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# Development and validation of a novel cell-based assay for potency determination of human parathyroid hormone (PTH)

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ABSTRACT

Parathyroid hormone (PTH) is the primary regulator of serum calcium homeostasis and plays a major role in bone metabolism. Its actions are mediated via the PTH1 receptor (PTH1R) resulting in adenylate cyclase activation and consequently production of cyclic adenosine mono-phosphate (cAMP). The latter stimulates cellular metabolic pathways. This study describes the development, validation and applications of a novel cell-based potency assay for PTH using HEK293 cells over-expressing PTH1R. PTH concentration-dependent cAMP formation in these cells was quantitatively analyzed employing time-resolved fluorescence technology (TR-FRET). The optimized assay was precise, reproducible and exhibited a high sensitivity to PTH with a limit of quantification in the low picogram range. The potencies of differently manufactured $PTH_{1-34}$ peptides, as well as a full-length variant ($PTH_{1-84}$), were all accurately measured. Since PTH activity is inhibited by neutralizing antibodies against PTH, the assay was adapted to detect and measure neutralizing antibodies in human serum. Thus, applications of this novel cell-based PTH potency assay were extended to immunogenicity testing of PTH preparations in non-clinical and clinical settings.



## 1. Introduction

Osteoporosis is a bone disease characterized by a decline in $Ca^{2+}$resorption leading to a reduction in bone mass density, deterioration in bone micro-architecture and increased fragility of bones with attendant increased risk of fractures [1]. After the age of 40, when bone mass densities have passed life peak levels, the risk of osteoporosis developing advances progressively. In particular, postmenopausal women, having imbalances in $Ca^{2+}$ metabolism due to hormonal changes, are primarily and often the most severely affected. It is estimated that 1 in 3 women over 50 will experience osteoporotic fractures [2]. Overall, it is estimated that 200 million women are affected worldwide [2,3]. Fast growing populations with increasing life expectancies suggest the numbers of osteoporosis sufferers will rise significantly in the future [3–5]. Based on studies of biochemical markers for bone resorption and formation, several medications, including bisphosphonates, selective estrogen receptor modulators, PTH and salmon calcitonin, have been indicated for halting progression of osteoporosis [6,7]. PTH is a polypeptide of 84 amino acids secreted by the parathyroid gland, which normalizes serum $Ca^{2+}$ levels by increasing $Ca^{2+}$ re-absorption in the kidney and stimulating the osteoclast- and osteocyte-mediated $Ca^{2+}$ release from bone. It plays a major role in normal bone homeostasis and remodeling, on the one hand by boosting osteoblastogenesis (anabolic response) leading to bone formation and on the other by indirectly boosting osteoclastogenesis (catabolic response) and bone resorption. However, beneficial treatment of osteoporosis has been achieved via intermittent administration of PTH. This approach results in systemic pharmacokinetics that follow a sharp "spike-dip" profile favoring osteoblastic bone formation over osteoclastic resorption, resulting in net gain of anabolic bone responses [8]. Persistently high levels of PTH, as observed in hyper-parathyroidism, have the opposite effect. Its anabolic effect on bones differentiates PTH from any other available drug indicated for osteoporosis: it can not only arrest the

*Abbreviations:* cAMP, cyclic adenosine mono-phosphate; DMEM, Dulbecco's Modified Eagle Medium; FCS, fetal calf serum; NAB, neutralizing antibody; NIBSC, National Institute for Biological Standards and Control; P, cell passage number; PTH, parathyroid hormone; PTHR1, parathyroid hormone receptor 1; PLA, parallel-line assay; TR-FRET, time resolved-fluorescence resonance energy transfer; WHO, World Health Organization.

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progression of the disease, but assist in actively reversing it through building up bone structure.

Two recombinant variants of PTH are currently commercially available: a full length variant containing all 84 amino acids of the polypeptide (brand name: PREOTACT; Nycomed) and a truncated variant consisting of the N-terminal 34 amino acids (brand name: FORTEO® [US], FORSTEO® [Europe]; Eli Lilly and Co.) [9–12]. Both variants have proven to be safe and clinically efficacious in osteoporosis patients, especially in affected postmenopausal women [9].

The mechanism of action of PTH has been well studied. In osteoblasts PTH acts via a cell surface membrane class II G-protein coupled receptor, known as PTH receptor 1 (PTHR1). Downstream of PTHR1, several distinct signal transduction pathways are activated. These include activation of adenyl cyclase signaling through cAMP, the PLC pathway [13,14], activation of PKC [15], release of intracellular stored $Ca^{2+}$ [16], the MAPK pathway [17,18] and phospholipase D [19,20]. There is strong evidence that, in the anabolic mode of action of PTH variants, cAMP plays an essential role. All PTH preparations exhibiting anabolic activity in animal models induce high titers of cAMP in vitro [21] and this forms the basis for development of functional cell-based assays for PTH potency determinations. The potency of the two commercial PTH products, FORTEO and PREOTACT, are currently evaluated in assays that use "PTH sensitive" cell lines [11,12]. Various cell lines naturally expressing PTHR1, such as the rat osteosarcoma cell line UMR-106 or SaOS-2, produce cAMP upon PTH induction and have proved suitable for potency assessment of PTH variants [22–24]. UMR-106-based assays were widely employed in the biological characterization of the WHO biological (potency) standard for $PTH_{1-34}$ (catalogue number 04-200, The National Institute for Biological Standardization and Control {NIBSC}, United Kingdom) [25]. However, since few data have been published concerning assay sensitivity, reproducibility and performance, there is an urgent need to provide accurate information on these criteria. Since the therapeutic window for PTH is very narrow, such informative data would be helpful both for pharmaceutical companies manufacturing PTH variants and for regulatory purposes.

The aim of this study was to develop and validate a sensitive and reproducible potency assay for PTH that could be used for several purposes, including PTH variant stability, marketing requirements for product release and immunogenicity assessment of PTH variant during preclinical and clinical development. Herein, we describe the development, validation and applications of a novel, stable, cell-based assay using PTHR1 overexpressing HEK293 cells, which is capable of measuring potencies of PTH variants from different sources ($PTH_{1-34}$ and $PTH_{1-84}$). The assay is validated according to guidelines of the international conference on harmonization (ICH) using the WHO biological (potency) standard for $PTH_{1-34}$ obtained from NIBSC as reference calibrant [26]. Further, its applicability for detecting and quantifying neutralizing antibodies (NABs) against PTH variants in human serum is investigated. Data showing the generality of its applicability to the quantitative evaluation of potency of PTH variants, including its usefulness in this respect for the development of a new $PTH_{1-34}$ therapeutic, as well as in assessment of the potential immunogenicity of such products, are presented.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Media and chemicals for cultivation of PTH sensitive cells were purchased from Invitrogen, USA. Human male serum that served as matrix for the NAB-assay and Isobutyl-methyl-xanthine stimulation medium was obtained from Sigma-Aldrich. A mouse-anti-human PTH monoclonal antibody (clone BGN/1F8) from Biotrend, Germany was used as positive control in the NAB-assay. $PTH_{1-34}$ from NIBSC (UK) (04/200) was used as a reference standard for assay validation. For potency comparisons, $PTH_{1-84}$ (95/646) from NIBSC (UK), Forteo (Eli-Lilly) and a new candidate $PTH_{1-34}$ product were analyzed. The latter was produced and characterized by Minapharm Pharmaceuticals, Egypt. The LANCE Ultra cAMP detection kit was supplied by Perkin-Elmer, USA.

### 2.2. Cell line generation and cultivation

Cloning of the lentiviral expression vector was done at Sirion Biotech, Germany. In detail, a 1899 bp fragment including human PTHR1 cDNA was amplified via PCR from plasmid BC1112221 (ImaGenes) and cloned via EcoR1 and Not1 into bicistronic lentiviral expression vector pLV-Ubic-MCS-IRES-Puro. Sequencing was performed to confirm correct cloning sequence. Lentiviral transduction particles were generated by cotransduction with the expression plasmid pLV-Ubic-PTHR1-IRES-Puro and lentiviral packaging plasmids in HEK293 T cells.

Hek293 cells were transduced with particles using a multiplicity of infection rate (MOI) of 5. Selection using 0.75 μg/ml puromycin was initiated 72 h after transduction. After death of control cells, puromycin concentration was reduced to 50% and cells were frozen in liquid nitrogen.

The stable cell pool was thawed and cultured for 3 further days. After washing with PBS, cells were lysed with RNA lysis buffer. Total RNA was extracted using Nucleospin RNA II Kit (Macherey und Nagel, Germany) and total RNA from host cells was used as control. 1 μg total RNA was used for reverse transcription into cDNA using Sprint RT complete-double preprimed Kit (Clonetech). qPCR was performed using a Light Cycler (Roche).

HEK293 cells for cloning and during the assay were grown in DMEM + Glutamax (cat. no. 32430, Invitrogen), 4.5 g/l glucose, 25 mM HEPES buffer, supplemented with 1% penicillin/streptomycin and 10% heat-inactivated FCS.

### 2.3. PTH potency determination using LANCE Ultra cAMP

HEK293-PTHR1 cells were trypsinized using triple select reagent diluted in complete medium and centrifuged at 400 rcf for 5 min at room temperature (RT). Cell pellets were resuspended in complete medium with 0.5 mM isobutyl-methyl-xanthine stimulation medium and cells diluted to a concentration of $1 \times 10^5$ cells/ml in stimulation medium. Aliquots of 10 μl of cell suspension were distributed into the wells (1000 cells/per well) of white-walled 96-well microtiter plates. PTH (reference standard and sample in triplicate) was serially diluted in 8 steps (dilution factor of 1.5) in stimulation medium from 150 to 8.76 pg/ml in a 96-well plate (i.e., in an equimolar range of serial dilutions of NIBSC $PTH_{1-84}$ beginning at 685 pg/ml). After assay plates had been incubated on ice for 10 min, serial dilutions of PTH (10 μl/well) were added to the cells using a multichannel pipette. Plates were incubated at 37 °C for 30 min using the heating option of an ELISA reader (Sunrise, Tecan, Austria) and then returned to ice incubation. 10 μl cAMP Tracer solution and 10 μl Ulight anti-cAMP (LANCE Ultra cAMP Kit) were added according to manufacturer's protocol. Plates were further incubated for 1 h at RT and the TR-FRET signal was detected by a multimode reader (Hidex Chameleon) using the following filters: excitation 330/80 nm, emission 616/8.5 nm, and 665/7.5 nm. Prompt time was set to 100 μs and TRF-time was set to 150 μs. Raw data was transferred to PLA 2.0 software (Steegmann Systems, Germany) for parallel lines analysis according to Ph. Eur. Chapter 5.3 using logarithmic data transformation of the TR-FRET signal at 665 nm as response.