MATERIAL AND METHOD:
The study covers to examine the females for genital tract infection clinically with the help of gynecologist department of Civil Hospital.

A) For microbiological work, samples of female vaginal discharge were collected, under advice of gynecologist.

B) The females, who had examine by the Physician & Dermatologist, for medical check-up, related to suspect HIV infection & also for skin genital lesion, blood was collected for the serological investigations.

C) Blood was collected for the serological test, from females who have history of abortion for, TORCH test.

Few patients were covered, in this study from private clinics.

The female's patient, were examined by consultant, clinical history cover, menstruation history, obstetrical history, past history, family history and functional history and physical examination, abdominal examination. In case of gynecological examination, the patient should be in lithotomy position.

The gynecological examination covers skin of pubic, vulva, perinal area and thigh, first. Inguinal glands were palpated and public hair examine, for lice and nits. After separating libia minora, discharge of introituses noted. Bartholin gland was palpated, with fingers, infected gland may be tender and beads of pus may come, was collected.

A bivalve vaginal speculum moistened with warm water was passed directed downwards of vaginal fornix, from wall of vagina and in vault of vagina, if any vaginal discharge present, and was collected with least contamination, by normal
Flora. For many genital pathogens, both the purulent exudates and cells need to be sampled. It was important to rub the swab on area rather than just dipping it in the pus.

Two vaginal/cervical swabs were collected, one for Microscopy and second in Stuart transport medium.

**pH:** Ph of vaginal carried out immediately during clinical procedure. Elevated vaginal Ph is significant in case of Bacterial vaginosis. *(Amsel’s Criteria).*

**KOH:** Characteristic odor after addition of 10% KOH solution to vaginal secretion is one of the diagnostic criteria’s in case of Bacterial vaginosis (The Whiff Test).

From one vaginal discharge; wet preparation, Gram stain, Giemsa stains, ZN Stain, KOH mounting and India Ink staining were carried out.

Wet preparation of vaginal discharge is carried out by addition of normal saline. After applying cover slip microscopic examination carried out under high power of microscope.

Presence of Clue cells (Epithelial cells with adherence, mostly Gardnerella vaginalis to the cells surface and its margin) identify the bacterial vaginosis.

Demonstration of motile trophozoite of Trichomonas vaginalis detects the infection of Trichomonas vaginalis (Motile protozoon 10 -12mu X 7-10 mu)
Wet mount so mucopurulent cervical discharge (inflammation of the cervix) shows more than 30 poly morphs per high power of microscopic examination favors Chlamydia infection.

Budding cells and pseudohyphae of yeast can be easily identified in wet preparation by adding 10%KOH thereby dissolving host gel protein and enhancing the visibility of fungal element.

**Gram Stain:** Roll the vaginal discharge over surface of clean microscopic slide Air dry and fixed with methanol.

Procedure: First apply gention violate for 1-2 min. Wash the smear with water. Apply grams iodine for 1-2 min. wash with water. Decolorized by acetone or methanol until violate colour come out. Stain with saffranine for 30-60 sec. Wash with water. Air dry and examine under oil immersion lens

**Ziehl Neelsen stain (Acid Fast stain):**
Fix vaginal smear was stain by Z.N.Stain.

Procedure: First of all cover smear with carbol fuschin over, the filter paper on smear.

Apply heat under the slide, for period of five minute. No boiling of stain Smear should not be dry.

Decolorized by 20% H2So4 until colour of primary stain come from smear.
Stain with Methylene blue or Melachine green for 1-2 min
Air dry smear and examine under oil immersion lens

Giemsan stain:
Methanol fixed smear was stain by Giemsa stain;

Procedure: Apply stain for period of 10 min
Apply buffer or distill water for 30 min.
Air dry and examine under oil immersion lens.

From second collected vaginal discharge swab, nutrient agar, MacConkey agar, blood agar, chocolate agar, strict and incubate at 37°C and 30°C in case of sabouraud’s agar, overnight. Also Specimens were inoculated Gonococcoal agar media. Inoculation was done at the O.P.D., immediately after collection of Sample.

Blood Agar: Medium was prepared by adding sterile blood to sterile neutriant agar that has been melted and cooled to 50°C. The concentration of the blood was 10% (ranging from 5-50%) either human or animal blood (Sheep or horse blood)

Thin layers of melted nutrient agar, about 7 ml for 4 inch pestidish was poured and allow setting. Then similar thin layer of 10% blood agar was prepared on the top of first layer (with out any air bubble).

Heated blood agar: Chocolate Agar: Medium was prepared by heating 10% sterile blood in a terile neutriant agar. Melt the agar; cool it in a water bath at 75°C
C. The blood was added to medium. Neutriant agar by gentle agitation from time to time until the blood becomes chocolate brown in colour within about 10 min. Then medium was poured as a plate. An alternative method of preparing plates of heater blood agar (Naylor 1961), by placing plate in to an incubator or hot air oven at 50\(^\circ\)C for 1-2 hours.

**Mac Conkey's Agar:** Medium was used for the cultivation of enteric medium. It contains bile salt to inhibit non intestinal bacteria. Lactose fermenters form pink colour colonies while the nonfermenter was colourless or pale colonies.

**Medium contents:**

- **Peptone**: 20 g
- **Sodium taurocholate**: 5 g
- **Water**: 1 liter
- **Agar**: 20 g
- **Neutral red solution**: 2% in 50% Ethanol: About 3-5 ml
- **Lactose 10% (Acq. Solu)**: 100 ml

Dissolve the peptone and sodium taurocholate (bile salt) in water by heating. Add the agar and dissolved in autoclave. Adjust pH to 7.5. Add lactose and the neutral red well shake and mix. Autoclave for 115\(^\circ\)C for 15 min.

**Contents of GC agar media**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Grams/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>15.00</td>
</tr>
<tr>
<td>Corn starch</td>
<td>1.00</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>4.00</td>
</tr>
</tbody>
</table>

217
Monopotassium phosphate 1.00
Sodium chloride 5.00
Agar 10.00

Preparation of media: 7.2 grams of GC agar base added to 100 ml distilled water, boiled to dissolve the medium completely, sterilized by autoclaving at 121 °C for 15 minute. Cool to 50° C and aseptically added sterile solution of 2% Hemoglobin (FDo22) and GC supplement (FDo21). To increase the selectivity of medium Linco T supplement (FDo26) was added. All the contents were mixed well and pour in to the sterile Petri dishes.

Final PH of media = 7.2 ± 0.2

• The inoculated plates were incubated at 35° - 36° C under 5-10% Co2, by using candle jar.

• Plates were examined after 24 hrs of incubation for the growth of gonococci, when there was no growth; the plates were incubated for next 24 hrs and examined on the third day of incubation.

On second day, the examination the colony characteristic of each above medium was carried out. Microscopic examination includes, motility (Hanging Drop Method), gram stain, KOH preparation, (particularly from the Saboraud’s agar medium to observe the morphology microscopically), Capsule straining (India Ink -negative staining) were carried out.

For confirmation of the organism various tests were carried out:

1) Catalase test: Place loopful hydrogen peroxide (H₂O₂) on colony of Nutrient agar or colony was collected from the Nutrient agar by Capillary and deep into the test tube containing hydrogen paroxide (H₂O₂)- prompt
effervescence indicate the catalase positive or liberation of air bubbles from H2O₂ into the test tube, indicate of Catalase positive.

2) The Coagulase test:
Coagulase test is done by two method (i) tube test (ii) slide test.
For tube test 0.1 ml of the growth culture or agar culture suspension of the isolated is added to 0.5 ml. human or rabbit plasma in narrow tubes. EDTA or Heparin is recommended as anticoagulant. Citrate is not preferable as anticoagulant for plasma.

The tube is put in water bath at 37° C for 3-6 hours. Plasma will clot after incubation indicate coagulase (free type) -positive test.
Where as in slide test, is performed by emulsified isolate in a drop of saline and a drop of plasma (human/rabbit) is mixed.
Prompt clumping of cocci indicate the coagulase (bound type) -positive test.

3) Oxidase test: 1.0 to 1.5% solution of tetramethyl phenylene diamine hydro chloride is poured over the colony or poured over the filter paper. Oxidase positive colony becomes maroon, purple within 30 to 60 seconds or colony smear over the filter paper change in purple paper test - positive.

4) Indole Production: The peptone water inoculated with isolated incubate for 40 to 96 hrs. at 37° C. After the addition of Kovac’s reagent red colour indicate the positive reaction.

5) Methyl Red test: Glucose phosphate broth was incubate with isolates for 30°C for five days
After addition of methyl red, in glucose phosphate broth -Red colour indicate positive reaction.
6) Voges proskaner test (VP): Peptone water was inoculated with isolates at 30°C for 5 days or 37°C for 48 hr. After addition of 0.6 ml of 5% solution of alpha nephthol (in ethanol and 0.2 ml of 40% HKOH -Pink colour appear in 2-5 min indicate positive reaction

7) Citrate utilization: Koser’s citrate medium streak with isolates - production of turbidity in the medium indicate positive reaction or colour change to blue in colour indicate (in slant) -positive reaction.

8) Triple sugar Iron test: The medium contain glucose lactose and sucrose and also contain ion sulphahate

The test indicates whether bacteria ferment glucose only or lactose and sucrose, also with or without gas formation beside H₂S production.

If slant remains red in colour butt become yellow in color all sugar glucose lactose and sucrose are ferment. Bubbles in butt indicate gas production. The test facilities, preliminary identification of Gram negative bacilli.

9) Sugar fermentation: 1% of different sugar e.g. glucose lactose sucrose maltose fructose galactose etc. solution is prepared. Test organism is inoculated 37°C after 24 hr Red colour indicate acid production and gas production indicated by collection of gas in Darham’s tube.

Colonies of Candida species on SDA agar were further differentiated by using tests like, Germ tube test, Chlamydsospore formation on cornmeal agar medium sugar fermentation and sugar assimilation tests and growth on CHROM agar media.
**Germ tube test:**
- The culture of Candida species is treated with normal human serum and incubated at 37°C for 2-4 hrs.
- A drop of suspension is examined on the slide under high power of microscope.
- The germ tubes are seen as long tube like projections extending from the yeast cells.
- There is no constriction at the point of attachment to the yeast cell as seen in case of Pseudohyphae.
- The germ tubes were formal within two hours of incubation in C. albicans and C. dubiliniensis.

**Chlamydosporere formation:**
- The suspected strain of the candida isolates is grown on cornmeal agar (CMA) and incubated at 25°C tempe.
- It shows the formation of large, highly refractile thick walled, and terminal chlamydospores after 2-3 days of incubation.
- C. albicans and C. dubliniensis form chlamydosposes.
- C. albicans and C. dubliniensis spp. were differentiated by presence of growth at 42-45°C.
- Calbicans-does not give growth at 42-45°C.
- C. dubliniensis grow at 42-45°C.

**Carbohydrate fermentation media:**
This medium contains peptone 1%, Nacl 0.5%, Andrade’s indicator 0.005% different sugars like Dextrose, Sucrose, Maltose etc. at a concentration of 2% are added to it. Inverted Durham’s tube is used for the detection of gas.
Carbohydrate Assimilation Media:

The media used was yeast nitrogen base, which was carbohydrate free medium.

Contents of media:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gm/liter</th>
<th>Ingredients</th>
<th>gm/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulphate</td>
<td>5.0</td>
<td>Thiamine hydrochloride</td>
<td>0.0004</td>
</tr>
<tr>
<td>K-Histidine hydrochloride</td>
<td>0.01</td>
<td>Boric acid</td>
<td>0.0005</td>
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<tr>
<td>DL-Methionie</td>
<td>0.02</td>
<td>Copper sulphate</td>
<td>0.00004</td>
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<tr>
<td>DL-Tryptophan</td>
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<td>Potassium iodide</td>
<td>0.0001</td>
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<td>Biotin</td>
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<td>Ferric chloride</td>
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</tr>
<tr>
<td>Calcium pantothenate</td>
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<td>Manganese sulphate</td>
<td>0.0004</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.000002</td>
<td>Sodium molybdate</td>
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<td>Inositol</td>
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<td>Monopotassium phosphate</td>
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</tr>
<tr>
<td>P-Amin benzoic acid</td>
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<td>Magnesium sulphate</td>
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<tr>
<td>Pyridoxine hydrochloride</td>
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<td>Sodium chloride</td>
<td>0.10</td>
</tr>
<tr>
<td>Riboflavin (Vit-B2)</td>
<td>0.0002</td>
<td>Calcium chloride</td>
<td>0.10</td>
</tr>
</tbody>
</table>

The medium was prepared in 10x strength, 6.7 gms. YNB suspended in 100 ml distilled water. Warm if necessary to dissolve the medium completely. Sterilized by membrane filter. Final medium made by taking 0.5 ml of medium into 4.5 ml of sterile distilled water pour into sterile Petri dishes.

Final PH of medium – 5.4 ± 0.2
Carbohydrate containing filter paper disks are added and utilization is determinate. Various carbohydrates used were Glucose, Maltose, Sucrose, Lactose, Cellobiose, Galactose, Trehalose, Raffinose, Melibiose, Xylose, Inositol, and Dulcitol.

**Urease test:**
It detects the ability of various yeasts to produce enzyme urease. In the presence of suitable substrate, urease splits urea producing ammonia, which raises the PH and colour changes from amber to pinkish red due to phenol red indicator. The test helps to differentiate the Candida Krusi, which is the only candida species giving urease test positive.

**Antibiotic Sensitivity:** A nutreit agar medium or Mueller Hinton medium was used (as it is free from substance inhibitory to action of antibiotic). The test organism (Isolates from the sample) was streak with help of swab to above medium. (Our aim to required confluent growth covering whole surface of plate) Disc of antibiotic (available from the market) were placed. Incubate the palte for 24 hr. at 37°C. Next day, the sensitivity (zone of inhibition) patterns (according to isolates and types of antibiotic used) were examined.

Blood samples were collectd for serological investigation includes; HIV screening test i.e HIV TRI-DOT and Comb AIDS-RS, ELISA Test for HIV and HpatitisB Antiegn (HBSAg), VDRL and TORCH Test.
SEROLOGY:

HIV: All the serum samples were screened by HIVASE I &II Microlisa a direct sandwich ELISA test for the detection of antibody to Human Immunodeficiency Virus type I and / or type II. (J. Mitra Co. Pvt. Ltd.)

**Principle:** A sandwich enzyme immuno essay which employs recombinant HIV I antibody gp\(^{120/41}\) and HIV-II antibodies (gp\(^{105/36}\)) antigen for the detection of antibody to HIV-I and II in human serum or plasma. These antigen are coated on to the micro-titer wells, when human serum or plasma (specimen) add will bind to the specific antigen, form a complex if anti HIV-I and II is present in human sera.

After washing, horseradish peroxidase (HRP) conjugate antihuman IgG is added to well allowing the formation of (HIV-I/II) (Anti HIV-I/II) (HIV \(\frac{1}{2}\) HRP) complex.

After washing, the substrate solution containing thechromogen and hydrogen peroxide is added to two wells for colour development. The absorbance of colour development is measured by EIA reader of the anti HIV-I and II content in sample. Serum sample negative by HIV ase 1+2 ELISA are reported as a negative for HIV.

**PREPARATION OF REAGENTS:**
Prepare the following reagents before or during assay procedures. Reagents and samples should be at room temperature (20-30\(^\circ\)C) before beginning the assay and can remain at room temperature during testing. Return reagents to 2-8\(^\circ\)C after
use. All containers used for preparation of reagents must be cleaned thoroughly and rinsed with distilled or deionized water. Prewarm the incubator at 37 °C.

1) Microlisa-HIV Strip: Bring foil pack to room temperature (20-30°C) before opening to prevent condensation on the microwell strips.

2) Break-off the required number of strips needed or the assay and place in the well holder. Take the strip holder with the required number of strips, taking into account that two negative and three positive controls should be included in the run while opening the fresh kit. However for one or two strips, one negative and two positive controls and for more strips at least two negative and three positive controls should be included in each subsequent runs.

3) Unused wells should be stored at 2-8°C, with dessicant in a aluminium pouch with clamp and rod.

4) Sample Preparation:
   Tube Dilution: Mark the tubes carefully for the proper indentification of the samples. Dilute the serum samples to be tested, with sample diluent (1:11 dilution) in separate tubes (200 µl diluent + 20 µl sample). Use a separate tip for each sample and then discard as biohazardous waste.

5) Microwell Dilution:
   a) Pipette 100 µl of sample diluent in to the microwell.
   b) Add 10 µl of serum sample to be tested.
   c) Ensure through mixing of the sample to be tested.

6) Preparation of Wash Buffer:
   a) Check the buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37°C until all crystals dissolve.
b) Prepare at least 50 ml. (2ml concentrated buffer with 48 ml. water) of buffer for each microlisa strip used. Mix well before use.

c) Mix 20 ml. 25 x 2ash buffer concentrate with 480 ml. of distilled or deionized water. Wash buffer is stable for 2 months when stored at 2-8°C.

7) Preparation of working conjugate: Dilute conjugate concentrate 1:100 in conjugate diluent. Do not store working conjugate. Prepare a fresh dilution for each assay in a clean glass vessel. Determine the quantity of working conjugate solution to be prepared from table given below. Mix solution thoroughly before use.

<table>
<thead>
<tr>
<th>No. of Strips</th>
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<tr>
<td>No. of Wells</td>
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<td>24</td>
<td>32</td>
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<td>72</td>
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<td>Enzyme</td>
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</tbody>
</table>

8) Prepare of working substrate solution: Dilute TMB concentrate 1:100 in substrate. TMB diluent. The 100X solution may crystallize during storage. Check for any crystals before use, if crystals are present resolubilize by warming at room temperature.
<table>
<thead>
<tr>
<th>No. of Strips</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>No. of Wells</td>
<td>8</td>
<td>16</td>
<td>24</td>
<td>32</td>
<td>40</td>
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<td>TMB Concentrate (μl)</td>
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<tr>
<td>TMB Diluent (ml)</td>
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<td>12</td>
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</tbody>
</table>

**Wash Procedure:**

1) Incomplete washing will adversely affect the test outcome.

2) Aspire the well contents completely into a waste container. Then fill the wells completely with wash buffer avoiding overflow of buffer from one well to another and allow to soak (approx. 30 seconds). Aspirate completely and repeat the wash and soak procedure 4 additional times for a total of 5 washes.

3) Automated washer if used should be well adjusted to fill each well completely without over filling.

4) Tap upside down on absorbent sheet till not droplets appear on the sheet, taking care not to dislodge the wells.

**Test Procedure:**

Once the assay has started, complete the procedure without interruption. All the reagents should be dispensed in the centre of the well and the tip of the pipette should not touch the wall of the microwell.

Fit the stripholder with the required number of Microlisa-HIV strips. The sequence of the procedure must be carefully followed. Arrange the assay control
wells so that well A-l is the reagent blank. From well A-1 arrange all controls in a horizontal or vertical configuration. Configuration is dependent upon reader software.

1) Add 100 µl sample diluent to A-1 well as blank.

2) Add 100 µl Negative control in each well no. B-1 and C-1 respectively. Negative Control is ready to use and hence no dilution is required.

3) Add 100 µl positive control in D-1, E-1 and F-1 wells. Positive control is ready to use and hence no dilution is required.

4) Add 100 µl sample diluent in each well starting from G-1 followed by addition of 10 µl sample. (Refer Microwell Dilution) alternatively transfer 100 µl of each sample diluted in sample diluent (1:11) in each well starting from G1 well (Refer Tube Dilution)

5) Apply cover seal.

6) Incubate at 37°C ± 2°C for 30 min. ± 2 min.

7) While the samples are incubating, prepare working wash solution and working conjugate as specified in Preparation of Reagents.

8) Take out the plate form the incubator after the incubation time is over and wash the wells 5 times with working wash solution according to the wash procedure given in the previous section (wash procedure).

9) Add 100 µl of working conjugate solution in each well including A-1.

10) Apply cover seal.

11) Incubate at 37°C ± 2°C for 30 min ± 2 min.

12) Aspirate and wash as described in step no. 8.

13) Add 100 µl of working substrate solution in each well including A-1.

14) Incubate at room temperature (20-30°C) for 30 min. in dark.

15) Add 50 µl of stop solution.
16) Read absorbance at 450 nm within 30 minutes in ELISA READER after blanking A-1 well. (Bichromatic absorbance measurement with a reference wavelength 600-650 nm is recommended when available).

17) Calculation of Results:

**Abbreviations:**

- NC: Absorbance of the Negative Control
- NCx: Mean Negative Control
- PC: Absorbance of the Positive Control
- PCx: Mean Positive Control

**Test Validity:**

**Blank Acceptance Criteria:**

Blank must be <0.100 in case of differential filter being used. In case differential filter is not available in the reader the blank value may go higher.

**Negative Control Acceptance Criteria:**

NC must be ≤0.150. If it is not so, the run is invalid and must be repeated.

**Positive Control Acceptance Criteria:**

1) PC must be ≥0.50

2) Determine the mean (PCx) value if one of three positive control values is outside of these limits, recalculate PCx based upon the two acceptable positive control values.

3) If two of the three positive control values are outside the limits, the assay is invalid and the test must be repeated.
Cust Off Value:

Absorbance

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>B1 Well</th>
<th>PC</th>
<th>D1 well</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>0.042</td>
<td>B1 Well</td>
<td>1.412</td>
<td>D1 Well</td>
</tr>
<tr>
<td>0.040</td>
<td>C1 Well</td>
<td>1.392</td>
<td>E1 Well</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.082</td>
<td>2 wells</td>
<td>1.407</td>
<td>FI Well</td>
</tr>
</tbody>
</table>

NCx = 0.082/2 = 0.041
PCx = 4.211/3 = 1.403

The cut off value is calculated by adding Mean Negative Control (NCx) and Mean Positive Control (PCx) as calculated above and the sum is divided by 6.

Cut off value = NCx + PCx
e.g. NCx = 0.041
PCx = 1.403

Cut off value - 0.041 + 1.403 = 1.444 = 0.240

Interpretation of Results:

1) Test specimens with absorbance value less than the cut off value are non-reactive and may be considered as negative of anti-HIV.
2) Test specimens with absorbance value greater than or equal to the cut off value are reactive for anti-HIV by Microlisa-HIV.
3) The O.D. for crystal clear negative samples can be in minus. However, the minus (-) O.D. does not in any way affect the result interpretation. It rather gives better specificity.
4) Specimens with absorbance value equal to or greater than the cut off value are considered initially reactive by the criteria of Microlisa-HIV. Original specimen should be retested in duplicate.
5) If both duplicate retest sample absorbance value is less than cut off value, the specimen is considered non reactive.

6) If any one of the duplicates retest sample absorbance value is equal to or greater than the cut off or both duplicate retest value are equal to or greater than the cut off, the specimen is considered reactive by the criteria of Microlisa HIV. Further confirmation by other EIA assays or confirmation assays including Western Blot or PCR is recommended.

Serum samples-positive by HIVase 1+2 ELISA were confirmed by using other 2 rapid tests.

a. HIV TRI-DOT (BIOTECH INC)

b. Comb AIDS-RS (SPAN Diagnostic Ltd.)

HIV TRI-DOT: Rapid visual test for the qualitative detection of antibodies to HIV-I and HIV-II in human serum/plasma.

HIV TRI-DOT has been developed and designed using gp-41, C terminal of gp-120 and gp-36 representing the immunodominant regions of HIV-I and HIV-II envelope gene structure respectively. There is immunofiltration membrane, which includes a "Built-in quality control Dot" which will develop colour during the test, there by confirming proper functioning of the device, reagents and corrects procedural application. This "CONTROL DOT" is the built in quality control.
**Principle:**

HIV antigens are immobilized on a porous immunofiltration membrane. Sample and regents pass through the membrane and are absorbed into the underlying absorbent.

As the patient's sample passes through the membrane, HIV antibodies, if present, bind to the immobilized antigens.

Conjugate binds to the FC portion of the HIV antibodies to give distinct pinkish purple DOT(S) against a white background.

**Test Procedure:**

1) Add 3 drops of buffer solution to the centre of the device.
2) Add 1 drop of patient's sample (serum/plasma) using the sample dropper.
3) Add 5 drops of buffer solution
4) Add 2 drops of liquid conjugate directly from the conjugate vial
5) Add 5 drops of buffer solution and read results.

**Interpretation of Results:**

Non-Reactive: If only DOT (only the control Dot) appears the specimen is non-reactive for antibodies either to HIV-I or HIV-II.

Reactive:

- If two DOT(S), one for the control and the other for HIV-I appear, the specimen is reactive for antibodies to HIV-I.
- If two DOT(S), one for the control and the other for HIV-II appear, the specimen is reactive for antibodies to HIV-II.
- If all the three DOTS, one each for control, HIV-I and HIV-II appear, the specimen is reactive for antibodies to HIV-I and HIV-II.

Comb AIDS-RS test: Dot immunoassay for the detection of antibody to HIV-I and HIV-II in serum or plasma.

Principle: Dot immunoassay employs the same principle an Enzyme Immuno Assay (EIA) whereby immobilized antigen-antibody complex is visualized by means of colour producing (chromogenic) reaction.

In Comb AIDS-RS the colored end-point is developed by a colloidal Gold-protein-A singal Reagent. Each arm of the comb is spotted with a circular spot near the tip, by an optimally standardized blend of HIV-I and HIV-II recombinant antigens and synthetic peptides.

When incubated with a specimen containing HIV-I and/or II antibodies, these antibodies bind specifically to the peptide antigen. The antibody-peptide complex is directly visualized after incubation with the protein. A colloidal Gold signal reagent.

A positive result is indicated by the presence of magenta red colored dot on the surface of the comb where peptides have been spotted.

Assay Procedure:

1) Mark the sample numbers on the micro test wells and add two drops of sample diluent to each micro test well that will be used for samples or control.
2) Add two drops (0.1 ml) sample or control to each of the above wells containing sample diluent. Mix sample with diluent by repeatedly aspirating and expelling with a micropipette or stirring with pipette tip. Record the position and identity of samples or controls as they are added.

3) Mark the sample numbers on the comb and place it in to rows of corresponding micro test wells. Incubate at room temperature for 10 minutes.

4) In the meantime dispense the colloidal Gold signal regent in to another set of wells which have not been previously used. Add 4 drops of colloidal Gold signal regent to each well.

5) Remove the comb, hold it vertically with tips pointing down and immerse in to the wash solution wash by carefully moving the comb forward and backward in the wash solution for a total of 10 times. Blot the tips of the arm.

6) Place the comb in to wells containing colloidal Gold signal regent. Incubate at room temperature for 10 minutes. After incubation, repeat the washing procedure.

7) Place the comb on a clean surface, reactive (labeled) side up. Do not blot or wipe the surface of the comb. Allow the comb to air dry completely before reading the result.

**Interpretation:**

Positive Result: A positive result is directed by the presence of magenta red coloured spot/dot near tip of the arm of the Comb. The absence of a colored spot/dot indicating, that the sample is free of HIV-I and II antibodies.
Negative Result: A negative result is directed by absence of any magenta-red colored spot-dot near the tip of the arm of comb, indicating absence of antibodies to HIV-I type I and II.
Sensitivity - 100%, Specificity - 100%

HBsAg - ELISA Test Kit - InTec Products, Inc.

Principle:
The Advanced HBsAg test is an ELISA based, double antibody "sandwich" immunoassay, which employs specific anti-HBsAg antibodies: monoclonal antibody to HBsAg immobilized at the bottom of the microtiter wells, and polyclonal antibodies to HBsAg coupled with horseradish peroxidase (HRP) as the conjugate solution.

During the assay, existing HBsAg in the specimen will react with these antibodies to form an "antibody-HBsAg-antibody-HRP" immuno-complex. After the unbound material is washed off during the assay procedure, substrate is applied to indicate the test result. The appearance of blue color in microtiter wells indicates HBsAg reactive result. The absence of the color indicates non-reactive result in the specimen.

Contents of the Kit:
Specimen collection: Separate the serum from the clot or plasma from the red cells as soon as possible to avoid hemolysis.

Preparation of Reagents: Enzyme conjugates working solution:

- **a.** Dilute enzyme conjugate to needed volume with enzyme conjugate diluent refer to the following table.
- **b.** Enzyme conjugate working solution may be stored at 1-8°C for 4 weeks.

1. Enzyme conjugate dilution table:

<table>
<thead>
<tr>
<th>Number of test</th>
<th>16</th>
<th>32</th>
<th>48</th>
<th>64</th>
<th>80</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme conjugate diluent (ml)</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Concentrated enzyme conjugate (μl)</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>200</td>
<td>250</td>
<td>300</td>
</tr>
</tbody>
</table>

2. Wash buffer:

- **a.** Dilute volume of concentrated wash buffer with 19 volumes of deionized or distilled water Mix well.
- **b.** Wash buffer may be stored at room temperature for 1 week.

Assay Procedures:

1. Bring all reagents and specimens to room temperature (18-25°C) before the assay. Swirl gently before use. Adjust incubator to 37±1°C.

2. Write down the numbers of specimens and the wells on the data sheet. One well for blank, five additional wells for the controls and one well, for each specimen.

3. Ad 50μl of control (3 negative controls and 2 positive controls) and 50μl of each specimen into wells respectively (Reserve 1 well for blank).

4. Add 50 μl enzyme conjugate working solution into each reaction well except for the blank.
5. Gently tap the plate to thoroughly mix the liquid in the wells; do not splash liquid onto the slip.

6. Incubate the plate in a 37°C incubator for 60 minutes.

7. Wash each well five times with wash buffer by wash procedure:
   a. Washing must be performed strictly according to the instructions; incomplete washing may bring out the false result.
   b. Aspirate the well contents completely into a waste flask. Then fill up the wells with wash buffer (330 μl or more), avoid overflow. Allow to soak (approx. 30-60 seconds). Aspirate completely and repeat the wash and soak procedure four additional times for a total of five washes.
   c. Make sure that no fluid remains on the strip-holder and strips after the last aspiration (e.g., by blotting with absorbent tissue).

8. Add 50 μl color A and 50 μl color B to each well.

9. Incubate the plate in a 37°C incubator for 15 minutes.

10. Add 50 μl 2M sulfuric acid into each well, gently tap the plate.

11. Measure OD with Micro-well reader at 450nm (single wavelength) or 450nm and 630nm as reference (dual wavelength).

Calculation and Result:

1) Negative Control Mean absorbance (NCx):
\[ NCx = (NC1+NC2+NC3)/3 \] Eliminate any NC greater than (> 0.100
2) Cut-off value: \[ \text{Cut-off} = 0.100 + NCx \]
3) Divide the sample absorbance by the cut-off Value
   Positive: Sample absorbance is greater than or equal (≥) to cut-off value
   Negative: Sample absorbance is less than (<) cut-off value.
Introduction: The One Step Strip Style HBsAg Test is a rapid, direct binding test for the visual detection of hepatitis B surface antigen (HBsAg) in serum. It is used as an aid in the diagnosis of hepatitis B infection. One Step HBsAg Test is based on the principle of sandwich immunoassay for determination of HBsAg in serum. Monoclonal and polyclonal antibodies are employed to identify HBsAg specifically. This one step test is very sensitive and only takes about 10-20 minutes. Test results are read visually without any instrument.

Test Procedure:

1) When you are ready to begin testing, open the sealed pouch by tearing along the notch. Remove the test kit from the pouch and use it as soon as possible.

2) Followed the illustration, dip the test strip with the arrow side pointing down into the vessel of serum for about 10 seconds. Do not immerse past the marker line. Take the strip out and lay it flat on a clean, dry and non-absorbent surface.

3) Wait for 10-20 minutes and read results. It is important that the background is clear before the result is read. Do not read results after more than 30 minutes.

Interpretation of Result:

Negative: Only one colored band appears on the control (C) region. No apparent band on the test (T) region.
Positive: In addition to a pink colored control (C) band, a distinct pink colored band will appear in the test (T) region.

Trans-RPR syphilis screening Test: (Regulus Marketing)

Principal: The RPR syphilis screening test is a macroscopic non-treponemal flocculation test for detection and quantitation of reagin, an antibody like substance present in serum or plasma and spinal fluid from syphilitic persons.

Specimen collection:
Unheated plasma or heated serum may be used. Avoid hemolysis. Serum samples are reportedly stable for 5 days if stored at +4°C. Plasma collected with EDTA is reportedly stable up to 24 hours.

Procedure Quantitative Test:
1) Bring all reagents and samples to room temperature.
2) Using the disposable sample dropper, dispense one drop of serum or plasma onto a separate circle on the test card. Use a fresh disposable sample dropper for each sample. Repeat step 2 using the positive and negative control sera.
3) Mix the carbon antigen well and Place one drop of “free fall” Antigen suspension onto each test specimen using needle dropper.
4) Place the card on rotator and rotate for 3 minutes at 100 rpm. Immediately after 3 minutes of rotation, read the results macroscopically in good light.

Procedure Quantitative Test:
1) Place 25μl of specimen onto test circle 1.
2) Place 25µl of 0.9% saline with a pipette into test circles, numbered 2 to 5. Do not spread saline.

3) Place 25µl of specimen onto the test circle 2. Prepare serial two-folds dilutions by drawing the mixture up and down the pipette 5-6 times (avoid any bubble formation). Transfer 25µl from circle 2 to 3, to 4, to 5. Dispose 25µl from circle 5 after mixing or save for further dilution.

4) Using a new stirring rod for each specimen, start at highest dilution of serum (circle 5). Proceed to circles 4, 3, 2 and 1.

5) Follow steps 4 to 5 in the Procedure of qualitative test.

Quantitative Test Result:

<table>
<thead>
<tr>
<th>Circle No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
<td>Undiluted</td>
<td>1/2</td>
<td>1/4</td>
<td>1/8</td>
<td>1/16</td>
</tr>
<tr>
<td>Reactive 1/2</td>
<td>R*</td>
<td>R</td>
<td>N**</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Reactive 1/4</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Reactive 1/8</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>N</td>
</tr>
<tr>
<td>Reactive 1/16</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

*: R = Reactive **: N = Non Reactive

If the last dilution (circle 5) 1:16 is reactive, proceed to test further dilutions of 1:32, 1:64, 1:128 and 1:256 as above.
Toxoplasma IgM was detected by Smart Comb™ Toxo IgM (qualitative detection of IgM antibodies to the Toxoplasma gondii):

**Principle:** The smart Comb™ Toxo Plasma IgM test is an indirect solid phase enzymes immuno assay. The solid phase is comb with projection (teeth). Each tooth is sensitized at two portions: (I) Upper spot IgM (control) (ii) Lower spot in activated Toxo Antigen. The developing plate has 6 rows A-F (of 12 wells) each road containing a reagent solution ready for use.

The test performed stepwise by moving the comb from raw to raw. The patient serum or plasma specimen is treated with antihuman IgG (Stripping) in order to prevent the interference as a result of competition by antitoxo IgG and Rheumatoid factor.

Pretreated specimen are further incubated in wells of raw A. Antitoxo IgM if present, in the specimens will specifically bind to the toxo antigen on lower spots on the teeth of the comb. Unbound component are washed away in raw B. In raw C the anti toxo IgM captured on the teeth and the human IgM on the upper spots. (Internal Central) will react with alkaline phosphatase AD labeled anti human IgM antibodies. In next two rows (C and D) unbound component are removed by washing.

In F raw the bound alkaline phosphatase will react with chromogenic subtract. The positive result was visible as a grey blue spots, on the surface of the teeth of the comb.
Developing Plates:
The kit contains 3 developing plates covered with aluminium foil. Each developing plate (figure-3) contains all reagents needed for the test. The developing plate consists of 6 rows (A-F) of 12 wells each.
The contents of each row are as follows:
Row A specimen diluent, containing goat antibodies to human IgG
Row B washing solution
Row C alkaline phosphatase-labeled goat anti-human IgM antibodies
Row D washing solution
Row E washing solution
Row F chromogenic substrate solution containing 5-bromo-4-chloro-3-indolyl (phosphate (BCIP) and nitro blue retnazolium (NBT)

Preparing the test:
Bring all components, developing plates, combs, reagents and specimens to room temperature and perform the test at room temperature (22-26°C).

Preparing the Developing Plate:
1) Incubate the Developing Plate in an incubator at 37°C for 20 minutes; or leave at room temperature (22°C-26°C) for 3 hours. Bring all other components reagents (Combs, stripping solution, controls and specimens) to room temperature.
2) Cover the work table with absorbent tissue to be discarded as biohazardous waste at the end of the test.
3) Mis the reagents by shaking the Developing Plate.

Test:
1) For each specimen and control, dispense 100 μl of stripping solution into a microtube or microtiter well.
2) To each microtube or well, add 10μl of a specimen, or of the Positive Control or Negative Control supplied with the kit. Mix by repeatedly refilling and ejecting the solution.

3) Set the timer and incubate for 10 minutes. (Adding Pretreated Specimens to Developing Plate)

4) Pipette 25μl of a pretreated specimen. Perforate the foil cover of one well in row A of the Developing Plate with the pipette tip or perforator and dispose the specimen at the bottom of the well. Mix by repeatedly refilling and ejecting the solution. Discard pipette tip.

5) Repeat step 4 for the other pretreated specimens and the two pretreated controls. Use a new well in row A and change pipette tips for each specimen or control.

6) Set the timer and incubate for 10 minutes. (Antigen-Antibody Reaction (Row A of the Developing Plate)

7) a) Insert the Cmb (printed side facing you) into the wells of row A containing specimens and controls. Mix: Withdraw and insert the Comb in the wells several times.

   b) Leave the Comb in row A for exactly 20 minutes. Set the timer. Near the end of 20 minutes, perforate the foil of row B using the perforator. Do not open more wells than needed.

   c) At the end of 20 minutes, take the Comb out of row A. Absorb adhering liquid from the pointed tips of the teeth on clean absorbent paper. Do not touch the front surface of the teeth.

8) Insert the Comb into the wells of row B. Agitate: Vigorously withdraw and insert the Comb in the wells for at least 10 seconds to achieve proper washing. Repeat agitation several times during the course of 2 minutes:
meanwhile perforate the foil of row C. After 2 minutes, withdraw the Comb and absorb adhering liquid as in step 7c.

9) Insert the Comb into the wells of row C. Mix as in step 7a. Set the timer for 20 minutes. Perforate the foil of row D. After 20 minutes, withdraw the Comb and absorb adhering liquid.

10) Insert the Comb into the wells of row D repeatedly agitate during 2 minutes, as in step 8. Meanwhile perforate the foil of row E. After 2 minutes, withdraw the Comb and absorb adhering liquid.

11) Insert the Comb into the wells of row E. Repeatedly agitate during 2 minutes. Meanwhile perforate the foil of row F. After 2 minutes, withdraw the Comb and absorb adhering liquid.

12) Insert the Comb into the wells of row F. Mix set the timer for 10 minutes. After 10 minutes, withdraw the Comb.

13) Insert the Comb again into row E. After 1 minute, withdraw the Comb and allow it to dry in the air.

Test Results:

Validation: In order to confirm that the test functions properly and to demonstrate that the results are valid the following three conditions must be fulfilled.

1) The positive control must produce two spots on the Comb tooth.

2) The negative control must produce an upper spot (internal control). The lower spot will either not appear or appear faintly, without affecting the interpretation of the results.

3) Each specimen tested must produce an upper spot (internal control).
Cytomegalovirus (CMV) IgM was detected by Smart Comb TM (by fast EIA test) for quantitative detection of IgM antibody to CMV

Principle:
The Smart Comb CMV IgM test is an indirect solid-phase enzyme immunoassay (EIA). The solid phase is a comb with 12 projections ("teeth"). Each tooth is sensitized at two positions:
Upper spot - human IgM (Internal Control)
Lower spot - inactivated CMV antigens

The developing plate has 6 rows (A-f) of 12 wells, each row containing a regent solution ready for use at a different step in the assay. The test is performed stepwise, by moving the comb from row to row, with incubation at each step.
At the outset of the test, serum or plasma specimens are pretreated with anti-human IgG (stripping), in order to prevent interference's as a result of competition by anti-CMV IgG, and by reteumatoid factor.

Pretreated specimens are further incubated in the wells of row A of the developing plate. The comb is then inserted in the wells of row A. Anti-CMV IgM, if present in the specimens, will specifically bind to the CMV antigens on the lower spots on the teeth or the comb (figure1). Unbound components are washed away in row B.

In row C, the IgM captured on the teeth and the human IgM, on the upper spots (Internal Control), will react with anti-human IgM antibodies labelled with alkaline phosphatase (AP).
In the next two rows (C and D), unbound components are removed by washing. In row F, the bound alkaline phosphatase will react with chromogenic substrate. The results are visible as gray-blue spots on the surface of the teeth of the Comb.

The kit includes a positive Control (ati-CMV IgM) and a negative control to be used in each assay run. Upon completion of the test, the tooth used with the positive control should show two gray-blue spots. The tooth used with the negative control should show the upper spot and either no lower spot or a faint lower spot. The upper spot should also appear on all other teeth, to confirm that the kit functions properly and that the test was performed correctly.

Test procedure and result (Interpretation) of Cytomegalovirus (CMV) IgM by Smart Comb™ (by fast EIA test) for quantitative detection of IgM antibody to CMV was similar to test procedure and result of Toxoplasma IgM by Smart Comb™ Toxo IgM (qualitative detection of IgM antibodies to the Toxoplasma gondii).

**ImmunoComb II - Rubella IgM: (ORGENICS)**

**Principle:** The ImmunoComb II Rubella IgM test is an indirect solid-phase enzyme immunoassay (EIA). The solid phase is a card with 12 projections ("teeth").

Each tooth is sensitized at two positions:
- Upper spot - human IgM (Internal Control)
- Lower spot - inactivated rubella antigens
The Developing Plate has 6 rows (A-F) of 12 wells, each row containing a reagent solution ready for use at a different step in the assay. The test is performed stepwise, by moving the Card from row to row, with incubation at each step.

At the outset of the test, serum of plasma specimens are pretreated with anti-human IgG (stripping), in order to prevent interferences as a result of competition by anti-rubells IgG, and by rheumatoid factor.

Pretreated specimens are further incubated with the solution in the wells of row A of the Developing Plate. The Card is then inserted in the wells of row A. Anti-rubella IgM, if present in the specimens, will specifically bind to the rubella antigens on the lower spot on the teeth of the Card.

Unbound components are washed away in row B. In row C, anti-rubells IgM captured on the lower spots of the teeth, and the human IgM on the upper spots (Internal Control), will react with alkaline phosphatase (AP) -labeled anti-human IgM antibodies.

In the next two rows, unbound components are removed by washing. In row F, the bound alkaline phosphatase will react with chromogenic components. The results are visible as gray-blue spots on the surface of the teeth of the Card.

Developing Plates: The kit contains 3 Developing Plates covered by aluminum foil. Each Developing Plate contains all reagents needed for the test. The Developing Plate consists of 6 rows (A-F) of 12 wells each. The contents of each row are as follows:

Row A specimen diluent, containing goat antibodies to human IgG
Row B  washing solution
Row C  alkaline phosphatase-labeled goat anti-human IgM antibodies
Row D  washing solution
Row E  washing solution
Row F  chromogenic substrate solution containing 5-bromo-4-cholor 3- indolyl phosphates (BCIP) and nitro blue tetrazolium (NBT)

Depending upon (requirement) test cut the teeth of the card and remaining teeth is kept in the pouch and closely tight.

Preparation of specimen and control: Take 100 mu of stripping solution and 25 mu. of each specimen and control (positive and negative) keep it for 10 minutes at room temperature.

Addition of the sample to developing plate: Pipette 25 mu. of specimen, break the aluminum foils cover of one well of Row A of the developing plate and dispense the specimen of the bottom of the well. Carried out, the same procedure for positive and negative control. Mix well incubates for 10 minutes at 37°C.

Antigen-Anti-Body reaction: Insert the card into the wells of row A. Containing specimen and control. Leave it for 37°C for 30 minutes. Near the end of 30 minutes, perforate the foiled of row B using perforator.

Row A absorbs the adhering liquid from the pointed tips of teeth on clear absorbent paper.
First wash: Insert the well of row B agitates vigorously for course of 2 minutes. Mean-time perforates the well C. Withdraw the card and absorbs liquid on absorbent paper.

Binding of conjugate: (Row C): Insert the card into the well of row C. Mix the card into the well and keep the card for 20 minutes at 37°C. After 20 minutes withdraw the card and absorb the adhering liquid.

Second wash (Row D): Insert the card into the row D repeatedly agitates during 2 minutes above step. After 2 minutes withdraw the card and absorb the liquid over absorbent paper.

Third Wash: Insert the card into the well of Row E. Repeatedly agitates during 2 minutes. Withdraw the card from Row E and absorb the card on absorbant paper.

Colour reaction (Row F): Insert the card into the well of row F. Incubate for 10 minutes at 37°C, after 10 minutes withdraw the card.

Stop reaction (Row E): Insert the card again into the row E. After 1 minute withdraw the card and allow it to dry in the air.

RESULT:
Positive Control must be produce two spots on the card tooth
Negative Control must be produce an upper spot (internal Control) The lower spot will either not appear or appear faintly without affecting the interpretation of the result
Each specimen tested must produce an upper spot (internal Control)

HERPES SIMPLEX VIRUS:
ELISA TEST HSV 1 and 2 IgM were detected by ELISA (Globe Diagnostic Srl. Italy):

Principle:
Microtiler strep well is coated with HSV-I and II specific antigen. The solid phase is first treated with the diluted sample. After washing step the IgM specifically banned to the antigen and detected with goat antihuman IgM antibodies conjugate to the peroxidise (HRP) after washing a substrate / chromogen is added an intensity of the generated colour is proportional to amount of the anti HSV-I and II IgM antibody in sample. The neutralizing reagent contains antihuman IgG blocking antibodies to prevent the assay from the interference due to the Rheumatoid factor and IgG.

Procedure:
1) Prediluted sample 1:50 with dilutent by mixing 10 μ sample with 500 μ dilutent in the mark dilution tube and control.
2) Cover the strep with adhesive film and incubate for 60 minute at 37° C.
3) Wash 4 to 5 times with 300 μ at diluted washing.
4) Add diluted tracer 100 μ in control and the test. After cover with adhesive keep it incubates for 60 minutes at 37° C.
5) Wash with 300 μ for 4-5 times.
6) Add the chromogen / substrate (1:1) of 100 μ to control and test and incubate at room temperature for 20 minutes. (Protect from light). Add the stop solution 100 μ.
7) Read the plate with ELISA automatic reader able to substrate the background at 620-630 nm and to the observance of samples and control at 450 nm.

**Calculation of Results:**

Calculate the mean value of the negative Control and the positive control (PC)

Calculate the cut-off value through the following formula

Cut-off = Mean NC + 0.250

Samples with OD 450 nm values higher than the cut-off are to be considered reactive to anti-HSV 1&2 IgM antibodies

Samples with OD 450 nm values lower than cutoff are to be considered not reactive.