A simple and compact smartphone accessory for quantitative chemiluminescence-based lateral flow immunoassay for salivary cortisol detection

A SIMPLE AND COMPACT SMARTPHONE ACCESSORY FOR QUANTITATIVE CHEMILUMINESCENCE-BASED LATERAL FLOW IMMUNOASSAY FOR SALIVARY CORTISOL DETECTION

Martina Zangheri[a], Luca Cevenini[a], Laura Anfossi[b], Claudio Baggiani[b], Patrizia Simoni[c], Fabio Di Nardo[b], Aldo Roda[a]

[a] Department of Chemistry, Alma Mater Studiorum – University of Bologna, Via Selmi 2, 40126 Bologna, Italy.

[b] Department of Chemistry, University of Turin, Via P. Giuria 5, 10125 Turin, Italy.

[c] Department of Medical and Surgical Sciences, Alma Mater Studiorum – University of Bologna, Via Massarenti 9, 40138 Bologna, Italy.

*Corresponding author

Prof. Aldo Roda

Department of Chemistry, Alma Mater Studiorum – University of Bologna, Via Selmi 2, 40126 Bologna, Italy.

Tel/fax +39 051343398

E-mail aldo.roda@unibo.it

Abstract
We have developed a simple and accurate biosensor based on a chemiluminescent (CL)-Lateral Flow Immunoassay (LFIA) method integrated in a smartphone to quantitatively detect salivary cortisol. The biosensor is based on a direct competitive immunoassay using peroxidase-cortisol conjugate, detected by adding the chemiluminescent substrate luminol/enhancer/hydrogen peroxide. The smartphone camera is used as light detector, for image acquisition and data handling via a specific application. We 3D-printed simple accessories to adapt the smartphone. The system comprises a cartridge, which houses the LFIA strip, and a smartphone adaptor with a plano-convex lens and a cartridge-insertion slot. This provides a mini-darkbox and aligned optical interface between the camera and the LFIA membrane for acquiring CL signals. The method is simple and fast, with a detection limit of 0.3 ng/mL. It provides quantitative analysis in the range of 0.3-60 ng/mL, which is adequate for detecting salivary cortisol in the clinically accepted range. It could thus find application in the growing area of home-self-diagnostic device technology for clinical biomarker monitoring, overcoming the current difficulties in achieving sensitive and quantitative information with conventional systems taking the advantage of smartphone connectivity and the enhanced performance of the included camera.

Keywords
Biosensor; chemiluminescence; lateral flow immunoassay; cortisol; point-of-care; smartphone.

1. Introduction

Developing an accurate and user-friendly diagnostic device for “point-of-care” (POC) applications is one of the most challenging objectives in the clinical field. Devices for POC analysis should be portable, quick, and easy to use. In an integrated, self-standing device, such devices should be able to perform the entire analytical process, from sample pre-treatment to measurement and data processing. Immunological methods are suitable for this because their high specificity and sensitivity makes it possible to detect clinical biomarkers even at low concentrations and in complex matrices. Within this context, paper-based immunoassays are an emerging alternative platform. This is because they are low cost and easy to handle, thanks to the use of capillary force for fluid transport and delivery without external power (Ballerini et al., 2012; Li et al., 2011; Martinez et al., 2008; Martinez et al., 2010; Posthuma-Trumpie et al., 2009; Zhao and Van der Berg, 2008). Of the paper-based analytical methods, lateral flow immunoassays (LFIAs) are considered one of the most commercially feasible analytical tools for rapid and portable clinical immunodiagnostics (Li et al., 2011; Qin et al., 2012), such as diagnosing blood infections or contamination, drug abuse, or for ascertaining pregnancy (Posthuma-Trumpie et al., 2009).
In LFIAs, the immunoassay is performed on nitrocellulose membrane. The immunoreagents are immobilized in specific positions, while other reagents are carried along the strip by the flow driven by capillary forces established upon sample addition. The most common detection principle uses bioreagents labelled with colloidal gold or latex particles. Due to label accumulation in specific positions along the strip, coloured bands form and are visually detected to provide a “yes/no” response on the analyte presence. However, in recent years, the LFIA format has been combined with portable instruments that provide quantitative sensing based on the use of portable photometric strip readers to measure the lines’ colour intensity (Anfossi et al., 2010; Molinelli et al., 2009; Salter et al., 2006) or by using alternative labels, such as enzymes (Cho et al., 2006), fluorescent nanoparticles (Li et al., 2010; Xia et al., 2009; Zou et al., 2010), electrochemical measurements (Fernandez-Sanchez et al., 2004; Muhammad-Tahir and Alocilja, 2003), and more recently chemiluminescence

detection of horseradish peroxidase (HRP) labelled reagents (Mirasoli M. et al., 2012a, 2012b; Roda et.al., 2003; Wang et al., 2007). Optical detection based on chemiluminescence (CL) is an ideal method for miniaturization and POC biosensor development because of its inherent sensitivity and simplicity. Miniaturized hydrogenated amorphous silicon photodiode array (Caputo et. al., 2013) and organic photodiodes (OPDs) (Pires and Dong, 2014) have recently been proposed as a low-cost and versatile technology for developing a miniaturised biosensor with high detectability and sensitivity. Another approach for CL detection is light imaging with a charge-coupled-device (CCD) or complementary metal-oxide semiconductor (CMOS) sensor, providing not only a measurement of emitted photons but also the 2D image of a single or an array of spots (Roda et al., 2011a). This allows spatial and, with one-shot colour cameras, spectral resolution. Nowadays, improvements in the sensitivity of the back side illuminated CMOS (BSI-CMOS) used in smartphone cameras mean it can be used to detect chemiluminescence. Moreover, the connectivity and data processing offered by smartphones can be exploited to perform analysis at home with simple procedures. The system could eventually be used to monitor patient health and directly notify the physician of the analysis results. This would decrease costs and increase healthcare availability and accessibility (Ozdalga et al., 2012; Zhu et. al., 2012).
Recent papers have shown it is possible to use a smartphone as a detector for a colorimetric LFIA method (Mudanyali et al., 2012; Sangdae et al., 2013). Until now, the use of CL detection and smartphone technology for LFIA methods has not been reported.
We therefore developed a portable analytical device that transforms a smartphone into a chemiluminescence detector for quantitative LFIA analysis. The analytical device comprises a smartphone equipped with custom-designed accessories made using a low-cost desktop 3D printer. The add-on assembly comprises two parts: a cartridge hosting the LFIA membrane and a smartphone adaptor, containing a plano-convex lens aligned with the camera and a slot for inserting the cartridge. Once the operator has carried out the assay on the LFIA strip, both the smartphone and the cartridge are inserted into the assembled cradle to perform the measurement. A built-in smartphone photography application and the camera's autofocus system were used to obtain an optimized image of the sensing surface.
We demonstrated the performance of the system by quantitative detection of salivary cortisol, which is considered a biomarker of stress, anxiety, and depression (Hellhammer et al., 2009; Tlili et al., 2011). Salivary levels of most steroids are very low; at least a factor 10 with respect to plasma levels (Roda et al., 1984). The developed assay must therefore be highly sensitive and accurate in order to detect disease-related variations at nanomolar levels. Normal cortisol level in saliva displays a circadian biorhythm, from a maximum observed in the morning of about 3-10 ng/mL to a nocturnal minimum of 0.6-2.5 ng/mL. Changes in these values or in cortisol biorhythm can be used as diagnostic indexes of stress-related diseases. We optimized a competitive immunoassay, in which cortisol in saliva and horseradish peroxidise (HRP)-cortisol conjugate compete for anti-cortisol antibody immobilized on the nitrocellulose membrane. The HRP-cortisol bound to antibody will be detected using a chemiluminescent substrate based on luminol/H_2O_2 and enhancers.
This system allows uniform and repeatable image acquisition of the strip and an objective and accurate quantification of cortisol in saliva samples.

2. Materials and methods

2.1 Reagents

Cortisol standard solution, polyclonal anti-peroxidase (HRP) antibody produced in rabbit, bovine serum albumin (BSA), and Tween-20 were purchased from Sigma Aldrich (St. Louis,

MO, USA). The Supersignal ELISA Femto CL substrate for HRP was purchased from Thermo Fisher Scientific Inc. (Rockford, IL). Polyclonal anti-cortisol antibody produced in rabbit was purchased from Analytical Antibodies (Bologna, Italy). HRP-conjugate cortisol was obtained from Diametra (Milano, Italy). The other reagents were of analytical grade and were used as received.
Salivettes cotton swabs for the saliva sampling were purchased from Sarstedt, Germany. The Silgard 184 polydimethylsiloxane prepolymer and the curing agent were obtained from Dow Corning (Midland, MI).
Assay strips for LFIA were prepared by immobilizing on nitrocellulose membranes the rabbit anti-cortisol antibody (T-line) and the rabbit anti-peroxidase antibody (C-line) keeping a distance of 5 mm between the two lines. The membranes were then assembled with a sample and an adsorbent pad and cut into sections. Details are available as Supplementary Material.

2.2 LFIA assay principle and format
An indirect competitive CL-LFIA assay was developed to achieve high salivary cortisol detectability and quantitative information. Cortisol in sample or standard solutions and HRP-cortisol conjugate migrate along the nitrocellulose membrane strip by capillarity. When reaching the area of the strip where the anti-cortisol antibody was immobilized (T-line), the cortisol in the sample competed with HRP-cortisol for binding a fixed and limited amount of immobilized anti-cortisol antibody. Unbound reagents continued to migrate until they reached the area of the strip where excess HRP-cortisol conjugate was captured by immobilized anti-HRP antibody (C-line). In the detection step, a CL substrate for HRP was added to the strip and the resulting CL signal was imaged using the smartphone camera.
The intensity of the CL signal of the T-line, which is inversely related to the amount of cortisol, allowed us to quantify the amount of cortisol in the sample. For quantitative analysis, the CL signal of the T-line was normalized with respect to that of the C-line of the same strip, improving the reproducibility of the assay. This allowed us to take into account environmental and matrix factors that might affect the intensity of the CL signal. These include changes in ambient temperature or the presence of HRP inhibitors in the sample. As with qualitative colorimetric LFIAs, the CL signal of the C-line was also used to assess the validity of the assay (i.e. an intense signal indicated the correct migration of the reactants along the strip).

2.3 Smartphone 3D printed accessory
A compact 3D-printed smartphone adaptor and an LFIA cartridge were made using the Makerbot Replicator 2X (Makerbot, Boston MA, USA). Thermoplastic black and yellow acrylonitrile butadiene styrene (ABS) polymers were used as printing material. 3D models were created using the browser-based 3D design platform Tinkercad. The MakerWare slicer software was used to define printing options and settings.
The smartphone accessory (Figure 1a) was designed for the Samsung Galaxy SII Plus smartphone. It holds a plano-convex lens of 6mm diameter (Edmund Optics, York, UK) in contact with the phone objective and houses an LFIA cartridge (8 cm length, 2.5 cm width and 1cm thickness), preventing ambient light interference in the camera objective. The LFIA cartridge supports a nitrocellulose strip and contains a 25 µl reservoir for HRP-cortisol conjugate solution, two separate 100 µl reservoirs for CL substrate and PBS, and an inlet for sample injection. The cartridge was printed in two separate pieces in order to insert the strip and reagents. A lid provides an optical window for signal acquisition and a grid allows evaporation of the absorbent pad (Figure 1b).

Since this approach has not previously been used to acquire CL signals from LFIA membranes, the analytical performance of the smartphone camera was compared with that of a conventional laboratory ultrasensitive imaging instrument, the Night OWL LB 981 luminograph (Berthold Technologies GmbH & Co KG, Bad Wildbad, Germany) equipped with a conventional lens-based optics and a highly sensitive, back-illuminated, double-Peltier-cooled, black and white CCD camera previously developed in our laboratory (Roda et al., 1996, 2002).

2.4 Sample and spiked sample preparation

Saliva samples were normally collected from healthy volunteers at 8 AM before eating, while the saliva used for the calibration curves was collected at 9 PM, when cortisol concentration is usually lower due to circadian variation. The confirmatory analysis for cortisol in saliva was performed using a commercial ELISA kit (Cortisol saliva ELISA, Diametra, Milan, Italy). To establish the calibration curves in saliva, several dilutions of cortisol were added to saliva to obtain the desired concentrations (between 0 and 100 ng/mL).

2.5 Assay procedure

Saliva samples were collected by resting an adsorbent swab inside the mouth until it was saturated (60-90 seconds). Then, the swab was placed into a syringe for immediate compression. The LFIA was begun by transferring a small volume (25 □L) of saliva into a prefilled well, positioned near to the sample pad, through a PDMS-based injection septum. The syringe needle penetrated through the PDMS layer and the sample was mixed up with the prefilled solution of HRP-cortisol conjugate diluted 1:100 (v/v) in PBS containing BSA 6% and Tween 0.2%.

Once injection of saliva was completed and the solution had reached the sample pad, it began to flow across the membrane where the immunoreactions take place. Upon complete migration of the solution (15 min), a washing step was performed by flowing 50 □L of PBS for 10 min. Then, the strip was wetted by 100 □L of CL substrate, stored in a reservoir placed near the nitrocellulose membrane. After inserting the cartridge into the slot on the smartphone's cover (Figure 1c), the CL signals were acquired (10 seconds, 0,3 MP 4:3 images) using a professional camera application for mobile devices (Camera FV-5 Lite). The timing of all the LFIA steps were controlled with a stopwatch application in the smartphone. The capture of the image was synchronized with a stopwatch set at 15 s and started with the injection of the CL substrate in order to acquire the image during the steady state CL emission.

To obtain quantitative information, a calibration curve was generated using cortisol standard solutions prepared in saliva (seven concentrations ranges from 0 ng/mL to 100 ng/mL). Using ImageJ software v.1.46 (National Institutes of Health, Bethesda, MD) for the elaboration of the signal, the mean photon emission intensity was measured in the areas corresponding to C-line and T-line of the LFIA strip. Each was subtracted from the mean background signal measured in adjacent areas. The T-line/C-line ratio was calculated for each concentration (7 points) and then converted into B/B0 ratio (B0, i.e. maximum T-line/C-line value) by dividing each value for the T-line/C-line ratio measured in the absence of cortisol. Calibration curves were obtained by plotting B/B0 values (6 points) against the log of analyte concentration and fitting the experimental data with a four-parameter logistic function.

To obtain the analyte concentration in real samples, B/B_0 value was calculated as described above and interpolated on a stored calibration curve.

3. Results and discussion

3.1 Smartphone camera detectability and resolution

The strips were imaged using both the reference LB-981 luminograph and the smartphone's camera (Figure 2a) using the developed mini darkbox accessory (see below). This was to evaluate the imaging resolution and assess the possibility of accurately measuring the photon emission from a T-line on the strip without any significant interference from the adjacent C-line. The smartphone camera allowed us to measure the CL intensity of each line without significant crosstalk, showing performances comparable to those obtained using a bench-top luminograph. The CL intensity profiles measured across the lines show that the peaks are resolved at the baseline, even in the case of a disproportion of CL intensity between the light intensities of the two adjacent lines.

The light detectability shown by the smartphone camera is only one decade less than those obtained using the CCD camera or organic photodiodes (Pires and Dong, 2014). This detector is less sensitive than a CCD because the latter is cooled at -40 °C and presents larger pixel size. However, the detectability offered by the BSI-CMOS smartphone camera is adequate for measuring the light signal from the LFIA strip at the clinically relevant cortisol concentration. In particular the Samsung Galaxy SII plus used in this work is equipped with a Sony IMX105 chip, 1/3.2" size, 1.4 μm backside illuminated pixels, and 8.13 MP.

The colour BSI-CMOS used in currently available smartphones is much more sensitive than previous front-illuminated sensors, with an increase in the amount of captured light from about 60% to over 90% (Swain and Cheskis, 2008), improving low-light imaging performance. Indeed, smartphones offer an integrated platform for signal acquisition and processing, together with direct connectivity (Bluetooth, Wi-Fi) for data managing and sharing.

3.2 LFIA optimization

3.2.1 Concentration of immunoreagents

Concentration of HRP-cortisol conjugate was optimized to generate an assay with a detection limit and dynamic range suitable for measuring physiological levels of cortisol in saliva samples and their variation in different diseases. The C-line and the T-line must have similar signals in order to use a single integration time to simultaneously measure their CL signals, preventing them from interfering with each other. The optimal concentration of HRP-cortisol conjugate (1:100 v/v), anti-cortisol antibody (1/50 v/v), and anti-HRP antibody (1/500, v/v) were selected as the dilution that provides the highest detectability and the best compromise between the two effects described above. Data referring to the HRP-cortisol conjugate dilution are reported in the Supplementary Material.

3.2.2 Chemiluminescent signal kinetics

The kinetics of the HRP-catalyzed CL emission in the presence of luminol-based substrates containing suitable enhancers reaches a steady state CL intensity in a short time (Marzocchi et al., 2008; Kricka et al., 2000). Migration along the whole LFIA membrane requires 15 minutes and might result in partial inactivation of HRP, especially in the presence of intense CL signals. We therefore applied the CL substrate directly to the nitrocellulose membrane, avoiding excessively long migration times along the whole strip. Measurement of the CL signals at different integration times (from 2 to 20s) indicated that 10 s was sufficient to achieve the maximum CL signal, thus this time was used for the analysis. Indeed, the intensity of the CL signal decreases considerably when using an integration time lower than 10 s, while a higher integration time does not significantly enhance the intensity of the signal itself. The steady state lasts for about 1 min (the kinetics of the CL signal obtained from LFIA membranes is available in the Supplementary Material). The reaching of a steady state

emission confirmed that an excess of substrate was present on the LFIA membrane, ensuring that the CL signal intensity was proportional to the enzyme amount.

3.3 Calibration curve

Calibration curves were generated using cortisol standard solutions in the range of 0.5 - 100 ng/mL. One calibration curve was constructed by adding known amounts of cortisol standard solutions to cortisol free saliva (Figure 2b). This was compared with a calibration curve obtained in PBS to evaluate the matrix effect. The calibration curves are reported in Figure 2c. Being a competitive type format, the decrease of the T-line/C-line ratio was directly proportional to the amount of analyte in the sample. The results of repeated calibration curves demonstrated a good reproducibility with an average of the relative standard deviation associated with each point of the calibration curve of 10% in buffer and 16% in saliva matrix. The limit of detection (LOD) was estimated as the concentration corresponding to the blank T-line/C-line value minus three times its standard deviation. The obtained values were 0.3 ng/mL in saliva matrix and 0.1 ng/mL in buffer while the dynamic ranges of the method extended from 0.3 to 60 ng/mL for saliva and from 0.1 to 60 ng/mL in PBS. It appeared that the LOD value was slightly influenced by matrix effect; nevertheless the value was adequate for evaluating cortisol concentrations in human saliva in both normal and pathological conditions.

3.4 Determination of cortisol in real samples

To further confirm the accuracy of the proposed method, saliva samples belonging to 11 subjects were analysed. To evaluate the concentration of cortisol in real saliva samples, the calibration curve obtained for the saliva matrix was used for interpolation by a nonlinear four-parameter logistic calibration plot. Experimental results of four independent measurements were compared with those obtained using a confirmatory method (Figure 3a). Chemiluminescence images of LFIA membranes used for analyses of saliva samples are reported in Figure 3b. A good agreement between CL-LFIA and commercial ELISA kit results was found for all samples. As can be seen, large error percentages were obtained at higher cortisol concentrations; in the competitive LFIA, infact, a lower signal obtained in the presence of high cortisol concentration can be more affected by the aspecific background signal leading to large errors. Recovery values were in the range from 88% to 116%, and a coefficient of variation below 20% was obtained, proving the reliability and practical applicability of the proposed CL-LFIA for quantitative determination of cortisol in saliva samples.

4. Conclusion

We have successfully developed and optimized a CL imaging system to quantify cortisol level in human saliva using a CL-LFIA method where the analytical membrane is inclosed in a simple cartridges placed in front to a smartphone camera. Because we used CL as a detection principle, the 3D-printed accessories used to adapt the smartphone are very simple and could be adapted for other smartphones or tablets.

The use of the 3D printing technology will allow to further improve the first prototype here described and to design different analytical formats even based on multiplex capability. The method is sensitive enough to detect and quantify cortisol in real saliva samples in the relevant physiological range. The method is rapid (total analysis time 30 min), simple, and could be performed without specialized personnel, allowing point-of-care analysis with reductions in cost and response time. The technology could be used to assess patient stress

levels as well as other pathologies, such as depression. The concept thus paves the way for a new generation of analytical devices in the clinical diagnostic field thanks to the ideal combination of sensibility a simplicity of the CL with the day-by-day increase in the performance of the new generation smartphone camera.

References

Anfossi, L., Calderara, M., Baggiani, C., Giovannoli, C., Arletti, E., Giraudi, G., 2010. Anal. Chim. Acta. 682, 104-109.

Ballerini, D.R., Li, X., Shen, W., 2012. Microfluid. Nanofluid. 13, 769-787.

Caputo, D., De Cesare, G., Dolci, L.S., Mirasoli, M., Nascetti, A., Roda, A., Scipinotti, R., 2013. IEEE Sensors J. 14, 2595-2601.

Cho, J.H., Han, S.M., Paek, E.H., Cho I.H., Paek S.H., 2006. Anal. Chem. 78, 793-800.

Fernandez-Sanchez, C., McNeil, C.J., Rawson, K., Nilsson, O., 2004. Anal. Chem. 76, 5649–5656.

Hellhammer, D.H., Wüst, S., Kudielka, B.M., 2009. Psychoneuroendocrino. 34, 163–171.

Kricka, L.J., Voyta, J.C., Broinstein, I., 2000. Methods Enzymol. 305, 370-390.

Li, Z., Wang, Y., Wang, J., Tang, Z., Pounds, J.G., Lin, Y., 2010. Anal. Chem. 82,7008–7014.

Li, C.Z., Vandenberg, K., Prabhulkar, S., Zhu, X., Schneper, L., Methee, K., Rosser, C.J., Almeide, E., 2011. Biosens. Bioelectron. 26,4342-4348.

Martinez, A.W., Phillips, S.T., Carrilho, E., Thomas, S.W., Sindi, H., Whitesides, G.M., 2008. Anal. Chem. 80, 3699-3707.

Martinez, A.W., Phillips, S.T., Whitesides, G.M., Carrilho, E., 2010. Anal. Chem. 82, 3-10.

Marzocchi, E., Grilli, S., Della Ciana, L., Prodi, L., Mirasoli, M., Roda A., 2008. Anal. Biochem. 377, 189–194.

Mirasoli, M., Buragina, A., Dolci, L.S., Simoni, P., Anfossi, L., Giraudi, G., Roda, A., 2012a. Biosens. Bioelectron. 32, 283– 287.

Mirasoli, M., Buragina, A., Dolci, L.S., Guardigli, M., Simoni, P., Montoya, A., Maiolini, E., Girotti, S., Roda A., 2012b. Anal. Chim. Acta. 721, 167– 172.

Molinelli, A., Grossalber, K., Krska, R., 2009. Anal. Bioanal. Chem. 395, 1309-1316

Mudanyali, O., Dimitrov, S., Sikora, U., Padmanabhan, S., Navruz, I., Ozcan, A., 2012. Lab Chip. 12, 2678–2686.

Muhammad-Tahir, Z., Alocilja, E.C., 2003. Biosens. Bioelectron. 18, 813–819.

Ozdalga, E., Ozdalga A., Ahuja, N., 2012. J. Med. Internet Res. 27, 14:e128.

Pires, N.M.M., Dong, T., 2014. Biomed. Mat. Eng. 24, 15-20.

Posthuma-Trumpie, G.A., Korf, J., Van Amerongen, A., 2009. Anal. Bioanal. Chem. 393, 569-582.

Qin, Z., Chan, W.C., Boulware, D.R., Akkin, T., Butler, E.K., Bischof, J.C., 2012. Angew. Chem. 51, 4358-4361.

Roda, A., Girotti, S., Lodi, S., Preti, S., 1984. Talanta. 31, 895-900.

Roda, A., Pasini, P., Musiani, M., Girotti, S., Baraldini, M., Carrea, G., Suozzi, A., 1996. Anal. Chem. 68, 1073–1080.

Roda, A., Mirasoli, M., Venturoli, S., Cricca, M., Bonvicini, F., Baraldini, M., Pasini, P., Zerbini, M., Musiani, M., 2002. Clin. Chem. 48, 1654–1660.

Roda, A., Guardigli, M., Michelini, E., Mirasoli, M., Pasini, P., 2003. Anal. Chem. 75,463A–470A.

Roda, A., Mirasoli, M., Dolci, L.S., Buragina, A., Bonvicini, F., Simoni, P., Guardigli, M., 2011a. Anal. Chem. 83, 3178–3185.

Roda, A., Cevenini, L., Michelini, E., Branchini, B.R., 2011b. Biosens. Bioelectron. 26, 3647-3653.

Salter, R., Douglas, D., Tess, M., Markovsky, B., Saul, S.J., 2006. J. Aoac. Int. 89, 1327-1334.
Sangdae, L., Giyoung, K., Jihea, M., 2013. Sensors. 13, 5109-5116
Swain P.K., Cheskis D., 2008, Photon. spectra. 42, 46-47.
Tlili, C., Myung, N.V., Shetty V., Mulchandani, A., 2011. Biosens. Bioelectron. 26 , 4382–4386.
Wang, X., Hofmann, O., Das, R., Barrett, E.M., De Mello, A.J., De Mello, J.C., Bradley, D.D.C., 2007. Lab Chip. 7, 58–6.
Xia, X.H., Xu, Y., Zhao, X.L., Li, Q.G., 2009. Clin. Chem. 55, 179–182.
Zhao, W., Van der Berg, A., 2008. Lab Chip. 8, 1988-1991.
Zhu, H., Isikman, S. O., Mudanyali, O., Greenbaum, A., Ozcan, A., 2012. Lab Chip. 13, 51–67.
Zou, Z.X., Du, D., Wang, J., Smith, J.N., Timchalk, C., Li, Y.Q., Lin, Y.H., 2010. Anal. Chem. 82, 5125–5133.

Figure 1. a) 3D printed (ABS) smartphone accessory comprising a plano-convex lens holder and the LFIA cartridge adaptor. **b)** 3D printed cartridge, housing the LFIA strip with control- (C-line) and test-line (T-line). The 3D model with the details of different compartements is also presented. **c)** The integrated cortisol LFIA smartphone-based device with running Camera FV-5 lite application for CL signal acquisition.

Figure 2. a) Comparison of CL images acquired with a Berthold Nigh Owl luminograph (left) or the smartphone camera (right). The insensity profile and image resolution of the two instrumentation are presented. **b)** CL images acquired with the smartphone at increasing concentrations of cortisol. **c)** Calibration curves for cortisol obtained in phosphate buffer and in saliva using the smartphone camera. Images were elaborated with imageJ software to quantify the CL signal of the C- and T-line and plotted as described in material and methods section.

Figure 3. a) Correlation between cortisol concentrations in saliva samples, obtained with commercial ELISA kit and the smartphone CL-LFIA device. **b)** CL images of saliva samples from healthy patients at low (I), medium (II) and high (III) cortisol concentration.