A method to estimate the rate constants of DNA annealing reaction from the experimental data:

What we need:

* Time evolution data of hybridized DNA,
* Initial concentration of the primer
* Initial concentration of the single strands
* It is preferred to have equal concentration of single strands and primer.

Curve fitting

From the information of initial concentration of the single strands, it is possible to determine the conversion of the reaction and it is defined as



Based on the number of measurement, a set of *x*(*t*) can be determined. Now, at each time t, the following quantity should be calculated.



*xe* is the equilibrium conversion which can be obtained once the reaction reaches steady state. Plot *y*(*t*) vs *t*. This plot should yield a straight line and the slope of the straight line is given as



Where *k1* is the forward rate constant of the annealing reaction and *CS0* is the initial single strands concentration. Once *k1* is estimated then using the equilibrium constant, it is possible to determine the reverse rate constant *k2*.

Next Step:

Calculate the melting temperature of the given oligonucleotides using Nearest Neighbor(NN) method and verify whether the theoretically obtained melting characteristics match with the experimental values. The experimental melting characteristics suggest that it follows two states or all or none model.(only one peak)

In protocol I and II, from the time of incubation we need to monitor the duplex concentration. Due to the instruments limitation as mentioned by Sudha, there is a decrease in duplex concentration in during the start of the incubation. After some time, the concentration however increases. We will have to start our analysis from this point onwards (80 seconds from the start of the incubation). If this has to be done, what value of concentration need to be assumed at this time. Is it corresponds to the RFU value at this time or corresponds to the RFU value at the start of the incubation.

If you start analysis from 80secs, you would have to take the RFU of this time point.

In protocol I, Compared to 74 deg C, 70 deg C produced less product for the same time of annealing. This could be due to the kinetics of the annealing reaction at two different temperatures (According to this data, 70 deg C is slow and 74 deg C is fast – But the current literature data does not support this observation). The equilibrium concentration of duplex, however, will be higher at 70 deg C and lower at 74 deg C. We will theoretically calculate the equilibrium concentration of dsDNA at these two temperatures using NN parameters and compare this with the corresponding experimentally obtained equilibrium dsDNA at these two different temperatures. This comparison will allow us to conclude which data may be corrupted.

Protocol 2 looks good. As it was mentioned before, we need to monitor the concentration of the duplex from the start of incubation till it reaches the equilibrium.

As I have mentioned before, the experiment was monitored till equilibrium.

In order to differentiate the difference between protocol I and II, we need to measure the steady state dsDNA concentration or the equilibrium dsDNA concentration by allowing the reaction to reach the equilibrium. (Allow sufficiently long time so that we get saturation in RFU values. I think this has been done). Now, we can theoretically calculate the equilibrium concentration at 70 deg C or any specific temperature that we have chosen to conduct our experiment. Find the deviation between this theoretical value and dsDNA concentration that was obtained using protocol 1 and 2. This will allow us to understand whether protocol I or II is better.

The given experimental fluorescence measurements (RFU values) need to be converted in to concentration data and curve fitting needs to be done as explained above.

Converting to concentration can be done by preparing a standard curve of RFU vs [dsDNA]. However, I am uncomfortable using such a standard in this case, given the discontinuity in RFU values between different steps and the as yet unexplained drop in RFU in the first few cycles of prolonged incubn of Protocol I.

We do know the initial conc of ssDNA (2uM) when we start the expt from 90oC at which temp all DNA will be single stranded.

Protocol II is probably more reliable if RFU needs to be converted to conc, since we have single measurements of RFU at 90o and 65oC (no unexplained drops).

To use the CFX96 data with a standard curve, it would best to normalize the curves (as I have done). The net change in RFU over time in the extended incubn step can be reliably correlated to [dsDNA].

Again, the program for the UV-Vis spectrophotometer is supposed to work as follows:

Melt Run Kinetics Run

Start temp End Temp to equilibration

1. 90oC 70oC
2. 60oC 70oC

It can be set up to either decrease from 90 to 70 and incubate to equilibrium or increase from 60 to 70 and incubate to equilibrium (or whatever temp we would like).

The only issue I see with correlating the spec reading to conc is that at any point the absorbance is a combination of [ssDNA] and [dsDNA]. (I am sure that given absorbance coefficients for ss and ds DNA, the contribution to the absorbance of both ss and ds DNA can be calculated). However, this issue will not arise if we do the expt on the fluorimeter instead of the spectrophotometer, because the fluorescence signal is a direct correlate of [dsDNA] ONLY.

So here are the advantages of repeating the expt on UV-Vis spectrophotometer:

1. Greater sensitivity than the PCR machine.
2. Raw measurements obtained that are not processed by the instrument software.
3. Continuity in measurements between the melt run and the kinetic run of the expt.

Additionally with a fluorimeter and the use of a fluorescent dye, the continuity in measurements will allow correlation with [dsDNA]. This would be done by performing a standard curve RFU vs [dsDNA] at different temperatures of interest.

I have a person in Agilent working on the program for the UV-Vis spectrophotometer. Once we find this to work reliably, it can also be suited for the fluorimeter. We could wait for these programs, repeat the experiments and see what the curves look like.

On the other hand, I could try doing a standard on the CFX96 and convert all the plots to normalized plots and try to calculate [dsDNA]. I am not sure whether this will work but I can take a look.

Karthik, you will have to tell me what temp.s you are interested in, the standards will have to be done at these temps.

Karthik will do all these calculations based on the data provided by Sudha. He will get Sudha’s help to convert RFU values in to dsDNA concentration. The other concentration values such as salt concentration, primer concentration will be obtained from the ppt. If any of these data is not available, Sudha will provide them to Karthik.