Supplementary Information for

Design of serum complement C9 immunoassays for more accurate risk prediction and early diagnosis of esophageal adenocarcinoma

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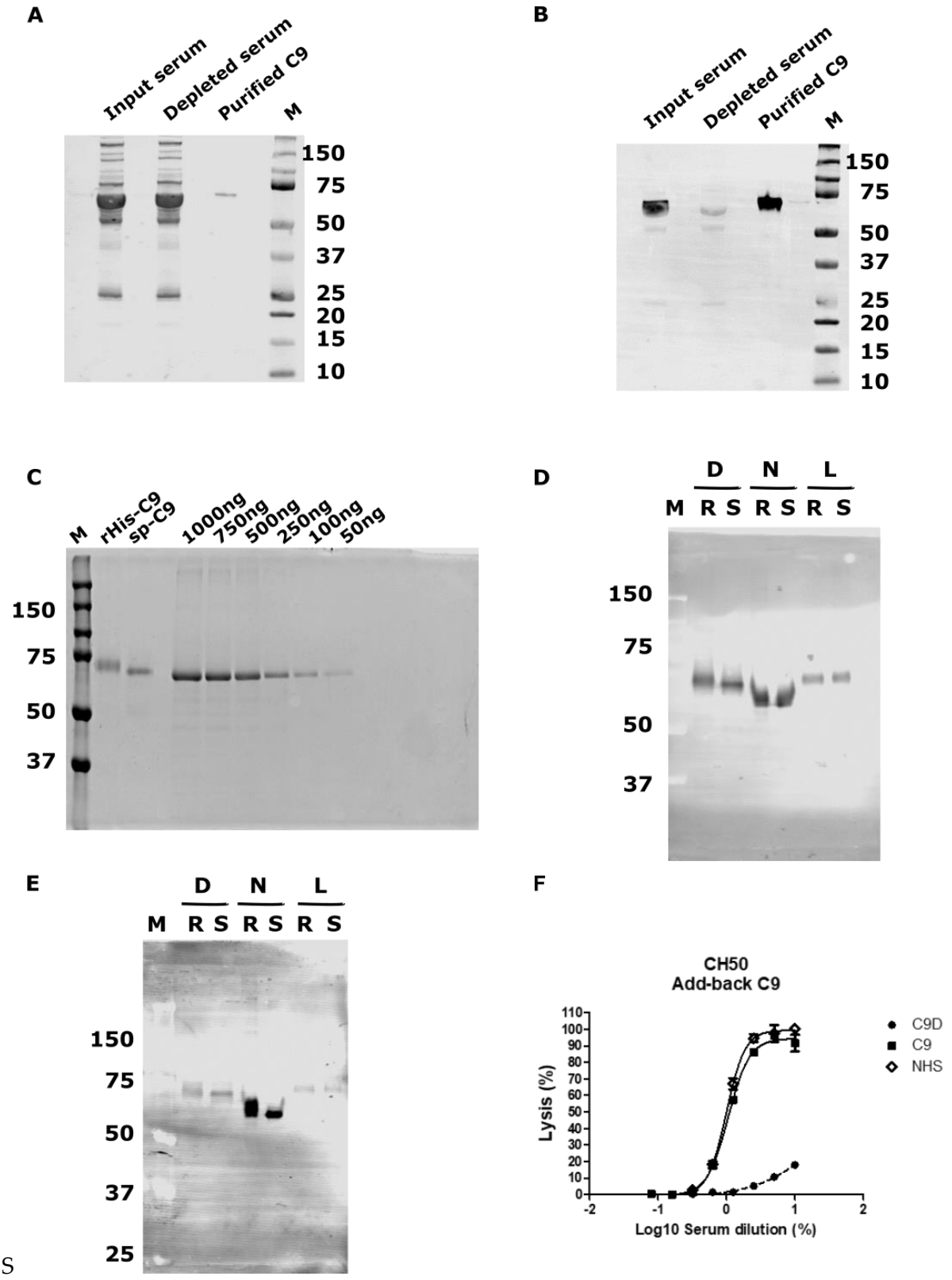

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Figure S1. Production and purification of C9 and antibody. *(A) Human serum was depleted of C9 using mAb26. Purity was checked using SDS-Page. Only one band was detected after purification and staining of the membrane using REVERT total protein stain, confirming no contaminants were present. (B) Western blot using mouse anti-C9 antibody (Abcam) confirmed the protein purified was C9 with minimal C9 detected in the depleted serum. (C) Recombinant C9 was produced in HEK cells*

then purified using FPLC on an AKTA system. Purified recombinant His-C9 and human C9 resolved on 10% PAGE gels under reducing conditions, stained with Colloidal Coomassie. Concentration was quantitated relative to dilutions of BSA. M: protein molecular weight marker. Gel shows that both types of C9 had no contaminating C9 or degradation. (D) 125 ng of C9 was resolved on and SDS-Page then analyzed by western blot. Recombinant C9 (R) was confirmed using mouse anti-C9 (Abcam) and was compared to serum (S) purified C9. Protein was either denatured (D=reduce/heat) or natured (N=not reduced/no heat) or denatured as per LeMBA (L) buffer. (E) mAb 26 antibody was characterized for detection of various preparations of C9 as per (D). mAb 26 had a high affinity for natured C9 relative to denatured C9, hence was highly suited for immunoprecipitation from serum. (F) mAb 26-purified C9 was titrated back into 10% C9 depleted serum to demonstrate the restored haemolysis. The dashed lines correspond to the C9 depleted serum (C9D) and open symbol to normal human serum (NHS). The assays were performed a minimum of three times with the comparable outcome. The error bars are standard errors of triplicates.

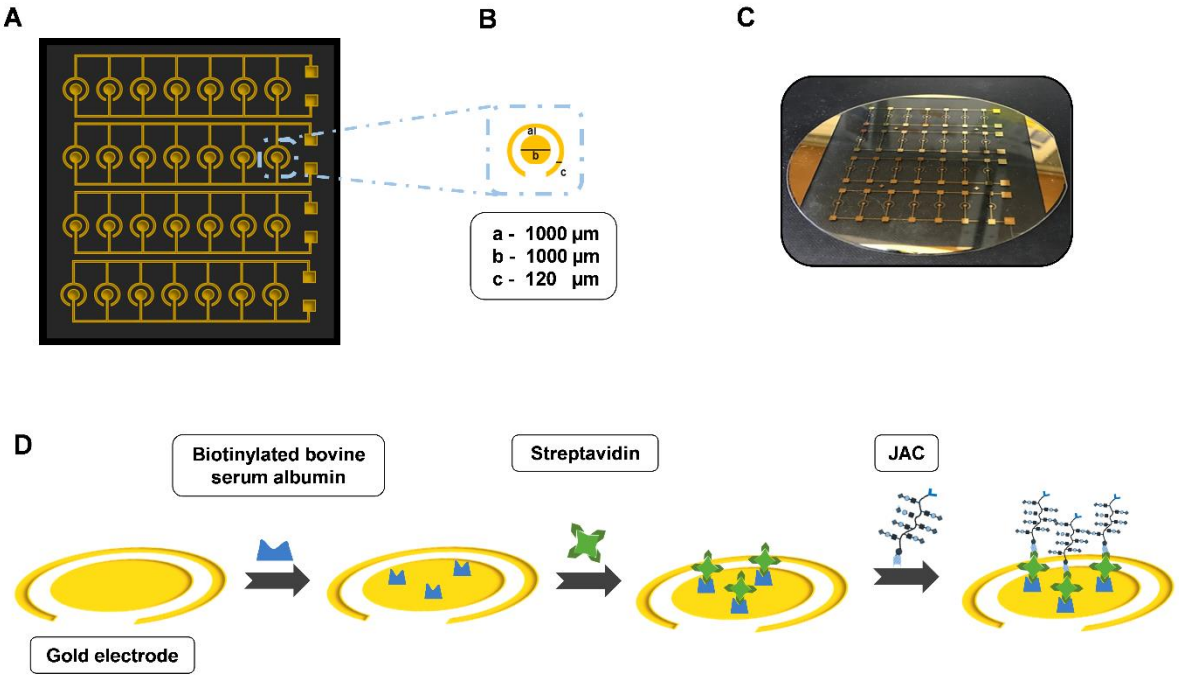

Figure S2. Endo Screen Chip design and functionalization. *(A) The Endo Screen Chip consists of an array of 28 electrode wells arranged in four rows to seven electrode wells each. (B) Each electrode was made of an inner circular electrode (1000 µm in diameter) and outer ring electrode (120 µm wide) that were separated by 1000 µm. The circular electrode and ring electrode acted as working and counter electrodes, respectively. (C) A photograph of the Endo Screen Chip. (D) Electrode functionalization with JAC using a biotin-streptavidin conjugation process.*

Table S1. Patient risk factors

		Healthy	BE	EAC	p value (Healthy vs BE vs EAC)
Number		15	16	15	
Age (years)	Median	64.39	61.89	61.72	0.146[a]
	Range	56-75	52-75	53-74	
BMI	Healthy wt (-<25)	10.9% (5)	6.5% (3)	2.2% (1)	0.054[b]
	Overweight(-<30)	10.9% (5)	23.9% (11)	10.9% (5)	
	Obese I (-<35)	10.9% (5)	4.3% (2)	17.4% (8)	
	Obese II (<40)	0.0% (0)	0.0% (0)	0.0% (0)	
	Obese III (>=40)	0.0% (0)	0.0% (0)	2.2% (1)	
Heartburn & Reflux History	Never	11.1% (5)	4.4% (2)	2.2% (1)	0.005[b]
	<Once/month	6.7% (3)	6.7% (3)	2.2% (1)	
	Monthly (few times/month)	8.9% (4)	13.3% (6)	2.2% (1)	
	Weekly (few times/wk)	2.2% (1)	0.0% (0)	15.6% (7)	
	Daily	2.2% (1)	11.1% (5)	11.1% (5)	

Percentage calculated is percentage relative to all cases. Brackets are the number of counts of each class. [a]Kruskal-Wallis Test. [b]Fisher's Exact Test.

Table S2. Comparison of disease classification by each model

Model 1: Patient risk factors

	Predicted				
Observed	Healthy	BE	EAC	Total	Percent Correct
Healthy	8	4	2	14	57.1%
BE	3	9	4	16	56.3%
EAC	1	3	11	15	73.3%
Total	12	16	17	45	
Overall Percentage	26.7%	35.6%	37.8%		62.2%

Model 2: Risk factors plus serum C9 and JAC-C9

	Predicted				
Observed	Healthy	BE	EAC	Total	Percent Correct
Healthy	6	5	3	14	42.9%
BE	4	11	1	16	68.8%
EAC	1	1	13	15	86.7%
Total	11	17	17	45	
Overall Percentage	24.4%	37.8%	37.8%		66.7%

Table S3. Properties of model 2

Comparison		*B*	Odds Ratio	Wald	95% Confidence Interval Odds Ratio	
					Lower Boundary	Upper Boundary
BE to Healthy	Intercept	6.617		1.449		
	Heartburn & Reflux History	0.481	1.618	1.929	0.821	3.189
	BMI	-0.358	0.699	0.256	0.175	2.794
	Age	-0.063	0.939	0.925	0.827	1.067
	C9	-0.273	0.761	4.268*	0.588	0.986
	JAC-C9	-0.127	0.881	0.069	0.342	2.270
EAC to Healthy	Intercept	-12.934		2.517		
	Heartburn & Reflux History	1.376	3.961	7.473**	1.476	10.626
	BMI	1.768	5.857	3.739	0.976	35.141
	Age	0.081	1.084	0.801	0.908	1.295
	C9	0.031	1.031	0.111	0.860	1.236
	JAC-C9	1.399	4.052	5.083*	1.201	13.676
EAC to BE	Intercept	-19.552		6.214		
	Heartburn & Reflux History	0.895	2.448	3.317	0.934	6.418
	BMI	2.125	8.374	5.435*	1.403	49.994
	Age	0.144	1.154	2.811	0.976	1.366
	C9	0.304	1.355	4.623*	1.027	1.787
	JAC-C9	1.526	4.602	5.989*	1.355	15.626

$\chi^2 = 37.983$ (10), $p < 0.001$, $R^2 = 0.570$ (Cox and Snell), 0.642 (Nagelkerke).

* $p < 0.05$

** $p < 0.01$

Table S4. Confidence intervals for multinomial logistic regression

		95% Confidence Intervals	
	AUC	Lower	Upper
Healthy			
Model 1	0.7350	0.5322	0.8712
Model 2	0.8272	0.6714	0.9181
BE			
Model 1	0.7435	0.5788	0.8595
Model 2	0.8405	0.6934	0.9247
EAC			
Model 1	0.8378	0.6774	0.9270
Model 2	0.9311	0.7936	0.9794

Model 1: Risk factors

Model 2: Risk factors plus serum C9 and JAC-C9