Cholesterol Depletion using Methyl-β-cyclodextrin

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Abstract

Cholesterol is an essential component of mammalian cells. It is the major lipid constituent of the plasma membrane and is also abundant in most other organelle membranes. In the plasma membrane cholesterol plays critical physical roles in the maintenance of membrane fluidity and membrane permeability. It is also important for membrane trafficking, cell signalling and lipid as well as protein sorting. Cholesterol is essential for the formation of liquid ordered domains in model membranes, which in cells are known as lipid nanodomains or lipid rafts. Cholesterol depletion is widely used to study the role of cholesterol in cellular processes and can be performed over days using inhibitors of its synthesis or acutely over minutes using chemical reagents. Acute cholesterol depletion by methyl-β-cyclodextrin (MBCD) is the most widely used method and here we describe how it should be performed to avoid the common side-effect cell death.

Key words: cholesterol, methyl-β-cyclodextrin

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1. Introduction

Cholesterol is essential for mammalian cells and is the major component of their plasma membrane [1]. Cholesterol has a small polar headgroup and large hydrophobic moiety which gives it a conical shape (**Fig. 1A, C**). In membranes, cholesterol associates with phospholipids in order to avoid unfavourable exposure to water [2]. Depletion or manipulation of cholesterol content is often used to study its role of various processes such as cell signalling, membrane fluidity, lipid sorting, membrane trafficking and membrane permeability. Methyl-β-cyclodextrin (MBCD) is also frequently used in lipid raft studies, but since it is not specific for a particular cholesterol pool this use should be discouraged [3].

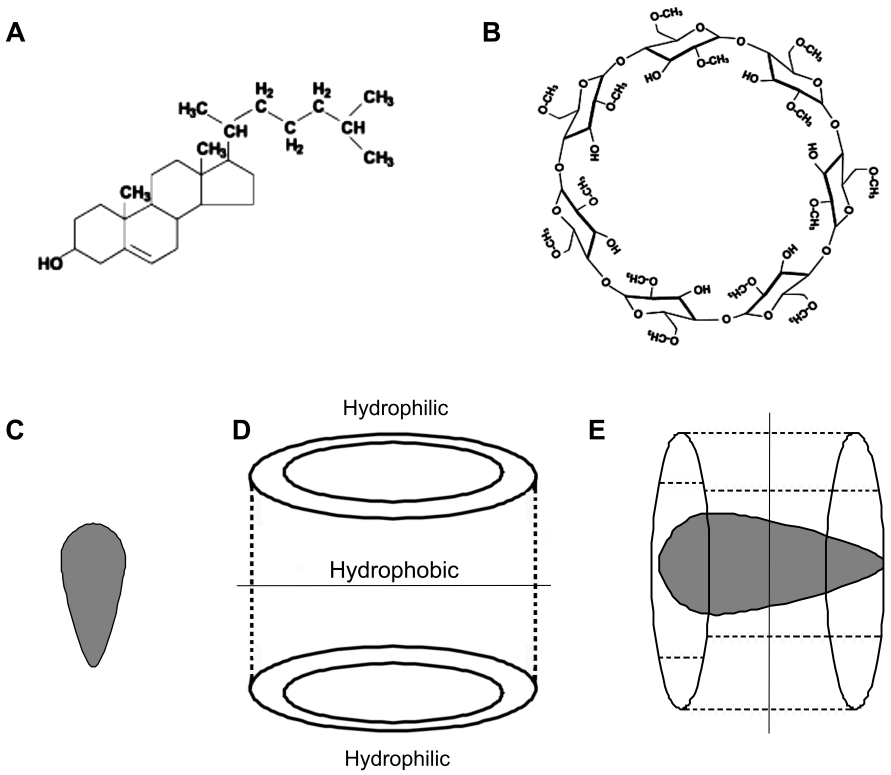

Fig. 1 The structures of cholesterol and MBCD and the formation of cholesterol-MBCD dimer complexes. (A) The structure of cholesterol. (B) The structure of MBCD. (C) Cholesterol represented as a cone. (D) A MBCD-dimer represented as a hollow cylinder. (E) A cholesterol-MBCD dimer complex.

Cellular cholesterol depletion can be achieved in several ways; treatment with metabolic inhibitors, growing cells in lipoprotein deficient medium or using chemicals to immobilize or to extract cholesterol. Examples of these methods are inhibition of cholesterol biosynthesis by statins [4], the culture of cells in the absence of exogenous cholesterol [5], the use of cholesterol binding agents such as digitonin, filipin and saponin [6, 7] and oxidation of cholesterol [8]. The most commonly used method is acute cholesterol depletion using MBCD. Unlike the cholesterol binding agents mentioned above that incorporate into membranes, MBCD has a central cavity able to form a 2:1 complex with cholesterol [9, 10]. Inhibition of *de novo* cholesterol synthesis or growing cells in lipoprotein deficient medium require treatment over days which is plenty of time to allow redistribution of the remaining cholesterol [3]. The cholesterol binding agents are not compatible with live cells; digitonin and saponin because they are detergents and filipin because it forms crystals with immobilized cholesterol resulting in leaky membranes [11]. Oxidation of cholesterol means the introduction of a new membrane component making it difficult to ascribe any changes to the depletion of one molecule or the gain of another. In comparison MBCD is faster, does not produce degradation products and, if used correctly, compatible with live cell studies. Moreover, MBCD has the advantage of acting strictly at the membrane surface [12]. A disadvantage with MBCD is that it is

not specific for cholesterol [13], but this limitation can be addressed by performing appropriate controls.

Cyclodextrins are water-soluble oligosaccharides containing hydrophobic cavities [14, 15]. Cyclodextrins contain a- (1-4) linked D-glycopyranose units which can form hexamers (α-CD), heptamers (β-CD) (**Fig. 1B**) or octamers (γ-CD). The number of glycopyranose units in the ring structure and the degree of polymerization determines the size of the hydrophobic cavity and the affinity towards specific compounds. The water solubility of cyclodextrins can be improved by various modifications including methylation [14, 15]. Based on the dimensions of their cavities, α-CD can form inclusion complexes only with low-molecular-weight molecules or compounds with aliphatic side chains, β-CD can form complexes aromatic or heterocyclic compounds, while γ-CD can accommodate a wider variety of large organic compounds such as macrocycles and steroids [16]. MBCD has a high capacity to bind cholesterol and is relatively cheap [17]. Moreover, it is highly water soluble [18]; properties which make MBCD the cyclodextrin the most widely used cyclodextrin for manipulating cholesterol levels in cells [17, 19]. In addition to cholesterol depletion, treatment of cells with MBCD complexed with cholesterol can be used to increase cholesterol levels or as equilibrium cholesterol controls [20, 21].

Due to the requirement for cholesterol in creating the plasma membrane permeability barrier, cells cannot cope with loosing too much of their cholesterol. How much will differ between cell types and for instance Jurkat T cells remain viable until they loose more than 50% of their total cholesterol (**Fig. 2**). Once a cell is dead, it is hardly surprising that a cellular process ceases to function and it is incorrect to ascribe this failure to the importance of cholesterol and/or lipid rafts. This was the case in the T cell signalling field [22] and care must be taken to make sure that cells remain viable.

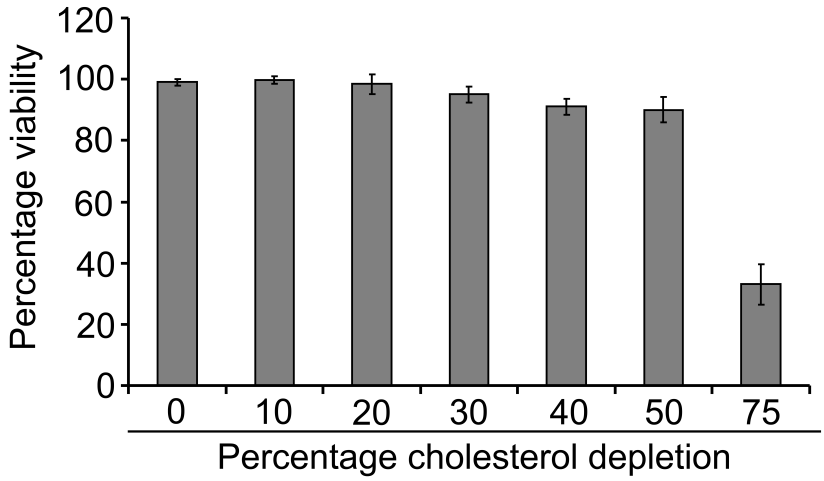

Fig. 2 Cell viability after MBCD treatment. Jurkat T cells were treated with MBCD at 37ºC to deplete progressive amounts of cholesterol and examined for viability by Trypan blue exclusion. Data shown are means ± SD from three experiments where 300 cells were counted for each condition (900 in total). The bulk of these data were originally published in BBA 1778, 1251-1258 [3].

2. Materials

To increase the reliability for the data, samples should be prepared in parallel. For instance if different levels of cholesterol depletion are desired, all samples should be prepared concurrently to minimize the risk that cell culture density/confluency or variations in protocols and reagents contribute to the results.

2.1 Cells

Grow cells of desired type under standard cell culture conditions. Make sure that cells that grow in suspension are not overgrown or that adherent cells are at no more than 80% confluency (*see* **Note 1**).

2.2 Reagents for Cholesterol Depletion

1. Methyl-β-cyclodextrin (Sigma, St Louis, MO).
2. Cholesterol (Nu-Chek Prep, Inc., Elysian, MN).
3. Cell culture medium.
4. HEPES (if not included in the cell culture medium).

2.3 Reagents for Viability Assessment

1. Trypan blue (Sigma, St Louis, MO).
2. Phosphate buffered saline.

2.4 Reagents for Assessment of the Level of Cholesterol Depletion by Radioactivity Tracing

1. [^{3}H]-Cholesterol (Perkin Elmer, Waltham, MA).
2. Scintillation fluid (Perkin Elmer, Waltham, MA).

2.5 Reagents for Assessment of the Level of Cholesterol Depletion by Cholesterol Oxidation

1. Chloroform.
2. Methanol.
3. Water.
4. 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) (Synchem OHG, Felsberg, Germany).
5. Potassium phosphate buffer, pH 7.4.
6. NaCl.
7. Cholic acid (Sigma, St Louis, MO).
8. Triton X-100 (Sigma, St Louis, MO).
9. Horse radish peroxidase (Sigma, St Louis, MO).
10. Cholesterol oxidase (Sigma, St Louis, MO).
11. Nitrogen gas.

2.6 Equipment for Preparing MBCD-Cholesterol Complexes

1. Glass tubes.
2. Microcaps.
3. Water bath.
4. Sonicator.
5. Vortex.

2.7 Equipment for Assessing Cell Viability

1. Hemocytometer.
2. Light microscope.

2.8 Equipment for Assessment of the Level of Cholesterol Depletion by Radioactivity Tracing

1. Glass tubes.
2. Scintillation counter.

2.9 Equipment for Assessment of the Level of Cholesterol Depletion by Cholesterol Oxidation

1. Glass tubes
2. Flouroscanner.
3. 96 well plates.
4. Microcaps.
5. Waterbath.

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3. Methods

3.1 Preparation of Cells for Cholesterol Depletion

1. Trypsinize the cells (*see* **Notes 2 and 3**).
2. Count the cells.
3. Transfer the desired number of cells to a tube.
4. Wash the cells in serum-free media. Repeat twice.
5. Suspend the cells in serum-free media at a concentration of $20x10^6$ cells/ml.

3.2 Preparation of Methyl-β-cyclodextrin Solutions

A freshly made MBCD solution should always be made (*see* **Note 4**).

1. Dissolve the desired amount of MBCD in medium supplemented with 25 mM HEPES (*see* **Notes 5 and 6**).

3.3 Cholesterol Depletion at Steady State

Cholesterol extraction is biphasic with an initial fast phase followed by a second slower phase that reaches a specific percentage extraction for each MBCD concentration [3] (**Fig. 3** and *see* **Note 7**).

1. Prepare the cells as described under section 3.1.
2. Mix the cell suspension with the MBCD solution at a 1:1 ratio. A summary of what percentage of cholesterol depletion different concentrations of MBCD can achieve is presented in Table 1.
3. Incubate at 37°C for 15 min. Tap the tubes intermittently during the MBCD-treatment so that the cells remain suspended in the solution and do not settle at the bottom of the tube.
4. Proceed with the subsequent analysis immediately after the MBCD-treatment (*see* **Note 8**).

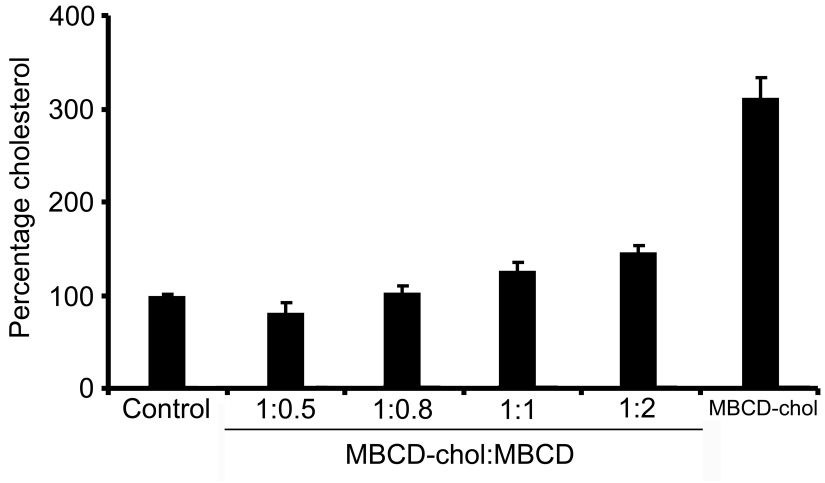

Fig. 3 Cholesterol extraction from cells by MBCD. Jurkat T cells ($10x10^6$/ml) labelled with [^{3}H]-cholesterol were treated with 2.5 mM MBCD in serum-free RPMI medium at 37 °C. At the indicated times, the cells were pelleted by a brief centrifugation and the MBCD-containing supernatant was transferred to a fresh tube. The cell pellet was resuspended in PBS and aliquots from both fractions were subjected to scintillation counting. Data shown are means±SD, n=3. These data were originally published in BBA 1778, 1251-1258 [3].

3.4 Preparation of Methyl-β-cyclodextrin-cholesterol Complexes

A freshly made MBCD-cholesterol solution, starting from point 2 below, should always be used (*see* **Note 4**).

1. Prepare a 25mg/ml cholesterol solution in chloroform:methanol (1:1, v:v) in a glass tube (*see* **Note 9**).
2. Transfer 200 μl of the 25 mg/ml cholesterol stock solution in a glass tube using a glass microcap.
3. Heat the tube to 80°C to evaporate the solvents.
4. Add 10.36 ml of 5 mM MBCD freshly made in serum-free medium.
5. Sonicate at 50-60% amplitude for 5 min in cycles of 4 s with 2 s on time and 2 s off time. After the sonication, the solution should appear clear.
6. Vortex the tube vigorously for 3 min.
7. Incubate the tube at 37°C overnight with constant stirring to avoid cholesterol crystallisation.

3.5 Establishing Extraction Conditions where no Net Cholesterol Extraction takes place

The hydrophobic pocket that forms when two MBCD molecules form a complex is of a suitable size to accommodate cholesterol. However, MBCD is by no means specific for cholesterol and other lipids may also be extracted [13]. Therefore control experiments where no net cholesterol extraction takes place need to be performed (*see* **Note 10**). Figure 4 illustrates how the level of cholesterol extraction varies in Jurkat T cells incubated with cholesterol-loaded MBCD and MBCD at different ratios.

1. Mix 5 mM MBCD with the 5 mM MBCD-cholesterol solution at different ratios.
2. Add the mixes at a 1:1 ratio to cells prepared as above, i. e. at a final cell density of $10x10^6$ cells/ml and a final MBCD concentration of 2.5 mM.
3. Incubate at 37°C for 15 min.
4. Assess the level of cholesterol depletion using either the radioactivity tracing or the cholesterol oxidation method.

Table 1. Cholesterol extraction by MBCD at 37°C

MBCD concentration (mM)	% cholesterol extracted
0.5	10
1.0	20
1.5	30
2.5	40
3.0	50

Jurkat T cells at 10×10^6 cells/ml were treated with different concentrations of MBCD at 37°C for 15 min to reach the maximal level of cholesterol extraction for each concentration. The bulk of these data were originally published in BBA 1778, 1251-1258 [3].

3.6 Assessment of Cell Viability

As with any treatment, it is important to check whether the cells remain viable after MBCD-treatment. There are many ways of doing this of which one method that is easy to perform is the Trypan blue exclusion method (*see* **Note 11**). Viable cells exclude the Trypan blue dye while dead cells are stained and appear blue.

1. Trypsinize the cells (if adherent cells).
2. Prepare a cell suspension (*see* **Note 12**).
3. Dilute the cell suspension 1:1 with 0.4% (w:v) Trypan blue in PBS. Mix well.
4. Fill the counting chambers of a hemocytometer or slides of an automatic counter with the cell suspension.
5. Count the number of both stained and unstained cells.
6. Calculate the percentage of unstained cells, which represents the percentage of viable cells (*see* **Note 13**).

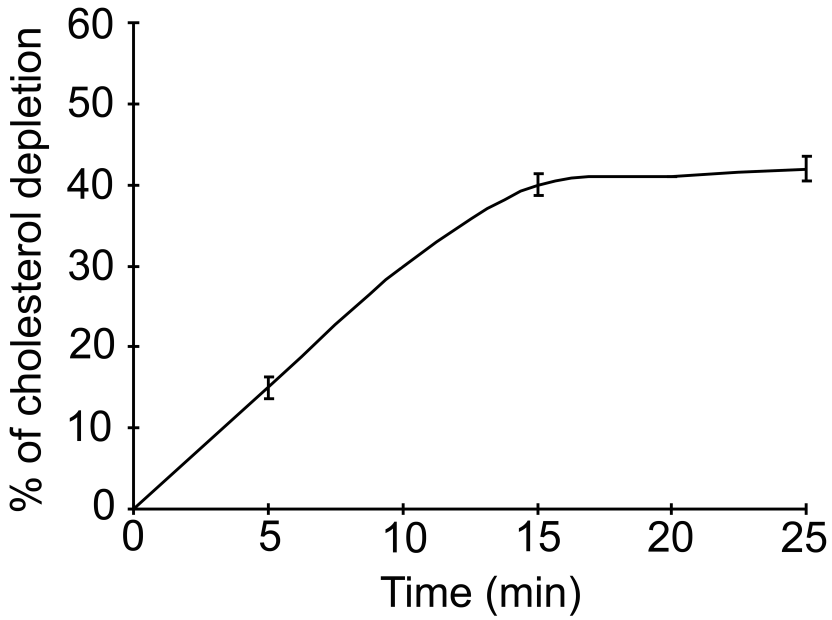

Fig. 4 Cholesterol equilibrium during MBCD-treatment. Jurkat T cells were treated for 15 min with 2.5 mM MBCD mixed with 2.5 mM MBCD–cholesterol complexes at 37 °C. The cells were then washed and lipids extracted. The cholesterol content was estimated using the Amplex red assay. Control cells were defined as containing 100% cholesterol. Data shown are means±s.e.m., n=4. These data were originally published in BBA 1801, 625-634 [22].

3.7 Assessment of Cholesterol Depletion

It is recommended that the extent of cholesterol extraction is always assessed. For many experiments, it may also be informative to vary the level of extraction to study its effect.

3.7.1 By Radioactivity Tracing

1. Prepare cells as described under section 3.1, steps 1-4.
2. Culture 2×10^6 cells in RPMI containing 2.5% FCS and 10 µCi [^{3}H]-cholesterol for 40 hr.
3. Wash the labelled cells with serum-free medium and mix them with non-labelled cells at a 2:3 ratio.
4. Resuspend the cells at 20×10^6 cells/ml in serum-free medium.
5. Perform cholesterol depletion with MBCD as outlined above.
6. Quickly pellet the cells by centrifugation.
7. Transfer the MBCD-containing supernatants to fresh tubes.
8. Resuspend the cell pellets in PBS.
9. Mix aliquots from the pellet and supernatant fractions with scintillation fluid.
10. Perform scintillation counting (*see* **Note 14**).

3.7.2 By Assessing Cholesterol Oxidation

1. Lyse the control and cholesterol depleted cells in 2.3 ml chloroform:methanol:water (1:1:0.3, v:v:v). Use glass tubes.

2. Add 2 ml chloroform, 1 ml methanol and 0.7 ml water to change the chloroform:methanol:water ratios to 3:2:1 (v:v:v). This composition will separate into two phases.
3. Transfer the lower organic phases to glass tubes.
4. Extract the water-phase with chloroform:methanol (3:1, v:v).
5. Pool the two organic phases.
6. Place the tubes in a heated (60°C) waterbath in a fume hood and evaporate the solvents under a stream of nitrogen.
7. Dissolve the residue in 200 μl of Amplex red assay buffer (0.1 M potassium phosphate pH 7.4, 50 mM NaCl, 5 mM cholic acid and 0.1% (w/v) Triton X-100 (TX)).
8. Incubate at 37°C for 2 hr.
9. Transfer 10 μl sample aliquots using microcaps to wells of a 96 well plate containing 90 μl Amplex red, horse radish peroxidase and cholesterol oxidase in Amplex red assay buffer. The final concentration should be 300 μM Amplex red and 2 U/ml each of horse radish peroxidase and cholesterol oxidase.
10. Incubate the 96 well plate at 37°C for 150 min (*see* **Note 15**).
11. Read the fluorescence by excitation at 544 nm and emission at 590 nm in a Fluoroscanner (*see* **Notes 16 and 17**).
12. The level of cholesterol depletion is calculated by comparing the values of control and MBCD treatment samples.

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4. Notes

1. In overgrown cells cholesterol may become a limiting factor for continued growth, which could effect its distribution in membranes and lipid bodies/droplets.
2. If may be tempting to perform the cholesterol depletion of adherent cells in plates, dishes or flasks, but it is crucial that the cell to MBCD ratio is kept constant. Assessing confluence simply is not as accurate as counting cells.
3. For cells that grow in suspension, trypsinization is not required. Using a system capable of correctly counting adherent cells without the need to first deattach them is a way to avoid the trypsinization step also for adherent cells.
4. Stored MBCD solutions are not as efficient at cholesterol extraction as are freshly made MBCD solutions. Aliquots of MBCD can be weighed concurrently and stored in enclosed tubes until the start of an experiment.
5. When dissolved in water MBCD is acidic and in order not to affect the cells a substantial amount of buffer, for instance 25 mM HEPES, is required.
6. The molecular mass of MBCD is rarely stated, but its average level of substitution is. The molecular mass ranges between 1,310.00 to 1,331.36 depending on the level of substitution.

7. The extractions stop at a steady level when equilibrium between free and cholesterol-bound MBCD in water solution is achieved. Once this happens, cholesterol still moves between the cell and MBCD-complexes but no net cholesterol extraction takes place.
8. The cholesterol will inevitably be extracted from the plasma membrane, but there is a continuous traffic of lipids in cells. Given the importance of cholesterol in maintaining the cell's permeability barrier and thus keeping it alive, cholesterol from intracellular compartments will be relocated to replenish the cholesterol that has been extracted from the plasma membrane [3].
9. Cholesterol, like any lipid, can adhere to plastic surfaces. To allow quantification of cholesterol it is therefore crucial to avoid plastic, including pipette tips.
10. An alternative to control experiments where no net cholesterol extraction takes place is to add cholesterol at the end of a depletion experiment using cholesterol loaded MBCD to see if the effect of cholesterol extraction is reversed. However, we do not favour this method since the cholesterol added may not distribute like the cholesterol removed and cholesterol addition can cause alterations in for instance cell signalling originating at the plasma membrane [23, 24].
11. The Trypan blue exclusion method is simple and provides a non-graded answer to the question whether a cell is alive or not. It will, however, not distinguish cells committed to dying from live cells, which require more elaborate methods like Annexin V staining of phosphatidyl serine [25].
12. If a hemocytometer is used cell densities of around $0.5\text{-}2\text{x}10^6$ are appropriate. For automatic cell counters, follow the protocol of the manufacturer.
13. It is crucial to account also for cells that are lost. If a short time series is performed, the assumption is that the cell density remains constant and that any lowering of the total cell count is attributable to lysed cells no longer present in the solution. Thorough suspension of the cells before cell aliquots are removed for viability assessment is of utmost importance.
14. Control cells should have 100% of scintillation counts in the cell pellet and no counts in the supernatant. For the MBCD-treated cells, the scintillation counts should decrease in cell pellet samples and increase in the supernatant as the concentration of MBCD is increased.
15. When many samples are run in parallel, it saves time and increases accuracy if components are first mixed and then added to each sample in a single pipetting step. Prepare a mix for the number of samples plus two since small drops of liquid tend to be lost both at the outside of the pipette tip and in the tube where the mix is prepared. If the mix is added before the sample aliquots, there is no need to change pipette tips between wells.

16. The levels of cholesterol are estimated using Amplex Red, which produces the fluorophore resorufin when combined with H_2O_2. Hydrogen peroxide is formed when cholesterol is subjected to cholesterol oxidase.

17. For comparison of samples, it is important that their cholesterol concentrations are within the response range of the assay. To test this, the control sample should be run at two different dilutions for instance 1X and 2X. If the response of the undiluted sample is not twice that of the 2X diluted sample, the samples need to be further diluted until a doubling in the response is achieved.

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