Electronic Supplementary Information

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Quantitative Electrochemical Metalloimmunoassay for TFF3 in Urine using a Paper Analytical Device

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Preparation of artificial urine

Artificial urine was prepared, following a procedure reported by Brooks and Keevil,[1] with some modifications, by mixing 2.0 mM citric acid monohydrate, 25 mM $NaHCO_3$, 170 mM urea, 2.5 mM $CaCl_2$, 90 mM NaCl, 2.0 mM $MgSO_4$, 10 mM Na_2SO_4, 7.0 mM KH_2PO_4, 7.0 mM K_2HPO_4, and 25 mM NH_4Cl in DI H_2O. The pH of the solution was adjusted to 6.0 using 1 M HCl.

Collection of urine samples

Fresh urine was obtained from 2 individuals in the afternoon. The collection of urine from these individuals was approved by the Human Subjects and Institutional Review Board at The University of Texas at Austin.

Preparation of MμB/spAb and AgNP/2°mpAb/mpAb conjugates

The MμB/spAb conjugate was prepared according to a protocol provided by the bead supplier (Invitrogen). Briefly, beads were weighed, incubated in PBS for 10 min, washed with PBS twice, and then incubated with the spAb in PBS containing 1 M $(NH_4)_2SO_4$ at 37 °C overnight. The following morning the beads were washed twice with 100 mM borate (pH 7.5) and then incubated in a blocking buffer composed of 1 wt% sodium casein in 100 mM borate (pH 7.5) for 2 h. The beads were then washed twice with the blocking buffer, resuspended in the blocking buffer, and stored at 4 °C until needed. The optimized assay required ~2.7 x 10^7 beads per replicate.

The AgNP/2°mpAb/mpAb conjugate was prepared following a previously reported procedure[2] with a few modifications. Briefly, 1.0 mL of 20 nm (nominal) citrate-capped AgNP stock solution was centrifuged at 16.6 xg and 20 °C for 20 min. The resulting pellet was then resuspended in 1.0 mL of 100 mM borate (pH 7.5) solution. The 2°mpAb was added until its concentration was 10

μg/mL, and then the mixture was agitated for 15 min, centrifuged for 20 min using the previously listed conditions, and finally the pellet was resuspended in 1 mL of 100 mM borate solution (pH 7.5). The mpAb was added to the mixture until its concentration was 10.0 μg/mL, and the mixture was then agitated for an additional 15 min. Casein (1 wt% in 100 mM borate, pH 7.5) was added to the mixture (final concentration: 0.10 wt%) to block the AgNPs and facilitate pellet resuspension after centrifugation. After 15 min, the mixture was centrifuged again using the aforementioned conditions and the pellet resuspended in 100 mM borate (pH 7.5) containing 0.1 wt% casein. After an additional 15 min, a fourth centrifugation was performed using the same conditions to wash away additional unconjugated Ab, and then the pellet was resuspended in 100 mM borate (pH 7.5) containing 1 wt% casein. The resulting conjugate was stored at 4 °C until needed. Each assay required ~2.0 x 10^{10} AgNPs per replicate.

Screening AgNP/anti-TFF3 antibody conjugates for TFF3 activity

AgNP/anti-TFF3 antibody conjugates were prepared following a procedure similar to the one described for the preparation of the AgNP/2°mpAb/mpAb conjugate. Two conditions were used to prepare the AgNP/anti-TFF3 antibody conjugates: 100 mM borate solution (pH 7.5) and 1 mM borate solution (pH 8).

The same procedure was followed for preparing the AgNP/anti-TFF3 antibody conjugates using either 100 mM borate solution (pH 7.5) or 1 mM borate solution (pH 8). 1.0 mL of 20 nm (nominal) citrate-capped AgNP stock solution was centrifuged at 16.6 xg and 20 °C for 20 min. The pellet was then resuspended in 1.0 mL of either 100 mM borate (pH 7.5) or 1 mM borate (pH 8) solution. An anti-TFF3 antibody was added, so that the final concentration was 10 μg/mL, and the mixture was agitated for 15

min. The mixture was then centrifuged for 20 min using the previously listed centrifugation conditions and the resulting pellet was again resuspended in either 1.0 mL of 100 mM borate (pH 7.5) or 1 mM borate (pH 8), each containing 0.1 wt% casein to block exposed surface on the AgNPs. After an additional 15 min incubation, a third centrifugation was performed, using the same conditions, to remove unconjugated antibody. Finally, the pellet was resuspended in 100 mM borate (pH 7.5) containing 1 wt% casein (for both conditions). The resulting conjugate was stored at 4 °C until needed.

After preparing the conjugates, their activities were screened using a TFF3 assay in a half-sandwich format. Briefly, 50 μL of 1.0 μg/mL TFF3 in 100 mM borate (pH 7.5) solution were incubated overnight at 4 °C in a Costar 3590 high-binding microtiter plate. After washing the plate 3 times with 100 mM borate (pH 7.5) solution, 300 μL 1 wt% casein in 100 mM borate (pH 7.5) solution was added to block the wells. The plate was incubated for 30 min with gentle agitation. After emptying the plate, 50 μL of the prepared AgNP/Ab conjugate was added and the plate was incubated for 15 min with gentle shaking. Next, the plate was washed 3 times with 100 mM borate (pH 7.5). After washing, ASV was performed on each of the wells as described in the Experimental Section (main text).

Table S1. List of 8 anti-TFF3 antibodies screened for activity against TFF3 after binding the antibodies to AgNPs under the 2 sets of conditions described in the previous section.

Supplier	Host Species	Polyclonal/ Monoclonal	Activity/ 100 mM borate pH 7.5	Activity/ 1 mM borate pH 8
R & D Systems	Mouse	Monoclonal	No	No
Abgent	Mouse	Monoclonal	No	No
Abcam	Rabbit	Monoclonal	No	No
RayBiotech	Rabbit	Polyclonal	No	No
Proteintech	Rabbit	Polyclonal	Some	Yes
ProSci	Rabbit	Polyclonal	No	No
LifeSpan BioSciences	Rabbit	Polyclonal	No	No
Abbiotech	Rabbit	Polyclonal	No	Some

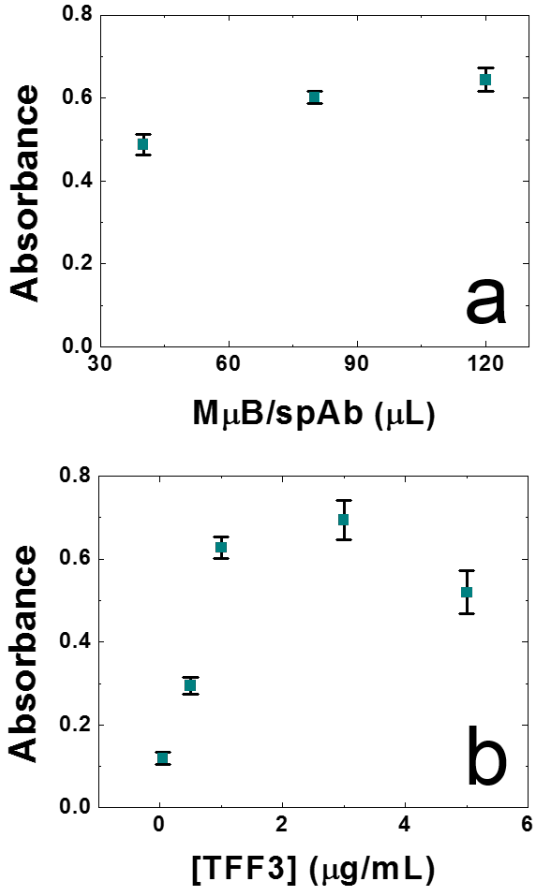

Figure S1. Partial optimization of the one-step incubation procedure using artificial urine. The data were obtained following the procedure for one-step assays with spectroscopic detection outlined in the Experimental section (main text). See Figure 3a (main text) for the unoptimized dose-response curve in artificial urine. (a) Plot of absorbance vs the volume of MμB/spAb used for partial optimization. The unoptimized assay reagents listed in Figure 3a were used, with the following exceptions: 10 μg/mL TFF3, 50 μg/mL spAb, and 5 μg/mL mpAb for all MμB/spAb volumes. (b) Plot of absorbance vs concentration of TFF3 for one-step incubation. The assay reagents are the same as for Figure 3b (main text) except spectroscopic detection was used. The assay reagents in (a) were used, but with different TFF3 concentrations and 80 μL of MμB/spAb. For all results in this figure, each data point represents the mean of 3 replicates and the error bars represent the standard deviation of the mean.

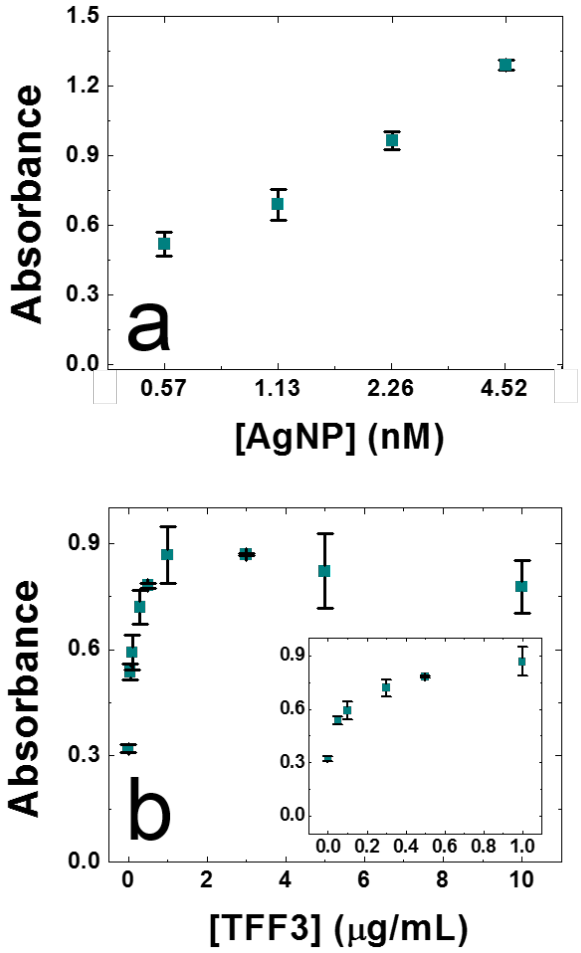

Figure S2. Final optimization of the one-step incubation procedure in artificial urine. The data were obtained following the procedure for one-step assays with spectroscopic detection outlined in the Experimental section (main text). (a) Plot of absorbance vs the AgNP conjugate concentration (which is equivalent to the AgNP concentration). The assay reagents are the same as in Figure S1b, but with the concentration of TFF3 fixed at 5 μg/mL. (b) Plot of absorbance vs the concentration of TFF3. This is the optimized one-step incubation dose-response curve, and it was obtained using the same conditions as in Figure 3c (main text), but the data were obtained spectroscopically. The conditions were the same as in (a), but using 20 μL of 3.39 nM AgNP conjugate and different TFF3 concentrations. The inset shows an expanded view of the low concentration range. For all results in this figure, each data point represents the mean of 3 replicates and the error bars represent the standard deviation of the mean.

Table S2. Determination of TFF3 concentrations in unspiked human urine samples using the R & D Systems TFF3 ELISA kit.

	Afternoon Urine – Sample 1	**Afternoon Urine – Sample 2**
[TFF3] Determined from R & D Systems Kit (µg/mL)	0.143	0.036

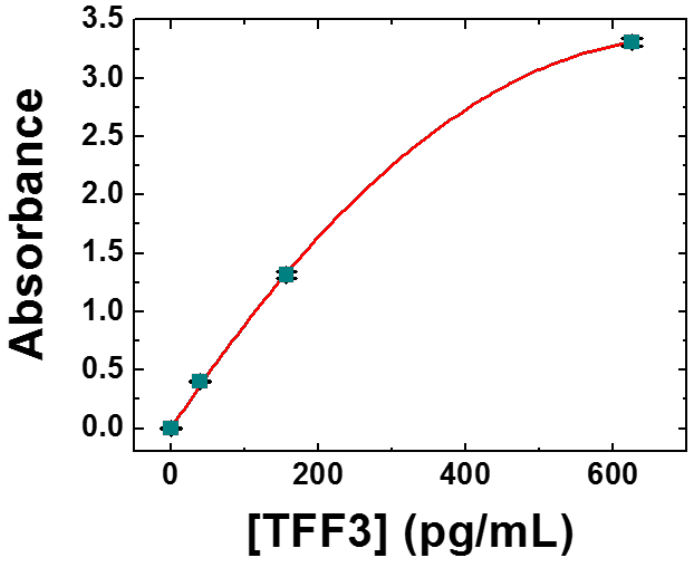

Figure S3. TFF3 dose-response curve generated using the R & D Systems kit. Artificial and human urine samples were spiked with a known concentration of TFF3 and diluted by a factor of 500 using the buffer provided with the kit.[3] A dose-response curve was generated following the kit protocol, and then the known TFF3 concentration in each sample was back-calculated from the dose-response curve. The data were fit with a second-order, zero-intercept regression model. Each data point represents the mean of 3 replicates and the error bars represent the standard deviation of the mean.

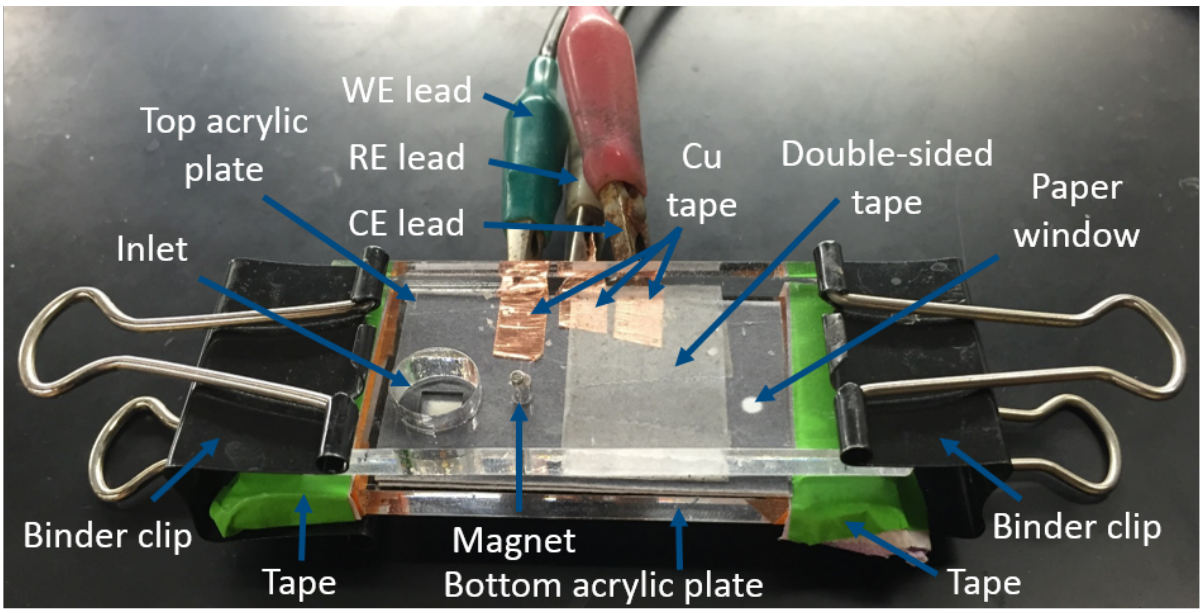

Figure S4. Photograph of assembled *o*Slip device.

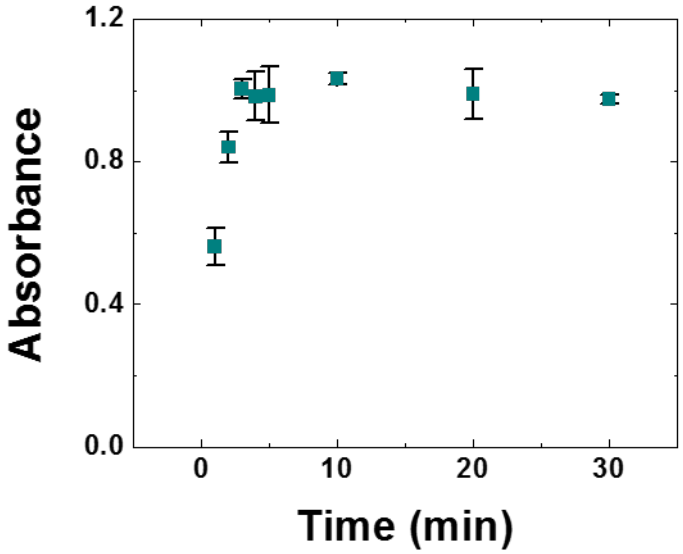

Figure S5. Variation of absorbance vs. assay incubation time for the one-step incubation. 20 µL of MµB/spAb containing 10 µg/mL spAb were used while the MµB concentration was 5 mg/mL. 1 µg/mL TFF3 prepared in 1 wt% casein and 0.565 nM of AgNP/2°mpAb/mpAb were used for all time points. After formation of the immunocomplex, a goat anti-rabbit 2° antibody conjugated to HRP (Invitrogen, Cat. No. 65-6120), diluted 1:2000 in 1 wt% casein, was incubated for 15 min. After washing, signal was generated via absorbance readings of the TMB reaction product at 450 nm.

References

1. T. Brooks and C. W. Keevil, *Lett. Appl. Microbiol.*, 1997, **24**, 203–206.

2. M. S. Szymanski and R. A. Porter, *J. Immunol. Methods*, 2013, **387**, 262–269.

3. R & D Systems Quantakine TFF3 ELISA Kit Manual