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Development of a high-throughput replicon assay for the identification of respiratory syncytial virus inhibitors

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ABSTRACT

Respiratory syncytial virus (RSV) drug discovery has been hindered by the lack of good chemistry starting points and would benefit from robust and convenient assays for high-throughput screening (HTS). In this paper, we present the development and optimization of a 384-well RSV replicon assay that enabled HTS for RSV replication inhibitors with a low bio-containment requirement. The established replicon assay was successfully implemented for high-throughput screening. A validation screen was performed which demonstrated high assay performance and reproducibility. Assay quality was further confirmed via demonstration of appropriate pharmacology for different classes of RSV replication tool inhibitors. RSV replicon and cytotoxicity assays were further developed into a multiplexed format that measured both inhibition of viral replication and cytotoxicity from the same well. This provided a time and cost efficient approach to support lead optimization. In summary, we have developed a robust RSV replicon assay to help expedite the discovery of novel RSV therapeutics.

1. Introduction

RSV is an enveloped, non-segmented negative-sense RNA virus in the *Paramyxoviridae* family. It is the leading cause of acute lower respiratory tract infections in young children and other high-risk populations (Collins and Melero, 2011). No efficacious RSV treatment or vaccine is available despite decades of research and drug discovery endeavor. Ribavirin, the only approved antiviral for RSV treatment, has limited clinical use due to toxicity and controversial efficacy. Immunoprohylaxis with RSV-neutralization antibodies is only effective as a preventive measure (Collins and Melero, 2011).

The urgent need to develop efficacious RSV treatments prompted several HTS campaigns for novel RSV inhibitors (Bonavia, 2011; Mason, 2004; Chung, 2013). These screens primarily used an RSV cytopathic effect (CPE) assay which evaluates compound protection against cell death induced by RSV infection (Bonavia, 2011). The CPE HTS protocol employs an extended assay time and requires biosafety level-2 containment which limits its robustness and practicality. Other approaches including cell-free RSV ribonucleoprotein complex (RNP) and minigenome assays have also been applied (Mason, 2004; Olivo, 1998). The RNP assay requires isolation of functional viral RNP from infected cells. The minigenome assay requires transient transfection of cells with five plasmids encoding each of the RNP components to reconstitute the viral RNP complex essential for viral RNA replication. The lack of robustness in both approaches has limited their utility as HTS screens.

A common alternative to screening for antivirals uses subgenomic replicon cell systems which have been proven to be robust and convenient tools for the discovery of viral replication inhibitors for many viruses. These autonomous replicons mimic the viral replication process in infected cells and do not release infectious virus particles, hence have the advantage of lowering the biosafety requirement (Rice, 2011; Yang, 2011; Masse, 2010). One prominent example is the HCV replicon system which has been the backbone of HCV drug discovery (Rice, 2011; Yang, 2011). The feasibility of achieving a stable and non-cytotoxic RSV replicon system was recently demonstrated, despite natural RSV infection being transient and cytopathic (Malykhina, 2011). This autonomous RSV replicon utilizes a modified viral RNA genome encoding the viral RNP proteins essential for RSV replication: The RNA-dependent-RNA polymerase (L), nucleoprotein (N), cofactor phosphoprotein (P), and second matrix protein (M2-1) (Fig. 1A). Viral genes involved in virus entry and assembly (G, F, SH) were deleted and replaced by the insertion of reporter and antibiotic-resistant genes. This allowed selection and enrichment of the stable replicon cells and generation of a robust assay readout. The cells can be passaged multiple times and still maintain the replicon RNA and protein expression, providing the foundation for a more efficient approach to large scale screening for RSV replication inhibitors.

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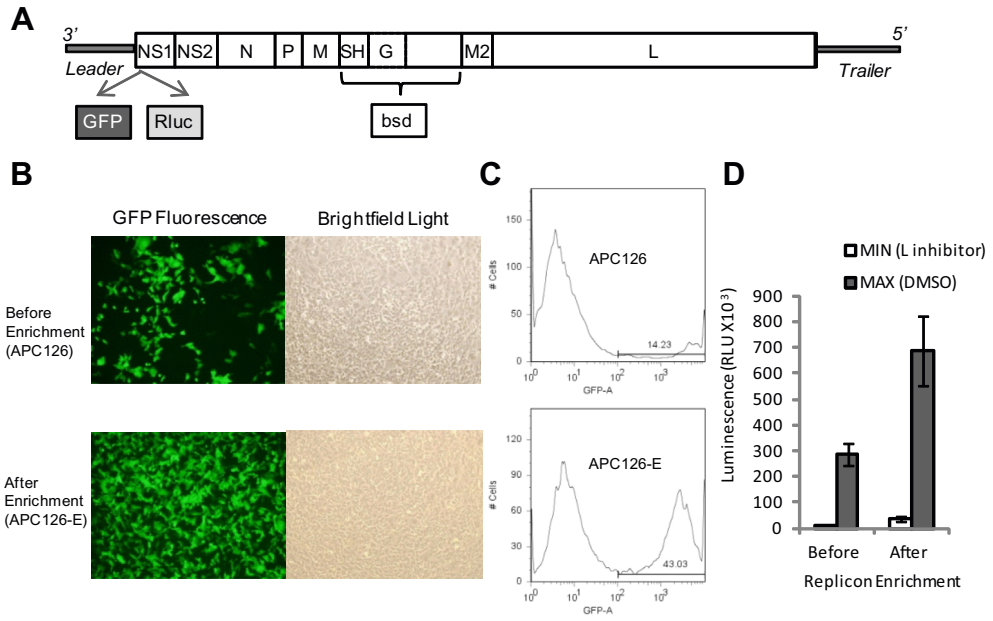

Fig. 1. Enrichment of reporter signal in RSV Replicon cells. (A) RSV replicon genome structure. Viral glycoprotein genes SH, G, F were replaced by selection marker bsd gene to acquire resistance to blasticidin. Green fluorescent protein (GFP) and renilla luciferase (Rluc) genes were inserted as reporters (Malykhina, 2011). (B, C) RSV replicon was enriched under the selection of blasticidin. Enrichment of replicon content in APC126-E cells was confirmed by increased GFP expression detected by fluorescence microscopy (B) and flow cytometry (C). (D) Enriched RSV replicon cells showed enhanced luciferase reporter signal and remained sensitive to RSV L inhibitor. APC126 and APC126-E replicon cells were treated with either DMSO (maximum signal) or a RSV L-inhibitor (minimum signal) for 48-h followed by luciferase activity detection. The data shown are mean values of the replicates from a representative experiment with standard deviation error bars.

Here we report the development and optimization of a robust HTS assay using this RSV replicon system. The fully optimized assay was further validated using known RSV replication inhibitors in a pilot screen. We also developed a multiplexed assay format which delivered both replicon and cytotoxicity readouts. These two robust versions of the RSV replicon assay provided new tools to advance HTS and chemistry campaigns towards the development of effective RSV treatments.

2. Materials and methods

2.1. Cells and media

2.1.1. RSV replicon cell lines

APC126 originating from Dr. Mark Peeples laboratory was licensed from Apath LLC. The cells were cultured in complete growth media consisting of Minimum Essential Medium (MEM) (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma), 2 mM L-glutamine (Sigma), 1 U/ml penicillin (Invitrogen), 1 μg/ml streptomycin (Invitrogen) and 10 μg/ml blasticidin-HCl (Invitrogen) at 37 °C in 5% CO_2. The enriched RSV Replicon Cell Line APC126-E was generated from APC126 cells following 4 weeks of culture and passaged in the presence of 40 μg/ml blasticidin-HCl.

2.1.2. Assay media

Assay optimization and validation work was performed using phenol red-free MEM supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine. HTS validation was carried out using the same assay media with the addition of 1 U/ml penicillin and 1 μg/ml streptomycin.

2.2. Compounds

Ribavirin and oseltamivir were purchased from ACME Bioscience and EMD Chemicals. RSV inhibitors: YM53403 (Sudo, 2005), an analog of YM53403 with equivalent anti-RSV potency (Compound 1 in Xiong, 2013), and BI cpd D (Liuzzi, 2005) were synthesized in-house. Compounds were solubilized in 100% v/v DMSO at 10 mM and further diluted in assay media to a final DMSO concentration of 0.1% v/v for screening. The RSV L inhibitor used in HTS assay development refers to YM53403 analog and was used at 10 μM to generate the minimum assay signal.

2.3. Large scale APC126-E cell cryobank for HTS

APC126-E cells were propagated in complete growth media containing 40 μg/ml blasticidin-HCl and cultured at scale in twenty 10 chamber Cell STACKS (Corning Life Sciences) to generate an assay ready cryobank of 1.2×10^{10} cells. Cells were detached using accutase, centrifuged, and re-suspended in complete growth media at a density of 6×10^7 cells/ml. An equal volume of cryopreservation media (80% FCS/20% DMSO) was added and the cells were aliquoted for cryopreservation using a controlled rate freezer (Planer Kryo).

2.4. RSV replicon assay optimization and characterization

Cryopreserved APC126-E cells were plated into 384-well white assay plates (Falcon) at a density of 3000 cells/well in a 54 μl volume and incubated at 37 °C with 5% CO_2 for 4 h before compound treatment. Compounds were diluted as described in the Compounds section and transferred to assay plates at 6 μl/well followed by 48-h incubation at 37 °C in 5% CO_2. For replicon reporter signal detection, the assay media was removed from the assay wells and replaced with 20 μl of 50 μM EnduRen™ Live Cell Substrate (Promega). Cells were incubated for 1.5 h at 37 °C prior to luminescence detection using a Synergy 2 plate reader (Biotek).

2.5. Validation of the HTS RSV replicon assay

150 nl each of 7000 compounds at 4 mM in DMSO was dispensed into white 384-well tissue-culture treated microplates

(Greiner Bio-One) using an Echo 555 acoustic dispenser (Labcyte Inc) (Turmel, 2010). This gave a final concentration of 0.25% v/v DMSO and a final assay compound concentration of 10 μM. These assay ready plates were thermally sealed and stored at room temperature. Cryopreserved APC126-E cells were dispensed into assay ready plates at a density of 3000 cells/well in 60 μl of assay media using a Multidrop Combi (Thermo Scientific). After 48-h at 37 °C in 5% CO_2, assay media was removed to leave a final volume of 10 μl, to which 20 μl of 25 μM EnduRen™ was added. Luminescence was measured using a Leadseeker plate reader (GE Healthcare Life Sciences).

2.6. Cytotoxicity assay

2.6.1. Standard format

Replicon cells were plated and treated with compounds as described in Section 2.4. Following compound treatment, assay media was aspirated and CellTiter-Glo® Luminescent substrate (diluted 1 to 1 in assay media) was added to the assay plates at 20 μl/well. Plates were incubated at ambient temperature in the dark for 5 min before luminescence detection.

2.6.2. Multiplexed format

EnduRen™ substrate and CellTiter-Glo® reagent were added sequentially to replicon cells to measure both luciferase activity and viability of the replicon expressing cells. Briefly, following detection of *Renilla* luciferase reporter of live replicon cells, EnduRen™ substrate was aspirated from each well, and replaced with 20 μl of CellTiter-Glo® reagent (diluted 1 to 1 in assay media) followed by luminescence detection as described in the standard format.

2.7. Data analysis

The percentage of maximum signal was calculated as: % control = (average of compound-treated cells)/(average of DMSO-treated cells). The quantification of cytotoxicity was calculated using:% cytotoxicity = 1−(average of compound-treated cells)/(average of DMSO-treated cells). EC_{50} and CC_{50} of compounds were extrapolated from% control and% cytotoxicity curves fitted using sigmoidal dose–response model 205 of XLfit software, respectively.

3. Results

3.1. RSV replicon enrichment

The RSV replicon APC126 cell line was originally passaged in the presence of 10 μg/ml blasticidin-HCl as recommended by Apath. Under this level of selection the replicon containing cells made up around 15% of the total population (Fig. 1B). A preliminary screen using APC126 cells showed high assay variability and Z' below 0.5 (data not shown). We enriched the replicon by culturing the cells under enhanced blasticidin-HCl selection, and detected a higher level of GFP expression in the enriched APC126-E cells

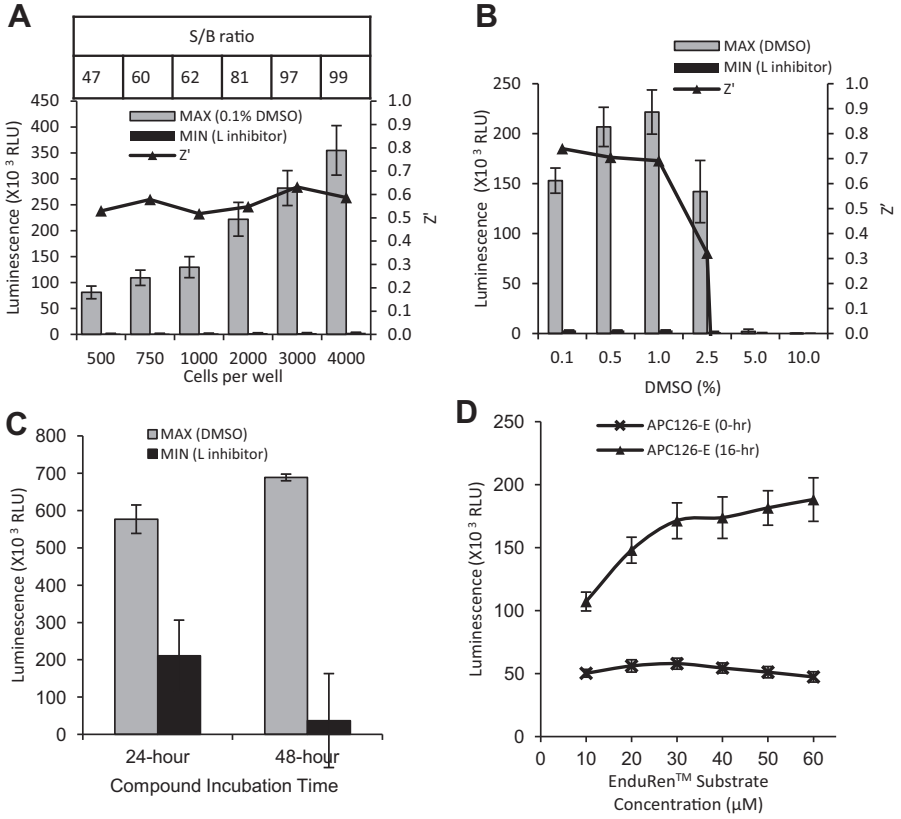

Fig. 2. Optimization of 384-well RSV Replicon Assay. Replicon luciferase assay maximum signal (DMSO treated), minimum signal (L inhibitor treated), and/or Z' were compared under the following assay conditions. (A) Cell seeding density: APC126-E cells were seeded at the indicated densities in 384-well plate and treated with DMSO or RSV L-inhibitor for 48-h. (B) Assay DMSO tolerance: APC126-E cells were seeded at density of 3000 cells per well and incubated for 48-h in assay media with the indicated DMSO concentrations. (C) Compound incubation time: APC126-E cells were seeded at density of 3000 cells per well and treated with DMSO or RSV L-inhibitor for 24 or 48-h. Differences in max and min signals from the two assays were significant with P values <0.0001 (student t-test). (D) Luciferase substrate concentration: Cryopreserved APC126-E cells were pre-seeded for 0 or 16 h at density of 3000 cells per well and incubated for 48 h in assay media with DMSO. Cells were incubated with luciferase substrate EnduRen™ at indicated concentrations to compare maximum luminescence signals. All DMSO controls were at 0.1% (v/v) and RSV L-inhibitor controls were at 10X EC_{50}. The data shown are mean values of the replicates from a representative experiment with standard deviation error bars.

by flow cytometry and fluorescence microscopy. The replicon expressing cells in APC126-E increased from 15% to 45% of the total population after the selection (Fig. 1B–C). The magnitude of luciferase reporter signal in APC126-E cells was also improved to more than two-fold (Fig. 1D).

3.2. 384-Well RSV replicon assay optimization

3.2.1. Cell density

To determine the optimal cell density for the replicon assay we titrated between 500 and 4000 APC126-E cells/well and measured the luciferase reporter signal after 48-h. A RSV L inhibitor with an EC50 of 0.3 μM in replicon assay was used at 10 μM to generate the minimum assay signal. Assay signal, signal to background (S/B) ratios and Z' from the different cell seeding conditions were compared. Both assay maximum signal and S/B ratios increased with higher cell densities (Fig. 2A). The Z' were consistently above 0.5, suggesting the APC126-E assay was robust at all cell densities tested (Fig. 2A).

3.2.2. The impact of DMSO

To ensure that the concentration of DMSO used in the assay had no significant impact on the replicon, luciferase reporter signal from replicon cells incubated with DMSO at final concentration of between 0.1% and 10% (v/v) were examined. The results showed that replicon cells tolerated DMSO up to a concentration of 1% without impacting the assay quality (Fig. 2B). At higher DMSO concentrations, assay variability became larger as Z' dropped to 0.3 at 2.5% DMSO. Replicon reporter signal was completely abolished when the DMSO concentration of the assay reached above 5% (Fig. 2B).

3.2.3. Compound incubation time

To determine whether the HTS assay could be adapted to a 24-h rather than 48-h compound incubation time, the assay signal window under these two conditions was compared. Larger assay window was found in the 48-h assay (Fig. 2C). The 24-h assay was able to detect inhibition of replicon replication by the RSV L inhibitor. However the level of inhibition was forty percent lower than that observed in the 48-h assay. The reduced inhibition by the RSV L compound in the 24-h assay was also observed in a concentration–response format, with an EC_{50} values (2.7 μM) two to four-fold higher than the 48-h assay (0.67 μM) and the RSV live virus assay (0.15 μM), suggesting that the 48-h assay is more sensitive in detecting the compound inhibitory effect.

3.2.4. EnduRen™ substrate concentration

A titration of EnduRen™ between 10 and 60 μM was carried out in the 48-h replicon assay. The assay luminescent signal plateaued at 10 and 30 μM of substrate using cryopreserved and proliferating replicon cells, respectively. An EnduRen™ concentration of 25 μM was selected for this assay (Fig. 2D).

3.3. Assay validation with RSV inhibitors

Activities of known RSV replication inhibitors in the replicon assay were evaluated. These included Ribavirin (a broad-spectrum antiviral) and two RSV L polymerase inhibitors, YM53403 and BI cpd D (Sudo, 2005; Liuzzi, 2005). Oseltamivir, an influenza inhibitor with no effect on RSV, was used as a negative control. All the RSV inhibitors tested, but not the influenza inhibitor, effectively blocked the RSV replicon replication. Potencies of these inhibitors in the replicon assay (EC_{50}) were comparable to their reported EC_{50} in RSV infectious virus assays, supporting that this assay is

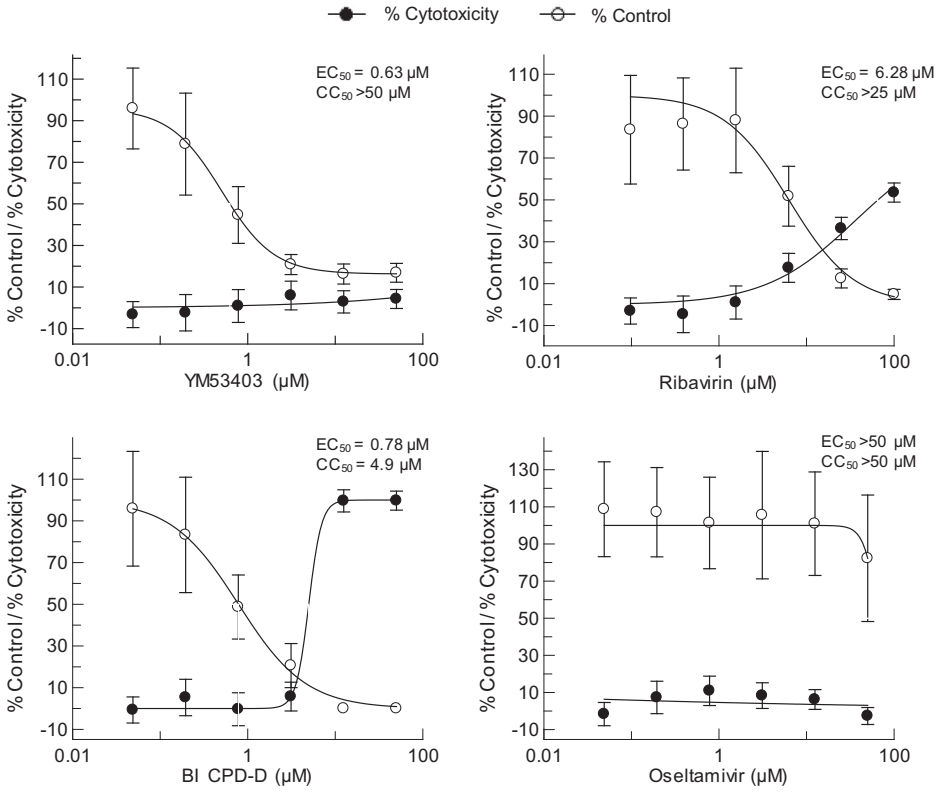

Fig. 3. Inhibitory and cytotoxic effects of antiviral inhibitors on RSV replicon cells. Serially diluted compounds were added to APC126-E seeded in 384-well assay plates at 3000 cells per well. Replicon cells viability (% cytotoxicity) and reporter signal (% control) affected by compounds were measured after 48-h of compound treatment. EC_{50} and CC_{50} were calculated as described in Section 2. The data are mean values with standard deviation error bars of the replicates within a representative experiment.

Table 1
Validation of assay-ready format for 384-well RSV replicon assay.

	Compounds	Proliferating[a] replicon cells	Cryopreserved replicon cells[b]		
			Pre-seeded[b] for 16 h	Pre-seeded[b] for 4 h	Assay ready[c] plate
EC_{50} (μM)	YM53403	0.52	0.69	0.59	0.88
	BI cpd D	0.29	0.44	0.25	0.63
	Ribavirin	4.22	5.22	5.09	3.18
	Negative	>50	>50	>50	>50
Assay performances	Z'	0.88	0.88	0.89	0.73
	S/B ratio	53	59	46	65

[a] Replicon in culture and pre-seeded 16 h prior to compound treatment.
[b] Cryopreserved replicon pre-seeded and recovered for 4 or 16 h prior to compound treatment.
[c] Cryopreserved replicon seeded directly into plates containing compounds.

suitable for screening for RSV replication inhibitors (Fig. 3). The CC_{50} values obtained in the replicon assay were comparable to the reported cytotoxicity values of YM53403 (82.3 μM) and BI cpd D (8.4 μM) (Fig. 3) (Sudo, 2005; Liuzzi, 2005).

3.4. Cryopreserved replicon cells

For HTS campaigns cryopreserved cells are often preferred over continuously cultured cells. The latter requires labor-intensive cell preparations and the homogeneity of the cell population across a screen can be challenging to maintain. Since the physiological state of the host cell is important to replicon replication, it was critical to confirm cryopreserved cells maintained a comparable level of replicon expression and drug sensitivity. Compared to cells cultured in assay plates overnight, cryopreserved cells expressed lower but reproducible level of luciferase reporter signal (Fig. 2D). The EC_{50} of the inhibitors obtained from assay using cryopreserved replicon cells were comparable to those from proliferating cells or recoverd cryopreserved cells, with no significant difference in Z' and S/B ratios (Table 1). This confirmed that the cryopreserved APC126 E cells were suitable for use in HTS campaign.

3.5. Use of assay-ready compound plates

To further streamline the RSV replicon assay for HTS, we investigated whether cryopreserved APC126-E cells could be added directly to assay ready plates where compounds are acoustically dispensed in advance. The data demonstrated that the assay Z', S/B ratios, and inhibitor pharmacology using assay ready plates were comparable to those observed when compound addition occurred following cell plating. This supported the use of assay ready plates and cryopreserved cells for identification of RSV replication inhibitors (Table 1).

3.6. HTS validation

The suitability of the RSV replicon assay for HTS was assessed using a diversity library of approximately 7000 small molecules selected from the AstraZeneca compound collection. Optimized RSV replicon assay conditions were applied, including the use of 3000 cryopreserved APC126-E cells/well and assay ready plates. This validation set was screened at a single compound concentration of 10 μM on two separate occasions with a randomized plate and well assignment (Fig. 4A). Good correlation was observed between the two independent screening runs, as evidenced by a linear fit of $Y = 0.969 + 0.993{*}X$ with an R^2 value of 0.859. In addition, control plates containing minimum ($10 \times EC_{50}$ of RSV L inhibitor), maximum signals (0.25% v/v DMSO) and reference ($1 \times EC_{50}$ of RSV L inhibitor) compounds were included in the runs to establish a Z' (Fig. 4B). An overall screening Z' of 0.58 ± 0.05 ($n = 23$) was achieved, demonstrating that the assay performance was robust and suitable for application in HTS.

3.7. Multiplexing replicon with cytotoxicity assay

To remove compounds that produce a diminished assay signal as a result of cytotoxicity rather than inhibition of viral replication, a separate cytoxicity assay is typically run as a counter screen. Since the Enduren Live Cell substrate of the replicon assay can allow the assay to be multiplexed with other lytic assay to deliver two readouts in a single assay, we investigated the possibility of combining the replicon and cytotoxicity assays. Comparison of

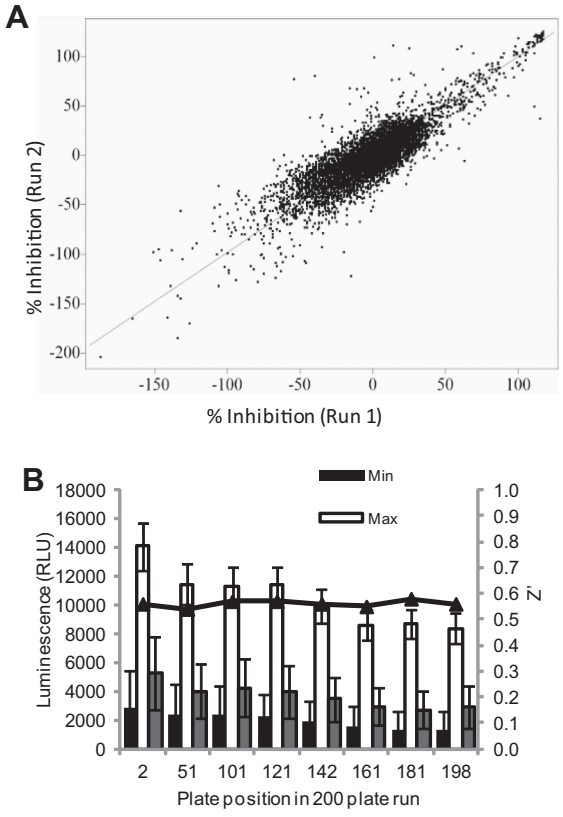

Fig. 4. Validation of 384-well HTS replicon assay. (A) A validation set of 7000 compounds was screened on 2 separate occasions at a concentration of 10 μM. Linear correlation between the two data sets was observed. (B) Graph showing data from 200 plate screening experiment; Min (10X EC_{50} of RSV L inhibitor), Max (DMSO) and Reference (EC_{50} of RSV L-inhibitor) controls were used to measure S/B and Z'. The data shown are representative data from a single experiment. The data points are the mean of the replicates ($n = 128$) from that experiment, and the error bars are the standard deviation of the replicates within the experiment.

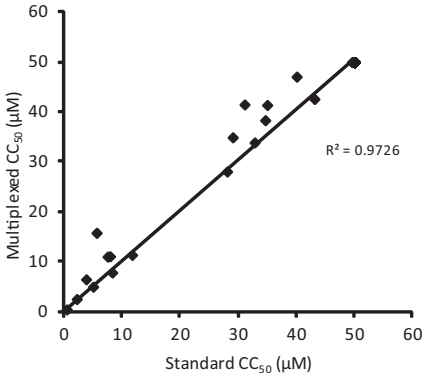

Fig. 5. Comparison of cytotoxicity measured using standard cytotoxicity assay and multiplexed replicon assay. Cytotoxic effects of seventy representative compounds were measured by standard and multiplexed cytotoxicity assays, as described in Section 2. Correlation between CC_{50} values measured by the two assays was observed. CC_{50} values $\geqslant 50$ μM are plotted as 50 μM. Data represented are the mean of triplicate measurements.

Table 2
Comparison of assay performances in standard and multiplexed cytotoxicity assays.

Assay format	Multiplexed	Standard
Z'[a]	0.9	0.9
Max (mean)[b]	679707	667461
Min (mean)[c]	2013	1896

[a] Data represent mean values from six replicate plates.
[b] Assay maximum signal from DMSO-treated replicon.
[c] Assay minimum signal from cell-free medium with DMSO.

the CC_{50} of 70 compounds obtained by multiplexed and standard assay formats showed results from the two assay formats were consistent (Fig. 5 and Table 2). The multiplexed method also showed Z' and percentage of coefficient variance comparable to the standard single detection formats, demonstrating the suitability of the multiplexed assay for efficient screening.

4. Discussion

Finding an effective treatment for RSV infection is an important public health priority. We established a robust RSV replicon assay for screening large compound libraries to identify novel RSV replication inhibitors. Our data demonstrated that the RSV replicon is a reliable tool system validated by multiple classes of RSV replication inhibitors. The engineered GFP reporter allows visualization of replicon expression in the cells, and can be utilized to guide the enrichment of replicon during assay optimization. The robust luciferase reporter signal of the replicon enabled a relatively simple HTS using cryopreserved cells and assay-ready compound plates without compromise to assay quality. The multiplexed RSV replicon/cytotoxicity assay format allows a more streamlined and cost-cutting HTS, and can also be applied to and benefit other screens.

Although the RSV replicon assay does not detect inhibitors of all key steps of the virus life cycle as provided by the conventional RSV live virus CPE assay (Wang, 2011), it has the strength of being a more target-focused assay. It has been suggested that small molecule inhibitors of RSV replication may have a greater potential to become efficacious RSV treatments compared to viral entry inhibitors (Chapman, 2007). If so, the RSV replicon assay would be a desirable tool to focus on the identification of viral replication inhibitors. The shorter incubation time needed for the replicon assay also makes the assay less susceptible to compound instability, screening batch size limit, and plate edge effects. The RSV replicon assay eliminates the need for transient transfection of cells with multiple DNA plasmids required by RSV minigenome system. This removes issues such as high assay variability, cytotoxicity in transfected cells, and identification of undesirable hits targeting DNA plasmid replication/expression. It also avoids the need to isolate HTS-scale functional viral proteins from infected cells that is required by the cell-free RSV RNP assay, which can be technically demanding (Mason, 2004; Liuzzi, 2005).

In conclusion, the RSV replicon assay is a suitable alternative to RSV CPE, RNP, or minigenome assay for HTS. Compared to these assays, it delivers to a faster cycle time, targets a more focused mechanism of inhibition, and has lower bio-containment requirements. Removing the requirement for live virus could potentially broaden RSV drug discovery by enabling organizations not equipped with the necessary large scale bio-containment facilities to also screen for RSV inhibitors.

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