Supporting Information

Flow Induced Dispersion Analysis for Probing Anti-dsDNA Antibody Binding Heterogeneity in Systemic Lupus Erythematosus Patients: Toward a New Approach for Diagnosis and Patient Stratification

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Injection procedure

Initial experiments indicated adsorption of the monoclonal antibody on the inner surface of the fused silica capillary. Only injecting the analyte in narrow zones around the indicator peak (see Figure S1) and using a polyethylene glycole (PEG)-coated capillary eliminated the adsorption problems. With this injection procedure it was observed that the indicator peak appeared broader when the same indicator and analyte vials were injected repeatedly. This was caused by contamination of the analyte vial by small volumes of indicator for each injection. After a number of injections this caused the analyte solution to become weakly fluorescent. To minimize this problem, injections from the indicator and analyte vials were performed a maximum of three times. In a further attempt to eliminate carryover the capillary inlet was dipped in pure buffer between the introduction of the analyte and the indicator (protocol II and III, described in the main manuscript).

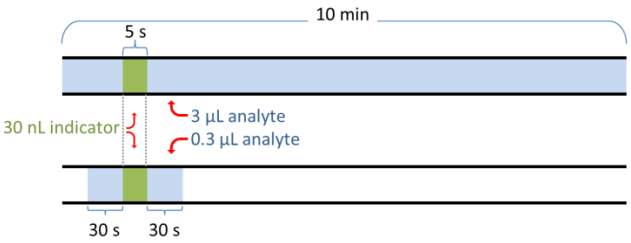

Figure S1: Schematic representation of the two different injection schemes used. In both cases 30 nL indicator is injected. In the first case the entire capillary is filled with the analyte and in the second case the analyte is only injected in the regions adjacent to the indicator peak reducing analyte consumption and adsorption. Capillary inner diameter of 75 µm and a total length of 75 cm and a mobilization pressure of 50 mbar.

Taylor conditions

In order for equation 1 in the main manuscript to be valid, a number of restrictions need to be imposed on flow rate and capillary dimensions. Basically, there should be time enough for the peak to adopt a Gaussian shape and conditions should be so that diffusive mass transport in the axial direction can be neglected. It is convenient to define a dimensionless time , τ, and use the peclet number, Pe, to quantify these conditions[1]:

$$\tau = \frac{Dt_R}{r^2} > 1.4 \quad \text{(S1)}$$

$$Pe = \frac{u \cdot r}{D} > 69 \quad \text{(S2)}$$

Where r is the capillary radius, D is the diffusivity, t_R is the peak retention time and u is the linear flow rate. These requirements are easily satisfied using small inner diameter silica capillaries.

Precipitation of dsDNA – antibody complex

It was hypothesized that pre-mixing analyte and indicator before the injection would result in a stronger binding effect as the binding equilibrium would be reached already before the experiment was started. This, however, resulted in a reduced indicator peak area due to immuno-precipitation in the vial prior to injection as shown in Figure S2. When the indicator peak area decreased the apparent indicator diffusivity increased, because the small fraction of unreacted fluorescent tag present in the indicator solution remained in solution. These results stress the importance of carefully monitoring the indicator peak area during method development as well as when performing the assays.

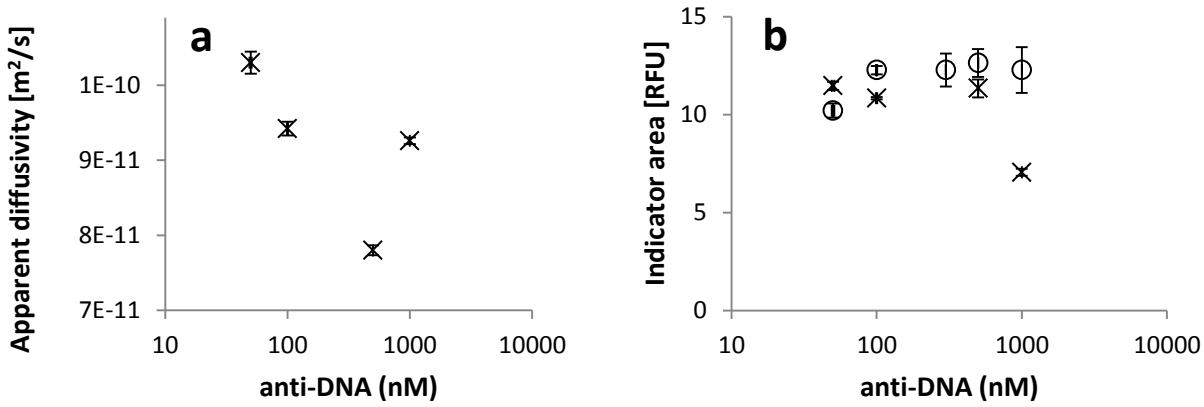

Figure S2: a) Apparent indicator (DNA-I) diffusivity as a function of model monoclonal antibody concentration. The indicator and analyte were mixed prior to injection. b) Indicator peak area as a function of antibody concentration. Crosses indicate data obtained with mixing prior to injection (diffusivities are given in Figure a) and circles represent data obtained with mixing in the capillary (the corresponding diffusivities are given in Figure 3 in the main paper). In each experiment the same amount of indicator was injected (30 nL, 50 nM), and thus the same peak area was expected.

Plasma auto-fluorescence

The diluted plasma was found to have significant auto-fluorescence relative to the 50 nM indicator, and thus the injection procedure with 30 s sample before and after the indicator used in buffer yielded distorted peak shapes due to the contribution from the auto-fluorescence from the diluted plasma. Instead it was found that injecting the sample for 300 s before introducing the indicator gave a stable, although increased, baseline before the indicator was detected as seen in Figure S3. The indicator was dissolved in buffer in all experiments and so the background fluorescence decreased when the diluted plasma was used in the solution surrounding the indicator zone. When 50 nM indicator was used in combination with 20% plasma this did not adversely influence the data. In order to obtain reliable peak shapes at higher plasma concentrations (85%) it was necessary to have similar plasma concentrations in indicator and sample solutions.

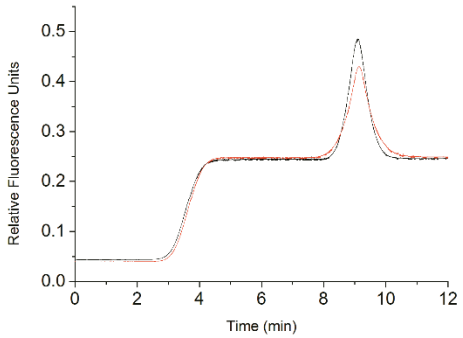

Figure S3: Representative concentration profiles (Taylorgrams) showing a peak of 29 nL 50 nM indicator in buffer spiked with (DNA-I) in 0 nM mAb (black) and 300 nM mAb (red). The rise in baseline is caused by phosphate buffer mixed with 20% plasma. This buffer was introduced 6 min prior to the indicator and used for the remainder of the experiment.

Viscosity corrections

When the capillary was uniformly filled, viscosity changes were corrected using equation 2[1]:

$$D_1 = D_2 \cdot \frac{t_{R,2}}{t_{R,1}} \qquad \text{(S3)}$$

where $t_{R,2}$ and D_2 are the peak appearance time and diffusivity at viscosity 2 and $t_{R,1}$ and D_1 is the peak appearance time and diffusivity at viscosity 1.

In the method used for measuring the patient samples the plasma sample was injected for 180 s before the introduction of the indicator and subsequently the plasma sample was used to drive the flow. The time for the plasma front to reach the detector did vary due to different viscosities in the blood plasma samples. The observed indicator diffusivities in the samples (D_s) were normalized to the diffusivity corresponding to the reference viscosity of pooled plasma from healthy donors (D_r) using equation S4:

$$D_r = D_s \frac{\eta_s}{\eta_r} \qquad \text{(S4)}$$

Where η_s and η_r is the viscosity of the sample and the reference respectively.

The η_s/η_r ratio is calculated from[2]:

$$\frac{\eta_s}{\eta_r} = \frac{2L}{l}\left(\frac{t_s - t_r}{t_r}\right) + 1 \qquad \text{(S5)}$$

where *L* is the total length of the capillary and *l* is the distance to the detector, and t_s and t_r are the times for the plasma to reach the detector for the sample and the reference respectively. The formula has been derived under the assumption that the mean viscosity during the introduction of the plasma is the average viscosity of the buffer initially inside the capillary and the viscosity of the plasma sample[2]. The reference viscosity was obtained immediately prior to the analysis of patient samples. Hereby the influence of long term drift in pump performance, and temperature variations in the small fraction of the capillary not being thermostated could be eliminated.

Data fitting procedure

The data presented in figure 3 were fitted to a 1:1 binding isotherm (equation 2 in the main manuscript). The fitting procedure gave rise to the data presented in table S1.

Table S1. Diffusivity of I, IA and affinity constant K corresponding to non-linear fit to the data in figure 3.

Parameter	D_I (m^2s^{-1})	D_{IA} (m^2s^{-1})	K (M^{-1})	R^2
0 % plasma	$1.03 \cdot 10^{-10}$ m^2 s^{-1}	$6.27 \cdot 10^{-11}$ m^2 s^{-1}	$4.24 \cdot 10^6$ M^{-1}	0.944
20 % plasma	$1.04 \cdot 10^{-10}$ m^2 s^{-1}	$6.07 \cdot 10^{-11}$ m^2 s^{-1}	$3.60 \cdot 10^6$ M^{-1}	0,975
85 % plasma	$9.68 \cdot 10^{-11}$ m^2 s^{-1}	$5.64 \cdot 10^{-11}$ m^2 s^{-1}	$2.76 \cdot 10^6$ M^{-1}	0.937

Statistical evaluation of detection limit

The limit of detection using the present methodology is based on a t-test. It is assumed that the standard deviation, s, for the diffusivity of the indicator is a good estimate of the standard deviation of the apparent indicator diffusivity in a sample with the lowest detectable concentration of anti-DNA antibodies. Based on this assumption the mean of a triplicate measurement that can be said to be different from the baseline measurement with 95% confidence can then be found by a t-test. The t-distribution can be used to find the probability that a given mean originates from the same normal distribution as the initial mean value with an estimated standard deviation. The mean of a triplicate measurement that is significantly different from the baseline value with a confidence of 95% is then used as an estimate of the limit of detection.

For the estimation of the limit of detection the indicator diffusivity at zero anti-DNA concentration obtained when preparing the standard curve in 20 % plasma is used. The triplicate measurement resulted in diffusivities of 1.05E-10 m^2/s, 1.07E-10 m^2/s and 1.07E-10 m^2/s respectively, resulting in a mean and standard deviation of 1.06E-10 m^2/s and 8.19E-13 m^2/s, respectively.

The largest one-sided error (*E*) of the estimate of the mean given with 95% confidence is obtained using a t-test. A one sided t-test is used as only a *lower* apparent diffusivity than the blank indicator diffusivity is interpreted as presence of the analyte (mAb). The deviation or Error (*E*) of the estimate of the mean for which there is 5% probability that it originates from the same population as the baseline value can be found from eq. S6.

$$E = t_\alpha \cdot \frac{s}{\sqrt{n}} \qquad \text{S6}$$

where t_α is a constant for a given number of degrees of freedom, *s* is the standard deviation and *n* is the number of experiments. The constant t_α for 2 degrees of freedom ($v = n-1$ and one sided t-test) is 2.920[3].

$$E = 2.920 \cdot \frac{8.19 \cdot 10^{-13} \frac{m^2}{s}}{\sqrt{3}} = 1.686 \cdot 8.19 \cdot 10^{-13} \frac{m^2}{s} = 1.38 \cdot 10^{-12} m^2/s$$

Thus the largest mean of a triplicate measurement that is significantly different from the baseline value with 95% confidence is: $(10.4 - 0.138) \cdot 10^{-11}$ m^2/s $= 10.26 \cdot 10^{-11}$ m^2/s.

For the model monoclonal antibody the standard curve obtained in 20% plasma can be used to calculate the antibody concentration corresponding to apparent indicator diffusivity.

$$c_A = \left(\frac{D_I - D_{AI}}{D_{app} - D_{AI}} - 1\right) \cdot \frac{1}{K} \qquad \text{S7}$$

$$c_A = \left(\frac{1.04 \cdot 10^{-10} - 6.07 \cdot 10^{-11}}{10.3 \cdot 10^{-11} - 6.07 \cdot 10^{-11}} - 1\right) \cdot \frac{1}{3.60 \cdot 10^6 M^{-1}} = 9.3\ nM$$

With the above approach the signal that is different from the baseline with 95% confidence is found under the assumption that the standard deviation is the same in a blank and at the limit of detection. The present analysis thus results in a limit of detection of 9.3 nM in 20% plasma. A similar analysis results in estimated limits of detection of 48 nM and 43 nM in 85% plasma and 0% plasma respectively.

Performance in uncoated fused silica capillaries

It is known that HSA can be used as a blocking agent to avoid unspecific adsorption[4]. It was hypothesized that performing the assay in 20% plasma would assist in blocking the capillary wall and thus remove the need for the PEG-coating. A standard curve in a pure silica capillary is given in Figure S4. Whereas the affinity appeared to be unaffected the standard deviation was found to increase showing the significant beneficial effect of the surface coating. It is hypothesized that the antibody may adsorb to the capillary wall and subsequently bind the DNA. As surface adsorption phenomena are often difficult to reproduce this is likely the cause for the increased standard deviation.

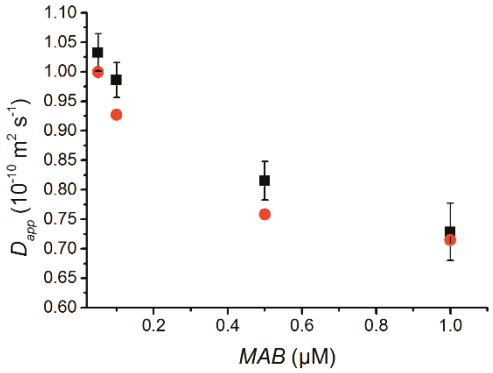

Figure S4: Diffusivity of the indicator DNA-I as a function of model monoclonal antibody concentration. The black squares have been obtained in a bare fused silica capillary and the red circles have been obtained in a PEG-coated capillary (reproduced from figure 3 in the main paper). The experiments were conducted using a phosphate buffer / 20% plasma mixture.

dsDNA Sequences

The selectivity of the monoclonal anti-dsDNA antibody against different DNA sequences was tested with two additional DNA-sequences. One (DNA-II) was hypothesized to be relevant for Lupus by Uccellini et al[5] and a randomly generated sequence (DNA-III). Different diffusivities ($D = 1.03 \cdot 10^{-10}$ m^2s^{-1}, $1.91 \cdot 10^{-10}$ m^2s^{-1} and $1.38 \cdot 10^{-10}$ m^2s^{-1} for DNA-I, II and III, respectively) were unexpectedly found for the three indicators

despite the fact that they all were 30-32 base pairs long. Therefore the purity was investigated by CE as shown in Figure S5. Here capillary electrophoresis (CE) was used for a separation of each indicator with the purpose of detecting any impurities present. For this work a 60 mM Tris buffer pH 8.4 was used. Initially the capillary was flushed with buffer for 2 min at 1 bar. Then the labelled dsDNA strand was injected at 25 mbar for 5 s followed by separation using a potential of 20 kV for 12 min in a PEG-coated capillary. Fluorescence detection was used as described for the FIDA experiments.

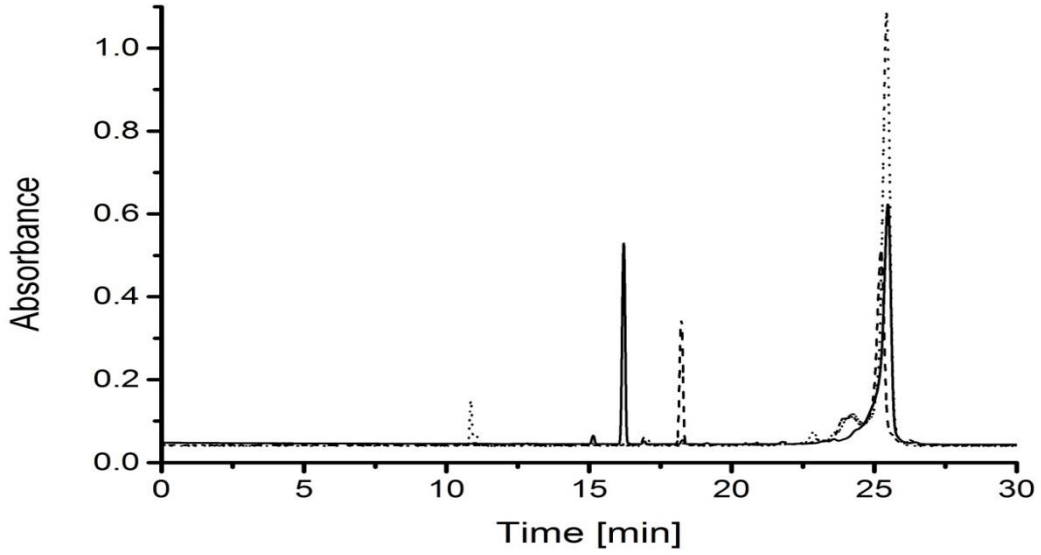

Figure S5. CE separation of the three indicators DNA-I (dotted), DNA-II (solid) and DNA-III (dashed). The smaller peaks appearing first in the electropherogram are the impurities and the larger peaks are the tagged indicators. The work was done using a positive potential of 20 kV.

An increasing content of contaminants correlated with a faster apparent diffusion for the indicators in TDA experiments. In TDA the diffusivity of the indicator will appear as the average of the faster impurities and the slower indicator and therefore be larger than expected for the pure indicator. A similar phenomenon has previously been observed[1] and while it may have an influence on sensitivity, it does not adversely affect the ability to obtain quantitative results on analyte concentrations.

Patient data

Table S2: Data for patients 1-6 at the time of the blood sampling.

patient	ELISA IU/mL	CLIFT 0-3	SLEDAI*	NEPHRITIS
1	≥200	3	5	No
2	≥200	3	4	No
3	≥200	3	12	Yes
4	0	0	0	No
5	0	0	4	No
6	0	0	0	No

*Systemic Lupus Erythematosus Disease Activity Index

References

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(5) Uccellini, M. B.; Busto, P.; Debatis, M.; Marshak-Rothstein, A.; Viglianti, G. A. *Immunology Letters* **2012**, *143*, 85-91.