# Evaluation of Apparent Non-Specific Protein Loss due to Adsorption on Sample Tube Surfaces and/ or Altered Immunogenicity

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**Supplementary theory and analysis**

In order to fully explain the approach used for calculation of signal losses enzyme-linked immunoassay (ELIA) was first chosen, as it involves losses associated with both non-specific adsorption of protein and altered immunogenicity. However, the equations are also applicable to BCA and SPR assays, although the former does not inherently detect losses due to compromised immunogenicity.

The percentage total signal loss was calculated using equation 1 (eq. 1) below,

$$\% \Delta S = X + Y \text{----------(eq. 1)}$$

where,
% ΔS is the total signal loss including losses due to adsorption and compromised immunogenicity, which was calculated from equation A (given in the main manuscript).
X is the percentage signal loss due to compromised immunogenicity, and,
Y is the percentage signal loss due to non-specific adsorption

The percentage signal loss at a particular incubation time ($A_n$) was calculated using eq. 2.

$$A_n = \text{amount corresponding to X + amount corresponding to Y----------(eq. 2)}$$

The total signal loss at 12 h incubation was thus calculated from eq. 2..

The signals obtained at the respective incubation times and temperatures in the specified sample tubes are provided below.

S0RT B (signal obtained for the samples incubated at 0 h RT in BSA-treated tubes) = 2.36
S12RT B (signal obtained for the samples incubated at 12 h RT in BSA-treated tubes) = 1.38
S12RT U (signal obtained for the samples incubated at 12 h RT in untreated tubes) = 0.98
S12 B 4 $^{o}$C (signal obtained for the samples incubated at 12 h at 4 $^{o}$C in BSA-treated tubes) = 1.59
S12 U 4 $^{o}$C (signal obtained for the samples incubated at 12 h at 4 $^{o}$C in untreated tubes) = 1.18

The total signal, loss including losses due to adsorption and compromised immunogenicity, was calculated from eq. A below (given in main manuscript).

$$\% \Delta S = [1 - (S_t / S_{0hB})] \, X \, 100 \text{----------(eq. A)}$$

where,
$S_t$ corresponds to the sample incubated in the untreated tube at a certain given incubation time (~ 12h unblocked for this example).
$S_{0hB}$ corresponds to the sample incubated in the BSA-treated tube at 0 h incubation time.

Therefore, for the sample incubated in the untreated tube for 12 h,

$$\% \Delta S = [1 - (0.98/2.36)] \text{ X } 100 = 59\%$$

The loss due to compromised immunogenicity was calculated from eq. B below. (also given in the main manuscript)

$$\%\Delta S_{\text{immunogenicity-associated}} = [1 - (S_{12\ B\ RT} / S_{12\ B\ 4\ ^{o}CB})] \, X \, 100 \text{---(eq. B)}$$

where,
$S_{12\ B\ RT}$ is signal obtained for the samples incubated at 12 h at RT in BSA-treated tubes
$S_{12\ B\ 4\ ^{o}CB}$ is signal obtained for the samples incubated at 12 h at 4 $^{o}$C in BSA-treated tubes

Therefore, for the sample incubated in the untreated tube for 12 h,

$\%\Delta S_{immunogenicity\text{-}associated} = [1 - (1.38/2.36)]X\ 100 = 42\%$

The loss obtained from eq. B only corresponds to the relative difference between the blocked samples incubated for 12 h at 4 $^{o}$C and the blocked samples incubated for 12 h at RT. Therefore, in order to calculate the exact signal loss contributed by compromised immunogenicity, the amount obtained from eq. B must be equated with the total signal loss (eq. A) obtained for the respective period.

The loss due to impaired immunogenicity (X) in the total signal loss ($A_n$) was calculated by eq. C

$X = [(B_n) . (A_n)]$----------(eq. C)

Where,
X is the loss due to impaired immunogenicity in the total signal loss
$B_n$: loss due to compromised immunogenicity
$A_n$: loss due to adsorption

$X = [42\% . 59\%] = [0.42 . 0.59] = 25\%$

Similarly, the total signal loss due to adsorption can be calculated by eq. D below

$Y = [(100 - B_n). A_n]$ ----------(eq. D)

Where,
Y is the loss due to adsorption in the total signal loss

$Y = [(100 - 42\%) . 59\%] = [58\% . 59\%] = [0.58 . 0.59] = 34\%$

Example:

We initially had 10 ng/mL of HFA in sample
The total amount lost as a function of signal loss (eq. A) = 0.59*10 = 5.9 ng/mL

| **Total amount (TA) = 10 ng/mL** | **Total loss (A) from eq. A % ΔS = X + Y** | **Total loss from eq. C Fraction ofX** | **Total loss from eq. D Y** |
|---|---|---|---|
| Percentage loss | 59% | 42% of A or 25% of TA | 58% of A or 34% of TA |
| Corresponding amount loss | 5.9 ng/mL | 2.5 ng/mL (42% of A)<br>2.5 ng/mL (25% of TA) | 3.4 ng/mL (58% of A)<br>3.4 ng/mL (34% of TA) |

Now if we put the values obtained for X and Y into eq. 2

$A_n$ should be equal to amount corresponding to X + amount corresponding to Y

$A_n$ = 5.9 ng/mL
X = 2.5 ng/mL
Y = 3.4 ng/mL
Therefore,

$A_n = 5.9 = X + Y = 2.5 + 3.4$

**Supplementary table 1.** Effect of varying incubation time of analyte on the sensitivity of the immunoassay as observed by the increase in $EC_{50}$.

| **Treatment ((Un)blocked) and Tube composition** | ***Increase in $EC_{50}$ values (ng/mL) over a period of 12 h of incubation ( $EC_{50}^{12h}$ - $EC_{50}^{0h}$) $\Delta EC_{50}$** | **Analytical error (% CV)** |
|---|---|---|
| **Unblocked PP** | 87 | 24 |
| **BSA-blocked PP** | 31 | 2 |
| **Unblocked GT** | 85 | 19 |
| **BSA-blocked GT** | 11 | 3 |

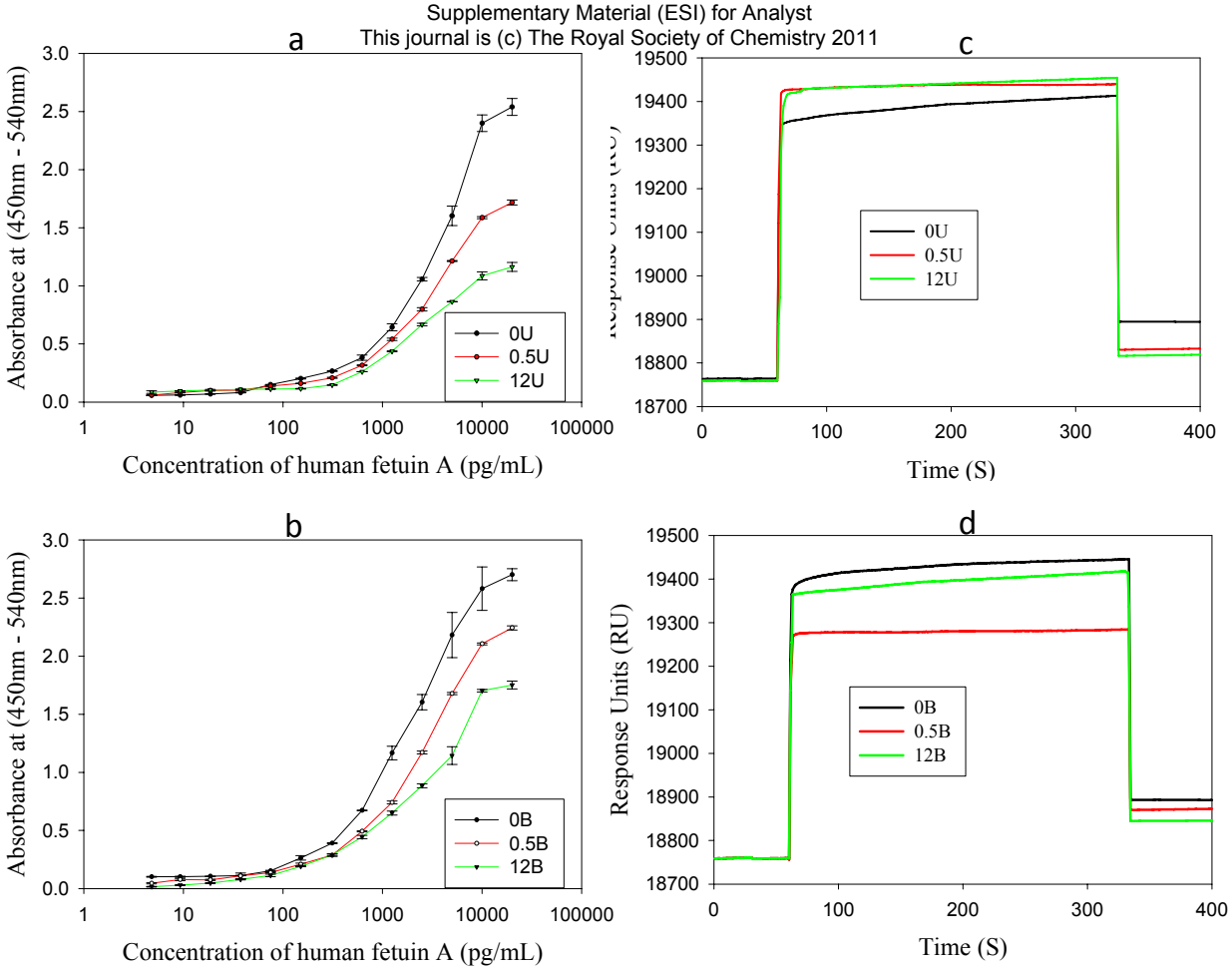
$EC_{50}$ for each set of assay was calculated using SigmaPlot version 11.0.

PP = polypropylene tubes; GT = glass tubes.

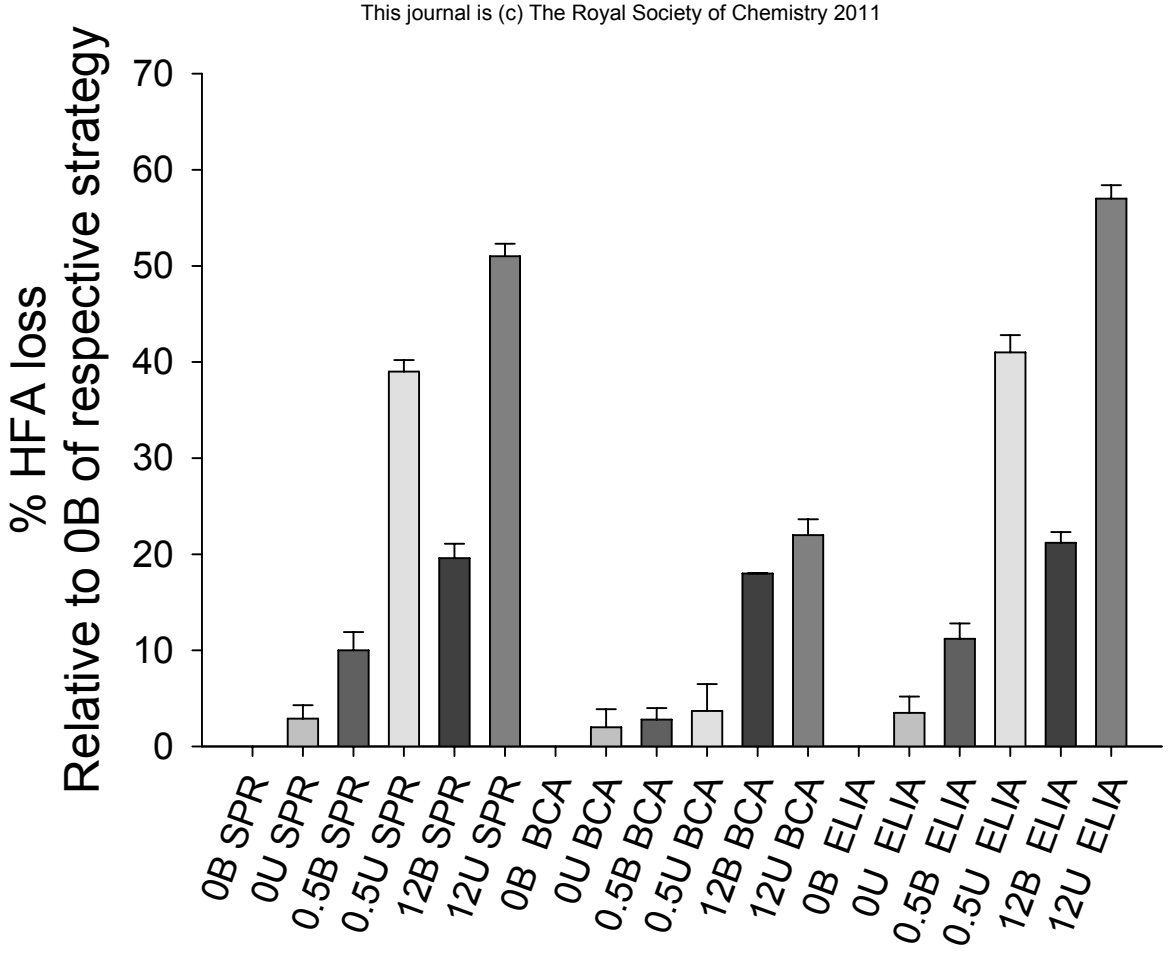
% CV corresponds to the coefficient of variation in all the assay repeats and is calculated as (SD/ Mean of the obtained signal for all repeats) X 100.

SD is standard deviation

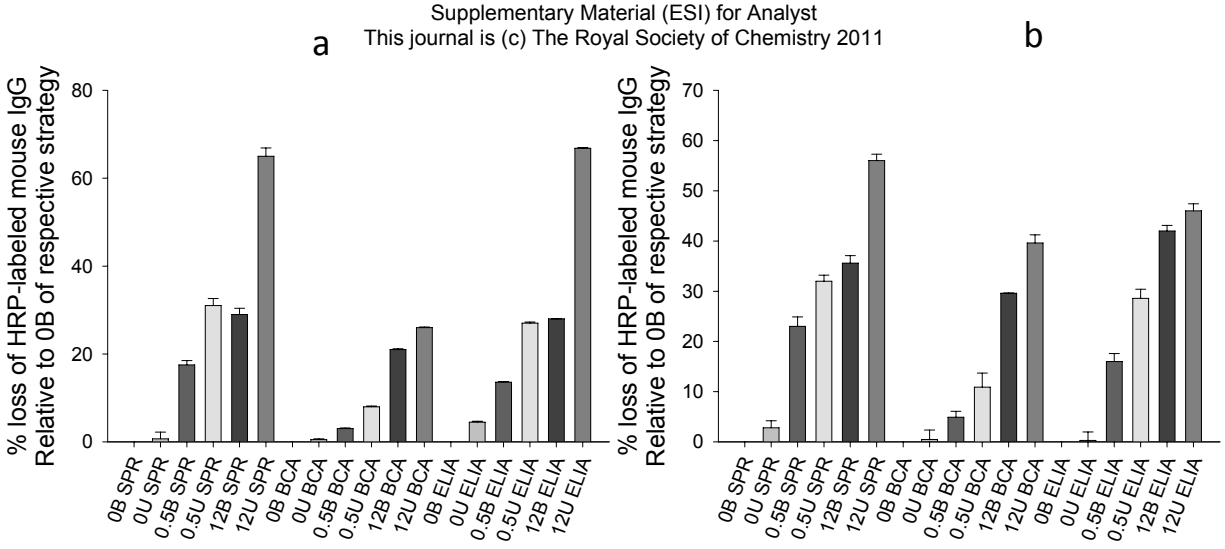
*$EC_{50}$ is a measure of assay performance and was described in the main paper.



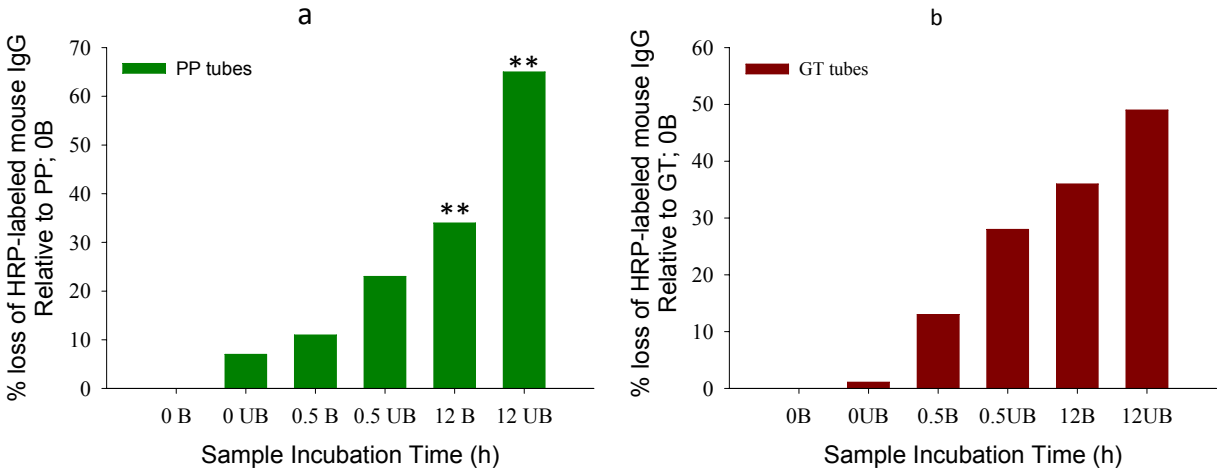

**Supplementary Figure 1.** Assay performance for the HFA samples incubated in BSA-treated (B) and untreated (U) PP tubes. ELIA was performed to analyze the effect of HFA loss on the assay performance with the sample incubated in untreated (a) and BSA-treated (b) PP tubes. It is evident that sampling performed in BSA-treated tubes efficiently reduces the adsorption-associated decline in absorbance. Similar results were obtained with the SPR assay performed with the samples incubated in BSA-treated (d) and untreated (c) PP tubes. Are you sure about this this seems incorrect A significant difference in assay signals obtained for incubation for 0.5h in BSA-treated and untreated tubes demonstrates the analyte loss as a function of incubation time as characterized by ELIA and SPR. Error bars in assay points correspond to standard deviations.




**Supplementary Figure 2.** Assay performance for the HFA samples incubated in BSA-treated (B) and untreated (U) glass tubes (GT tubes). The BCA assay was performed to analyze the total protein content available in solution. The protein loss can be quantified by relative analysis with respect to samples incubated in blocked tubes at 0h (0B). Results for the BCA assay are designated as '0B BCA' to '12U BCA'. ELIA and SPR were performed to analyse the effect of HFA loss on assay performance with both sandwich and direct immunoassay detection approaches, respectively. The samples results corresponding to ELIA and SPR are suffixed as 'ELIA' or 'SPR' on the X-axis. Since losses due to adsorption and altered immunogenicity are determined in both the ELIA and SPR-based detection approaches, therefore, the percentage signal loss obtained for the HFA in all the sets of ELIA and SPR is higher than that obtained with the BCA assay. Error bars correspond to standard deviations.




**Supplementary Figure 3.** Assay performance analysis for HRP-labeled mouse IgG incubated in BSA-treated (B) and untreated (U) (a) PP and (b) GT tubes. A similar analyte loss pattern was obtained for PP and GT tubes. However, this loss was higher with the use of GT tubes. The symbols used are as in previous diagrams




**Supplementary Figure 4.** Analysis of the effect of the nature of the sample tube and non-specific losses due to HRP-labeled mouse IgG adsorption. HRP-labeled mouse IgG was incubated in the BSA-treated and untreated polypropylene (PP) and glass (GT) tubes. The BSA-treated and untreated are designated as 'B' and 'U', respectively. Performance of the assay for samples in GT and PP was similar. However, comparatively less signal and hence less protein analyte was lost in both treated and untreated set of PP tubes. The relative signal loss was calculated using the formula (A) described in experimental section. ** The fractional contribution of compromised functionality in the total signal loss was also calculated for samples incubated in PP tubes (Table 1 & Table 2).