

Two Sides of the Same Coin: Development and Integration of Nontreponemal and Treponemal Immunoassays on a Single Automated System

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1. Purpose and Goals

- Syphilis remains a growing public health concern**, with the rate of congenital syphilis currently over ten times the targeted reduction goal set by the WHO.
- 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 integrate treponemal and nontreponemal testing onto a **single automated platform**, enabling assessment of both *T. pallidum* exposure and disease activity within a unified workflow that can be readily integrated into clinical laboratory settings.

3. Background

Syphilis is a sexually transmitted infection caused by the *Treponema pallidum* bacterium. Global incidence continues to rise, with **eight million new cases reported** in 2022, including nearly **700,000 cases of congenital syphilis** (Figure 1).^{1,2} In the US, 2023 marked the highest number of reported cases since 1992, highlighting a growing public health concern.³

Syphilis is diagnosed through a multi-test algorithm consisting of **treponemal and nontreponemal tests** (Figure 2). Treponemal tests detect *T. pallidum*-specific antibodies and provide evidence of exposure to the pathogen, while nontreponemal tests detect reagin antibodies produced in response to cellular damage associated with active infection. Nontreponemal titers can decline following treatment, making these useful tests for assessing disease activity and monitoring treatment response. Together, these two testing modalities provide evidence of both *T. pallidum* exposure and disease activity needed for syphilis diagnosis and patient management.

Treponemal testing has increasingly transitioned to **automated immunoassay platforms**, whereas nontreponemal testing has remained largely dependent on the **manual rapid plasma reagin (RPR) card test**, which requires serial dilution and visual interpretation of macroscopic agglutination. Automated RPR readers have improved workflows but are typically standalone instruments that retain the underlying agglutination chemistry and reliance on visual assessment of the reaction.

A **fully automated, integrated immunoassay platform for both treponemal and nontreponemal testing** represents an opportunity to streamline syphilis testing and provide evidence of pathogen exposure and disease activity in a single workflow.

There is an **unmet need** in syphilis diagnostics for a **fully automated, single-platform syphilis testing method that integrates both treponemal and nontreponemal testing** for streamlined integration into clinical laboratory workflows.

Background: Syphilis is a serious public health concern, with 8 million new cases diagnosed globally in 2022. It is diagnosed through a multi-test algorithm consisting of a treponemal test to detect bacterium-specific antibodies, and a nontreponemal (NT) test to detect nonspecific reagin antibodies produced in response to host cellular damage. Treponemal tests are typically automated and provide objective results, whereas manual NT tests are known to be highly subjective, relying on visual (human or camera-based) interpretations of card-based carbon particle agglutination tests. Currently, there are no commercially available systems that can simultaneously and objectively run both treponema and NT tests from the same sample. This is a critical unmet need in modern STI testing clinics that would allow for faster diagnosis and treatment with greater consistency.

Method: We developed 1) a Treponema assay utilizing specific membrane proteins of the bacterium as the capture antigen and 2) a NT assay using a novel method to attach the lipoidal reagin antigen to magnetic beads. Using 200 characterized human serum samples, we compared our ALPCO CLIA Treponema and NT immunoassays against well-established methods such as DiaSorin's LIAISON Treponema Screen assay and Gold Standard's AIX1000 RPR assay.

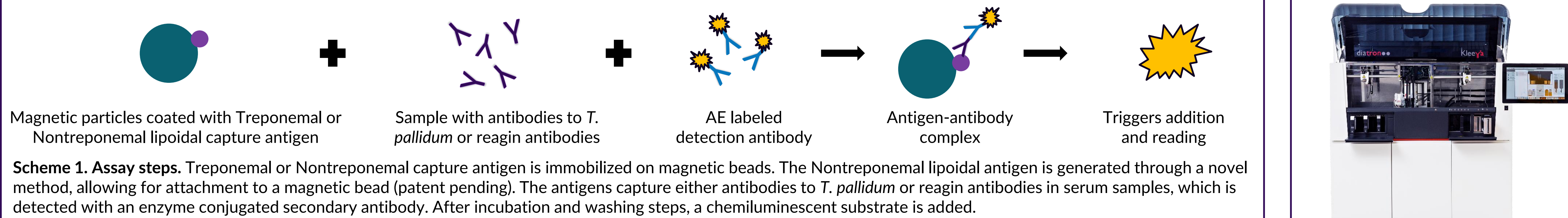
Results: The PPA and NPA of our Treponemal assay are both 99% relative to the DiaSorin system. Our NT immunoassay showed 85.5% overall agreement to the AIX1000 system, and our assay enables titer prediction without the need for serial dilution. Among reactive samples with antibody titers $\geq 1:2$, 97% were correctly identified as reactive. Our assay performs equivalently to RPR card tests at the low 1:1 titer of reagin antibody, where interpretation is most variable with agglutination-based methods. Negative samples fall within an acceptable range on our NT assay.

Conclusion: We developed both Treponema and NT assays on our automated ALPCO CLIA platform that have comparable clinical performance with established methods. Importantly, both assays can be run on a single system using one sample without manual dilution or subjective interpretation. A single platform workflow will consolidate syphilis testing resulting in faster diagnosis and treatment initiation, thus significantly advancing syphilis diagnostics.

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.

Treponema antigen for antibody detection. Recombinant *Treponema pallidum* membrane proteins are immobilized on magnetic beads to capture anti-*T. pallidum* antibodies from patient specimens. Captured IgG and IgM are detected using an IgG+IgM detector antibody, enabling total antibody detection. The same detector system is utilized in the nontreponemal assay, providing a common detection system across both assays.



Automated immunoassay. Both Treponemal and nontreponemal immunoassays are run on the KleeYa[®] platform (Figure 4, STRATEC SE., Birkenfeld, Germany). A patient serum sample is loaded onto the instrument, which generates an index value based on the relative light intensity of the sample to a cut off control. Index values are proportional to captured antibody levels, enabling automated titer determination without manual dilutions for the nontreponemal assay and reflecting *T. pallidum* antibody levels in the treponemal assay. Fully automated sample and reagent handling supports 60 tests/hour for treponemal testing and 120 tests/hour for nontreponemal testing.

Clinical correlation. A cohort of serum samples characterized using the DiaSorin LIAISON Treponema Screen and Gold Standard's AIX1000 automated RPR instrument were tested on ALPCO's Treponemal and Nontreponemal immunoassays (Figure 5). Samples with available volume were also tested in-house on Arlington Scientific's RPR card test. The reported titers by AIX1000 were used to determine the relationship of index value to titer. An additional cohort of highly characterized reactive samples acquired from the DMID BEI repository (contract #75N93022C00024) were also tested on both ALPCO assays in conjunction with the nonreactive cohort to further validate assay performance (Figure 7). These samples were also tested in-house on Arlington Scientific's RPR card test and resulting endpoint titers were compared to the ALPCO-NT reported titer.

Analytical testing. Precision was assessed using four to five samples spanning the assay ranges, with 16 replicates measured over two days. Intra- and inter-day %CVs were calculated for each sample.



Figure 4. KleeYa[®] instrument. These assays are 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 immunoassays run on a single platform.

5. Results

Comparator Testing

DiaSorin Treponema				AIX1000 RPR			
ALPCO-T	R	99	1	100	R	118	13
	NR	1	99	100	NR	19	75
	Total	100	100	200	Total	137	88
PPA: 99.0%, NPA: 99.0%				PPA: 86.1%, NPA: 85.2%			

Alignment within +/- 1 titer of reactive samples: 88%

Figure 5. Qualitative alignment of ALPCO's automated treponemal and nontreponemal immunoassays to the DiaSorin LIAISON Treponema Screen assay and Gold Standard's AIX1000, respectively. Procured samples previously tested on Gold Standard's AIX1000 and DiaSorin LIAISON Treponema Screen assay were analyzed on ALPCO's assays. Semi-quantitative data between reported titers by both nontreponemal tests show good agreement within +/-1 titer, with most discordance being in the 1:1 titer zone. No clinical data outside of treponema and RPR results were available for these samples.

(a) Samples characterized reactive with 1:1 titer on AIX1000			(b) Samples characterized reactive with 1:1 titer on AIX1000		
ALPCO - NT Assay	R	26	In-house RPR card	R	17
	NR	16		NR	10
	Total	42		Total	27
% Agreement: 62%			% Agreement: 63%		

(c) Samples characterized reactive with 1:1 titer on ALPCO-NT assay		
In-house RPR card	R	23
	NR	14
	Total	37
% Agreement: 62%		

Figure 6. 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.

During comparator testing, a set of serum samples were tested on both ALPCO's treponema and nontreponemal assays on the KleeYa platform. Qualitative agreement reached 99% and 85% for treponema and nontreponemal assays, respectively.

Demonstration of Alignment in Well-Characterized Syphilis Reactive Samples

Treponema				Nontreponema				Treponema				Nontreponema			
Sample ID	ALPCO CLIA Index Value	ALPCO Titer	RPR Card Titer	Sample ID	ALPCO CLIA Index Value	ALPCO Titer	RPR Card Titer	Sample ID	ALPCO CLIA Index Value	ALPCO Titer	RPR Card Titer	Sample ID	ALPCO CLIA Index Value	ALPCO Titer	RPR Card Titer
BA1-25RY	28.0	219.86	128	128	BA5-1Y67	15.0	1.83	1	2	319	0.28	0.66	NR	NR	NR
BA1-128P	23.6	167.46	128	128	BA5-2G64	3.07	1.78	1	1	338	0.06	0.66	NR	NR	NR
BA2-03BY	32.1	158.96	128	64	BA6-70W2	8.40	1.49	1	1	355	0.54	0.66	NR	NR	NR
BA5-2ERL	22.2	128.85	128	64	BA5-34SA	7.50	1.41	1	1	353	0.11	0.66	NR	NR	NR
BA3-06PS	31.0	117.30	64	128	BA7-18WE	14.1	1.40	1	1	608	0.11	0.66	NR	NR	NR
BA7-0PI9	28.3	89.55	64	64	BA5-21ZP	5.89	1.23	1	1	622	0.20	0.65	NR	NR	NR
BA1-21DT	16.5	87.27	64	128	318	0.90	2.97	2	NR	315	0.78	0.65	NR	NR	NR
BA7-3HT9	25.2	84.01	64	256	331	0.44	2.36	2	NR	522	0.05	0.64	NR	NR	NR
BA2-0W3R	16.2	81.12	64	32	523	0.51	1.79	1	NR	605	0.05	0.64	NR	NR	NR
BA4-0NGG	30.1	80.82	64	512	617	0.08	1.35	1	NR	529	0.32	0.64	NR	NR	NR
BA2-09AR	26.7	80.52	64	128	347	0.16	1.72	1	NR	357	0.09	0.64	NR	NR	NR
BA7-22CK	28.7	79.65	64	512	329	0.04	1.45	1	NR	532	0.11	0.61	NR	NR	NR
BA7-0TEF	22.5	75.37	64	512	601	0.06	1.26	1	NR	602	0.17	0.61	NR	NR	NR
BA1-1PCT	17.9	68.38	64	32	614	0.34	1.11	NR	NR	619	0.30	0.60	NR	NR	NR
BA1-0ZAU	21.7	67.65	64	64	344	0.09	1.05	NR	NR	345	0.93	0.59	NR	NR	NR
BA6-4VTT	16.0	58.71	32	16	535	0.15	1.03	NR	NR	312	0.27	0.59	NR	NR	NR
BA2-0FNK	12.9	53.17	32	32	328	0.50	1.02	NR	NR	528	0.09	0.59	NR	NR	NR
BA2-0VBV	23.4	47.25	32	32	352	0.44	1.01	NR	NR	333	0.27	0.59	NR	NR	NR
BA5-2810	27.6	34.85	32	64	332	0.13	1.00	NR	NR	348	0.14	0.59	NR	NR	NR
BA2-0EPG	14.8	33.83	32	32	628	0.11	1.00	NR	NR	603	0.22	0.58	NR	NR	NR
BA5-1HAF	28.4	29.52	16	64	526	0.13	0.98	NR	NR	606	0.18	0.57	NR	NR	NR
BA1-1VEA	3.74	24.36	16	64	609	0.06	0.98	NR	NR	623	0.07	0.57	NR	NR	NR
BA5-3RCB	11.2	19.81	16	8	337	0.07	0.98	NR	NR	627	0.09	0.57	NR	NR	NR
BA3-0WJ5	26.8	16.70	16	32	322	0.24	0.97	NR	NR	330	0.22	0.57	NR	NR	NR
BA1-0T8D	19.8	16.09	16	32	336	0.19	0.96	NR	NR	354	0.12	0.57	NR	NR	NR
BA5-1FG5	13.2	14.02	8	8	341	0.39	0.94	NR	NR	519	0.09	0.56	NR	NR	NR
BA1-0N69	32.0	11.79	8	16	351	0.10	0.93	NR	NR	610	0.05	0.56	NR	NR	NR
BA7-2531	16.9	11.74	8	16	326	0.28	0.92	NR	NR	615	0.06	0.54	NR	NR	NR
BA3-0G1D	12.6	11.33	4	8	334	0.16	0.92	NR	NR	320	0.09	0.54	NR	NR	NR
BA3-101Y	11.8	9.40	8	4	538	0.20	0.91	NR	NR	612	0.04	0.53	NR	NR	NR
BA7-45LD	18.6	9.21	8	8	616	0.17	0.90	NR	NR	621	0.22	0.51	NR	NR	NR
BA1-0DWV	14.1	8.96	8	16	359	0.26	0.89	NR	NR	520	0.12	0.51	NR	NR	NR
BA1-1BB6	14.7	8.64	8	16	360	0.05	0.89	NR	NR	356	0.14	0.50	NR	NR	NR
BA7-2WYV	25.0	8.52	8	16	631	0.37	0.88	NR	NR	346	0.34	0.50	NR	NR	NR
BA4-0GTP	9.51	8.46	8	4	625	0.19	0.85	NR	NR	518	0.05	0.49	NR	NR	NR
BA7-3ASK	23.3	8.36	8	16	537	0.35	0.84	NR	NR	358	0.13	0.48	NR	NR	NR
BA7-0NAM	21.9	8.28	8	16	314	0.29	0.83	NR	NR	343	0.22	0.48	NR	NR	NR
BA6-6HEZ	23.6	7.86	4	16	340	0.34	0.83	NR	NR	618	0.04	0.46	NR	NR	NR
BA7-47GE	21.2	7.28	4	16	336	0.18	0.78	NR	NR	324	0.10	0.46	NR	NR	NR
BA4-02XG	29.3	7.07	4	16	527	0.15	0.78	NR	NR	531	0.29	0.42	NR	NR	NR
BA5-3H0B	11.9	6.96	4	16	321	0.07	0.78	NR	NR	626	0.26	0.40	NR	NR	NR
BA5-3CHV	24.4	6.86	4	16	339	0.13	0.76	NR	NR						
BA6-6W67	20.2	6.21	4	8	539	0.26	0.76	NR	NR						
BA6-4LVY	11.2	6.18	4	16	604	0.14	0.75	NR	NR						
BA7-1MUU	11.0	6.00	4	4	642	0.17	0.75	NR	NR						
BA5-3FY9	16.5	5.21	4	8	327	0.07	0.74	NR	NR						
BA5-3009	8.14	5.20	4	4	530	0.11	0.74	NR	NR						
BA5-26UE	18.4	4.74	4	16	335	0.06	0.72	NR	NR						
BA3-0CYN	12.8	3.65	2	8	533	0.20	0.72	NR	NR						
BA2-0LUT	11.3	2.58	2	4	624	0.86	0.71	NR	NR						
BA4-0XV5	24.5	2.45	2	2	317	0.12	0.71	NR	NR						
BA6-6PPO	9.82	2.41	2	8	540	0.07	0.71	NR	NR						
BA5-2VYR	14.2	2.33	2	4	534	0.13	0.71	NR	NR						
BA5-2YHX	12.5	2.26	2	4	521	0.18	0.70	NR	NR						
BA5-3681	5.61	2.07	2	2	611	0.19	0.70	NR	NR						
BA7-17ZV	21.1	2.04	2	4	525	0.27	0.69	NR	NR						
BA5-3RV6	8.12	1.99	1	2	349	0.08	0.68	NR	NR						
BA4-0DNX	15.9	1.85	1	4	620	0.06	0.68	NR	NR						

Figure 7. Alignment of ALPCO's automated nontreponemal and treponemal immunoassays to well characterized syphilis reactive samples. Reactive Treponema samples were characterized by the DMID BEI repository while Nonreactive Treponema samples were characterized by the DiaSorin LIAISON Treponema Screen assay. All samples were characterized by an in-house RPR card screen at ALPCO.

NR: Not reactive; pink shade is indicative of sample classification as reactive on predicate, grey shade is indicative of sample classification as nonreactive on predicate