Interactomics: Protein Arrays and Label-Free Biosensors.
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Lecture-10.
Proetein-Small Molecule Interaction Study: Immobilization and Binding Analysis.

Welcome to MOOC NPTEL course on intractomics, identification of small molecules which bind specifically two proteins has become crucial in the discovery and development of new pharmaceutical drugs and for elucidating complex pathways in biological systems. The primary goal of small molecule screening in pharmaceutical development is to identify suitable candidates on the basis of their binding to the selected target molecules.

The first stage of the process often involves a screening of large candidate libraries to identify promising binders against a protein target. Usually positive control to test the binding activity is appropriate if a suitable control analyte molecule is available. The small molecules are often dissolved in organic solvents to maintain analyte solubility and hence, matching the organic solvent concentration in samples and running buffers becomes very important. The response obtained from small molecule analytes is inherently low because of their molecular weight. Therefore, the sensor surface for small molecules screening are prepared with high level of ligand 8000 to 10000 RU for average size proteins.

As we have performed the immobilization of proteins in earlier lecture we are not going to repeat the process here to simultaneously immobilize two proteins carbonic anhydrase and human serum albumin on two different flow channel of a CM5 sensor chip. This will be demonstrated by small molecule screening assay where 8 small drug molecules including digitoxin, warfarin, naproxen and phenytoin will be tested. Should we now proceed with the immobilization and screening analysis of these small molecules with these two selected target proteins?

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Lecture Outline

- Perform immobilization of Carbonic anhydrase (CA) and Human serum albumin (HSA) on two different flow channels of a CM5 sensor chip.
- Data analysis for ligand immobilization on the sensor chip surfaces.
- Screening assay set up for eight small drug molecules against the immobilized proteins.
- Data analysis for the screening assay.

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Immobilization of Carbonic anhydrase (CA) and Human serum albumin (HSA)

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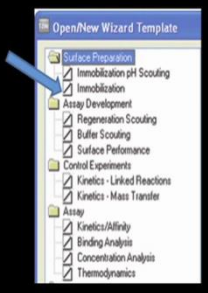
Immobilization assay set-up



Immobilization of human serum albumin (HSA: 67 KDa) and carbonic anhydrase (CA: 30 KDa) for small molecule screening.

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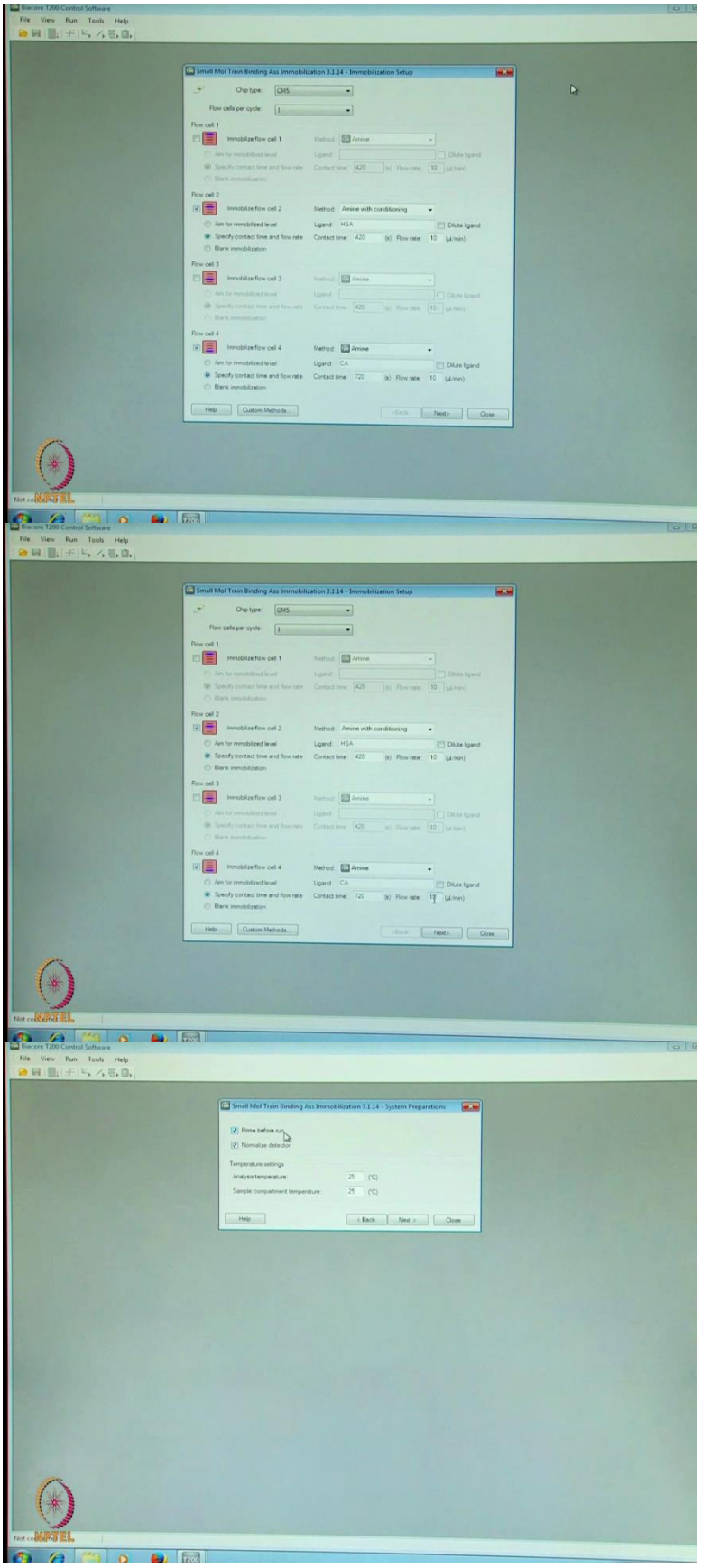
Biacore T200 Control Software
File View Run Tools Help
Small Mol Train Binding Ass Immobilization 3.1.14 - Immobilization Setup
Chip type: CM5
Flow cells per cycle: 1
Flow cell 1
Immobilize flow cell 1
Method: Amine
Flow cell 2
Immobilize flow cell 2
Method: Amine with conditioning
Ligand: HSA
Dilute ligand
Aim for immobilized level
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
Flow cell 4
Immobilize flow cell 4
Method: Amine
Ligand: CA
Contact time: 720 (s) Flow rate: 10 (µl/min)
Help
Custom Methods...
< Back
Next >
Close
Small Mol Train Binding Ass Immobilization 3.1.14 - System Preparations
Prime before run
Normalize detector
Temperature settings
Analysis temperature: 25 (°C)
Sample compartment temperature: 25 (°C)
Help
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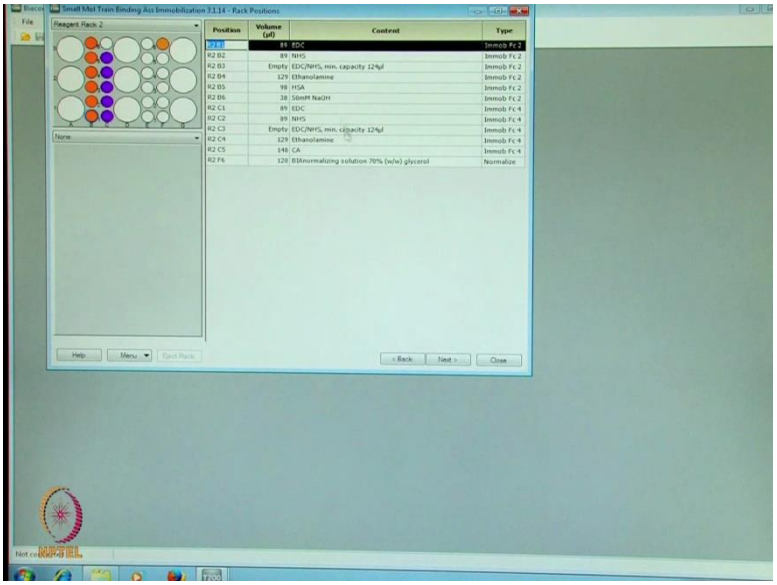

So, let us have the lab experimental session now. In this session, we will understand the interaction of proteins with small molecules. So, to start with small molecule interaction we have to start again with the immobilization we go into binding or screening and we go into kinetic mode to determine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

So, the chip selected is CM5, flow channels for the cycle is one, on the flow channel one we leave it blank and flow channel 3 also we leave it blank, flow channel two human serum albumin is immobilized with the specify 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 with a specify contact time and flow rate mode for 720 seconds and 10 flow rate.

We now go into the next tab, we will highlight the prime before run and normalize detector and temperature will be at 55 degree centigrade and we go to the next tab and we have the rack positions with the required amount of reagents shown here. 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.

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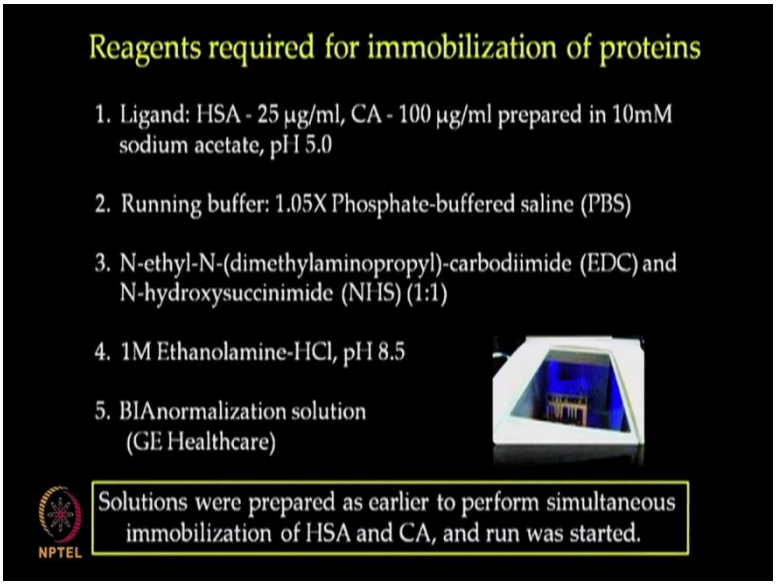

The proteins are prepared at working concentration of 25 microgram per ml for human serum albumin and 100 microgram per ml of carbonic anhydrase using an immobilization buffer of pH 5. We will be using 1.05xPBS buffer as the running buffer which will be connected to the system followed by priming. EDC and NHS as prepared and suggested in the amine coupling kit for a surface activation will be used in one is to one ratio.

One molar ethanol amine HCl pH 8.5 is prepared for blocking the three 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 difference between individual sensor chips. After preparation of these samples and reagents we transfer the sample tubes to the appropriate rack and start the run as shown beta two macroglobulin immobilization in earlier lecture.

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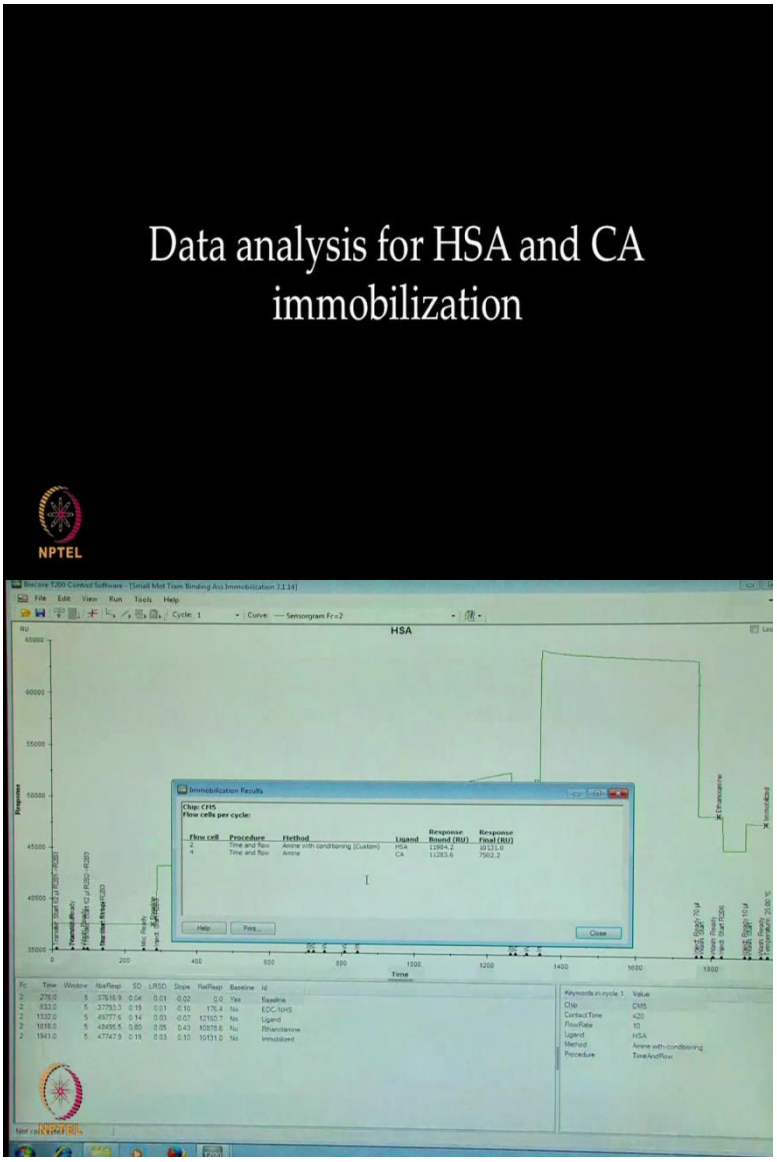

We will hence move forward to discuss the immobilization results obtained from this run. From our experimentation the following are the results for the immobilization of human serum albumin and carbonic anhydrase on two different flow channels, we see here from the immobilization results. Here, you can 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 ethanol amine and immobilization.

On the cycle two similarly you have activation with EDC-NHS mixture, passage of ligand and blocking of the surface with ethanol amine 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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Points to ponder

- Immobilization was successfully performed for both the proteins on two different flow channels of the same chip.
- The final immobilization levels achieved for the two proteins were as follows:
  - HSA: 10131 RU
  - CA: 7502 RU

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Screening of small molecule binding to HSA and CA

- Molecules to be screened for binding to HSA and CA:
  - Digitoxin (765 Da), Warfarin (308 Da), Naproxen (230 Da), Phenytoin (252 Da), Sulfanilamide (172 Da), CBSA (Carboxybenzene sulphonamide, 201 Da), Furosemide (331 Da), Azosulfamide (543 Da)
- Molecules specific to HSA: Digitoxin, Warfarin, Naproxen, Furosemide, Azosulfamide, Phenytoin
- Molecules specific to CA: Azosulfamide, Furosemide Sulfanilamide, CBSA
- Positive Control: Warfarin and Furosemide
- Negative Control: 5% DMSO in 1.05X PBS

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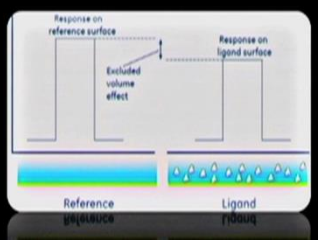
Solvent correction

- Small molecules are often soluble in organic solvents such as DMSO.
- DMSO added to the samples and running buffer can result in large bulk response.



Reference flow cell (Fc) has room for more DMSO than ligand Fc

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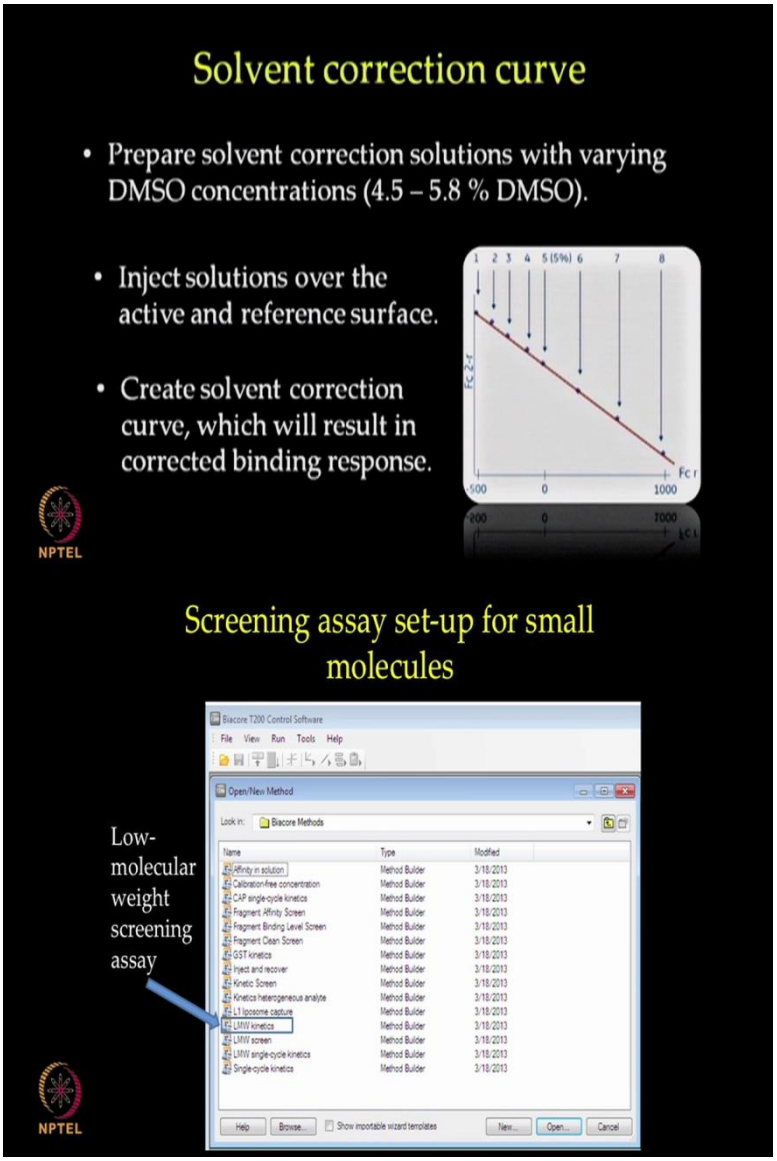

These small molecule to be screened for binding will include digitoxin, warfarin, naproxen, phenytoin, sulfanilamide, carboxybenzene salphonamide, furosemide and azosulfamide. Some of these molecules are specific to HSA whereas rests are specific to CA, furosemide being specific to both. Warfarin will 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. Since, small molecules are often soluble in organic solvents such as DMSO and so are these drug molecules. It can results in large bulk responses since reference flow cell has room for more DMSO than active flow cell with ligand that is why we need to prepare a solvent correction curve by preparing solvent correction solutions 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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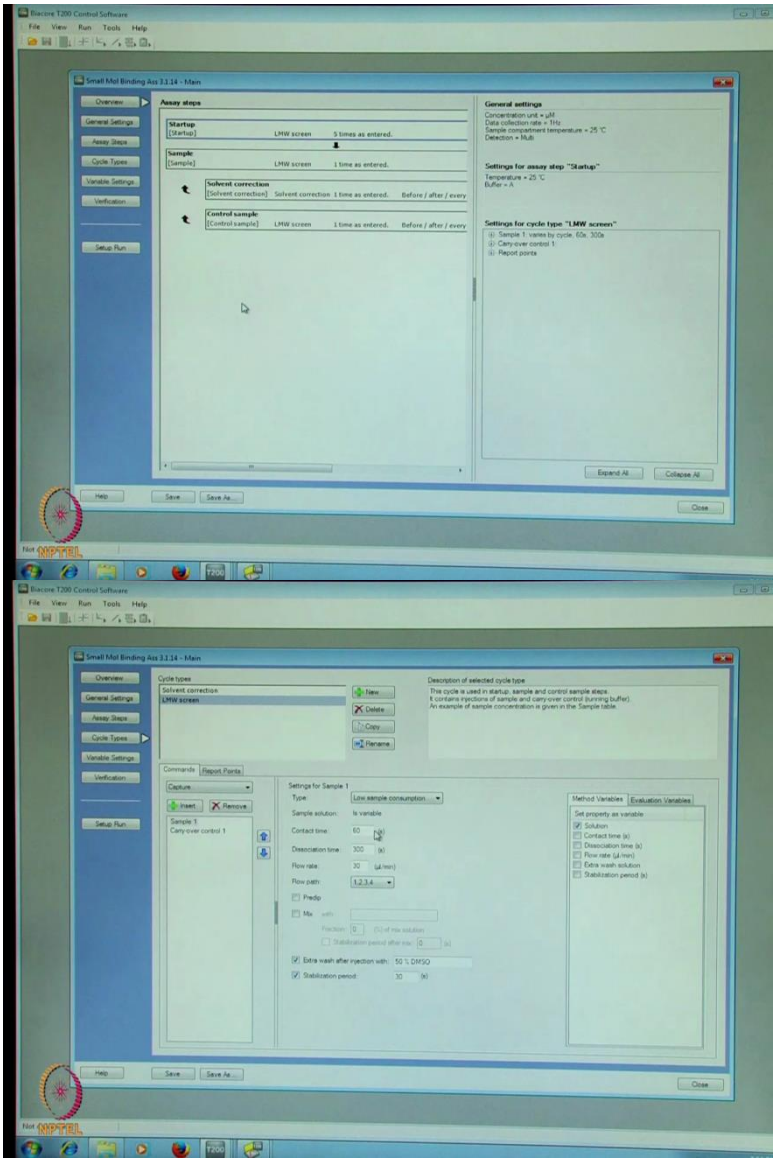

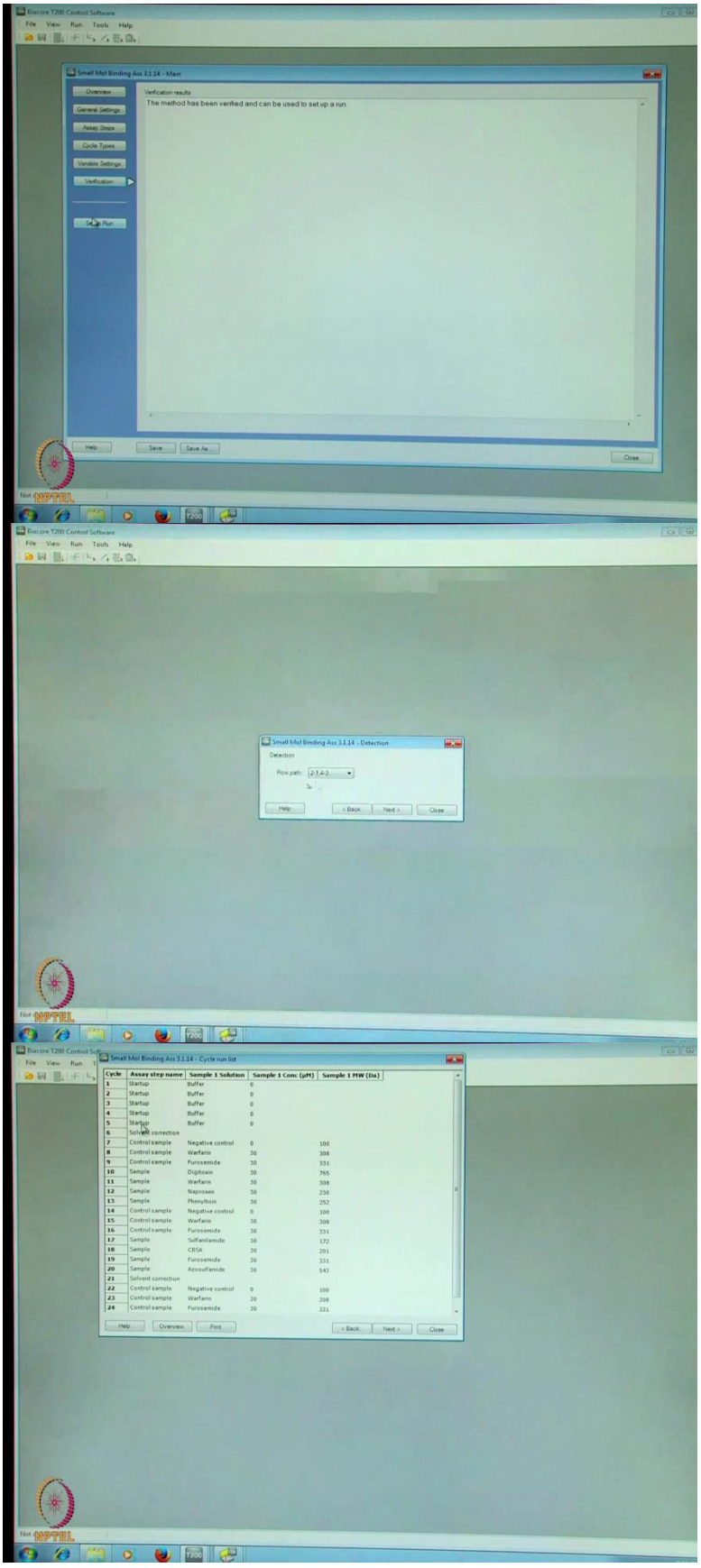
Biacore T200 Control Software
File View Run Tools Help
Small Mol Binding Ass 3.1.14 - Main
Overview
General Settings
Assay Steps
Cycle Types
Variable Settings
Verification
Run
Verification results
The method has been verified and can be used to set up a run.
Help
Save
Save As ...
Close
Small Mol Binding Ass 3.1.14 - Detection
Detection
Flow path:
2-1,4-3
Help
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Next >
Close
Small Mol Binding Ass 3.1.14 - Cycle run list
Cycle
Assay step name
Sample 1 Solution
Sample 1 Conc (µM)
Sample 1 MW (Da)
1 Startup Buffer 0
2 Startup Buffer 0
3 Startup Buffer 0
4 Startup Buffer 0
5 Startup Buffer 0
6 Solvent correction
7 Control sample Negative control 0 100
8 Control sample Warfarin 30 308
9 Control sample Furosemide 30 331
10 Sample Digitoxin 30 765
11 Sample Warfarin 30 308
12 Sample Naproxen 30 230
13 Sample Phenytoin 30 252
14 Control sample Negative control 0 100
15 Control sample Warfarin 30 308
16 Control sample Furosemide 30 331
17 Sample Sulfanilamide 30 172
18 Sample CBSA 30 201
19 Sample Furosemide 30 331
20 Sample Azosulfamide 30 543
21 Solvent correction
22 Control sample Negative control 0 100
23 Control sample Warfarin 30 308
24 Control sample Furosemide 30 331
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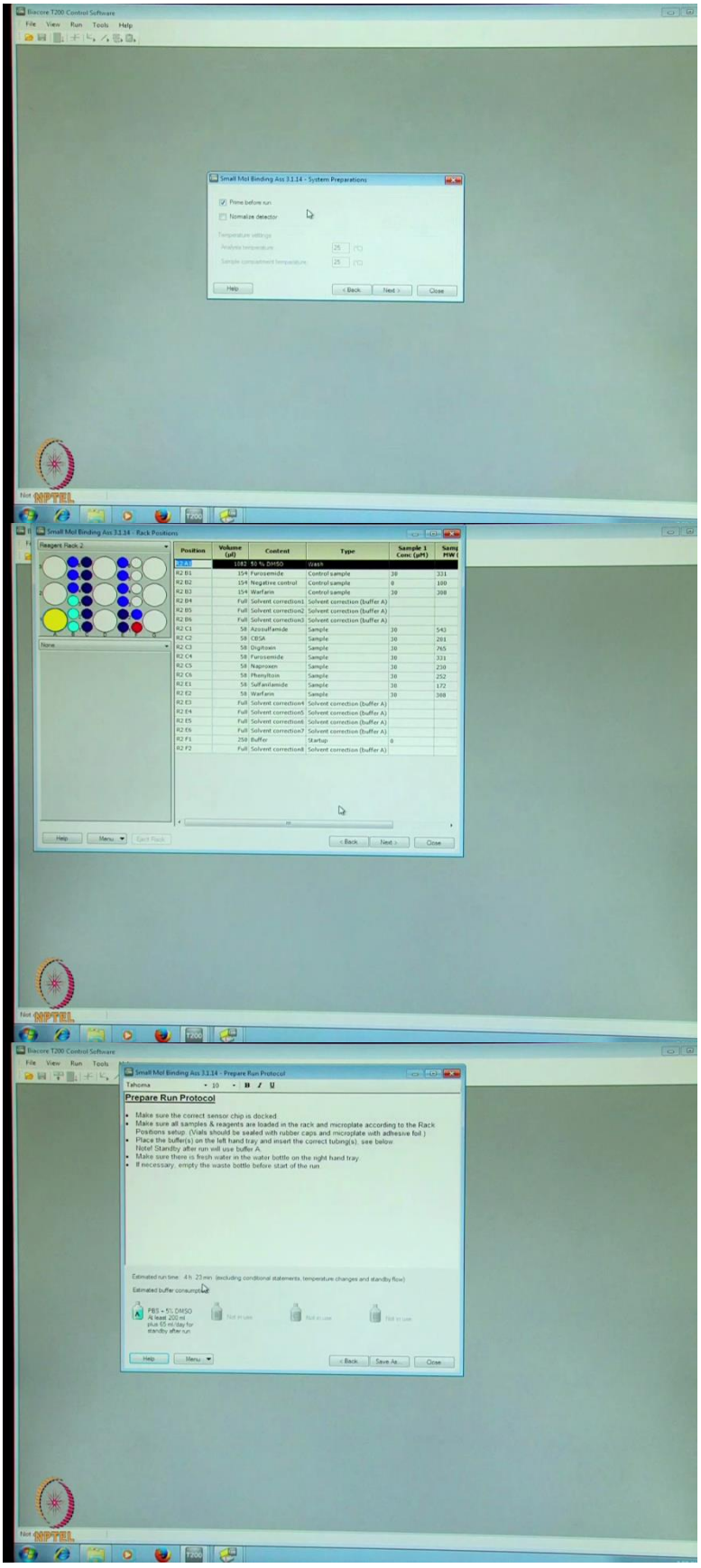

We will now move forward to set up the binding assay result to screen small molecules for binding to human serum albumin and carbonic anhydrase. Here, we will setup our experiment

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

Control samples like positive and negative controls were also included in this mode. 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 carry over effects, stabilization time was 30 seconds.

We verify this method and setup a run in which it will have a subtraction of active surface 2 was subtracted from the 1 reference surface. Active surface 4 was subtracted to 3 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, warfarin, naproxen, phenytoin and a native 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 next control sample injected.

So, once we are done with that we highlight prime before run and this is how we prepare the template for the experiments. With the solvents corrections provided, buffer for the startup provided, various samples pipetted out in DMSO and warfarin, furosemide as positive and negative control and 50 percent DMSO for wash in between each cycle. We will save next and the whole binding experiment including solvent correction takes 4 hour and 23 minutes and requires at least 200 ml of phosphate buffer saline with 5 percent DMSO included in the running buffer.

Preparation of the running buffer, preparation of the samples, 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 one 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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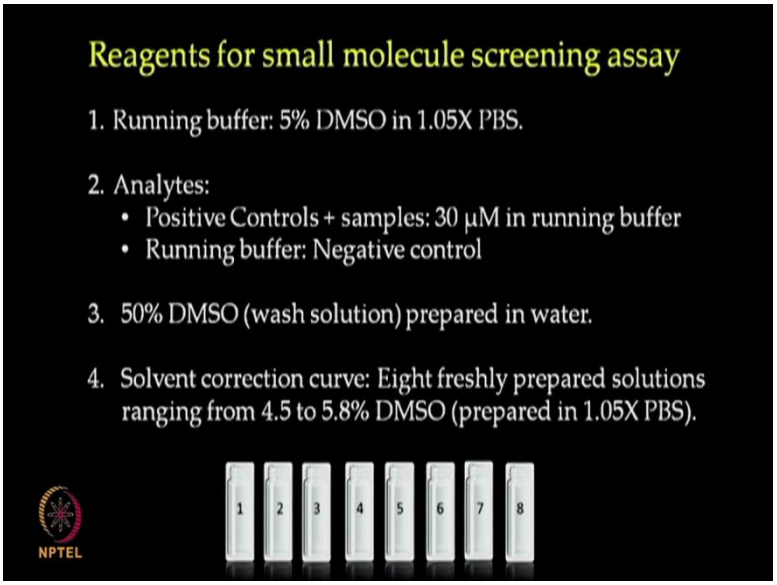

As per the volumes listed in the table we will prepare the following samples and reagents. Running buffer will be 5 percent DMSO in 1.05xPBS. Positive control and samples at 30 micromolar concentration in running buffer and running buffer will be used as negative control. A wash solution is prepared 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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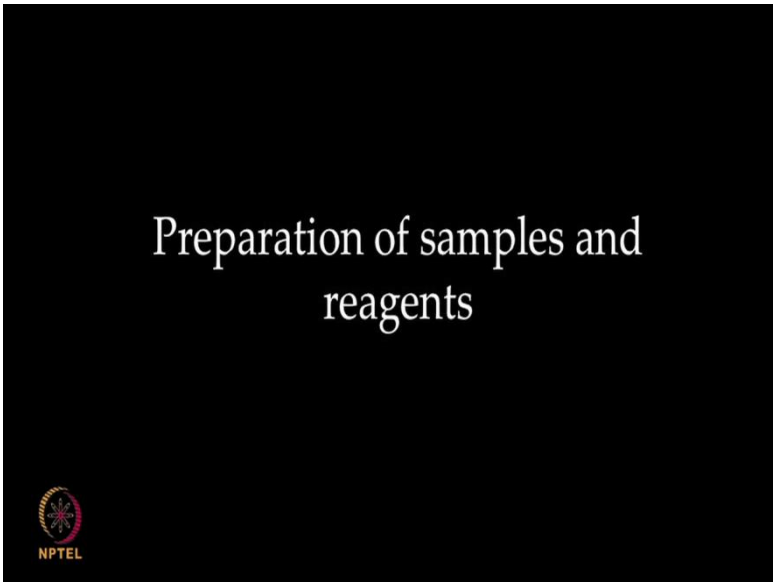

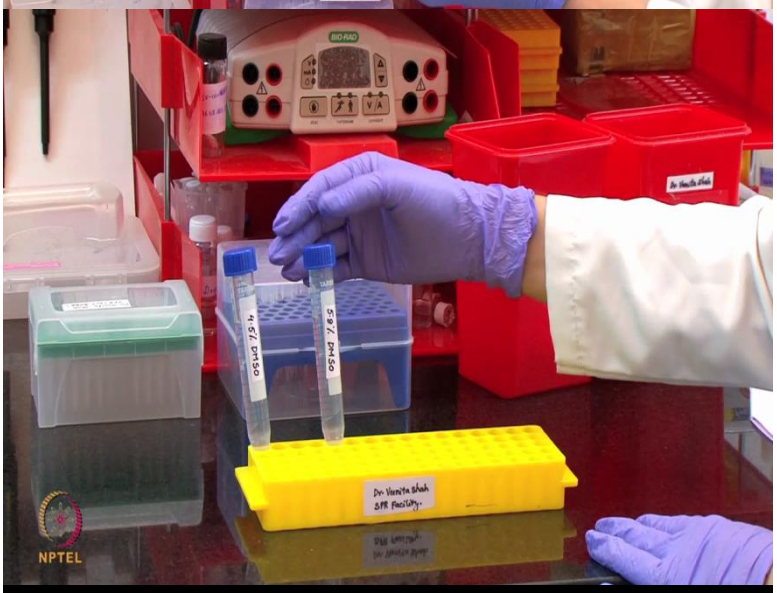

Preparation of solvent correction working solutions

Buffer\Vial	1	2	3	4	5	6	7	8
4.5% DMSO		200	400	600	800	1000	1200	1400
5.8% DMSO	1400	1200	1000	800	600	400	200	
Total volume	1400	1400	1400	1400	1400	1400	1400	1400

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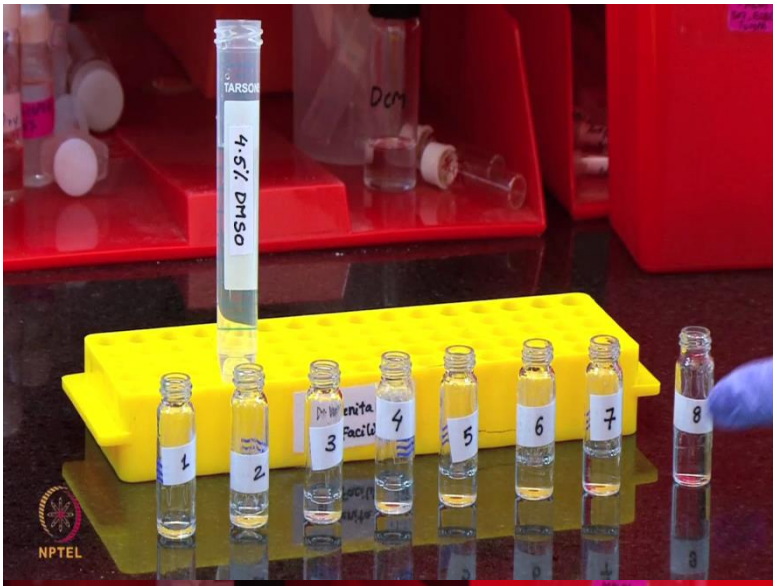

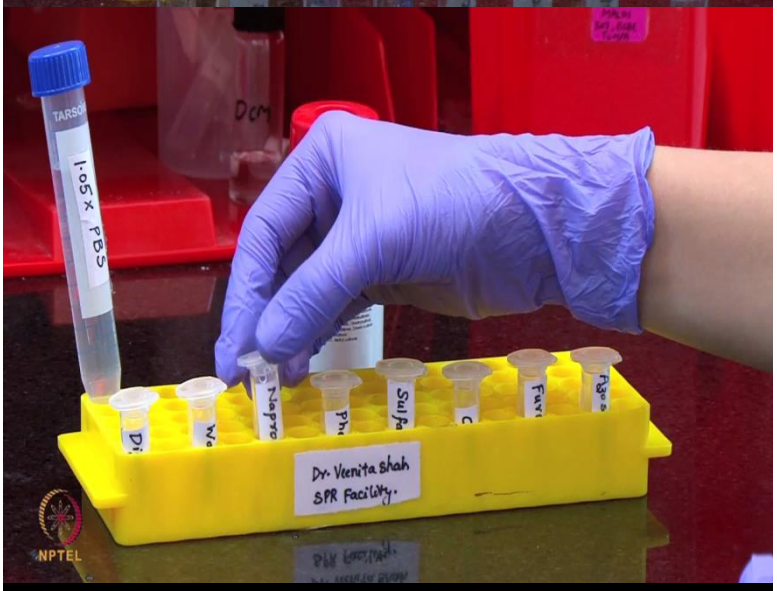

Preparation of sample solution

	30 µM
10 mM stock	3 µl
100% DMSO	47 µl
1.05x PBS	950 µl
Final volume	1 ml

We will now work on the reagent require for the screening assay for binding of proteins human serum albumin and carbonic anhydrase against several small drug molecule. In this protein small molecule study we will be using 5 percent DMSO in 1.05xPBS as the running buffer, which will also be used for the initial startup cycles. 8 different solvent correct solutions will be 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.

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 tube 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. We will be preparing all small molecule samples including digitoxin, warfarin, naproxen, phenytoin, sulfanilamide, carboxybenzene salphonamide, furosemide and azosulfamide from 1.05xPBS and 10 milimolar stock solution of these drug molecule using DMSO solution according to this table shown here.

So, final concentration of 30 micromolar of small molecules will be used for the screening assay. For washing we have also prepared 50 percent DMSO in water using 600 microliter of DMSO and 600 microliter of water in a 16 mm vial. Running buffer will also be used as negative control in this experiment. All of these samples and reagents have now been allicoted in the required volume in these specialized tubes.

We will now proceed to insert these tubes including the samples are 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 proteins small molecules screening assay. We have now placed all our tubes inside the rack including the wash solution, the samples, the solvent correction solutions, the startup solution and the negative control. We are placing this rack inside the system to start with the screening assay. We will now start with our run as demonstrated in the earlier lectures.

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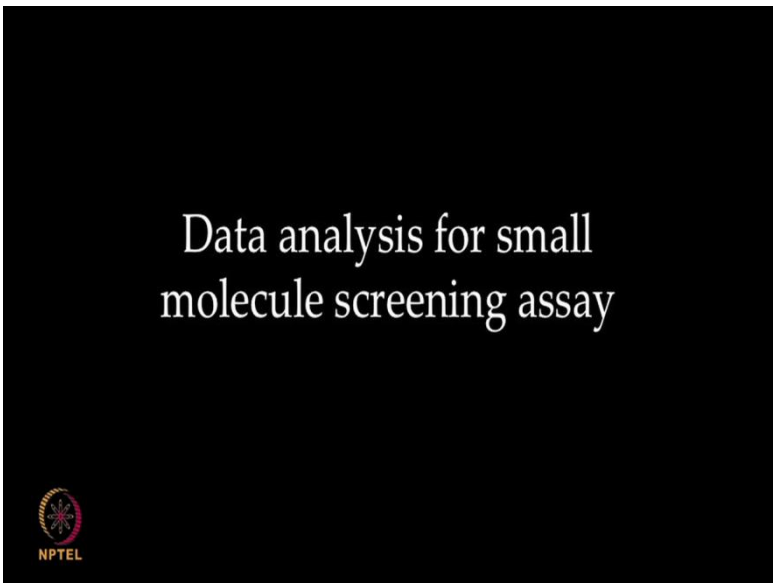

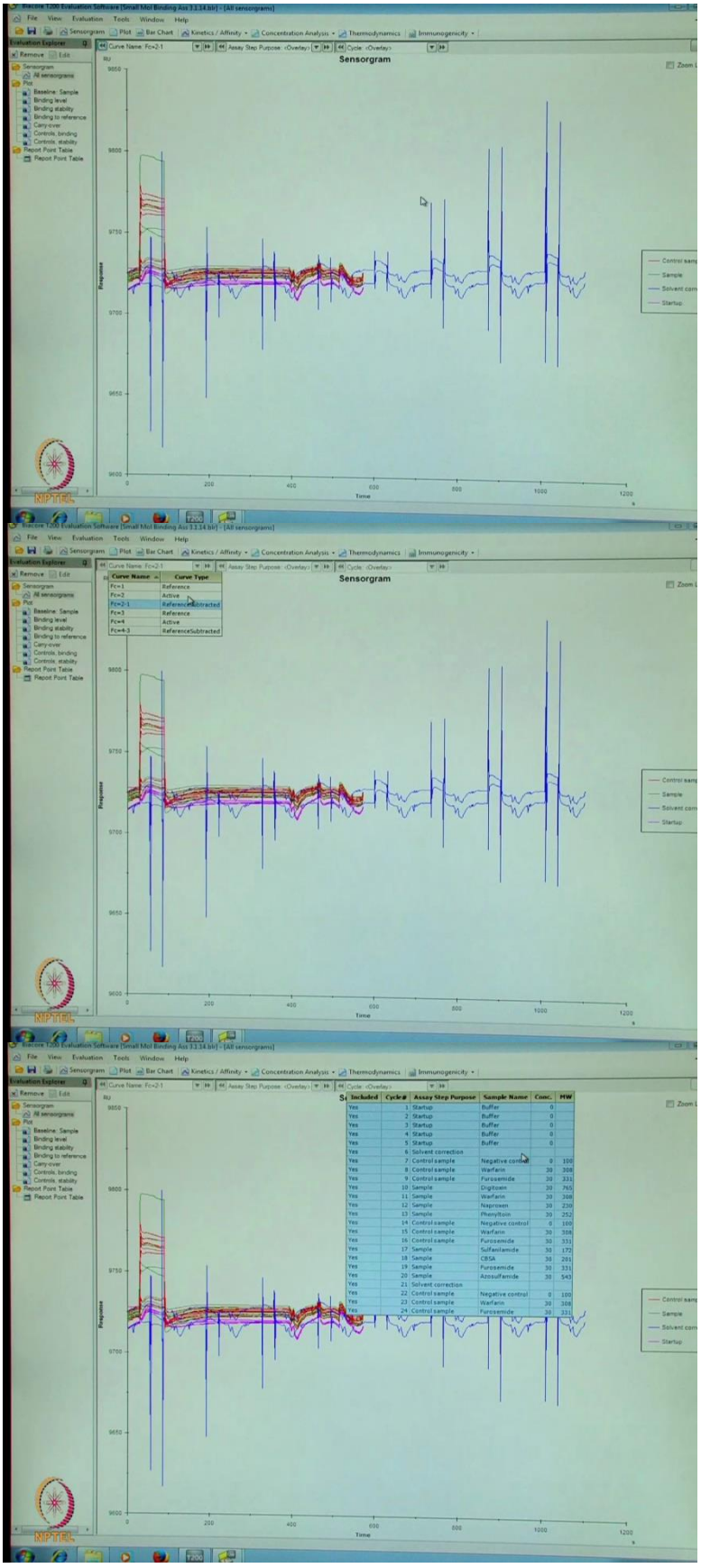
Sensorgram
Response
Time
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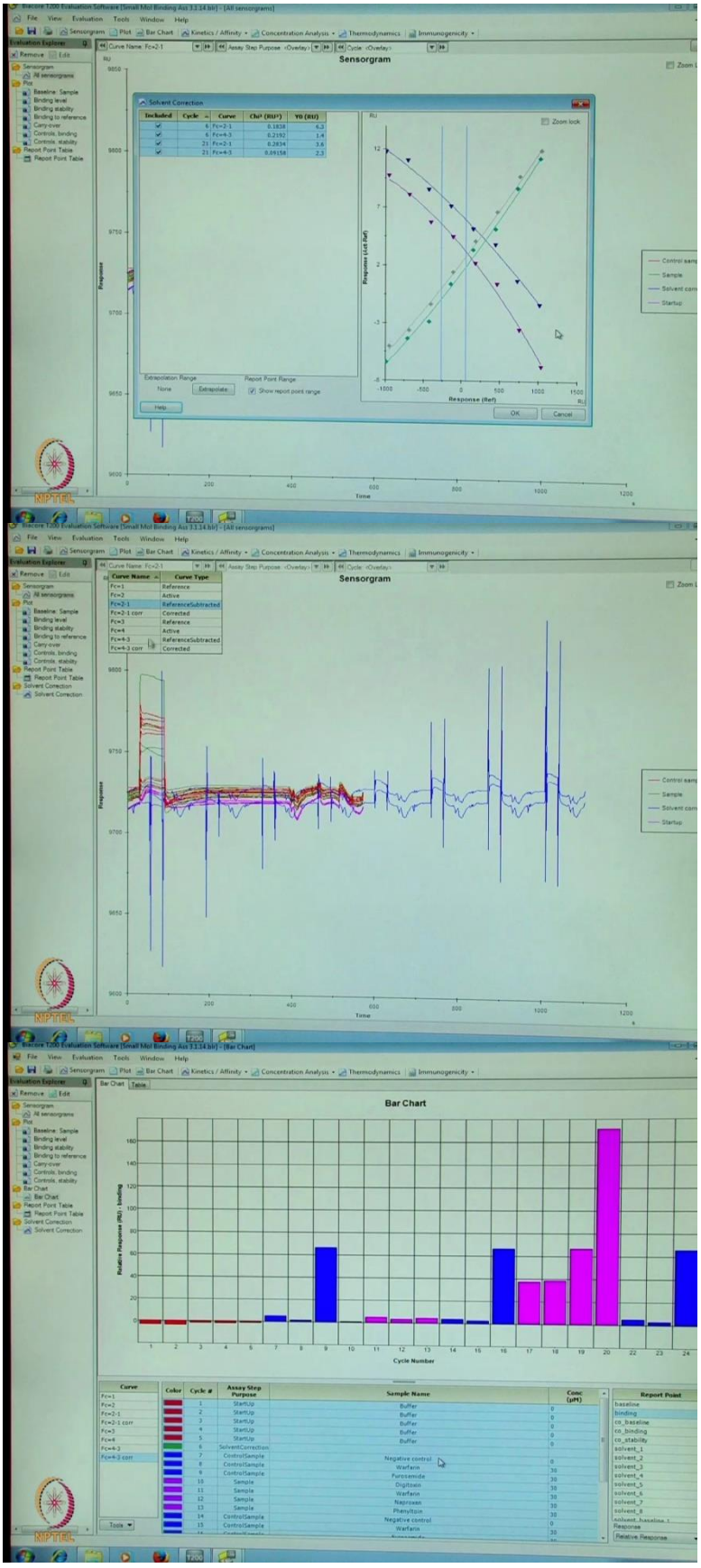
Sensorgram
Solvent Correction
Sensorgram
Bar Chart
Cycle Number

Small molecule binding, once the wizard is setup and the experiment is done for at least 4 to 6 hours, we will open the data file to see further data. So, this how the data look like with all the control sample solvent correction and startups on 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 of the carbonic anhydrase and the reference subtracted 4 minus 3 surfaces when we go here on the overlay, we see startup cycles, solvents correction, negative and positive control samples and various samples that were injected on the surface of the two active ligands.

Here, first we have to go on add solvent correction, check the solvent correction data and if the data is good enough we can save ok and the data is subtracted for the solvent effect of DMSO and we can see now in the our sensorgrams, it shows 2 minus 1 corrected and simultaneously, 4 minus 1 corrected with the solvent correction data that we have generated. 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 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 two different surfaces behave differently while interacting with the small molecules. This will end the session on solvent correction and binding of small molecules to two ligands immobilized on the surface.

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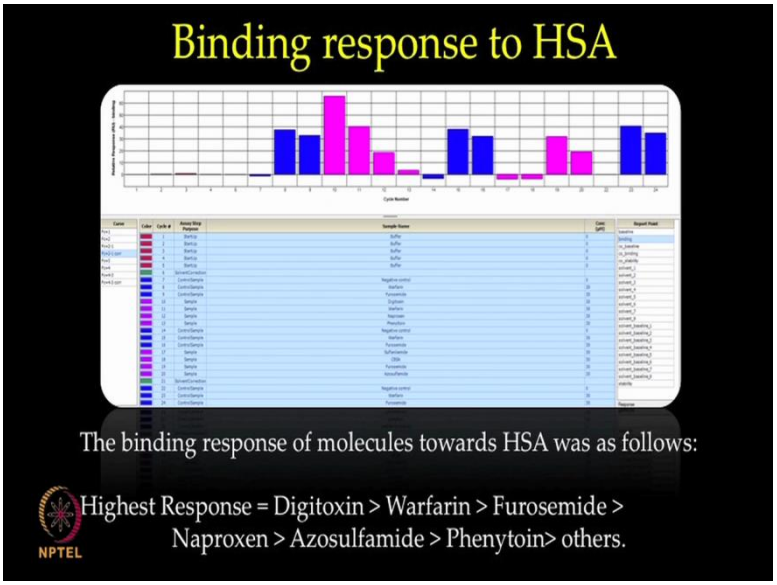

Different drug molecules bound to human serum albumin with different binding responses based on their respective affinities. In this case, digitoxin shows the highest affinity towards HSA followed by warfarin, furosemide, naproxen, azosulfamide, phenytoin and others. The binding response for these molecules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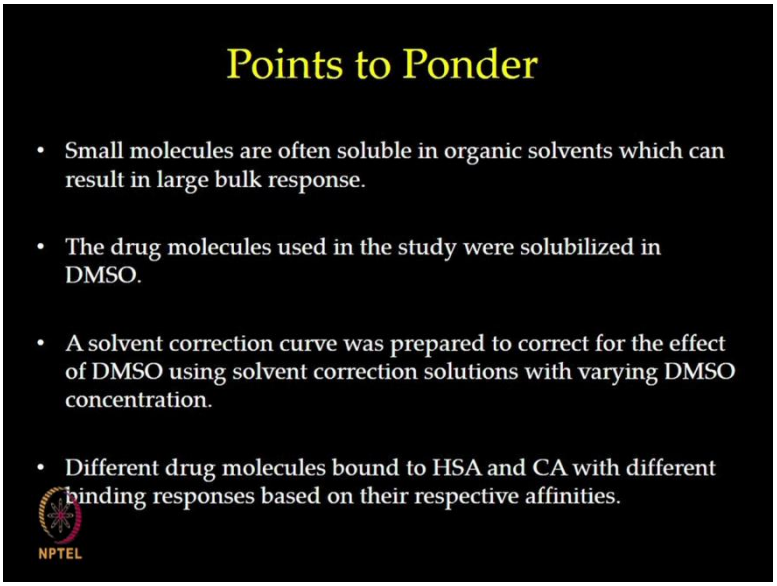

We will continue this experiment on protein, a small molecule interaction for kinetic analysis in next lecture. Thank you.

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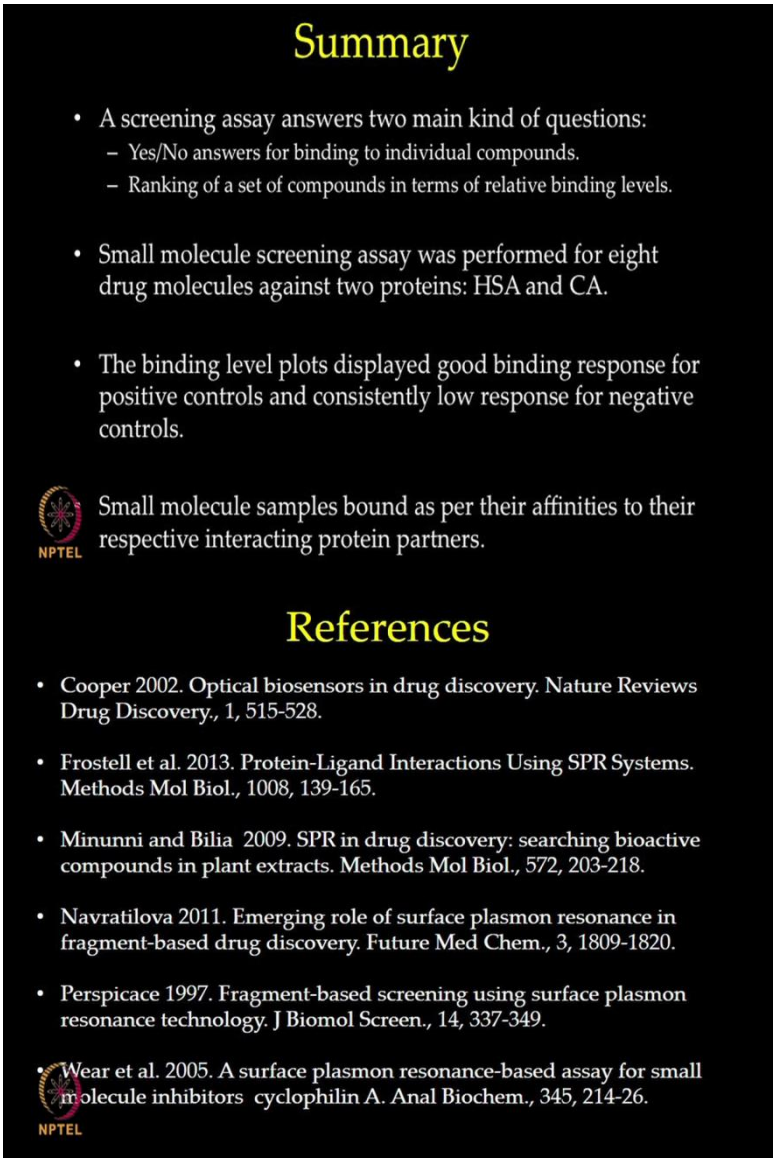

Acknowledgment

Experimental demonstration and commentary by Dr. Veenita Shah (IIT Bombay) and Dr. Srinivas Sistla (GE Healthcare).