**Michaelis Menten Kinetics Parameter determination**

**A and B et al presented the following reaction mechanism for the enzymatic addition of nucleotide in a DNA amplification reaction.**

****

**In the above reaction, *E*, *SP*, *E.SP*, *E.Di.N* and *E.Di* represent free enzyme, single strand-primer duplex, enzyme bound single strand-primer duplex, a product intermediate that has *i* extra nucleotide compared to single strand-primer duplex and the product that has *i* extra nucleotide compared to the single strand primer duplex respectively. The rate constants, ke and k-e are enzyme binding rate constants and in this study we don't focus on the estimation of these rate constants. The rate constants, k1, k-1 and kcat are the typical Michaelis Menten rate constants that represents the rate of enzymatic nucleotide addition on the SP molecules. We assume that the dissociation of *E.Di* is not significant (mention the reference) as the nucleotide concentration is kept excess and it will quickly react with *E.D­i* and does not allow it to dissociate. so that In this work we focus on the estimation of these rate constants.**

**Though the enzymatic addition of nucleotide is represented based on Michaelis Menten kinetics, the reaction mechanism that is represented by equation 1, is not similar to that of regular MM kinetics mechanism. Here, *N* is a substrate and *E.Di* is similar to the enzyme molecules. As it can be seen there are a series of nucleotide addition and simultaneously there is an enzyme binding reaction as well. Therefore, a careful theoretical investigation on the above series of reaction needs to be done to derive an experimental protocol that can be used to estimate the MM kinetics parameters.**

**The overall rate of formation of the products, E.Di can be written as**



Since the substrate, N concentration is high as it is typically assumed in MM kinetics formulation, steady state approximation for the intermediates can be assumed and hence,



Where 

Therefore,





Also,







During the initial stage of the reaction, assuming there is no [E.Dn] in the product, it is possible to measure the summation of E.D­i. We also know the concentration of the nucleotide and rate of the reaction. Now,



For various [N] concentration, it is possible to measure the initial rate and plot vs 

Slope is  and the intercept is 

**Protocol:**

1) If the initial SP concentration is 1 μM and the enzyme concentration is 1 nM, we get more than 95% equilibrium conversion and that leaves 10-12 M of Enzyme in the reaction mixture. Here we need to convert the Units of enzyme in to Molar concentration. Typically 1 enzyme unit = 1 μmol/ min.

2) For the above fixed SP and enzyme concentration, Equilibrate the SP and enzymes for long time, so that almost all enzyme molecules bind with the SP molecules.

3) Fix the length of the target DNA to be high ( please read the below 3rd comment ).

4) Now, add a specific amount of nucleotide with E.SP molecules and measure the rate of the reaction with respect to the time.

5) Repeat the above step for different concentration of Nucleotide

6) The initial concentration of N can be chosen in such a way that it is within a particular limit from the typical nucleotide concentration that was chosen in our previous experiments. In other words, initial concentration of nucleotide can be N +/- ε and ε can be chosen based on experimental convenience.

7) Also, in order to check whether the chosen SP concentration is enough to use all the enzyme molecule, assume one other SP concentration which is higher than the original SP concentration and do an extension reaction (for one single N concentration) and compare the Fluorescence data. Now we have Fluorescence data for each SP concentration and compare them. If they are equal, then, the chosen SP concentration can attract all the enzyme molecules.

**Comments:**

1) Based on the above experiments, the initial rate for various initial concentration of the N is measured. The time series data (step 4 of the above protocol) that will be obtained for each nucleotide concentration will be fit using the Prism software to estimate the initial rate of the reaction.

2) Since N typically in excess compared to the SP molecules, it does not allow E.Di molecules to dissociate back to give Enzyme. Even if there is a dissociation of E from E.Di or E.Dn, those enzyme molecules will bind with the SP molecules ( note that we have excess SP) and produce E.SP. Therefore, at any time during the initial stage of the reaction, all the enzyme molecules will be associated with E.SP and E.Di.

3) Further, since we measure the initial rate of the reaction we can neglect the concentration of E.Dn molecules. This assumption can be well validated if we can consider an extension reaction with sufficiently a long target DNA ( probably more than 100 base pairs length).

4) With the above assumptions, it is possible to approximate that the summation of the E.Di and E.Di.N is the initial concentration of the enzyme. Using this, it is possible to derive a typical MM kinetic equation.