President's DNA Initiative
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Advancing Justice Through DNA Technology

DNA Analyst Training

Confirmatory Tests

Many different tests have been used to confirm that a stain contains blood. The oldest is chemical confirmation of the presence of hemoglobin or its derivatives by the formation of specific crystals. For example, the Takayama or hemochromogen test, in which ferrous iron from hemoglobin reacts with pyridine to produce red feathery crystals of pyridine ferroprotoporphyrin. Another confirmatory test uses the Teichman reagent, consisting of a solution of potassium bromide, potassium chloride and potassium iodide in glacial acetic acid, and is heated to react with hemoglobin. The reaction first converts the hemoglobin to hemin, and then the halides react with the hemin to form characteristic brownish-yellow rhomboid crystals.

Blood can be identified as being of human origin by precipitin reactions with antisera specific for components of human blood. Usually this is an anti-human serum serum - that is, an antiserum to human serum. Strictly speaking, this is a test for human origin not for human blood, as serum constituents such as albumin and some globulins are found in the extra-vascular space.

The original precipitin reaction was carried out by layering a solution of antibody on top of a solution of stain extract in a tube, and left for a period of time to allow the development of a precipitin band at the interface. This is referred to as the tube method, and is still used in a few laboratories today.

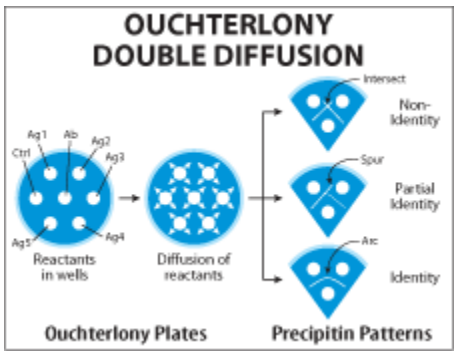
Ouchterlony

However, most species identification uses radial diffusion of antigen and antibody through agar gel. This is the Ouchterlony test. A variant of the Ouchterlony test, called cross-over electrophoresis, uses an electric field rather than diffusion to move the extract and antibody through the gel. Ouchterlony plates can be purchased or made in the laboratory.

Extracts are made from stained areas of interest, and from nearby unstained areas (substrate controls). *Note that the use of unstained controls is a fundamental principle in forensic immunologic testing.*

Stain and controls samples are loaded in the outer wells and a drop of anti-

human antiserum is loaded into the center well. The process is repeated for antisera to other species, such as dog, cat, and cow; this may include the species from which the antiserum was obtained (e.g., rabbit).



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The plates are left at 4°C for a suitable period (which can range from a few hours to overnight) and the serum proteins and antibody molecules diffuse outward from the wells. A precipitin band is formed when the diffusing stain contains proteins that are recognized by IgG molecules in the diffusing antiserum. The precipitin band is sometimes clearly visible to the naked eye, but it is normal to stain the plates with amido black or other general protein stain, to enhance sensitivity and clarity.

Cross-over Electrophoresis

Cross-over electrophoresis for species identification is conducted using agar at a pH of 8.6. Stain extracts are loaded into wells arranged in a line at the cathode end of the plate and the antiserum is loaded into wells at the anode end. During electrophoresis, the electric field drives the serum proteins towards the anode, but the IgG molecules, which are essentially neutral at this pH, are driven to the cathode by the process of electroendosmosis. The antigen-antibody precipitation occurs at the interface between the two rows of wells. Electroendosmosis occurs because the supporting medium acquires a net negative charge. If free, the negatively charged molecules would migrate to the anode, but this is not possible because the agar is immobilized on the plate. Instead, the effect is countered by positively charged water molecules migrating to the cathode. The migrating water molecules carry any dissolved neutral molecules (such as IgG) with them.

ABAcard®

The method of choice today is the ABAcard® HemaTrace test strips manufactured by Abacus Diagnostics, Inc. Stain extract is applied to the bottom of the test strip, where any human hemoglobin present in the extract will combine with a monoclonal antihuman hemoglobin antibody. The antibody is labeled with a dye. Any antibody-antigen formed then migrates through an absorbent membrane to the test area of the strip. The test area has an immobilized polyclonal antihuman hemoglobin that will capture the Ag-Ab complex to form an Ab-Ag-Ab sandwich. The pink dye becomes visible as a band in the test region at

concentrations of human hemoglobin above about 0.05 µg/ml. An internal control consisting of human hemoglobin antibody–dye conjugate cannot bind to the antibody in the test area but is captured by an antibody in the control area. A correctly functioning positive test will therefore show two pink bands, one in the test area and one in the control area. A correctly functioning negative test will show only one pink band, in the control area. If there is any problem with the test there will be no visible bands.

The ABAcard® test has been extensively validated and shown to be sensitive, specific and rapid.

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