

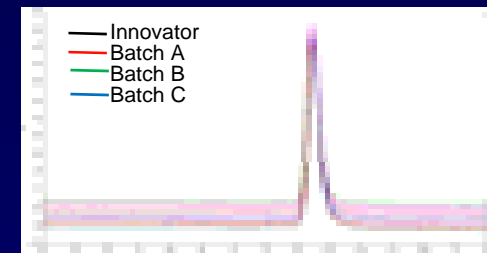
- ❖ Size exclusion (SEC)
- ❖ Weak Cation Exchange (WCEX)
- ❖ RP-HPLC



SEC HPLC

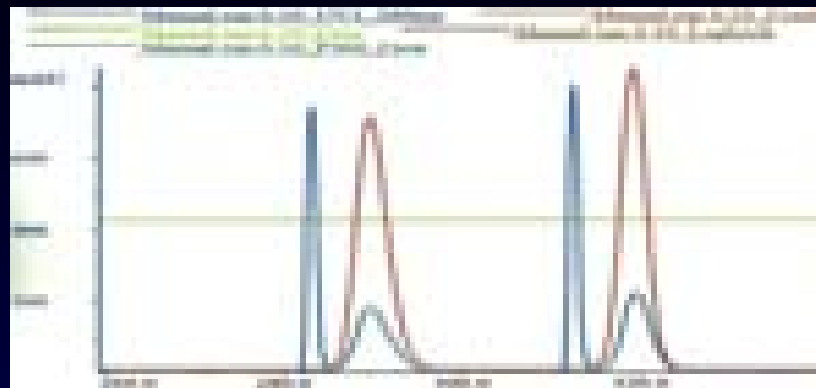


CE HPLC

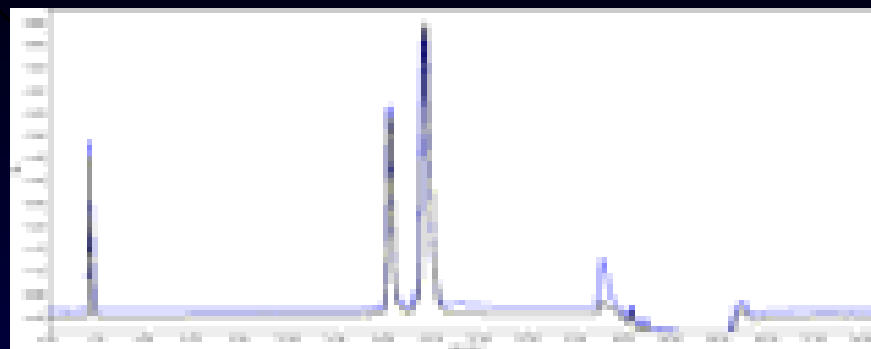


SEC HPLC

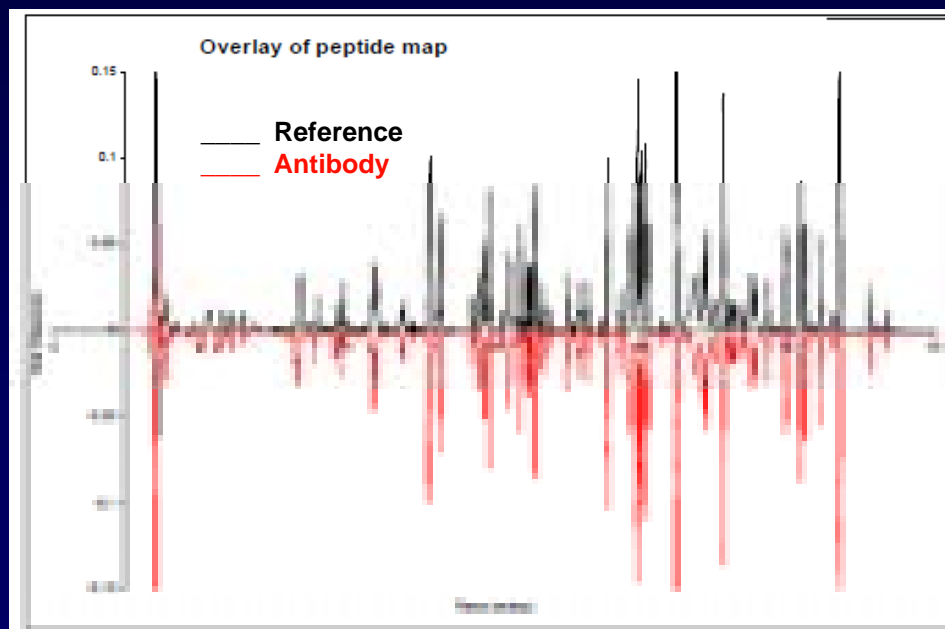
HPLC based characterization



Desalting chromatogram



RP-HPLC for chain separation



Peptide mapping of antibody molecule

Amino acid analysis



❖ Two step method

❖ Acid hydrolysis

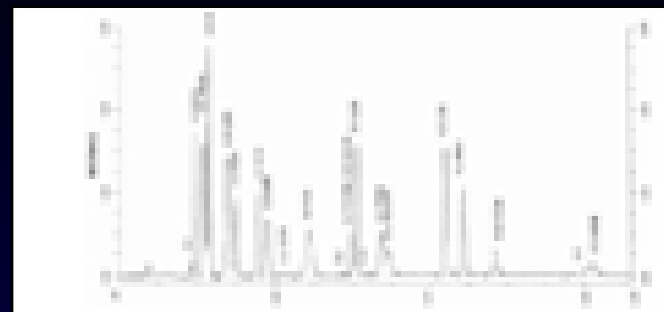
❖ CE HPLC

❖ Post column Ninhydrin reaction

❖ Detection at 570nm for most amino acids

❖ Detection at 440nm for Glu and Pro

❖ Compare biosimilar with Innovator



Amino acid	Predicted	Observed
Asp+Asn	49	51.9
Thr	56	54.6
Ser	88	87.9
Glu+Gln	61	58.1
Pro	48	45.7
Gly	44	44.1
Ala	40	40.5
Val	58	57.0
Met	6	0.8
Ile	14	13.5
Leu	45	47.3
Tyr	31	27.8
Phe	20	19.3
Lys	49	48.7
His	13	12.4
Arg	14	13.1
Cys	16	3.7
Trp	13	
Total	665	626..4

Mass spectrometry based Analysis



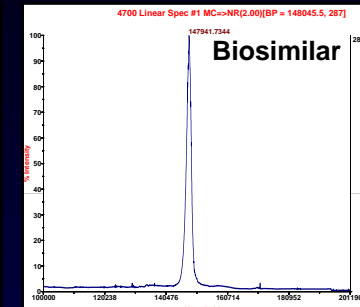
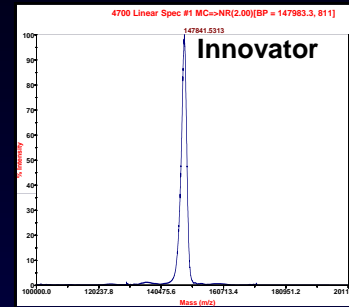
❖ Intact mass analysis

- ❖ Comparison- innovator vs. biosimilar

❖ Peptide mass fingerprinting

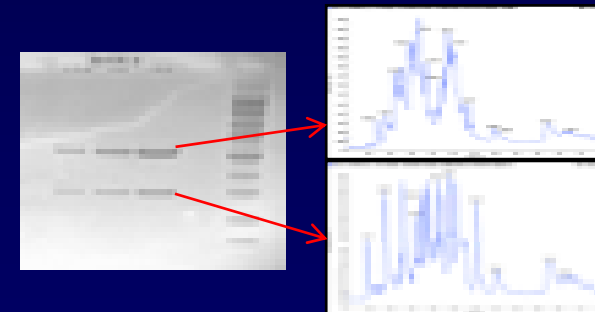
❖ Post translational modification

- ❖ Glycan profiling (1.4-2.4 kDa change)
- ❖ N terminal blocking (-17/-18 Da change)
- ❖ Deamidation (+1Da change)
- ❖ Succinimide (-17Da change)
- ❖ Lysine glycation (+162 Da change)
- ❖ C- terminal clipping (-128 Da change)



Biosimilar MAb	Intact Mass (Da)
Expected	147841.53
Observed	147941.73

Intact Mass analysis



Peptide mass Fingerprinting

Types of glycosylation



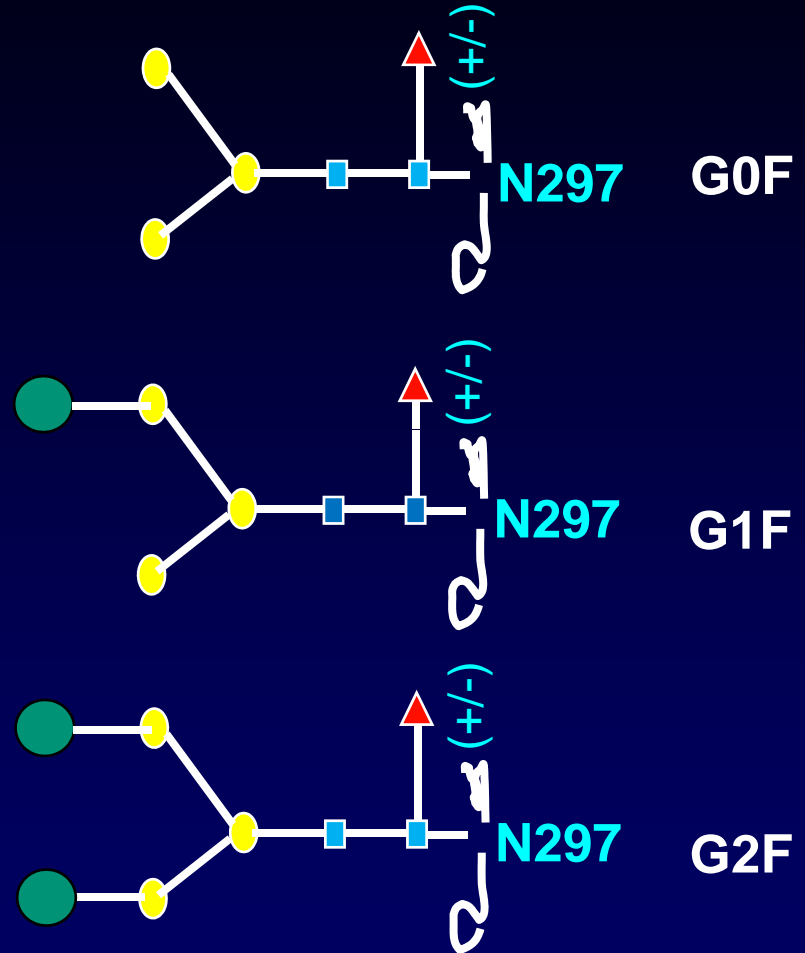
■ N- acetyl glucosamine (GlcNAc)

● Mannose

▲ Fucose

⬠ Sialic acid

● Galactose



Characterization of post translational modification



❖ Highly consistent post translational modification profile

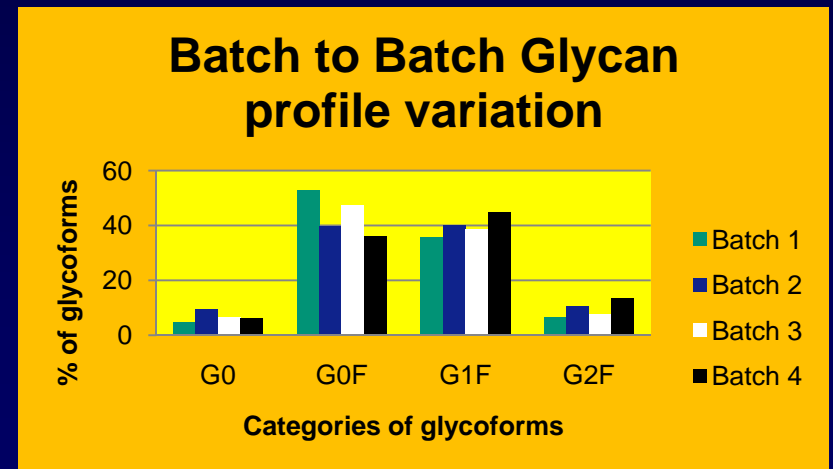
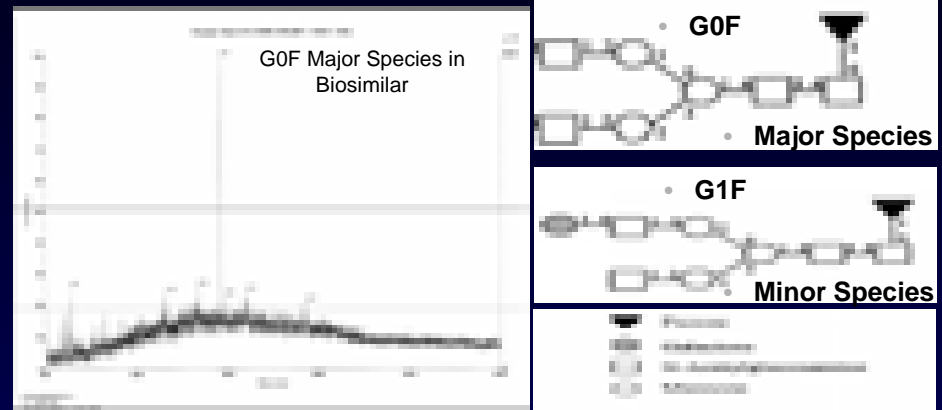
❖ Impact

- ❖ Potency
- ❖ Pharmacokinetics
- ❖ Pharmacodynamics
- ❖ Immunogenicity

❖ Increased production levels might compromise PTM machinery

❖ Impact

- ❖ Lower stability
- ❖ Batch to batch inconsistency



Adapted: J Am Soc Mass Spectrom 2009, 20, 2021–2033

Binding affinity characterization



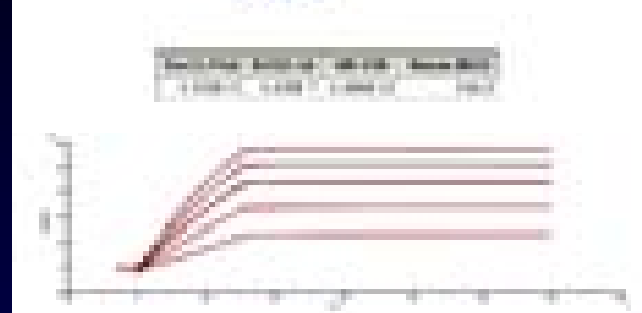
❖ Surface plasmon resonance (SPR)

- ❖ Measures binding affinity constant ' K_D '
 - ❖ Ratio of association constant ' K_a ' and dissociation constant ' K_d '
- ❖ Reflects strength of binding
- ❖ Lower ' K_D ' more potent
- ❖ Biosimilar comparability
- ❖ Novel mAbs
- ❖ Immunogenicity studies

Innovator

KD: 1.6×10^{-12}

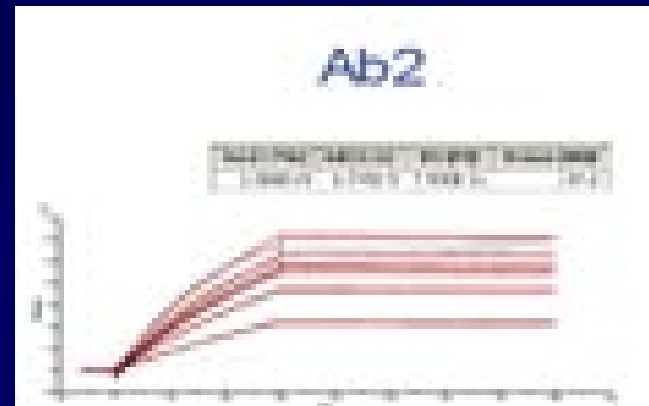
Ab1



Biosimilar T75 flask

KD: 8.0×10^{-11}

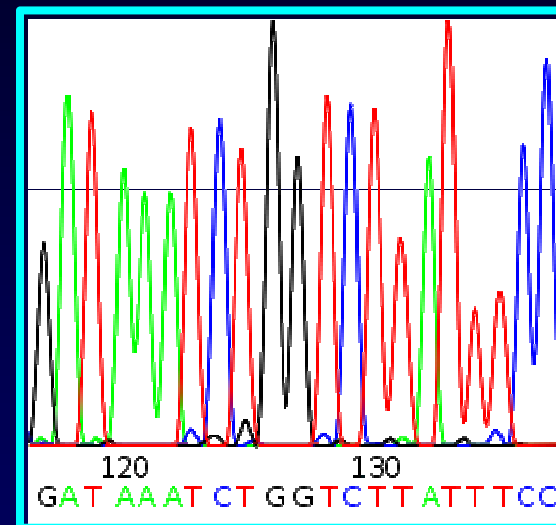
Ab2



DNA sequencing



- ❖ Cloning vector/ PCR product
- ❖ Expression vector
- ❖ Integrated cassette
- ❖ mRNA/ cDNA



upload.wikimedia.org

Clone characterization



- ❖ Copy number
- ❖ Real time PCR
- ❖ Southern blotting
- ❖ Northern blotting



Southern blot analysis

Lane	Id
1	Plasmid with gene of interest(GOI) - RE 1 digest
2	Plasmid with GOI- RE1 + 2 digest
M	Mol. Wt marker
3	CHOK1SV + GOI RE1 digest
4	CHOK1SV + GOI RE1 +2 digest digest
5	CHOK1 SV only

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Summary



- ❖ Selection of a range of techniques
- ❖ Understanding the product early
- ❖ Generation of data to get a range
- ❖ Use of Mass spectrometry
- ❖ Robust comparability program



Thank You!

