

Highly Reproducible Femtomolar Detection of an Antisense Oligo in Human Plasma Using Electrochemiluminescence

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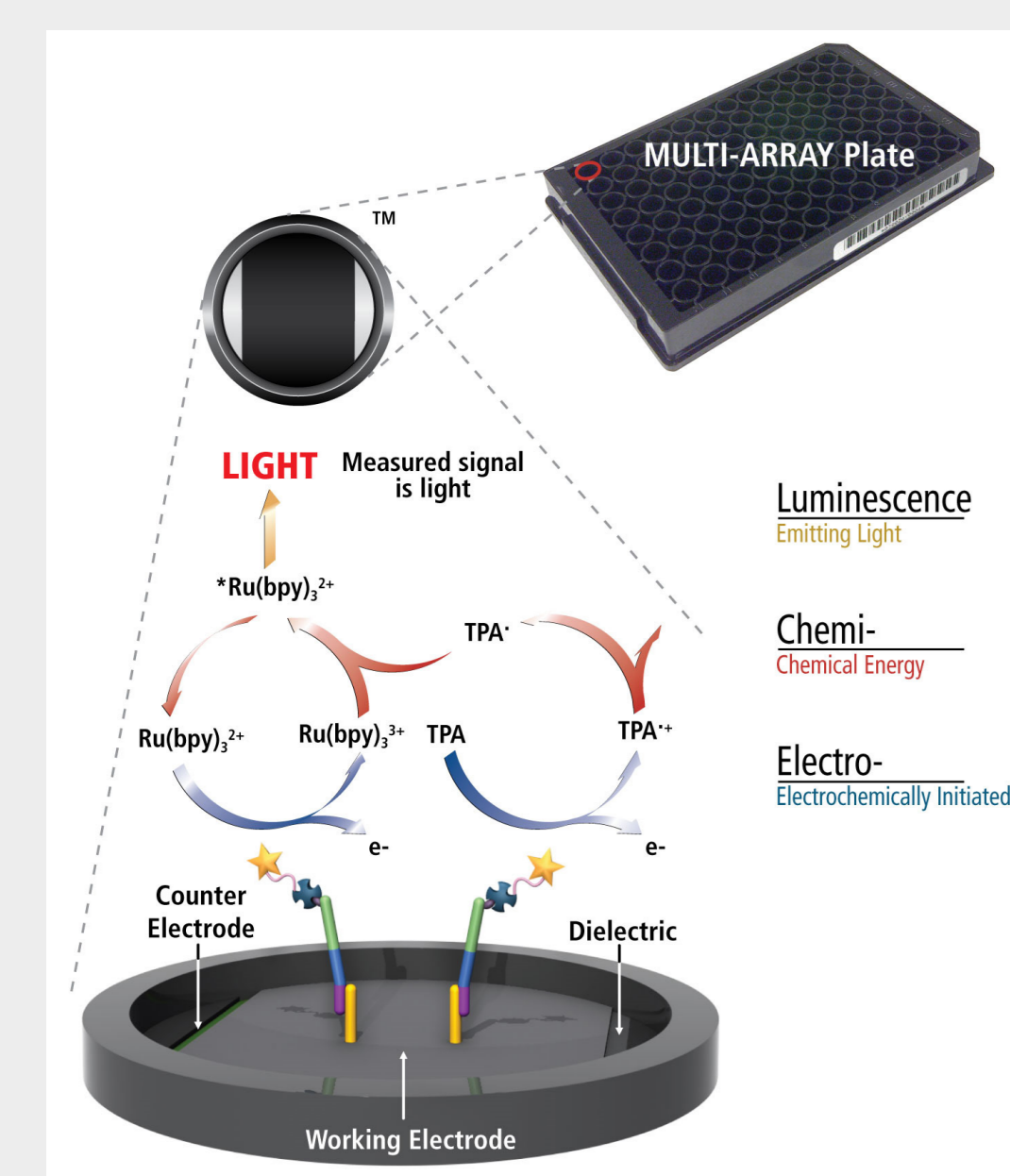


PURPOSE

There has been a rapid rise in the number of antisense oligonucleotide (ASO) therapeutics being developed to regulate the expression of genes associated with metabolic, neurologic, cardiovascular, and other diseases. These modified DNA or RNA molecules are usually shorter than 30 nucleotides and dosed at low levels to minimize toxicity, making them difficult to measure *in vivo*. HPLC-MS/MS and hybridization ELISA are used to detect ASOs but these techniques lack the femtomolar sensitivity needed to assess the full pharmacokinetic profile of ASOs in biological matrices.

METHODS

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Surface coatings can be customized.

Basis of the N-PLEX platform

N-PLEX® plates contain 10 unique capture oligonucleotides that are bound to their corresponding spots on the electrode surface. Detection of a nucleic acid sequence of interest is accomplished by hybridization of one or more probes with sequence complementary to these capture oligos and the nucleic acid of interest, followed by detection via electrochemiluminescence (i.e. biotin/streptavidin SULFO-TAG interactions). Blocking, hybridization, and detection are completed using MSD™ proprietary buffers and diluents.

ASO Assay Development

Two assays were developed for detecting unconjugated and conjugated forms of a promising PCSK9-ASO drug candidate in human plasma on the N-PLEX platform.

Plasma samples

Human plasma samples in sodium citrate were purchased from BioIVT. For all experiments involving the use of plasma, exogenous ASO was spiked into human plasma and serially diluted in the same matrix to generate calibration curves.

ASO detection via two-probe approach

The two-probe detection assay used probes that were complementary to the nucleotides of one half of the ASO. One probe contained a spot-specific sequence at the 5' end that allowed for hybridization to the N-PLEX plates, while the other probe contained a biotin on the 3' end for detection. The probes were hybridized to the ASO and then to spot-specific capture oligos on the N-PLEX plates. Streptavidin-bound SULFO-TAG was then used for detection of the captured ASO.

ASO detection via RNase protection assay

The RNase protection assay (RPA) utilized a single chimeric probe for the detection of the ASO on the N-PLEX platform. This chimeric probe contained a 5' DNA sequence that was complementary to the plate-bound capture oligo followed by an RNA portion that was complementary to the ASO and a biotin on the 3' end for detection via streptavidin-bound SULFO-TAG. Plasma samples were pretreated with RNasease reagent (ThermoFisher Scientific) and heated to 60°C for 10 minutes to inactivate endogenous RNases. Once the probe was hybridized to both the ASO and the plate, an RNase cocktail was added to degrade any single-stranded RNA sequence. Therefore, any RNA in the probe not fully protected by the ASO would be degraded and the biotin released from the DNA portion of the probe, rendering it undetectable via streptavidin-bound SULFO-TAG.

RESULTS

Technical Overview and Calibration Curves (Two-Probe Approach)

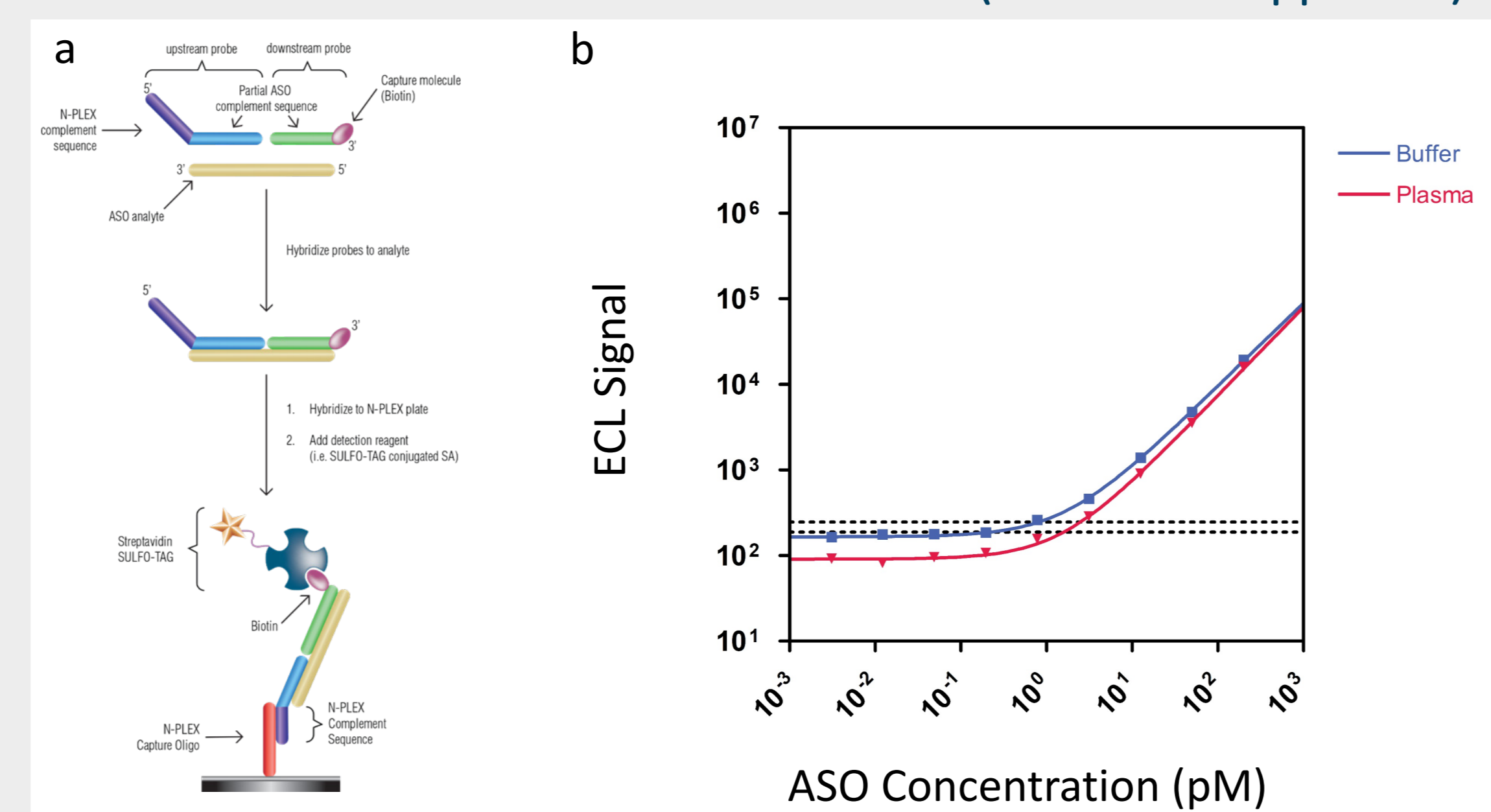


Figure 1. Schematic for ASO detection via the two-probe approach (a) and comparison of calibration curves generated in buffer or human plasma (b).

Conjugated and Unconjugated Mix Testing (Two-Probe Approach)

Conjugated/Unconjugated Ratio	Conc. (pM)	Average Signal	% of 50/50
100/0	100/0	12,713	95%
80/20	80/20	13,140	98%
60/40	60/40	12,882	96%
50/50	50/50	13,358	100%
40/60	40/60	13,492	101%
20/80	20/80	14,900	112%
0/100	0/100	14,060	105%
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Table 1. The unconjugated and conjugated forms of the ASO drug were mixed at different ratios to assess whether any conjugation bias was observed using the two-probe approach. The signals derived from the mixtures were compared to a 50/50 mix normalization.

Dilution Linearity and Spike Recovery (Two-Probe Approach)

Dilution Factor (Conc.)	Average Signal	% Recovery	Spike Concentration	Average Signal	% Recovery
1 (200 pM)	31,507	100%	64 pM (High spike)	8,634	101%
2 (100 pM)	14,757	94%	8 pM (Mid spike)	1,227	116%
4 (50 pM)	6,881	87%	1 pM (Low spike)	317	119%
8 (25 pM)	3,532	90%			
16 (12.5 pM)	1,779	90%			

Table 3. A high concentration of the ASO drug was spiked into plasma and diluted 2-fold 4 times. The linearity of each dilution was assessed by comparing experimental signal to expected signal using the two-probe approach.

Control Recovery in Reproducibility Testing (Two-Probe Approach)

Control	Conc. (pM)	Intra-plate Average Concentration (n=2)									Inter-run Average Conc. (n=18)	Inter-run Conc. CV
		Day 1			Day 2			Day 3				
High	128	116.5	131.0	122.3	146.6	126.6	129.5	114.8	119.7	118.2	125.0	7.9%
Mid	32	24.8	29.9	28.3	28.5	25.0	24.5	21.9	23.5	22.7	25.4	10.9%
Low	8	5.4	6.3	6.7	8.2	7.1	6.8	6.9	7.8	7.1	6.9	11.7%

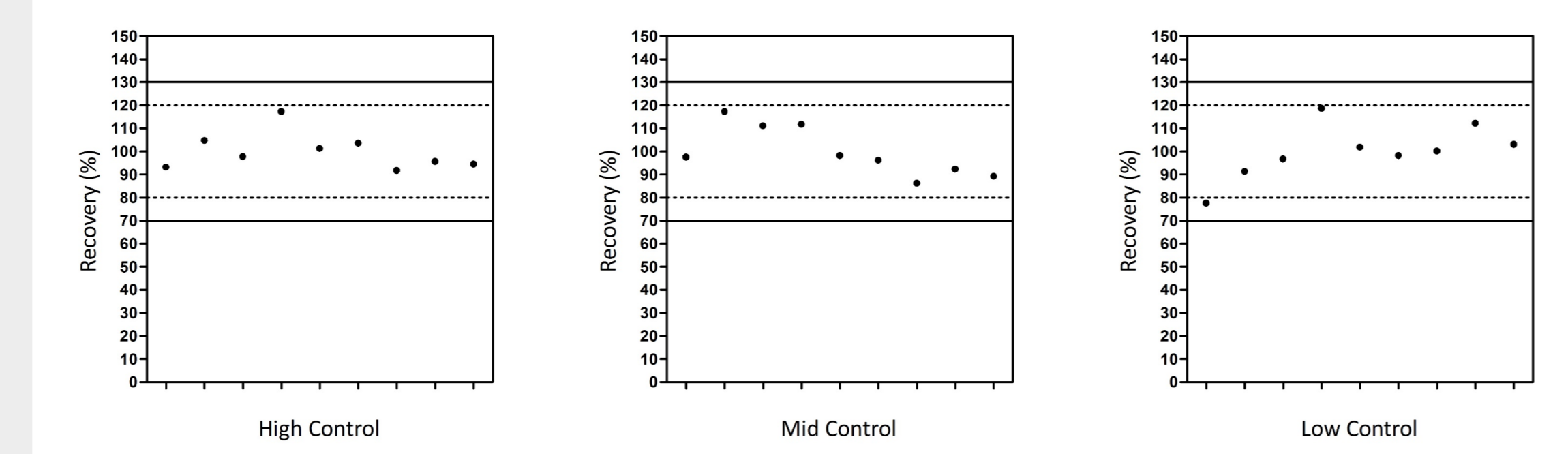


Figure 3. Controls were made at a high (128 pM), mid (32 pM), and low (8 pM) concentration prior to reproducibility testing. Three separate preparations of each control were run each day for a total of three days. The calculated concentrations were determined using the two-probe approach and the inter-run concentrations and concentration CVs were compiled across all three days of testing (Table). Each data point was also plotted and gated based on 20% (dotted line) and 30% (solid line) of experimental mean (Graphs).

RESULTS

Technical Overview and Calibration Curves (RPA)

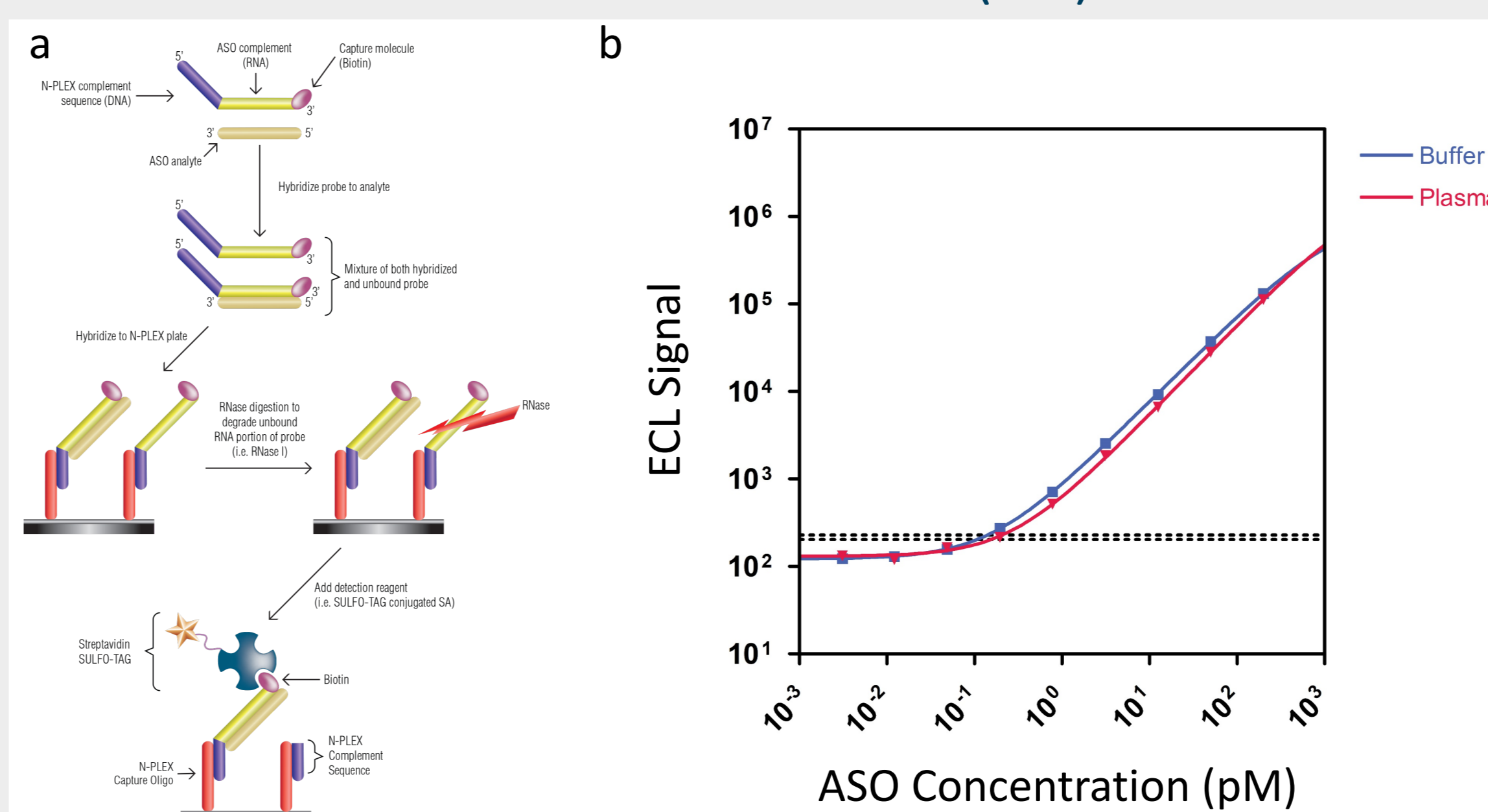


Figure 2. Schematic for ASO detection via the one-probe RNase protection assay (RPA) (a) and comparison of calibration curves generated in buffer or human plasma (b).

Conjugated and Unconjugated Mix Testing (RPA)

Conjugated/Unconjugated Ratio	Conc. (pM)	Average Signal	% of 50/50
100/0	100/0	44,263	97%
80/20	80/20	43,483	95%
60/40	60/40	44,240	97%
50/50	50/50	45,570	100%
40/60	40/60	40,773	90%
20/80	20/80	38,366	84%
0/100	0/100	45,785	101%
Blank	0/0	112	-

Table 2. The unconjugated and conjugated forms of the ASO drug were mixed at different ratios to assess whether any conjugation bias was observed using RPA. The signals derived from the mixtures were compared to a 50/50 mix normalization.

Dilution Linearity and Spike Recovery (RPA)

Dilution Factor (Conc.)	Average Signal	% Recovery	Spike Concentration	Average Signal	% Recovery
1 (200 pM)	112,705	100%	64 pM (High spike)	31,999	82%
2 (100 pM)	49,257	87%	8 pM (Mid spike)	4,072	81%
4 (50 pM)	23,600	84%	1 pM (Low spike)	899	105%
8 (25 pM)	14,247	101%			
16 (12.5 pM)	7,278	103%			

Table 4. The ASO drug was spiked into plasma at a high, mid, and low spike concentration. The recovery of each spike was assessed by comparing experimental signal to expected signal using the two-probe approach.

Control Recovery in Reproducibility Testing (RPA)

Control	Conc. (pM)	Intra-plate Average Concentration (n=2)									Inter-run Average Conc. (n=16)	Inter-run Conc. CV
		Day 1			Day 2			Day 3				
High	64	56.9	76.8	62.2	74.6	68.4	53.9	58.7	73.8	65.7	13.5%	
Mid	8	6.0	7.4	7.1	7.3	6.7	6.2	7.1	8.8	7.1	12.5%	
Low	1	0.7	0.7	0.9	0.8	0.7	0.7	0.9	1.1	0.8	16.3%	

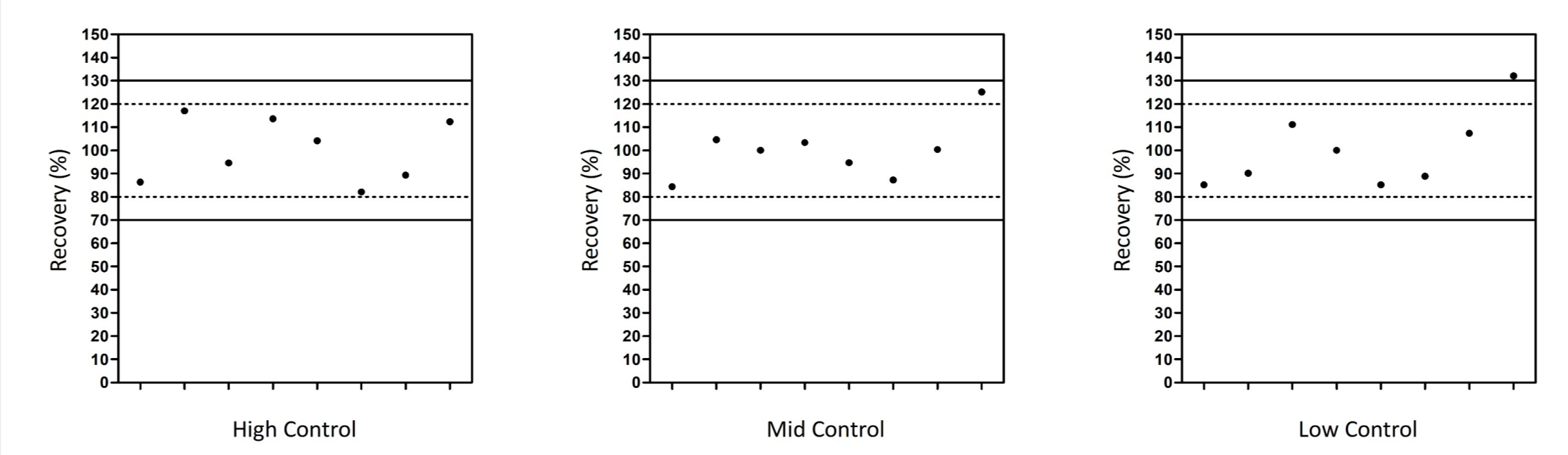


Figure 4. Controls were made at a high (64 pM), mid (8 pM), and low (1 pM) concentration prior to reproducibility testing. Two or three separate preparations of each control were run each day for a total of three days. The calculated concentrations were determined using RPA and the inter-run concentrations and concentration CVs were compiled across all three days of testing (Table). Each data point was also plotted and gated based on 20% (dotted line) and 30% (solid line) of experimental mean (Graphs).

RESULTS

Calibration Curve Reproducibility and LLOD / LLOQ Determinations (Two-Probe Approach)

Calibrator	Conc. (pM)	Intra-plate Average Signal (n=2)									Inter-run Average Signal (n=18)	Inter-run Signal CV
		Day 1			Day 2			Day 3				
Cal-1	200	30,025	28,794	29,329	29,000	33,387	32,433	30,486	28,638	29,974	30,229	5.5%
Cal-2	50	7,798	6,716	7,018	7,368	8,446	8,935	8,141	7,526	7,803	7,750	8.9%
Cal-3	12.5	2,252	1,758	1,842	1,993	2,229	2,270	2,224	2,132	2,206	2,101	9.1%
Cal-4	3,125	600	563	540	623	773	711	735	671	675	654	12.1%
Cal-5	0.781	212	216	178	244	315	259	230	254	275	242	16.4%
Cal-6	0.195	147	187	154	189	241	183	133	154	165	172	18.7%
Cal-7	0.0488	142	156	134	128	250	207	83	132	97	147	35.4%
Cal-8	0	109	134	140	192	231	113	89	204	173	154	31.5%
Inter-run Average												
Hill Slope		1.04	1.03	1.12	1.02	1.04	0.98	0.95	1.03	0.95	1.03	
r ² value		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
LLOD (pM)		0.54	0.83	0.79	2.05	0.53	0.39	0.35	0.93	0.38	0.47	

Table 7. Three separate calibration curves were prepared and run across three days to assess the reproducibility of the two-probe approach. The inter-run average signal and signal CV were determined across the three days of testing. The lower limit of detection (LLOD) was determined using these calibration curves and 30 blank wells per plate. Based on these metrics, the estimated lower limit of quantification (LLOQ) was calculated.

LLOQ (pM)	3.01
Signal at LLOQ	594
Background	154
Signal/Background	3.9
Signal-Background	440

Calibration Curve Reproducibility and LLOD / LLOQ Determinations (RPA)

Calibrator	Conc. (pM)	Intra-plate Average Signal (n=2)								Inter-run Average Signal (n=16)	Inter-run Signal CV
		Day 1		Day 2		Day 3		Day 3			
Cal-1	200	103,001	106,578	109,486	130,971	159,100	137,194	133,711	111,811	123,981	15.7%
Cal-2	50	35,586	22,679	28,804	38,372	38,588	37,768	33,510	27,931	32,905	17.8%
Cal-3	12.5	8,791	7,429	8,729	10,655	11,828	10,585	9,476	7,473	9,370	16.8%
Cal-4	3,125	2,719	2,209	2,275	2,864	3,501	3,153	2,648	2,068	2,679	18.4%
Cal-5	0.781	802	814	611	852	924	842	872	657	796	13.5%
Cal-6	0.195	330	358	276	352	382	381	284	318	335	12.1%
Cal-7	0.0488	202	173	150	182	217	199	183	174	185	11.3%
Cal-8	0	195	179	163	162	170	157	164	149	167	8.5%
Inter-run Average											
Hill Slope		1.00	0.91	1.01	0.98	0.95	0.95	0.97	0.96	0.96	
r ² value		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
LLOD (pM)		0.10	0.08	0.12	0.08	0.08	0.07	0.15	0.11	0.09	

Table 8. Two or three separate calibration curves were prepared and run across three days to assess the reproducibility of RPA. The inter-run average signal and signal CV were determined across the three days of testing. The lower limit of detection (LLOD) was determined using these calibration curves and 30 blank wells per plate. Based on these metrics, the estimated lower limit of quantification (LLOQ) was calculated.

LLOQ (pM)	0.42
Signal at LLOQ	510
Background	167
Signal/Background	3.1
Signal-Background	343

CONCLUSION

These data highlight the utility of the N-PLEX platform for highly sensitive and reproducible ASO detection in plasma using 96-well plate processes that are amenable to high throughput testing.



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