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Lecture – 15
Fluorescence Spectra of Amino Acids and Proteins

Now, let us start with the fluorescence experiment. In the UV visible absorption spectroscopy, we have noted down the absorption maxima of various proteins. Now, we are interested in finding the emission spectra of those proteins, how it looks actually, so for that what we need to do we need a fluorometer. So, what is the fluorometer? This is a fluorometers. A fluorometer is basically an instrument which is utilised for measuring the fluorescence spectra of a protein or any compounds. So, this fluorometer has compartment like the UV one and the screen where we can completely monitor our UV spectra.

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Now, compared to our UV spectra, this fluorometer uses only one cuvette that is only one cuvette. Here, this cuvette is the quartz cuvette and it is much more costlier than the UV cuvette. And one of the important thing to note down in this cuvette is that all the four sides of this cuvette, here we can see a transparent there is no frost side out here, this is because there is we have in seen in the theory classes. In fluorescence

spectroscopy, the light is transmitted in the right angle direction once it goes out here, it is light transmitter right angle direction the fluorescence light, so that is why here we have taken the cuvette and all the sites are equally transparent.

Now, this may cuvette should be cleaned completely cleaned, it is better to clean this cuvette with acetone and dried completely before using it, now we have washed with water for the time being. Now, let us start with our experiment now where we are filing this cuvette initially with double distilled water and taking up spectra first of all. Now, we have taken this cuvette and filled it with 3 ml of double distilled water.

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Now, we will clean all the sides properly. One important thing to note down here is that please do not touch any of the sides of this cuvette, you can touch here in this top or you can touch those corners out here, but do not try not to touch this all those four sides; we can touch the top layer, whether the solution is not there. See weather if bubbles are here or not's, we can see a few bubbles does touch it with the pipette, basically this is not any protein solution, this is pure water.

Now, you have taken double distilled water in the fluorescence cuvette and we will measure the fluorescence for double distilled water for the time being. Now, this is the fluorometer instrument, this one is the lid of the instrument will open this lid.

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And here we can say compartment, now in this compartment interesting thing note down here is that only one holder is present, only the UV instrument where two holders are present for reference and for sample, and that is why we have to repeat this for two times. Now, this one is for the reference we are using this solvent water, now we have placing it in this holder.

However, here we can see one is the entrance slit near the entrance slit excitation light will come, here the monochromator is there, excitation monochromator just behind the slit and it will go through the sample and fluorescence emission will be detected at right angle to with. So, another slit out your exit slit is present here, where it will detect the fluorescence emission range which we will place in the method part and the exit in the exit part the emission monochromator will scan the emission range.

Now, we are placing this cuvette gently out here in the holder and here one thing we can see here the two pipes are there. So, a curious mind may acts what is the reason for this two pipes, basically it is for other experiment in which we are doing any temperature dependence study, here these are the water inlet and outlet pipe; water comes from one place and goes out from the other basically it heats of the sample. We are not interested kindly in this one; we are interested in basic spectroscopic techniques. So, after we have kept this solution in this holder we will just place down this lid.

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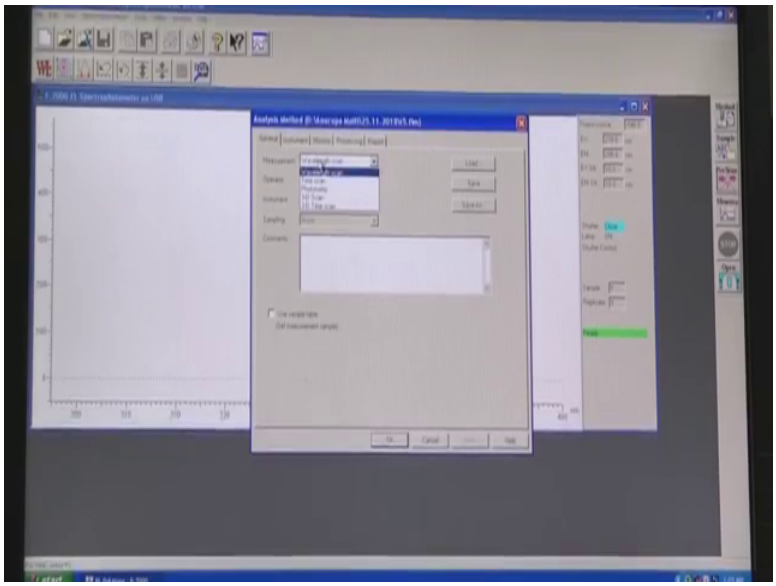

So, this is the software for this instrument where we are doing the fluorescence spectroscopy analysis. Now, different software's are there for different types of instrument for this instrument this is FL solution is a software. Now, we want to go to the method, initial part where to set we have to give information to this software how we want to proceed with our experiment. For this we have to go to in a method part, in the method part we want to do a wavelength scan, Other parts are there like 3D scan, 3D time scan, but you currently we are doing wavelength scan.

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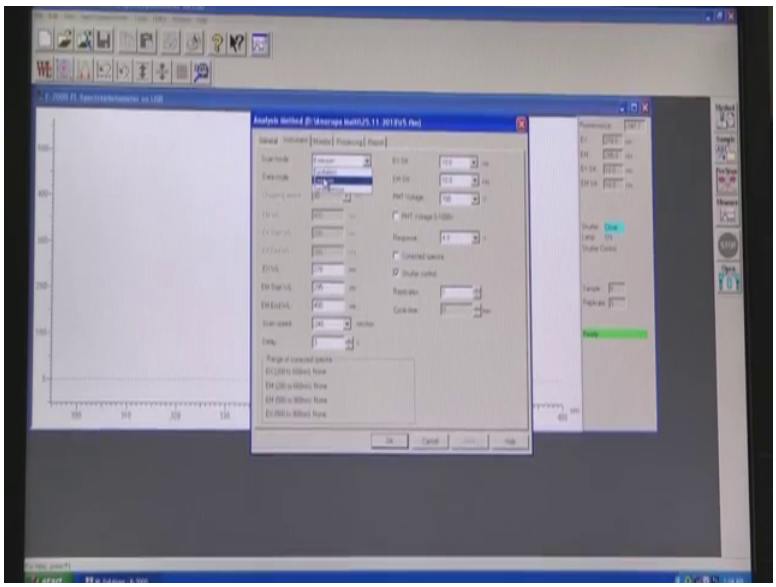

In the instrument part, we are interested in emission. Other things we can do like excitation or synchronous, but currently we are doing emission and data mode is fluorescence not luminous and for phosphorus for the time being, but fluorescence emission we are interested in. The excitation wavelength out here is 278 nano meter, the emission wavelength is our range of wavelength, where we can start from at the emission wavelength start is around 295 nano meter.

Generally, it is kept around 10 to 11 nano meters hard then that of the excitation wavelength. The emission wavelength will end it around 400 nano meter, so we have to give banned series of banned in which the emission we will take place. Now, the scan speed is set around 240 we can vary according to a requirements, but delay is the 3 nanosecond.

Now, you slit width; here the exit and the entrance slit is basically due to the excitation slit of the emission slit, it is from 10 nano meter it is 6. However it depends upon instrument, instrument for instrument with much more higher resolution or much more advance fluorescence, spectrometer instrument, the slit width is basically kept at around 2 to 5. And slit will varies in depending upon the type of fluorophore at type of component out here in the instrument or out here for analysis and also upon the sensitivity of this instrument. Fluorescence slit width for excitation is on 10 nano meter and here its emission is also 10 nano meter.

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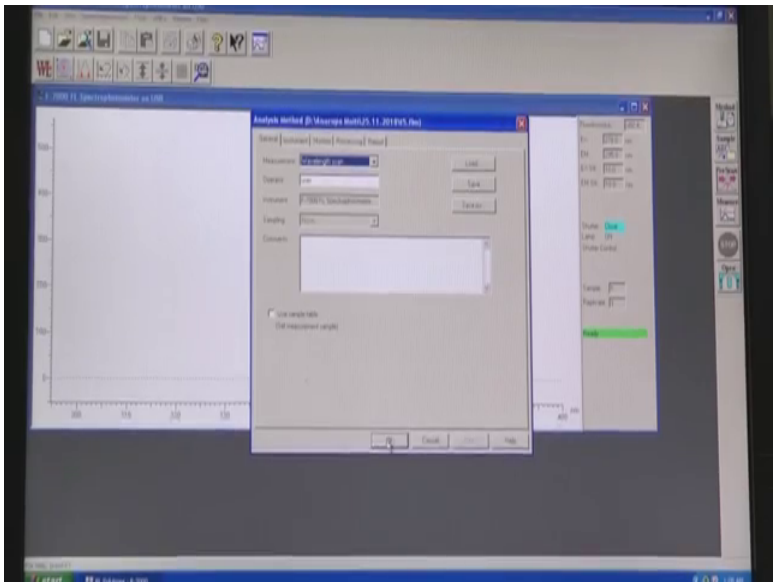

And other things are kept as default, now we are clicking ok.

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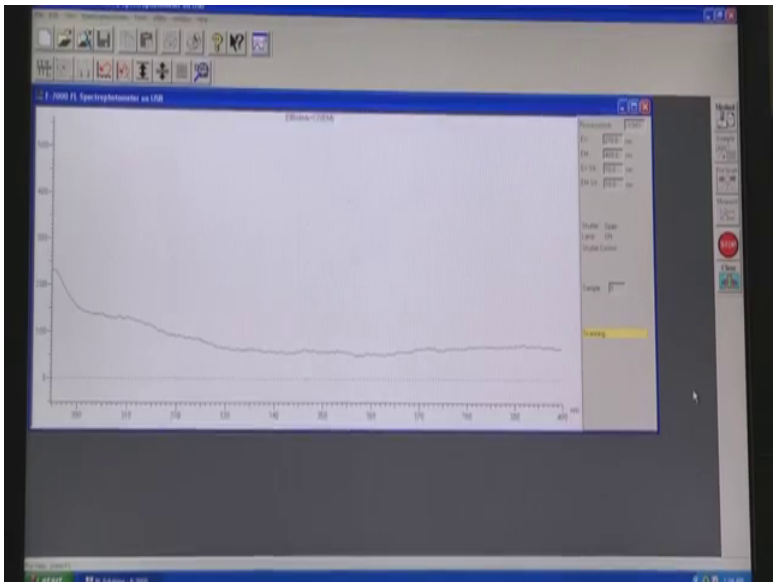

The a machine will just take a few second to initialise open itself, now we will give a pre scan this is a requirement for this particular software and machine. And the pre scan part, what the machine basically we initialize of itself in depending upon the instruction it is said in the method region. After pre scan what we need to do, we need to go to measure; now once I click measure, where the scanning has started and we can see a type of spectra a non-uniform spectra basically appears, because its we are taking the spectra for double distilled water, which has no fluorophore present in it, just a background spectra for this one. Background spectra is required that is the solvents of the spectra required in which the protein of the fluorophore is dissolved.

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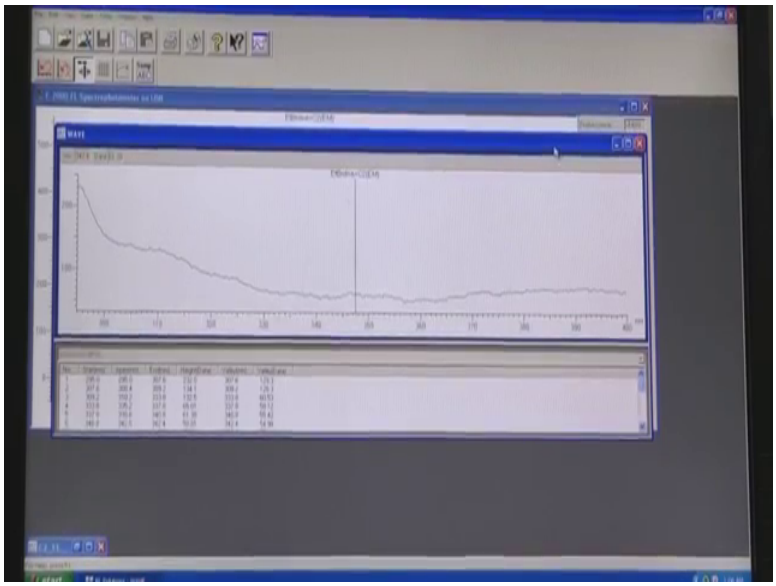

Now, we are done with it. Now, we will proceed to the next observation. Now, we will load protein solution and see it spectra. Now, we will see the fluorescence emission for a protein having tyrosine residues only that is hardness a, we will open this lid take out.

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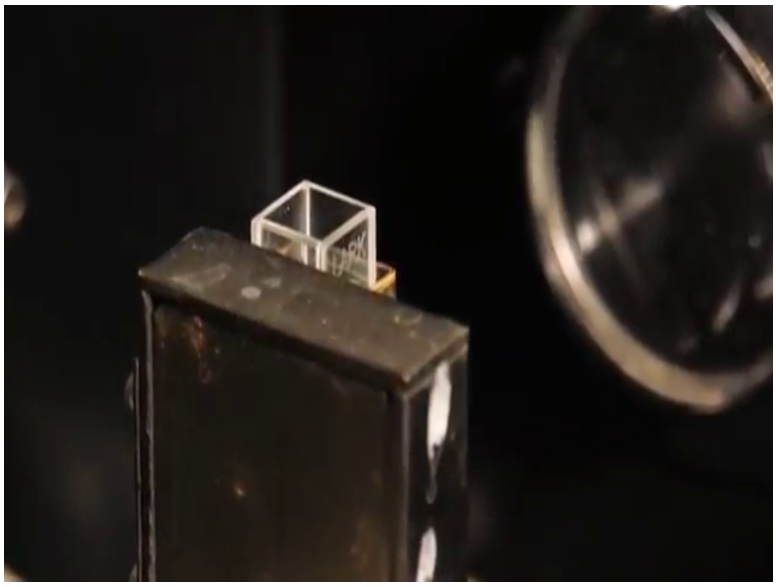

This double distilled water basically, now what we need to do is that we now let to pipette out around 10 micro litre of double distilled water.

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Out here, we are taking a 10 micro litre pipette. So, we are taken 10 micro double distilled water and now we will be taking this r n s a solution, we have taken 10 micro litre of r n of a solution and slowly adding this in the cuvette and mixing it properly. Generally, the amount of protein or rather the concentration of protein used for fluorescence should be much lower than that of the UV, instrument because fluorescence are much more sensitive than that of UV.

So, now we will again place this one in the compartment. One easy method is that you do not have to basically consider which side to phase this or which such a phase this one, you can keep it in any site any way you like unlike the UV part, we will close it.

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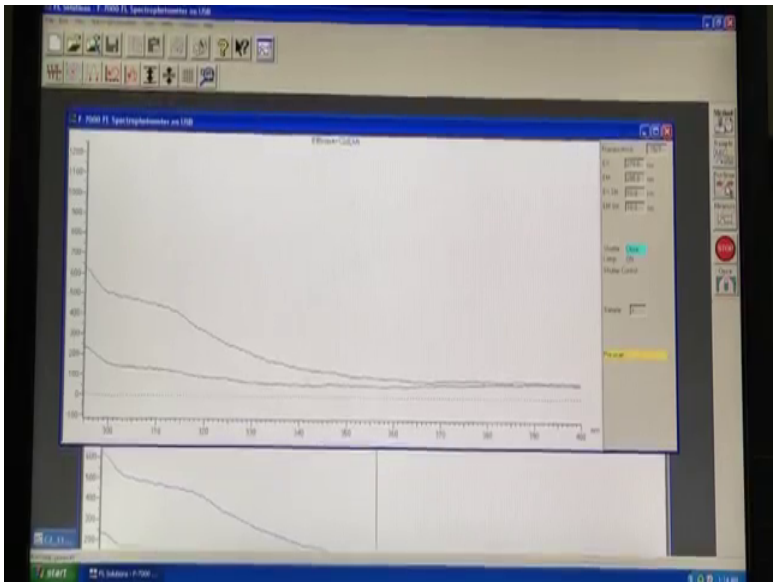

So, now you have taken and it is a solution and let us it is fluorescence, initially are pre scan required. After pre scan let us see the measurement part.

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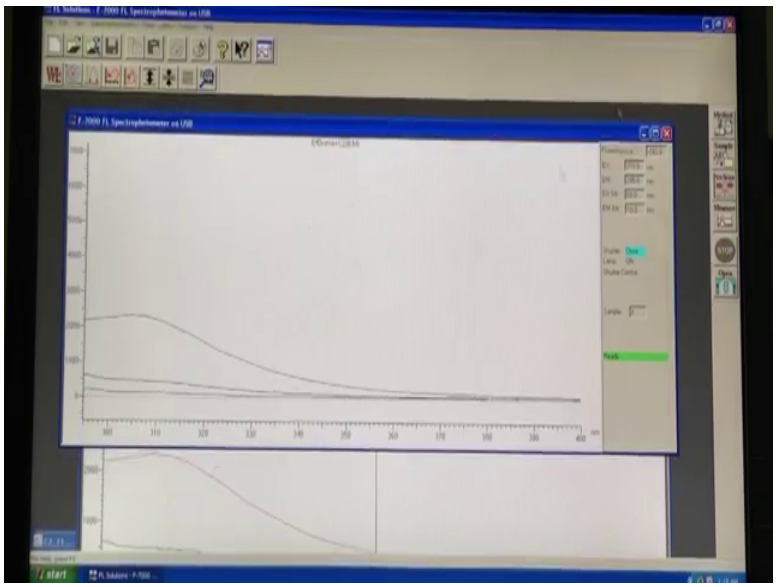

Here, we can see in this is the emission max lambda max for emission of this tyrosine residue and its them a fluorescence spectra for r n s a. Now, we can actually see it like this way.

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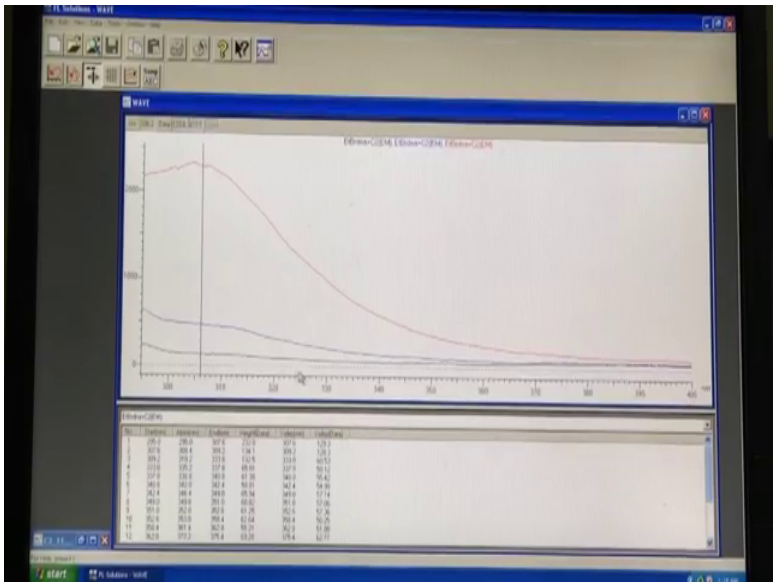

Increase it here, we can see the fluorescence spectra basically for r n s a something like this way and this one is the sort the base line or not base line, this one is for actually the solvent and this is the fluorescence with the maximum around comes a value is around here we can see the maximum value, it is around 304 to 305. So, this is the fluorescence spectra for r n s a which is for tyrosine residue and no tryptophan is present. Now, we have seen the fluorescence emission for r n s a protein which continuous tyrosine residues. The r n s a protein have in tyrosine residues give the fluorescence emission of around 304 nano meter.

Now, you are shifting to another protein that is h s a whose a lambda max we have checked from UV. This h s a has to tryptophan residues in it, now we have taken the cuvette containing r n s a solution, cleaned it completely with double distilled water. And then we have taken this solution of h s a in it, now putting this solution in the fluorescence chamber out here closing the slate gently. Now, let us see the spectra how it comes.

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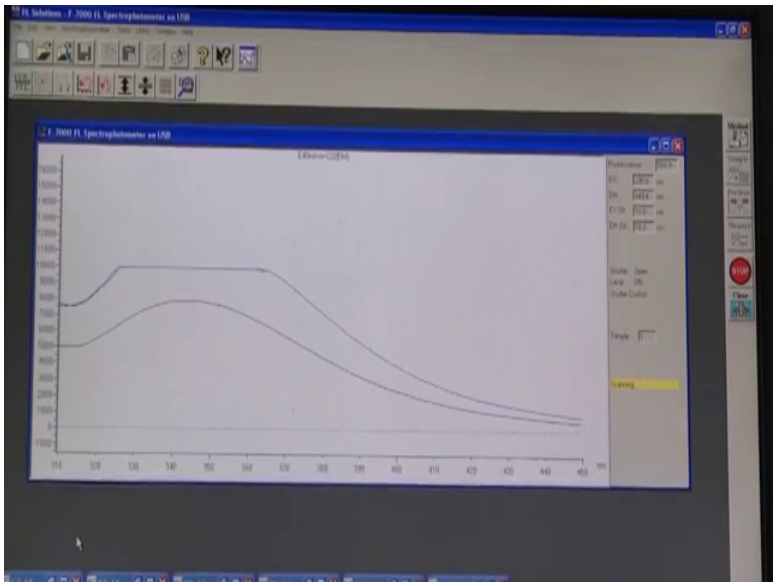

Now, we have made analysis the measurement, here we can see plateau type appears out here. So, basically what is this? This is not the correct analysis, basically here the problem mainly we might estimate that saturation as taken place the intensity of the lamp is not sufficient enough to make this analysis. So, what we need to do in this case, once you find a plateau not only for fluorescence and also for UV one, you need to dilute the solution. So, we have to take the solution out and diluted. Now, we have taken the solution once again, we will do a pre scan.

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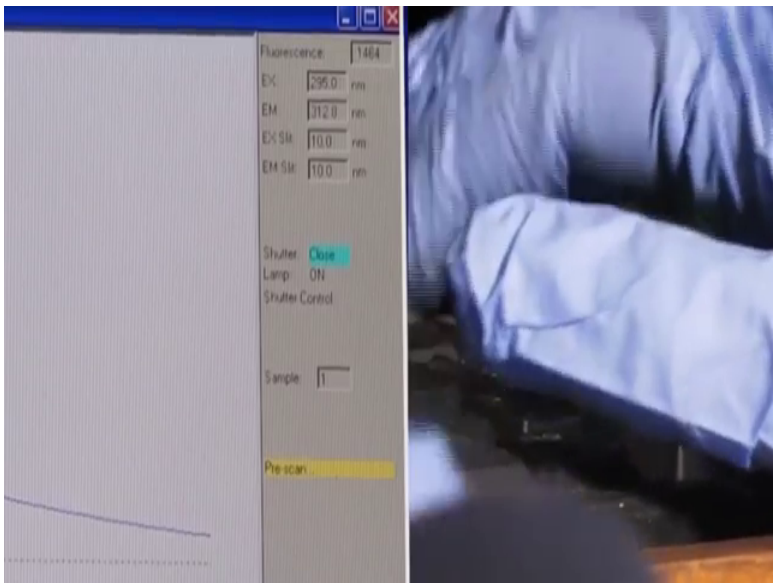

After which we will measure, now here we can see the plateau is no longer there or rather a spectrum has taken place by this way. So, we are diluted it by 50 percent, now this is the emission spectra.

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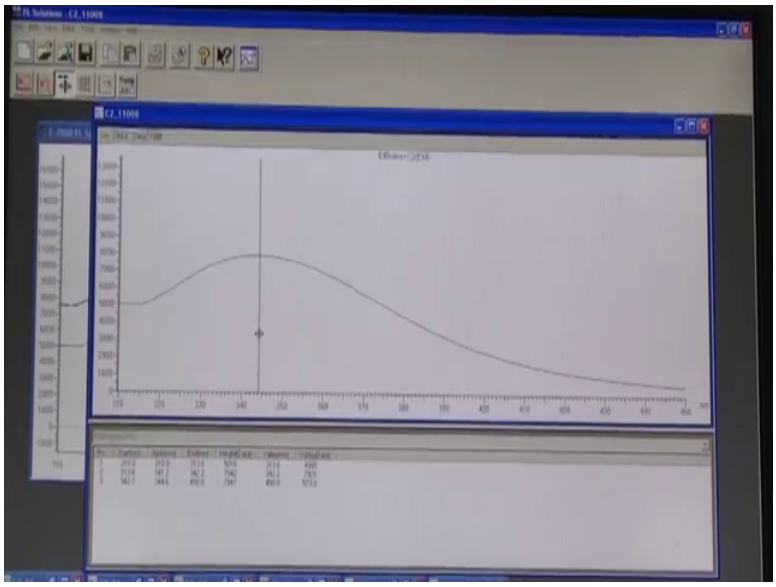

Here we can see, this is the actual emission spectra for our h s a. Now, one important into note down here is that the lambda max here we can see comes generally around 300 just about 340 to 345 or 347, it ranges upend defined proteins and for each is a it is around 340 4 or 45 like this ok.

So, now the next protein we will consider is lysosome. Now, we have seen the emission spectra for r n s a continent r n s a and h s a continent tryptophan. Now, we have seen the emission spectra for lysosome which have both tyrosine and both tryptophan. Now, interestingly we will see how the emission spectra changes, when you change the excitation wavelength. Now, this is the solution for lysosome, we are taking it in this lid out here.

So, we have seen emission spectra for tyrosine excitation of around 278 nano meter, expected emission spectra to given emission maxima to around 204 to 205 nano meter, where has we have obtained defined emission spectra with a maxima of around 340 with 345 nano meter. Now, this emission spectra basically resembles to that of tryptophan residue, why this is the keys.

Basically in absorbance spectra, we have seen in for lysosome it gives a broad absorbance spectra. So, tryptophan basically have absorbance around 280 and it might the excitation is although around 295, but tryptophan is observed around 280 nano meter. So, once you are exciting the sample around 270 nano meter apart from tyrosine residues the tryptophan residues are also being excited.

Now, why cannot we see that tyrosine residues that a emission from tyrosine residues although it is getting excited. A reason is simple has you have studying the theoretical classes, basically what happens the quantum yield place an important role out here. The quantum yield for tryptophan is much much higher than that of tyrosine residues. So, all the tyrosine residues are giving a fluorescence emission spectra, but how it is completely being overshadowed by tryptophan residues.

So, we can see here once the protein contain the tyrosine residues only, we can deal with tyrosine's emission, but once the protein content book tyrosine as well as tryptophan we should always considered with tryptophan emission. So, in conclusion what we can say from today's experiment. We have seen the uses the absorbance spectra of simple amino acid residues as well as the protein composing of defined amino acid. We have seen the r n s a, UV absorbance containing only tyrosine residues, we have seen for h s a containing tryptophan and lysosome containing boot, tyrosine and tryptophan.

From UV absorbance we can measure the concentration as well as we can know the excitation wavelength for fluorescence from the lambda max value. Now, comment fluorescence what we can see the fluorescence emission we have to set the excitation wavelength at a particular value and a emission range. We generally start from around a value which is 10 to 12 nano meter ahead of excitation. After that we have seen the emission for tyrosine, fluorescence from r n s a protein, for h s a tryptophan fluorescence.

And the most important factor for lysosome what we can see is that for protein containing both tyrosine tryptophan, we should stick to tryptophan fluorescence; this will be utilised in studying the protein ligand complexes and also protein denaturation studies. So, fluorescence method is a widely used technique for studying protein complexes and also protein structure. Thank you, I hope you had a great time in this course.

So, we are going to see how to measure the absorbance of a sample a protein sample using a nano drop using instrument. We have seen, how to major the absorbance using conversional UV in spectra photometer. And even more, advance spectra photometer here is the nano drop. So, what is the importance of nano dropping? importance of nano drop lysine the fact that we can use very small amount of sample, previous when we have seen that we have this is a cuvette out layer and cuvette generally requires around 600 to around one m 1 of protein samples or protein solution, but here we can use around one drop and which is equivalent to want to micro meter and not at all millilitre.

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So, we just need a pipette, we just need a pipette here and around 2 micro litre pipette and we can add here.

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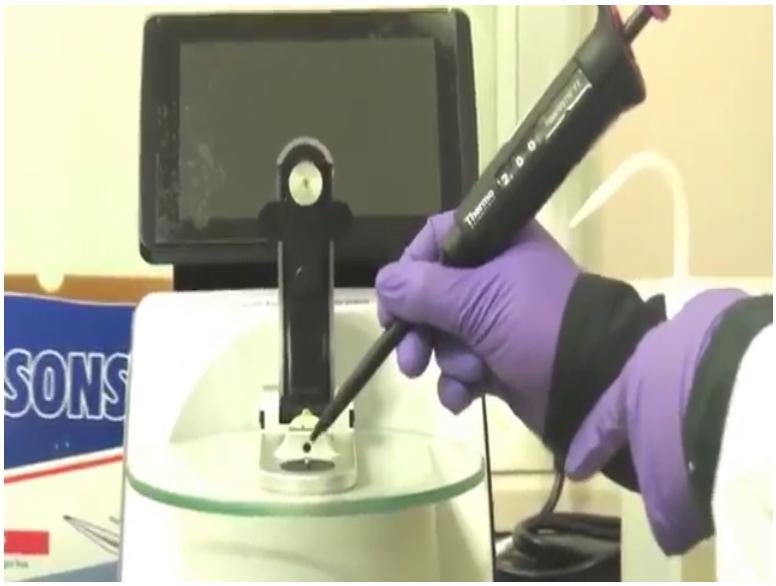

And we can measure the absorbance. So, let us get familiar with this new advanced micro, so let us gets familiar with this new advance narrow drop UV instrument. This instrument basically has one two major components out here, one is the screen where we can see the values; once you operate it we can come across this part. And the second part is this basement, here in this basement we can see two components out here, once again this one cough part and once in if the this cough part, you can see there are again two spaces out here. So, this cough curve part is generally known as the handle of this instrument and this part is known as the pedestal.

So, what basically goes out here it that we add this drop this nano drop or rather small 1, 2 drop of the sample out here, in this part of the instrument this is on the pedestal and then we actually lower down this handle and the machine takes its absorbance. In addition, there is also facility for adding cuvette out here.

Here there is a small compartment, which is currently shielded because we are using it for nano drop only. So, here we can also at a cuvette. So, this instrument so as two purposes one for this nano drop and the second is also for cuvette, where you can use around 200 to 300 micro meter up to 200, 300 micrometer of sample.

So, let us start with this instrument for that you have to first term on the main switch, and then there is a body switch just behind of instrument, which you are directly operate. So, we just switch down this instrument [FL]. So, we switch down this instrument and we

can seeing here there is light and started doing and instrument is undergoing initialisation, like other previous give a instrument. So, it will take some times for initialisation and in between initialisation this keep in mind as a previous solution we should not take on the lid of the UV instrument.

So, similarly we should not open this handle, while the machine is initialising. So, instrument is initializing and the option is given, so we should not lift this on, here we can see in the option do not lift armed during initialisation [FL].

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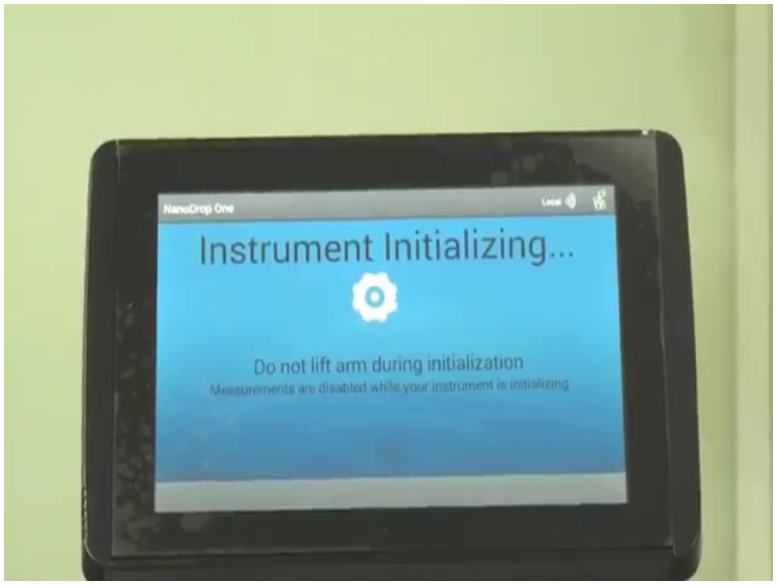

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So, the machine is initialised and this is the home screen of this instrument, here we can see various types of options cutting from nucleic acid, proteins and other things. In the nucleic acid part we can see, but we can measure the absorbance of dove standard d n a, this is very simple standard d n a r n a and other now different types of d n a for d n a experiments.

Now, coming to protein part here we can see we can measure the absorbance of protein at 280 that label is protein if we need, then some preference you did not have those tryptophan or tyrosine here we can measure the protein absorbance a 205. Apart from this, we can also do BCA acid and other Bradford, lowry, etcetera things.

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And in addition here, we can also use this OD600 part. In this OD600 part we are measuring the bacteria culture and one thing to remember is that we should use cuvette for this and not the nano drop, it is preferable to use a cuvette for this 600. For this current experiment, you will be concentrated one protein and how in this protein here we are using this protein 280, because the protein of a concern has tyrosine received.

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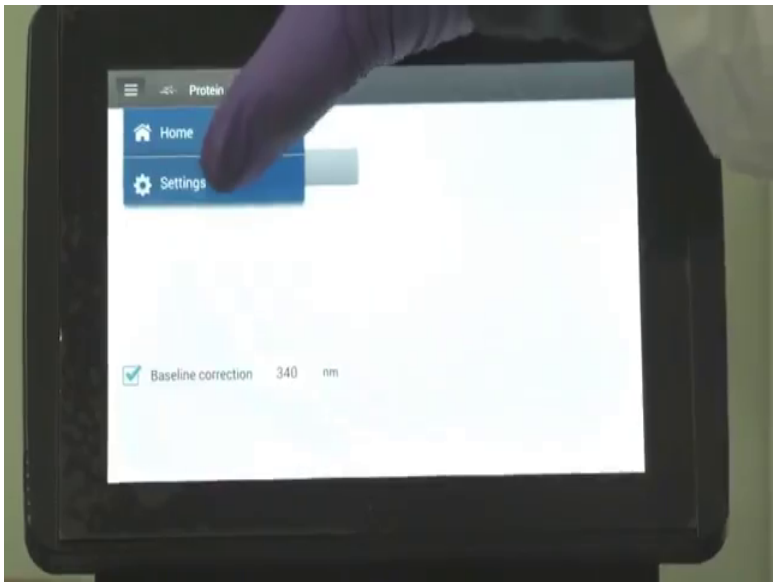

Now, once we have click this protein part here may in go to this option and in the second's option, we will show.

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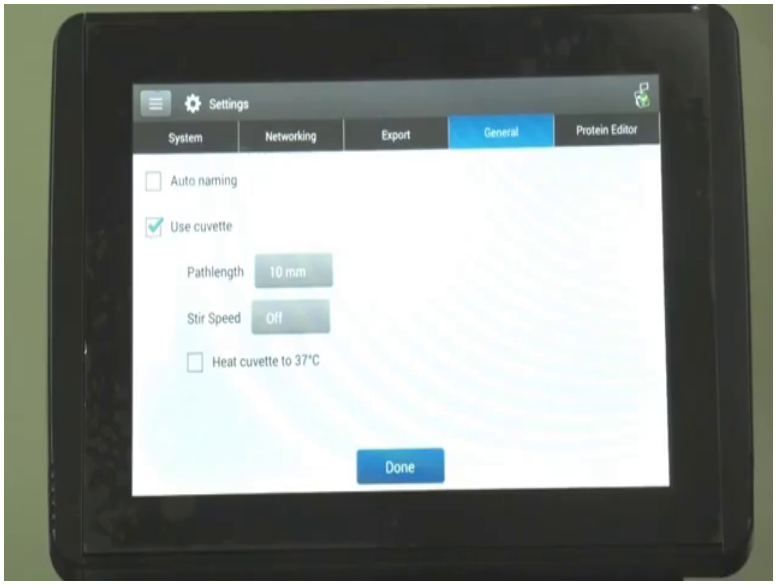

This open in it and use cuvette, and if we use cuvette we will tick this option here, here so in this path length and everything; however, for our this nano however, for this nano UV experiment we are not use in cuvette. So, we will go take it and simply without taking whether checking this boxes here, press done.

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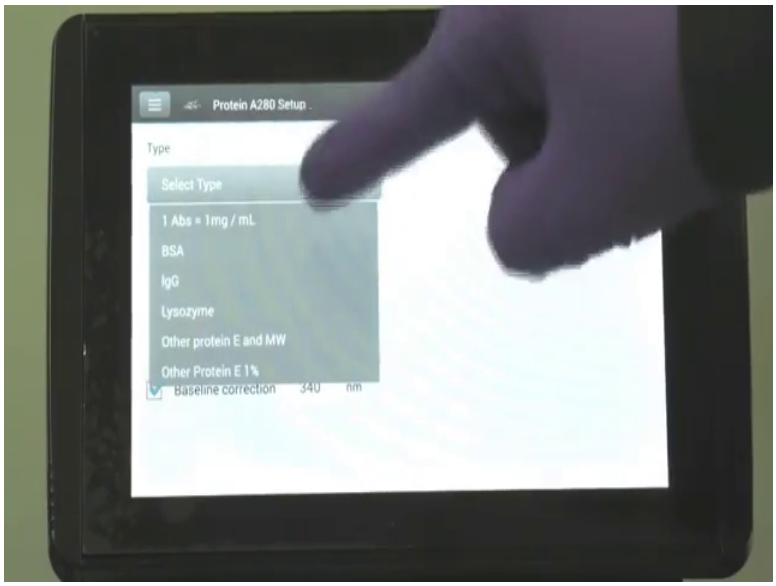

Now, we will go to this type select. Once we click this one, we can see the dropdown menu it showing BSA and lysosome and other different types of proteins. However, our protein is not listed here and great use this default path these are 1 absorbance 1 per millilitre, this is just a custom link and we are just click it.

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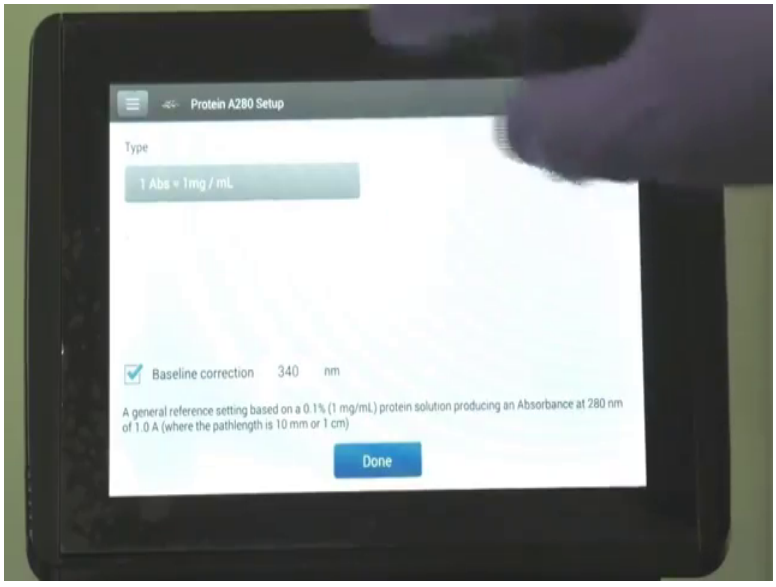

And to show this baseline correction, these are some default option so this instrument and way they will click done.

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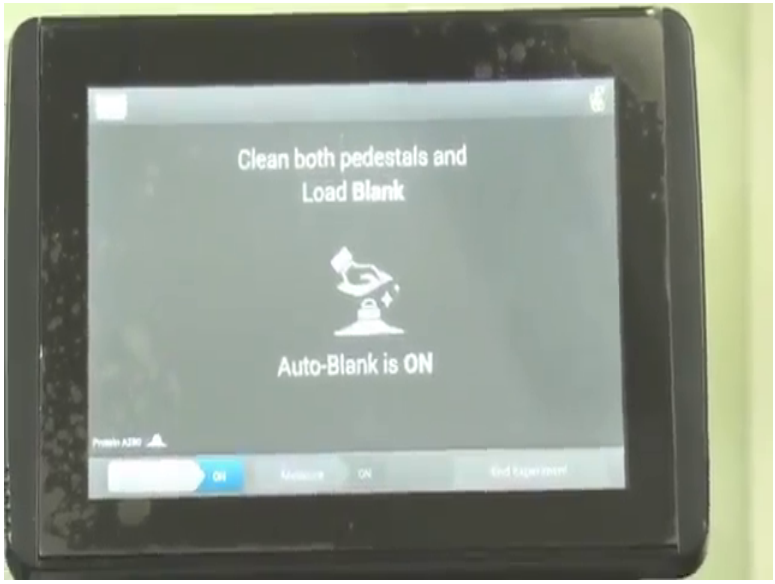

Now, once we click done the machine is showing to in both pedestals and both blank that is we have to clean this part, for cleaning gradient keep white tissue papers.

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Not normal tissue papers, this clean white tissue papers are specifically made for sensitive instruments. Now, we will take this tissue paper.

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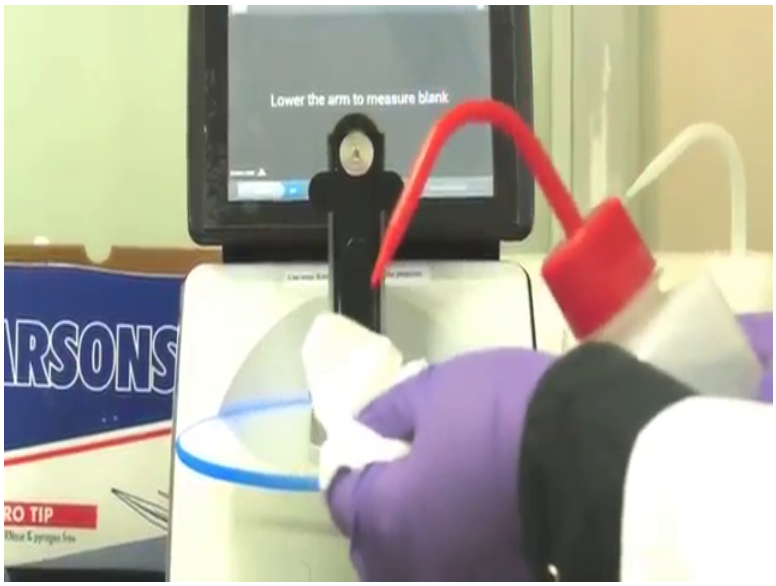

We will gently lift up this handle and here we can see it is given the lower the arm to measure the blank. So, before measuring the blank we will just moist, this we shall moist tissue paper and gently gravid out here, this is the pedestal where the sample is to be given out here and again this one. It is preferred not to directly hot water out here in this pedestal, because sometimes there are sensitive parts or sensitive instrumental things begin here, then which might get effected. How it is recommended to voice to use of voice tissue paper in order to clean this.

Now, once we are use this tissue paper to clean our pedestal we will just use the blank for the current part, we are using simple water as blank. So, we are taking this 2 micro litre pipette, this 2 micro litre pipette and this is the tip, now here is a pure water, there is in milky water, 2 micro litre milky water. And gently at the tip, there is a tip here we will just give a drop the single drop and keep it mind and just sheet this one that there is no here bubble form out here.

Now, after that what to happen is the do we just lower down this handle and this measurement we will a carry down, [FL] a lower down the [FL] [FL] check it. So, here the blanking is done [FL] hm. So, we can see the blanking has been done automatically and the option is this coming the clean both pedestal and load sample.

And one important being as auto measurement is on that is if you want to make the measure automatic and if you want to make the measurements manually, they off to in

the defined option, all went is convenient to you auto measurement on in that. So, again we will just lift out this handle, take this clean white tissue paper, clean this one, no need it use white tissue paper currently, because it is already look at their solution the (Refer Time: 26:34) liquid in which the protein is dissolved. We will be taking a clean take place no do not use the previously, previously sometimes you may use because the tip has been used in the solvent in which report in is dissolved, but in biochemistry practical it is better to use a clean tip each and every time, whenever doing and do a experiment.

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So, here we are taken the protein solution dissolved in the dissolved in water torchy, again we will be taking 2 micro litre a protein sample. (Refer Time: 27:23) will be put in a drop, yes here in a drop. And now we will again lower down this handle carefully, see now measuring this we showing measuring data automatically its measuring the data.

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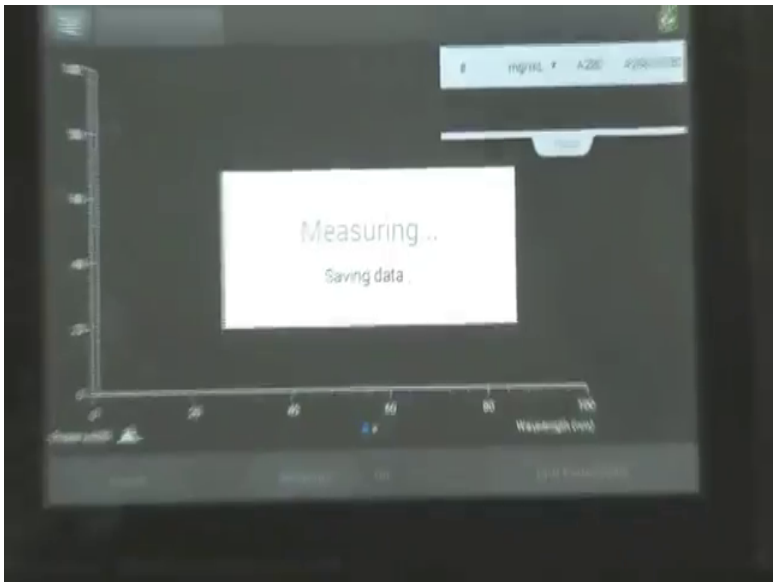

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Here, we can see this absorbance spectra Mathews out here, and as we have previous miss in the UV spectra, there we can take an absorbance spectra this similar to that only. Here we can see this band around 280 which shows the protein absorbance spectra. Now, one important thing to note from here is that the absorbance 280 count here, we assume the absorbance 280.77, this is the absorbance some which we can calculate the concentration, moving the extinction coefficient at those wavelength.

And now one another important thing is absorbance 260 by 280, those are this 0.64 which nearly suggests that our this protein solution is free from any sort of d n a or nucleic acid scan, this is the actually may use the measure the concentration nucleic acid, they extent the nucleic acid (Refer Time: 28:45) hm. So, we have obtain this spectra, here we can see this is the absorbance us of wavelength measurement here we can see the hump or the here we can see the hump, which is for the protein absorbance around 280 from here we can see options are given we are take 280, significance the absorbance at 280 nano meter.

And once we know the extinction coefficient of this protein at 280 nano meter, which is easily form the literature here we can calculate the concentration from the under beers law, which is absorbance equal to epsilon in to concentration in to length. And the path length here is standard this one, now a 260 and by 280 a first whether in a d n a or nucleic acid a performance in the solution. The value point 6 4 state that its protein solution is free from any sort of nucleic acid. So, from here we can carry out our concentration measurement of this protein by using a very small amount of protein solution.

Now, we can end this experiment we can is the save the data on in the experiment. It is recommended to note down this absorbance value, once the experiment is over. Now, here it click this one.

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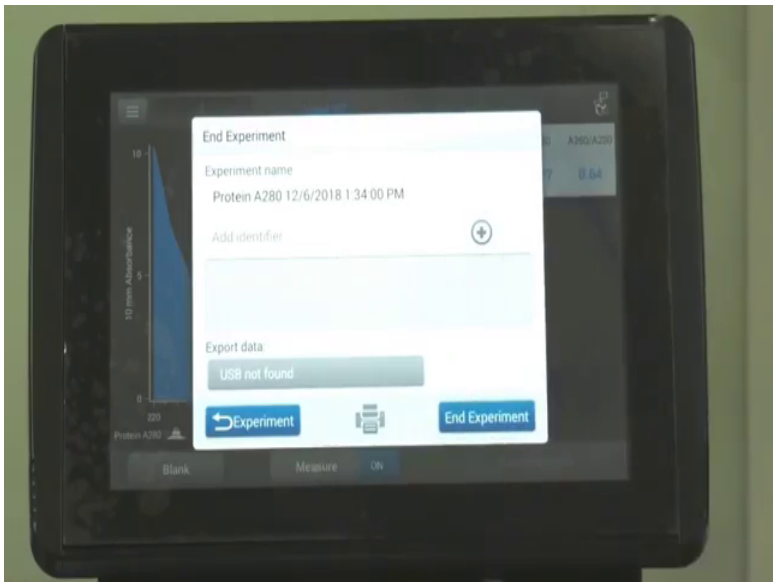

And here is should being show then option, where we can save this one and if what click this one [FL] ready. So, what? So, we can either save this data or we can noted down now we can end the experiment we can click this end experiment part. And once you click it is showing that whether in what to save this data, if we need we can save it by for the time being. Once you need to estimate the concentration of protein, you just at note the down the absorbance done and no need to save this data, if you required, you should said it. Now, here the end of experiment.

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Again, the option is showing, please clean the sample area. Now, how to clean this one? We have to again lift out this handle carefully, take this clean white wised it use in distilled water and clean the region the pedestal, where we have given the sample carefully. And once you have use this protein sample, kindly note to clean the pedestals so that it does not contaminate with the next sample which you or your lab mate might be using.

After cleaning with water on the with honest tissue paper, it is recommended to use the white tissue paper or clean white to make the surface try once again free from water. And now we can you are down this handle, and if you wish to carry out any further experiment, we can click this on touch to continue and again we are directed to the home screen.

So, we can see how we can easily measured the concentration of any protein solution by using this nano UV. And unlike the previous UV experiment where we need around 200, 300 micro litre protein sample, we can measure the concentration easily by using single drop; this generally requires for measurement the protein generally all right. Generally a few proteins are there where you cannot actually afford huge concentration a huge amount, their proteins are quite expense and for that in the small amount of protein. And now this instrument comes in that state, where you can easily use the small amount of protein to measure the UV and find the concentration.