Biochemical / Biophysical Kinetics "Made Easy"

Software DYNAFIT in drug discovery research

Petr Kuzmič, Ph.D.
BioKin, Ltd.

1. **Theory**: differential equation models

   - **DYNAFIT** software

2. **Example**: lanthaScreen® Eu assay in "kinetic" mode

   - **p38a** kinase / antibody / tracer
   - **p38a** kinase / antibody / tracer / **desatinib**

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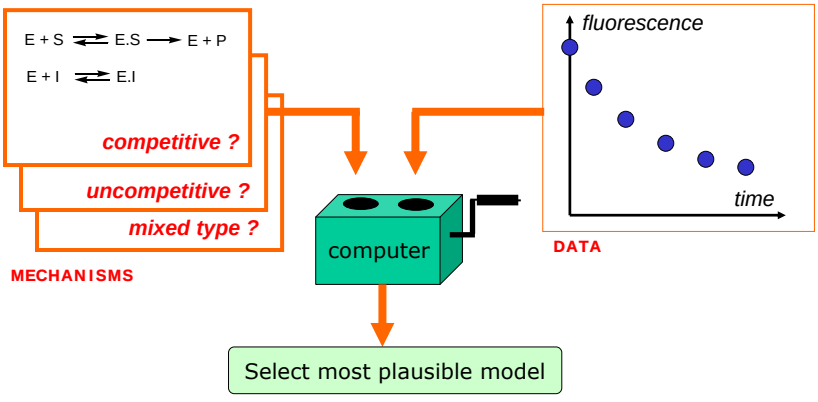
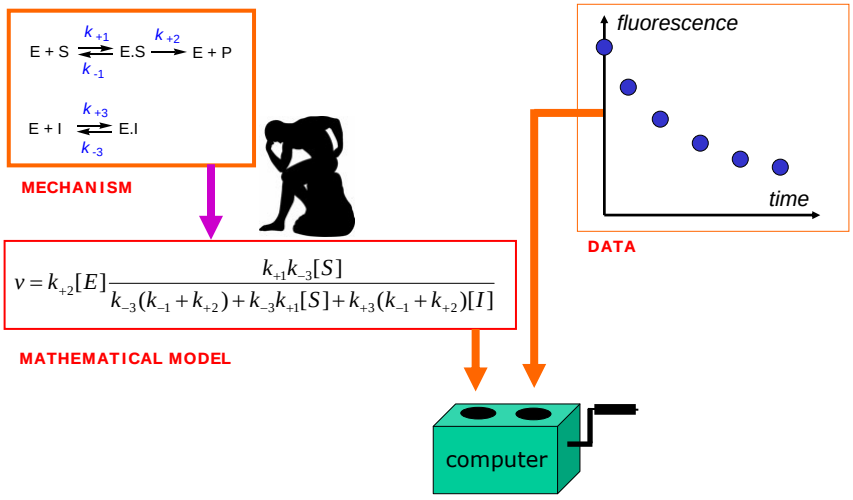
The task of mechanistic kinetics

SELECT AMONG MULTIPLE CANDIDATE MECHANISMS



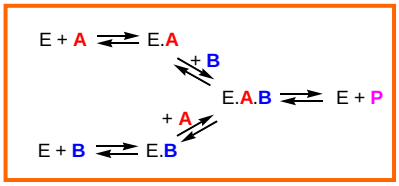
From mechanistic to mathematical models

DERIVE A MATHEMATICAL MODEL FROM BIOCHEMICAL/BIOPHYSICAL IDEAS



Problem: Simple mechanisms ...

MERELY FIVE REACTIONS ...



"RANDOM BI-UNI" MECHANISM

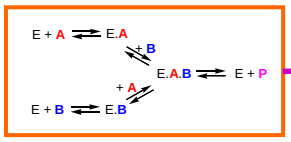
- 2 reactants (A, B)
- 1 product (P)

- 5 reversible reactions
- 10 rate constant

... lead to complex algebraic models

MERELY FIVE REACTIONS ...

Segel, I. (1975) *Enzyme Kinetics.* John Wiley, New York, p. 646.



"RANDOM BI-UNI" MECHANISM

$$\frac{v}{[E]_t} = \frac{K_1[A][B] + K_2[A]^2[B] + K_3[A][B]^2 - K_4[P] - K_5[A][P] - K_6[B][P]}{K_7 + K_8[A] + K_9[B] + K_{10}[A][B] + K_{11}[A]^2 + K_{12}[B]^2 + K_{13}[A]^2[B]}$$

$$+ K_{14}[A][B]^2 + K_{15}[P] + K_{16}[A][P] + K_{17}[B][P] + K_{18}[A][B][P] \qquad (IX\text{-}181)$$

$(k_{-5} = 0)$

where K_1 through K_{18} represent combinations of rate constants:

$K_1 = k_1k_{-2}k_3 + k_{-1}k_2k_4k_5$, $K_2 = k_1k_3k_4k_5$, $K_3 = k_2k_3k_4k_5$,

$K_4 = k_{-1}k_{-2}k_{-3}k_{-5} + k_{-1}k_{-2}k_{-4}k_{-5}$, $K_5 = k_{-1}k_{-3}k_4k_{-5}$,

$K_6 = k_{-2}k_3k_{-4}k_{-5}$, $K_7 = k_{-1}k_{-2}k_{-3} + k_{-1}k_{-2}k_{-4} + k_{-1}k_{-2}k_5$,

$K_8 = k_1k_{-2}k_{-3} + k_1k_{-2}k_{-4} + k_1k_{-2}k_5 + k_{-1}k_{-3}k_4 + k_{-1}k_4k_5$,

$K_9 = k_{-1}k_2k_{-3} + k_{-1}k_2k_{-4} + k_{-1}k_2k_5 + k_{-2}k_3k_{-4} + k_{-2}k_3k_5$,

$K_{10} = k_1k_{-2}k_3 + k_{-1}k_2k_4 + k_1k_3k_{-4} + k_2k_{-3}k_4 + k_3k_4k_5$,

$K_{11} = k_1k_{-3}k_4 + k_1k_4k_5$, $K_{12} = k_2k_3k_{-4} + k_2k_3k_5$, $K_{13} = k_1k_3k_4$,

$K_{14} = k_2k_3k_4$, $K_{15} = k_{-1}k_{-2}k_{-5} + k_{-1}k_{-4}k_{-5} + k_{-2}k_{-3}k_{-5}$,

$K_{16} = k_{-1}k_4k_{-5} + k_{-3}k_4k_{-5}$, $K_{17} = k_{-2}k_3k_{-5} + k_3k_{-4}k_{-5}$, $K_{18} = k_3k_4k_{-5}$

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New approach: Numerical Kinetics

NO MORE ALGEBRA: LET THE **COMPUTER** DEAL WITH IT !

Theoretical foundations: *Mass Action Law*

RATE IS PROPORTIONAL TO CONCENTRATION(S)

MONOMOLECULAR REACTIONS

A ⟶ products

rate is proportional to **[A]**

$-\,d[A]/dt = k[A]$

BIMOLECULAR REACTIONS

A + B ⟶ products

rate is proportional to **[A] × [B]**

$-\,d[A]/dt = -\,d[B]/dt = k[A] \times [B]$

Theoretical foundations: *Mass Conservation Law*

PRODUCTS ARE FORMED WITH THE SAME RATE AS REACTANTS DISAPPEAR

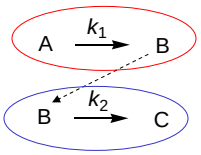
EXAMPLE

A ⟶ P + Q

$-\,d[\mathbf{A}]/dt = +\,d[\mathbf{P}]/dt = +\,d[\mathbf{Q}]/dt$

COMPOSITION RULE ADDITIVITY OF TERMS FROM SEPARATE REACTIONS

mechanism:



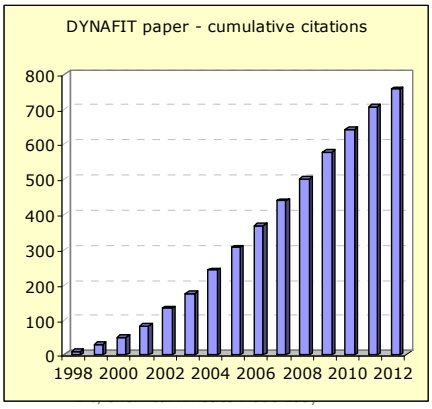
$d[B]/dt = +k_1[A] - k_2[B]$

Software package DYNAFIT

REFERENCES

1. Kuzmic P. (1996) *Anal. Biochem.* **237**, 260-273.
"Program DYNAFIT for the analysis of enzyme kinetic data"

2. Kuzmic P. (2009) *Meth. Enzymol.*, **467**, 247-280.
"DYNAFIT – A software package for enzymology"



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9

A "Kinetic Compiler"

HOW DYNAFIT PROCESSES YOUR BIOCHEMICAL EQUATIONS

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E.S \xrightarrow{k_3} E + P$$

Input (plain text file):

```
E + S ---> ES   :  k1
ES ---> E + S   :  k2
ES ---> E + P   :  k3
```

Rate terms:

$k_1 \times [E] \times [S]$

$k_2 \times [ES]$

$k_3 \times [ES]$

Rate equations:

$d[\boldsymbol{E}] / dt = - k_1 \times [E] \times [S] + k_2 \times [ES] + k_3 \times [ES]$

$d[\boldsymbol{ES}] / dt = + k_1 \times [E] \times [S] - k_2 \times [ES] - k_3 \times [ES]$

Similarly for other species...

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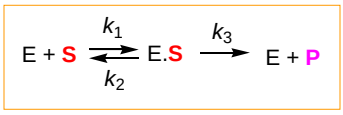
System of Simple, Simultaneous Equations

HOW DYNAFIT PROCESSES YOUR BIOCHEMICAL EQUATIONS



"The LEGO method"
of deriving rate equations

Input (plain text file):	Rate terms:	Rate equations:
E + S ---> ES : k1 ES ---> E + S : k2 ES ---> E + P : k3	$k_1 \times [E] \times [S]$ $k_2 \times [ES]$ $k_3 \times [ES]$	

DynaFit can analyze many types of experiments

MASS ACTION LAW AND MASS CONSERVATION LAW IS APPLIED IN THE SAME WAY

chemistry
biophysics
pharmacology
enzymology

EXPERIMENT	DYNAFIT DERIVES A SYSTEM OF ...
Kinetics (time-course)	Ordinary differential equations (ODE)
Equilibrium binding	Nonlinear algebraic equations
Initial reaction rates	Nonlinear algebraic equations

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1. **Theory**: differential equation models

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2. **Example**: lanthaScreen® Eu assay in "kinetic" mode

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   - **p38α** kinase / antibody / tracer / **desatinib**

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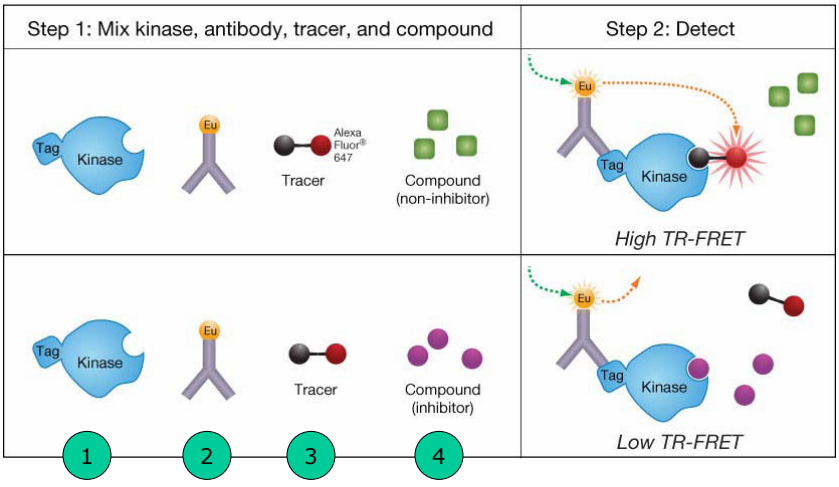
Kinase – Antibody – Tracer – Inhibitor assay

A **FOUR-COMPONENT** MIXTURE



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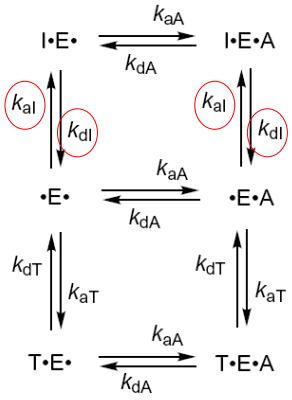
Kinase – Antibody – Tracer – Inhibitor: mechanism

PURPOSE: OBTAIN **RATE CONSTANTS** FOR INHIBITOR ASSOCIATION & DISSOCIATION



E … **e**nzyme
A … **a**ntibody (FRET donor)
T … **t**racer (FRET acceptor)
I … **i**nhibitor

- **four** components
- **five** complexes (3 binary, 2 ternary)
- **six** *unique* rate constants

Rate constants and receptor-ligand residence time

IS IT WORTH CHASING AFTER **RATE** CONSTANTS?

Mbalaviele *et al.* (2009) *J. Pharm. Exp. Ther.* **329**, 14-25

"PHA-408 is an ATP competitive inhibitor, which binds **IKK-2** tightly with a relatively slow **off rate**."

Puttini *et al.* (2008) *haematologica* **93**, 653-61

"The present results suggest a slower **off-rate** (dissociation rate) of [a novel **Abl kinase** inhibitor] compared to **imatinib** as an explanation for the increased cellular activity of the former."

Tummino & Copeland (2008) *Biochemistry* **47**, 5481-92

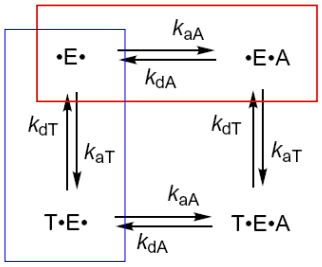
"... the extent and duration of responses to receptor-ligand interactions depend greatly on the **time period** over which the ligand is in **residence** on its receptor."

Let's look at Kinase + Antibody + Tracer only.

No Inhibitor yet.

Research plan: Stepwise model building

ASSUME THAT THE ANTIBODY AND THE PROBE BIND **INDEPENDENTLY**



1. get k_{aA} and k_{dA}
2. get k_{aT} and k_{dT}

- only then add the "unknown" inhibitor

Try to find conditions that might allow treating this as a simple "A + B" (two-component) system.

Classical method: "k(obs)" assuming [Antibody] >> [Kinase]

GOODRICH & KUGEL (2006) "Binding and Kinetics for Molecular Biologists", pages 91-95

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB$$

$$AB_t = AB_{max}(1 - e^{-k_{obs}t}) \quad (27)$$

$$k_{obs} = k_1[B]_i + k_{-1} \quad (25)$$

Summary: How to experimentally measure k_1 and k_{-1}

1. Develop an assay to monitor AB.
2. Combine A and B at $t = 0$ ($[B]_i >> [AB]_{max}$).
3. Measure accumulation of AB over time.
4. Plot data and determine k_{obs} (using Equation 24 or 27).
5. Repeat Steps 2–4 at multiple different $[B]_i$.
6. Plot k_{obs} versus $[B]_i$ and determine k_1 (and k_{-1}) (Equation 25).
7. Consider whether k_1 is diffusion limited.

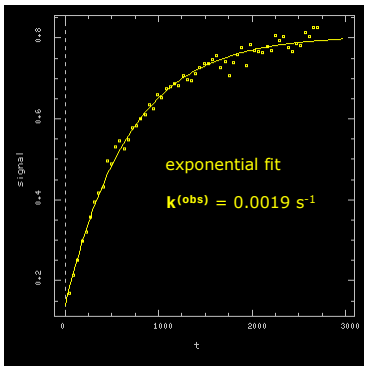
Enzyme + Antibody at very large excess of [Tracer] (pt. 1)

THIS **MIGHT** ALLOW US TO TREAT THIS AS A SIMPLE "A + B" (TWO-COMPONENT) SYSTEM

METHOD:

1. Incubate Tracer & Kinase
   - [Tracer] fixed, very large excess
   - [Kinase] varied
2. Wait 10 minutes to equilibrate
3. Add [Antibody]
4. Measure increase in fluorescence (T.E.A)

$$T{\bullet}E{\bullet} \underset{k_{dA}}{\overset{k_{aA}}{\rightleftharpoons}} T{\bullet}E{\bullet}A$$



[E] = 1.613 nM
[A] = 0.2 nM
[T] = 200 nM

Enzyme + Antibody at very large excess of [Tracer] (pt. 2)

DYNAFIT INPUT "SCRIPT": WE **CAN** USE SIMPLE ALGEBRAIC MODELS AS WELL

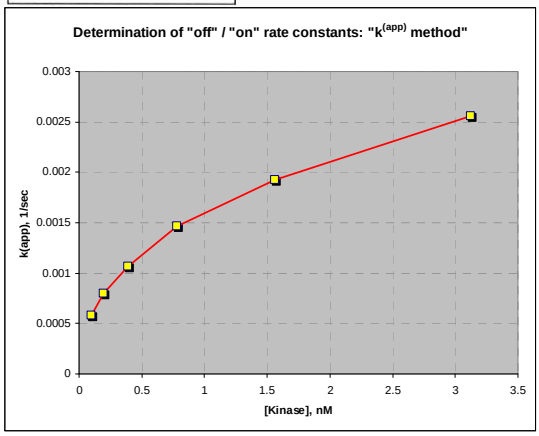
```
[task]

   task = fit
   data = generic

[parameters]

   t
   Ao, A, k

[model]

   Ao =   0.1 ?
   A  =     1 ?
   k  = 0.001 ?

   F = Ao + A*(1 - exp(-k*t))
```

$$AB_t = AB_{max}(1 - e^{-k_{obs}t}) \qquad (27)$$

Enzyme + Antibody at very large excess of [Tracer] (pt. 3)

TRY TO FIT $k^{(obs)}$ TO THE STRAIGHT LINE MODEL EQUATION

$$k_{obs} = k_1[B]_i + k_{-1} \qquad (25)$$



?!

not a straight line

Enzyme + Antibody at very large excess of [Tracer] (pt. 4)

POSSIBLE REASONS FOR THE **NONLINEARITY** OF k(app) VS. [Kinase] PLOT

1. [Kinase] is *not* at very large excess over [Antibody] at all times

	A	B	C	D
	assay #	[E], nM	[Ab], nM	[E] / [Ab]
	1	3.13	0.20	15.6
	2	1.56	0.20	7.8
	3	0.78	0.20	3.9
	4	0.39	0.20	2.0
	5	0.20	0.20	1.0
	6	0.10	0.20	0.5

Summary: How to experimentally measure k_1

1. Develop an assay to monitor AB.
2. Combine A and B at $t = 0$ ($[B]_i >> [AB]_{max}$).
3. Measure accumulation of AB over time.

Goodrich & Kugel (2006), p. 95

2. Signal in LanthaScreen® not strictly proportional to concentrations ?

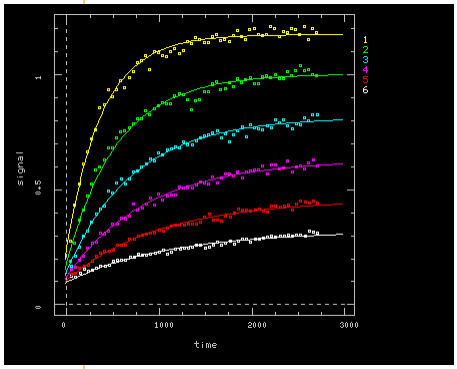
The "emission ratio" is calculated as the 665 nm signal **divided** by the 615 nm signal.

3. Kinase concentrations being "off" their nominal values ?

Enzyme + Antibody at very large excess of [Tracer] (pt. 5)

GLOBAL FIT TO A MECHANISTIC MODEL

```
[task]
   task = fit
   data = progress

[mechanism]
   E + Ab <===> E.Ab   :   kaA   kdA

[constants]
   kaA = 0.001 ?
   kdA = 0.001 ?

[concentrations]
   Ab = 0.2

[responses]
   E.Ab = 3 ?

[data]
...
   file d07 | concentration E =  3.1250 ?
   file d06 | concentration E =  1.5625 ?
   file d05 | concentration E =  0.7813 ?
...
```



Enzyme + Antibody at very large excess of [Tracer] (pt. 6)

GLOBAL FIT TO A MECHANISTIC MODEL: BEST-FIT MODEL PARAMETERS

Optimized Parameters

No.	Par#Set	Initial	Final
#1	kaA	0.001	0.000920853
#2	kdA	0.001	0.000614225
#3	[E]#1	3.125	2.18796
#4	r(E.Ab)	3	7.23505
#5	offset	0.1	0.0858441
#6	[E]#2	1.5625	1.28789
#7	[E]#4	0.3906	0.478694
#8	[E]#5	0.1953	0.281575
#9	[E]#6	0.0977	0.162183

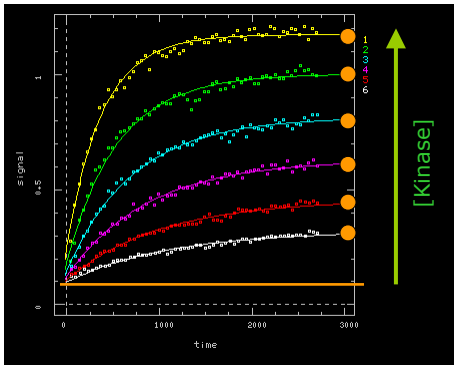
$k_{"on"} = 0.92 \times 10^6\ M^{-1}\ s^{-1}$
$k_{"off"} = 0.00061\ s^{-1}$
$K_d = k_{"off"} / k_{"on"} = 0.66$ nM

[E], nM nominal	[E], nM best-fit	Ratio
3.13	2.19	**1.4**
1.56	1.29	**1.2**
0.78		
0.39	0.48	**0.8**
0.20	0.28	**0.7**
0.10	0.16	**0.6**

Signal in LanthaScreen® not strictly proportional to concentrations ?

Enzyme + Antibody at very large excess of [Tracer] (pt. 7)

FIT AN **"EQUILIBRIUM"** BINDING MODEL TO END-OF-TRACE SIGNAL VALUES




```
[task]

   task = fit
   data = equilibria

[mechanism]

   E + Ab <===> E.Ab    :    KdA dissoc

[constants] ; nM

   KdA = 0.7 ?

...
```

$K_{dA} = 0.6$ nM

Enzyme + Antibody at very large excess of [Tracer] (pt. 8)

SUMMARY

RESULTS

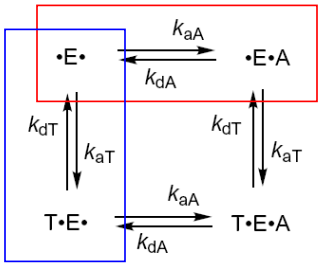
- Kinase–Antibody dissociation equilibrium constant is around 0.7 nM
- The association rate constant is $0.9 \times 10^6\ M^{-1}s^{-1}$ ("diffusion control")
- The half-time for dissociation is about 20 minutes ("slow")

CONCLUSIONS

- At nanomolar concentrations of [Kinase] and [Antibody] there is always a mixture of all three species (E, Ab, E.Ab)
- Diluting any kinase/antibody stock solution will cause the antibody to "fall off" the E.Ab complex
- This "falling off" of the antibody will be slow, measured in minutes

Research plan: Stepwise model building

ASSUME THAT THE ANTIBODY AND THE PROBE BIND **INDEPENDENTLY**



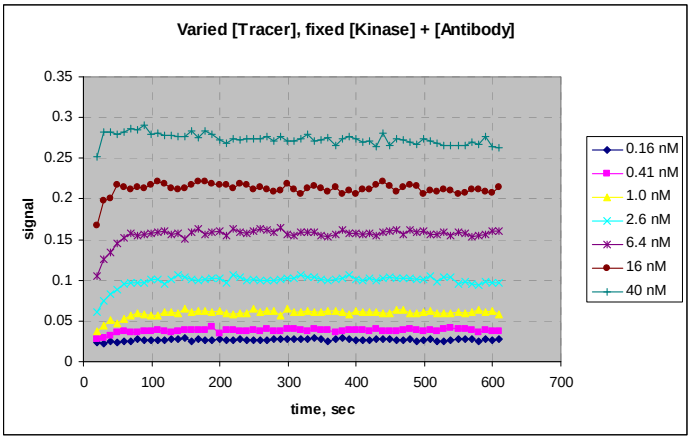
1. get k_{aA} and k_{dA} ✓

2. get k_{aT} and k_{dT}

Try to find conditions that might allow treating this as a simple "A + B" (two-component) system.

Kinase - Antibody - Tracer: varied [Tracer] (pt. 1)

RAW DATA



1. 5 µL 1.5 nM Kinase + 6 nM Antibody
2. 5 µL solvent
3. incubate 30 min
4. 5 µL Tracer, varied final concentration

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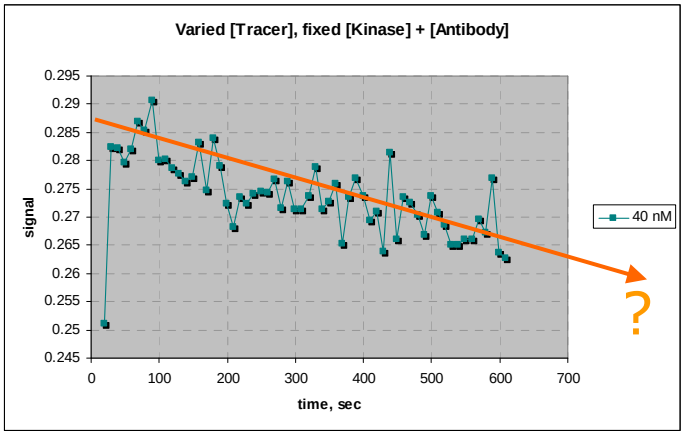
29

Kinase - Antibody - Tracer: varied [Tracer] (pt. 1)

RAW DATA: CLOSER LOOK AT HIGH [TRACER] CONCENTRATION ASSAY



1. 5 µL 1.5 nM Kinase + 6 nM Antibody
2. 5 µL solvent
3. incubate 30 min
4. 5 µL Tracer, 40 nM final concentration

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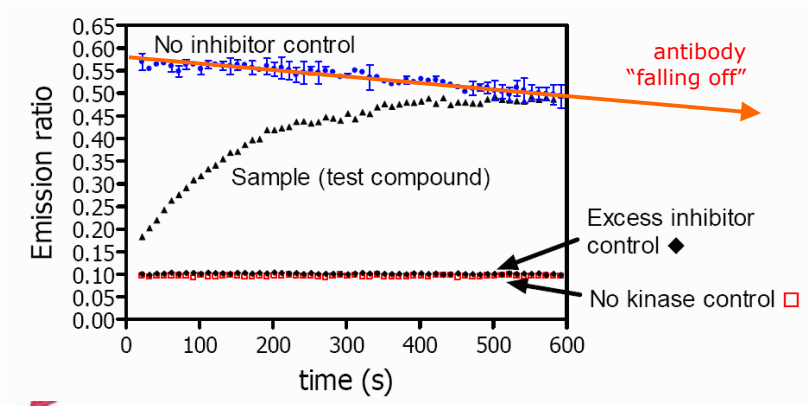
30

Gradual "loss of signal" upon dilution: Invitrogen literature

RESULTS FROM POSTER PRESENTED BY INVITROGEN

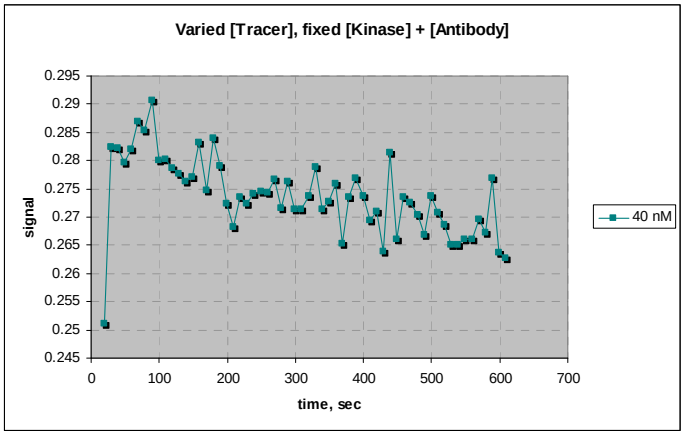


Determination of Drug-Kinase Residence Time in a Homogenous, Low-Volume Format

Bryan Marks, Connie Lebakken, Steve Riddle, Richard Somberg, Sara Hensley

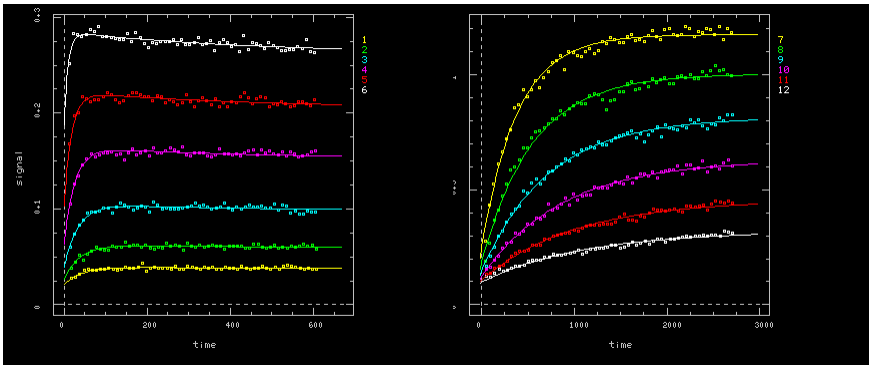
Problem: Can't use "classical" method based on $k^{(app)}$

THIS IS DEFINITELY **NOT A RISING EXPONENTIAL**



Solution: Global fit of both sets of experiments (pt. 1)

EXPERIMENT #1: VARIED [**TRACER**] EXPERIMENT #2: VARIED [**KINASE**]



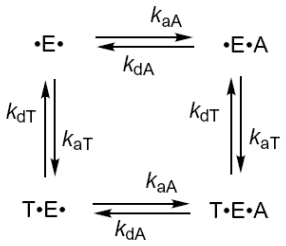
[A] = 2 nM
[E] = 0.5 nM
[T] = 0.4 ... 40 nM

[A] = 0.2 nM
[E] = 0.098 ... 3.13 nM
[T] = 200 nM

Solution: Global fit of both sets of experiments (pt. 2)

DYNAFIT INPUT ("SCRIPT") FILE

```
[task]
   task = fit
   data = progress

[mechanism]
   E + Tr <===> E.Tr          :   kaT   kdT
   E + Ab <===> E.Ab          :   kaA   kdA

   E.Tr + Ab <===> E.Tr.Ab    :   kaA   kdA
   E.Ab + Tr <===> E.Tr.Ab    :   kaT   kdT

[constants] ; nM
   kaA = 0.001 ?
   kdA = 0.001 ?

   kaT = 0.001 ?
   kdT =  0.01 ?
...
...
...
```



Solution: Global fit of both sets of experiments (pt. 3)

DYNAFIT OUTPUT: BEST-FIT PARAMETERS

Optimized Parameters

No.	Par#Set	Initial	Final
#1	kaT	0.001	0.00265125
#2	kdT	0.01	0.0209196
#3	kaA	0.001	0.000920579
#4	kdA	0.001	0.00061413

$k_{on"T} = 2.7 \times 10^6\ M^{-1}\ s^{-1}$
$k_{off"T} = 0.021\ s^{-1}$

$K_{dT} = 7.9$ nM

$k_{on"A} = 0.92 \times 10^6\ M^{-1}\ s^{-1}$
$k_{off"A} = 0.00061\ s^{-1}$

$K_{dA} = 0.66$ nM

CONCLUSIONS

- Tracer is tight binding but "not that tight":
  It will be **impossible to *completely* prevent inhibitor rebinding** by competition.
- Tracer is relatively "fast off" but "not that fast" ($t_{1/2}$ = 0.5 min):
  In certain experiments there could transients due to tracer interactions.
- It's a fairly complicated system:
  Once inhibitor is added *all six rate constants* need to be considered.

Now for the complete four-component system:

Kinase + Antibody + Tracer + Inhibitor

Kinase - Antibody - Tracer - Inhibitor: data

KINASE: p38α | ANTIBODY: anti-GST | TRACER: Invitrogen "Tracer-199" | **INHIBITOR: desatinib**

Data: **Bryan Marks**, *Invitrogen*

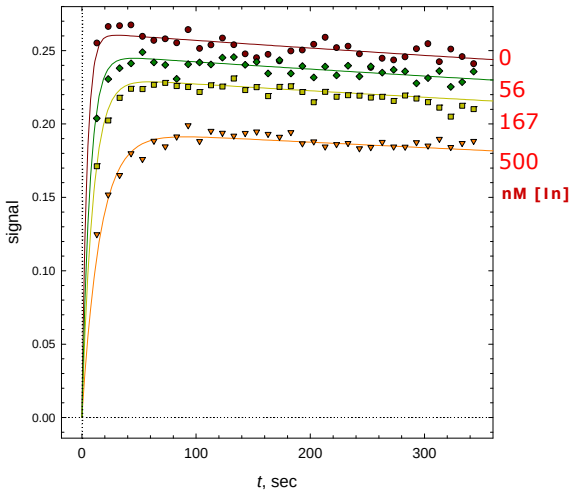
EXPERIMENT:

1. incubate
   [E] = 4 nM
   [Ab] = 40 nM
   [In] = *varied*

   30 minutes

2. dilute 1:20 with Tracer
   final concentrations

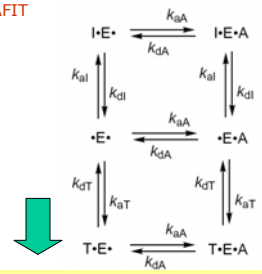
   [E] = 0.2 nM
   [Ab] = 2 nM
   [Tr] = 100 nM
   [In] = *varied*



Kinase - Antibody - Tracer - Inhibitor: fitting model

AUTOMATICALLY DERIVED BY DYNAFIT

system of simultaneous
ordinary
differential equations



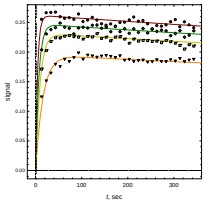
DynaFit Input

```
[mechanism]

   E + In <===> E.In          :   kaI   kdI
   E + Tr <===> E.Tr          :   kaT   kdT
   E + Ab <===> E.Ab          :   kaA   kdA

   E.In + Ab <===> E.In.Ab    :   kaA   kdA
   E.Ab + In <===> E.In.Ab    :   kaI   kdI
   E.Tr + Ab <===> E.Tr.Ab    :   kaA   kdA
   E.Ab + Tr <===> E.Tr.Ab    :   kaT   kdT
```

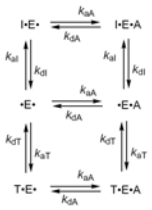
Kinase - Antibody - Tracer - Inhibitor: rate constants

ASSUMPTION: **INDEPENDENT** BINDING SITES – ONLY **TWO ADDITIONAL** RATE CONSTANTS

DATA



+



MODEL

LEAST-SQUARES FIT

k_{aI} = 2.1 × 10^9 $M^{-1}.s^{-1}$

k_{dI} = 19 s^{-1}

PARAMETERS

"RESIDENCE TIME"

τ = 0.05 sec

Kinase - Antibody - Tracer - Inhibitor: state variables

EVOLUTION OF SPECIES CONCENTRATIONS DURING THE KINETIC EXPERIMENT

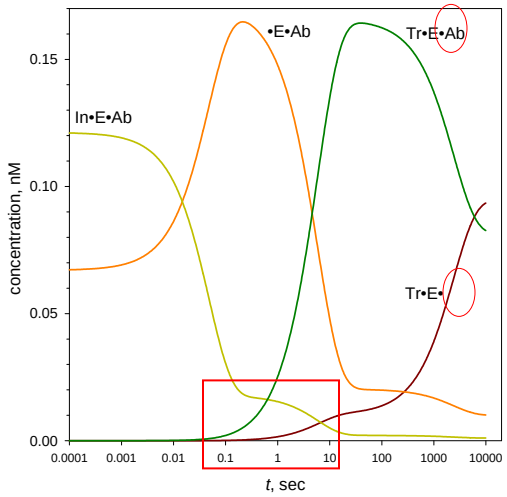
EXPERIMENT:

1. incubate
   [E] = 4 nM
   [Ab] = 40 nM
   [In] = 370 nM
   30 minutes

2. dilute 1:20 with Tracer
   final concentrations

   [E] = 0.2 nM
   [Ab] = 2 nM
   [Tr] = 100 nM
   [In] = 18.5 nM

optimize design!



Acknowledgments

- **Bryan Marks**: all kinase experiments
  Invitrogen, a.k.a. Life Technologies, Madison, Wisconsin

Questions ?

http://www.biokin.com