

Introduction

Tropomyosin-related receptor kinase B (TrkB) is a receptor for brain-derived neurotrophic factor (BDNF); its signaling aids in neuronal survival, plasticity, differentiation, and growth via activation of several downstream cascades. Overexpression of a truncated isoform lacking the intracellular kinase domain (TrkB.T1) has been associated with the development and persistence of chronic pain. Published data show that TrkB.T1 knockout in mouse models restores motor function and reduces pain after spinal cord injury. The goal of our project is to identify small molecules that suppress expression of TrkB.T1 as potential therapeutics for chronic pain, focusing on two regulatory mechanisms: (1) the differential post-transcriptional processing of TrkB pre-mRNA, and (2) post-transcriptional regulation of TrkB.T1 expression via the 3' untranslated region (3'UTR) of its mRNA. For the first, we hypothesize that the proportions of the two major TrkB isoforms are principally controlled by cleavage and polyadenylation (pA) site recognition at an upstream (T1) pA site, so drugs inhibiting 3'-processing at that site should suppress TrkB.T1 synthesis. For the second, we hypothesize that the TrkB.T1 mRNA 3'UTR contains regulatory sequences whose functions can be modulated by compounds that manipulate the function, expression, or RNA-binding activities of key trans-acting factors, which can suppress TrkB.T1 production by accelerating decay and/or inhibiting translation of TrkB.T1 mRNA. For each mechanism, we developed independent live cell high throughput screening (HTS) assays to identify small molecules that could (1) block 3'-cleavage and polyadenylation at the TrkB.T1 pA site, or (2) suppress gene expression through the TrkB.T1 mRNA 3'UTR. With these findings, we aim to discover one or more novel drugs capable of suppressing TrkB.T1 expression that can be tested as novel analgesics in mouse models of chronic pain.

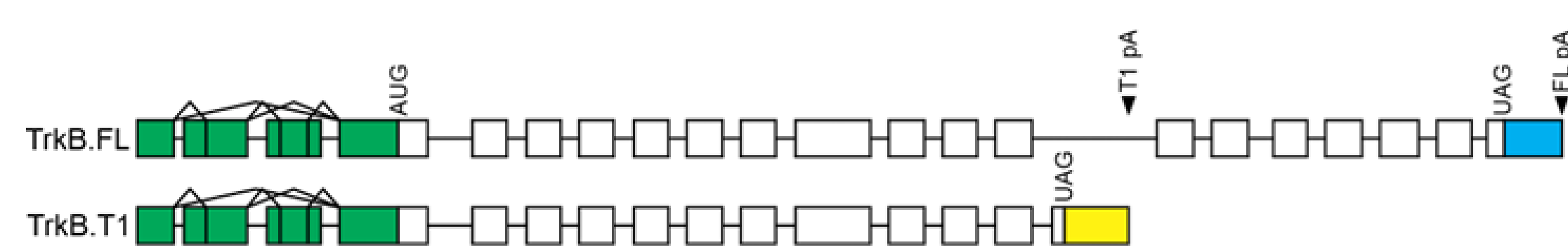


Fig 1. Exon organization of the major trkB mRNA isoforms. Figure adapted from Matyas et al, 2017, *J. Neurosci.*

Method

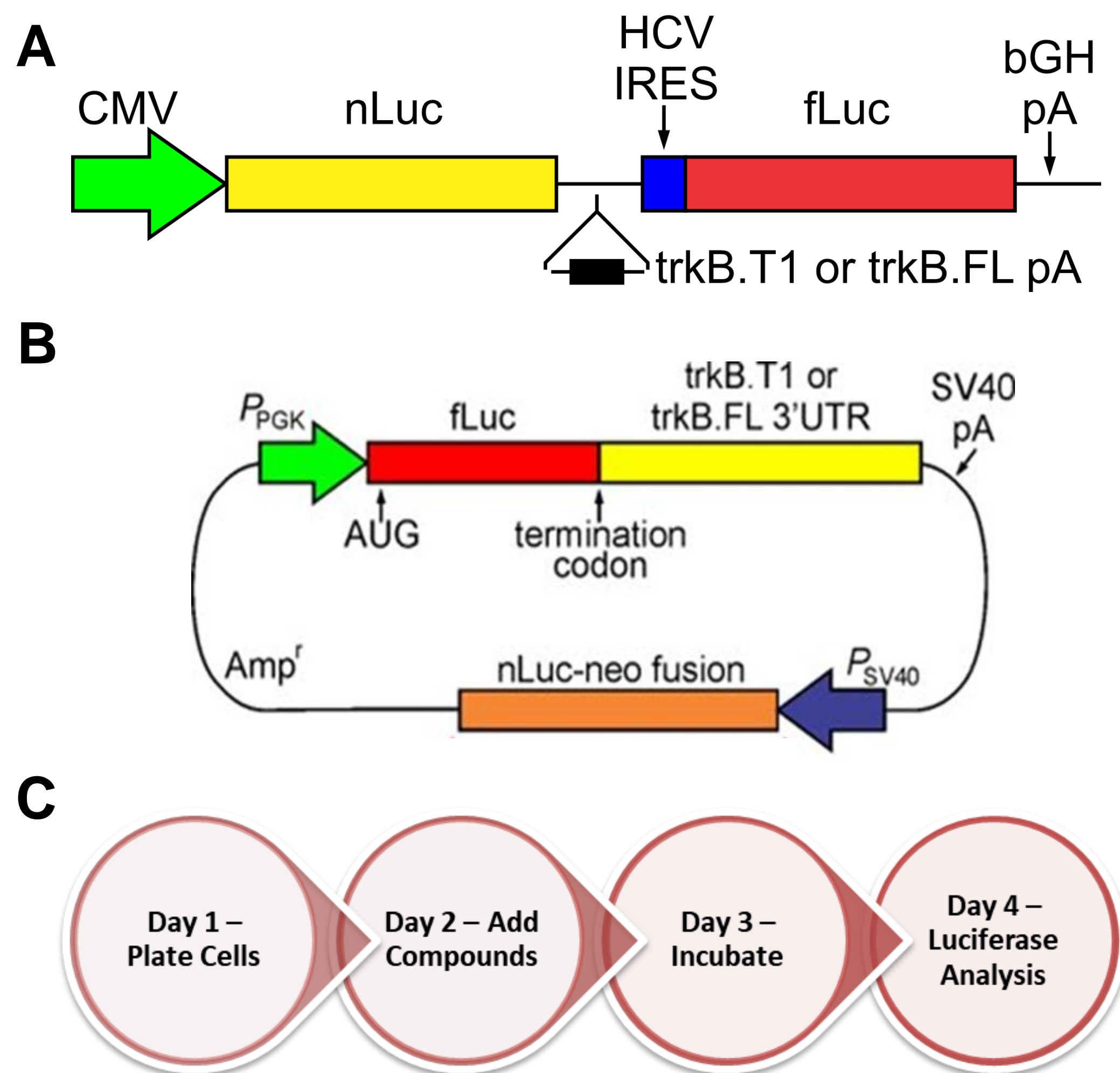


Fig 2. HTS design. (A) TrkB pA site assay reporter construct; (B) TrkB mRNA 3'UTR assay reporter construct; (C) HTS workflow.

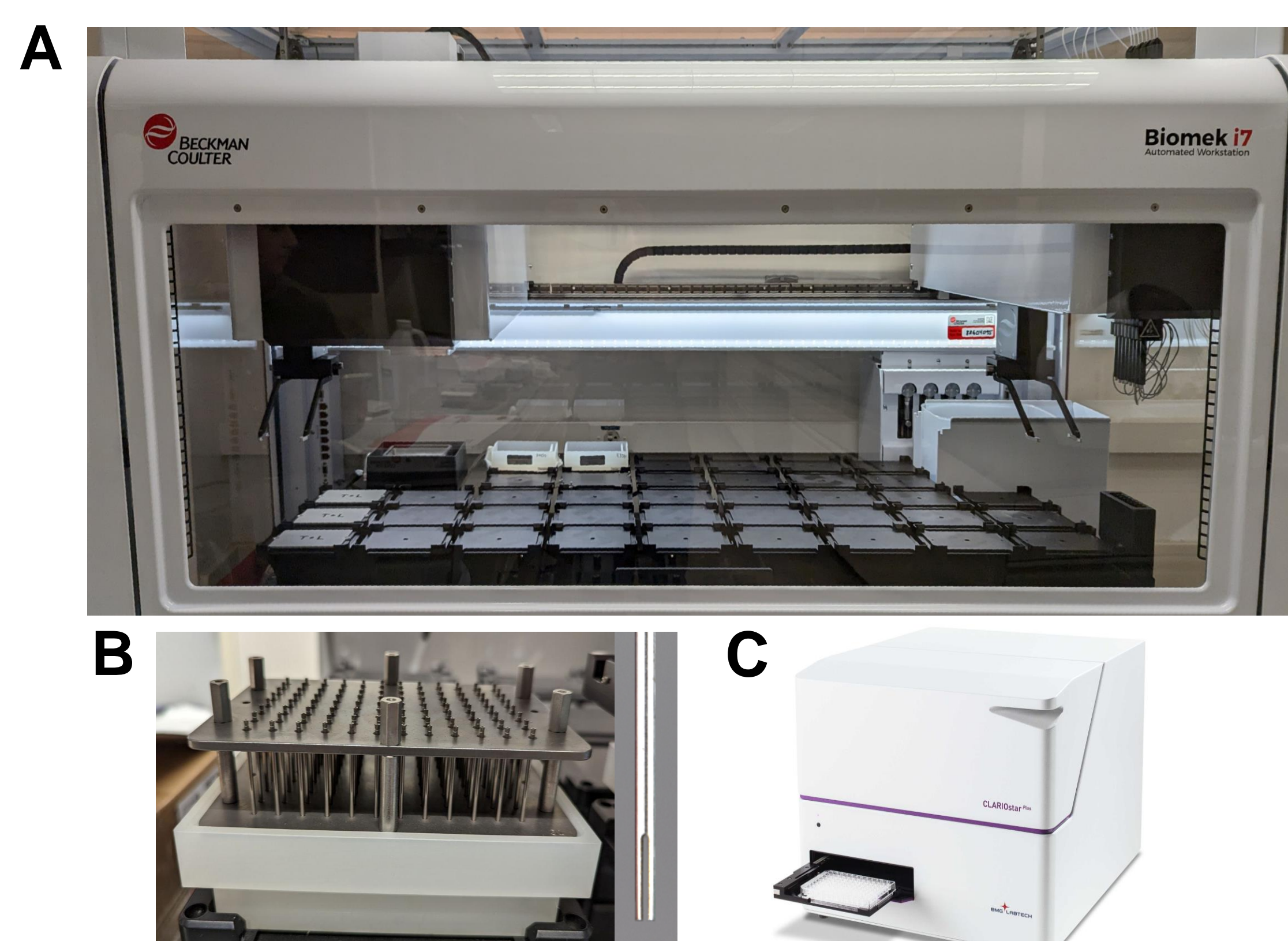


Fig 3. Instruments used for HTS. (A) Biomek i7 from Beckman Coulter, (B) 96 pintool from V&P Scientific, (C) ClarioStar Plus from BMG.

Results

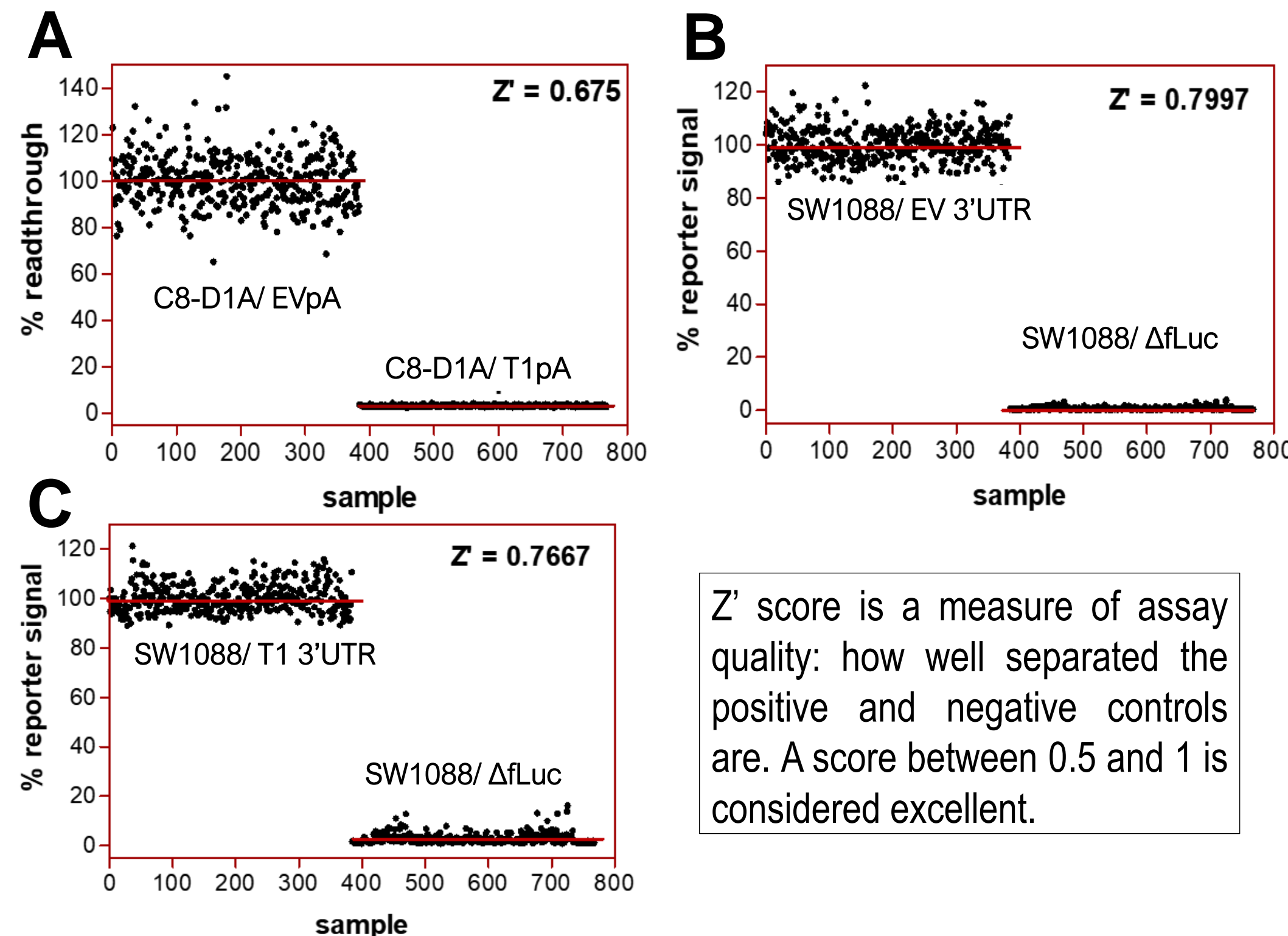


Fig 4. Z' estimates of (A) pA assay, (B) empty vector 3'UTR assay, and (C) TrkB.T1 mRNA 3'UTR assay reporter cell lines, in the absence of compounds.

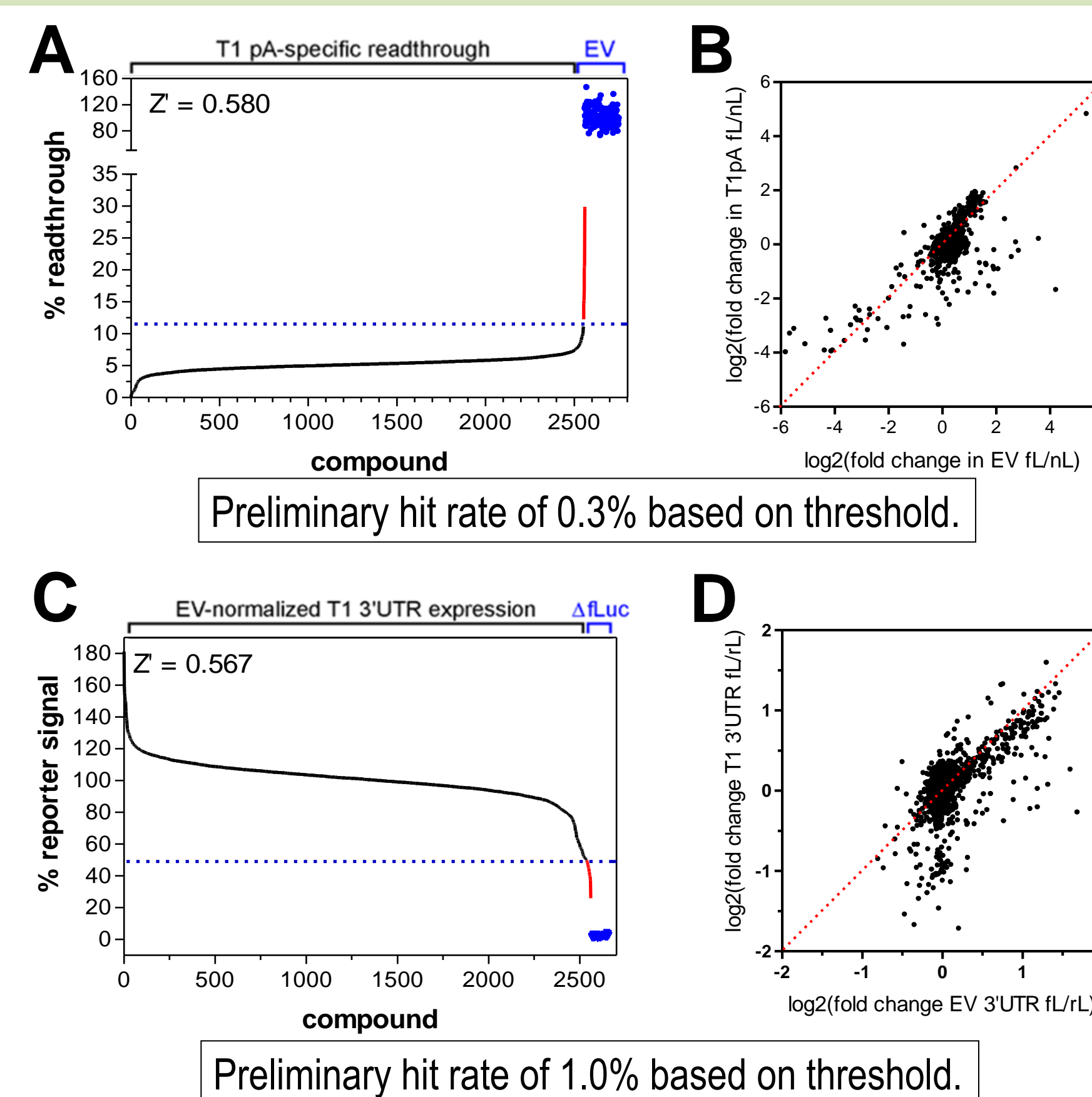


Fig 5. HTS in 96-well format using the Spectrum Collection compound library from MicroSource. (A) pA assay hits plot, (B) correlation plot of drug-dependent effects on T1pA and EVpA Luc ratios, (C) T1 3'UTR assay hits plot, (D) correlation plot of T1 3'UTR and EV 3'UTR.

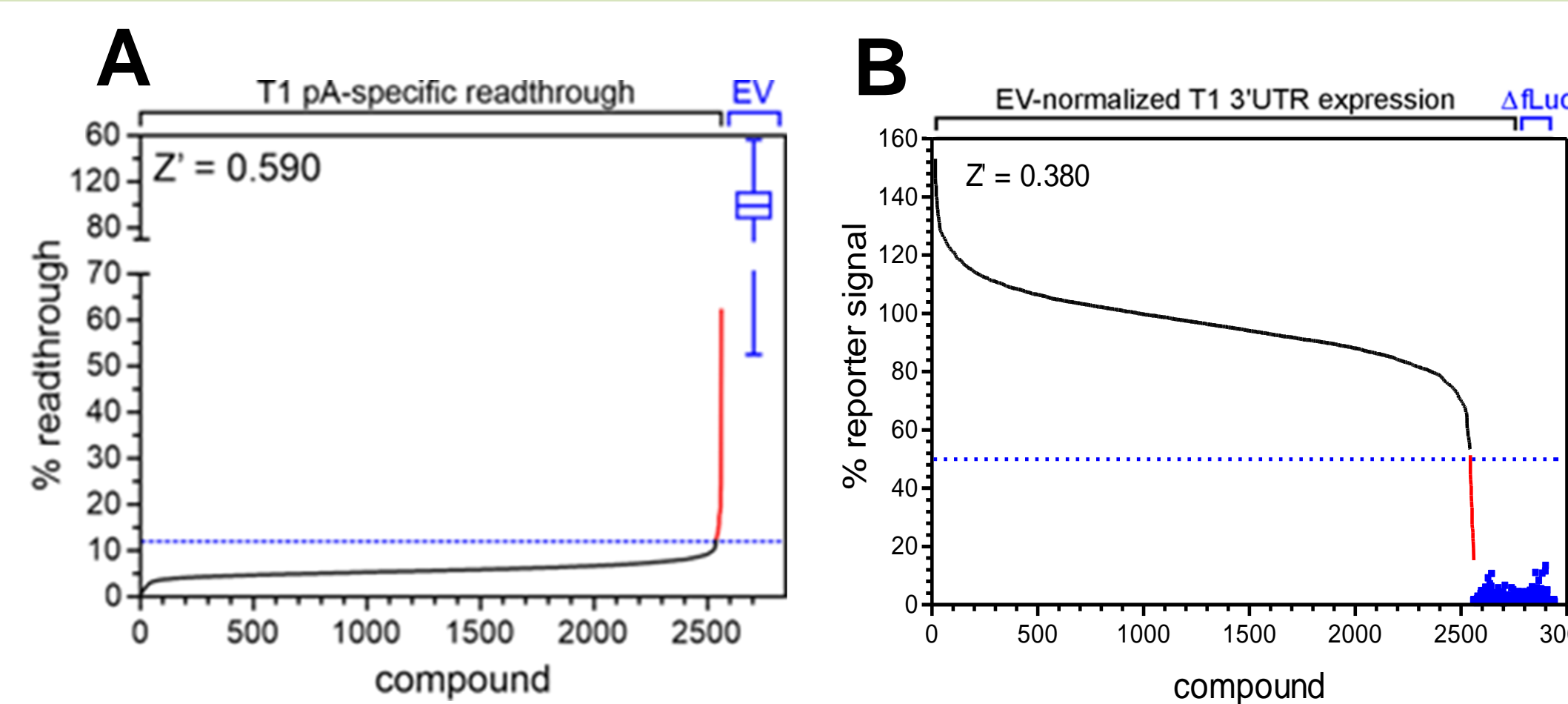


Fig 6. HTS in 384-well format using the Spectrum Collection compound library from MicroSource. (A) pA assay hits plot; (B) T1 3'UTR assay plot.

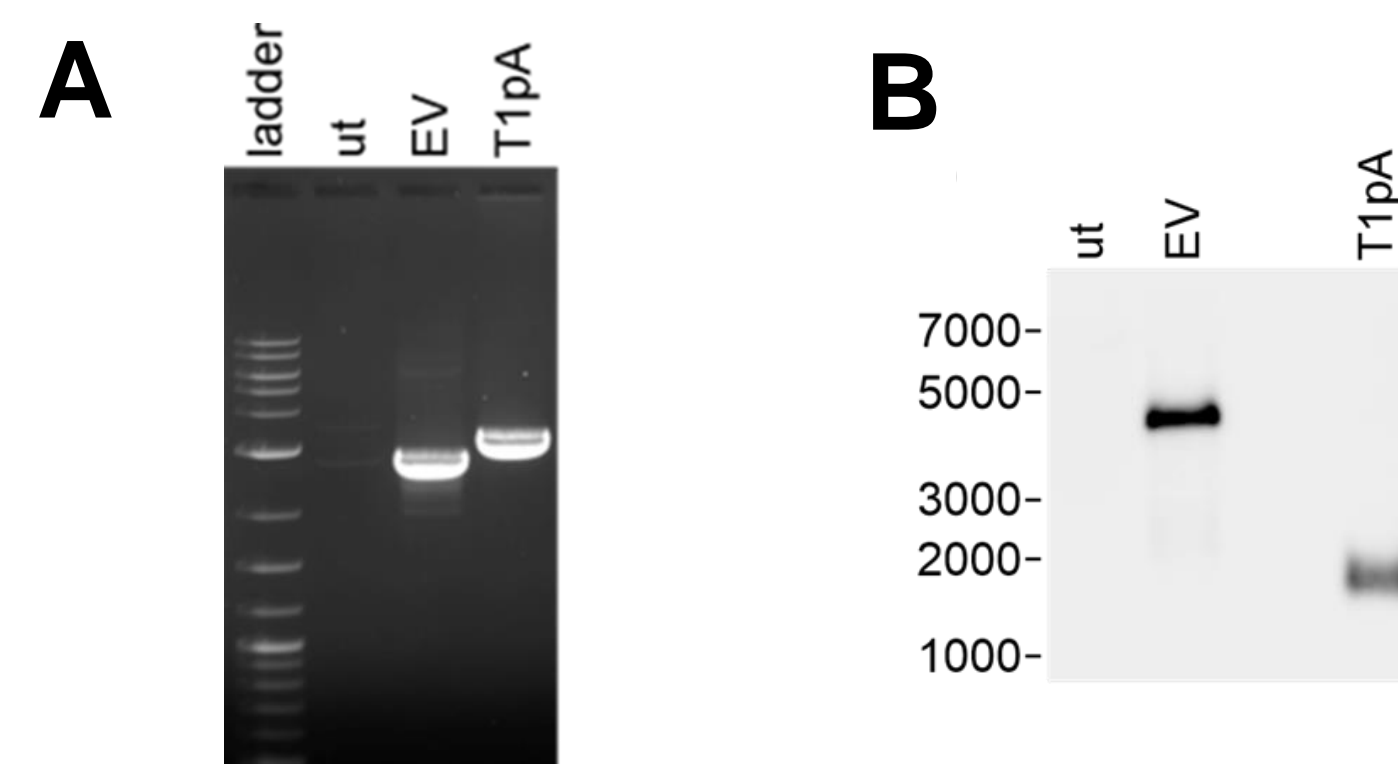
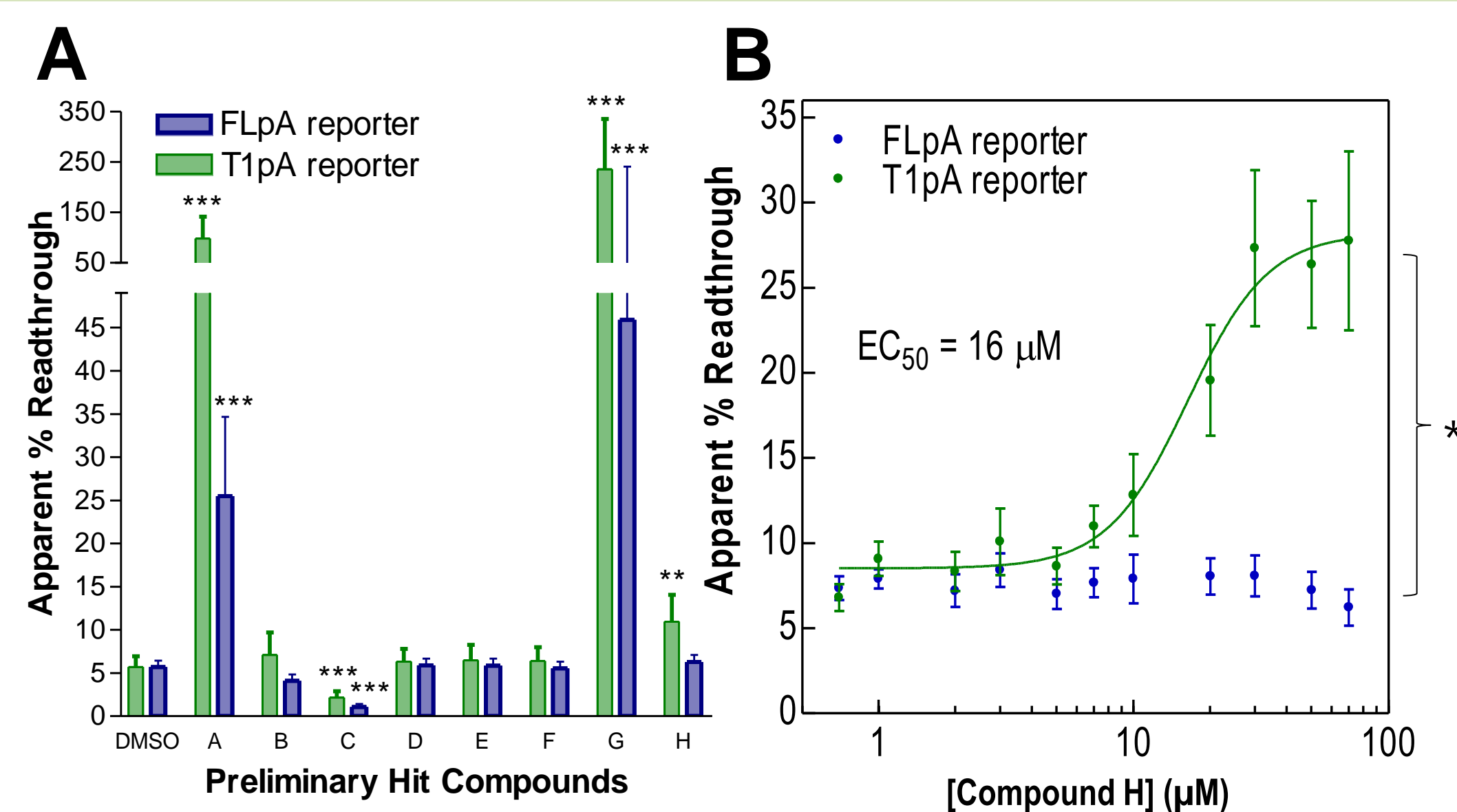


Fig 7. Validation of pA reporter construct by (A) PCR amplification of transgene sequences between the CMV promoter and bGH pA region and (B) northern blot analysis of total RNA from C8-D1A clonal cell lines, nLuc probe.



All data are presented as mean \pm SD, statistical significance from two-tailed unpaired Student's *t* test, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$ vs DMSO.

Fig 8. Preliminary hit validation. (A) HTS validation of preliminary hits at 10µM in 96-well format looking at both T1pA and FLpA reporter signal (n=6), (B) drug response curve of compound H (0.02-50 µM) looking at both T1pA and FLpA reporter signal (n=4).

Results

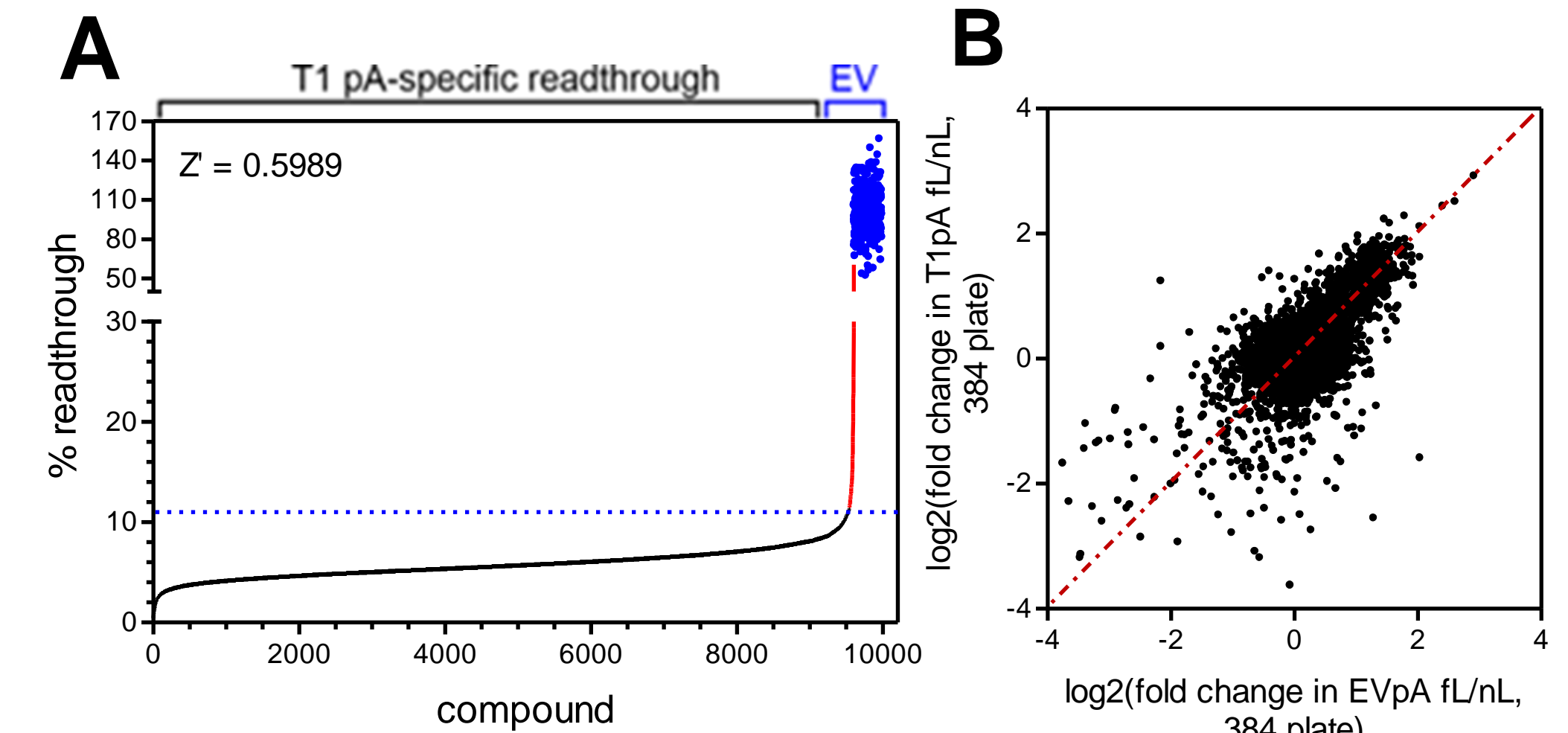


Fig 9. HTS of pA assay in 384-well format using the 100k Diverse Compound Library from ChemDiv. Figure represents the analysis of the first 9.6k compounds.

Conclusions

- Vector-independent drug effects on luciferase expression/activity necessitate parallel screens of experimental and control cell lines.
- The polyA reporter assay is sufficiently robust in both 96 and 384-well format drug screens.
- Compound H appears to selectively block 3'-cleavage and polyadenylation at the TrkB.T1 pA site.
- The current 3'UTR reporter construct (fLuc/nLuc) is not sensitive enough to give $Z' > 0.5$ in 384-well format in true experimental conditions.

Future Directions

- Develop new 3'UTR reporter cell lines using an nLuc/fLuc system and repeat the Spectrum Collection screen in 384-well format to interrogate sensitivity.
- Continue the HTS of the 100k Diverse Compound Library from ChemDiv and interrogate the current preliminary hits, using the pipeline in Figure 10.
- Identify mechanism(s) regulating 3'-cleavage and polyadenylation of TrkB isoforms using validated hits obtained from the polyA assay.
- Characterize mechanism(s) of action for trkB.T1-suppressing compounds using validated hit compounds obtained from the 3'UTR assay.

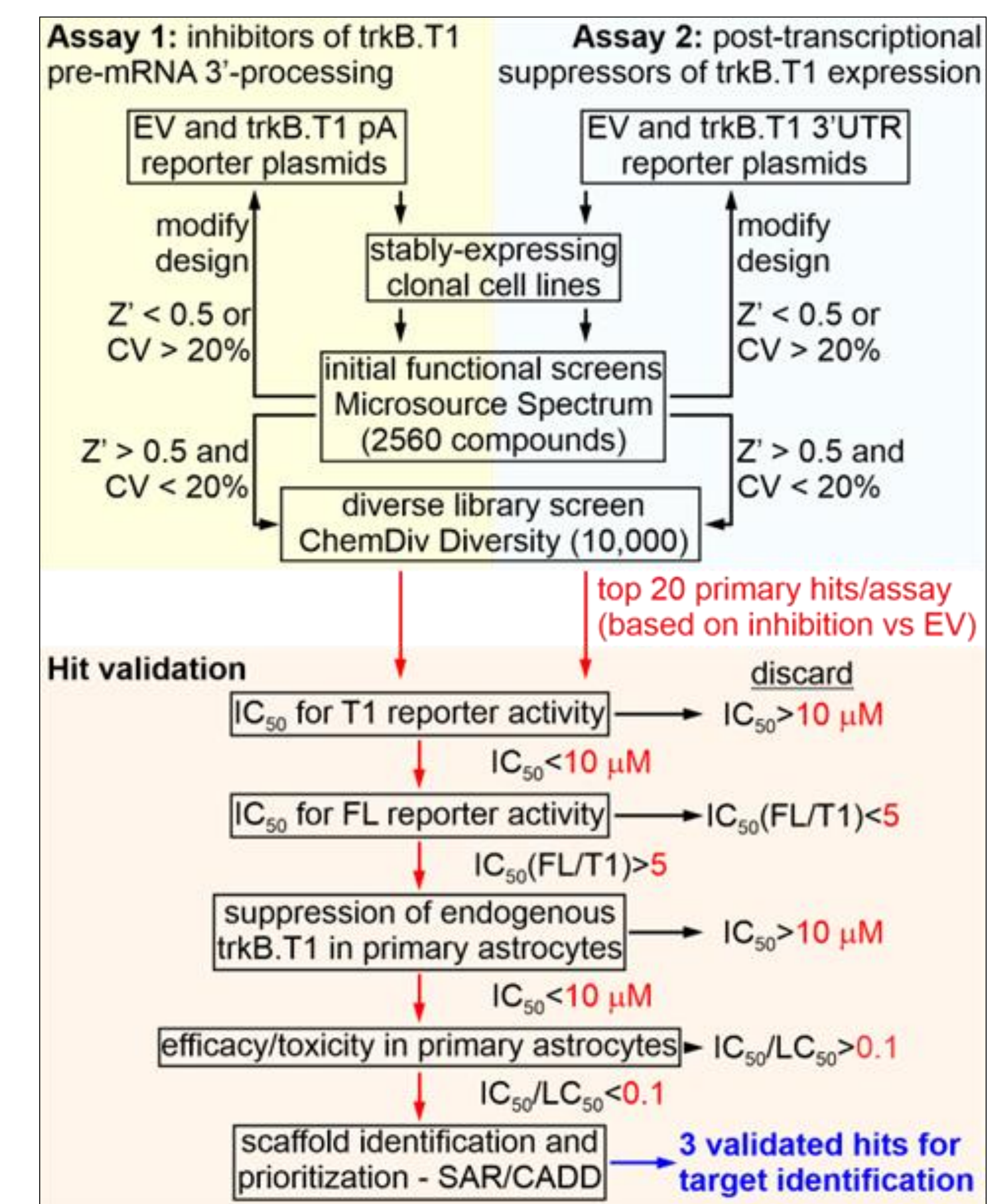


Fig 10. Long-term drug screen workflow.

References

1. Renn CL, Leitch CC, Dorsey SG. In vivo evidence that truncated trkB.T1 participates in nociception. *Mol Pain*. 2009;5:61.
2. Matyas JJ, O'Driscoll CM, Yu L, et al. Truncated trkB.T1-mediated astrocyte dysfunction contributes to impaired motor function and neuropathic pain after spinal cord injury. *The Journal of Neuroscience*. 2017;37:3956-3971.
3. Yamashita A, Takeuchi O. Translational control of mRNAs by 3'-untranslated region binding proteins. *BMB Rep*. 2017;50:194-200.
4. Klein R, Conway D, Parada LF, Barbacid M. The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell*. 1990;61(4):647-656.
5. Mayr C. Regulation by 3'-untranslated regions. *Annu Rev Genet*. 2017;51:171-194.

Acknowledgements

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