Hilly village lanes,
Whitewashed sunlit walls.
Cerulean sea.
The laughter of children.

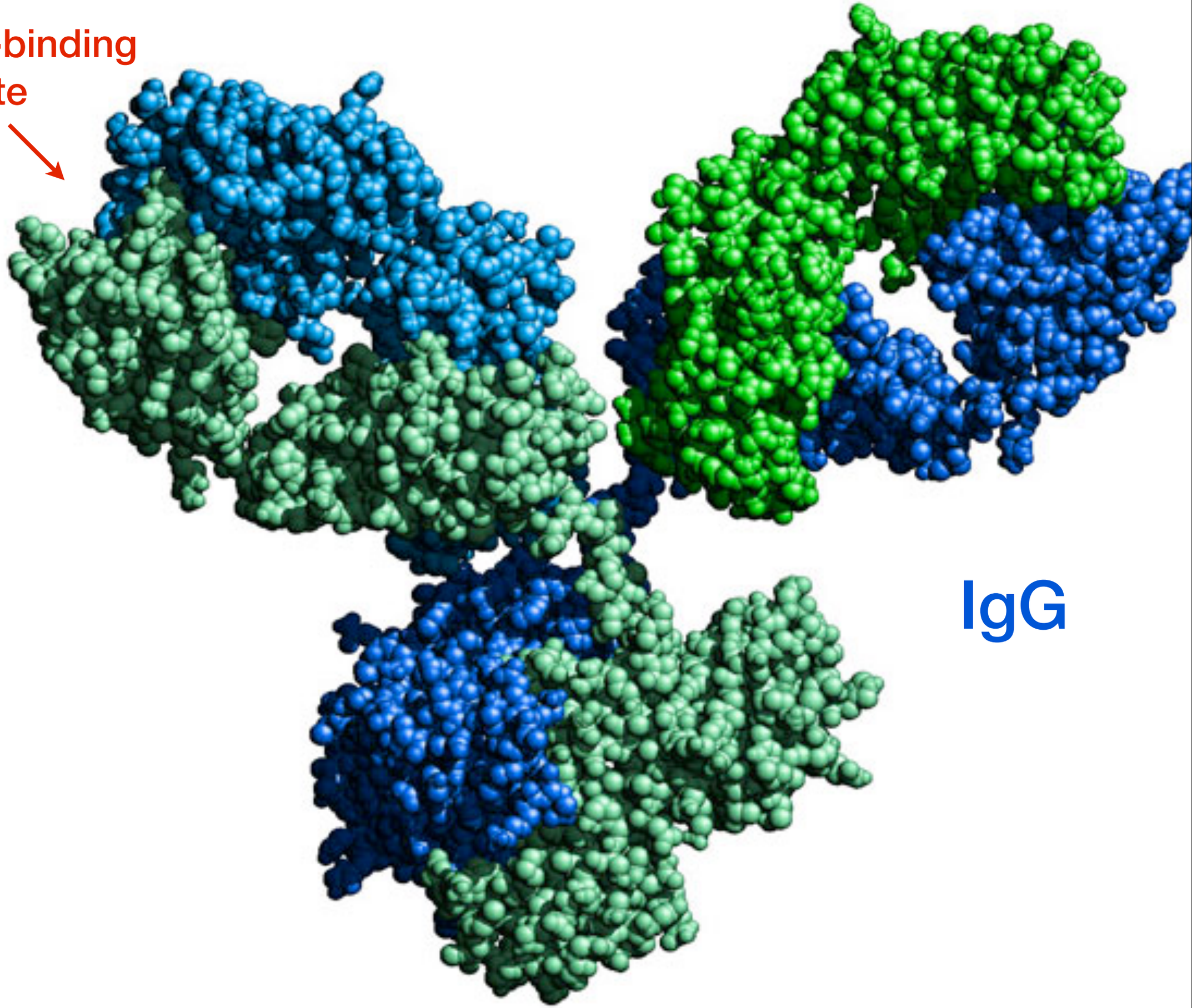
–Deng Ming-Dao

Application of LC-MS/MS and LBA methods in concert for bioanalysis of monoclonal antibody oncology drugs and associated soluble target receptors

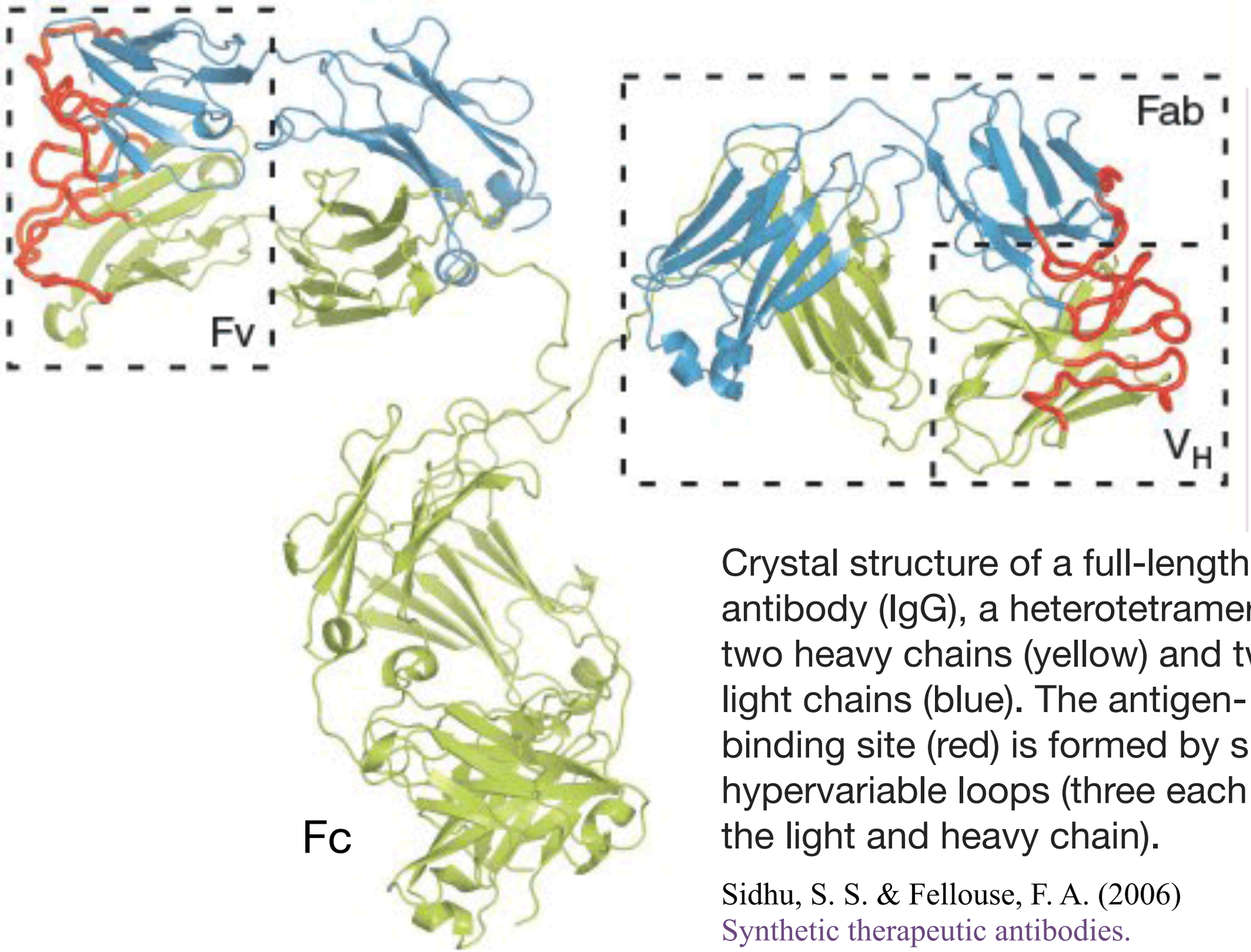
EBF 6th Open Meeting, Barcelona Spain, 20 Nov 2013

Rand Jenkins

antigen-binding site



IgG

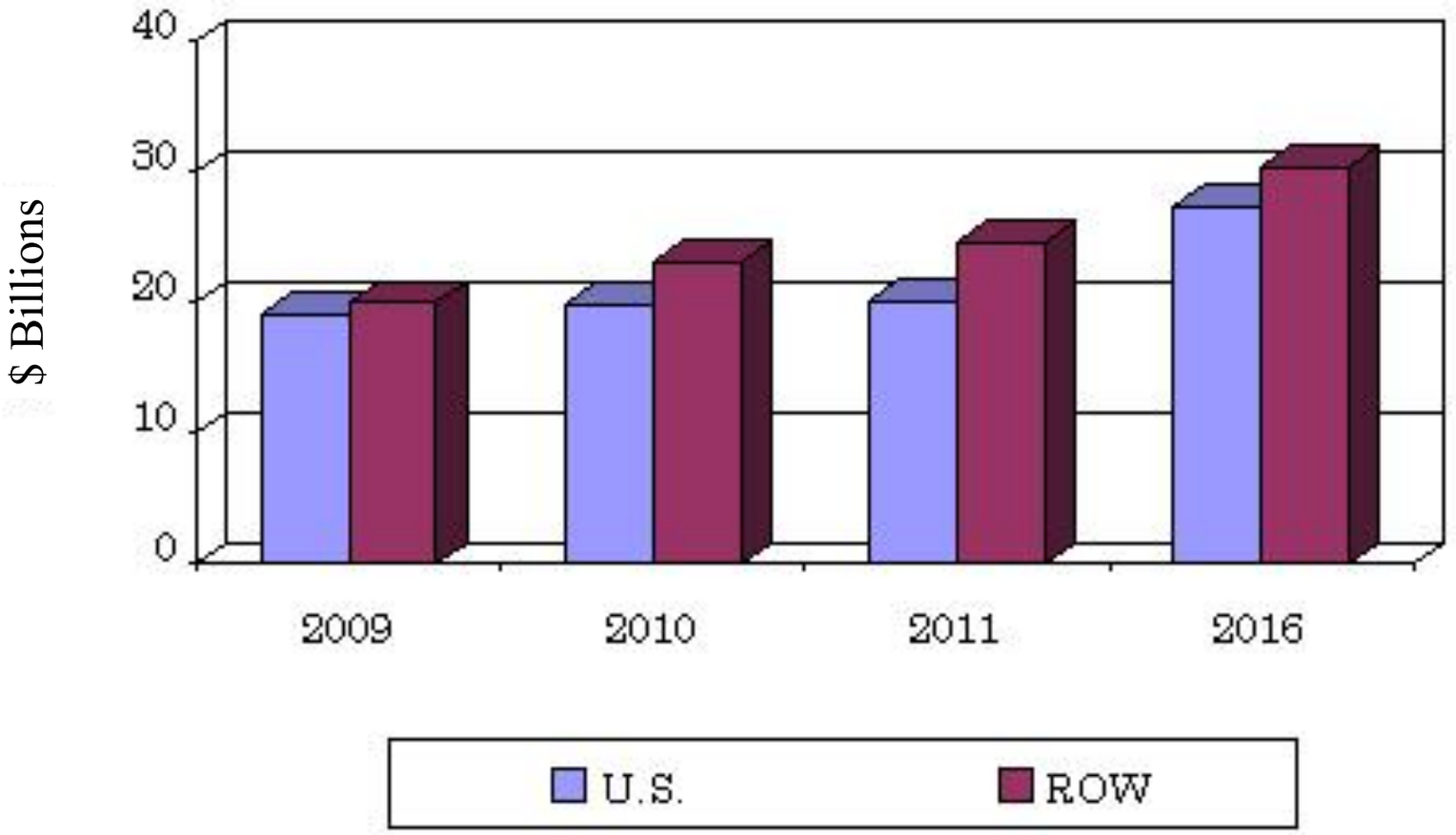


Crystal structure of a full-length antibody (IgG), a heterotetramer of two heavy chains (yellow) and two light chains (blue). The antigen-binding site (red) is formed by six hypervariable loops (three each from the light and heavy chain).

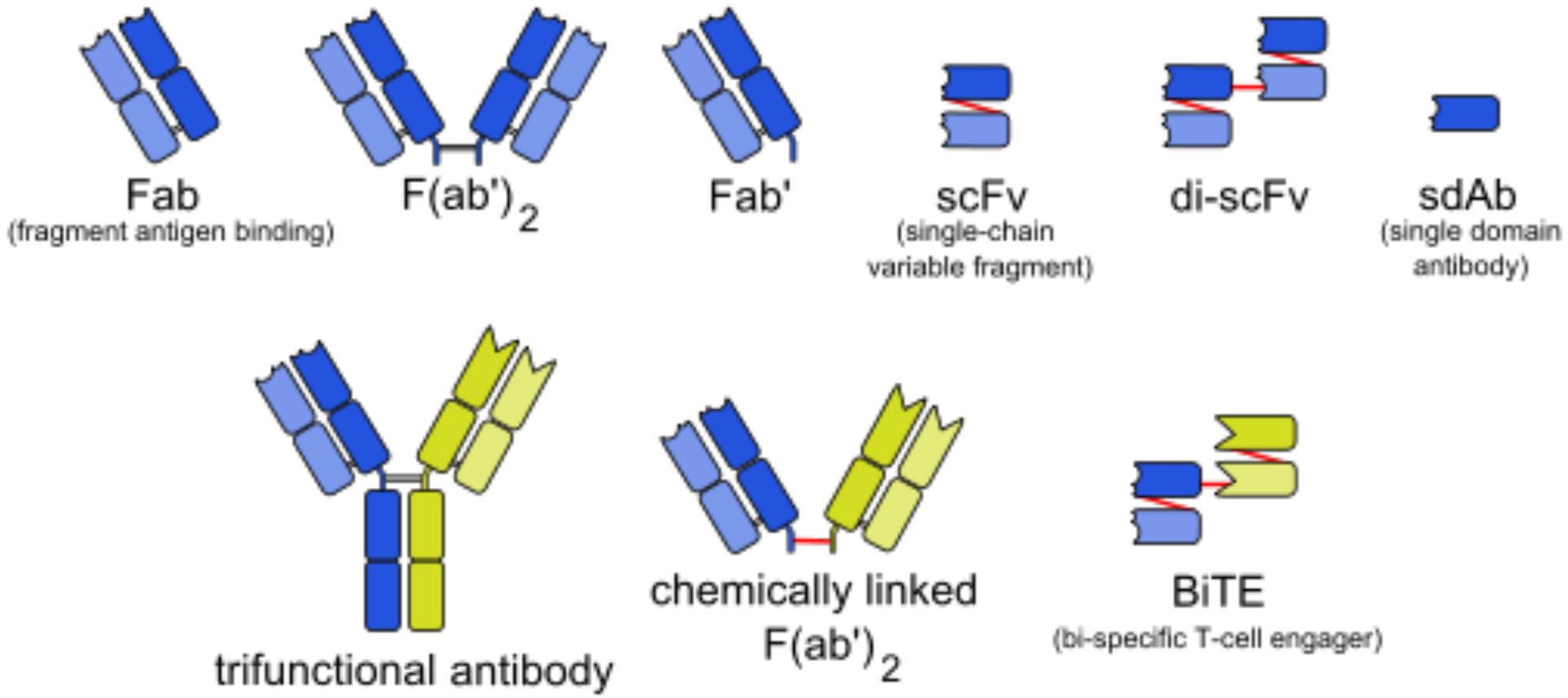
Sidhu, S. S. & Fellouse, F. A. (2006)
Synthetic therapeutic antibodies.
Nat. Chem. Biol. 12, 682-8.

"The global market for therapeutic monoclonal antibodies (mAbs) is expected to rise at a compound annual growth rate (CAGR) of 5.3% to nearly $58 billion in 2016."

GLOBAL SALES OF THERAPEUTIC MONOCLONAL ANTIBODIES, 2009-2016
($ BILLIONS)

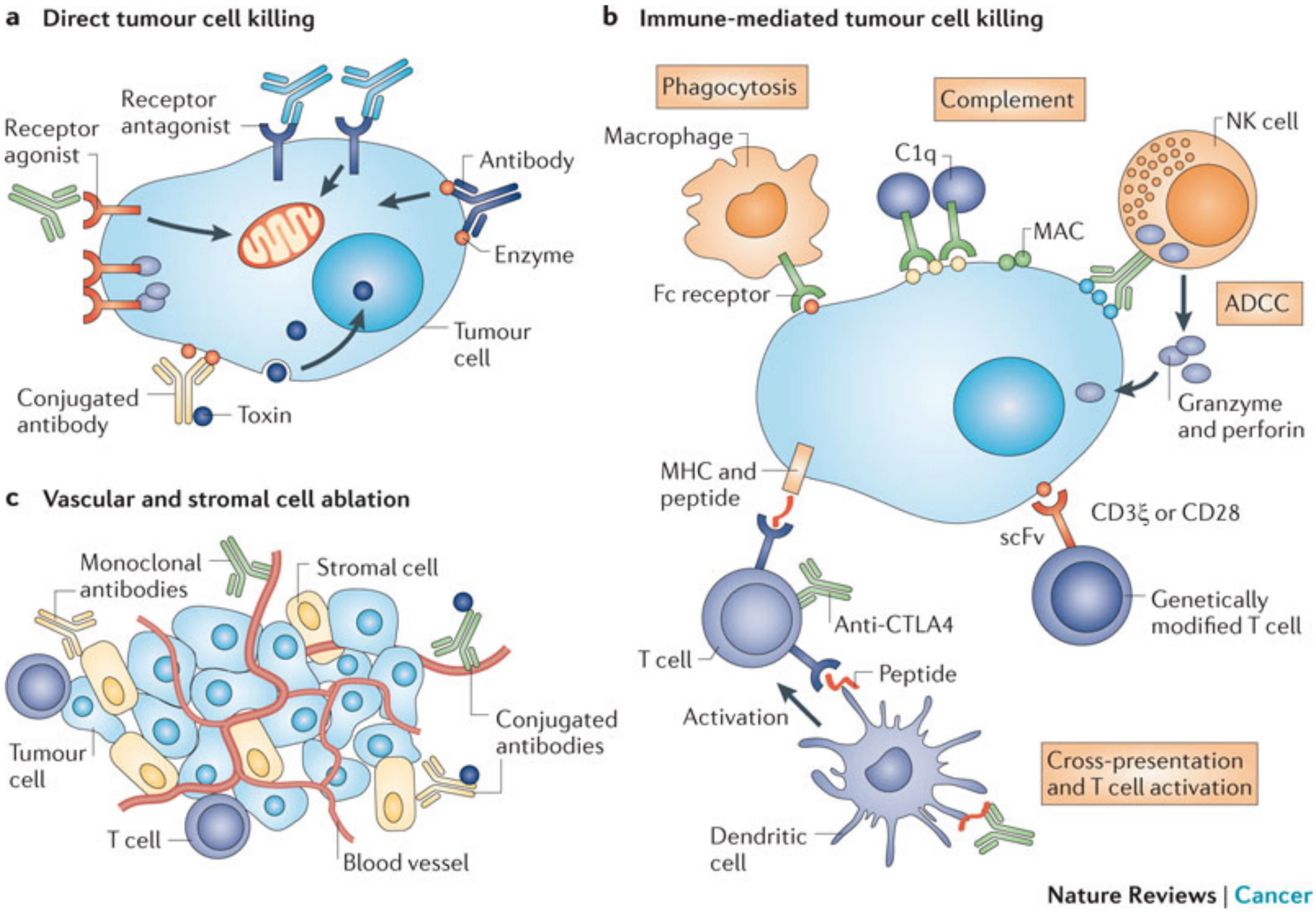


Many types of “non-natural” mAbs also being pursued



mAb therapeutics in Oncology

Mechanisms of tumour cell killing by antibodies.



Scott AM, Wolchok JD, & Old LJ, Antibody therapy of cancer, *Nature Reviews Cancer* **12**, 278-287 (April 2012)

Table 3. Monoclonal antibodies currently FDA-approved in oncology

Antibody	Target	FDA-Approved indication	Mechanism of action
Trastuzumab (Herceptin®) * humanized IgG1	HER2 (ErbB2)	HER2-positive breast cancer, as single agent or in combination with chemotherapy for (i) adjuvant or (ii) palliative treatment; HER2-positive gastric or gastroesophageal junction carcinoma, as first-line treatment in combination with cisplatin and capecitabine/5-FU	Inhibition of HER2 signaling; ADCC
Bevacizumab (Avastin®) humanized IgG1	VEGF	For the palliative treatment of colorectal cancer, non-squamous non-small cell lung cancer, glioblastoma, or renal cell carcinoma	Inhibition of VEGF signaling
Cetuximab (Erbitux®)* chimeric human/murine IgG1	EGFR (ErbB1)	In combination with radiation therapy for the initial treatment of locally or regionally advanced squamous cell cancer of the head and neck (SCCHN); As a single agent for SCCHN patients with whom prior platinum-based therapy has failed; Palliative treatment of pre-treated metastatic EGFR-positive colorectal cancer	Inhibition of EGFR signaling; ADCC
Panitumumab (Vectibix®)* human IgG2	EGFR (ErbB1)	As a single agent for the treatment of pre-treated EGFR-expressing, metastatic colorectal carcinoma	Inhibition of EGFR signaling
Ipilimumab (Yervoy®) IgG1	CTLA-4	For the treatment of unresectable or metastatic melanoma	Inhibition of CTLA-4 signaling
Rituximab (Rituxan® and Mabthera®) * chimeric human/murine IgG1	CD20	For the treatment of CD20-positive B cell non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukemia (CLL), and for maintenance therapy for untreated follicular CD20-positive NHL	ADCC; direct induction of apoptosis; CDC
Alemtuzumab (Campath®) humanized IgG1	CD52	As a single agent for the treatment of B cell CLL	Direct induction of apoptosis; CDC
Ofatumumab (Arzerra®) human IgG1	CD20	Treatment of patients with CLL refractory to fludarabine and alemtuzumab	ADCC; CDC
Gemtuzumab ozogamicin (Mylotarg®) humanized IgG4	CD33	For the treatment of patients with CD33-positive acute myeloid leukemia in first relapse who are 60 years of age or older and who are not considered candidates for other cytotoxic chemotherapy (withdrawn from use in June 2010)	Delivery of toxic payload, calicheamicin toxin
Brentuximab vedotin (Adcetris®) chimeric IgG1	CD30	For the treatment of relapsed or refractory Hodgkin lymphoma and systemic anaplastic lymphoma	Delivery of toxic payload, auristatin toxin
^{90}Y-Ibritumomab Tiuxetan (Zevalin®) murine IgG1	CD20	Treatment of relapsed or refractory, low-grade, or follicular B cell NHL; Previously untreated follicular NHL in patients who achieve a partial or complete response to first-line chemotherapy	Delivery of the radio-isotope yttrium-90
^{131}I-Tositumomab (Bexxar®) murine IgG2	CD20	Treatment of patients with CD20 antigen-expressing relapsed or refractory low-grade, follicular, or transformed NHL	Delivery of the radio-isotope iodine-131; ADCC; direct induction of apoptosis

*Not recommended in colorectal cancer patients whose tumors express mutated KRas

Scott AM, Allison JP, and Wolchok JD. Monoclonal antibodies in cancer therapy, *Cancer Immunity* (2012) Vol. 12, p. 14

Table 3. Monoclonal antibodies currently FDA-approved in oncology

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Bevacizumab humanized Ig			EGF signaling
Cetuximab (E chimeric hum			GFR signaling;
Panitumumal human IgG2			GFR signaling
Ipilimumab (Y IgG1			TLA-4 signaling
Rituximab (R Mabthera®) chimeric hum			induction of DC
Alemtuzumal humanized Ig			on of apoptosis;
Ofatumumab human IgG1			
Gemtuzumab (Mylotarg®) humanized Ig			xic payload, toxin
Brentuximab (Adcetris®) chimeric IgG			xic payload, n
^{90}Y-Ibritumon (Zevalin®) murine IgG1		complete response to first-line chemotherapy	e radio-isotope
^{131}I-Tositumomab (Bexxar®) murine IgG2	CD20	Treatment of patients with CD20 antigen-expressing relapsed or refractory low-grade, follicular, or transformed NHL	Delivery of the radio-isotope iodine-131; ADCC; direct induction of apoptosis

Antibody	Target	FDA-Approved indication
Trastuzumab (Herceptin®) * humanized IgG1	HER2 (ErbB2)	HER2-positive breast cancer, as single chemotherapy for (i) adjuvant or (ii) pall gastric or gastroesophageal junction ca combination with cisplatin and capecital
Bevacizumab (Avastin®) humanized IgG1	VEGF	For the palliative treatment of colorectal cell lung cancer, glioblastoma, or renal
Cetuximab (Erbitux®)* chimeric human/murine IgG1	EGFR (ErbB1)	In combination with radiation therapy fo regionally advanced squamous cell can As a single agent for SCCHN patients v therapy has failed; Palliative treatment positive colorectal cancer
Panitumumab (Vectibix®)* human IgG2	EGFR (ErbB1)	As a single agent for the treatment of pr metastatic colorectal carcinoma
Ipilimumab (Yervoy®) IgG1	CTLA-4	For the treatment of unresectable or me
Rituximab (Rituxan® and Mabthera®) chimeric human/murine IgG1 *	CD20	For the treatment of CD20-positive B ce and chronic lymphocytic leukemia (CLL untreated follicular CD20-positive NHL

*Not recommended in colorectal cancer patients whose tumors express mutated KRas

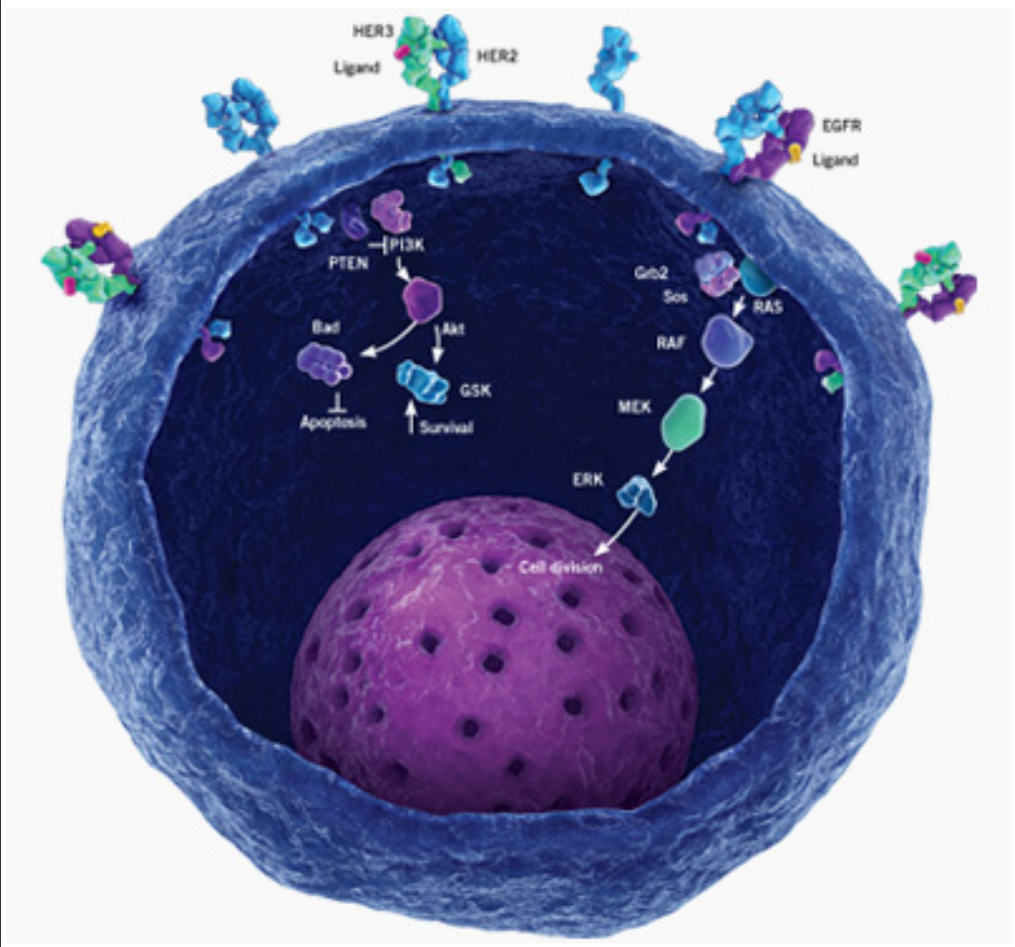
Scott AM, Allison JP, and Wolchok JD. Monoclonal antibodies in cancer therapy, *Cancer Immunity* (2012) Vol. 12, p. 14

Model mAb/receptor project

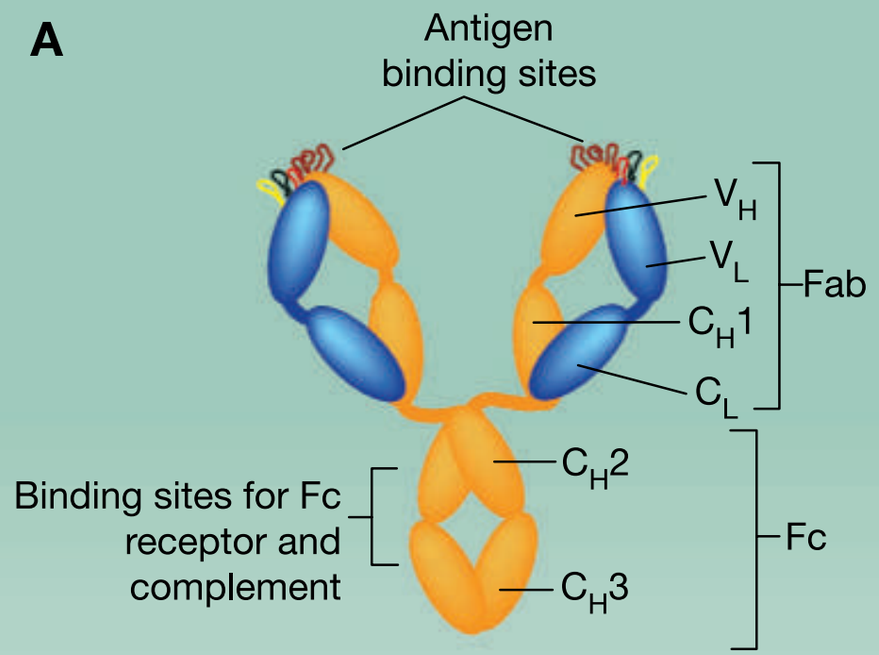
Trastuzumab (Herceptin®) is a humanized immunoglobulin gamma 1 (IgG1) mAb, directed against the **human epidermal growth factor receptor-2 (HER2/neu)**, which is over-expressed in about 25% of breast cancer patients.

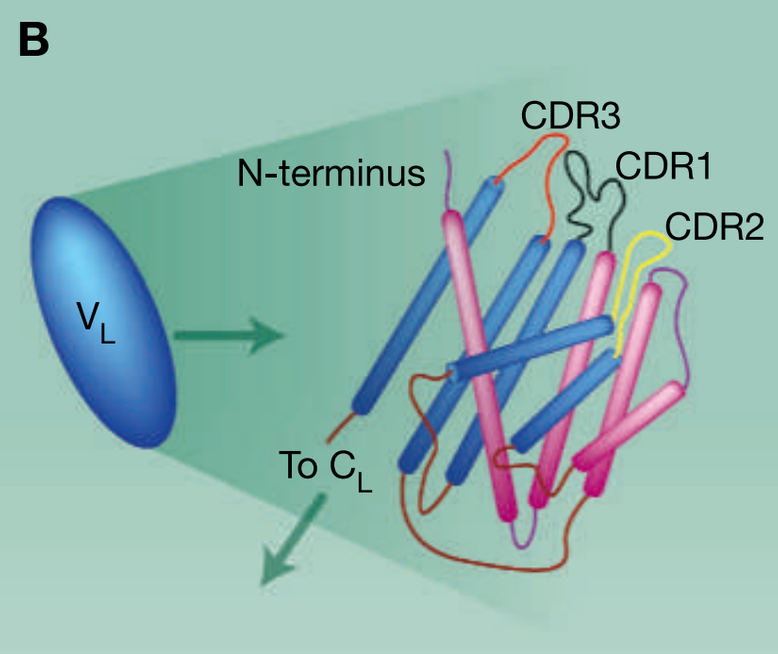
Trastuzumab binds to the juxtamembrane portion of the extracellular domain of the HER2 receptor, prevents the activation of its intracellular tyrosine kinase, and recruits immune effector cells that are responsible for antibody-dependent cytotoxicity (ADCC).

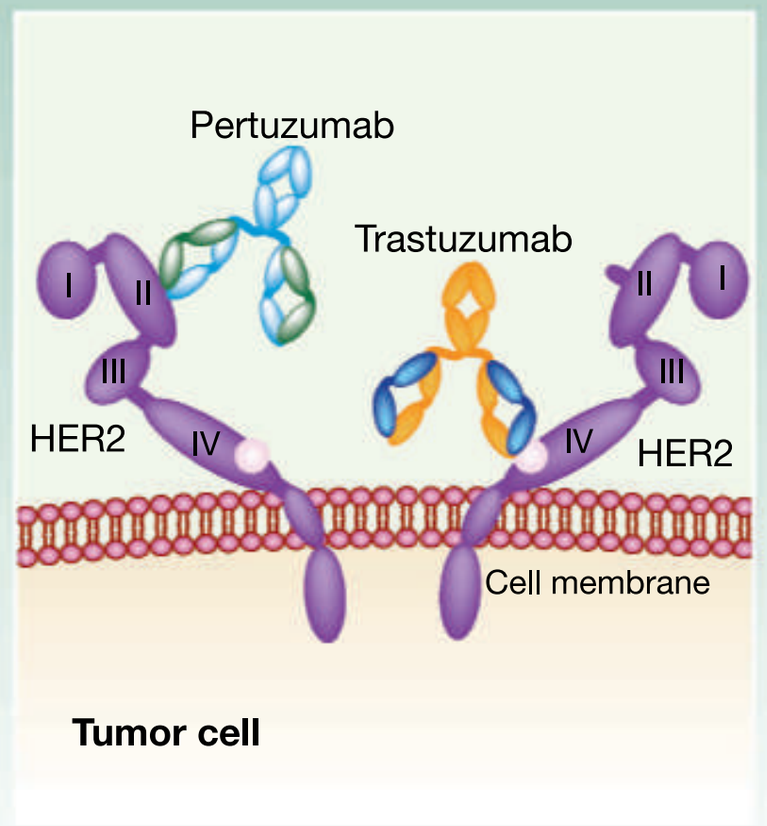
Hudis CA. Drug Therapy: Trastuzumab — Mechanism of Action and Use in Clinical Practice, *N Engl J Med* 2007;357:39-51.

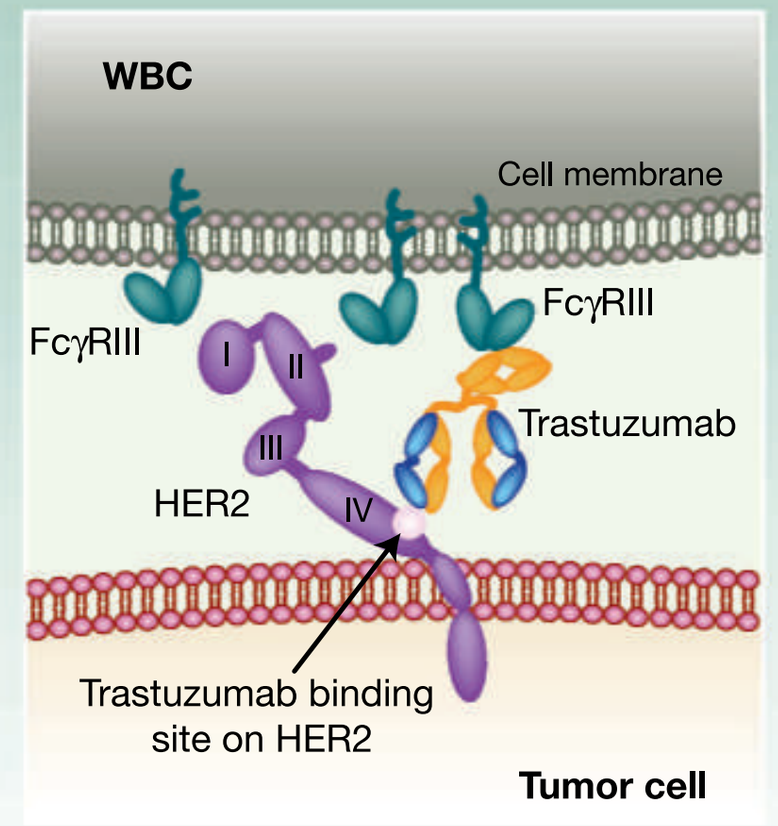


Human epidermal growth factor receptor (HER) pathways play a critical role in cancer biology. Dysregulation of HER-mediated signaling pathways results in the growth and spread of cancer cells. The HER family consists of 4 structurally related receptors: HER1 (EGFR), HER2, HER3, and HER4. HER family receptors are activated by ligand-induced dimerization, or receptor pairing.[Genentech]









CCR Focus

Pohlmann PR, Mayer IA, and Mernaugh R, Resistance to Trastuzumab in Breast Cancer, *Clin Cancer Res* 2009;15(24):7479-7491

Shed or soluble receptors as biomarkers?

Serum HER-2/neu Test Utility at a Glance

The Serum HER-2/neu Test is used to monitor a patient's HER-2/neu status once a diagnosis of metastatic breast cancer has been established. The chart summarizes the clinical utility of the Serum HER-2/neu Test as a monitoring tool complementary to tissue testing.

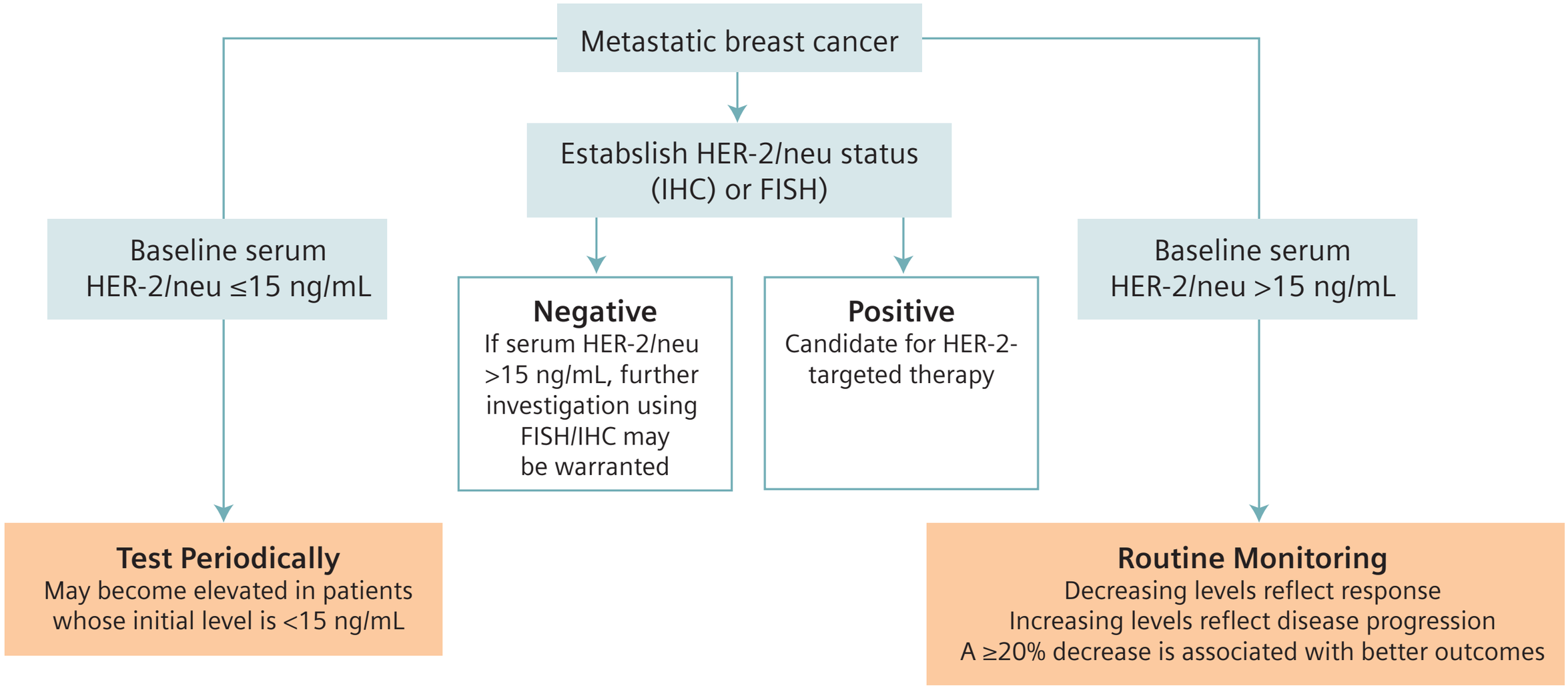


Figure 1. A Serum HER-2/neu testing algorithm for metastatic breast cancer.

Siemens Healthcare Diagnostics
ADVIA Centaur® Serum HER-2/neu test

FDA approved serum HER2/neu assay

Siemens Healthcare Diagnostics
ADVIA Centaur® Serum HER-2/neu test

Outstanding Assay Performance

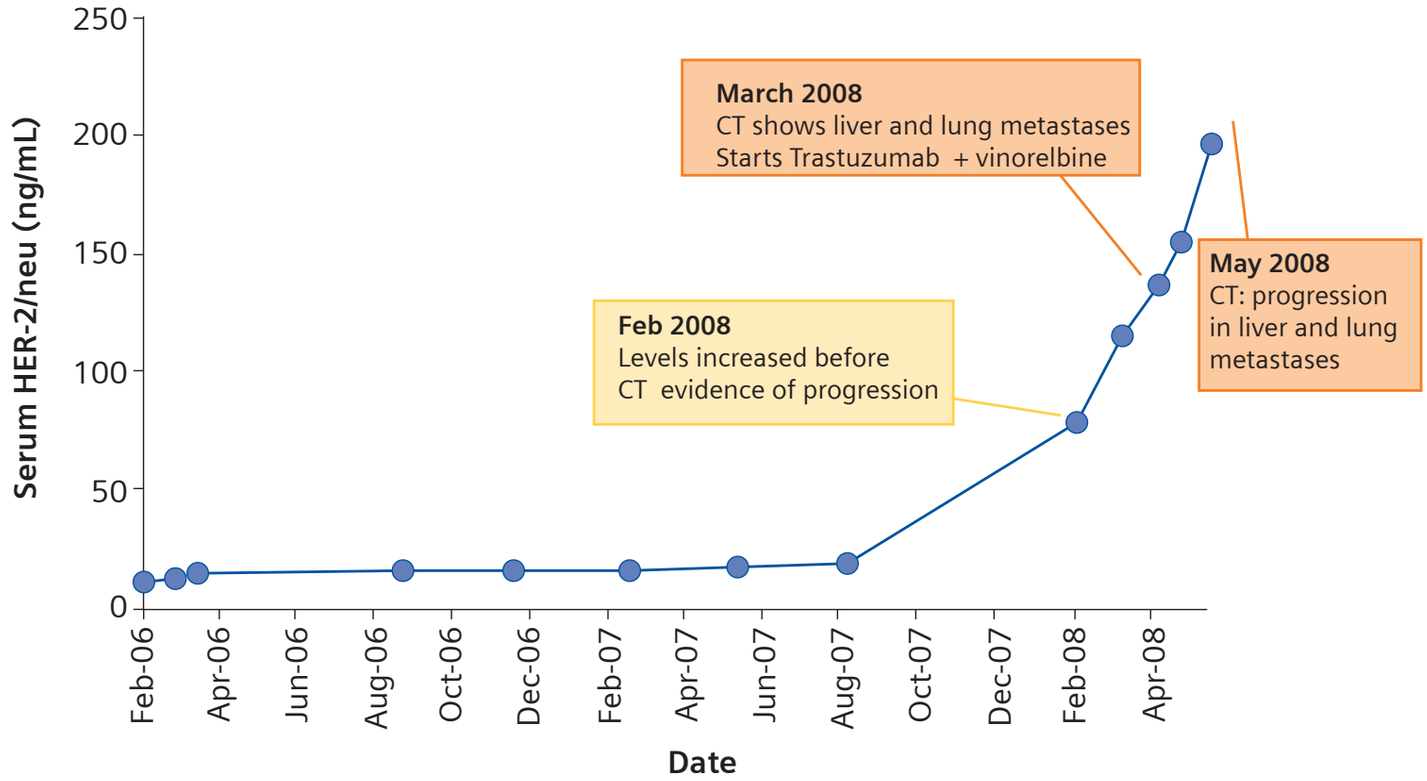
- Broad assay range (**0.5-350 ng/mL**)
- Excellent reproducibility
(Total %CV Range 3.2-5.7%)

Clinical Utility

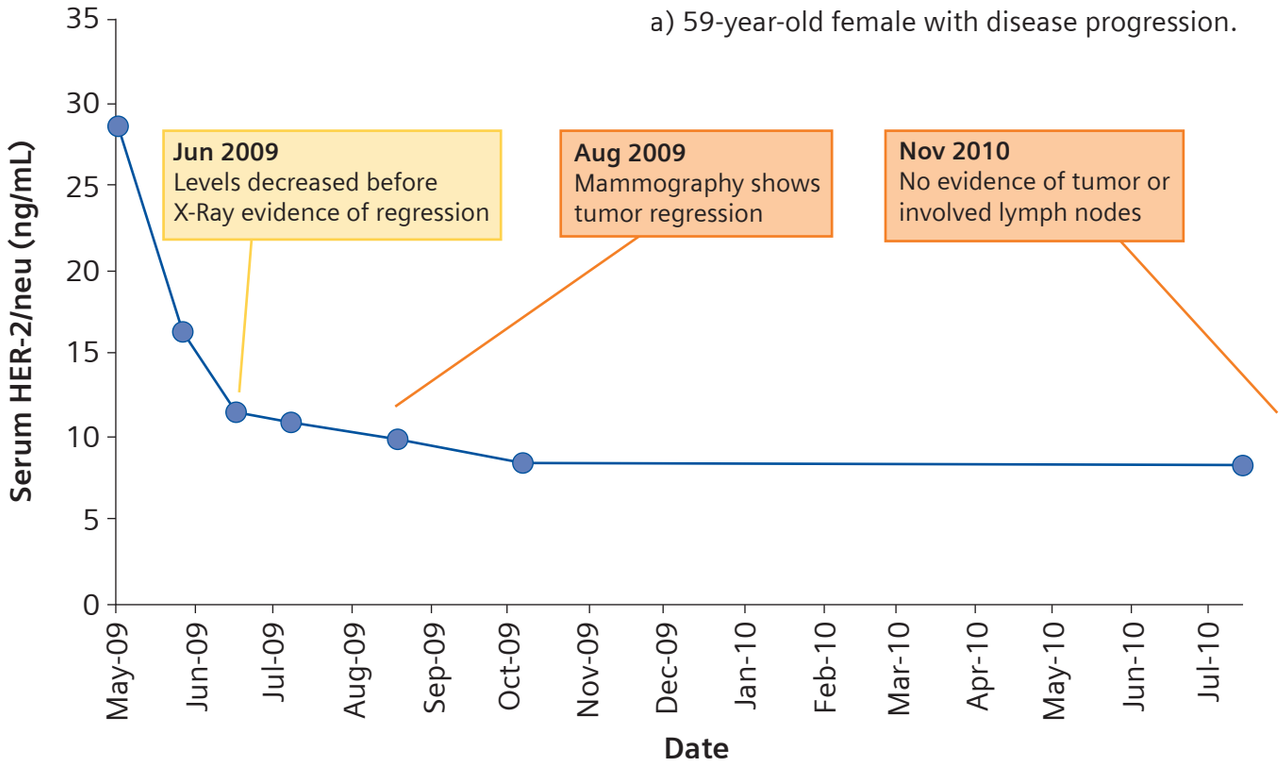
- HER-2/neu values obtained may be used in the follow-up and monitoring of patients with metastatic breast cancer whose initial Serum HER-2/neu level is greater than 15 ng/mL
- No interference from HER-2/neu-based therapy
- **95% of normal patients have Serum HER-2/neu levels below 15.2 ng/mL**
- Serial changes in Serum HER-2/neu have been correlated with changes in clinical status for all patients whose pre-treatment Serum HER-2/neu values exceeded 15 ng/mL
- **For each pair of serial measurements, an increase of equal or greater than 15% is considered to indicate progression**
(predictive value of 67%)
- For each pair of serial measurements, a change of less than 15% increase is considered to indicate a lack of progression
(predictive value 71%)

Example serum HER2/neu clinical profiles

Disease progression



a) 59-year-old female with disease progression.



Response to treatment

(a) A 54-year-old female with response to treatment.

Example serum HER2/neu clinical profiles

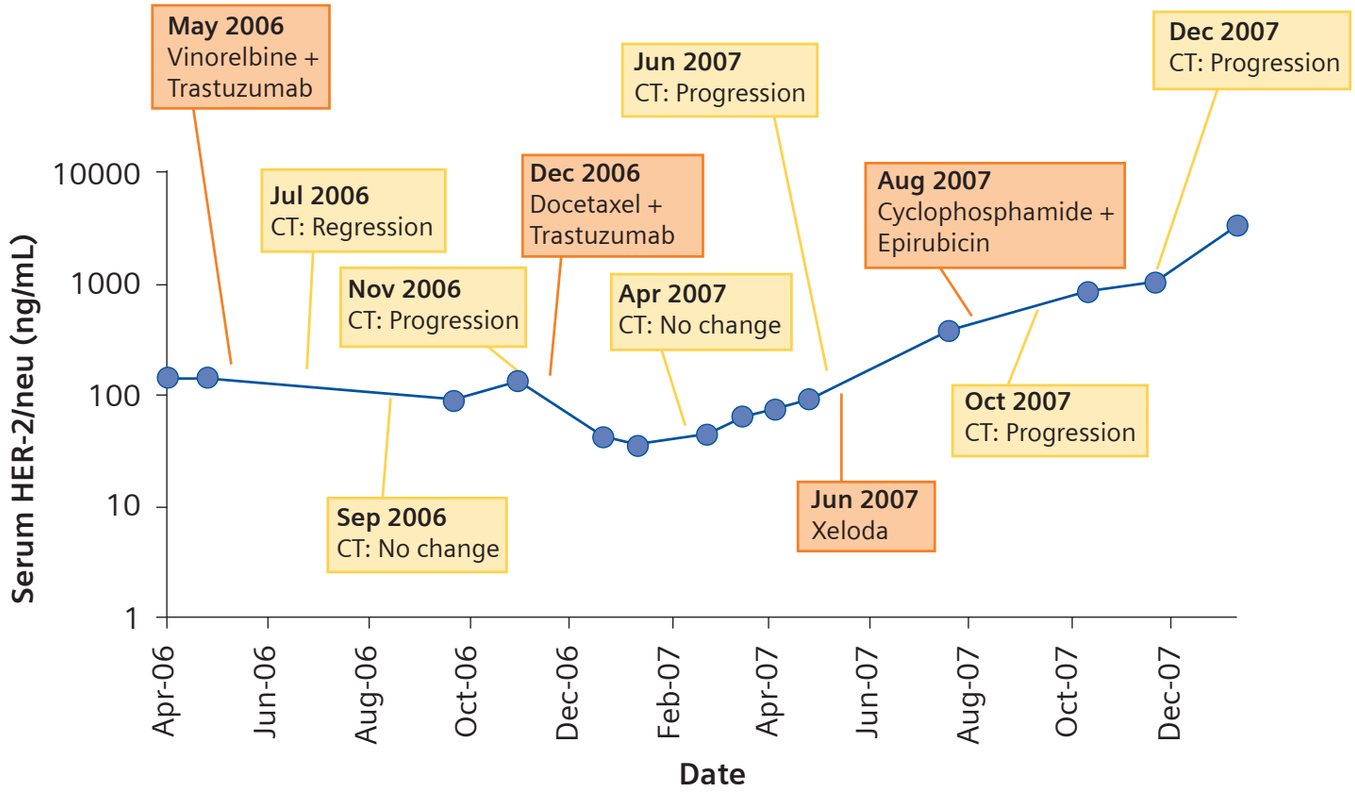


Figure 3. Clinical course and serum HER-2/neu levels (logarithmic scale).

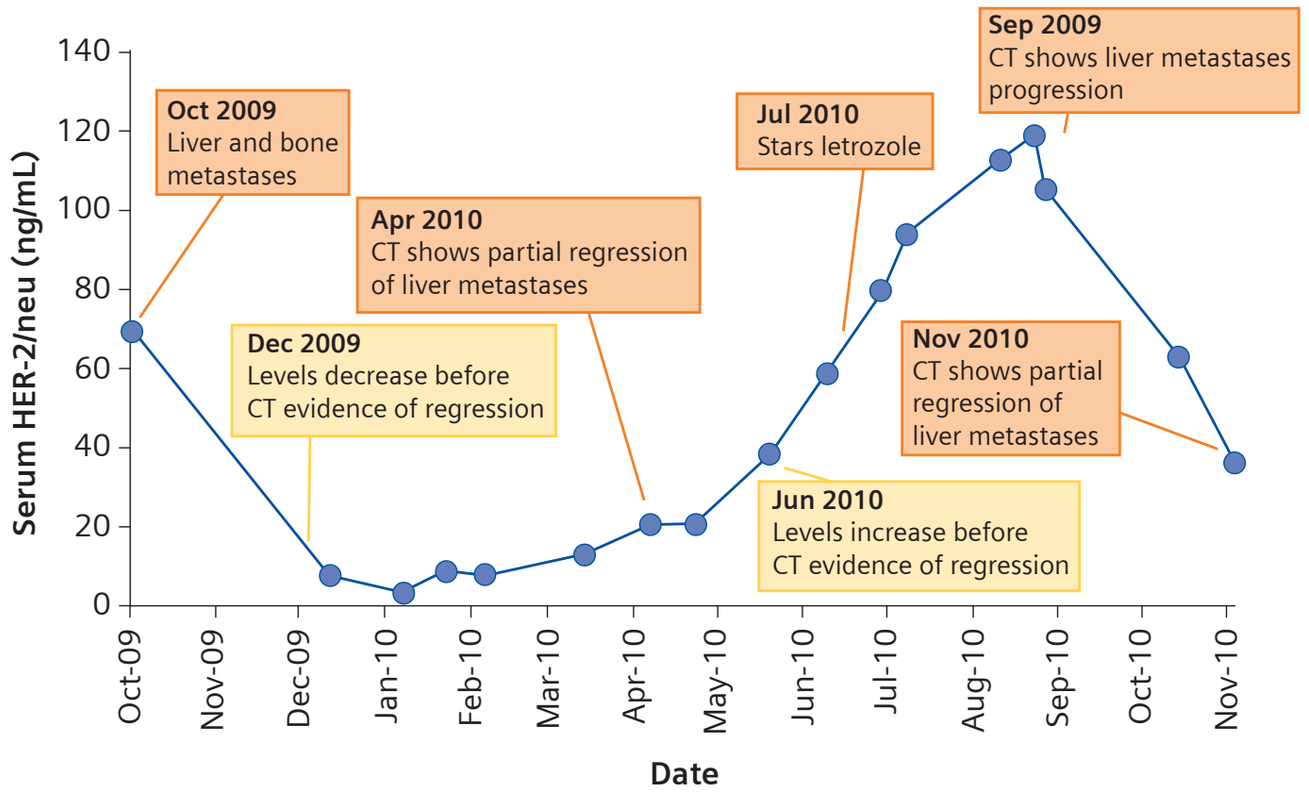


Figure 6. Clinical course and serum HER-2/neu levels.

Serum HER-2: predictive values for detecting metastatic recurrence in breast cancer patients

Serum HER-2 values from healthy controls (age 30–56) ranged from 5.4 to 15.5 µg/L; higher values were seen in postmenopausal women compared with premenopausal ($p = 0.01$).

Serum HER-2 increased in 69 % of patients up to several months before the detection of symptomatic metastatic disease by the conventional clinical methods. **We conclude that serum HER-2 is a useful marker for monitoring tissue HER-2-positive patients in order to detect metastatic recurrence.**

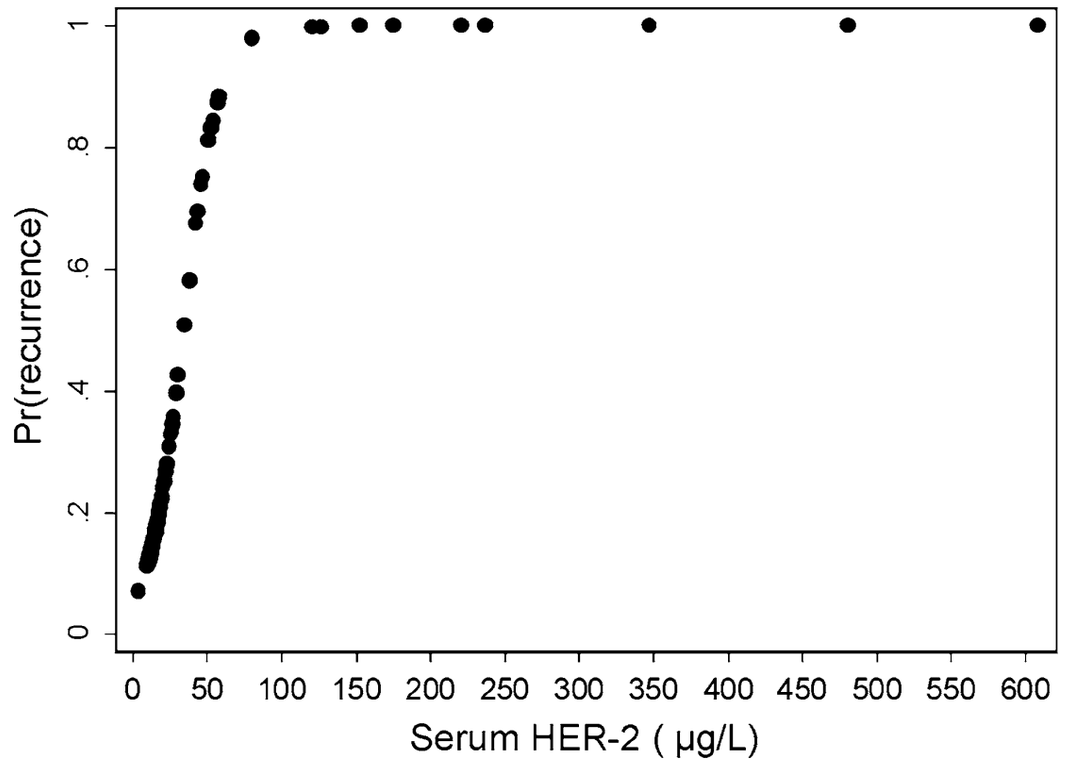
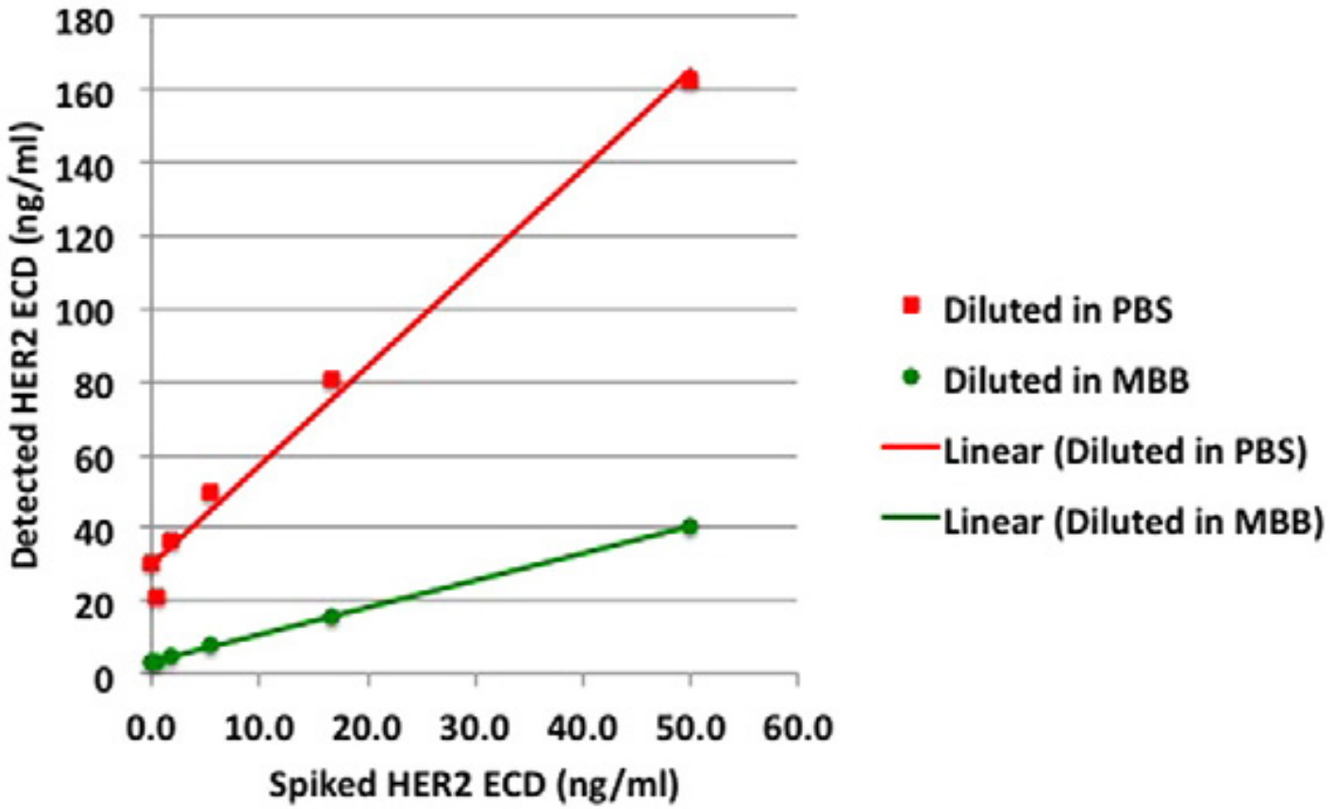


Fig. 2 Serum HER-2: estimated probabilities for metastatic recurrence in tissue HER-2-positive patients

Sørensen PD, Jakobsen EH, Madsen JS et al, Serum HER-2: sensitivity, specificity, and predictive values for detecting metastatic recurrence in breast cancer patients, *J Cancer Res Clin Oncol* (2013) 139:1005–1013

False ELISA results caused by heterophilic human anti-animal immunoglobulin antibodies (HAIA)



… the calculated concentrations of HER2 ECD in the spiked samples were statistically different from the expected concentrations.

Considering the lack of information on the serum interference issue in a considerable number of previous studies, ***it is highly possible that some HER2 ECD levels in the literature might be misleading.***

…, it is highly recommended that future HER2 ECD tests should include a HAIA-preventing mechanism such as the MBB buffer.

Fig. 2. Detection of recombinant HER2 ECD in samples containing HAIA serum. Recombinant HER2 ECD (eBioscience, Inc.) at different concentrations but with consistent levels of HAIA serum (5%) was detected using anti HER2/neu monoclonal antibodies 6E2 and biotin-A21. The presence of MBB buffer corrected the influence of the HAIA serum and showed good correlation between detected and expected HER2 ECD levels

Lam L et al. *Biochimica et Biophysica Acta* 1826 (2012) 199–208

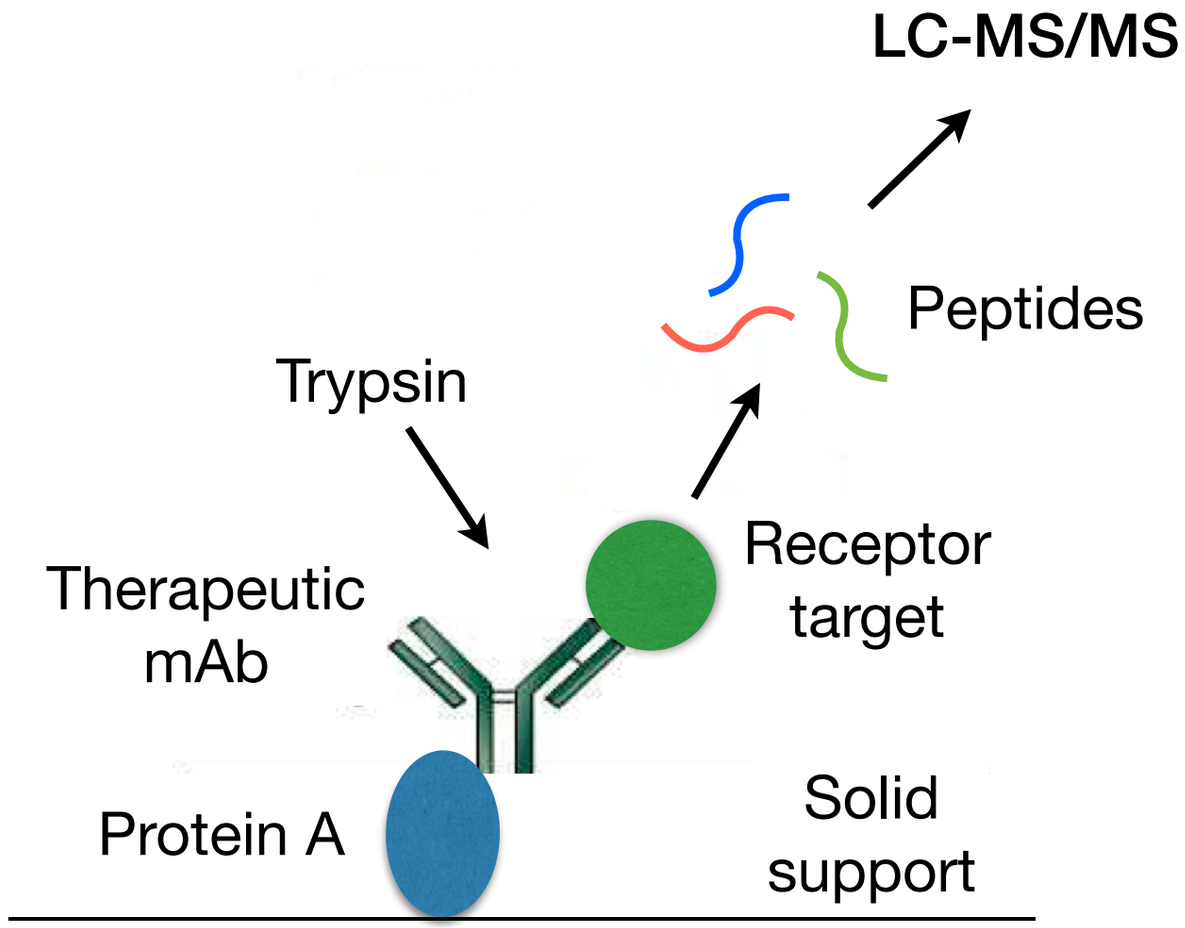
Why consider LC-MS/MS?

LB-LC-MS/MS approach

goals

- Simultaneous analysis - *mAb and receptor*
- Trastuzumab - *range: 100 ng/mL to 100 µg/mL*
- HER2/neu - *range: 1.00 ng/mL to 1000 ng/mL*
- Generic IgG extraction - *Fc-affinity capture*
- Analysis of unique peptides - *CDR and other*
- Freedom from LBA interferences - *e.g. HAIA*

Hybrid LB-LC-MS approach



mAb-receptor complex capture

Affinity capture

alternatives

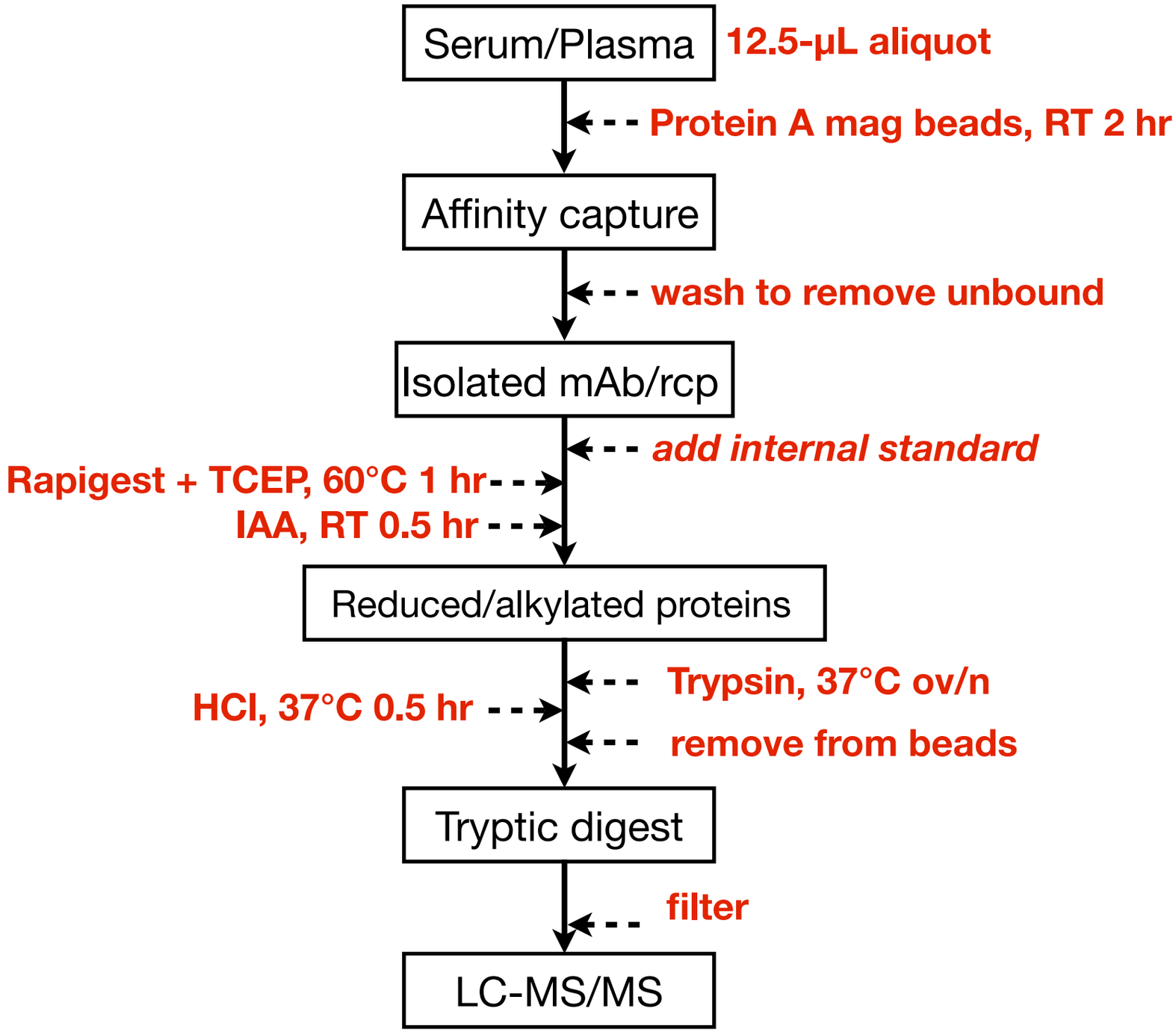
Generic

- Protein A, G, A/G (binds to Fc of IgGs)
- Protein L (binds to kappa light chains VκI, VκIII and VκIV subtypes)
- Anti-human IgG, anti-human Fc, anti-human kappa (nonclinical)

Analyte-specific

- Anti-idiotype (anti-ID) mAbs (not to receptor binding paratope)
- Anti-target mAb (biotinylated) *for receptor only assay*

Assay workflow



LC-MS/MS method

LC-MS/MS System

Waters nano-Acquity

Waters Xevo TQ-S triple quadrupole with TRIZAIC source

Electrospray positive ionization (+ESI) multiple reaction monitoring (MRM) mode

HPLC Columns

Trapping: Waters PEEK-SIL, 300 µm x 50 mm, 5 µm Symmetry C18

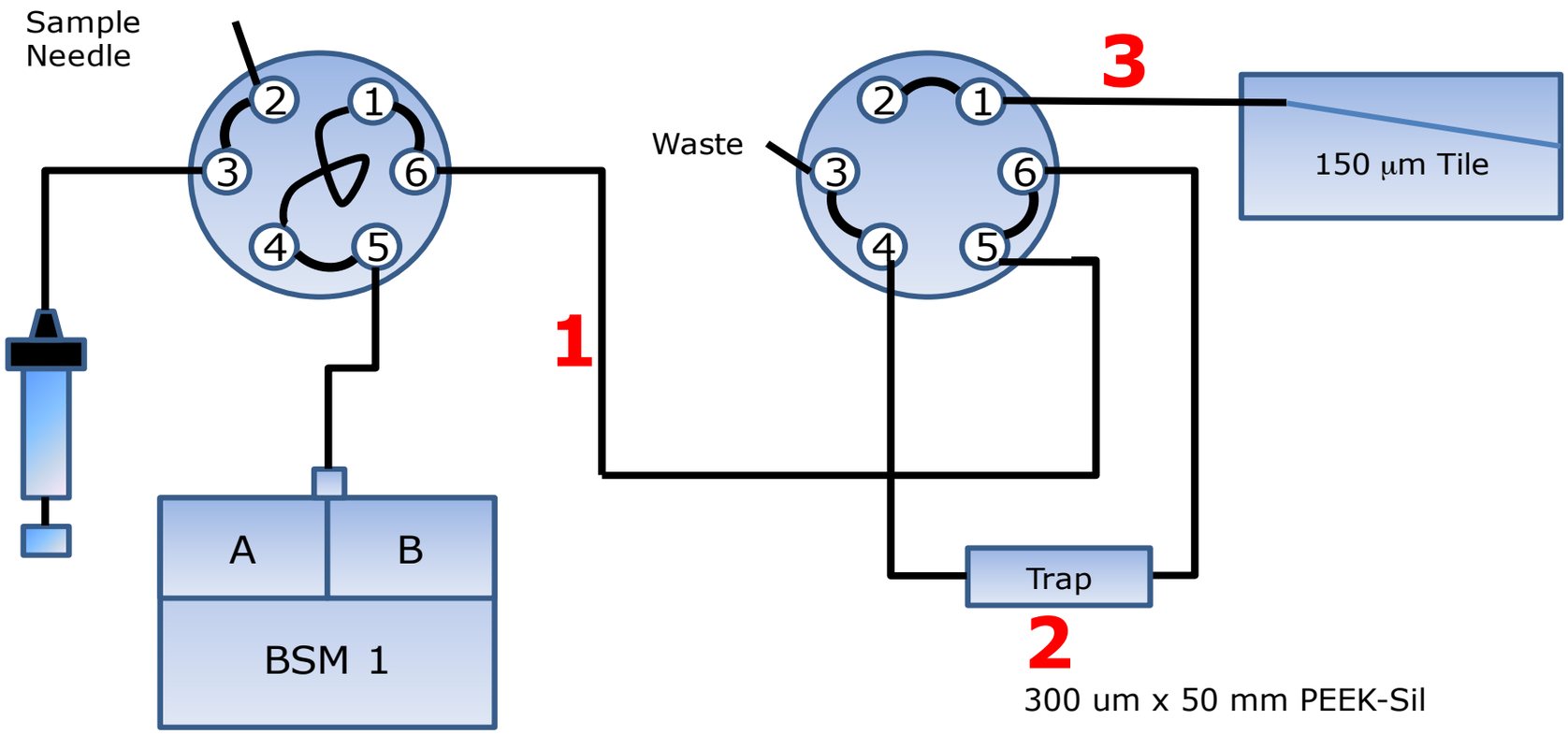
Analytical: Waters T150 Nanotile (*prototype*), 150 µm x 50 mm, 1.7 µm BEH C18

Mobile phases

A: 99.5:0.5:0.1 Water / ACN / Formic Acid, v/v/v

B: 90:5:5:0.1 Acetonitrile / DMSO / Water / Formic Acid, v/v/v/v

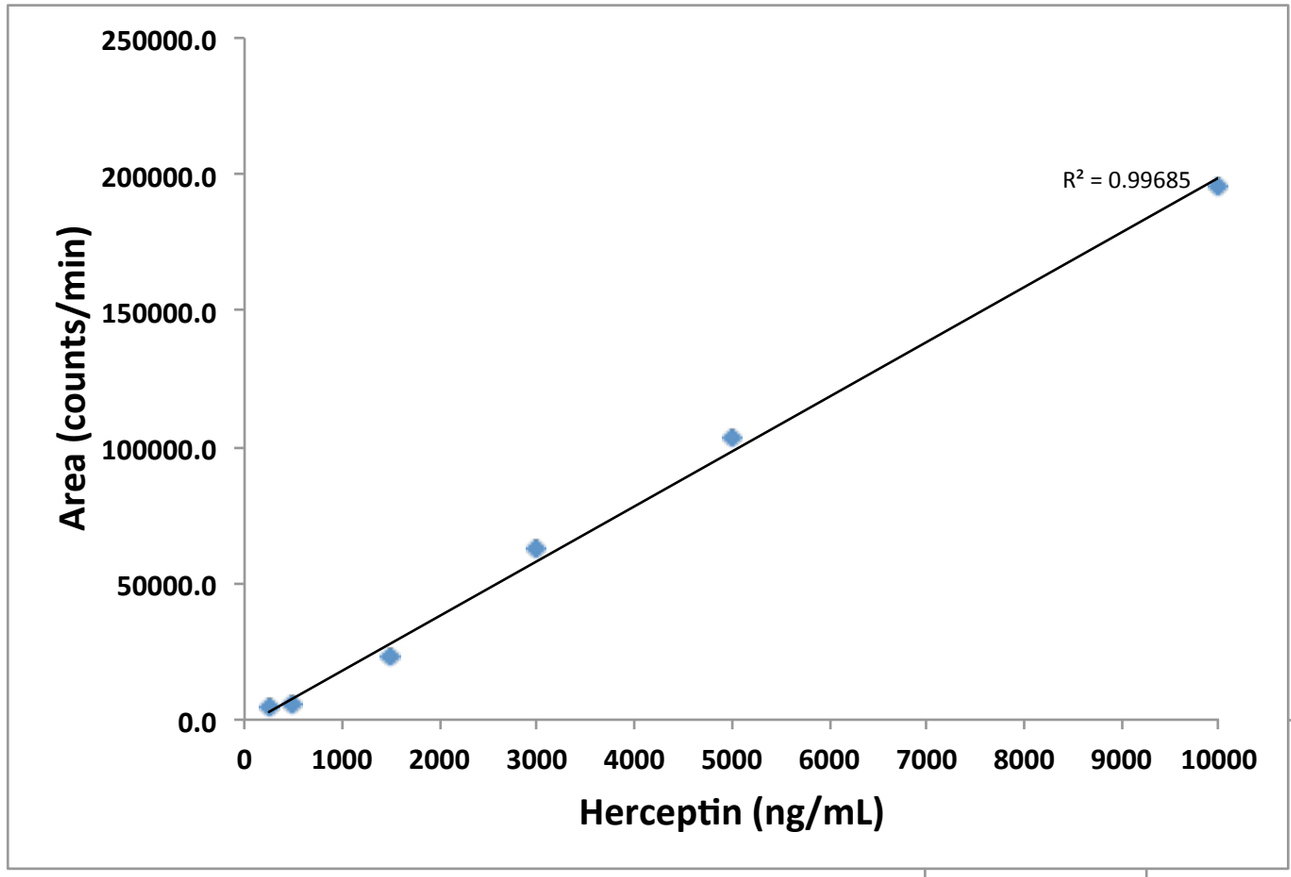
LC trapping scheme with Waters TRIZAIC 150



1. Injection of 2-10 µL sample
2. Trapping on 300 µm x 50 mm column
3. Back-transfer to 150 µm x 50 mm T150 Nanotile analytical column

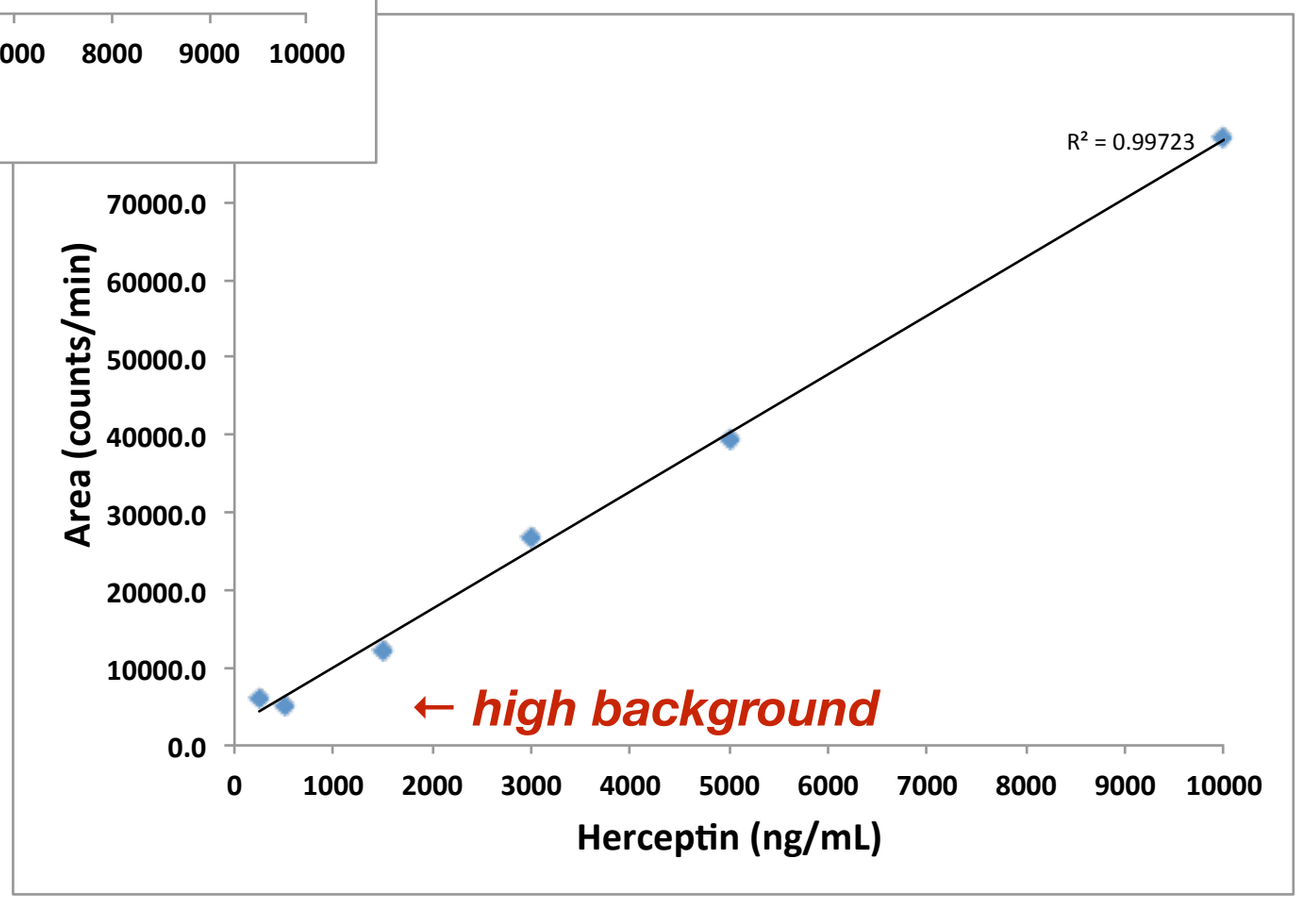
Some results to date

Trastuzumab alternate peptides

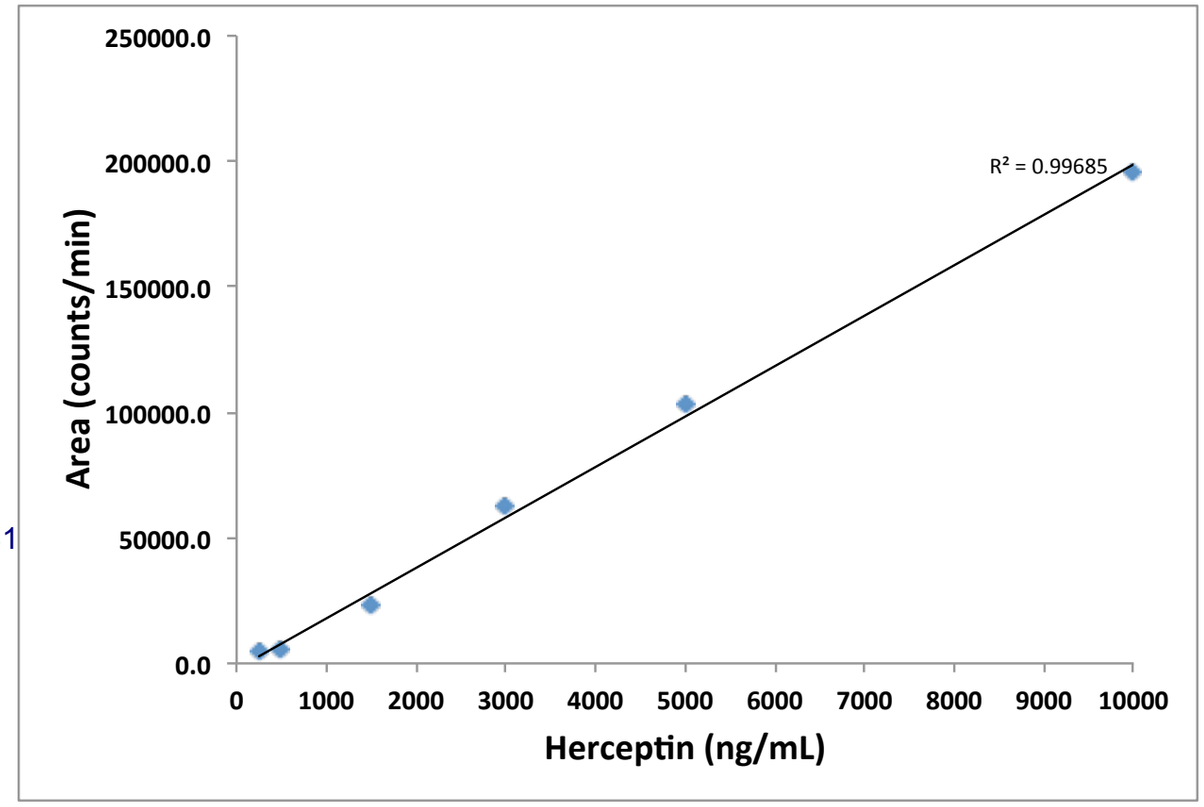


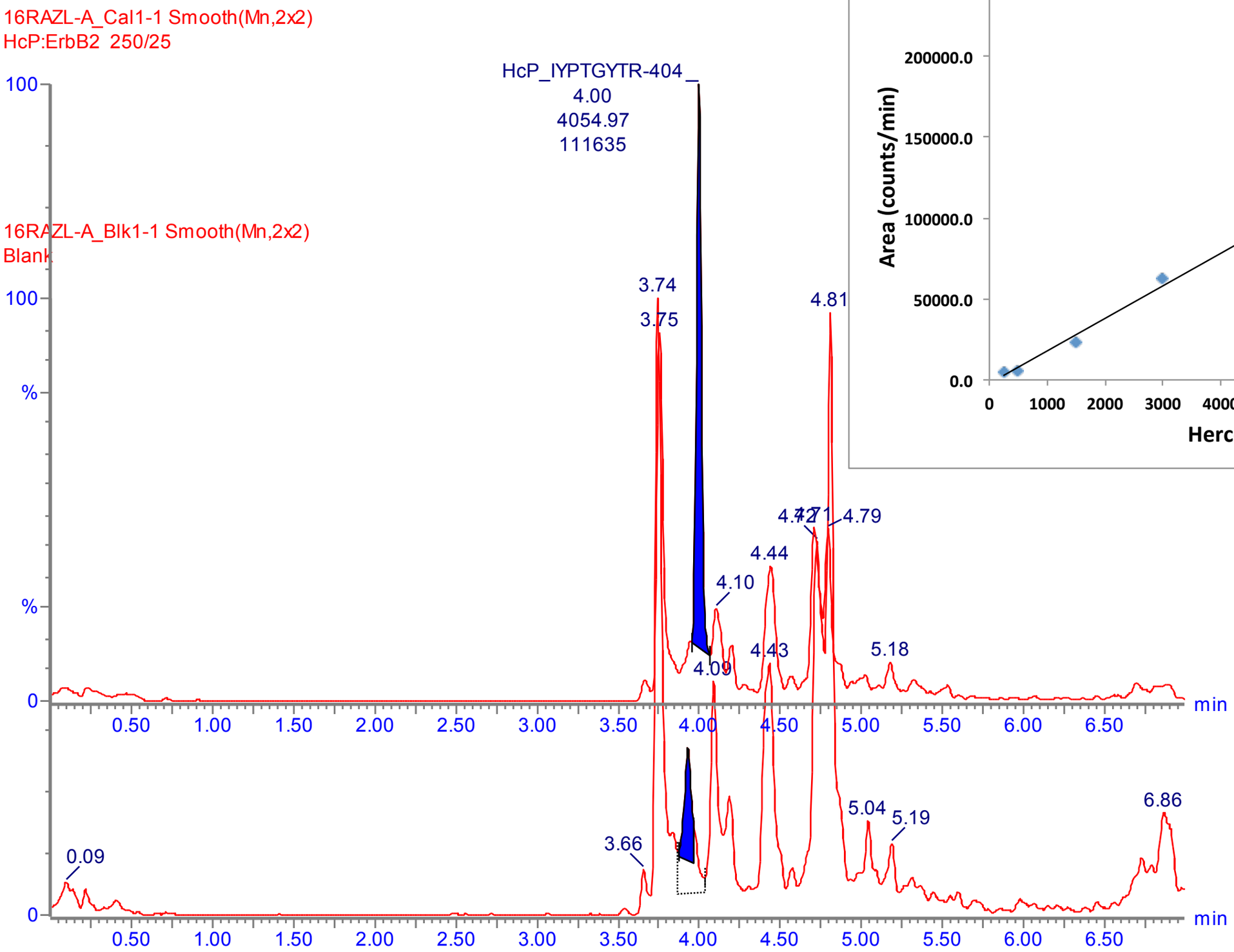
IYPTGYTR
m/z 542.8>404.7

FTISADTSK
m/z 485.2>721.4



Trastuzumab
IYPTGYTR m/z 542.8>404.7





250 ng/mL

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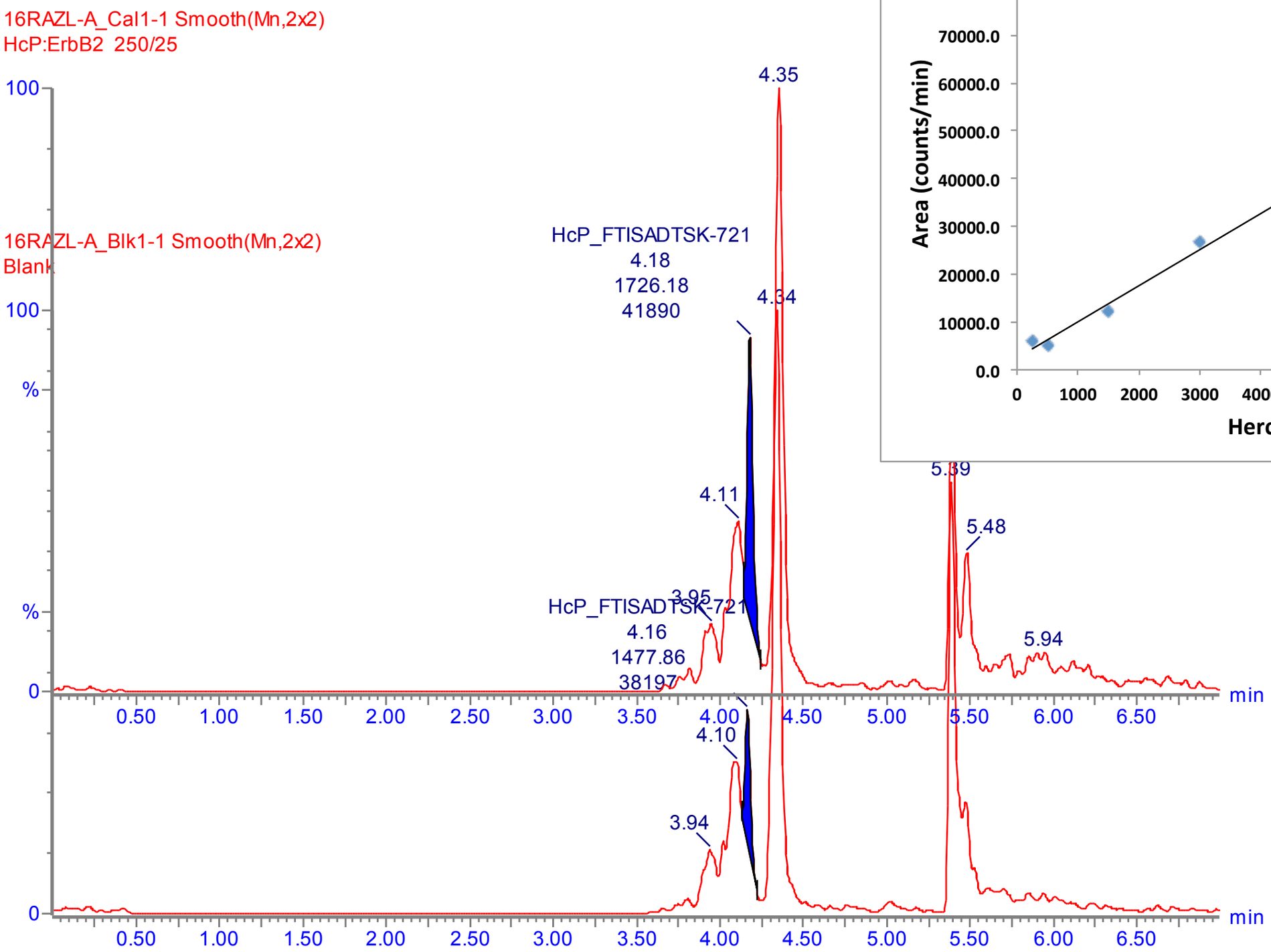
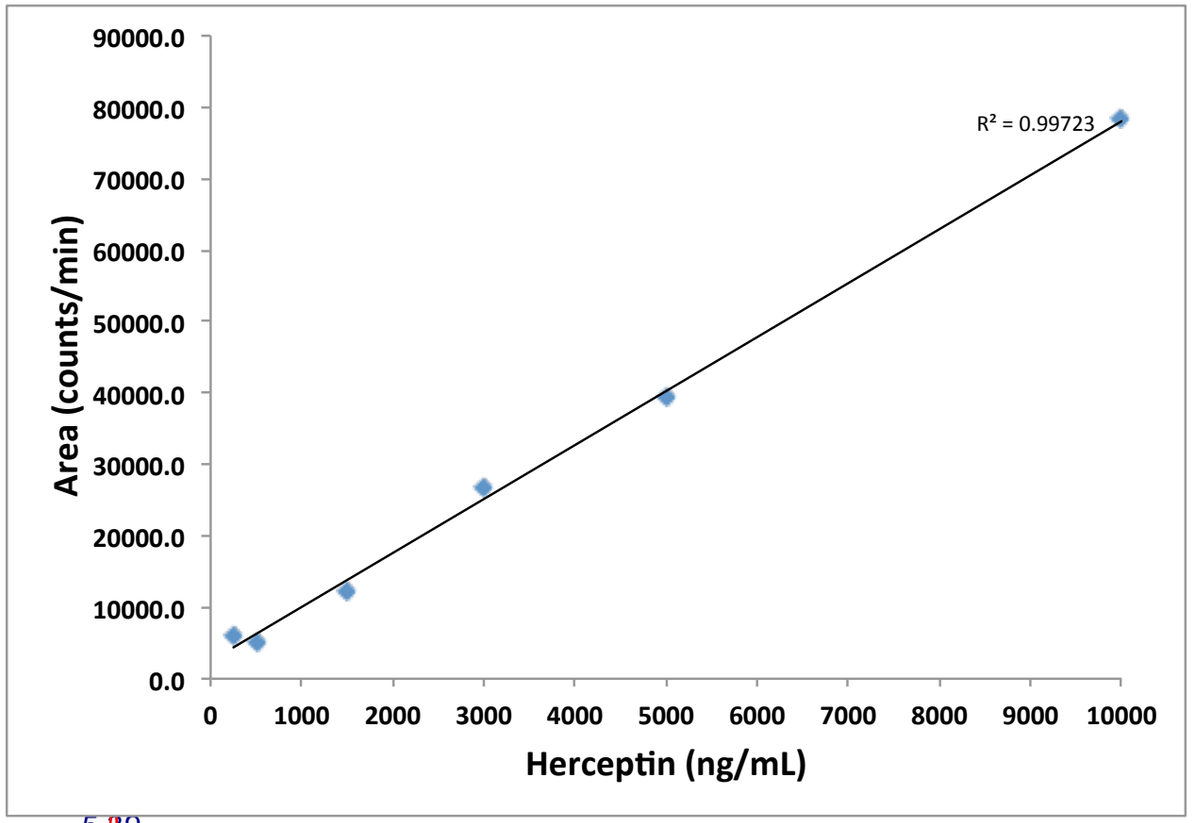
Trastuzumab
FTISADTSK m/z 485.2>721.4





250 ng/mL

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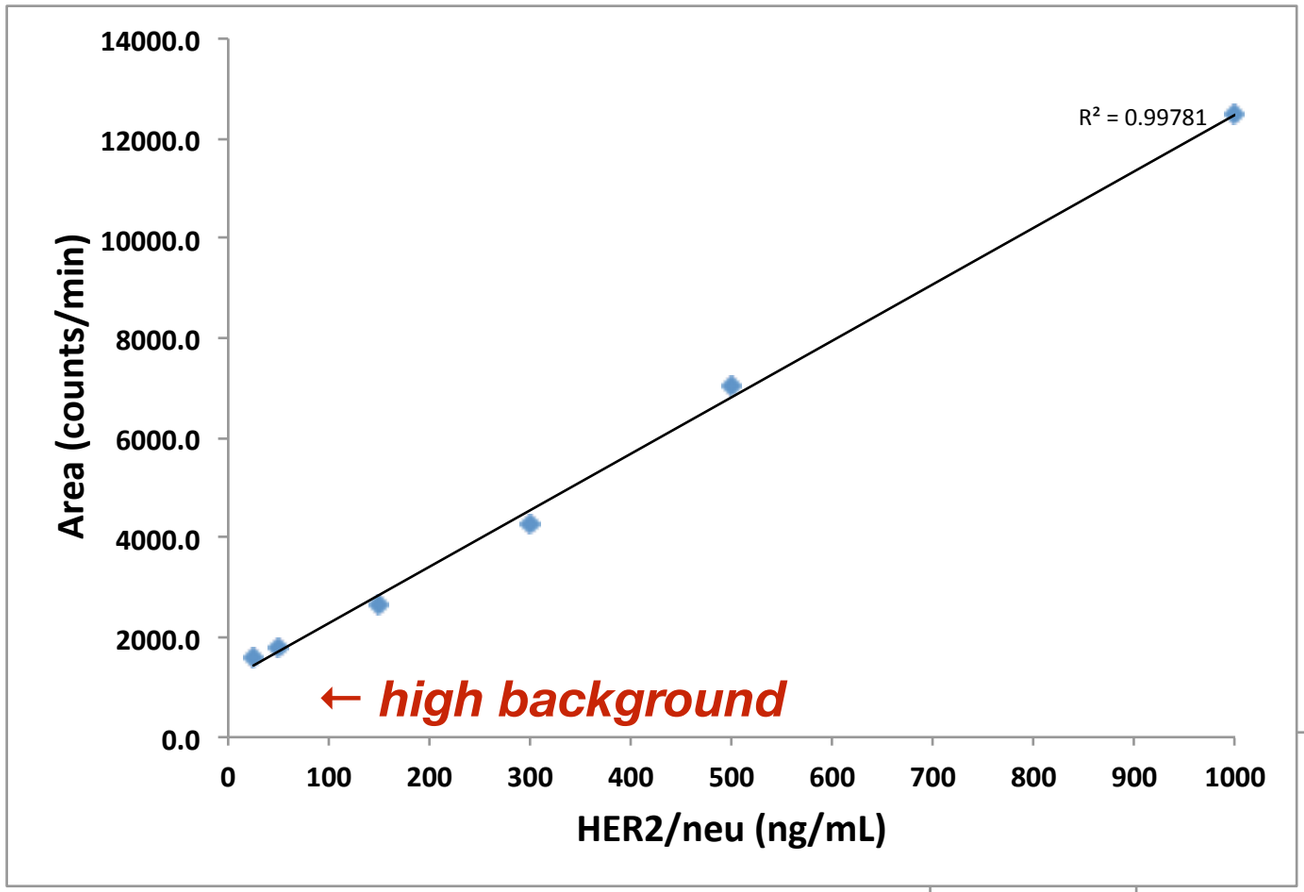
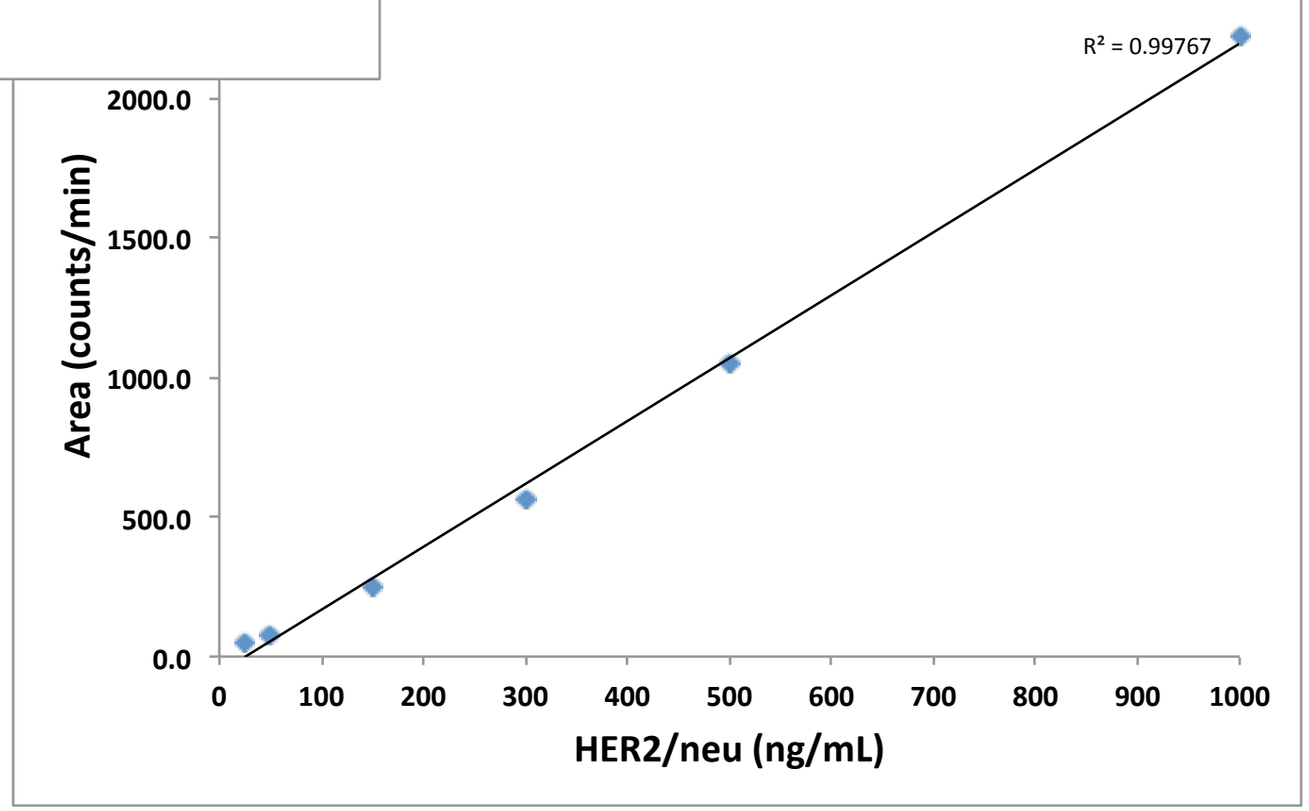
HER2/neu
alternate SRM transitions

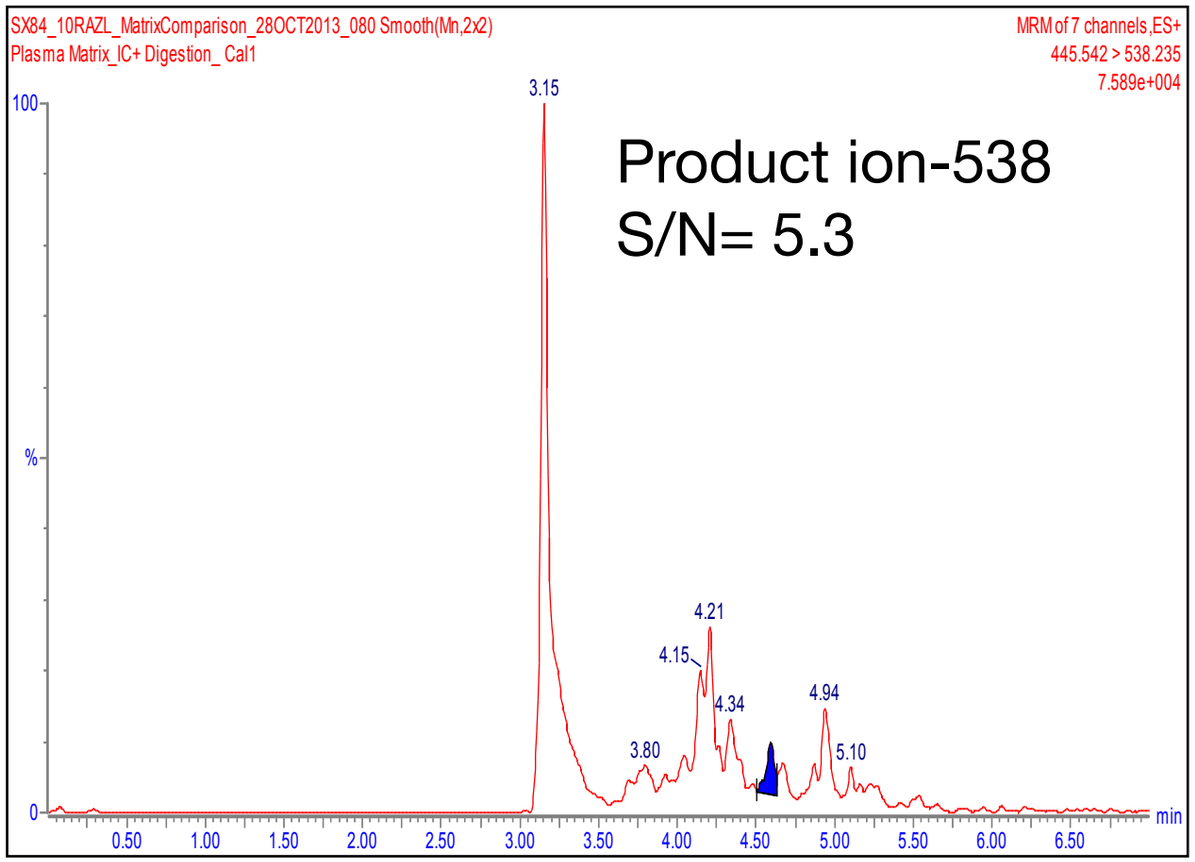
VCYGLGFMEHLR
m/z 445.5>538.2

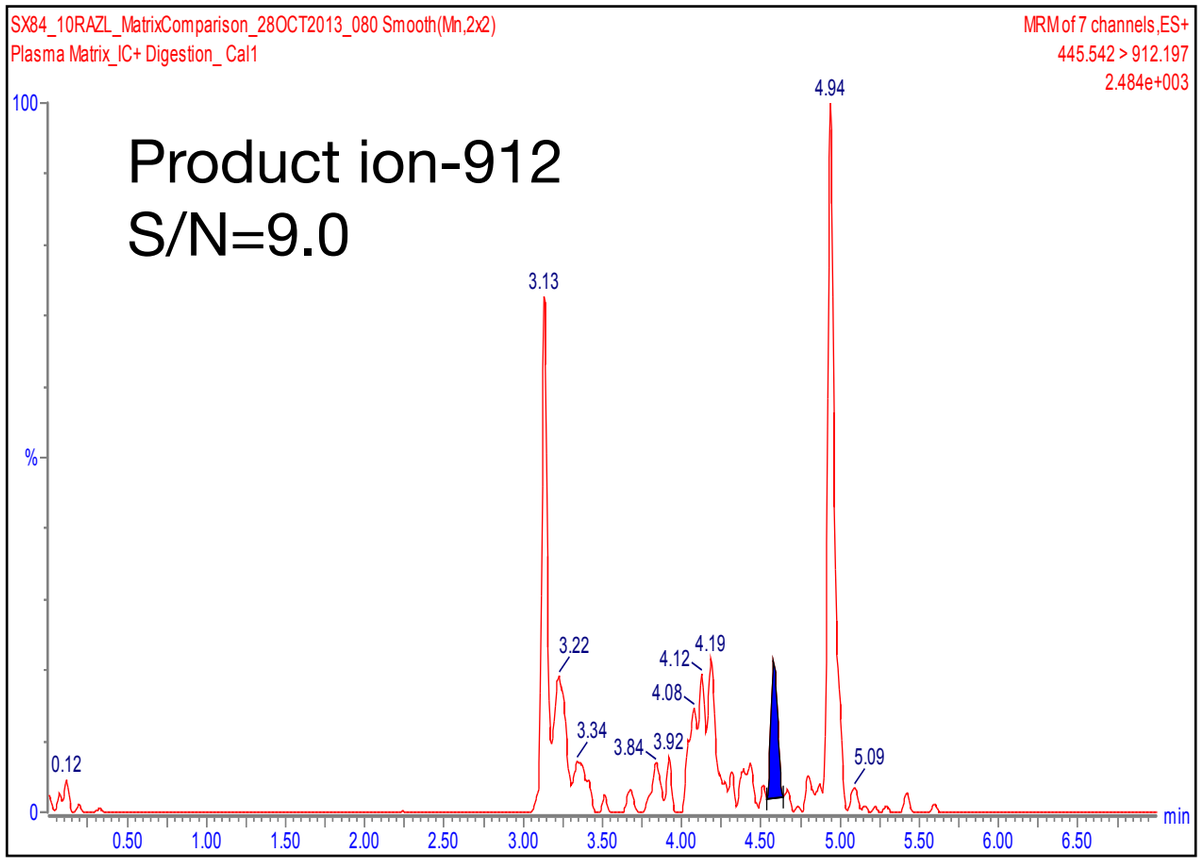


VCYGLGFMEHLR
m/z 445.5>912.5

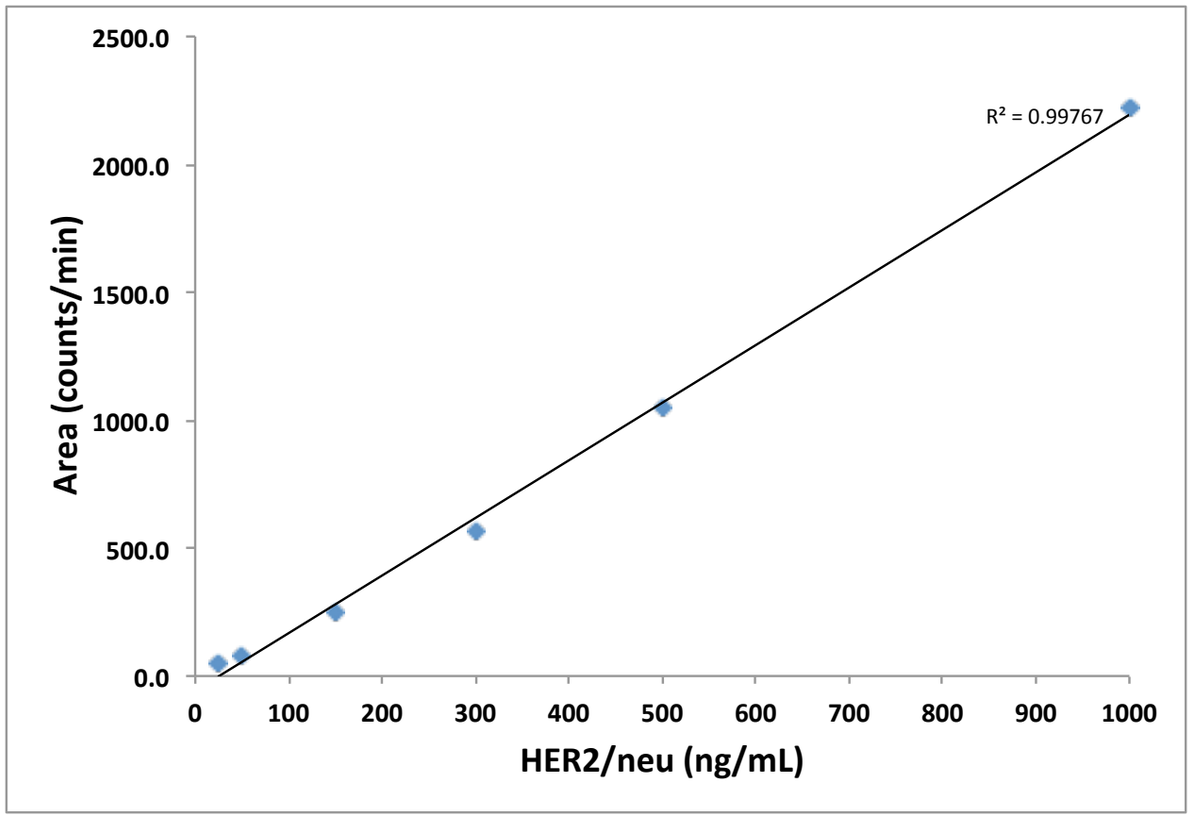


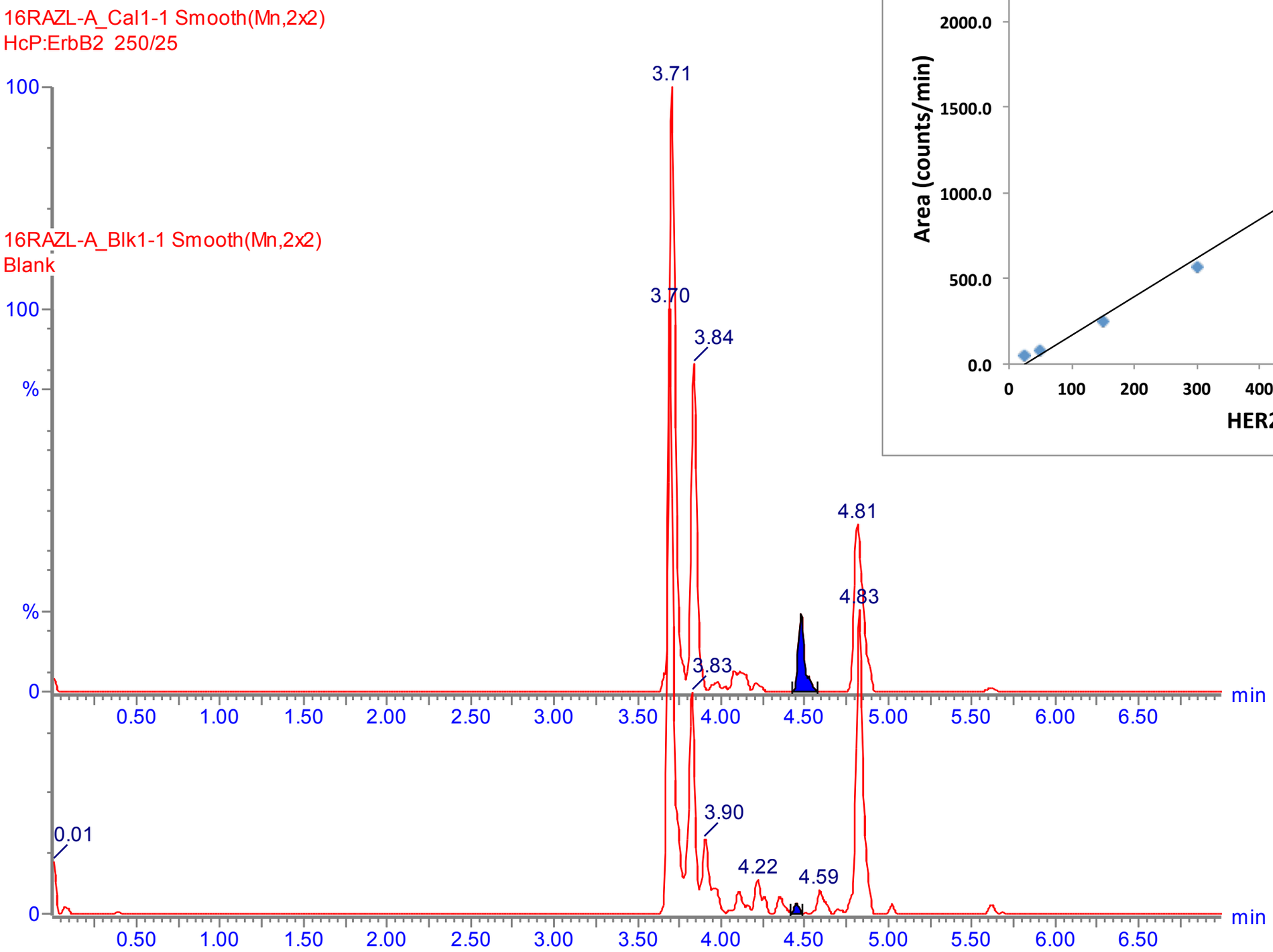
Comparison of 25.0 ng/mL HER2/neu SRM signals





HER2/neu
VCYGLGFMEHLR m/z 445.5>912.5



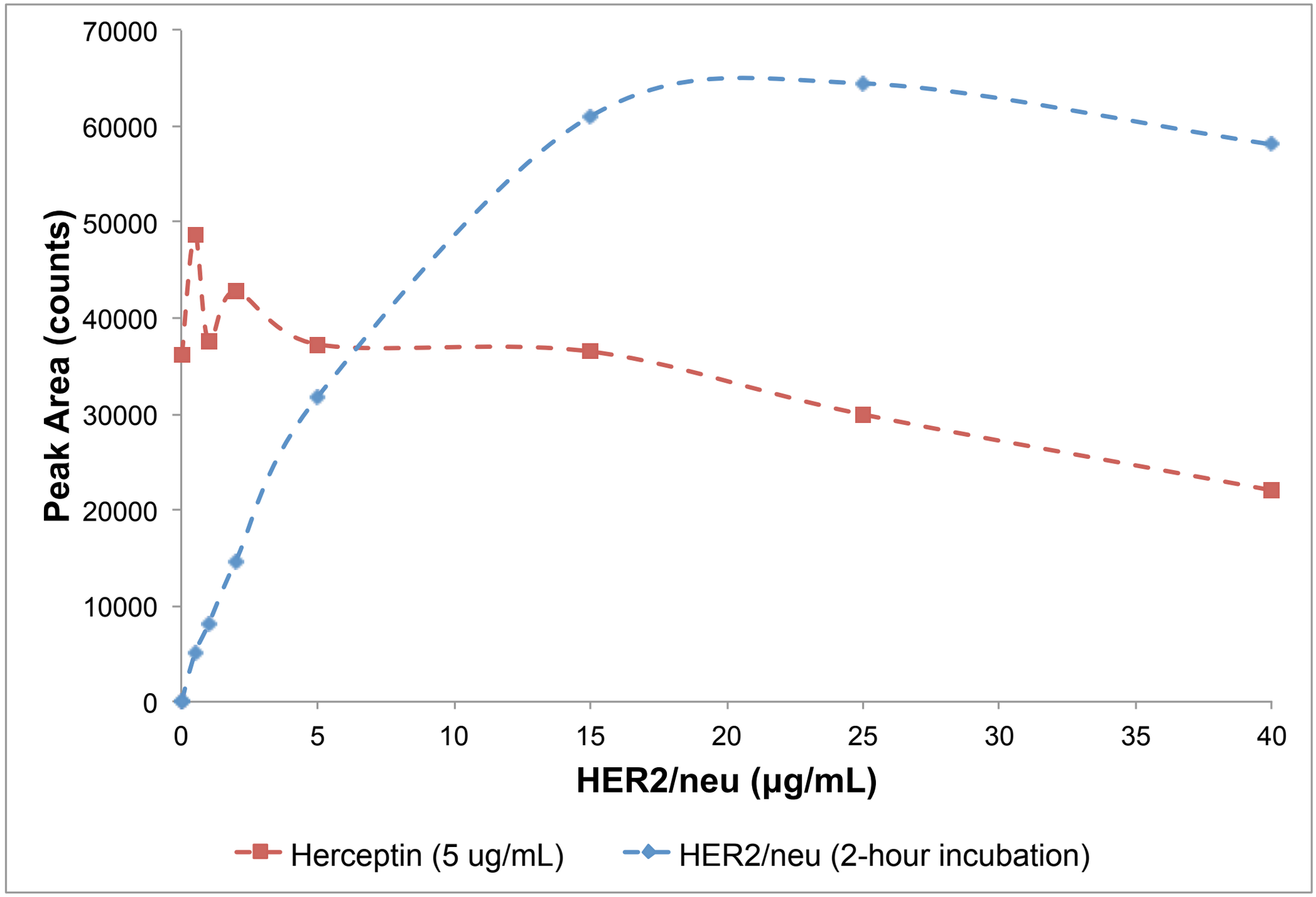


25.0 ng/mL

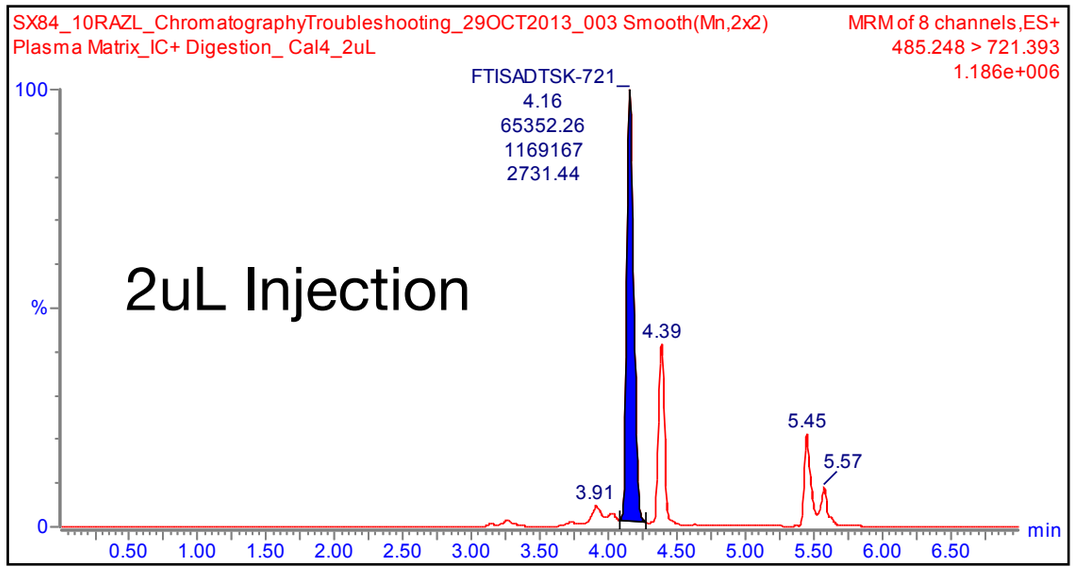
blank

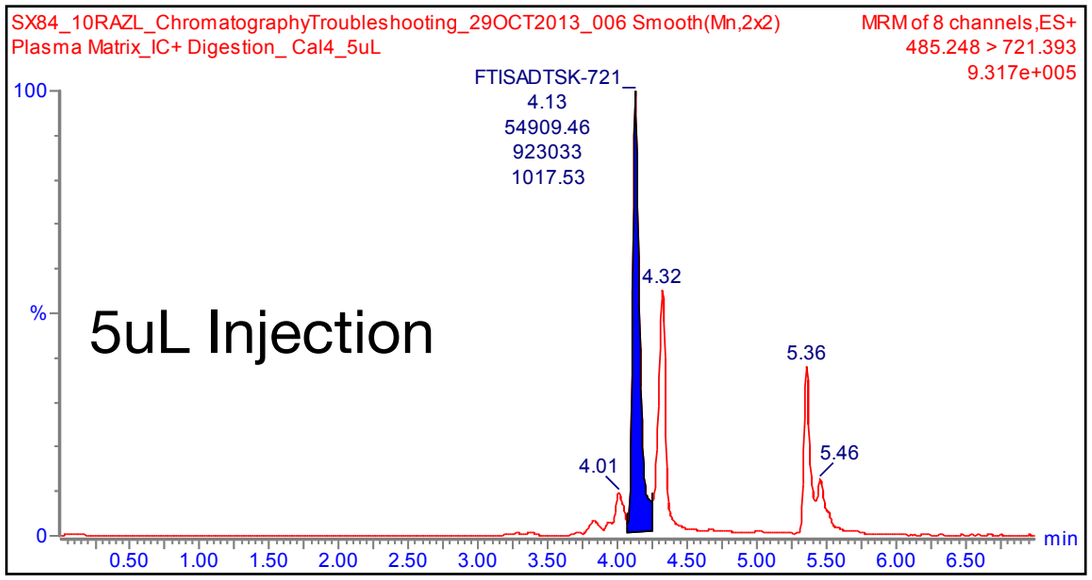
Receptor-mAb saturation effects

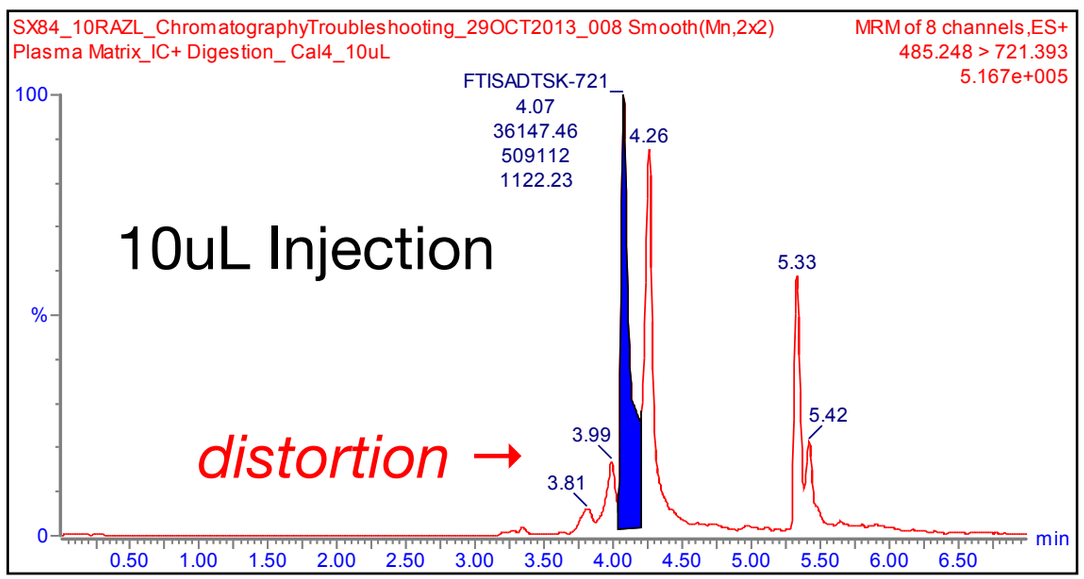
HER2/neu vs. constant Trastuzumab



Trastuzumab - injection volume impact with undiluted plasma digests

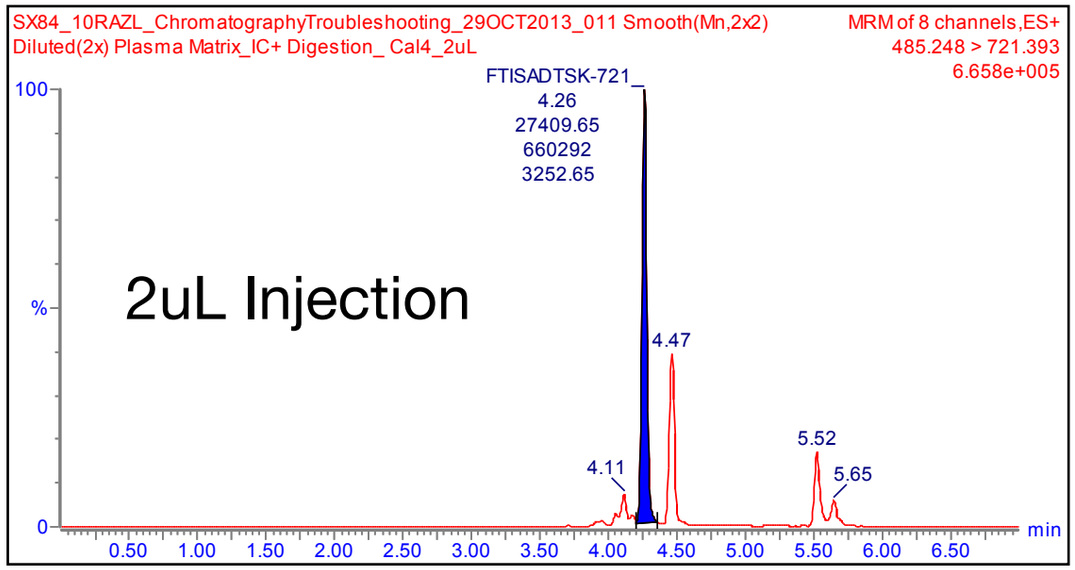


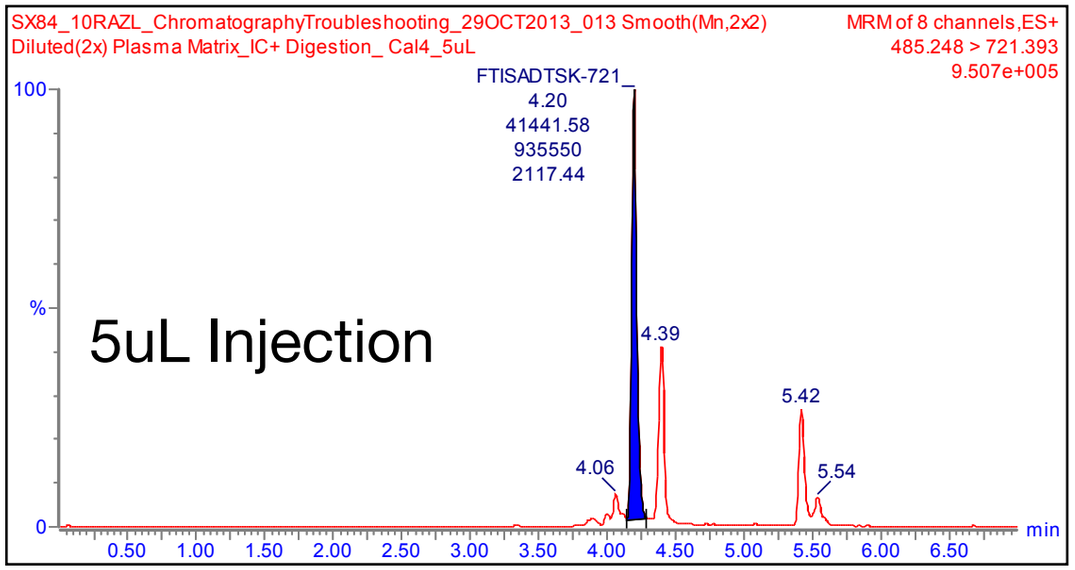


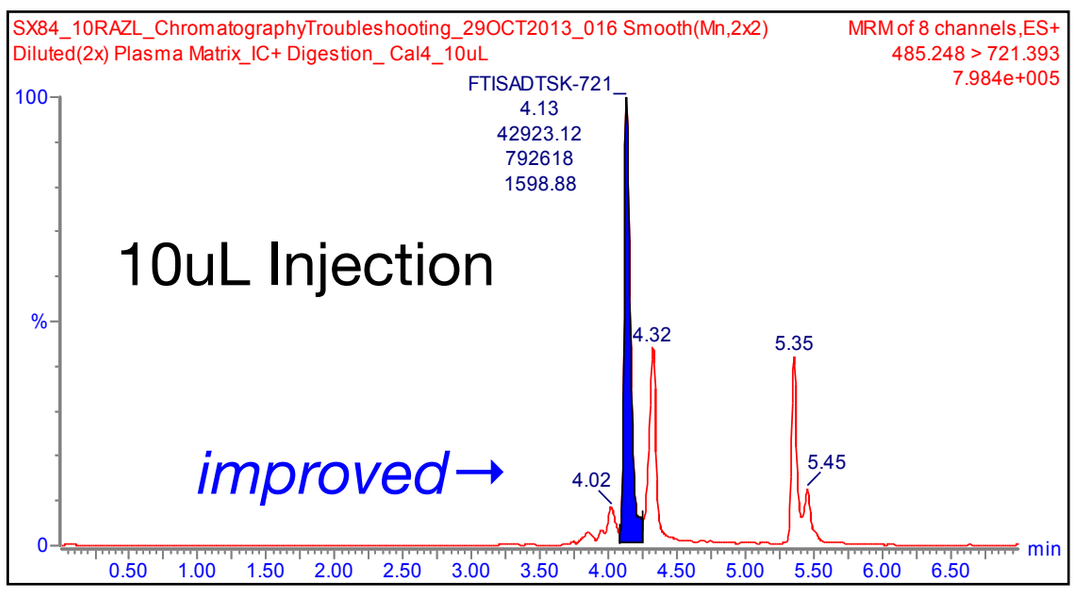


PLAMSA- HcP				Diluted		
Injection Number	**2uL**	**5uL**	**10uL**	**2uL**	**5uL**	**10uL**
1	65352	63376	41472	27410	41442	46311
2	52205	54909	36147	27563	38102	42923
Average	58779	59143	38810	27487	39772	44617
% Increase over 2uL injection		0.6	-33.8		44.7	43.1
% Increase over 2uL injection (undiluted)				-53.2	-32.3	-24.1

Trastuzumab - injection volume impact with 2x-diluted plasma digests







	PLAMSA- HcP			Diluted		
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LB-LC-MS/MS approach

issues

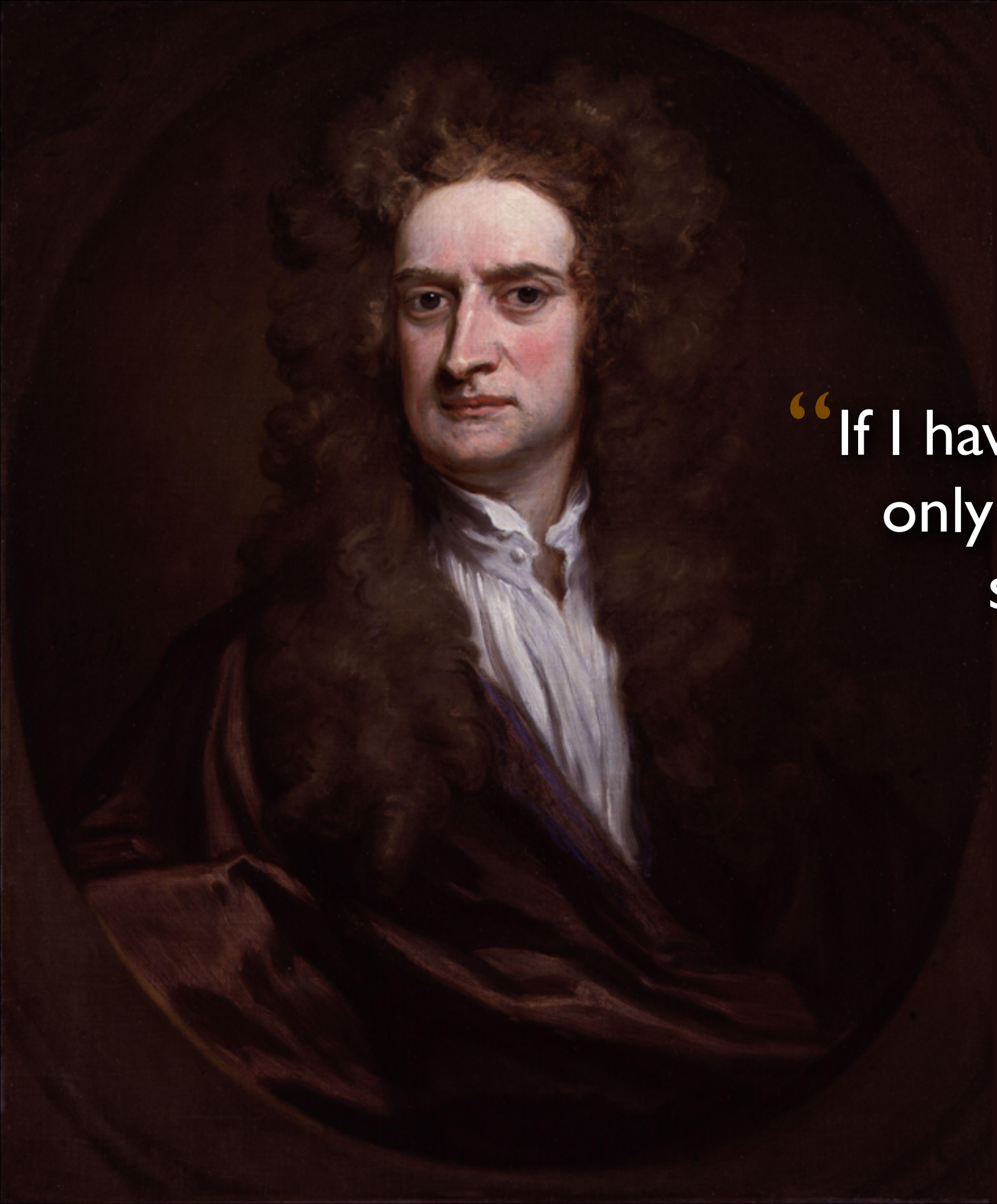
- Sensitivity/ranges - *1.0 ng/mL(?) for HER2/neu, with 100-fold difference in analyte ranges*
- Sample volume - *restricted by total IgG load with generic capture media (use agarose beads?)*
- Overload effects - *on chromatography and total eluting peptides impact on ESI microspray*
- Comparability with approved assays? - *unknown*
- Potential value of combo assays? - *unknown*

LB-LC-MS/MS approach

future plans

- Implement 2D-UPLC/µLC heart-cut techniques
- Evaluate other capture reagent formats
- Extend concept to other mAb/receptor models
- Analyze incurred patient samples
- Compare LC-MS/MS to LBA formats

acknowledgements



“If I have seen further it is only by standing on the shoulders of giants.”

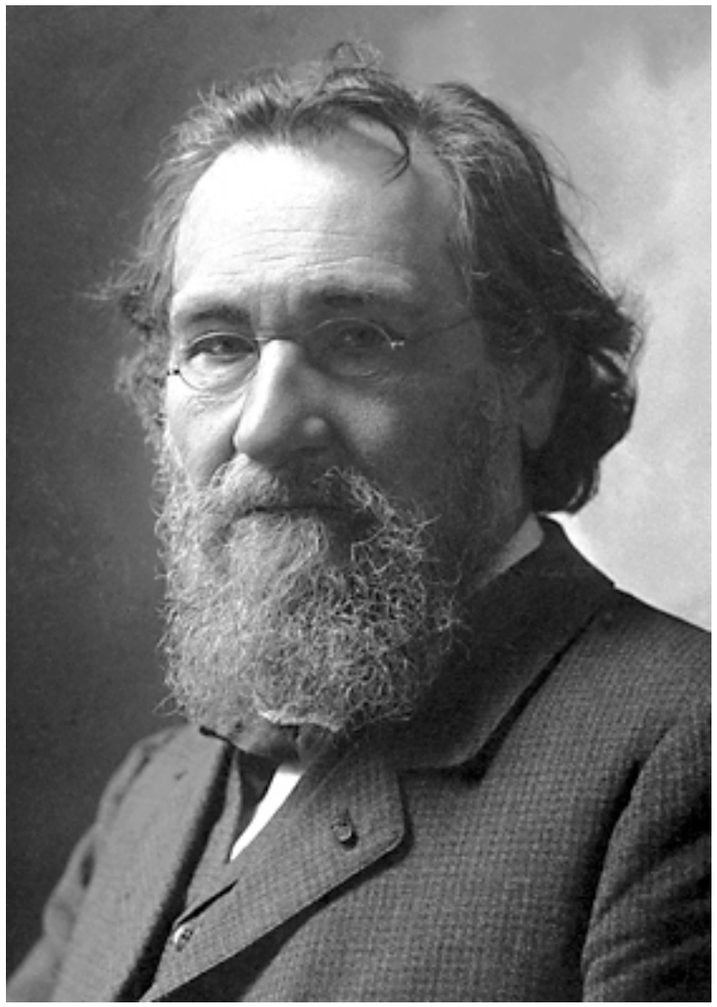
— Sir Isaac Newton

PIONEERS

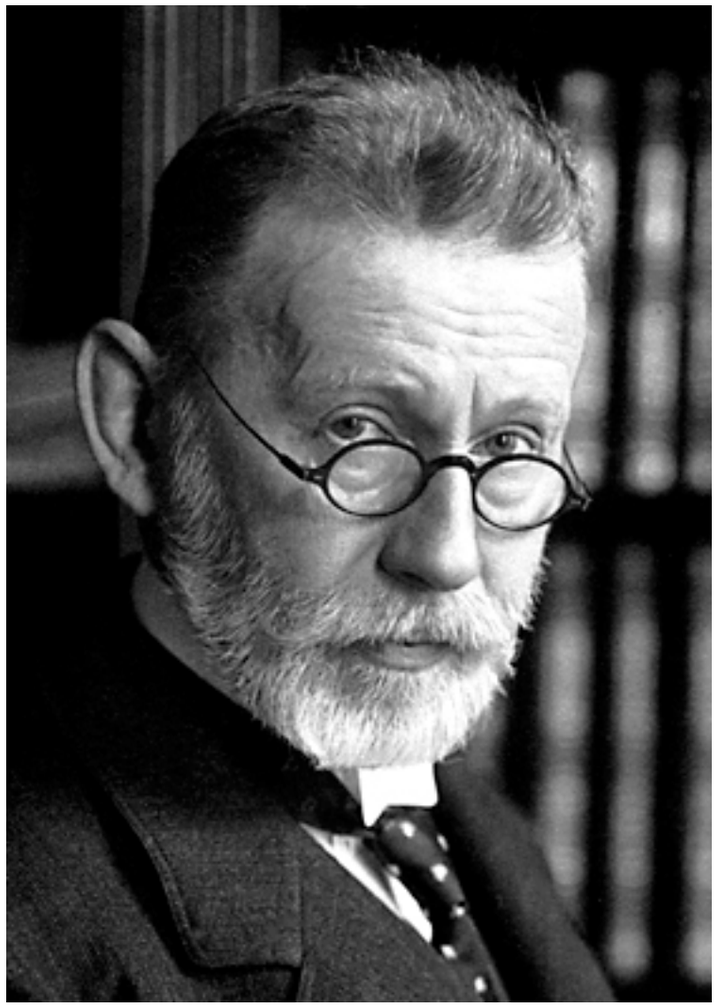
who helped make
therapeutic mAbs possible

The Nobel Prize in Physiology or Medicine 1908

"*in recognition of their work on immunity*"



Ilya Ilyich Mechnikov

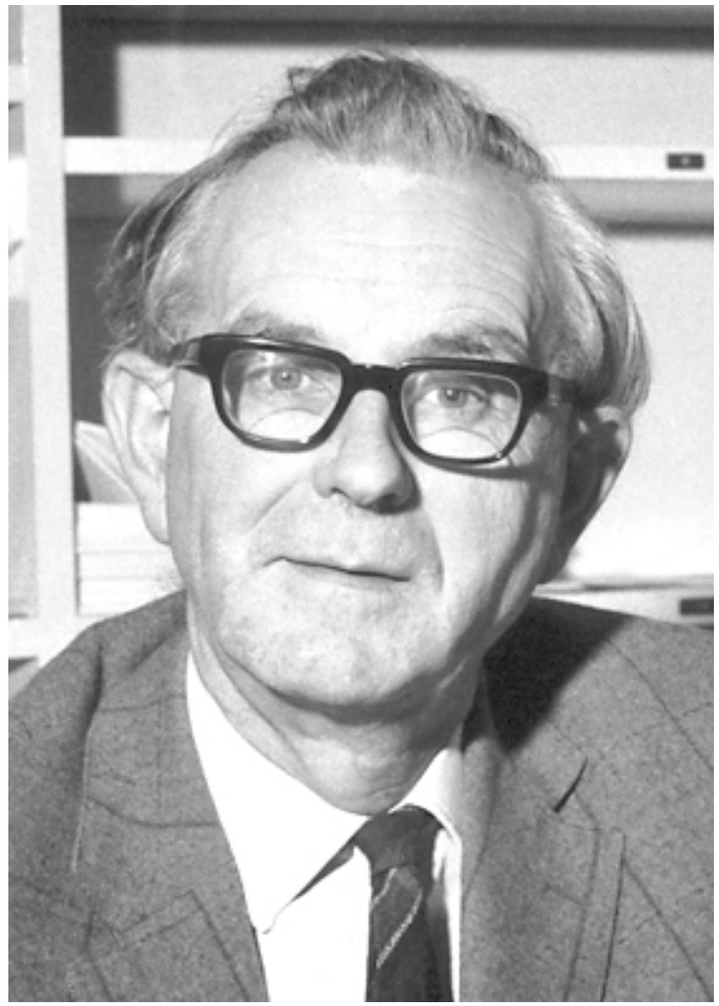


Paul Ehrlich

The Nobel Prize in Physiology or Medicine 1972

"*for their discoveries concerning the chemical structure of antibodies*"

Gerald M. Edelman

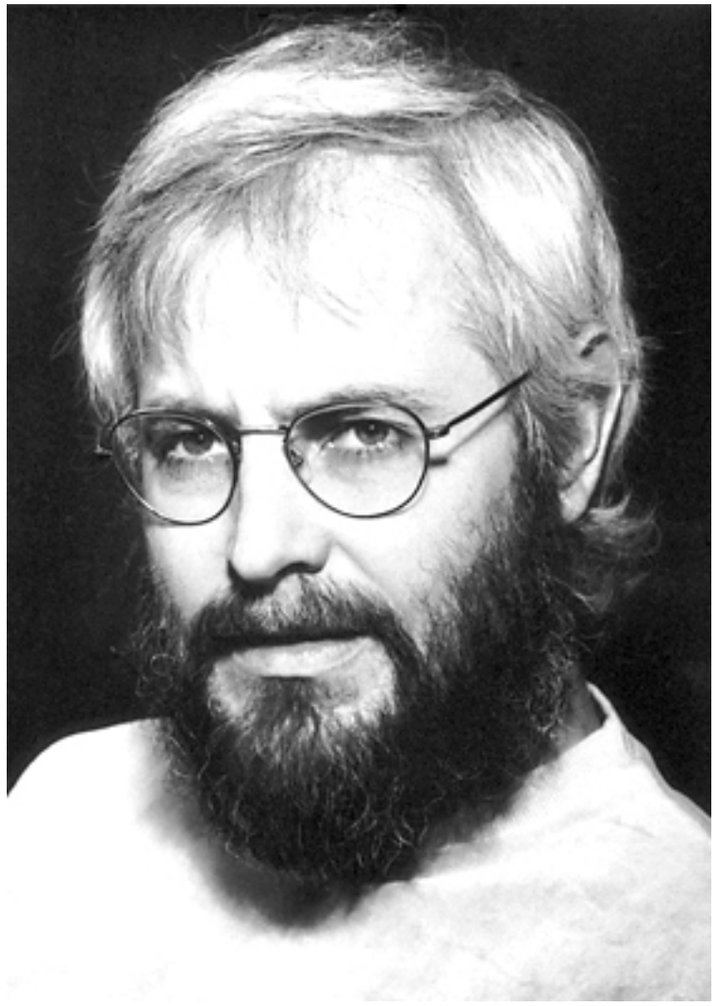


Rodney R. Porter

The Nobel Prize in Physiology or Medicine 1984

"for theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of monoclonal antibodies"

Niels K. Jerne



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