Constructing Lentiviral NSD3-Short-3xFLAG Constructs to Transduce AML Cell Lines

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Objective. To engineer an NSD3short-3xFLAG lentiviral expression vector for generating stable transgene expressing acute myeloid leukemia (AML)-derived cell lines.

Rationale. There is currently a lack of high quality antibodies to endogenous NSD3. FLAG-tagged NSD3 constructs will be useful for performing co-immunoprecipitation and ChIP experiments to validate previous findings regarding NSD3-short's association with enhancers and promoter regions.

Experimental Details

Cloning Strategy. The NSD3short gene was PCR amplified, adding a 5' BamHI site and 3' 3x-FLAGtag/SalI site, from a mammalian CMV-driven NSD3 expression plasmid generously provided by a collaborator. The PCR fragment was cloned into a lentiviral vector modified from pRRLSIN.cPPT.PGK-GFP.WPRE, a gift from Didier Trono (Addgene plasmid # 12252). The plasmid was modified by D. Barsyte to replace the CMV promoter with an EF-1a promoter and introduce an IRES PuroR cassette downstream of the MCS for bicistronic expression of a gene of interest and a selection marker for puromycin. The plasmid map is shown below (Figure 1).

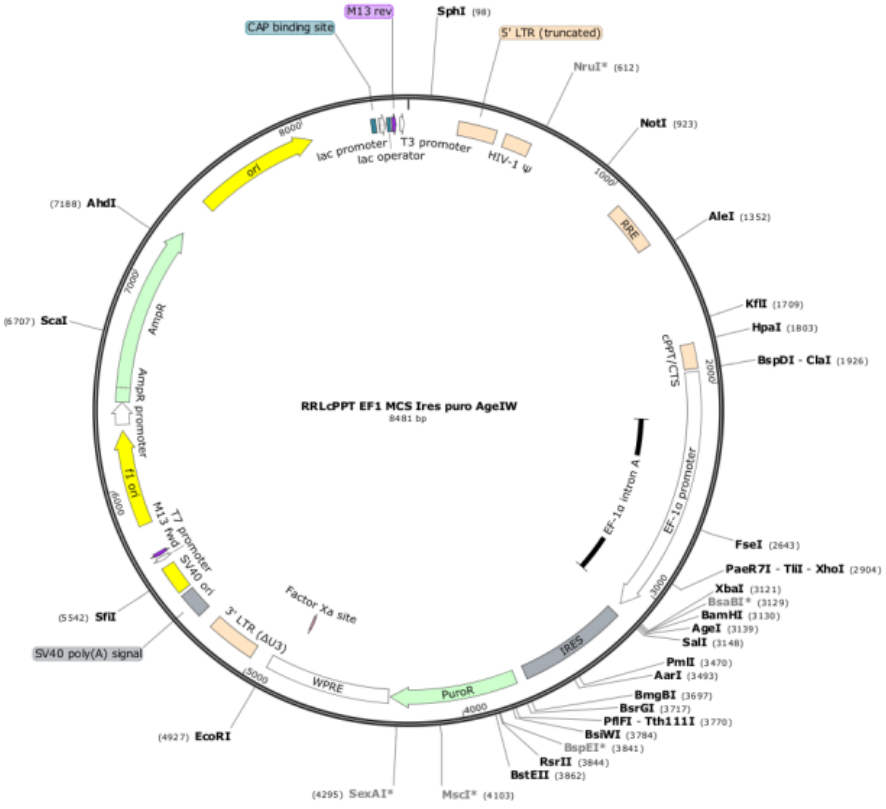

Figure 1: *- RRLcPPT EF1 MCS Ires puro AgeIW map.*

1. PCR

PCR reaction to generate 5'BamHI-NSD3short-3xFLAG-SalI 3' fragments for cloning using NEB Q5 High-Fidelity DNA Polymerase (M0491) following manufacturer's instruction.

Primers

Primer	Sequence	Tm (Celsius)
BamHI_NSD3_Fwd	TTCGGATCCATGGATTTCTCTTTCTCTTTCATGCAAGG	73
SalI_FLAG_Rev	TAAGTCGACTCACTTGTCATCGTCATCCTTGTAATCGATATCATGATCTTTATAAT CACCGTCATGGTCTTTGTAATCGCCTCCGTCCACAGTTTCCTCAATCGCTGCGG	83

Oligo Melting Temp Calculated with NEB Tm Calculator v1.9.7

Cycling Conditions

1. Initial Denaturation
   95°C - 60s
2. First Amplification - Low Temp - 5x
   98°C - 10s
   68°C - 20s
   72°C - 2min
3. Second Amplification - High Temp - 25x
   98°C - 10s
   72°C - 2min
4. Final Extension
   72°C - 2min

0.5 µL Dpn1 (NEB - R0176) added to PCR reaction and incubated for an additional 5 min at 37 °C to clear template DNA, then PCR purified (MACHEREY-NAGEL - cat740609). Product was run on a 1% gel (Figure 2), matching the expected size of ~2000 kb.

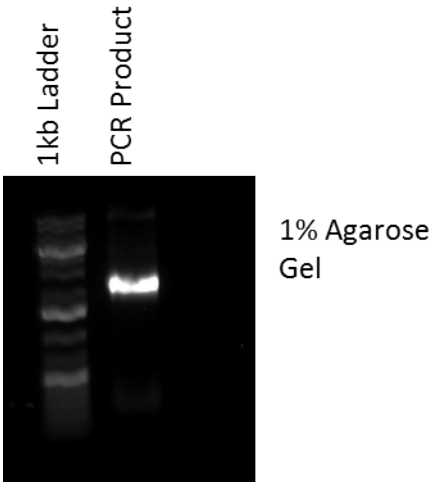

Figure 2: *PCR Prodcut Run Agrose Gel. Ladder = GeneRuler 1kb (Thermo Scientific - SM0312)*

2. Digest

Reaction Setup:
* 20 µL DNA (1 µg)
* 1.25 µL BamHI-HF (NEB - R3136)
* 1.25 µL SalI-HF (NEB - R3138)
* 2.5 µL 10x NEB Smart Cut

Incubate 1 hr at 37 ºC, add 1 µL CIP phostaptase (NEB - M0290) to cut vector and incubate for an additional 5 min at 37 ºC. Cut vector and insert then PCR purified (MACHEREY-NAGEL - cat740609) and run on a 1% agarose gel to verify (Figure 3).

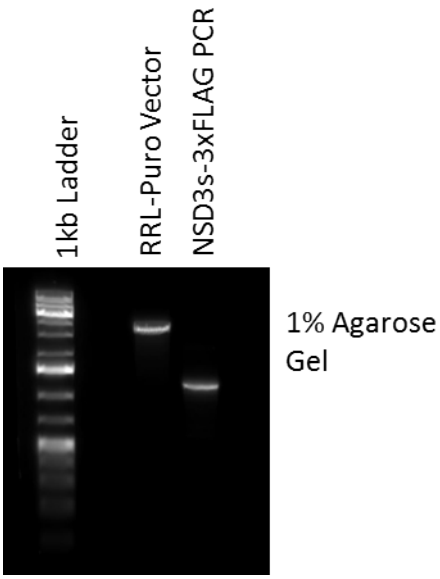

Figure 3: *BamHI-SalI Digested Vector and Insert. Ladder = GeneRuler 1kb (Thermo Scientific - SM0312)*

3. Ligation

10 µL Ligation Reaction Setup:

	3:1 Insert to Vector	No Insert CNTRL	No Vector CNTRL
50 ng Vector	6	6	-
35 ng Insert	1.75	-	1.75
Buffer	1	1	1
Ligase	1	1	1
dH2O	0.25	2	6.25

- Incubate ligation reaction for 10 min at room-temperature.
- Transform 5 µL in 50 µL of DH5a competent cells (Thermo Fisher - cat18265017)
- The following morning several clones were selected, grown in 5 mL overnight cultures, plasmids purified using QIAprep Spin Miniprep Kit (Qiagen - Cat#27104), and screend by diagnostic restriction digest with BamHI & SalI.
- Putative constructs were sent for sequencing.

4. Sequencing

Samples were sent for sequencing at ACGT Corporation, Toronto, Ontario. Several constructs contained the correct sequence. Sequencing runs of the selected clone are shown below.

Primer Name: EF1aFwd
Primer Sequence: TCAAGCCTCAGACAGTGGTTC

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##
GtgatactagatTCGGATCCatGGatTTcTctTTCtCTTTCATGCAAGGGaTCAtGGGAAACACAATTCAGC
AACCACCTCAACTCATTGACTCCGCCAACATCCGTCaGGAGGATGCCTTTGATAACAACAGtGACNT
TGCTGAAgATGGtGGCCAgACaCCATATGAAGcTACTTTGCAGCAAGGcTTtCAGtACCCAGCTACA
ACAgAAgATCTTCCTCCACTCACAAatGGGtATCCaTCATCAATCAGtGtGNATGAAACTCAAACCAAA
TACCAGTCATATAATCAGtATCCTAAtGGGTCAGCCAAtGGcTTTGGtGCAGTTAgAAACTTTAGCCCC
ACTGACTATtATCATTCAgAAATTCCAAACACAAgACCACATGAAATTCTGGAAAAACCTTCCCCTCC
ACAGCCACCACCTCCTCCTTCGGtACCACAAACTGTGATTCCAAAgAAgACTGGcTCACCTGAAATT
AAACTAAAAATAACCAAAACTATCCAgAAtGGCAGGGAATTGTTTGAGTCTTCCCTTTGTGGAGACC
TTTTAAATGAAGTACAGGCAAGTGAGCACACGAAATCAAAGCATGAAAGCAGAAAAGAAAAGAGG
AAAAAAAGCAACAAGCATGACTCATCAAGATCTGAAGAGCGCAAGTCACACAAAATCCCCAAATTA
GAACCAGAGGAACAAAATAGACCAAATGAGAGGGTTGACACTGTATCAgAAAAACCAAGGGAAgA
AcCAGTACTAAAAGAGGAAGCCCCAGTTCAgCcAtACTATCTTCTGtTCCAACAACGGAAGTGTCCA
CtgtGtTAAGTTTCaGgtgG
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Primer Name: IRESR
Primer Sequence: CCTCACATTGCCAAAAGACG

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##
atagACAACGCAcaCCGGCctTatTCcAaGcGGTTCGGccagtAACgtTAGGGAGAGGGGcGGAATTTA
CGGTCGACTCACTTGTCATCGTCATCCTTGTAATCGATATCATGATCTTTATAATCACCGTCATGGTCT
TTGTAATCGCCTCCGTCCACAGTTTCCTCAATCGCTGCGGAGACGGAGCTGTCACTGAATCTGACA
GAGCCCTGCACTCCCCGGTCCGCCGACCCTTTCTGTAATCCAGTCTTCACTGTAGCCTGAGGAACT
GTTTCAACCTGCTCCTTTTTGATCTTCTTCTTTGGCACAACCTCAGTGGATTTCTCTGATTCAGAACG
AGTTCTAATTGATCTTCTCTGTTGCTTCTTTTCTACTGAGCCTGTAGAACCAGATGTTGCTTCAGGAG
ATGATACACTCTGCGTTGGCTTTTCATTTCTCTGGTTTGGTGTAGAAATTATAAGCCTGTCTTGCCCC
CTGACACTTATTTCTGTTTTGTTACCAATTCCCTTTGTTGAATAAACAAATTGATCGATAAATTTCCCA
TCCCCTGTAGCATTCTGAAGAGCAAACACTTGTTCAATTTTCACAACTGgAGACATGTTACACTTCTG
CAAATCCAGGCTCCCTTTGTGCATCGTAATGgAAGCTGGTAAGGAtTTCCTTGCTGCCGCAGtTTTC
CAgGcTATTTTaACaGGCGGtGGctCTTCCTCTTCCGCACTTGtctGCcgCcTCTGGcTATgtcTcCgAAtT
TCAgtActTGAgAg
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