- **Dipstick for haemoglobin in urine**
  Principle: redox-reaction with substrate (TMB)
  Procedure: dilute saliva 1 : 5 with bidist. water
  Adavantages - easy performance
  - not very expensive
  - indicates also peroxidase in saliva

- **Transferrin in saliva**
  Principle: ELISA
  cut off value: 0.5 mg/dl
  (nomal range in serum: 200 - 350 mg/dl)
  Disadvantages: - very expensive (reagents ≥ 1 USD)
  - time consuming
  (ca. 45 min. incubation time)

- **DHEA-S in saliva** (Lac G., N. Lac and A. Robert, 1993)
  Principle: RIA
  cut off value: 24.1 nmol/l
  (normal range in serum: ca. 7500 nmol/l)

Table 5: Methods for evaluation of blood contamination in saliva

Measurement of Steroids in Saliva

General remarks

Since the first assay for measuring steroids in saliva was described in 1964, different and improved methods have been established.

In former times, assays for serum samples were adapted for use with saliva. Because of smaller concentrations of the analytes in saliva and matrix effects several extraction procedures were evaluated.

Currently, scientists have developed more sensitive assays for use with salivary diagnostics. In addition to chromatographic procedures, different immunological methods are used: radioimmunoassays (RIA), enzyme linked immunosorbent assay (ELISA), fluorescence immuno assay (FIA) and chemiluminescence immunoassay (LIA).

Among the various assay designs, there are differences in the use of radioactive or non-radioactive material, the sensitivity of the method and differing needs for equipment. The non-radioactive assays in microtiter plate format can be used in a

wider variety of laboratory settings and offers the flexibility of running the assays manually or on fully automated equipment.

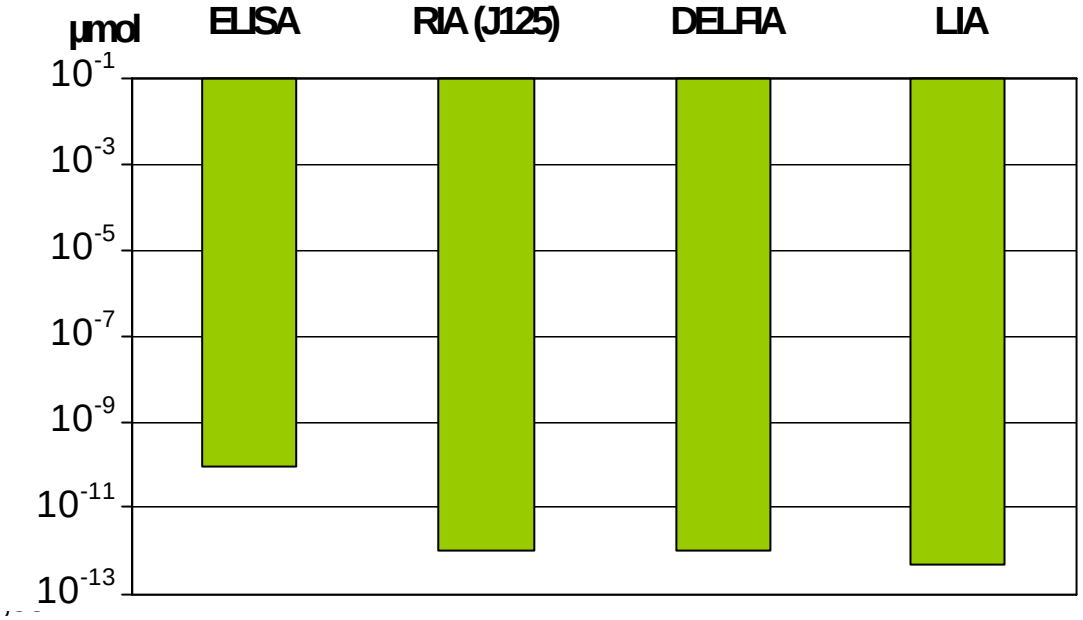


Fig. 17: Sensitivity of the tracer of different immunoassays. ELISA = Enzyme with dye detection; RIA (^{125}I) = detection of radioactive ^{125}I molecules; DELFIA = detection of time resolved fluorescence with Europium-complexes; LIA = Peroxidase with luminol detection (S. Albrecht et al., 1997).

Chemiluminescence

Chemiluminescence is a scientific term describing light emission as a release of energy created during chemical reactions. There are two kinds of luminescence reactions used in immunoassays in medical sciences:

Flash luminescence: The luminophor (e.g. aryl acridinium ester) is directly bound to the antigen or antibody. The luminescence reaction is started by addition of a starting reagent. The emitted flash of light during the chemical reaction lasts only a few seconds. This type of chemiluminescence is normally used in molecular biology.

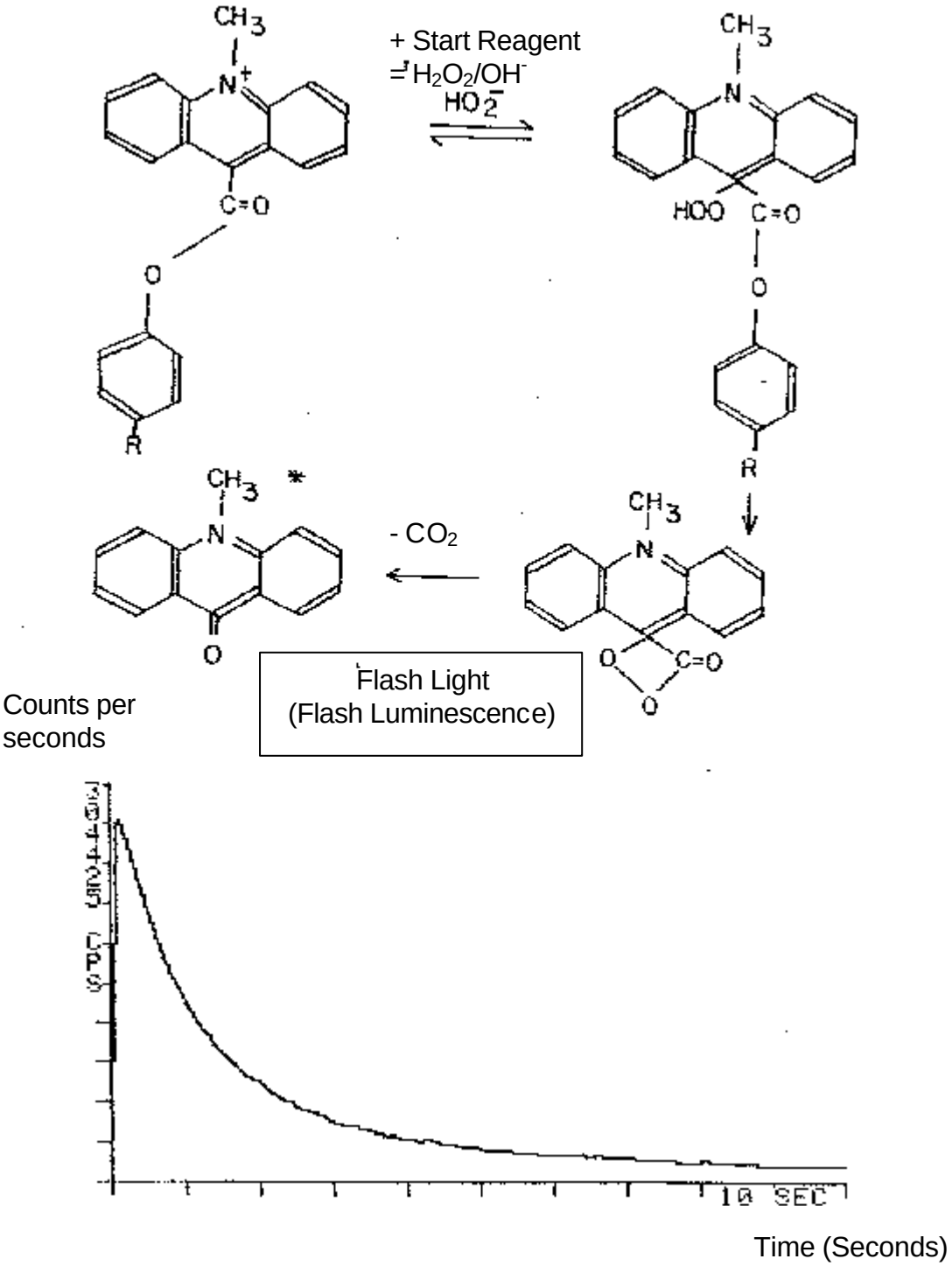


Fig. 18: Flash luminescence: chemiluminescence reaction of aryl acridinium esters.

Glow luminescence: The antigen or antibody is bound to an enzyme (e.g. peroxidase). Luminophor (e.g. luminol) which is subsequently added, is altered by the enzyme in a chemical reaction in which light is emitted. Because the enzyme can metabolize many molecules, a glowing light is emitted for a span of minutes up to approximately an hour. This glow luminescence is used in many immunoassays in clinical pathology.

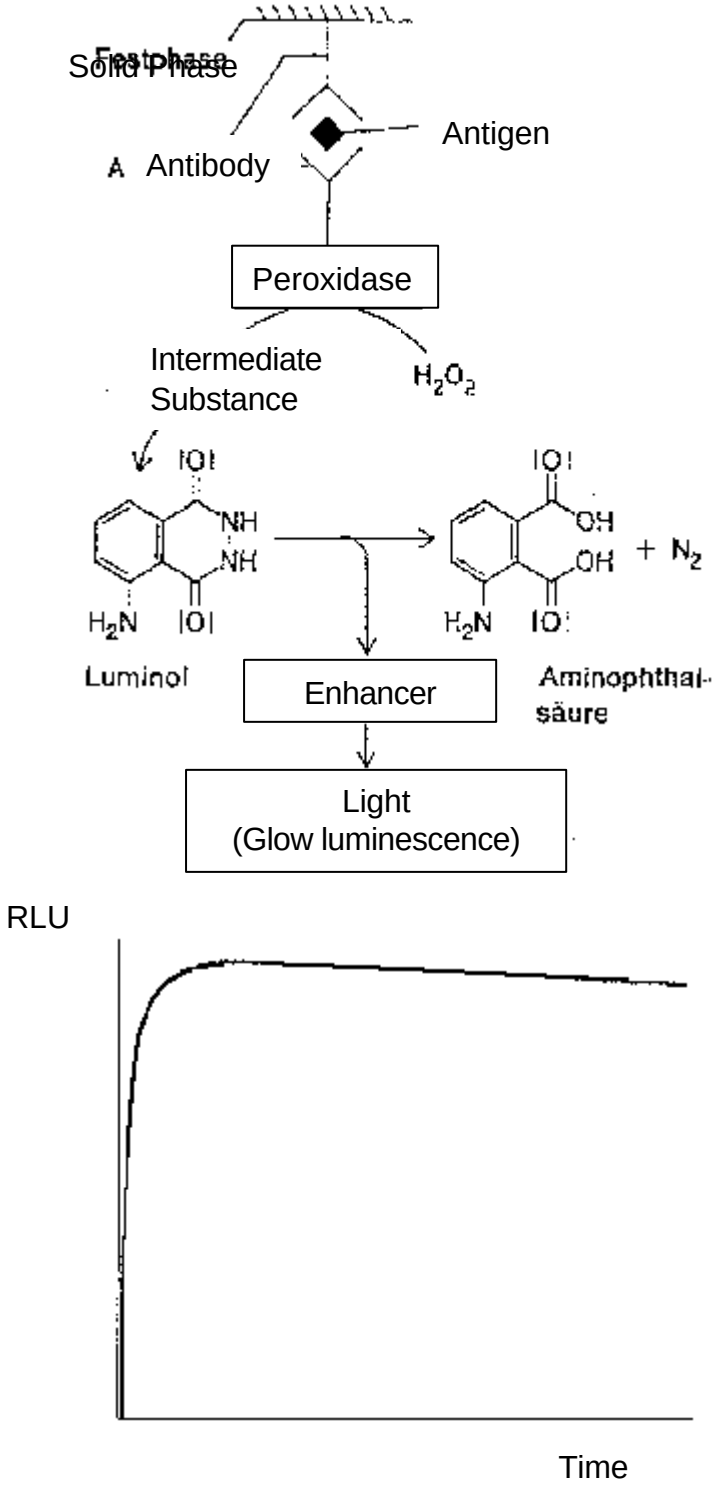


Fig. 19: Glow Luminescence: peroxidase catalysed chemiluminescence reaction.

For measuring the luminescence of immunoassays, special instruments are necessary. The detection unit of such a luminometer may be a photomultiplier tube (PMT). The luminescent light is focussed into this PMT. The PMT transforms the light into an electronic signal which is proportional to the intensity of the light. For measuring flash luminescence, an additional injector module is recommended which times the addition of the starting reagent and the measurement of the emitted flash of light.

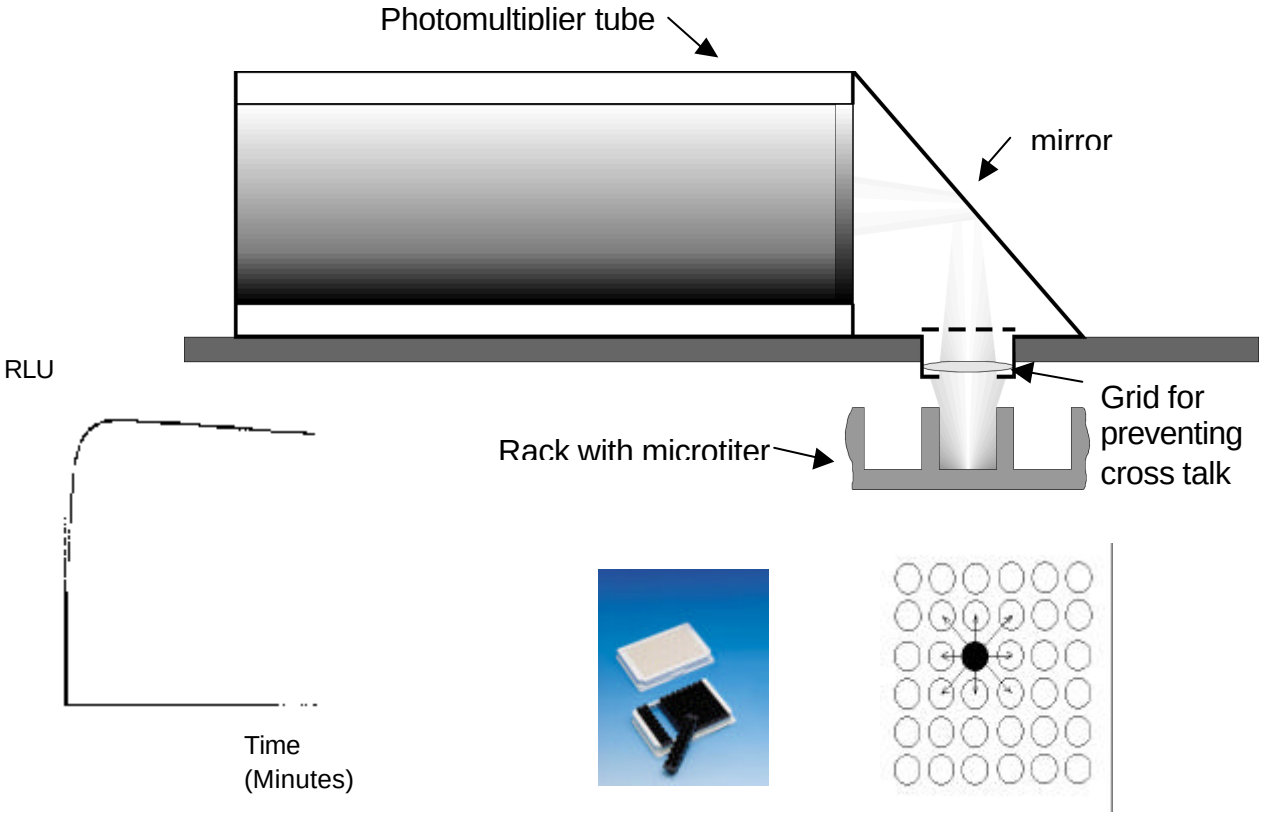


Fig. 20: Principle of a glow luminometer

There are many manufacturers of luminometers on the market. The chart below is a list of well known manufacturers. This is not a comprehensive list of all manufacturers.

Manufacturer / web page	Luminometer	**Multifunctional Reader**
Anthos http://www.anthos-labtec.com		***Lucy 1, 2, 3*** ***AutoLucy*** (luminescence; absorbance)
Berthold-Detection Systems http://www.berthold-ds.com	***MPL1*** ***MPL2*** ***Orion***	
Berthold Technologies http://www.bertholdtech.com	***Centro LB960*** ***MicroLumatPlus*** ***LB96V***	***Mithras LB940*** (luminescence, fluorescence)
Bio-Rad Laboratories http://www.bio-rad.com		***Lumimark Mikroplate*** (luminescence, absorbance)
Bio-Tek Instruments GmbH http://www.biotek.com		***FLX800*** (luminescence; fluorescence) ***Synergy; FL600*** (luminescence ; fluorescence ; absorbance)
BMG Labtechnologies http://www.bmg-labtechnologies.com	***LUMIstar Galaxy***	***FLUOstar Galaxy*** (luminescence, fluorescence ; absorbance)
HARTA Corporation http://www.hartacorporation.com	***MicroLumi 96***	
Hidex Oy http://www.hidex.com		***Plate Chameleon*** (luminescence ; fluorescence ; absorbance ; scintillation counting)
Molecular Devices Corporation http://www.moleculardevices.com	***Lmax***	***Analyst AD / Analyst HT*** ***Acquest*** (luminescence; fluorescence; absorbance)
Packard http://www.packardbioscience.com	***LumiCount***	***Fusion Universal Microplate Analyzer*** (luminescence ; fluorescence ; absorbance)
Perkin Elmer/Wallac http://www.perkinelmer.com		***1420 Victor*** (luminescence ; fluorescence ; absorbance)
SFRI Laboratoire http://www.sfri.com	***Lumax2*** ***Lumax3***	
Tecan Schweiz AG http://www.tecan.ch		***GENios*** ***Spectra FluorPlus*** ***Safire*** ***Ultra*** (luminescence, fluorescence, absorbance)
ThermoLabsystems http://www.labsystems.fi	***Luminoskan Ascent***	***Fluoroskan Ascent FL*** (luminescence and fluorescence)
Turner Designs Inc. http://www.turnerdesigns.com	***The Reporter***	
UltraSource, Inc. http://www.ultrasourceinc.com	***Stripwell Luminometer***	

Table 6: Manufacturers of luminometer

LIAs of IBL-Hamburg

Several criteria are highly significant in evaluating the overall quality and utility of any laboratory assay for the quantitation of hormone levels in saliva:

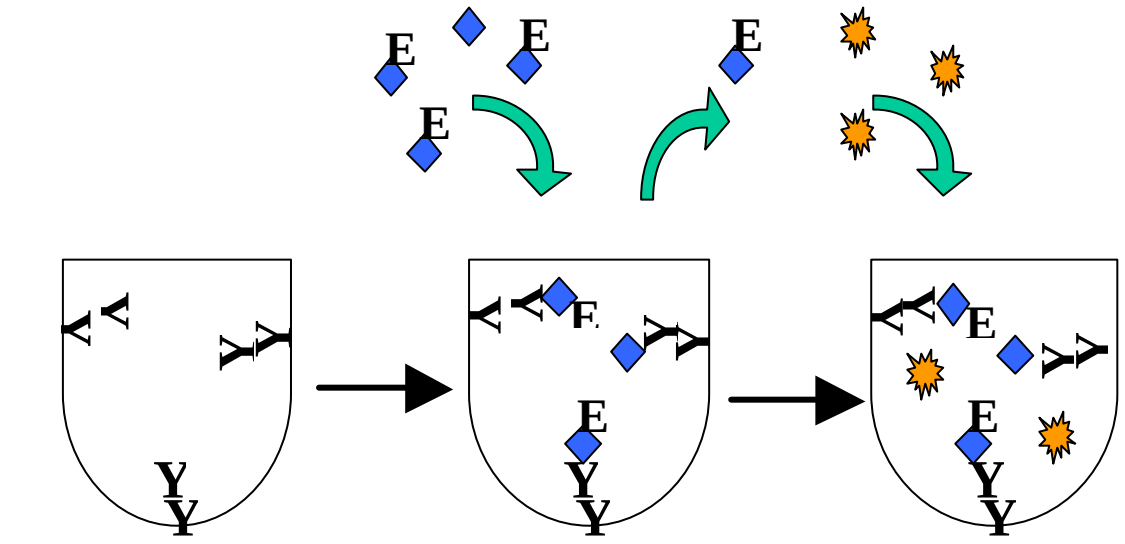
Sensitivity: It is common to mention the **analytical sensitivity** of any quantitative immunoassay. Analytical Sensitivity is determined by measuring replicates of the zero standard, calculating the mean value and the standard deviation (SD) of the signal, and assessing a concentration of the analyte which corresponds to the mean value ± 2 SD. This parameter can be influenced by the matrix of the zero standard. If it is not a saliva matrix but a highly diluted serum matrix, or only a buffer solution, the calculated analytical sensitivity will be artificially and erroneously low, and will not be an accurate reflection of the true sensitivity of that particular assay.
Therefore, it is important to evaluate the **functional sensitivity** by using saliva samples with low concentrations of analyte and calculating the coefficient of variation of replicate measurements at these low concentrations.

Range of linearity of the assay (shape of the standard curve): The concentrations of analyte in physiological, pathological and eventually therapeutic situations should be within the linear range of the standard curve of the assay. Typically the standard curves generated by immunoassays, that were designed for serum applications and then later modified for salivary diagnostics, do not have linear slope characteristics at that part of the standard curve, where the majority of the saliva samples are found.

Cross reactivity of the antibody to hormones similar to that of the analyte: In some salivary steroid assays, a very low cross reactivity is important to distinguish the target analyte from other hormones which are common in saliva in high concentrations (e.g. in the cortisol assay the cross reactivity to cortisone which has a 2 to 5 times higher level in saliva than cortisol).

Saliva controls: In order to recognize matrix effects of the samples, the use of kit controls, which are designed with the same matrix, is essential. Therefore for saliva assays the use of “true” saliva controls instead of diluted serum controls or controls with a buffer matrix is necessary.

The following diagrams show the scheme of the IBL-Hamburg LIAs for the free steroids in saliva, and a summary of the LIA assay advantages are also provided:



Y = goat anti rabbit Ab

Y = rabbit anti steroid Ab

E = steroid peroxidase or alkaline phosphatase conjugate

= steroid from sample

= luminol or acridan based substrate

Typical Standard Curve

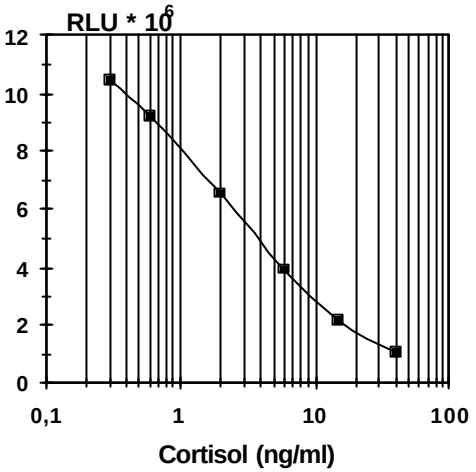


Fig. 21: Scheme of the IBL-Hamburg LIAs for steroid hormones in saliva

Advantages of the LIAs of IBL-Hamburg

- High sensitivity
- Good precision at low concentrations
- low cross reactivity to related metabolites
- Extended range of linearity
- Only 20 - 50 µl saliva sample necessary
- no extraction procedure
- Most kit reagents ready for use
- saliva kit controls (2 levels)
- easy test procedure
- Cortisol LIA FDA cleared
- simple reader for glow luminescence
- Convenient software also used for ELISA reader
- Applicable to automates

Table 7: Summary of the advantages of the Steroid in Saliva LIAs of IBL-Hamburg