

Multiplexing of Protein Detection Capabilities

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Multiplexing of

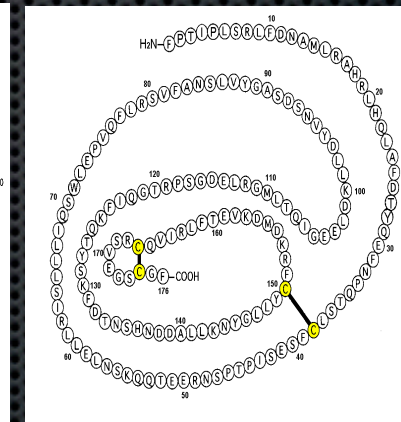
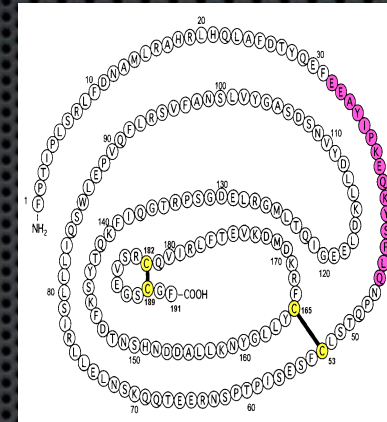
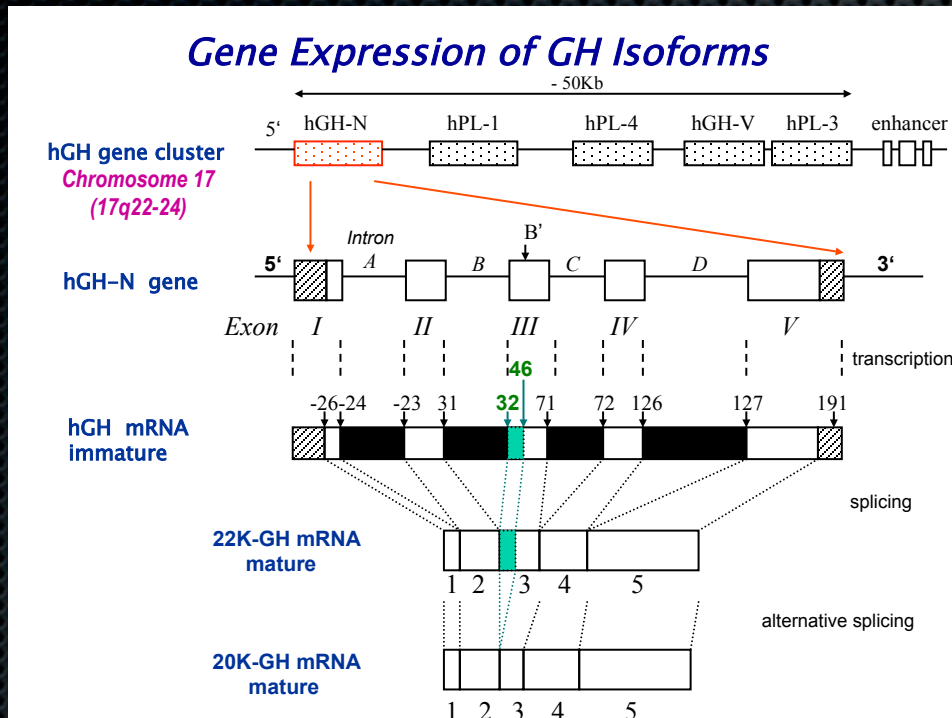
- Protein Assays:

Bead arrays for hGH isoforms

- Glycoprotein Assays:

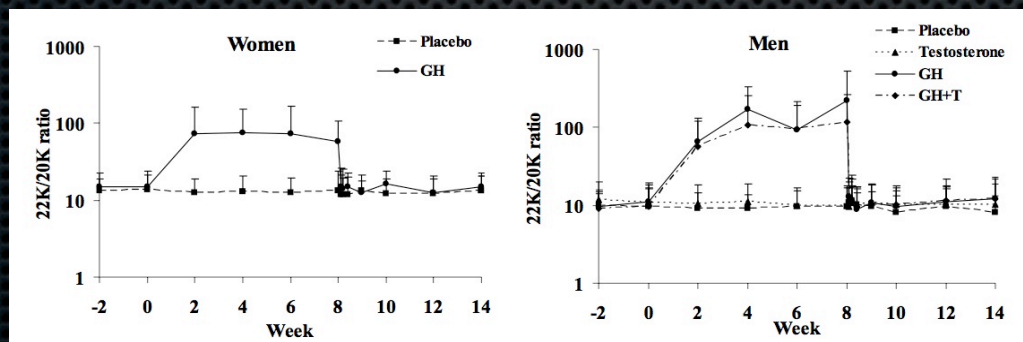
Lectin microarrays for glycoprotein hormones

Gene expression of hGH isoforms, and detection of hGH doping based on the compositions



22K-hGH

20K-hGH



GH application study on young athletes
Australia-Japan study

Assay Multiplexing

- **Advantage**

- Minimizing workload

- Less sample volume (Single aliquot)

- Less errors for calculating parameters

- Unification of operation

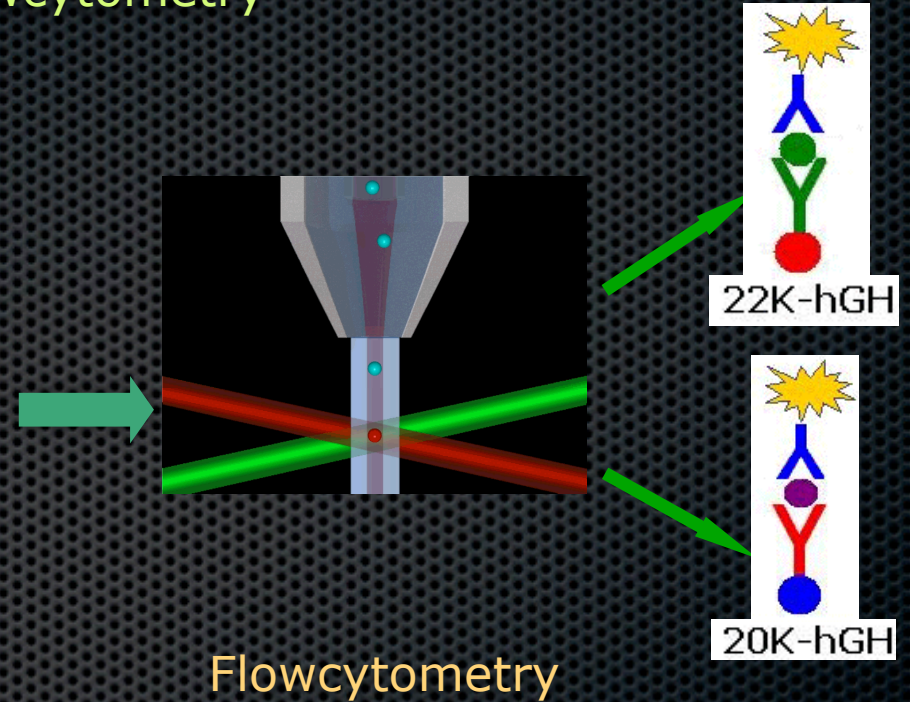
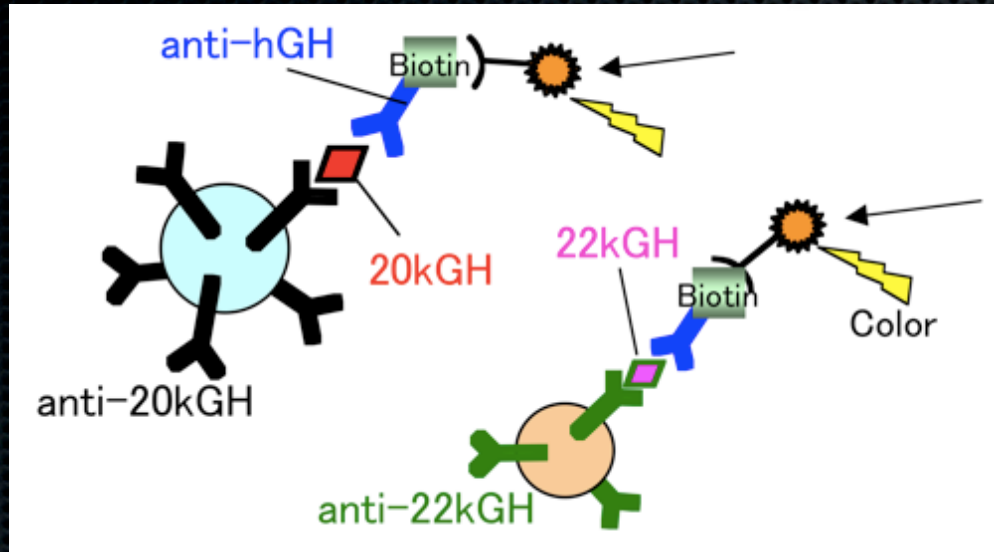
- Sensitivity (relative to mass spectrometry)

- **Disadvantage**

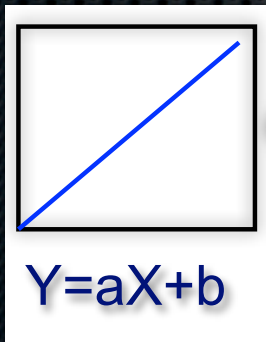
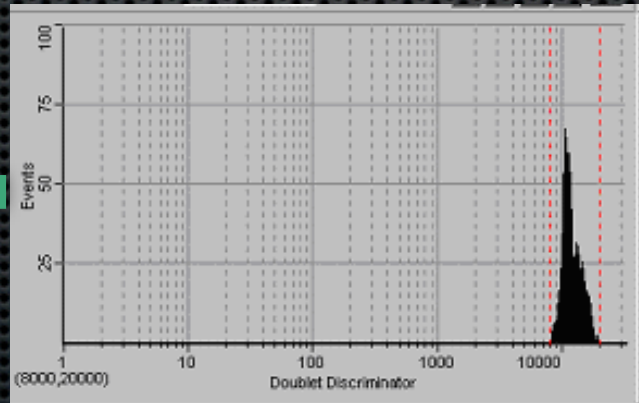
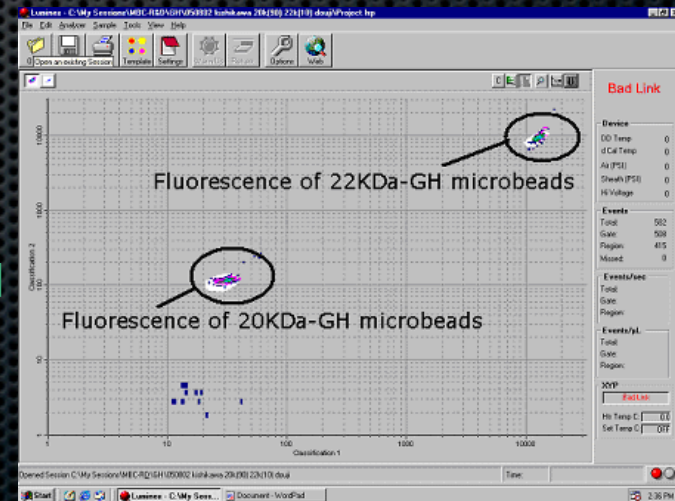
- Less flexible assay conditions

- Expandability (e.g. Micro chip)

Multiplexed hGH isoform assay by means of Flowcytometry



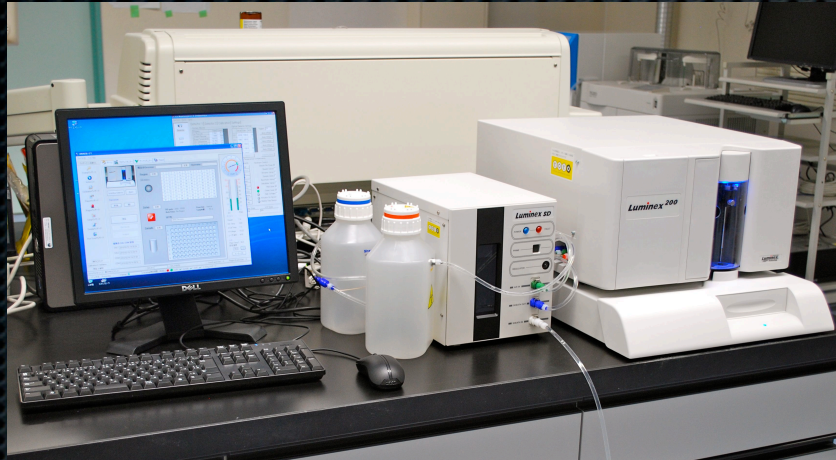
Flowcytometry



Calibration

Integration

Gating



Same principle as ELISA

Easily expandable & customizable

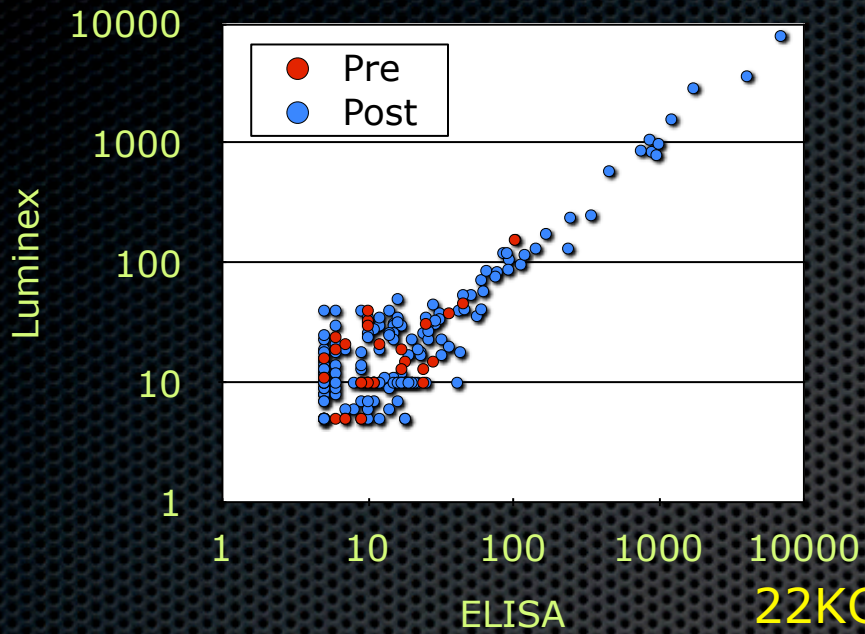
Similar system can be implemented to
normal flowcytometer

Assay Profiles

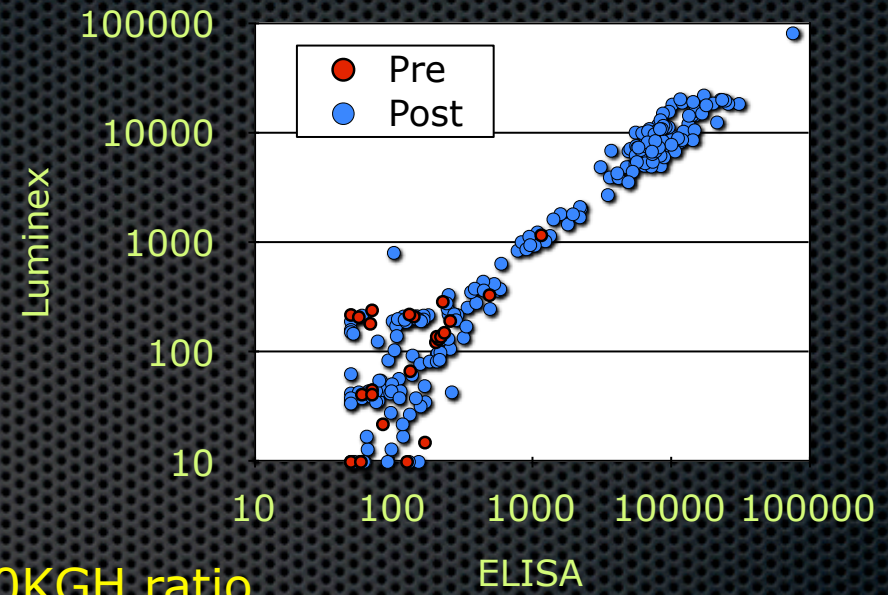
	ELISA		Luminex Multiplexed Assay	
	22K-GH	20K-GH	22K-GH	20K-GH
Capture Ab	A36030	D05	A36030	D05
Detection Ab	Biotinyl-anti-hGH AF1047		Biotinyl-anti-hGH AF1047	
Conjugate	Streptavidin-HRP Conjugate		Streptavidin R-phycoerythrin Conjugate	
Reaction	TMBZ			
Measurement	D 450nm / 620nm reference		Ex 532nm / Em 580 nm	
Reaction Reagent	TMBZ: 3,3',5,5'-tetramethylbenzidine (Peroxidase substrate "2-component system", KPL Cat No. 50-76-00)		Streptavidin, R-phycoerythrin conjugate (SAPE) 1 mg/mL Invitrogen Cat. No. S866	
A36030	Purified anti-hGH mouse Mab (Clone: A36030047P, IgG1κ from Biospecific)			
D05	Purified anti-20K-GH mouse Mab (Recloned in MCM, IgG1κ)			
22K-GH	WHO98/574 from NIBSC Lot.050413			
20K-GH	Recombinant 20KD-hGH. (Uchida et.al. <i>J Biotechnol.</i> 55 , 101-112,1997)			
Streptavidin	AMDEX® Streptavidin (Amersham Bioscience, Cat No. RPN4401V)			
Calibrator, QC	Sheep serum fortified with known ammounts of hGH isoforms			
Biotin	Biotin-XX Protein Labeling Kit (Molecular Probes, Cat No. F-4367)			

hGH application samples - ELISA vs Luminex -

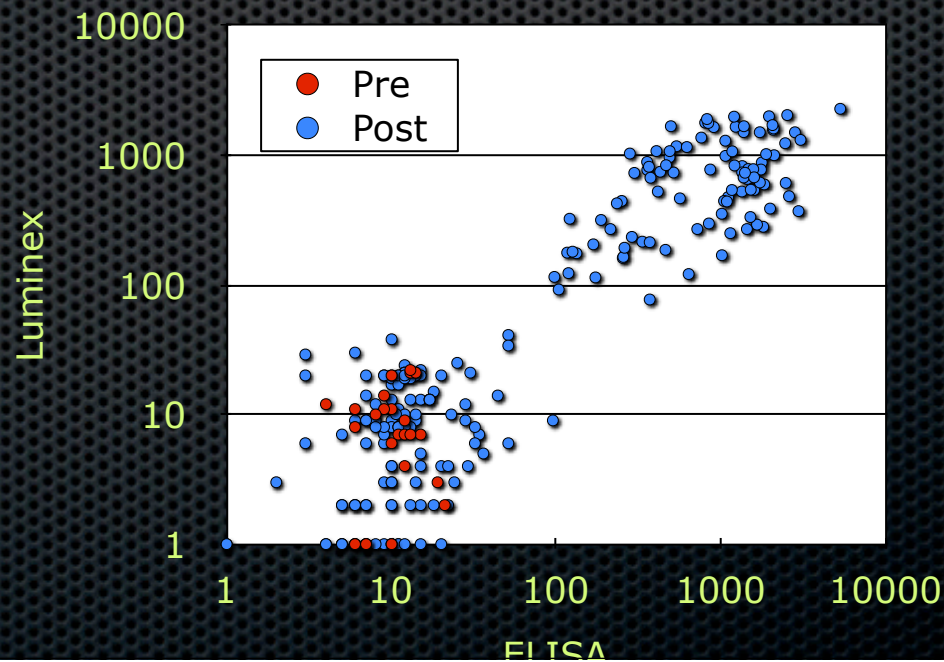
20K-GH



22K-GH

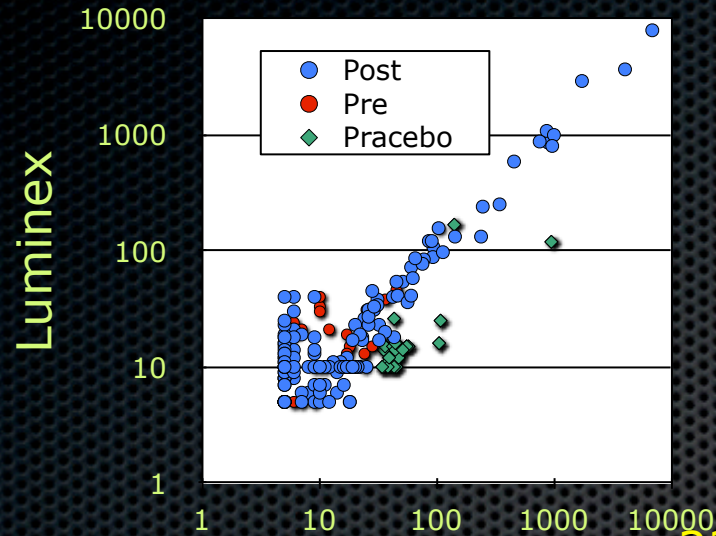


22KGH/20KGH ratio

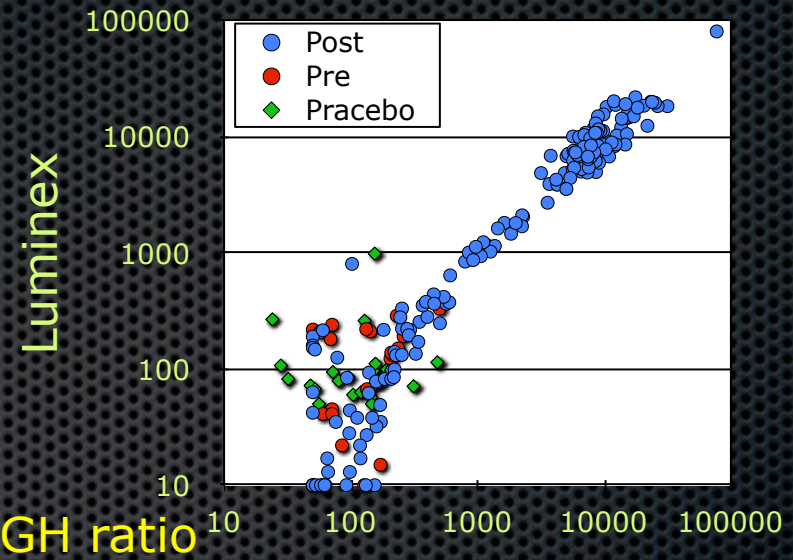


hGH application samples - ELISA vs Luminex -

20K-GH



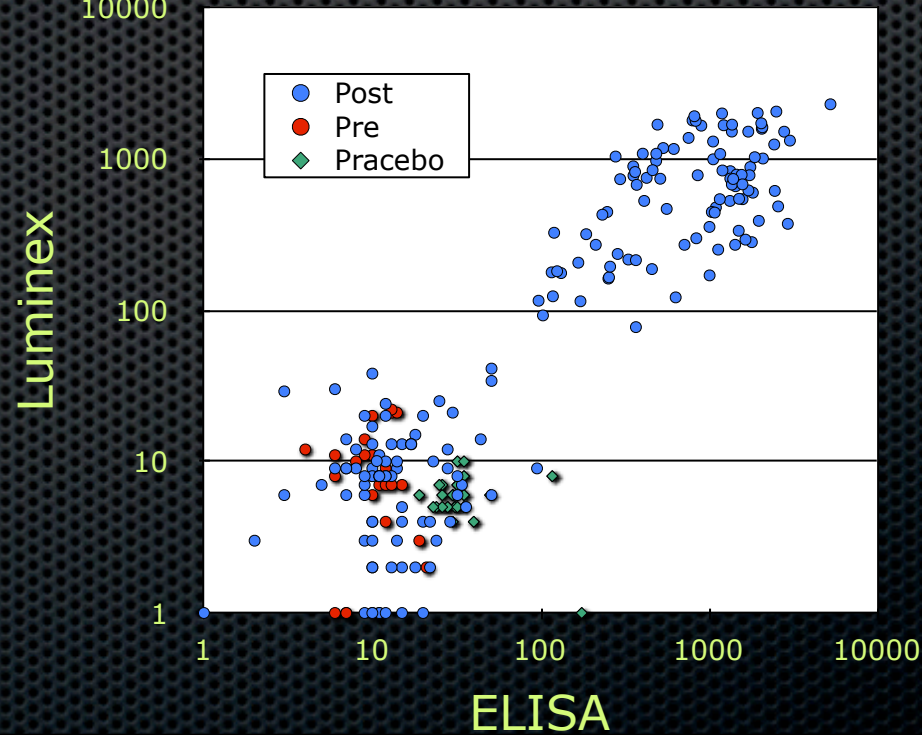
22K-GH



ELISA

22KGH/20KGH ratio

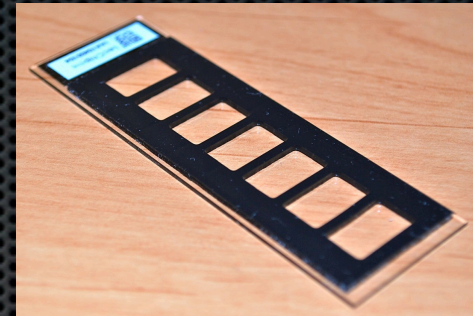
ELISA



Multiplexing by bead arrays

- Two or more targets in one measurement
- Essentially no lot-to-lot difference
- Need single sample volume only
- Adding new item does not increase manpower
- Ratio calculation can compensate errors

Lectin microarrays



- No doping test for glycanes was available.
- allow one step glycane profiling
- can identify glycoprotein hormones
- differentiate stereo isomers of saccharides
and the linkage

N-glycane profiles of EPO and the biosimilars by MS

n-Glycane	m/z	EPO-BRP	EPO- α	EPO- β	EPO- κ	ChinaEPO	NESP	CERA
BisAntennary	2,217	0.02	0.11	0.11	0.20	0.17	0.14	0.21
BisAntennary Sia1	2,522	1.01	0.31	0.37	0.12	0.77	0.34	0.56
BisAntennary Sia2	2,827	0.46	0.17	0.19	0.04	0.49	0.13	0.19
TriAntennary	2,582	0.05	0.08	0.14	0.12	0.08	0.23	0.16
TriAntennary Sia1	2,887	0.24	0.29	0.44	0.48	0.21	0.79	0.44
TriAntennary Sia2	3,192	0.53	0.40	0.50	0.33	0.38	0.71	0.47
TriAntennary Sia3	3,497	0.35	0.15	0.15	0.05	0.27	0.16	0.10
TetraAntennary	2,947	0.00	0.06	0.06	0.10	0.05	0.15	0.07
TetraAntennary Sia1	3,252	0.13	0.18	0.29	0.29	0.09	0.56	0.27
TetraAntennary Sia2	3,557	0.32	0.42	0.38	0.37	0.11	0.96	0.30
TetraAntennary Sia3	3,862	0.29	0.35	0.26	0.17	0.29	0.57	0.15
TetraAntennary Sia4	4,167	0.11	0.10	0.05	0.03	0.01	0.10	0.02
TetraAnt Lac1 Sia1	3,617	0.00	0.06	0.03	0.13	0.02	0.04	0.02
TetraAnt Lac1 Sia2	3,922	0.01	0.11	0.02	0.17	0.02	0.07	0.00
TetraAnt Lac1 Sia3	4,228	0.01	0.16	0.00	0.19	0.03	0.04	0.01
TetraAnt Lac1 Sia4	4,533	0.04	0.02	0.00	0.05	0.01	0.00	0.00
TetraAnt Lac2 Sia2	4,287	0.16	0.00	0.00	0.05	0.00	0.00	0.00
TetraAnt Lac2 Sia3	4,593	0.00	0.03	0.01	0.11	0.00	0.00	0.00
No of N-Glycanes		3	3	3	3	3	5	3
Acidity Index		6.88	5.58	4.76	4.82	4.95	8.19	4.10
					Biosimilars			

Acidity index: NESP > EPOBRP > EPO- α > EPO- β , EPO- κ , China-EPO > CERA

Biosimilar EPO does have different N-glycane composition and different terminal sialic acid structure.

Acetylation of EPO O-glycanes

O-Glycan	Fragment	mass	Contents of O-Glycopeptide					
			BRP	EPO- α	EPO- β	EPO- κ	NESP	CERA
Hex ₁ HexNAc ₁	I	1830	0.02	0.02	0.04	0.02	0.02	0.02
Hex ₁ HexNAc ₁ Sia ₁	I	2121	0.48	0.50	0.54	0.45	0.34	0.52
Hex ₁ HexNAc ₁ Sia ₁ monoAcetylated	I	2163	0.03	0.02	0.01	0.02	0.03	0.02
Hex ₁ HexNAc ₁ Sia ₂	I	2412	0.35	0.40	0.39	0.44	0.40	0.37
Hex ₁ HexNAc ₁ Sia ₂ monoAcetylated	I	2454	0.07	0.04	0.01	0.05	0.14	0.05
Hex ₁ HexNAc ₁ Sia ₂ bisAcetylated	I	2496	0.05	0.01	0.00	0.03	0.08	0.02

Certain amounts of O-glycanes of recombinant EPO was detected as the acetate.

Human cell vs CHO

CHO cells do not express β -galactoside α 2,6-sialyl transferase (α 2,6-STN)

Leland et.al. *J.Biol.Chem* **269**(14), 10628 (1994)

CHO

$(\text{NeuAc}\alpha 2 \rightarrow 3)_{1-3} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1-6$ $>$ Man-R
 $(\text{NeuAc}\alpha 2 \rightarrow 3)_{1-3} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1-3$ $>$ Man-R

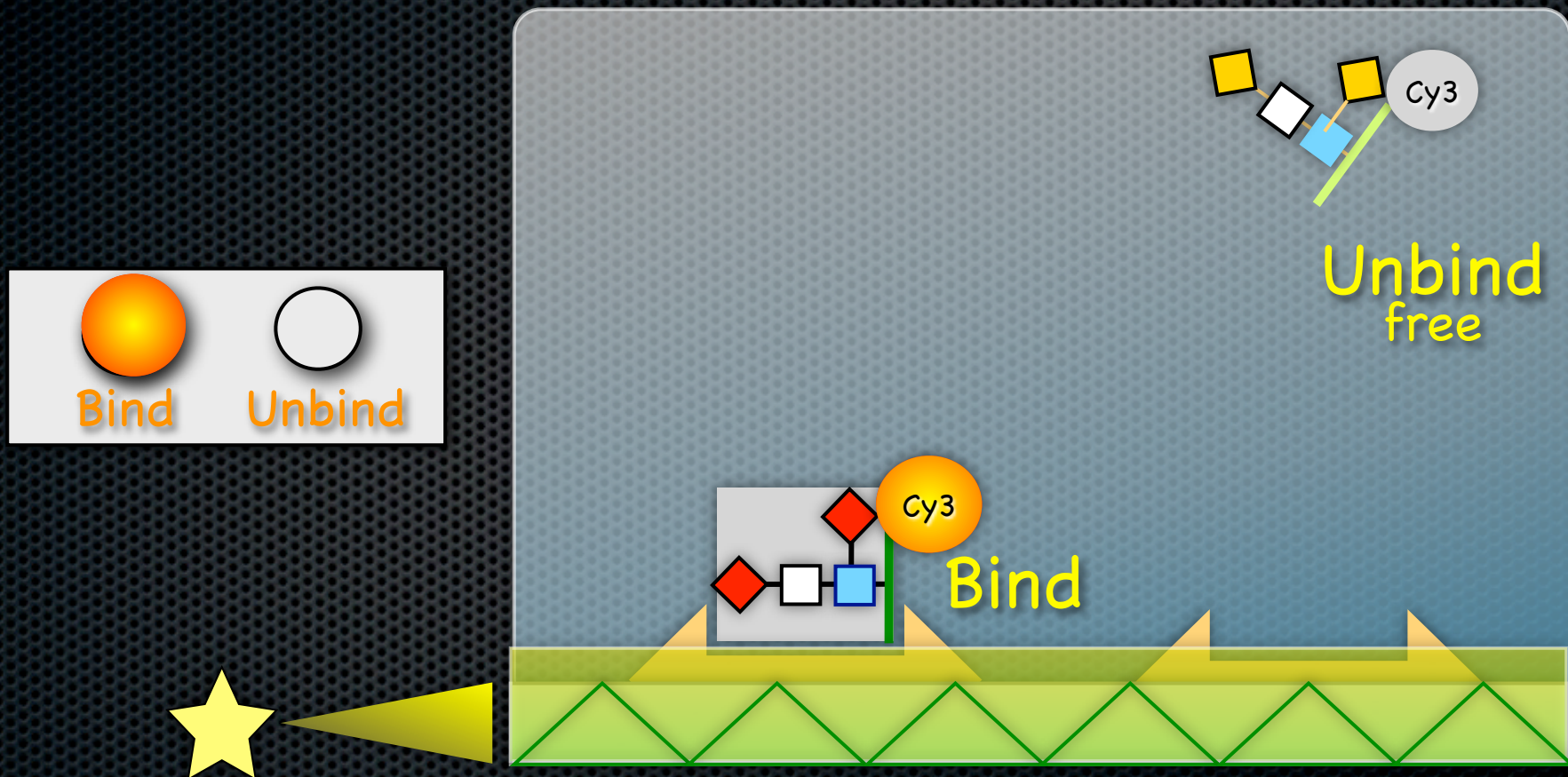
Human

$(\text{NeuAc}\alpha 2 \rightarrow 6)_{1-3} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1-6$ $>$ Man-R
 $(\text{NeuAc}\alpha 2 \rightarrow 3)_{1-3} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1-3$ $>$ Man-R

Kihata Y, *Tanpakushitsu Kakusan Koso (In Japanese)*, **36**(5), 775-788 (1991)

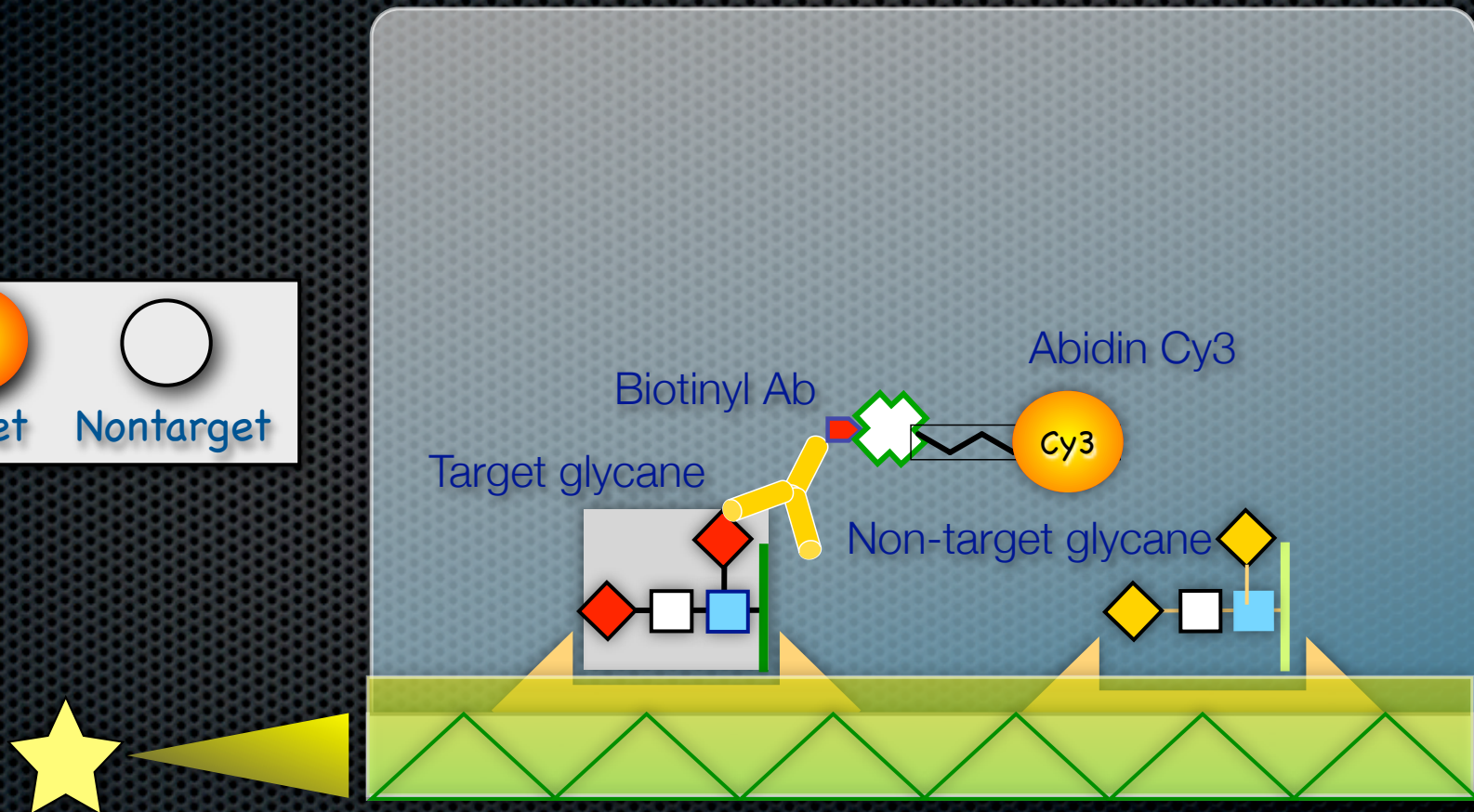
Lectin Microarray Chip technology

Measurement of Evanescent Fluorescence
without washing and B/F separation

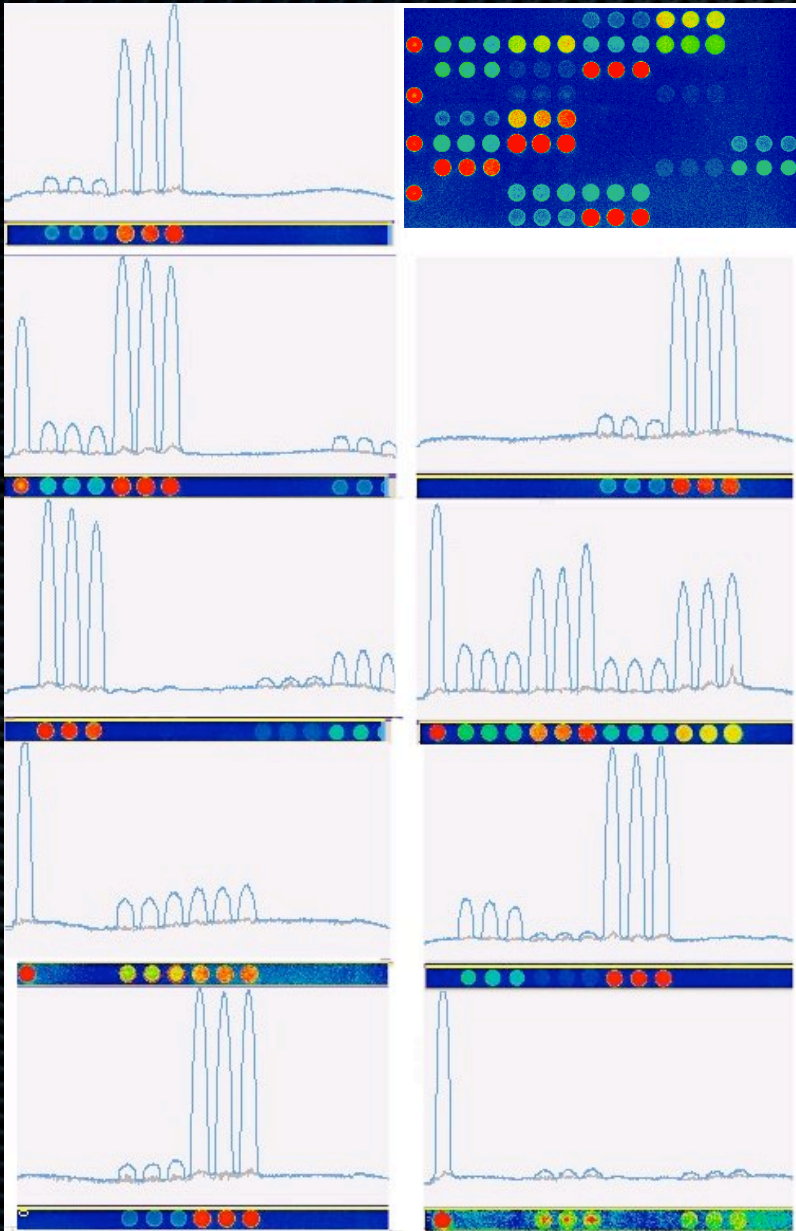


Lectin Microarray Chip technology

Antibody assisted detection of targetted glycanes
- Lectin-Ab sandwich detection mode-



Lectin microarray detection



Glycopeptide



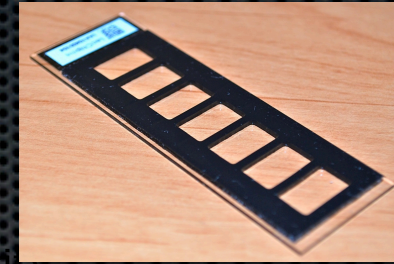
Buffer Exchange
Cy3 labeling
Remove excess Cy3
20°C 3hrs on chip

Image Scanning

(Measurement of evanescent fluorescence)

Integration of detector response

Layout of lectin microarrays



A list of lectins on sensor chip and the specificity

	1. LTL	10. TJA-I	19. GNA	28. STL	37. VVA
PC	2. PSA	11. PHAL	20. HHL	29. UDA	38. DBA
	3. LCA	12. ECA	21. ACG	30. PWM	39. SBA
PC	4. UEA-I	13. RCA120	22. TxLCI	31. Jacalin	40. Calsepa
	5. AOL	14. PHAE	23. BPL	32. PNA	41. PTL-I
PC	6. AAL	15. DSA	24. TJA-II	33. WFA	42. MAH
	7. MAL	16. GSL-II	25. EEL	34. ACA	43. WGA
PC	8. SNA	17. NPA	26. ABA	35. MPA	44. GSL-I A4
	9. SSA	18. ConA	27. LEL	36. HPA	45. GSL-I B4

SNA & SSA recognize NeuAc α 2-6 Gal/GalNAc



Epoetin- α
(CHO cell line)



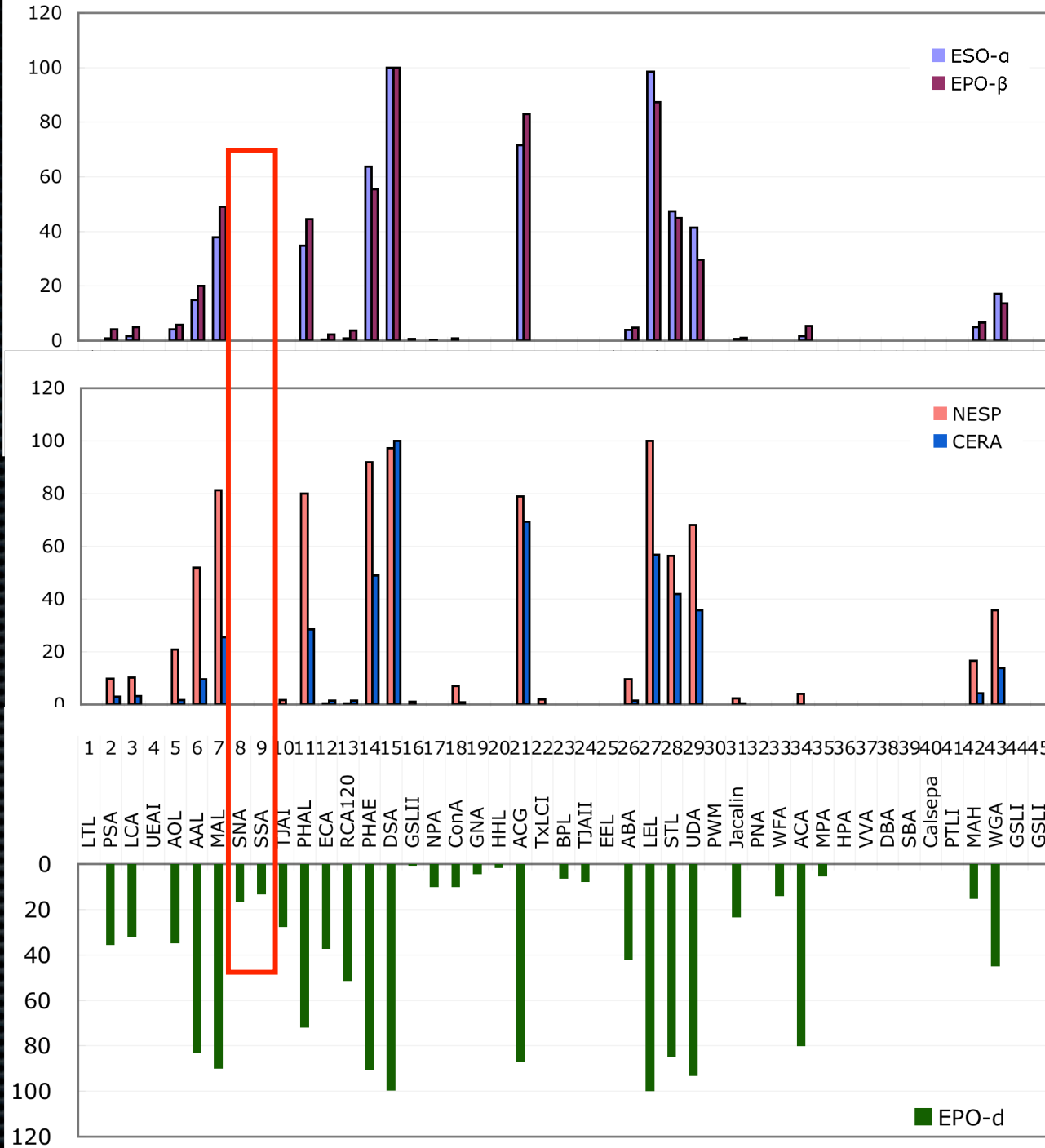
Epoetin- δ
(Human cell line)

SNA/SSA

ESA

CHO cell line

Human cells



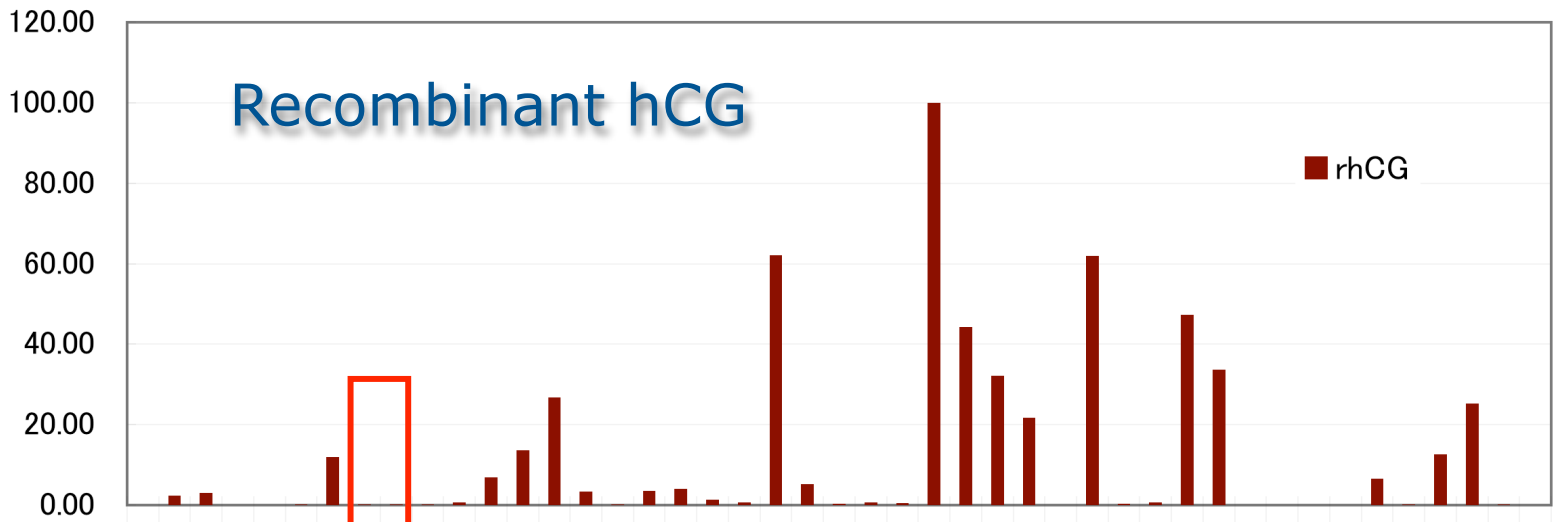
EPO-α
EPO-β

NESP
CERA

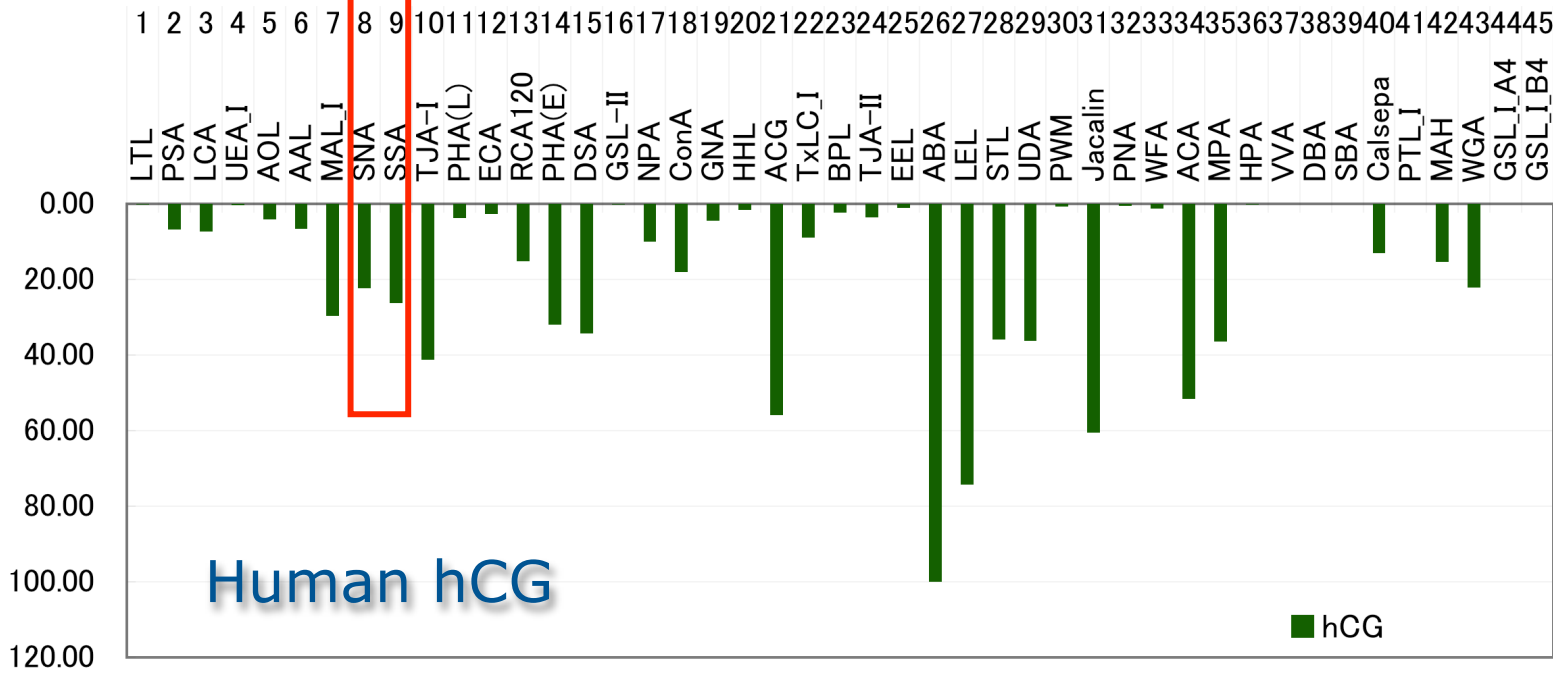
EPO-δ

Choriogonadotropin

CHO cell line



Pregnant urine



Lectin Microarray

(lectin-glycan interaction)

- Glycoprotein shows identical lectin spectra.
- identifies origin of recombinant products based on the inter-species difference of glycan expression.
- recognizes stereo isomers and the linkage
which cannot be differentiated by mass spectrometry.
- is robust and sensitive.
- has high sample throughput.