Interactomics: Basics and Applications

Prof. Sanjeeva Srivatsava
Department of Biosciences and Bioengineering
Indian Institute of Technology, Bombay

Lecture - 37
Protein - small Molecule Interaction Study: Immobilization & Binding Analysis

Hello students. We are going to focus today on a small Molecule based Analysis. In the previous lecture you have learnt their identification of a small molecules, which binds specifically to the proteins or hotspot regions of the proteins; can become crucial in discovery. And development of new pharmaceutical drugs and for elucidating the complex pathways in biological systems.

The primary goal of a small molecule screening in pharmaceutical industry is to identify suitable candidates, on the basis of their binding to the selected target molecules. The first stage of the process often involves screening large candidate libraries to identify the promising candidates against the protein target. Usually the positive controls are used to test the suitable binding activities if a control analyte is available.

The small molecules are often dissolved in organic solvents. Therefore, to maintain the analyte solubility the concentration of organic solvent in samples and in the running buffer becomes very important. The response obtained from a small molecule analytes is inherently low because of their molecular weight. Therefore, the sensor surface for a small molecule screening are prepared with high level of ligand, ranging from a 1000 to 10000 RU for a average sized protein.

Similar to the previous lecture, where we have performed the immobilization of proteins. In this lecture we will simultaneously immobilize 2 proteins carbonic anhydrase and human serum albumin on 2 different flow channel of a CM 5 sensor surface. The chip will be used to demonstrate a small molecule screening where 8 drug molecules will be tested. We will now proceed with the immobilization and a screening analysis of the small molecules on these two selected target proteins. So, let us have this lab experimental session now.

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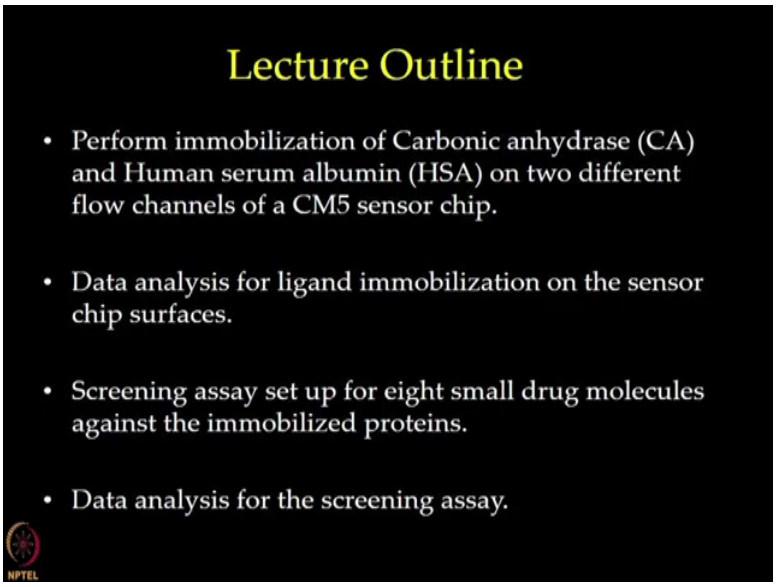

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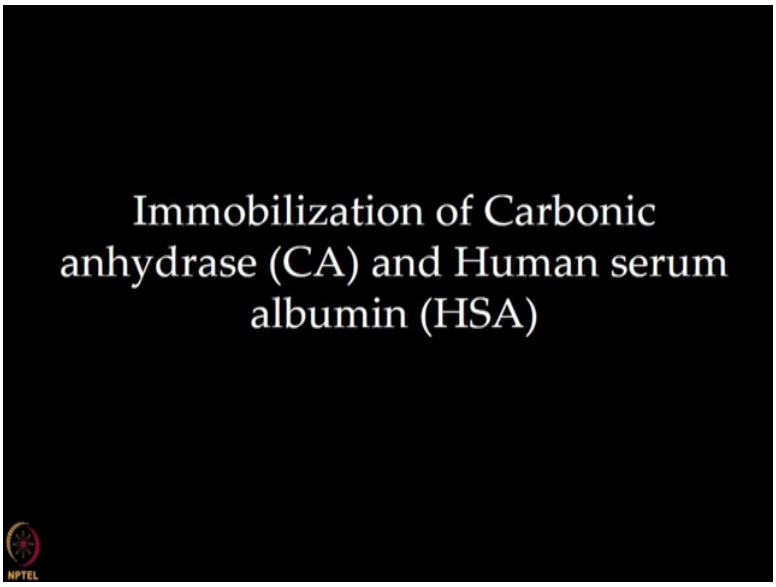

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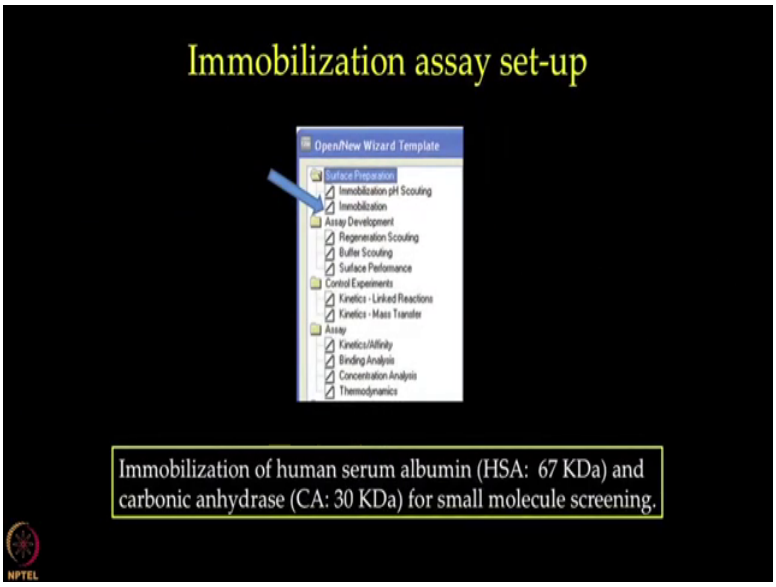

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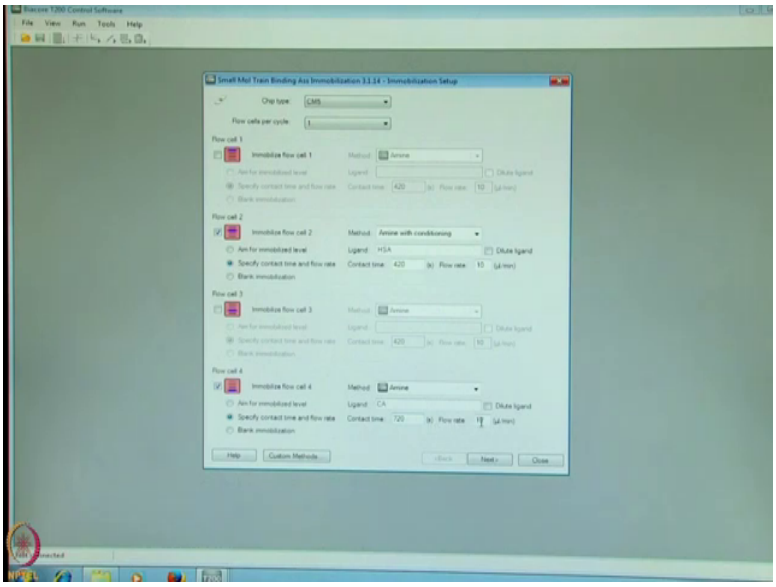

In this session, we will understand the interaction of proteins with small molecules. So, to start with small molecule interaction, we have to start again with the immobilization we go into binding or screening and we go into kinetic mode to determine the affinity of small molecule. Here we have opened a new immobilization wizard for the immobilization of human serum albumin. And carbonic anhydrase on 2 different flow channels separated with 2 reference channels.

So, the chip selected is CM 5; flow channels per cycle is 1 on the flow channel 1 we leave it blank. And flow channel 3 also we leave it blank, flow channel 2 human serum albumin is immobilized, with a specified contact time and flow rate of 420 seconds. And flow rate of 10 microliter per minute. On flow channel 4 by amine coupling we will immobilize carbonic

anhydrase. Again we specify contact time and flow rate mode for 720 seconds and 10 flow rate.

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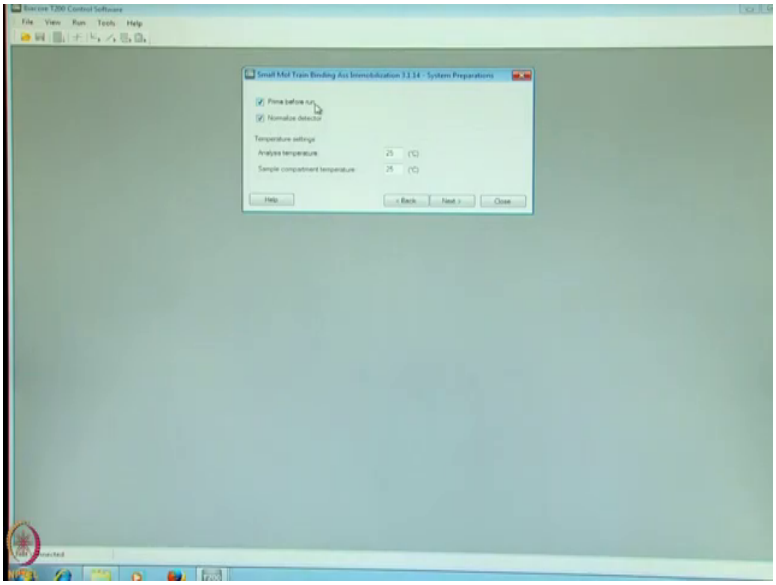

We now, go into the next tab we will highlight the prime before run. And normalize detector and temperature will be at 25 centigrade.

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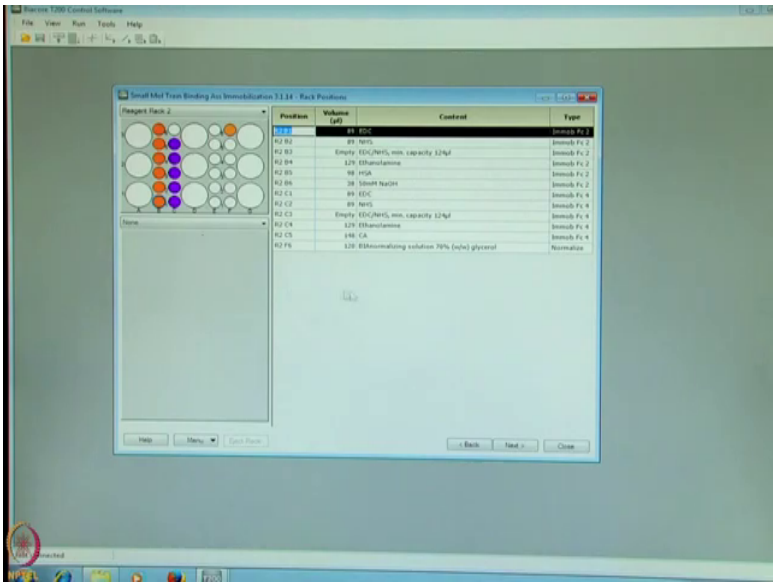

And we go the next tab and we have the rack positions, with the required amount of reagents shown here.

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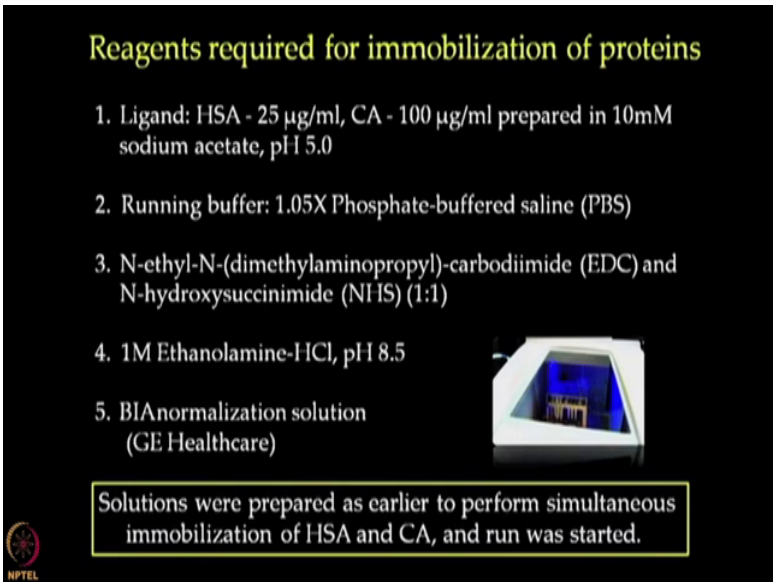

As per the volumes listed on the table we will now prepare buffers and reagents required for immobilization of these two proteins on the sensor chip. The proteins are prepared at working concentration of 25 micrograms per m l for human serum albumin and 100 microgram per m l of carbonic anhydrase using an immobilization buffer of p H 5.

We will be using 1.05X PBS buffer as the running buffer, which will be connected to the system followed by priming. EDC and NHS as prepared and suggested in the amine coupling kit, for surface activation will be used in 1 is to 1 ratio. 1 molar ethanolamine HCL p H 8.5 is prepared for blocking the free ester groups on the surface.

Lastly we would require BIA normalization solution to normalize the resonance signal in all flow channels to compensate for small differences between individual sensor chips. After preparation of these samples and reagents, we transfer the sample tube to the appropriate

racks and start the run as shown for beta 2 microglobulin immobilization in earlier lectures. We will hence move forward to discuss the immobilization results obtained from this run.

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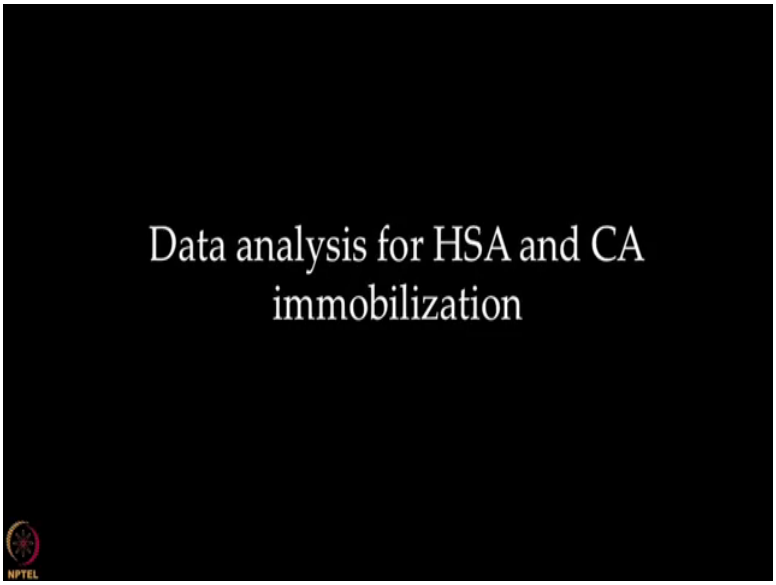

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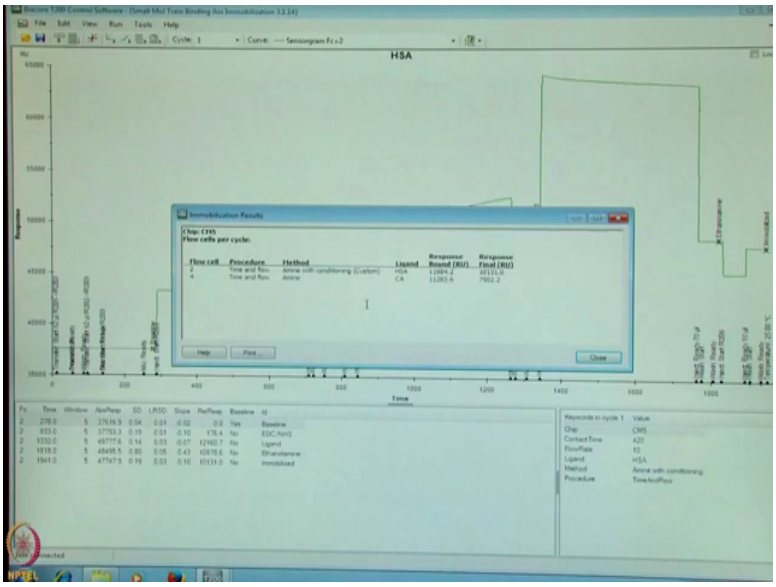

From our experimentation, the following are the results for the immobilization of human serum albumin and carbonic anhydrase on two different flow channels. We see here from the immobilization results.

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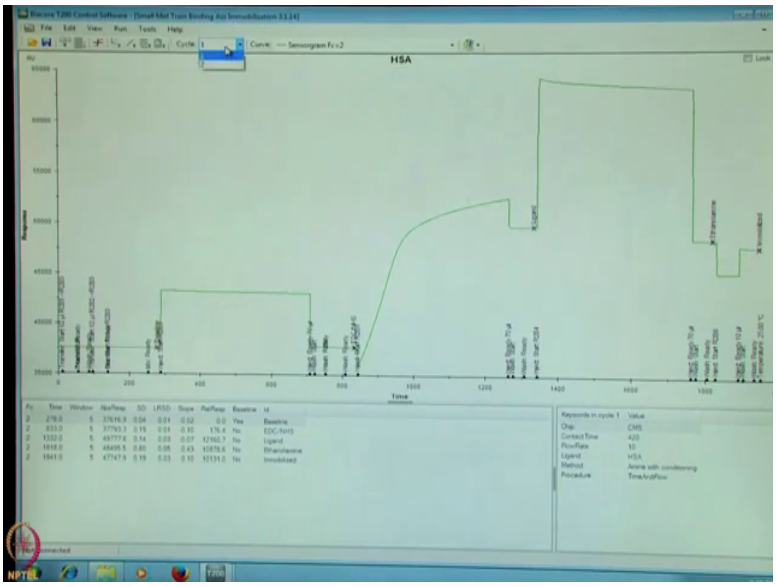

Here you can see on the screen immobilization of human serum albumin by activation of the surface with EDC NHS. Passage of the ligand to the sufficient amount required blocking with ethanolamine and immobilization.

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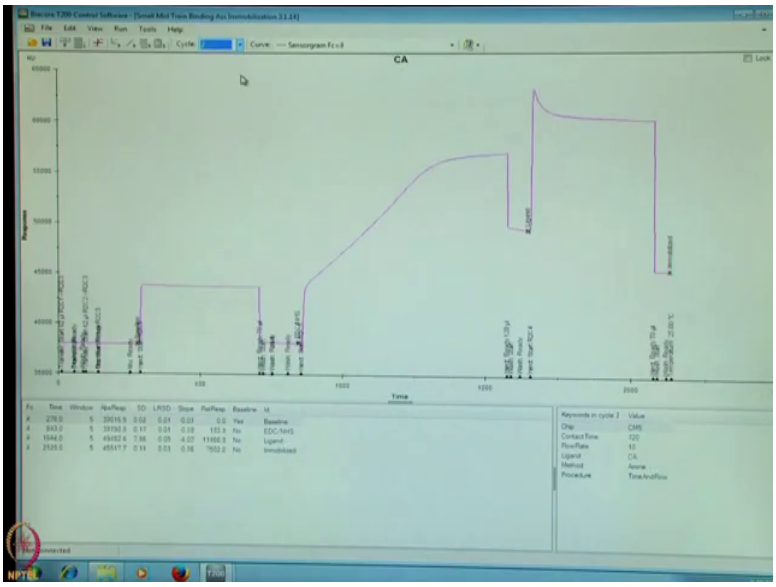

On the cycle 2 similarly you have activation with EDC NHS mixture, passage of ligand and blocking of the surface with ethanolamine to reach an immobilization level of 11466. With this we are done for the immobilization of HSA which is Human Serum Albumin and CA Carbonic Anhydrase; on the surface of the chip and the chip is ready to go for the binding experiment.

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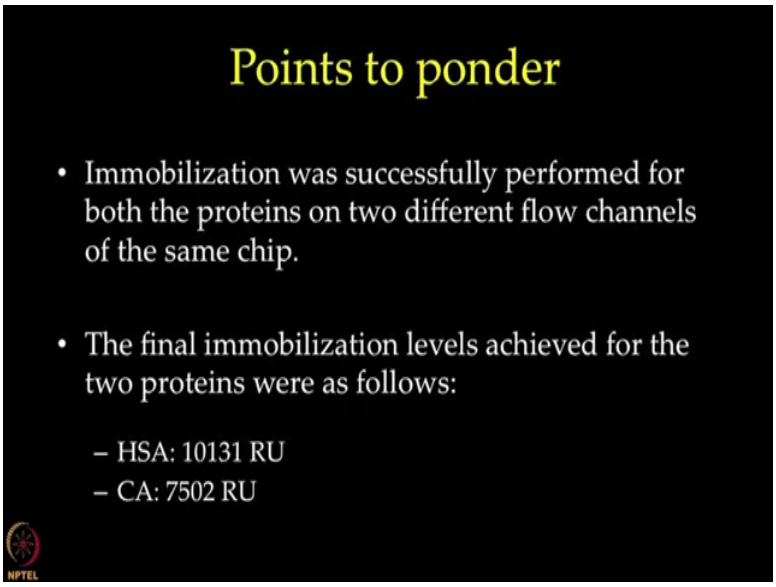

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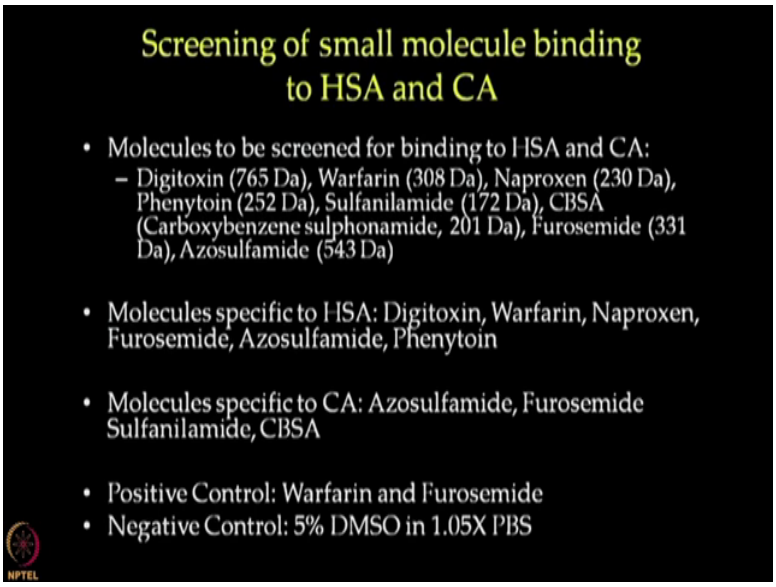

The small molecules to be screened for binding will include, digitoxin, warfarin, naproxen, phenytoin, sulfanilamide, carboxybenzene, sulphonamide, furosemide, azosulfamide. Some of these molecules are specific to HAS; whereas, rest are specific to CA; furosemides being specific to both. Warfarin will be used as a positive control for human serum albumin and furosemide for carbonic anhydrase. We will also use 5 percent DMSO in running buffer as negative control.

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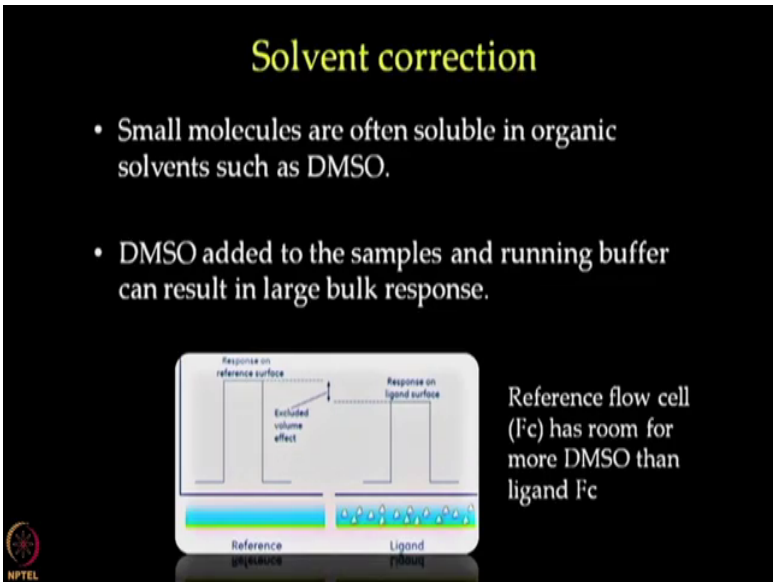

Since small molecules are often soluble in organic solvents such as DMSO and so are these drug molecules. It can result in large bulk responses, since reference flow cell has room from more DMSO than active flow cells with ligand.

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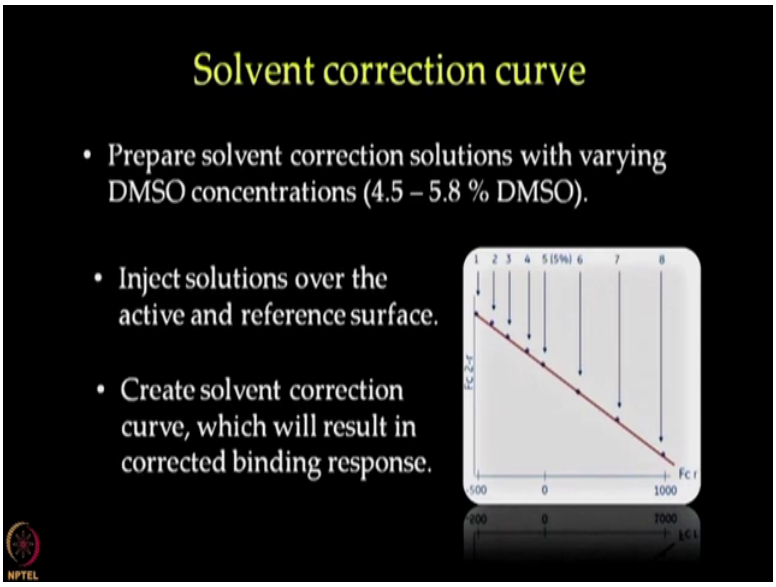

That is why we need to prepare a solvent correction curve by preparing solvent correction solution with varying DMSO concentrations; which will range from 4.5 percent to 5.8 percent DMSO. We inject these solutions over this active and reference surface. And then create a solvent correction curve which will result in corrected binding responses.

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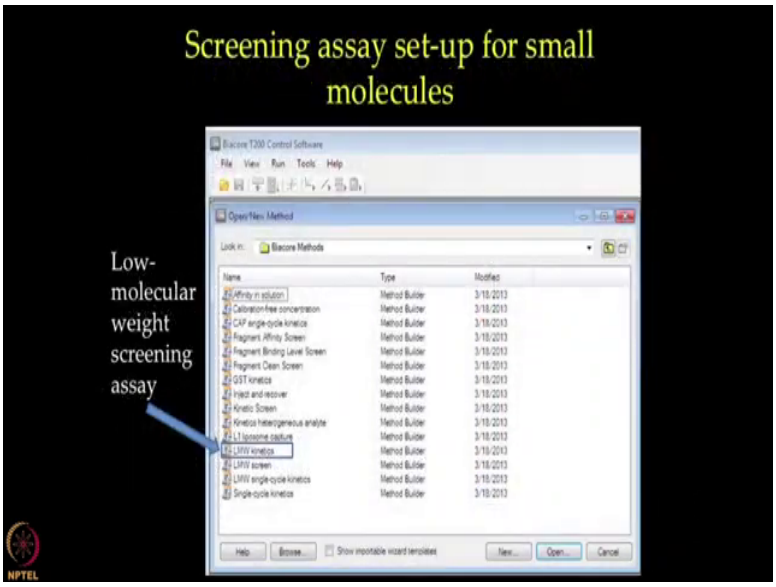

We will now move forward to set up the binding assay wizard to screen small molecules for binding to human serum albumins and carbonic anhydrase.

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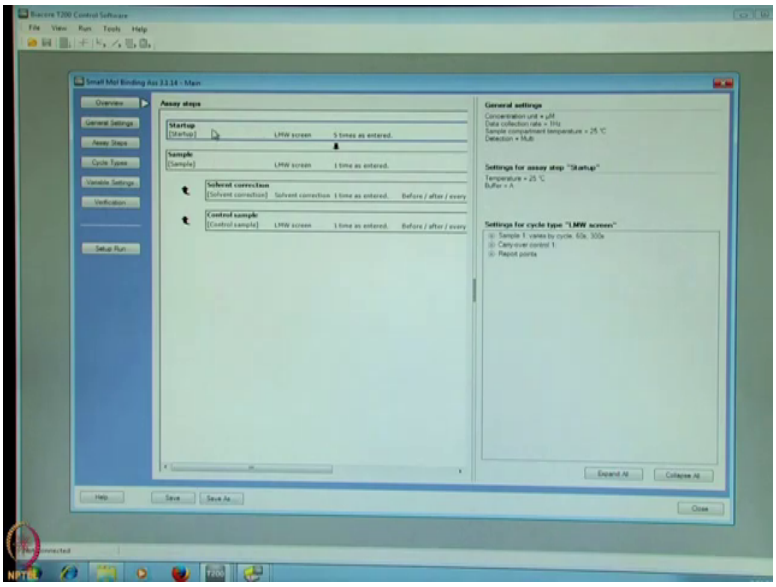

Here we will setup our experiment to check the interaction of some small molecules binding to carbonic anhydrase and human serum albumin. So, for that we need to prepare a method and the method actually has the startup 5, one round of sample injection.

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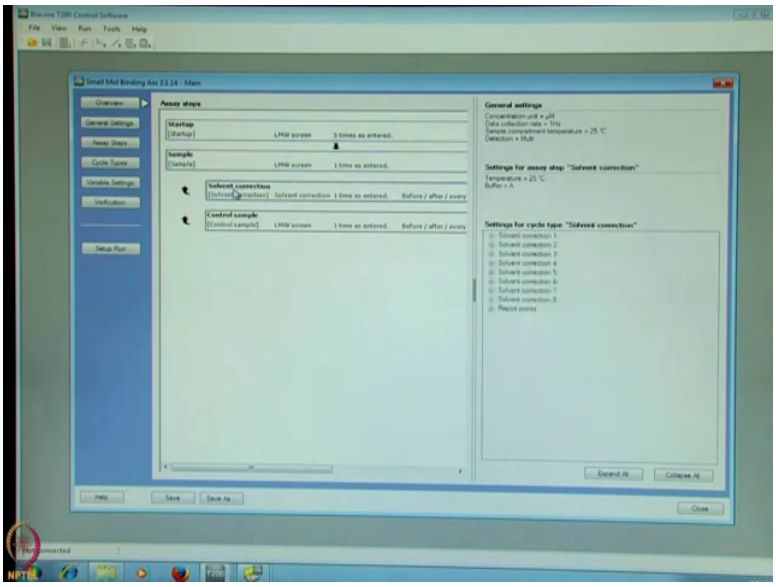

Solvent correction, which are 8 different correction solutions are passed on the surface and the DMSO effect on the response is subtracted.

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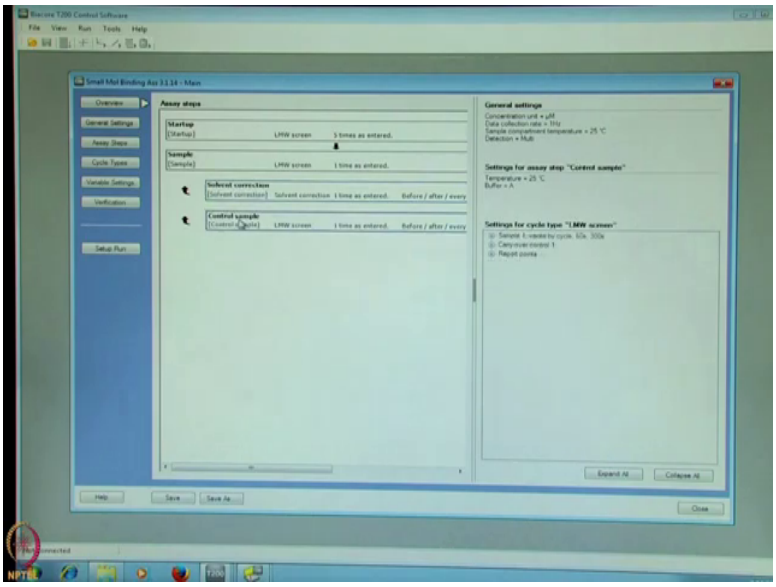

Control samples like positive and negative controls were also included in this mode.

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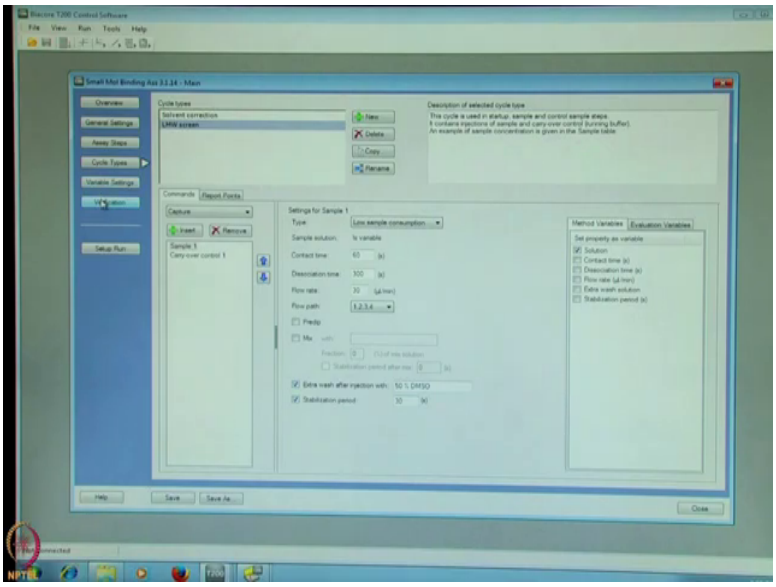

In the low molecular weight screen mode, we have the contact time of 60 seconds dissociation time of 300 seconds flow rate of 30 microliter per minute. And the molecules were flown on 1 2 3 and 4 channels. With an extra wash with 50 percent DMSO to prevent carryover effects stabilization time was 30 seconds.

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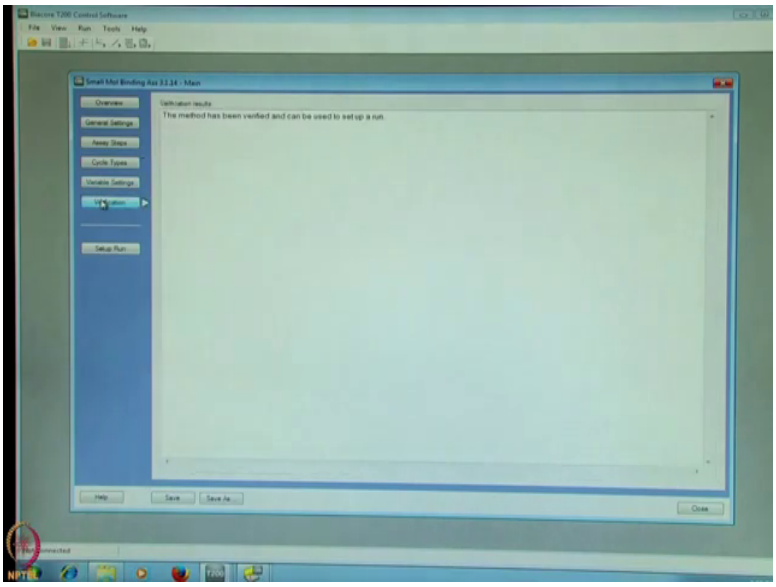

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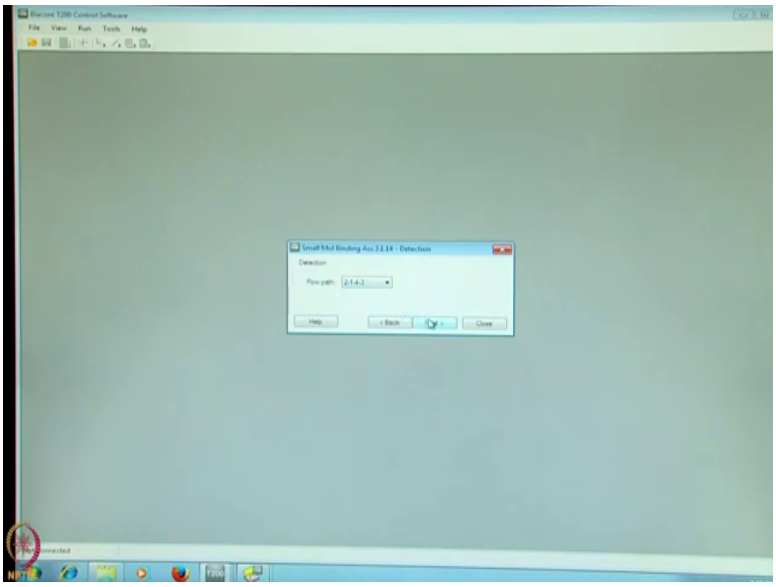

We verify this method and set up a run in which, it will have a subtraction of active surface 2 was subtracted from the one reference surface active surface 4 was subtracted to 3.

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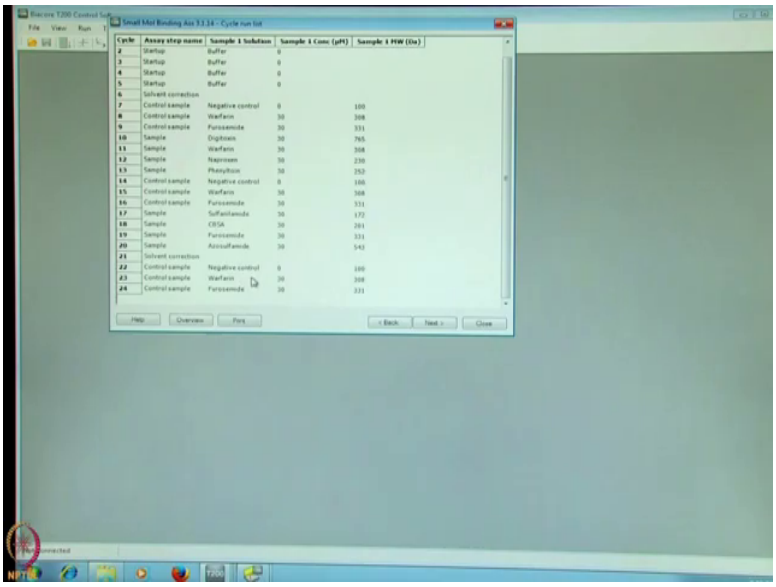

And we have the following effect of experiment setup here we have 5 startups; one set of solvent correction of 8 runs injected here 3 sets of control samples which include negative and positive control. Samples like digitoxin, warfaren, naproxen, phenytoin and a negative control were injected again and successively. Sample correction at every 30 cycles 10 cycles or 20 cycles depending upon how you setup the experiment. And finally, another round of control sample injected.

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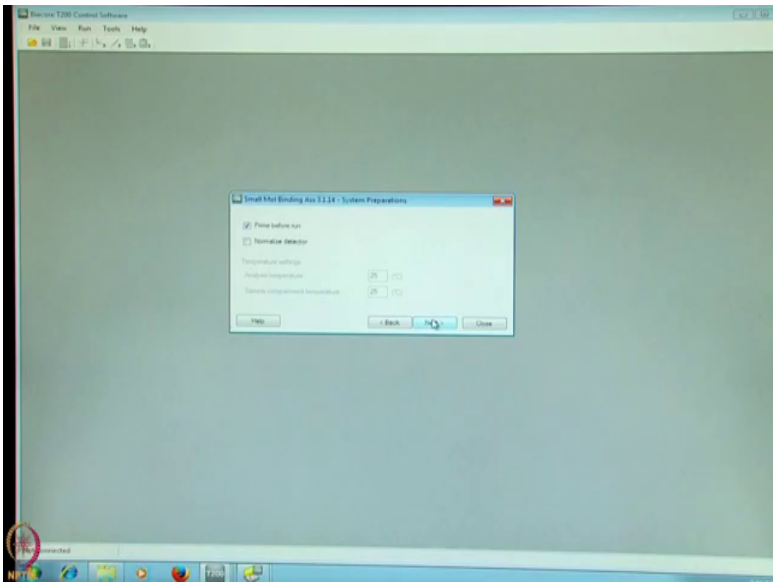

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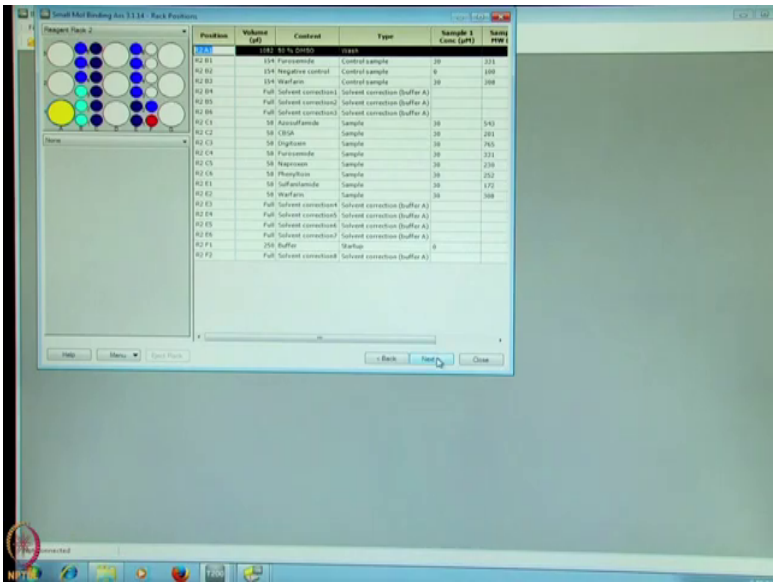

So, once we are done with that we highlight prime before run. And this is how we prepare the template, for the experiments. With solvent corrections provided buffer for the startup provided various samples pipetted out in DMSO and warfarin, furosemide as positive and negative controls.

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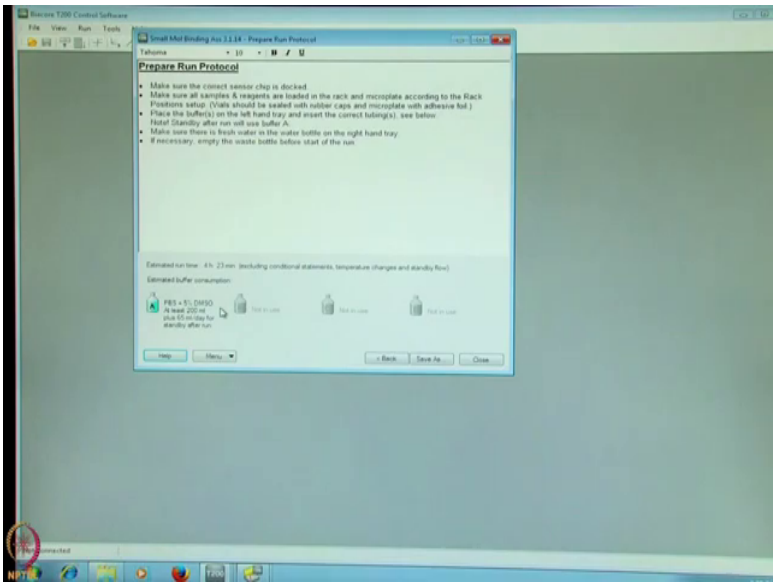

And 50 percent DMSO for wash in between each cycle. We will say next and the whole binding experiment including solvent cont correction takes 4 hours and 23 minutes. And requires at least 200 m l of phosphate buffer saline with 5 percent DMSO included in the running buffer.

Preparation of the running buffer preparation of the sample, checking for the solubility, checking for the aggregation is very important when we setup our small molecule interactions. Immobilization level should be high, machine should be cleaned at least or desorbed 1 day prior instrument should be in a equilibrated with the running buffer for at least 6 hours before start of the experiment.

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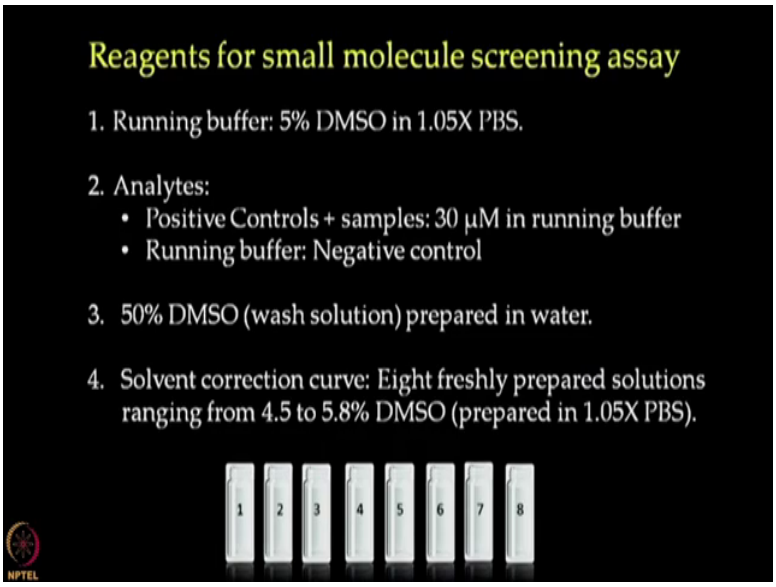

As per the volumes listed in the table, we will prepare the following samples and reagents. Running buffer will be 5 percent DMSO in 1.O5X PBS. Positive controls and samples at 30 micromolar concentration in running buffer and running buffer will be used as negative control. A wash solution is prepared with 50 percent DMSO in water. And lastly 8 freshly prepared DMSO solutions ranging from 4.5 percent DMSO to 5.8 percent DMSO are prepared for solvent correction.

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We will now work on the reagent required for the screening assay for binding of proteins human serum albumin and carbonic anhydrase against several small drug molecules.

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In this protein small molecules are evenly used in 5 percent DMSO in 1.O5X PBS as the running buffer which will also be used for the initial startup cycles 8 different solvent correct solutions.

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Maybe prepared ranging from 4.5 percent DMSO in PBS to 5.8 percent DMSO in PBS and these solutions will be prepared by mixing these solutions in different ratios as shown here.

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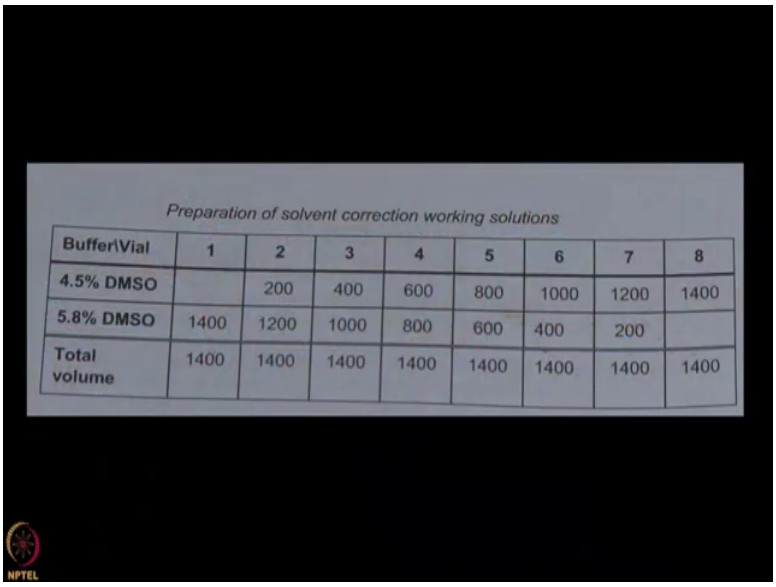

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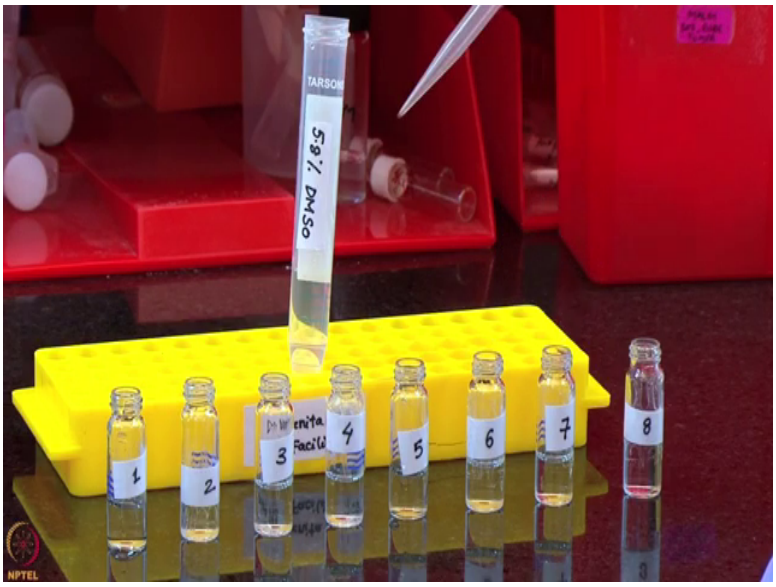

Thus we will number these tubes as 1 to 8 and add 200, 400, 600, 800, 1000, 1200 and 1400 microliter of 4.5 percent DMSO; in tubes 2 to 8 respectively. Following this we will add 1400, 1200, 1000, 800, 600, 400 and 200 microliter of 5.8 percent DMSO in tubes 1 to 7 respectively.

We will mix these solutions vigorously and put the lids on and keep them in dark until we are ready with other samples.

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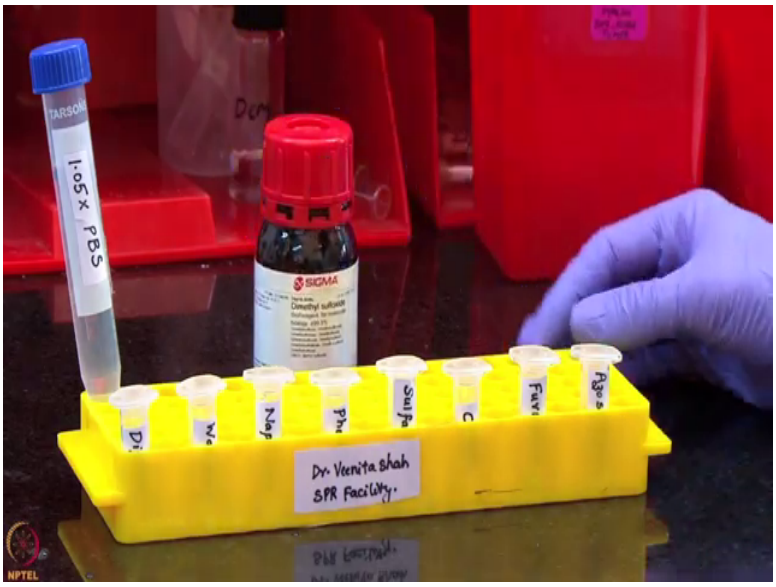

We will be preparing all small molecules samples including digitoxin, warfarin, naproxen, phenytoin, sulfanilamide, carboxybenzene, sulphonamide, furosemide and azosulfamide. From 1.O5X PBS and 10 millimolar stock solutions of these drug molecules using DMSO solution according to this table shown here.

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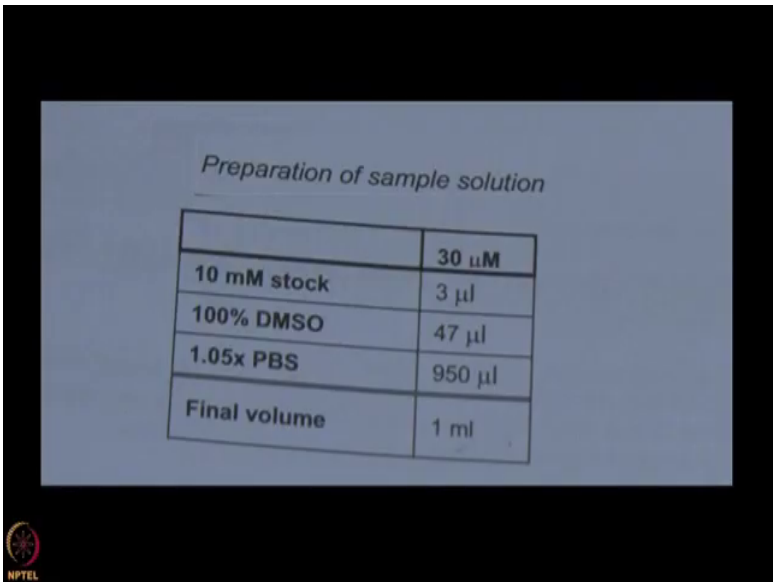

So, final concentration of 30 micromolar of small molecules will be used for the screening assay. For washing we had also prepared 15 percent DMSO in water using 600 microliter of DMSO and 600 microliter of water in a 16 m m vial.

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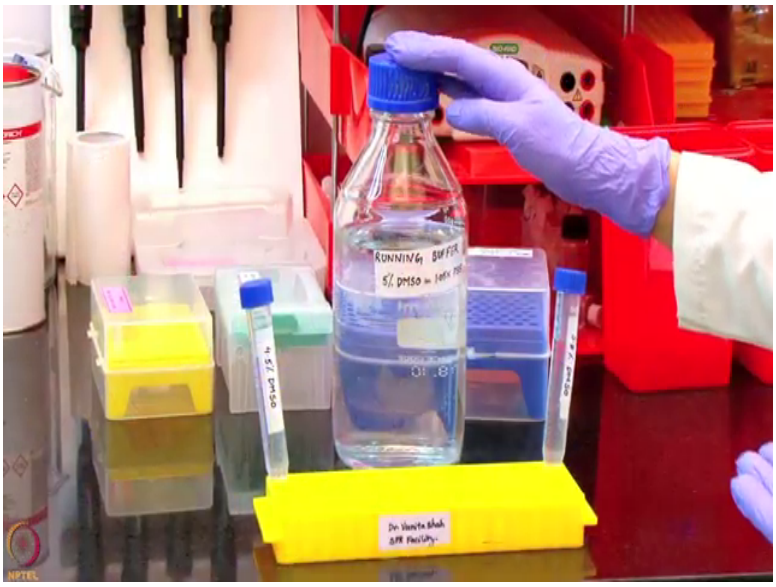

Running buffer will also be used as negative control in this experiment.

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All of these samples and reagents have now been aliquoted in the required volume in the specialized tubes.

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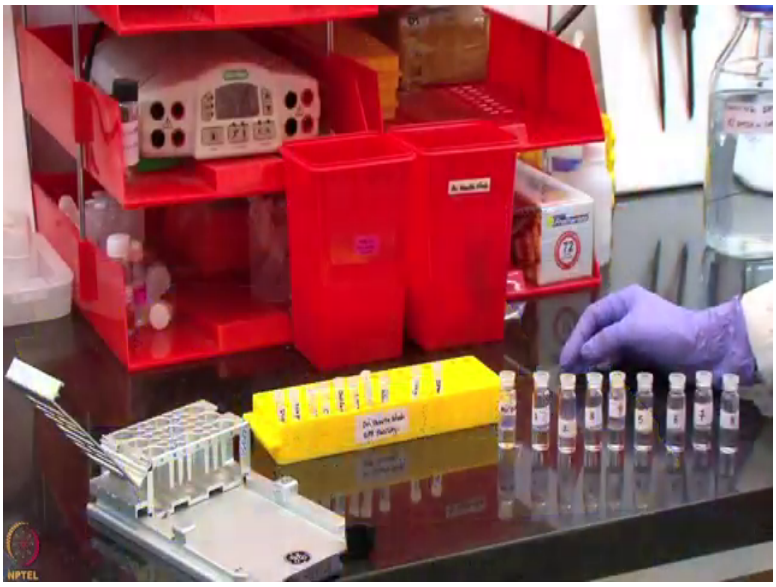

We will now proceed to insert these tubes including the samples at 30 micromolar concentration. The negative control the startup 50 percent DMSO as wash solution and the solvent correction solution into the appropriate rack and then into the system to perform the protein small molecules screening assay.

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We have now placed all our tubes inside the rack including the wash solution the sample the solvent correction solution the startup solution and the negative control.

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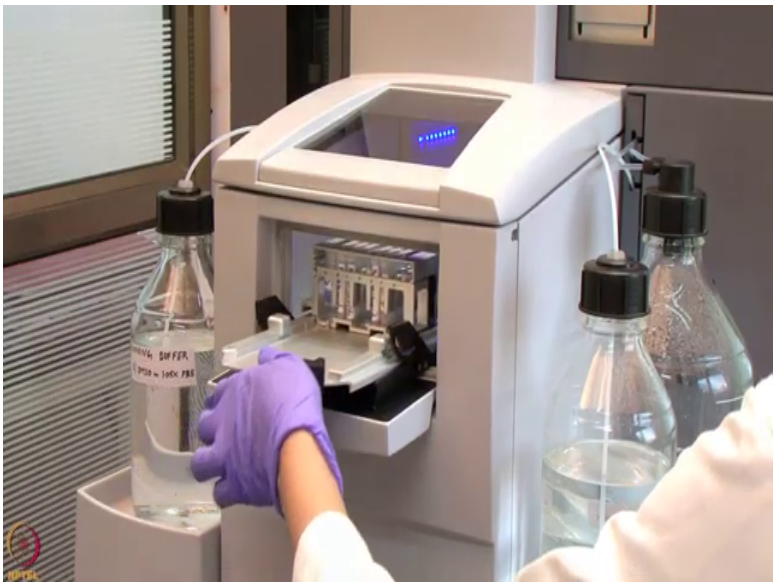

We are placing this rack inside the system to start with our screening assay. We will now start with our run as demonstrated in the earlier lectures.

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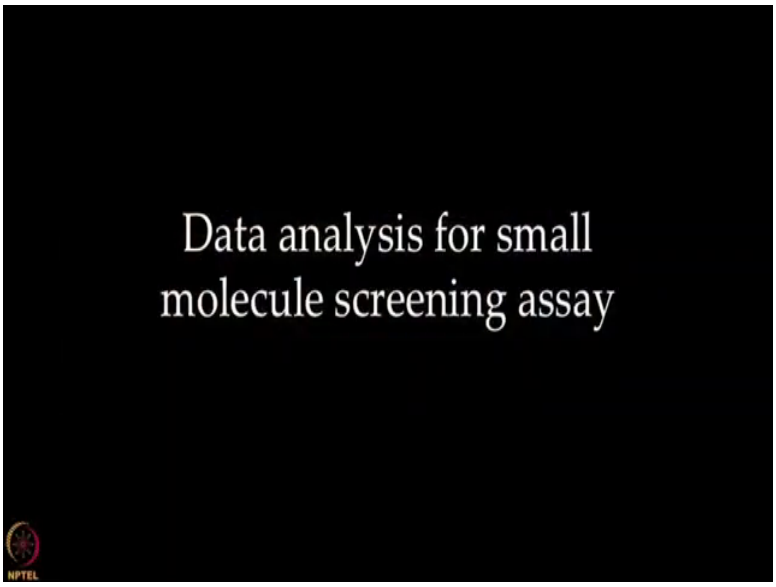

Small molecule binding once the wizard is setup.

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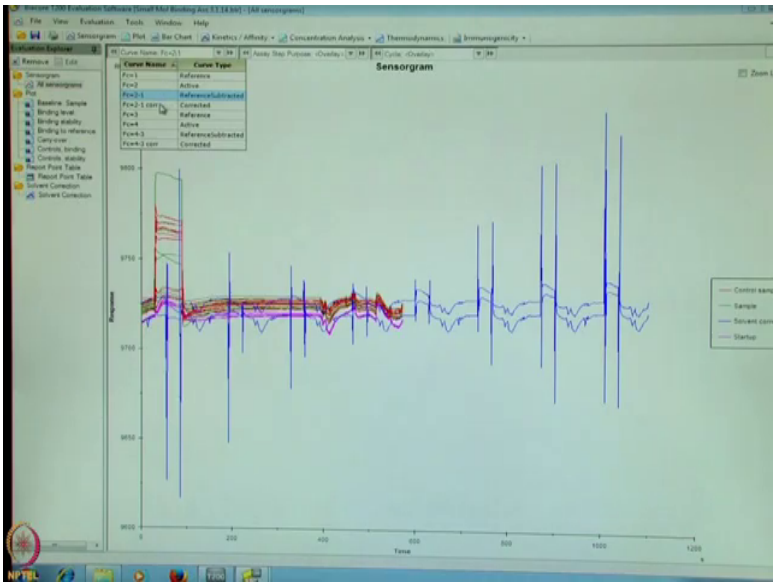

And the experiment is done for at least 4 to 6 hours. We will open the data file to see for the data. So, this is how the data look like, with the all the control samples sample solvent correction and startups all overlaid on each other. Here we can see we have the reference surface active surface as our human serum albumin. Again we have a reference subtracted surface 2 minus 1. And we have the reference surface 3, again an active surface for the carbonic anhydrase and the reference subtracted 4 minus 3 substances.

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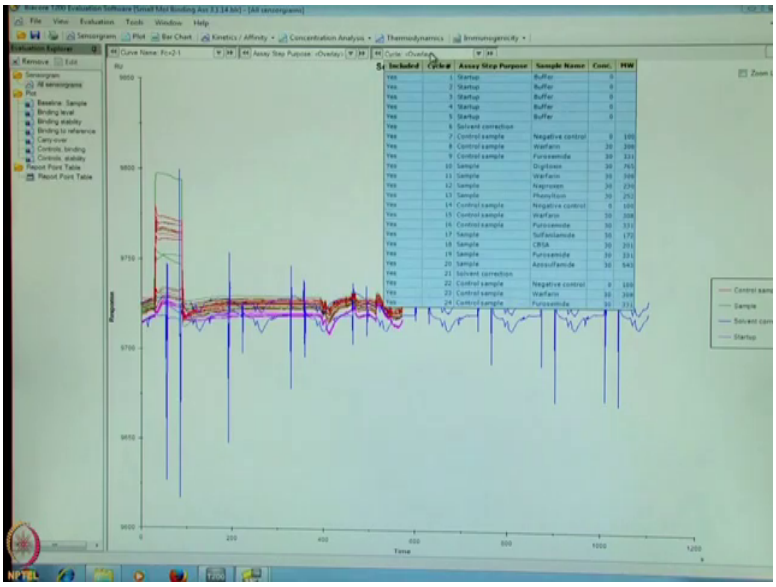

When we go here on the overlay, we see startup cycles solvent correction negative and positive control samples. And various samples that were injected on the surface of the 2 active ligands.

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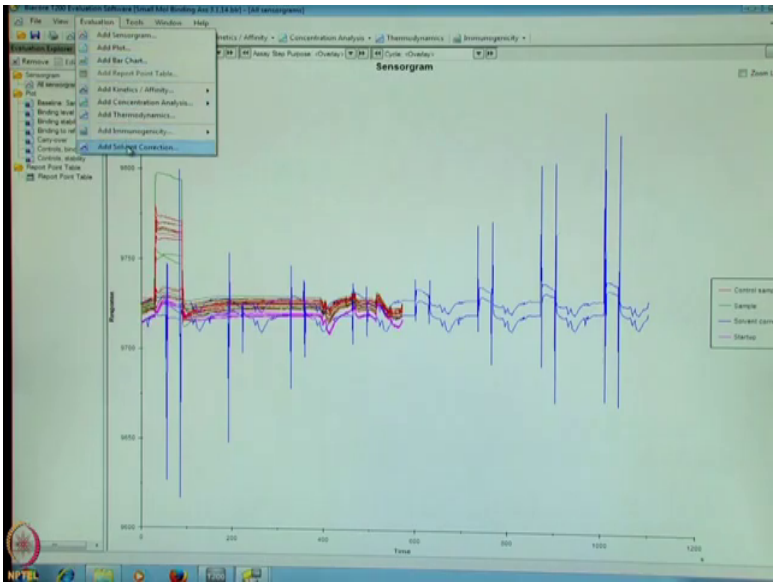

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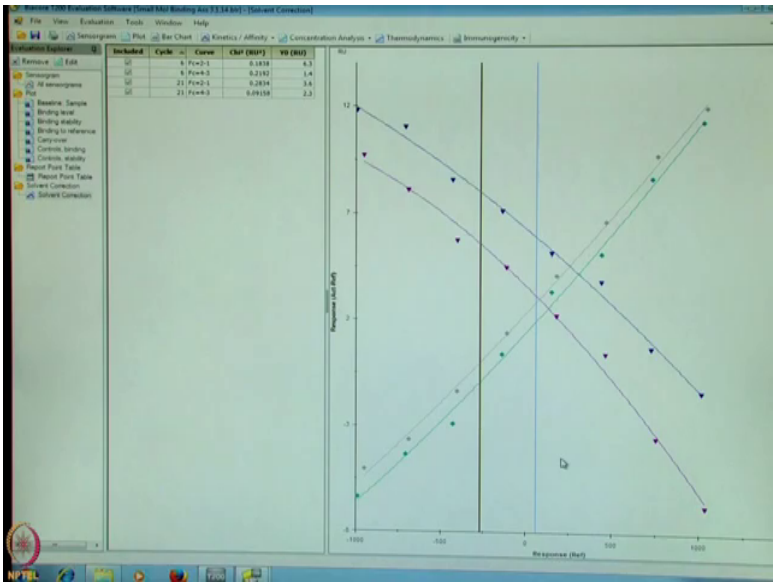

Here first we have to go on add solvent correction. Check the solvent correction data and if the data is good enough we can say and the data is subtracted for the solvent effect of DMSO. And we can see now in the all sensorgrams it shows 2 minus 1 corrected and simultaneously 4 minus 1 corrected with the solvent correction data that we have generated.

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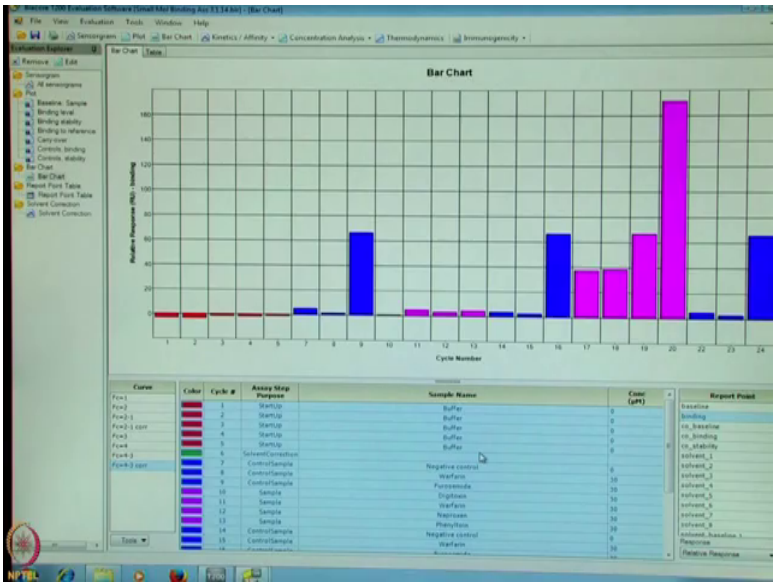

Here another point is that we could evaluate the data in a bar chart mode. We have various startups solvent correction control samples and various components injected here and their interaction with the surfaces; also similarly at 2 minus 1 corrected position.

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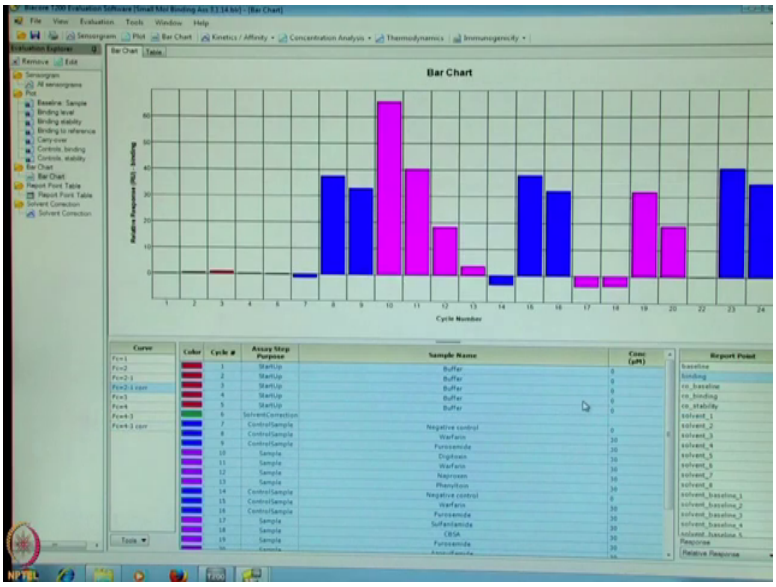

We can see similar kind of a data. With this we can conclude some molecules have no binding; whereas, some molecules have some amount of binding from the data. And this indicates that the 2 different surfaces behave differently, while interacting with the small molecules. This will end at the session on solvent correction and binding of small molecules to 2 ligands immobilized on the surface.

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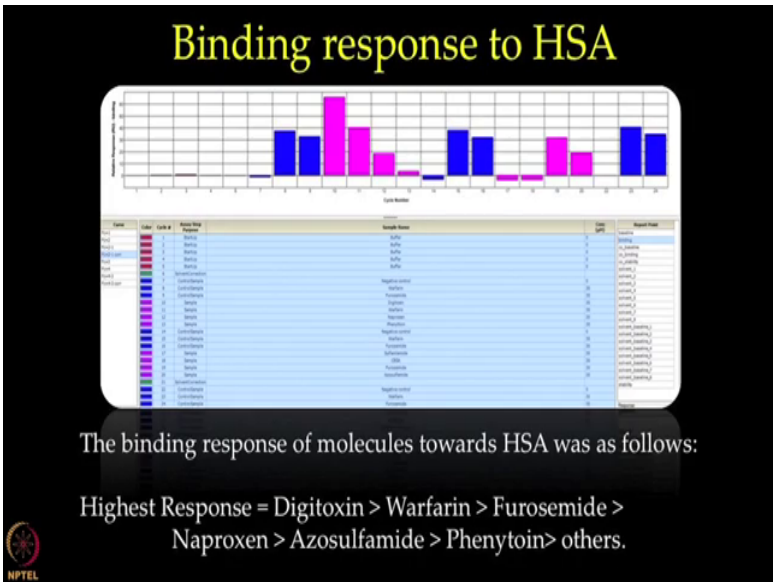

Different drug molecules bound to human serum albumin with different binding responses based on their respective affinity. In this case, digitoxin shows the highest affinity towards HSA followed by wals warfarin, furosemide, naproxen, azosulfamide, phenytoins and others. The binding response for these molecules observed for human serum albumin correlated well with their expected affinities.

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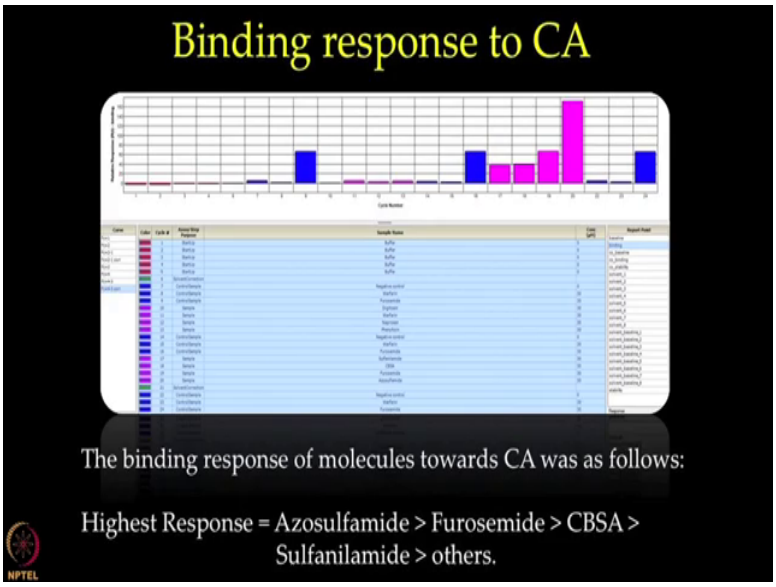

Similarly, different drug molecules also bound to carbonic anhydrase with different binding responses based on their respective affinity. In this case, azosulfamide shows the highest affinity towards carbonic anhydrase followed by furosemide, CBSA, sulfanilamide and others. The binding response for these molecules observed for carbonic anhydrase also correlated well with their expected affinities.

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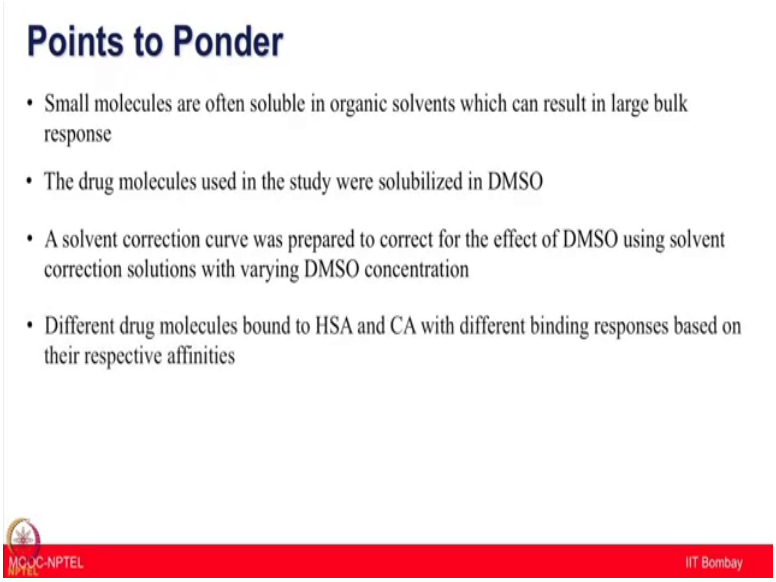

. In today's lecture, you must have observed that different small molecules have different binding response. And affinities for the two bound protein that is serum albumin and carbonic anhydrase. We will continue this experiment on protein a small molecule interactions for kinetic analysis in the next lecture.

Thank you.