Method Sheet 09

Crystal violet assay for mammalian cell biomass

Overview

This method sheet explains how to perform a basic assay of adherent mammalian cell biomass using the crystal violet reagent. This colorimetric assay is based on the ability of crystal violet, a cationic dye, to bind to DNA and proteins within adherent cells that remain attached to a plastic surface after fixation and washing. Absorbance of the dye is proportional to the number of adherent mammalian cells on a plastic surface, which is used as a proxy for cell proliferation and/or viability. The washing step circumvents issues relating to interference of absorbance measurements by plant pigments. However, the assay does not distinguish between live and dead adherent cells, so is also sensitive to loss of cellular attachment for reasons other than low viability.

Reagents

- Crystal violet reagent (0.2% crystal violet in 20% methanol, see Notes section for how to prepare)
- 20% acetic acid in dH_2O (see Notes section for how to prepare)

Equipment

- Squeezable wash bottle dispensing distilled H_2O
- A large plastic tray to collect waste runoff from plate washing
- Multichannel pipette capable of dispensing 50 - 100 µl
- Waste stream for halogenated chemicals
- Microplate reader capable of measuring a wavelength between 540 and 600 nm

Method

1) Cell culture methods preceding the staining step should be performed in a sterile environment with attention to sterile technique.
2) However, after the cells have been challenged, the staining part of the crystal violet assay does not need to be performed in a sterile environment - the following steps can be performed in any laboratory with a suitable chemical waste stream.
3) This assay can be messy, so it may be helpful to avoid stains from accidental spills by covering the bench surface with protective paper (such as Benchkote).
4) It is essential to wear appropriate PPE for this protocol, including lab coat, gloves and safety glasses.
5) Aliquot 6 ml crystal violet solution per microplate to a suitable reservoir.
6) Remove all medium from the wells by inverting vigorously over the plastic tray, then patting dry with paper tissue.
7) Adjust a multichannel pipette to dispense 50 µl.

8) Firmly press the pipette into a row of sterile pipette tips (8 or 12, depending on the type of pipette), ensuring all are securely attached.
9) If the tips are loose or fall off during use, use gloved finger and thumb to pull up and seat firmly each tip individually, being very careful to touch only the upper part of the tip, and not the lower part of the tip which must remain sterile before use.
10) Push the plunger on the pipette down to the first stop (not all the way to the second stop).
11) Insert the tips into the liquid in the reservoir, ensuring all tips are below the surface of the liquid.
12) Slowly release your thumb to allow the plunger to return to the top position.
13) Look carefully across all the tips to ensure the level of liquid is the same in each tip, if not, dispense the suspension back into the reservoir and try again.
14) Likewise, if there are any large air bubbles in any of the tips, dispense back into the reservoir and try again.
15) Move the pipette to the plate containing cells and dispense all the liquid into the wells of the next available column, pipetting past the first stop all the way to the second stop of the plunger this time.
16) Repeat this process until every well in the plate has received crystal violet reagent.
17) Replace the lid on the plate and move the plate gently backwards and forwards to ensure the reagent covers completely the bottom of every well.
18) Incubate the plate at room temperature for **15 minutes**.
19) Gently wash all crystal violet solution from every well using distilled water from a squeezable wash bottle (tap water is also acceptable for this step).
20) Invert the plate over a large plastic waste tray until all liquid has been expelled.
21) Pat the plate dry after each wash by inverting the plate onto tissue.
22) Repeat the process of filling the wells gently with water, inverting the plate to remove the water and then drying with paper tissue, three more times.
23) The plate is correctly washed when little or no more blue reagent appears on the tissue.
24) Use a multichannel pipette to add 100 µl of 20% acetic acid solution to every well.
25) Pat the plate gently at the side to solubilise the cells until the colour in each well reaches visible homogeneity (about 30 seconds).
26) Use a microplate reader to measure absorbance of every well at 570 nm (filters between 550 and 600 nm will also work well for this purpose).
27) Retrieve the results from the microplate reader software for later analysis.
28) Empty the liquid contents of the plate into the waste tray, and discard the waste plastic to a suitable dry waste stream.
29) Discard the contents of the waste tray into a waste bottle that has been designated for the removal of chlorinated chemical waste (often found in a fume cabinet).
30) Wipe the waste tray dry with tissue for re-use.
31) Clean up any spills by first wiping up any excess with tissue, then spraying the stain with 20% acetic acid, wiping with tissue, then spraying with 100% H_2O, then wiping again, repeat until all stain is removed.

Notes

- The crystal violet assay is very sensitive to the washing step - if washing too vigorously, the adherent cells can be washed away, or if washed too little, excess dye outwith cells confounds the reading. Be very careful with how vigorously you wash the cells when adding water to remove excess crystal violet dye from the plate after staining. Do not point the nozzle of your water bottle directly at the cell monolayers - direct it instead gently against the side walls of the wells. Make sure every well in the plate is filled completely with water during each wash step, slowly and carefully. After inverting the plate to expel the water, remove the excess by patting gently on a paper towel. Keep repeating this process until the blue dye no longer appears on the paper towel. Only then should you move on to adding the solubiliser to read the plate. By way of comparison, the MTT assay generally has higher reproducibility because it does not include a washing step.
- It is essential to wear appropriate PPE for this protocol, including lab coat, gloves and safety glasses.
- Be very careful not to get this stain on the bench, your skin or clothes - the stain can be messy and is very difficult to remove.
- If you do spill crystal violet stain on a surface, wipe up the excess with tissue, then spray with 20% acetic acid, wipe up with tissue, then with 100% H_2O, wipe again with tissue, repeat until all stain is removed.
- The methanol in the crystal violet reagent is the fixative that hardens the adherent cells.
- Dead cells are washed away during the washing steps as they are non-adherent.
- The acetic acid in the resuspension reagent solubilises the crystal violet dye, allowing a uniform colour to be measured by a microplate spectrophotometer.
- Be careful to not wash the cells too vigorously - point the nozzle of the wash bottle at an angle away from the bottom of the wells by holding the plate at a 45^o angle from vertical, and squeeze gently.
- Excessive pressure during the washing step will result in loss of adherent cells and poor reproducibility.