Interactomics: Protein Arrays and Label-Free Biosensors.
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Lecture-9.
Protein-Protein Interaction Study: Kinetic Analysis.

Welcome to MOOC NPTEL course on intractomics, determination of interaction kinetics is one of the most striking application for SPR biosensors and understanding of kinetics is essential to evaluate the strength of a bio-molecular complex interaction. The label-free real time detection allows kinetic analysis to be performed by monitoring interaction as a function of time over a range of analyte concentrations and then fitting the whole dataset to the mathematical model described in the interaction presenting the kinetic parameters, for example, association and dissociation rate constants.

Affinity constants which exhibit the strength of binding between the molecules can be derived either from the rate constants or from steady state binding analysis. A kinetic analysis experiment can be setup in a biacore experiment in two ways; multicycle kinetics where each analyte concentration is run as a separate cycle, regenerating the surface after each sample injection and single cycle kinetics which runs a series of analyte concentration in one cycle with no regeneration between sample injections.

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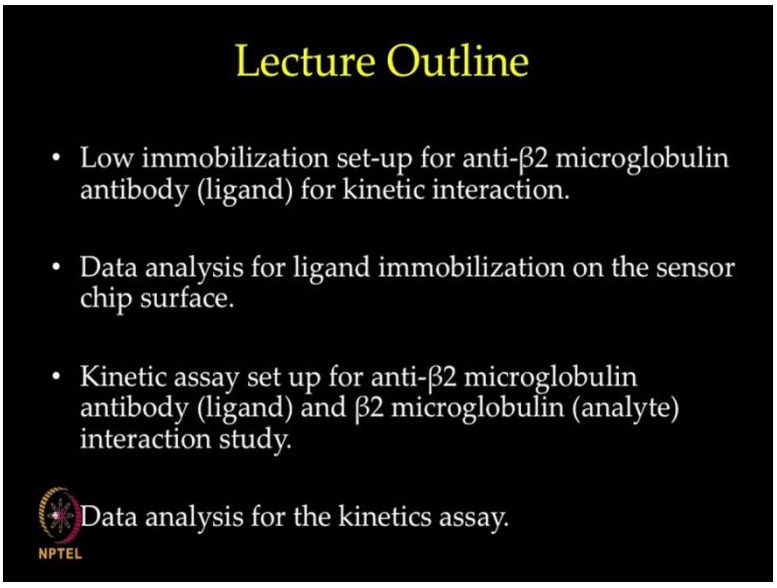

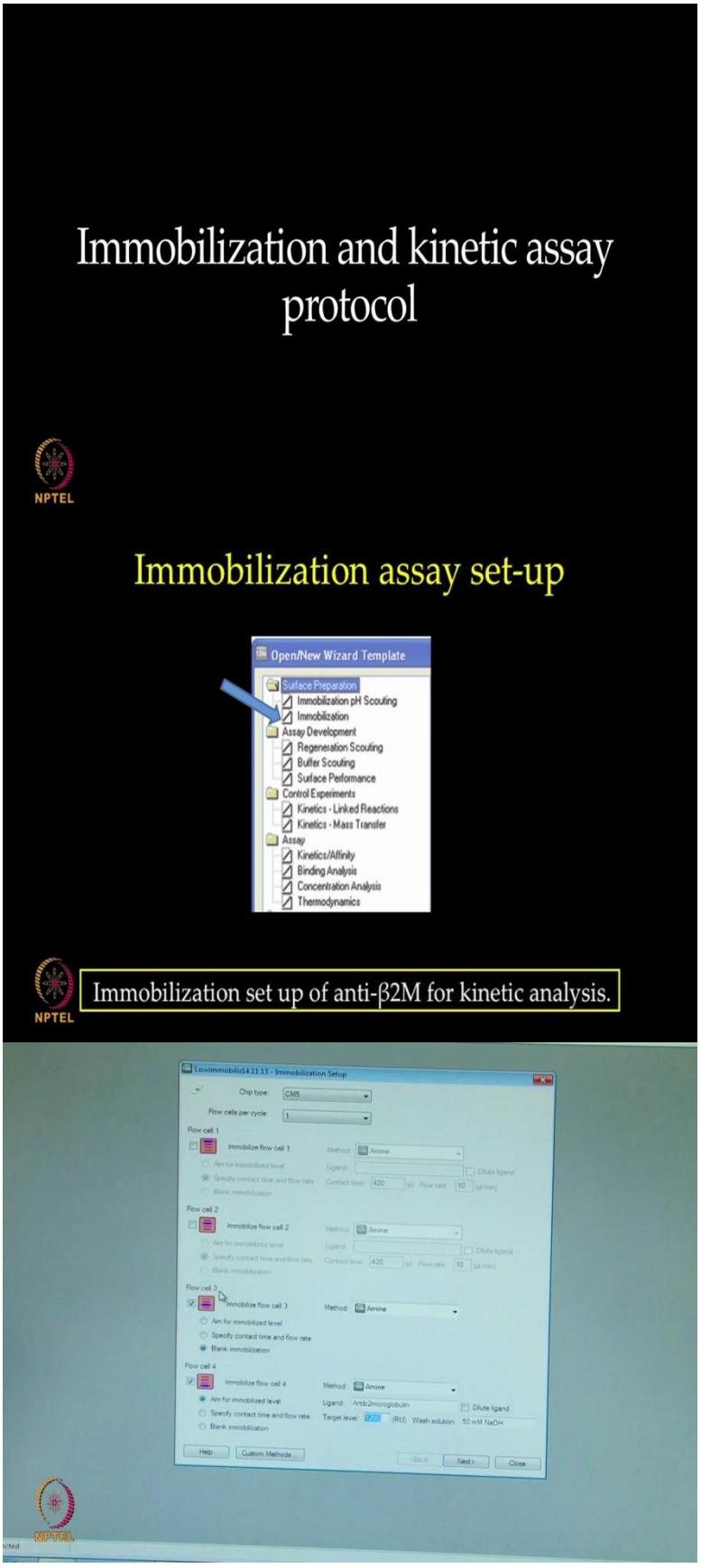

Today, we are going to perform the kinetic analysis for the protein beta two microglobulin with anti-beta two microglobulin antibody using multicycle kinetics. So, let us have the lab

experimental sessions now. We will start with low immobilization protocol setup for anti-beta two microglobulin as a lower immobilization level will be beneficial in this kinetic interaction analysis. We have to re-do 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 two microglobulin at 1200 RU for a kinetic experiment.

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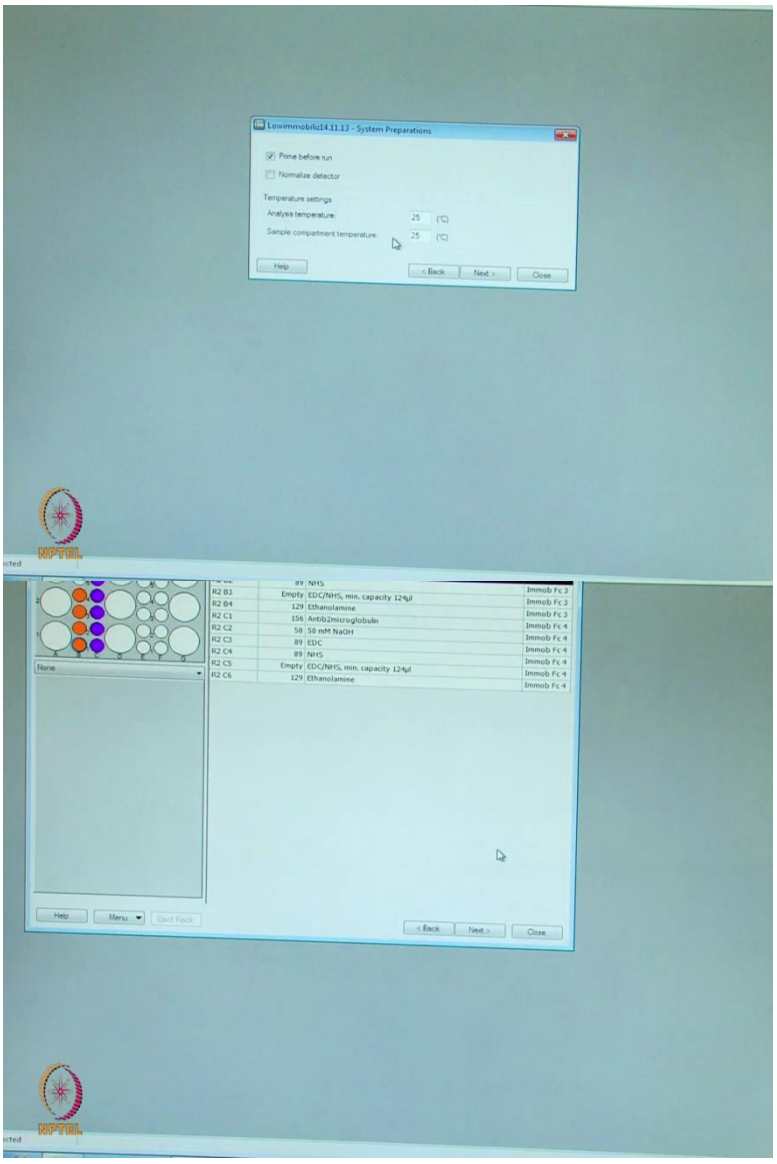

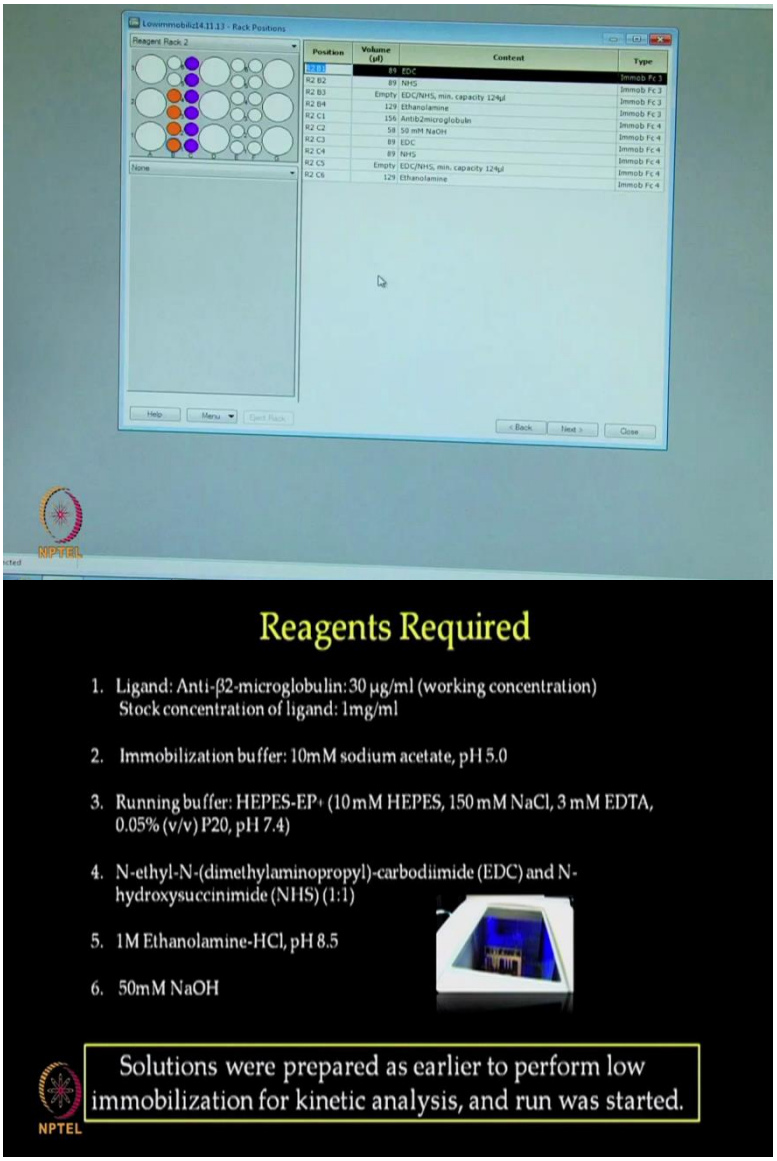

Here, again we will highlight prime before run and keep the sample compartment temperature at 25 degree centigrade and we will go to the next tab. Now, we will add vials with the rack positions and the volumes. As per the volumes listed in the table we will prepare the reagents for immobilization in a similar manner as demonstrated earlier in lecture 33. We prepared a working concentration of 30 microgram per ml of beta two microglobulin antibody from a stock concentration of 1 mg per ml using an immobilization buffer of 10 milimolar 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 one is to one ratio for surface activation. One molar ethanol amine HCl pH 8.5 was prepared for blocking the free ester group on the surface. Lastly, 50 milimolar NaOH was prepared which will be used to remove the electrostatically bound ligand. We prepare the above mentioned solutions,

transfer them to appropriate sample vial, place them in the suitable rack 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 two microglobulin antibody.

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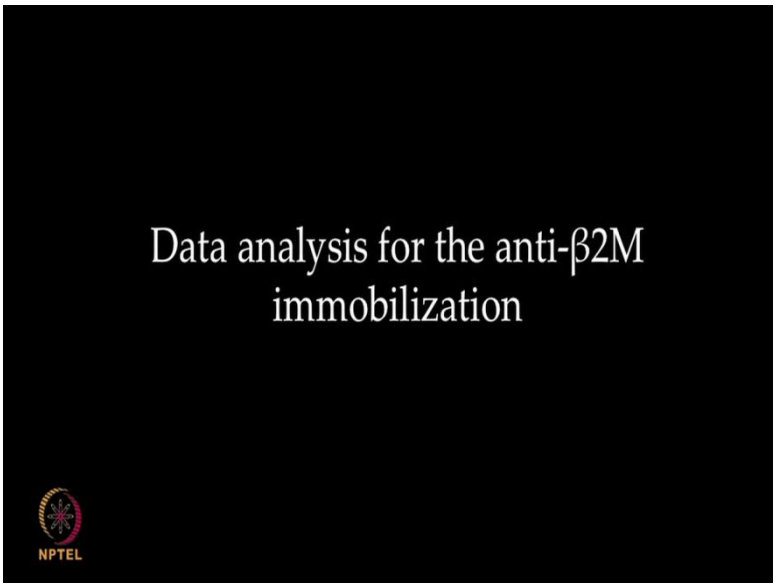

So, this is a blank sensorgram, after immobilization we will see the reference surface is blank immobilize. Here, you see the activation of the chip by EDC-NHS and blocking of the surface with ethanol amine. So, this will be the blank immobilization. Now, we will go to another surface or another flow channel, going to the next flow channel FC4 highlighted. This is a sensorgram for active surface immobilization involving protein, to start with there is a pre-concentration estimation of the required amount of ligand washed with the sodium hydroxide, activation of the surface with EDC and NHS mixture, pulsing of the protein for the required number of RUs and then block off the extra sides with ethanol amine. 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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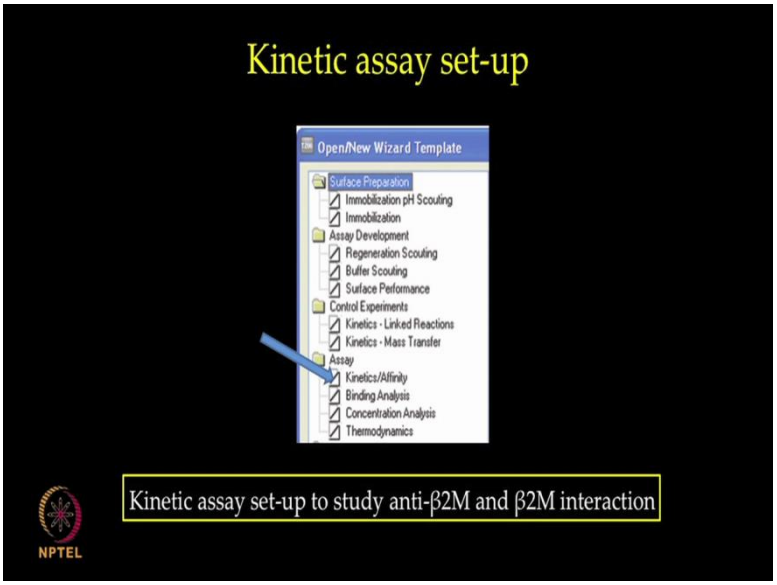

We will now setup a wizard for kinetic analysis, to start with we have selected from the wizard kinetic template. Selected the flow path 4 minus 3, chip type CM5 sample and the flow is to regeneration. Once, we have setup with this template we say next and startup cycles are selected at 5 with HEPES-EP plus buffer. The sample contract time of 118 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 simulization time of zero seconds and we now go on to the next tab and fill in the name of the sample as beta two microglobulin if the molecular weight of 11800 and concentration with 0, 2, 4, 8, 16, 32 and the repeat of 8 nanomolar again, we say next and prime before run is highlighted.

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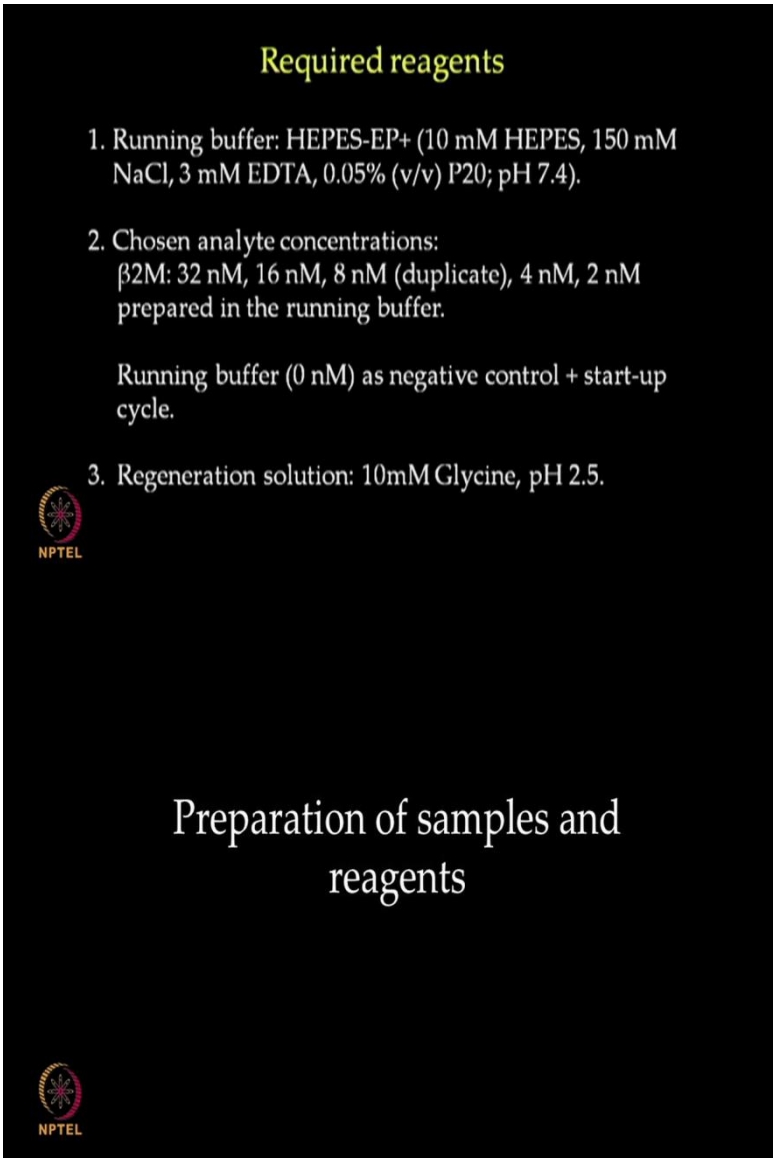

Now, we have the rack positions this way, we have regeneration buffer here, startup here, with the samples here. In today's kinetic assay experimental setup, we will be using HEPES-EP plus as the running buffer. We will be preparing a total of 5 concentrations of beta microglobulin starting from 32 nanomolar,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

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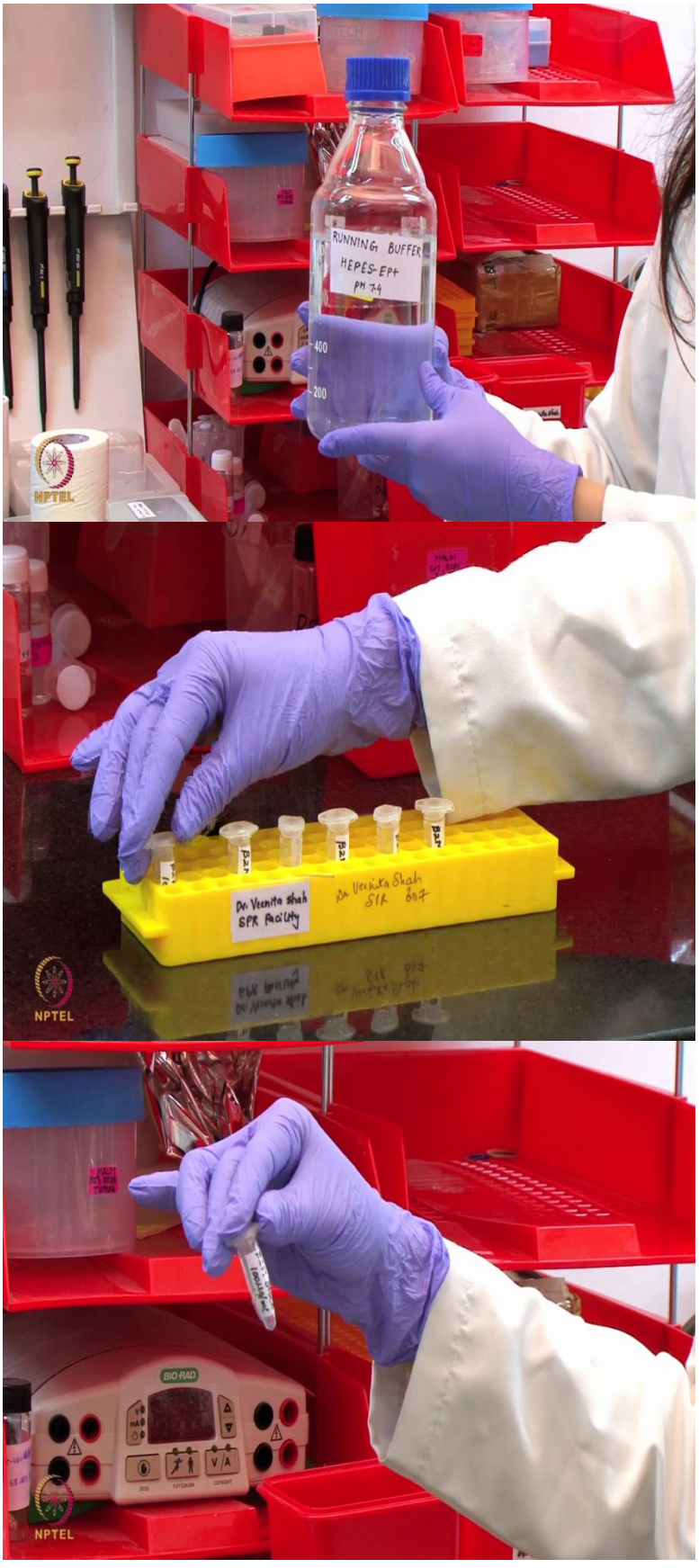

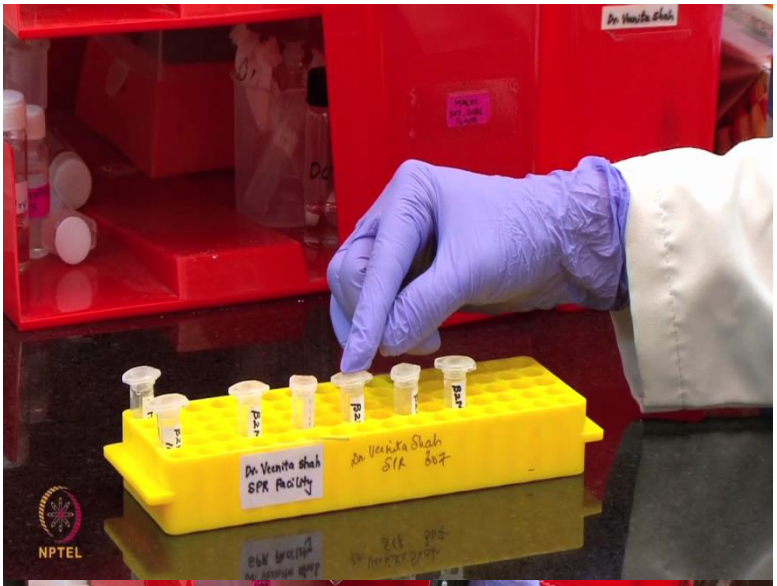
Dr Vennita Shah
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Dr Vennita Shah
SIR 807
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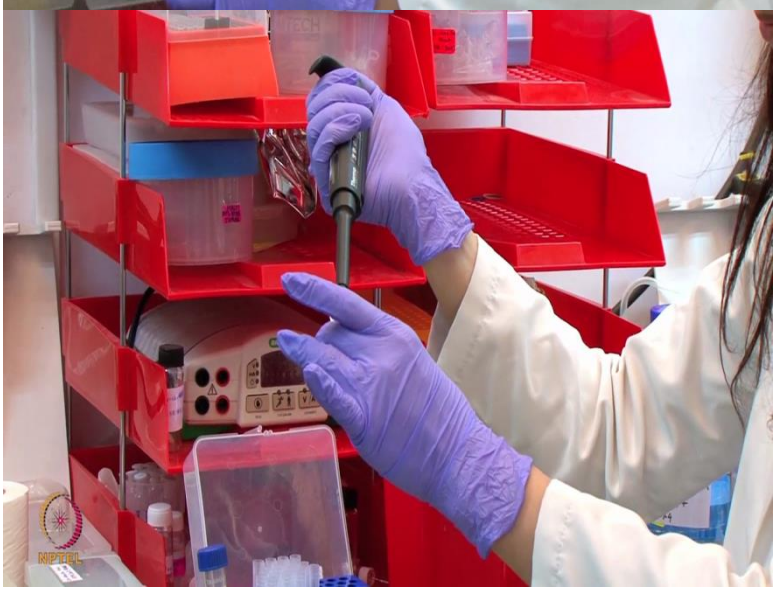
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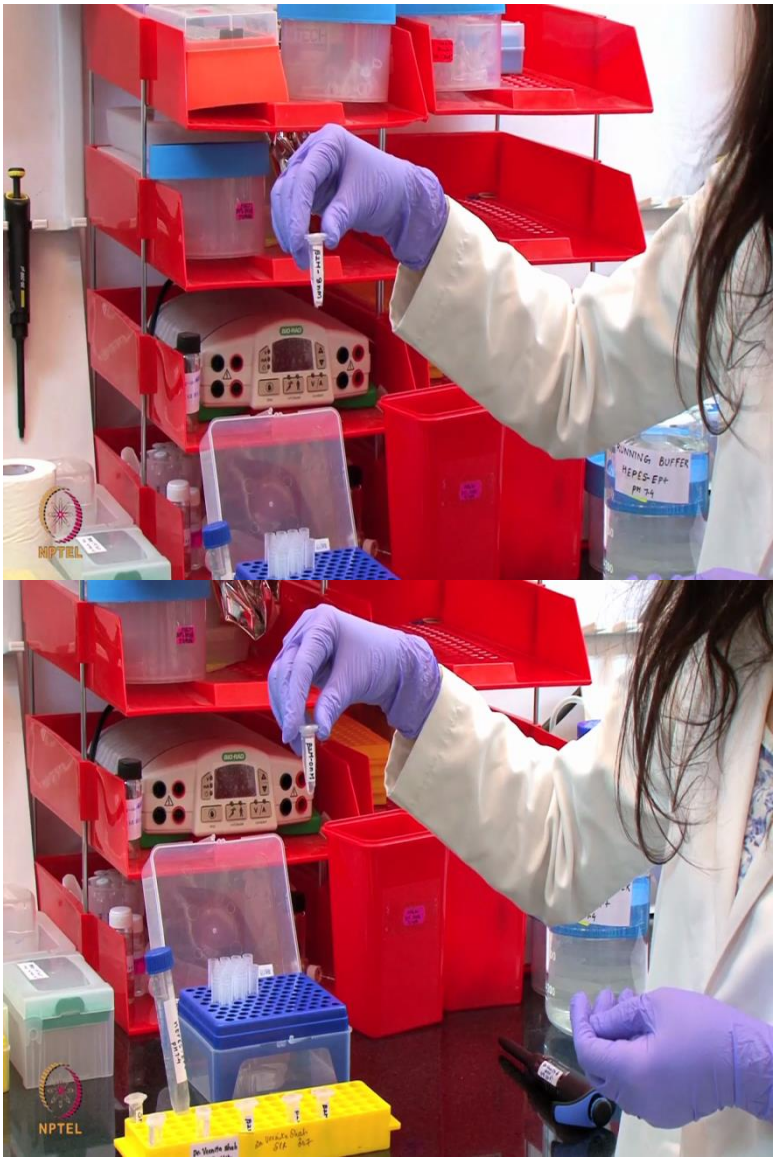
RUNNING BUFFER
HEPES-EPP
PH 7.4
NPTEL

We will now work on the reagents required for the kinetic interaction analysis between anti-beta two microglobulin and beta two microglobulin. Once again, we will be using HEPES-EP plus as the running buffer which will also be used for the initial startup cycle. 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.

We will be preparing this concentration series in two poor dilutions using the running buffer starting from 32 nanomolar solution which means we will add 300 microliter of 32 nanomolar concentration with 300 microliter of buffer to prepare 16 nanomolar concentration. We similarly continue with the dilution series for rest of the concentration. One of the concentrations that is 8 nanomolar will be run in duplicate and we will also include a zero nanomolar concentration in the experiment.

For regeneration we will be using glycine HCl pH 2.5 and the regeneration solution. All these samples and reagents have been transfer in the required volumes in these specialized tubes and then now, we inserted in the appropriate rack and then into the system for the kinetic interaction analysis.

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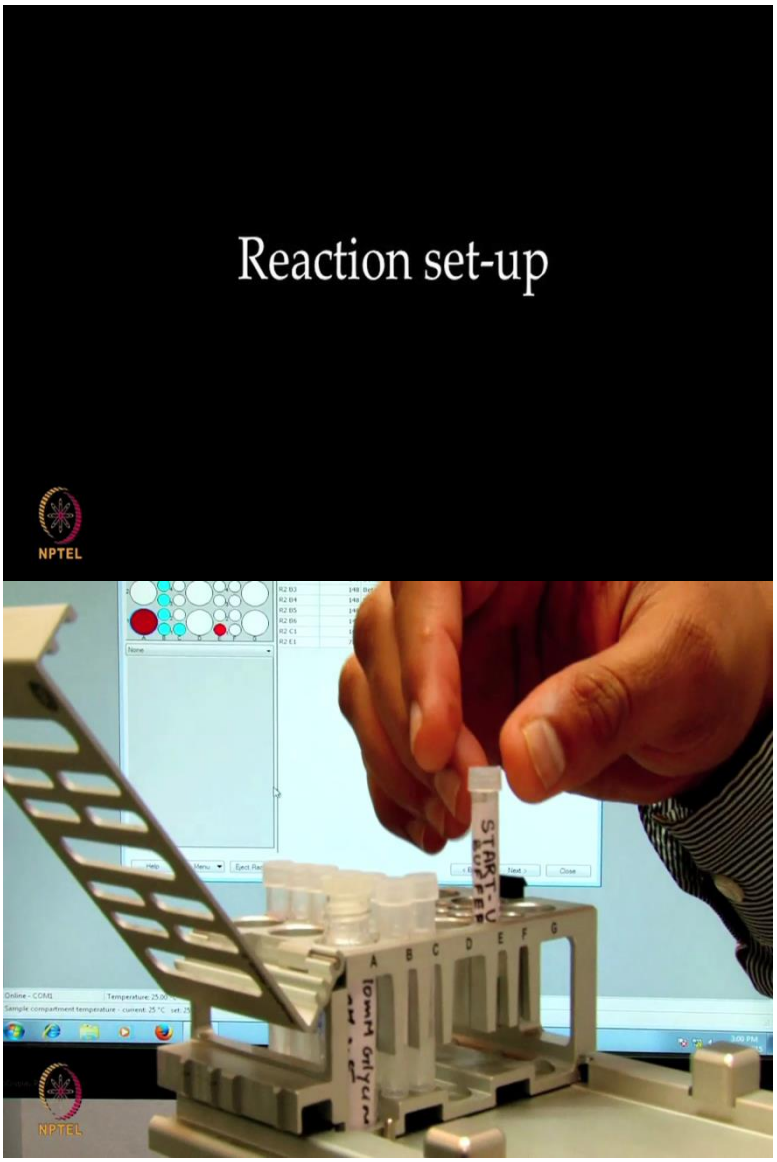
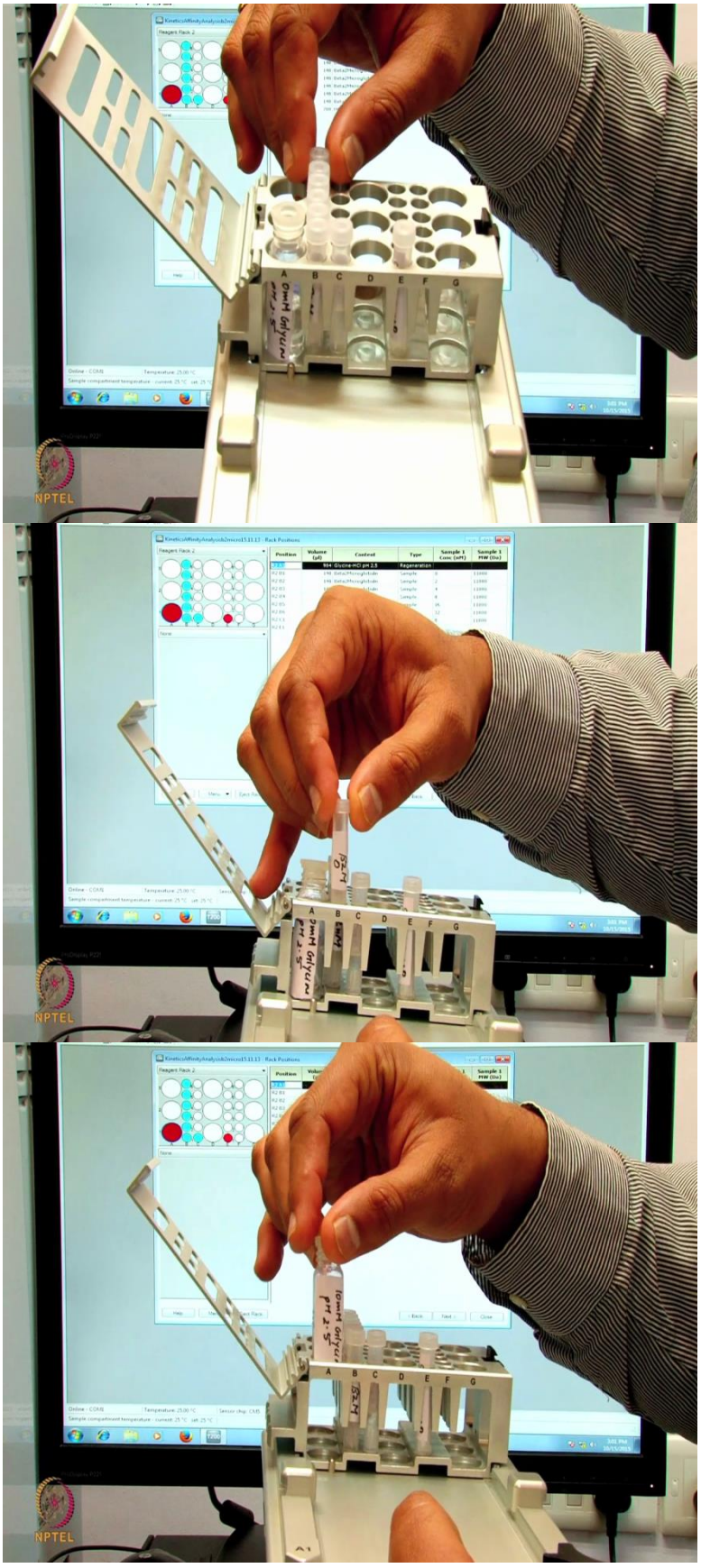
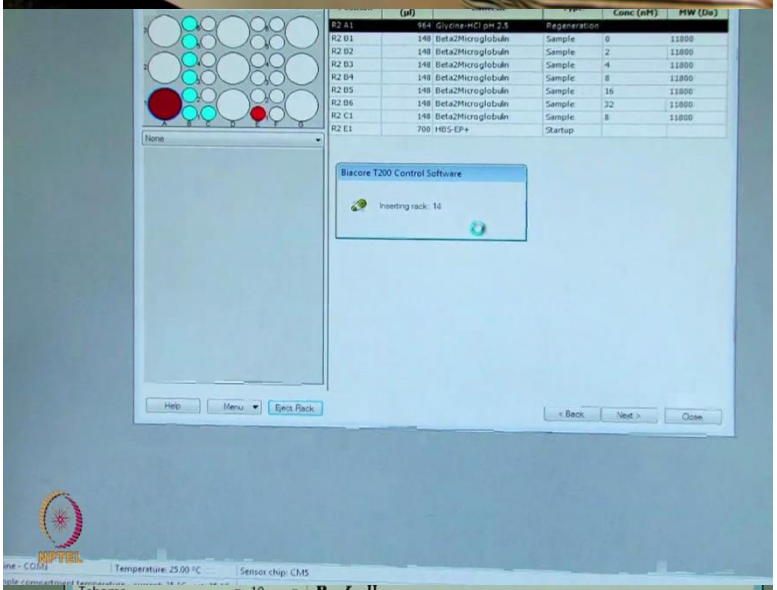
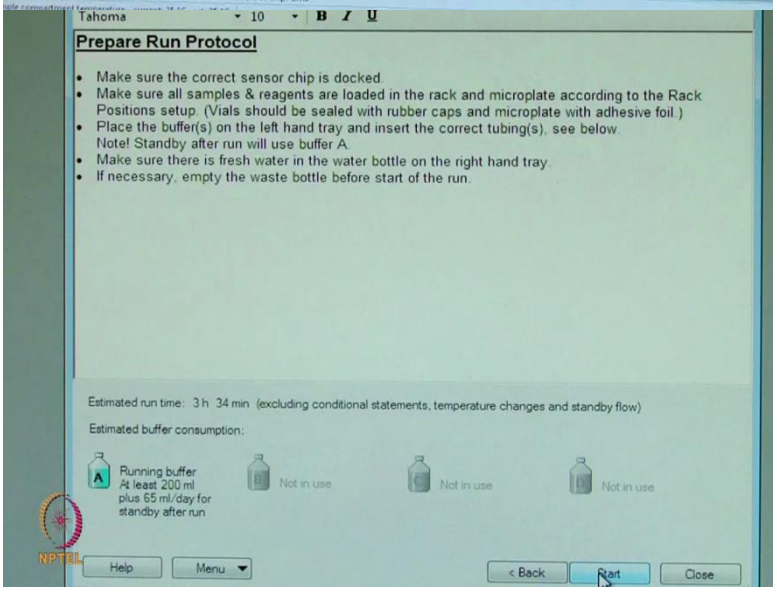
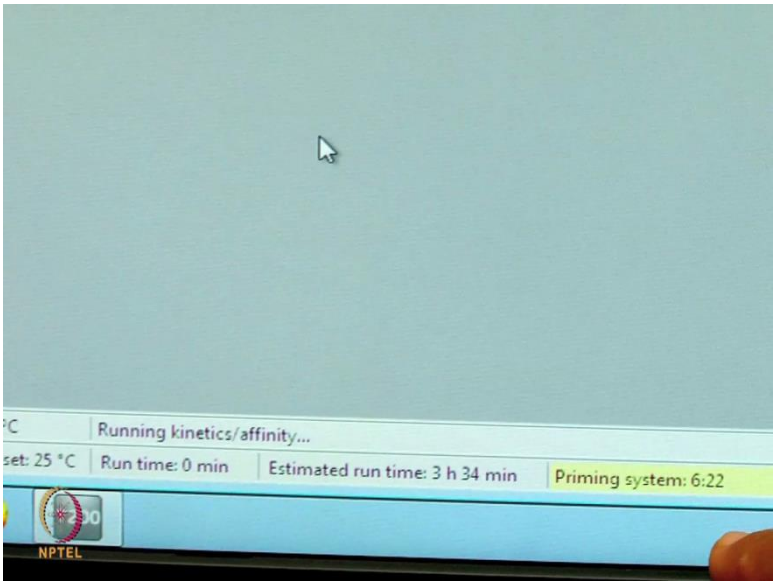





Biacore T200 Control Software
Inserting rack: 14
Help
Menu
Eject Rack
< Back
Next >
Close


Tahoma
10
Prepare Run Protocol
Make sure the correct sensor chip is docked.
Make sure all samples & reagents are loaded in the rack and microplate according to the Rack Positions setup. (Vials should be sealed with rubber caps and microplate with adhesive foil.)
Place the buffer(s) on the left hand tray and insert the correct tubing(s), see below.
Note! Standby after run will use buffer A.
Make sure there is fresh water in the water bottle on the right hand tray.
If necessary, empty the waste bottle before start of the run.
Estimated run time: 3 h 34 min (excluding conditional statements, temperature changes and standby flow)
Estimated buffer consumption:
Running buffer
At least 200 ml
plus 65 ml/day for
standby after run
Not in use
Not in use
Not in use
Help
Menu
< Back
Start
Close



So, from the template here we have prepared the sample rack to the different vials, to start with we have startup placed at E1 position and we have the different concentrations of beta two M with a concentration mark from positions HC1, B1 to B6. So, 32, 16, 8, 4, 2 nanomolar and again zero. Once, we have placed them we will also have glycine 10 milimolar pH 2.5 regeneration solution put at the A1 position.

Now, we close the rack, we go on the screen eject rack save ok. We say next on the tab, it takes 3 hours 34 minutes with 200 ml of minimum buffer and say start. You can see here, this says running kinetics affinity takes 3 hours and 34 minutes with a priming of 6 minutes.

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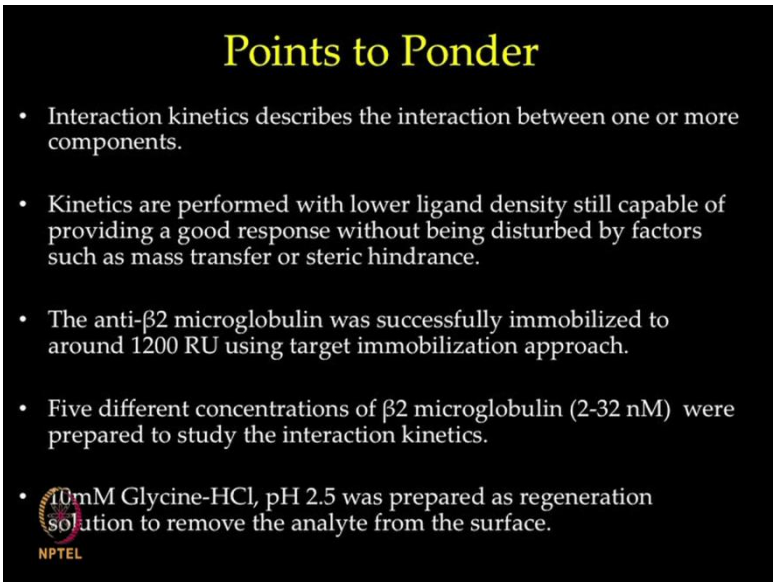
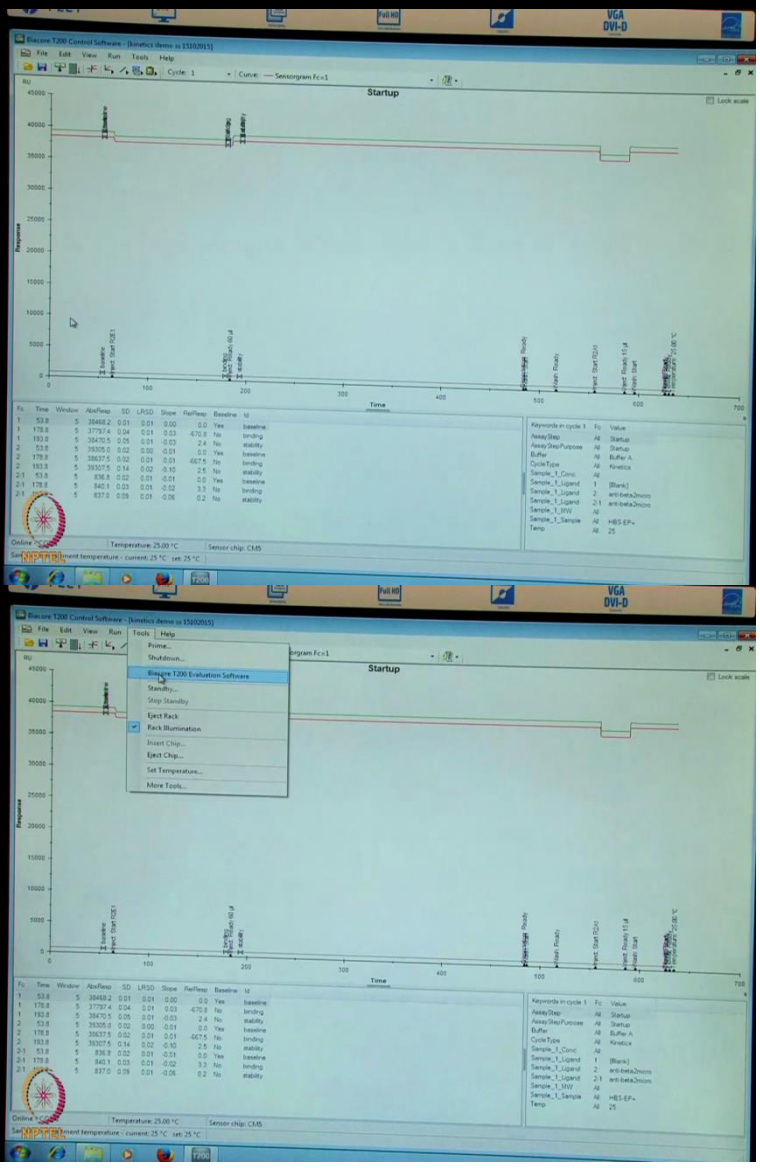
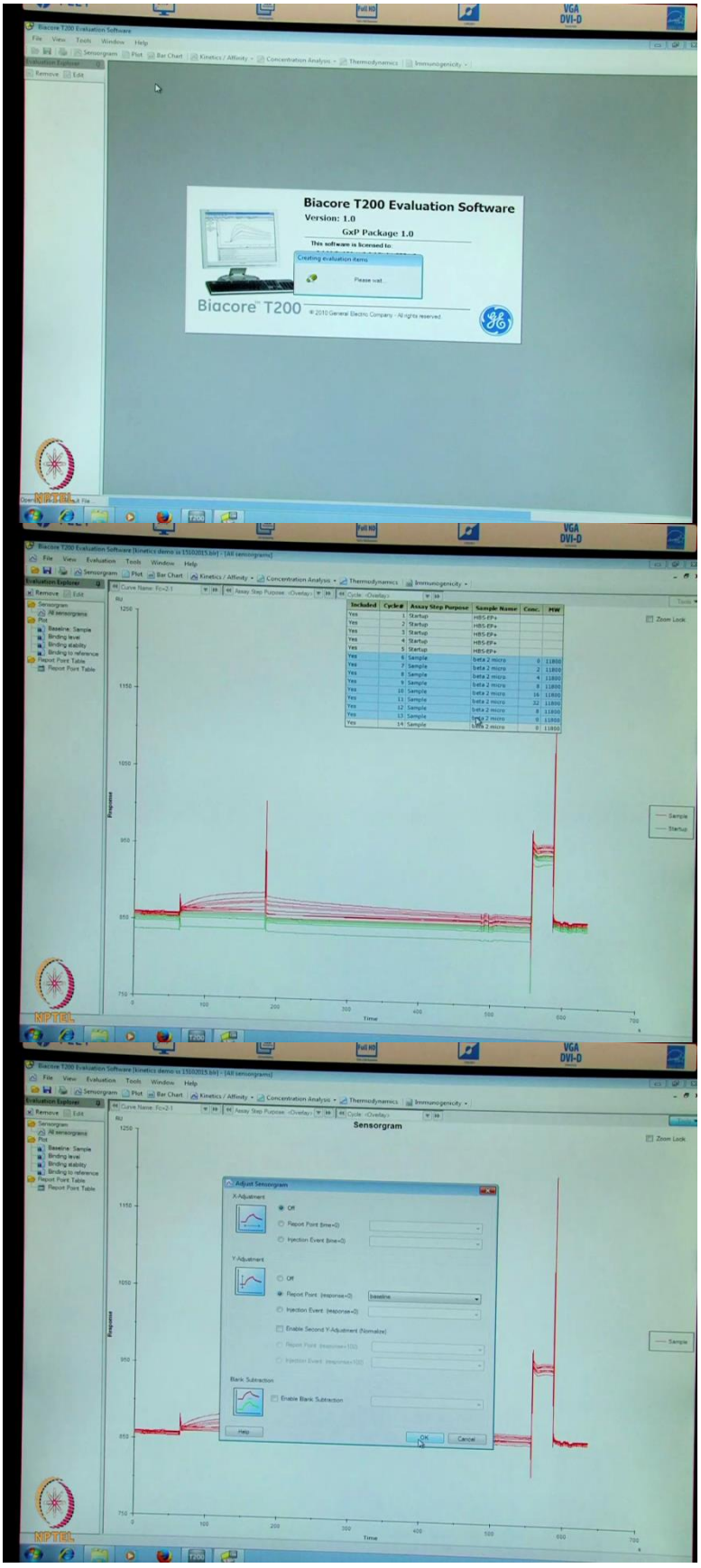
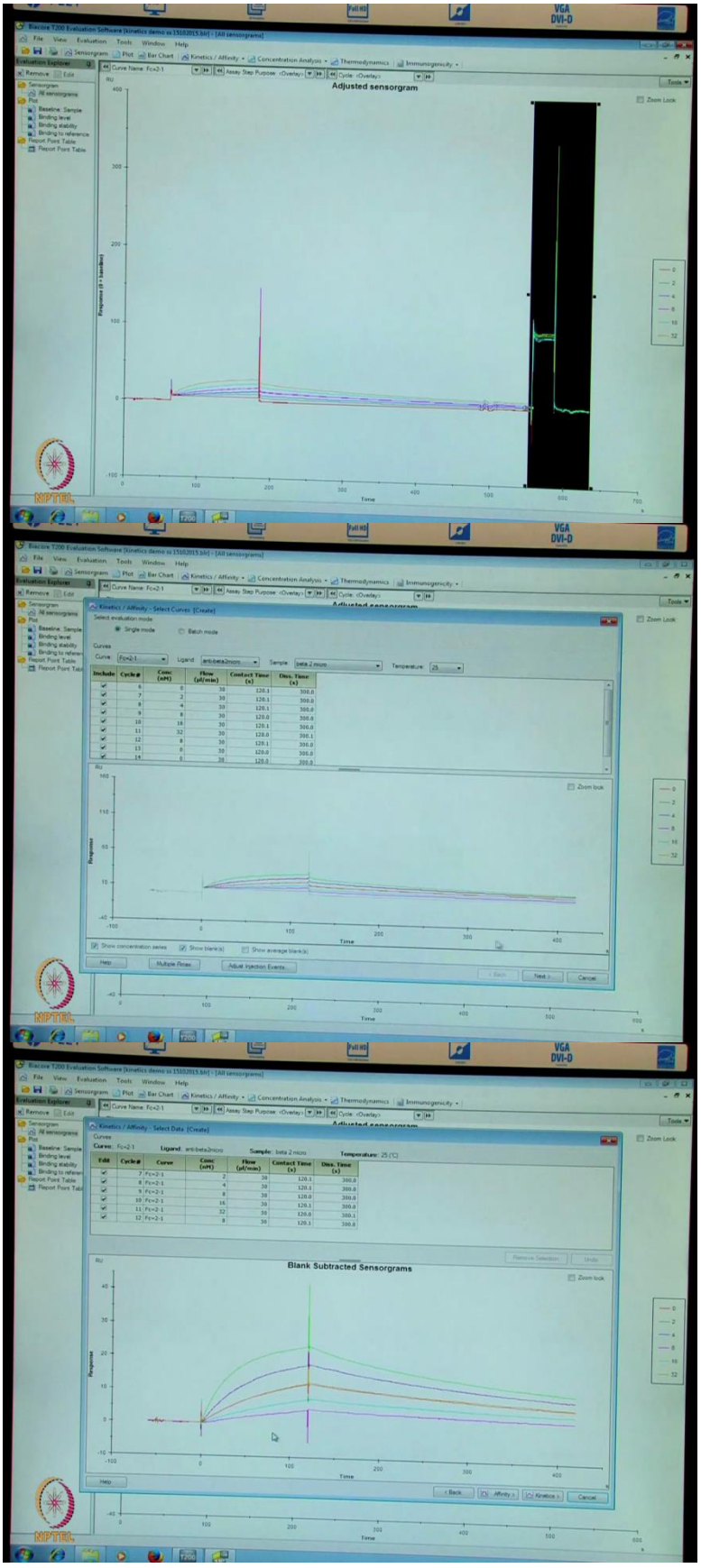
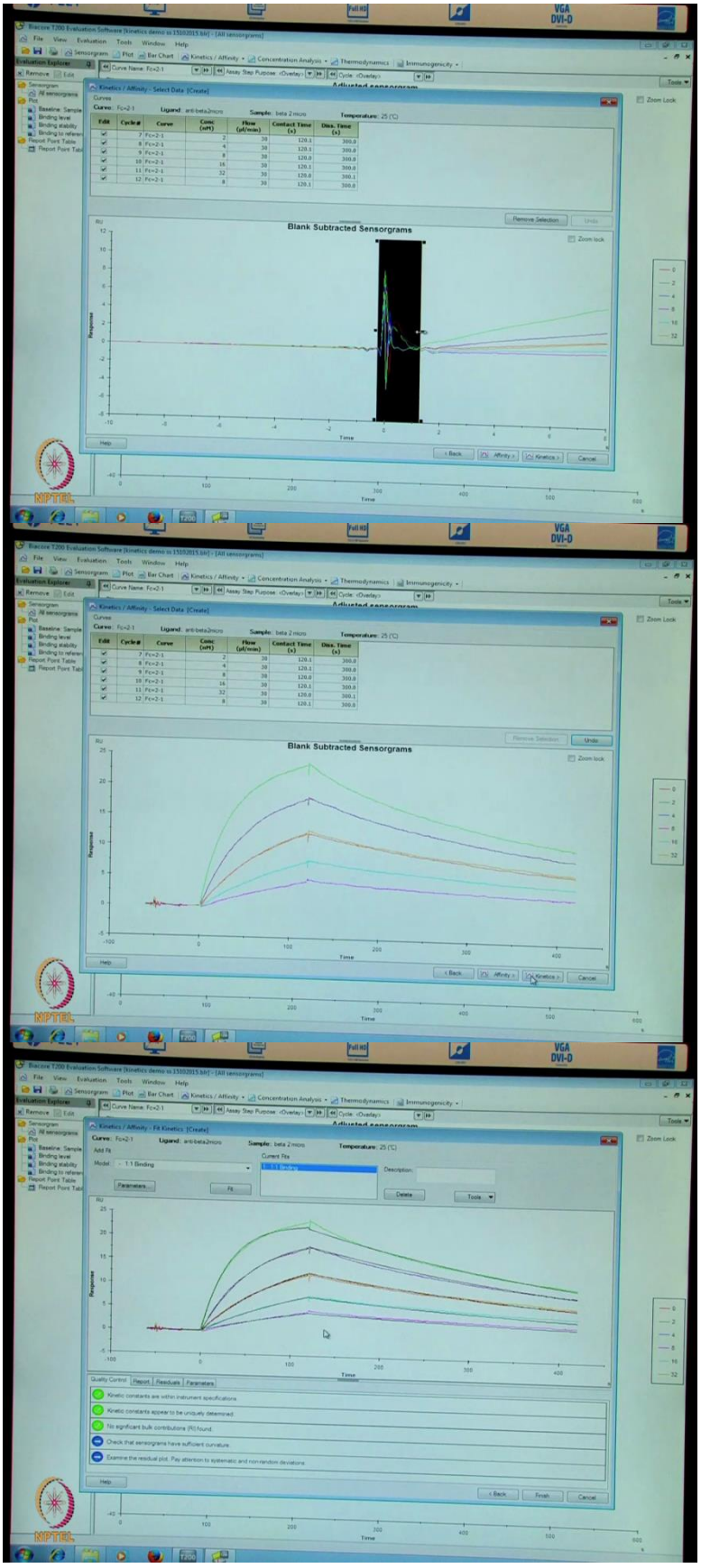


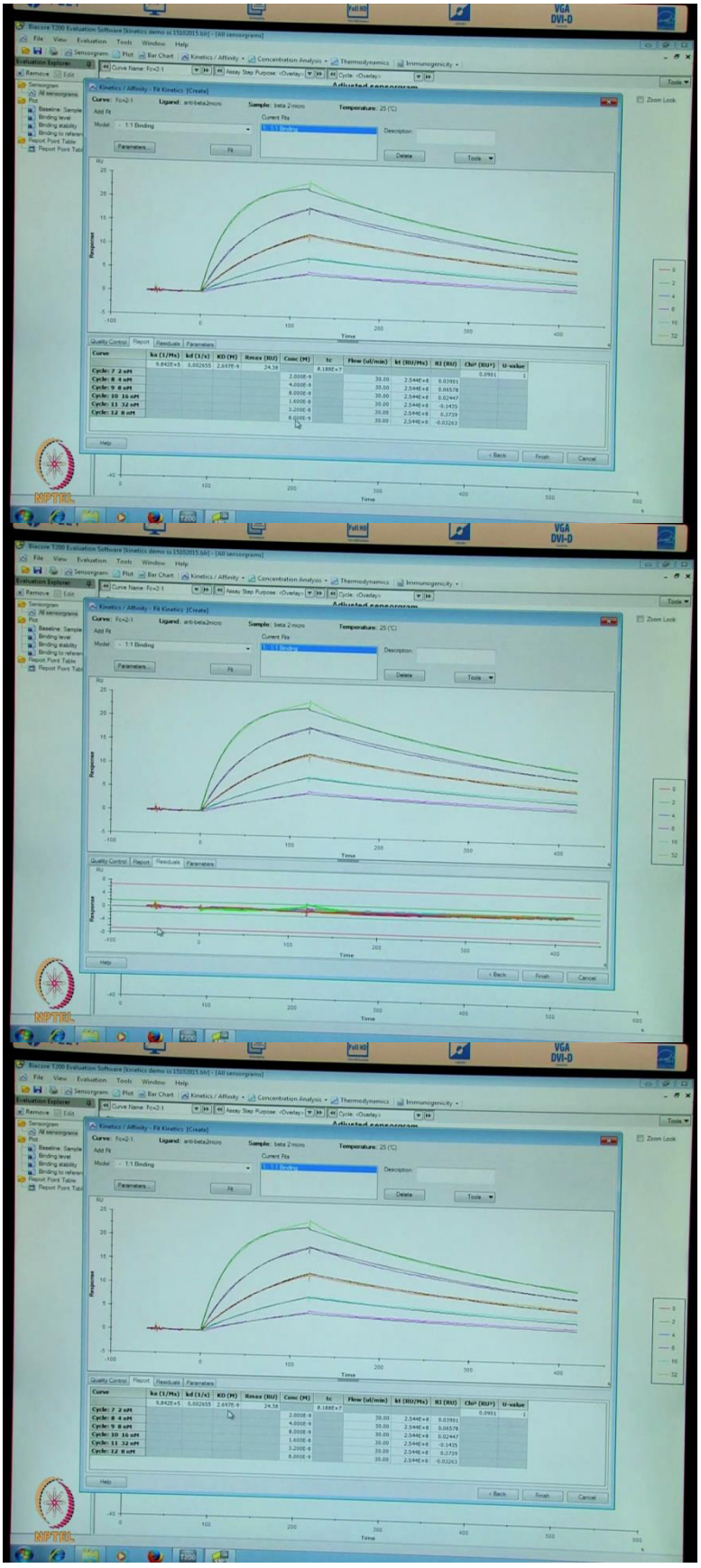
Data analysis for the kinetic assay




Biacore T200 Evaluation Software
Version: 1.0
GxP Package 1.0
This software is licensed to:
Creating evaluation items
Please wait...
Biacore T200
© 2010 General Electric Company - All rights reserved
Sensorgram
Adjust Sensorgram
X-Adjustment
Y-Adjustment
Blank Subtraction
Enable Blank Subtraction
Help
OK
Cancel
Response
Time
NPTEL


Adjusted sensorgram
Blank Subtracted Sensorgrams
Time
Response


Blacore T200 Evaluation Software
Kinetics / Affinity - Select Data [Create]
Blank Subtracted Sensorgrams
Response
Time
Kinetics / Affinity - Fit Kinetics [Create]
Quality Control
Report
Residuals
Parameters
Kinetic constants are within instrument specifications
Kinetic constants appear to be uniquely determined
No significant bulk contributions (RI) found
Check that sensorgrams have sufficient curvature
Examine the residual plot. Pay attention to systematic and non-random deviations
Back
Finish
Cancel
NPTEL



Once, the immobilization is done and the experiment is been run. This is how the data will end here and from the data we can go to tools, biacore T200 evaluation software. So, this is

how the data is generated, you see all the startup runs here and we go on overlay to select sampled data. Now, we go on tools sensorgram adjustment, report point, base line, again to the tools color by concentration.

Now, you can see all the data is the differently colored, you see the rate of association, dissociation and regeneration with the legend of the different concentrations of beta two M injected. Here as well like binding we will right click on the mouse and cut away this portion and now, this is our actual data with the different concentrations of beta two M injected on the surface. To evaluate this data, we can go into the kinetic and affinity mode, select surface bound.

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

Now, we go on to this and see next if at all there are unwanted spike, can be removed in this region or in this tab by a simply highlighting that area and right click and highlight that unwanted region and say remove selection. Similarly, we could away these spikes by highlighting that area to the right click of the mouse and say removed selection. Now, that there are no spikes we can proceed to fit this data in the kinetic mode as you can see on the tab.

Now, you see here one to one binding and parameters here fit here. So, we could place mouse on the fit and press start. So, data is fit to the black lines superimposing on the colored lines and the following information is generated. If you see the quality control tab, report tab, the residuals tab and parameters each of them has data from this experiment, to start with we go on to the control, quality control tab and see here all the three are in green with kinetic constants are within instruments specifications or uniquely determined and no bulk contributions found.

We have learned from our theoretical sessions bulk contribution will be more if there is a mismatch between buffer and sample. Now, we go to the next tab report. We can see lot of information that is generated here. The on rate is given here, similarly there is an off rate and there is affinity or binding constant provided here, the Rmax is also given. The various

concentrations that was used to set up the experiment are provided here, flow rate for this experiment is provided here. The change in refractive index due to differences in the sample and running buffer are provided here and a quality control parameter like I 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. Rmax or a maximum number of binding sides available at 24, chi square of 0.0901 should be below 1 at least give value below 25 is always considered to be good. 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 line on the colored lines is very close.

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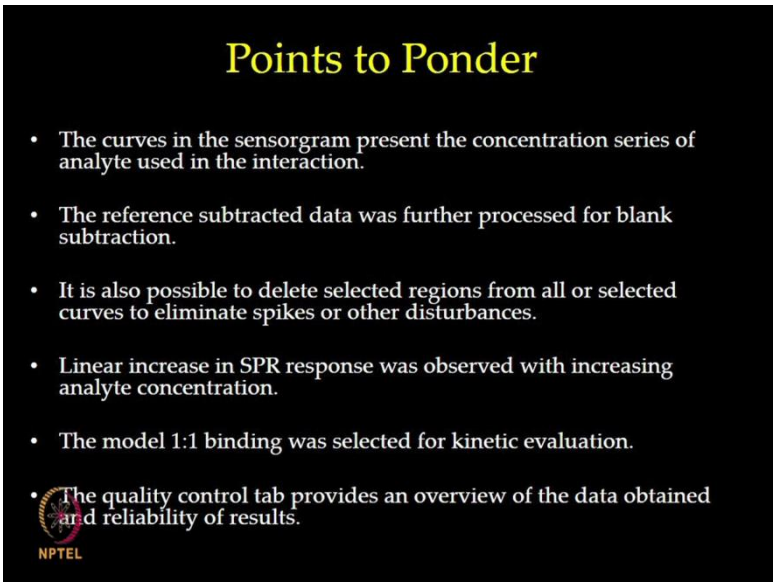

Now, we go on to another tab parameter and find the lot of information which are nothing but the standard error calculated for the data. With this we conclude, the interaction of anti-beta two M to beta two microglobulin at 2.6 nanomolar affinity. We will start a new topic on protein, a small molecule interactional study in next lecture. Thank you.

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