

# An Evaluation of Newmarket Syphilis EIA I and EIA II

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## Background

The aim of this evaluation was to assess and compare the ability of Newmarket Laboratories Syphilis EIA I (60088) and EIA II (60093) to detect anti-treponemal antibodies in human serum and plasma. The EIA II assay was evaluated as part of a MHRA (Medicines and Healthcare products Regulatory Agency) evaluation. Newmarket Laboratories approached the Evaluations Unit to evaluate the EIA I assay privately.

## Description of the assays

Both EIAs use three treponemal recombinant antigens in a sandwich test to detect anti-treponemal IgG, IgM and IgA antibodies. The assay incorporates a 96 well microplate coated with the recombinant antigens. The conjugate contains the three recombinant treponemal antigens which are conjugated with horseradish-peroxidase (HRP). The conjugate binds to any antibodies in the test specimen which have bound to the solid phase recombinant proteins. Tetramethylbenzidine (TMB) in the substrate reacts with the HRP to produce a blue colour if anti-treponemal antibodies are present in the test specimen. Any blue colour reaction will turn yellow on the addition of the stopping solution (0.5M sulphuric acid). The reactivities are read photometrically at 450nm with a 630nm reference filter.

For the EIA I assay the conjugate and specimen are added sequentially, whereas for the EIA II assay the conjugate and specimen are added into the same well at the same time. All other stages are identical. See the *Appendix* (Figure 6) for the assay protocols and incubation times.

## Evaluation method and specimen panel

The evaluation followed a protocol that was agreed with the manufacturer. All equipment was available at the Evaluations Unit. The manufacturer was offered the opportunity to train the evaluator, but decided that this was not necessary. The assays were performed according to manufacturer's instructions. The microplates were read on a Bio-Tek EL808 plate reader, which was linked to a computer with KC4 software. Lot number (60088) was used for the EIA I evaluation, and lot number (60093) for the EIA II evaluation.

The sensitivity was assessed by testing each assay against a panel of 114 specimens from individuals with known disease stage and treatment status, along with 121 specimens from individuals whose disease stage and treatment status was unknown. The positive specimens were either serum or plasma. The specificity was assessed by testing each assay against 249 negative blood donor specimens (serum). Details of the specimen panel are shown in Table 1. A quality control was used throughout the evaluation to monitor inter-run variability. All specimens had less than two freeze-thaw cycles. False negative, false positive and discordant specimens were retested in duplicate.

Specimens that gave discordant results were tested by other supplementary assays, including Murex ICE Syphilis EIA (Abbott Laboratories, product code 500E-8E04-01), Newmarket TPHA (Newmarket Laboratories, product code 60005a), Serodia TPPA (Fujirebio, product code 9241) Mercia Syphilis IgM EIA (Microgen Bioproducts, product code M404), and INNO-LIA Syphilis (Innogenetics, product code K-1089).

Table 1. Specimen panel

| Sample category   | Number                    |
|---|---------------------------|
| 1. Blood donors' sera   | 249                       |
| 2. Positive samples from Impath-BCP (stages known)                  |                           |
| Primary syphilis  | 7 treated<br>16 untreated |
| Secondary syphilis  | 5 treated<br>4 untreated  |
| Early latent syphilis   | 4 treated<br>3 untreated  |
| Late latent syphilis  | 8 treated<br>4 untreated  |
| 3. Positive samples from CPHL (stages known)                        |                           |
| Primary syphilis  | 17 untreated              |
| Secondary syphilis  | 34 untreated              |
| Early latent syphilis   | 12 untreated              |
| <b>Total where disease stage and treatment status known = 114</b>   |                           |
| 4. Positive samples (syphilis stage not known)                      |                           |
| Profile specimens   | 78                        |
| SNBTS plasma  | 33                        |
| CPHL specimens  | 10                        |
| <b>Total where disease stage and treatment status unknown = 121</b> |                           |
| 5. QCRU quality control sample                                      | 1                         |
| <b>TOTAL (number of specimens)</b>                                  | <b>485</b>                |
| <b>Notes:</b>   |                           |
| BCP = Impath-BioClinical Partners Inc., USA                         |                           |
| CPHL = Central Public Health Laboratory, HPA, UK                    |                           |
| Profile = Profile Diagnostics Inc., USA                             |                           |
| SNBTS = Scottish National Blood Transfusion Service                 |                           |
| QCRU = Quality Control Reagents Unit, HPA, UK                       |                           |

## Results

### *Specificity*

The specificity was assessed by testing each assay against 249 blood donors. The EIA I assay had one initially reactive specimen, that was unreactive after retesting, giving a final specificity of 100% (Table 2). The EIA II assay had no reactive specimens, giving a specificity of 100%.

Table 2. Specificity

| Assay  | Number tested | Number initially reactive | Number repeatedly reactive | % Specificity (95% confidence intervals) |
|--------|---------------|---------------------------|----------------------------|--|
| EIA I  | 249           | 1                         | 0                          | 100 (98.5 - 100)                         |
| EIA II | 249           | 0                         | 0                          | 100 (98.5 - 100)                         |

### Sensitivity

Of the 114 specimens from individuals with known disease stage and treatment status, 112 were detected by EIA I, giving a sensitivity of 98.2%. The EIA II detected 113 of the 114 specimens giving a sensitivity of 99.1% (Table 3). Both EIAs repeatedly failed to detect a specimen from an individual who had untreated primary syphilis. The second specimen that was not detected by the EIA I assay, was also from an individual who had untreated primary syphilis.

Table 3. Sensitivity at different disease stages

| Assay  | Treatment Status | Number positive (OD/CO > 1.0) at different disease stages |           |              |             | % Sensitivity (95% confidence interval) |
|--------|------------------|---|-----------|--------------|-------------|---|
|        |                  | Primary   | Secondary | Early latent | Late latent |   |
| EIA I  | Untreated        | 31/33   | 38/38     | 15/15        | 4/4         | 98.2 (93.8 - 99.8)                      |
|        | Treated          | 7/7   | 5/5       | 4/4          | 8/8         |   |
| EIA II | Untreated        | 32/33   | 38/38     | 15/15        | 4/4         | 99.1 (95.2 - 100)                       |
|        | Treated          | 7/7   | 5/5       | 4/4          | 8/8         |   |

There were three specimens that initially gave weak reactions (OD/CO 1 - 2) in the EIA I assay, so these were repeated in duplicate. All but one still gave weak reactions and the one specimen with an initial OD/CO of 1.1, gave OD/COs of 0.83 and 0.86 after retesting in duplicate. This specimen was also from an individual who had untreated primary syphilis and therefore reduces the EIA I sensitivity to 97.4%. Due to the need to conserve specimen volume for further MHRA evaluations, low positives from the EIA II assay have not yet been repeated.

A summary of positive reactions and the overall sensitivity, when the disease stage and treatment status is unknown, is shown Table 4. Both EIAs gave a sensitivity of 100%

Table 4. Sensitivity when disease stage and treatment status was unknown

| Assay  | Number positive (OD/CO >1.0)<br>Total = 121 | % Sensitivity (95% confidence interval) |
|--------|---|---|
| EIA I  | 121   | 100 (97 - 100)                          |
| EIA II | 121   | 100 (97 - 100)                          |

On the basis of results from other syphilis tests (see Evaluation method), all discordant specimens were deemed to be positive. These results are shown in Table 8. The only specimen not confirmed positive by these methods was positive only by darkfield microscopy only. This specimen was from a patient that had been treated for primary syphilis; and was the only specimen not to be detected by the EIA II assay, when the disease stage was known.

### Distribution of initial reactivities

The distribution of initial reactivities for the 235 syphilis positive specimens and 249 specimens from blood donors are presented in Figure 2 and 3. The EIA I assay gave rise to one initial false reactive specimen, two initial false negative specimens and there were three positive specimens that had a low OD/CO ratio of 1 - 2. The EIA II gave rise to no false reactive specimens, one false negative specimen and there was one positive specimen that had a low OD/CO ratio of 1 - 2. Assays with good discrimination between positives and negatives have few, or no, specimens wrongly classified and few reactions in close proximity to the cut off. The range, mean and median OD/CO ratios for the positive and blood donor specimens for both assays are shown in Table 5.

Figure 2. Distribution of initial reactivities for the EIA I assay

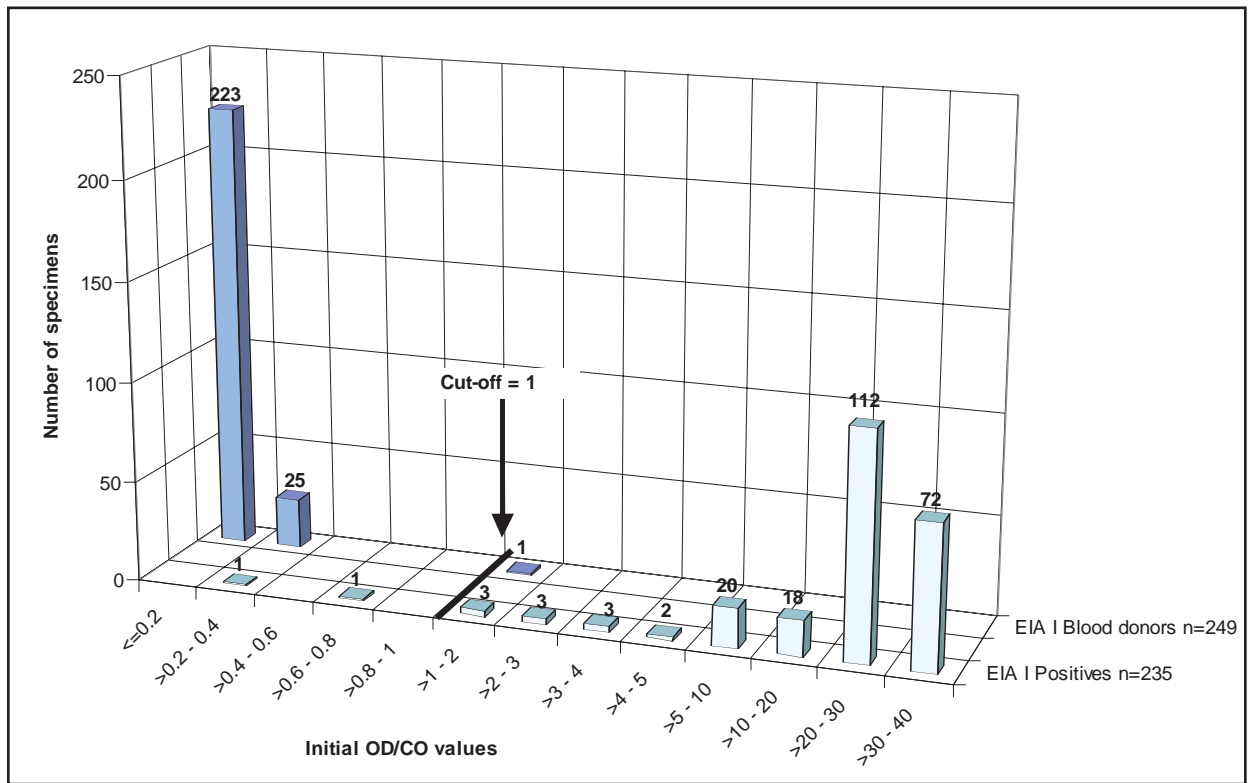


Figure 3. Distribution of initial reactivities for the EIA II assay

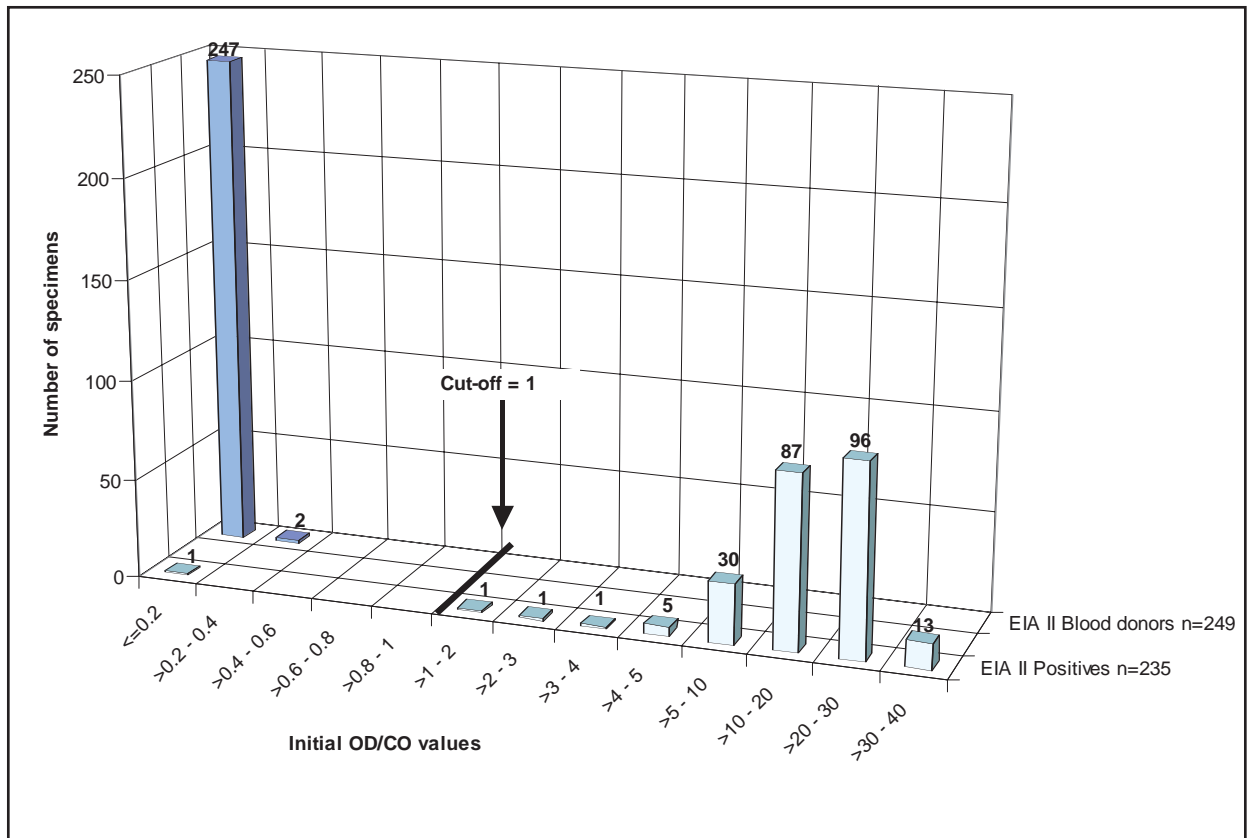


Table 5. Range, mean and median OD/CO ratios

| Specimen type | OD/CO           |      |        |                  |       |        |
|---------------|-----------------|------|--------|------------------|-------|--------|
|               | Newmarket EIA I |      |        | Newmarket EIA II |       |        |
|               | Range           | Mean | Median | Range            | Mean  | Median |
| Blood donors  | 0.10 - 1.69     | 0.17 | 0.16   | -0.01 - 0.38     | 0.1   | 0.09   |
| Positives     | 0.22 - 31.73    | 24.7 | 28.73  | 0.12 - 33.05     | 18.65 | 19.07  |

#### Quality Control Specimens

The inter-run reproducibility of the Newmarket Laboratories EIA I and EIA II was determined using a quality control sample that gave a reactivity within the linear dynamic range of the assay. The quality control used was the HPA anti-*Treponema pallidum* QC (catalogue number QCRSYPHC1). This sample was added sequentially along with the test specimens, three times on each plate. The QC sample was positioned at the start of the plate immediately after the kit controls, in the middle and at the end of the plate. Figures 4 and 5, and Table 6 show that both kits gave good reproducibility, with the EIA I assay giving slightly better reproducibility. The quality control that was positioned at the end of the microplate, always gave lower OD/CO ratios than the other two. Interestingly, the replicate that was positioned in the middle of the EIA I microplate always had a slightly higher OD/CO ratio than the first replicate. The three replicates from the EIA II assay gave decreasing reactivities from replicate one to three throughout all runs.

The HPA quality control sample was always within two standard deviations of the mean for each replicate over the six runs. All the manufacturer's assay controls for both assays were always within the specified limits described in the kit inserts.

Figure 4. Inter-run reproducibility of the Syphilis HPA QC on the EIA I assay

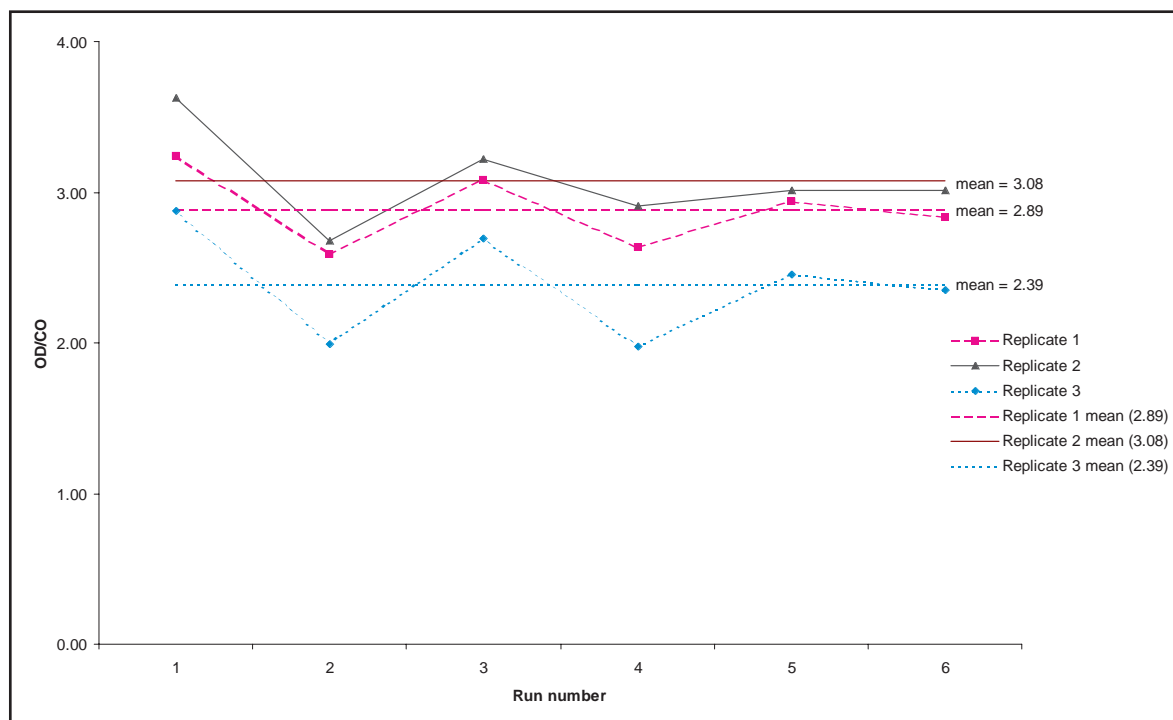


Figure 5. Inter-run reproducibility of the Syphilis HPA QC on the EIA II assay

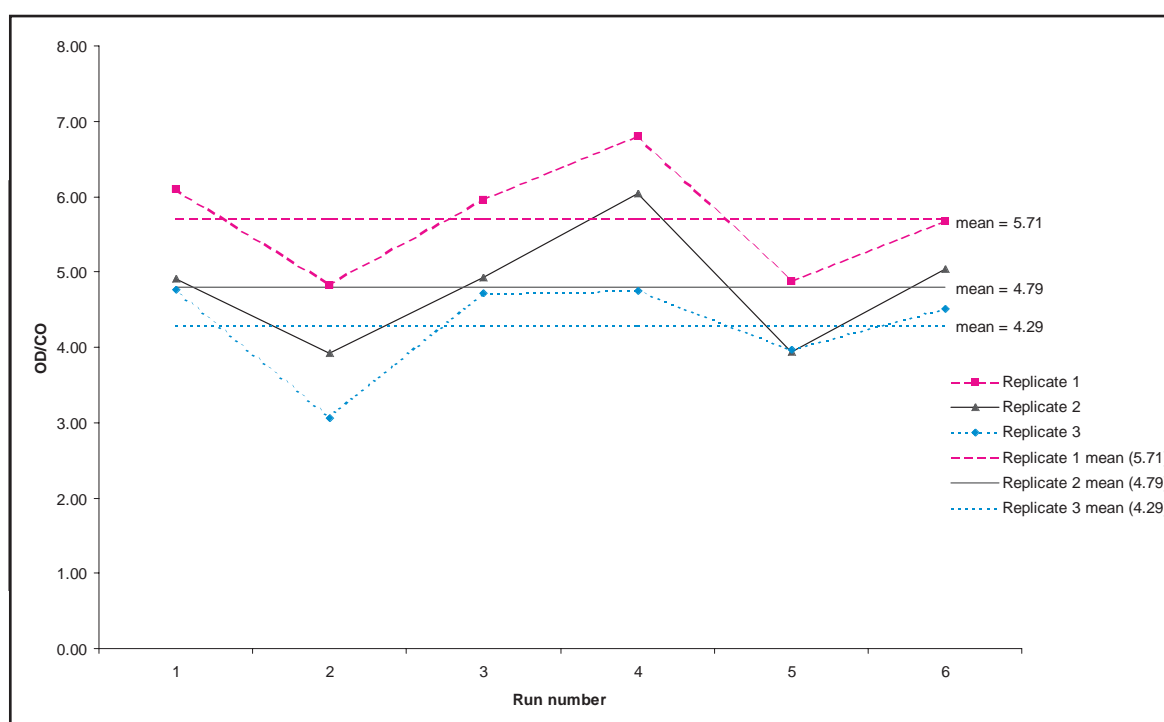


Table 6. Inter-run reproducibility

|                         | HPA QCRU (OD/CO) |             |             |             |             |             |
|-------------------------|------------------|-------------|-------------|-------------|-------------|-------------|
|                         | EIA I            |             |             | EIA II      |             |             |
|                         | Replicate 1      | Replicate 2 | Replicate 3 | Replicate 1 | Replicate 2 | Replicate 3 |
| Range                   | 2.59 - 3.24      | 2.68 - 3.62 | 1.97 - 2.88 | 4.84 - 6.81 | 3.91 - 6.04 | 3.06 - 4.76 |
| Mean                    | 2.89             | 3.08        | 2.39        | 5.71        | 4.79        | 4.29        |
| Median                  | 2.89             | 3.02        | 2.39        | 5.81        | 4.92        | 4.61        |
| No. of runs             | 6                | 6           | 6           | 6           | 6           | 6           |
| Standard deviation (sd) | 0.25             | 0.32        | 0.36        | 0.76        | 0.80        | 0.68        |
| -1sd                    | 2.63             | 2.75        | 2.03        | 4.95        | 4.00        | 3.62        |
| +1sd                    | 3.14             | 3.40        | 2.75        | 6.47        | 5.59        | 4.97        |
| -2sd                    | 2.38             | 2.43        | 1.66        | 4.19        | 3.20        | 2.94        |
| +2sd                    | 3.40             | 3.72        | 3.12        | 7.23        | 6.39        | 5.65        |

### Technical appraisal

Both assays were similar and very easy to use, a summary of the technical appraisal can be found in Table 7. It would be useful if the kit insert stated how much wash buffer should be used during washes. Information given on the handling of potential biological hazards was adequate, but there was no safety information either in the kit insert, or on the reagent packaging, describing the potential hazards of the chemical reagents used *e.g.* sulphuric acid, TMB/peroxide. The EIA II assay required one wash procedure per plate and contained two bottles of wash buffer (20x concentration). The EIA I assay required two wash procedures per plate and also contained two bottles of wash buffer, but at a lower concentration (10x). There was very little wash buffer left at the end of running the EIA I assay; this situation could be improved by placing the 20x concentrated wash buffer in the EIA I kit.

Table 7. Technical appraisal

|  | EIA I |   |      |   |   | EIA II |   |      |   |   |
|--|-------|---|------|---|---|--------|---|------|---|---|
|  | Good  |   | Poor |   |   | Good   |   | Poor |   |   |
| <b>Kit instructions</b>                      | 1     | 2 | 3    | 4 | 5 | 1      | 2 | 3    | 4 | 5 |
| Clarity                                      |       | x |      |   |   |        | x |      |   |   |
| Presentation                                 | x     |   |      |   |   | x      |   |      |   |   |
| Content                                      |       |   | x    |   |   |        |   | x    |   |   |
| Safety instructions                          |       |   |      | x |   |        |   |      | x |   |
| <b>Kit and reagent packaging / labelling</b> |       |   |      |   |   |        |   |      |   |   |
| Clear identification                         | x     |   |      |   |   | x      |   |      |   |   |
| Labelling                                    | x     |   |      |   |   | x      |   |      |   |   |
| Reagent packaging                            | x     |   |      |   |   | x      |   |      |   |   |
| Safety ( <i>i.e.</i> contains sodium azide)  |       |   |      | x |   |        |   |      | x |   |
| <b>Ease of use</b>                           |       |   |      |   |   |        |   |      |   |   |
| Performing test                              | x     |   |      |   |   | x      |   |      |   |   |
| Sample preparation                           | x     |   |      |   |   | x      |   |      |   |   |
| Reagent preparation                          | x     |   |      |   |   | x      |   |      |   |   |

## Conclusion

The final specificities for both the EIA I and EIA II was 100%.

When the disease stage and treatment status were known the EIA I and EIA II gave initial sensitivities of 98.2% and 99.1 % respectively. The final sensitivity for the EIA I assay was 97.4%. When the disease stage and treatment status was unknown the EIA I and EIA II assays gave sensitivities of 100%.

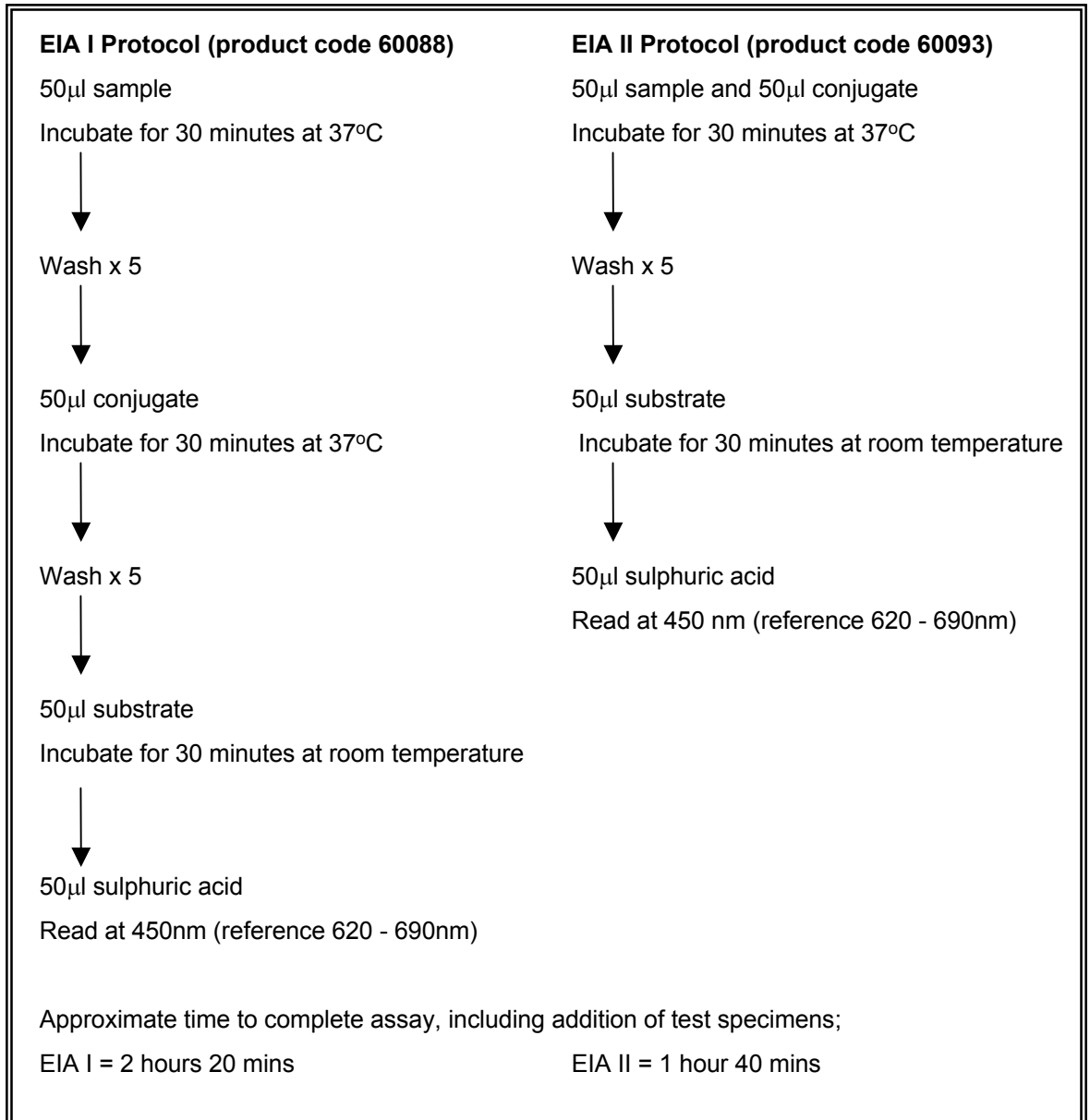
The EIA II assay was better than the EIA I assay at detecting the early primary specimens.

Both kits were similar and very easy to use. The EIA II assay could be completed more rapidly since the conjugate and test specimen was added at the same time. All the manufacturer's controls and the external quality controls gave the expected results on all runs.

An MHRA report on the evaluation of syphilis agglutination assays is in preparation and will be available early in April 2004. An MHRA evaluation of syphilis EIAs is also in progress. Once the reports are published it will be possible to compare the performance of Newmarket EIA I and EIA II assays with other syphilis assays.

## Appendix

**Figure 6. Assay protocols**



Appendix

Table 8. Discordant specimen results and supplementary testing

| Evaluations Unit No. | Disease stage | Treatment Status | Newmarket assay evaluation data (OD/CO) |           |           |            |            |            | Supplementary data |            |                |              |         |       |       |      |        |
|----------------------|---------------|------------------|---|-----------|-----------|------------|------------|------------|--------------------|------------|----------------|--------------|---------|-------|-------|------|--------|
|                      |               |                  | Newmarket assay evaluation data (OD/CO) |           |           |            |            |            | EIAs (OD/CO)       |            | Newmarket TPHA | Serodia TPPA | Innolia |       |       |      | Result |
|                      |               |                  | EIA I (a)                               | EIA I (b) | EIA I (c) | EIA II (a) | EIA II (b) | EIA II (c) | MUREX ICE          | Mercia IgM |                |              | TpN47   | TpN17 | TpN15 | TmpA |        |
| 02S0040*             | Primary       | Untreated        | 0.22                                    | 0.15      | 0.15      | 0.12       | 0.08       | 0.08       | 0.32               | 0.20       | 0              | 0            | -       | -     | -     | -    | NEG    |
| 03S0281              | Primary       | Untreated        | 1.10                                    | 0.83      | 0.86      | 2.91       |            |            | 1.29               | 1.19       | 2              | 2            | 1       | +/-   | -     | -    | POS    |
| 03S0287              | Primary       | Untreated        | 0.71                                    | 0.52      | 0.64      | 1.91       |            |            | 0.72               | 1.49       | 0              | 2            | +/-     | +/-   | +/-   | -    | POS    |

When OD/CO > 1, the result is positive  
 Scoring for the TPHA and TPPA; 0 = negative, 1 = indeterminate, 2 = weakly reaction, 3 = medium reaction, 4 = strong reaction  
 Innolia - scores are the intensities of the antigen lines when compared with the control lines  
 \* Positive by darkfield microscopy  
 \*\*Samples not yet tested by IgM EIA and Innolia LIA

Table 9. Reactive blood donor specimens

| Evaluations Unit No. | Newmarket assay evaluation data (OD/CO) |           |           |        |            |            |
|----------------------|---|-----------|-----------|--------|------------|------------|
|                      | EIA I                                   | EIA I (2) | EIA I (3) | EIA II | EIA II (2) | EIA II (3) |
| 03S0192              | 1.69                                    | 0.15      | 0.13      | 0.11   |            |            |