TRIPLE-TEST

PRINCIPLES/DISCUSSION:
Some bacteria produce enzymes which hydrolyze various chromogenic substrates. When these substrates are bound to another base, the hydrolysis will produce a color reaction, some apparent immediately and others by the use of special equipment or the addition of color developers. The triple test makes use of these principles by combining non-interfering substrates, producing three chromogenic reactions in one product. This reduces the cost per test and, in many cases, allows organism confirmation from a single tube.

1. The primary tests are nitro-phenol bound substrates. Organisms containing the necessary enzymes hydrolyze these substrates, releasing the yellow nitro-phenol which is visible without addition of any reagent. The nitro-phenol test will be the one listed first in the product name.
2. Enzymes acting on the secondary tests (methylumbelliferyl based) release fluorescent methylumbelliferone (4-MU) which can be observed under a Wood's lamp, also without addition of any reagent. These will be the second tests listed in the name of a product.
3. The last tests are aminopeptidase tests. These are located on a disc in the tube. Organisms containing the enzymes necessary for hydrolysis of the applicable arylamide release free beta-naphthylamide which is detected by the addition of aminopeptidase reagent. These will be listed last on the labels.

MATERIAL SAFETY DATA:
Each tablet consists of approximately 0.5 mgs. each of the applicable substrates with inert fillers and tableting compounds. The discs contain the applicable substrate dissolved in an inorganic solvent. None of the ingredients is hazardous in this form. Tests contain the following chemicals in different combinations:

Nitrophenol substrates: (1st test - yellow)
- ONPG (o-nitro-phenol-ß-D galactopyranoside)
- NAG (p-nitro-phenol-N-acetyl-ß-D-glucosaminide)
- AFU (p-nitrophenol-alpha-D-fucopyranoside)
- AGLU (0-nitrophenl-alpha-D-glucopyranoside)
- PO4 -(alkaline phosphatase)

Methylumbelliferyl substrates: (2nd test - blue fluorescence)
- MUG (4-methylumbelliferyl-ß-D-glucuronide)
- MBGL (4-methylumbelliferyl-ß-D-glucoside)
- MBGA (4-methylumbelliferyl-ß-d-galactoside)
- MAGA (4-methylumbelliferyl-alpha-d galactoside)
- MAGL (4-methylumbelliferyl-alpha-D-glucoside)

Naphthylamide substrates:(3rd test(disc)) - red or purple aminopeptidase)
- ARG (arginine ß-naphthylamide)
- PRO (proline-ß-naphthylamide)
- PYR (pyrrolidonyl-ß-naphthylamide)
- TRY (Na-Benzoyl-DL-arginine-ß-naphtylamide-" trypsin")
- CHY (N-Glutaryl-Gly-Gly-Phe-ß-naphthylamide-"chymotrypsin")
- LGY (Leucyl glycine ß naphthylamide)

COMBINATIONS:
K1048 PO4/MAGL/ARG
K1237 AFU/MAGA/CHY
K1238 AFU/MAGA/ARG
K1263 AGLU/MAGA/LGY
K1464 NAG/MBGL/PRO
K1466 NAG/MBGA/TRY
K1496 ONPG/MUG/PYR

STORAGE:
Store according to label instructions.

MATERIALS REQUIRED:
Each type of test is sold separately, ready to use in test tubes, 28 tubes per bottle. Usage requires 24 hour growth on media appropriate for the specimen. Consult a current reference manual for the correct media to use. The following items are also required but not provided:
- Microbiology loop, needle, or sterile swab for harvesting
- Purified water, pH 6.5-7.5
- Long-wave fluorescent light
- K982375 or K2375 PEP reagent

PROCEDURE:
1) Add at least 0.25 ml but not more than 0.5 ml (about 5 drops) of water to the tube containing the tablet.
2) Inoculate heavily from fresh 24 hour growth on a plate or slant. The heavier the inoculum the brighter and faster the reaction: use a visible loopful of paste. Mix well by using the loop to move the tablet around in the tube. It is not necessary for the tablet to dissolve.
3) Incubate for at least 2 hours @35-37C. If test cannot be read within 4 hours, incubate in the dark at room temperature for 8 or more hours. Do not read after more than 24 hours as false positives may occur.
4) As an alternative, you may remove the disc from the tube and do as a spot test. Moisten the disc on a slide and smear with organism. Wait 2 minutes and add the reagent.

INTERPRETATION:
(Read and interpret in the exact sequence listed)
1) **Nitro-phenol tests:** Observe for a yellow color indicating a positive test. Colorless is negative.
2) **4-MU**: Observe the tube for fluorescence by holding **under** a long-wave (360nm) ultra-violet light. Holding the light at any other angle could cause interference with true positive reactions. A positive 4-MU shows a **bright** bluish green fluorescence. Since some organisms tend to have a slight fluorescence of their own, if pale fluorescence or fluorescence of a different color is noted, it is best to compare with a known positive as a control. **Disregard** fluorescence when the aminopeptidase reaction is positive; this pale fluorescence is related to the aminopeptidase reaction only.
3) **AMINOPEPTIDASE:** Perform the aminopeptidase test last by adding 2 drops of PEP reagent directly to the tube and reincubating for 15 minutes. Ambiguous results of the aminopeptidase may be intensified by vortexing a few seconds after addition of the reagent. Red, blue, or purple is positive. Green, turquoise, or yellow is negative. The color may be seen most readily on the disc. It may or may not be evident in the water.

REFERENCES:

(1) Manual of Clinical Microbiology, Fifth Edition
(2) Kilian, M and Bulow, P. 1976. Rapid Diagnosis of Enterobacteriacae, Acta path. microbio. Scan, Sect B, 84:245-251
(3) Wadsworth Anaerobic Bacteriology Manual, 5th Edition, 1993,Glucosidase tests, page 152.

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