Minigenome-Based Reporter System Suitable for High-Throughput Screening of Compounds Able To Inhibit *Ebolavirus* Replication and/or Transcription[▽]

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We describe an *Ebolavirus* minigenome-based system that is suitable for high-throughput screening of compounds able to impair *Ebolavirus* virus replication and/or transcription. The assay is robust (Z′ factor, >0.6) and can be carried out in low-biosafety containment. Results from a pilot screen of 960 compounds are presented.

Ebolaviruses and the closely related *Marburgviruses* cause hemorrhagic fever in humans and nonhuman primates, with high mortality rates (18). Currently, treatment of these infections is limited to supportive care (1). The need for efficacious therapeutics is further underscored by the increasing frequency of *Ebolavirus* outbreaks in both humans and endangered great ape populations (2, 20, 21).

High-throughput screening (HTS) systems should be robust with high reproducibility and should be amenable to work in low-biosafety containment. *Ebolavirus* is categorized as a biosafety level 4 agent, and HTS systems that rely on live *Ebolavirus* are thus impractical. Here, we describe a minireplicon system to screen for compounds that interfere with viral replication and/or transcription. In these systems, a virus-like RNA encoding a reporter protein is amplified by the viral proteins required for replication and transcription, i.e., the *Ebolavirus* polymerase (L), nucleoprotein (NP), and VP30 and VP35 proteins (10, 14).

A minireplicon system based on *Zaire Ebolavirus* developed in our laboratory was taken as a starting point (22). First, we generated a reporter construct (p3E5E-Luc) in which the open reading frame (ORF) for the luciferase gene (in antisense orientation) is flanked by the *Zaire Ebolavirus* leader and trailer regions (which contain the viral promoters for replication and transcription) and by T7 RNA promoter and terminator sequences (Fig. 1A). Next, we transfected human embryonic kidney (293T) cells with p3E5E-Luc, pC-T7 (for T7 polymerase expression [15]), and pCEZ-NP, pCEZ-VP35, pCEZ-VP30, and pCEZ-L (for the expression of the *Zaire Ebolavirus* NP, VP35, VP30, and L proteins, respectively [15])

using Trans-IT LT1 reagent (Mirus); the amounts of the respective plasmids are listed in Table 1, Setup number 1. To determine background expression levels of luciferase in the absence of a functional nucleocapsid complex (negative control), we substituted empty vector for pCEZ-L. Forty-eight hours posttransfection, an equal volume of Steady-Glo luciferase assay substrate (Promega) was mixed with each sample and luminescence was measured. Mean luciferase expression and standard deviations were obtained by measuring luminescence in eight separate wells for both the positive (with pCEZ-L) and negative (without pCEZ-L) controls. For this and all subsequent experiments, we next calculated the Z′ factor (24) as a measure of the robustness of the assay: $Z' = 1 - [3 \times (\sigma_p + \sigma_n)/(\mu_p - \mu_n)]$, where σ_p is the standard deviation of the positive control (reporter protein expression from the minireplicon), σ_n is the standard deviation of the negative control (reporter protein expression in the absence of the L protein), μ_p is the mean of the positive control, and μ_n is the mean of the negative control. The Z′ factor incorporates the dynamic range between positive and negative controls, as well as their well-to-well variability. A Z′ factor of >0.5 is recommended for a robust screen, and values above 0.75 are preferred before transfer to a 96-well platform because the Z′ factor usually declines during adaptation to smaller formats (24).

In this assay, we found high well-to-well variability for the positive control and marked luciferase expression in the negative control (i.e., in the absence of the polymerase protein) (Fig. 2A). The signal-to-background ratio was 88:1 and the Z′ factor was 0.53, which is considered too low for transfer to a HTS protocol.

To improve the signal-to-background ratio and the Z′ factor, we next tested a minireplicon system that utilizes cellular RNA polymerase I for the transcription of virus-like RNAs (10). In this approach, the RNA polymerase used for the synthesis of virus-like RNAs is no longer provided from a plasmid, thus reducing the number of plasmids needed and thereby potentially increasing the robustness of the system.

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A.

p3E5E-Luc

B.

pHH21-3E5E-Luc
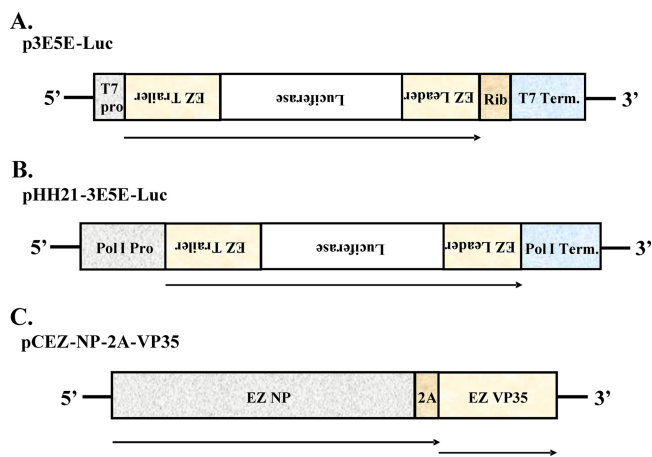

C.

pCEZ-NP-2A-VP35

FIG. 1. Schematic representation of transcription cassettes for the synthesis of *Ebolavirus*-like RNAs. (A) *Ebolavirus* minigenome transcribed by T7 RNA polymerase, resulting in negative-sense, virus-like RNA. The HDV ribozyme (Rib) creates the 3′ end of the minigenome through self-cleavage. (B) The same minigenome as in panel A inserted between the RNA polymerase I (pol I) promoter and terminator sequences. (C) Bicistronic cassette for synthesis of NP and VP35 from a single transcript. The 2A site mediates self-cleavage of the polypeptide chain, resulting in individual NP and VP35 products.

To establish an RNA polymerase I-driven minigenome, we cloned the luciferase gene and the *Ebolavirus* leader and trailer regions between RNA polymerase I promoter and terminator sequences (Fig. 1B). When 293T cells were transfected with this reporter construct (pHH21-3E5E-Luc; see Setup number 2 in Table 1) and the indicated amounts of protein expression plasmids, the signal-to-background ratio rose to 171:1 and the Z′ factor improved to 0.80, indicating markedly reduced well-to-well variability (Fig. 2B). However, appreciable background luminescence in the negative control remained a concern (Fig. 2B).

To determine if the use of a different cell line would result in lower background luminescence, we tested COS-1 cells. These cells, like 293T cells, constitutively express the simian virus 40 (SV40) large T antigen, which enhances protein expression levels from plasmids possessing the SV40 origin of replication as used here. Using Setup number 3 in Table 1, we found that background luminescence was significantly reduced in COS-1 cells; however, well-to-well variability was increased compared to that in 293T cells, resulting in a Z′ factor of only 0.52 (Fig. 2C).

TABLE 1. Plasmid cocktails used for different assay setups

Plasmid	DNA amt (μg used per well)[a]			
	Setup 1	Setup 2	Setup 3	Setup 4
pCEZ-NP	0.05	0.05	0.05	
pCEZ-VP35	0.05	0.05	0.05	
pCEZ-NP-2A-VP35				0.05
pCEZ-VP30	0.03	0.03	0.03	0.03
pCEZ-L	0.3	0.3	0.3	0.3
pC-T7pol	0.1			
p3E5E-Luc	0.1			
pHH21-3E5E-Luc		0.025	0.025	0.025

[a] A 48-well tissue culture plate was used.

A.

Signal:background – 88:1
Z' factor = 0.53

Mean = 3.7 x 10⁵   Mean = 4.2 x 10³
SD = 5.7 x 10⁴   SD = 3.5 x 10²

B.

Signal:background -171:1
Z' factor = 0.80

Mean = 2.9 x 10⁵   Mean = 1.7 x 10³
SD = 1.9 x 10⁴   SD = 0.6 x 10²

C.

Signal:background - 3,700:1
Z' factor = 0.52

Mean = 1.1 x 10⁵   Mean = 0.3 x 10²
SD = 1.7 x 10⁴   SD = 0.04 x 10²

D.

Signal:background - 7,000:1
Z' factor = 0.83

Mean = 3.5 x 10⁵   Mean = 0.5 x 10²
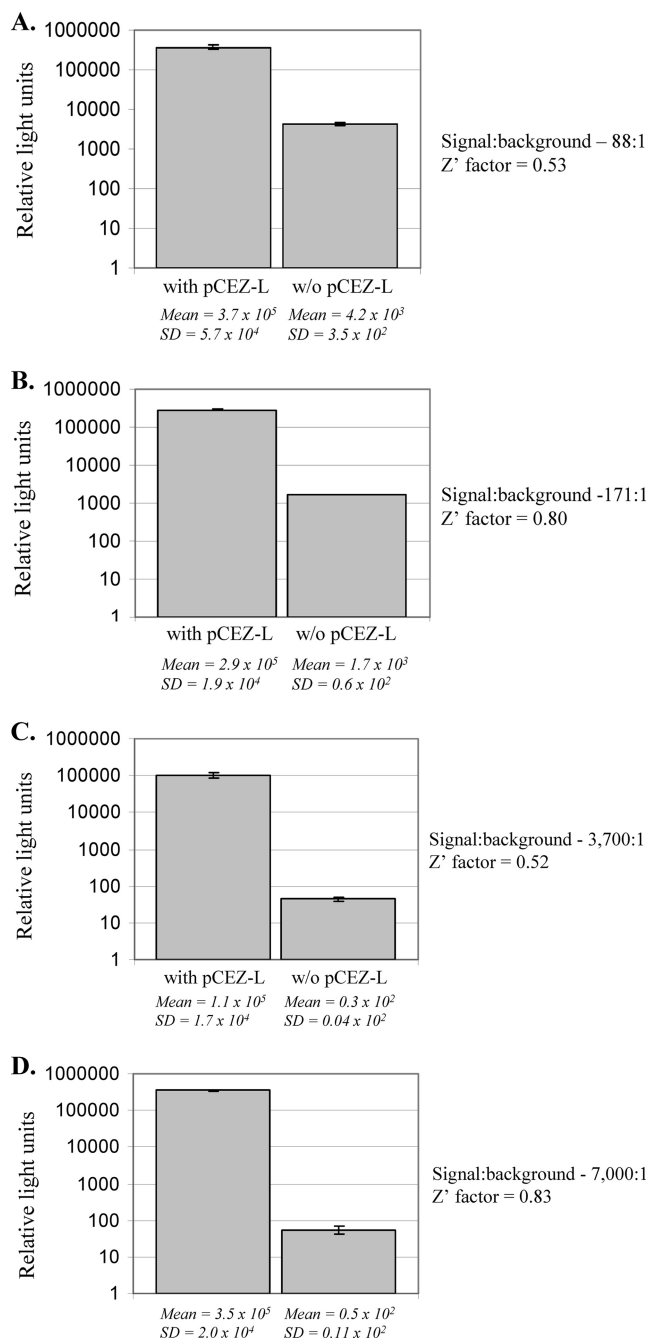*SD = 2.0 x 10⁴   SD = 0.11 x 10²*

FIG. 2. Establishment of *Ebolavirus* minireplicon assays for HTS. Shown are results from four different assay setups. Mean luciferase expression and standard deviations were obtained by measuring luminescence from eight transfection replicates for both the positive (with pCEZ-L) and negative (without pCEZ-L) controls. (A) 293T cells were transfected with a plasmid cocktail consisting of pCEZ-NP, pCEZ-VP35, pCEZ-VP30, pCEZ-L or empty control vector pC-T7, and p3E5E-Luc (see Fig. 1A) as shown for Setup number 1 in Table 1. (B) 293T cells were transfected with a plasmid cocktail consisting of pCEZ-NP, pCEZ-VP35, pCEZ-VP30, pCEZ-L or empty control vector, and pHH21-3E5E-Luc (see Fig. 1B) as shown for Setup number 2 in Table 1. (C) COS-1 cells were transfected with a plasmid cocktail consisting of pCEZ-NP, pCEZ-VP35, pCEZ-VP30, pCEZ-L or empty control vector, and pHH21-3E5E-Luc as shown for Setup number 3 in Table 1. (D) COS-1 cells were transfected with plasmids pCEZ-NP-2A-VP35, pCEZ-VP30, pCEZ-L or empty control vector, and pHH21-3E5E-Luc as shown for Setup number 4 in Table 1.

TABLE 2. Compounds that inhibit reporter gene expression by >75% and reduce cell viability by <25%

Compound	Luciferase inhibition (%)	Reduction in cell viability (%)
Cephaeline dihydrochloride	99	14
Doxorubicin hydrochloride	97	25
Ellipticine	99	8
Emetine dihydrochloride	99	3
Patulin	89	20
Salinomycin	86	17

To further increase the robustness of the screening assay, we attempted to reduce the number of plasmids required for minigenome replication and transcription by constructing a bicistronic vector expressing NP and VP35 (pCEZ-NP-2A-NP) (Fig. 1C). In this construct, a picornavirus-derived 2A-like self-cleaving peptide (5, 6, 17) separates the coding regions for NP and VP35. These viral proteins were chosen because they are required in equivalent amounts for optimal minigenome transcription (22). Next, we performed minireplicon assays with pCEZ-NP-2A-VP35 in place of pCEZ-NP and pCEZ-VP35 in COS-1 cells (Setup number 4 in Table 1). The background-to-noise ratio was 7,000:1 with a Z′ factor of 0.83 (Fig. 2D), indicative of a robust assay that is compatible with HTS requirements.

We transferred our optimized assay to a 96-well format in a pilot study. To simplify the screening protocol, a batch transfection procedure was used: COS-1 cells in suspension were mixed with transfection reagent-DNA complexes and mechanically plated. This protocol provides two advantages: (i) it combines cell plating and transfection into a single step, reducing the impact of pipetting errors, and (ii) it reduces the assay time by 1 day. For the pilot assay, we tested 960 well-characterized compounds from the Prestwick Chemical Known Bioactives library. Compounds suspended in dimethyl sulfoxide (DMSO) (10 μM final concentration/well) were added, and, after incubation for 32 h, luciferase expression was measured. Several wells on each plate received DMSO only (negative controls). To eliminate compounds that reduced *Ebolavirus* replication due to cell cytotoxicity, parallel plates were treated with the test compounds and cell viability was measured using the CellTiter-Glo luminescent cell viability assay (Promega). The percent inhibition of luciferase expression by each compound was calculated as follows: $1 - $ (luciferase signal of compound-treated well/mean luciferase signal of wells treated with DMSO) $\times 100$.

Adapting the assay to the 96-well format impacted its robustness modestly (overall Z′ factor of 0.64). Six compounds were found to inhibit luciferase expression by >75% and reduce cell viability by <25% (Table 2). Cephaeline and emetine are closely related alkaloid components of ipecac (19) (a well-known substance used to induce vomiting), and emetine has been shown to inhibit protein synthesis and promote apoptosis in mammalian cells (3, 9, 13). Emetine appears to be an especially potent inhibitor of translation of viral mRNA in some systems (16). Patulin and salinomycin have antibacterial properties and are also proapoptotic compounds (7, 12, 23). It has been shown that patulin can specifically inhibit replication of a fungal double-stranded RNA (dsRNA) virus (4). Doxorubicin

and ellipticine appear to suppress DNA replication in eukaryotic cells through several mechanisms, including DNA intercalation and inhibition of DNA topoisomerase II activity (8, 25). Doxorubicin can also block Tat-mediated enhancement of HIV long terminal repeat (LTR)-driven transcription by recruiting suppressive nuclear factors to the transactivation response element of the HIV promoter (11). The relative impact of these compounds on COS-1 cell metabolism and architecture versus *Ebolavirus* minigenome replication, as well as live *Ebolavirus* replication, is currently under investigation, and additional compound libraries are being tested.

In conclusion, the *Ebolavirus* minigenome-based assay described here enables rapid screening of compounds for inhibitors of *Ebolavirus* replication and/or transcription.

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