Interactomics: Basics and Applications

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Lecture - 33
Protein Immobilization for Protein-protein Interaction Studies

Today, we will talk about Protein Immobilization for Protein-protein Interaction Studies. Surface plasmon resonance has transformed the study of biomolecular interactions by developing a platform that does not require the ligand or the analyte to be labeled. SPR measures this interaction between a ligand which is immobilized on the sensor chip surface and a analyte, which is passed in the solution form. This measurement takes place in a real time, label free environment which provides kinetics, affinity and concentration based ligand substrate interaction information. In the last lecture, we focused on the important parameters that are to be considered while designing an spr based assay.

In today's lecture, we will talk about immobilization of the ligand on the sensor chip surface to study the protein-protein interactions. In today's lecture on protein immobilization, the ligand anti beta 2 microglobulin will be covalently immobilized on the surface of the sensor chip using amine coupling chemistry. The direct immobilization of the ligand onto the chip surface is known as direct coupling. Another way of immobilizing a biomolecule is by using capture chemistry, where the ligand is not covalently immobilized to the sensor chip surface; but is captured through the electrostatic interactions.

The major steps involved in the immobilization of anti beta 2 microglobulin antibody will involve activation, preparation of amine coupling, immobilization and deactivation. So, let us have an experimental lab session on protein immobilization.

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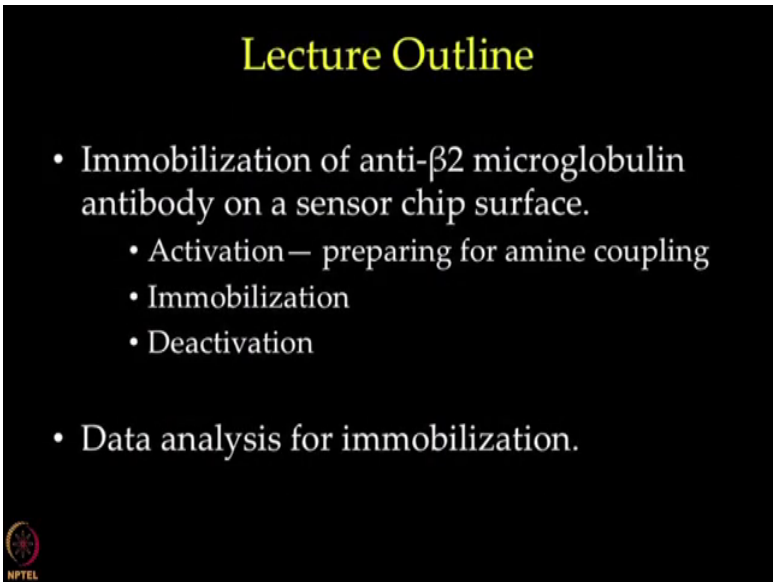

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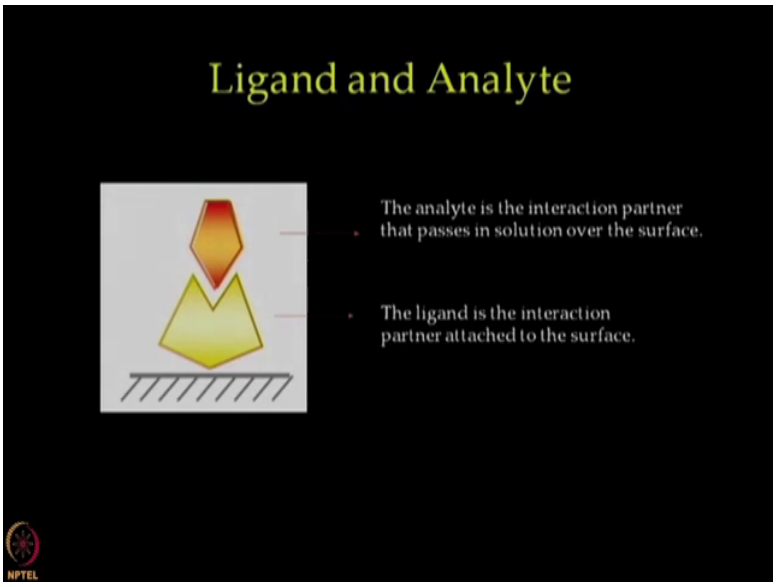

Let us learn a little about the basics of immobilization and SPR assay. In the molecular interaction study using surface plasmon resonance, we will immobilize one of the interacting molecule that is anti beta 2 microglobulin on the gold sensor chip surface; while the protein beta 2 microglobulin will 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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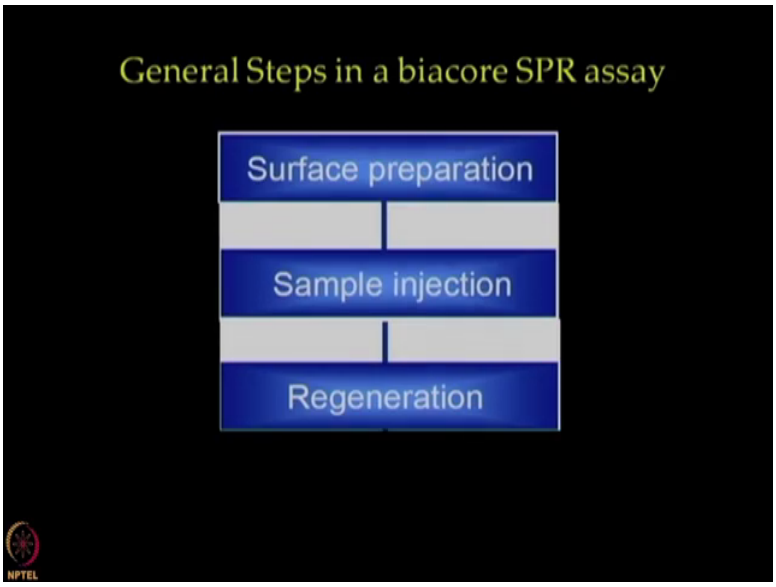

The three major steps in a biacore, SPR assay involve immobilization, the process by which ligand is attached to the sensor chip surface; interaction analysis, where the analyte is injected over the sensor chip surface and the interaction between the analyte and the immobilized ligand is monitored and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

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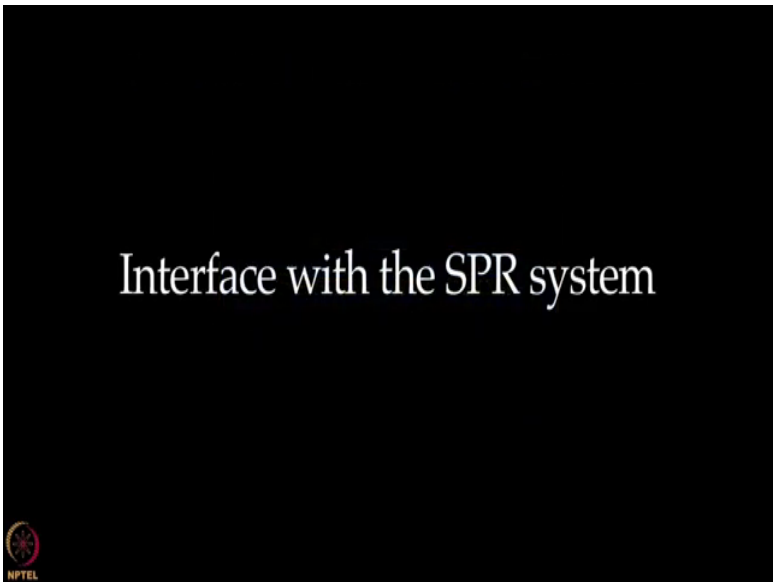

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So, this is a surface plasmon resonance and it is a Biacore T 200. So, this machine has the various parts like, the running buffer is connected here and any of the biological buffers can be connected as a running buffer. They could be have any pH from 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 needles 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 at 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.

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Here are some kind of indications are provided; when the machine is ready or the system is calibrating the 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.

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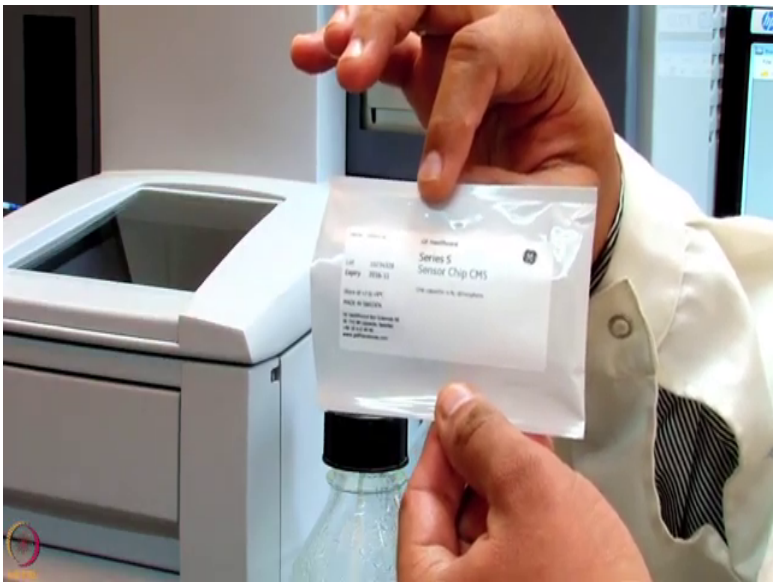

So, this is a new chip, the chips are generally provided in these cassettes.

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And the new CM 5 chip will look like this.

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This chip will be inserted in the chip docking area. From the control software, we will eject the sensor chip that is connected.

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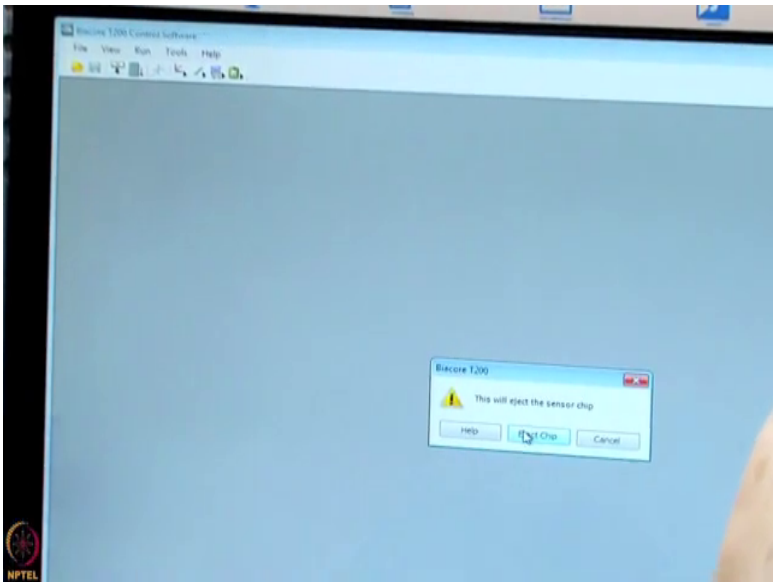

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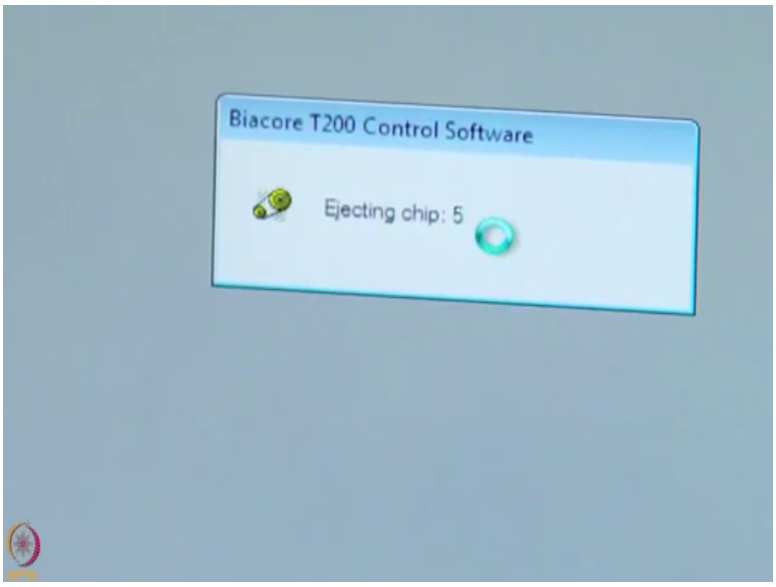

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So, now the old chip will be removed and we will insert a new biacore chip CM 5 here. And the insertions or the way orientation of the chip is shown on the chip in arrows. And we will close the compartment door and identify the chip from the chip type here.

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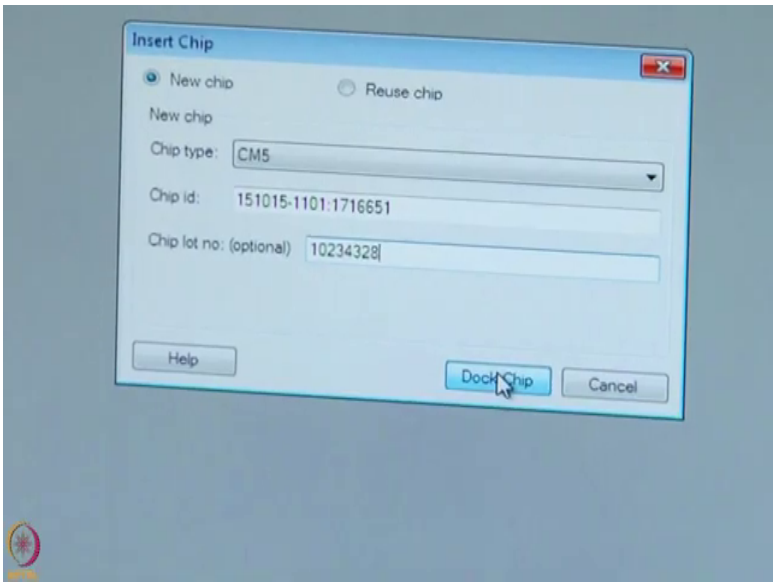

And chip will be given a new id and sometimes it is very essential to add the name for the chip and also the lot number and say dock chip.

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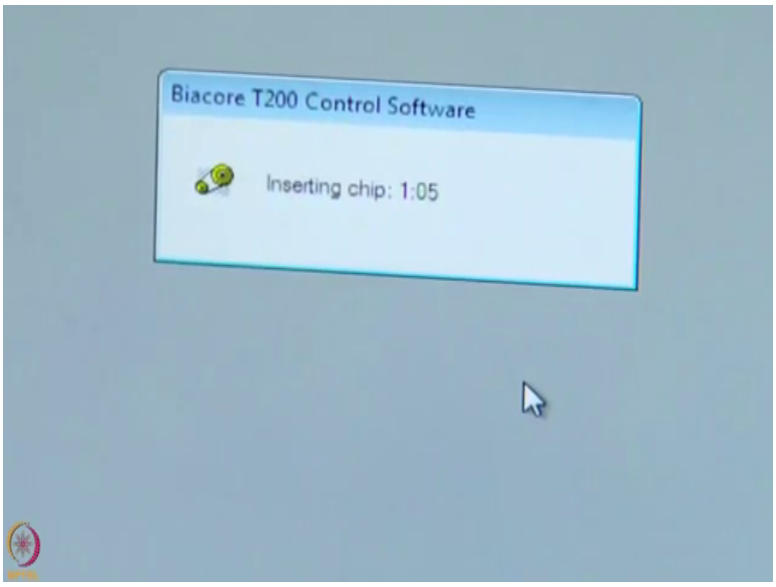

Now, the chip is getting docked. So, we have connected a new buffer, we will prime the system. Priming is the process of sending buffer through the IFC and equilibrate the system before our experimental. Generally in any biacore experiments, buffer should be connected and equilibrated overnight. Or if there is no time a minimum of 3 hours of equilibration is essential; otherwise when to start a new experiment do at least 6 primes on the system.

Now, that the chip is docked, we will prime the system.

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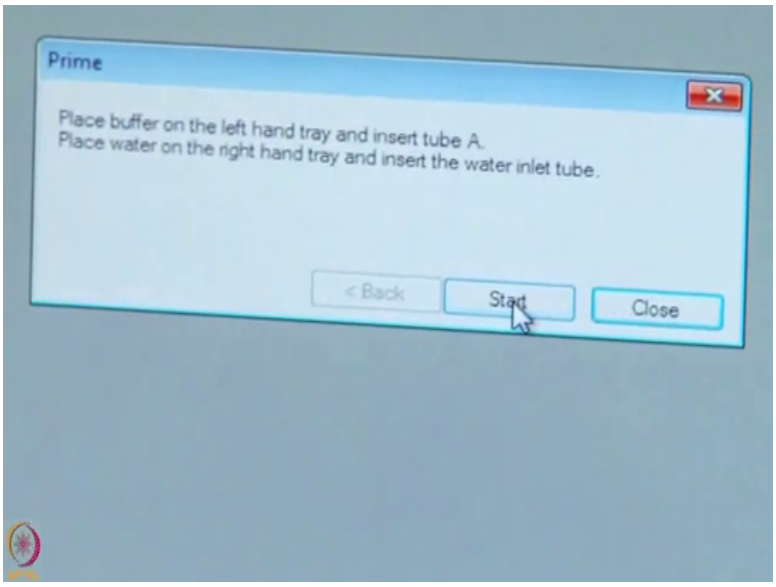

Finally we have connected the buffer. So, we will just click start.

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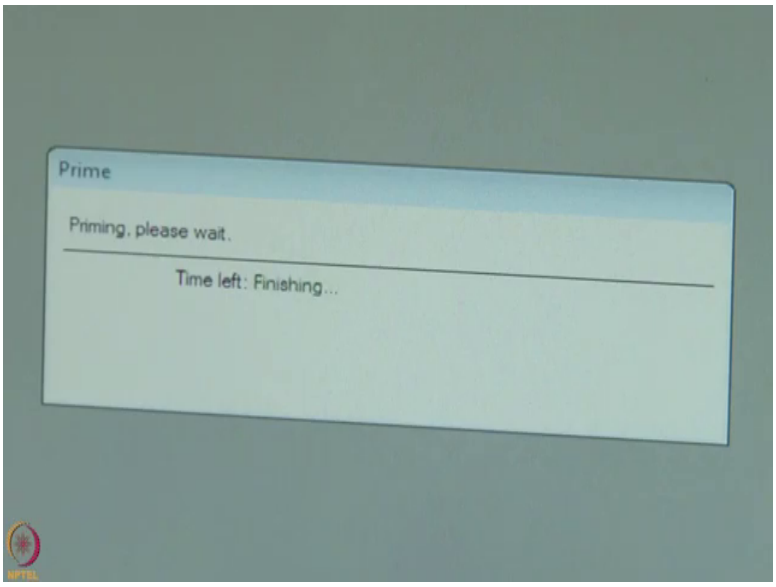

And it takes 6 minutes for the system to prime.

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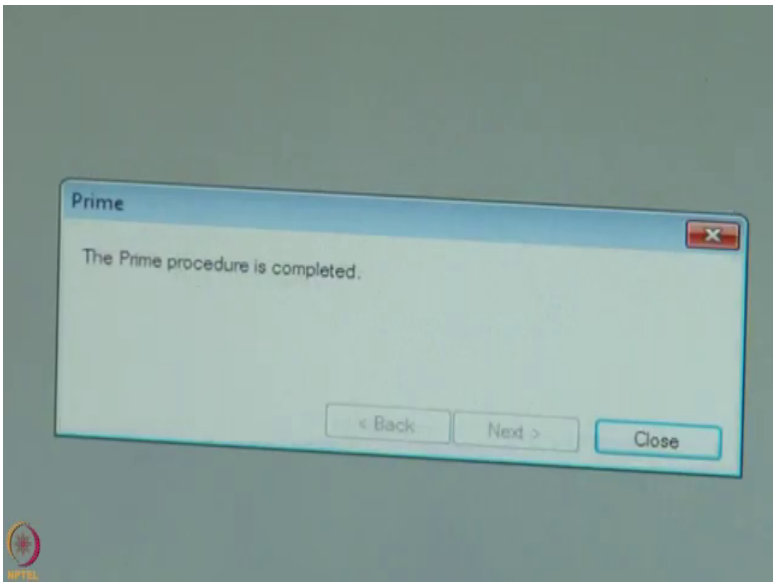

Now, the prime procedure is complete, we will do an immobilization of a ligand. In this case today, we are actually immobilizing anti beta 2 M antibody for that immobilization. Let us prepare a wizard.

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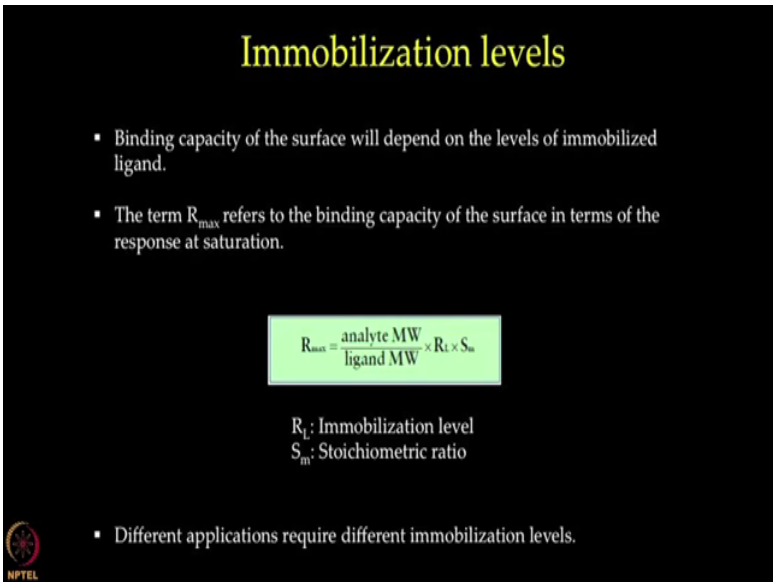

Before we set up an immobilization protocol, let us understand a little about immobilization levels.

The binding capacity of the chip surface will depend on the levels of immobilized ligand. The term maximum response referred to as R max is described as the binding capacity of the surface in terms of the response at saturation. A theoretical R max value can be calculated using the formula shown below, where R L is the immobilization level and S m is the stoichiometric ratio.

A theoretical calculated R max is often higher than the experimentally derived R max 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.

Different applications may require different binding capacities and thus different immobilization levels. A low R max is often beneficial in kinetic analysis, while higher immobilization levels are advantageous in binding analysis and concentration measurements.

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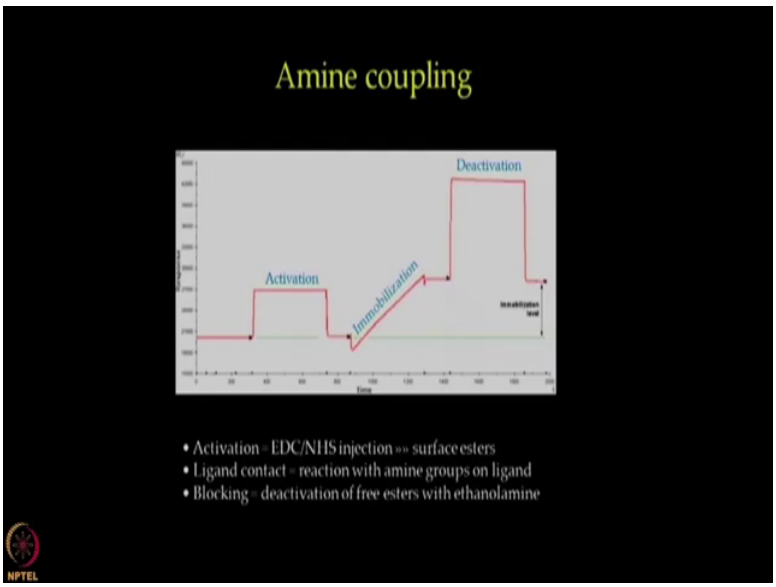

Today we are going to immobilize the antibody on a CM 5 chip using a mean coupling chemistry and the figure here shows a typical immobilization sensor gram using amine coupling. The three major steps involved here are; activation of the surface, esters using EDC and NHS, covalent coupling of the ligand on the sensor chip using amine groups of the ligand and deactivation of free esters with ethanol amine. We will analyze the results of anti beta 2 microglobulin immobilization later in the lecture.

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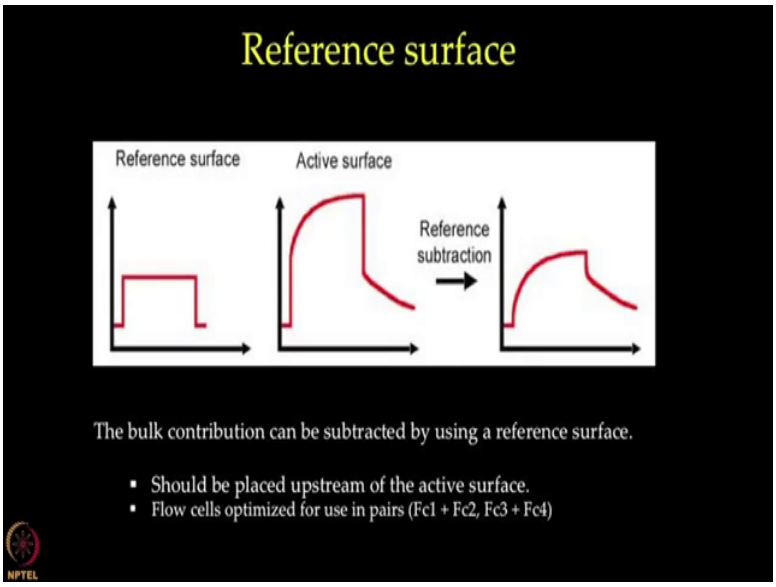

Reference subtraction is particularly important for assays, where measurement is taken during the sample injection. The bulk contribution due to any difference in the sample matrix and running buffer can be subtracted by using a reference surface. This reference surface is typically placed upstream of the active surface. The flow cells on the chip surface are optimized accordingly for use in pairs that is flow cell 1 with flow cell 2 and flow cell 3 with flow cell 4.

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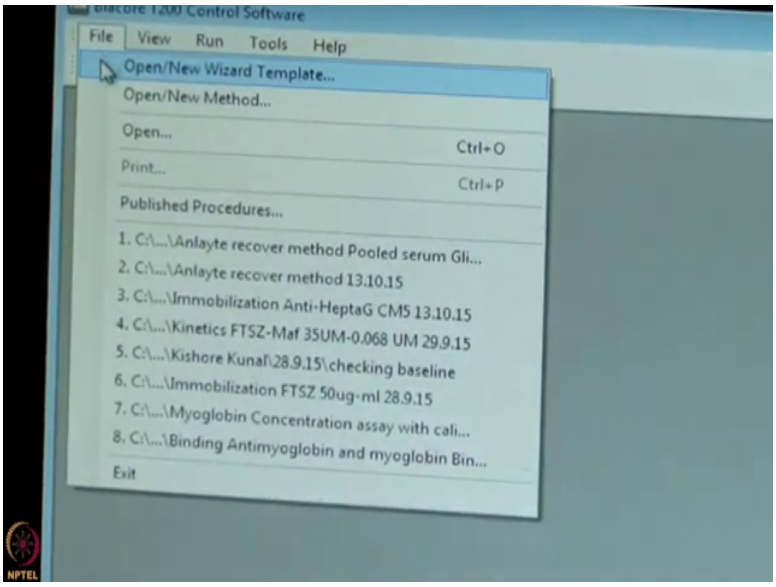

File, open new wizard template.

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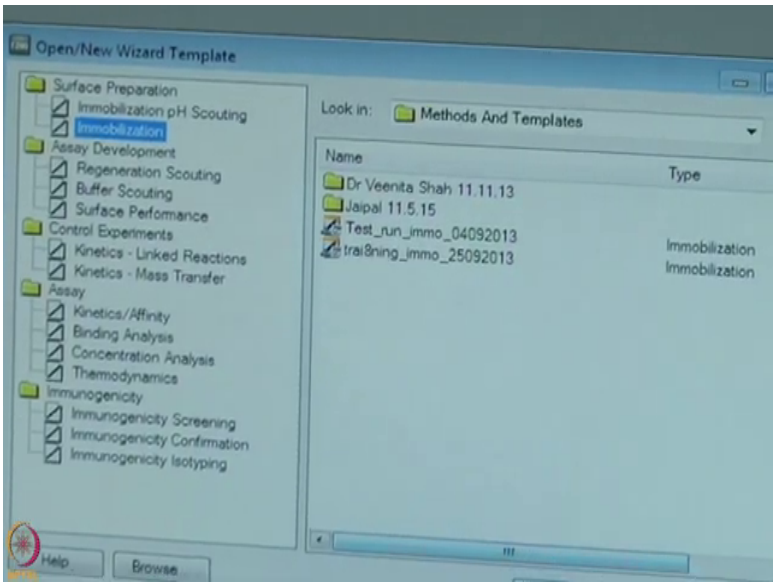

Identify Immobilization from surface preparation, say new.

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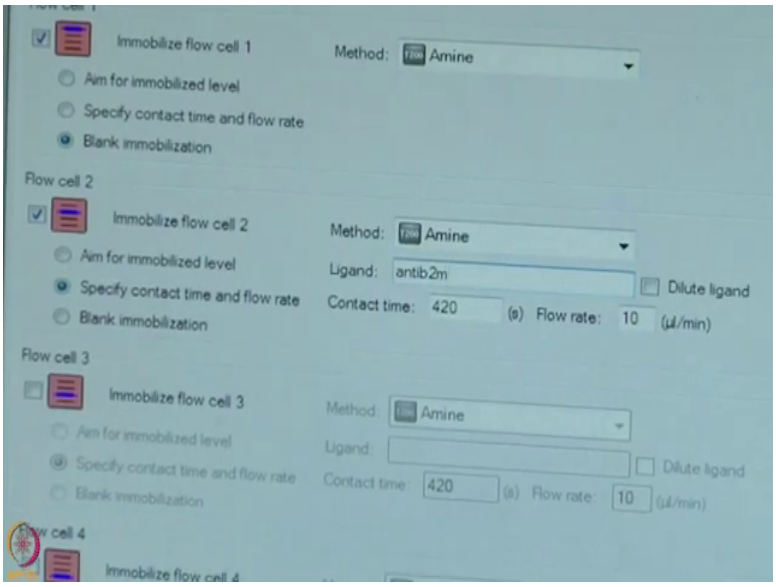

So, we will immobilize for binding; flow cell 1 and 2 for binding and 3 and 4 for kinetics.

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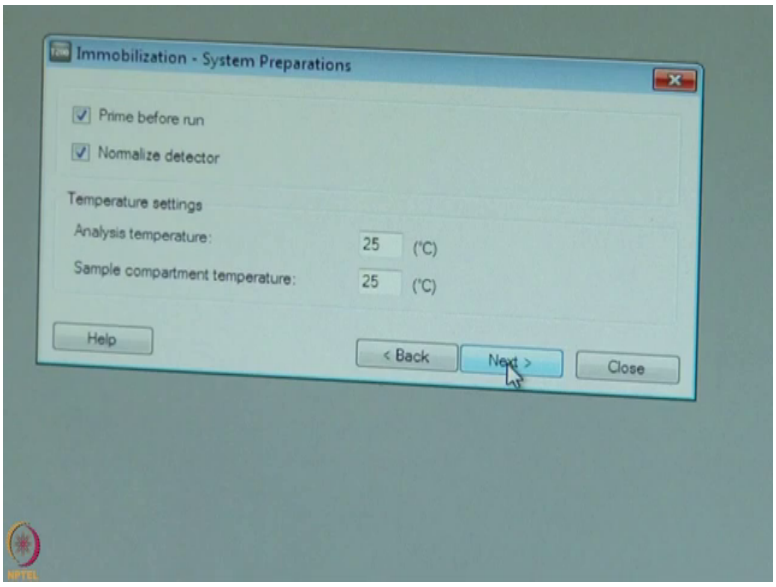

Prime before run, normalize detector, say Next.

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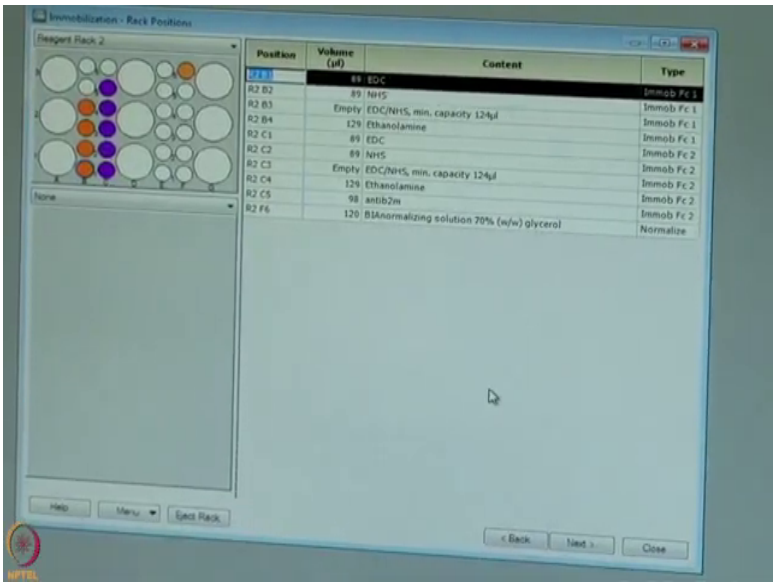

And the rack positions are displayed with the number of vials and the Volumes and the Positions list.

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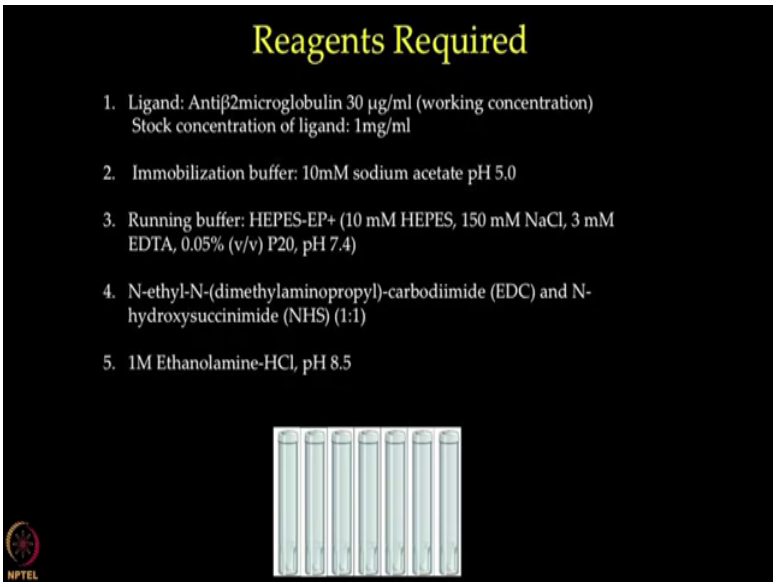

As was listed on the table, we will now have a closer look on the buffers and reagents required for immobilization of anti-beta 2 microglobulin. The reagents include a stock concentration of anti-beta 2 microglobulin, from which a working concentration of 30 microgram per m l will be made using an immobilization buffer of 10 millimolar sodium acetate pH 5. We need HEPES EP plus p H 7.4 which will include 10 millimolar HEPES, 150 millimolar N a C l, 3 millimolar EDTA, and 0.05 percent P 20. 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 1 is to 1 ratio for surface activation. Lastly, we also require 1 molar ethanolamine HCL pH 8.5 for blocking the free ester groups on the surface.

We shall now proceed to use the above mentioned reagents for our immobilization experiment.

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We will now work on the reagents required for the immobilization of anti beta 2 microglobulin on the cellular chip surface.

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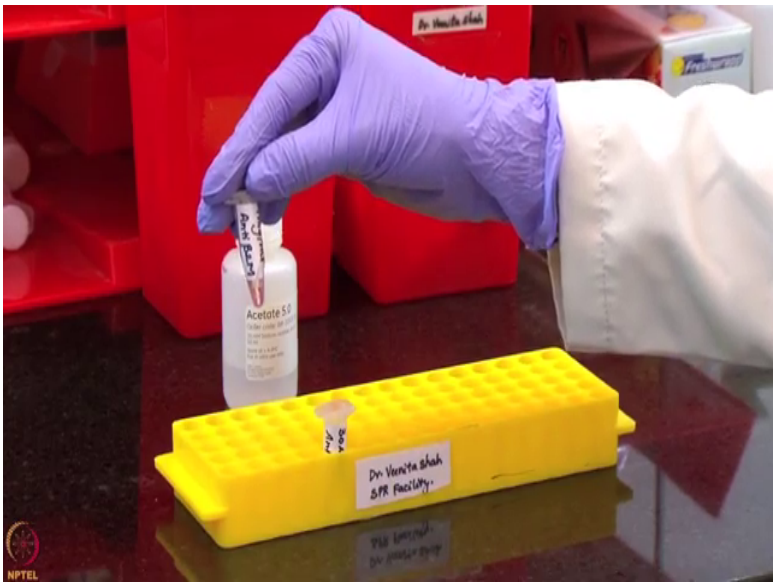

We will first tell you this stock concentration of the antibody which is 1 mg per ml of anti beta 2 microglobulin.

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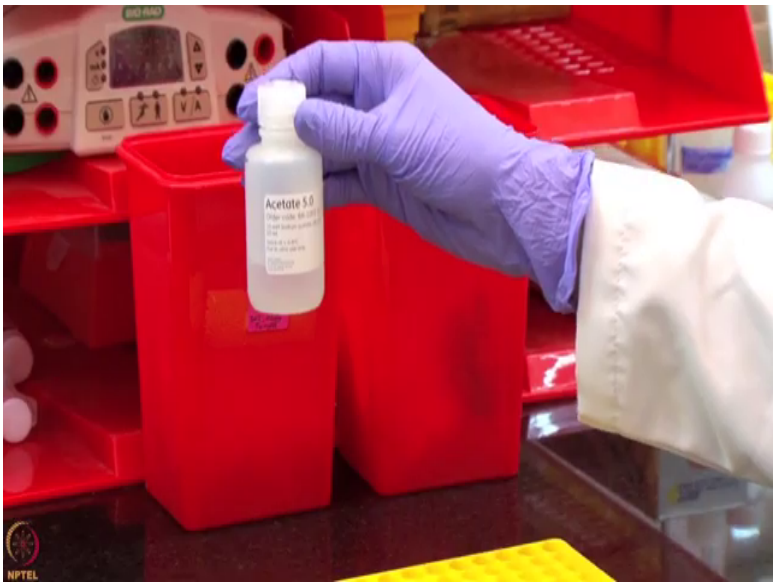

In 10 milli molar sodium acetate pH 5 to make a working antibody solution of 30 microgram per ml.

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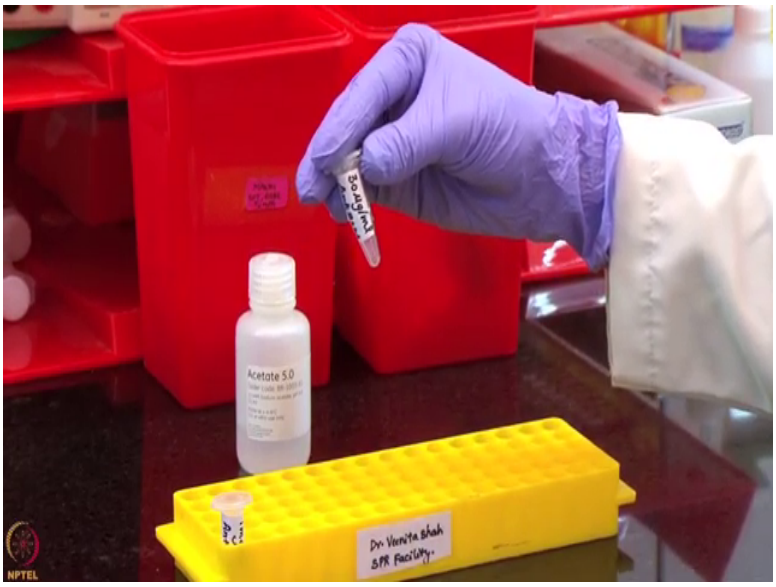

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For this, we will take 6 microliter of ligand stock and mix with 194 microliter of sodium acetate pH 5.

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The choice of the correct immobilization buffers is an important parameter to consider and the p H scouting features of the system can help in choosing the correct p H of the immobilization buffer.

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This is our 30 microgram per ml of ligand pH.

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Aliquots of NHS, EDC and ethanol amine are prepared and transferred to the specialized tubes used for the system.

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We have now transferred all the reagents into these specialized tubes. So, we have two NHS tubes, two EDC, two empty tubes for longer stability of EDC, NHS which will be mixed inside the system; two ethanol tubes, one for the blank flow cell and the other for the active flow channel and one ligand solution.

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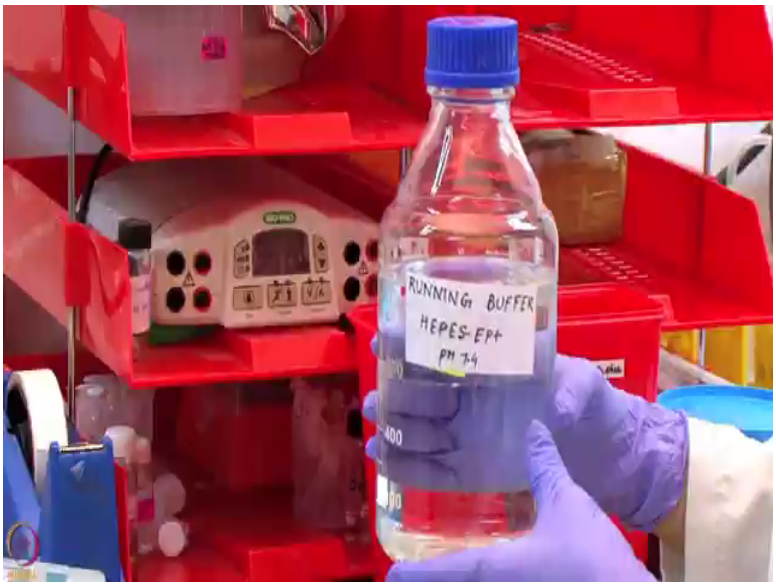

We will be using HEPES EP plus as the running buffer, which will be connected to the system prior to the initialization of the immobilization gram.

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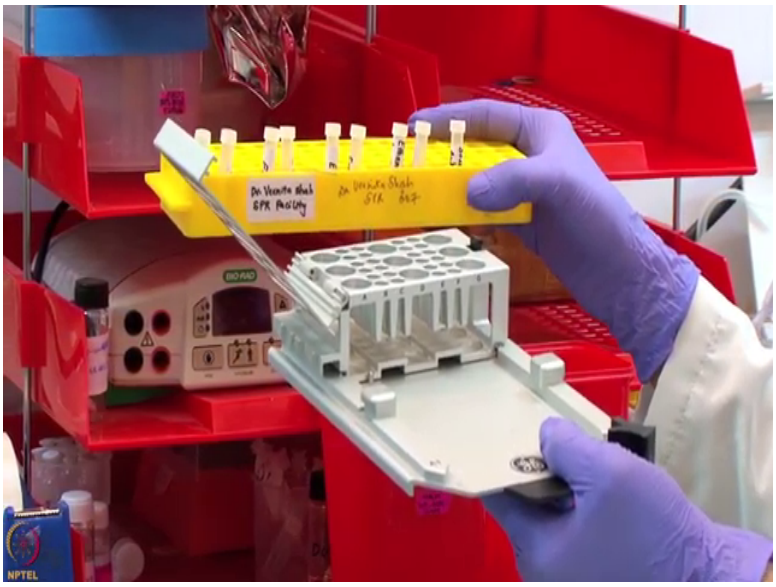

We will now proceed to insert these tubes into the appropriate rack and then into the system for immobilization of anti beta 2 microglobulin on the sensor chip surface.

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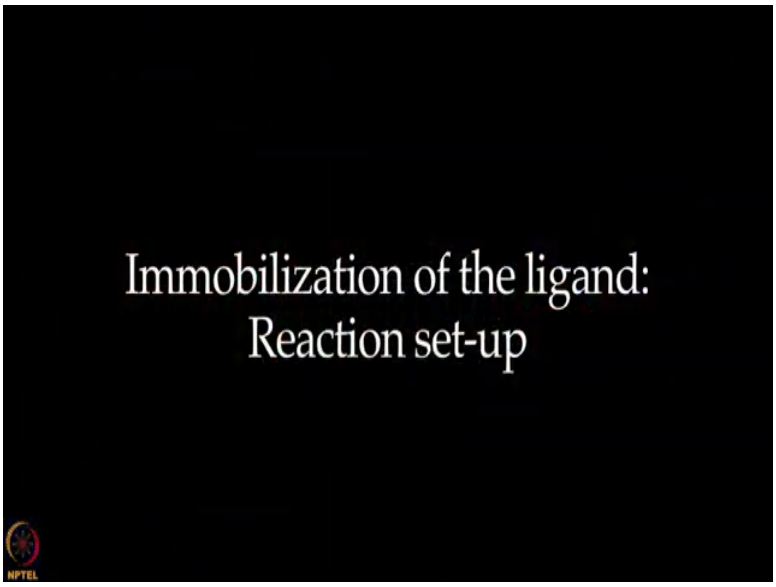

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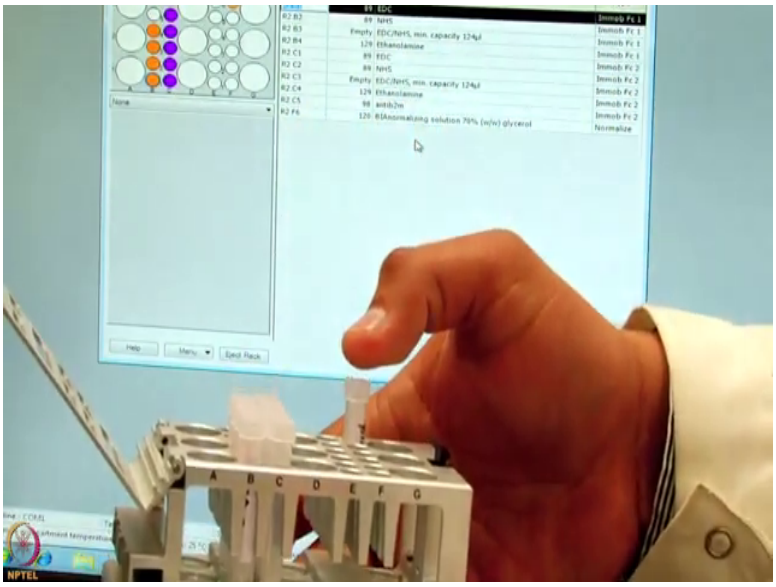

Now, that we made the template, we will insert the vial in the right position. We just filled them with the required volumes of different chemicals to start with the EDC is positioned here, the NHS is positioned here, the ethanol amine is positioned here, and there is an empty vial. In a similar way another row is also made; but so one for the reference surface, another for the active surface. To normalize the chip, normalization solution for the machine; to normalize the discrepancies on the RU responses on four different flow channels.

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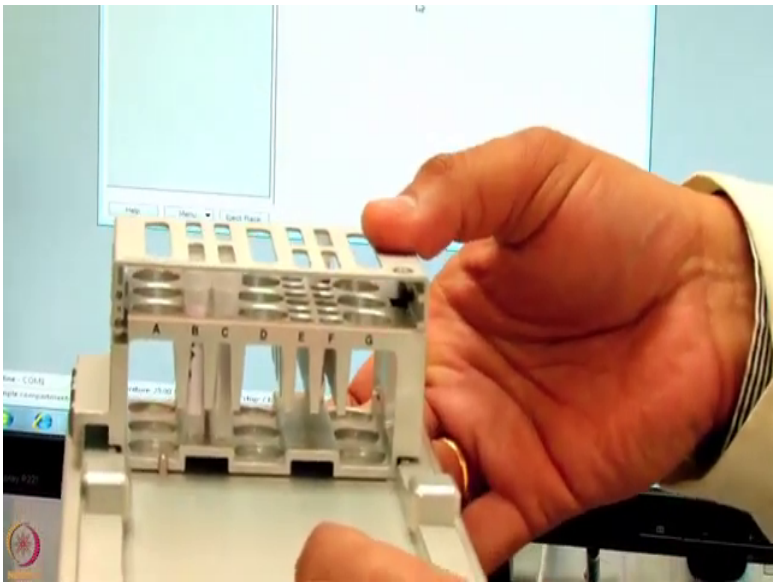

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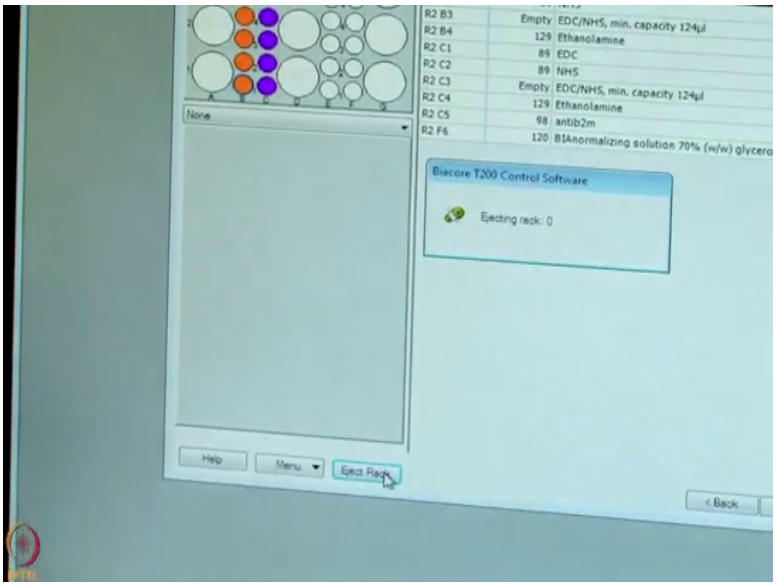

We close the door this way and go on the screen here. We eject the rack.

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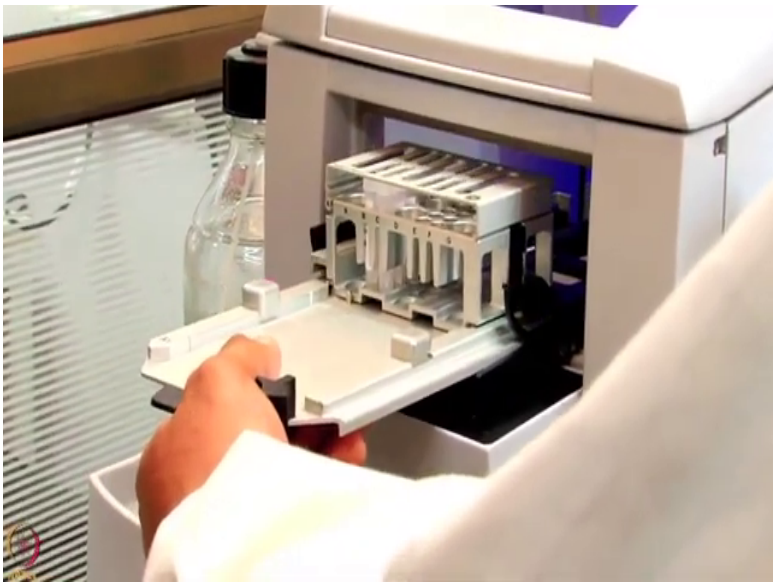

We will insert the rack into the sample compartment this way and we need to lock it inside. Lock it inside and then go on the screen. Close the compartment. We go to the Next tab.

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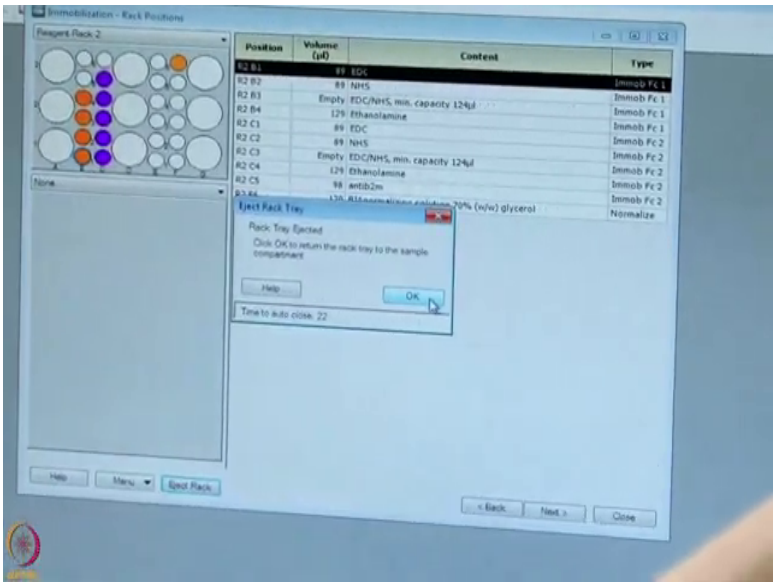

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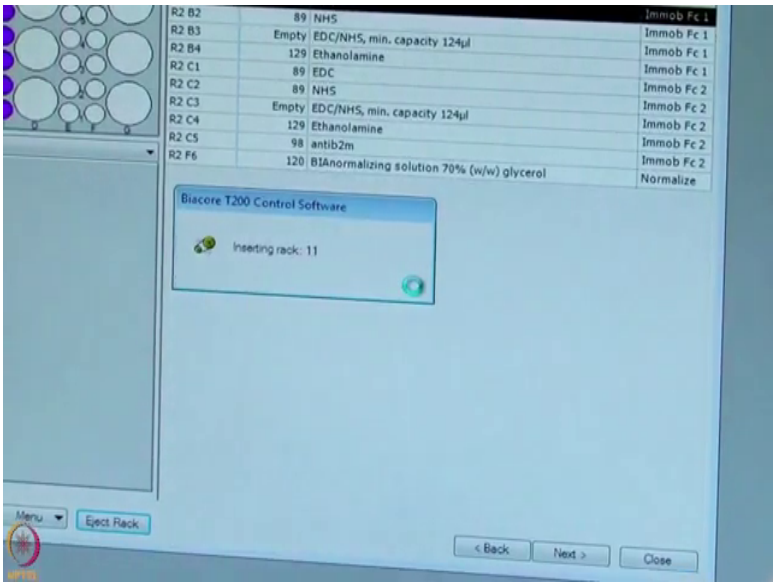

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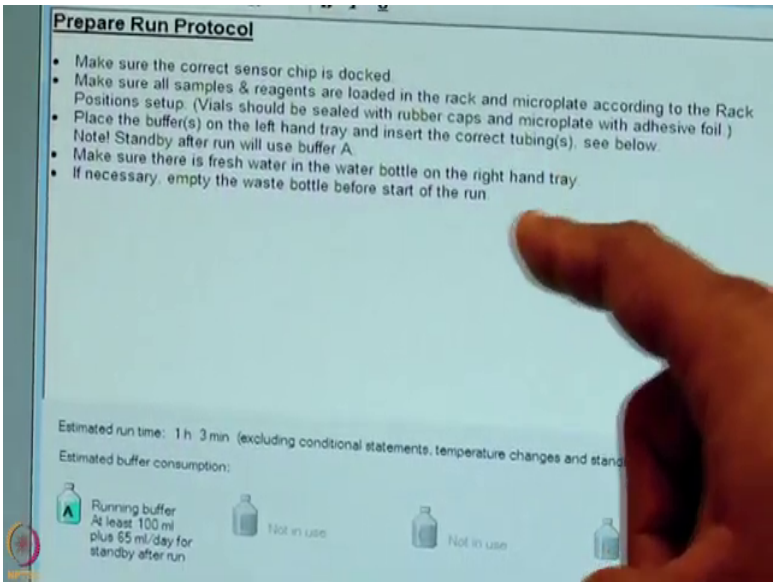

So, here there are some points we need to take care before start of the run. Make sure the correct sensor chip is docked, make sure all sample regents are loaded in the rack and microplate according to the rack position set up. Vials should be sealed with caps, place the buffer, place water and make sure there is sufficient amount of water and buffer.

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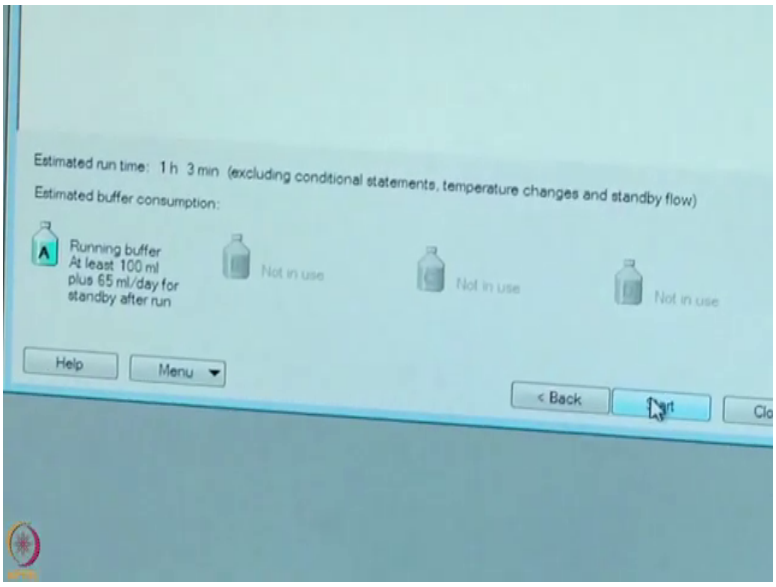

So, once we go through all these and everything is set in the machine; it shows the estimated run time as 1 hour 3 minutes and we have connected the running buffer. Now we will start the experiment. This will prepare the chip for the immobilization process, and immobilize the antibody or the ligand of our choice today the anti beta 2 m. We need to save this as and now the immobilization process is running.

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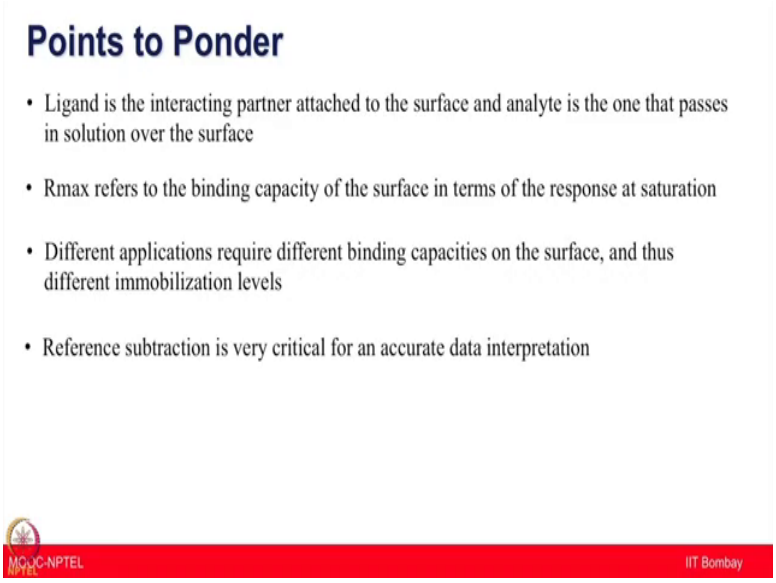

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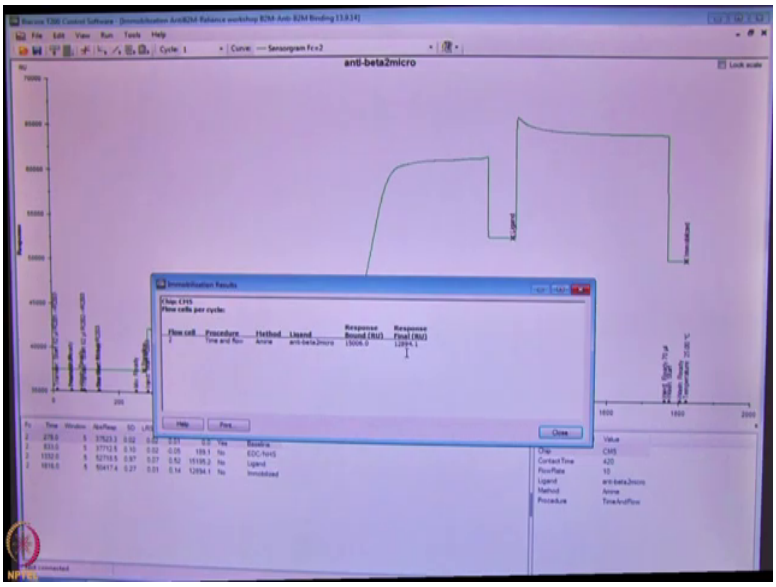

We will now look at the results of our anti beta 2 microglobulin immobilization performed on a CM 5 chip. Looking at the immobilization results, dialog box there are two response levels calculated from the sensorgram. The response bound which represents the amount of ligands bound to the surface after ligand injection; whereas, the response final test represents the amount of ligand covalently bound to the surface. So, here as we observed 12894 RU of anti beta 2 microglobulin is immobilized on the surface of the chip.

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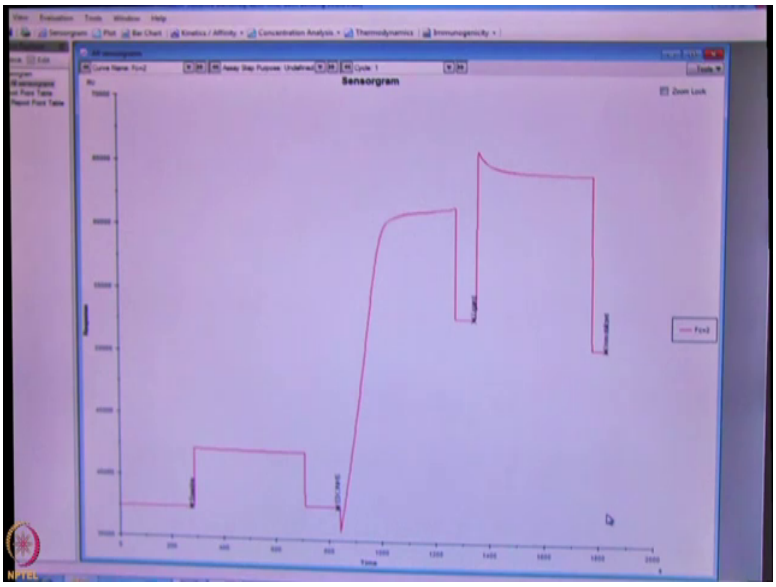

Now, analyzing the immobilization sensorgram, we observe the baseline here followed by EDC, NHS activation of the dextran matrix, which is again followed by baseline after activation of the surface. This is followed by covalent coupling of the ligand to the dextran matrix. The buffer washes away the 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 2 microglobulin immobilized.

So, as we observed, we have successfully immobilized anti beta 2 microglobulin and will now proceed for our binding experiment in our next lecture.

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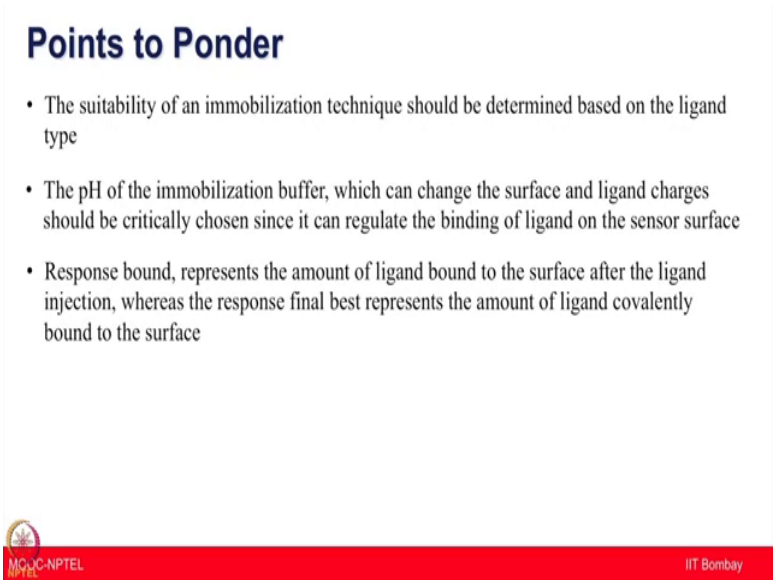

I hope today's lab session was informative and you got an idea how to perform these experiments in the laboratory settings. Performing an interaction analysis on active and a stable ligand surface is a key to generate the robust data set. Today we have witnessed this procedure for immobilizing anti beta 2 microglobulin antibody on CM 5 sensor chip surface. In the next lecture, we will discuss about the binding analysis that is performed during protein-protein interaction studies

Some of these reagents which we are talking these are standard reagents like anti beta 2 microglobulin and purified protein; but you can definitely try out the similar procedure on your protein of interest and the antibody of interest. The essential guideline for performing the experiments will remains the same.

I hope these sessions are going to build the confidence for you that you can utilize this knowledge to perform your own experiments on your own protein and antibodies of interest, will continue more of the lab demonstration in the next lecture.

Thank you.