TITLE: Detection of Parathyroid Hormone-like Hormone in Cancer Cell Cultures by Gold Nanoparticle-based Lateral Flow Immunoassays

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ABSTRACT

Parathyroid Hormone-like Hormone (PTHLH) exerts relevant roles in the progression and dissemination of several tumours. However, factors influencing its production and secretion have not been fully characterized. The main limitation is the lack of specific, sensitive and widely available techniques to detect and quantify PTHLH. We have developed a Lateral Flow Immunoassay using gold nanoparticles label for the fast and easy detection of PTHLH in lysates and culture media of three human cell lines (HaCaT, LA-N-1, SK-N-AS). Levels in culture media and lysates ranged from 11 to 20 ng/mL and 0.66 to 0.87 µg/mL respectively. Results for HaCaT are in agreement to the previously reported, whereas LA-N-1 and SK-N-AS have been evaluated for the first time. The system also exhibits good performance in human serum samples. This methodology represents a helpful tool for future *in-vitro* and *in-vivo* studies of mechanisms involved in PTHLH production as well as for diagnostics.

KEYWORDS: Parathyroid Related Hormone (PTHLH), Lateral Flow Immunoassay (LFIA), HaCaT, LA-N-1, SK-N-AS.

ABBREVIATIONS

Parathyroid Hormone-like Hormone (PTHLH), Humoral hypercalcemia of malignancy (HHM), Lateral Flow Immunoassay (LFIA), Radio Immunoassay (RIA), Immunoradiometricassays (IRMAs), Gold Nanoparticles (AuNP), Gold aggregation test (GAT), Fetal Bovine Serum (FBS), Phosphate buffered saline (PBS), Optical Density (OD), Absorbance Units (AU).

BACKGROUND

Parathyroid Hormone-like Hormone (PTHLH) is a secreted factor that is present in virtually all organs and tissues of the body. It was initially identified in cancer patients with hypercalcemia. The syndrome was named humoral hypercalcemia of malignancy (HHM) and was apparently caused by a factor released by the tumour [1]. Parathyroid hormone (PTH) was first proposed as the factor responsible of these effects, but it was never demonstrated that increased levels of PTH could cause HHM. The sequencing and cloning of the PTHLH gene in 1987 showed the high homology of the N-terminal region between PTH and PTHLH. This homology allows PTHLH to signal through the PTH receptor [2]. Once isolated and characterized as the factor responsible for HHM [3], PTHLH was reported to exert other functions in cancer as well as in normal tissues [2-7]. In cancer, it has been reported to be involved in the initiation, survival and progression of primary tumours [8, 9], as well as in the generation and development of metastases [10-12]. Moreover, novel functions of this cytokine are still being described in recent papers [5, 11]. Nevertheless, the mechanisms that control its production and secretion are only partially understood.

One of the limitations to characterize the multiple mechanisms that control its production and secretion is the lack of specific, sensitive and easy techniques to detect and quantify this molecule in *in vitro* models. After the description and characterization of PTHLH, few methodologies were adapted to detect circulating PTHLH[13], mostly radioimmunoassays (RIAs) and immunoradiometric assays (IRMAs) which have been successfully applied in cancer patients [14, 15]. Advantages of the radioactive assays rely in the ease of isotope conjugation, signal detection with little optimization and low limit of detection, around 0.7 pmol/L. Nevertheless, these assays have serious drawbacks mostly related to the half-life of the isotope,

the potential hazard of radioactivity and, consequently the strict requirements needed to implement these procedures. For this reason, in most cell biology and molecular research laboratories PTHLH is analysed at the mRNA level by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and the protein is evaluated by immunocyto/histochemistry [16], Western blot [17] and ELISA[11, 18]. However, these methods are expensive, time-consuming and require trained personnel, thus making alternative approaches of potential interest in a number of settings.

In this context, biosensors based on nanomaterials, or nanobiosensors, constitute an outstanding alternative for a low-cost, fast, efficient, large scaling and user-friendly analysis [19, 20]. For example, an electrochemical immunoassay based on nanoparticles and nanochannels for the detection of standard PTHLH solutions (spiked in cell culture media) has been very recently reported by our group [21]. However, the developed immunosensing system requires the assembling of nanoporous membranes on the electrode surface together with different incubation/washing steps before the electrochemical reading, being a non-integrated and relatively time-consuming system.

Lateral-flow immunoassays (LFIAs), very well-known for the popular pregnancy test[22], are highly integrated systems that appear as ideal candidates for rapid PTHLH analysis, thanks to their characteristics of simple use, rapid result, low cost, good specificity and long shelf life[23]. In these systems, all the reagents are preloaded in paper strips, the samples are casted on a pad of the strip and flow by capillarity through all the components, mixing with the reagents and reaching the detection zones. As a result, the assay is done by just adding the sample on the corresponding pad, and adding washing solution afterwards. Finally qualitative results can be read

by naked eye and, in case that quantification is required, only a simple colorimetric reader is needed. In addition to the pregnancy test, LFIAs have been extensively applied for the detection of different analytes such as cancer biomarkers [24], DNA [25, 26], toxins [27] and metals [28].

The inherent limitations of LFIA technology in terms of sensitivity and reproducibility have been overcome in the last years thanks to the tools offered by the nanotechnology, mostly taking advantage of the plasmonic properties and large surface area of gold nanoparticle (AuNPs) tags [29-33].

In the aforementioned context of increasing relevance of PTHLH in cancer the objective of this work is to develop and optimize a rapid, sensitive, cheap, easy-to-use and non-hazardous technique to detect and quantify PTHLH secreted in cell cultures, with the aim of offering a new tool for the study of fundamental aspects related to the mechanisms and the factors associated to PTHLH secretion, which can be of great importance for therapeutic applications. We here present a novel LFIA approach based on AuNPs for the rapid and simple determination of this protein production and secretion in three human cell lines: two neuroblastoma cell lines (SK-N-AS and LA-N-1), a tumour where high PTHLH expression has been associated with benign subgroups [34] and immortalized keratinocytes (HaCaT) as a control of high PTHLH production.

The proposed LFIA method allows for the quantification of PTHLH in cell lysates as well as in cell culture media. This is relevant because PTHLH exerts different functions inside the cell (as an intracrine, autocrine factor) and as a secreted protein that mediates a cross-talk with neighbour cells (paracrine functions) [2, 4]. Finally, the suitability of the system to perform detection in a complex matrix (human serum) spiked with commercial PTHLH has been proved.

METHODS

PTHLH recombinant was purchased to Sigma Aldrich (Spain).Polyclonal rabbit antibody (38-208) against PTHLH was purchased to Pro-Sci (USA). Polyclonal goat antibody (Ab6702) against rabbit IgG was purchased to Abcam (UK).SK-N-AS cells were purchased from the European Collection of Cell Cultures. LA-N-1 cells were kindly provided by Dr.Nai-Kong Cheung (Memorial-Sloan Kettering Cancer Center, New York, USA) and HaCaT cells by Dr. Joan Guinovart (Institut de Recerca Biomèdica, Barcelona, Spain). Serum samples for the spiking and recovery experiments were obtained from blood samples (1-3 mL) from healthy donors collected during a minor surgery. Residual volumes were processed and stored for research purposes after all necessary diagnostic procedures. Written informed consent was obtained to store these samples in the Tumour Bank and to use them as anonymized controls. Serum was obtained from blood as per Standard Operating Protocols at Developmental Tumour Biology laboratory, Hospital Sant Joan de Déu, Barcelona. An IsoFlow reagent dispensing system (Imagene Technology, USA) was used to dispense the detection and control lines. A commercially available strip reader, model ESE Quant Lateral Flow reader (Qiagen Inc., Germany) was used for quantitative measurements. Rest of general reagents and instruments are detailed at the Supplemental Material.

Synthesis of gold nanoparticles (AuNP) and preparation of AuNP/antibody conjugates

AuNP of 15 nm in diameter were prepared by reduction of $HAuCl_4 \cdot 3H_2O$ following the procedure described by Turkevich *et al.*[35] and modified with antibodies accordingly to the conditions optimized by a Gold Aggregation Test (GAT),following the experimental procedure already reported by the group[36]. Experimental details about the AuNPs synthesis, GAT and AuNP/antibody complex formation are given in the Supplemental Material.

Preparation of the conjugate, sample and detection pads

In order to prepare the conjugate pads, the fibre glass strips were soaked by drop casting with the solution of AuNP/antibody conjugates. The resulting conjugate containing fibre glass was dried in a vacuum chamber for 1.5 hours.

The sample pad was prepared by dipping the cellulose fibre strips in a 0.5% (w/v) BSA, 0.05% (v/v) Tween 20, 10mM pH7.4 PBS solution, and dried in the oven at 70ºC for 30 minutes.

Capturing antibodies for both the test and control line were deposited on the nitrocellulose pad using the isoflow dispenser. The polyclonal antibody anti-PTHLH at 100 µg/mL in 1 mg/mL BSA 10mM pH 7.4 Phosphate buffer (PB) was deposited in the test line, while a polyclonal antibody against rabbit IgG at 1mg/mL in 10mM pH 7.4 PB was deposited in the control line. The substrates were dried in an oven at 37ºC for 1.5 hours. Last step was the assembly of the 4 the components of the LFIA on the backing card: detection pad, conjugate pad, sample pad and absorbent pad were placed by this order. Pads were placed so one pad overlaps the next one where they meet in order to assure the flow of the liquid from one pad to the other.

The resulting assemblies are cut in 7 mm wide strips and stored in a sealed flask with desiccating pearls in the fridge until the moment of use.

Cell lines culture, lysates preparation and human serum samples

Cells were grown in Roswell Park Memorial Institute (RPMI)-1640, 10% fetal bovine serum (Invitrogen, CA, USA), 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL), at 37ºC and 5% CO_2. Mycoplasma polymerase chain reaction (PCR) tests were routinely performed. Cells were seeded in T-75 flasks, and after reaching 80% confluence, supernatants (also referred to as cell culture media) were collected by decantation. Cells were trypsinized and counted (see Supplemental **Figure** S2). To obtain cell lysates, cell suspensions were centrifuged for 5 min at 250 x*g*. Pellets were re-suspended in 1 mL of PBS, centrifuged 5 min at 91 x*g* and then re-suspended in 100 µL of extraction buffer (25mM HEPES, 300mM NaCl, 0.1mM Orthovanadate, 10mM sodium fluoride, 1.5mM magnesium chloride, 0.1% Triton X100, 0.2mM EDTA, 0.5mM phenylmethylsulfonyl fluoride, 0.5mM dithiothreitol in milliQ water). Following incubation on ice for 10 min, mixtures were centrifuged 15 min at 13000 x*g* and lysates were then stored at -80ºC.

A pool of serum samples was obtained by mixing 500µL of serum from 6 different patients. The resulting mixture was used as serum matrix to perform the spiking of commercial PTHLH in order to carry out the signal recovery study.

Immunoblotting

Total protein amount was quantified according to the Bradford method [37]. Indicated quantities of proteins were electrophoresed in 15% SDS-PAGE gels and transferred onto nitrocellulose membranes. Incubation with primary polyclonal antibody anti-PTHLH was followed by HRP-conjugated secondary antibodies. Immunoreactive bands were detected with enhanced chemiluminiscence reagents.

Analysis of the samples by Lateral Flow Immunoassay (LFIA)

Samples were analysed following a LFIA, in which 150µL of the sample were added on the sample pad. After 4 minutes, 50µL of Tween 20 at 0.05% in PBS (washing solution) were added on the conjugate pad. This process was repeated 3 times in order to wash the excess of conjugates and the non-specific adsorbed conjugates onto the nitrocellulose and printed lines. The total time required for the tests was between 15 and 20 minutes. The strips were let dry at room conditions for 20 minutes and afterwards read using a colorimetric reader for lateral flow strips. For all the measurements the background signal of PBS was subtracted. All the measurements were repeated 3 times, and the corresponding error bar calculated.

First a calibration curve using standard solutions of PTHLH in PBS was performed. The cell lysate was diluted to 1:250 in PBS (see dilution optimization and **Figure** S3 at the Supplemental Material) and measured by LFIA, being the content of PTHLH extrapolated from the calibration curve).

For the determination of PTHLH in cell culture media, the method of the standard additions was applied for samples without any previous dilution. Additions of 0, 10 and 50 ng/mL of the standard PTHLH solution were evaluated.

The regular experimental procedure of lateral flow analysis was applied for the spiked PTHLH (to reach final concentrations of 0, 5 and 100 ng/mL) in both PBS and human serum samples (the background signal was not subtracted from the measurement in order to calculate the recoveries).

RESULTS

Assay principle

A LFIA based on AuNPs is designed (see details at **Figure 1**) and applied for the quantification of PTHLH in HaCaT, LA-N-1 and SK-N-AS cell culture media and cell extracts. Samples are casted onto the pre-treated cellulose fibre (sample pad) and flows by capillarity towards the other end (adsorption pad) of the strip. In this pathway, sample meets the conjugate pad, where conjugates of 15nm AuNPs modified with polyclonal anti-PTHLH antibodies have been pre-stored. The PTHLH is recognized by the conjugates thereby forming an AuNP/Antibody/PTHLH conjugate. These conjugates keep flowing through the nitrocellulose. Immobilized antibodies anti-PTHLH bind the PTHLH forming an immunosandwich with the AuNP/Antibody/PTHLH, holding the conjugates in the detection line and making a red band (detection line) due to the accumulation of AuNPs. Excess of AuNP/Antibody conjugates migrate further, and are captured on the control line, where antibodies against the anti-PTHLH are previously immobilized. The appearing of the control line is necessary to confirm the good performance of the assay and the migration of the reagents along the strip. The colour density or optical density (OD) of the detection line is proportional to the analyte concentration. A schematic view of both the LFIA working principle and the experiments procedure can be observed in

Figure 1. Three different cell lines were evaluated and two types of samples from each one were analysed: cell culture media and cell lysates. A calibration curve using standard solutions of the protein was made so as to quantify PTHLH in cell lysates. As the low levels of PTHLH in cell media did not allow the sample dilution before analysis, standard additions technique was applied so as to avoid matrix effects. Cell lysates were also analysed by means of Western Blot as reference method.

It is important to note that the polyclonal antibody used in this work recognizes the central region of the PTHLH (amino acids 37-122).

Characterization of AuNPs and AuNP/antibody conjugates

Homemade AuNP were characterized by UV-Vis spectroscopy showing the typical absorption band at 520 nm which evidences the formation of a well-dispersed colloidal suspension. TEM analysis confirms a narrow size distribution of spherical AuNPs of 15 ± 2 nm and (**Figure 2A and 2B**).

Antibodies are conjugated onto the AuNP surface through a simple adsorption, with a random orientation, as extensively previously reported [26,30-33, 36]. The well-known affinity of the thiol groups contained in the cysteine (present in the constant region of the antibodies) to gold substrates also contributes to this binding. Gold Aggregation Test (GAT) is preliminarily performed to determine the minimum antibody concentration to use for conjugation (see details at Supplemental Material). NaCl dislocates surface charges on AuNPs causing their aggregation while antibody-coated AuNPs do not aggregate since NaCl is not able to break the Au–S bond [36]. The minimum antibody concentration for avoiding AuNP aggregation was determined by measuring the difference between the absorbance at 520 nm

(dispersed AuNPs) and at 580 nm (aggregated AuNPs) for different antibody concentrations. Different pHs were also evaluated. Based on the results shown in **Figure2C** and **2D**, the optimum conditions were an anti-PTHLH concentration of 100 µg/mL and incubation at pH 9 (see BSA concentration optimization and related Supplementary **Figure S1**).

PTHLH detection in cell lysates

The proposed LFIA was used to calculate the amount of PTHLH present in cell lysates from three different cell lines: HaCaT, LA-N-1, SK-N-AS. As far as we know, PTHLH levels in neuroblastoma (LA-N-1 and SK-N-AS cell lines) have not been evaluated as yet, while the levels in HaCaT lysates are expected to be at ng/mL order [11, 38]. Real-time quantitative PCR performed in these three cell lines showed a wide range of PTHLH expression varying from low (LA-N-1), medium (SK-N-AS) to high (HaCaT) (data not shown).

Standard solutions of PTHLH in PBS buffer were initially used to evaluate the effect of the PTHLH concentration on the analytical signal (Optical density). As shown in **Figure 3A** (left), a gradual increase in the colour intensity of the test line was observed for increasing concentrations of PTHLH**,** being possible to detect up to 5 ng/mL even with the naked eye. As shown in **Figure 3B,** a logarithmic relationship between the value of the Optical density (measured with a strip reader) and the protein concentration was found in the range from 5 ng/mL to 100 ng/mL (correlation coefficient: 0.995), as adjusted to the following equation:

$$\text{Optical density (AU)} = 48.201 \cdot \text{Ln}[\text{PTHLH}]\ (\text{ng/mL}) - 29.935 \quad (n=3) \quad \text{(Eq. 1)}.$$

where n corresponds to the number of individual assays.

The limit of detection (LOD), calculated by extrapolating from the calibration plot the concentration of analyte required to give a signal equal to the blank plus three times its standard deviation was 1.42 ng/mL [39], while the limit of quantification (LOQ) calculated as the smallest amount of detectable analyte with a CV<20% was 2 ng/mL [40]. The reproducibility of responses of 5 ng/mL (n=10) gave a relative standard deviation (RSD) of 4%.

Since high concentrations of PTHLH are expected to be found in cell lysates (in the order of μg/mL) 1:250 dilutions of lysates were prepared with PBS buffer before LFIA analysis to obtain analytical signals within the calibration curve range. This approach produced significant signals for the three cell lines (HaCaT, SK-N-AS and LA-N-1), as shown in **Figure 3A** (right). The corresponding values of optical density are shown in the graph of **Figure 3B** as discrete dots. As matrix effects are not relevant in these highly diluted samples, the concentration of PTHLH in each lysate was directly extrapolated from the calibration curve made with standard solutions. In this way, PTHLH concentrations between 0.66 and 0.87 μg/mL were estimated in cell lysates for the three lines evaluated, as summarized in **Table 1.**

Total protein content in each sample was calculated by Bradford method (see Supplemental Material and related **Figure S4**. The ratio PTHLH (measured by LFIA)/total protein (Bradford) amount was also calculated (**Table 1**).

For an independent detection of PTHLH we performed immunoblot (or Western Blots) to detectthe protein. As **Figure 3C** (left panel) shows, when the gels are loaded with the same concentration of total protein, different signals are observed being the weakest signal corresponding to the LA-N-1 sample. But the relative differences did not precisely match those observed with LFIA. As a semi-quantitative technique, Western Blot provides only relative information about differences in

PTHLH content between different samples. Its accuracy relies on several factors as protein quantity, primary and secondary antibodies concentration or even the housekeeping protein selected [38]. So a proper quantification by this technique would require many different tests for each sample. If the quantification performed with the easier, quicker method of LFIA is trustworthy, we could correct the loading of gels to an equal PTHLH amount that we could observe in a Western Blot. **Figure 3C** (right panel) shows that, when we load equal PTHLH quantity, calculated with data in **Table 1**, we obtain bands with similar intensity, despite the preliminary and apparent disagreement of the numbers. These results validate the LFIA quantification as a reliable method for PTHLH quantification in a complex mixture of proteins as a cell lysate sample.

This assay to detect PTHLH production in cell lysates could also be applied for the detection of PTHLH in tissue samples, including tumours. Thus, it might be a useful tool, together with mRNA expression analysis, to analyse PTHLH regulation *in vitro* and also in *in vivo* models.

PTHLH detection in cell culture media

Presence of secreted PTHLH in HaCaT, LA-N-1 and SK-N-AS cell culture media was calculated by LFIA. Cells morphology before collecting cell media can be observed in images shown in **Figure 4**.

Cell culture media represents a very complex matrix composed by the supplemented media (proteins mainly coming from Fetal Bovine Serum) and also proteins secreted by the cells. Since the expected amount of secreted PTHLH found in the cell culture media is at ng/mL levels [41], which is close to the LOD of the developed LFIA, the sample itself cannot be diluted before analysis. As matrix effects are expected to

play a significant role in these non-diluted samples, PTHLH standard solution was added several times to a sample aliquot to estimate the PTHLH levels in cell culture media [42].

PTHLH standards (0, 10 and 50 ng/mL) were added to samples as detailed in the Materials and Methods section. As shown in **Figure 4,** the gradual increase in the obtained analytical signal is proportional to the added concentration of PTHLH according to the linear relationships shown in the inset.

Extrapolating from such equations the point on the *x*-axis at which OD = 0, the negative intercept on the *x*-axis corresponds to the amount of the PTHLH (ng/mL) in the sample. In this way, the amounts of secreted PTHLH present in cell media were 20.16 ng/mL, 14.19 ng/mL and 11.73 ng/mL respectively. As in the case of cell lysates, the highest levels of PTHLH were found in HaCaT cells media, while the other cell lines showed similar amounts of secreted PTHLH. Hence, secreted PTHLH seems to be in correlation with the PTHLH present inside the cell in these particular cell contexts.

The advantages of our methodology mainly rely in the fact of having all the reagents preloaded in the tests, making it possible to perform a quick detection (less than 20 minutes) even with the naked eye, without incubations and washing steps in between, in which only the samples have to be drop casted onto the sample pad of the already made LFIA strips. Other reported techniques require long incubations (hours or even overnight), washing steps, and usually reaction kinetics are involved in the final reading step. Furthermore no hazardous reagents are necessary, specialized facilities (RIA and IRMA) are not required and simple and cheap equipment is used.

Performance in real samples: spike and recovery

Although the main goal of this work is the quick and easy detection of PTHLH in cell cultures, with the aim to give researchers a new resource to help the studies of the expression levels and secretion of this PTHLH in cell cultures, the performance of the LFIA assay in human serum was also evaluated so as to demonstrate the potential of the developed system for diagnostics. Since the levels of PTHLH in human serum of healthy donors (few pg/mL) [14, 15] are below the LOD of our system, the native amount of PTHLH present in the serum is neglected and only the spiked PTHLH is considered. A pool of human serum from 6 different donors was used to spike 0, 5 and 100 ng/mL. The obtained analytical signals and their comparison to those obtained in PBS buffer are shown in **Table 2**. Recoveries around 90% for all the concentrations assayed were achieved. These results suggest that the developed LFIA has acceptable recovery rates for human serum samples making it a promising candidate to perform detection of PTHLH in a scenario of great interest for future diagnostic applications.

DISCUSSION

In summary, a LFIA for the detection of PTHLH has been designed and tested, being capable to perform the detection of such factor at ng/mL levels in real samples obtained from HaCaT, LA-N-1 and SK-N-AS cell cultures (cell lysates and cell media). Secreted PTHLH in cell lysates and cell culture media has been evaluated. These results are in agreement with the obtained by Western Blot analysis, used as a semi-quantitative reference method.

Our approach represents a fast and easy methodology to detect PTHLH in cell lysates and cell culture media, As far as we know this is the first time that LFIA has been applied for the detection of PTHLH taking advantage of the properties that paper based nanobiosensors offer (easy to use, quick tests, good sensitivity). We would like to remark the advantage that means the possibility of taking a sample from cell culture media and quantify PTHLH in less than one hour. This pioneer study offers a novel alternative for the quick and robust detection of PTHLH avoiding the need of complex or expensive requirements. Besides, LFIA represents a versatile and easy adaptable technique. Components of the assay can be easily modified with the aim of adapting the assay to a different detection context. This kind of paper-based diagnostics are suitable for largescale production, making them a very cheap and efficient technology.

The developed method offers a new tool for the study of fundamental aspects related to the mechanisms and the factors associated to PTHLH secretion, which can be of great importance for therapeutic applications. Furthermore the low matrix effects found when analysing a human serum sample, demonstrate the ability of the developed system to perform analysis in scenarios of interest for future diagnostic applications.

The present study opens the way to future applications of LFIA for the rapid determination of a relevant protein in the biology and treatment of several malignancies.

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FIGURE LEGENDS

Figure 1: (**Top**) Schematic view of the experimental procedure for PTHLH quantitation in cell culture media and cell lysates using a gold nanoparticle (AuNP)-based lateral flow immunoassay (LFIA).(**Bottom**) Depiction of sequential steps involved, and materials and reagents used in the designed LFIA. TL and CL stand for test line and control line, respectively.

Figure 2: Characterization of gold nanoparticles (AuNPs) and AuNP/anti-PTHLH conjugates. (**A**) TEM image (inset graph corresponds to size distribution diagram) and (**B**) UV-Vis spectrum of the AuNPs suspension. (**C**) Difference in absorbance (Abs 520-Abs 580) plotted against anti-PTHLH concentration in the gold aggregation test (GAT) for different pH values. (**D**) Pictures of the corresponding solutions (incubation at pH 9).

Figure 3: PTHLH evaluation in cell lysates. (**A**) Pictures of the LF strips after assays run with different standard solutions of PTHLH and with lysates(diluted at 1:250). (**B**)Effect of PTHLH concentration (standard solutions) on the optical density. The signals obtained for the three cell lines, diluted at 1:250 are inserted. (**C**) Western blot experiments for cell lysates.

Figure 4: PTHLH quantification in HaCaT, LA-N-1 and SK-N-AS cells culture media. (Left) Optical microscope images of cell cultures before the assay.(Right) Standard

additions of PTHLH in cell culture media of the three cell lines. Insets in graphics show the strips after each addition (in ng/mL).