Interactomics: Basics and Applications

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Lecture - 38
Protein - small Molecule Interaction Study: Kinetic Analysis

We have been discussing about the use of surface plasmon resonance technology in different applications. And discussed in the previous lecture, SPR is increasingly being used in drug discovery and validation studies using small molecules. SPR biosensors offer exceptional sensitivity and high quality kinetics enabling the most challenging assays of analyzing the low molecular weight compounds with target molecules.

It allows determination of broad range of affinity, different parameters like off rate and on rate constants, the ability to work with samples containing organic solvents like DMSO is a challenging task which is not supported by these SPR biosensors. In our previous lecture, we have screened the binding of 8 different small molecules to carbonic anhydrase and serum albumin.

And the results obtained were evaluated. This performance was studied and binding response at active and reference surfaces were examined. One of these drug molecules furosemide dissolved in DMSO from the previous screening assay will further be characterized for rapid kinetic analysis with carbonic anhydrase as immobilized target using a standard compound concentration series and DMSO solvent correction curve.

We will now proceed with the immobilization and kinetic characterization of a small molecule with target protein. Let us have the experimental demonstration today.

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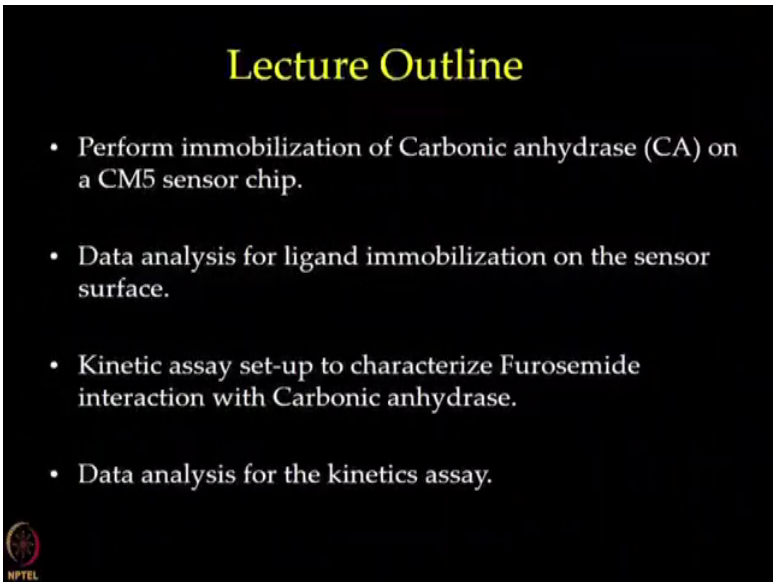

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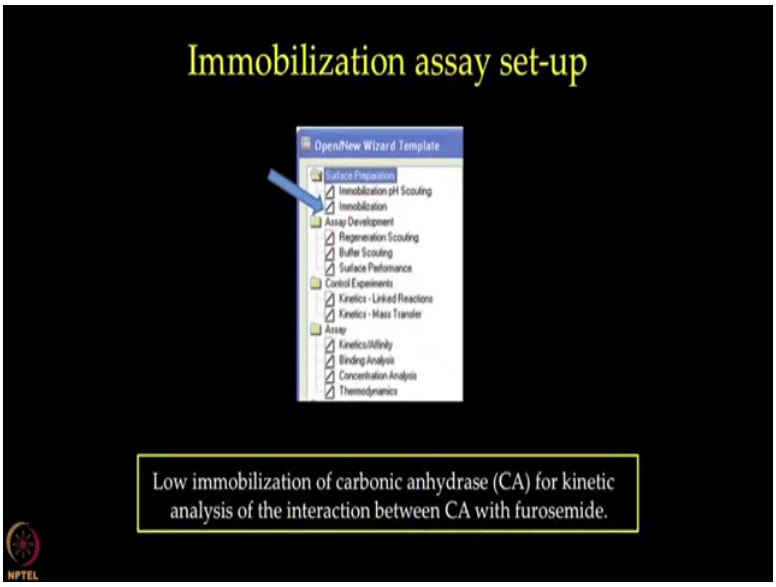

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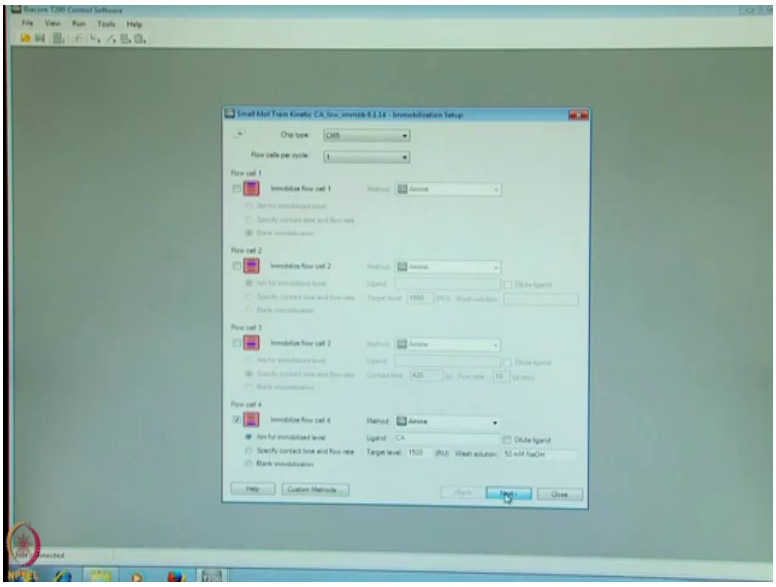

Now, we will setup our immobilization for the kinetic template. For this kinetic experiment, we will have the carbonic anhydrase immobilized by amine coupling on a flow channel 4. So, the target level of 1500 RUs and we leave immobilization as 0 on flow cell 3.

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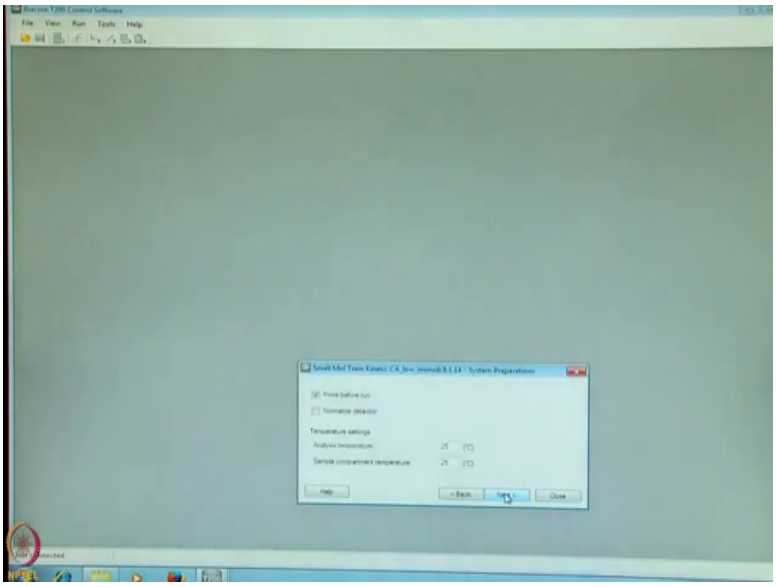

We go to the next tab, prime before run is highlighted here analysis temperature at 25.

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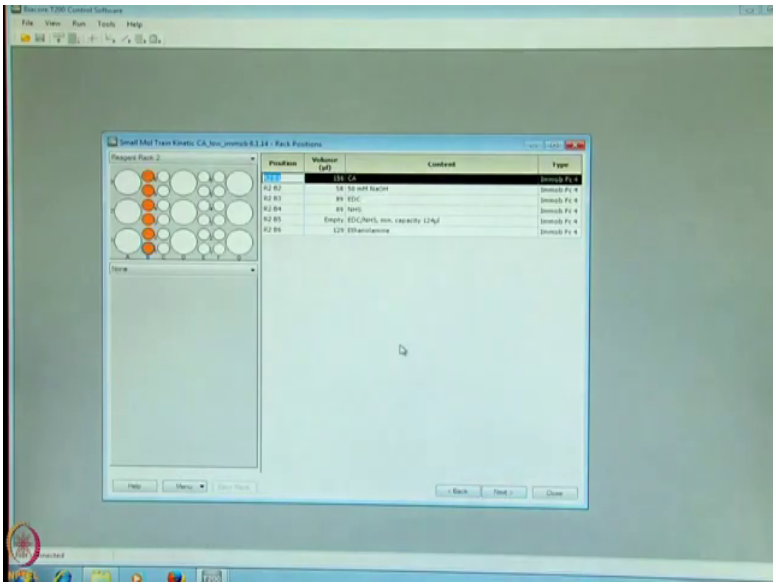

Now, we will have the following reagents prepared for the kinetic analysis of the interaction of carbonic anhydrase with various small molecules.

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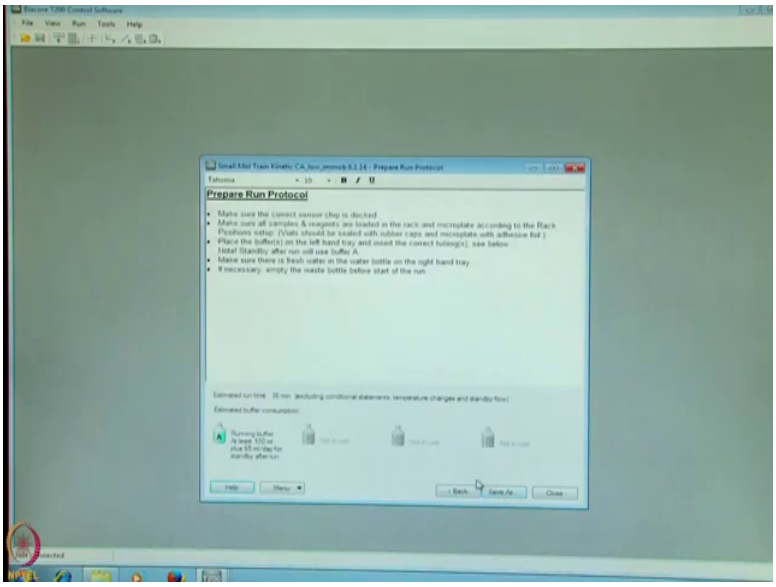

Once the template is prepared, we go onto the next tab and we identify the runtime of 36 minutes with a buffer consumption of at least 100 ml for this particular session.

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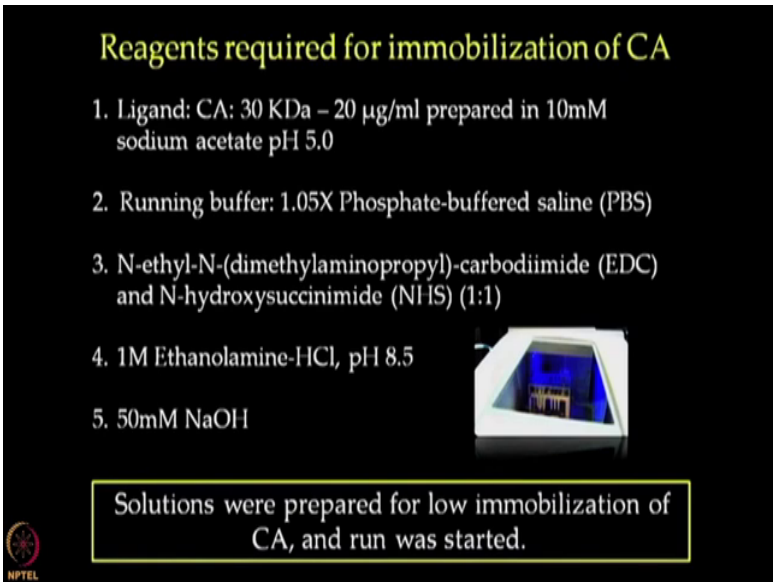

As per the volume listed on the table, we will prepare the buffers and reagents required for immobilization of carbonic anhydrase on the sensor chip. The ligand is prepared at a working concentration of 20 micrograms per m l using an immobilization buffer of 10 millimolar sodium acetate pH 5.

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 are prepared as suggested in the amine coupling kit for surface activation. 1 molar ethanolamine HCl, pH 8.5 is prepared for blocking the free ester group on the surface. Lastly, 50 millimolar NaOH was prepared which will be used to remove the electrostatically bound ligand.

After preparation of these samples and reagents, we transfer the sample tubes to the appropriate rack and start the run as shown for beta 2 microglobulin immobilization in earlier lecture.

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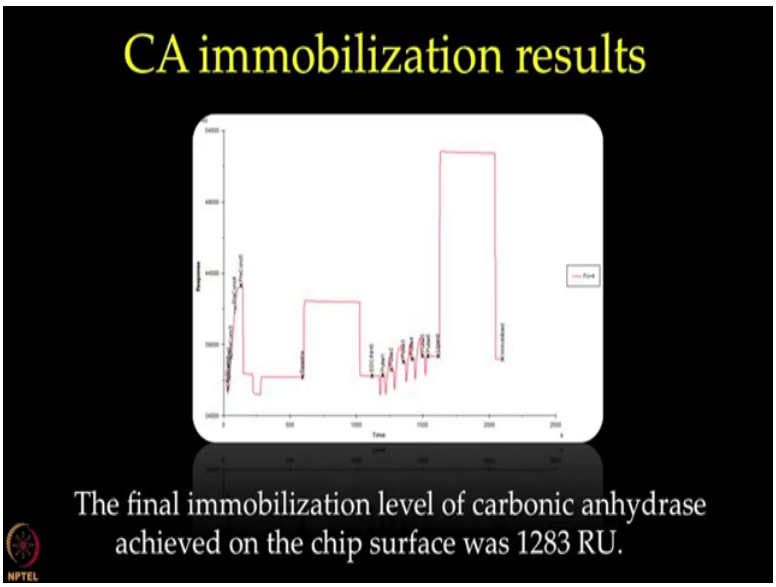

We will hence look at the immobilization results obtained from this run. Since, it is recommended to use lower immobilization levels for kinetics assay compared to screening assays. We targeted an immobilization level of 1500 RU for carbonic anhydrase. As shown in the figure the immobilization procedure resulted its final immobilization level of 1283 RU s.

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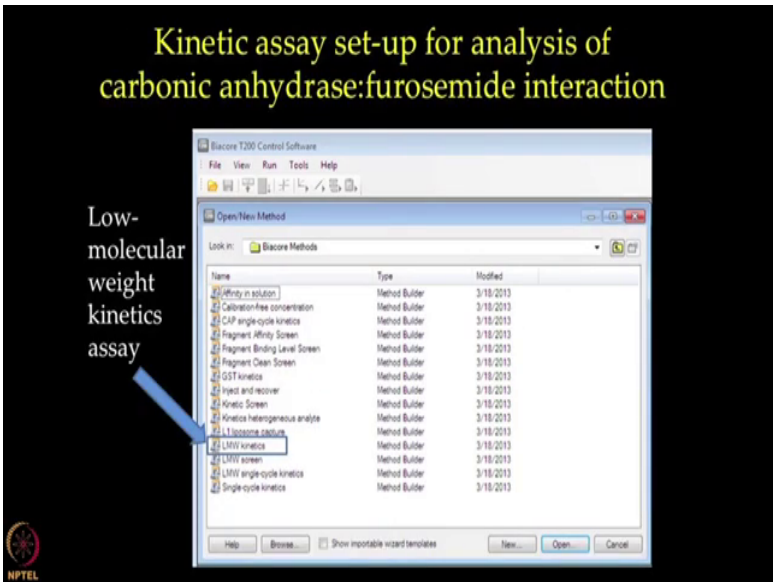

With this, we proceed further with setting up of kinetic assay wizard for low molecule weight compounds to study the interaction between carbonic anhydrase and furosemide.

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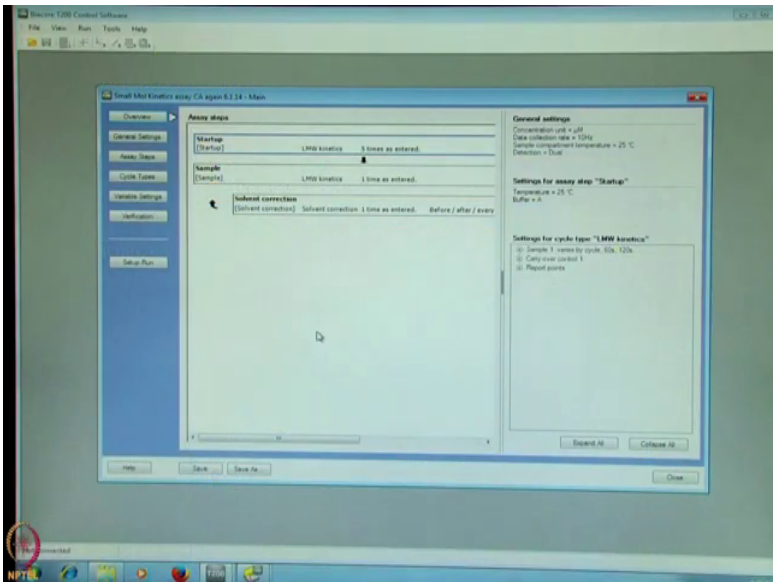

We now see how a small molecule kinetic template is setup. Similarly, here as well we have the startup we have a sample flown at least once on the surface and a solvent correction with 8 different solvent correction points set here, here also.

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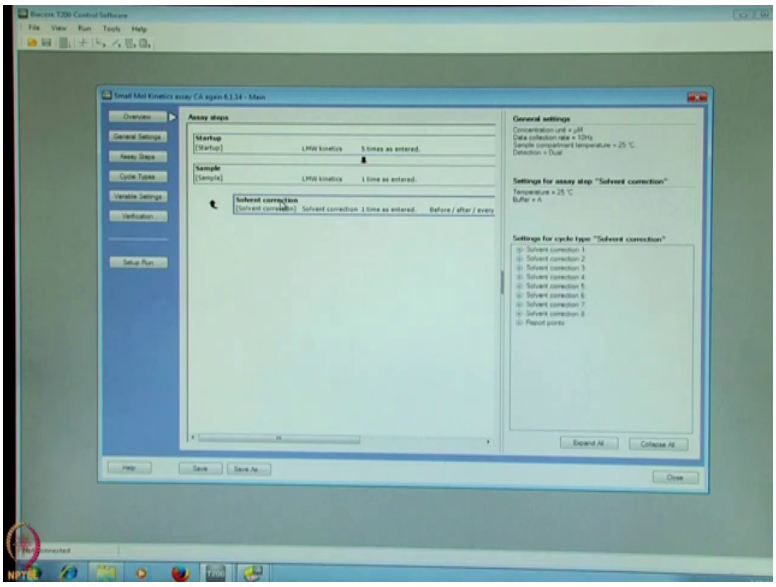

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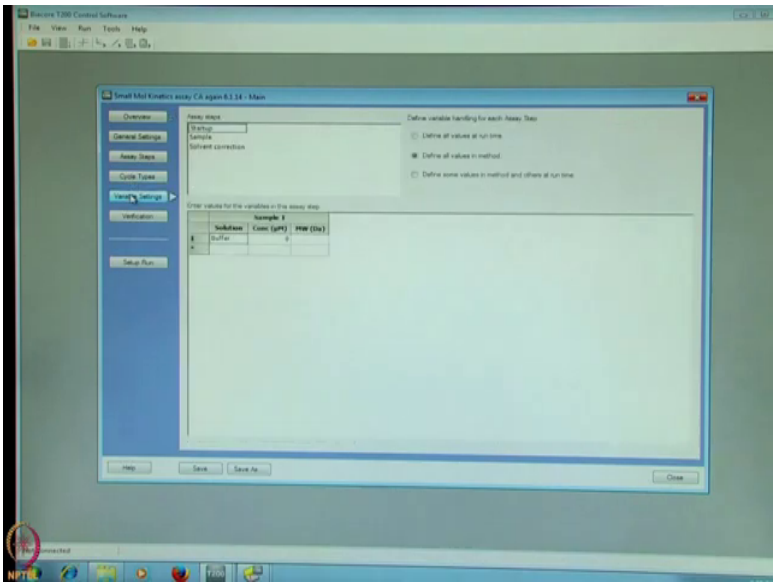
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Small Mol Kinetics assay CA again 6.1.14 - Main
Overview
General Settings
Assay Steps
Cycle Types
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Verification
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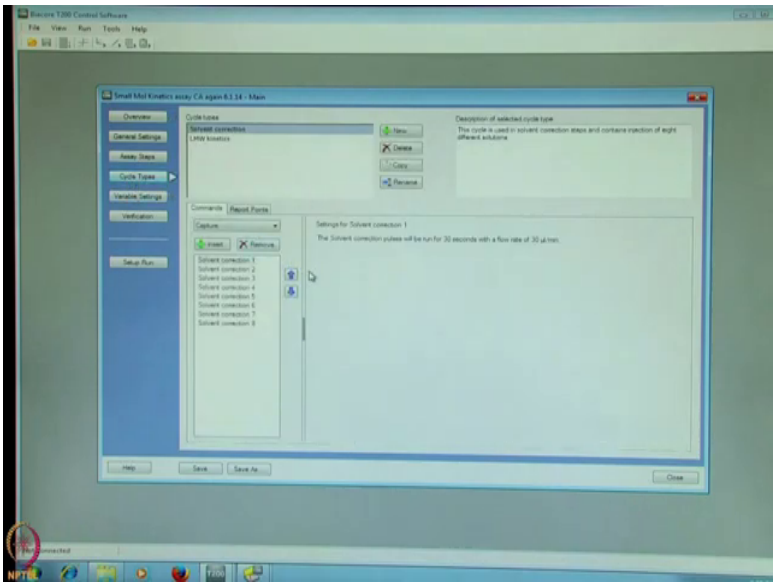

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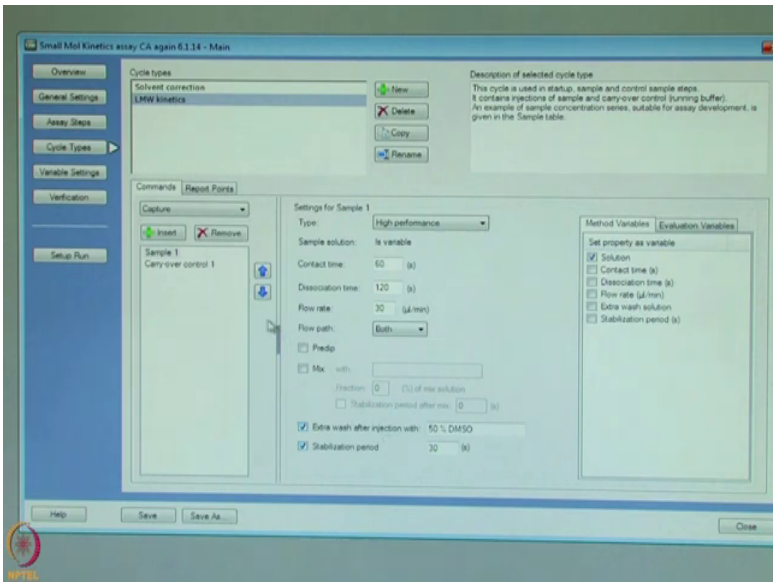

In the variable setting or in the cycle type we go to the low molecular weight kinetic wizard check that the type is high performance. Contact time is 60 seconds, dissociation time is 120 seconds, flow rate at 30 microliter per minute, flow path both, extra wash with 50 percent DMSO, stabilization time of 30 seconds is provided here.

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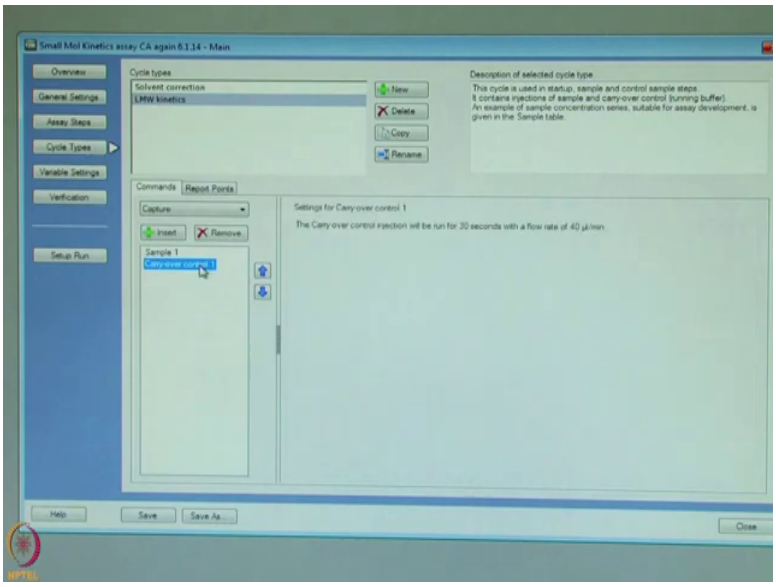

Also, there is a carryover control injection will be given at a flow rate of 40 microliter per minute.

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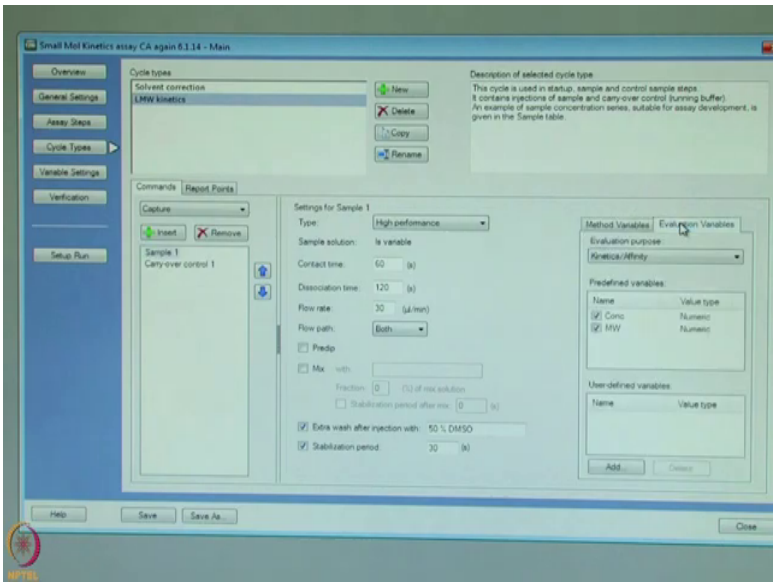

Similarly, the sample solution is a variable here also the concentration and the molecular weight are variables here.

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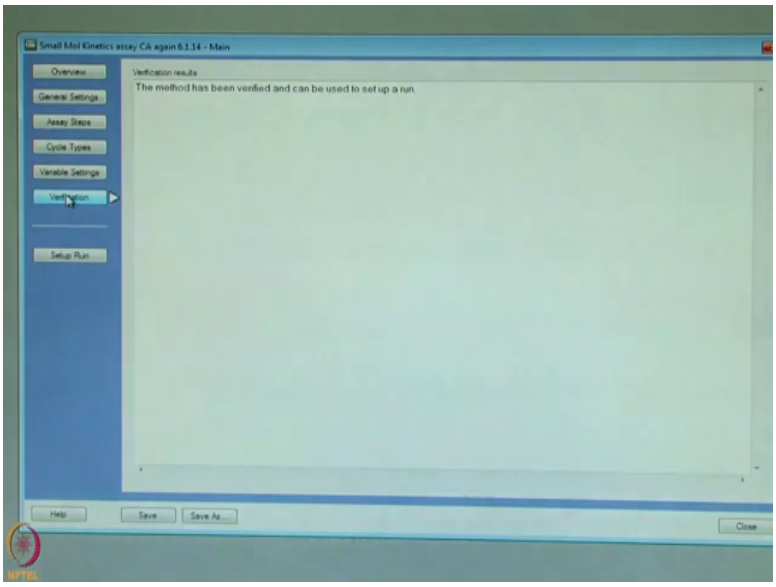

So, we go into the verification mode and see that the method has been verified and can be used to setup a run, we go into the setup, here the flow path is 4 minus 3.

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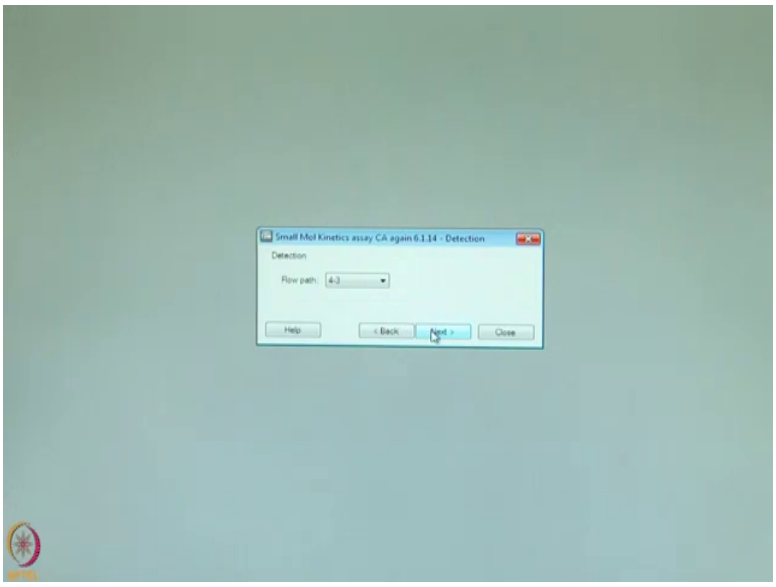

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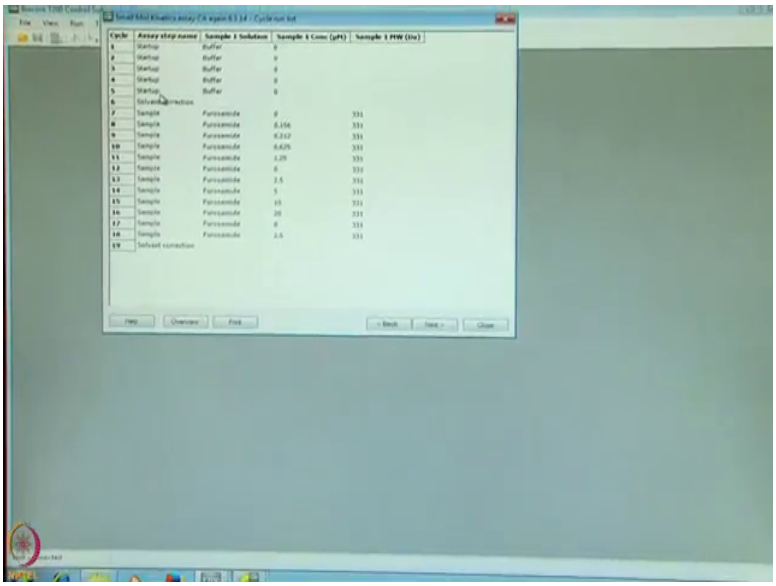

We go on next, we have 5 startup cycles. One set of solvent correction before the run and another select solvent correction after the run; in between, we have the furosemide injected at various concentrations on the surface of the carbonic anhydrase and the kinetic data is accumulated here.

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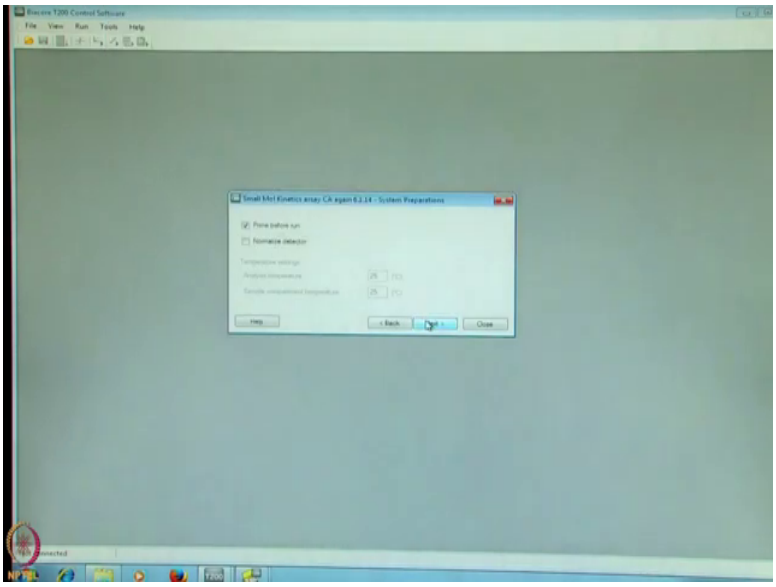

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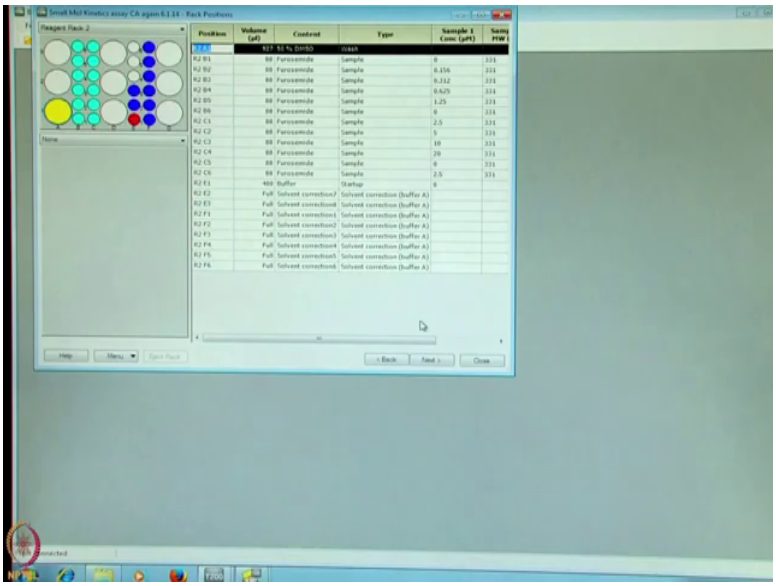

We have highlighted prime before run and the following are the rack positions for various samples with furosemide at different concentrations. 50 percent DMSO buffer for the startup, solvent correction. 8 different solvent correction sample vials provided for successful kinetic experimentation of furosemide binding to carbonic anhydrase.

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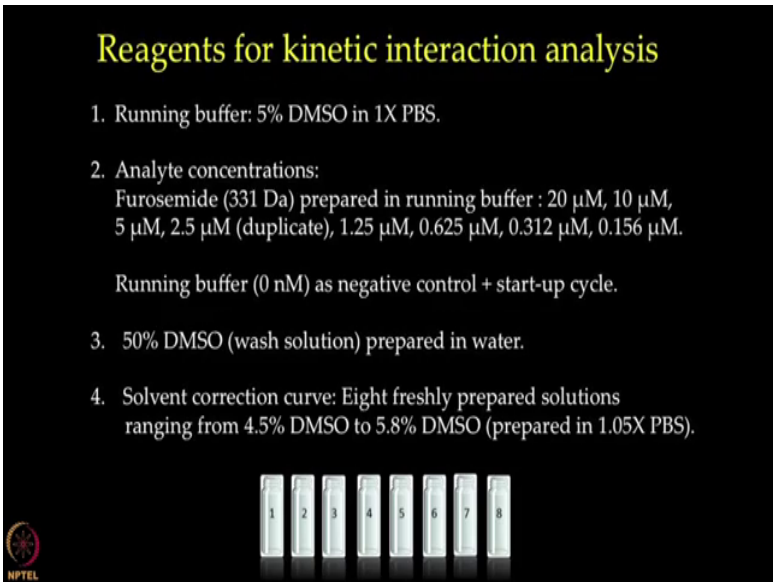

As per the volumes listed in the table, we prepared the following samples and reagents. We prepare a running buffer of 5 percent DMSO in 1X PBS, we will be preparing a total of 5 concentrations of furosemides starting from 20 micromolar, 10 micromolar, 5 micromolar, 2.5 micromolar, 1.25 micromolar, 0.625 micromolar, 0.312 micromolar and 0.156 micromolar using a twofold serial dilution in the running buffer with 2.5 micromolar concentration in duplicates.

Running buffer will be used as a 0 concentration negative control as well as in startup cycles. A wash solution is prepared with 50 percent DMSO in water. Lastly, 8 freshly prepared DMSO solutions ranging from 4.5 percent to 5.8 percent DMSO are prepared in 1.05X PBS for solvent correction.

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We will now work on the reagents required for the kinetic assay to study the interaction between human serum albumin and furosemide.

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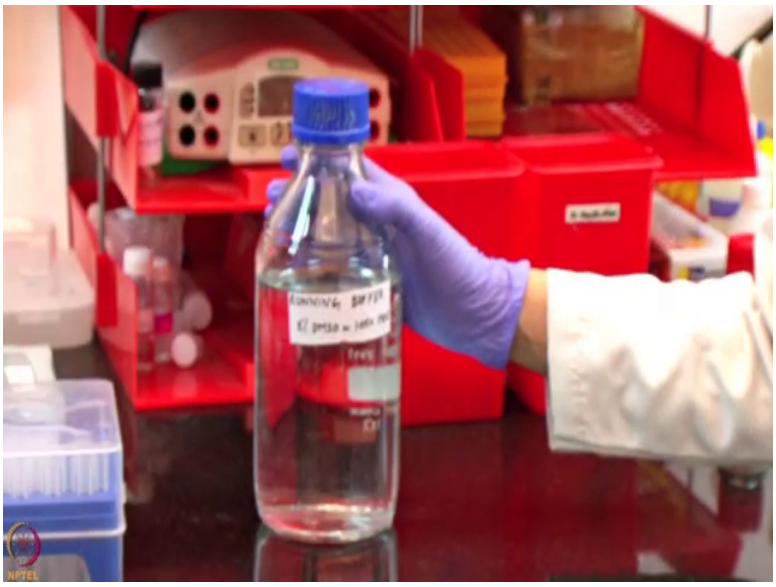

In this study, we will again be using 5 percent DMSO in 1.05X PBS as the running buffer which will also be used for the initial startup cycle.

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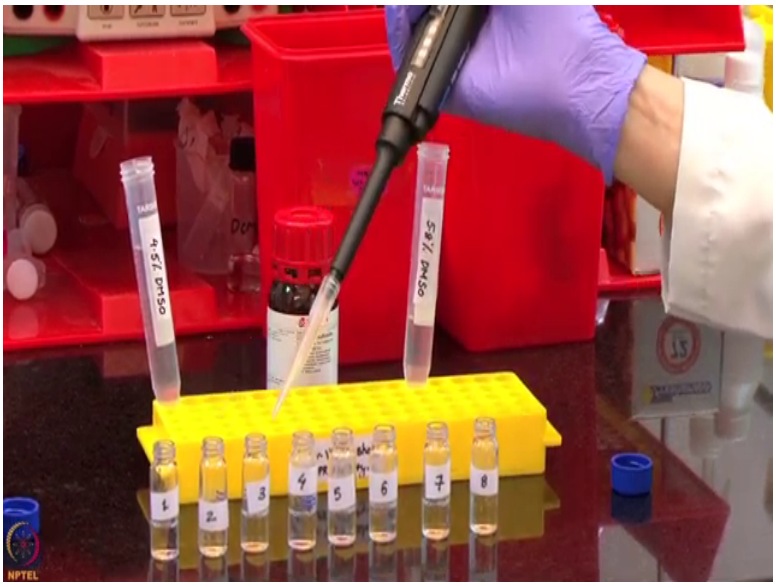

As prepared in the screening assay; we will again prepare 8 different solvent correct solutions ranging from 4.5 percent DMSO in PBS to 5.8 percent DMSO in PBS by mixing these two solutions in different ratios.

To reiterate, we have numbered the tubes as 1 to 8 and added 200, 400, 600, 800, 1000, 1200 and 1400 microliter of 4.5 percent DMSO in tubes 2 to 8 respectively. Following this, we added 1400, 1200 1000, 800, 600, 400 and 200 microliters of 5.8 percent DMSO in tubes 1 to 7 respectively. We will make these solutions rigorously and put the lids on and keep them in dark till we are ready with all the other samples.

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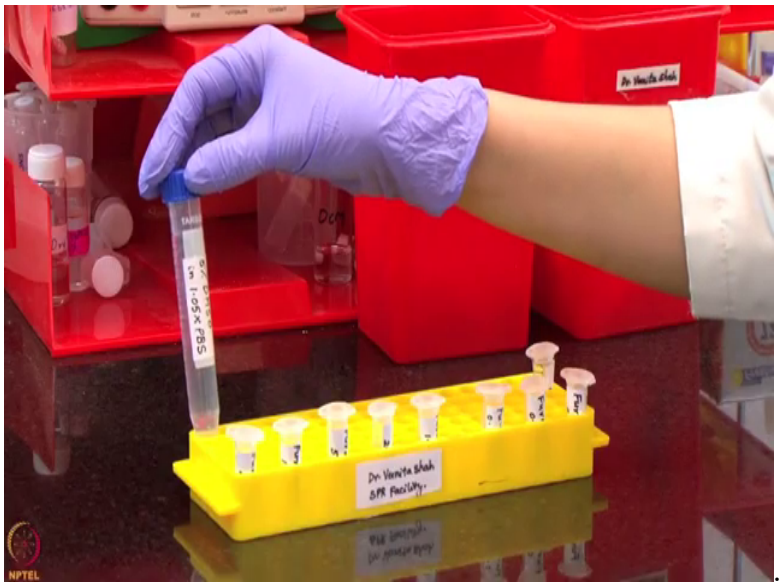

We will be using 8 different concentrations of the analyte furosemide in the running buffer including 20 micromolar, 10 micromolar, 5 micromolar, 2.5 micromolar, 1.25 micromolar, 0.625 micromolar, 0.312 micromolar and 0.156 micromolar.

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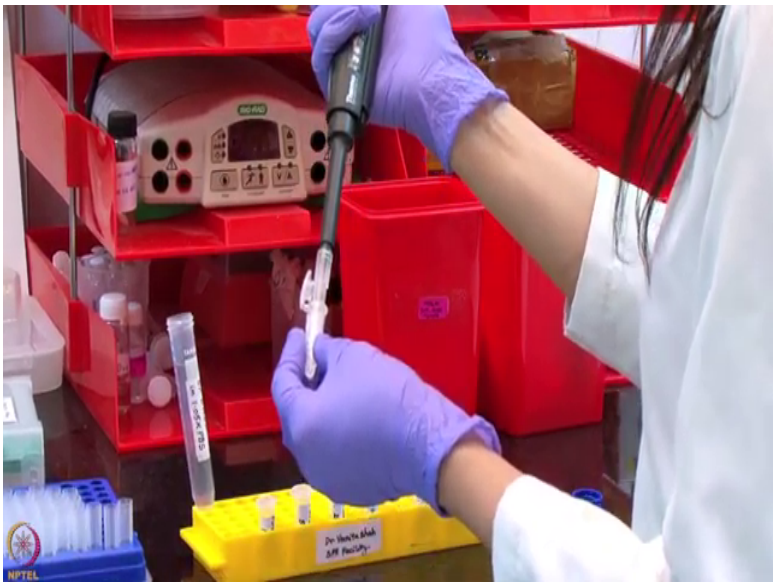

We will prepare this concentration series in twofold dilution using running buffer starting from 20 micromolar; which means, we will make 200 microliter of 20 micromolar protein with 200 microliter of running buffer to get the 10 micromolar concentration.

We similarly continue the twofold dilution to obtain the rest of the concentration series. One of the concentration should be running duplicate. So, here in this experiment we will be running 2.5 micromolar concentration in duplicate. And we will also include 3, 0 concentrations which be nothing but the running buffer. All of these samples and reagents have now been aliquoted in the required volumes in these specialized tubes.

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We will now proceed to insert these tubes including the sample the startup 50 percent DMSO and the solvent correction solution into the appropriate racks and then into the system to perform the protein small molecule screening assay.

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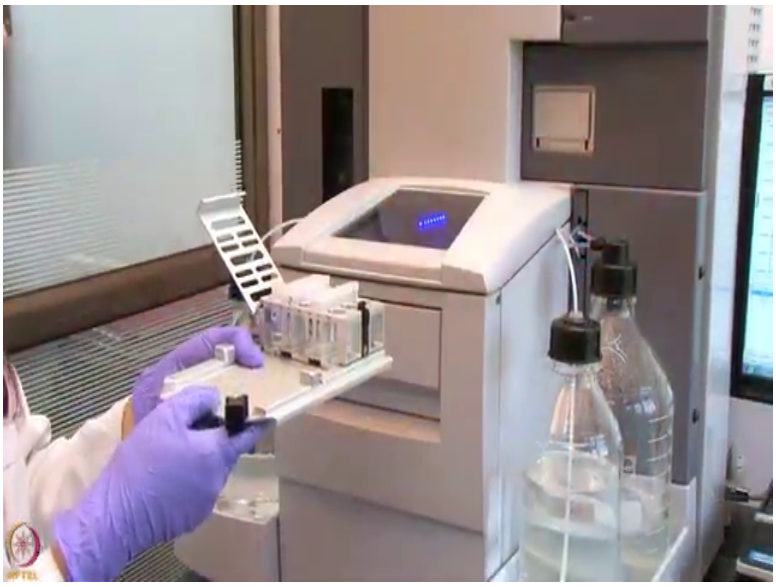

We have now placed all the tubes inside the rack including the wash solution, the furosemide sample, the startup and the solvent correction solutions. And now we will place this rack inside the system to start with our kinetic assay. We will Now, start with our run as demonstrated in our earlier lectures.

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Points to Ponder

- Low ligand immobilization was performed for kinetic interaction assay between carbonic anhydrase and furosemide
- The carbonic anhydrase was successfully immobilized to 1283 RU using target immobilization approach
- Eight different concentrations of furosemide (0.156-20 µM) were prepared to study the interaction kinetics
- No regeneration solution was required for this protein-small molecule assay

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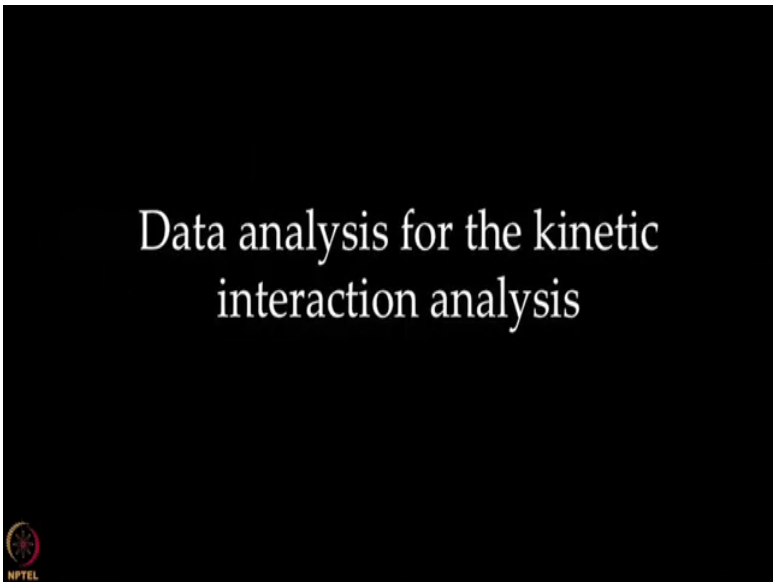

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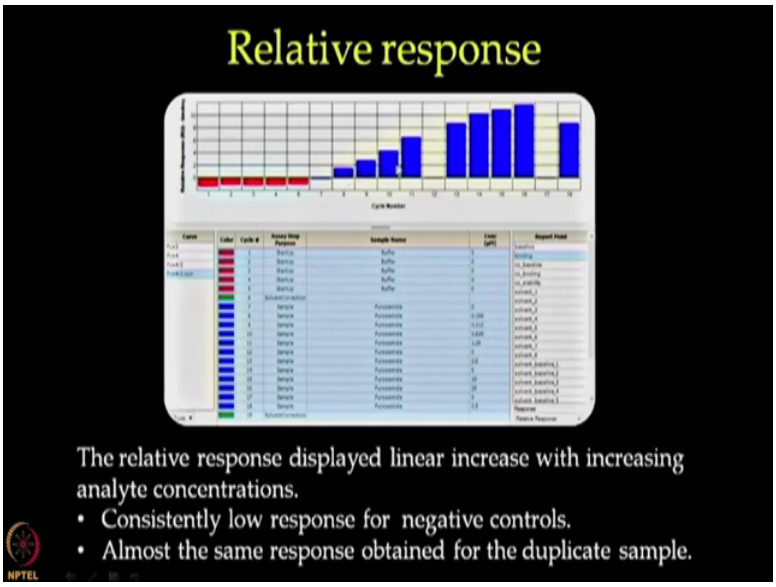

Before starting with the data analysis on kinetics of the interaction between carbonic anhydrase and furosemide. We would like to show the bar graph obtained from the sensorgram which represents the relative response for report point bindings against cycle number for samples.

As reflected in the graph, the relative response displayed linear increase with increasing analyte concentration with consistently low response for negative controls shown by cycles 7, 12 and 17. And almost the same response was obtained for duplicate sample that is 2.5 micromolar shown by cycles 13 and 18. Let us now have a more detailed data analysis on the kinetics assay.

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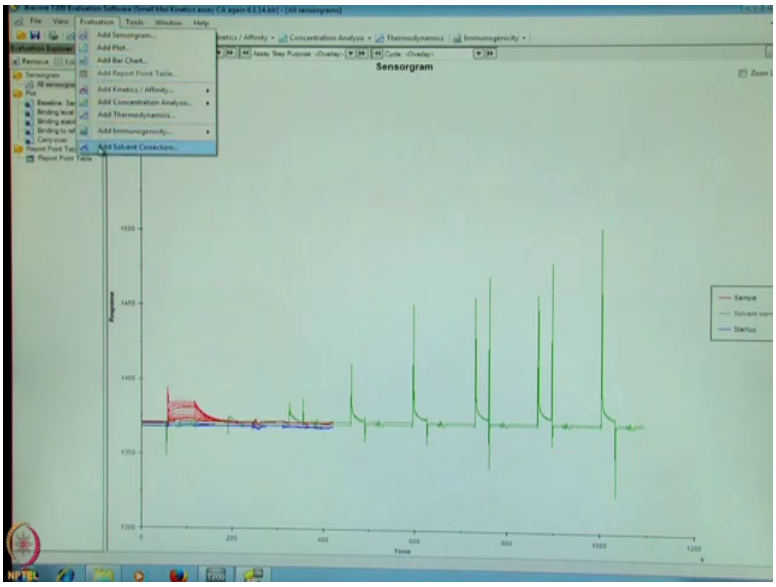

Now, we will look at the kinetic data generated from the interaction of carbonic anhydrase with furosemide. The data looks like this way with a sample solvent correction and a startup. Now to evaluate this data we have to go into the evaluation add solvent correction and once the solvent correction data looks ok, say ok. And the data is now subtracted from the sensorgram.

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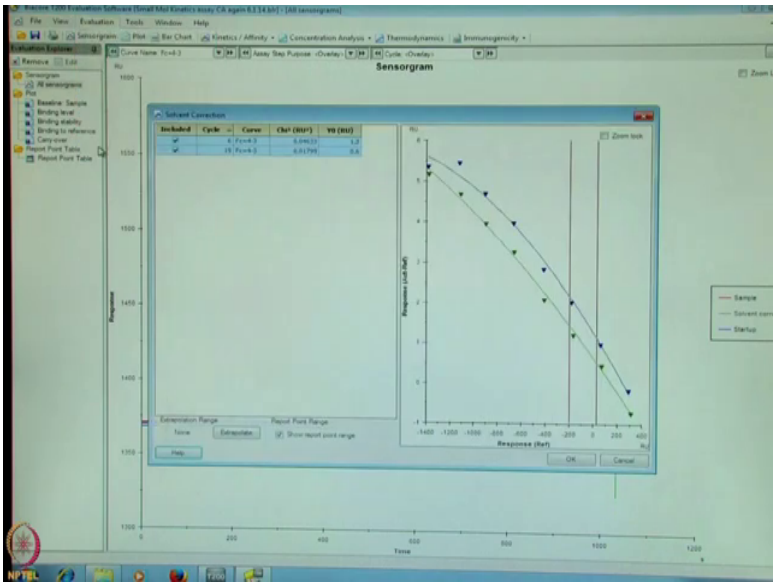

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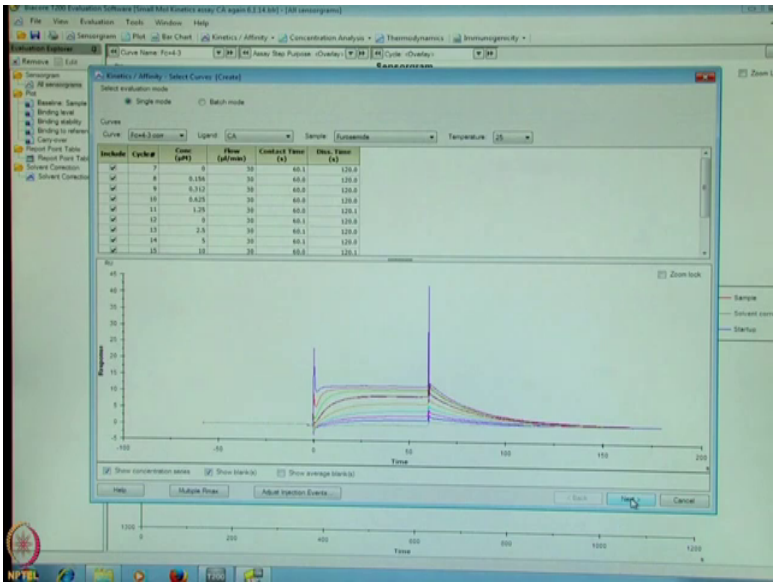

From this again we go into the kinetic affinity mode, surface bound data is shown here with a different colours for furosemide concentrations.

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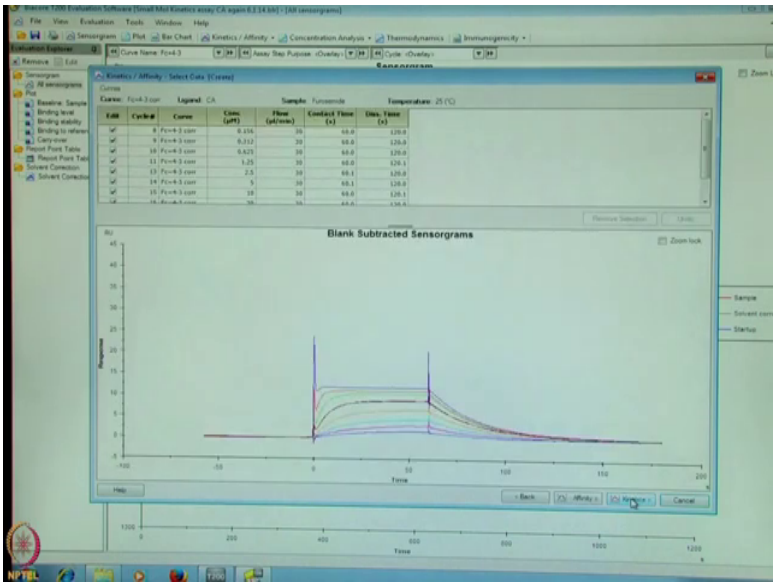

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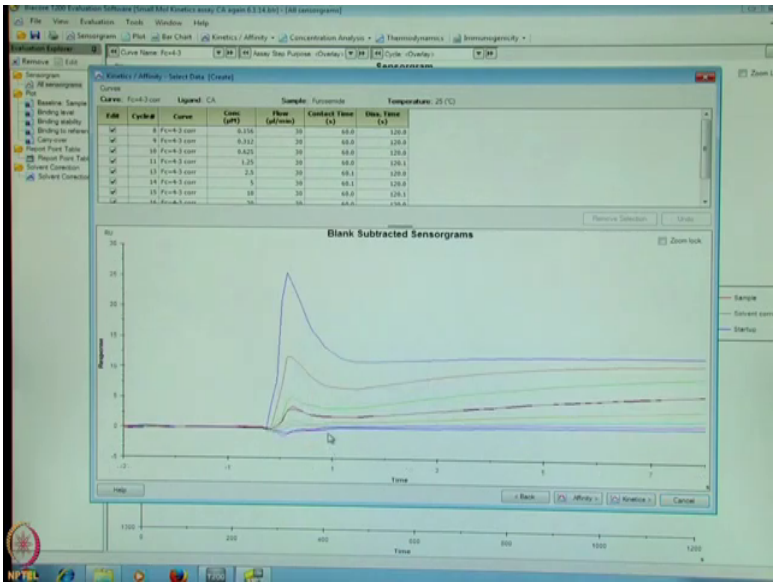

We say next and we can clearly see the data having some spikes. So, using the mouse button we can highlight that range, subtract or remove the selected areas. Similarly, we can do that here as well.

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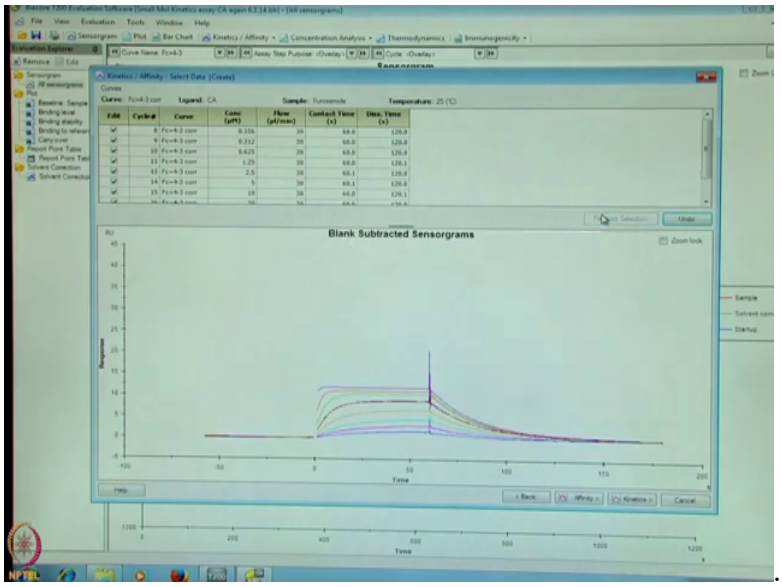

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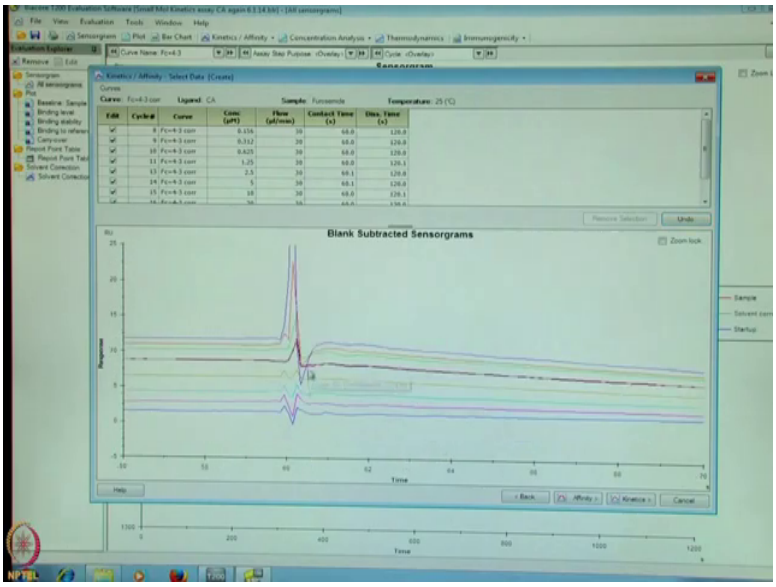

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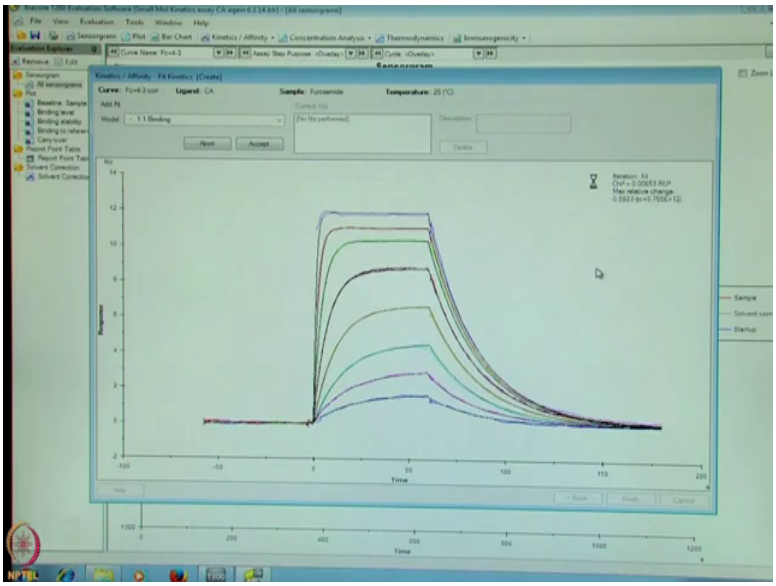

Once the subtraction is done, we can go further to evaluate in the kinetic mode and fit the data.

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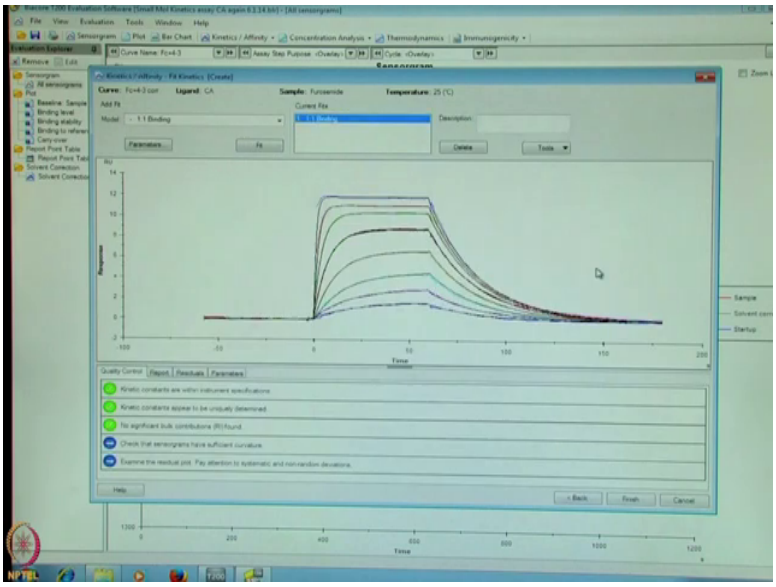

So, here on the quality control tabs you see all of them green and there is no significant bulk contribution found and data is uniquely determined and the data points or the kinetic on and off rates are within the range of the machine.

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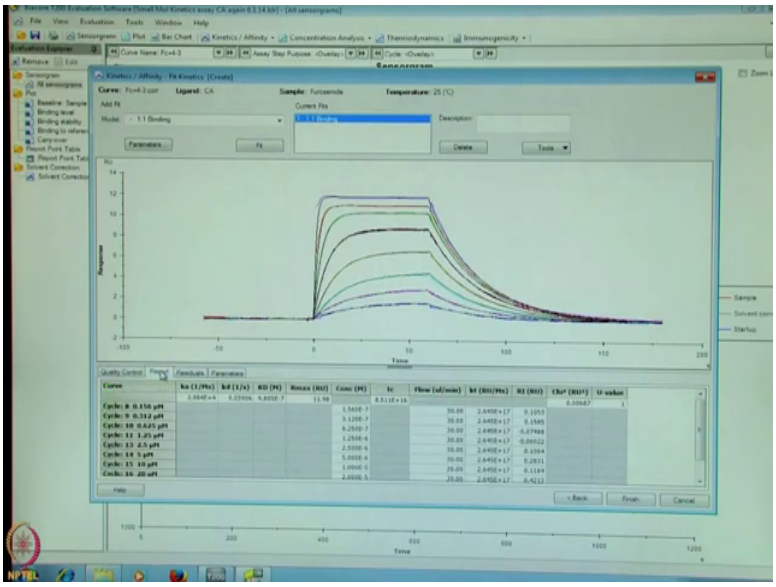

The report shows on rate, off rate affinity constant or binding constant R max of 12 RU, various injected concentrations flow rate. Differences in the refractive index of the sample and the running buffer chi square below 1 and u value below 25.

So, this data shows the interaction of furosemide with carbonic anhydrase is 9.8 into 10 to the power of minus 7 molar. With this we go to the next tab and analyze the data.

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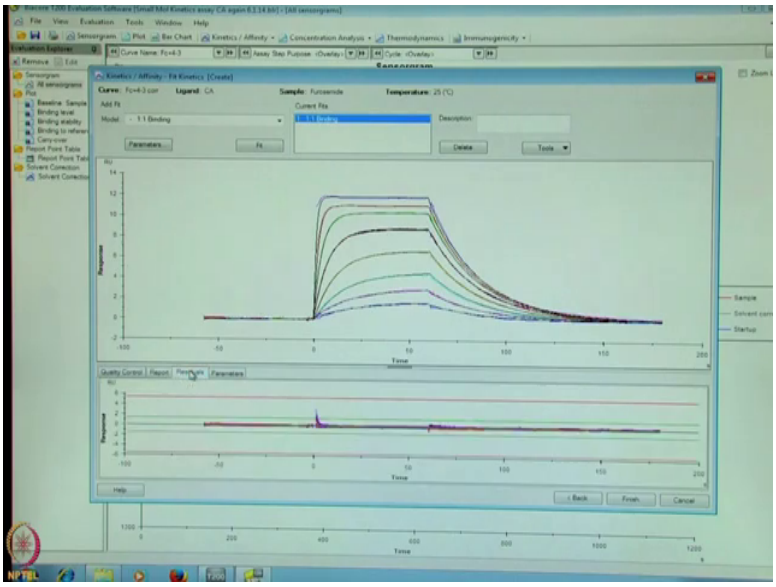
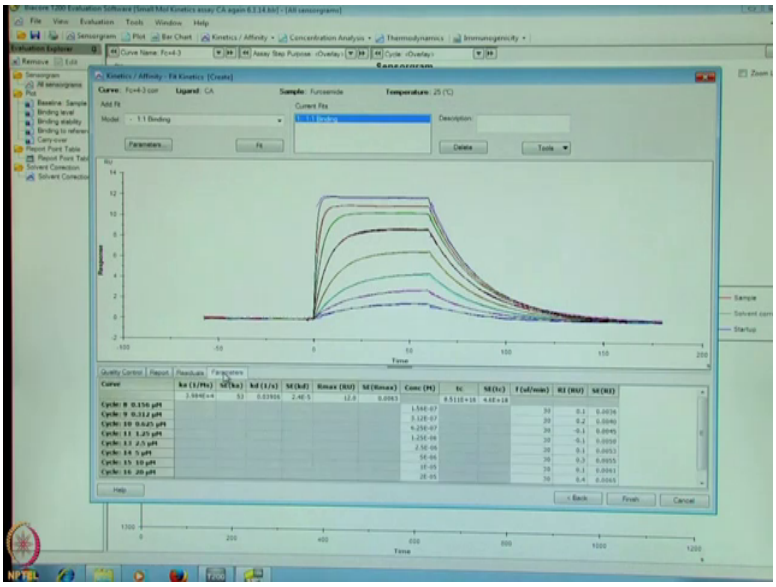


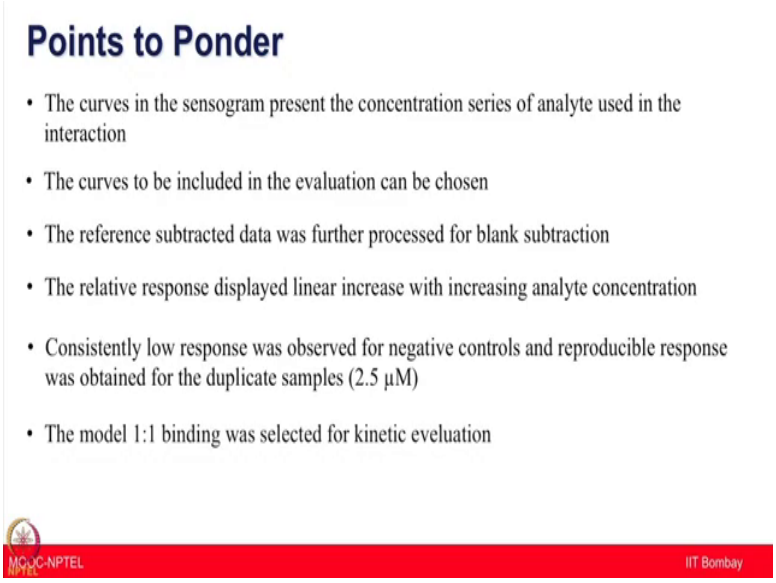
Here with the one to one fit of the data, the residuals or the differences from the black line to the coloured line should fall within the green areas or at least within the red area. Here they are within the green areas and looks absolutely good to proceed with this data.

(Refer Slide Time: 20:12)



Similarly, other parameters like the standard error is provided here. With this, we will conclude the kinetic information generated for the interaction of furosemide with the ligand carbonic anhydrase recorded at 25 degree centigrade and solvent corrected for the DMSO presence in the running and sample buffers.

(Refer Slide Time: 20:44)



I hope by now, you also got a good understanding of SPR based assays and to analyze the data obtained from these binding experiments. In the next lecture we will discuss the basics of another label free platform which is biolayer interferometry and its applications in protein research.

Thank you.