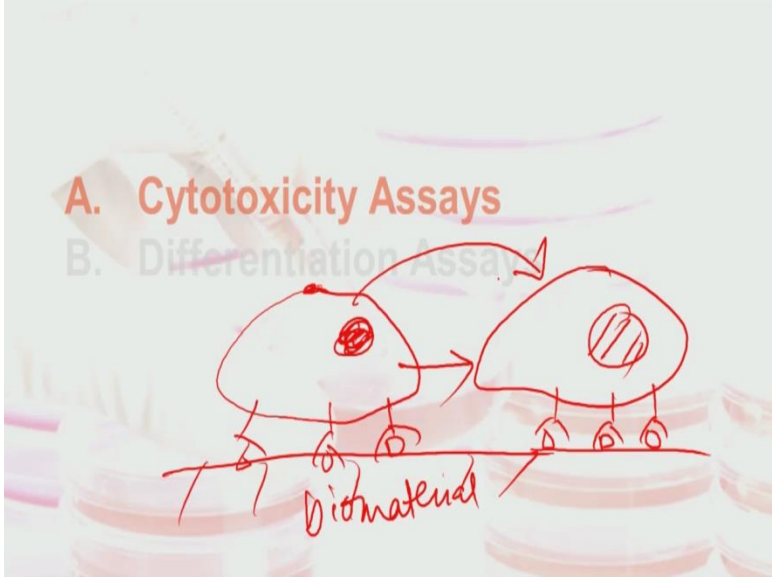
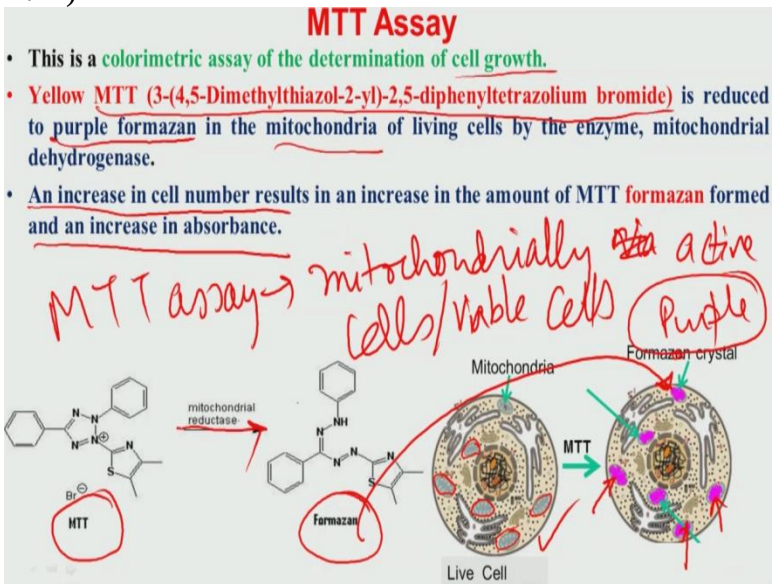
Biomaterials for Bone Tissue Engineering Applications
Professor Bikramjit Basu
Materials Research Centre
Indian Institute of Science Bangalore
Module 5
Lecture No 23

(Refer Slide Time: 0:29)



In this module we will discuss some of the in vitro assays to quantify the whether a cell when they adhere on a material substrate whether the cell are kind of viable, they are like metabolically active cells or whether the cell, the cell is already dead, that is number one. Number two is that whether after adhering on a material substrate, whether the cell is changing its functionality. Remember, I am showing here two different cells with different nucleus being said a different name just to show that they are, show that it is a differentiated cell. So it is more matured cell.

(Refer Slide Time: 1:17)



Now the first one which is most widely used in the biomaterials community is that MTT assay. So if one can go through most of the published papers in the field of biomaterials, they would notice that this MTT assay is most widely used as one of the biochemical assay for determination of the for the, to quantify the cell viability and subsequently if it is measured at different time point, then it can also give an idea about cell growth on a material substrate. So before I start discussing on this, any biochemical assays, let me tell you that the fundamental principle of any biochemical assay is that you use certain reagent which will essentially interact with some of the cellular organelles.

It can be mitochondria, or it can be, it can introculate with DNA of the cells and so on. And once it (inter) interacts with some of the cellular organelles or some biomolecules or some biological macromolecules within the cell, then what will happen? It will change the colour of that particular organelles, or that (parti) or that specific organelles in case the assay is a colorimetric assay. That means you need to change, you need to determine that what is the change in optical density before you add that specific biochemical reagent and after it is being added to the, after it is being added to a culture medium.

So I repeat whenever you use certain certain biochemical assays it interacts with some of the organelles within the cells or some biological macro molecules or protein or or protein molecules, proteins in the cell, cell structure. And After it interacts with that specific organelles

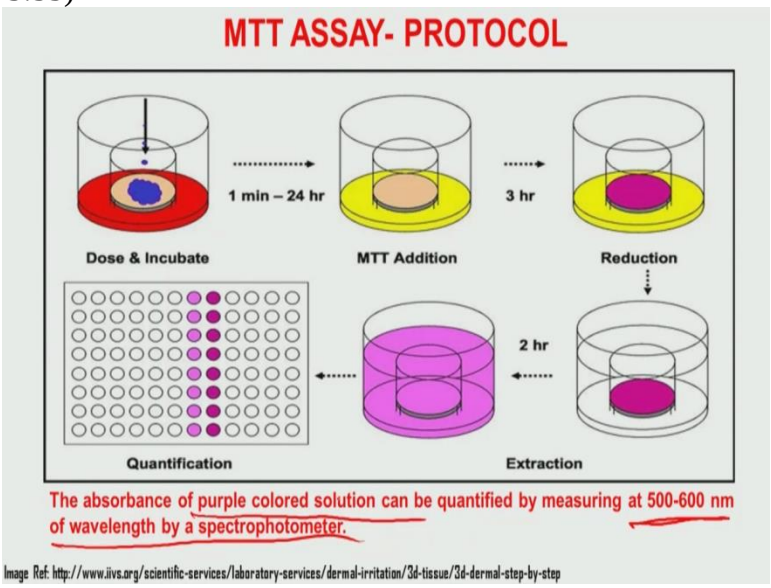
or biological macromolecules, then what it will do? It will change the colour and hen change in the optical density is recorded by some microplate reader or so on and that optical density, change in optical density will reflect on certain aspects, whether it is a CTV cell viability or whether it is a cell death, something they will, some something will be detected.

So now in the case of this MTT assay, as I said it is a colorimetric assay, that means it is based on this colour change. So this is a yellow MTT and it has a very large expanded chemical formula. It is reduced to purple formazen, once it interacts with the mitochondria of the cell. So essentially MTT assay, this MTT reagent interacts with the mitochondria of the living, of the live cells or living cells . And then it, then the process of mitochondrial dehydrogenase takes place and then formazan crystals forms.

Now formazan crystals have a purple colour. So certainly this purple colour, with this purple colour this optical density now would be, now would be, quantified in terms of the increase in absorbance and this increase in absorbance will be proportional to the number of mitochondrially viable cells. So therefore, MTT assay essentially determines mitochondrially viable cells or mitochondrially active cells or in one word it actually determines the number of viable cells in solution. So this is the MTT reagent formula.

After the mitochondrial reductase then it forms these formazan crystals, this formazan crystals purple in colour and it is shown that this is the live cells and then after the MTT reacts then it is, it forms this purple coloured formazan crystals and their absorbance is kind of recorded.

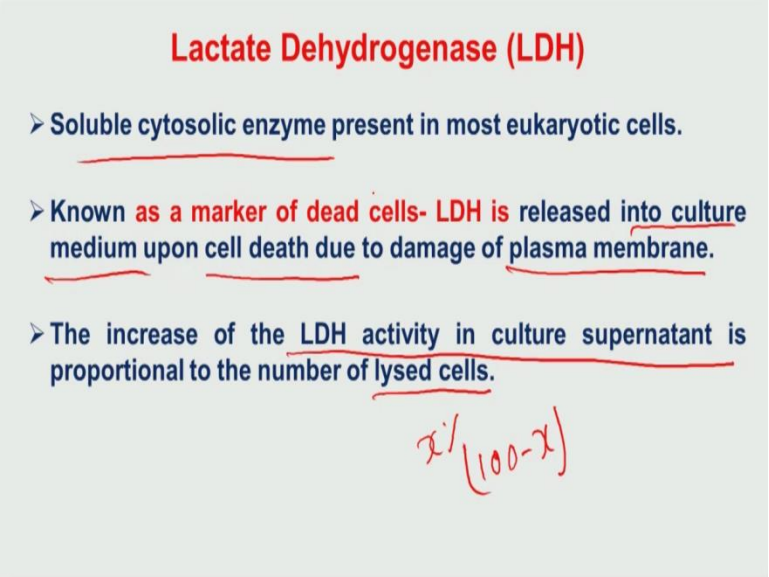
(Refer Slide Time: 5:53)



So this is the protocol in a schematic. So what what normally people do that you can take some particle eluates or you can use the word bulk samples. Now this is, it typically shows the culture plates, well like, you can use 8 well plates or you can use 96 well plates and so on. So you put this so first, first you have to add this yellow MTT reagent to the cell to the cell suspension containing the particles or containing this bulk materials.

Now once it is reduced then you can extract this one and then put it in the the extracted solution, you put it into the 96 well plate and then you can have some blank one or some control sample and then you can have this is actually that test sample. This absorbance of the purple colour solution then for this quantified by measuring this absorption is particular range that is 500-600 nanometer wavelength by spectrophotometer. Now once this measured that will that will give you that what is the number, what would be the relative number of this mitochondrially viable cells.

(Refer Slide Time: 7:14)

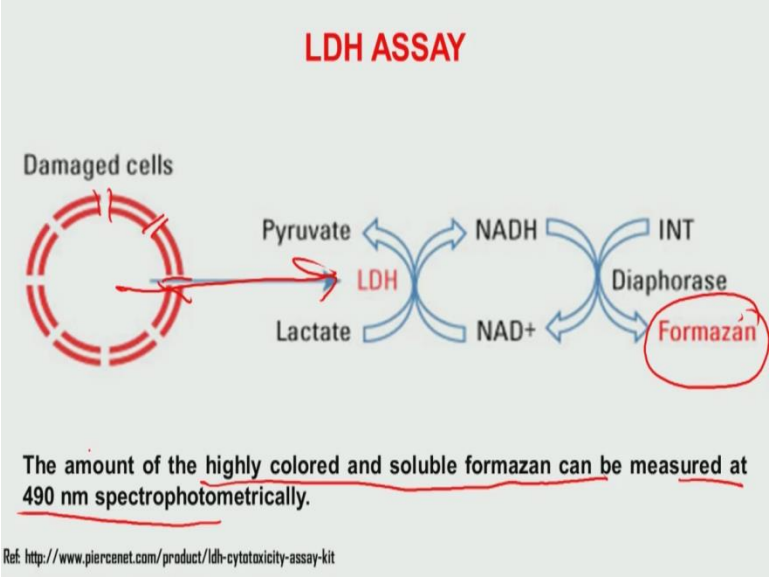


Now the in cell biology often people use the complimentary assays. Now one of the examples of this complimentary assays is this lactate dehydrogenase assay, that is LDH assay. So what LDH actually does, it quantifies the number of dead cells. Now one of the things I have I think emphasised in one of the earlier modules that in all this biological assays, you have to state whatever this biological assay actually is meant for. One should not over stretch it, one should not project it to a large extend.

For example, I repeat it that if the X percentage cells are mitochondrially viable then one cannot simply write that 100 - x cells are deal. They may be dead, they may not be dead because 100 - X cells out of that certain fraction is dead or certain fraction is in different state of their cell fate processes. So this LDH it is a marker for the dead cells and it is released in the culture medium. one cell dies due to the damage of the plasma membrane.

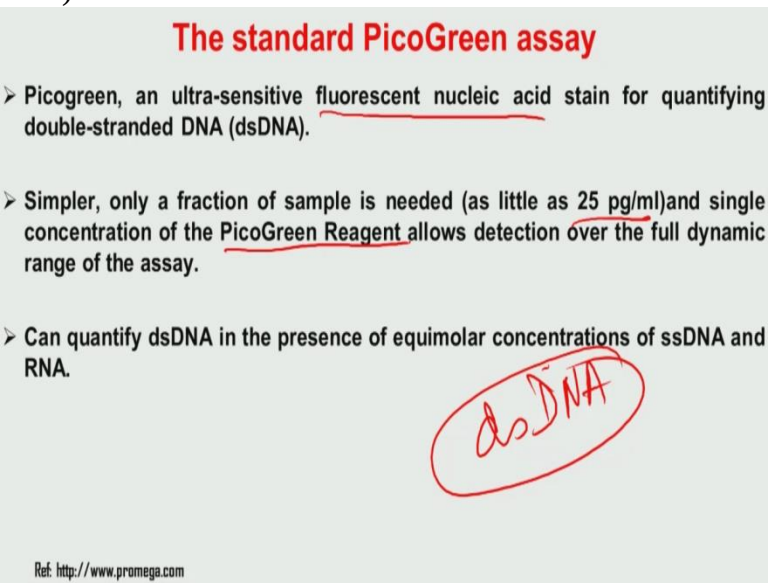
And the increase in therefore the LDH activity in the culture supernatant is proportional to the number of live cells. So this is a typical, this is a typical rational of using that LDH assay and this LDH is typically a cytosolic enzyme which is present in most eukaryotic cells. So once it is coming out, that means your cell lyses have taken place and that is why that LDH is coming out from the cytosol to the exocellular region.

(Refer Slide Time: 9:00)



So this is how the schematic looks like. So you have a (dama) Damaged cells. That means that cell membrane lost its integrity. As a result this LDH comes out and then it forms again the formazan crystal. Now this is again a highly coloured supernatant and soluble formazan crystals can be measured at similar wavelength region like you know 490 nanometer spectrophotometrically and this will aid in the quantification of the number of dead cells.

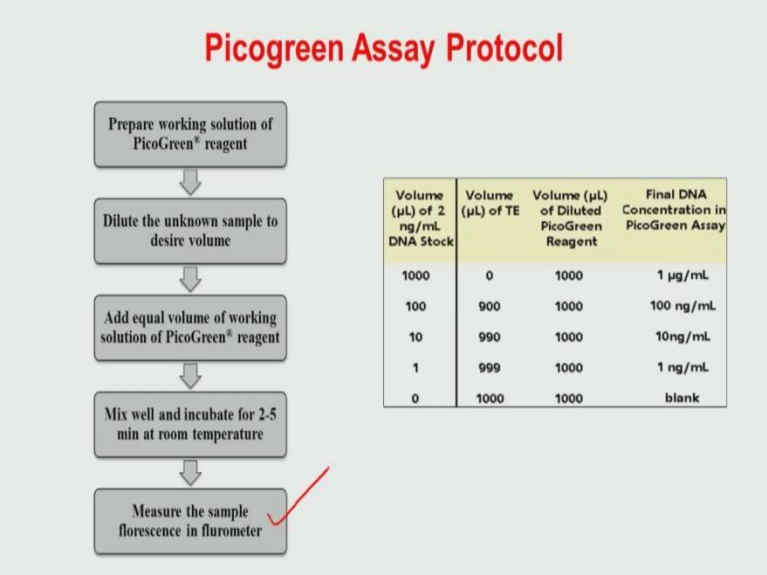
(Refer Slide Time: 9:32)



Third one is the PicoGreen assay. PicoGreen is actually a fluorescent nucleic acid, so it is a very ultra-sensitive florescent nucleic acid stain for quantifying double standard DNA. So remember

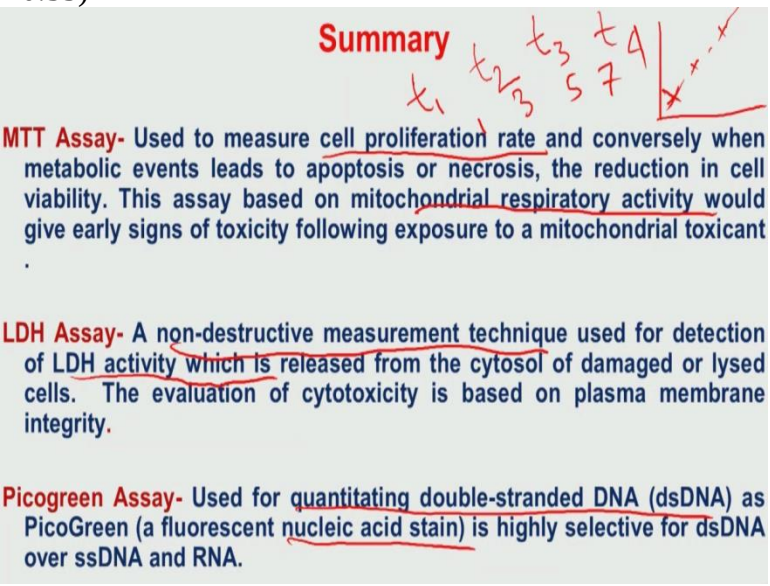
this is only used for the double stranded DNA. It is very simpler and only a fraction of the sample is needed like you know it is in the order of 25 picogram per millilitre and single (con) concentration of PicoGreen reagent allows detection over the full dynamic range of the assay.

(Refer Slide Time: 10:06)



And so this is, this is the typical protocol. So one has to first start, one has to first prepare the working solution of PicoGreen reagent. After that the similar to other assays that one has to dilute with that unknown sample of a desired volume, then add equal volume of the working solutions and measure the sample fluorescence in the fluorometer. So this is how this PicoGreen assay that works.

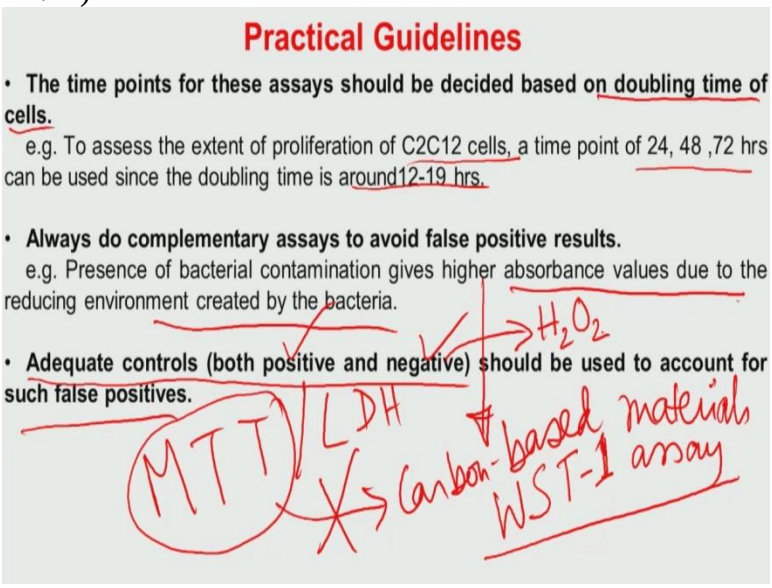
(Refer Slide Time: 10:35)



So let me summarise these three different assays that I have just discussed in last 10 minutes or so. So first one is the MTT assay which is (mes) which is used to measure the metabolically active cells and if that metabolically active cells is measured at 3 or 4 different time points in culture like T1, T2, T3, T4 or in other words let us say 1 day 3 day, 5 day, 7 days then if you can plot this one, that how this, how this cell number is increasing then you can see that how the cell growth takes place, that kind of quantification is possible. When metabolic events lead to necrosis or the reduction in cell viability, this assay is based on mitochondrial respiratory activity and would give early signs of toxicity.

LHD assay it is a non-destructive again measurement technique and this increase in the LDH activity is the solution essentially is the measure of the cell lysed cells. PicoGreen assay, it is just to quantify the double- stranded DNA and is highly selective for double stranded over single stranded DNA and RNA.

(Refer Slide Time: 11:47)



Now some practical guidelines, I mean if one does experiments in cell biology particularly in the context of biomaterial research. So the time point of this assays, or the culture period should be based on the doubling time of cells. Now some of the doubling time of cells has been mentioned before, (bu) but on the basis of the doubling time let us say if one has to study the proliferation of the C2C12 mouse myoblast cells, then a time point, a typical time point of 24, 48, 72 hours is fine because their typically the C2C12 cells their doubling time is 12 to 19 hours.

And one has to always do complimentary assays to avoid any false positive results. For example, presence of bacterial contamination gives higher absorbance value due to reducing environment created by the bacteria. Now another thing that is quite important to mention like one can use this MTT assays or LDH assays. So particularly this MTT assay is not useful for carbon based materials.

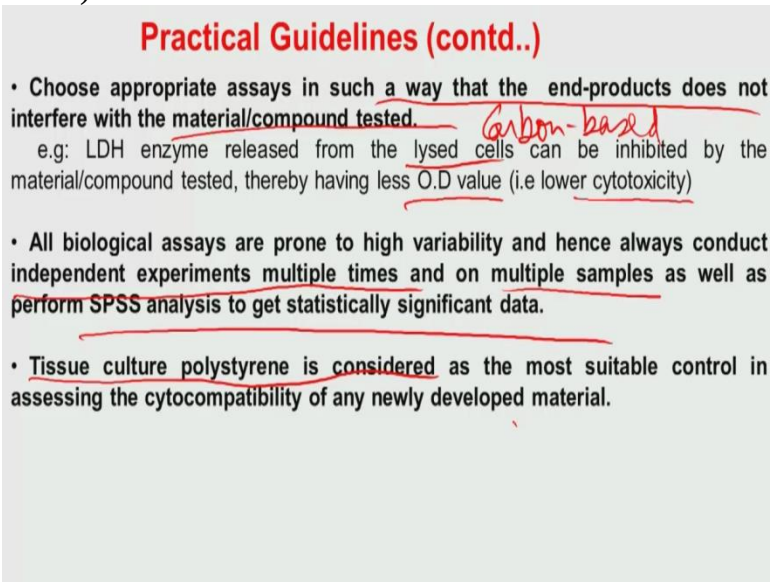
Now if you use this carbon nano tube based materials or any other carbon based materials for example graphene based materials you want to understand their, you want to understand their toxicity towards specific cell lines, one cannot use the MTT assay. So for that you need to use certain other specific assays, so because that MTT reagent interacts with carbon and therefore it gives a false reading which may not reflect the mitochondrially active cells in that particular solution.

So as long as this biological reagents or biochemical reagents, they do not interact with the materials per se, then you can use, but once it starts reacting with the materials, also material composition then one has to be very careful. For carbon based material there is another assay, what is called WST- 1 assay and this WST-1 assay can be used and the WST-1 assay actually provides reliable measure of the mitochondrially active cells on the carbon based materials.

The second thing, the third thing that have mentioned is that, that appropriate controls like both positive and negative control should be used to account for such false positives. Now many time in that biomaterials people use the tissue culture polystyrene or tissue cultured plastic cover slip as the one of the control where cells normally grow. Now another positive control one can use is like hydroxyapatite as such in case of the bio ceramic materials because hydroxyapatite is known to be the most biocompatible and bioactive materials and therefore different cell, different cell types can grow very easily on hydroxyapatite based materials.

And what about negative control? Negative control often people use like they simply use the culture solution and they add H2O2 and if you use Hydrogen peroxide, then normally it kills all the cells and this H2O2 added cells also can be used as a negative control.

(Refer Slide Time: 15:26)



Now choose appropriate assays in such a way that the end product does not interfere with the material or compound tested. This is the point that I just explained you with some examples of

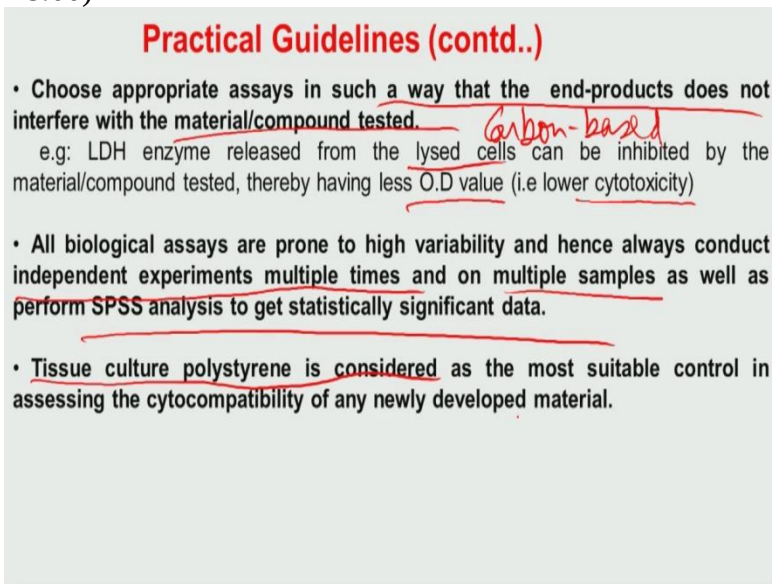
the carbon based materials right. Then this, another example is that LDH enzyme realised from lysed cell can be inhibited by the material or compound tested and thereby having less optical density values or lower cytotoxicity.

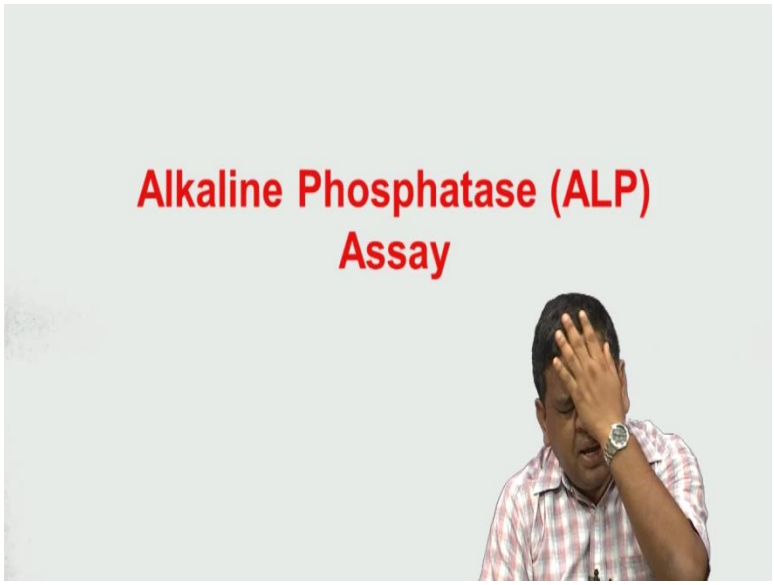
So although most of the cells are lysed but the origin of this LDH assay is, that LDH is an enzyme which is present in the cytoplasm or the cytosol, that has to be coming out of the cells to the medium and unless that LHD in an appropriate amount is released into the medium, you do not have any way to measure their optical density values. So any compound which is present in the culture medium or whose toxicity you want to asses, if that compound inhibits the the inhibits the release of the LDH enzyme to the solution then automatically you can get lower optical density values.

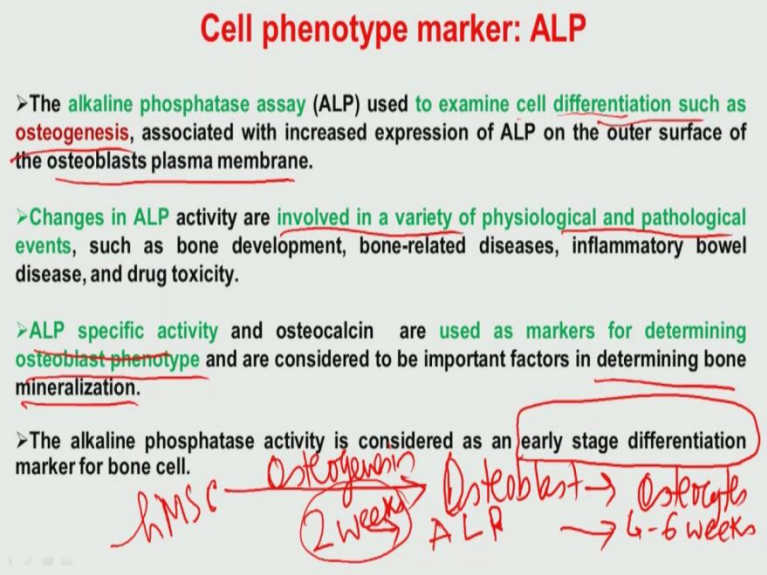
Ok, so other points that I have mentioned is that all biological assays are prone to high variability hence always conduct independent experiments multiple times and are multiple samples as well as perform SPSS analysis to get statistically significant data. Because if you do that some, cell culture experiments today on the same materials, and if you do it tomorrow, because since cells are continuously growing in the medium and you are culturing those cells on another non-living (Sub) substrate, therefore you may get some variable cell culture, variable results from different biological assays.

So definitely one would have to use this assays multiple times and multiple samples and do statistical analysis which is very very important in the context of cell biology research because one has to say one has to do this appropriate statistical tools so that they can convince the they can convince others that yes indeed that statistically significant difference can be obtained as far as the cell functionality is concerned. The other things I have mentioned already that tissue culture polystyrene or cover slip is used as the most suitable control in cytocompatibility.

(Refer Slide Time: 18:00)







Now coming to the differentiation assays, so differentiation means the differential gene expression which I have mentioned earlier and one of the, in the context of bone tissue engineering applications or orthopaedic applications what is the, what is the theme of this course. There are two assays which are important. One is the alkaline phosphatase assay. So alkaline phosphatase assay is to, is used to examine cell differentiation such as osteogenesis like as I said that it is, it is for the bone cells and it is associated with increased expression of ALP on the outer surface of the osteoblasts plasma membrane.

Essentially this ALP is expressed more heavily, more, more high level at the outer surface of the plasma membrane of the osteoblast and changes in ALP activity also involved in the various physiological and pathological events like bone development or bone related diseases or inflammatory bowel diseases or drug toxicity. Third one the ALP specific activity is used for the osteoblastic phenotype and this is important in determining bone mineralisation. And fourth one it is an early stage differentiation.

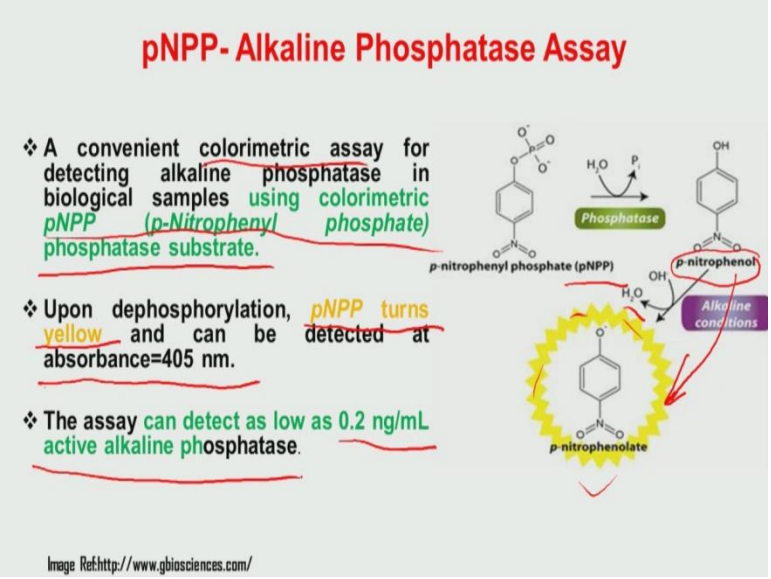
So for example if you grow human mesenchymal stem cells which I will cover in one of the module and then you are following that osteogenesis lineage of the human mesenchymal stem cells. And then ultimately it will go osteoblast and subsequently osteocytes. So this, all these differentiation aspect needs to, you need to grow this human mesenchymal stem cells on certain osteoinductive materials for let us say two weeks then after that you go for 4-6 weeks and so on.

So you can see that at different time frame you are essentially detecting that whether there is any signature of the differentiation of human mesenchymal stem cells to osteoblast.

So that early stage means, let us say from two week to three weeks you can see whether ALP expressed increased on not. If ALP expression is increased at the early stage then you can be very sure that this human mesenchymal stem cells is undergoing osteogenesis. Now after the late, after this osteoblast cells form (())(20:37) osteoblast, matured osteoblast and finally osteocytes. Then you see that the expression of the osteocalcin which is essentially late (oste) differentiation marker.

So essentially the progress of differentiation is to be assessed by two or three different markers and this different, this complimentary assays will give you some quantification that how this differentiation process is taking a place at different time points in culture.

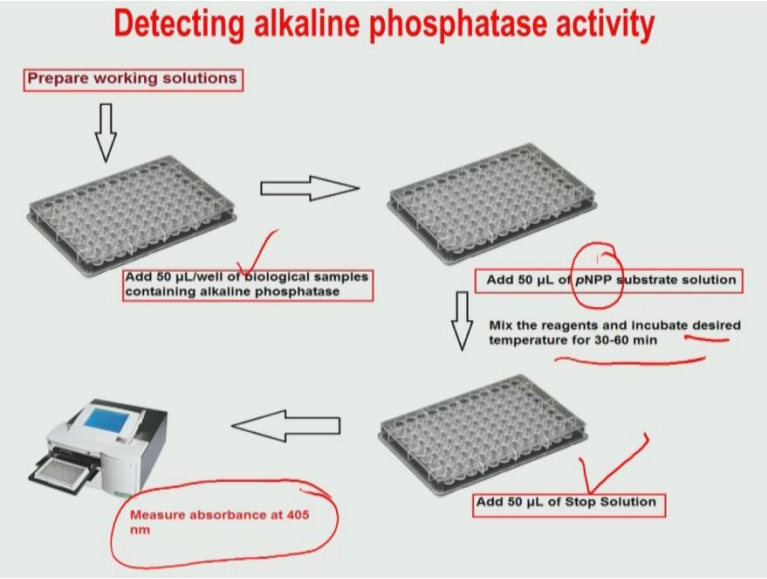
(Refer Slide Time: 21:08)



So this is little bit more details of the ALP assay. So you, you have this pNPP that is the p nitrophenyl phosphate and once you add this alkaline under this alkaline conditions that p nitrophenyl will undergo (trans) transformation to p-nitrophenolate and you can see that there is a certain colour change and this is again a colorimetric assay so you can see essentially based on the determination of the optical density change and this this optical density change is essentially monitored by this pNPP activity. Now upon dephosphorylation this pNPP essentially turns yellow.

So like that you have you remember that in case of MTT your formazan crystal that turns purple coloured and then purple colour is measured at certain wavelength using spectrophotometer spectrophotometrically. So similarly during the ALP, upon dephosphorylation this pNPP turns yellow and this is placed at 405 nanometer absorbance and this assay can detect, this is very sensitive assay. This assay can detect 0.2 nanogram per millilitre active alkaline phosphatase. So even if this alkaline phosphatase is present in very small amount ALP assay can pick up this small amount of this alkaline phosphatase and then it can quantify.

(Refer Slide Time: 22:51)



So this is little bit more detail on this, how this assay is conducted. So you first prepare the working solution like as I discussed, mentioned for other assays. Then add at least 50 microlitre per well biological samples containing alkaline phosphatase, then you add 50 microlitre pNPP as I mentioned before in the last slide. Then mix the agents and into a desired temperate for, desired temperature means 37 degree Celsius for 62, 30-60 minutes roughly up to 1 hour. Then 50 micro litre of the stop solution is added and finally this absorbance is measured at 405 nano meters.

So from this simple description of the different assays what you can notice is that there are 3 or 4 distinctive things that you have to understand that each assay depends on certain (spectropho) particularly this colorimetric assay depends on the final measurement of the optical density changes at a specific wavelength.

Second thing is that, that you have to understand what is the sensitivity of this assays and third thing is that for what kind of, what kind of purposes that you are essentially measuring that, and what is the origin for all this assays like whether it interest with the specific organelles or certain enzymes in this kind of leached out of the particular cytoplasm of the cell and so on. So once you know the sufficient background of each and every assay, then only you will be in a position to you will be in a position to analyse and interpret the different values that is coming out of all these assays. So I will continue this differentiation in the next module and complete this one.