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Chapter 10

Evans blue dye exclusion assay: Mechanisms, protocols, and applications in cell viability analysis

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1. Introduction:

Evans blue is a dye that has the ability to penetrate the damaged cell membranes of deceased cells, resulting in a distinct blue staining. The Evans Blue assay is a widely used technique in biology and medicine to assess vascular permeability, particularly in studies involving inflammation, injury, or pathological conditions affecting blood vessel integrity. Named after the scientist Herbert M. Evans who first described its application in 1936, the assay utilizes Evans Blue dye, a synthetic azo dye with high affinity for serum albumin. In the Evans Blue assay, the dye is intravenously injected into experimental animals, where it quickly binds to circulating albumin in the bloodstream (Gaff et al., 1971). Albumin serves as a carrier protein, facilitating the distribution of the dye throughout the vascular system. As the dye-albumin complex circulates, it remains confined within the intact blood vessels under normal physiological conditions (Masters, 2000)).

However, in situations where vascular permeability is compromised, such as during inflammation or tissue injury, the dye-albumin complex leaks out of the blood vessels and accumulates in the extravascular space. This extravasation of Evans Blue dye serves as a marker of increased vascular permeability, allowing researchers to quantify the extent of leakage as an indicator of vascular dysfunction. After a suitable period of time following dye injection, the animals are euthanized, and tissues of interest are harvested. Evans Blue dye is then extracted from the tissues by various methods, such as perfusion or homogenization, and its concentration is determined spectrophotometrically. The amount of dye present in the tissue correlates with the degree of vascular permeability, providing a quantitative measure of vascular leakage. The Evans Blue assay has broad applications in preclinical research, including studies of inflammatory diseases, vascular disorders, ischemia-reperfusion injury, and drug-induced toxicity. It provides valuable insights into the pathophysiology of vascular dysfunction and helps evaluate the efficacy of therapeutic interventions aimed at preserving vascular integrity. This assay is used to

distinguish deceased cells in algal cultures of *Chlorella* and *Chlamydomonas* (the same procedure can be applied to other green algae) (Baker and Mock 1994; Tripathi et al., 2018).

2. Preparations of Evans Blue stain:

1. Make 1% Evans blue solution by dissolving 0.1 gm of the dye in 10 milliliters of MilliQ water.
2. Filter the dye through 0.2 Micron filters using a syringe and filter assembly to remove any large undissolved particles. The solution remains stable at room temperature for several months when covered with aluminum foil.

3. Procedure:

1. Utilize 1.5 milliliter Eppendorf tubes for proper cell pelleting.
2. For algal cultures with lower density (<1 million cells/milliliter), centrifuge a small amount of culture to form a cell pellet.
3. The resultant cell pellet should be combined and suspended in 900 µl of nutrient medium.
4. Dispense 100 µl of the prepared 1% Evans blue dye suspension and allow the mixture to rest at room temperature for 20 minutes, preferably in darkness.
5. Wash the cells thoroughly with MilliQ water to remove any remaining dye, beginning by centrifuging the stained cells.
6. Repeat steps 2-3 times, extracting 900-950 µl of supernatant, adding MilliQ water, and centrifuging. Keep the final cell pellet in a small volume (10-20 µl).
7. Collect a small portion (approximately 5 µl) of the cell pellet and carefully place it onto a clean glass slide. Apply a coverslip gently to disperse the cells and observe them under a microscope.
8. Preferably, use an immersion lens with 100X magnification.
9. Calculate the number of blue cells and determine the percentage of dead cells relative to the total cell count.

4. Precautions:

1. Ensure all solutions and materials used are sterile to prevent contamination of cell samples.
2. Use accurate and consistent volumes of Evans Blue dye to avoid variability in assay results.
3. Maintain precise incubation times and conditions to ensure reliable dye uptake by non-viable cells.
4. Handle cells gently during the assay process to minimize cell damage or loss.
5. Perform the assay in a controlled environment to avoid temperature fluctuations that could affect cell viability.

6. Prepare controls (e.g., known viable and non-viable cell samples) to validate the assay results.

5. References:

1. Freshney, R. I. (2015). *Culture of animal cells: A manual of basic technique and specialized applications* (7th ed.). Wiley-Blackwell.
2. Gaff, D. F., & Okong'o-Ogola, O. (1971). The use of non-permeating pigments for testing the survival of cells. *Journal of Experimental Botany, 22*(1), 756–758.
3. Baker, G. L., & Mock, N. M. (1994). An improved method for monitoring cell death in plant tissues. *Plant Physiology, 105*(2), 520–523.
4. Tripathi, K., Kumar, N., & Abraham, G. (Eds.). (2018). *The role of photosynthetic microbes in agriculture and industry*. Nova Science Publishers, USA.