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TITLE: LIVE/DEAD VIABILITY FOR CLINICAL SAMPLES

PRINCIPLES OF THE PROCEDURE:

This protocol is utilized to differentially stain live and dead cells. The assay is based on a reaction of a fluorescent dye with cellular amines. The reactive dye can permeate the membranes of necrotic cells and react with free amines both in the interior and on the cell surface, resulting in intense fluorescent staining. In contrast, only the cell surface amines of viable cells are available to react with the dye, resulting in relatively dim staining. This method allows for a better determination of large populations of dead cells which may exist within the samples.

SPECIMEN REQUIREMENTS:

1. Bone marrow or whole blood collected in sodium heparin or EDTA tubes and kept at room temperature. Specimens must be processed within 48 hours of collection time; EDTA specimens within 24 hours.
2. Cerebral spinal fluid, pleural fluid and bronchial washes collected in sterile containers kept at room temperature. Specimens must be processed within 6 hours of collection.
3. Tissue and lymph node specimens should be sterilely minced using a scalpel and cut into sections from 0.01 to 0.25 cubic cm in size. They should be placed into a screw top, leak proof container with RPMI 1640 or alternatively HANKS balanced salt solution (HBSS). Both should be supplemented with gentamicin sulfate, 50µg/mlm to retard bacterial growth. Lymph nodes should be processed within 12 hours of collection. Tissue samples should be processed within 6 hours of collection and should be kept on ice or at 4˚C. Samples with a viability of less than 85% will not be reported.
4. All requisitions must indicate:
    a. Patient name and ID number
    b. Date and time of collection
    c. Sample type

REAGENTS:

1. Live/Dead® Fixable Green Dead Cell Stain Kit for Flow Cytometry (Invitrogen Molecular Probes cat# L-23101)
2. Phosphate Buffered Saline Solution (PBS) Non-sterile filtered (Gibco Cat# 21300-058).

EQUIPMENT & INSTRUMENTATION:

12 by 75 test tubes
Pipettors and tips
Centrifuge adaptors
Absorbent towels for blotting
Vortex
Centrifuge
Test tube racks
Timer

PROCEDURE:

The live/dead dye is prepared for a week's worth of test (i.e. 100 tests/week). The working stock is prepared at a 1:50 dilution in PBS. The test samples will receive 5µl of the working stock. This working stock is stored in the refrigerator at 4-8˚C in the dark for no longer than 7 days.

Reconstituted Stock Preparation:

1. Bring one vial of the supplied fluorescent reactive dye (Component A) and one vial of anhydrous DMSO (Component B) to room temperature before removing caps.

2. Add 50µl of DMSO to the vial of reactive dye. Mix well and visually confirm that all the dye has gone into solution.

3. Immediately aliquot the reconstituted stock solution into 10µL aliquots and store at -20˚C and protect from light.

4. When stored correctly the reconstituted stock aliquots should be stable for at least 6 months.

Working Stock Preparation

1. Remove a reconstituted stock aliquot from the clinical lab freezer.
2. Add 490µl of PBS to the stock aliquot to make a 1:50 dilution. This is enough for 100 tests.
3. Initial and date the vial. The vial will be stored in the clinical lab refrigerator in the 4 color panel box for up to 1 week.

Usage Procedure

1. Add 5µl of the 1:50 dilution working solution to the appropriately labeled tube(s).
2. Add 100µl of appropriately washed specimen sample to the tube(s) and vortex.
3. Incubate the tube(s) on ice in the dark for 20 minutes.
4. Vortex the sample(s) after the incubation period is complete and place into centrifuge adaptors.
5. Add 3.5 ml of the lysing reagent to the tube(s) and place parafilm securely over the top of each adaptor. Invert twice. Let stand for 5 minutes at room temperature.
6. Centrifuge @ 1400rpm (423 x g) for 5 minutes at 7˚C. Carefully remove parafilm. Decant and blot each tube. Vortex tube(s) and return to adaptors.
7. Add 3.5 ml of PBS to each tube and centrifuge as above. Decant and blot as above. Vortex tube(s) and place back into original test tube rack.
8. Add between 0.3ml – 0.5ml of 2% formaldehyde to the fix the sample. Cover with parafilm or cap the tube(s) and place in the refrigerator until acquisition on the flow cytometer.

PROCEDURAL NOTES:

INTERFERENCES: Protect stained cells from light to prevent quenching.

QUALITY CONTROL GUIDELINES:

Blood from a healthy donor should be stained weekly with the live/dead green dye to confirm that it is working properly. As a new lot of reagent is prepared, it should be tested with that day's healthy donor or similar sample in parallel with the current lot to confirm that both give comparable results.

EXPECTED VALUES: N/A

REPORTING RESULTS & CALCULATIONS:

Each sample processed in the clinical laboratory will have this test run. The results will be included in the individual report.

REFERENCES:

Invitrogen Molecular Probes Live/Dead Kit Insert

VERSION HISTORY:

Version	Effective Date	Section	Description of Revisions/Justifications
2007.01.07	2007.01.12	SRP	•
2007.08.03	2007.08.03	SRP	Reviewed and updated
2009.06.18	2009.06.18	SRP	New format
2011.06.03		SRP	Reviewed and updated
FL-SRP-2090.00	7/1/11	SRP	Reviewed

Name / Title	Signature	Date
Author: Title:		
Reviewer: Title:		
Approved: Paul Wallace, Ph.D. Title: Director		