Testing RRL-NSD3-Short-3xFLAG-IRES-Puro for Stable Expression in H1299 Cells

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Overview. In this experiment, I tested a lentiviral construct for NSD3-Short-3xFLAG expression. This vector contains an EF1a promoter driving NSD3-3xFLAG-IRES-Puro_Resistance bicistronic transcript (RRL). Also included is an empty vector control and eGFP expression in the same backbone, however lacking the ires-puro resistance sequence.

1. Experimental Details

1.1. Transfection of HEK293-T Cells

1. 24 hours prior to transfection 2 x 10^6 HEK293T cells were plated in 10 cm dishes.
2. Following day cells were transfected with lentiviral construct and packaging vectors using jetPrime (Polyplus - Cat # 114-15), following manufacture's instructions. Briefly, DNA added to jetPrime Buffer, then vortexed 10 sec. Add jetprime, vortexed 10 sec. Incubated for 10 min at RT, then added transfection complexes to cells.
3. Cells were incubated overnight and the following morning HEPES (pH 7.5) was added to 10 mM to ensure pH was maintained.
4. 48 hours following transfection media was collected and the HEK293T cells bleached and discarded.
5. Media containing virus was then spun at 1500 x G for 10 min at 4^o C to clear cell debris and transferred to a new falcon tube.

Transfection Mix for 2x 10 cm Dish

Lenti Plasmid	Concentration - Volume (10ug)	Lenti Packaging (10ug)	jetPrime Buffer	jetPrime
EmptyVector	458 ng/ul - 22 uL	10 ul	1 mL	40 uL
NSD3-3xFlag	508 ng/ul - 19.7 uL	10 ul	1 mL	40 uL
eGFP	505 ng/ul - 19.8 uL	10 ul	1 mL	40 uL

Lenti Packaging Mix 1:1:1 - 1ug/ul (Provided by Dalia)
pMDG - Envelope - Addgene #12259
REV pRSV - Rev - Addgene #12253
RRE pMDLg - Gag & Pol - Addgene #12251

1.2 PEG Concentration of Virus

5x PEG Solution
* 20% PEG8000
* 1.2 M NaCl
* 1 mM EDTA pH 8
-Dissolve in water, and filter 0.2 /*mu*m.

1. PEG solution was added to clarified media to 1x (5 ml to 20 ml viral media)
2. Virus was incubated overnight at 4 ^{o} C.
3. Following day, samples were centrifuged at 1500 x G for 30 min to pellet PEG/viral particles.
4. The supernatant was poured off, then spun again for 5 min and any remaining sup pippetted off.
5. Viral pellet was than resuspended in 2 ml cold 1x PBS, with a portion being used directly to transduce H1299 cells and the remainder stored at -80^o C.

1.3 Transduction of H1299 Cells

To transduce cells for stable I selection, I followed the protocol outlined by addgene and found here https://www.addgene.org/protocols/generating-stable-cell-lines/. I've briefly outlined the protocol below;

1. Prepare media (RPMI - 10% FBS + Pen/Strep) containing 2μg/mL polybrene.
2. The 10x virus prep was diluted into polybrene containing media at the following dilutions and added (500 μ/L per 6 well dish). The dilutions take into account concentration step.

Dilution Setup:

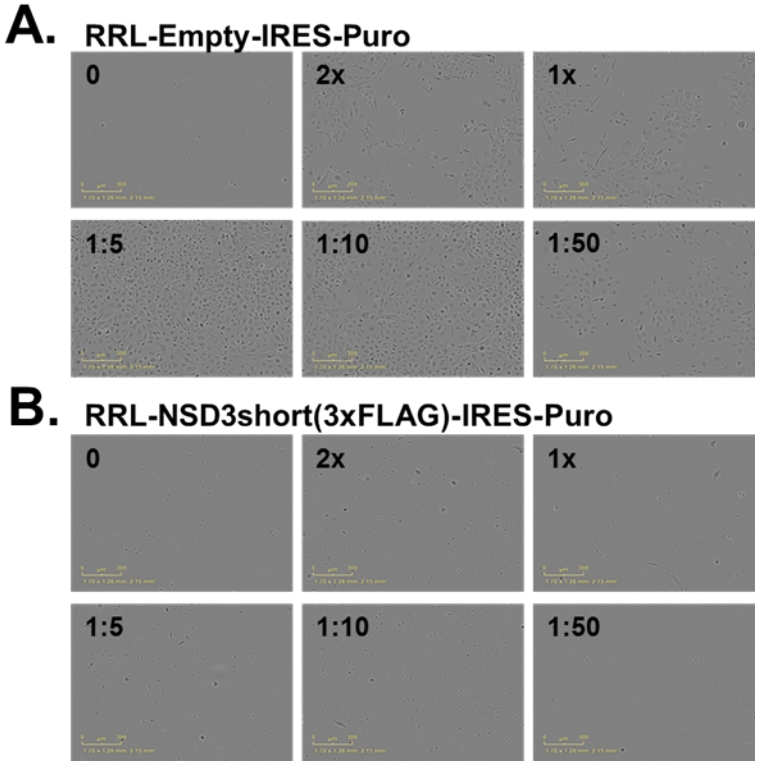
Final Dilution	Volume 10x Virus	Volume Media (uL)
0	0	500
2x	300	200
1x	150	350
1:5	30	470
1:10	15	485
1:50	3	497

3. To reverse transduce H1299 cells, H1299 cells were trypsinized, counted and resuspended at 100 000 cells /ml in polybrene containing media (2 μ/g/mL).
4. 1 mL of cell suspension was added to each well containing diluted virus and incubated overnight.
5. The following morning cells were washed in 1x warmed PBS and fresh media added.
6. Approximately 48 hrs following transfection, media was changed and fresh media containing 2 μ/g/mL puromycin (Sigma) was added, with the exception GFP transduced cells.
7. 72 hours after transduction. GFP transduced cells were harvested, washed several times in 1x PBS and run on a MACSQuant® VYB (Miltenyi Biotec) flow cytometer to assess GFP expression. Puromycin cells were imaged several days after puromycin treatment using the IncuCyte system. Results shown below.

2. Result

Table showing GFP expression levels in H1299 cells transduced with RRL-eGFP virus.

Dilution	Percent GFP-Positive
0	0.38
2x	85.86
1x	85.27
1:5	70.42
1:10	56.10
1:50	25.64



3. Observations

The NSD3 lentiviral constructs I have designed do not appear to be generating infectious particles. From my GFP and empty vector controls, I can assume this is caused by the construct itself and not my protocol for producing lentiviral particles in HEK293T cells. The RRL-NSD3-IRES-Puro construct is 10 488bp, which may be exceeding the packaging limit of our virus, typically less than 10kb. To address this I have looked into several ways to decrease the size without losing functionality. One solution may be to change the bicistronic element that allows expression of NSD3 and the puromycin resistance marker from the same promoter. If I replace the IRES sequence (~500) with a P2A self-cleaving site the plasmid size will be reduced to 9896 bp, hopefully improving titre.

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