An end to subjective RPR reading? Development of a novel nontreponemal immunoassay for syphilis

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1. Purpose and Goals

- **Syphilis is a growing public health concern**, with the rate of congenital syphilis currently over ten times the targeted reduction goal set by the WHO.
- Current nontreponemal testing for active syphilis infection is **laborious and subjective**, which can lead to inter- and intra-laboratory variability.
- We present the **development of a fully automated nontreponemal immunoassay** using a novel approach to anchor the nontreponemal lipoidal antigen to a solid support.
- Our goal is to develop a **fully automated solution** for nontreponemal testing that detects reagin antibodies in patients, indicating an active syphilis infection.

2. Abstract

Background: Syphilis is a serious public health concern, with eight million new cases diagnosed globally in 2022.[1] Congenital syphilis resulting from vertical transmission can cause severe adverse pregnancy outcomes. The WHO's "triple elimination initiative" aims to reduce the current rate of vertical transmission by over 10-fold.[2] Nontreponemal tests indicate an active syphilis infection and are commonly done manually through RPR or VDRL methods. Results can be highly subjective, as they are determined by visual interpretations that require trained technicians, causing delays in treatment. To meet the WHO initiative, nontreponemal assays must be transitioned to automated systems with quick turnaround times.

Methods: We have identified a novel method to attach a lipoidal antigen to a magnetic bead that allows the antigenic component of the nontreponemal assay to be integrated into an automated immunoassay with a turnaround time of under 30 minutes. Using human serum samples characterized as reactive, and not reactive, we present a comparison of our novel immunoassay against traditional RPR cards (Arlington Scientific) and to an automated card reading platform (Gold Standard's AIX1000).

Results: Our nontreponemal immunoassay has an overall agreement of 85.5% to the AIX1000 system. Of reactive samples with moderately low to very high antibody levels (i.e. titers 1:2 to 1:1024), 97% are reactive on our assay. Our assay performs equivalently to RPR card tests when reactive samples contain the lowest level of reagin antibodies (1:1 titer), which are the most subjective and difficult to identify samples using card-based methods. Negative samples fall within a low, acceptable range on our assay. Using a single dilution, 91% of all reactive samples are classified at an acceptable titer when compared to the predicate. Ongoing work aims to improve recognition at the lowest titer.

Conclusion: We have developed a novel nontreponemal immunoassay that successfully identifies moderately low to very high reactive syphilis samples and correctly classifies titer with no manual dilutions. Converting a historically manual test to a fully automated immunoassay with a quick turnaround time can improve treatment timeframes and potentially decrease rates of congenital syphilis.

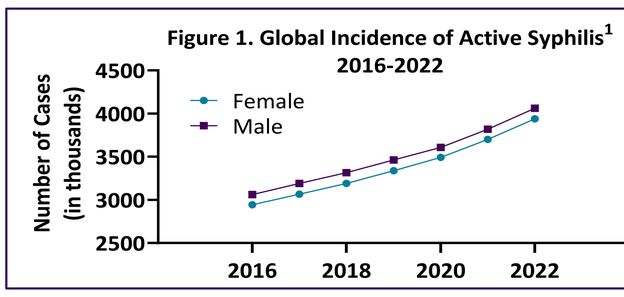
3. Background

Syphilis is a sexually transmitted infection cause by the *Treponema pallidum* bacterium. An increase in global incidence led to **eight million new cases** in 2022 (Figure 1).[1] Syphilis is primary transmitted through contact with sores or lesions and can be transmitted vertically between an infected mother and unborn baby, termed congenital syphilis, which can result in severe health complications for the child. Nearly **700,000 cases of congenital syphilis** were reported across the globe in 2022. In the US, 2023 saw the largest number of cases since 1992.[3] This continued rise in cases of syphilis and congenital syphilis has led to serious public health concerns.

Syphilis is diagnosed through a multi-test algorithm consisting of **treponemal and nontreponemal tests**. Treponemal tests detect specific antibodies produced against the bacteria and have been widely transitioned to numerous automated platforms. However, these tests remain reactive after successful treatment, **rendering them ineffective to diagnose an active infection** or to monitor response to antibiotic treatment. **Nontreponemal tests detect non-specific reagin antibodies** that are produced during the immune system's response to cellular damage caused by the syphilis infection. Reagin antibodies are known to be present during active infection and subside with treatment, allowing nontreponemal tests to **indicate an active infection and monitor treatment response**.

Nontreponemal testing centers around the historically manual **rapid plasma reagin (RPR) card test** (Figure 2). Manual RPR tests are **subject to high variability** due to the subjective visual reading of the charcoal spots. Nontreponemal tests are **semi-quantitative**, as reactive samples are diluted two-fold on the RPR card to determine the endpoint titer. A four-fold decrease in endpoint titer is indicative of successful treatment. Recent advances have included development of automated card readers for these charcoal-based agglutination card tests, but they rely on the same macroscopic chemistry that is difficult to transition into a fully automated immunoassay, and they require stand alone instrumentation.

Figure 1. Global Incidence of Active Syphilis[1] 2016-2022



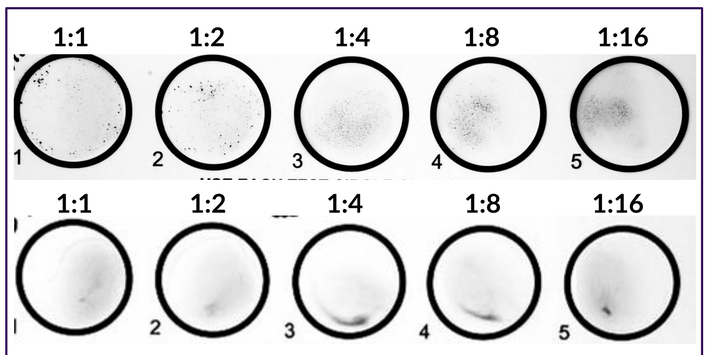
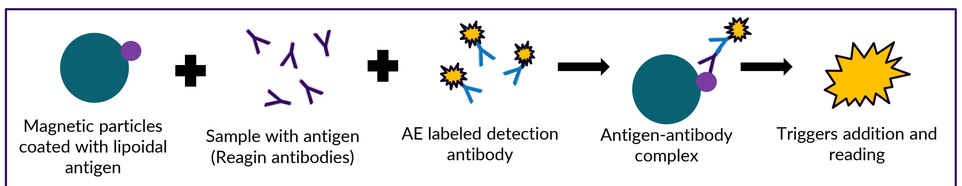


Figure 2. RPR card test examples. Nontreponemal testing centers around the rapid plasma reagin (RPR) card test, which utilizes charcoal particles and micellular lipoidal antigens to allow for macroscopic visualization of the agglutination reaction in the presence of reagin antibodies. Reactive samples are diluted two-fold on the RPR card. The last titer deemed to be reactive is the reported titer and a four-fold decrease in titer is indicative of successful treatment. The example on top is a high reactive, with visible carbon aggregates through the 1:16 dilution. The example on the bottom is a low reactive, demonstrating a lack of carbon aggregates that can lead to challenges in interpretations of weakly reactive samples.

There is an **unmet need** in the field of syphilis diagnostics to develop a **fully automated nontreponemal immunoassay** that can be readily integrated into hospital lab settings.

4. Methods

Lipoidal antigen for reagin antibody detection. Lipids akin to those in the current RPR card test (cholesterol, lecithin, and cardiolipin) are stabilized in a protein assisted complex that forms the lipoidal antigen required for reagin antibody capture (Scheme 1). This lipoidal antigen moiety is then attached to a magnetic bead for use on an automated instrument. The novel method to generate the lipoidal antigen and attach it to a magnetic bead has a patent pending.



Scheme 1. Assay steps. The lipoidal antigen is generated through a novel method, allowing for attachment to a magnetic bead (patent pending). The lipoidal antigen captures the reagin antibody in serum samples, which is then detected with an enzyme conjugated secondary antibody. After incubation and washing steps, a chemiluminescent substrate is added.

Automated immunoassay. The assay is run on the KleeYa® platform (Figure 3, STRATEC SE., Birkenfeld, Germany). A patient serum sample is loaded onto the instrument, and an index value is generated based on the relative light intensity of the sample to a cut off control. The index value is proportional to the amount of captured reagin antibody and is used to determine the titer of each sample, with no manual dilutions necessary. All sample and reagent handling is completed by the instrument at a rate of 120 tests/hour and with a time to result under 30 minutes.

Clinical correlation and sample linearity. A cohort of 225 human serum samples (n=137 reactive) and (n=88 nonreactive) that were previously tested on Gold Standard's AIX1000 automated RPR instrument were tested on our novel assay. Samples with available volume were tested in-house on the Arlington Scientific's RPR card test. The reported titers by AIX1000 were used to determine the relationship of index value to titer. Samples were diluted linearly using two-fold dilutions to determine the span of the assay.

Figure 3. KleeYa® instrument. This assay is performed on the KleeYa® platform (STRATEC SE., Birkenfeld, Germany), a chemiluminescence analyzer that allows for complete automation of an immunoassay. The platform performs the sample processing, reagent dispensing, incubations, wash processes and sample measurement evaluation. The KleeYa® platform is semi-open access, with ALPCO's suite of immunoassay solutions, including our treponemal assay, able to run on a single platform.

Correlation to Predicate

Reactive samples by current ALPCO nontreponemal immunoassay

Sample	ALPCO Index	Titer ALPCO	Titer AIX1000
P117	542.4	1:256+	1:128
P118	448.7	1:256+	1:128
P111	447.8	1:256+	1:1024
P113	356.4	1:256+	1:256
P120	355.5	1:256+	1:128
P110	318.9	1:256+	1:1024
P121	224.0	1:128	1:128
P132	211.3	1:128	1:32
P112	199.8	1:128	1:256
P119	170.8	1:128	1:128
P139	155.3	1:128	1:16
P116	145.3	1:128	1:128
P115	141.9	1:128	1:128
P127	127.4	1:64	1:64
P122	116.8	1:64	1:128
P124	109.8	1:64	1:64
P126	104.4	1:64	1:64
P130	96.1	1:64	1:32
P74	94.2	1:64	1:32
P134	88.1	1:64	1:32
P114	86.2	1:64	1:256
P125	77.8	1:64	1:64
P135	76.9	1:64	1:32
P143	73.5	1:64	1:16
P131	65.5	1:64	1:32
P129	63.4	1:32	1:32
P133	63.2	1:32	1:32
P137	55.5	1:32	1:16
P123	51.7	1:32	1:64
P142	42.5	1:32	1:16
P128	36.5	1:32	1:32
P140	32.7	1:32	1:16
P138	28.9	1:16	1:16
P59	25.3	1:16	1:8
P141	24.6	1:16	1:16
P66	20.0	1:16	1:4
P97	18.6	1:16	1:8
P43	15.8	1:8	1:16
P159	13.9	1:8	1:4
P148	12.6	1:8	1:8
P41	10.7	1:8	1:4
P149	10.6	1:8	1:8
P156	10.6	1:8	1:4
P150	8.8	1:8	1:8
P158	8.7	1:8	1:4
P136	7.9	1:4	1:16
P38	7.8	1:4	1:4
P79	6.9	1:4	1:8
P40	6.8	1:4	1:1
P76	6.6	1:4	1:8
P152	6.4	1:4	1:8
P162	6.1	1:4	1:4
P101	5.9	1:4	1:8
P145	5.5	1:4	1:8
P82	4.7	1:4	1:2
P50	4.5	1:4	1:8
P95	4.4	1:4	1:8
P153	3.9	1:2	1:8
P151	3.7	1:2	1:8
P163	3.7	1:2	1:4
P52	3.7	1:2	1:2
P64	3.7	1:2	1:1
P29	3.6	1:2	1:2
P23	3.5	1:2	1:1
P91	3.4	1:2	1:4
P68	3.3	1:2	1:2
P34	3.3	1:2	1:2
P72	3.3	1:2	1:1
P85	3.1	1:2	1:1
P6	3.0	1:2	1:8
318	2.9	1:2	NR
P99	2.9	1:2	1:1
P32	2.8	1:2	1:2
P155	2.8	1:2	1:4
P22	2.8	1:2	1:2
P69	2.7	1:2	1:2
P160	2.7	1:2	1:4
P87	2.5	1:2	1:4
P19	2.5	1:2	1:1
P21	2.4	1:2	1:2
P56	2.4	1:2	1:2
P25	2.4	1:2	1:2
P46	2.3	1:2	1:2
P4	2.2	1:2	1:2
P84	2.1	1:2	1:1
P9	2.1	1:2	1:4
P27	2.1	1:2	1:2
347	2.0	1:2	NR
P77	1.9	1:1	1:1
P42	1.9	1:1	1:1
331	1.9	1:1	NR
P45	1.7	1:1	1:2
P100	1.7	1:1	1:2
P88	1.7	1:1	1:2
P61	1.6	1:1	1:1
P71	1.6	1:1	1:2
P8	1.5	1:1	1:1
P157	1.5	1:1	1:4
P24	1.5	1:1	1:1
P3	1.5	1:1	1:1
527	1.5	1:1	NR
325	1.5	1:1	NR
P53	1.5	1:1	1:2
P17	1.4	1:1	1:2
P37	1.4	1:1	1:2
P75	1.4	1:1	1:2
P35	1.4	1:1	1:2
P60	1.3	1:1	1:2
P39	1.3	1:1	1:1
P30	1.3	1:1	1:1
533	1.3	1:1	NR
P154	1.3	1:1	1:4
P36	1.3	1:1	1:1
P93	1.2	1:1	1:1
P26	1.2	1:1	1:2
P73	1.2	1:1	1:1
P48	1.2	1:1	1:1
P65	1.2	1:1	1:1
P103	1.1	1:1	1:1
525	1.1	1:1	NR
329	1.1	1:1	NR
313	1.1	1:1	NR
314	1.1	1:1	NR
P49	1.1	1:1	1:2
P80	1.1	1:1	1:1
P7	1.1	1:1	1:1
526	1.0	1:1	NR
540	1.0	1:1	NR
P51	1.0	1:1	1:1
352	1.0	1:1	NR
P57	1.0	1:1	1:1

Non-reactive samples by current ALPCO nontreponemal immunoassay

Sample	ALPCO Index	Titer ALPCO	Titer AIX1000
519	1.0	NR	NR
P78	1.0	NR	1:1
350	1.0	NR	NR
614	1.0	NR	NR
359	0.9	NR	NR
348	0.9	NR	NR
P2	0.9	NR	1:1
P47	0.9	NR	1:1
337	0.9	NR	NR
332	0.9	NR	NR
328	0.9	NR	NR
334	0.9	NR	NR
336	0.9	NR	NR
P67	0.9	NR	1:1
360	0.9	NR	NR
628	0.9	NR	NR
321	0.9	NR	NR
P83	0.9	NR	1:1
P161	0.9	NR	1:4
617	0.9	NR	NR
339	0.8	NR	NR
613	0.8	NR	NR
537	0.8	NR	NR
317	0.8	NR	NR
616	0.8	NR	NR
523	0.8	NR	NR
341	0.8	NR	NR
323	0.8	NR	NR
326	0.8	NR	NR
P94	0.8	NR	1:1
316	0.8	NR	NR
322	0.8	NR	NR
349	0.8	NR	NR
P5	0.8	NR	1:1
P55	0.8	NR	1:1
P54	0.8	NR	1:1
P147	0.8	NR	1:8
P92	0.8	NR	1:2
P62	0.8	NR	1:1
P58	0.7	NR	1:1
335	0.7	NR	NR
625	0.7	NR	NR
327	0.7	NR	NR
539	0.7	NR	NR
344	0.7	NR	NR
351	0.7	NR	NR
345	0.7	NR	NR
605	0.7	NR	NR
534	0.7	NR	NR
357	0.7	NR	NR
342	0.7	NR	NR
601	0.7	NR	NR
538	0.7	NR	NR
P70	0.7	NR	1:1
535	0.7	NR	NR
355	0.7	NR	NR
P63	0.7	NR	1:1
624	0.6	NR	NR
530	0.6	NR	NR
319	0.6	NR	NR
521	0.6	NR	NR
P102	0.6	NR	1:1
353	0.6	NR	NR
P96	0.6	NR	1:1
P98	0.6	NR	1:1
356	0.6	NR	NR
609	0.6	NR	NR
330	0.6	NR	NR
315	0.6	NR	NR
338	0.6	NR	NR
608	0.6	NR	NR
627	0.6	NR	NR
611	0.6	NR	NR
536	0.6	NR	NR
619	0.6	NR	NR
606	0.6	NR	NR
358	0.6	NR	NR
604	0.6	NR	NR
602	0.6	NR	NR
528	0.6	NR	NR
312	0.6	NR	NR
532	0.5	NR	NR
524	0.5	NR	NR
522	0.5	NR	NR
603	0.5	NR	NR
320	0.5	NR	NR
615	0.5	NR	NR
343	0.5	NR	NR
622	0.5	NR	NR
333	0.5	NR	NR
520	0.5	NR	NR
354	0.5	NR	NR
324	0.5	NR	NR
531	0.4	NR	NR

		AIX1000 R	AIX1000 NR	AIX1000 Total
ALPCO	R	118	13	131
	NR	19	75	94
	Total	137	88	225

PPA: 86.1%, NPA: 85.2%

Figure 4. Alignment of ALPCO's automated nontreponemal immunoassay to samples characterized by Gold Standard's AIX1000. Samples previously tested on the AIX1000 instrument were tested on the current design of ALPCO's automated nontreponemal assay.

NR: Not reactive; pink shade is indicative of sample classification as reactive, grey shade is indicative of sample classification as nonreactive. Index values are shaded to indicate classification on ALPCO's assay, while sample names are shaded to indicate classification on AIX1000.

ALPCO's current automated nontreponemal immunoassay performs with a positive percent agreement (**PPA) of 86.1%** and negative percent agreement **(NPA) of 85.2%** using samples previously characterized by the AIX1000 system.

Of note, 92/95 (**96.8%**) samples classified as reactive with a **1:2 titer or higher** by AIX1000, were also classified as reactive on the ALPCO assay, suggesting nearly all the discordance lies in the low positive 1:1 titer zone.

Titer Classification

ALPCO assay \ AIX1000	NR	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
NR	**75**	16	1	1	1							
1	11	**18**	12	2								
2	2	7	**13**	6	3							
4		1	1	**2**	7	1						
8				4	**3**	1						
16				1	2	**2**						
32						3	**3**	1				
64						1	5	**4**	1	1		
128						1	1		**4**	1		
256+									3	**1**		2
Agreement within +/- 1 titer		60%	96%	75%	75%	67%	89%	100%	100%	67%	NA	100%

Figure 5. Titer classification comparison between ALPCO's nontreponemal assay and AIX1000 titer classification. The endpoint titers of each sample were determined. Alignment within +/- 1 titer is an accepted practice in titer determination. The low % agreement in the 1:1 titer bin is driven by the false nonreactive samples, or samples classified as reactive by AIX100 but nonreactive by ALPCO. While agreement to predicate titer allows us to understand the capability of the ALPCO assay to report a titer, it is known that endpoint titers between currently available assays may vary by test and by laboratory.[4] Thus, standard practice is to use the same nontreponemal test when monitoring treatment response.

Overall, the ALPCO assay and AIX1000 agree on sample titer **within +/- 1 dilution in 83%** of the tested samples.

Sample 1 Dilution	ALPCO Index	ALPCO Titer	%Recovery
Neat	171	1:128	
2	93.1	1:64	92%
4	55.7	1:32	83%
8	28.2	1:16	99%
16	15.3	1:8	92%
32	7.53	1:4	102%
64	4.27	1:4	88%
128	2.66	1:2	80%
256	1.80	1:1	74%

Sample 2 Dilution	ALPCO Index	ALPCO Titer	%Recovery
Neat	468	1:256+	
2	268	1:256+	87%
4	145	1:128	93%
8	85.5	1:64	85%
16	42.3	1:32	101%
32	23.8	1:16	89%
64	11.3	1:8	106%
128	6.47	1:4	87%
256	3.48	1:2	93%
512	2.34	1:2	74%
1024	1.50	1:1	78%

Figure 6. Examples of dilution linearity of reactive samples. Samples were diluted 2-fold, in a manor similar to RPR titering, and tested on the ALPCO assay. Percent recovery was calculated by dividing the signal for each dilution by one-half of the signal from the prior dilution.

Within the analytical range of the assay, a 2-fold dilution generally results in an index value that puts the sample in the next titer, akin to a manual RPR card. The neat dilution and the dilution at which the sample crosses into the 1:1 zone are within +/1 titer. Percent recoveries are all within 30% of expected, with both samples resulting in an average recovery of 89%.

The current assay design has an analytical range spanning from the equivalent of **1:1 to 1:256 titers**, using just one sample result. The assay performs similarly to RPR cards in that a two-fold dilution results in a decrease of one titer bin.

Taken together, this data supports the ALPCO assay can be used to monitor treatment, **as a four-fold dilution in titer is able to be differentiated.**

The 1:1 Conundrum in RPR Testing

(a) Samples characterized reactive with 1:1 titer on AIX1000		
ALPCO Assay	R	26
	NR	16
	Total	42

% Agreement: 62%

(b) Samples characterized reactive with 1:1 titer on AIX1000		
In-house RPR card	R	17
	NR	10
	Total	27

% Agreement: 63%

(c) Samples characterized reactive with 1:1 titer on ALPCO assay		
In-house RPR card	R	23
	NR	14
	Total	37

% Agreement: 62%

Figure 7. Low titer (1:1) reactive samples compared on three different methods. Samples with enough volume were tested in house on an RPR card from Arlington Scientific, Inc. (ASI). This subset of samples were compared for reactivity on (a) AIX1000 vs ALPCO's assay, (b) AIX1000 and manual RPR card, and (c) manual RPR card and ALPCO's assay. The similarities in agreement highlight the difficult nature of detecting the lowest reactive samples.

The main area of discordance in our assay is the zone of reactive samples with 1:1 titers, with 11/13 (84.6%) of false reactive samples being classified as 1:1 on ALPCO's assay and 16/19 (84.2%) of the false nonreactive samples being classified as 1:1 on the AIX1000.

These results highlight the **variability in the 1:1 titer range**.

6. Conclusion and Future Directions

We have developed a nontreponemal immunoassay on a fully automated platform that can be run in tandem with ALPCO's treponema assay, or any other assay in the ALPCO test suite. Our current assay design boasts an 85.8% overall agreement to Gold Standard's AIX1000 classification of reactivity, with nearly 97% concordance for reactive samples over a 1:2 titer. Sample dilutions suggest this assay is appropriate for monitoring treatment response, as two-fold dilutions result in a sample decreasing in index by one titer bin. Future directions are focused on improving alignment at the lower end of the assay, between reactive samples with 1:1 titers and non-reactive samples; however, the agreement of detecting low reactive samples is similar between the AIX1000 system and either in-house manual RPR cards or the ALPCO assay.

7. References

1. Global HIV, Hepatitis and STIs Programmes (HHS), WHO, 2024.
2. Country guidance for planning triple elimination of mother-to-child transmission of HIV, syphilis and hepatitis B virus programmes. Geneva: World Health Organization and the United Nations Children's Fund (UNICEF), 2025.
3. Centers for Disease Control and Prevention. Sexually Transmitted Infections Surveillance 2023. Atlanta: US Department of Health and Human Services; 2024.
4. Papp JR, Park IU, Fakile Y, Pereira L, Pillay A, Bolan GA. CDC Laboratory Recommendations for Syphilis Testing, United States, 2024. MMWR Recomm Rep 2024;73(No. RR-1):1–32.