Interactomics: Protein Arrays and Label-Free Biosensors.
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Lecture-7.
Protein Immobilization for Protein-Protein Interaction Studies.

Welcome to MOOC NPTEL course on intractomics, today we will talk about protein-protein interactions study, immobilization of the ligand on sensor chip. Surface Plasmon resonance has transformed the study of bio-molecular interactions by delivering a platform that does not require the ligand or analyte to be labeled. SPR measures the interaction between a ligand which is immobilized on the sensor chip surface and an analyte which is passed in the solution. This measurement takes place in real time label-free environment providing kinetic, equilibrium and concentration data.

In today's SPR immobilization experiment, the ligand anti beta 2 microglobulin will be covalently immobilized to the surface of sensor chip using amine coupling chemistry. The direct immobilization of the ligand is known as direct coupled. Another way of immobilizing a bio-molecule is by using capturing chemistry. In this case, the ligand is not covalently immobilized to the sensor chip surface but is captured through the electrostatic interactions.

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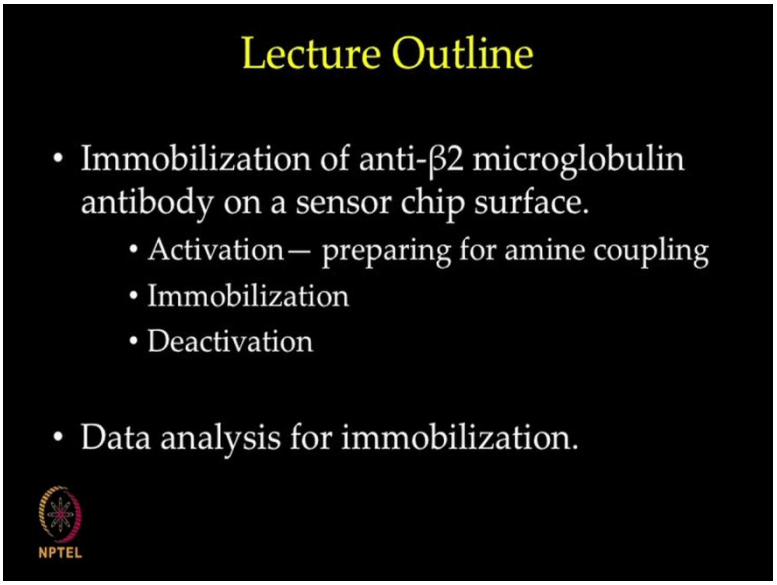

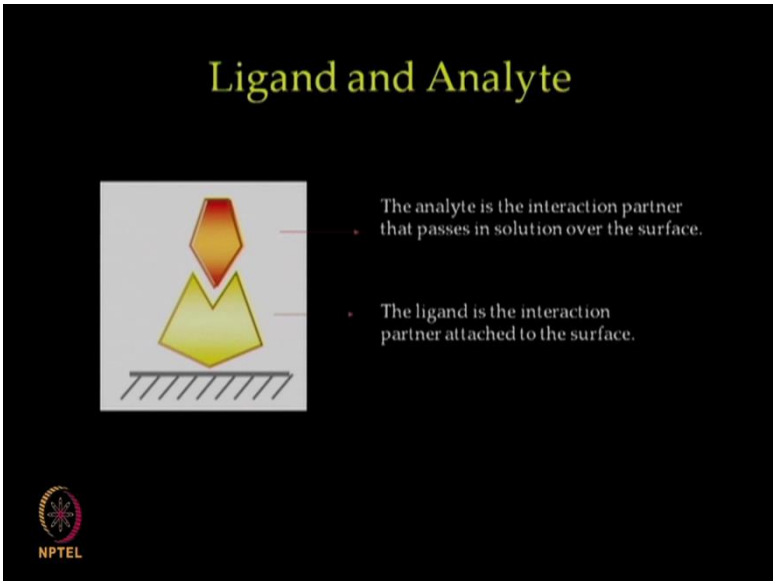

The major steps involved in the immobilization of anti beta 2 microglobulin antibody will involve activation preparing for amine coupling immobilization and deactivation. So, let us have, the lab experimental session now. Let us learn a little about the basics of immobilization and SPR assay. In the molecular interaction study using surface plasmon resonance we will immobilize one of the interacting molecule that is anti-beta two microglobulin on the gold sensor chip surface while the protein beta two microglobulin will be passed over that surface in solution. Here, the ligand refers to the immobilized component and the interacting partner in the sample injected over the surface is referred to as the analyte.

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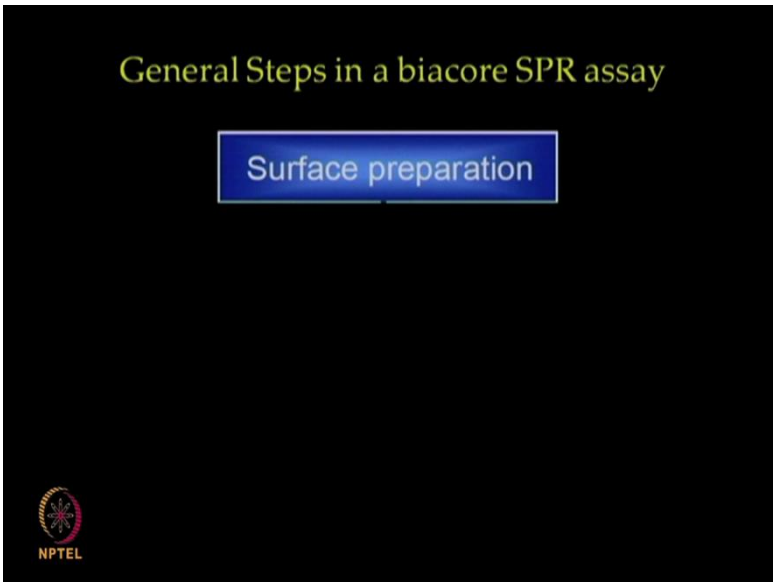

The three major steps in a biacore SPR assay involved; immobilization, the process by which ligand is attached to the sensor chip surface, interaction analysis where the analyte is injected over the sensor chip surface and the interaction between the analyte and immobilized ligand is monitored. And regeneration, the process of removing bound analyte from the ligand on the surface.

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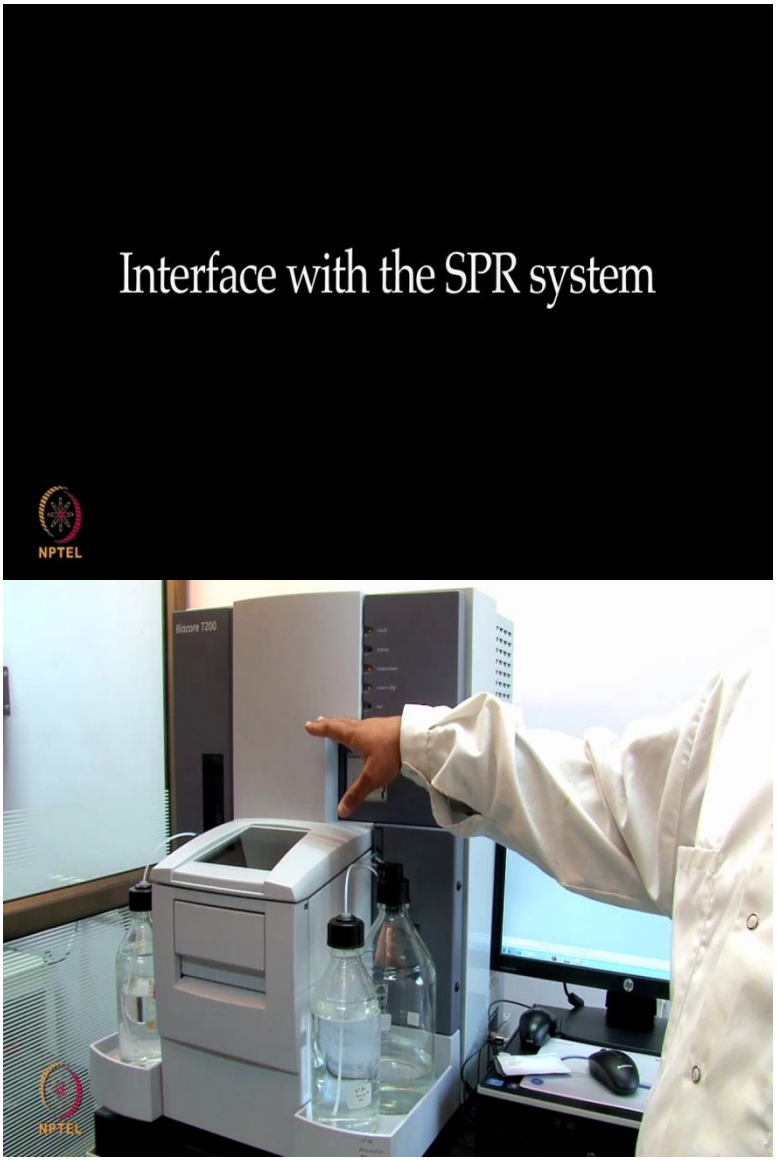
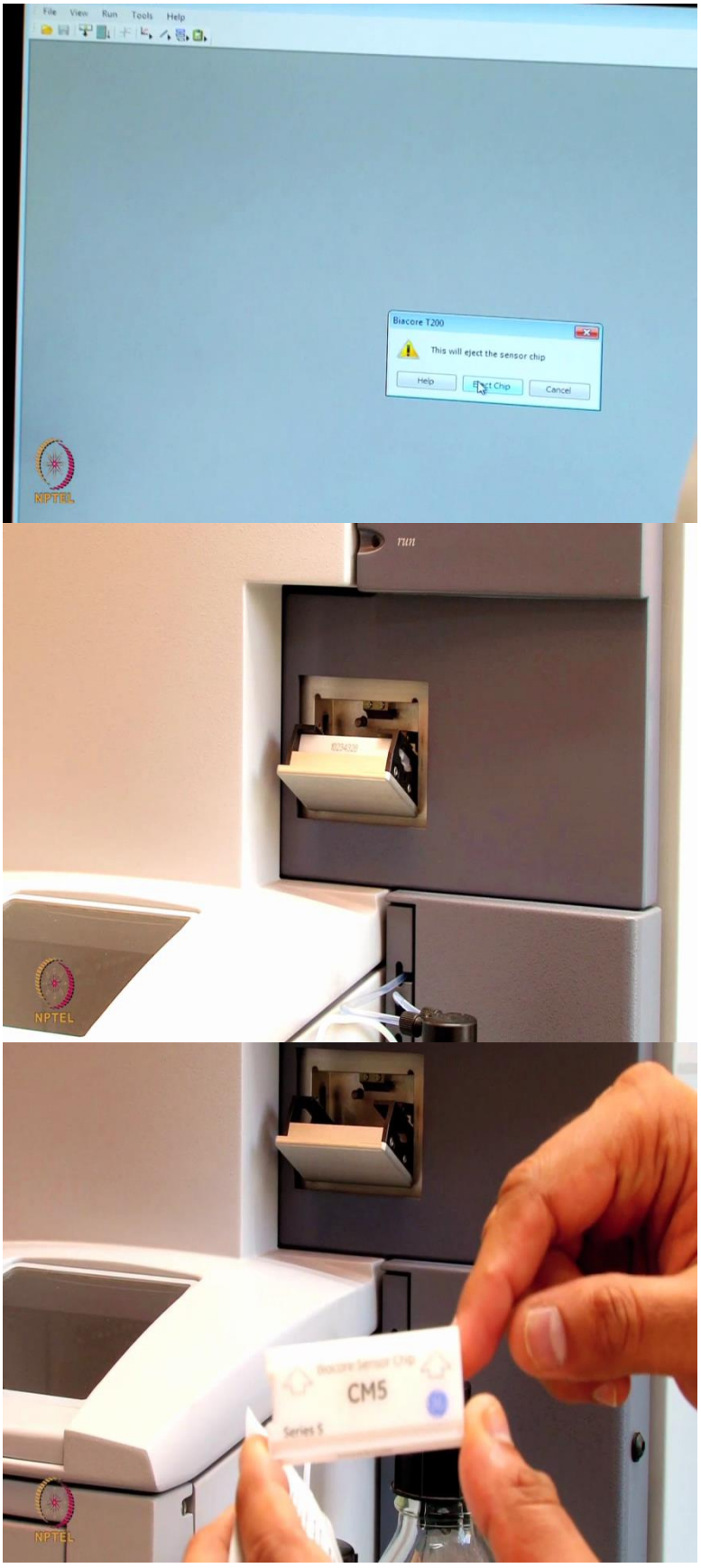



Series S
Sensor Chip CM5


Biacore Sensor Chip
CM5
Series S


File View Run Tools Help
Biacore T200
This will eject the sensor chip
Help
Eject Chip
Cancel
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run
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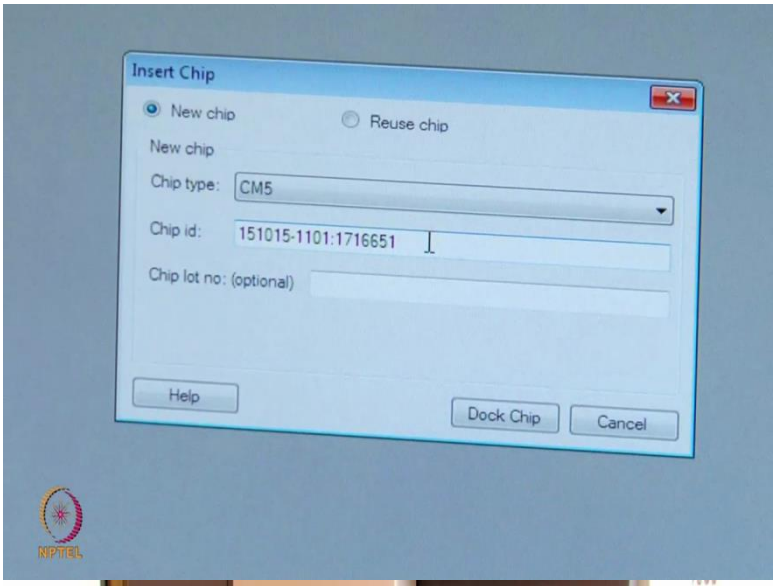
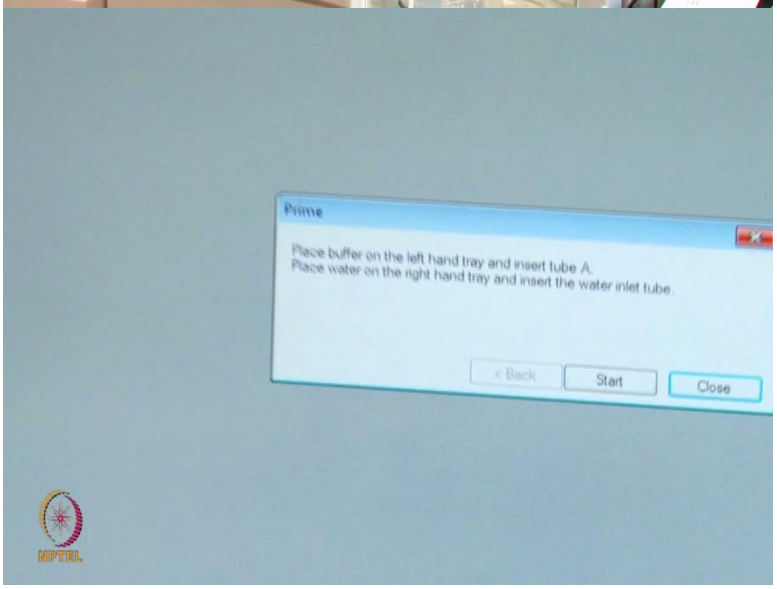
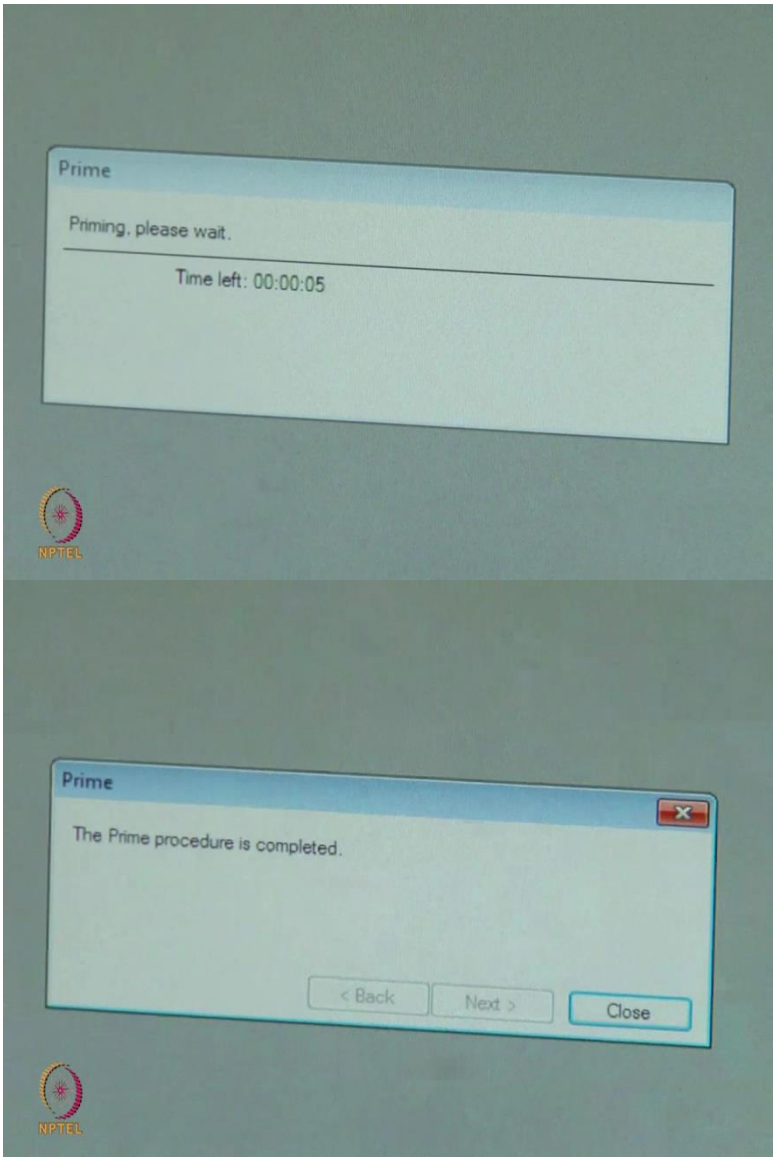
CM5
Series S
NPTEL


Insert Chip
New chip
Reuse chip
New chip
Chip type:
CM5
Chip id:
151015-1101:1716651
Chip lot no: (optional)
Help
Dock Chip
Cancel


Prime
Place buffer on the left hand tray and insert tube A.
Place water on the right hand tray and insert the water inlet tube.
< Back
Start
Close



So, this is a surface Plasmon resonance and it is a biacore T200. So, this machine has the various parts like the running buffer is connected here and any of the biological buffers can be connected as a running buffer. There could be have any pH from low to high. So, the regular buffers used in a biacore experiment are HBS EP-HBSN PBS for small molecule experiments.

Coming here, we have a water reservoir. The water reservoir is useful for cleaning niddles and syringe and we have a waste reservoir here and this waste reservoir collects all the waste. The samples are actually sent in this compartment, chip is docked in this compartment. So, the experiment starts by picking up samples from this compartment and transferring them here at the interaction side and then the experiment is recorded on the screen. Here, some

kind of indications are provided when the machine is ready or the system is calibrating temperature, when the new chip is docked and the run is actually happening.

So, we will now, connect a new running buffer here and prime the system before our immobilization experiment. So, here the new HBS-EP plus buffer is connected and the new chip will be docked now. So, let us look at the chip now. So, this is a new chip, the chips are generally provided in these cassettes and then new CM5 chip will look like this. This chip will be inserted in chip docking area from the control software, we will eject to the sensor chip that is connected.

So, now the old chip will be removed and we will insert a new biacore chip CM5 here and the insertion or the way orientation of the chip is shown on the chip in arrows and we will close the compartment door and identify the chip from the chip type here and chip will be given a new id and sometimes it is very essential to add the name for the chip and also, the lot number and save dock chip.

Now, the chip is getting docked. So, we have connected a new buffer, we will prime the system. Priming is the process of sending buffer through the IRC and equilibrate the system before our experiment. Generally, in any biacore experiments, buffer should be connected and equilibrated or if there is no time, a minimum of three hours of equilibration is essential, otherwise when to start a new experiment do at least six primes on the system.

Now, that the chip is docked we will prime the system. Primly, we have connected the buffer. So, we just say start and it takes six minutes for the system to prime. Now, the prime procedure is complete, we will do an immobilization of a ligand. In this case today, we are actually immobilizing anti-beta two M antibody for that immobilization. Let us prepare a wizard, before we setup an immobilization protocol, let us understand a little about immobilization levels.

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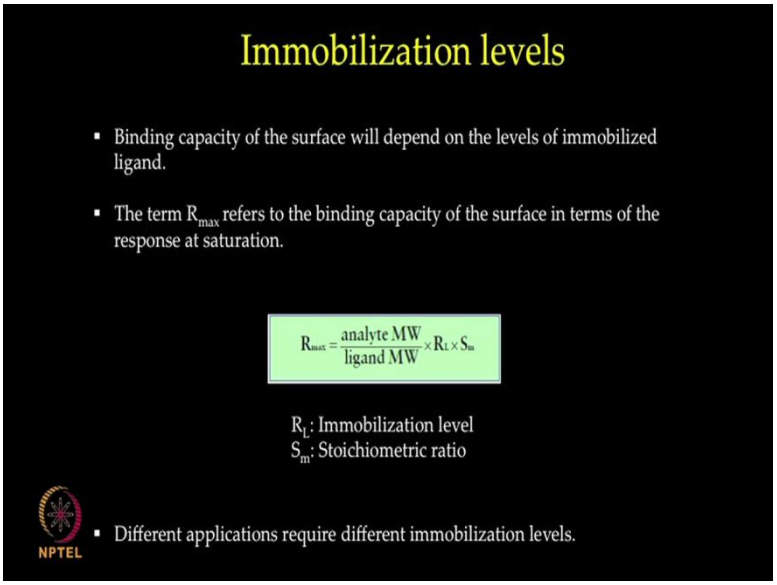

The binding capacity of the chip surface will depend on the levels of immobilized ligand. The term maximum response refer to as Rmax is described as the binding capacity of the surface in terms of the response at saturation. A theoretical Rmax value can be calculated using the formula shown below where RL is the immobilization level and the Sm is the stoichiometric ration. A theoretical calculated Rmax is often higher that the experimentally derived Rmax for the same interaction. This could be because of several reasons such as the ligand is not fully active or that there is steric hindrance in the interaction.

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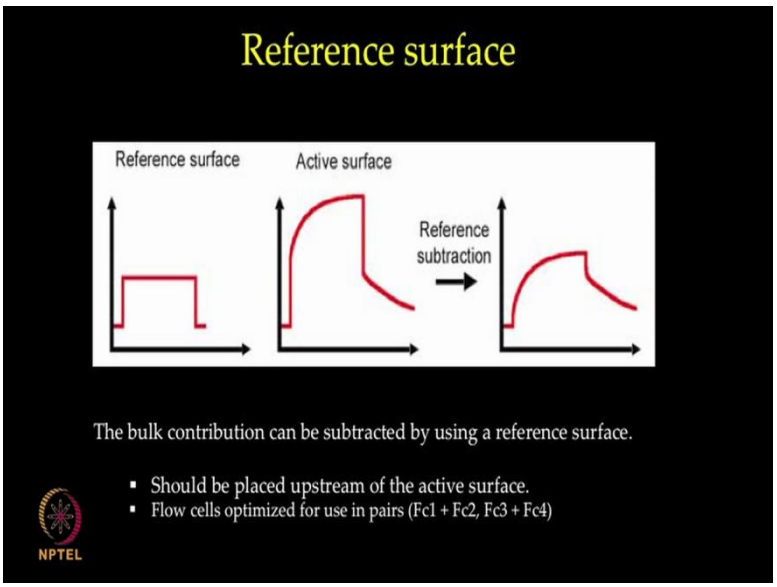

Different applications may require different binding capacities and thus, different immobilization levels. A low Rmax is often beneficial in kinetics analysis while higher

immobilization levels are advantageous in binding analysis and concentration measurements. Today, we are going to immobilize antibody on a CM5 chip using amine coupling chemistry and the figure here, shows a typical immobilization sensor gram using amine coupling.

The three major steps involved here are activation of the surface esters using EDC and NHS. Covalent coupling of the ligand on the sensor chip using amine groups of the ligand and deactivation of free esters with ethanol amine, we will analyse the results of anti-beta two microglobulin immobilization later in the lecture. Reference subtraction is particularly important for assays where measurement is taken during the sample injection.

The bulk contribution due to any difference in the sample matrix and running buffer can be subtracted by using a reference surface. This reference surface is typically placed upstream of the active surface. The flow cells on the chip surface are optimized accordingly for use in pairs that is flow cell one with flow cell two and flow cell three with flow cell four.

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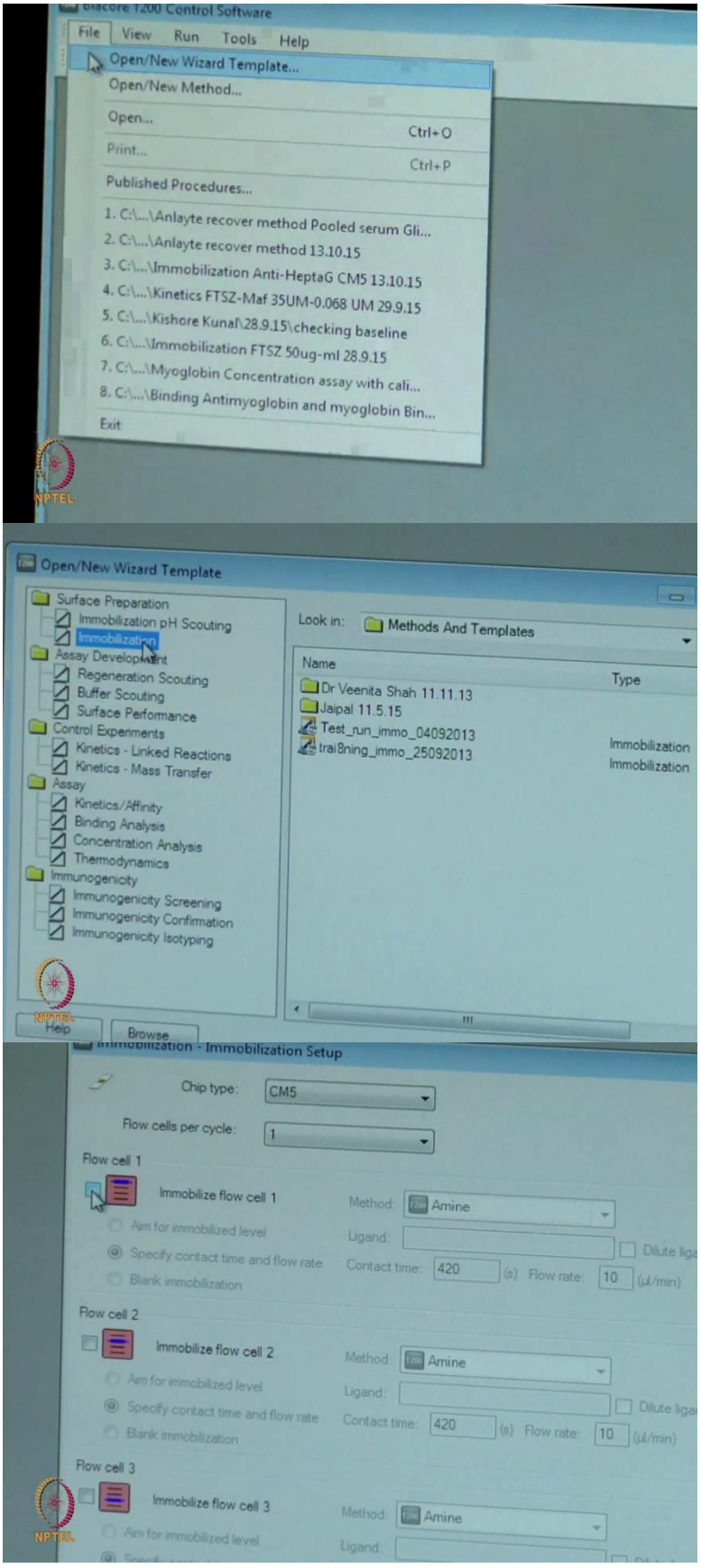
Biacore T200 Control Software
File View Run Tools Help
Open/New Wizard Template...
Open/New Method...
Open... Ctrl+O
Print... Ctrl+P
Published Procedures...
1. C:\...\Anlayte recover method Pooled serum Gli...
2. C:\...\Anlayte recover method 13.10.15
3. C:\...\Immobilization Anti-HeptaG CM5 13.10.15
4. C:\...\Kinetics FTSZ-Maf 35UM-0.068 UM 29.9.15
5. C:\...\Kishore Kunal\28.9.15\checking baseline
6. C:\...\Immobilization FTSZ 50ug-ml 28.9.15
7. C:\...\Myoglobin Concentration assay with cali...
8. C:\...\Binding Antimyoglobin and myoglobin Bin...
Exit
Open/New Wizard Template
Surface Preparation
Immobilization pH Scouting
Immobilization
Assay Development
Regeneration Scouting
Buffer Scouting
Surface Performance
Control Experiments
Kinetics - Linked Reactions
Kinetics - Mass Transfer
Assay
Kinetics/Affinity
Binding Analysis
Concentration Analysis
Thermodynamics
Immunogenicity
Immunogenicity Screening
Immunogenicity Confirmation
Immunogenicity Isotyping
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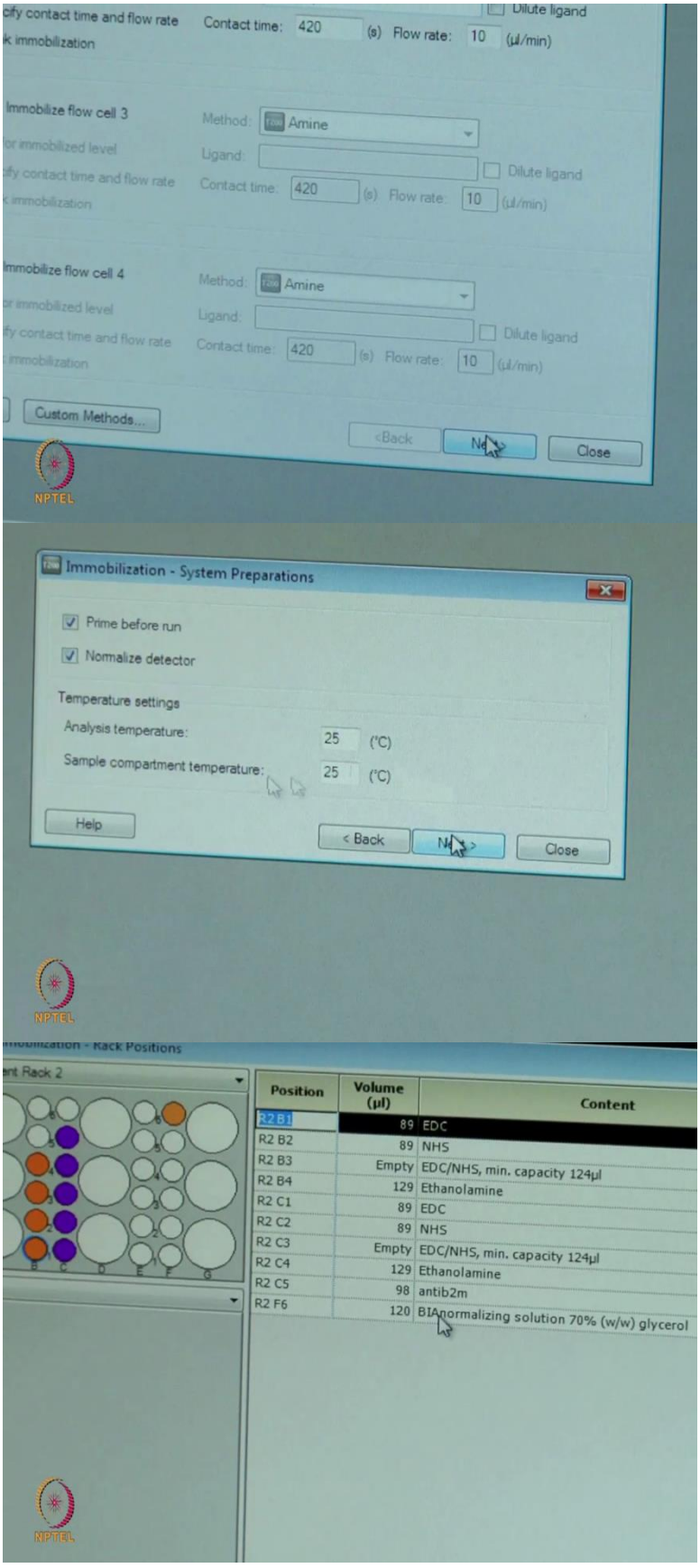
Dr Veenita Shah 11.11.13
Jaipal 11.5.15
Test_run_immo_04092013
Immobilization
trai8ning_immo_25092013
Immobilization
Help
Browse...
Immobilization - Immobilization Setup
Chip type: CM5
Flow cells per cycle: 1
Flow cell 1
Immobilize flow cell 1
Method: Amine
Aim for immobilized level
Ligand:
Specify contact time and flow rate
Contact time: 420 (s) Flow rate: 10 (µl/min)
Blank immobilization
Flow cell 2
Immobilize flow cell 2
Method: Amine
Aim for immobilized level
Ligand:
Specify contact time and flow rate
Contact time: 420 (s) Flow rate: 10 (µl/min)
Blank immobilization
Flow cell 3
Immobilize flow cell 3
Method: Amine
Aim for immobilized level
Ligand:


Dilute ligand
Contact time: 420 (s) Flow rate: 10 (µl/min)
Immobilize flow cell 3
Method: Amine
Ligand:
Dilute ligand
Contact time: 420 (s) Flow rate: 10 (µl/min)
Immobilize flow cell 4
Method: Amine
Ligand:
Dilute ligand
Contact time: 420 (s) Flow rate: 10 (µl/min)
Custom Methods...
<Back
Close
NPTEL
Immobilization - System Preparations
Prime before run
Normalize detector
Temperature settings
Analysis temperature: 25 (°C)
Sample compartment temperature: 25 (°C)
Help
< Back
Close
NPTEL
Position
Volume (µl)
Content
R2 B1 89 EDC
R2 B2 89 NHS
R2 B3 Empty EDC/NHS, min. capacity 124µl
R2 B4 129 Ethanolamine
R2 C1 89 EDC
R2 C2 89 NHS
R2 C3 Empty EDC/NHS, min. capacity 124µl
R2 C4 129 Ethanolamine
R2 C5 98 antib2m
R2 F6 120 BIAnormalizing solution 70% (w/w) glycerol
NPTEL

File, open new wizard template, identify immobilization from surface preparation say new. So, we will immobilize for binding, flow cell one and two for binding and three and four for kinetics. Prime before run; normalize detector save next and the rack positions are displayed with the number of vials and the volume and the positions.

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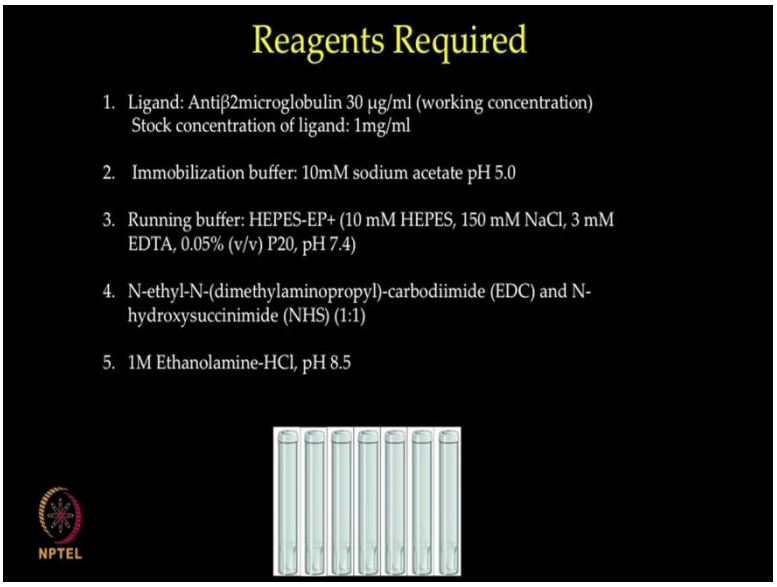

As was listed on the table, we will now have a closer look on the buffers and reagents required for immobilization of anti-beta two microglobulin . The reagents include a stock concentration of anti-beta two microglobulin from which a working concentration of 30 microgram per ml will be made using an immobilization buffer of 10 milimolar sodium acetate pH 5, we need HEPES-EP plus pH 7.4 which will include 10 milimolar HEPES, 150 milimolar NaCL, 3 milimolar EDTA and 0.05 percent P20. This will be used as the running buffer which is already connected to the system followed by priming of the system.

EDC and NHS in the amine coupling kit are used in one is to one ratio for surface activation. Lastly, we also require one molar ethanol amine HCl pH 8.5 for blocking the pre-ester groups on the surface. We shall now proceed to use the above mentioned reagents for our immobilization experiment. We will now work on the reagents required the immobilization of anti-beta two microglobulin on cellular chip surface.

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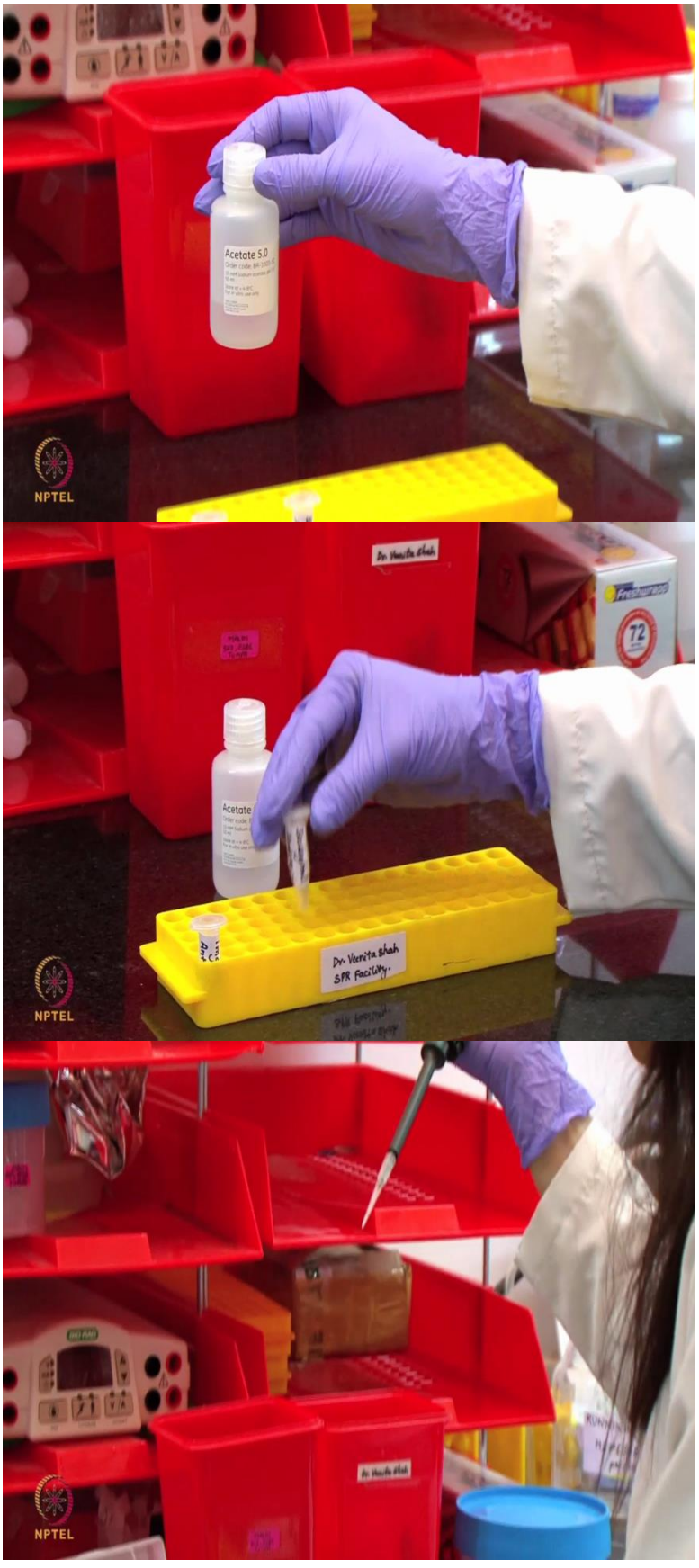

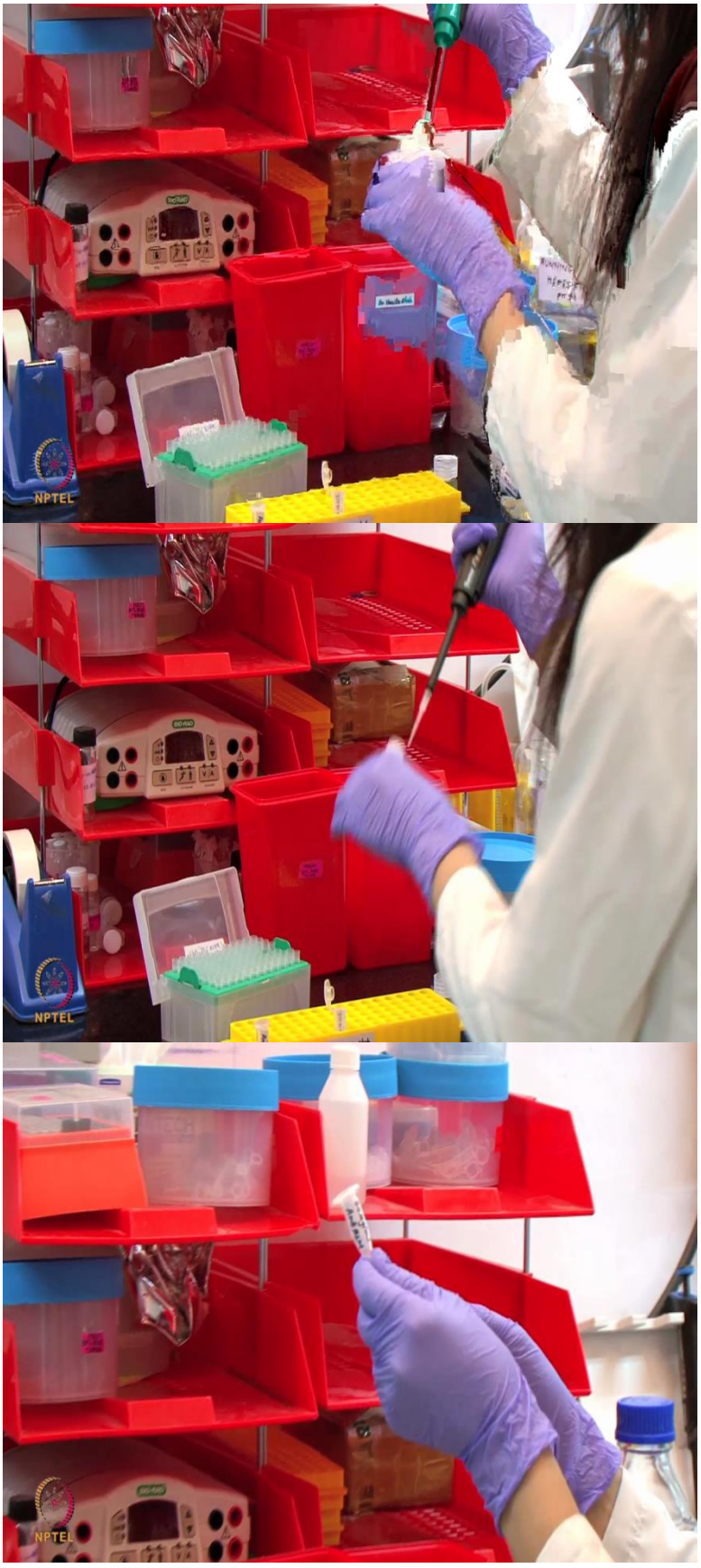
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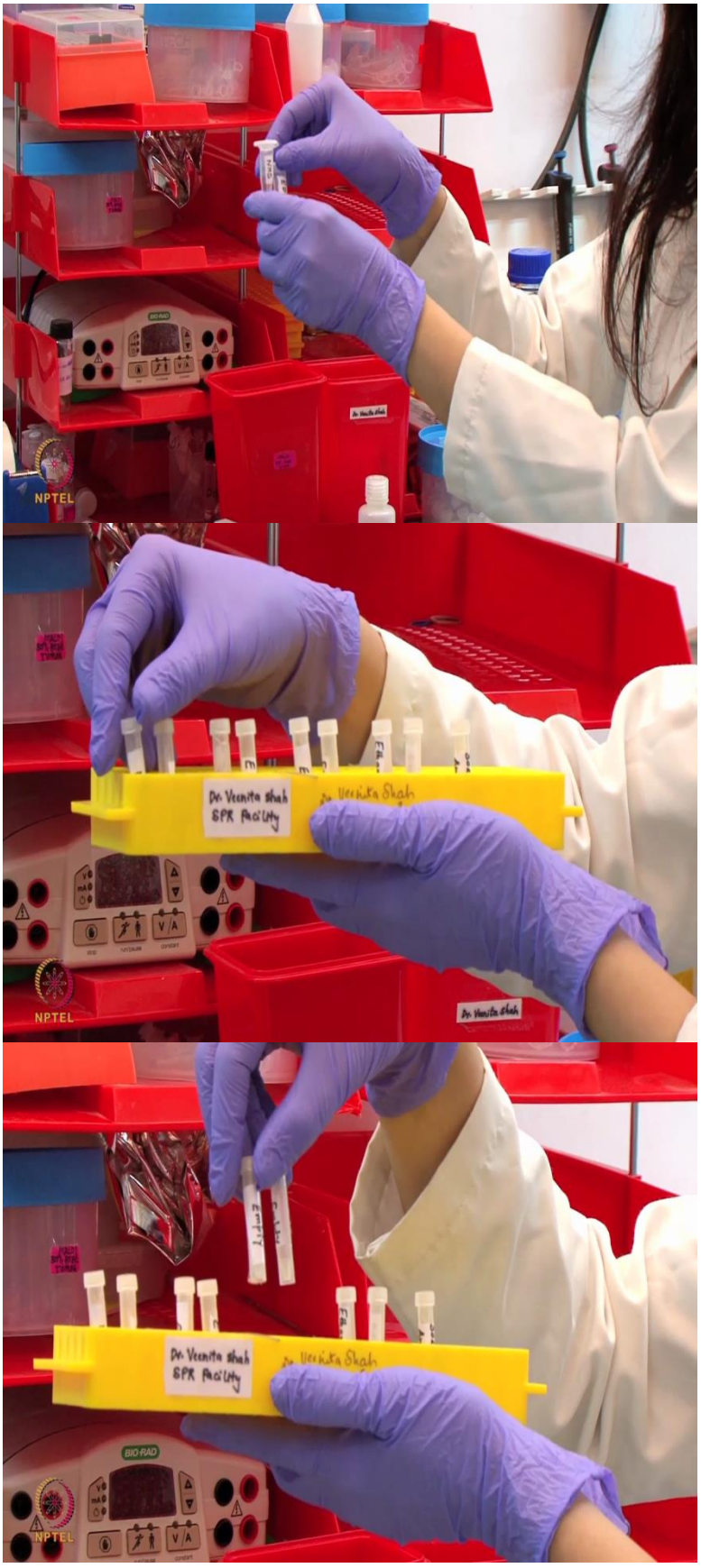
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Dr. Veenita Shah
SPR Facility
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Dr. Veenita Shah
SPR Facility
BIO-RAD
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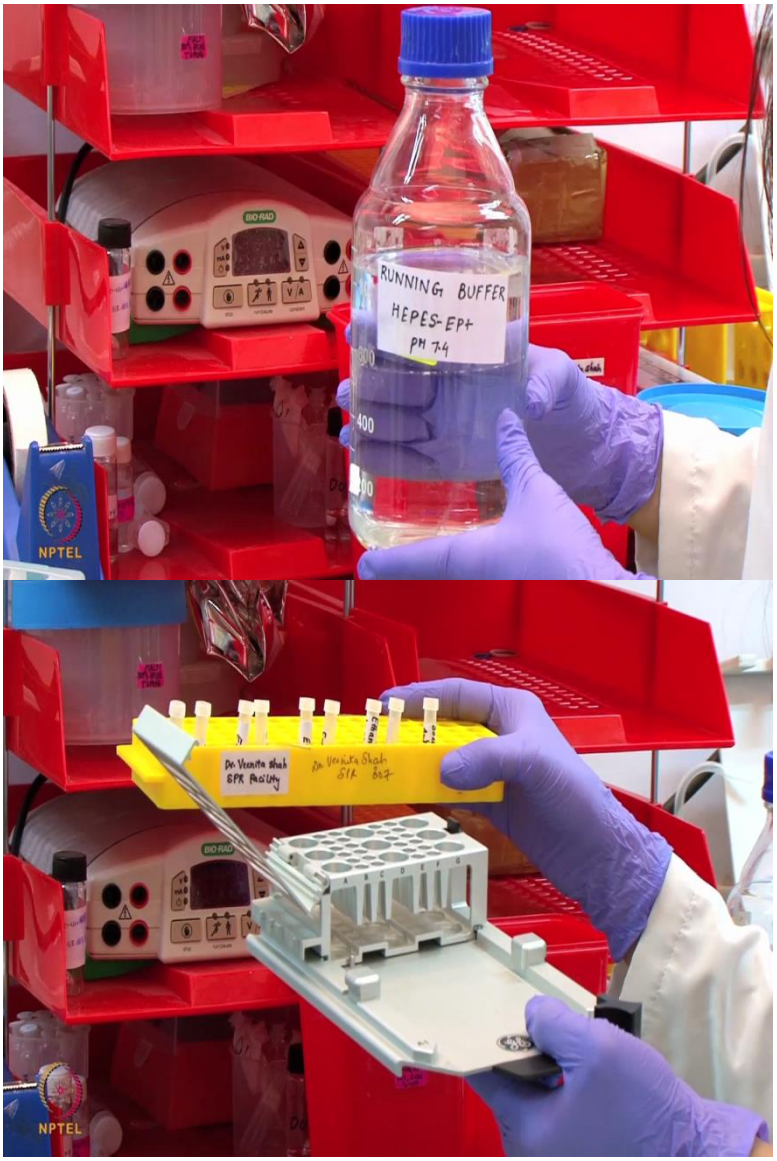

We will first tell you the stock concentration of the antibody which is 1 mg per ml of anti-beta two microglobulin . In 10 milimolar sodium acetate pH 5 to make a working antibody solution of 30 microgram per ml for this we will take 6 microliter of the ligand stock and mix it in 194 microliter of sodium acetate pH5. The choice of the correct immobilization buffer is an important parameter to consider and the pH scouting feature of the system will help in choosing the correct pH of the immobilization buffer. This is our 30 microgram per ml of ligand pH.

(())(17:47) of NHS, EDC and ethanol amine are prepared and transferred to this specialized tubes used for the system. We have now transferred all the reagents into this specialized tube. So, we have two NHS tube, Two EDC, two empty tubes for longer stability of EDC, NHS which will the makes inside the system two ethanol tube, one for the blank flow cell and the other for the active flow channel and one ligand solution. We will be using HEPES-EP plus

as the running buffer which will be connected to the system prior to the initialization of the immobilization run.

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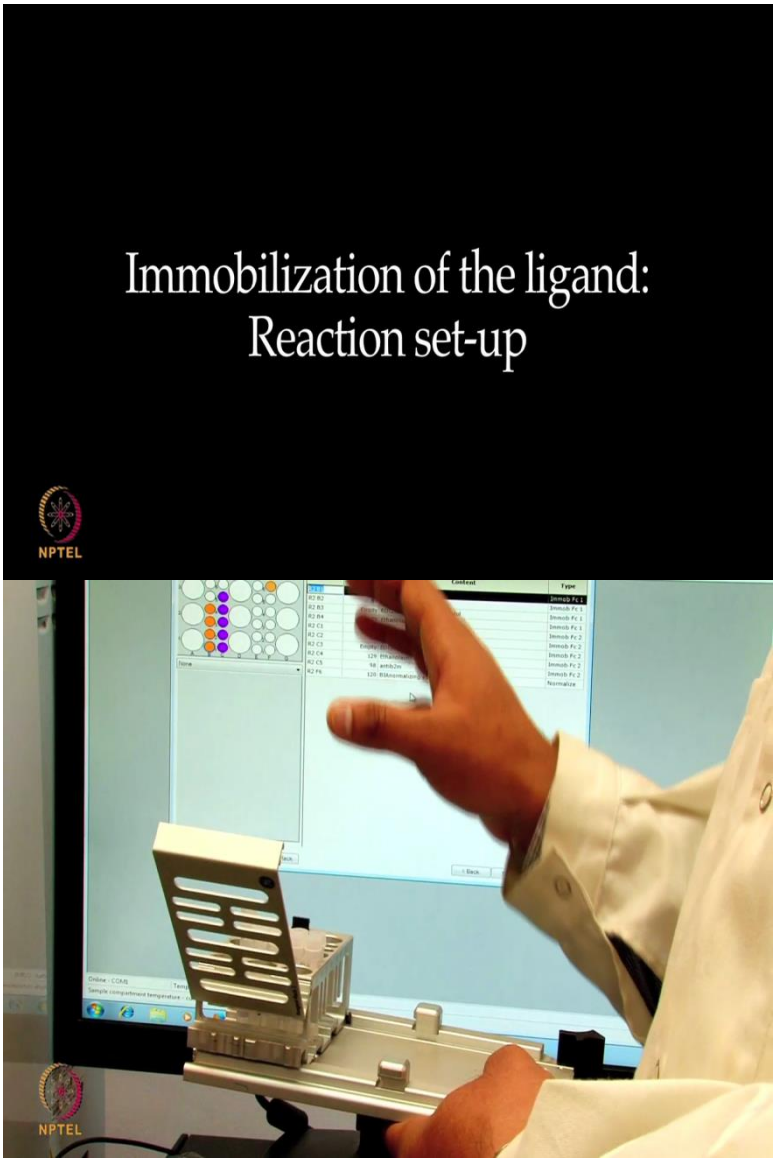
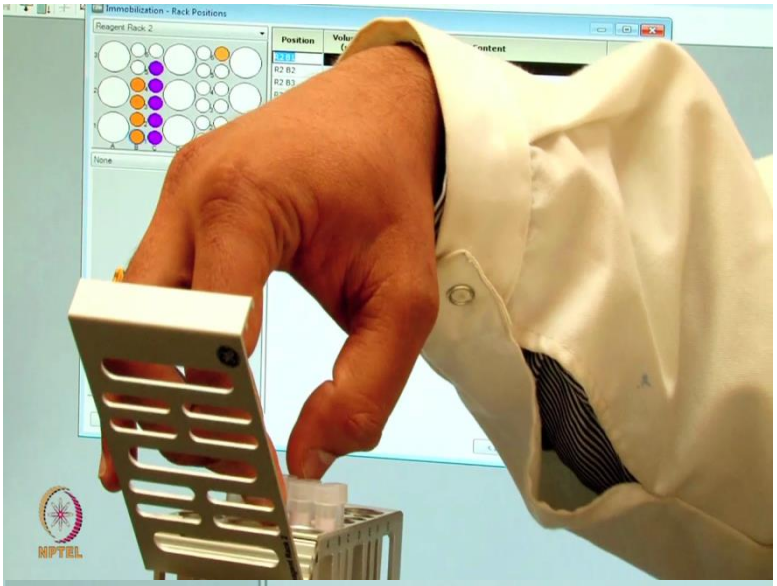
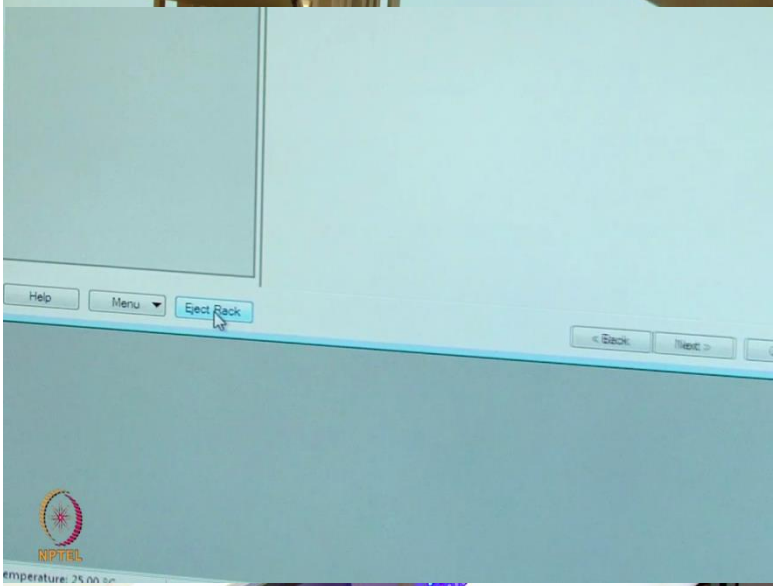



Immobilization - Rack Positions
Reagent Rack 2
Position
None
NPTEL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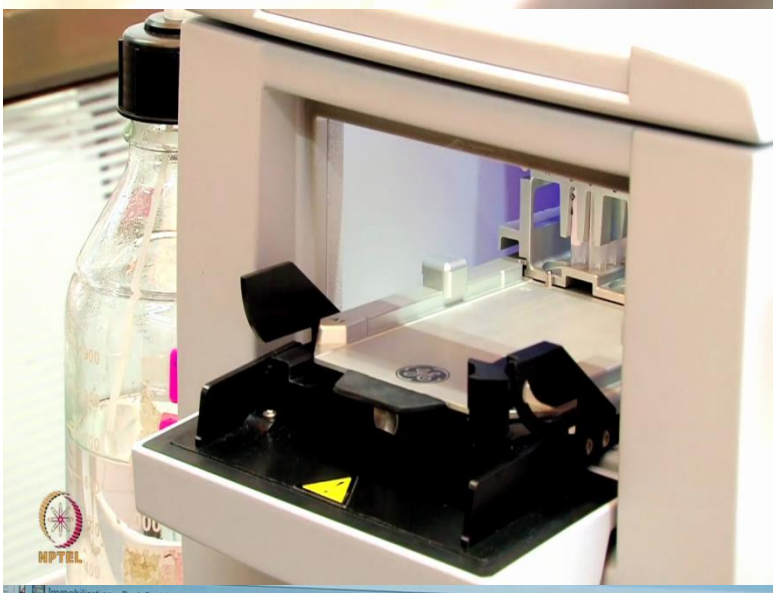

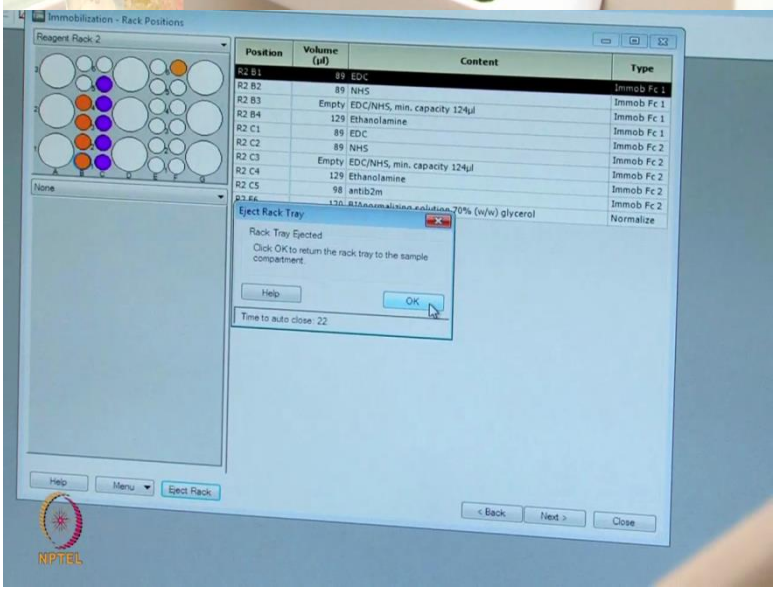
Immobilization - Rack Positions
Reagent Rack 2
None
Position
Volume (µl)
Content
Type
R2 B1 89 EDC
R2 B2 89 NHS Immob Fc 1
R2 B3 Empty EDC/NHS, min. capacity 124µl Immob Fc 1
R2 B4 129 Ethanolamine Immob Fc 1
R2 C1 89 EDC Immob Fc 1
R2 C2 89 NHS Immob Fc 2
R2 C3 Empty EDC/NHS, min. capacity 124µl Immob Fc 2
R2 C4 129 Ethanolamine Immob Fc 2
R2 C5 98 antib2m Immob Fc 2
70% (w/w) glycerol Normalize
Eject Rack Tray
Rack Tray Ejected
Click OK to return the rack tray to the sample compartment.
Help
OK
Time to auto close: 22
Help
Menu
Eject Rack
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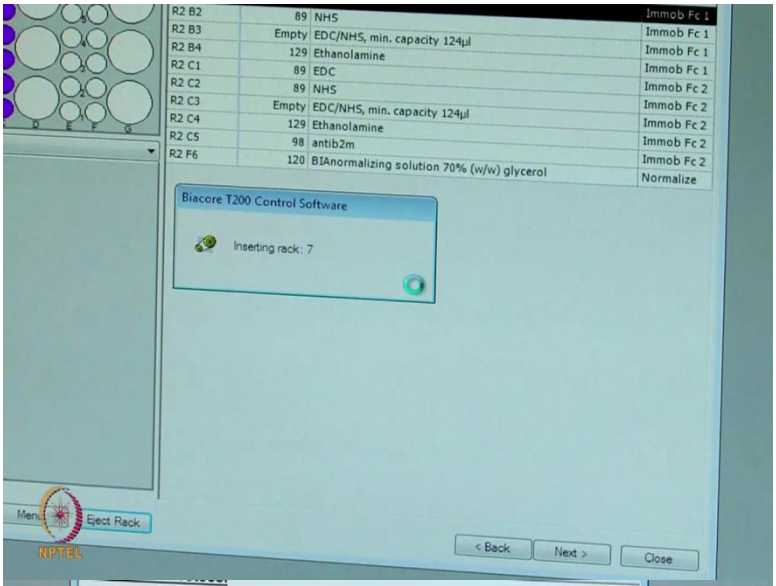
Close


R2 B2 89 NHS Immob Fc 1
R2 B3 Empty EDC/NHS, min. capacity 124µl Immob Fc 1
R2 B4 129 Ethanolamine Immob Fc 1
R2 C1 89 EDC Immob Fc 1
R2 C2 89 NHS Immob Fc 2
R2 C3 Empty EDC/NHS, min. capacity 124µl Immob Fc 2
R2 C4 129 Ethanolamine Immob Fc 2
R2 C5 98 antib2m Immob Fc 2
R2 F6 120 BIAnormalizing solution 70% (w/w) glycerol Immob Fc 2
Normalize
Biacore T200 Control Software
Inserting rack: 7
Eject Rack
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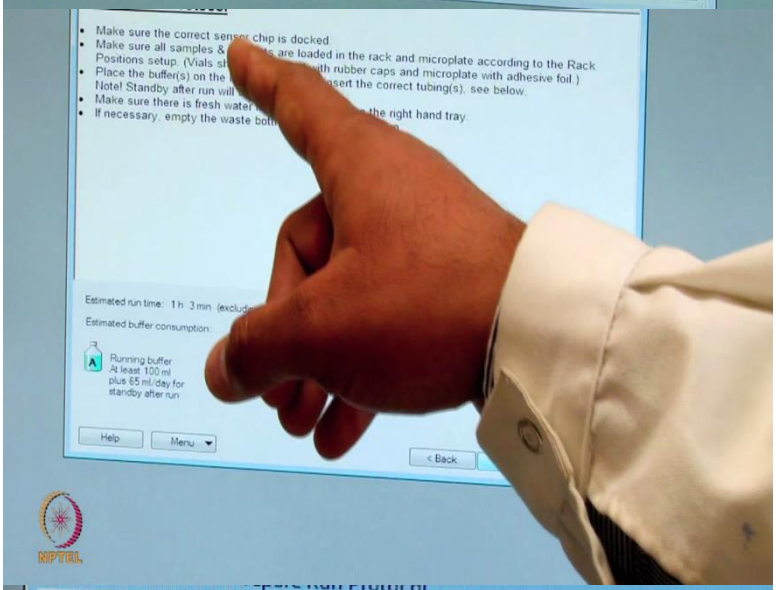
Make sure the correct sensor chip is docked
Make sure all samples & reagents are loaded in the rack and microplate according to the Rack Positions setup. (Vials should be sealed with rubber caps and microplate with adhesive foil.)
Place the buffer(s) on the left hand tray and insert the correct tubing(s). 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: 1 h 3 min (excluding
Estimated buffer consumption:
A Running buffer At least 100 ml plus 65 ml/day for standby after run
Help
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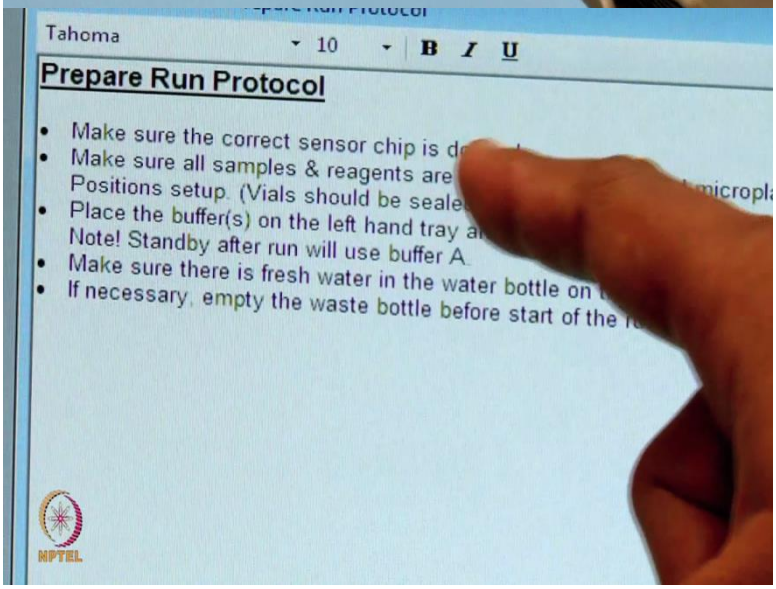
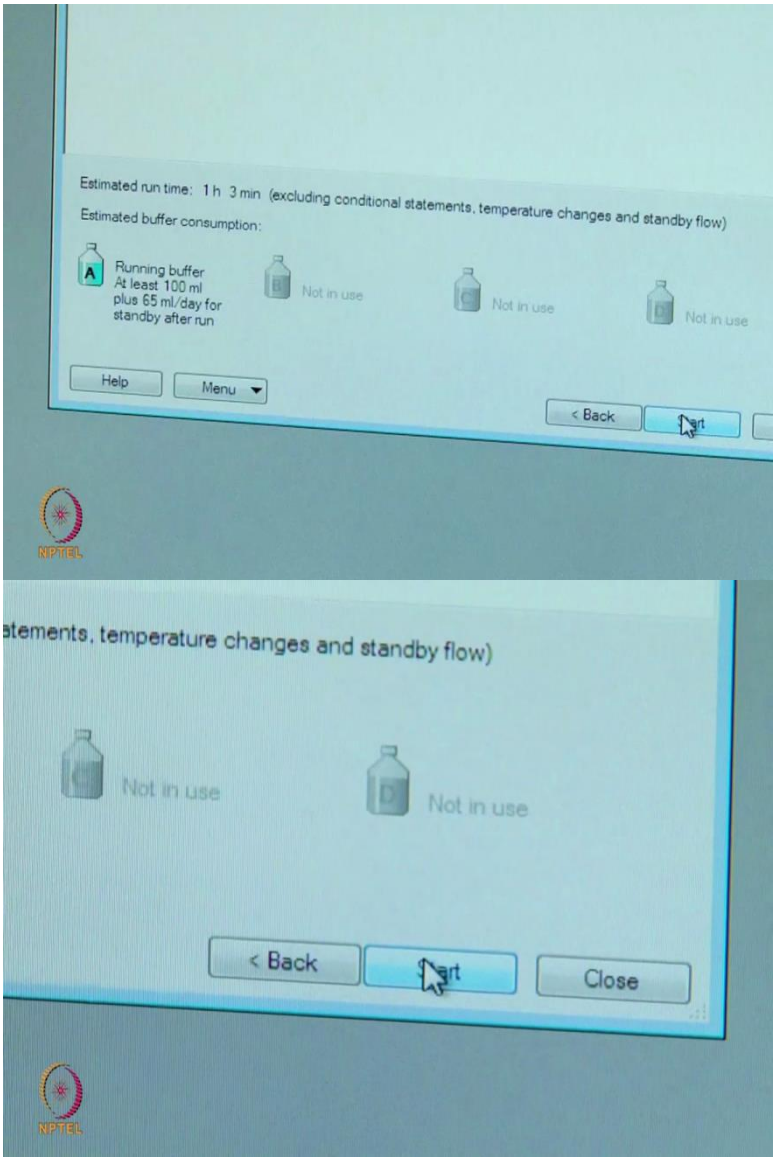

Tahoma
10
B I U
Prepare Run Protocol
Make sure the correct sensor chip is d
Make sure all samples & reagents are
Positions setup. (Vials should be seale
Place the buffer(s) on the left hand tray a
Note! Standby after run will use buffer A
Make sure there is fresh water in the water bottle on
If necessary, empty the waste bottle before start of the
NPTEL



We will now proceed to insert these tubes into the appropriate rack and then into this system for immobilization of anti-beta two microglobulin on the sensor chip surface. Now, the we made the template, we will insert the vial in the right position, we just fitted them in the required volumes of different chemicals to start with the EDC is positioned here, the NHS is positioned here, the ethanol amine is positioned here and there is empty vial , in a similar way another row is also made. So, one for the reference surface and other for the active surface to normalize the chip, normalization solution for the machine, to normalize the discrepancies on the RU responses on four different close channel.

We close the door this way and go on the screen here, we eject the rack, we will insert the rack into the sample compartment this way and need to lock it inside, lock it inside and then go on the screen, close the compartment. We go to the next tab. So here there some points we need to take a before start of the run. Make sure the correct sensor chip is docked. Make

sure all sample reagents are loaded in the rack and micro plate according to the rack position setup, vials should be sealed with caps, place the buffer, place water and make sure there is sufficient amount of water and buffer.

So, once we go to all these and everything is set in the machine, it shows the estimated run time as 1hour 3 minutes and we have connected the running buffer. Now, we will start the experiment. This will prepare the chip for the immobilization process and immobilize the antibody or the ligand of our choice today the anti-beta two M. We need to save this as and now, the immobilization process is running.

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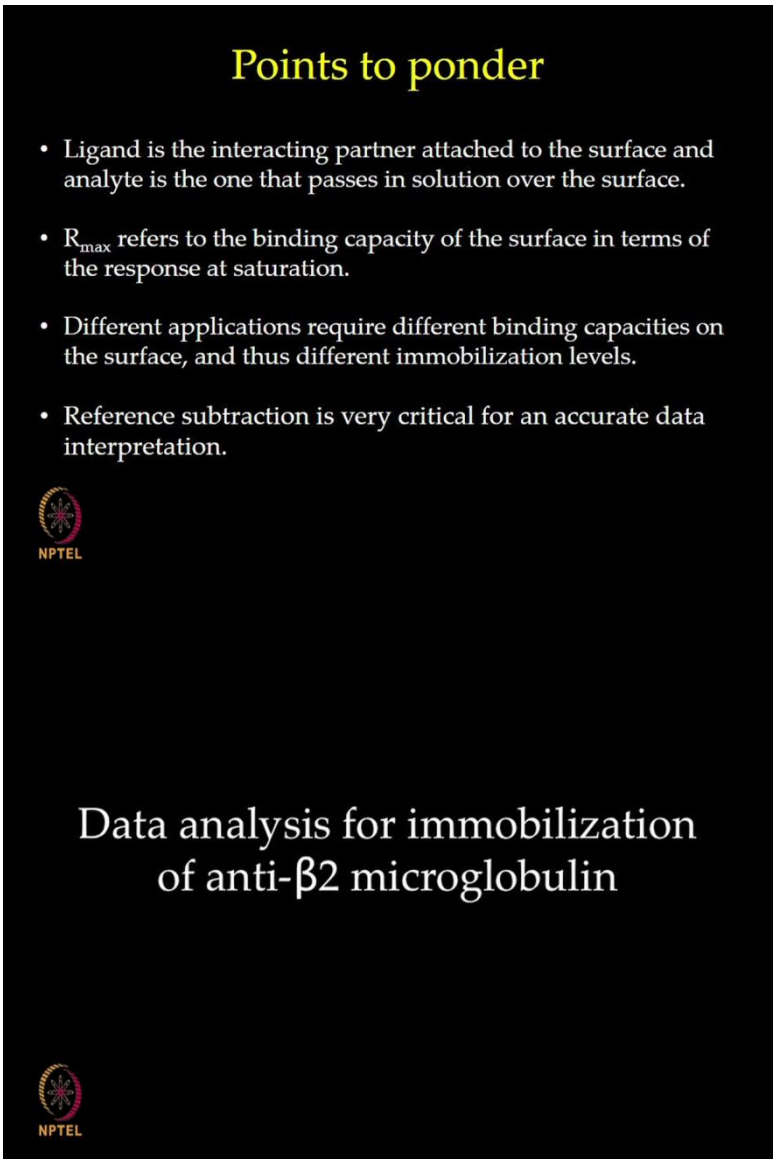

We will now look at the results of our anti-beta two microglobulin immobilization performed on CM5 chip. Looking at the immobilization results dialog box, there are two response levels calculated from the sensor gram. The response bound which represents, the amount of ligand bound to the surface after ligand injection whereas the response final test represents the amount of ligand covalently bound to the surface.

So, here as we observe 12894 RU of anti-beta two microglobulin is immobilized on the surface of the chip. Now, analyzing the immobilization sensor gram, we observe the baseline here followed by EDC-NHS activation of the dextran matrix which is again followed by baseline after activation of the surface. This is followed by covalent coupling of the ligand to the dextran matrix. The buffer washes away the loosely associated ligand molecules. Deactivation and further washing away of loosely associated ligand happens and the

difference in response between these two points reflect the amount of anti-beta two microglobulin immobilized.

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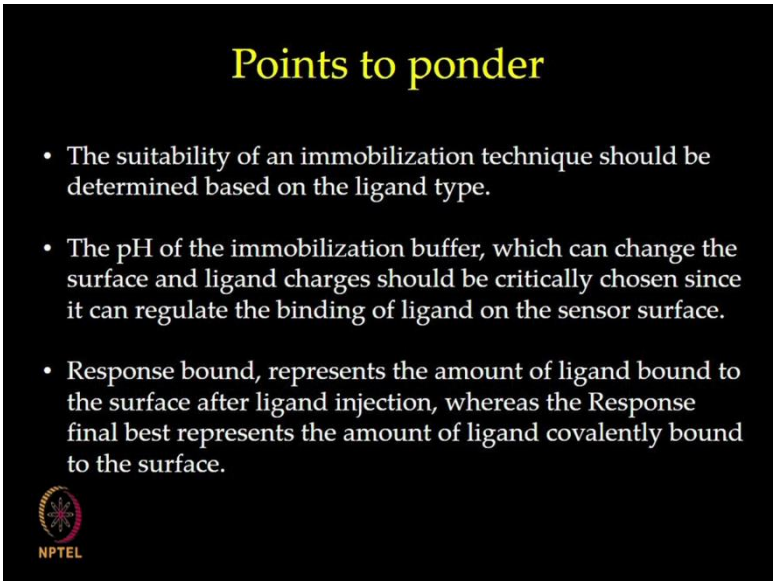

So, as we observed we have successfully immobilized anti-bets two microglobulin and we will now proceed for our binding experiment in our next lecture. Performing an interaction analysis on active and stable ligand surface is key to generate robust data set. We have witnessed this procedure for immobilizing anti-beta two microglobulin antibody on CM5 sensor chip surface. In next lecture, we will talk about protein-protein interactions study binding analysis. Thank you.

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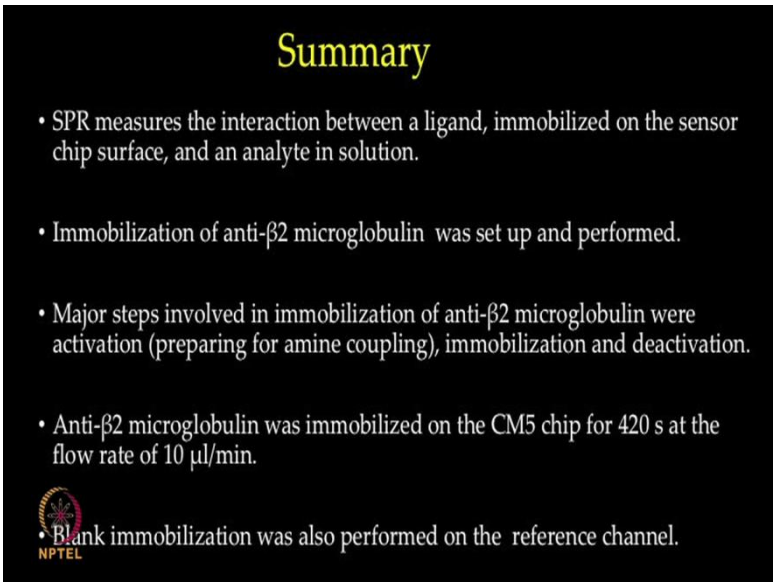

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