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Preface

Tulip Group of companies believes in offering our valued customers the technical support and scientific information to keep updated with the latest international standards and trends in diagnostic testing.

Laboratory results play a pivotal role in providing the clinician the scientific data in diagnosing, monitoring and prophylaxis of deserving patients. Keeping in mind our valuable customers, Tulip Group will offer periodically a series of Tech Notes presented with a short, summarized overview pertaining to a specific technique/product/disease related information.

We hope that this series of Tech Notes on "**Perspectives on membrane-based Rapid DiagnosticTests & detection of hCG using these tests"** will aid the laboratory personnel to understand the limitations of immunochromatography technique and assay design for pregnancy testing thereby ensuring accurate and confident reporting of results.

Yours faithfully,
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Perspectives on membrane-based Rapid DiagnosticTests & detection of hCG using these tests

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Perspectives on membrane-based Rapid DiagnosticTests & detection of hCG using these tests

The need for a rapid, reliable, simple, sensitive in-vitro diagnostic assay for use at point-of-care, have lead to the commercialization of in vitro Rapid Diagnostic Tests based on the principle of Immunochromatography.

Rapid Diagnostic Tests are membrane-based immunoassays that allow visual detection of an analyte in liquid specimens. In clinical assays, specimens such as urine, whole blood, serum or plasma, saliva and other body fluids may be employed.

What are the principles of membrane-based Rapid Diagnostic Tests?

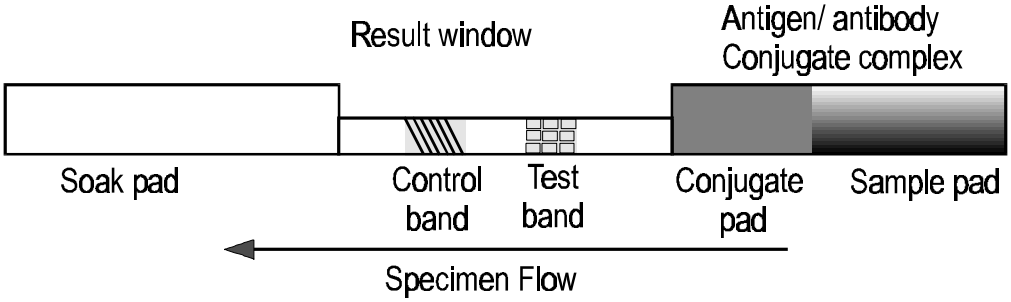
Currently available Rapid Diagnostic Tests comprise of a base membrane such as nitrocellulose. A detector reagent (antigen/antibody–indicator complex) specific to the analyte, impregnated at one end of the membrane. A capture reagent is coated on the membrane at the test region.

When the specimen is added to the sample pad, it rapidly flows through the conjugate pad. Analyte if present in the specimen, binds to the detector reagent. As the specimen passes over the test band to which the capture reagent is coated, the analyte-detector reagent complex is immobilized. A colored band proportional to the amount of analyte present in the sample, develops. The excess unbound detector reagent moves further up the membrane and is immobilized at the control band.

What are the components of membrane-based Rapid Diagnostic Tests and how are they constructed?

Rapid Diagnostic Tests consists of:

1. Sample pad
2. Detector reagent/conjugate: Antigen/antibody–indicator complex specific to the analyte, impregnated in the conjugate pad but remains unbound
3. Test band: Coated on nitrocellulose membrane; specific to the analyte
4. Control band: Usually anti-detector antibodies coated on the membrane, serves to validate the test results
5. Soak pad



Construction of Rapid Diagnostic Tests

Currently immunochromatography tests are available in two formats; “Lateral flow” and “Transverse flow or Flow through”. The lateral flow are available in device or dipstick format. The lateral flow formats are commonly employed where rapid detection of pregnancy, drug abuse, infectious disease or parasitology is required, and serve as qualitative screening assay at laboratories, physician’s office or at homes due to their simplicity and ease of performance. The flow through format is less common as the assay requires greater operator involvement. However, some of these assays enable semi-quantitative estimation of the analyte by visual comparison with an in-built reference.

Regardless of the format used, the desired specificity, sensitivity and assay performance depends upon reliable formulation and proper assay assembly.

What are the limitations and effects of various components on the performance of membrane-based Rapid Diagnostic Tests?

This section highlights the role of various components of Rapid Diagnostic Tests and their effect on attaining the desired performance characteristics.

How does the nitrocellulose membrane affect the sensitivity of Rapid Diagnostic Tests?

Rapid Diagnostic Tests are fabricated on a solid support membrane, usually made of nitrocellulose. Membranes employed in Rapid Diagnostic Tests are porous. Depending upon the porosity, some membranes are better suited for applications with certain specimens than others. This is because, the pore size of the membrane has significant effect on the capture reagent binding properties and the lateral flow rate. The combined effects of these two-phenomenon in turn determine the sensitivity and performance of the test assay.

Pore size and capture reagent binding properties

It has been observed that as the pore size decreases the effective surface area available for binding of capture reagent increases. Greater effective surface area available for binding, results in optimal coating of the capture reagent, which is essential for attaining the desired sensitivity of the assay.

Pore size and lateral flow rate

It has also been observed that as the pore size increases, the lateral flow rate increases. However slower flow rate increases the effective concentration (concentration required for interaction) of the analyte, since a slower flow rate allows the analyte and the capture reagent to be in close proximity for a longer time. As it is well known, immunological reactions are time-dependent and prolonged exposure of the analyte with the capture reagent allows better interaction and thus results in increased sensitivity. The flow rate is important when the analyte is present in low concentrations, such as borderline samples. The relationship between lateral flow rate and effective analyte concentration is: effective analyte concentration $\alpha \frac{1}{(\text{flow rate})^2}$

Thus it is important to optimize the membranes such that Rapid Diagnostic Tests can achieve rapid results which are also reliable and accurate

Why are colloidal gold sol particles commonly employed in the detector reagent in membrane-based Rapid Diagnostic Tests?

Interpretation of results in Rapid Diagnostic Tests depends upon the development of a signal at the stipulated time. A signal is generated when capture reagent – analyte - detector reagent complex is formed. The detector reagent/ conjugate consists of an antibody or antigen bound to the indicator. The indicator imparts color to the signal, enabling visual interpretation of results.

Colored latex particles, colloidal gold sol particles, dyes, enzymes and carbon particles are some of the indicators used in immunochromatographic assays. However, stability, protein-binding properties, particle size are critical factors that determine their use in immunochromatographic assays. The most popular indicators used in immunochromatographic assays is the colloidal gold sol particle.

Colloidal gold sol particles as indicator

Homogenous colloidal gold sol particles are inert and can couple with antibody / antigen, which is stable in dried as well as in liquid form. All the above mentioned parameters are determined by the particle shape and size of colloidal gold.

Effect of shape of colloidal gold sol particles on stability

Colloidal gold sol particles have a net negative charge called “Zeta Potential”. This Zeta Potential maintains the minimal distance between two particles resulting in long term stability. Ideally, colloidal

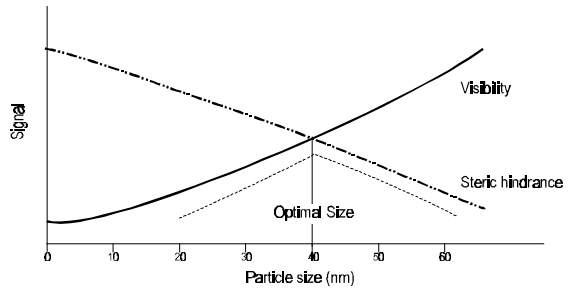
gold sol particles should be spherical in shape, since this shape allows uniform distribution of Zeta Potential at the surface. In case of non-homogenous particles, the Zeta Potential is not uniformly distributed thus the particles may come together to form aggregates. These aggregates may permanently get impregnated into the conjugate pad, or during the test assay may deposit on the nitrocellulose membrane leading to discrepant results. Such non-homogenous colloidal gold is usually blue/black in colour.

Effect of shape of colloidal gold sol particles on sensitivity

Spherical, homogenous colloidal gold sol particles also allow uniform coating of the detector reagent at their surface. Whereas non-homogenous colloidal gold sol particles do not allow uniform coating of detector reagent, resulting in decreased assay sensitivity and specificity.

Effect of size on color of colloidal gold sol particles

It has been observed that as the colloidal gold sol particles increase in size, the colour turns from light pink to cherry red to red-purple to blue-black to grey-black. Darker colored particles are preferred in Rapid Diagnostic Tests since darker colors allow easy interpretations of results. However, as the colloidal gold sol particles increase in size, these particles are less stable and aggregate together. Secondly due to the steric hindrance, the larger colloidal gold sol particles tend to dwarf the coated antigen/antibody making interaction with the analyte difficult.

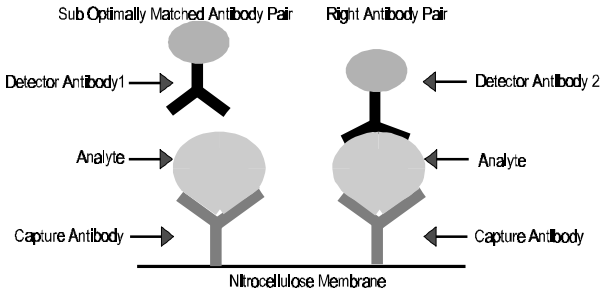


Graph of particle size v/s signal colour of colloidal gold sol

Ideally, the colloidal gold sol used in immunochromatographic assays is ≈ 40nm in size and imparts a cherry red color, which enables optimal visualization of results against a clear white background and is stable in dry and liquid form. However, purple colored colloidal gold sol particles if properly stabilized, can also be used in Rapid Diagnostic Tests

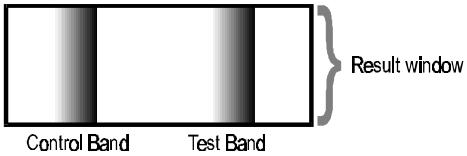
Why are variations in band appearance commonly observed in membrane-based Rapid Diagnostic Tests employed for antigen detection?

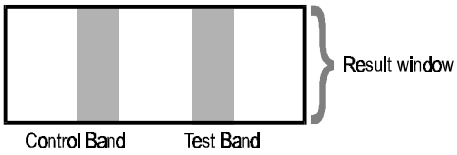
The sensitivity / specificity of Rapid Diagnostic Tests primarily depends upon the detector and capture reagent pair. Ideally, the detector reagent should be specific to one epitope of the analyte and the capture reagent specific to another epitope of the same analyte, thereby enabling two-site sandwich immunoassay. To illustrate the same please refer to the diagram below:



Two-site sandwich immunoassay

For higher analyte sensitivity, manufacturers of commercial Rapid Diagnostic Tests for antigen detection depend on the use of various combinations of capture reagents at the test and control band. Avid capture reagents have a high affinity for the analyte. When the sample containing the analyte reaches the avid capture reagent at the test band, due to high affinity, the avid reagent at the edge of the band captures most of the analyte. Thus resulting in a distinct thin colored line at the edge of the test band.





Band appearance due to avid antibodies **Band appearance due to less avid antibodies**

On the other hand, use of less avid capture reagent (lesser affinity for the analyte) results in capture of the analyte uniformly across the test or control band. Thus broader bands are generated by less avid antibodies.

Variations in band appearance in different assays is due to use of varying avidity of the antibodies at the test / control band

What is the role of sample pad in membrane-based Rapid Diagnostic Tests?

Rapid Diagnostic Tests enable detection of the analyte in several specimen such as urine, whole blood, serum or plasma. However, the pH, viscosity, ionic concentration, turbidity, total protein content may vary from specimen to specimen. Variations in these factors can cause alterations in the colloidal gold particles or the capture reagent leading to non-specific results. For example, highly turbid specimens can cause invalid results since the particles from the specimen may block the membrane preventing sample flow. Urine specimen becomes acidic on storage due to bacterial growth. Due to a shift in the pH, the colloidal gold particles come together to form aggregates which may interfere in the performance of the test.

Rapid Diagnostic Tests incorporating serum as specimen may give false positive results due to the presence of Heterophillic Antibodies. These antibodies have multispecificity and bind the capture reagent to the detector reagent leading to false positive results. Use of Rapid Diagnostic Tests incorporating Heterophillic Blocking Reagents (HBR) is recommended to avoid this interference.
A sample pad with a bed volume of minimum retention capacity facilitates transfer of the entire specimen dispensed. This not only ensures minimal wastage of specimen but the excess specimen can be used to wash away unbound conjugate from the test region for better visualization of results.

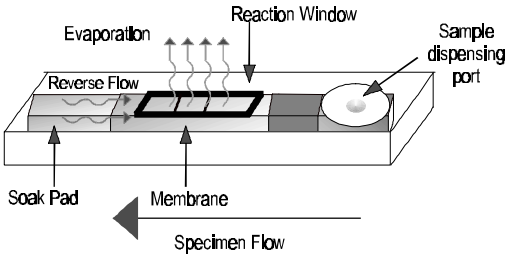
Thus use of sample pad that allows incorporation of buffer salts, stabilizers and HBR, to a large extent eliminates variation in pH, ionic concentration and interference of Heterophillic Antibodies

What is the role of soak pad in membrane-based Rapid Diagnostic Tests?

Use of a soak pad with high bed volume is preferred in Rapid Diagnostic Tests because the total volume of specimen that enters the test assay can be increased. This increased volume can be used to dislodge the conjugate as well as wash away the unbound/unreacted conjugate from the test region contributing to clearer background and better visualization of results.

Why do "Faint Ghost Bands" appear at the test region if the device is left out on the worktable?

A common phenomenon observed in the device format is appearance of faint ghost bands at the test region after some time. After completion of the test, if the device is exposed to warm ambient temperatures, evaporation occurs from the result window. Due to evaporation, the excess sample along with unreacted / unbound conjugate from the soak pad flows back to the reaction area. This unreacted or unbound conjugate may then get deposited on the test band resulting in appearance of a **"Faint Ghost Band"** after some time.



Appearance of "Faint Ghost Band"

Results must be recorded <u>at the end</u> of the recommended reaction time for correct interpretation

How do we interpret "Broken Bands" at the test / control region?

To prevent evaporation of the specimen from the test window, the membrane of the device is laminated with the help of a thin transparent tape. Sometimes, during the process of lamination, air pockets may be formed between the membrane and the tape. These air pockets, prevent uniform sample flow, which may result in appearance of broken bands at the test/control region.

However, appearance of even a broken band at the test region indicates positive results

In the following section we shall discuss the role of hCG as a marker for diagnosing pregnancy and certain conditions that may give discrepant results.

What is pregnancy testing?

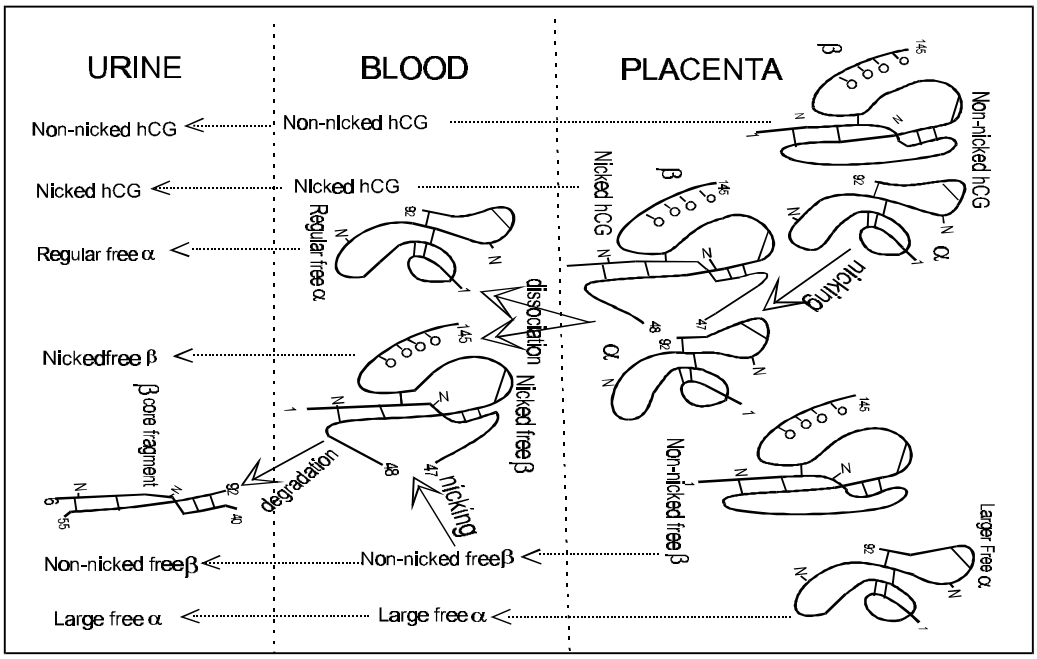
Presence of human Chorionic Gonadotrophin (hCG) and its sub units in urine/serum is an important marker in diagnosis of early pregnancy and determining problem pregnancies. Most pregnancy tests employed today, are based on two site sandwich immunoassay that utilize polyclonal/monoclonal antibodies raised against different epitopes on the hCG molecule.

What is human Chorionic Gonadotrophin (hCG)?

Human Chorionic Gonadotrophin (hCG) is a complex glycoprotein hormone consisting of two dissimilar polypeptide subunits, α and β, linked non-covalently. The α subunit of hCG is similar to that of the other pituitary glycoprotein hormones; Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH) Thyroid Stimulating Hormone (TSH). It is the β subunit that provides the differentiating characteristics of hCG from LH, FSH and TSH. hCG is secreted by the synctiotrophoblastic tissue of the placenta into the maternal circulation, shortly after implantation of the zygote. The biological role of hCG is to facilitate implantation of the zygote as well as maintain and develop the corpus luteum to secrete progesterone.

What are the levels of hCG observed during normal pregnancy?

In a normal 28-day menstrual cycle, with ovulation occurring at day 14, serum hCG can be detected within 24 hours post implantation, whereas hCG can be detected in urine in minute quantities around day 23. The concentration of hCG doubles approximately every 1.7 to 2 days and peaks between 8 to 12 weeks after last normal menstrual period (LMP) and reaches a concentration of about 100,000 mIU/L. Subsequently the levels of hCG start to decline by the second trimester and remain constant until parturition. Levels of hCG during pregnancy are expressed in relation to days after the last normal menstrual period (LMP). However, rate of increase of hCG following implantation varies among different individuals, thus the time at which a test becomes positive may also vary. During the 1st trimester, 96-98% hCG circulates as an intact molecule in the serum of women with uncomplicated pregnancy. The hCG molecule is cleaved rapidly and cleared by the kidneys resulting in the formation of a variety of hCG metabolites. Thus urine contains intact hCG, nicked hCG, α subunit, nicked/ free β subunit and β core fragment.



hCG and its metabolites during pregnancy

Adapted from :Immunoassay of human Chorionic Gonadotropin, its free subunits, and metabolites" L.A. Cole; Clinical Chemistry, 43:12; 2233-2243; 1997.

What is the effect of antibody pair used in the membrane-based Rapid Diagnostic pregnancy Tests for detection of hCG?

Anti-hCG antibodies used in the detector or capture reagent are specific to certain epitopes on the hCG molecule. Different species of antibodies detect different epitopes of hCG molecule. Since, different antibodies are used by different manufactures, thus different brands do not recognize the same epitope of the hCG molecule. Secondly, since hCG and its metabolites are present in the maternal circulation and in the urine, depending on the epitope specificity of the antibody pair used as the detector or capture reagent, different brands may detect either intact hCG, nicked hCG, α subunit, nicked/ free β subunit or β core fragment. This can result in discrepancies between brands in few cases of hCG detection.

What is the clinical significance of hCG?

In normal, healthy women of child-bearing age, elevated levels of hCG are associated with pregnancy. However, certain other clinical conditions such as trophoblastic diseases and non-trophoblastic disease such as hydatidiform mole, choriocarcinoma, testicular tumor are associated with high levels of hCG and its dissociated or degraded sub units in the patient's circulation.

Thus the clinical significance of measuring hCG and its subunits are:

1. To diagnose early pregnancy
2. To monitor pregnancy prognosis
3. Diagnose and monitor trophoblastic tumors

What are the implications of sensitivity and specificity on membrane-based Rapid Diagnostic Tests for pregnancy?

An ideal pregnancy test is one, which can detect and is sensitive to the lowest levels of hCG in the patient's urine/serum. On an average hCG levels of 25-100 mIU/ml are reached on the first day of missed period. The ideal cut-off of pregnancy test should be adjusted so as to detect hCG levels greater than **10 mIU/ml**, since, it has been observed that hCG levels of < 10 mIU/ml may be found in healthy non-pregnant women and even normal, healthy men. Since the α subunit of hCG is similar to that of LH, FSH and TSH, Rapid Diagnostic Tests for pregnancy should be specific to the β **subunit of hCG only**, ensuring no false positive reactions due to cross reactivity with other hormones.

What is the ideal specimen to be employed for pregnancy testing?
Urine is the most widely used specimen for pregnancy assays. However, hCG can be detected in serum within 24 hours post implantation. Thus serum as a specimen can be used for early detection of hCG and monitoring fertility treatment. Concentration of hCG in urine specimens depends on time of urine collection, volume of urine excreted, fluid intake where as the level of hCG is uniformly distributed in serum and hence it can be collected at any time of the day. hCG is a very stable molecule if preserved or kept in blood or serum, however hCG dissociates at a much faster rate when left in urine. Since urine facilitates bacterial growth it may drastically alter the pH, ionic concentration as well as increase turbidity of the specimen, which may affect the hCG concentration.

Why are discrepant results observed during problem pregnancies?
Abnormal pregnancies such as ectopic pregnancies, threatened abortions, spontaneous abortions and early pregnancy loss are associated with low levels of hCG and its subunits.

Ectopic Pregnancy
Implantation of the zygote outside the uterus is known as ectopic pregnancy. It has been observed that during ectopic pregnancies the hCG levels are below the expected values for the same gestation period. In such conditions, appearance of faint bands in Rapid Diagnostic Tests, may result.

Spontaneous abortions
Spontaneous abortion or Miscarriage is natural (non-induced) termination of pregnancy within 20 weeks of gestation. It has been estimated that about 15-20% of recognized pregnancies have resulted into spontaneous abortions. Abortion occurring within the first 16 weeks of pregnancy, are termed as early miscarriage or early abortion. The cause of early spontaneous abortion is usually due to defect in the fetal chromosome. **Spontaneous abortions in early pregnancy can occur several weeks after the embryo or fetus has actually died.** In such cases, the woman may demonstrate initial positive hCG tests which may be come negative in a few day/weeks and she may still not have her menses. Late spontaneous abortions occur between the 17^{th}-20^{th} week of pregnancy. The main causes are often related to the mother's state of health. The probable causes are severe malaria infection, Rh incompatibility, hormonal imbalance, Venereal diseases, chronic diabetes, high blood pressure, abnormalities of the immune system or abnormalities of the uterus or cervix.

Early pregnancy loss
Early pregnancy loss is a clinical condition where in the embryo fails to implant into the uterus or it is rejected by uterus in the form of a normal or slightly heavy menses. This occurs during the 2^{nd} week after implantation of the embryo. Thus most women are unaware that they had an early pregnancy loss. hCG levels rise during the first week post implantation as in the case of a normal pregnancy, peak at second week of implantation (28^{th} day from LMP) and then rapidly decline with the occurrence of bleeding along with fetal loss. An hCG assay, if performed during the 1^{st} or 2^{nd} day of missed period (28^{th}-29^{th} day from LMP) would indeed give a positive result due to the presence of hCG. However, negative results are obtained if the test assay is repeated after an interval of 1 to 2 days, i.e around the 31^{st} day after LMP.

During which conditions other than pregnancy are detectable levels of hCG observed?
Detectable levels of hCG has been observed in the following cases:

Normal, healthy men and normal, healthy non-pregnant women
Low levels of hCG have been detected in normal, healthy men and normal, healthy non-pregnant women. Low levels of hCG are secreted from the gonadotroph cells of the pituitary. It has been observed that hCG levels of ≈ 5 mIU/ml are found in healthy men and pre-menopause women and levels of < 10 mIU/ml are found in healthy post menopause women.

Exogenous administration of hCG
hCG injections are administered to women on fertility treatment to trigger ovulation or prolong the luteal phase of the menstrual cycle. This hCG in the circulation may take upto 5-14 days to be cleared depending upon the dose and individual's clearance rate.

Trophoblastic tumors and non-trophoblastic tumors
Trophoblastic tumors such as trophoblastic neoplasm, hydatidiform mole, choricarcinoma have been shown to secrete variable ratios of intact hCG and its metabolites. Also non-trophoblastic diseases such as testicular/ovarian and bladder cancers are associated with high levels of hCG and its sub units. Thus prior to performing a hCG test, trophoblastic and non-trophoblastic disease conditions should be ruled out as these conditions.

Phantom hCG-like immuno-reactivity
Certain trypsin-like molecules, cholera toxin or certain bacterial secretions produce phantom hCG-like immuno-reactivity in serum, which may then interfere in the serum based test assay. Serum dilution can rule out this phenomenon.

After normal delivery
Low levels of hCG have been found in the maternal circulation after a normal delivery. However, persistence of low hCG levels may indicate an underlying pathological condition such as retained pieces of the placenta.

How do procedural errors affect Rapid Diagnostic Tests?
Improper storage conditions
As recommended in the manufacturers pack insert, all reagents should be stored at the recommended temperature 2-30^{0}C. Exposing the reagents to excess freeze-thaw can result in deterioration of the detector and capture antibodies, leading to invalid results.

Procedural errors
All specimens/reagents should be brought to room temperature prior to testing. In Rapid Diagnostic pregnancy Tests, the reaction time between antigen and antibody is short (» 5 to 15 minutes), thus the sensitivity of the test assay depends up on the temperature of the assay. As such, the antigen-antibody reactions are optimized at R.T. Hence it is important that the device/dipstick be brought to R.T. prior to testing.

Dropper and drop size
Always use the droppers provided with the kit to dispense the sample specimen while performing the test. Secondly, it is important to hold the dropper vertically straight, perpendicular to the sample pad/ sample dispensing port. Variation in the force required to dispense the liquid and the angle at which the dropper is held leads to variations in the drop size. This may affect the sensitivity of the test assay, especially in borderline samples. It is important that **exactly** the recommended drop size should be used for correct assay performance.

Excess sample volume dispensed
Adding excess sample in no way improves the performance of the test. The excess sample added, cannot be absorbed by the sample pad and thus flows out through the sides of the device. Sometimes, the excess sample may flow out along with the conjugate. The amount of the conjugate left in the device is insufficient to perform the assay, leading to invalid results. Secondly, once the specimen flows through the device, the soak pad cannot retain the excess volume of the sample, which then may flow out through the sides of the device or may also flow back to the membrane along with unreacted/ unbound conjugate. This unreacted / unbound conjugate may then deposit onto the membrane resulting in apparently discrepant results.

Damage in packing

Check the device/dipstick pouch for pinholes and observe any colour change in the desiccant (should be bluish). Once opened, the test is to be performed immediately. Prolonged exposure of the nitrocellulose membrane to the surroundings can lead to absorption of moisture. The water molecules fill up the pores preventing sample-detector reagent flow, through the device/dipstick.

Reading results at the recommended time

Since the test band completion in an immunochromatographic test is based on the membrane capillary action, the detector–analyte complex travels onto the test (capture) band. In routine, depending upon the analyte concentration the test band formation starts rapidly enough. However when **borderline** concentration at the limit of assay sensitivity are encountered, the test band appearance is delayed. Sometimes to the extent of reaching the time of completion of the test itself. It is thus recommended to retest such samples subsequently by recollecting the sample instead of reporting the results based on reading the test beyond the recommended assay time.

Understanding the limitations of Rapid Diagnostic Test, sample and procedure related limitations, would aid the laboratory personnel minimize erroneous reporting and troubleshoot confidently for confident result reporting

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