Experimental Biochemistry
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Lecture - 19
Urea Denaturation of HSA Studied By UV/VIS Absorbance

Hello everyone welcome to another Experimental Biochemistry class. Today, we are going to study about protein folding and denaturation studies. So, basically in the previous experimental part we have seen that protein gives an UV Absorbance and also fluorescence. Basically this is due to the protein residues specially the aromatics residues present in the protein.

Now, what happens basically protein scales at physiological condition within our body? So, it retains it is structure. And, when it is subject to some sort of stress like excess acid, or some denaturating agent, or temperature something like that, it denaturates. What denaturation means basically it means it loses it is tertiary structure, that if the entire study structure the protein breaks to some extent partially or fully, it determines the degree of denaturation.

The details of denaturation you have studied in the theoretical classes. Now, we are going to see how to basically experimentally absorb protein denaturation? Now, various ways are there in order to monitor the denaturation process in protein. Now, the process we are going to add up in this case is spectrum of matrix studies. Mainly were first going to absorb the absorbent study using UV spectrophotometer, there are various types of denaturating agents which are commonly used, mainly they are one of temperature the other is denaturating agents like wanted in hydra chloride urea.

So, we will start initially with urea as a denaturating agent. As a very well know that urea basically denaturates structure the protein breaks with structure and they are by the change in absorbance of the protein can be noted down. So, let us see whether the structure of the protein have any role to play on it is absorbance value. For that basically we need urea solution and the protein we are going to use is human serum albumin. The protein having one tryptophan residue and we are going to monitor the change in absorbance of the 55 residue.

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We have taken an urea solution. So, urea solution we have prepared around 8 molar urea solution. However, the concentration you may vary generally for the denaturation the maximum concentration we are using generally in biochemistry labs it is around 8 molar urea.

However, in this things, since the maximum concentration at 8 molar. So, we cannot reach this concentration we will keep our concentration up to 5 or 6 whatever we can manage.

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So, we will initially take the urea solution prepared in a volumetric flask in a small beaker. This beaker should be initially washed cleanly and dried small amount of urea that will be required for our experimental purpose.

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Before starting our experiment, we have to initialize the UV spectrometer and do the baseline correction. Now, initialization has already been done in the instrument, for baseline correction as we have previously seen that we need to fill this to qubits, that is the UV Qubit with water which of the solvent in our present case.

Now, as we have already seen in the previous video that the Qubit has 2 sides; one is the clean side, very transparent side, and the other is actually the frost side. We have always to start touch the frost side while taking the solution.

Now, we will take water that is in the pipet and fill it properly before using and preferably it should be dried. In this case we require 3 ml of double distilled water. Similarly I will be filling the another Qubit with double distilled water.

Now, once we have fill this Qubit you have to wash this transparent side properly and I will put this in the chamber.

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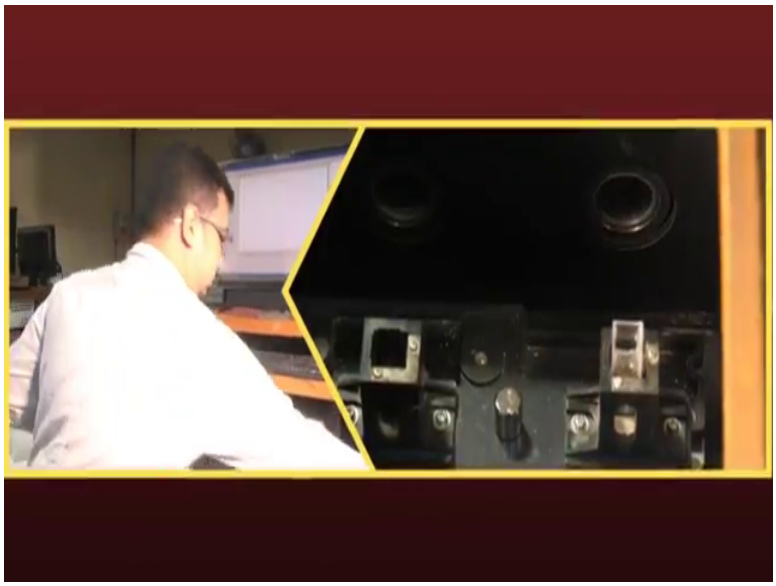

Now, this is the UV chamber here this is the compartment and those to the holder for the qubit, this has been previously discussed in the last video. Now, when you are placing this Qubit you should keep in mind that this transparent side should face the passage of light. This is the light travels from here to here for the transparent side should face here, this one is for reference and this part is for sample.

So, we will put this Qubit in the reference part and we will take this Qubit this is the sample qubit, currently it is filled with the solvent no sample is present here. And, this will be used for baseline correction.

So, we will keep this in the sample compartment.

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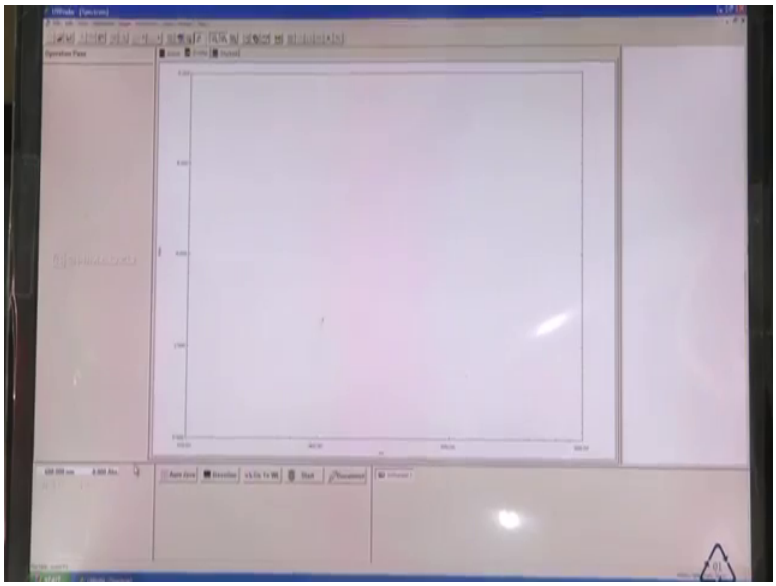

And, then well close the lid. So, we have kept the 2 qubits within the CV chamber. Now, this is the softer as we have seen previously. First, we have to do is to go to the method part noted by M this is the method part.

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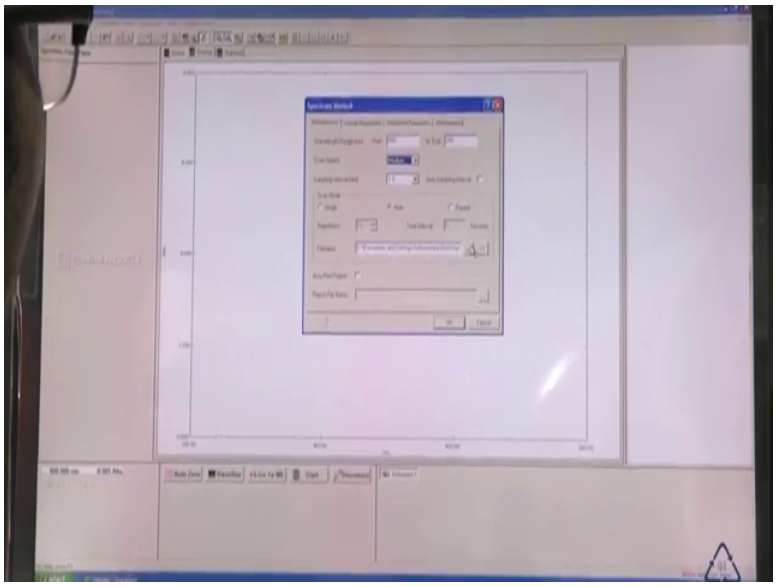

Click here and here comes the spectrum method. And, in this part we have to change the wavelength and here it will start from 200, and this has been changed to 600, and it was initially different part, but for the current analysis we will require 600. The scan speed is medium sampling interval one nanometer wavelength reading will be taken.

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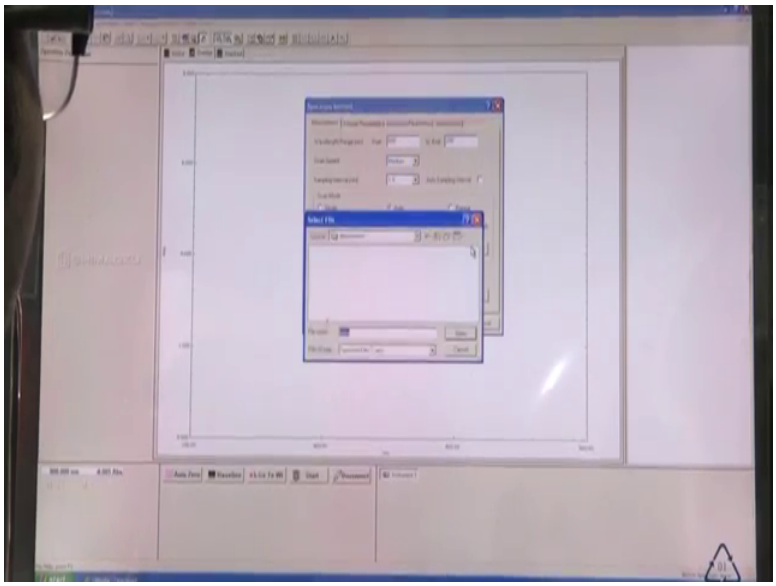

The file name we have currently set up in under in PTL denaturation.

And, other parameters are more or less same in the slit width is to and here let us click ok. Now, if you want to change the wavelength range and you might change the spot, but currently since protein, we have to analyze the absorbance around 200 to 300 nanometers. So, it is better to stick to 600 nanometer. So, let us click ok. And, now we have to go to this baseline, initially we have to do baseline correction this is the baseline part just next to auto 0.

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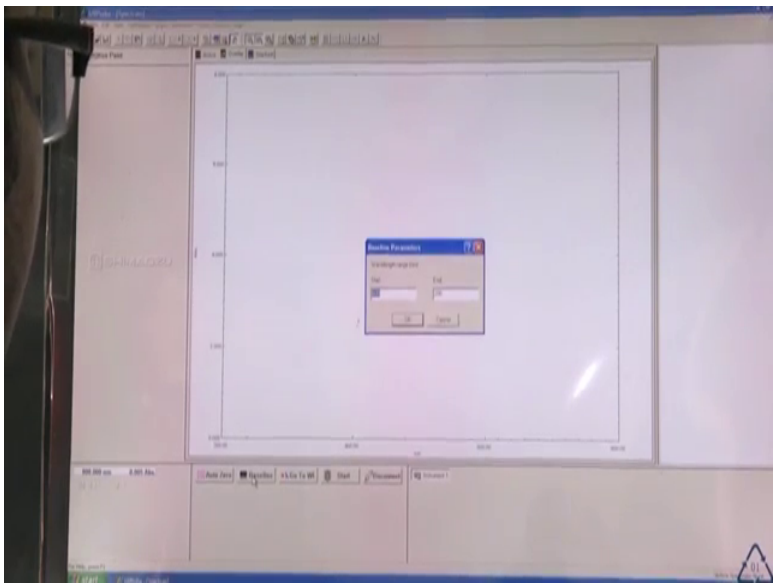

Let us click this base line and it will prompt the another option where you can change the start and the end wavelength, this has been set 600 and 200 So, let us click.

And, the machine will slowly initialize itself for this line correction. In this part you can see that the wavelengths are being scanned, here that is the baseline correction has been scanned from here to here. Do not do anything, do not press or go to any options, while baseline correction is going on. Now, it is slowly reached to this 200 nanometer and the instrument will be ready soon, we can say again this options are enabled. So, our baseline correction is done we can start with the experiment.

Now, we have to see the change in the absorbent spectra of protein in driven of urea. However, before that let us check how the absorbent spectra of urea looks like ok, for that what we will do will take urea solution in the qubit.

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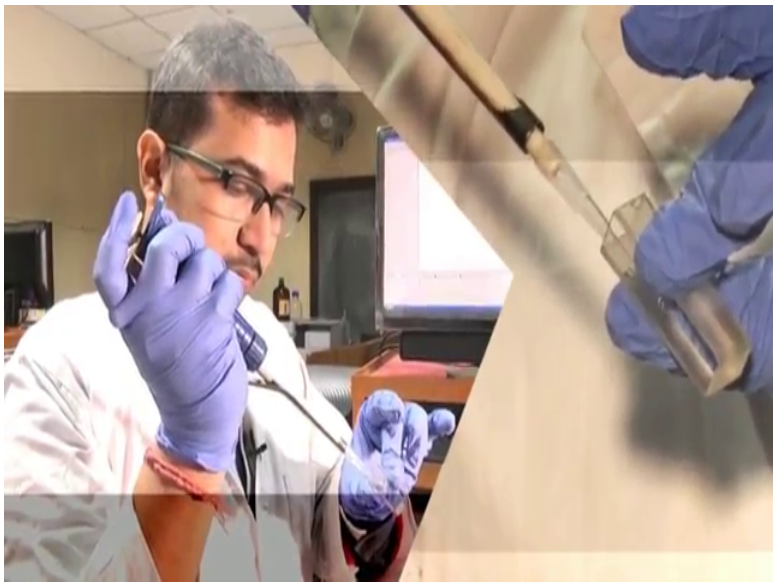

Now, in the Qubit we have initial of water. So, we will take small amount of water out from this Qubit and fill this with urea solution and mix it. So, that the solution becomes homogeneous leave free that air bubbles are not found within those so, Qubit. Once that prepared with this urea solution the Qubit will fit absorbance of this urea solution.

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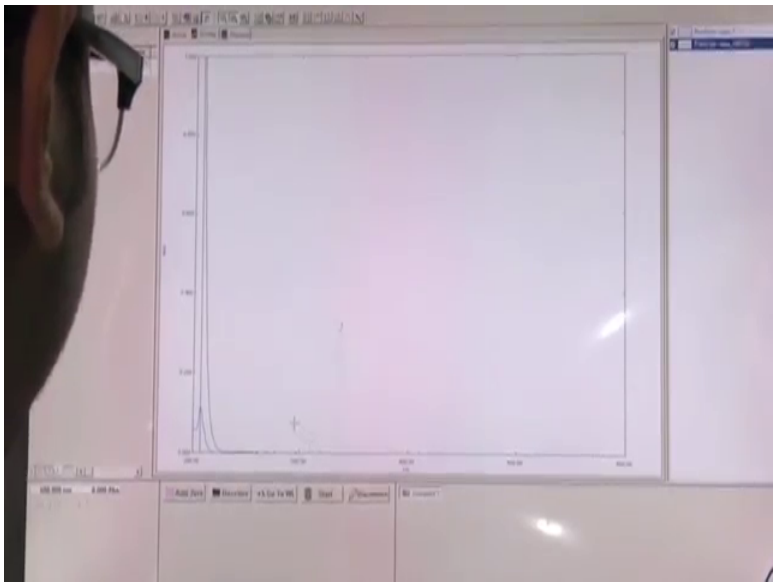

So, you have taken the urea in the sample compartment. Now, we will do auto flow initially, for that the value at 600 nanometer becomes 0 I observed it is value. If, it is done we consider absorbent and set to 0 and now we can start with our measurement.

And, let us click start and here we can change it. Let us change the value bit around 0.5. For here we can see peek at around 205 nanometer from here we can see, know remarkable peaks are obtained in this region 200 to 300.

So, let us check this one by lowering those value around 0.1 we can see this lower range here we can see some arbitrary spectras like there, but it is not a smooth in peak are obtained out here. So, we can say that there are no significant peaks in the region 200 to 300 for urea. And, this might this peak might overlap with the protein amide bond peak, now let us check if we add this urea in the reference compartment what happens basically. For that will add the same amount of urea in the reference compartment.

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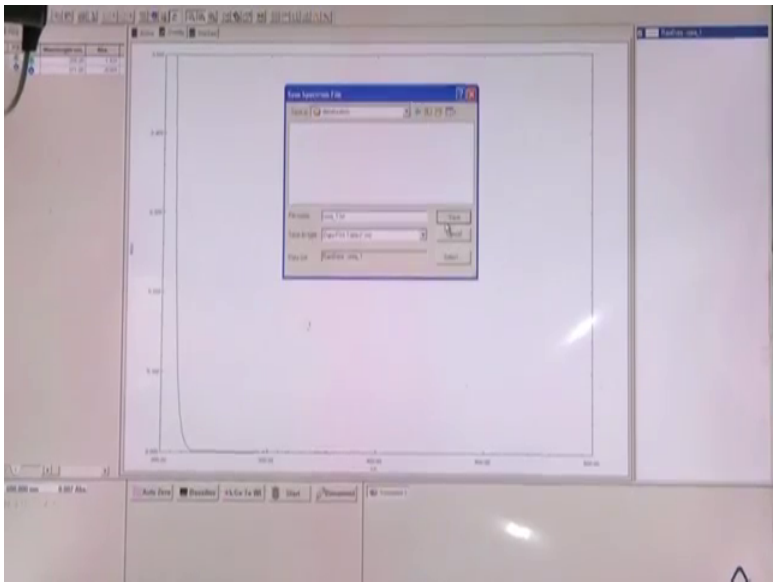

So, let us first save this data as urea one in a spectrum file and you may also save it as data text file denaturation part. Now, we have added uria in the reference compartment and let us see what happens? We will explain why you have added it in the reference compartment, now we have to click the auto 0.

Now, let us click start. So, the scan is already started out here we can see that the wavelength scan is been taking place, we can change for our convenience if we want to see the spectra. It gives an absorbent value of around 207 simulacra last one, over here one important thing to note down out here is that, we have seen that this black line indicates only urea.

And, here the blue line basically indicates urea when added in the sample and in reference compartment. What is basically does is that basically nullifies the effect of urea in both part the small absorbance out here. Basically maybe due to the manual error which might have occurred in while taking the solution, the solution the volume of may not be exactly the same or the concentration might differ a bit. So, this small range of error might occur in the lower wavelength range.

However, since I not much constant in this case so, what we will do? We will just consider out here in the 200 300 region, where we can see no significant peaks I have been shown for urea. So, we can easily carry out our protein denaturation studies.

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Now, we are going to start with our actual experiment, that if the change in absorbance of protein. The protein we have taken here is human serum albumin. The human serum albumin is basically protein in a known as HAS. Now, HSA has one tryptophan residue and as we have discussed will monitor the change in the tryptophan absorbance.

Now, this HSA we are going to take a small amount of HSA in the Qubit and we are gradually going to add their urea and see how the absorbance spectrum changes?

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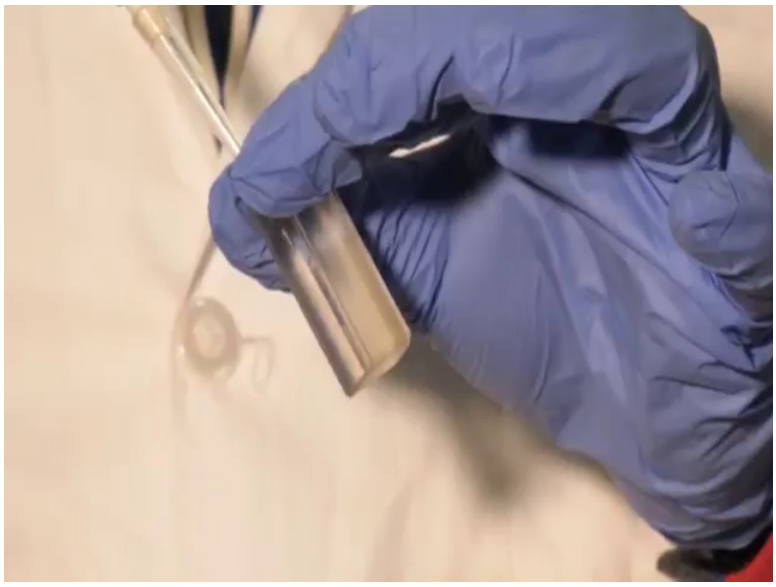

Now, this is the Qubit out here, we are taking small amount of water in the Qubit. And, filling equal amount of HSA solution out here, now please remember to wash this Qubit properly. After this previous observation, I have cleaned this Qubit very well. The Qubit should be properly cleaned and if possible to be dried and then we have to take the next solution.

So, we have taken HSA were going to homogeneous it as much as possible try to avoid bubble formation, bubbles are being formed out here currently now we have to just remove those. You can remove the bubble with Qubit try to mix them homogeneous now the bubbles are being removed.

Now, this is HSA solution we had to see the absorbance of this he full solution.

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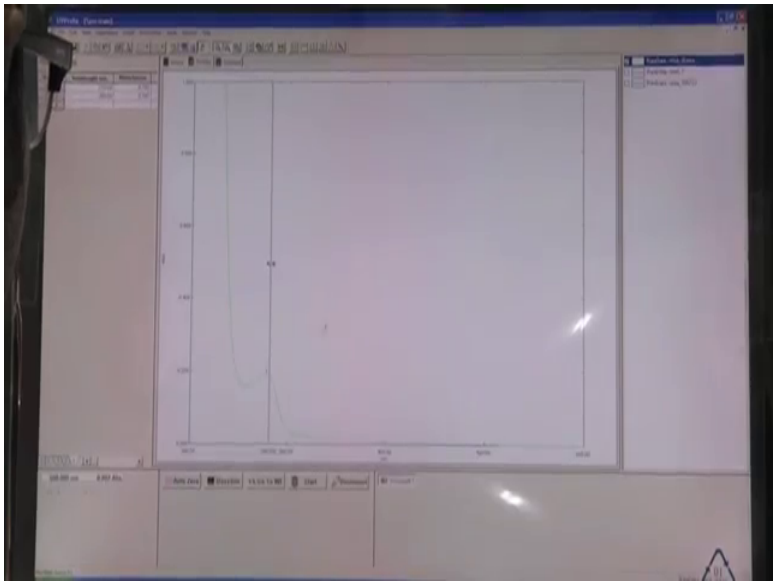

So, we have taken HSA in the sample Qubit and reference group it is currently filled with water double distilled water. Now, we can see some sort of absorbance is coming out here. So, we have to do auto 0, it will take a bit few seconds. Now, we can see the absorbance is currently set to 0. Now, we can start this experiment and 0 scan has already started. And, whenever scan is going on it is recommended or rather it is advice please do not open this lid. Now, here we can see a gradual appearance on the spectral line. Here we can see the major peak for the amino acids and there goes the spectra for the peptide bands.

Now, this one here is the region of concern. Now, the first thing we need to do is to save this part denaturation, you have to save this one as HSA 0 urea, that if we have not added urea this is the normal HAS so, 0 urea. So, saving this as spectrum files and after that we are going to save as in the data printable dot txt, we are serving it as a text file with the same name 0 urea. Now, let us see how it comes basically if we drag this line vertical line, we can see around 278 were having a value 0.091.

And, however, if you want to see or if you want to study what is the value of around 280, which we can monitor, we have to just click here 280, we have to just enter 280 out here and press enter and it will give the value around 280. So, you may consider anywhere around 270 to 280 depending upon the situation, which I will discuss later on.

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So, this is the HSA solution we are just taking out from the sample compartment.

Now, we will be adding urea to it the urea will be around 0.2 molar urea, the concentration currently set out here. So, this is the urea solution we are going to take definite volume. So, the contribution is 0.2 molar. And, we are going to add it out here and make sit properly try to make it homogeneous, see no bubbles are there wash the 2 transparent sides with the tissue paper. And, let us transfer it in the chamber.

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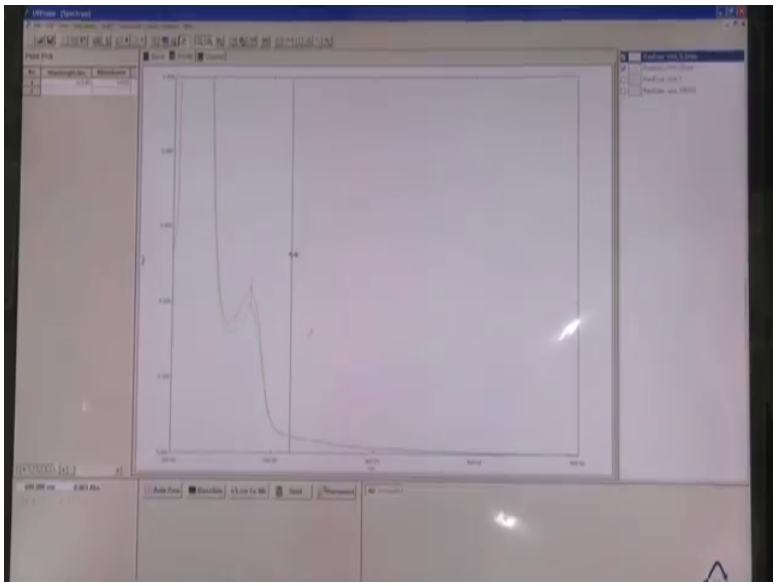

So, let us click on auto 0 although you can see the absorbance out here is all set to 0, but we can again do it auto 0, auto 0 is complete and now we will start with the scan let us click start and the samples scan is already been started. Here we can see the absorbance likely increases from the previous one. Now, once complete let us save this one and then we will go for analysis to save denaturation, now you can click here HSA on the score 0.2 molar urea click save, and then go to save in the same name save it as text format ok.

So, now coming to this analysis, what is basically happening out here we can see if we change it scale a bit around 0.5. Here we can see the green line is basically for protein without any denaturant or urea, and this red line is when 1 2 molar urea has been added and we can see there is a change in the absorbence value out here.

So, what we need to know is that we basically need to analyze this change where the change is actually occurring. Now, the change we can see if occurring mainly around 278 the maximum of observed around 278 out here. And, then what is the intensity of this red line we can consider from here point to 1 4. It is recommended to current currently write down this intensity whatever you get for a particular concentration.

However, if you miss intensity of the first one say if you did not note down this for pure HAS. What you can do is that, you can click here this are the options, you can click out here you have this is the raw data HSA 0 urea double click out here.

Once you double click this will be selected and you can go to this active part, out here we can see active overlay start and if we click the active part. Now, the active part will be shown the part will be shown which is activated that is only HAS. Here we can see at 278 the values around 191 point sorry which in the activated part here we can see the HSA con value the absorbance value is 0.191.

Now, if we click this HSA 0.2 molar here we can see around 278. The value is around 0.214 that is from 191 it is increased 0.214. So, let us click on this overlay and what the stack do the stack actually shows you different types of spectra one above the another it is stacking them, and it is better to analyze in the overlay part where you can basically see the changes in the absorbance. Now, we will add another concentration. Now, we are adding the next urea to the solution for this we are setting the concentration 0.4.

So, we are adding the reclusive among volume of urea to this HSA solution. Now, the 2 ways of adding one you can just take this puppet and directly add it out here kept in the sample holder. Now, we can add it out here, but; however, it is recommended not to do that although sometimes people add out here, but it is recommended to take this Qubit out [FL] (Refer Time: 21:48).

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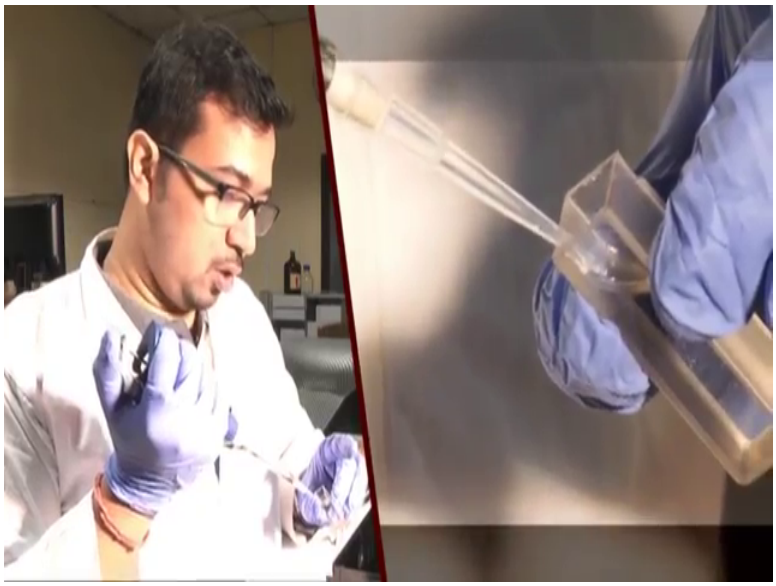

So, we have taken out this Qubit from the compartment and we are adding the required volume of urea to it, please note that change the tip of this puppet from the last 2 via solution we have added never use a tip. Because the solution of urea one you have added

here you have tipped the tip into the solution. So, what happens it has been contaminated with protein as well? So, never emerge this tip once again in the urea solution. So, we are now making those homogeneous.

Now, why it is better to add the solution not in the cham not in the chamber and, but rather outside. So, what happens is that when you take the solution or any solution added in the chamber, sometimes it may happen that the solution might not fall in the Qubit or it might get spilled and fall outside. So, it is very costly instrument the spectrophotometer.

So, it is better to avoid any sort of liquid contamination with it. So, better you take out the Qubit and you can basically control it while you are adding. And, now we have made it quite homogeneous will remove the sort of bubbles formed out here. Now, we will go for the analysis.

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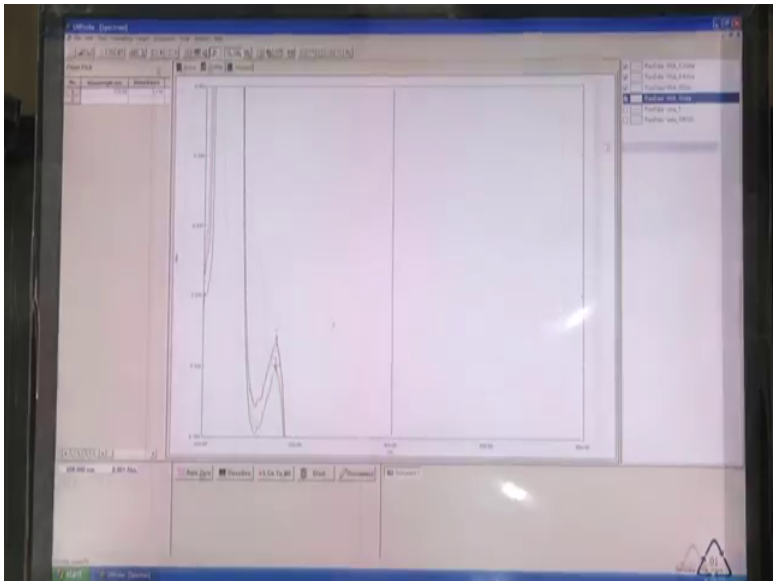

So, now we will do auto 0 each time you take a reading perform this auto 0 part ok. So, it is currently one again set to 0. So, it is values coming one. So, once we are again doing auto 0. Somewhere the value ya it is now 0. So, let us start. Now, let us save this one save as we can click here and the name will appear and we can change this name to 0.4 urea and in the spectrum file. Another file you saved in the name of 0.4 urea and again we can click this save as go there we can change to get up dot txt data file and again save.

Now, coming to this part what we can see, if we change the scale to 0.3. Here we can see the shift or rather the incremental quite low out here, because here the line above the red line the gap is not that much we can also visualize the gap by lowering this or increasing the lower value of an increasing the lower value to around 0.15. Here we can very well observe the change out here. Now, this part this is the green this is the red and the, but just above this red line this the black line is for 0.4 molar urea.

Basically what is happening if that upon giving 0.2 molar urea, the dean denaturation is started. So, the absorbent increases, but 0.4 molar of urea the de denaturation process does not vary that much from 0.2 molar of urea. So, the change in absorbance is not that much prominent.

Now, let us add another concentration of urea. So, what basically has happened out here, that the this part has gradually decreased, from the previous part. This may be basically due to the dilution process. Since, we are adding it to has we are basically doing a dilution that if we are adding urea constantly to it. So, the concentration of HSA might be diluted and that is where we can see a gradual decrease in the absorbent value or matt fluctuations might occur out here. So, it is recommended to subtract the control after you have done the experiment I will show it once.

Now, we have to save this one save as one molar urea. So, let us move it a bit 4 0 or it is around better to save 0.1 5. So, here we can see the green line about this green line this again decreases. So, basically the decrease should not be there, it is basically the dilution factor the solution of getting diluted. So, that is why the absorbant decreases. However, once we subtract the control which is having equal amount of urea in it, then we will get the actual solution.

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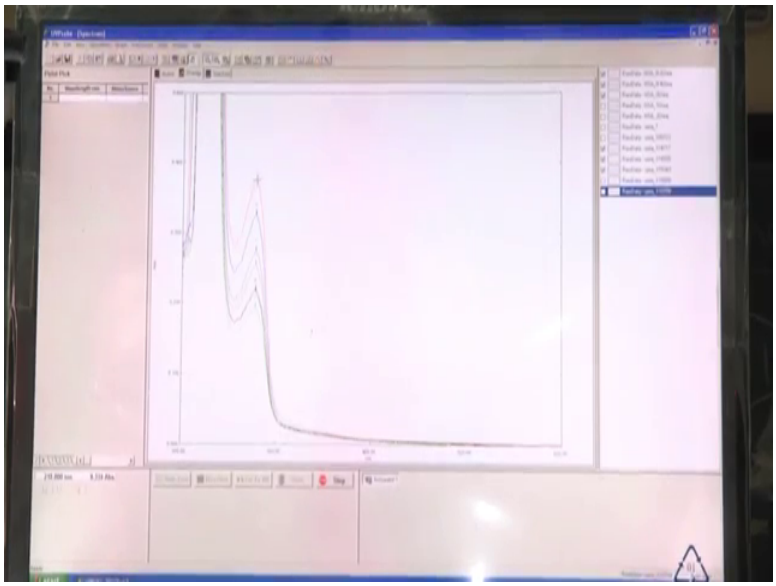

Now, we have taken one molar urea bring the auto 0. Now, it is done let us start here we can figure the increase in the peak intensity. This is for one molar urea solution and we can calculate the intensity at around 278.

Now, as we have discussed earlier basically you can take the intensity at 278 or 280 wherever you fill, but you have to note down the change. The change where the maximum change occurs, generally the maximum change might occur around 278 or the maximum change might occur around 280 or 281. Basically a 270 to 281 280, where the maximum change between those HSA without urea and HSA with urea occurs, and there you have to take the change in the absorbance value.

Now, we will go to 2 milli molar. [FL] Now, we will go to 2 molar urea solution, we have taken for 2 molar urea solution, here we can see the change again for one to 2 is not that much as for 0.421. So, we can see the change once again coming to 0.3 out here. And, this red line the spot is basically for 2 molar urea. Now, we will be adding 4 molar of urea 4 we have done auto 0 again this is for 4 molar.

And, start at least you should take 5 to 6 reading for this plot, you know this plot use the concentration of the denaturant versus the change in absorbance at 278 or 280, wherever it comes maximum. Now, here we can see it is for 4 molar urea. Now, we are doing it for 6 molar urea solution, the effect of urea of contribution 6 molar on HSA protein. Here we can see the intensity increases in a absorbance of this HSA solution.

So, we have seen how the change in concentration of urea caucused change in absorbance value, with increasing concentration of urea the absorbance increases. Basically what happens if that upon adding urea, the protein unfolds and the tryptophan residue comes out in the medium? So, the extent of tryptophan residue exposed in the solvent medium increases it absorbance value actually it was folded, that you all are the tryptophan where not exposed and tryptophan residue is basically buried in HSA.

And, once it we opens up upon adding a denaturant more and more tryptophan residues of different proteins in the solution are being exposed. And, now as we increase the concentration 2 things occur more and more protein are getting denaturate. And, also the extent of denaturation increases and the extent basically increases maximum at around 8 molar of urea, which we are not currently performing here we are restricting ourselves to around 6 molar urea. The reason is that we have prepared the solution of 8 molar. And, now we cannot actually achieve the solution at 8 molar in the Qubit finishes stop solution of 8 molar.

Anyway so, what is happening is that as you are increasing from 1 to 2, 4 to 6 again getting an increase in the absorance value and increasing absorbance value is much higher. So, what is happening is that at higher concentration the protein is unfolding at a maximum rate. And this way what is happening is that more and more tryptophan residues are getting exposed with thereby increasing the absorbance value. Now, were going to see the temperature effect on protein denaturation.