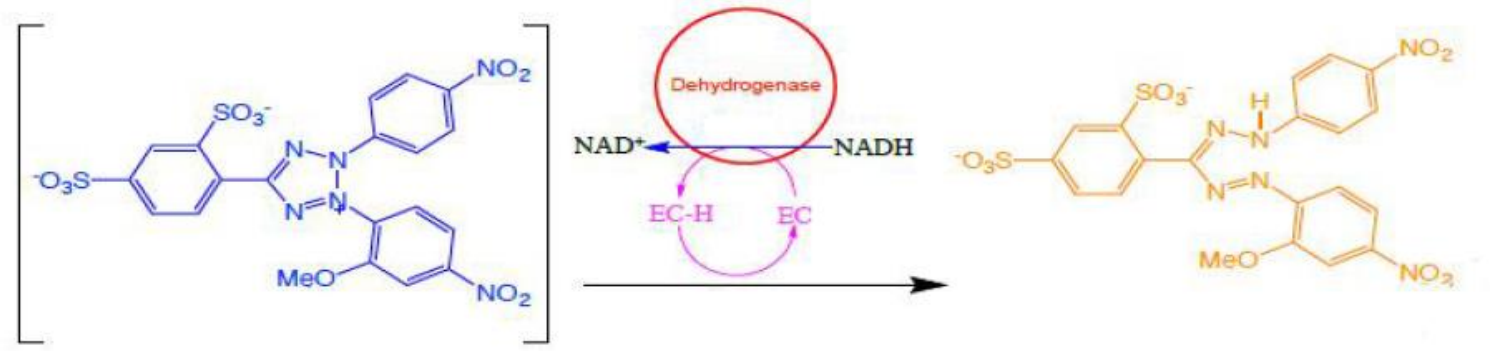
Cell Counting Kit-8 (CCK-8)

Cat#: orb1154736 (User Manual)

2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazoliumsodiumsalt is a analogue to MTT. With the existence of coupling reagent of electronics, this compound can be reduced to orange hydrophilic formazan (following picture) by dehydrogenase and solved directly into medium. The faster cells grows and the more cells in the medium, the deeper of the medium color is, in contrast, the bigger cytotoxicity cells have, the lighter of the color is. As for the same cell type, the medium color and the live cells have a linear relationship. Taking advantage of this, we can do analysis of cell proliferation and cytotoxicity through this kit.



Methods comparation of CCK-8 and MTT

CCK8 kit assay provide a highly sensitive, easy to use, safe, good repeatability method to detect cell proliferation and activity. Compared to MTT, CCK8 do not need organic reagent and radio isotope with less procedure and no loss during detection, leading to an exactly result.

1. The product of MTT assay is hydrophobic, which need to be solved in organic solvent such as DMSO; The Product of CCK-8 is hydrophilic, so the procedure of solving can be skipped, reducing the error.
2. Compared to MTT, measurement range of CCK-8 is wider and has a higher sensitivity.
3. CCK-8 kit has no cytotoxicity, so multiple measurements can be available to select the best time to detect.
4. The reagent of CCK-8 is more stable than those in MTT, leading to a good repeatability.
5. MTT has cytotoxicity with its product need to be solved, bringing about the possible harm to conductor. However, it is a safe way that reagents contained in CCK-8 kit will do no harm to researchers with our organic solvent.
6. CCK-8 kit can preserve for a long period at 4℃ without light, ready to use.

	CCK-8	MTT
1	The product formazan is hydrophilic, it do not need to be solved	The reduced product formazan is hydrophobic, it need to be solved.
2	Good repeatability	Poor repeatability
3	Less procedure	More procedure
4	Measurement wavelength: 450～490nm	Measurement wavelength : 550～600nm
5	Only one bottle, ready to use.	Extra organic reagents needed, inconvenient

Application:

CCK-8 kit can be applied to detect activities of bio-active factors, selection of drug for tumor treatment, assessment of proliferating cells, drug sensitivity, and the experiments related to cell activities and proliferation. This kit is ready to use and very convenient, including a bottle of CCK-8 solution. There is no need to solve the product during the experiment. It is suitable for large scale detection with 96-well plate or 384-well plate with microplate reader.

Conservation :

Store at 0-5℃ without light. Avoid freeze and thaw. Term of validity is one year since production. CCK-8 can be stored at -20℃ for two years. Please store at 0-5℃ if frequently used

Instruction of CCK-8 :

1. Draw standard curve (when measure the particular cell amount)
(1) Count cell in the suspension with cell count plate, and seed the cells;
(2) Dilute the cells with the medium according to a certain ratio(e.g. 1/2). It is usually set 3-5 gradient of cell Concentration, 3-6 duplicates each group.
(3) To make the seeded cell adherent, a 2-4 hours culture is needed. Then measure absorbance after adding CCK reagent for some time. Draw a standard curve with cell amount on its X-axis and O.D value on its Y-axis (The sample and the standard should be conducted under the same condition in order to ensure the amount of seeded cells.)

2. Detection of cell activity
(1) Add 100 µL cell suspension to 96-well each well. Put the culture plate into incubator (37℃, 5% CO2) for a pre-culture;
(2) Add 10 µL CCK solution into each well(bubbles should not exist in wells, they will affect the measurement);
(3) Incubate the culture plate into the incubator for 1-4 hours;
(4) Measure the O.D value at 450 nm;
(5) If it is inconvenient to measure absorbance, please add 10 µL 0.1M HCl solution each well or 1%(w/v)SDS solution and then store at R.T. without light. The absorbance changes little within 24 hours.

3. Detection of cytotoxicity
This example is 96-well, other culture plate size should be arranged according to the fact;

(1) Add 100 µl cell suspension into 96-well plate(the usual density of cells to assess proliferation is 2000 cell/100 µl, the density of cells to assess cytotoxicity is 5000 cells/100 µl. the amount of cells for each well should be determined by factors such as the volume of cells and the rate of proliferation, etc). according to the experiment, pre-culture the 96-well plate in the incubator(37°C, 5% CO2) for 24 hours;
(2) Add 1-10 µl different concentration of drug into the plate to stimulate the cells;
(3) Incubate the plate in the incubator for a period (e.g. 6, 12, 24, or 48 hours).
(4) Add 10 µl CCK-8 solution into each well(No bubbles! It will affect the absorbance). If the initial volume of medium is 200 µl, you need to add 20 µLcck-8 solution. Apply this ratio to other cases. Wells with corresponding medium but without cells can be regarded as the blank control. If the drug probably affect the absorbance, the medium with the drug, CCK-8 and corresponding medium can be regarded as the blank control
(5) Incubate 1-4 hours in the incubator continually. For the most common cases, an incubation of an hour is enough. Time for incubation should be determined by the cell type and density. Time spot for incubation(e.g. 0.5, 1, 2, 4 hours) can be predicted by a series of detection when carrying out the first experiment and the most suitable one can be applied to the following experiment.
(6) Measure the absorbance at 450 nm(can be replaced by 420-480 nm filter). Sub-wavelength longer than 600 nm can be applied to measure the absorbance as dual wavelength spectrophotometry.
(7)If it is inconvenient to measure absorbance, please add 10 µL 0.1M HCl solution each well or 1%(w/v)SDS solution and then store at R.T. without light. The absorbance changes little within 24 hours.
(8) Note: if the materials to be measured have a stronger oxidation and reduction, you can replace with fresh medium before CKK adding in order to exclude the effect of drug.

Activity calculation

Cell activity (%) = [A(drug treated)-A(blank)]/[A(non-treated)-A(blank)]*100%
A(drug treated): absorbance of wells with cells , CCK solution and certain concentration of drug;
A(blank): absorbance of wells with medium and CCK solution but without cells;
A(non-treated): absorbance of wells with cells, CCK solution but without drug;
Cell activity: activity of cell proliferation or cytotoxicity.

Notes:

1. Evaporation must be taken into account when culturing for a longtime with 96-well plate; On one hand, owing to the outer wells is the easiest wells to evaporate, you add water or PBS into these wells which abandoned; on the other hand, 96-well plate can be put close to water avoiding evaporation;
2. The principle of CCK-8 kit is detection of activity of cell dehydrogenase. Any reduction such as certain antioxidants in the system should be excluded, or they will affect the result.
3. the amount of the cells seeded in the wells and time of culturing after adding CCK-8 should be explored by setting a series of gradient.
4. Multichannel pipette has been suggested to utilize to reduce the error between wells. Do not put the pipette under liquid surface, easily making bubbles, affecting the absorbance. We recommend a declining adding method along the walls of plate.
5. The minimum of seeded cells is 1000 cells/well. The sensitivity to detect white blood cell is relatively low, so the recommended amount of seeded cells is more than 2500 cells/well and the culturing time can be

lengthened appropriately. If 24-well or 6-well plate is need, please calculate the amount of seeding, and the volume of CCK-8 solution need to be added is the one in ten of total medium amount.
6. please replace with fresh medium if a long culturing time or the coloring of medium changes or pH changes occurred.
7. It is allowed to replace 450 nm filter with 430-490 nm filter, but 450 nm filter has a highest sensitivity.
8. Serum and phenol red have no obvious effect on this kit. The effect of phenol red in the medium can be counteracted by the blank control, so it will not affect the result.
9. For your safety and health, please wear protective clothes and disposal gloves.

Analysis of cell proliferation

Device：incubator, microplate Reader, pipette

Procedure	Notes
Prepare cell suspension	Note1: Seeded cells need a further 2-4 hour to culture to adherent, if there is no need for adherent, you can skip this procedure.
↓	Note2: Owing to few amount of CCK-8 added to each well, errors may be cause by the reagents on the wall of the well.
Seed cells into 96-well plate	Tap the plate to help to mix well after adding reagents.
Culture in 37℃ incubator (Note 1)	
	Note3: different amount of formazan forms according to different cell types.
↓	If the color is lighter than expected, a longer culturing may be needed to confirm the most suitable condition
Add 10ul CCK-8 solution(Note 2)	Blood cells need more time(5-6hours) to develop color due to few formazan formation.
Culture for 1-4 hours(Note 3, Note 4)	Note4: Tap the plate if the color is uneven.
Measure absorbance at 450nm (Note 5)	Note 5: Dual wavelength spectrophotometry has been suggested to measure: Main wavelength: 450-490 nm; sub-wavelength: 600-650 nm.

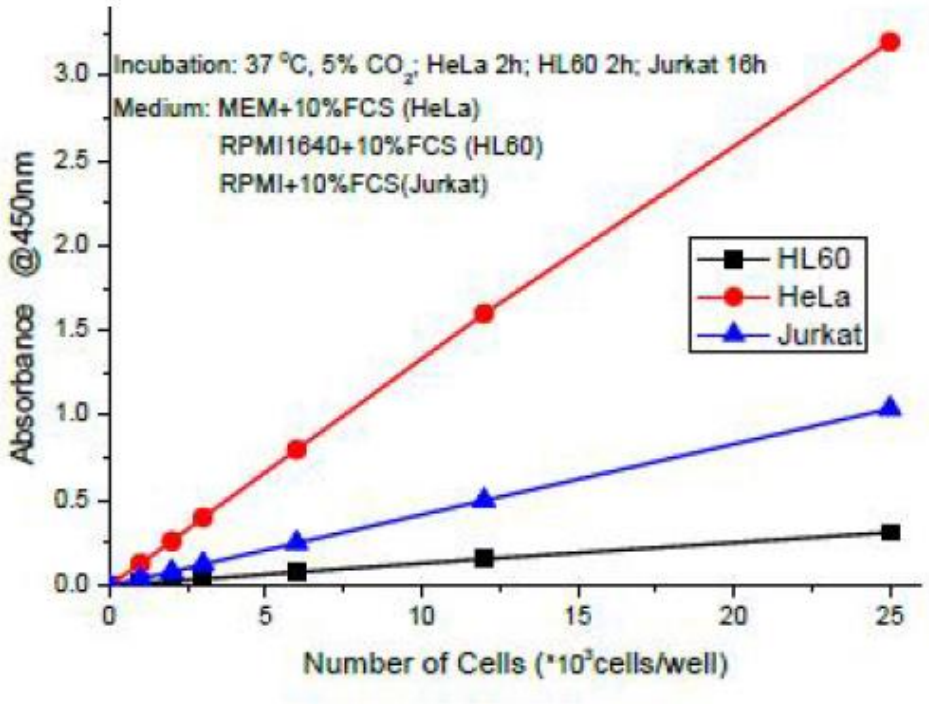


Figure 1 Correlation curve of cell amount and absorbance.

Analysis of cell proliferation

Device ： incubator, microplate Reader, pipette

Procedure	Notes
Prepare cell suspension	Note1: Seeded cells need a further 2-4 hour to culture to adherent, if there is no need for adherent, you can skip this procedure.
↓	Note2: the time of incubation with drug should be determined by the property of the drug, the sensitivity of the cells and the cell cycle.
Seed cells into 96-well plate Culture in 37℃ incubator (Note 1)	Note3: Owing to few amount of CCK-8 added to each well, errors may be cause by the reagents on the wall of the well. Tap the plate to help to mix well after adding reagents.
Add different concentration of drug Add 10 μl CCK-8 solution(Note 2)	Note4: different amount of formazan forms according to different cell types. If the color is lighter than expected, a longer culturing may be needed to confirm the most suitable condition.
Culture for 1-4 hours (Note 3 ,Note 4)	Note 5: Blood cells need more time(5-6hours) to develop color due to few formazan formation. Tap the plate if the color is uneven.
Measure absorbance at 450nm (Note 5,	

Note6)

Note 6: Dual wavelength spectrophotometry has been suggested to measure:
Main wavelength: 450-490 nm; sub-wavelength: 600-650 nm.

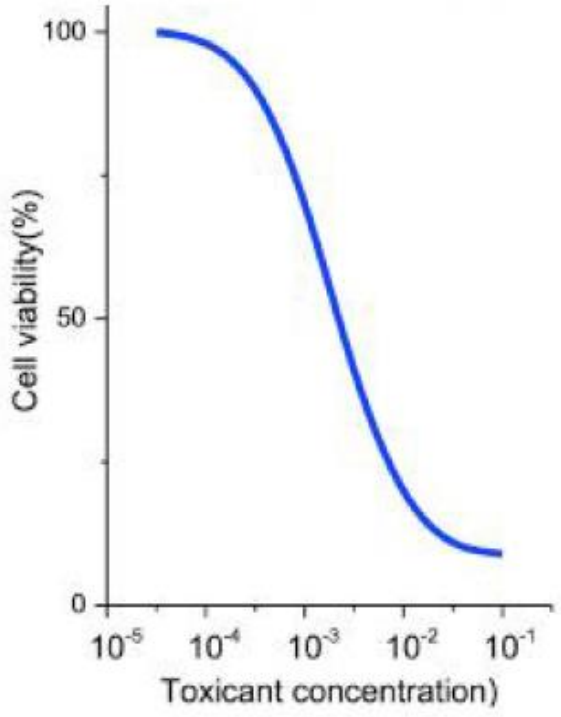


Figure 2 Curve of cytotoxicity

Calculate according to the formula below and draw the curve, the value of toxicant concentration corresponds to the
50% of cells' viability is IC50
Cell viability =[(AS-Ab)/(Ac-Ab)] X100%
AS: experimental group(wells with cells, medium, CCK-8, drug)
Ac: control(wells with cells, medium, CCK-8, but without drug)
Ab: blank(wells with medium, CCK-8, drug but without cells)

Common problems and Solutions

Q1: What is the purpose of the setting of sub-wavelength? Is it necessary ?
A1: it is not necessary to set a sub–wavelength. There is no absorbance of CCK-8 at sub–wavelength. The setting of sub-wavelength is to exclude the effect of turbidity of samples.
Q2: how to set a blank control?
A2: Add CCK-8 into wells with the medium, drug but without cells. These wells are blank controls, and then measure at 450 nm.
Q3: do my drug have effect on measurement when adding a drug into the system? How to solve it ?
A3: Sometimes, it will influence the result. If your drug have a stronger oxidation or reduction, the absorbance will be changed. Solutions: our first step is to confirm whether it takes effect on the absorbance. Adding the CCK-8 into the wells with medium, drug; and then measure the absorbance at 450 nm of the wells. If the

absorbance is obviously changed than the wells without drug, it proves that this drug does have an effect on measurement. We should exclude this effect by replacing with fresh medium before adding CCK-8.
Q4: How about no replace with the CCK-8 medium? Is there any influence?
A4: Normally, there is no problems. But if there is materials with stronger oxidation or reduction, we recommend you replace with fresh medium avoiding errors. Following medium will influence the measurement: IMDM(blank is 1.0), McCoy/s(blank is 0.3).
Q5: what kind of materials will influence the result?
A5: CCK-8 measurement will be influenced when there is any reduction in the system, leading to the absorbance increased. When there is any material with stronger oxidation, the reaction will be inhibited, leading to the absorbance decreased. When there is phenol red in the system, it will not influence the measurement, although the absorbance increased. Serum has no effect on measurement.
Q6: There is only a method for measurement of 96-well plate. How about the mount of CCK-8 I should add into the 24-well plate or 12-well plate ?
A6: It is usually added one of ten of the total volume of medium.
Q7: How to avoid the errors because of the residue of CCK-8 in tip and on walls of the well?
A7: Dilute before adding CCK-8 reagents. Mix it well after adding.
Q8: How to solve the poor repeatability of several experiments?
A8: there are several case:
(1) When culturing in the incubator, the outer plate is easy to evaporate, leading to a different volume of medium and an increase of errors. We recommend you the outer wells adding some PBS or water, not for measurement.
(2) It is probably caused by the CCK-8 on the walls of the well, we recommend tapping the plate after adding to help to mix well.
(3) the amount of the cells in wells is too much or too little. Please explore the best condition between 1000-100000/well.
Q9: How to stop the reaction during developing the color?
A9: There are several ways to stop(96-well plate):
(1) Add 10 µl 0.1M HCl solution to each well;
(2) Put the plate into 4℃ refrigerator;
(3) Add 10 µl 1%SDS solution to each well.
Q10: How about the high absorbance in the experiment if the amount of the cells cannot be reduced?
A10: Shorten the time of culturing after adding CCK-8.
Q11: Is it necessary to pre-culture cells?
A11: Not necessary. If you want your cells keep in a good state, we recommend you pre-culture cells. If there is no pre-culture, the dehydrogenase in the cells may be not very stable. Some researchers do not pre-culture, but they keep the samples and standards in the same condition when detecting.
Q12: If the drug contains metal component, will it influence the result?
A12: Metal components do influences the color development. The final concentration of 1mM $FeCl_3$, $CuSO_4$ will inhibit the 15% and 90% percent of chromogenic reaction, respectively, leading to a decrease of sensitivity. 100% inhibition occurs when the final concentration is 10 mM.
Q13: How about the term of validity of CCK-8?
A13: CCK-8 will be effective within one year under 0-5 ℃ , and within two years under -20 ℃ , without light. It if recommended to store at -20℃ for long term preservation. The common color of CCK-8 is pink. If there is

any obvious changes of the color, it may have a deterioration. Freeze and thaw may cause an increase of blank, taking effect on the result. Store at 0-5℃ if it is often used.
Q14: Is it necessary to count cells if replacing the medium after pre-culture ?
A14: It is usually count cells growing in logarithmic growth period with hemocytometer. Cells should be treated with trypsin and be made cell suspension. You can get a exactly amount of cells with hemocytometer.
Q15: Will the CCK-8 stains the cells ?
A15: CCK-8 will not stains cells. CCK-8 in medium will not pass through the cell membrane, and it is reduced by dehydrogenase into a highly hydrophilic yellow formazan, which is a chromogenic reaction. After chromogenic reaction, you can replace with the fresh medium and culture the cells continually.
Q16: Is it the same sensitivity to different cells.
A16: Owing to the different dehydrogenase activity, color will vary, so that sensitivity is different.
Q17: What is the difference between the amount of the suspended cells and adherent cells?
A17: most suspended cells is hard to develop color than adherent cells with a lower absorbance. We usually extend the culturing timer after adding CCK-8 or adding the seeded cells. It is easy to develop color for adherent cells. The absorbance sometimes will be beyond the range of microplate Reader if there is too much cells.
Q18: Is it necessary to draw a standard curve for every experiment?
A18: It is strongly suggested to draw a standard curve every time. Although the cells may be the same, the state of cells may be different. We recommend to draw a standard curve every time for different cell state. If the batch of the regents is different, the sensitivity may be a bit different, we also recommend to draw standard curve for different batch.
Q19: Sometimes cells died with the drug treatment, but its dehydrogenase may be active, can I calculate the cell amount ?
A19: No. Owing to the reaction of dehydrogenase within cells reflecting the amount of cells indirectly, the measurement value will be higher than the real one.
Q20: What is the color of CCK-8?
A20: CCK-8 should looks like pink. Measurement will be affected if the color is different.
Q21: Can I use CCK-8 to detect live cells after importing a gene?
A21: Yes.
Q22: Can I use CCK-8 to detect cells in a pH5 medium?
A22: Theoretically, it can work, but it should be tested with standard samples.
Q23: What is the expected range of absorbance?
A23: Usually, it range from 0.1 to 2.0. It's best to localize around 1.0.
Q24: Will bacteria react with CCK-8?
A24: CCK-8 will not react with bacteria. The defensive system of bacteria is stronger, so it will not have a reduced reaction with CCK-8.
Q25: CCK-8 is used to detect live cells, how about its influence to the cells in proliferating and static period?
A25: Usually, CCK-8 is used to detect cells growing in logarithmic growth period. If the activity of dehydrogenase of cells in proliferating and static period vary a lot, the result will shows a big difference
Q26: What should I do if the absorbance is too low?
A26: There are two ways to solve this problem. 1. Add cells appropriately; 2. Extending the time of reaction after adding CCK-8.
Q27: What is the reason that the color do not change the absorbance of which is same as blank when detecting drug's cytotoxicity after adding CCK-8 and a period of culturing?

A27: There are two possible reasons. 1. The concentration of the drug is so high that no cells live in these wells; 2. Chromogenic reaction of CCK-8 has been inhibited by the drug's oxidation. We recommend to replace with fresh medium to exclude the drug, and CCK-8 is added after no existence of drug.
Q28: How to avoid the aggregation of cells?
A28:1. Lengthen the time of digestion; 2. Shake by hand or machinery to help to mix well; 3. Pipette repeatedly avoiding bubbles.
Q29: What is the suitable time point to detect suspended cells?
A29: Owing to unchanged medium which has been used for a long time, the oxygen and nutrients will lose, affecting the state of cells. We recommend to culture for 4-5 days to ensure whether the amount of cells has been saturated. If a longer period need to be cultured, we recommend the outer well blank without cells avoiding evaporating.
Q30: How to draw a standard curve?
A30: Take some cell suspension with an kwon density into the plate, and then dilute with medium according to a certain ratio. Thus, there are a series of wells with different cell density. After a period of culturing, add CCK-8 into wells. Measure the absorbance with a microplate reader. Draw a line with the amount of cells on X-axis and absorbance on Y-axis.
Q31: What is the result of too much cells?
A31: If there are too much adherent cells, cells may overlap, leading to a poor state of cells even death. Besides, too much cells may cause the high absorbance (even beyond the range of microplate reader).
Q32: What is the difference between BrdU and CCK-8?
A32: CCK-8 measure the activity of dehydrogenase in cells growing in logarithmic growth period to reflect the activity of cells indirectly. BrdU is to detect the synthesis of DNA. BrdU needs not only reagents but also a fixation of cells through the antigen-antibody reaction by fluorescence. It is convenient to use CCK-8 to simply detect cell proliferation.
Q33: The drug has an inhibition effect on cells during cytotoxicity, why does the measurement result increase?
A33: The drug may react with CCK-8. We recommend to set a blank control. If the absorbance is higher than the normal, it indicates that the drug react with CCK-8. You can replace with fresh medium to solve this problem.
Q34: After adding CCK-8 , why do the value vary at different time point(e.g. at 3 hours and 3.5 hours)?
A34: Owing to the reaction of CCK-8 and dehydrogenase in the cells is a cyclic reaction. With time elapse, the color become deeper and the absorbance become higher. But the amount of cells do not change. We recommend to ensure the best condition of time and absorbance, and fix the time of CCK-8 incubation in order to a stable data for following experiments.
Q35: Why the data between groups or within a group vary, and there is no regular pattern?
A35: There may be a CCK-8 residue in tip or on the walls of wells. It will be better to adding samples faster avoiding the residue. We recommend to dilute the CCK-8 to reduce the errors: dilute CCK-8 with the same volume of medium, mix well and add 20 µl to each well.
Q36: When detect cytotoxicity with CCK-8, 1.How is the suitable time for pre-culturing? 2. How to calculate the amount of cells when cells divides? 3. What if a longer time for pre-culture than normal ?
A36: Usually, the time of pre-culture for adherent cells is 24 hours. Cell division can be ignored if you only care about LC50, but you should do a control with the same cells. If the time for pre-culture is too long, cells may be saturated and the absorbance sometimes will be beyond the range of microplate Reader if there is too much cells.

Q37: Why the amount of cells do not increase (absorbance do not increase) when a stimulator of cell division added into experiment ?
A37: You should observe with microscope to ensure the cells increase or not. (The possibility that added simulator reacts with CCK-8 and the absorbance do not change is too low). If the amount of cells indeed increase under the observation of microscope, please check if the drug has oxidation. It may inhibit the reaction of CCK-8. The method is that do a comparation with wells with medium, cells, and one add the drug, and the other do not. Then add CCK-8 to test its absorbance. If the absorbance of no drug is higher than the one the drug treated, it indicates that the drug has oxidation.