Laboratorio di chimica bioorganica

Misure di binding e di inibizione

Federico Berti

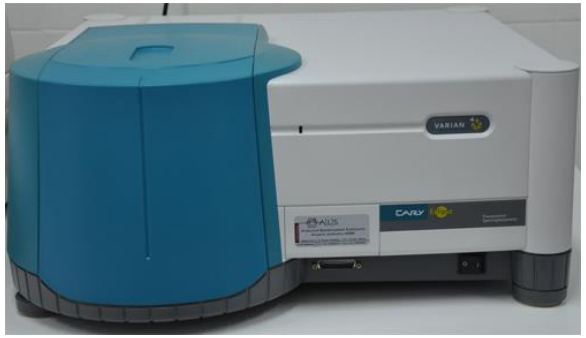
Misura di una costante di equilibrio

$$R + L \underset{k_{off}}{\overset{k_{on}}{=}} \mathrm{RL}$$

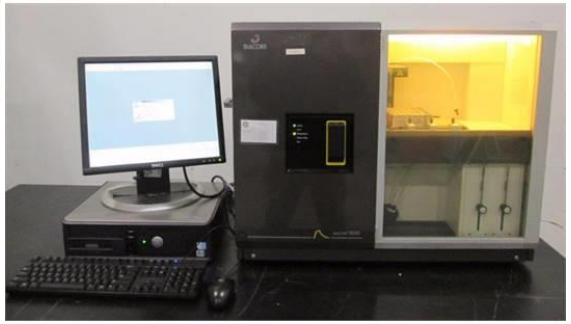
Free **Bound**

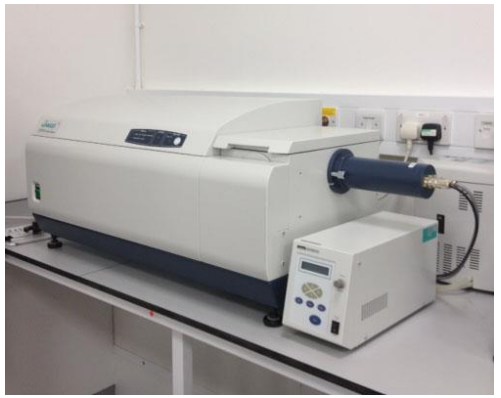
$$K_{Ass} = \frac{[RL]}{[R][L]}$$

$$K_D = \frac{[R][L]}{[RL]} = \frac{k_{off}}{k_{on}}$$



Libero contro legato





Misure all'equilibrio: l'isoterma di Langmuir

$$R + L = \mathrm{RL}$$

$$K_D = \frac{[R][L]}{[RL]}$$

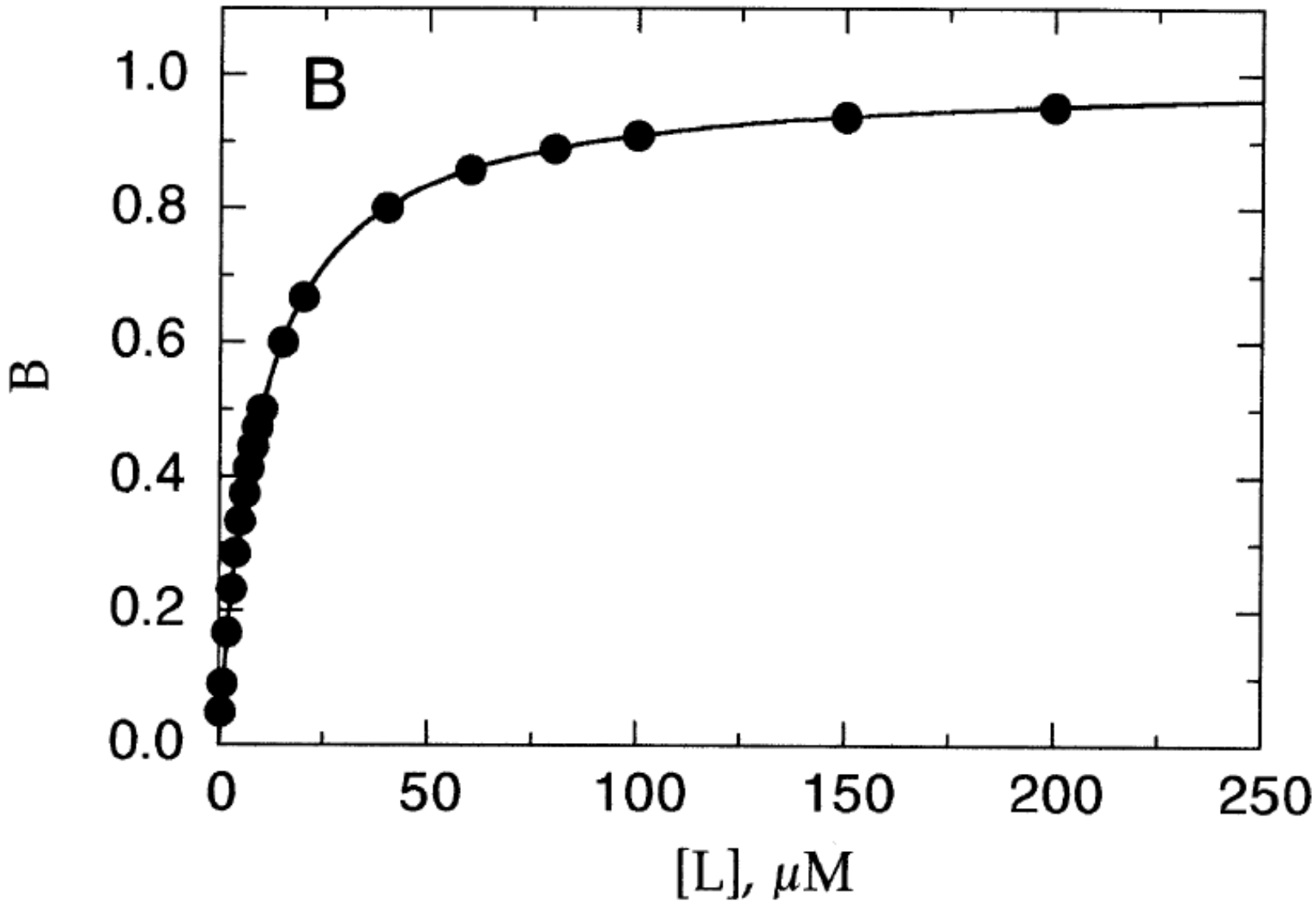
$$[RL] = K_D[R][L]$$

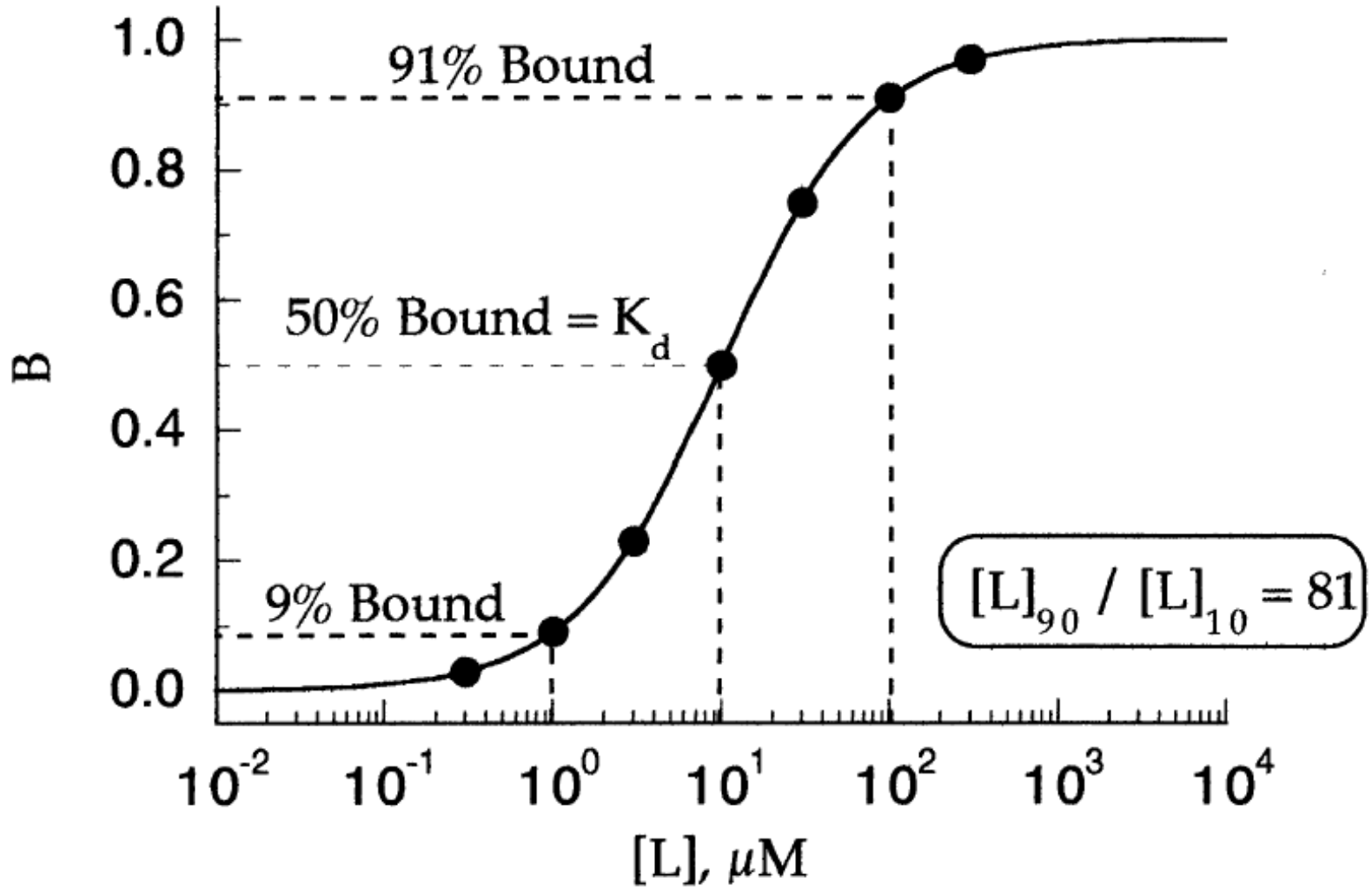
$$[R] = [R]_0 + [RL]$$

$$[L] \approx [L]_0$$

$$\boldsymbol{[RL] = \frac{[R]_0[L]_0}{K_D + [L]_0}}$$

$$\frac{[RL]}{[R]_0} = \boldsymbol{B} = \boldsymbol{\frac{[L]_0}{K_D + [L]_0}}$$




1.0
0.8
0.6
0.4
0.2
0.0
B
91% Bound
50% Bound = K_d
9% Bound
[L]_90 / [L]_10 = 81
10^-2
10^-1
10^0
10^1
10^2
10^3
10^4
[L], μM

The Langmuir isothermal is valid only if $[L] \approx [L]_0$ and gives the true value of K_D only if $[R]_0 \leq 2K_D$

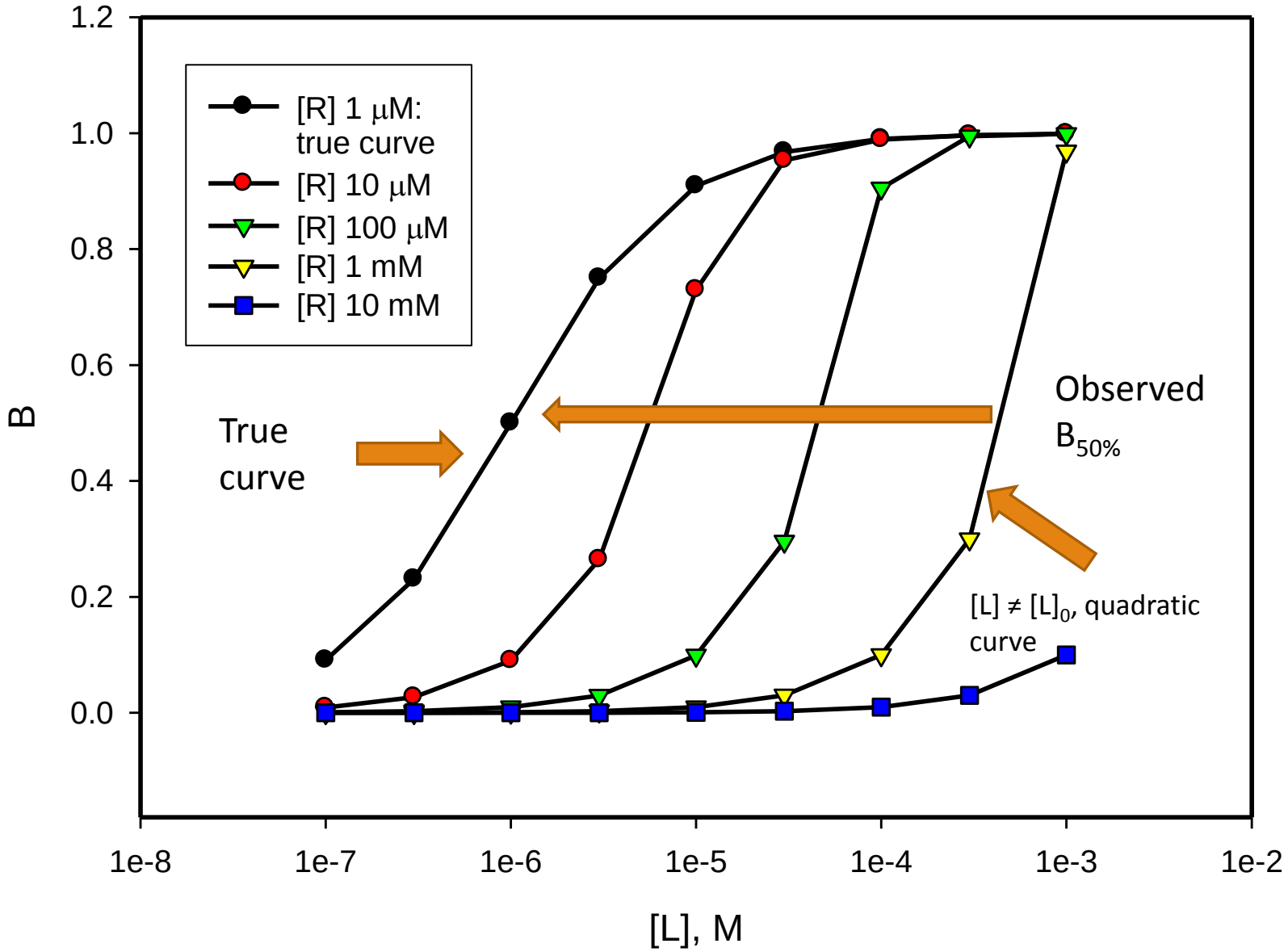
Otherwise, a fit to the Langmuir isothermal will give physically meaningless results, and apparent values of K_D

Fitting the binding data to the «quadratic curve» will always yield the true affinity:

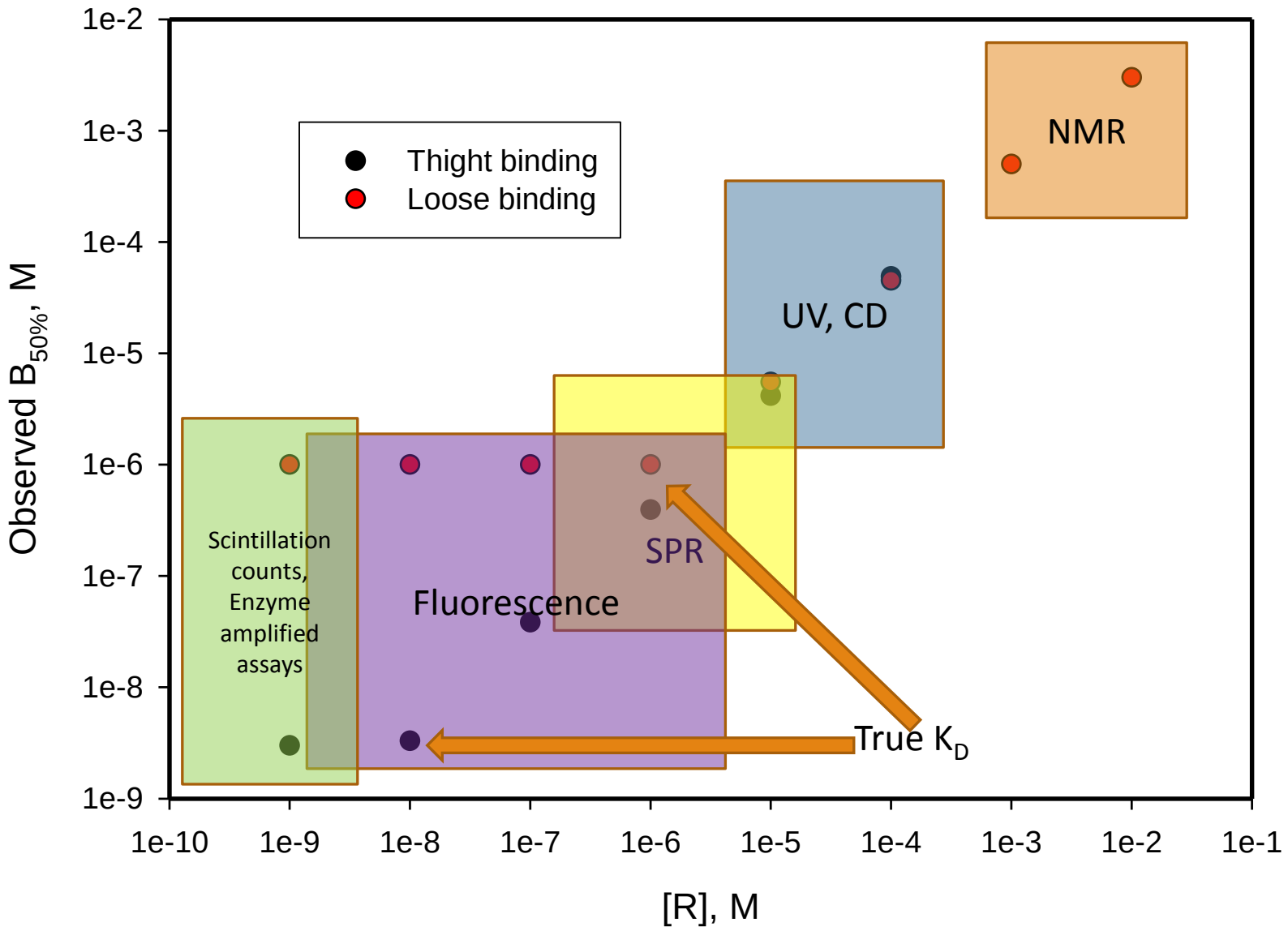
$$R + L = RL$$

$$K_D = \frac{([R]_0 - [RL])([L]_0 - [RL])}{[RL]}$$

$$[RL] = \frac{([R]_0 + [L]_0 + K_D) - \sqrt{([R]_0 + [L]_0 + K_D)^2 - 4[R]_0[L]_0}}{2}$$



One should fit the data to the Langmuir isothermal only if $[R]_0$ is safely below K_D.
The meaure can be done only with high sensitivity techniques.
In any other case, the quadratic curve must be used to fit the data



Transforms of the binding isotherms

3. Hill equation:

$$RL_n = R + nL$$

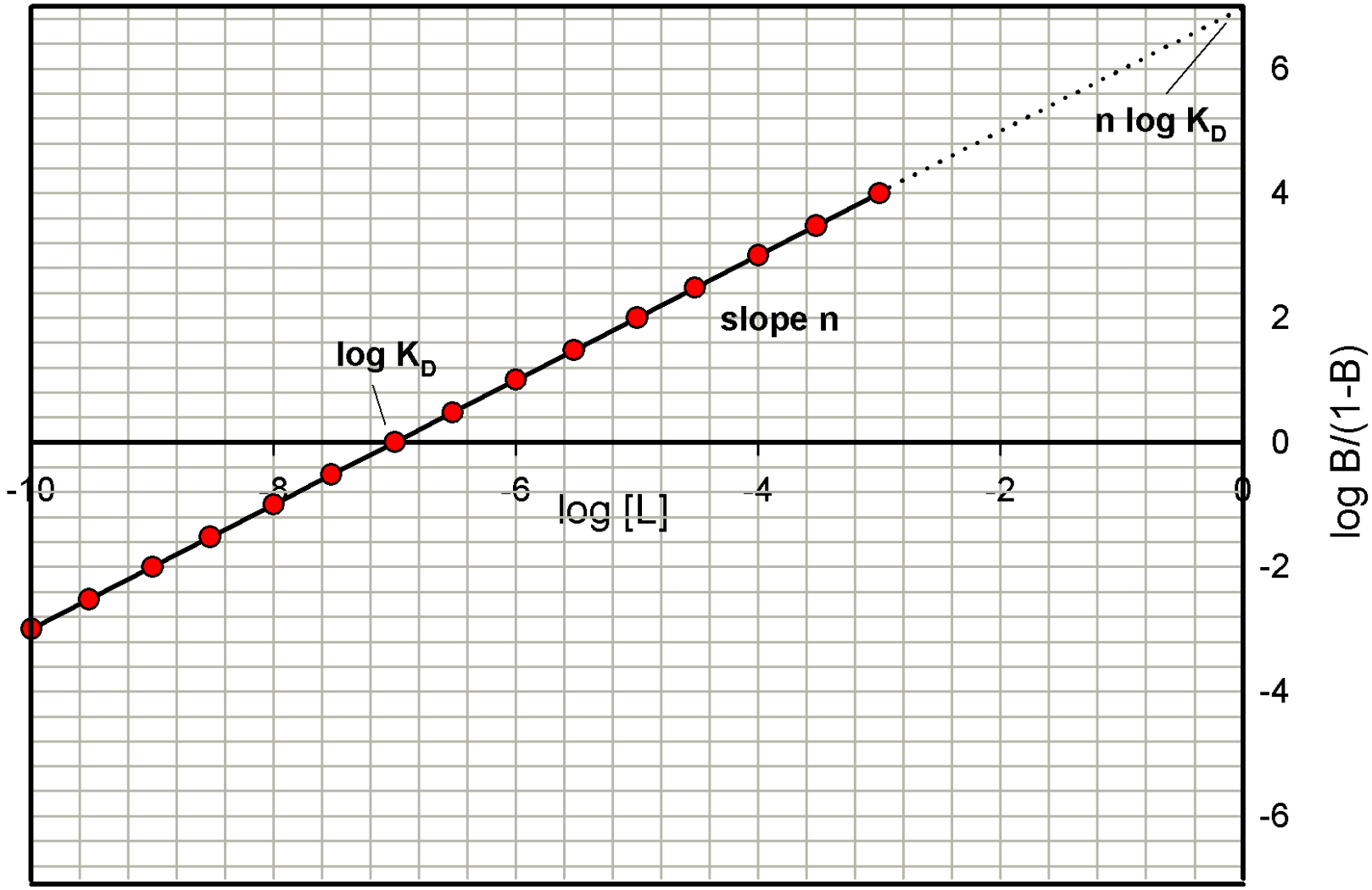
$$B = \frac{[L]^n}{{K_D}^n + [L]^n}$$

$$\frac{1}{[B]} = 1 + \left(\frac{K_D}{[L]}\right)^n$$

$$\frac{B}{1-B} = \left(\frac{[L]}{K_D}\right)^n$$

$$\log\frac{B}{1-B} = n\log[L] - n\log K_D$$

In the examples K_D is always 100 nM and $[R]_0$ is always 10 nM



Binding energetics

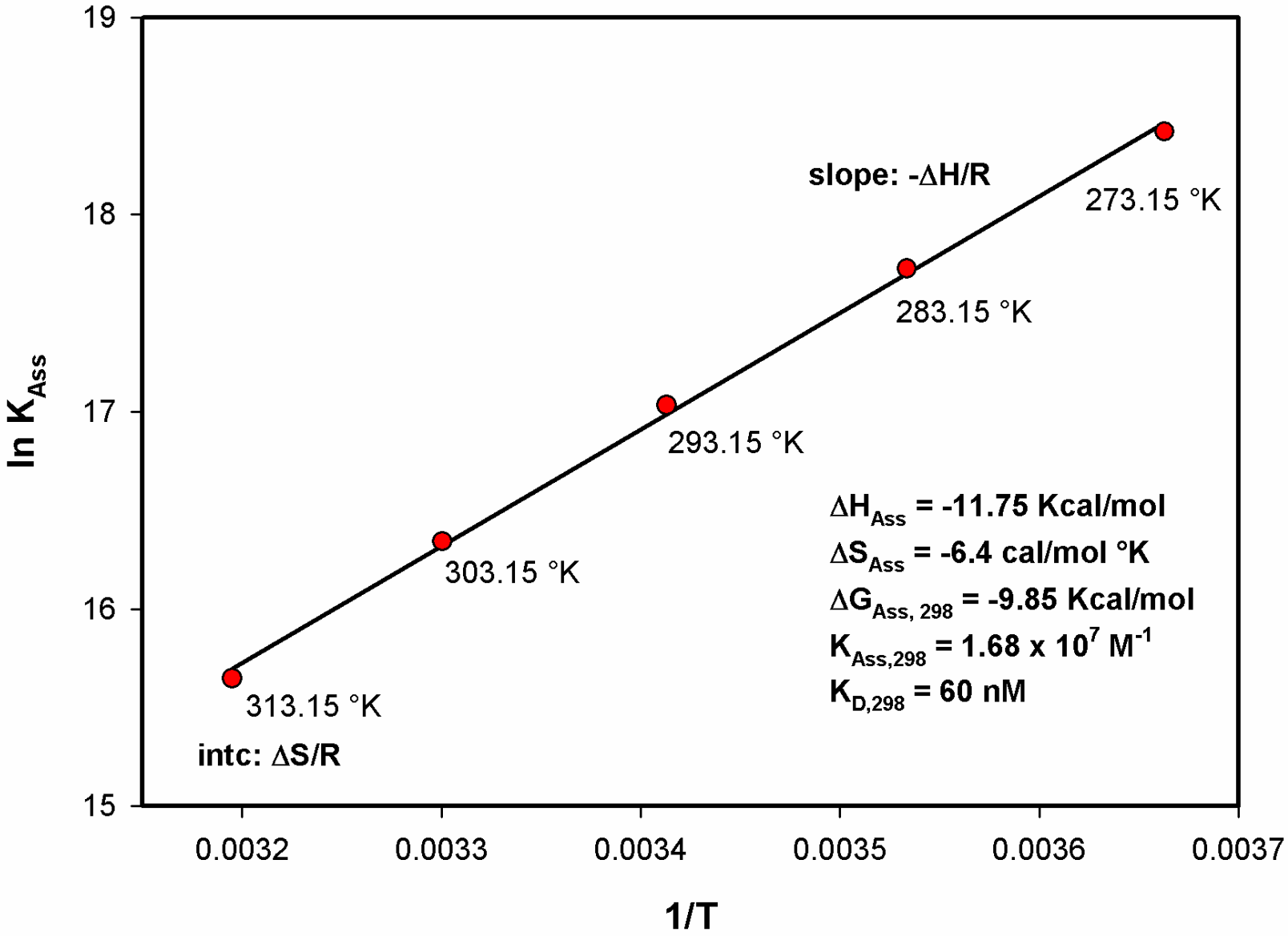
Although binding equilibria are most often defined on K_D, binding energies are always referred to the association process.

The Van't Hoff plot is commonly used to obtain the association enthalpy and entropy.

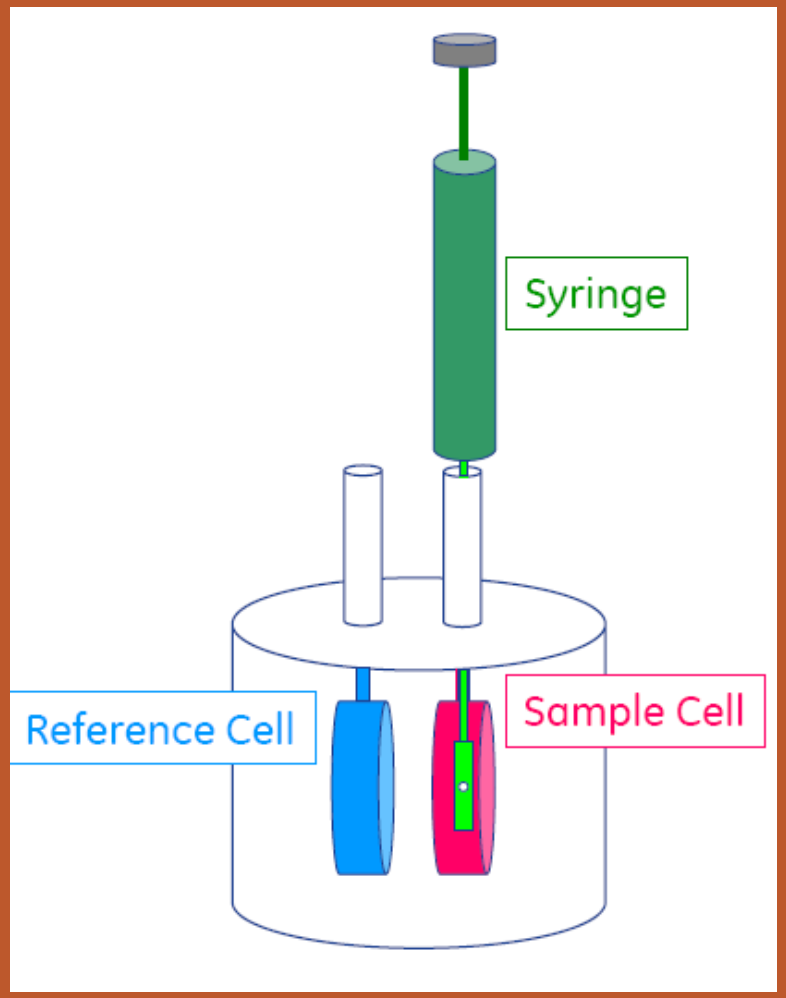
$$\ln K_{Ass} = -\frac{\Delta G}{RT}$$

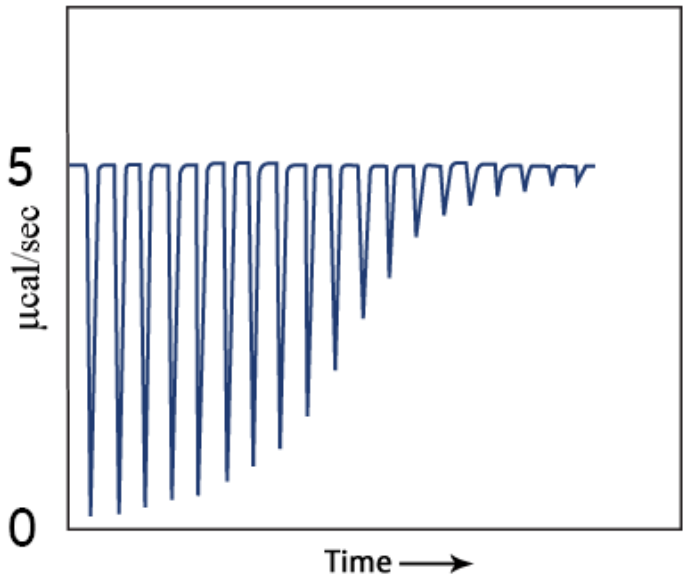
$$\ln K_{Ass} = \frac{\Delta H}{RT} + \frac{\Delta S}{R}$$

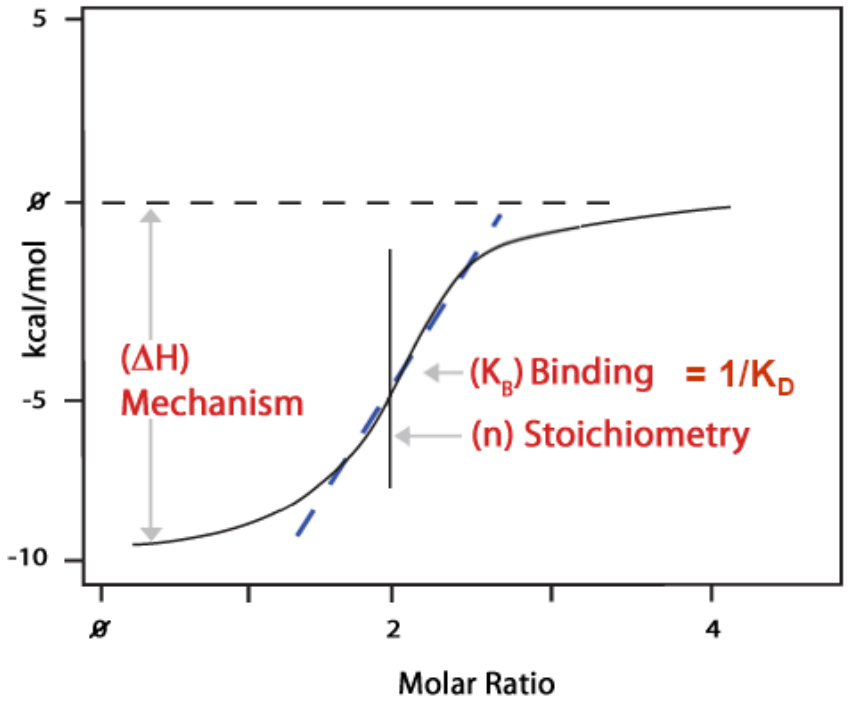
Binding energies are affected by large errors! A 5% error on K_D measured in a 40°K range leads to a 11% error on ΔH values around 10 Kcal/mol and to a >50% error on ΔS values around 10 cal/mol°K.



Isothermal titration calorimetry







Measuring B vs. F without isolating B or F

- In a homogeneous / colloidal system
- In a heterogenous system with immobilized R or L

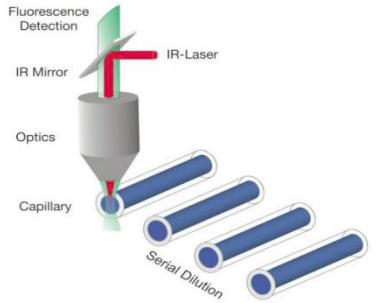
- From a spectral change:
  - Appearance of a new signal due to RL
  - Change of a signal due to R (decrease / increase of intensity, shift)
  - Displacement of a label from R
- From a change of a general physical property of the sensor (plasmon resonance, r.i. at the interface …)

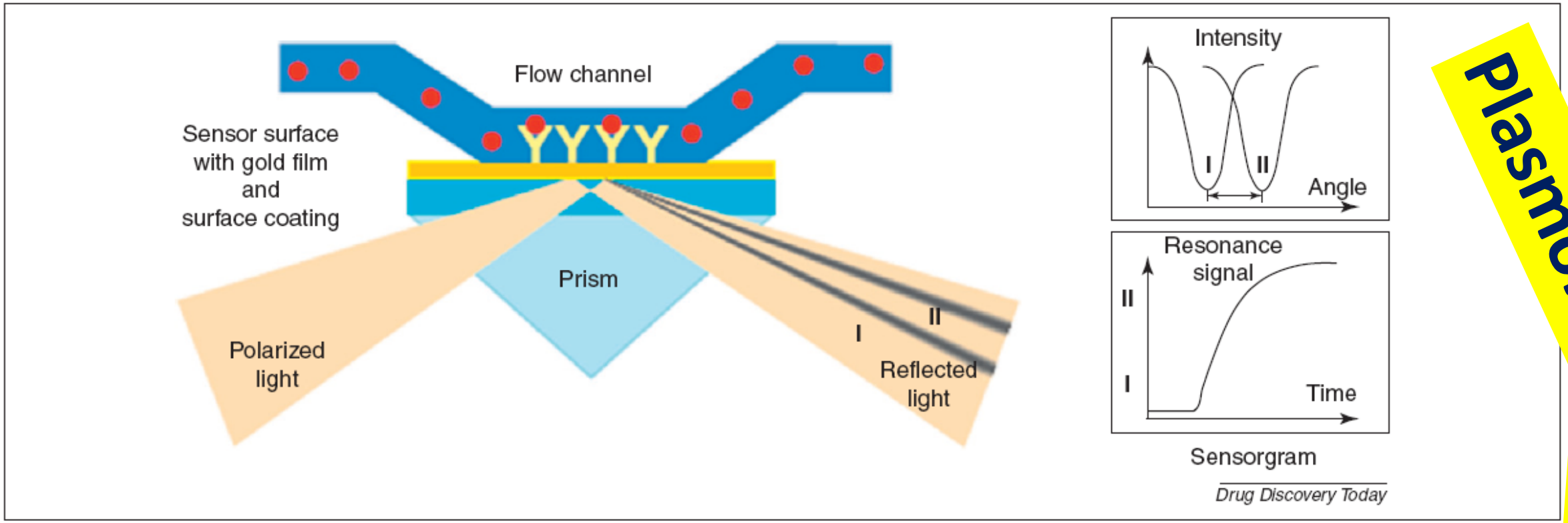
L mother solution(s) in a co-solvent

Limit the added volume to 5%! Check the effect of the co-solvent alone

	Precision %	Accuracy %
P10	1.25	2.5
P20	1.5	5
P200	0.4	1
P1000	0.3	1.5
Hamilton 10 µL	1	1

main problems are due to
- Different density and mixing times
- Different temperatures
- **Your hands**
- **Your attention**





Plasmon Resonance

FIGURE 1

Basic configuration of a surface plasmon resonance (SPR) sensor. It consists of a prism mounted on a sensor chip with a thin gold film. The surface of the gold film on the side opposite the prism is coated and contains the immobilized target protein. The latter is exposed to the drug via a microfluidic flow channel. In essence, SPR makes use of the excitability of oscillating electrons at the metal film surface by light. When the quantum energy carried by light photons exactly equals the quantum energy level of the metal electrons, a plasmon is created. This is a group of excited electrons that behave as a single electrical entity. Under the conditions where incident light energy is absorbed and plasmons are created (i.e. attenuated total reflection), a dip in the intensity of reflected light is observed. The wavelength at which this occurs is termed the 'plasmon resonance wavelength'. The plasmon created generates an electrical field known as an evanescent field, on both sides of the metal surface. This field decays exponentially, the strongest being at the metal surface with a limited range of approximately 300 nm. SPR instrumentation exploits a unique feature of this plasmon field: any change in the chemical composition of the environment within the range of the evanescent field causes a change in the conditions at which light couples with the plasmon. This includes biomolecular interactions occurring at the sensor surface impacting the refractive index within the evanescent wave. The resulting shift in the wavelength of light, which is absorbed rather than reflected, can be measured as a change in resonance angle or resonance wavelength. The magnitude of the shift is quantitatively related to the magnitude of the chemical change. SPR instrumentation exploits exactly this dependency of the SPR signal on the chemical environment of the metal film carrying immobilized ligand exposed to potential binding partners.

SPR

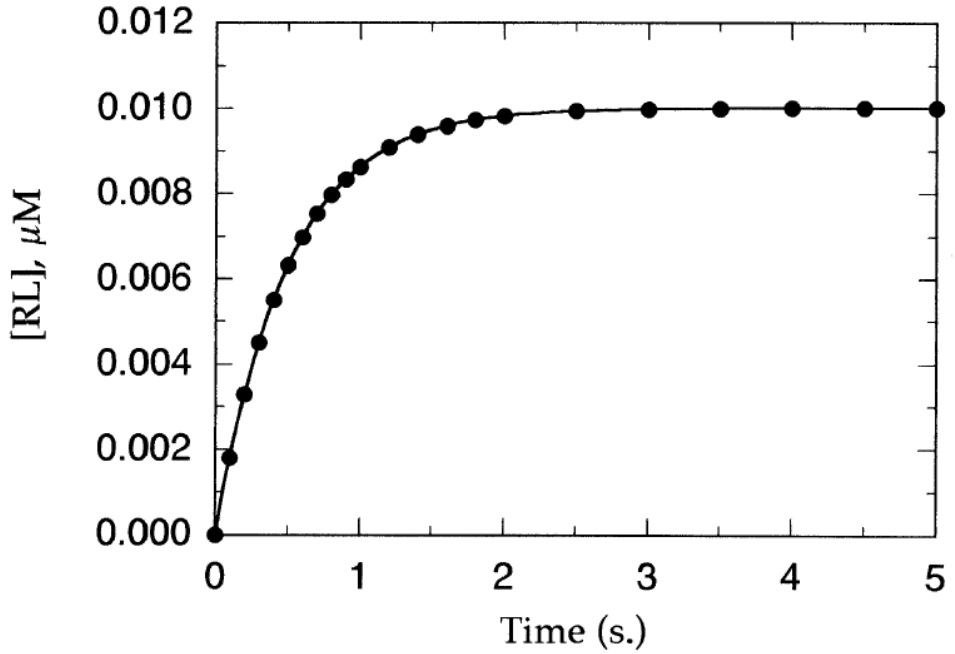
The kinetic approach to equilibrium

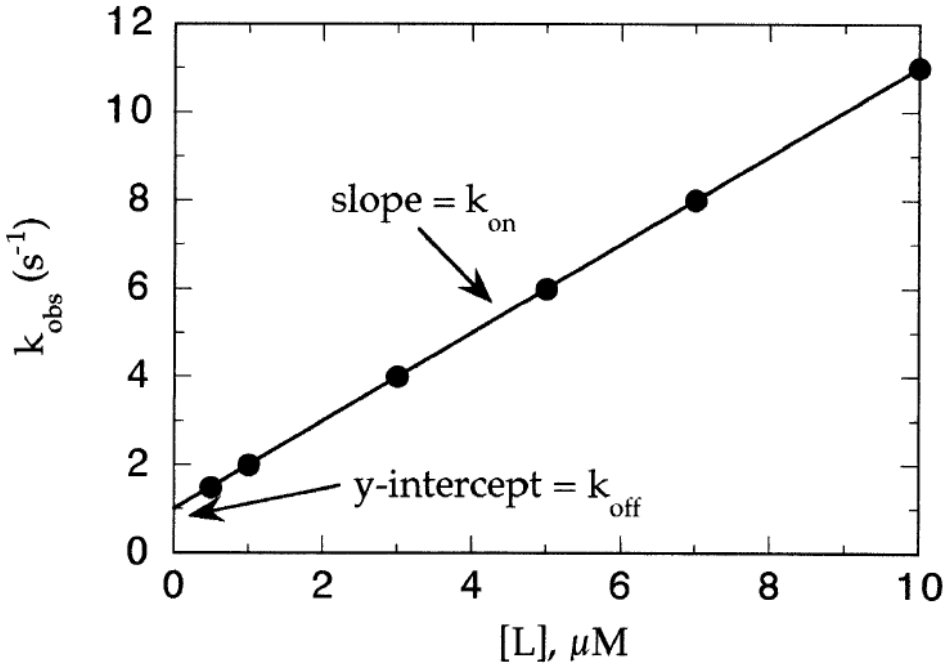
$$R + L \underset{k_{off}}{\overset{k_{on}}{=}} \mathrm{RL}$$

$$K_D = \frac{k_{off}}{k_{on}}$$

$$[RL] = [RL]_{eq}[1 - \exp(-k_{obs}t)]$$

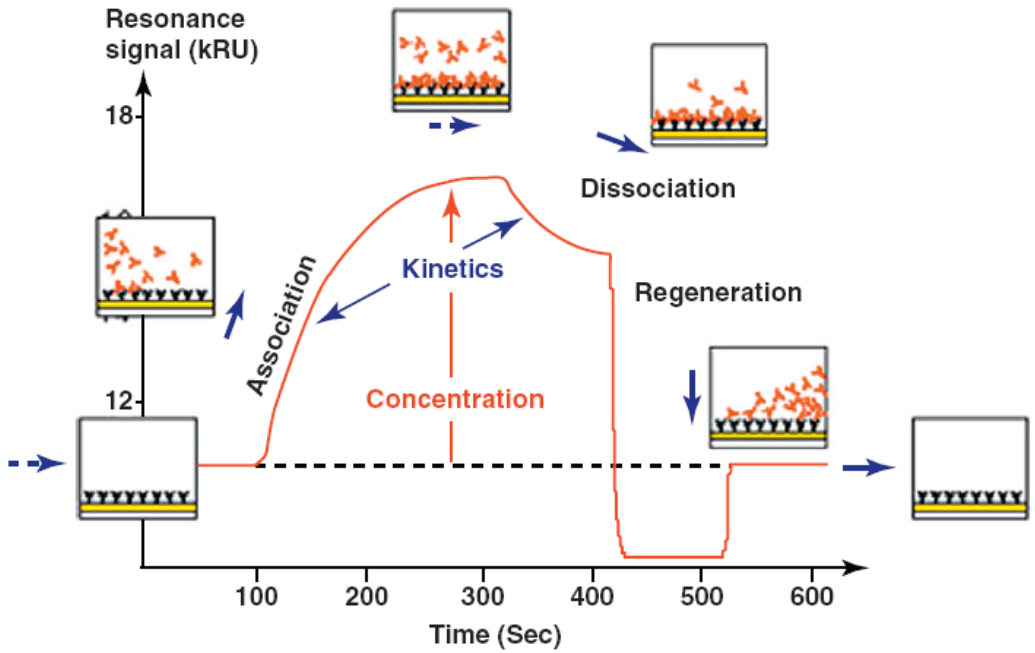
$$k_{obs} = k_{off} + k_{on}[L]$$

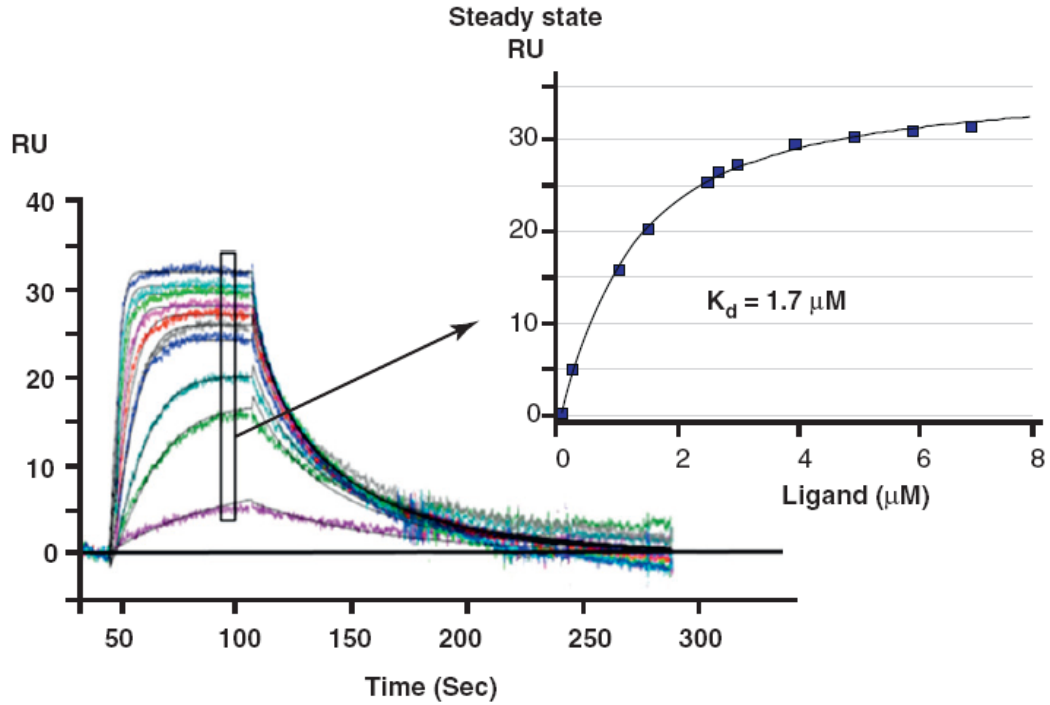




SPR

The kinetic approach to equilibrium





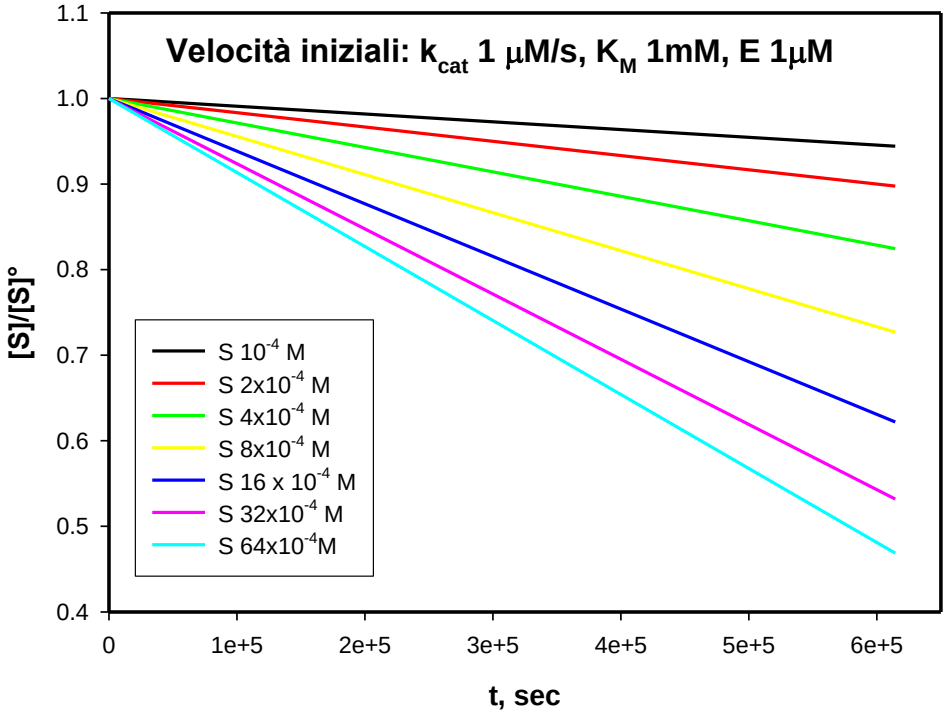
Misure di tipo cinetico: inibitori enzimatici

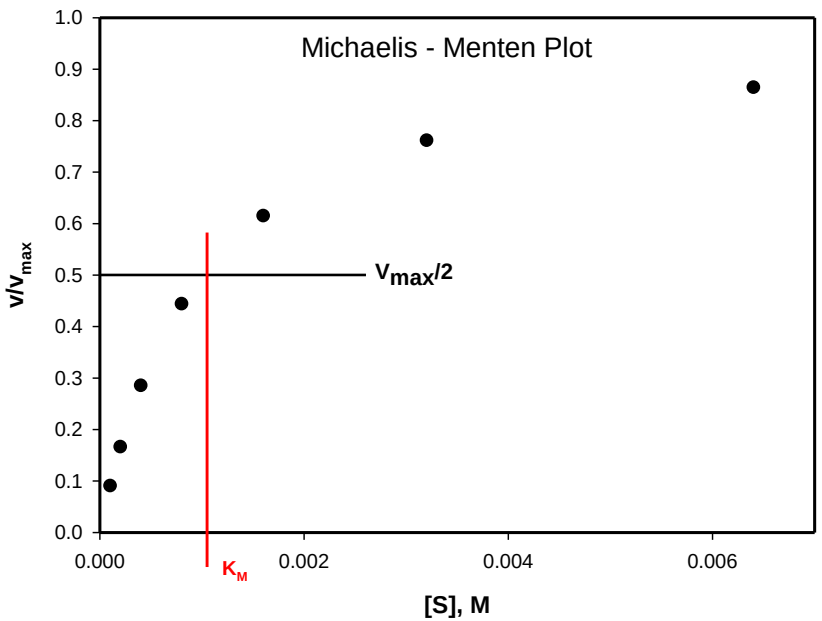
$$E + S \underset{k_{-1}}{\overset{k_1}{=}} ES \xrightarrow{k_2} E + P$$

$$\frac{d[S]}{dt} = -\frac{k_2\,[E][S]}{[S] + K_M}$$

$$\int_{S_0}^{S} \frac{[S] + K_M}{[S]}\, d[S] = \int_0^t k_2\,[E]\, dt$$

$$-\Delta[S] + K_M \ln\frac{[S]_0}{[S]} = k_2\,[E]\,t$$





La misura di IC_{50}

$$IC_{50} = K_i \left(1 + \frac{[S]}{K_M}\right)$$

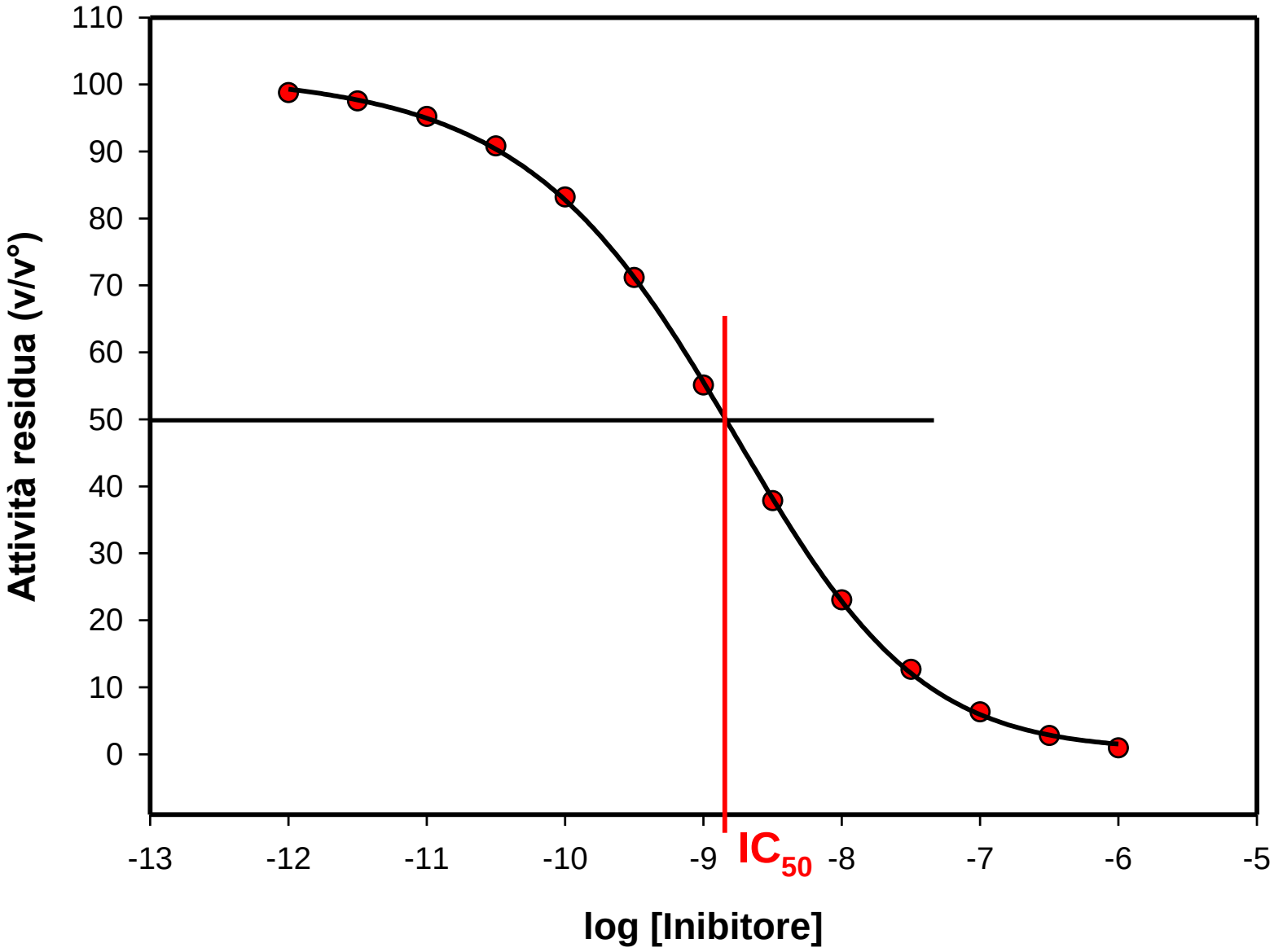
Nonlinear Regression
[Equation] f = min + (max-min)/(1+(x/logEC50)^Hillslope) fit f to y
tolerance=0.000001 stepsize=10 iterations=200
R = 0.99994783 Rsqr = 0.99989566 Adj Rsqr = 0.99986088
Standard Error of Estimate = 0.4654

	Coefficient	Std. Error	t	P
min	0.6930	0.3566	1.9431	0.0839
max	101.7382	0.4270	238.2415	<0.0001
logEC50	-8.8738	0.0114	-777.0828	<0.0001
Hillslope	-12.2548	0.1933	-63.4109	<0.0001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	3	18683.3928	6227.7976	28748.4423	<0.0001
Residual	9	1.9497	0.2166		
Total	12	18685.3425	1557.1119		

Durbin-Watson Statistic = 0.8641
Normality Test: K-S Statistic = 0.1509 Significance Level = 0.9049
Constant Variance Test: Passed (P = 0.8632)
Power of performed test with alpha = 0.0500: 1.0000



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Product Information

Human Calpain 1 Inhibitor Screening Kit

Catalog Number **MAK210**
Storage Temperature –70 °C