Interactomics: Basics and Applications

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Lecture - 35
Protein-protein Interaction Study: Kinetic Analysis

Hello students. We were discussing about different experiments which are related to the SPR. Determination of interaction kinetics is one of the most striking applications for SPR bio-sensors. Understanding the kinetics is essential to evaluate the strength of a biomolecular interaction. The label-free real time detection allows the kinetics analysis to be performed by monitoring interaction as a function of time over a range of analyte concentrations.

If you think about previously the microarray experiments, what is the SPR experiment? You may realize that for many of the binding experiment you can get an idea from the microarray experiment whether a binding happens or does not happen. You will get yes or no answer. Whereas, in case of SPR not only you get an answer that a binding happens, but also you can measure its kinetics you can measure on rate, off rate and you can look at the kd which is dissociation constant.

The data set obtained is then fit to a mathematical model and leads to determination of kinetic parameters like association and dissociation rate constants. Affinity constants exhibit the strength of binding between the molecules and it can be derived either from the rate constants or from a steady state binding analysis.

A kinetic analysis experiment can be setup in two different ways. First it can be multi cycle kinetics where each analyte concentration is run as a separate cycle, regenerating the surface after sample injection or second it can be single cycle kinetics which runs a series of analyte concentration in a one cycle with no regeneration between sample injections.

Today we are going to perform the kinetic analysis for our standard protein pair which is protein beta 2 microglobulin with anti-beta 2 microglobulin antibody using multi-cycle kinetics. So, let us continue with the lab experimental session.

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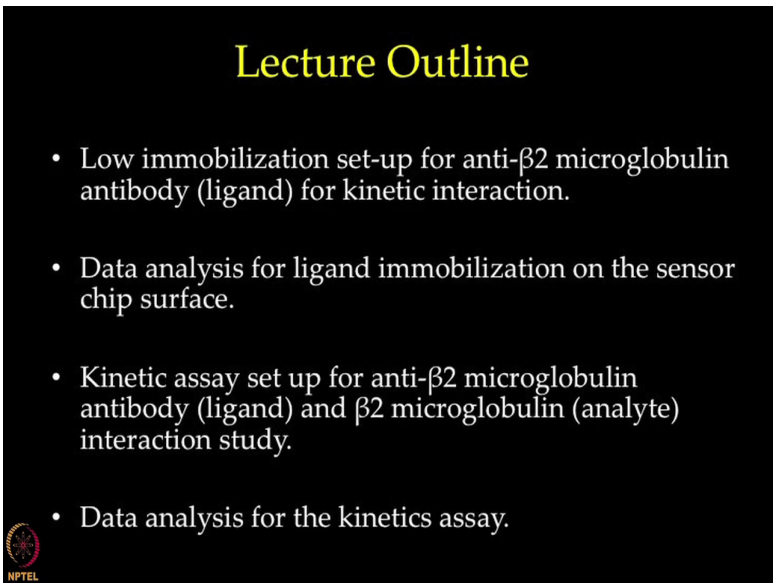

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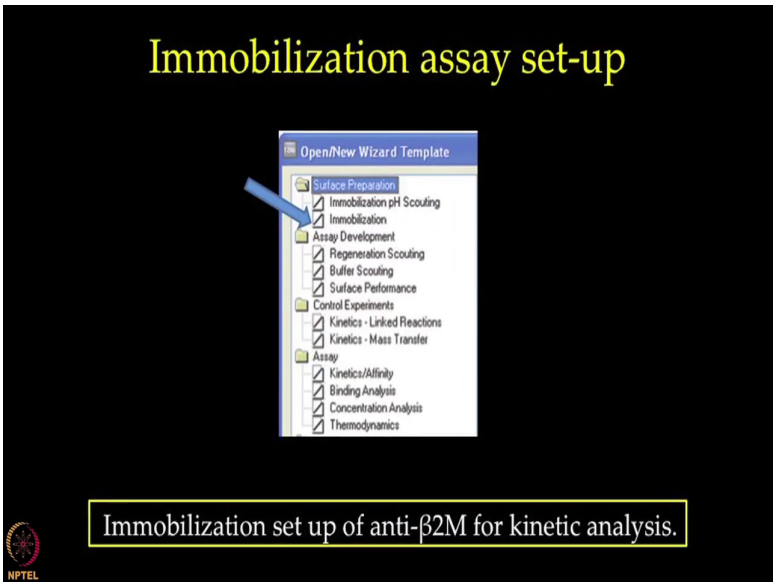

We will start with low immobilization protocol setup for anti-beta 2 microglobulin as a lower immobilization level will be beneficial in this kinetic interaction analysis.

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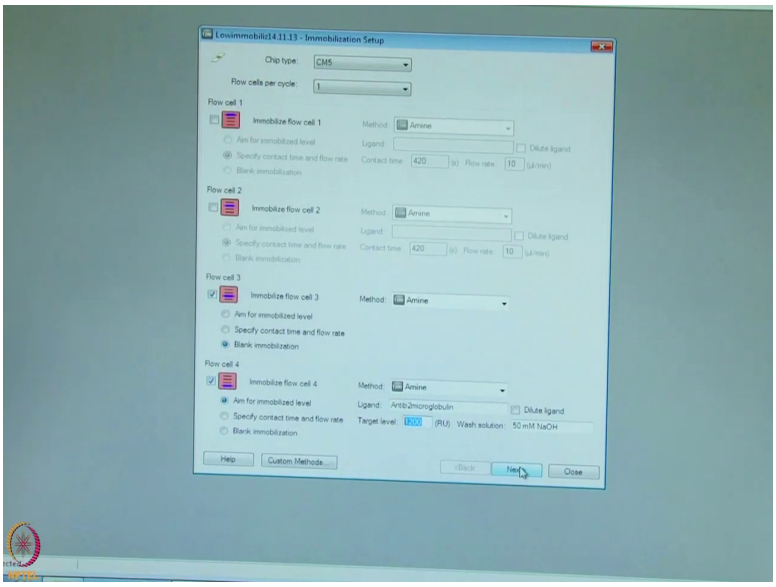

We have to redo our immobilization. In this case now we are selecting flow channel 3 and selecting flow channel 4. Flow channel 3 will be a blank immobilization, and flow channel 4 we will immobilize anti-beta 2 microglobulin at 1200 RU for a kinetic experiment.

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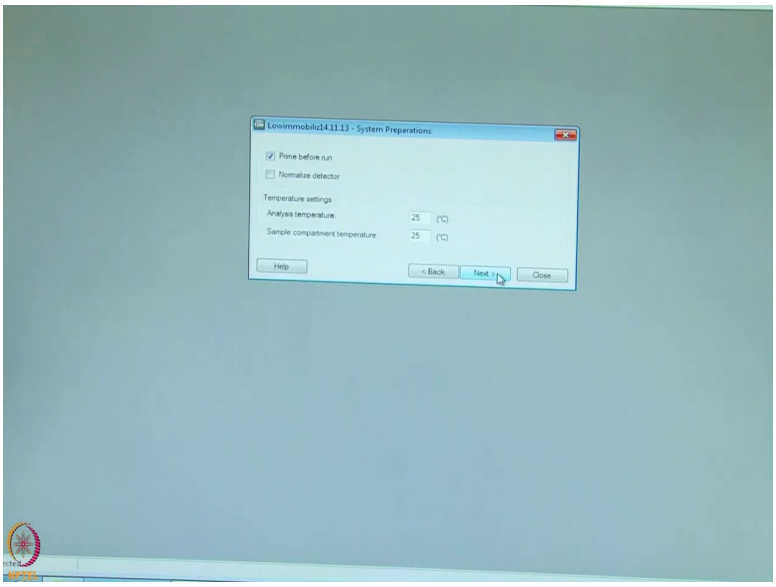

Here again we will highlight prime before run and keep the sample compartment temperature at 25 degree centigrade and we will go to the next pair.

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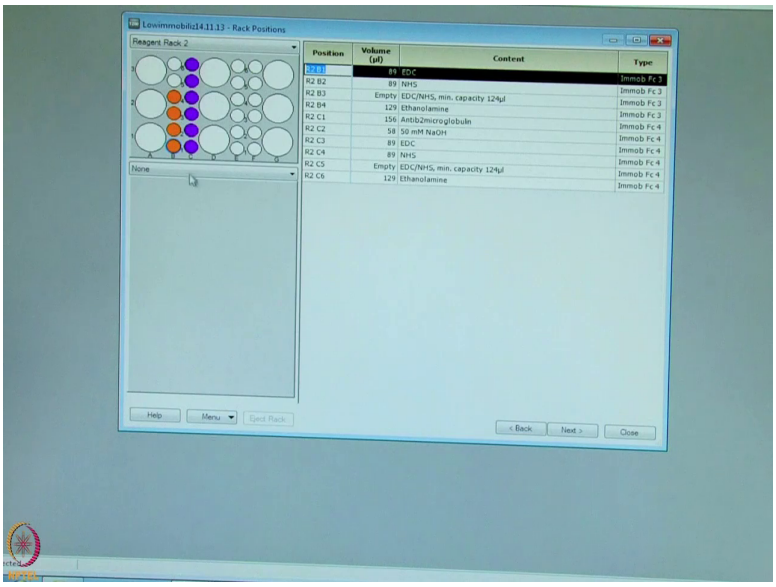

Now, we will add vials with the rack positions and the volumes.

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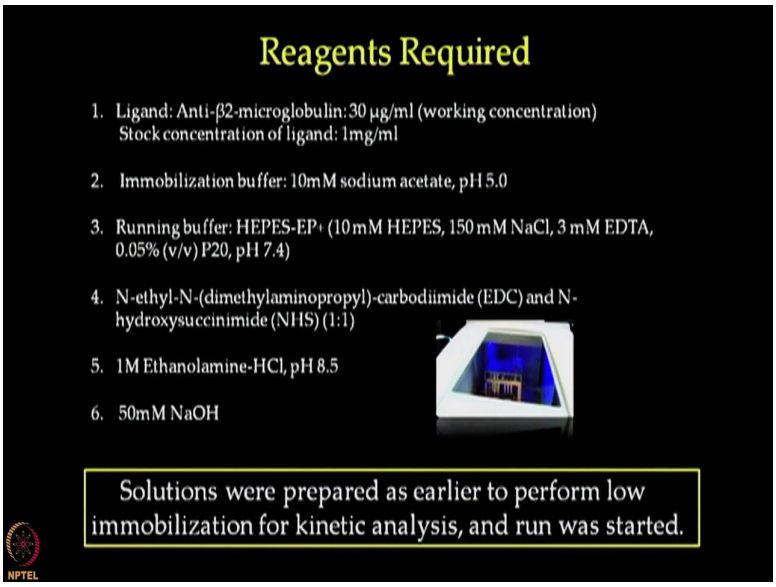

As per the volumes listed in the table we will prepare the reagents for immobilization in a similar manner as demonstrated earlier in lecture 33. We prepared a working concentration of 30 microgram per ml of beta 2 microglobulin antibody from a stock concentration of 1 mg per ml, using an immobilization buffer of 10 millimolar sodium acetate pH 5. We prepared HEPES EP plus as the running buffer which was connected to the system followed by priming. EDC and NHS were prepared to be used in 1 is to 1 ratio for surface activation. One molar ethanolamine HCL pH 8.5 was prepared for blocking the free extra groups on the surface. Lastly 50 millimolar NaOH was prepared which will be used to remove the electrostatically bound ligand.

We prepared the above mentioned solutions, transport them to appropriate sample vials place them in the suitable racks and start the immobilization run as we did in lecture 33. To save

time we shall now proceed directly to analyze the data obtained from immobilization of beta 2 microglobulin antibody.

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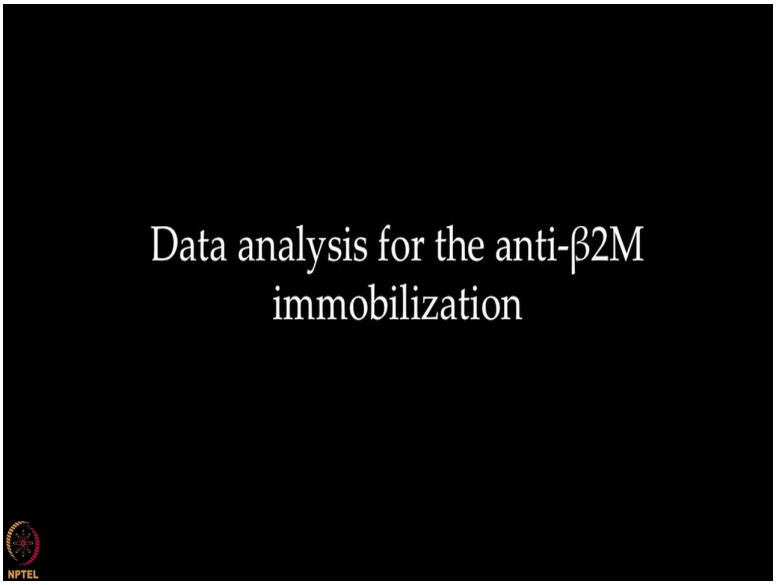

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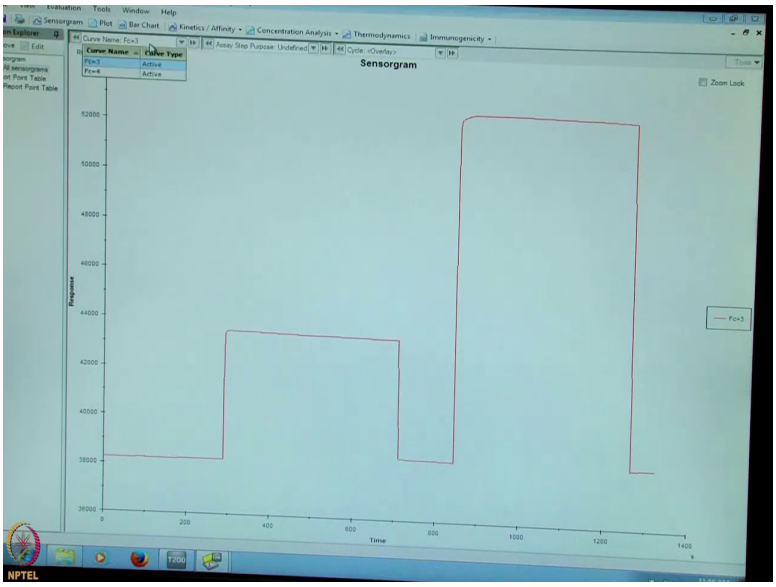

So, this is the blank sensorgram.

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After immobilization we will see the reference surface is blank immobilized here you see the activation of the chip by EDC NHS and blocking of that surface with ethanolamine. So, this will be the blank immobilization.

Now, we will go to another surface or another flow channel.

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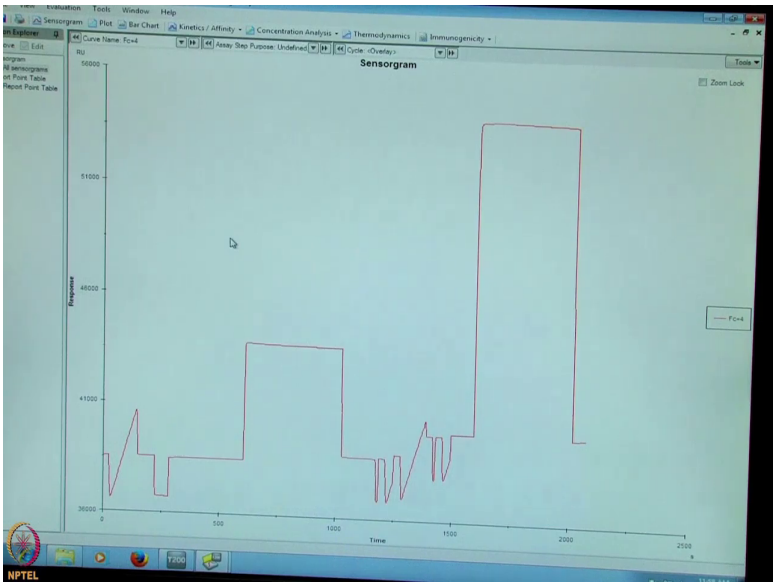

Going to the next flow channel FC 4, highlighted. This is a sensorgram for active surface immobilization involving protein. To start with very a preconcentration estimation of the required amount of the ligand, wash with the sodium hydroxide, activation of the surface with EDC and NHS mixture, falsing of the protein for the required number of RUs, and then block of the extra sites with ethanolamine. The immobilization level is actually determined from this level subtracted to this level. Now, this will conclude our immobilization procedure. We have successfully immobilized the required level of ligand for our next experiment.

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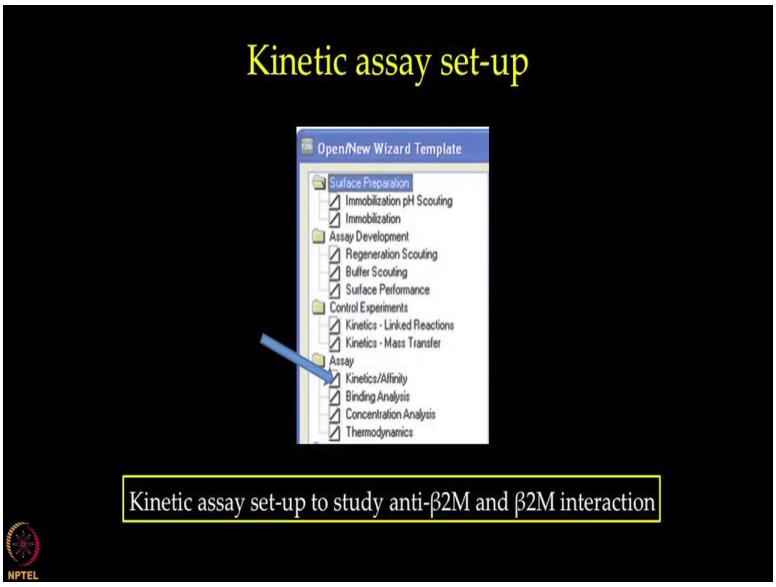

We will now setup a wizard file kinetic analysis.

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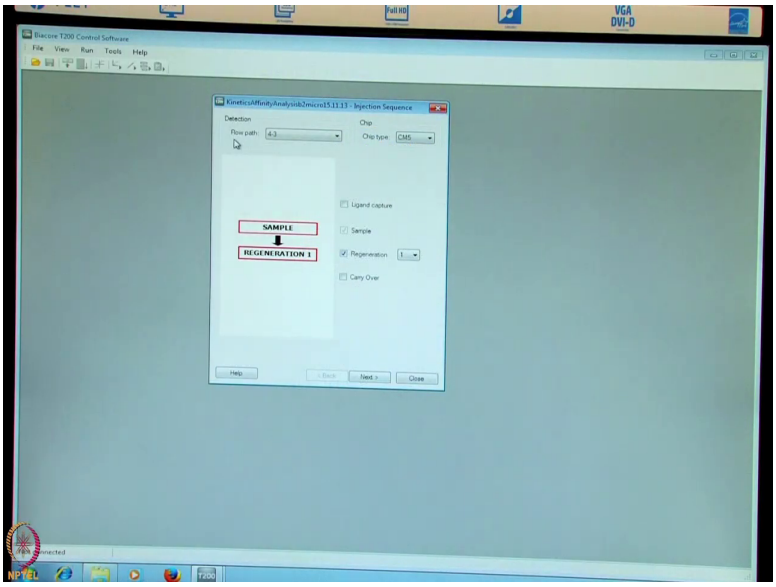

To start with we have selected from the wizard kinetic template selected the flow path 4 minus 3, chip type CM 5, sample and the flow is to regeneration. Once we have setup with this template we say next.

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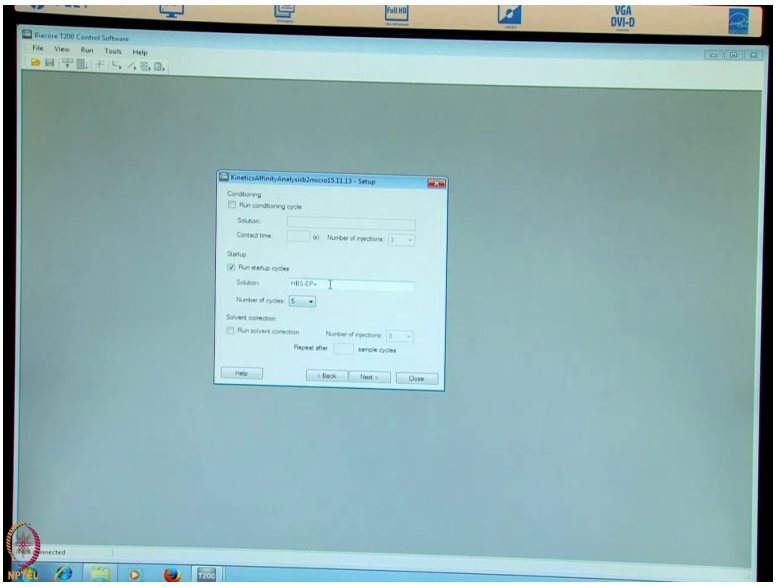

And startup cycles are selected at 5 with HPSEP plus buffer.

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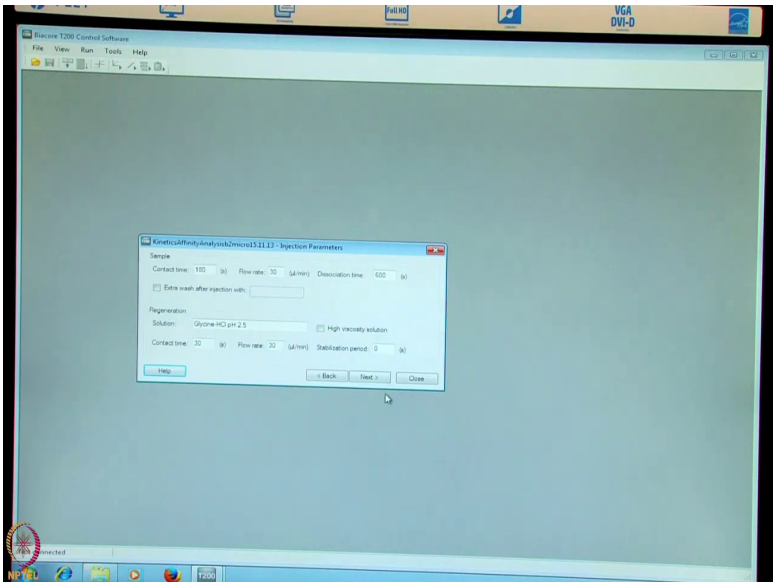

The sample contact time of 180 seconds is selected. Flow rate of 30 microliter per minute is selected. Dissociation time of 600 seconds is selected.

Coming to the regeneration the regeneration solution is glycine HCL pH 2.5 with a contact time of 30 seconds, flow rate of 30 microliter per minute, stabilization time of 0 seconds.

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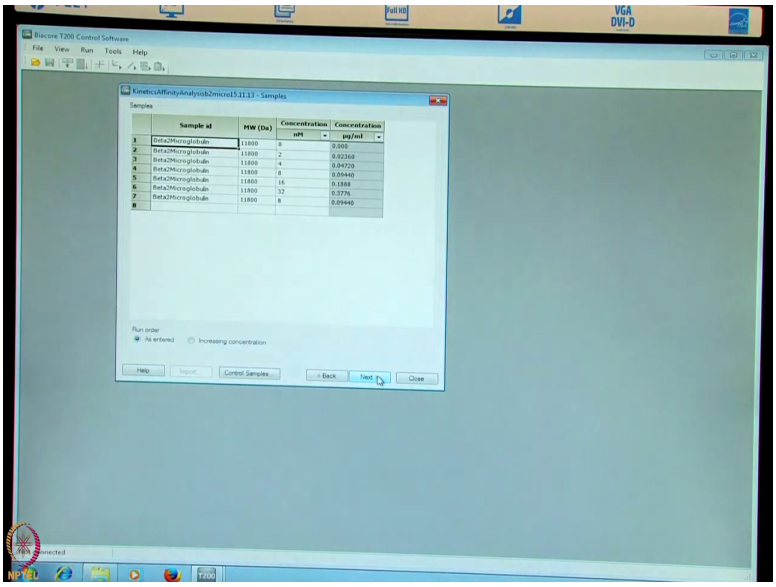

And we now go onto the next tab and fill in the name of the sample as beta 2 microglobulin with a molecular weight of 11800 and concentration with 0, 2, 4, 8, 16, 32 and the repeat of 8 nanomolar again. We say next.

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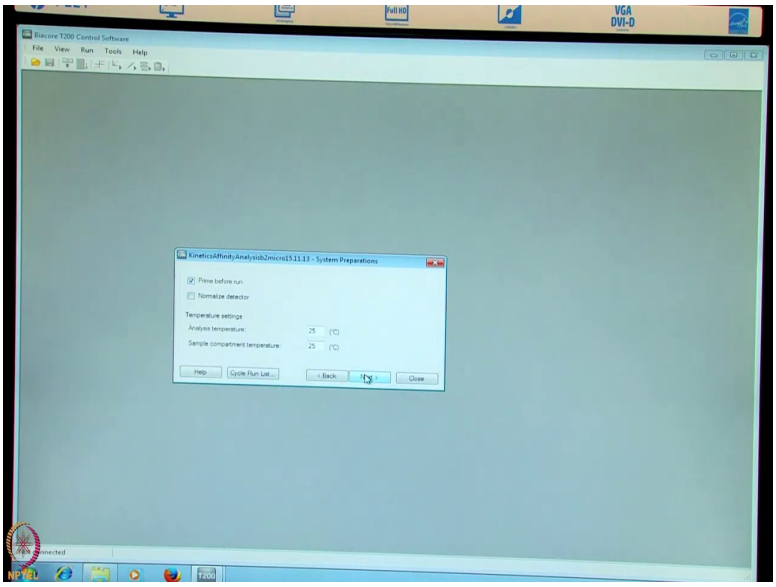

And prime before run is highlighted.

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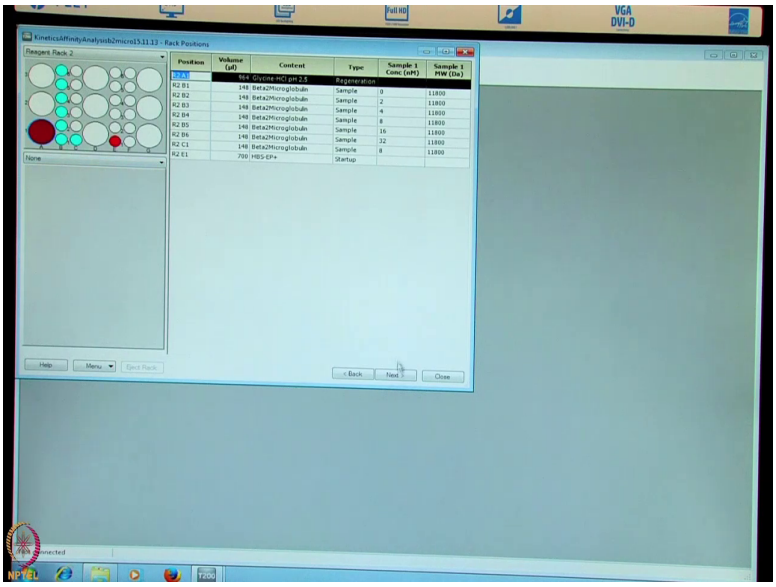

Now, we have the rack positions this way. We have regeneration buffer here. Startup here with the sample here.

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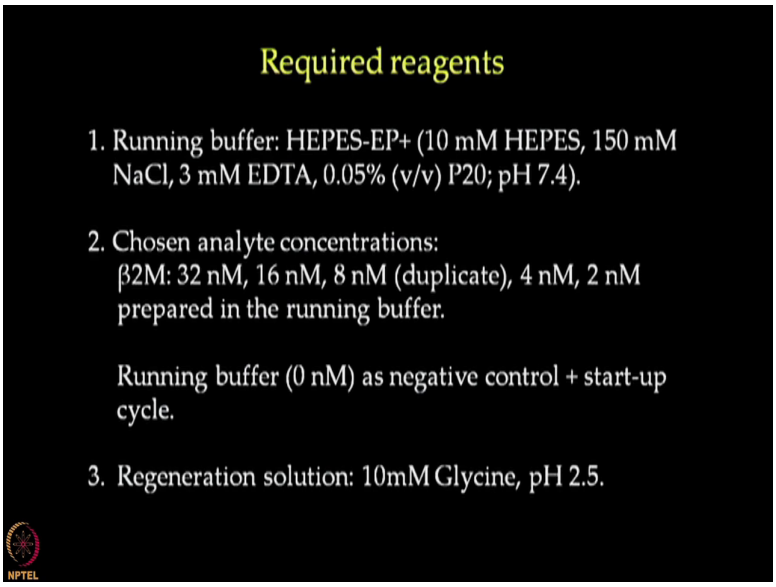

In today's kinetic assay experimental setup, we will be using HEPES EP plus as the running buffer. We will be preparing a total of 5 concentrations of beta microglobulin starting from 32 nanomolar, 16 nanomolar, 8 nanomolar, 4 nanomolar and 2 nanomolar using two-fold serial dilution in the running buffer and 8 nanomolar concentration in duplicate. We will be using HEPES as 0 concentration negative control for the reaction as well as for the initial startup cycles. For regeneration solution we will be using 10 millimolar glycine pH 2.5. We shall now proceed to setup our experiment for kinetic analysis.

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We will now work under reagents requires, for the kinetic interaction analysis between anti-beta 2 microglobulin and beta 2 microglobulin.

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Once again we will be using HEPES EP plus as a running buffer which will also be used for the initial startup cycle.

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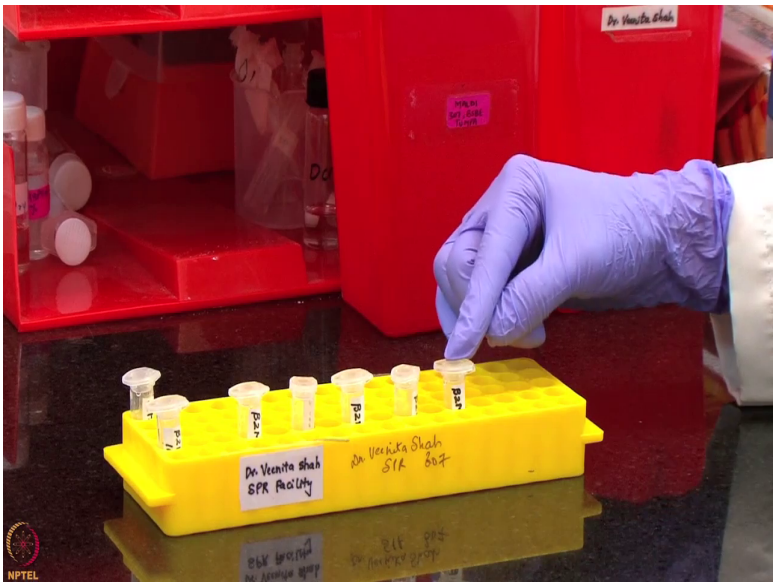

We will be using our protein stock concentration of 100 microgram per ml to prepare 5 different concentration of protein including 32 nanomolar, 16 nanomolar, 8 nanomolar, 4 nanomolar and 2 nanomolar.

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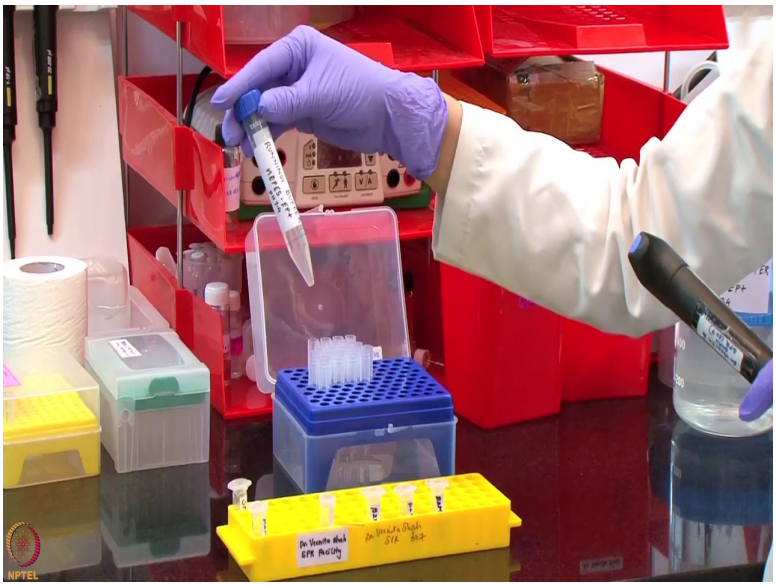

We will be preparing this concentration series is two-fold dilution using the running buffer starting from 32 nanomolar solution which mean we will add 300 microliter of 32 nanomolar concentration with 300 microliter of buffer to prepare 16 nanomolar concentration.

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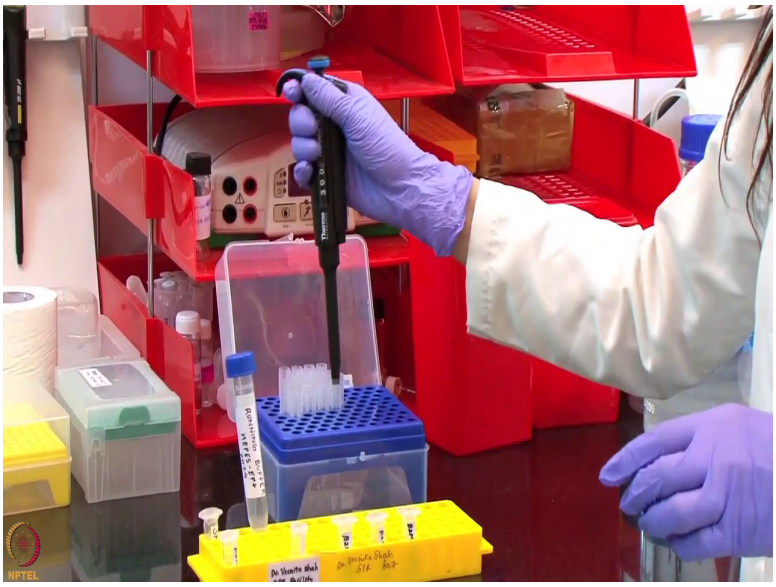

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We similarly continue with the dilution series for rest of the concentrations.

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One of the concentration that is 8 nanomolar will be run in duplicate.

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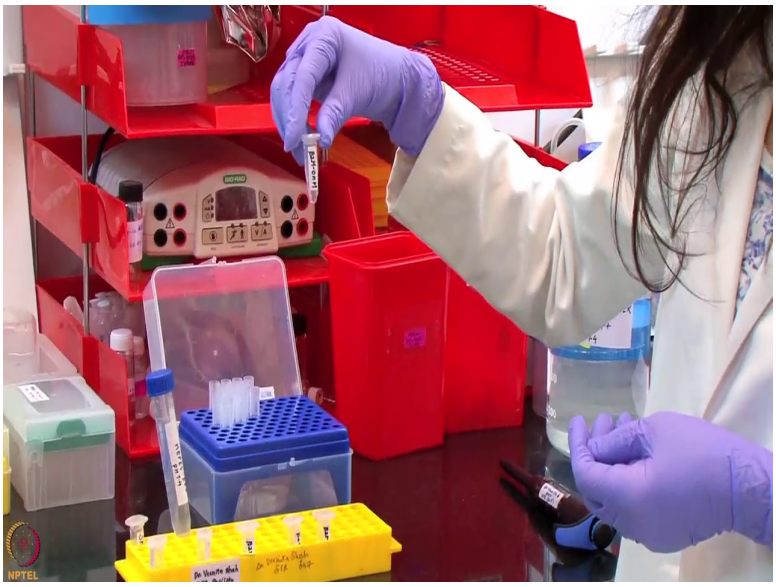

And we will also include a 0 nanomolar concentration in the experiment.

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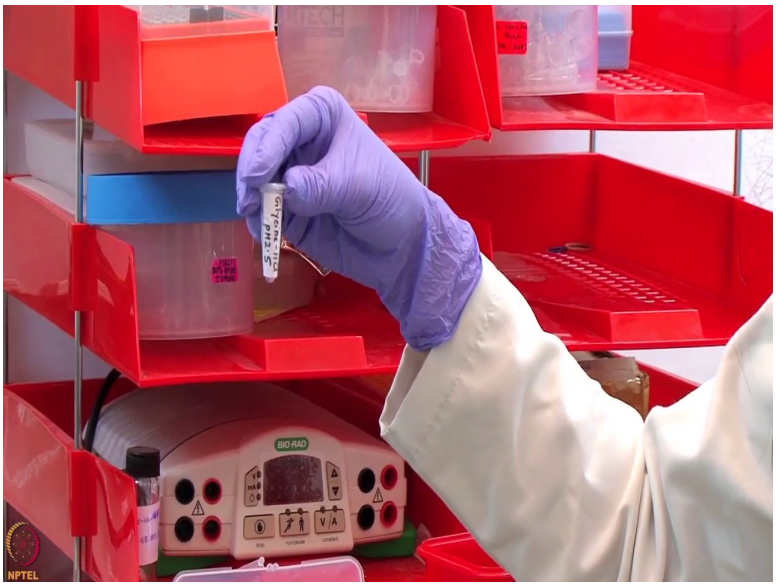

For regeneration we will be using glycine HCL pH 2.5 as the regeneration solution.

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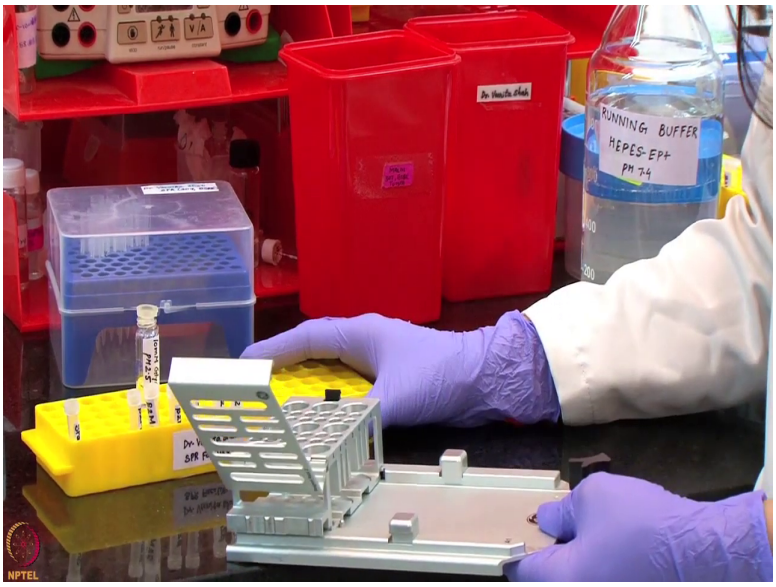

All the samples and reagents have been transferred in the required volume in the specialized tubes and will now be inserted in the appropriate rack and then into the system for the kinetic interaction analysis.

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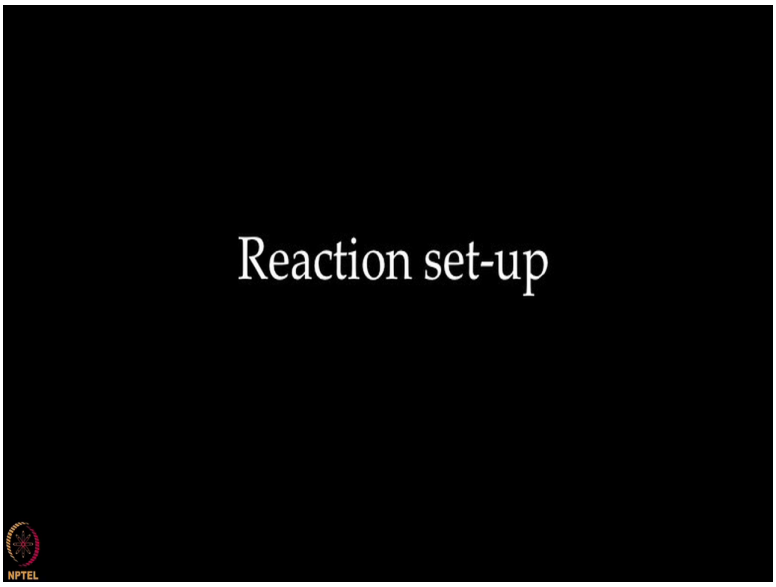

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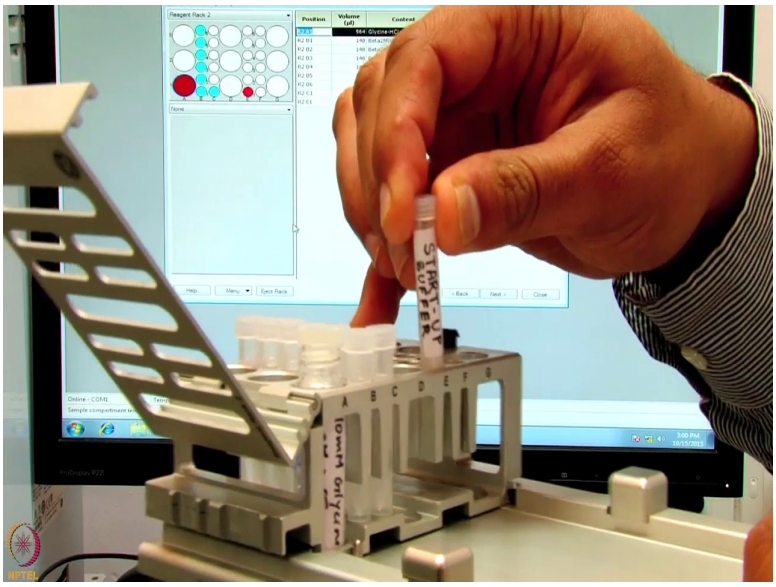

So, from the template here we have prepared the sample rack with the different vials. To start with we have startup placed at E 1 position.

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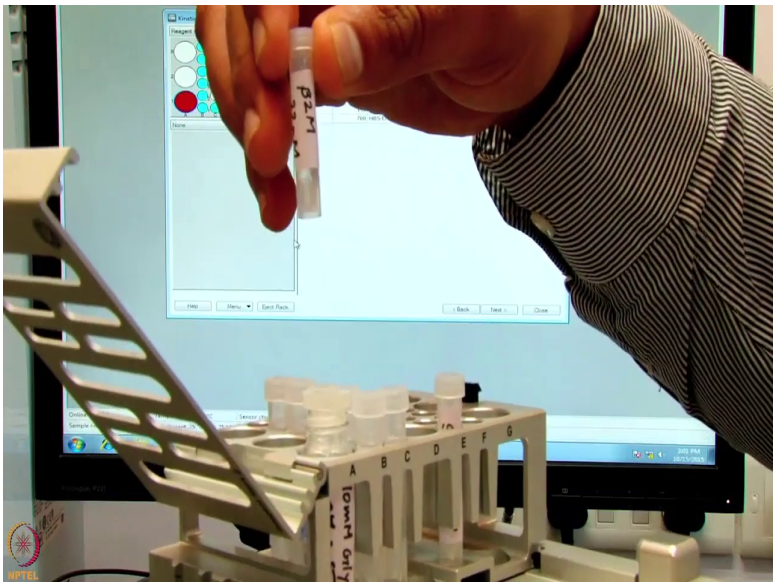

And we have the different concentrations of beta 2M with a concentration mark from positions at C 1, B 1 to B 6.

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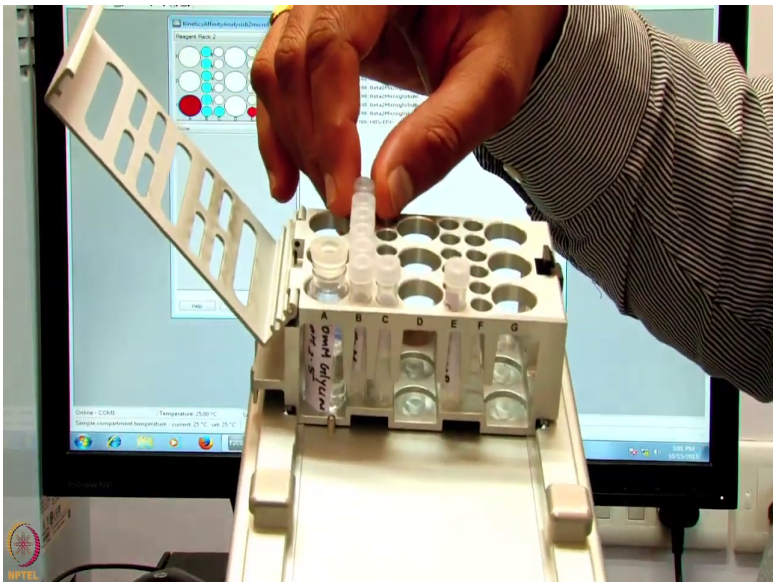

So, 32, 16, 8, 4, 2 nanomolar and again 0.

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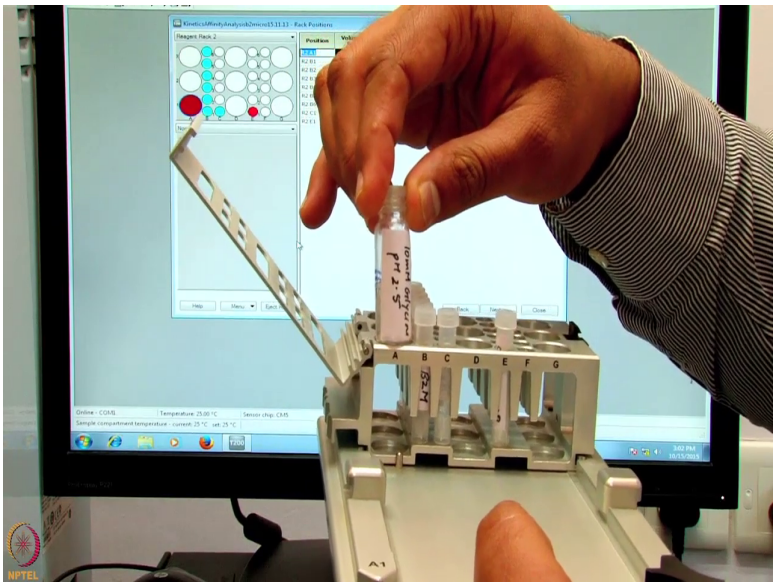

Once we have placed them, we will also have glycine 10 millimolar, pH 2.5 regeneration solution put at the A 1 position.

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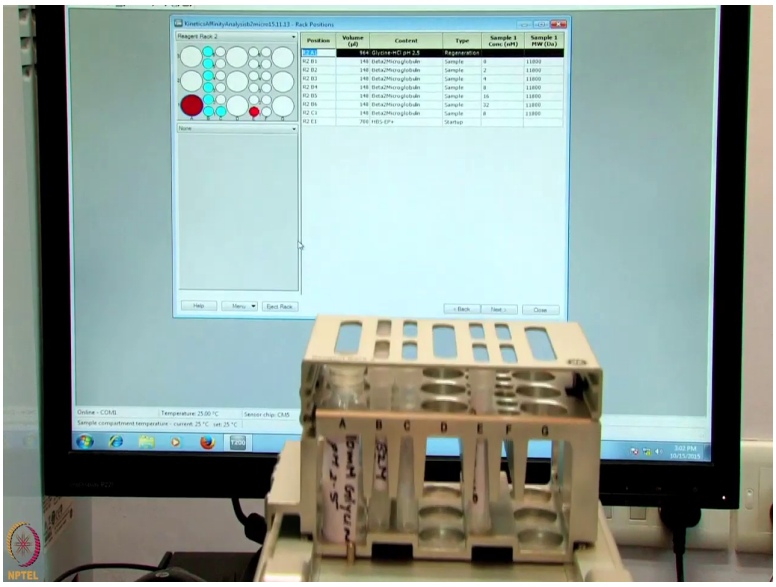

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Now, we close the rack, we go on the screen, eject rack, say ok.

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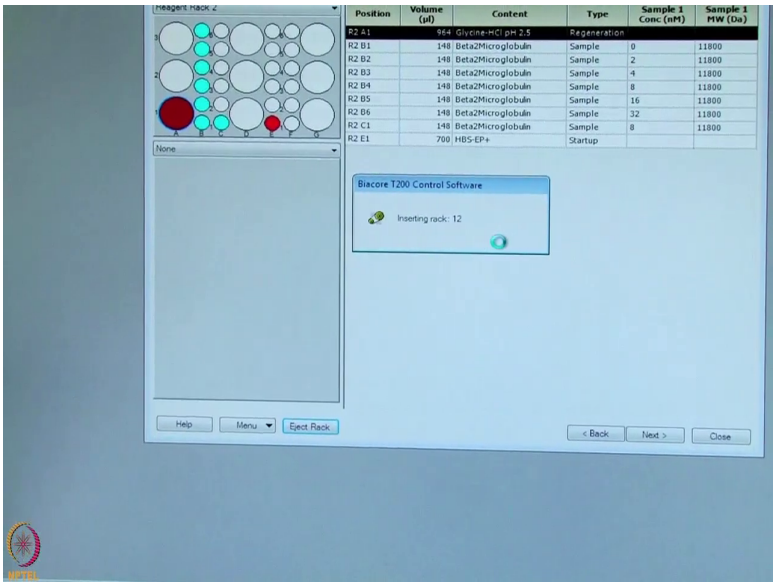

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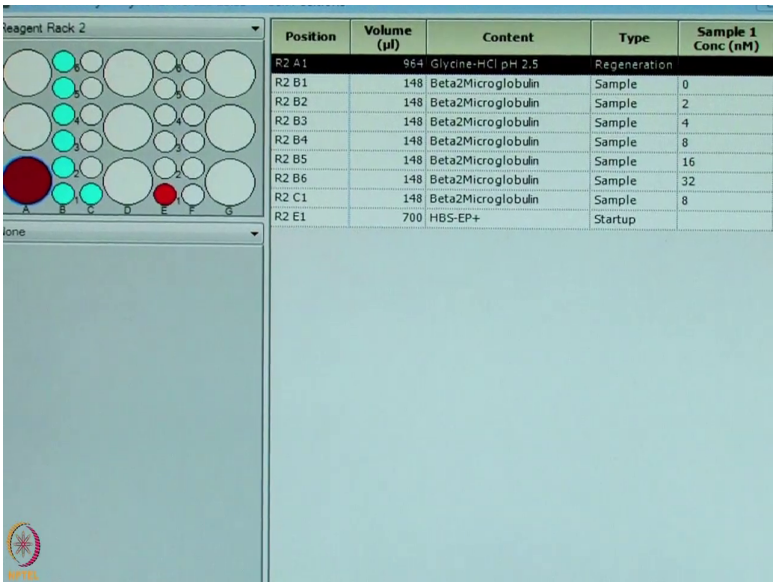

We say next on the tab.

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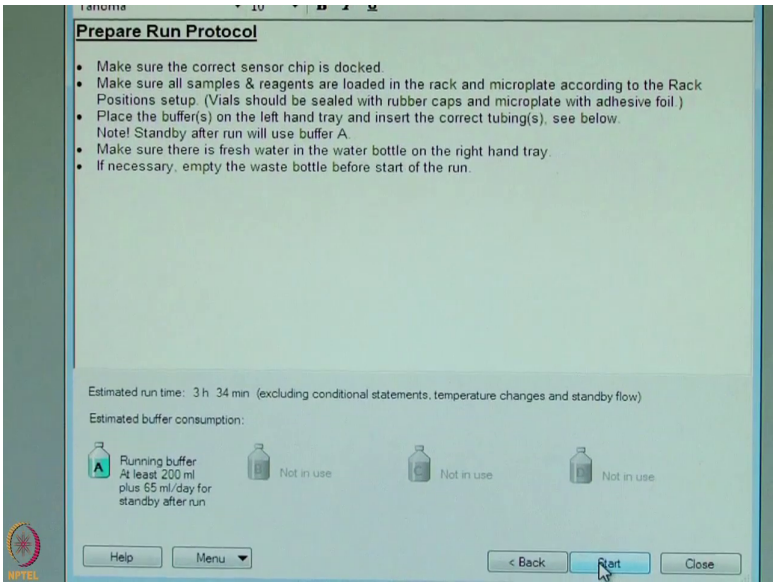

That takes 3 hour 34 minutes, the 200 ml of minimum buffer and say start.

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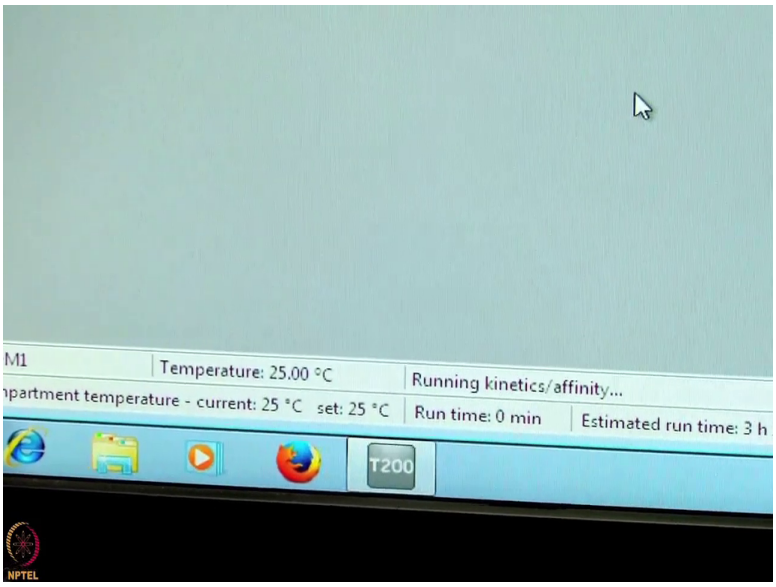

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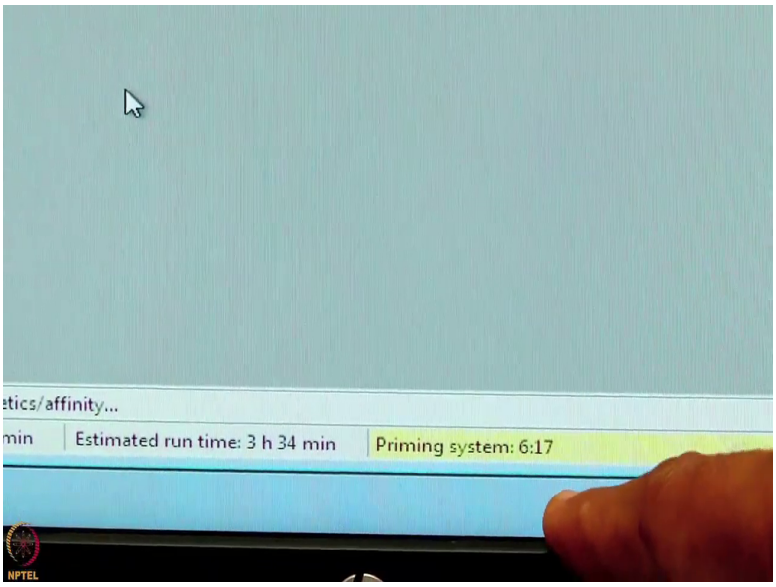

You can see here it says running kinetics affinity takes 3 hours and 34 minutes with a priming of 6 minutes.

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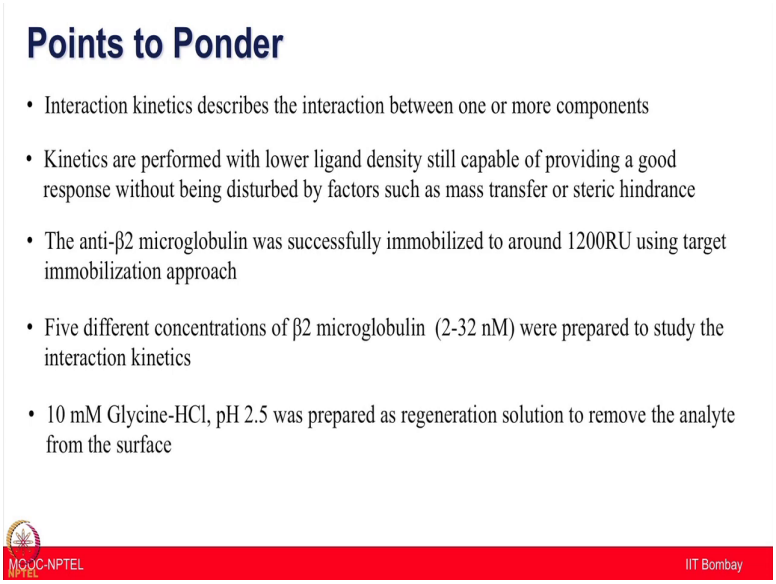

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Once the immobilization is done and the experiment is been run this is how the data will end here.

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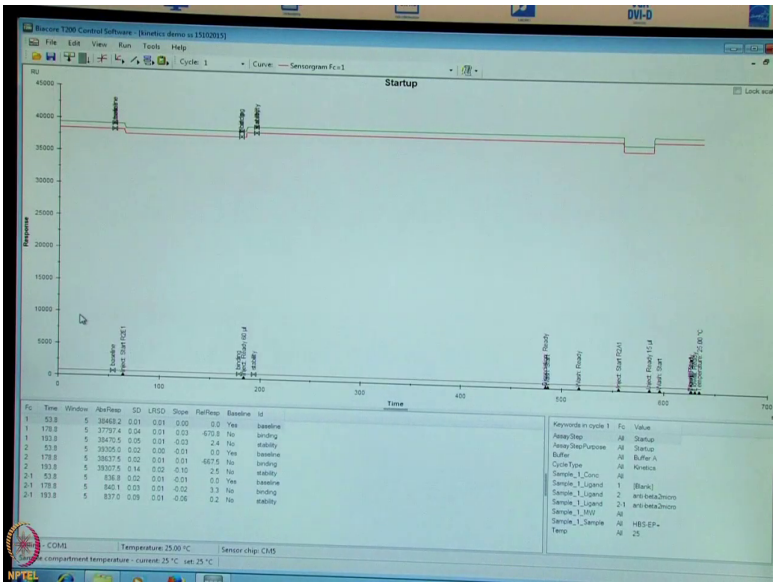

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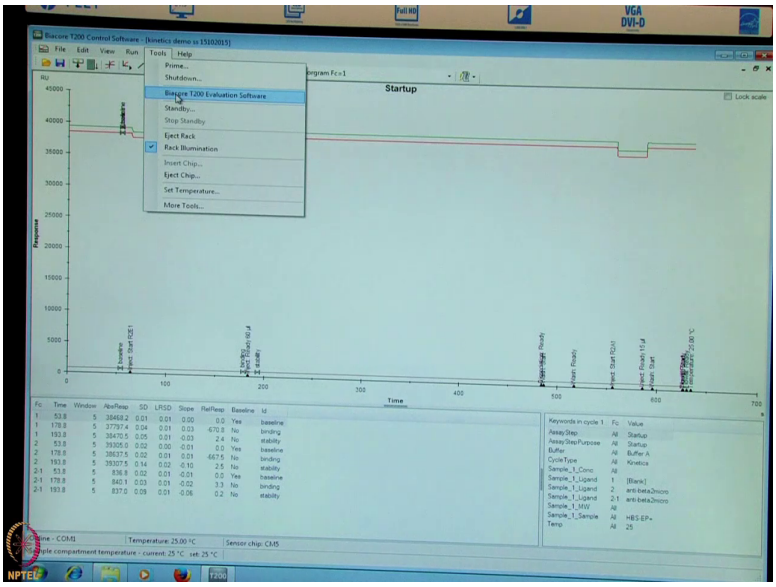

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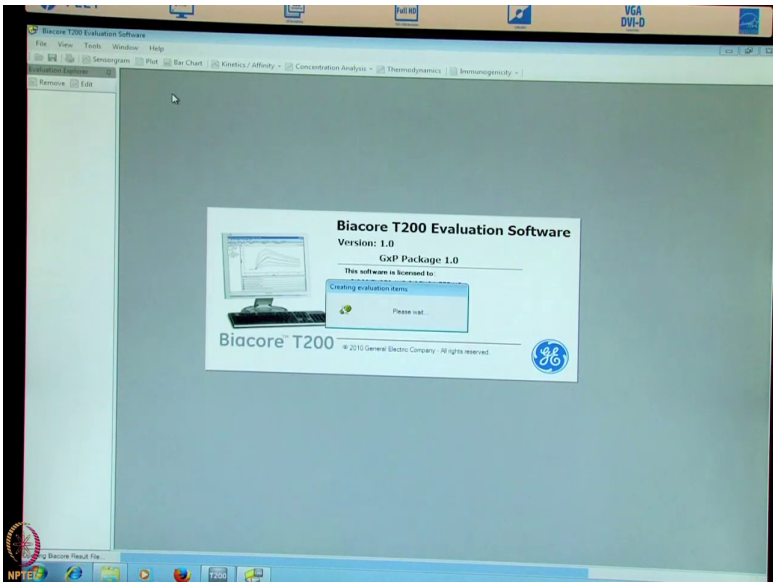

And from the data we can go to tools.

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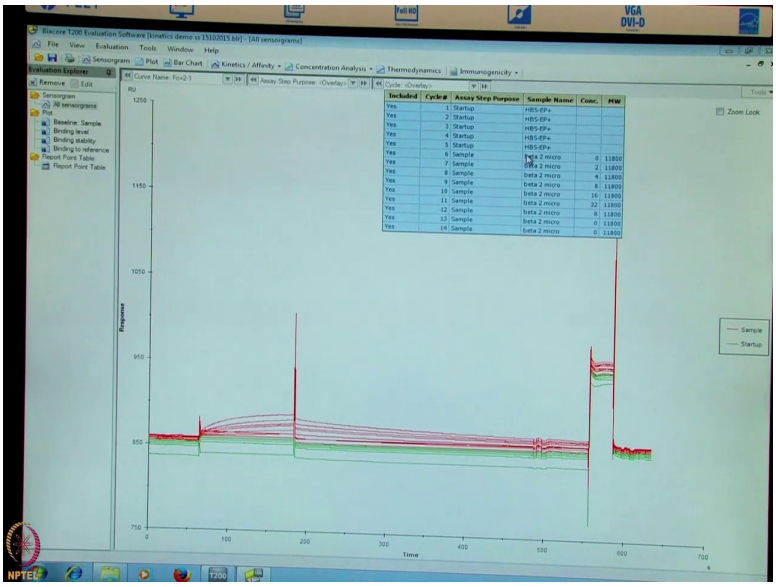

Biacore T200 evaluation software.

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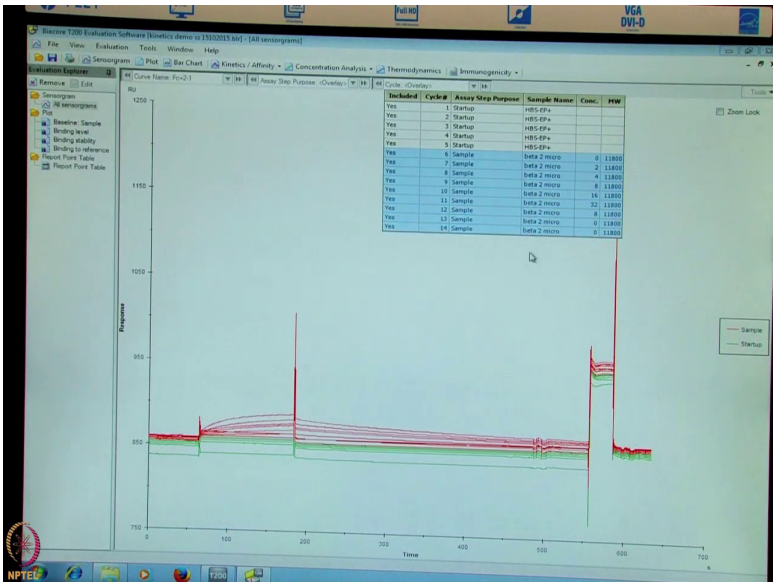

So, this is how the data is generated. You see all the startup runs here and we go on overlay to select sample data.

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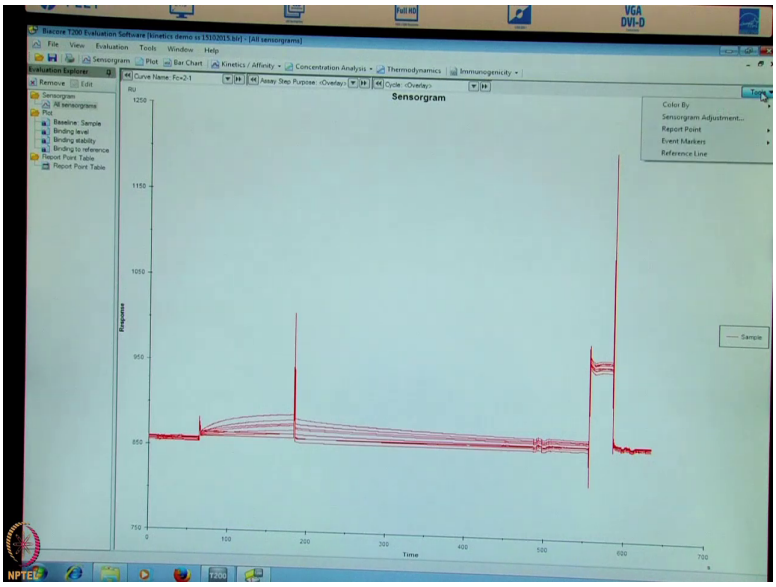

Now, we go on tools, sensorgram adjustment.

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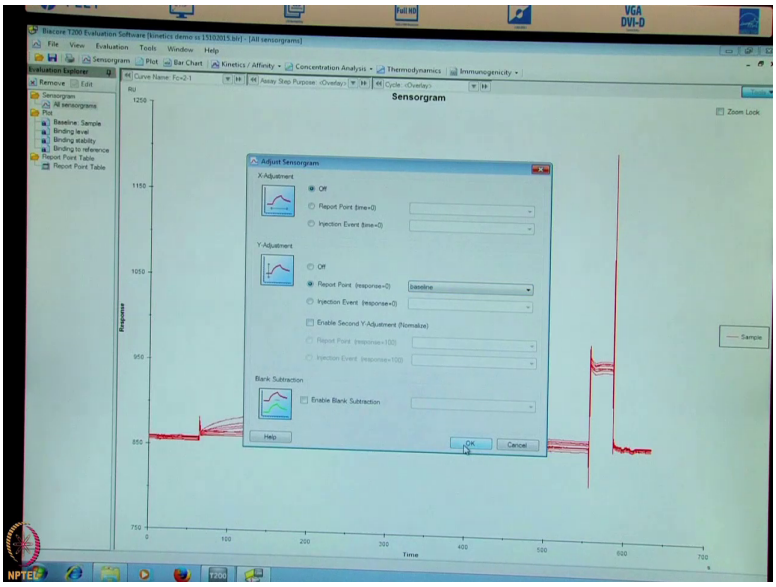

Report point. Baseline.

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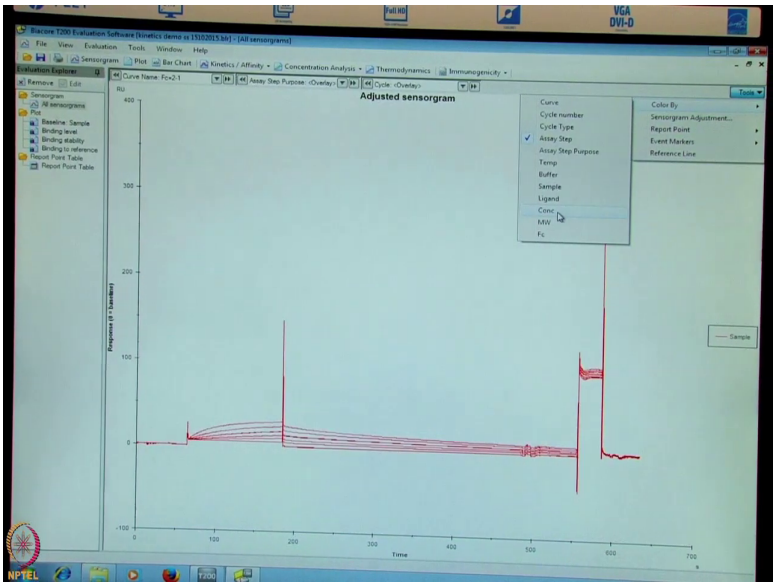
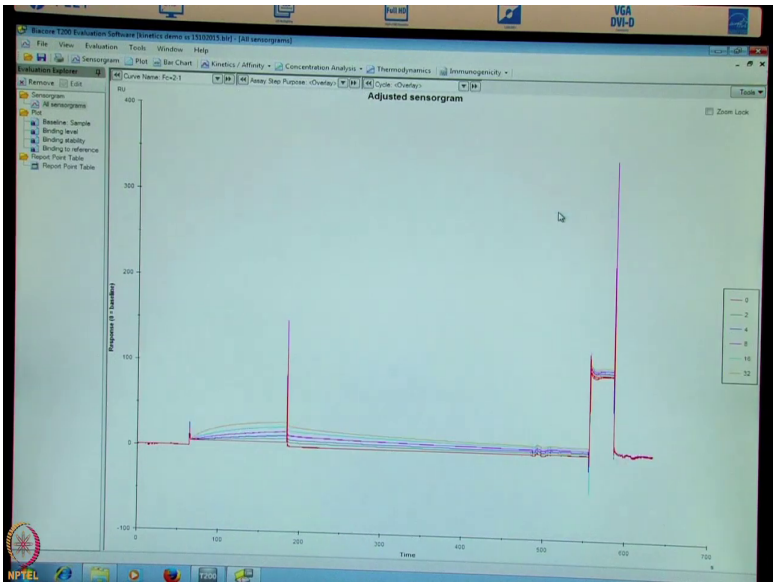


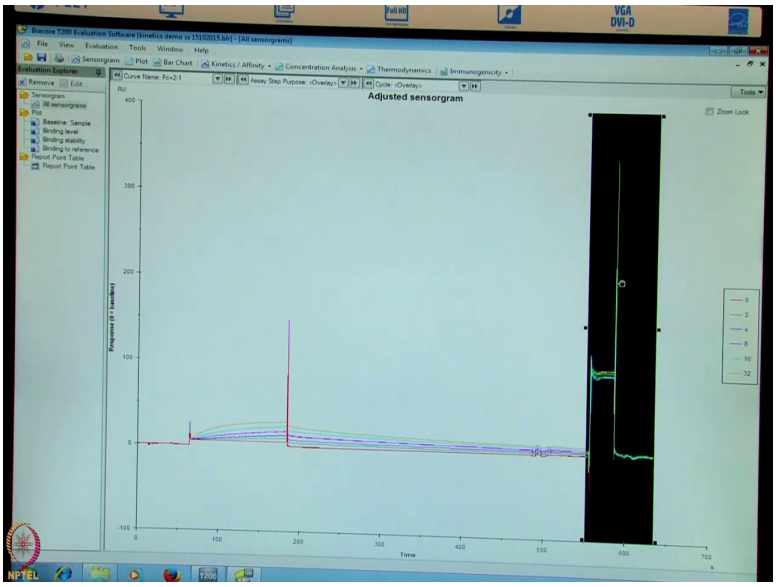
Again to the tool. Colour by concentration.

(Refer Slide Time: 21:00)



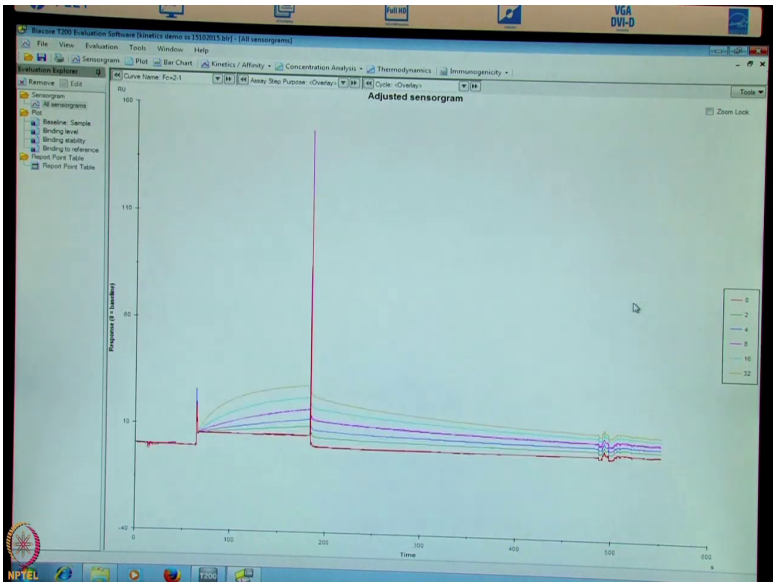
Now, you can see all the data is differently coloured. You see the rate of association, dissociation and regeneration with the ligand of the different concentrations of beta 2M injected.

(Refer Slide Time: 21:37)



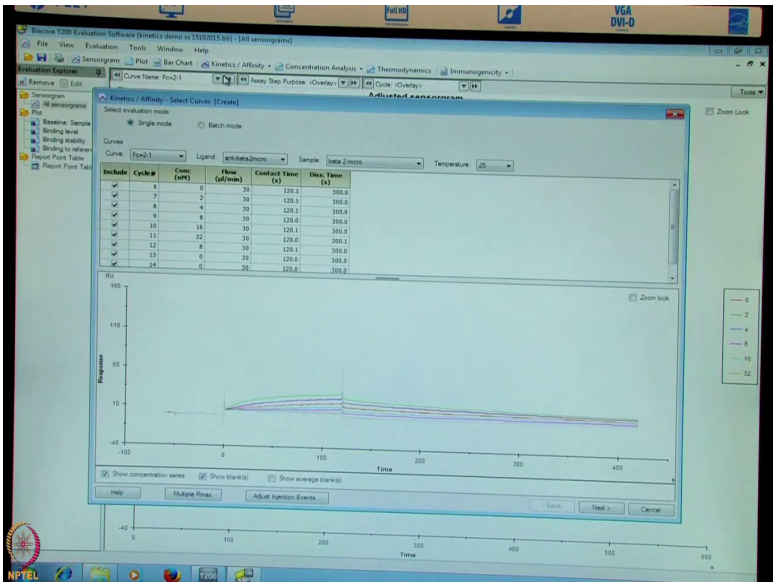
Here as well like binding, we will write click on the mouse and cut away this portion.

(Refer Slide Time: 21:46)



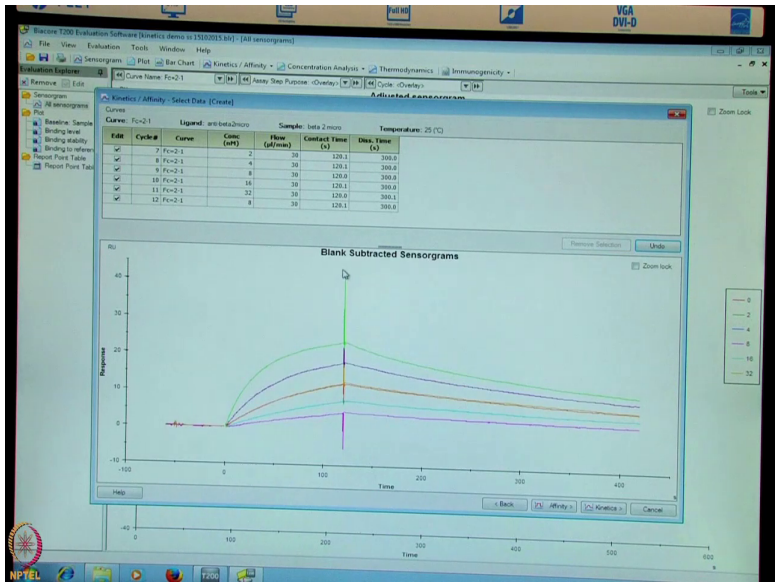
And now this is our actual data with a different concentrations of beta 2M injected on the surface. To evaluate this data we can go into the kinetic and affinity mode, select surface bound.

(Refer Slide Time: 22:21)



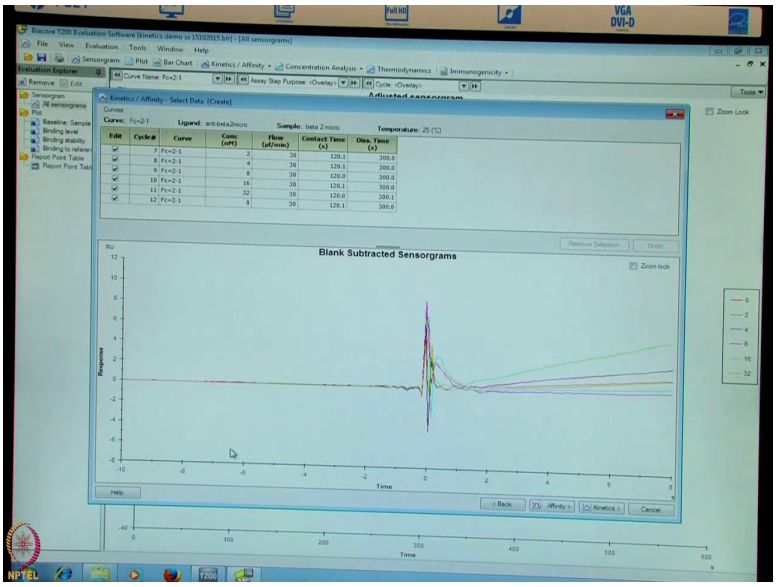
The data is shown here. We can go to them, here you can see the different cycle number different concentrations of the beta 2M. At a flow rate injected was 30 microliter per minute, contact time of 120 second, and dissociation time of 300 second. We have selected 2 minus 1 surface the ligand that is immobilized these anti-beta 2 microglobulin and the sample is beta 2 microglobulin or analyte, interaction performed at 25 degree centigrade. Now, we go onto this and say next.

(Refer Slide Time: 23:25)

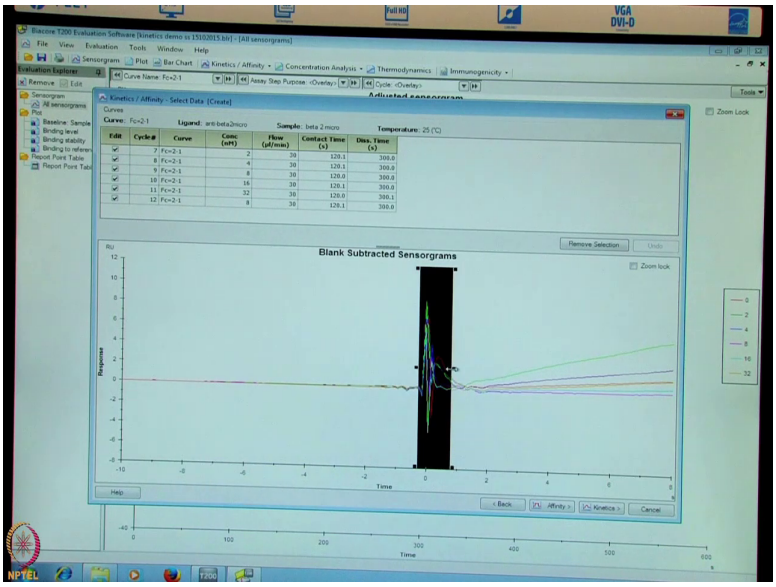


If at all there are unwanted spikes can be removed in this region or in this tab by simply highlighting that area.

(Refer Slide Time: 23:42)

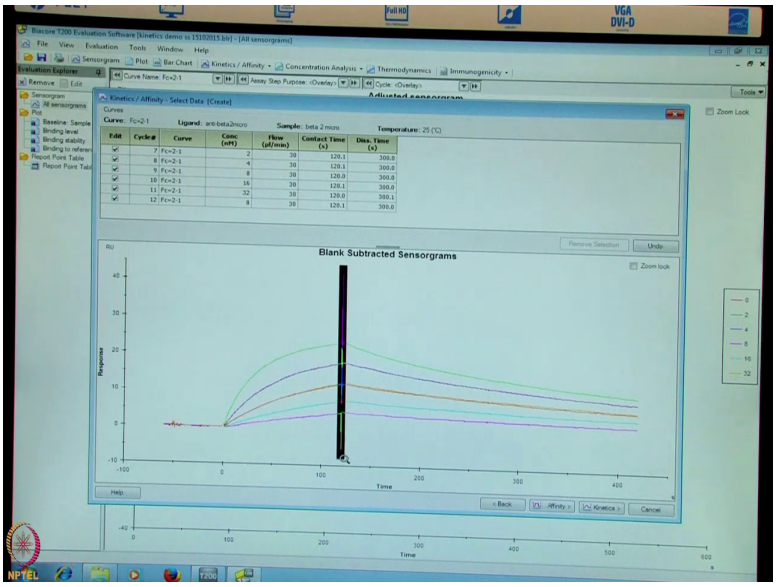


(Refer Slide Time: 23:47)



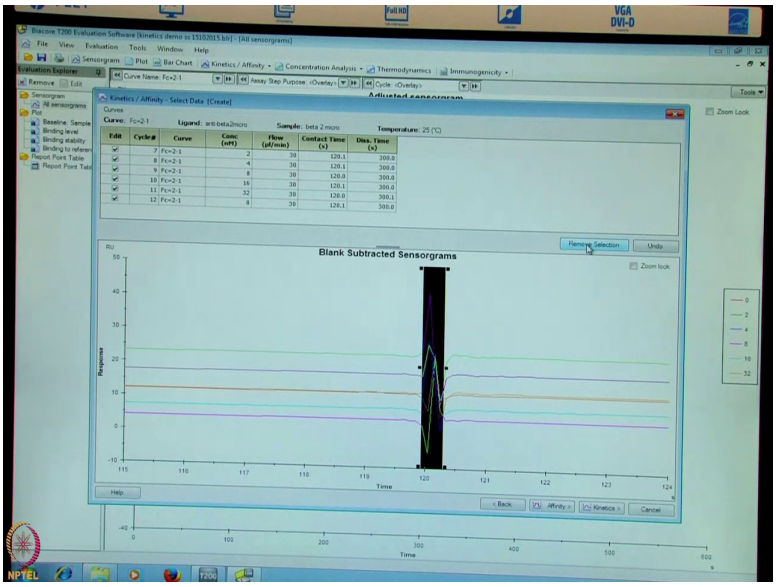
And right click, and highlight that unwanted region and say remove selection.

(Refer Slide Time: 24:04)



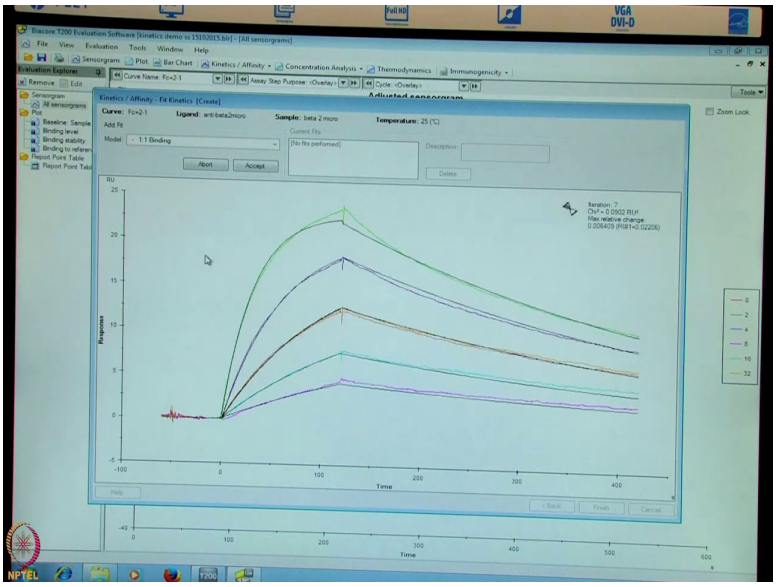
Similarly, we could take away these spikes by highlighting that area, is the right click of the mouse and say remove selection.

(Refer Slide Time: 24:07)



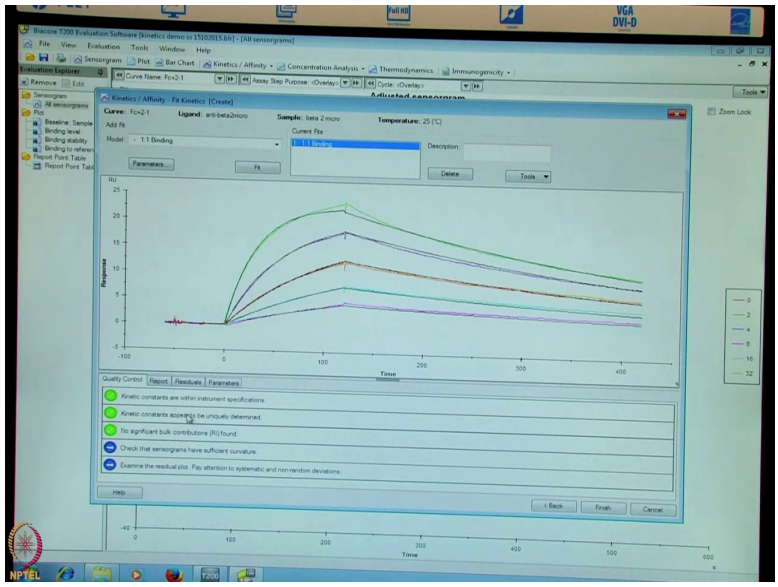
Now, that there are no spikes we can proceed to fit this data in the kinetic mode as you can see on the tab.

(Refer Slide Time: 24:15)



Now, you see here one to one binding and parameters here, fit here. So, we could place mouse on the fit and press start.

(Refer Slide Time: 25:00)



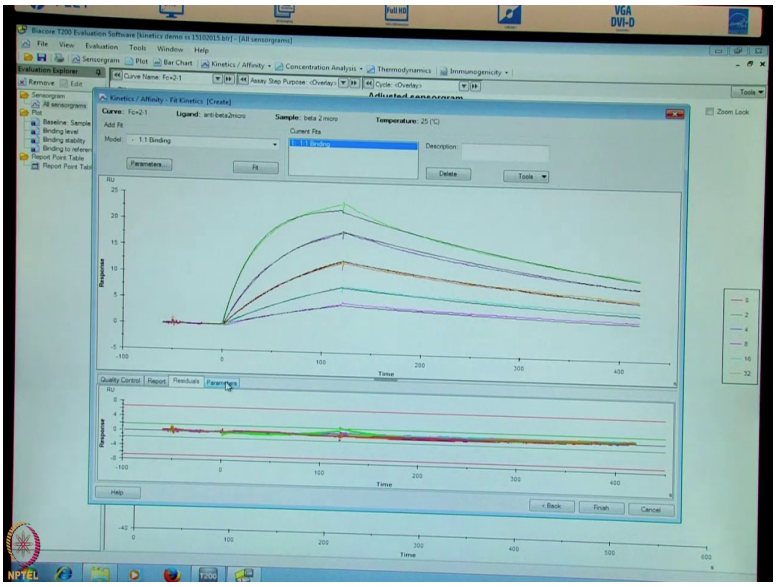
So, data is fit with the black lines superimposing on the coloured lines, and the following information is generated. If you see the quality control tab, report tab, the residual tab and parameters, each of them has data from this experiment. To start with we go onto the control quality control tab and see here all the 3 are in green with kinetic constants are within instrument specifications or uniquely determined and no bulk contributions found. We have learnt from our theoretical sessions bulk contribution will be more if there is a mismatch between buffer and sample.

(Refer Slide Time: 26:25)

Now, we go to the next tab report. You can see lot of information that is generated here. The on rate is given here. Similarly, there is an off rate, and there is a affinity or a binding constant provided here. The R max is also given. The various concentrations that was used to setup the experiment are provided here. Flow rate for this experiment is provided here.

The change in the refractive index due to differences in the sample and running buffer are provided here and a quality control parameter like chi square and u value are also shown. So, kd refers to the affinity of the two molecules which is 2.6 into 10 to the power of minus 9 molar or 2.69 nanomolar affinity. R max are a maximum number of binding sites available at 24. Chi square of 0.0901, should be below 1 at least. u value below 25 is always considered to be good.

(Refer Slide Time: 28:08)



We go to the next tab residuals. The residual should be aligned between the red line, preferably between the green lines and here you can see that the data is completely within green lines and that indicates the superimposition of the black lines on the coloured lines is very close.

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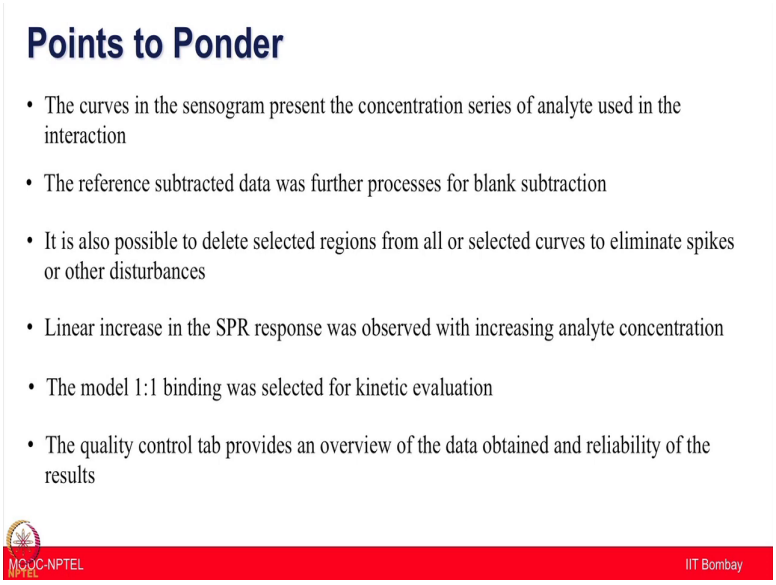
Now, we go onto another tab, parameters and find lot of information which are nothing but the standard error calculated for the data.

With this we conclude the interaction of anti-beta 2 and to beta 2 microglobulin at 2.6 nanomolar affinity.

(Refer Slide Time: 29:23)



I hope that the lab demonstrations of different steps involved to study and analyze the biomolecular interactions would have helped you in better understanding the SPR and the workflow involved. In the next lecture we are going to have a scientist from (Refer Time: 30:24) Center (Refer Time: 30:25) who is going to talk to you about applications of SPR technology in biologically relevant problems.

Thank you.