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Validation of a quantitative cell-based relative potency assay for LUXTURNA

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Voretigene neparvovec-rzyl (Luxturna) is an AAV2 vector (AAV2-hRPE65v2) that expresses a cDNA encoding the human retinal pigment epithelium-specific 65 kDa protein (RPE65). It has been approved for the treatment of visual deficits associated with biallelic mutations in human RPE65 in the US, European Union (EU), and multiple other countries. To achieve regulatory approval, it was necessary to validate an assay demonstrating its biological activity or potency. The assay measures AAV2-h*RPE65*v2 transduction in HEK293 cells and the subsequent biological activity of the vector-encoded RPE65 protein in cell lysates. RPE65 converts all-*trans*-retinol to 11-*cis*-retinol, which is quantified using liquid chromatography with tandem mass spectrometry (LC-MS/MS). The assay was validated for seven characteristics, namely system and sample suitability, specificity, linearity, precision, relative accuracy, range, and robustness. The validated assay can be used to confirm the relative potency levels of different lots of Luxturna in the range of 50%–150% of a reference standard (defined as 100% potent). This represents the first report of validation studies supporting an *in vitro* cell-based relative potency assay for an AAV vector, which was used to evaluate lot-to-lot consistency, stability, and comparability following manufacturing changes and to successfully launch Luxturna, the first gene therapy approved in the US for a genetic disease.

INTRODUCTION

Currently, the largest category of gene therapies for genetic disease are adeno-associated virus (AAV) vectors.[1] AAV-mediated gene therapies, with US Food and Drug Administration (FDA) and/or European Medicines Agency (EMA) approval, are now available for a rare genetic retinal dystrophy (Luxturna [Spark/Roche]), spinal muscular atrophy type 1 (Zolgensma [Novartis]), hemophilia B (Hemgenix [Uniqure/CSL Behring] and Beqvez [Spark/Pfizer]), hemophilia A (Roctavian [BioMarin]), Duchenne muscular dystrophy (Elevidys [Sarepta]), and aromatic amino acid decarboxylase deficiency (Upstaza [PTC Therapeutics]).

In addition to clinical safety and efficacy, regulatory approval has required advances in AAV manufacturing and quality systems. As with all investigational products, AAV manufacturers are required to demonstrate product safety, identity, quality, purity and potency, and specificity to demonstrate the consistency of these parameters from lot to lot in the manufacturing process. Thus, it is critical to develop robust and reproducible assays that can measure these quality attributes. Although it is recognized by regulatory authorities that analytical methods will evolve and may even change during product development, to obtain a biologics license in the US and a marketing authorization in the European Union (EU), validated assays with defined acceptance criteria are required.[2–4]

In contrast to safety and purity assays, identity and potency assays are product specific. Identity assays for gene-therapy products confirm the physical properties of vectors (for example, the capsid serotype and DNA sequence), while vector potency assays determine the specific ability or capacity of the product to achieve a defined biological effect.[5] These assays should provide a quantitative measure of biological activity, which is linked to the relevant biological properties. Although not essential, the potency assay will ideally represent the mechanism of action (MOA) of the product.[4,6,7] The MOA of a gene-therapy product depends on a series of steps including gene transfer, gene expression, and biological activity of the therapeutic transgene product. Although both *in vivo* and *ex vivo* assays are acceptable, the goal for a licensed product is to develop a cell-based potency assay with a measurable, quantitative endpoint. Due to the inherent variability in test systems, an absolute measure of potency may not be possible, which has led to the adoption of relative potency methods (US Pharmacopeia [USP] <1032>).[8] This methodology relies on the inclusion of a reference-standard (RS) vector. Test vectors are expected to show dose-response curves that parallel the RS dose-response curve, and the horizontal displacement between the curves is interpreted as the log of the relative potency. FDA regulations allow

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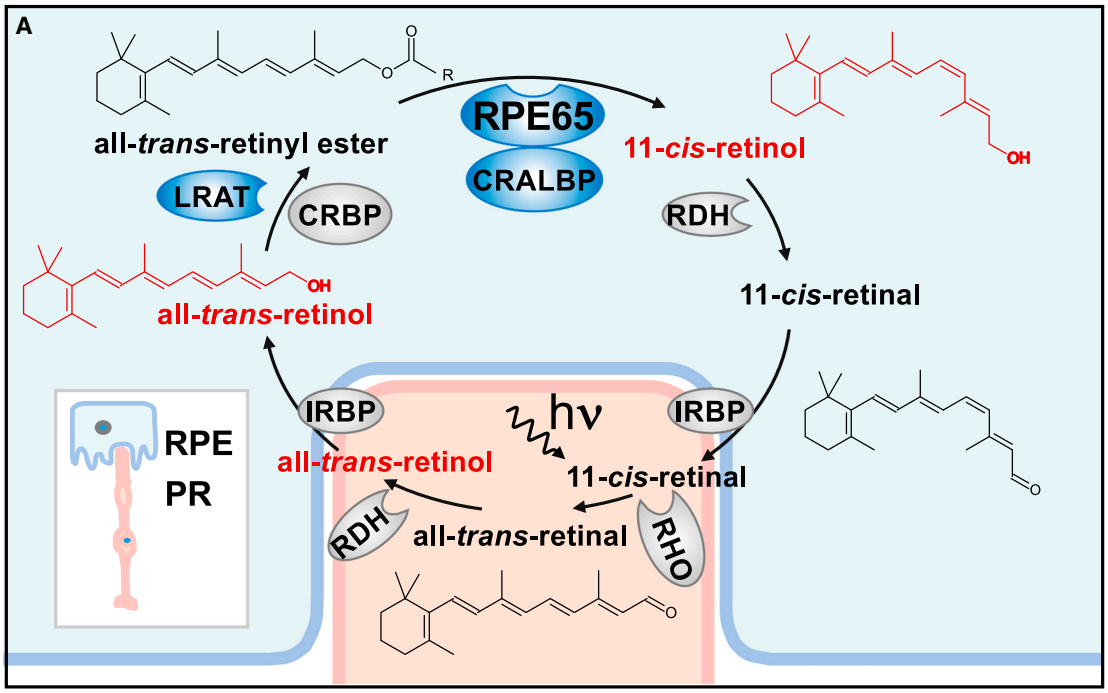

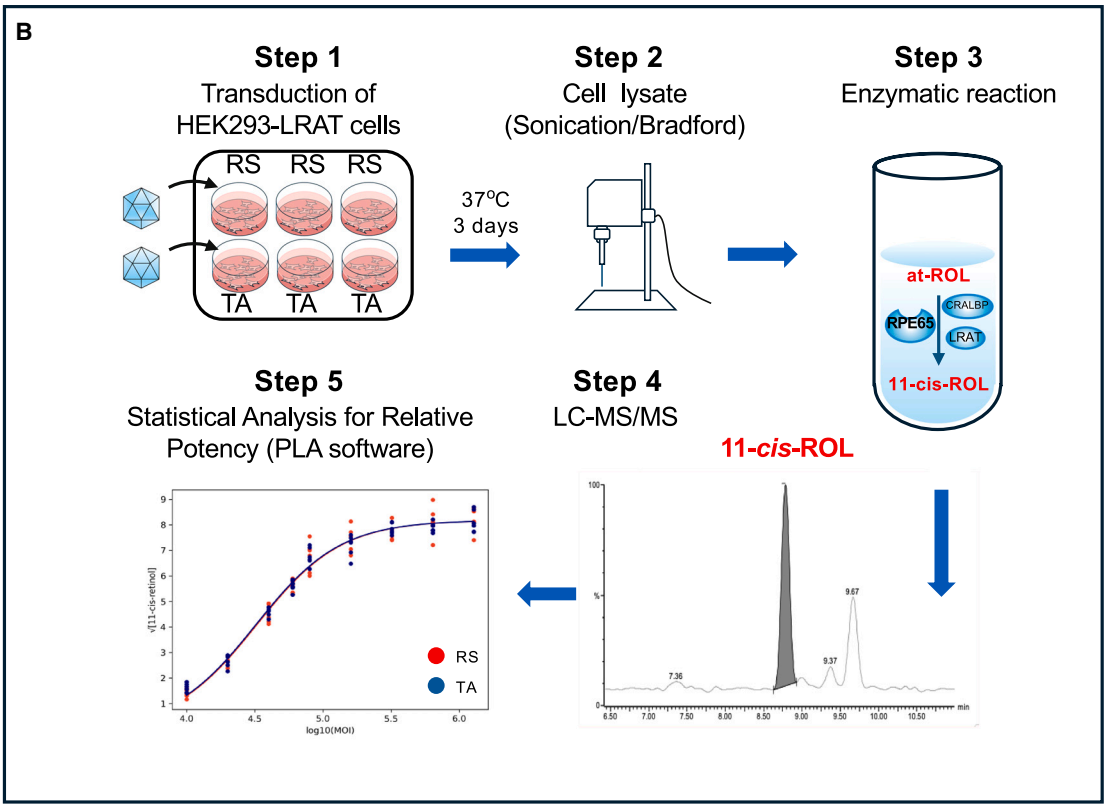

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for flexibility in determining the potency of investigational products, but the assay used for release of a licensed biological drug product must comply with multiple biologics and current good manufacturing practices (cGMP) regulations.[6] Validation of a potency assay involves the evaluation of the assay performance characteristics, including accuracy, precision, specificity, linearity, system and sample suitability, and robustness. In addition, sound statistical methods should be justified and applied to ensure data are valid and the reportable result is fit for purpose.

Luxturna, a recombinant AAV2 vector that expresses the cDNA encoding the human retinal pigment epithelium-specific 65-kDa protein (AAV2-h*RPE65*v2), is used to treat the visual deficit associated with biallelic mutations in RPE65. Based on a randomized controlled trial demonstrating improvement in a functional clinical endpoint measuring mobility, and in visual function tests including light sensitivity, visual acuity, and visual fields,[9] Luxturna was approved by the US FDA in 2017 (the first approved gene therapy for genetic disease in the US) and by EMA in 2018, with subsequent worldwide approvals.

During early clinical studies, the potency of AAV2-h*RPE65*v2 (Luxturna) was evaluated using an *in vivo* assay, which relied on subretinal injection in the rd12 mouse model[10] and utilized detection of the pupillary light reflex 6 weeks later as a qualitative measure of vector activity. Shortcomings of the *in vivo* assay included the lack of a quantitative readout, a long turn-around time, and the labor-intensive nature of the measurements; thus, the regulatory agencies had uniformly encouraged the sponsor to develop and validate a quantitative *in vitro* cell-based biochemical assay to assess the potency of Luxturna.

The *RPE65* gene encodes a retinoid isomerohydrolase enzyme,[11–13] which is normally expressed in retinal pigment epithelial (RPE) cells and has an essential role in the visual cycle (Figure 1A). The visual cycle[14] begins in photoreceptor cells when 11-*cis*-retinal (11-*cis*-RAL), acting as a chromophore when bound to opsin, absorbs a photon of light and is isomerized to all-*trans*-retinal (at-RAL). This results in a conformational change in opsin and triggers the phototransduction cascade. To sustain normal vision, efficient regeneration of 11-*cis*-RAL is required. This process occurs by a series of reactions in photoreceptor outer segments and RPE cells. at-RAL is first reduced to all-*trans*-retinol (at-ROL) by at-ROL dehydrogenase (RDH) in photoreceptors. It is subsequently transported to RPE cells by interphotoreceptor retinoid binding protein (IRBP), where cellular retinol binding protein (CRBP) facilitates its esterification to retinyl ester by lecithin retinol acyltransferase (LRAT). RPE65 acts as an isomerohydrolase to convert all-*trans*-retinyl ester to 11-*cis*-retinol (11-*cis*-ROL), in the presence of cellular retinaldehyde binding protein (CRALBP). Finally, oxidation of 11-*cis*-ROL by *cis*-specific RDH regenerates the visual pigment, 11-*cis*-RAL, which is transported back to photoreceptor cells, completing the visual cycle.

The validated *in vitro* potency assay described in this paper was designed based on the seminal work of Moiseyev et al.,[11] who were among the first to demonstrate that RPE65 is the isomerohydrolase responsible for regeneration of the chromophore, 11-*cis*-RAL, in the visual cycle. These investigators showed that lysates from cells transduced with an adenoviral vector expressing RPE65, when incubated with a radioactive substrate (at-ROL), generated 11-*cis*-ROL, which was detected and quantified using high-performance liquid chromatography (HPLC) and scintillation counting. In addition to adapting the assay for use with an AAV vector, we chose to develop a non-radioactive assay to avoid the environmental and health concerns related to working with radioactive isotopes and to eliminate the requirement for a specialized radioactive facility to conduct the assay. Thus, the validated assay utilizes non-radioactive at-ROL as the substrate and liquid chromatography with tandem mass spectrometry (LC-MS/MS) for detection and quantification of the product, 11-*cis*-ROL. This paper outlines the development and validation of this potency assay, which is used to evaluate lot-to-lot consistency, to track stability of the product over time, and to allow lot release of AAV2-h*RPE65*v2 (Luxturna).

RESULTS

Overall design of the potency assay

The AAV2-h*RPE65*v2 vector depends on a series of steps to confer its biological activity, including gene transfer, transcription and

Figure 1. Visual cycle and format of *in vitro* cell-based relative potency assay

(A) Anatomical localization of the visual cycle in the retina, which initiates in the PR outer segments and is completed in the RPE. (B) When light strikes the PR, 11-*cis*-RAL is isomerized to at-RAL. To sustain normal vision, regeneration of 11-*cis*-RAL is required. This process occurs by a series of reactions in PR outer segments and RPE cells. at-RAL is first reduced to at-ROL (red) by at-ROL dehydrogenase (RDH) in PRs. It is subsequently transported to RPE cells by IRBP, where cellular retinol binding protein (CRBP) facilitates its esterification to retinyl ester by lecithin retinol acyltransferase (LRAT) (blue). RPE65 acts as an isomerohydrolase to convert all-*trans*-retinyl ester to 11-*cis*-ROL (red), in the presence of cellular retinaldehyde binding protein (CRALBP) (blue). Finally, oxidation of 11-*cis*-ROL by *cis*-specific RDH re-generates the visual pigment, 11-*cis*-RAL, which is transported back to PR cells, completing the visual cycle. (B) Steps involved in *in vitro* cell-based RP assay: (1) HEK293-LRAT cells (see text) were transduced in triplicate at nine different MOIs (1×10^4 – 1.28×10^6 vg/cell) (for 100% RP) of a reference standard (RS) and test article (TA), both of which were the same lot of AAV2-hRPE65v2. (2) Cells were harvested 3 days later, lysates were prepared, and total cellular protein was quantified by Bradford assay. (3) Duplicate enzymatic reactions were prepared containing 100 μg of total cellular protein. The cellular protein contained RPE65 (blue), expressed from AAV, and LRAT (blue), expressed in stably transfected HEK-293-LRAT cells. To each lysate, was added 5 μM at-ROL (red) and 25 μM CRALBP (blue), and the reactions were incubated at 37°C in the dark under dim yellow light for 2 h. (4) The reaction mixtures were extracted and the product of the reaction,11-*cis*-ROL (red), was separated from other retinol isomers by LC-MS/MS and quantitated. (5) The square roots of the 11-*cis*-ROL measurements ($n = 6$ per MOI) were plotted versus the log10 of the MOI using 3PL logistics curves. The calculation of the RP of the TA was determined using PLA software. PR, photoreceptor; RPE, retinal pigment epithelial; 11-*cis*-RAL, 11-*cis*-retinal; 11-*cis*-ROL, 11-*cis*-retinol; at-RAL, all-*trans*-retinal; at-ROL, all-*trans*-retinol; LRAT, lecithin retinol acyltransferase; CRALBP, cellular retinaldehyde binding protein.

Table 1. RP for each of the 20 validation assays

Assay	Analyst	Day	Target level	RP reported by PLA v3.0	ln(RP)
2V2[a]	1	1	0.5	0.549	−0.6
5v4B (repeat)	1	2	0.5	0.523	−0.649
1V1[a]	2	1	0.5	0.500	−0.692
4V3B (repeat)	2	2	0.5	0.547	−0.604
16V14[a]	1	1	0.75	0.741	−0.3
18V16	1	2	0.75	0.729	−0.317
14V12[a]	2	1	0.75	0.751	−0.287
17V15	2	2	0.75	0.814	−0.206
7V6[a]	1	1	1.00	1.017	0.016
11V9	1	2	1.00	1.072	0.069
6V5B (repeat)	2	1	1.00	0.943	−0.059
8V7B (repeat)[a]	2	2	1.00	0.970	−0.03
20V18	1	1	1.25	1.193	0.177
22V20[a]	1	2	1.25	1.322	0.279
19V17[a]	2	1	1.25	1.351	0.301
21V19	2	2	1.25	1.469	0.384
13V11[a]	1	1	1.50	1.483	0.394
15V13	1	2	1.50	1.352	0.301
10V8B (repeat)[a]	2	1	1.50	1.512	0.414
12V10	2	2	1.50	1.237	0.213

[a]Dose-response curves from these assays are plotted in Figures 3 and 4.

translation of the h*RPE65* transgene, and finally conversion of at-ROL to 11-*cis*-ROL by the isomerohydrolase activity of the RPE65 protein (the transgene product). The last step in the process (generation of 11-*cis*-ROL) is completely dependent on the initial step (gene transfer), so we utilized the final step as the quantitative readout for the potency of AAV2-h*RPE65*v2. We chose HEK293 cells as the target cells in the assay because AAV2 efficiently transduces these cells. However, it was necessary to modify these cells by stable transfection of LRAT in order to support the visual cycle. HEK293-LRAT cells were transduced with multiple multiplicities of infection (MOIs) of AAV2-*RPE65*v2 in order to define the linear and asymptotic portions of the dose-response curve. Following transduction, cell lysates were used in an isomerohydrolase assay. The product of the reaction, 11-*cis*-ROL, was separated from the substrate (at-ROL) and from other *cis*-retinol isoforms using liquid chromatography (LC) and was quantified using tandem mass spectrometry (MS/MS). The assay was validated, and relative potency levels were determined using a biostatistical software package.

Prior to validation of the assay, optimization of cell culture, the enzymatic assay and the LC-MS/MS method was conducted. The variables analyzed, and final assay format are summarized in Table S1. With respect to cell culture, the cell culture plate size, culture time, number and values of MOIs, and inclusion of etoposide to enhance transduction were all evaluated. The isomerohydrolase enzymatic assay involves incubation of cell lysates with at-ROL (substrate) and CRALBP. Therefore, various concentrations of these reagents were evaluated and optimized for 11-*cis*-ROL generation. Other enzymatic assay variables evaluated included the substrate solvent, dose volume, and total protein lysate concentration. LC-MS/MS method parameters that were optimized, including the LC mobile phase and the ionization method, allowed for the generation of a sensitive standard curve that ranged from 2 to 200 nM 11-*cis*-ROL. The final conditions were also capable of cleanly separating 11-*cis*-ROL from other retinol isomers, including 9-*cis*-ROL, 13-*cis*-ROL, and the substrate at-ROL.

Detailed description of potency assay

The final validated potency assay can be divided into five major steps (Figure 1B): (1) transduction of HEK293-LRAT cells with AAV2-h*RPE65*v2, (2) preparation of cell lysates and determination of total protein concentration, (3) performance of an enzymatic assay on cell lysates using non-radioactive at-ROL as a substrate, (4) detection and quantitation of the 11-*cis*-ROL product by LC-MS/MS, and (5) statistical analysis using version 3.0 of the Stegmann Systems PLA bioassay platform and determination of relative potency.[15] Performance of the assay (steps 1–4) requires 4 days. Briefly, HEK293-LRAT cells were seeded in 6-well plates with Dulbecco's modified Eagle's medium (DMEM) containing blasticidin (to provide selective pressure on the LRAT plasmid). The following day, the cells were transduced in triplicate with nine different MOIs of AAV2-h*RPE65*v2 (1×10^4, 2×10^4, 4×10^4, 6×10^4, 8×10^4, 1.6×10^5, 3.2×10^5, 6.4×10^5, and 1.28×10^6 vector genomes [vg] per cell). Negative controls included three wells of cells treated with formulation buffer and three untreated wells. Three days later, cells were harvested, lysates were prepared by sonication, and their total protein concentrations were determined by the Bradford assay. Duplicate enzymatic reactions containing 100 μg of total cellular protein, 5 μM at-ROL, and 25 μM CRALBP were incubated at 37°C in the dark under dim yellow light for 2 h. Both the substrate (at-ROL) and the binding protein (CRALBP) were present in excess, so the limiting factor for production of the product was the concentration of RPE65 expressed by the AAV vector. CRALBP was initially sourced from three different vendors, but only one of these (EyeCRO, Oklahoma City, OK) produced material that was biologically active in the assay system. The enzymatic reactions were stopped by the addition of methanol and were extracted with hexane. LC-MS/MS was used to separate 11-*cis*-ROL from other retinol isomers in the reaction and to quantitate the amount of 11-*cis*-ROL produced. Six determinations of 11-*cis*-ROL per MOI were obtained (*vide infra*) and used for statistical analyses. The response profile of a given sample consisted of a 3-parameter logistic (3PL) fit (*vide infra*) to a square-root transformation of measured 11-*cis*-ROL as a function of log10(MOI).

The relative potency methodology described in USP <1032>[8] and <1033>[16] was adopted to avoid the inherent variability of absolute 11-*cis*-ROL measurements. During validation, the same lot of AAV2-h*RPE65*v2 served as both the RS and the test article (TA).

The expectation is that TA will perform similarly to the RS, with a response profile being parallel to the RS, which has an assigned relative potency value of 1 (or 100% potent). According to USP <1033>,[16] validation of a relative potency assay requires a minimum of three relative potency levels, and five are recommended. The Luxturna assay included five different levels of vector potency (50%, 75%, 100%, 125%, and 150%). The RS was used at the same nine MOIs described above, but the TA was used at either the same, higher, or lower MOIs to validate the detection accuracy of similar-, higher-, or lower-potency vectors, respectively. For example, to evaluate vectors that differ by 50% potency, the RS was utilized at nine MOIs ranging from 1×10^4 to 1.28×10^6 vg/cell, while the TA was utilized at nine MOIs ranging from 0.5×10^4 to 6.4×10^5 vg/cell. See Table S2 for a summary of the MOIs used to evaluate 50%, 75%, 100%, 125%, and 150% relative potency, and Figure S1 for a map of the 6-well plate cell-culture setup for evaluation of the 50% relative potency level. The average of six 11-*cis*-ROL determinations per all nine MOIs was calculated for both RS and TA. The 3PL response profiles for 2RS and TA were generated by plotting the square-root transformation of the 11 *cis*-ROL concentrations versus the log10(MOI), and the relative potency between TA and RS was determined using PLA software.

Validation

Formal validation studies consisted of completion of 20 successful assays. They were performed by two different analysts, each analyst performing the assays on two different days and at the five relative potency levels: 50%, 75%, 100%, 125%, and 150% (Table 1). During validation, five assays failed and were therefore repeated. The deviations associated with these five assays are summarized in Table S3 and included failures to generate a relative potency result due to system and sample suitability failures and failures due to errors in determining the correct protein concentrations using the Bradford assay. The assays that were repeated are indicated in Table 1 with a "B" appended to the assay name. Thus, the data from 20 successful assays were used for statistical analyses. Assay reliability was assured according to regulatory guidance documents[6,16,17] for the following seven characteristics: (1) system and sample suitability, (2) specificity, (3) linearity, (4) precision (repeatability, intermediate precision), (5) relative accuracy, (6) range, and (7) robustness.

System and sample suitability

Four parameter logistic curves are often used to analyze bioassays because the sigmoidal shaped dose-response curve is only approximately linear across a specific range of concentrations. Beyond this linear range, the response quickly plateaus and approaches a minimum and maximum. For Luxturna, the usual fourth parameter D (lower asymptote) was indistinguishable from 0, thus the dose-response profile was found to be best described by a three-parameter logistic (3PL) model. Initial investigations also determined that a square-root transformation of the measured 11-*cis*-ROL levels yielded an approximately homogeneous normal distribution of measurement errors and thus was used in the 3PL model to generate the dose-response curves.

3PL model:

$$Y = \frac{A}{1+10^{-B\cdot(\log\ 10(MOI)-C)}} \quad \text{(Equation 1)}$$

where:

$$Y = \sqrt{[11 - cis - \text{retinol}]} \quad \text{(Equation 2)}$$

and MOI level corresponds to the 11-*cis*-retinol result.

The response profile shape, Y vs. log10(MOI), is governed by the values of the following three parameters:

A = upper asymptote parameter.

B = Hill coefficient parameter (proportional to the slope of the response profile at the inflection point).

C = log10(EC_{50}) parameter (MOI dose at inflection point).

Fitting a parallel profile model requires a sufficient number of concentrations or in the case of gene-therapy vectors, MOIs. According to USP <1032>,[8] it is preferable to have at least four more concentrations than the number of parameters that will be estimated in the model. Thus, for a 3PL model, seven concentrations are recommended. Furthermore, two concentrations are recommended to support each asymptote. For Luxturna, we chose to use nine doses or MOIs to fit the 3PL model, even though the lower MOI asymptote is fixed at zero (Table S2).

System and sample suitability testing is an integral part of many analytical procedures and is based on the concept that the equipment, reagents, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such.[18] In order to ensure that the reportable result from a given bioassay execution is reliable, limits are placed on key performance metrics. These metrics and their associated limits are referred to as suitability criteria. Multiple suitability criteria control the shape of the response profiles of the RS and TA. System (or assay) suitability criteria, such as the general shape of the RS curve, the degree of agreement with the assumed 3PL model, and an acceptable level of outliers, are meant to ensure that the assay procedure is properly executed. Sample suitability criteria are meant to assure that the shape of the TA and RS response profiles are sufficiently similar (e.g., equivalent upper asymptote and Hill coefficients). All suitability metrics must be within their acceptable ranges in order for the relative potency to be considered valid and reportable for decision making. With the full (unrestricted) 3PL model, the upper asymptote (A) and the Hill coefficient (B) are allowed to have different values for RS and TA. However, with the reduced (restricted) 3PL model, the upper asymptote (A) and the Hill coefficient (B) are constrained to have the same values for both RS and TA. Similar response profile shapes provide evidence of equivalence between the RS and TA, making it possible

Table 2. System and sample suitability requirements

Suitability type	Sample data	Metric (statistical estimate)	Lower limit	Upper limit	Test type
System	RS	A (upper asymptote)[a]	3.4 nM	13.6 nM	equivalence
		B (Hill coefficient)	0.23	9	equivalence
		C (log10(EC_{50}))[b]	3.4	5.9	equivalence
		number of outliers	0	5	count
		R^2	0.74	1.0	point estimate[c]
Sample	TA	A (upper asymptote)[a]	3.4 nM	13.6 nM	equivalence
		B (Hill coefficient)	0.23	9	equivalence
		A (difference from RS)[a]	−2.8 nM	+2.8 nM	equivalence
		B (difference from RS)[a]	−3.6 nM	+3.6 nM	equivalence
		number of outliers	0	5	count
		R^2	0.74	1.0	point estimate[c]
		90% CI for RP	76%	130%	interval estimate[c]
		relative RP range	0%	60%	point estimate[c]
	TA & RS	sum of squares (lack of fit)	0	90	point estimate

[a]Square-root transformation of 11-*cis*-ROL concentration (nM).
[b]Log10(EC_{50}) of the MOI.
[c]For information only.

to estimate relative potency. The horizontal distance between the curves may be different, and this distance is related to true potency of the TA.

A potency assay included 54 measurements of 11-*cis*-ROL concentrations (six replicates for each of nine MOI levels) for each of RS and TA. All measurements were assumed to be independent. All analyses were performed using the Stegmann Systems PLA bioassay platform.[15]

During the development phase of the bioassay, the system suitability requirements for each execution of the bioassay were identified and are detailed in Table 2.

Associated with each of the 14 suitability requirements is a test type. Equivalence tests[19] require the 90% confidence interval (CI) for the metric to be entirely contained within the limits. Thus, the upper asymptotes are required to be between 3.4 and 13.6 nM 11 *cis*-ROL (square-root transformed), the Hill coefficient for each curve must be between 0.23 and 9, and the log10 of the half maximal effective concentration (EC_{50}) of both curves must be between 3.4 and 5.6 (or 2.51×10^3 and 7.94×10^5 vg/cell) to demonstrate equivalence. Passing an equivalence test provides strong evidence that the suitability limits are met. The consequences of failing an equivalence test are software rejection of the data and an inability to report the relative potency. Count tests refer to the total number of outliers identified in either RS- or TA-based assays. No more than five outliers per each assay (five out 108 11-*cis*-ROL measurements) are acceptable in order to meet sample suitability requirements. Point and interval estimate tests only require that the estimate be within the limits.

The relative potency and its 90% CI are obtained using Fieller's method,[16] with the 90% CI estimate required to be contained within the range of 76%–130% of the expected relative potency. The relative potency range is the width of the CI expressed relative to the relative potency point estimate and is calculated by subtracting the lower end of the 90% CI from the higher end and dividing by the relative potency point estimate. For the assay to be acceptable, this range must be between 0 and 60%.

The sum of squares (non-linearity), a measure of the inconsistency between the observed data and the fitted model, is obtained from the analysis of variance. Some suitability metrics (R^2, 90% CI for relative potency, and relative potency range) were for information only. An information-level test failure still allows a reportable relative potency but identifies a metric that deserves careful monitoring.

Specificity

Specificity is the ability to unequivocally assess the TA response in the presence of components, which may be expected to be present during sample analysis. Typically, these include impurities, degradants, and sample matrix. Specificity shows a lack of interference from these matrix components. The specificity of the assay was evaluated by preparing and analyzing the effects of the AAV2-*hRPE65*v2 formulation buffer. The same dilution volumes as those used for the RS were used for formulation buffer, and it was treated as the TA for all nine MOIs (100% relative potency level). In order for the assay to meet specificity requirements, the formulation buffer must show no dose response compared to the response elicited by the RS. In addition, to affirm that the bioassay is capable of identifying samples whose response profiles do not match that of the RS, the formulation

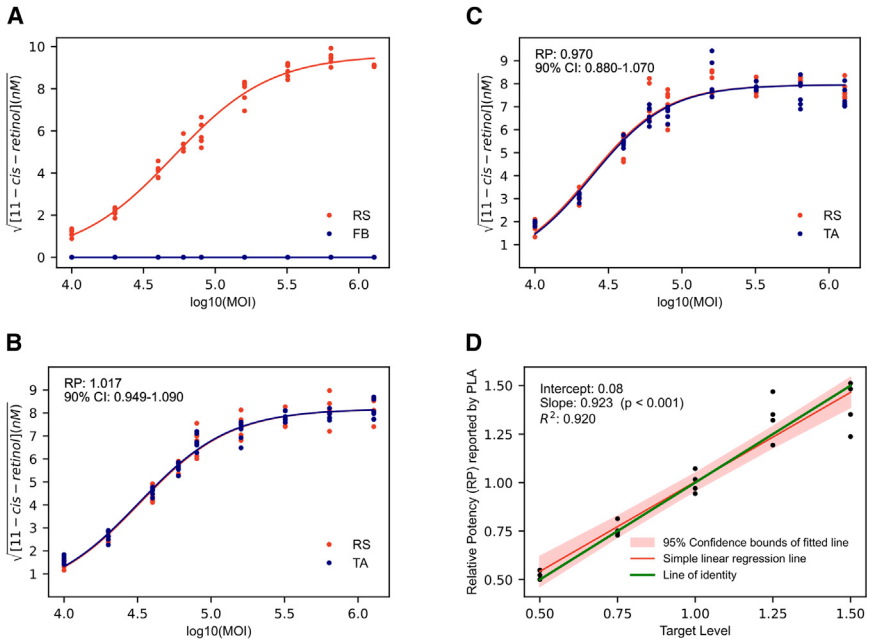

Figure 2. Dose-response curves demonstrating specificity, 100% RP, IP, and dilutional linearity

Standard assay conditions (see Figure 2) were used in these studies. (A) To evaluate specificity, HEK293-LRAT cells were transduced in triplicate with nine different MOIs of AAV2-hRPE65v2 (RS) from 1 × 10^4 to 1.28 × 10^6 vg/cell or with formulation buffer (FB). The lack of a response when using FB demonstrates assay specificity. (B) To evaluate 100% RP, HEK293-LRAT cells were transduced in triplicate with nine different MOIs (1 × 10^4 to 1.28 × 10^6 vg/cell) of AAV2-hRPE65v2 (RS) or the same nine MOIs of AAV2-hRPE65v2 (TA) by analyst 1. Similar and overlapping dose-response curves were observed, and the potency of TA was determined to be 101.7% relative to RS (100%). (C) To evaluate IP, HEK293-LRAT cells were transduced in triplicate with nine different MOIs (1 × 10^4 to 1.28 × 10^6 vg/cell) of AAV2-hRPE65v2 (RS) or the same nine MOIs of AAV2-hRPE65v2 (TA) by analyst 2. RP of TA was determined to be 97% relative to RS. The similar levels of RP determined by analysts 1 and 2 demonstrate IP of the assay. (D) To evaluate dilutional linearity, the calculated RP values from the 20 assays listed in Table 1 (column 5) were plotted against their respective target potency values. The fitted linear regression line had a slope of 0.92 ($p < 0.001$), an intercept of 0.08, and an R^2 value of 0.929. The plot demonstrates dilutional linearity between the experimental and the expected (theoretical) relative potencies at five concentration levels (50%, 75%, 100%, 125%, and 150%).

buffer is expected to fail the sample suitability criteria in Table 2, particularly the A and/or B metric difference equivalence tests.

The average 11-*cis*-ROL levels produced following transduction of HEK293-LRAT cells with the RS (AAV2-h*RPE65*v2) at nine MOIs or with formulation buffer are shown in Table S4. Representative LC-MS/MS chromatograms showing the levels of 11-*cis*-ROL produced in HEK293-LRAT cells transduced with RS at three of the nine different MOIs are shown in Figure S2. The 11 *cis*-ROL standard curve used for quantification, with a calibration range of 2–200 nM, is also shown. When compared to the dose-response elicited by the RS, no dose response was observed with the AAV2-h*RPE65*v2 formulation buffer (Figure 2A). The PLA v3.0 software provided an estimate of zero for relative potency. The AAV2-h*RPE65*v2 formulation buffer sample failed the sample slope criterion and also failed the equivalence test. These results demonstrate that the method is specific to AAV2-h*RPE65*v2.

In contrast, when AAV2-h*RPE65*v2 was used as both RS and TA and evaluated at the 100% accuracy level, similar and overlapping dose-response curves were observed (Figure 2B). As expected, the TA response was parallel to that of the RS, and in this assay the TA had 101.7% potency relative to RS. The average 11-*cis*-ROL levels observed in this assay (7V6) are listed in Table S5.

Dilutional linearity

The linearity of an analytical method assesses the ability of the assay to obtain test results that are directly proportional to the concentration of the sample. To demonstrate dilutional linearity, the observed relative potency must meet the expected relative potency (i.e., 50%, 75%, 100%, 125%, and 150%), with the fitted line close to the line of identity, and a coefficient of determination (R^2) $\geq$0.85.

The experimental relative potency reported by PLA v3.0 software for each of the 20 assays with target relative potency levels of 50%, 75%, 100%, 125%, and 150% are provided in Table 1 (column 5). The experimental relative potency values were plotted against their respective theoretical, target potency values. As shown in Figure 2D, the slope of the line was 0.923 ($p < 0.001$) with an intercept of 0.08. The dilutional linearity of the assay was confirmed because the 95% confidence bounds on the fitted line include the line of identity. The 95% CI estimate of the intercept of the simple linear regression was −0.053 to 0.214, and the 95% CI estimate of the slope was 0.797–1.049. Since the CI for the intercept includes zero and the CI for the slope includes one, dilutional linearity was demonstrated. The R^2 of the fitted line (0.929) was above 0.85 and thus met the validation acceptance criteria, and this indicates that dilutional linearity was demonstrated between the experimental and the expected (theoretical) relative potencies at five concentration levels (50%, 75%, 100%, 125%, and 150%). These data demonstrate that the assay can reliably differentiate relative potency (RP) levels of TA between 50% and 150% relative to the RS.

Intermediate precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample

Table 3. The IP and estimate of accuracy (relative bias) for each target level using the ln(Relative Potency) values

Target level	Average ln(RP)	SD ln(RP)	90% CI for mean ln(RP)		Geometric mean	90% CI for geometric mean RP		Geometric %CV (% GCV)	Relative bias (%) (the point estimate)	90%CI for relative bias (%)	
0.5	−0.636	0.044	−0.69	0.58	0.53	0.5	0.56	4.5	5.9	0.6	11.4
0.75	−0.277	0.049	−0.34	−0.23	0.76	0.72	0.8	5	1	−4.6	7
1	−0.001	0.056	0.07	0.07	1	0.94	1.07	5.8	−0.1	−6.5	6.7
1.25	0.285	0.086	0.18	0.39	1.33	1.2	1.47	8.9	6.4	−3.8	17.7
1.5	0.330	0.092	0.22	0.44	1.39	1.25	1.55	9.7	−7.2	−16.8	3.4

under the prescribed conditions.[18] The precision of an analytical procedure is usually expressed as the variance, standard deviation, or coefficient of variation of a series of measurements. Intermediate precision (IP), a measure of intra-run and inter-run variability, expresses the laboratory variations of the analytical method (e.g., different days, different analysts, and different equipment). The IP of the method was determined using the natural log of the RP (ln(RP) (Table 1, column 6) and a variance component analysis, as described in USP <1033>[16] and USP <1010>.[19] IP is expressed as percentage geometric coefficient of variation (%GCV). The IP for each concentration level must be $\leq$30% for assay acceptance. As seen in Table 3, column 9, the IP values (%GCV) for RP levels of 50%, 75%, 100%, 125%, and 150% were 4.5%, 5.0%, 5.8%, 8.9%, and 9.7%, respectively, and therefore met the validation acceptance criteria (i.e., $\leq$30%). The pooled IP value for all 20 RP values calculated, also referred to as an overall estimation of % geometric coefficient of variation (%GCV) (see materials and methods), was 8.2% and also met the validation acceptance criteria (i.e., $\leq$30%) (Table S6).

The dose-response curves used to estimate IP for the AAV2-h*RPE65*v2 RS and TA at the 100% potency level for analyst 1 (assay 7V6) and analyst 2 (assay 8V7B) are shown in Figures 2B and 2C, respectively. The PLA v3.0 selected dose-response curves used to estimate IP for AAV2-h*RPE65*v2 RS and TA at the 50%, 75%, 125% and 150% potency levels for both analysts are shown in Figure 3.

Relative accuracy

The relative accuracy of an RP assay is the relationship between measured RP and known RP and is calculated as 100 × (observed potency/target potency − 1)%. The 20 dilutional linearity assays listed in Table 1 were used to determine relative accuracy (or relative bias). The relative bias was determined at each of the five concentrations, using the average RP values (ln RP) obtained from the IP testing (Table 3, column 1). The relative bias for each accuracy level must be ±15% for the accuracy to be acceptable. The point and 90% CI estimates of relative bias are provided in the last three columns of Table 3. The point estimates for relative bias at the 50%, 75%, 100%, 125%, and 150% RP levels were +5.9%, +1.0%, −0.1%, +6.4%, and −7.2%, respectively, and met the validation acceptance criteria of ±15%. In addition, all the 90% CIs, except for the 50% level (90% Cl is 0.6–11.4), included zero; thus, from a statistical point of view, there is no evidence for any bias at these target levels.

Range

The range of the analytical method describes the interval between the upper and lower concentration, for which it has been demonstrated that the analytical method has a suitable level of precision, accuracy, and linearity. The analysis and conclusions derived from the assessment of the IP, relative accuracy, and dilutional linearity were used to establish the range over which results can be reliably reported.

The range of 50%–150% of the nominal vector concentrations (MOIs: 1×10^4, 2×10^4, 4×10^4, 6×10^4, 8×10^4, 1.6×10^5, 3.2×10^5, 6.4×10^5, and 1.28×10^6 vg/cell) was supported by the linearity, accuracy, and precision data (shown above). Results supporting the range are reported in dilutional linearity, intermediate precision, and relative accuracy sections of the paper. Based on these data, the assay was validated between a range from 50% to 150% RP.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters and provides an indication of its reliability during normal usage. The robustness of this method was evaluated by varying the standard assay conditions for the following parameters: time to transduction (i.e., cell seeding time), number of sonication pulses, and CRALBP concentration. To evaluate the influence of cell seeding time on the assay, the time allowed for HEK293-LRAT cells to be grown in the 6-well plates prior to AAV2-h*RPE65*v2 transduction was altered from the normal time of 20–24 h to either 18 or 26 h for both RS and TA. The second parameter, number of sonication pulses used to generate cell lysates, was maintained at two pulses for RS but was changed to either one or three pulses for cell lysates treated with TA. Lastly, the impact of CRALBP concentration on the enzymatic activity was altered from the normal concentration of 25 μM CRALBP to either a lower (20 μM) or higher concentration (30 μM). The target accuracy level used in this experiment was 100%; i.e., both RS and TA were used at MOIs of 1×10^4, 2×10^4, 4×10^4, 6×10^4, 8×10^4, 1.6×10^5, 3.2×10^5, 6.4×10^5, and 1.28×10^6 vg/cell. The results from these experiments determine the operating range of the potency assay, and any condition that results in a system suitability failure is considered a limiting condition and would need to be strictly controlled in the method. The system and sample suitability criteria were met for all the robustness experiments and conditions.

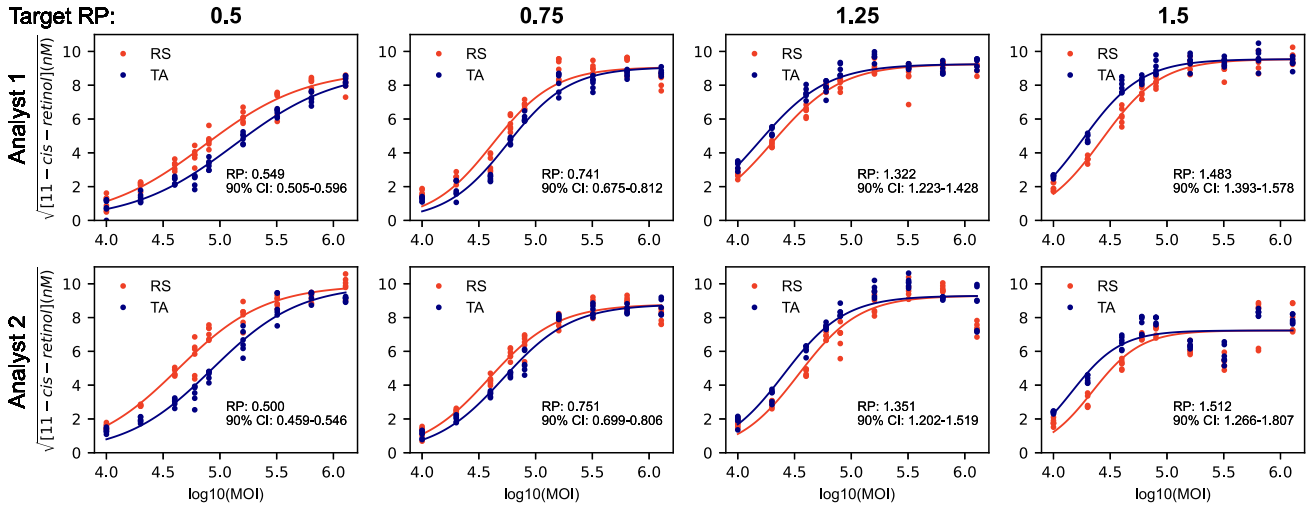

Figure 3. Dose-response curves evaluating 50%, 75%, 125%, and 150% relative potency

HEK293-LRAT cells were transduced in triplicate with nine different MOIs of AAV2-hRPE65v2. The RS was used at nine MOIs ranging from 1×10^4 to 1.28×10^6 vg/cell, but the TA was used at 50%, 75%, 125%, or 150% of the MOIs used for the RS. Assays were performed by two different analysts. Following transduction, duplicate cell lysates were prepared and the RPE65 enzymatic reaction was performed. The product of the reaction, 11-*cis*-ROL, was separated from other retinol isomers by LC-MS/MS and quantified. The square roots of the 11-*cis*-ROL measurements (n = 6 per MOI) were plotted versus the log10 of the MOI. TA, test article; 11-*cis*-ROL, 11-*cis*-retinol.

The dose-response curves for all the robustness conditions are displayed in Figure 4, and the RP results for all the robustness assays (assays 24ROB1, 26ROB3, 25 ROB2, 27ROB4, 28ROB5, 29ROB6) are shown in Table S7. For comparison, the RP results from four assays used to determine IP, using the standard conditions, are also summarized in Table S7. None of the robustness modifications affected the potency of the TA versus the RS, which ranged from 94.3% to 112% potent, among all 10 assays. The relative standard deviation (RSD) of the overall RP was 6.89%, demonstrating that the method is robust to varying the conditions for transduction time (22 ± 4 h), sonication pulses (2 ± 1 pulse), and CRALBP concentration (25 ± 5 μM).

DISCUSSION

After decades of development work, several AAV vectors are now therapeutic products, with Luxturna being the first AAV vector approved in the US. Approval of these vectors required clear demonstration of clinical efficacy and safety, demonstration that manufacturing processes and related quality systems met commercial standards, and development and validation of numerous assays to demonstrate consistency of vector characteristics from lot to lot. One of the most challenging assays for gene-therapy developers is a potency assay that can evaluate the MOA and measure the biological activity of the vector. During the early phases of clinical development, we utilized an *in vivo* potency assay, based on restoration of pupillary light reflexes in rd12 mice. However, the regulatory agencies have encouraged the use of quantitative cell-based assays and an RP methodology.

The AAV2-h*RPE65*v2 vector depends on a series of biological steps to confer activity. First, transfer of the vector genome to cells and transcription of the h*RPE65* transgene is required. This is followed by production of biologically active hRPE65 protein. The last step in the process, conversion of at-ROL to 11-*cis*-ROL, relies on the enzymatic activity of RPE65. This step is completely dependent on gene transfer, so we utilized the final step to develop a functional potency assay. The *in vitro* cell-based assay is based on the isomerohydrolase activity of the RPE65 protein, which converts at-ROL to 11-*cis*-ROL. We initially considered using induced pluripotent stem cell (iPSC)-derived mutant RPE65 cells, but the cost of developing and maintaining such a cell line was excessive, and a thorough evaluation of iPSC growth characteristics and AAV transduction efficiency would be required. We chose instead to develop an assay using an HEK293 cell line, as these cells have been successfully used for many bioassays and have a rapid growth rate, increasing throughput, and AAV2 vectors are known to transduce HEK293 cells very efficiently. Importantly, these cells do not express RPE65; thus, no background activity was present in the absence of vector. A genetic modification of these cells was required to perform the RPE65 enzymatic reaction. That is, it was modified to stably express LRAT, a protein required for conversion of at-ROL to all-*trans*-retinyl ester, the precursor of 11-*cis*-ROL. Attempts were made to generate a cell line that also stably expressed CRALBP, another protein required for generation of 11-*cis*-ROL, but we were unsuccessful in identifying clones that expressed sufficient amounts of the protein to carry out the reaction. Thus, HEK293-LRAT cells were transduced with AAV2-h*RPE65*v2, and CRALBP was added to the transduced cell lysates along with the substrate at-ROL to perform the enzymatic reaction. Following transduction, 11-*cis*-ROL was resolved from other retinol isomers present in the lysate and its concentration was quantified by LC-MS/MS.

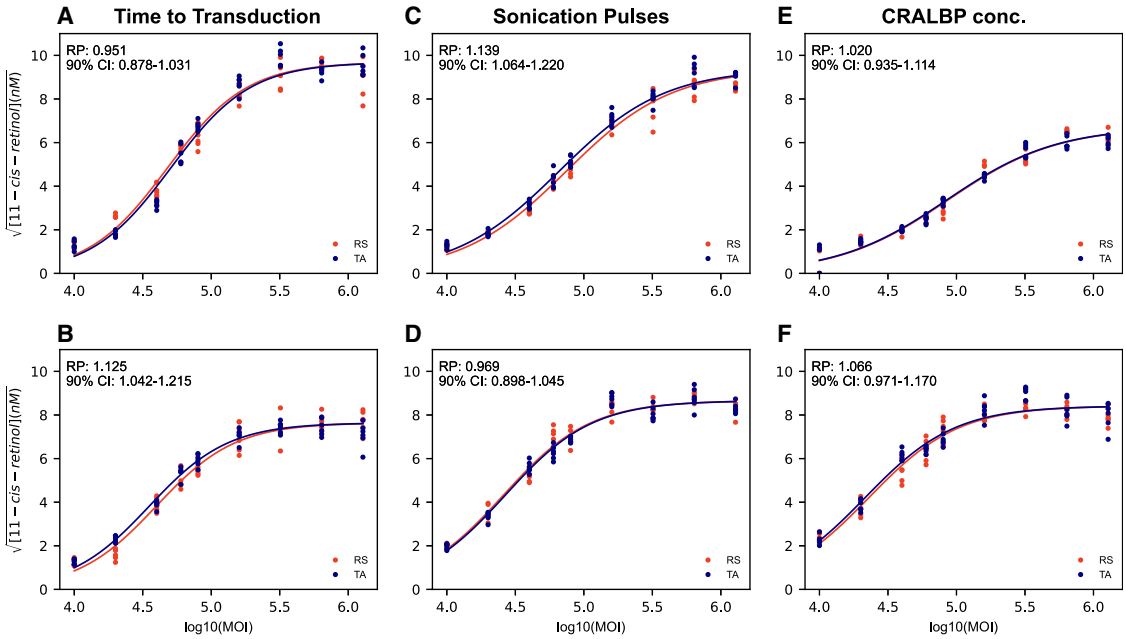

Figure 4. RP assay demonstrates robustness

HEK293-LRAT cells were transduced in triplicate with nine different MOIs of AAV2-hRPE65v2 from 1×10^4 to 1.28×10^6 vg/cell. AAV2-hRPE65v2 was used as both the RS and the TA at a target accuracy level of 100%. Following transduction, duplicate cell lysates were prepared and the RPE65 enzymatic reaction was performed. The product of the reaction, 11-*cis*-ROL, was separated from other retinols by LC-MS/MS and quantified. The square roots of the 11-*cis*-ROL measurements ($n = 6$ per MOI) were plotted versus the log10 of the MOI. The standard assay conditions were modified to evaluate the robustness of the assay as follows: time to transduction (i.e., cell seeding time), number of sonication pulses, and CRALBP concentration. To evaluate the influence of cell seeding time on the assay, the time allowed for HEK293-LRAT cells to be grown in the 6-well plates prior to AAV2-hRPE65v2 transduction was altered from the normal time of 20–24 h, to either 18 h (A) or 26 h (B), for both RS and TA. The second parameter, number of sonication pulses used to generate cell lysates, was maintained at two pulses for RS but was changed to either one (C) or three pulses (D) for cell lysates treated with TA. The impact of CRALBP concentration on the enzymatic activity was evaluated at 20 μM CRALBP (E) or 30 μM CRALBP (F) for both RS and TA. TA, test article; 11-*cis*-ROL, 11-*cis*-retinol.

The development of potency assays for enzymes such as this isomerohydrolase is in some ways more straightforward than for structural proteins, inhibitors, or cofactors. In addition, the Luxturna vector encodes the naturally occurring enzyme rather than, for example, a truncated or mutated variant, which meant that previously developed assays for the wild-type protein could be used as a template for the potency assay. Nevertheless, we encountered a number of challenges as we sought to bring the assay to standards required for product release. For example, choosing the appropriate number of MOIs to generate 3PL dose-response curves used for measuring RP was guided by recommendations in USP document <1032>[8] Once MOIs were identified, it was essential to confirm that the concentrations of reagents (at-ROL and CRALBP) used in pilot isomerohydrolase assays were sufficient for complete conversion to 11-*cis*-ROL at all nine vector concentrations used. A published analytical method used for quantifying 11-*cis*-ROL relied on a radioactive substrate and standard HPLC/scintillation counting for quantification.[11] However, a more environmentally appropriate assay was desired, so we developed a sensitive LC-MS/MS method capable of resolving and quantifying non-radioactive 11-*cis*-ROL from other retinol isoforms. Finally, sophisticated statistical analyses were required to determine RP levels using a commercially available PLA software package.

A validated potency assay is required for evaluating lot-to-lot consistency, stability over time, and for release of commercial lots of AAV2-h*RPE65*v2 (Luxturna). Validation of the assay must comply with the guidance documents that are applied to other biological therapies and must demonstrate system and sample suitability, specificity, linearity, precision, relative accuracy, range, and robustness. Twenty valid RP assays, each consisting of 108 unique 11-*cis*-ROL determinations, were performed using AAV2-h*RPE65*v2 as both the RS and TA at nine MOIs each. A summary of the assay validation parameters and results is shown in Table 4.

The dose-response profile for Luxturna was found to be best described by a 3PL model, and, thus, in order to validate the RP assay, the system and sample suitability parameters required that the upper asymptotes, Hill coefficients, and log10(EC_{50}) of the RS and TA dose-response profiles show equivalence. Other requirements of each assay were that no more than five out of 108 11-*cis*-ROL measurements be identified as outliers and that the 90% relative potency range for each measured RP level be between 76% and 130% of the expected RP level. All of these suitability parameters were met in 20 total assays conducted during validation. Based on these 20

Table 4. Summary of validation parameters and results

Parameter	Results
System and sample suitability	all criteria were met
Specificity	no dose response observed for formulation buffer
Dilutional linearity	reported RP is linear to expected RP (R^2 = 0.93)
Intermediate precision	50%: % IP = 4.5% 75%: % IP = 5.0% 100%: % IP = 5.8% 125%: % IP = 8.9% 150%: % IP = 9.7% pooled: % IP = 8.2%
Relative accuracy	50%: % bias = 5.9% 75%: % bias = 1.0% 100%: % bias = −0.1% 125%: % bias = 6.4% 150%: % bias = 7.2%
Range	50%–150% of nominal method concentration
Robustness	method is robust for: time to transduce: 22 ± 4 h sonication pulses: 2 ± 1 pulse CRALBP concentration: 25 ± 5 μM

suitable assays, six other assay characteristics were evaluated and validated. The assay was shown to be specific for AAV2-h*RPE65*v2, as no 11-*cis*-ROL response was observed when formulation buffer was used as the TA to treat cells. Dilutional linearity of the assay was also confirmed because the theoretical RP levels (50%, 75%, 100%, 125%, and 150%) were shown to be directly proportional to the experimental RP levels, with an R_2 value of 0.93 for the fitted line. Another measure of the relationship between the experimental and theoretical relative potencies is relative accuracy, and it was determined that the relative accuracies of measuring these five levels of potency, were all within ± 15% of the theoretical level. IP, which represents laboratory variations in assays due to performance on different days, by different analysts and on different pieces of equipment, ranged from 4.5% to 9.7% for the five RP levels and thus met the requirement of <30% for validation of the assay. The combined data established that the range over which the results can be reliably reported is 50%–150%. Finally, the assay was unaffected by deliberate changes in cell culture growth time, concentration of CRALBP, and changes to the preparation of the cell lysate, thereby demonstrating robustness of the potency assay.

In summary, the results demonstrate that 11-*cis*-ROL is produced in a dose-dependent manner and the dose-response profiles of the RS and TA conform to 3PL curves and demonstrate equivalence. By evaluating the displacement of the parallel RS and TA curves, the assay can reliably determine the RP of Luxturna at levels between 50% and 150% of an RS. Although there is at least one published report of a validated potency assay suitable for release of a clinical-grade AAV vector,[20] to our knowledge, this is the first report of a validated RP assay for release of a commercial AAV vector product. The methodology used to develop and validate the RP assay for Luxturna can serve as a model for other AAV-based therapeutics.

MATERIALS AND METHODS

Reagents

Cell culture reagents including DMEM high glucose (catalog [Cat.] no. 1965-092), penicillin-streptomycin-glutamine (100×) (cat. no. 10378-016), fetal bovine serum, certified, heat inactivated (HI FBS), US origin (cat. no. 10082-147), and blasticidin S HCl (10 mg/mL) (cat. no. Al 1139-03) were purchased from Thermo Scientific. Human CRALBP protein was obtained from EyeCRO (Oklahoma City, OK). Organic solvents 2, 6-di-tert-butyl-4- methylphenol (also known as butylated hydroxytoluene [BHT]) (cat. no. B1378-100G), N,N-dimethylformamide (DMF), HPLC grade, ≥99.9% (cat. no. 270547-lL), 1,3-bis[tris(hydroxymethyl)methyl amino]propane) (also known as Bis-Tris propane [BTP]) (cat. no. B6755-100G), and hexane HPLC grade ≥98.5% (cat. no. 293253-2L) were purchased from Sigma-Aldrich. Acetonitrile, UHPLC grade (cat. no. A955-4) and methanol, UHPLC grade (cat. no. A456-4) were purchased from Fisher Scientific, and isopropyl alcohol, HPLC grade EMD (cat. no.PX1838-1) was obtained from EMD Millipore. The retinol isomers 11-*cis*-ROL (cat. no. R252105) and at-ROL-deuterium 5 (at-ROL-d5) (cat. no. R252002) were purchased from Toronto Research Chemicals. at-ROL (cat. no. R7632) was purchased from Sigma-Aldrich. AAV2-h*RPE65*v2 was produced as described in Maguire et al.[21]

Cell culture

HEK293-LRAT cells were provided by EyeCRO (Oklahoma City, OK). To generate this cell line, HEK293 cells were cultured until 75% confluency and a linearized plasmid (pcDNA6, Thermo Fisher Scientific, Fremont, CA), containing human LRAT, was transfected into cells using Fugene 6 (Promega, Madison, WI) using the manufacturer's protocol. Stably expressing cells were selected using blasticidin-containing medium and subcloned to generate a stably expressing cell line. Cells were certified to be mycoplasma free prior to preparation of master and working cell banks. Immediately after thawing, cells were cultured in complete DMEM (DMEM, 1× penicillin-streptomycin-glutamine, 10% fetal bovine serum). Cells were subsequently passaged in complete DMEM containing blasticidin (10 μg/mL).

AAV transduction *in vitro*

HEK293-LRAT cells were seeded in 6-well plates at 1×10^6 cells/well with 2 mL Complete DMEM containing blasticidin (10 μg/mL). On the following day (20–24 h for all assays except robustness assays, where cells were grown for either 18 or 26 h), a series (series A) of nine different dilutions of AAV2-h*RPE65*v2 were prepared in media. This series of dilutions was considered the RS and was used to transduce HEK293-LRAT cells at nine different MOIs. A separate series (series B) of nine dilutions of the same lot of AAV2-h*RPE65*v2 was considered the TA and was used to transduce cells at nine different MOIs. Three replicate wells of HEK293-LRAT cells were transduced at each MOI in a total volume of 2 mL. To evaluate 100% RP, the MOIs used for RS and TA were identical: 1×10^4, 2×10^4, 4×10^4, 6×10^4, 8×10^4, 1.6×10^5, 3.2×10^5, 6.4×10^5, and 1.28×10^6 vg/cell (Table 2). To evaluate 50% RP, the MOIs of the RS remained the same, but the MOIs of the TA were 2-fold lower.

The MOIs of RS and TA used to evaluate five levels of RP are shown in Table 2. A separate 6-well plate was used for control wells, which included three wells treated with formulation buffer, and three untreated wells, with one well used for cell counting prior to transduction. Plates were incubated at 37°C for 2.5–3 days.

Preparation of transduced cell lysates

Following transduction, cells were "power washed" by pipetting the contents of each of the wells of the 6-well plates up and down several times. No additional buffer was added. The three MOI replicates were kept independent, collected in separate 15-mL polypropylene tubes, and centrifuged at room temperature for 5 min at 1,200 rpm. Medium was aspirated from the tubes and cell pellets were resuspended in 100 μL of reaction buffer (10 mM BTP, pH8.0/100 mM NaCl). Cells were lysed using an ultrasonicator (VirTis Virsonic 100) that was set between levels 10 and 11 and pulsed twice for 0.5 s. In robustness assays, one or three pulses were evaluated. The total protein concentrations of the cell lysates were determined relative to a BSA standard curve (25–2000 μg/mL) using a Pierce Bradford assay (Thermo Scientific; cat. no. 23200), following the manufacturer's instructions using a quadratic curve fit. Spike recovery samples were prepared using lysates from cells treated with formulation buffer and containing a known concentration of the BSA standard (e.g., 7.5 μL of 2,000 μg/mL standard; final concentration 750 μg/mL). Acceptance criteria for the Bradford assay required that the standard curve have a $R^2 \geq 0.985$ and the spike recovery be 75%–125%. The remaining cell lysates (98 μL) were frozen at −80°C until used in the enzymatic assay.

RPE65 isomerohydrolase activity assay

The transduced cell lysates were thawed and re-sonicated, as described above, and gently mixed. Duplicate enzymatic reactions were performed on triplicate cell pellets, so six independent 11-*cis*-ROL measurements were obtained at each MOI. For each reaction, 100 μg of total cell lysate was added into 200 μL of reaction buffer (10 mM BTP, pH8.0/100 mM NaCl) containing 0.5% BSA and 25 μM CRALBP in a 1.5-mL polypropylene tube. The following steps were performed in the dark under dim light to prevent retinol instability. Two μL of 500 μM at-ROL dissolved in 100% DMF was added to each reaction, and tubes were briefly vortexed and incubated at 37°C for 2 h. The reactions were stopped by addition of 300 μL of 10 mM BHT in methanol (BHT-MeOH). The samples were covered with foil to protect them from light until processing for 11-*cis*-ROL analysis by LC-MS/MS, which was performed on the same day.

Determination of 11-*cis*-retinol product

Processing of the samples involved addition of 20 μL of reconstitution solution (6 mM BHT in 60% methanol) and 20 μL of 2,000 nM at-ROL-d5, which was used as an internal standard (see below for preparation). This was followed by addition of 300 μL of hexane. The samples were then vortexed for 5 min and centrifuged at 13,000 rpm for 5 min. The upper organic phase (∼250 μL) was transferred to a clean 96 well plate (VWR 96-well deep well microplates, 2.0 mL, cat. no. 40002-012). The samples were evaporated to dryness at 40°C under a gentle N_2 flow using a TurboVap for ∼30 min. Seventy-five microliters of reconstitution solution was added to each well and the plate was filled with N_2 using the TurboVap (∼30 s at flow setting of ∼25). The plate was covered with a VWR lid (Mat Lids for 2.0-mL microplates (cat. no. 40002-016) and vortexed for ∼3 min. The plate was then centrifuged at 3,000 rpm for ∼2 min. Ten microliters of each sample was injected onto a liquid chromatography column, and the concentration of 11-*cis*-ROL in each cell lysate was determined by the quantitative LC-MS/MS method.

Retinol RSs for 11-*cis*-ROL and the internal RS (at-ROL-d5) were prepared under dim yellow light for the quantitative LC-MS/MS. RSs and quality-control (QC) solutions of 11-*cis*-ROL were prepared from a 5 mM stock solution of 11-*cis*-ROL in 10 mM BHT in DMF. The stock solution was aliquoted in amber glass vials with inert gas and stored at −80°C until use. A set of working-standard solutions was prepared in reconstitution solution by serial dilution of the 5 mM 11-*cis*-ROL stock. The eight working standards ranged from 20 to 2,000 nM 11-*cis*-ROL. A set of QC solutions were also prepared in reconstitution solution by serial dilution of the 5 mM 11-*cis*-ROL stock. The concentrations of the working QC solutions were 1,600 nM (QC-H), 500 nM (QC-M), 50 nM (QC-L), and 20 nM (QC-LL).

Prior to LC/MS-MS, the working standards and QC solutions were reconstituted in a manner similar to that of the samples. That is, 20 μL of the eight individual working standards and four QC solutions were aliquoted into 1.5-mL polypropylene tubes. A reaction matrix (200 μL 1× BTP-NaCl-BSA reaction buffer) was added to each tube, followed by 300 μL of BHT-MeOH solution, 20 μL of internal RS (2,000 nM at-ROL-d5 in reconstitution solution), and 300 μL of hexane. The tubes were vortexed for 5 min, centrifuged at 13,000 rpm for 5 min, and the upper organic phase (∼250 μL) was transferred to a clean 96-well plate. The samples were evaporated to dryness at 40°C, reconstitution solution (75 μL) was added to each well, and the plate was filled with N_2 using the TurboVap. The plate was covered with a VWR lid, vortexed, and centrifuged at 3,000 rpm for ∼2 min. Ten microliters of each sample were injected onto an LC column. Thus, the final concentrations of the working standards analyzed by LC-MS/MS ranged from 2 to 200 nM 11-*cis*-ROL and the final concentrations of the QC solutions were 160, 50, 5, and 2 nM 11-*cis*-ROL.

The at-ROL RSs were prepared by pre-weighing aliquots of the powder and storing the vials at −80°C. A 2 mM stock solution prepared in 100% DMF was stored at −80°C for up to 1 month, and dosing solutions of 500 μM (or 100 μM used for assay optimization), also in 100% DMF, were prepared fresh for each assay. A stock solution of 1 mM at-ROL-d5 was prepared in DMF, and a working solution of 2,000 nM at-ROL-d5 was made in reconstitution solution, which was stored at −80°C.

A Waters Acquity UPLC system equipped with a BEH Shield RP18 column (1.7 μm, 2.1 × 150 mm) (part no. 186003376) was used for

resolution of the retinol isoforms. Samples in a volume of 10 μL were injected onto the column (40°C) and separated using an isocratic mobile phase of acetonitrile:methanol:isopropyl alcohol:-water (45:20:5:30 v/v/v/v) at a flow rate of 350 μL/min. The total run time was 11 min, and the retention times of the retinol isomers were as follows: 11-*cis*-ROL (∼8.8 min), at-ROL (∼9.7 min), and at-ROL-d5 (IS) (∼9.5 min).

A Waters XEVO TQ-S mass spectrometer utilizing atmospheric pressure chemical ionization (APCI) was used for ionization in the mass spectrometry, and Waters MassLynx v4.1 software was used to integrate the chromatographic peaks. Automatic integration was used to integrate the 11-*cis*-ROL peak throughout the assay batch samples, although manual integration was allowed for low-MOI samples.

The standard curve was generated by plotting 11-*cis*-retinol peak areas to internal standard peak ratios for each of the eight standards (2, 5, 10, 20, 50, 100, 160, and 200 nM), against their theoretical concentration. The concentrations of 11-*cis*-ROL produced in the lysates were determined by plotting the ratio of the 11-*cis*-ROL peak areas to the internal standard peak areas against the 11-*cis*-ROL standard concentrations for each MOI.

Prior to performing sample analysis, certain LC-MS/MS criteria were required to be met, including system suitability requirements, standard requirements, and QC requirements. Standard curve calibrations were obtained using a linear regression using peak area ratios. The slope, y intercept, and the correlation coefficient (R^2) were determined, and the concentration of each calibration standard was back-calculated from the calibration curve to determine the accuracy against its nominal concentrations. The acceptance criteria included a $R^2 \geq 0.960$. In addition, the accuracy of the 2 nM standard must be within (100 ± 30)% of its nominal concentration, the accuracy of the 5 nM standard must be within (100 ± 25)% of the nominal concentration, and the accuracy of the standards above 10 nM must be within (100 ± 15)% of their nominal concentrations. Other requirements included system suitability for instrument sensitivity and precision prior to injecting samples. For system sensitivity, the signal-to-noise ratio of three independently injected sensitivity neat solution samples (4 nM 11-*cis*-ROL) must be ≥ 3. For instrument precision, the injection of three independent mid-QC samples (50 nM 11-*cis*-ROL) must meet a precision criterion; i.e., the RSD of the peak area for the 11-*cis*-ROL peak must be $\leq 10\%$. Acceptance criteria for QC samples were also required for the LC-MS/MS data to be valid. The concentrations of the QC samples were calculated from the standard curve and their accuracy was determined against their nominal concentrations. Accuracy must be within (100 ± 30)% of the nominal concentration for LLOQ QC (2 nM), within (100 ± 25)% of the nominal concentration at low QC (5 nM) level, and within (100 ± 15)% of the nominal concentration at mid-QC (50 nM) and high-QC (160 nM) levels. The samples from cells treated with formulation buffer and the untreated cell samples were analyzed by LC-MS/MS but were not included in the PLA template because there was no generation of 11-*cis*-ROL in the samples. The samples from untreated wells were reported as a negative control or non-transduced cells (NTCs) in the LC-MS/MS runs. The negative samples from the formulation buffer wells were used for spike recovery analyses in the Bradford assay and for preparing recovery QC samples for LC-MS/MS.

Statistical analysis

In the isomerohydrolase activity potency assay, six independent measurements of 11-*cis*-ROL were obtained at nine MOIs for the RS and six independent measurements at nine MOIs were also measured for the TA. The statistical analyses of these measurements were carried out with PLA software version 3.0[15] using a four-parameter logistical (4PL) model. In this model the lower asymptote was fixed at zero because of the low 11-*cis*-ROL response observed at low MOIs, thus becoming a three-parameter model (3PL). PLA 3 software system for biostatistical analysis comes fitted with a fully featured bioassay package capable of running all types of biological assays described by the USP <1010>, <1032>, <1033>, and <1034>,[8,15–17] as well as European Pharmacopoeia fourth-ninth editions, chapter 5.3.[22] The square-root transformation was employed in PLA to render the error variance approximately constant across the response range. The first step in the analysis of the quantitative potency assay is to create a PLA template using a 3PL model with the lower asymptote fixed at zero. Using the template, the square roots of the 11-*cis*-ROL concentrations are entered, excluding technical outliers that were identified during execution of the assays. The PLA template includes five assay-suitability tests and nine sample-suitability tests (Table 4).

No more than five outliers per each assay (five out 108 11-*cis*-ROL measurements) are acceptable in order to meet sample-suitability requirements. The PLA template employed both objective and subjective criteria for outlier identification. Objective criteria utilized a non-recursive Dixon test performed on groups of six measurements at an alpha level of 1%. Any single value in the group of six that failed this test was automatically deemed an outlier and omitted from any further analysis. The alpha level of 1% was intended to assure that no more than 1% of values were incorrectly identified as outliers. Subjective criteria used to identify outliers relied on the expertise and due diligence of the analyst, and they involved identifying laboratory errors or deviations of any kind and excluding the unrepresentative data from any further analysis. The PLA analysis produced a response profile with individual data values plotted. If values were observed that clearly deviated markedly from the fitted curve and from other values, such values were deemed unrepresentative and were removed to avoid biasing results. Such deviations are encoded in the PLA observations tables and were not used for analysis.

DATA AVAILABILITY

All non-proprietary data are available upon request to the corresponding author.

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AUTHOR CONTRIBUTIONS

L.B.C. and K.A.H. contributed to conception and design, data analysis and interpretation, and manuscript writing. K.D., D.L., DC, I.I., and G.B. contributed to collection and assembly of data and data analysis and interpretation. D.L.B. conducted the statistical analyses and assisted with manuscript preparation. R.F. provided valuable suggestions and oversaw production of CRALBP. All authors reviewed and approved the manuscript.

DECLARATION OF INTERESTS

L.B.C. is an inventor on a patent application that describes the potency assay. The patent was licensed to Spark Therapeutics.

SUPPLEMENTAL INFORMATION

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