Interactomics: Protein Arrays and Label-Free Biosensors.
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Indian Institute of Technology, Bombay.
Lecture-8.
Protein-Protein Interaction Study: Binding Analysis.

Welcome to the MOOC NPTEL course on intractomics. To completely understand the function of a protein, it is important to look beyond its expression pattern and identify its potential interaction partners and determine their interaction dynamics. To study the interaction between two binding partners in SPR experiment, one partner is attached to the surface and other is passed over the surface in a continuous flow of sample solution.

The interaction of ligand and analyte is measured by the SPR instrument as a change in the refractive index over time and response observed is directly proportional to the change in mass concentration close to the surface. In the previous lecture, we completed immobilization procedure of anti-beta two microglobulin antibody on the surface of a CM5 chip, we will now move forward to perform the binding analysis of anti-beta two microglobulin antibody with beta two microglobulin protein.

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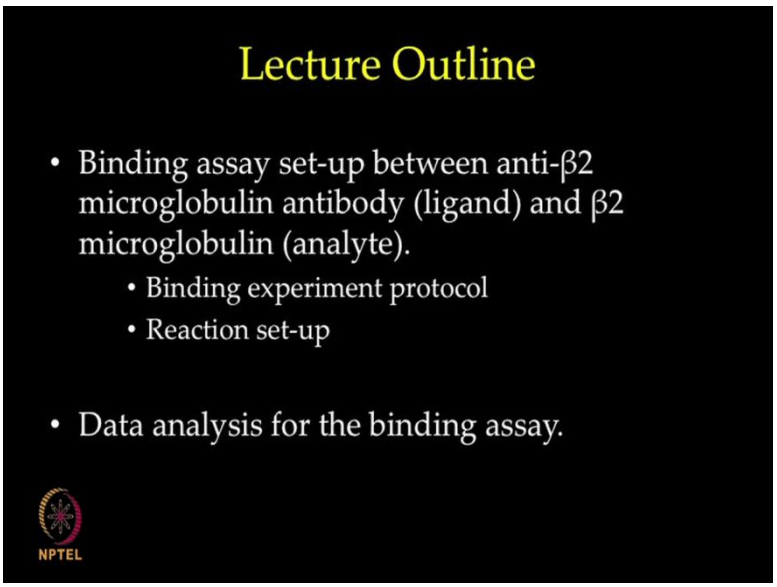

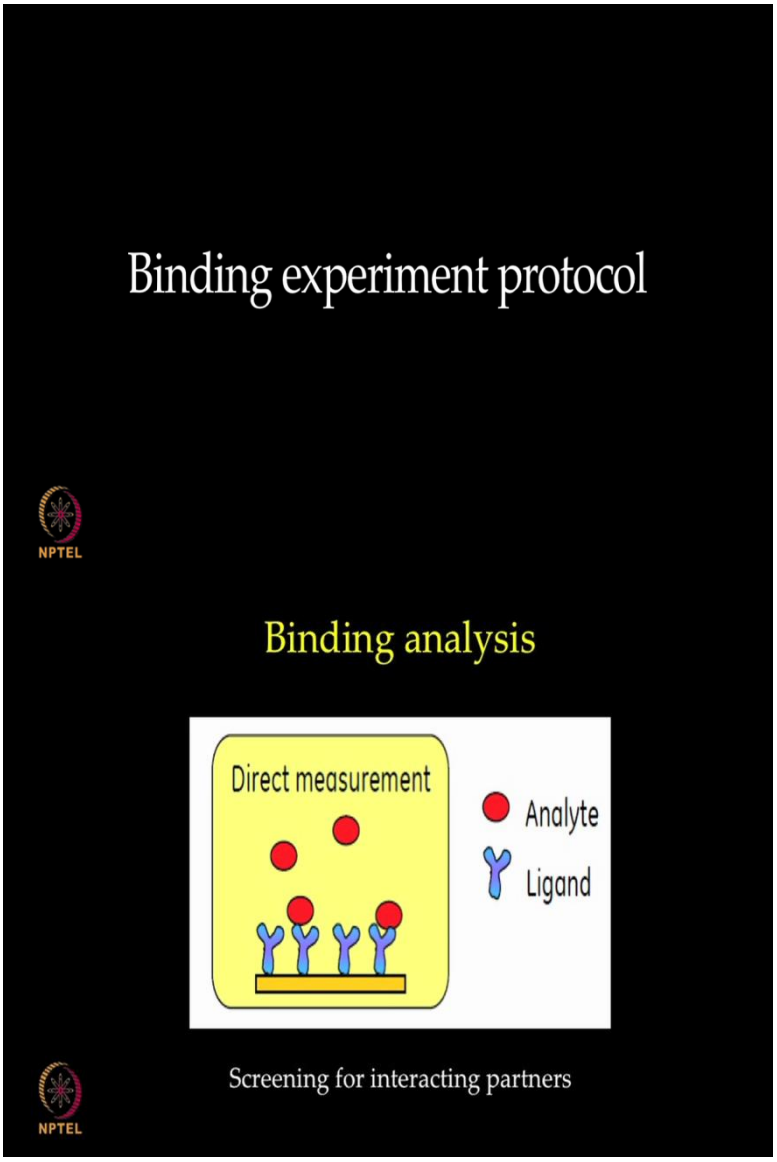

So, let us have the experimental session now. We will go into the second session now and we will use our immobilized chip for a binding experiment. So, here we will first prepare a template for the binding experiment. A binding experiment actually deals with screening or a single concentration screening of different compounds on the immobilized ligand and look for its ability to interact with the target that is immobilized on the surface.

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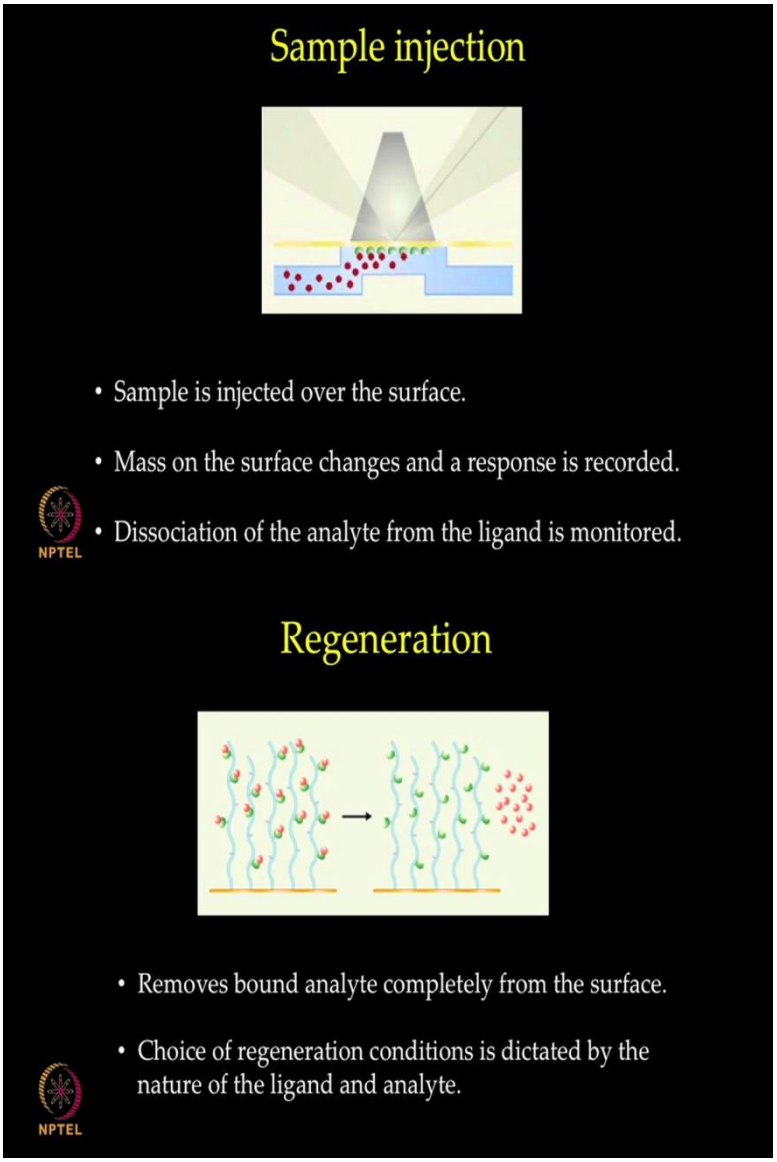

Before we go ahead with the binding experiment, let us understand some important considerations, during sample injection the analyte an injected over the surface with a constant flow and concentration. Analyte in the sample binds to the immobilized ligand on the surface, the mass on the surface changes and the response is recorded. After sample injection buffer flows over the surface to allow monitoring of analyte dissociation from the ligand.

Regeneration as already discussed by Dr. Srinivas, is the process of removing bound analyte from the ligand on the sensor chip surface after analysis of a sample. Efficient regeneration which means removing bound analyte without affecting the ligand activity is crucial to a successful assay. If the regeneration is incomplete or the binding activity of the surface is reduced, the performance of the assay is impaired.

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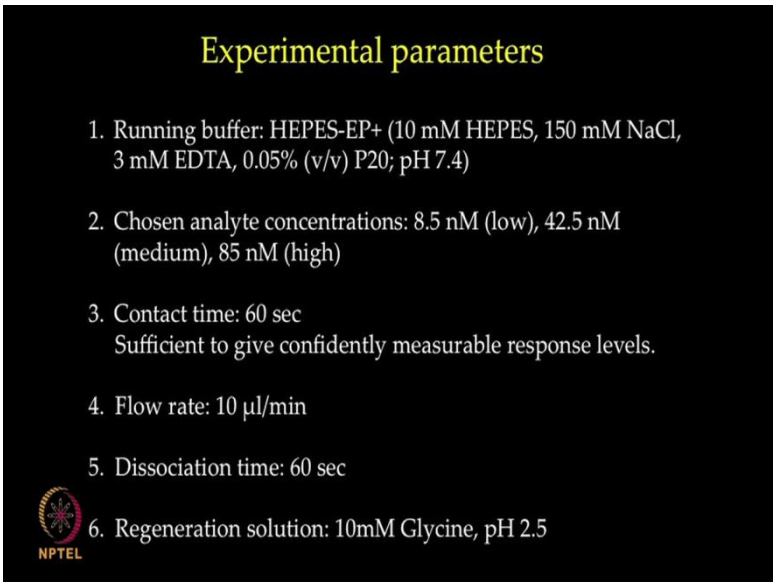

The choice of conditions for regeneration is dictated by the stability and nature of the ligand and analyte. In today's binding experimental setup, we will be using HEPES-EP plus as the running buffer, we will preparing three different concentrations of beta two microglobulin protein which are 8.5 nanomolar of 42.5 nanomolar and 85 nanomolar for evaluating it is binding with the antibody having 8.5 nanomolar concentration in duplicate. These three concentrations will be referred to as low, medium and high.

Contact time between the sample and the sensor surface should be sufficient to give confidently measurable response levels without compromising screening throughput. Contact time of 22 minutes are usually sufficient for a binding experiment. Here, we will provide a contact time of 60 second at the flow rate of 10 microliter per minute with the dissociation time of 60 seconds. And ideal regeneration condition is the one their analyte response of the same concentration is constant after repeated injections.

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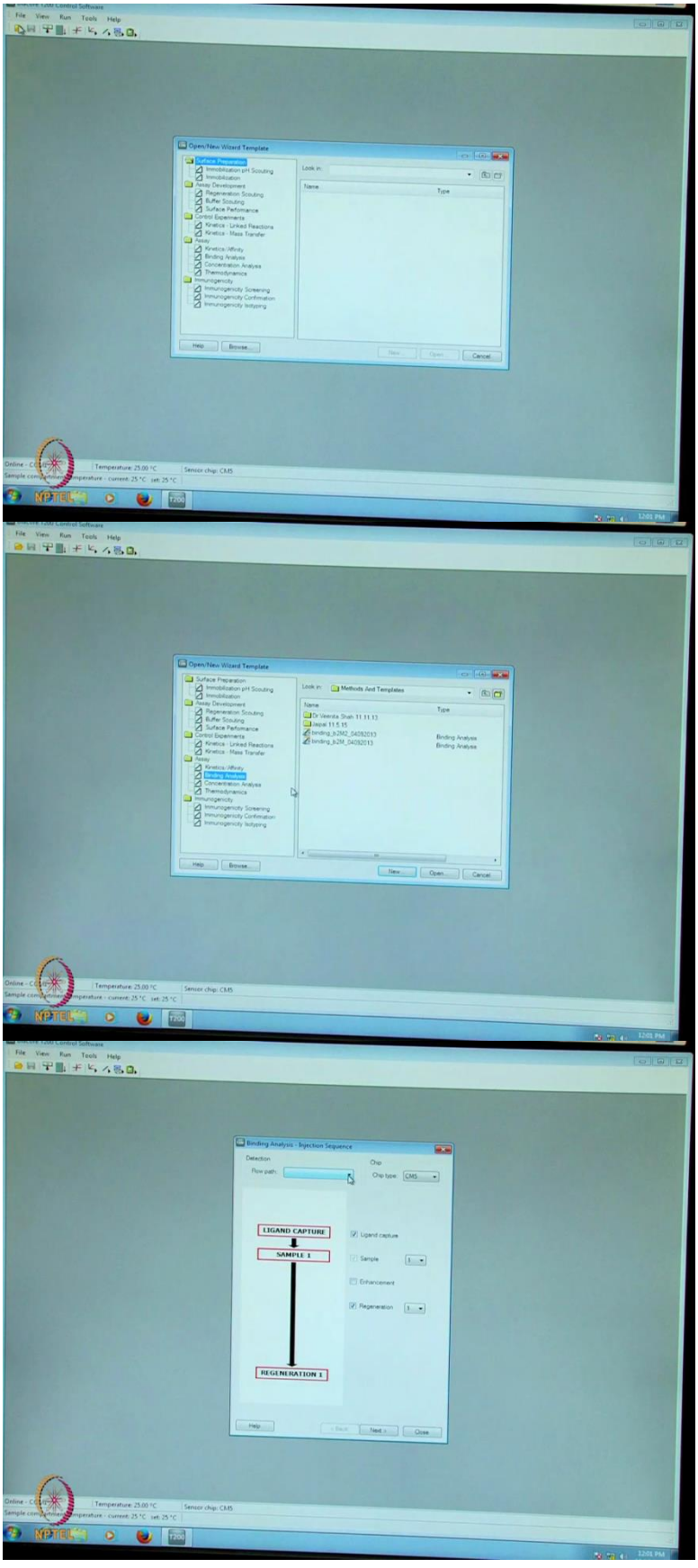
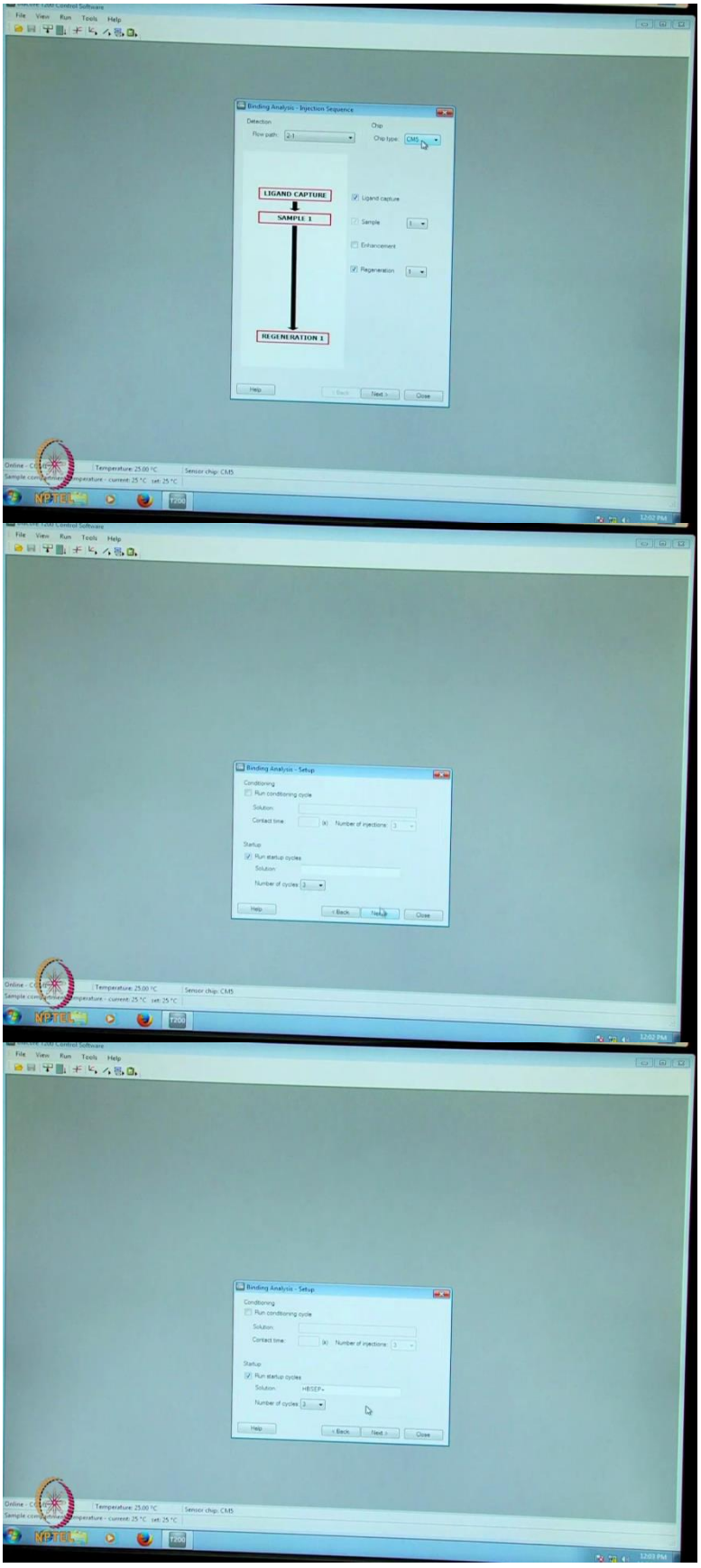
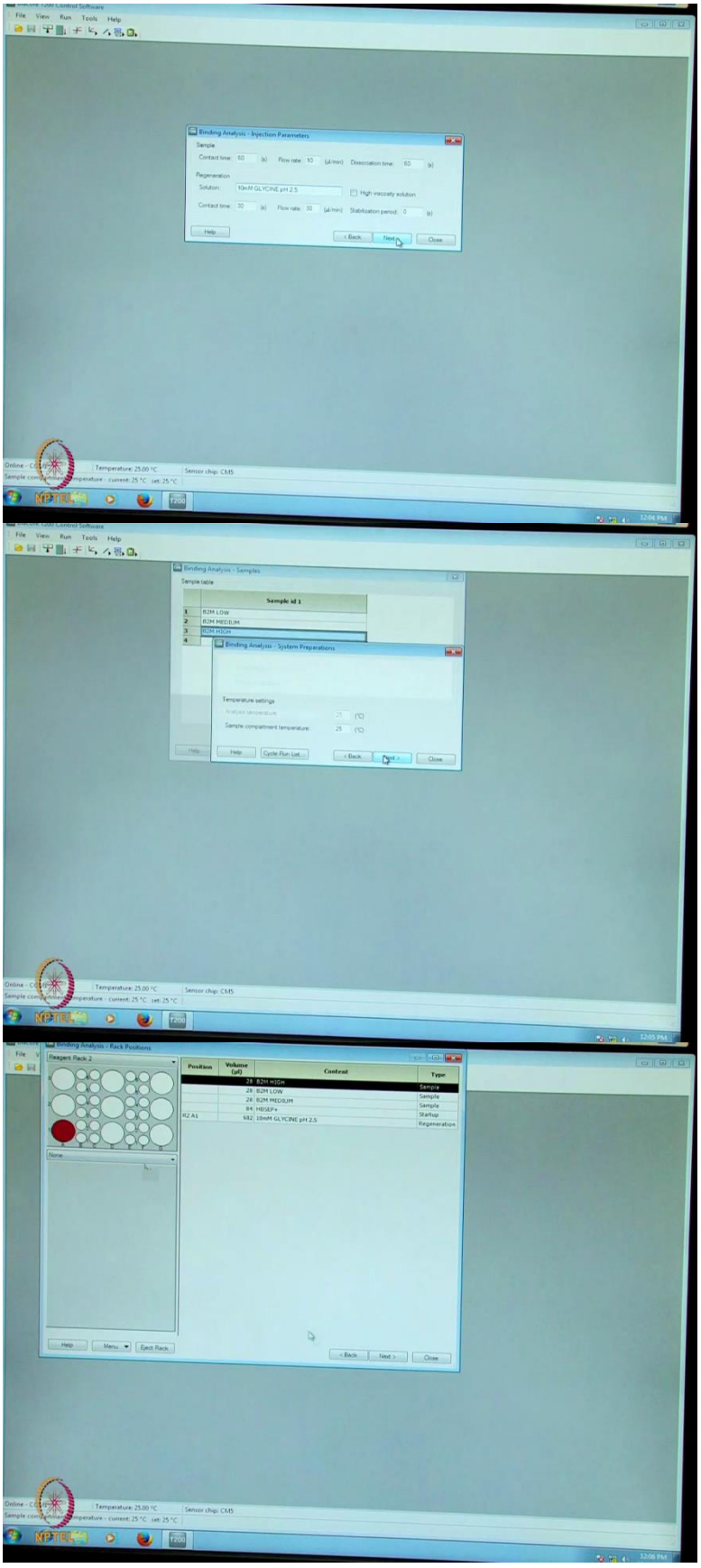
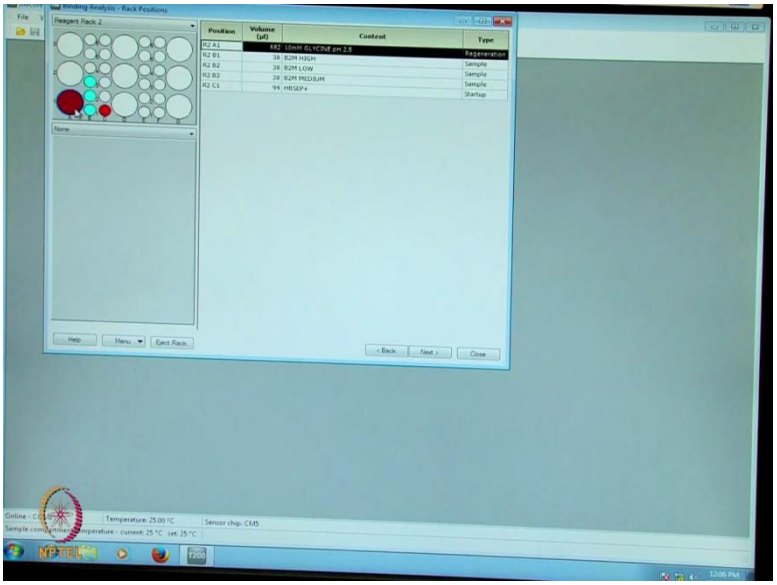



Binding Analysis - Injection Sequence
LIGAND CAPTURE
SAMPLE 1
REGENERATION 1
Binding Analysis - Setup
Binding Analysis - Setup


Binding Analysis - Injection Parameters
Sample
Contact time: 60 (s) Flow rate: 10 (µl/min) Dissociation time: 60 (s)
Regeneration
Solution: 10mM GLYCINE pH 2.5
High viscosity solution
Contact time: 30 (s) Flow rate: 30 (µl/min) Stabilization period: 0 (s)
Help
< Back
Next >
Close
Temperature: 25.00 °C
Sensor chip: CM5
Binding Analysis - Samples
Sample table
Sample id 1
1 B2M LOW
2 B2M MEDIUM
3 B2M HIGH
4
Binding Analysis - System Preparations
Temperature settings
Sample compartment temperature: 25 (°C)
Help
Cycle Run List...
< Back
Next >
Close
Binding Analysis - Rack Positions
Reagent Rack 2
None
Position
Volume (µl)
Content
Type
28 B2M HIGH
Sample
28 B2M LOW
Sample
28 B2M MEDIUM
Sample
84 HBSEP+
Startup
A1 A1
682 10mM GLYCINE pH 2.5
Regeneration
Help
Menu
Eject Rack
< Back
Next >
Close



Today, we will be using 10 milimolar glycine pH 2.5 for regeneration of the surface; we will now proceed with our binding experiment protocol. Well before making the template we will open the file wizard again, binding analysis new identify the flow path as 2 minus 1 as we have done our immobilization on 2 minus 1, chip will be CM5 that is docked already, we will not have ligand capture, sample and regeneration.

We will go to the next tab, here we are not using any conditional cycle, we will start with the startup cycle. Startup cycles are cycles of buffer used for equilibrating the system. So, here basically buffer is used as analyte. So, we can type as HEPES-EP plus buffer and from the pull down menu, we will select 3 cycle. Generally for binding experiments, 3 cycles are selected, going to the next tab for setting up of binding, we need to specify the contact time as 60 seconds default flow rate of 10 microliter per minutes. Dissociation time of any minute or any second by default we can consider 60 seconds.

The regeneration solution we would prefer here would be 10 milimolar glycine pH 2.5 with the default contact time of 30 seconds, flow rate of 30 microliter per Minuit and with no stabilization time. We go to the next tab; here we need to fill in the name of all single concentration compounds. So, here we would select our analyte as beta two M, just that we have one analyte we will take it in three different concentrations. So, we will name as low, beta two M medium and beta two M high.

So, low indicates lower concentration, medium medium concentration, high concentration and we will go to the next tab we will collect prime before run and normalize is not required here because the chip is already immobilized and we will go with the default temperatures

and we will go to the next tab here, we will not select a micro-titer plate and this is our rack positions for a binding experiment. Here, we have at the C1 position HEPES-EP plus buffer for three different startups. We have three concentrations of analyte; high, medium and low and we have the regeneration solution here which is 10 milimolar glycine pH 2.5 and we will prepare our solutions and start the binding experiment.

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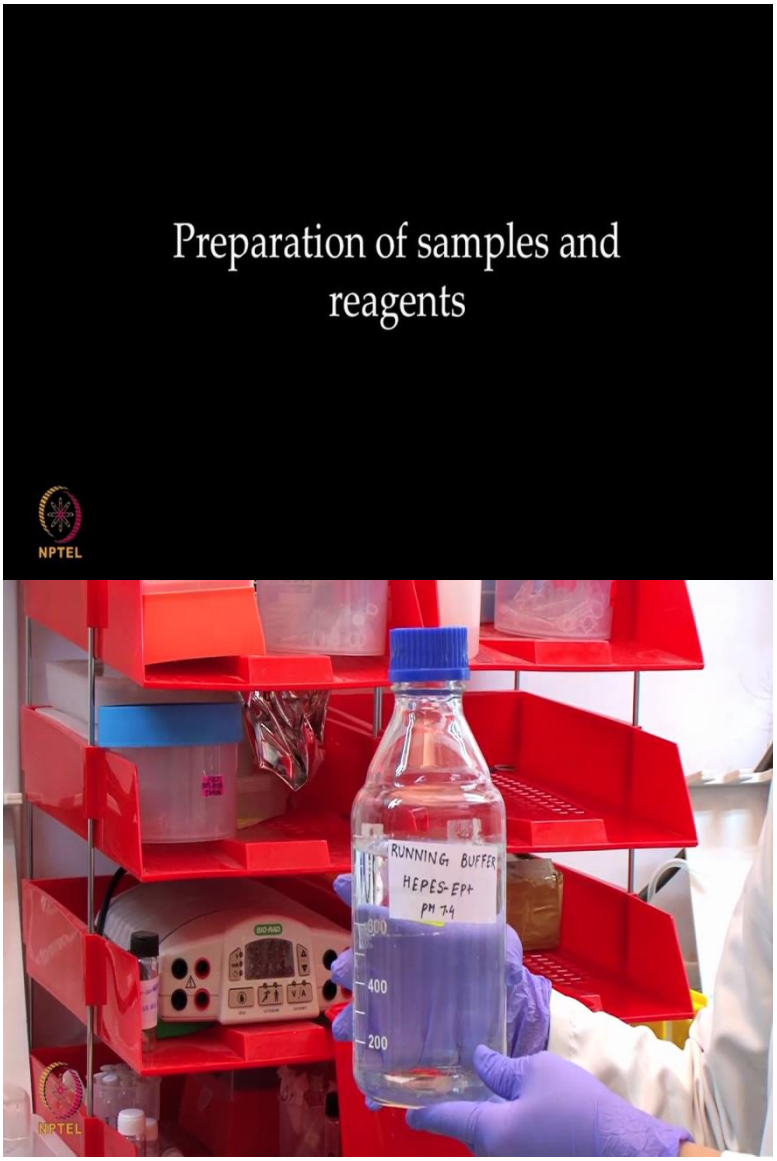

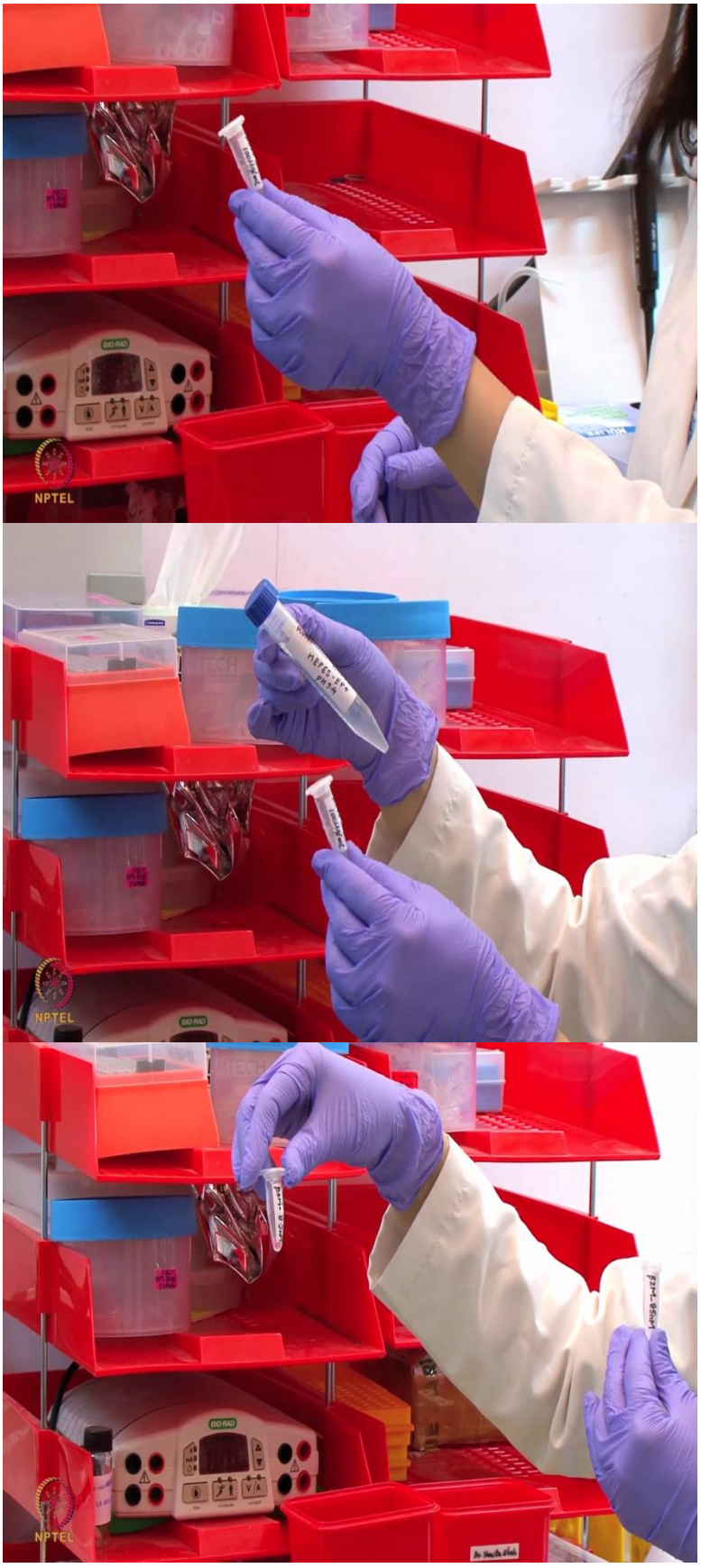
BIO-RAD
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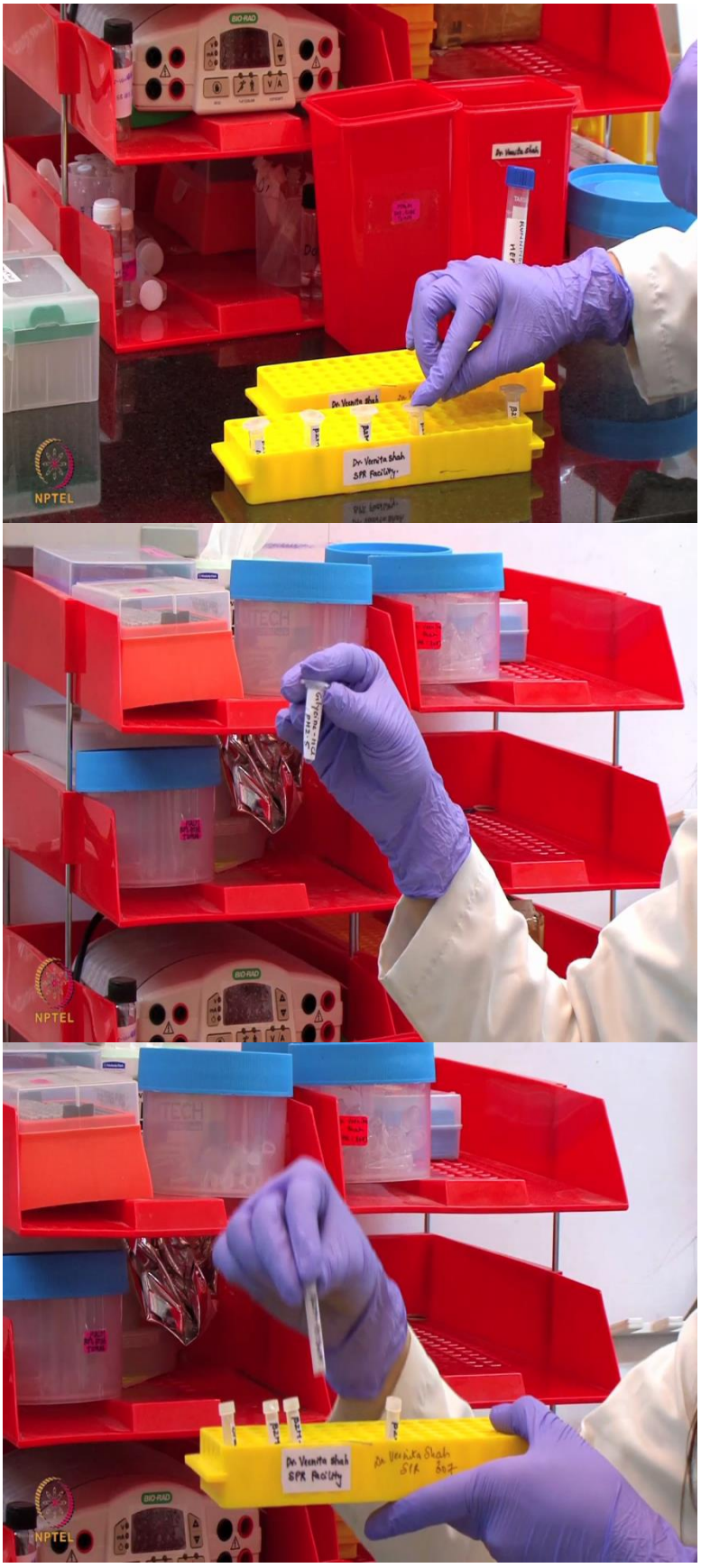
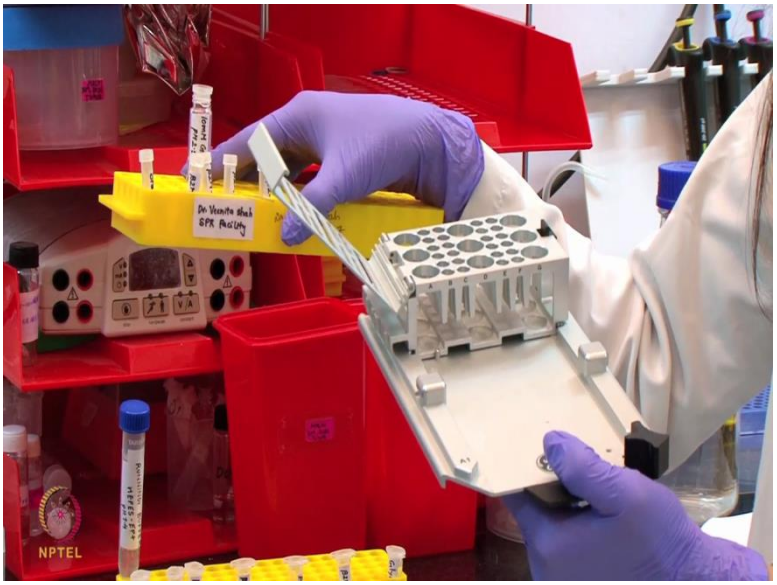
NPTEL


BIO-RAD
Dr Vinita Shah
SPR Facility
NPTEL



We will now work on the reagent require for the binding analysis of anti-beta two microglobulin with beta two microglobulin proteins. We will be using HEPES-EP plus as the running buffer which will also be used for the initial startup cycles. We will tell you the stock solution of protein that is 100 microgram per ml in the running buffer HEPES-EP plus to prepare 100 microliter of three different concentrations that is 85 nanomolar, 42.5 nanomolar and 8.5 nanomolar which are referred to as high, medium and low concentrations in the experiment.

We will also include one zero nanomolar concentration in the experiment which will be nothing but the running buffer. For the regeneration of the surface we have prepared glycine HCl pH 2.5 as the regeneration solution, we have transferred all the solution in the specialized tube starting from the startup, beta two M concentrations starting from 85 nanomolar, 42.5 nanomolar, 8.5 nanomolar and zero nanomolar.

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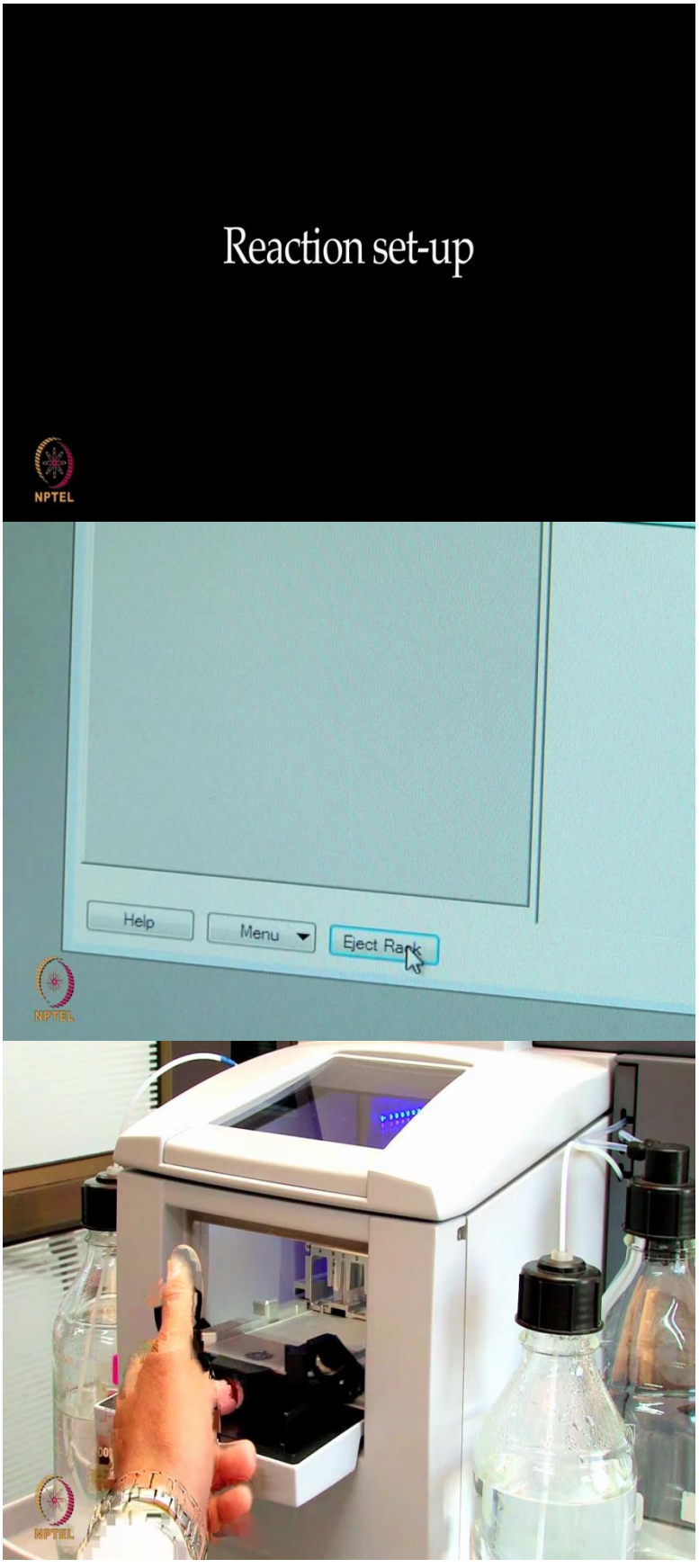
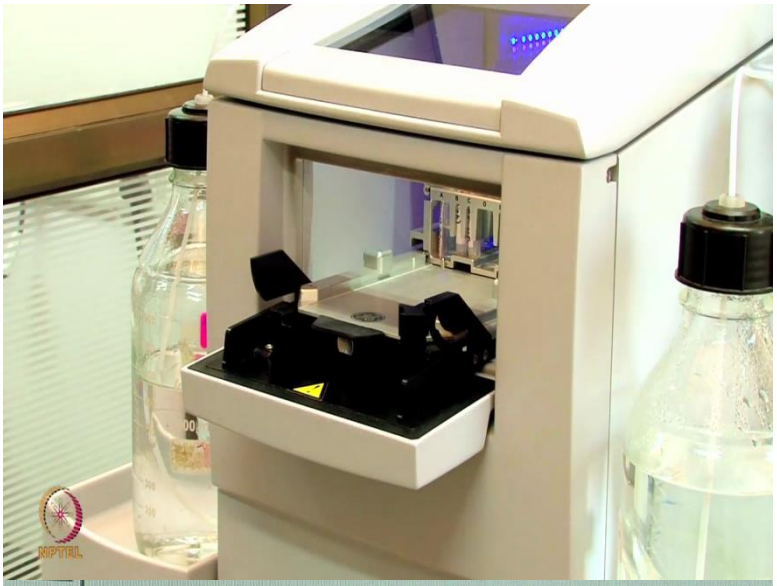
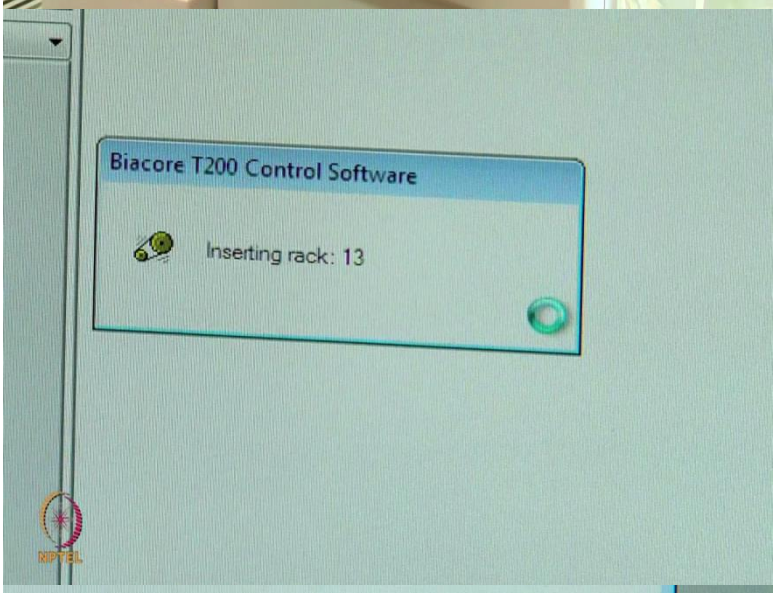


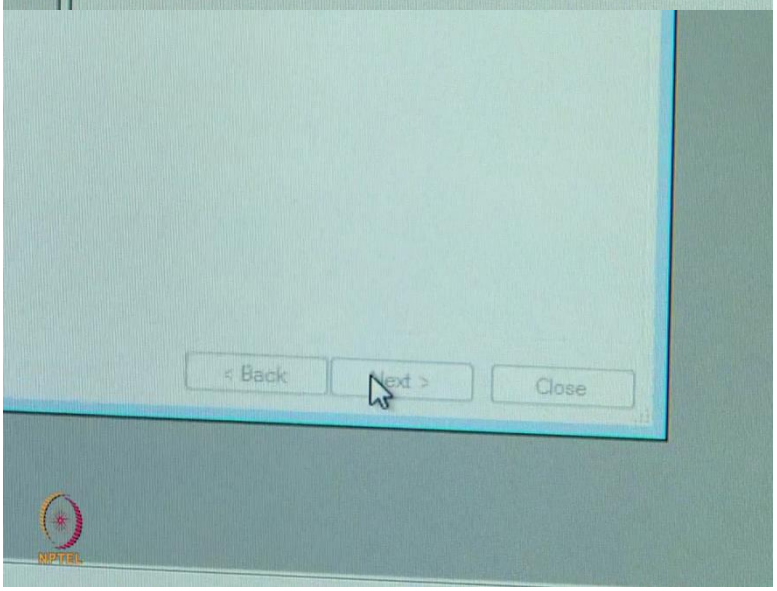

Biacore T200 Control Software
File View Run Tools Help
Binding Analysis - Rack Positions
Reagent Rack 2
Content
Type
Regeneration
Sample
Sample
Sample
Startup
A B C D E F G
NPTEL


P221
VGA
DVI-D
NPTEL


NPTEL




Biacore T200 Control Software
Inserting rack: 13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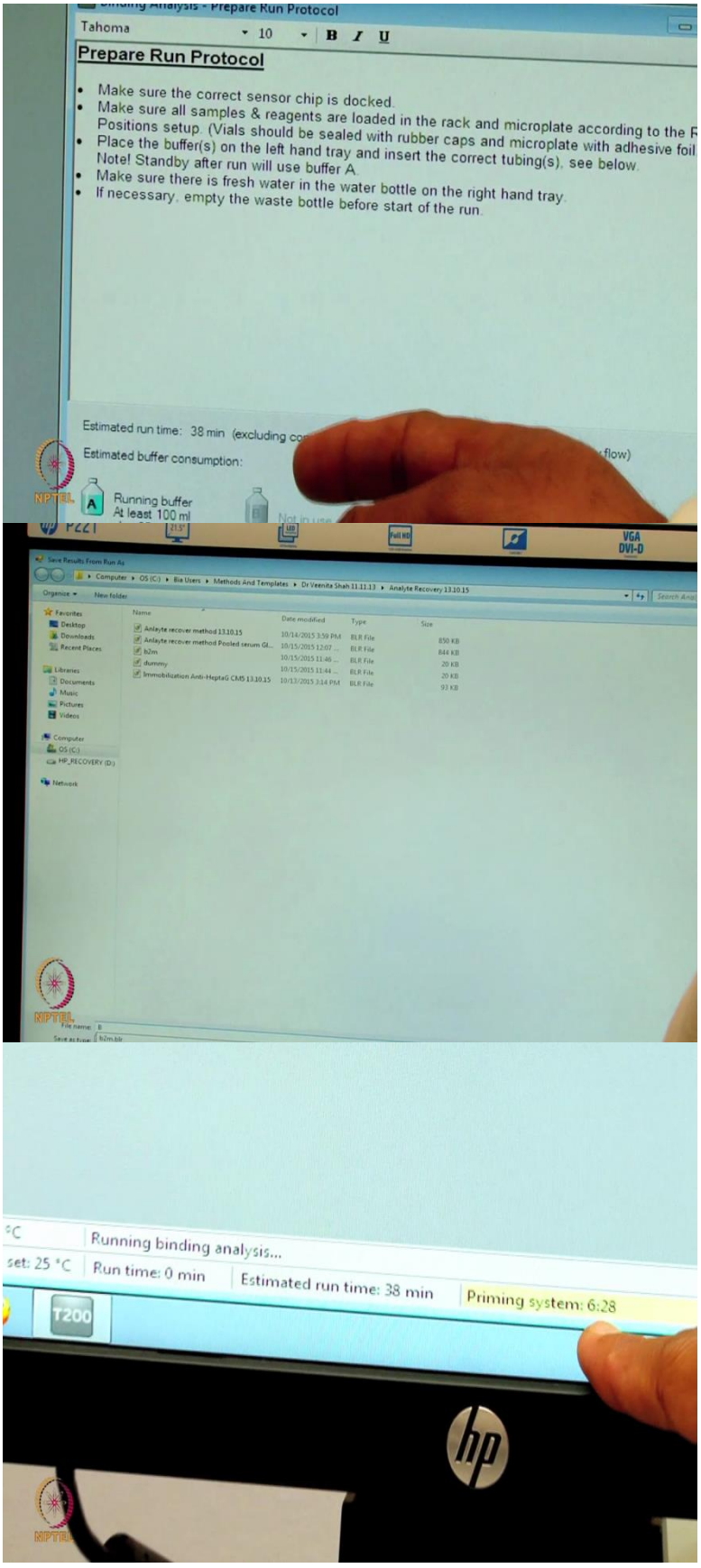
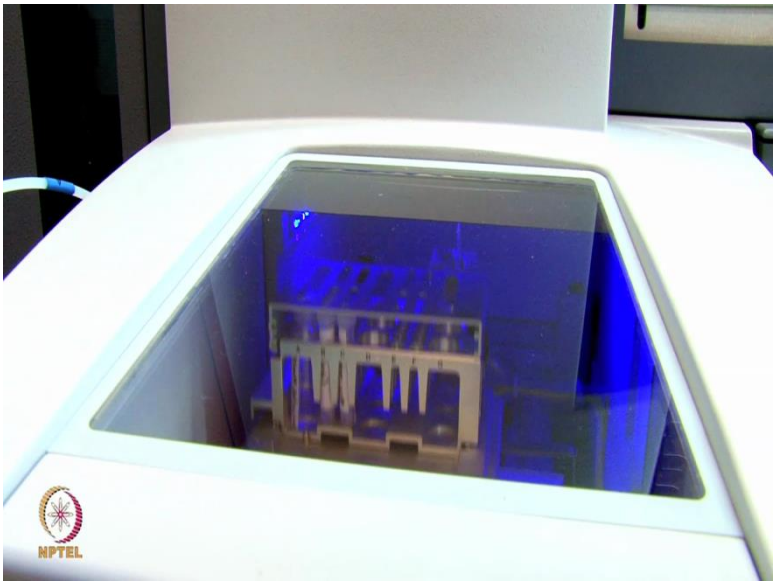
Next >
Close


Prepare Run Protocol
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 R
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: 38 min (excluding co
Estimated buffer consumption:
Running buffer
At least 100 ml
Not in use
VGA
DVI-D
Save Results From Run As
Computer ▸ OS (C:) ▸ Bia Users ▸ Methods And Templates ▸ Dr Veenita Shah 11.11.13 ▸ Analyte Recovery 13.10.15
Organize
New folder
Favorites
Desktop
Downloads
Recent Places
Libraries
Documents
Music
Pictures
Videos
Computer
OS (C:)
HP_RECOVERY (D:)
Network
Name
Date modified
Type
Size
Analyte recover method 13.10.15
Analyte recover method Pooled serum Gl...
b2m
dummy
Immobilization Anti-HeptaG CM5 13.10.15
BLR File
File name:
Save as type:
b2m.blr
Running binding analysis...
set: 25 °C
Run time: 0 min
Estimated run time: 38 min
Priming system: 6:28
T200



The regeneration solution is placed in this glass vial. We will now insert these tubes into the appropriate rack and then into this system to start with the binding analysis of anti-beta two microglobulin with beta two microglobulin protein. We will eject the rack now, to insert new vials, eject rack and take the plate out of the sample rack and we will create with the binding vials.

So, the vials positions filled with different samples as you can see on this screen, the startup is here. So, beta two M medium, beta two M low, beta two M high are put at their respective positions and vial for regeneration of 10 milimolar glycine pH 2.5 is here. Now, we close the rack and will be inserted in the sample compartment by ejecting the rack compartment, inserting the plate next tab again we need to do all these checks, check the prime.

The estimated run time of 38 minutes and we have sufficient amount of buffer and we will now, start the experiment; we will save this template as binding save. Now, we will save the result vial again as binding. And now, the experiment has started, shows running binding analysis with an estimated time of 38 minutes. System is priming now and once we finish off the binding experiment we will take a look at the data.

(Refer Slide Time: 17:47)

Points to Ponder

- After immobilization, the ligand surface is ready to use for an SPR assay.
- The analyte concentration has a direct influence on the association phase, so three different concentrations of β2 microglobulin were tested for binding.
- It is a good practise to start the binding experiment with few buffer injections to prime the system.
- The interaction time (contact time) should be long enough to provide sufficient curvature for the association curve and dissociation curve to analyse the data.

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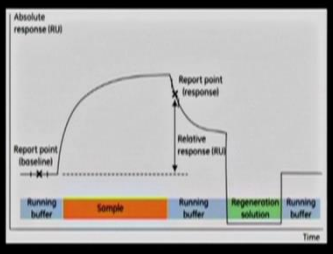
DATA ANALYSIS

Binding of anti-β2-microglobulin with β2-microglobulin

NPTEL

Sensorgram



Sensorgram is a plot of response against time, showing the progress of interaction.

Report point records the response on a sensorgram at a specific time averaged over a short time window.

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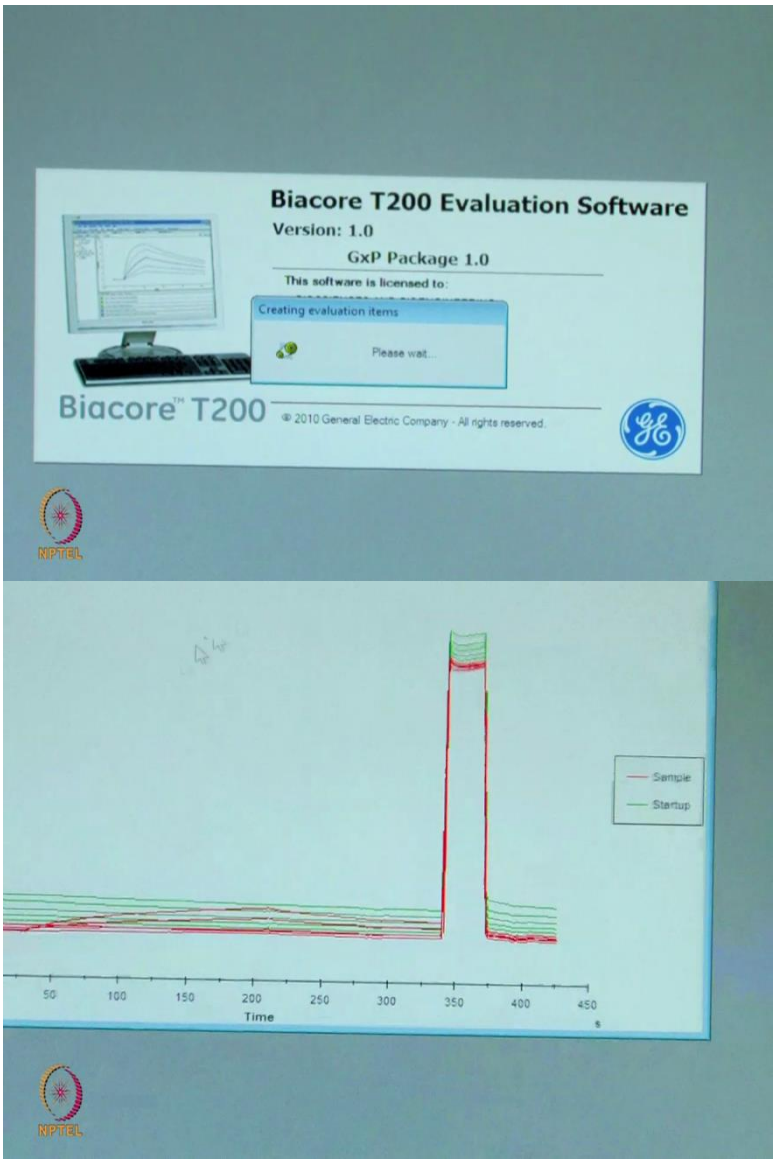
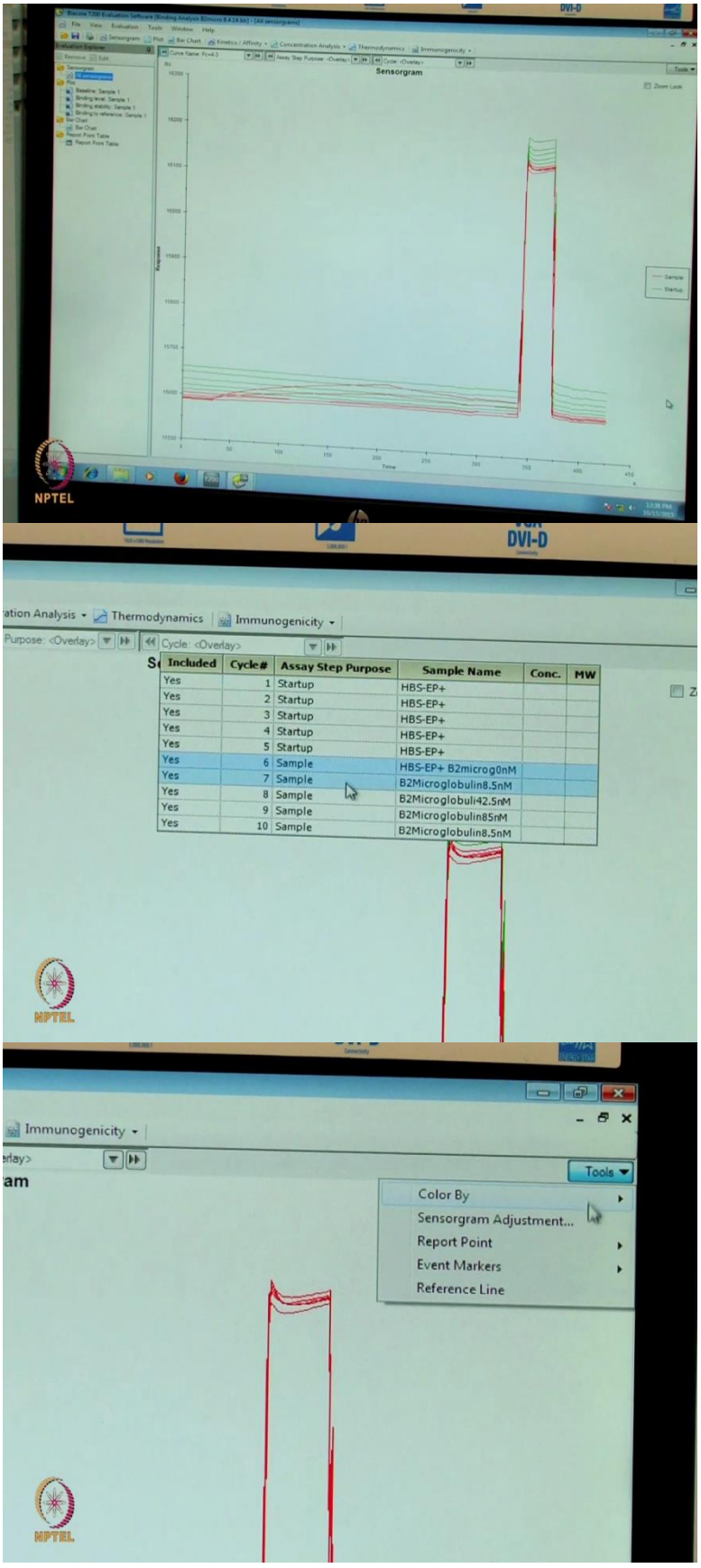
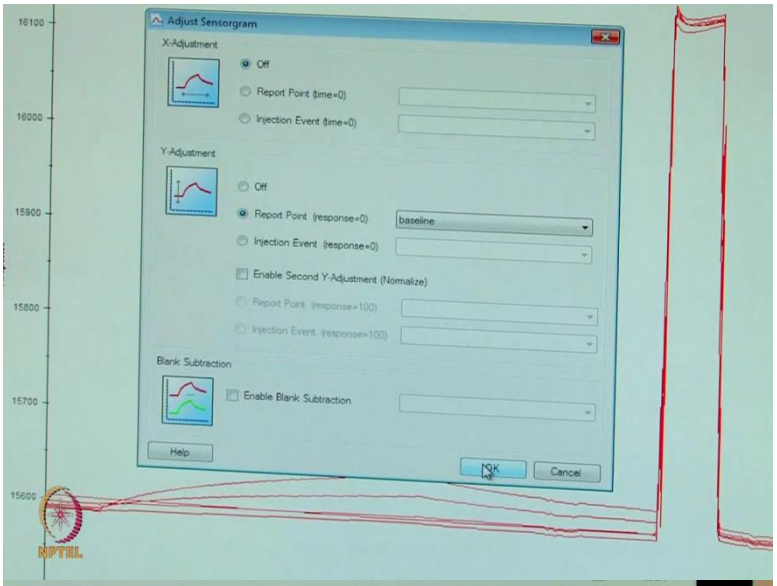
Before we analyse the binding data from the experiment, let us look at a typical sensorgram for binding between a ligand and an analyte. A sensorgram as shown here is a plot of response against time showing the progress of interaction. This curve is displayed on the system during the course of experiment, we observe the base line followed by the injection of analyte which leads to increase in the binding response during the association phase.

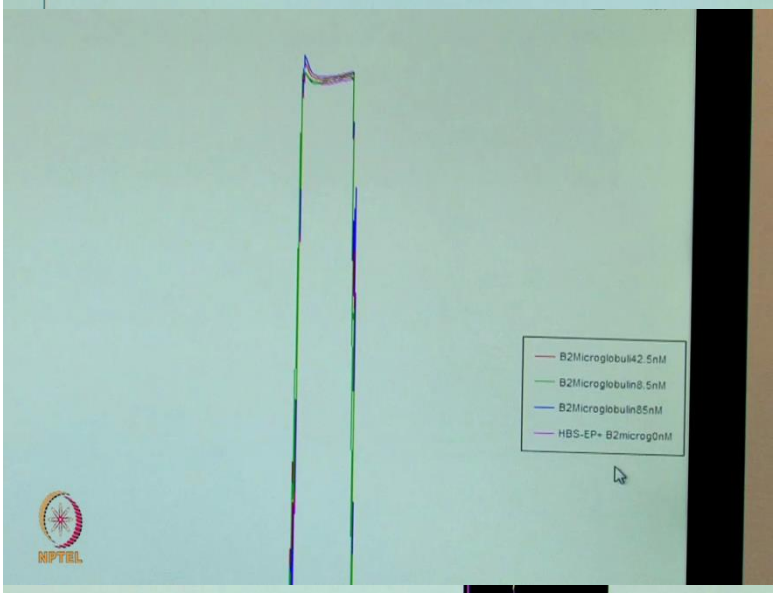
Just after this top of the sample injection, we observe report point which records the response on a sensorgram at a specific time averaged over a short time window. This is followed by dissociation phase regeneration and then back to baseline.

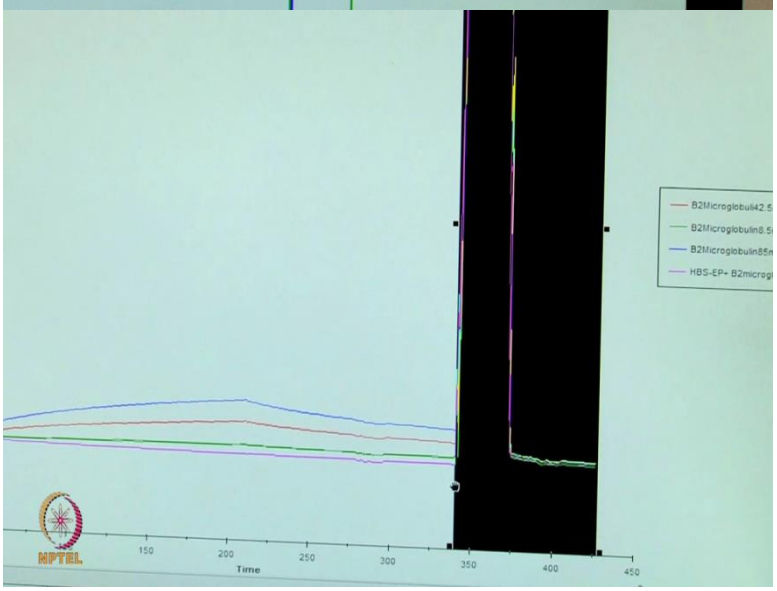
(Refer Slide Time: 19:37)

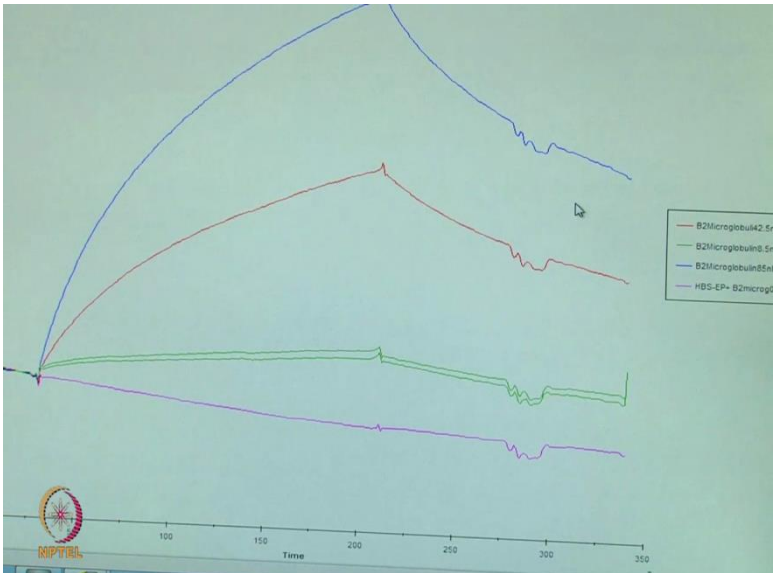


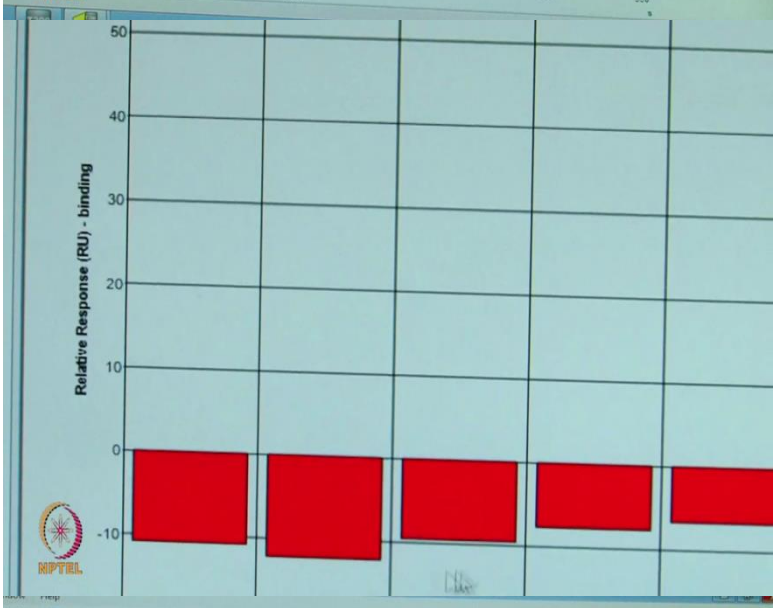

Sensorgram
NPTEL
DVI-D
Thermodynamics
Immunogenicity
Cycle: <Overlay>
Included
Cycle#
Assay Step Purpose
Sample Name
Conc.
MW
Yes 1 Startup HBS-EP+
Yes 2 Startup HBS-EP+
Yes 3 Startup HBS-EP+
Yes 4 Startup HBS-EP+
Yes 5 Startup HBS-EP+
Yes 6 Sample HBS-EP+ B2microg0nM
Yes 7 Sample B2Microglobulin8.5nM
Yes 8 Sample B2Microglobuli42.5nM
Yes 9 Sample B2Microglobulin85nM
Yes 10 Sample B2Microglobulin8.5nM
Immunogenicity
Tools
Color By
Sensorgram Adjustment...
Report Point
Event Markers
Reference Line


Adjust Sensorgram
X-Adjustment
Off
Report Point (time=0)
Injection Event (time=0)
Y-Adjustment
Off
Report Point (response=0)
baseline
Injection Event (response=0)
Enable Second Y-Adjustment (Normalize)
Report Point (response=100)
Injection Event (response=100)
Blank Subtraction
Enable Blank Subtraction
Help
OK
Cancel
16100
16000
15900
15800
15700
15600


B2Microglobul42.5nM
B2Microglobulin8.5nM
B2Microglobulin85nM
HBS-EP+ B2microg0nM

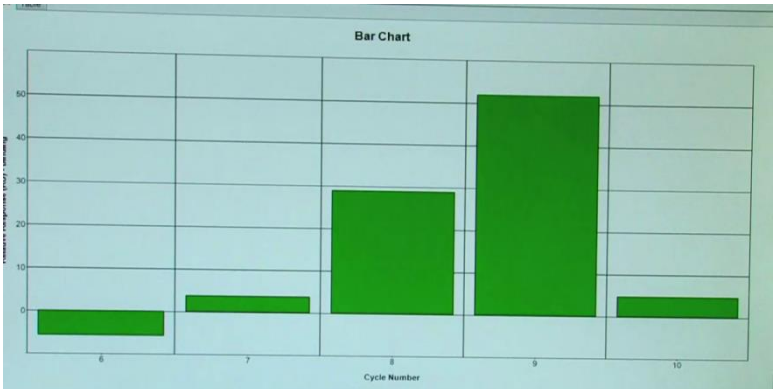

150
200
250
300
350
400
450
Time

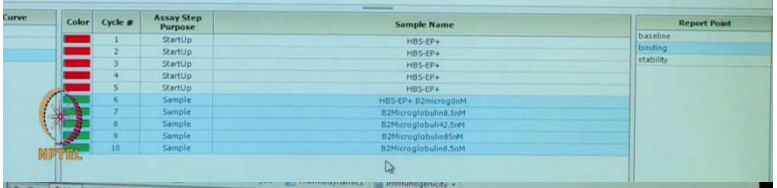




Color	Cycle #	Assay Step Purpose	Sample Name
	1	StartUp	HBS-EP+
	2	StartUp	HBS-EP+
	3	StartUp	HBS-EP+
	4	StartUp	HBS-EP+
	5	StartUp	HBS-EP+
	6	Sample	HBS-EP+ B2microg0nM
	7	Sample	B2Microglobulin8.5nM
	8	Sample	B2Microglobuli42.5nM
	9	Sample	B2Microglobulin85nM

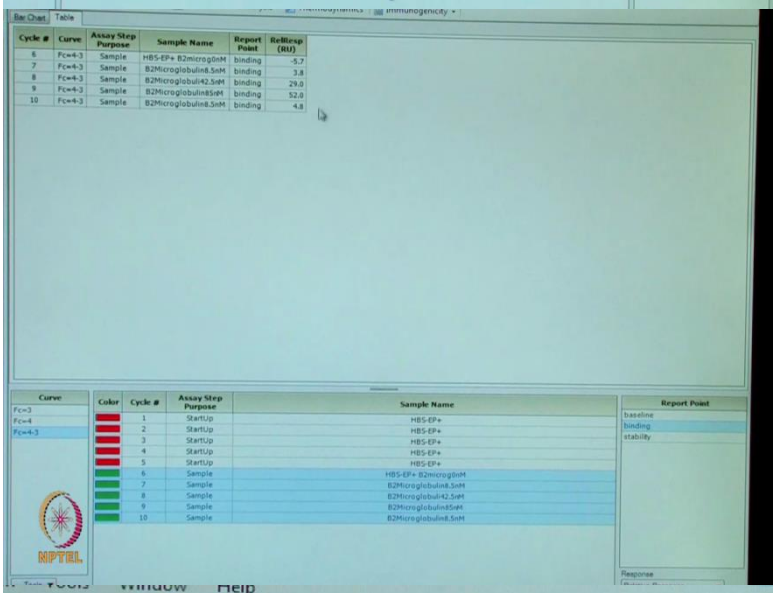
Report Point
baseline
binding
stability



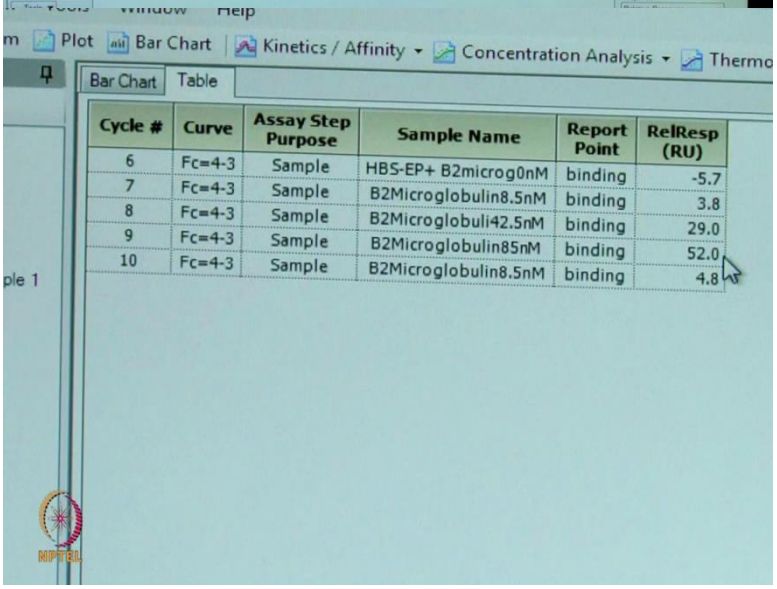


Color	Cycle #	Assay Step Purpose	Sample Name
	1	StartUp	HBS-EP+
	2	StartUp	HBS-EP+
	3	StartUp	HBS-EP+
	4	StartUp	HBS-EP+
	5	StartUp	HBS-EP+
	6	Sample	HBS-EP+ B2microg0nM
	7	Sample	B2Microglobulin8.5nM
	8	Sample	B2Microglobuli42.5nM
	9	Sample	B2Microglobulin85nM
	10	Sample	B2Microglobulin8.5nM

Report Point: baseline, binding, stability



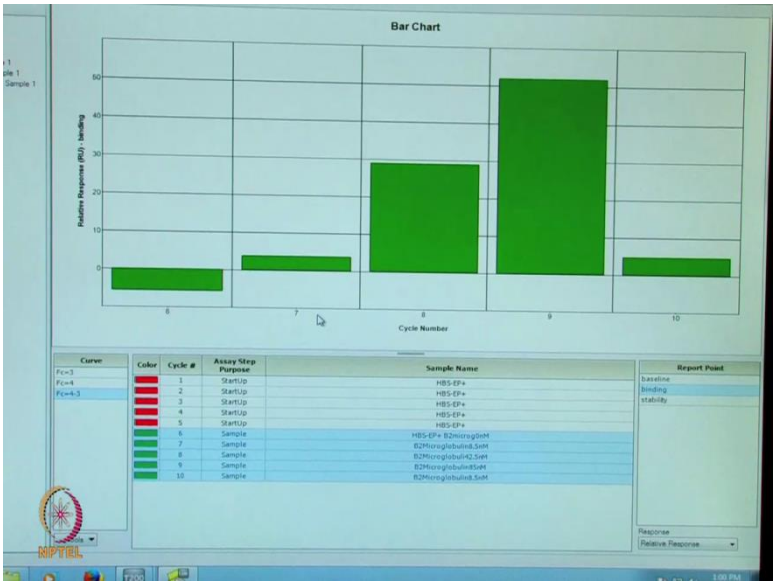
Cycle #	Curve	Assay Step Purpose	Sample Name	Report Point	RelResp (RU)
6	Fc=4-3	Sample	HBS-EP+ B2microg0nM	binding	-5.7
7	Fc=4-3	Sample	B2Microglobulin8.5nM	binding	3.8
8	Fc=4-3	Sample	B2Microglobuli42.5nM	binding	29.0
9	Fc=4-3	Sample	B2Microglobulin85nM	binding	52.0
10	Fc=4-3	Sample	B2Microglobulin8.5nM	binding	4.8



Plot | Bar Chart | Kinetics / Affinity | Concentration Analysis | Thermo

Bar Chart | Table

Cycle #	Curve	Assay Step Purpose	Sample Name	Report Point	RelResp (RU)
6	Fc=4-3	Sample	HBS-EP+ B2microg0nM	binding	-5.7
7	Fc=4-3	Sample	B2Microglobulin8.5nM	binding	3.8
8	Fc=4-3	Sample	B2Microglobuli42.5nM	binding	29.0
9	Fc=4-3	Sample	B2Microglobulin85nM	binding	52.0
10	Fc=4-3	Sample	B2Microglobulin8.5nM	binding	4.8



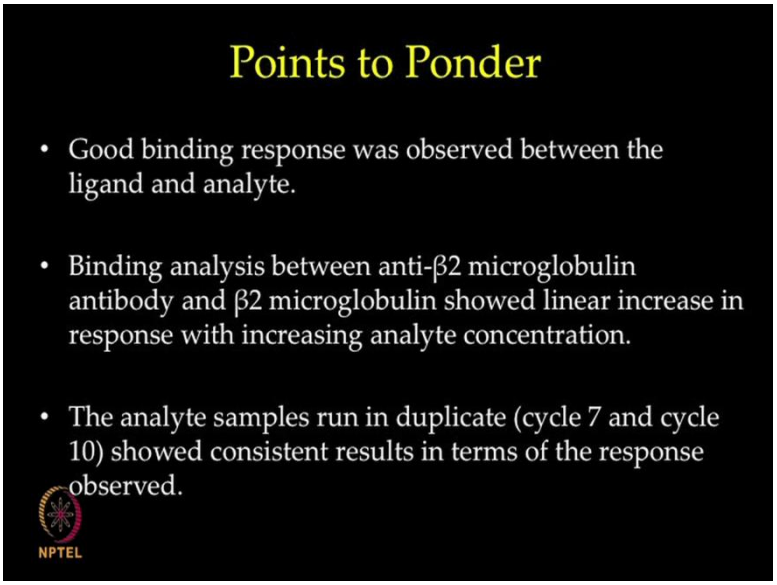
We will now proceed to analyse the data obtained from binding of anti-beta two microglobulin with beta two microglobulin protein, after finish of our binding experiment by double click on the file, the file is open now, we will see here from our binding experiment, it shows all sensorgram here. So, here the green ones are our startup. So, we have setup 3 or 5 different startups and the red ones are our actual data from beta two microglobulin. What we will do is we will highlight only our sampled data.

And now, you see the data for beta two M. We go on to sensorgram adjustment to report point on the vial adjustment baseline and save ok. Now our data is baseline to zero if we want we can as well go on to color sample and we will see the different samples in different color with the ligand on one side of the screen. Here, we have low, medium and high concentrations of beta two microglobulin injected over anti-beta two M; we could subtract or delete the regeneration area cut. Now, we can see our different concentrations of beta two M with one of them in duplicate.

So, there is definitely binding of the beta two M to anti-beta two M antibody in a dose dependent manner. The data can also be shown in the form of a bar chart with all our red startup runs and green as our sample runs. Here, we will highlight the sample runs only and see cycle 7,8 and 9 are our low, medium and high concentrated data which are dose dependent binding.

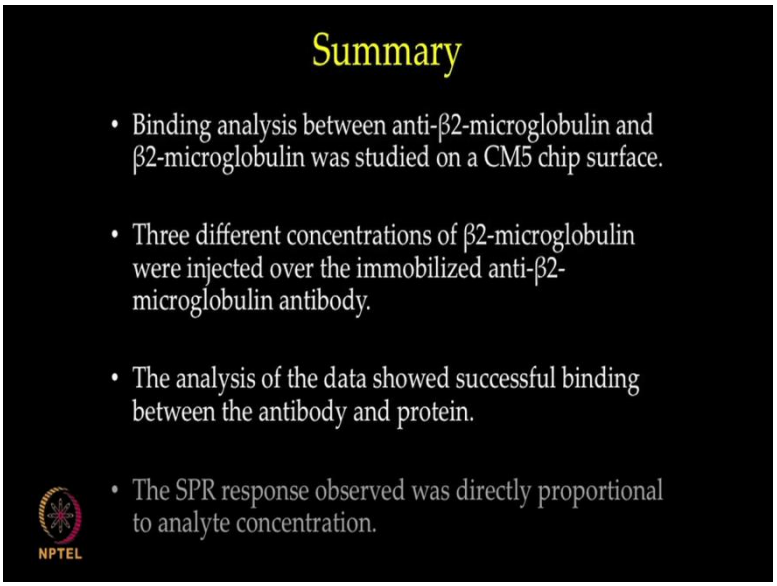
Going to another tab we can see the relative responses of each molecule from the binding at 4 for the duplicates and medium at 29 and high at 52. With this we will conclude our binding session and we prepare now for a kinetics experiment.

(Refer Slide Time: 24:40)



Protein interactions are identified using a wide array of applications however what is also required is a understanding of the extent to which these interactions actually occur. Therefore, performing protein-protein interactions studies and calculating their kinetics values becomes very crucial. Let us continue our discussion and SPR experiment for the kinetics analysis in next lecture. Thank you.

(Refer Slide Time: 25:31)



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