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Optimized luciferase assay for cell-penetrating peptide-mediated delivery of short oligonucleotides

Henrik Helmfors *, Jonas Eriksson, Ülo Langel

Department of Neurochemistry, Stockholm University, Stockholm 10691, Sweden

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ABSTRACT

An improved assay for screening for the intracellular delivery efficacy of short oligonucleotides using cell-penetrating peptides is suggested. This assay is an improvement over previous assays that use luciferase reporters for cell-penetrating peptides because it has been scaled up from a 24-well format to a 96-well format and no longer relies on a luciferin reagent that has been commercially sourced. In addition, the homemade luciferin reagent is useful in multiple cell lines and in different assays that rely on altering the expression of luciferase. To establish a new protocol, the composition of the luciferin reagent was optimized for both signal strength and longevity by multiple two-factorial experiments varying the concentrations of adenosine triphosphate, luciferin, coenzyme A, and dithiothreitol. In addition, the optimal conditions with respect to cell number and time of transfection for both short interfering RNA (siRNA) and splice-correcting oligonucleotides (SCOs) are established. Optimal transfection of siRNA and SCOs was achieved using the reverse transfection method where the oligonucleotide complexes are already present in the wells before the cells are plated. Z' scores were 0.73 for the siRNA assay and 0.71 for the SCO assay, indicating that both assays are suitable for high-throughput screening.

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An increasing portion of the discovery phase in life science research is moving toward an automated high-throughput screening (HTS)[1] approach to assays. This development leads to a quicker gain of data, allowing a researcher to swiftly weed out the uninteresting and quickly discover what may work and what may be a waste of time. However, automation up to the HTS level in academia is available only to those academic labs with the resources to use one of the HTS facilities available in some countries and universities. Most academic labs seldom use any automated approaches.

During the past decades, a new class of peptide-based carriers that have the ability to cross the cellular membrane has emerged. These peptides are called cell-penetrating peptides (CPPs). These peptides are (mostly) cationic (and/or amphipathic) and, in addition to self-translocation, also have the ability to carry cargo with them into the intracellular environment [1,2]. The cargo that these peptides are able to carry is diverse, ranging from small molecules [3] to plasmids [4] and other oligo- and polynucleotides such as short interfering RNA (siRNA) and splice-correcting oligonucleotides (SCOs) [5,6] all the way to proteins [7]. The therapeutic potential seems unlimited, but the CPP technology has so far not led to any clinical trials. Studies investigating toxicity and biodistribution are ongoing, and CPP oligonucleotide therapies are anticipated to reach clinical trials in the near future [8].

So far, the standardized way to describe the internalization properties of CPPs has been to measure the uptake of fluorescently labeled peptides either through a fluorimetric measurement or by confocal microscopy [9]. This is a good way to measure the uptake of individual CPPs with small hydrophobic (the fluorophore) covalently coupled cargoes; however, this is less interesting if the main objective is to use the CPPs as delivery vehicles for macromolecules.

Successful delivery of a macromolecule should preferably result in some form of biological activity that is easy to measure. Many assays rely on either one of the two most popular reporter genes: green fluorescent protein [10,11] or luciferase [12]. The main benefits of luciferase-based assays are that the readout is simple and the signal-to-background ratio is very high. One such assay that we previously found to be practical [13] is the splice-correction assay developed by Kole and coworkers [14]. We previously investigated the oligonucleotide cotranslocation potency of various peptide vectors using this assay [5,6,15,16]. It is a cellular assay

* Corresponding author.
E-mail address: henrik.helmfors@neurochem.su.se (H. Helmfors).

[1] *Abbreviations used:* HTS, high-throughput screening; CPP, cell-penetrating peptide; siRNA, short interfering RNA; SCO, splice-correcting oligonucleotide; DTT, dithiothreitol; CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine triphosphate; HPLC, high-performance liquid chromatography; mRNA, messenger RNA; RNAi, RNA interference; LH_2, D-luciferin; CV, coefficient of variation.

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in which the activity of an antisense oligonucleotide results in upregulation of luciferase gene expression. In the past, we have relied on small-scale use of 24-well cell culture plates for all of our experiments. This has been adequate when assessing one or at most a few CPPs at the same time.

Many publications about CPPs report on the discovery of, and sometimes applications for, a single new peptide [5,6,15–17], whereas there are fewer publications that present a series of peptides [18,19]. Being able to compare multiple peptides and transfection conditions simultaneously over a shorter time would increase the rate of discovery. For this purpose, here we present a method that aims to reduce the size of our previously very successful 24-well assay for splice correction [13] into a 96-well format. At this format, the reagent usage for CPPs and oligonucleotides is the same as for a 24-well plate with the added benefit of being able to test up to four times as many experimental conditions simultaneously; however, the luciferase reagent is consumed at a four times higher amount. That rate of reagent consumption makes a commercial reagent almost prohibitively expensive. Taking four times as many measurements simultaneously reveals a bottleneck for efficiency. The simplest luminometers, which lack reagent injectors, read one well at a time and take more than 3 min to measure one 96-well plate. During this time, the signal may change by up to 20% between the first and last measurements of a 96-well plate.

Here we present a method for evaluating transfection via CPPs that has the potential to scale up to true HTS. It is based on a cost-effective homemade reagent for the firefly luminescence assay. The work presented is optimized for transfection of short oligonucleotides such as SCOs and siRNAs. This journal recently published a miniaturized gene transfection assay using luciferase plasmid in 384- and 1536-well plates [20]. The transfection was done using $CaPO_4$–DNA or PEI–DNA with automated robotic liquid handling and was read using high-end equipment and a commercial luciferase reagent. Our assay was done manually using handheld pipettes, making it also useful for those without specialized equipment. In addition, our assay reagent significantly reduces the cost of one assay; for example, according to current list price (April 2015), the Promega ONE-Glo reagent costs approximately 90 euro per 96-well plate, whereas our assay reagent costs approximately 3 euro per 96-well plate, which is a 30-fold cost savings.

Materials and methods

Reagents

For delivery experiments and luciferin assay buffer, PS-2′-OMe splice-correcting oligonucleotides (5′-CCU CUU ACC UCA GUU ACA-3′) were purchased from RiboTask (Denmark), D-luciferin was purchased from PerkinElmer (Sweden), and siRNA against firefly luciferase [ACGCCAAAAACAUAAAGAAAG(dTdT)] was purchased from Eurofins Genomics (Germany). All other reagents, $MgCO_3$, $MgSO_4$, Tricine, dithiothreitol (DTT), coenzyme A (CoA), ethylenediaminetetraacetic acid (EDTA), and adenosine triphosphate (ATP) were obtained from Sigma–Aldrich (Sweden).

Peptide synthesis

PepFect14 [4,6] was synthesized on a Rink Amide ChemMatrix resin (PCAS Biomatrix, Canada), on a Biotage Initiator + Alstra peptide synthesis machine (Biotage, Sweden), using *N,N′*-diisopropylcarbodiimide (DIC) and OxymaPure as coupling reagents and standard Fmoc protected amino acid monomers (Iris Biotech, Germany). Peptide was cleaved using 95% trifluoroacetic acid (TFA, Iris Biotech), 2.5% H_2O, and 2.5% triisopropylsilane (Sigma–Aldrich) and was precipitated in cold diethyl ether. The obtained crude peptide was dissolved in 5% acetic acid and lyophilized. The peptide was purified by high-performance liquid chromatography (HPLC) using a preparative BioBasic C8 column (ThermoFisher, Sweden) with a gradient elution made up of acetonitrile and water. The identity of the purified product was verified using matrix-assisted laser desorption ionization mass spectrometry (MALDI–MS) (Voyager-DE STR, Applied Biosystems). After HPLC purification, the peptide was lyophilized and peptide solutions used later were based on dilutions of accurately weighed substance.

Cell cultures

HeLa pLuc 705 and U-87 MG-luc2 cells were both maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with glutamax, 1 mM sodium pyruvate, 10% fetal bovine serum (FBS, Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin in a water-jacketed incubator at 37 °C and 5% CO_2.

For experiments that investigated the composition of the luciferin reagent, 1×10^4 HeLa pLuc 705 cells were seeded into white, tissue culture-treated, clear flat-bottom 96-well plates (Corning) 24 h prior to transfection. On the day of transfection, the cell culture medium was replaced with fresh medium prior to treatment with peptide/oligonucleotide complexes.

Optimal results in the splice-correcting assay were achieved when 7×10^3 cells were seeded into the same kind of 96-well plates as above that already contained the peptide/oligonucleotide complexes—so-called reverse transfection. After 24 h, most of the cell medium was carefully aspirated, and the plates were then centrifuged upside down at low (200–300) rpm for 15 s in a plate centrifuge to completely empty them of cell medium. Cells were subsequently frozen at −80 °C and lysed with a single freeze–thaw cycle, the frozen plates were allowed to reach room temperature before being assayed for luciferase activity.

For siRNA experiments, 7×10^3 U-87 MG-luc2 cells stably expressing an enhanced version of the firefly luciferase enzyme were seeded into the same type of plates as above, with the plates already containing the peptide/siRNA complexes. Then, 24 h post-transfection, the plates were treated the same way as for the SCO experiments, aspirated, centrifuged upside-down, freeze–thawed, and assayed as above.

In vitro transfection

SCOs and PF14 were mixed differently than as described previously. The peptide was dissolved in ultrapure water (MilliQ) at 1 mM concentration, and aliquots of this solution were kept frozen at −20 °C. Aliquots were thawed on the day of transfection and diluted in ultrapure water to 100 μM. SCOs were delivered lyophilized, and on arrival they were dissolved in RNase-free ultrapure water at 100 μM concentration and aliquoted. On the day of transfection, aliquots were thawed and diluted to 10 μM concentration. Peptide and SCO were mixed at a 5:1 M ratio in a phosphate/sodium buffer at pH 7.4 to a final concentration of 7.5/1.5 μM, and 10 μl of the complex solution was added to the 96-well plate prior to plating 90 μl of HeLa pLuc 705 cell suspension, resulting in a final concentration of 150 nM SCO and 750 nM PF14.

The principle behind the SCO delivery assay is as follows. A plasmid, HeLa pLuc 705, carrying the luciferase gene is interrupted with an insertion of intron 2 from β-globin pre-mRNA (messenger RNA) carrying a cryptic splice site. If the aberrant splice site is not masked by antisense oligonucleotides (SCOs), the pre-mRNA of luciferase will be improperly processed. Successful delivery leads to an increase in luminescence.

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