



REPLY TO  
ATTENTION OF

**DEPARTMENT OF THE ARMY**  
**PROGRAM EXECUTIVE OFFICE**  
**FOR CHEMICAL AND BIOLOGICAL DEFENSE**  
**5203 LEESBURG PIKE**  
**SKYLINE #2, SUITE 1609**  
**FALLS CHURCH, VA 22041-3203**



July 23, 2002

This white paper is in response to the July 19, 2002 memorandum from the Executive Office of the President Office of Science and Technology Policy (OSTP) for Federal Mail Managers and First Responders to Federal Mail Centers. The OSTP memorandum discusses the use of commercially available detection and identification technologies for use in determining if unknown substances resembling *Bacillus anthracis* powder do in fact contain this agent. It advises that actions taken for the procurement of such commercial technologies should cease. The memorandum states that the only valid method for the detection and identification of *Bacillus anthracis* is to transport suspect samples to an accredited Centers for Disease Control (CDC) Laboratory Response Network (LRN) Laboratory for microbiological culture. The policy memorandum addresses only *Bacillus anthracis* detection. It does not address any other threat agents. The memorandum also appears to focus on single technology testing with no context to the deployment of that technology. However, the memorandum also states that the Department of Defense (DOD) will continue to procure capabilities in addressing DOD requirements.

While this memorandum is clearly directed towards the detection of *Bacillus anthracis* in mailrooms and not directing the DOD to alter its current detection and identification strategies, it does raise questions about the capability of several technologies that the DOD currently employs. Specifically, the memorandum is directed towards Polymerase Chain Reaction (PCR) and Hand Held Assay (HHA) technologies. It is the intent of this document to put some context to the employment of these technologies and to succinctly describe how these multiple pieces fit into the overall detection strategy.

The DOD has recognized since the inception of its Biological Warfare (BW) Detection program that the lowest risk methodology for the detection and identification of BW warfare agents involves a layered, multi-technology strategy. Each of those single technologies has its strengths and limitations. The choice of how and when those technologies are employed involves many factors that must be taken into account through command risk analyses. These multiple technologies are fielded to the user community along with adequate training and information about their capabilities. It is then command responsibility to define how and when these capabilities are employed, based on the operational situation and what actions can be taken based on their results.

HHAs and PCR play vital roles in the DOD biodetection strategy. HHAs are effective when employed as part of a systematic, layered detection approach supported by additional levels of confirmation and with a variety of technologies. PCR is an integral piece of that laboratory confirmation. The OSTP recommends an integrated response with threat assessment protocols, which the DOD has adopted as military doctrine and put into effect.

HHAs can be properly employed to provide information in an expedient fashion when they are used as intended and are supported by additional technologies. HHAs were designed to provide data quickly, to enhance early command assessment and response to a given scenario. To improve upon

HHA generated data, the DOD has invested aggressively in fielding other technologies such as freeze-dried ECL immunoassays and DNA-based PCR assays, the results of which can be combined with other data to produce supporting evidence of the presence and identification of a biological agent and increase the confidence in the data generated. These investments in new technologies are being fielded and integrated into the overall biodetection strategy and will further enhance our ability to protect the warfighter and contribute to the Homeland Defense effort.

HHAs provided the first indication of the presence of *Bacillus anthracis* in the letter sent to Senator Daschle. Command decisions were made based on the high probability that the substance in question was *Bacillus anthracis*. These decisions did not include immediate medical treatment but did include movement of personnel from potentially contaminated areas, the shutting down of the building ventilation system to prevent more wide-spread contamination and the notification of medical personnel that there may be an incident. Furthermore, HHA test results prompted expedient sample transport to USAMRIID for laboratory based testing that included PCR, ECL, and traditional microbiological culture analysis.

The DOD investment in fielding new technologies such as advanced immunological assays and PCR is focusing on the highest standards of quality testing, from reagent manufacture, to Quality Control and Assurance testing, to proper operator training and certification. The DoD acquisition, scientific and testing communities, to include the the Program Executive Office for Chemical and Biological Defense (PEOCBD), US Army Medical Research Institute of Infectious Disease (USAMRIID), the Armed Forces Institute of Pathology (AFIP), the Naval Medical Research Center (NMRC), the AF Institute of Environment, Safety and Occupational Health Risk Analysis (AFIERA) the US Army Chemical School (USACMLS) and the Soldier Biological Chemical Command (SBCCOM) are working towards a common technological and procedural approach to BW agent testing and surveillance. Improved ties with additional CDC certified laboratories and proper fielding and implementation of advanced testing technologies will further enhance first response with HHAs.

The DOD has instituted an operationally tested and suitable biological warfare agent detection strategy consistent with countering BW threats. When used properly, the use of immunological and PCR assays are valid and necessary tools to provide first tier, presumptive agent identification information as part of this overall detection strategy. For more information please contact either Dr. David Cullin at [dave.culling@peocbd.army.mil](mailto:dave.culling@peocbd.army.mil) or Dr. Peter Emanuel at [peter.emanuel@us.army.mil](mailto:peter.emanuel@us.army.mil) .

Prepared By: Dr. David Cullin, (703) 681-9607  
Approved By: COL(P) Steven V. Reeves, (703) 681-9600

# INFORMATION PAPER: Hand Held Assay (HHA)

## INTRODUCTION

The HHA is a simple, antibody - based assay (test) used to presumptively identify Biological Warfare (BW) agents (Figure 1).

- HHAs are the primary identification component of several fielded (BIDS, IBAD, Portal Shield, Dry Filter Units) and developmental DOD (JBPDS) BW detection systems.
- HHAs are inexpensive, reliable, and easy to use.
- An HHA is a one-time use capability designed to presumptively identify one agent. The current capability allows for presumptive identification of 10 different BW threat and 4 stimulant agents. Positive and negative trainer HHAs are also available.
- The HHA is designed to be used only on non-porous surfaces (example - metal, plastic and glass)
- The best results can be achieved when samples are taken from an area where the concentrations are believed to be the highest.
- The results can be utilized to advise and assist in facilitating the resolution of a biological incident. It is only after an agent's identity can be ascertained that an effective outer perimeter can be established, neutralization plans formulated, decontamination procedures enacted, emergency medical treatment plans made, and environmental preservation precautions taken.
- HHAs are not designed to be the sole method of identification and are not for diagnostic use.

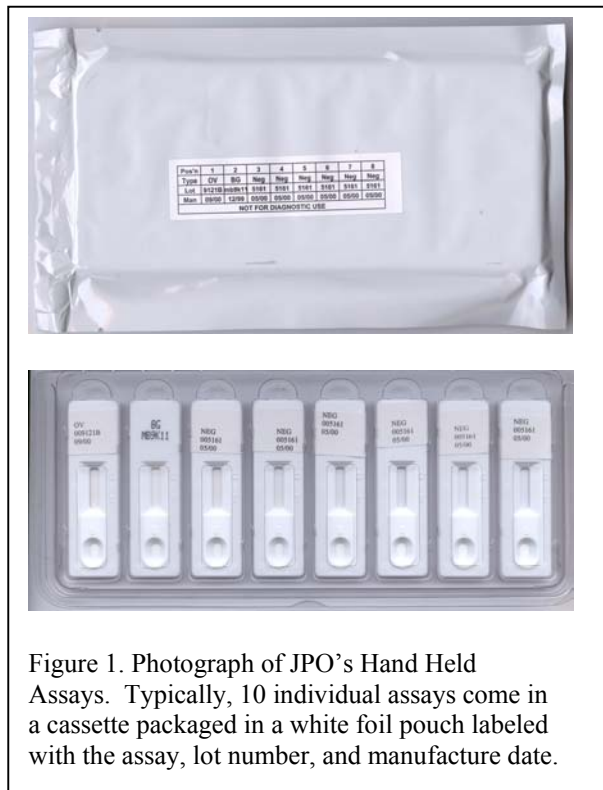


Figure 1. Photograph of JPO's Hand Held Assays. Typically, 10 individual assays come in a cassette packaged in a white foil pouch labeled with the assay, lot number, and manufacture date.

- The ongoing research and development efforts are producing additional BW agent assays.

## THE SCIENCE BEHIND THE HAND HELD ASSAY

The HHA is a form of biological assay called immunochromatography and is designed to provide a quick and accurate presumptive identification of selected biological warfare agents. The HHA works on the principle of antigen/antibody interactions.

- Antigens are any foreign substance that when introduced into the host are capable of eliciting an immune response, which ultimately results in antibody production.
- Antibodies are molecules that are found in the blood and tissue fluids of mammals that are produced in response to a given antigen. Biologically, the role of the antibody is to bind the intruding foreign substance and facilitate its removal from the body.

Typically, an organism carries many different complex antigens on its surface. The differing antigens are called epitopes and it is not uncommon for many different antibodies to be produced in response to an infection (Figure 2). An epitope is unique to a given antigen and correlates with the genetic diversity of varying species of microorganisms.

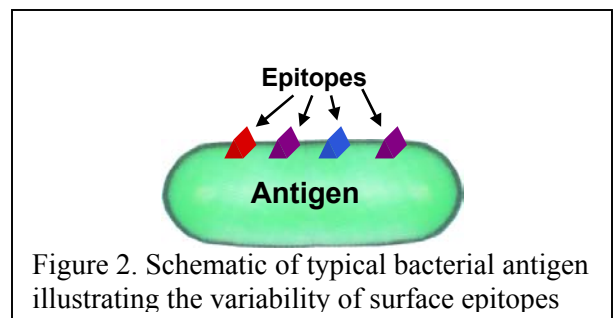


Figure 2. Schematic of typical bacterial antigen illustrating the variability of surface epitopes

HHA's exploit the exquisite sensitivity and specificity of antibodies to detect and differentiate microorganisms. These antibodies are able to physically grab on to a portion of an antigen with their antigen-binding site. Two categories of antibodies are typically used in immunoassays:

- polyclonal antibodies (PAB's) - Polyclonals represent a population of many antibodies which bind to numerous different antigens (epitopes) (Figure 3).
- monoclonal antibodies (MAB's) - Monoclonals represent a single type of antibody which bind to one specific antigen (epitope) (Figure 3).

Polyclonal antibodies are typically used for immunoassays because of their ease of production and their superior sensitivity. What makes polyclonal antibody assays more sensitive is that they can cover the surface of a complex antigen such as a microorganism more uniformly thus improving the detection capability (Figure 3). Monoclonals represent a single type of antibody which bind to one specific epitope.

A high degree of sensitivity and specificity against a particular biological agent can be achieved by careful screening and selection of a monoclonal antibody.

However, monoclonal antibodies can bind to only one type of epitope on the surface of the cell, possibly reducing the level of coating. The potential then exists

to give up a certain level of sensitivity. Polyclonal antibodies are far easier, faster, and cheaper to produce. However, in general, polyclonal antibodies do not have the specificity of a monoclonal. Efforts

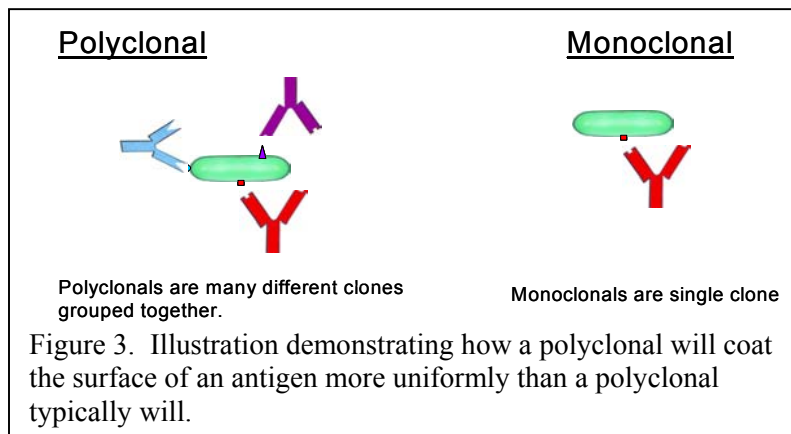


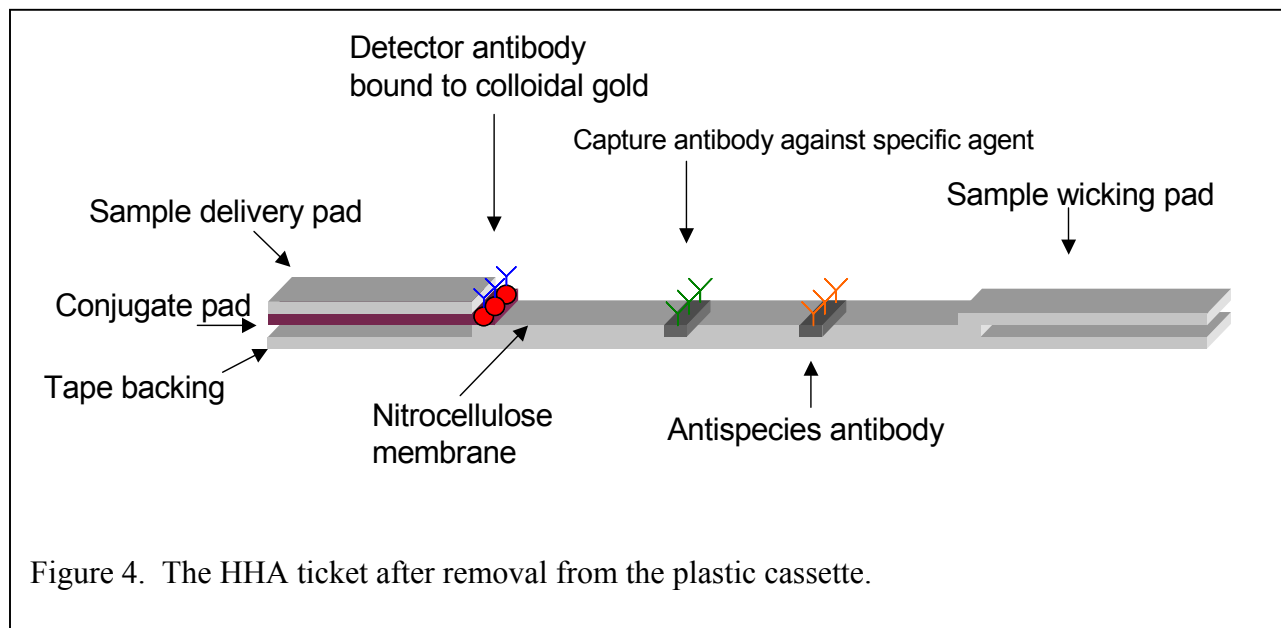
Figure 3. Illustration demonstrating how a polyclonal will coat the surface of an antigen more uniformly than a monoclonal typically will.

to combine monoclonals are being successfully employed to improve new HHAs through balancing sensitivity and specificity.

## HHA COMPONENTS

The components of the HHA are as follows (Figure 4):

- **Sample delivery pad:** When the sample is added to the sample delivery well, it contacts the sample delivery pad first. The sample delivery pad functions to filter out any large particulate matter in the sample and to hold the sample so that it can slowly wick through into the conjugate release pad.
- **Conjugate release pad:** The conjugate pad contains the detector antibody which is conjugated to colloidal gold. This allows for visualization of the antibody. If sample is added to the assay that contains compatible antigen, the colloidal gold labeled antibody will bind to target antigen and allow for detection of the antigen when it subsequently binds to the capture antibody.
- **Nitrocellulose Membrane:** The sample enters the nitrocellulose membrane via capillary action towards the sample wicking pad. Bound to the membrane are the capture antibody and the anti-species antibody which are sprayed in discrete lines on the membrane about halfway up the ticket.
- **Capture antibody:** The capture antibody is what makes up the test line on the ticket. The test line is adjacent to the letter "T" on the plastic cassette. The capture antibody is bound to the membrane and when antigen flows past it serves to capture the antigen.
- **Antispecies antibody:** The anti-species antibody will bind the colloidal gold labeled antibody regardless whether antigen is present or not. This serves as the control to indicate whether the assay is functioning properly and is adjacent to the letter "C" on the plastic cassette. It is called an anti-species antibody because it is made in one species of animal that has been immunized with the antibody from another species. For example, if the detector antibody was made in goat then the anti-species antibody would be a rabbit immunized with goat antibodies to produce a rabbit anti-goat antibodies.
- **Sample wicking pad:** The sample wicking pad serves as a reservoir to hold the sample after it has wicked across the nitrocellulose membrane. The sample wicking pad will only hold the sample for a short period of time before the sample will begin to flow back across the membrane towards the sample delivery pad during which time nonspecific binding can occur producing false positives. That is the capture antibody and detector will adhere to each other whether antigen is present or not. For this reason it is important to read the HHA at the 15 minute time point.
- **Tape backing:** The tape backing serves simply to hold the above components in place.
- **HHa buffer:** A component of the HHA which is not part of the HHA device, but a critical part of the kit is the HHA sample dilution buffer. The solution added to the HHA must be aqueous for the assay to function. The HHA buffer contains PBS, Triton X-100, and sodium azide. The PBS serves to adjust the sample to a neutral pH so that the antibodies are able to function properly. Any significant deviation from a pH of 7 will change the conformation of the antibodies and they will no longer have the ability to bind antigen. The Triton X-100 is a surfactant that helps to prevent aggregates from forming which do not flow well across the nitrocellulose membrane. Sodium azide acts as a preservative to prevent growth of any microbial contaminants during storage of the buffer.



## HHA RESULTS AND LIMITATIONS

When reading an assay, any visible test line, even a very faint one, should be considered real. There are four potential outcomes that may be observed after running an HHA.

- The first potential outcome is two red lines indicating a positive assay. This may also be a result of matrix effects (see below) so running the sample a second time following diluting 1:10 and 1:100 in HHA buffer would be prudent (Figure 5.a).
- The second outcome is a single line, the control line. This may be a valid negative or may be the result of the “hook effect” (see below). Again, running the sample a second time following diluting 1:10 and 1:100 in HHA buffer is advised (Figure 5.b).
- The third outcome is a positive test line but no control line. This is probably due to a faulty assay which requires running the sample again with a new set of HHA’s (Figure 5.c).
- The fourth outcome is where no lines show up. This can be the result of a faulty assay, a matrix effect, or the assay may have been exposed to moisture. The nitrocellulose membrane must be dry in order to wick the sample. If an assay has an incomplete control or positive line after running, the assay is also faulty. To resolve this a new HHA is used utilizing sample dilutions of 1:10 and 1:100 in HHA buffer (Figure 5.d).

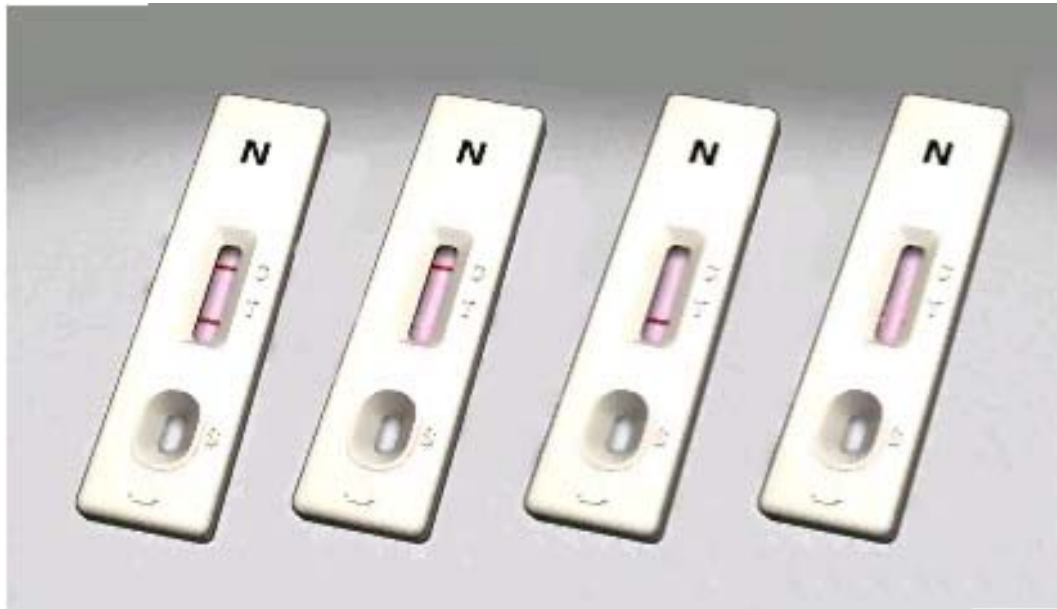
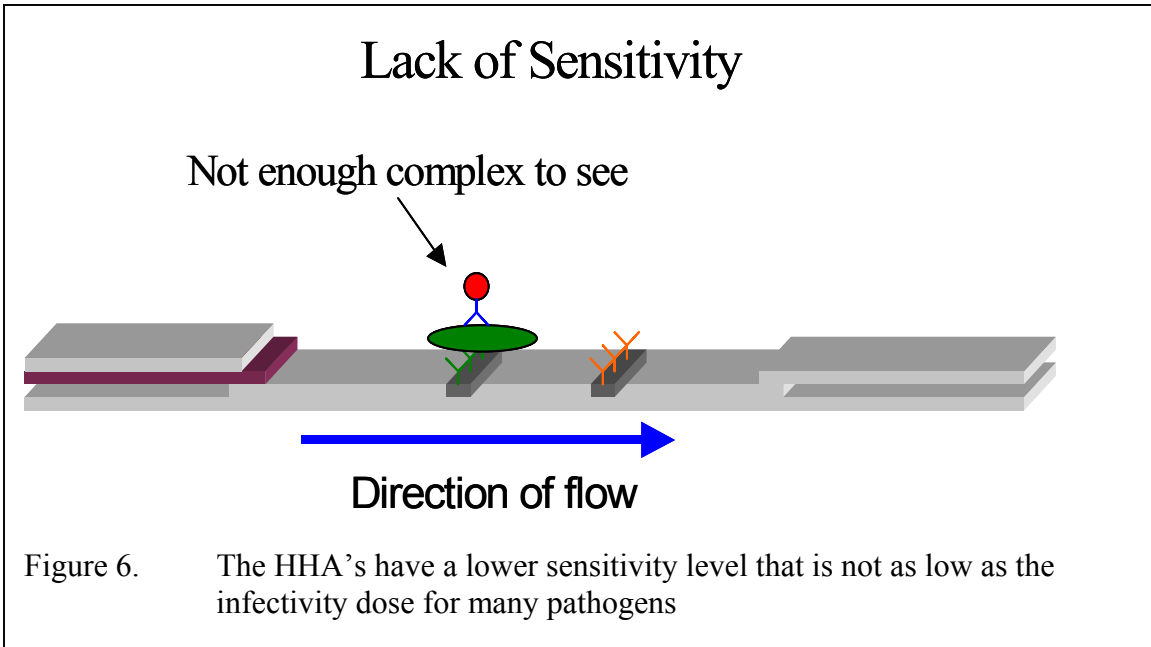


Figure 5. Potential results following use of HHA's. From left to right: A positive Assay (a); A negative assay (b); A faulty assay (c); A faulty assay, or potential matrix effects (d).

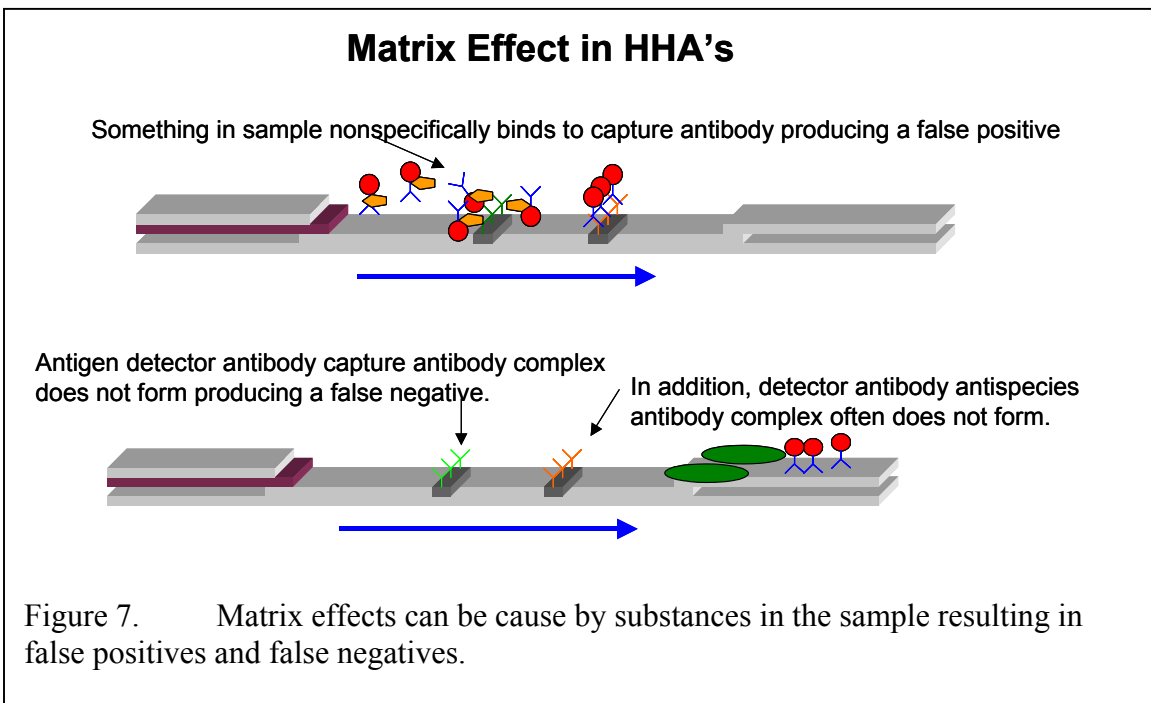
All results whether positive, negative or inconclusive should be documented. It is important to keep in mind that no matter what the outcome of an HHA, these tests provide only a presumptive identification and that the samples will need further evaluation at a confirmatory lab.

Although HHA's are fairly reliable, accurate, and sensitive assaying environmental samples is exceedingly difficult and some of the technological limits may surface. An awareness of possible deficiencies/limitations will help the operator recognize and hopefully avoid any potential problems. There are four major issues with immunochromatographic assays that could affect the accuracy of an analysis. An understanding of these limits will help to decrease their occurrence and mitigate possible detrimental effects on the accuracy of a sample analysis.



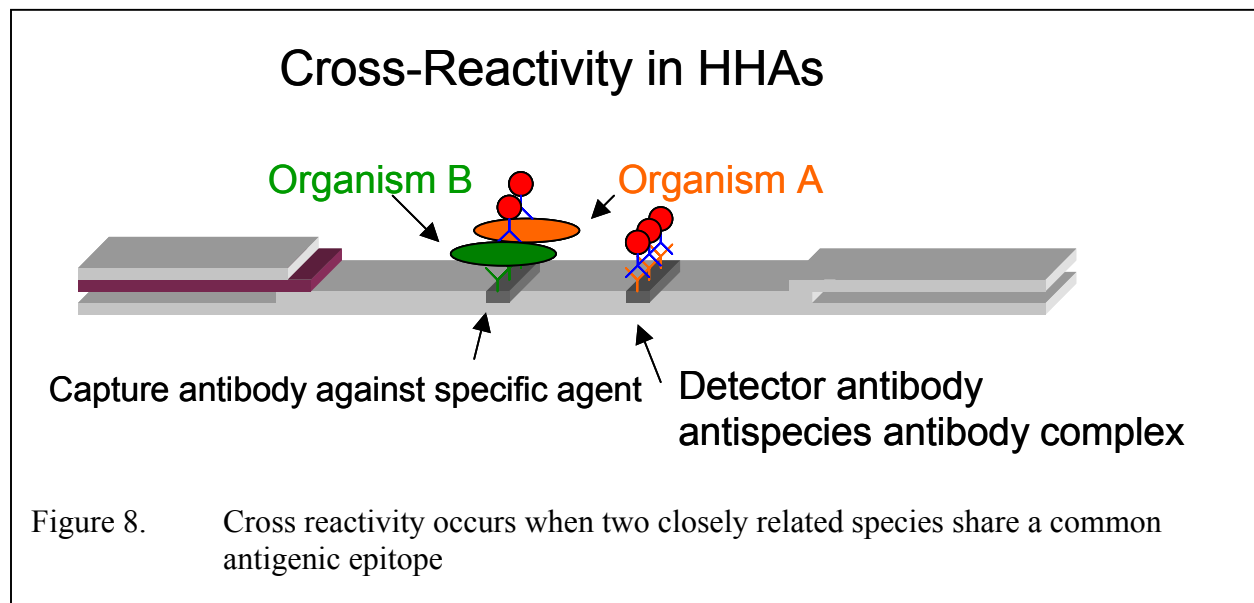


- Sensitivity Cutoff (Figure 6). HHA's, like all biological assays, have a sensitivity cutoff. This means that for each different agent assay there is a threshold concentration that below this concentration the assay it will not be able to detect the presence of the antigen. Although HHA's are very sensitive, the infective dose for most pathogens is far lower than the sensitivity of the HHA's. Therefore, if a sample is tested and the result appears to be negative (*false negative*), there may still be enough biological agent in the sample to cause illness. You may give false information if you state that the sample does not have a particular agent in it because it may very well have.

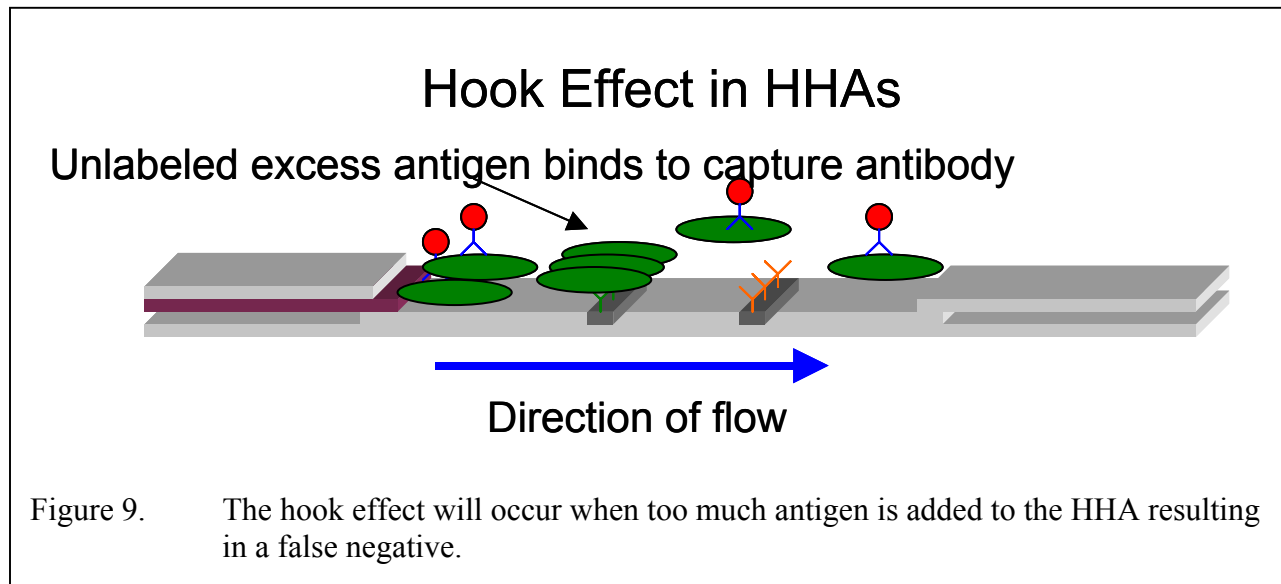




- Matrix effect (Figure 7). The matrix effect is often encountered when assaying environmental samples in HHA's. It can not be predetermined what type of sample will have to be analyzed prior to an incident. Sometimes a sample will not be compatible with the HHA's. This can result in false negatives or false positives. A false negative will occur if there is biological agent in the sample, but something else in the sample or some property of that sample prevents the antibodies from binding to the antigen. Conversely a false positive can occur if there is no biological agent in the sample, but something else in the sample or some property of that sample causes the detector and capture antibodies to bind together non-specifically. The HHA's are screened using several common matrices (dust, tap water, sewage, human sera, and soil) to ensure that they will be less likely to pose a problem, but these matrices and others may still pose a problem. Typically, the substance causing the matrix effect can be diluted out while leaving enough of the specific antigen to react in the HHA to see a true positive. If a matrix effect is suspected, it is recommended that a 1:10 dilution of the sample in HHA buffer be run on a second HHA. This remedy also applies if you find the pH of your sample to be significantly above or below neutral (pH 7.0). It is important to note the control line when running samples. If the control line does not form, there may be a sample matrix problem.



- Cross-Reactivity (Figure 8). Cross-reactivity is most often seen with the use of polyclonal antibodies in HHA's but can occur even if monoclonal antibodies are employed. Cross-reactivity usually occurs when an antibody binds to the species it was designed for but it also binds specifically to close relatives of that species. This occurs when two closely related species share a common antigenic epitope allowing the antibodies in the HHA to bind to both species. It is seen most often with PAB's because they potentially can bind to many different epitopes on a given antigen (thus the likelihood of crossreactivity is increased). Cross-reactivity occurs with the *Bacillus anthracis* HHA in which the antibodies bind not only *Bacillus anthracis* but also other *Bacillus* such as *Bacillus thuringiensis*. Unfortunately these other *Bacillus* are normal constituents of soil therefore soil is incompatible with the Anthrax HHA. At this time, there is no monoclonal antibody in production for *Bacillus anthracis*.



- Hook Effect (Figure 9). The hook effect occurs when too much antigen is added to the HHA which then results in a false negative. What occurs is the amount of antigen exceeds the finite amount of colloidal gold antibody. The excess unbound antigen migrates across the nitrocellulose membrane more rapidly than the heavier labeled antigen where it saturates all the binding sites on the capture antibodies. When the labeled antigen arrives there are no binding sites remaining on the capture line so it continues on to the sample wicking pad. Fortunately this problem can be easily overcome as long as the operator is aware of it and take the appropriate steps.

The HHA is easy to use but, for it to be effective, it must be used correctly and under the right circumstances. The HHA is not designed to be used in all circumstances. HHAs should not be used under the following conditions:

- Sampling porous surfaces. Porous surfaces contain grooves that can trap an agent thereby lowering the concentration available for testing. In addition, the grooves may contain dirt or other interferences that could hinder the effectiveness of the assay.
- Sampling in areas where a lot of dust/dirt has collected. As previously mentioned, dirt and dust may contain inhibitors that effect the reliability of the HHA.
- Sampling where there is an excessive concentration of suspected agent. This may cause clogging (hook effect) and lead to an inconclusive result. Where excessive amounts of sample exist, diluting the sample in HHA buffer 1:10 or 1:100 should be sufficient to obtain a reliable result.
- Soil sampling. Soil may contain microorganisms that have similar antigenic properties to a bio warfare agent and cause a false positive. Soil also contains numerous inhibitors that could adversely effect the HHA result.
- When the HHA has been removed from its protective packaging prior to initiating a test. The nitrocellulose membrane can absorb humidity from the air and lead to an inconclusive test result.