Colorimetric Plasminogen Activation Assay

Stack Lab Protocol

(see next page for calculations)

Abbreviations:

VLKpNA - Val-Leu-Lys-para-nitroanilide (Sigma V-0882)
NA - nitrophenolate anion
Pg - plasminogen
Pm - plasmin
PA - plasminogen activator
K_m - Michaelis constant
k_{cat} - catalytic rate constant

To assay PA activity in conditioned medium using a 96 well plate microtiter plate reader:

1. Turn on plate reader before starting. On our reader, choose KINETICS405. This monitors the change in absorbance at 405 nm over a 2 hr time course at 37 C. [Turn on incubator]. Assay each sample in triplicate.

2. Reaction:
120 ul of 20 mM Hepes pH 7.4
17.5 ul of 3 uM Pg
18 ul of 3 mM VLK-pNA
Start reaction by addition of 20 ul conditioned medium sample.
Monitor A405/time.

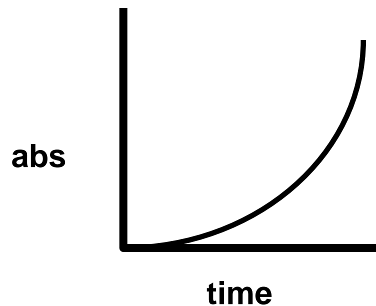
3. To calculate mol Pm/min, see below.

To calculate the <u>velocity of plasminogen activation</u>
(derived by Wohl et al., (1980) J. Biol. Chem 255:2005-2013)

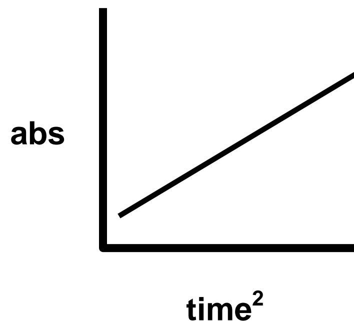
The reaction being measured is:

This is analyzed using a secondary reaction:

When Pg is activated by PA, cleavage of VLK-pNA is monitored by absorbance at 405 nm. If you plot absorbance *vs* time, a curved line results:



This is because Pm is generated continuously, so the amount of Pm in the secondary reaction is not constant. To obtain linear data, plot A_{405} vs time^2:



This gives a straight line with a slope of b/2. This slope can now be used to calculate the initial velocity of Pm formation according to the equation:

$$v = \frac{b\,(1 + K_m/S_o)}{\varepsilon\, k_{cat}}$$

v = initial velocity of Pg activation

K_m = Michaelis constant for hydrolysis of VLK-pNA by Pm
= 3×10^{-4}M

k_{cat} = catalytic rate constant for hydrolysis of VLK-pNA by plasmin. This constant is empirically determined in 20 mM Hepes, pH 7.4 to be 1.35×10^3 M/min*mol Pm. [Note - this number may vary if your buffer is very different]

ε = extinction coefficient of nitrophenolate anion which absorbs at 405 nm
= 1×10^4 M^{-1} [Erlanger et al., (1961) Arch. Biochem. Biophys. 95:271-278]

S_o = initial concentration of VLKpNA (0.3 mM in our assays)
(3×10^{-4} M)

So for example, if we plot Abs *vs* time^2, the slope of the line in A/min^2 = b/2 = (for example) 1.01×10^{-4}; then b = 2.02×10^{-4}

Plugging this into the velocity equation:

$$v = \frac{(2.02 \times 10\text{-}^{4}\ \text{abs/min}^2)\,[1 + (3\text{x}10^{-4}\ \text{M}/\ 3 \times 10^{-4}\ \text{M})]}{(1 \times 10^4\ \text{M}^{-1})\,(1.32 \times 10^3\ \text{M min}^{-1}\ \text{mol Pm}^{-1})}$$

$$= 3 \times 10^{-11}\ \text{mol Pm/min}$$

Converting microtitre plate reader data

Our Molecular Devices microtitre plate reader gives data in milliO.D./min

1. x mO.D./min x 1 O.D./1000 mO.D. = x' O.D./min
2. Run time - example 120 min
   x' O.D./min x 120 min = x" O.D.

3. convert to O.D./min^2:

   x" O.D./120^{2}min = x''' O.D./min^2

Plug this into equation above to get mol Pm/min. [If using our standard assay conditions of 0.3 mM VLK-pNA and 3 mM Pg in 20 mM Hepes, pH 7.4, can just multiply by 1.5×10^{-7})

Example: Plate reader gives you 10 mO.D./min for a 2 hr run

1. 10 mO.D./min x 1 O.D./1000 mO.D. = 0.01 O.D./min
2. 0.01 O.D./min x 120 min = 1.2. O.D.
3. 1.2 O.D./120^{2}min^2 = 8.3×10^{-5} O.D./min^2

Plug into equation and result is 1.25×10^{-11} mol Pm/min