Supplementary material

2. Materials and methods

2.3 Buffers for cortisol-HRP conjugate working solution

Composition of buffers tested to achieve long-term stability of cortisol-HRP conjugate working solution.

- Buffer A: 100 mmol L^{-1} 2-(N-morpholino)ethanesulfonic acid (MES), 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 1 mmol L^{-1} $MgCl_2$, 0.01% Thimerosal, 0.1% streptomycin sulfate, 1 mmol L^{-1} $SrCl_2$, 1:1000 Proclin 300, 0.1% gum arabic, 3% BSA, 0.1% Tween 20, pH 6.0.
- Buffer B: 16 mmol L^{-1} Na_2HPO_4, 4 mmol L^{-1} NaH_2PO_4, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} $SrCl_2$, 0.1% streptomycin sulphate, 0.1% Proclin 300, 0.1%, Gum Arabic, 3% BSA, 0.1% Tween 20, pH 7.4.
- Buffer C: 25 mmol L^{-1} Na_2HPO_4, 75 mmol L^{-1} NaH_2PO_4, 0.015% 8-anilino-1-naphthalenesulfonic acid, 3% BSA, 0.1% Tween 20, pH 6.5.
- Buffer D: 100 mmol L^{-1} 2-(N-morpholino)ethanesulfonic acid (MES), 1 mmol L^{-1} EDTA, 1 mmol L^{-1} $MgCl_2$, 0.1% streptomycin sulfate, 1 mmol L^{-1} $SrCl_2$, 10 µmol L^{-1} hemin, 50 mg L^{-1} cytochrome c, 1:1000 Proclin 300, 0.1% gum arabic, 3% BSA, 0.1% Tween 20, pH 6.0.
- Buffer E: 50 mmol L^{-1} boric acid, 50 mmol L^{-1} sodium citrate, 0.1% BSA, 0.05% Tween 20, 0.01% thimerosal, 8 mmol L^{-1} EDTA, pH 6.0.

3. Results and discussion

3.1 Optimization of the immunoassay

3.1.1 Anti-cortisol antibody

The anti-cortisol antibody selected for LFIA development was immobilized at different dilutions (ranging from 1:500 to 1:10,000 v/v) on LFIA nitrocellulose membranes, then tested by analysing cortisol-free oral fluid samples and cortisol-spiked (1 and 10 ng mL^{-1}) oral fluid samples. Results are shown in Figure S1.

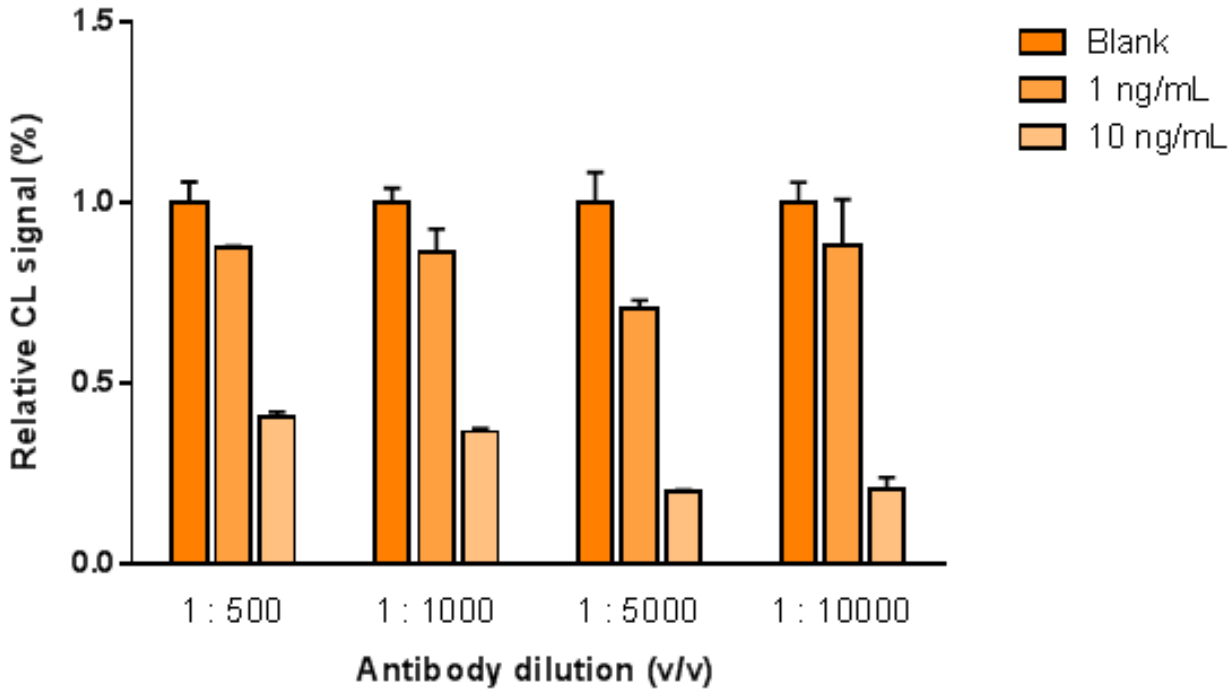

Figure S1: CL signals obtained upon immobilizing anti-cortisol antibody at different concentrations on LFIA nitrocellulose membranes and then analysing oral fluid samples at different cortisol concentrations (0, 1 and 10 ng mL^{-1}). Results are normalized for the signal obtained in the absence of cortisol.

3.1.4 LFIA strip materials

Different commercial sample pads were tested by analysing cortisol-free oral fluid samples. As shown in Figure S2, the highest signal/background ratio was obtained using the saliva pad II, which was thus selected for assay development.

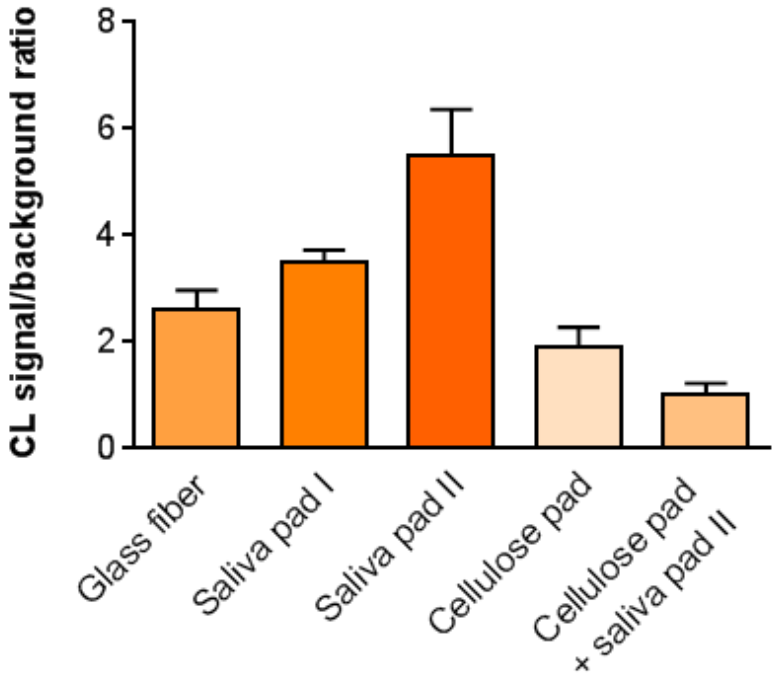

Figure S2: CL signals/background ratio values obtained upon analysing cortisol-free oral fluid samples on LFIA strips equipped with different commercial sample pads.

3.1.5 Shelf-life of reagents: stability of HRP-cortisol conjugate

Different conservation buffers (A - E) were evaluated by performing an accelerated storage test on cortisol-HRP conjugate solutions at three concentration levels, namely 1:250, 1:500 and 1:1000 (v/v) dilutions.

As shown in Figure S3, buffers D and E provided a satisfactory stability of cortisol-HRP conjugate (up to 3 weeks at 37°C) at all concentration levels, while buffers A, B and C buffers were not able to preserve its activity.

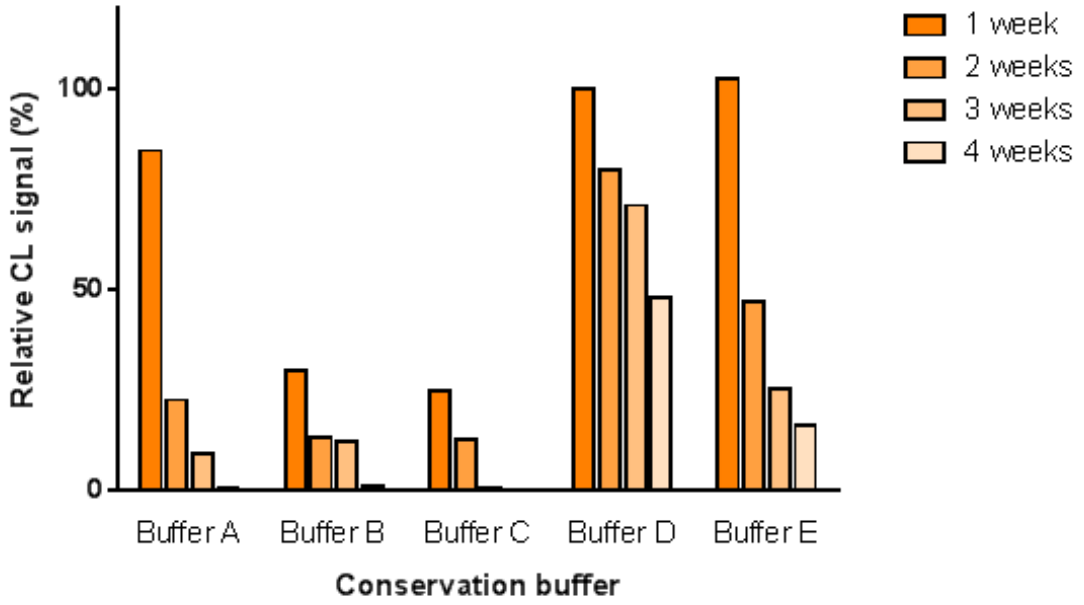

Figure S3: CL signals obtained upon analysing cortisol-free oral fluid samples employing cortisol-HRP conjugate solutions prepared at three concentration levels (1:250, 1:500 and 1:1000 (v/v) dilutions) in different conservation buffers (A - E) and stored at 37°C for up to 4 weeks. Results are compared with those obtained using a freshly prepared cortisol-HRP conjugate solution in the same buffer.

3.1.6 Washing

To eliminate the washing step prior to CL measurement we evaluated the possibility to increase the amount of CL substrate loaded onto the LFIA strip and exploit the flow of the CL substrate also for washing.

As shown in Figure S4, both washing of the LFIA strip with PBS (50 µl) prior to the CL measurement and increase up to 70 µl of the amount of the CL substrate loaded in the sample pad eliminated the interference of the oral fluid matrix on the CL reaction.

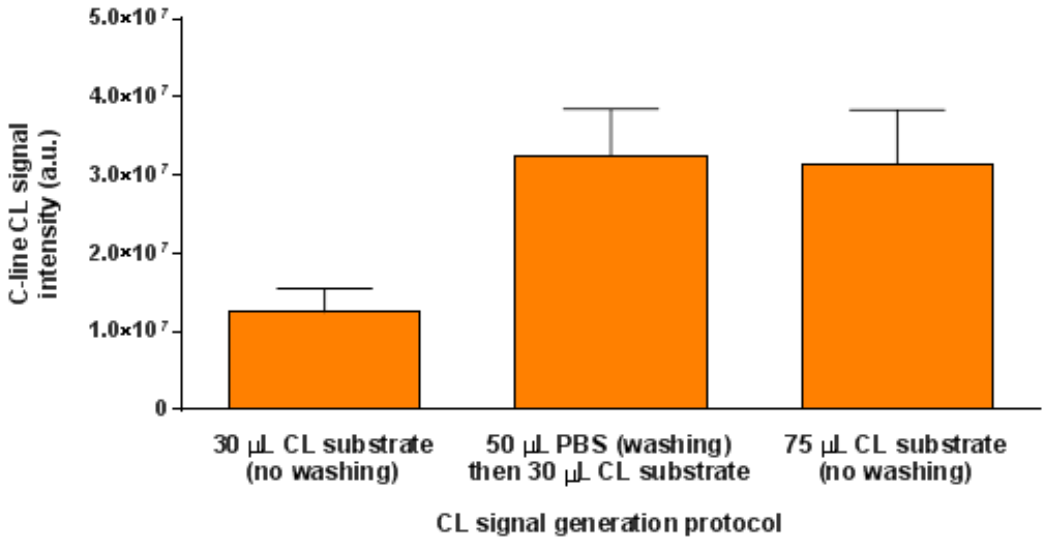

Figure S4: Mean CL signals (n = 3) measured in the C-line during analysis of cortisol-free oral fluid samples using different protocols for CL signal generation: (a) loading of 30 µl of CL substrate on the sample pad (no washing); (b) washing with 50 µl of PBS followed by loading of 30 µl of CL substrate on the sample pad; (c) loading of 75 µl of CL substrate on the sample pad (no washing).

3.1.7 Method performance

Assay accuracy was also evaluated by analysing 10 saliva samples and comparing the results with those obtained using a commercial ELISA kit for salivary cortisol obtained from Diametra. The results shown in Figure S5 showed a good correlation between the two assays, with recovery values comprised between 84% and 112%.

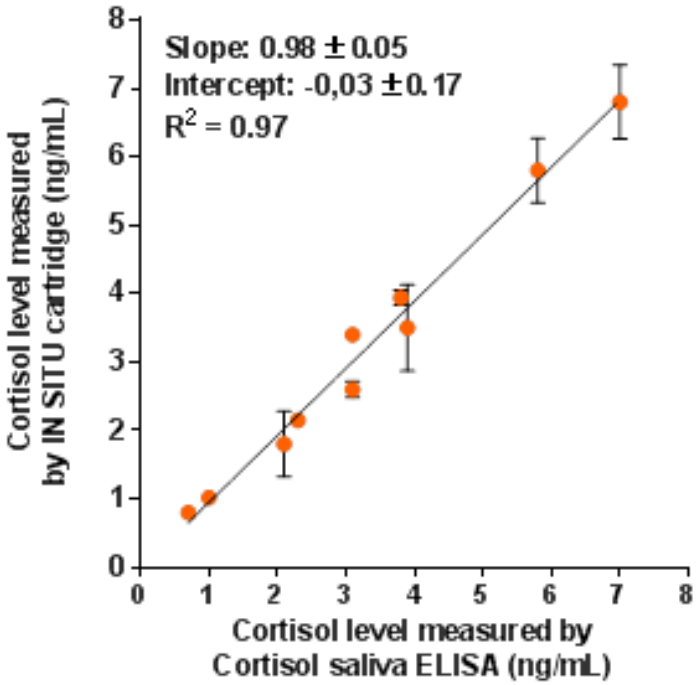

Figure S5: Comparison of the cortisol levels measured in 10 saliva samples using the IN SITU LFIA cartridge and the commercial ELISA kit for salivary cortisol.