Making Ultrasensitive Endogenous NanoLuc® Luciferase Reporter Cell Lines Using CRISPR

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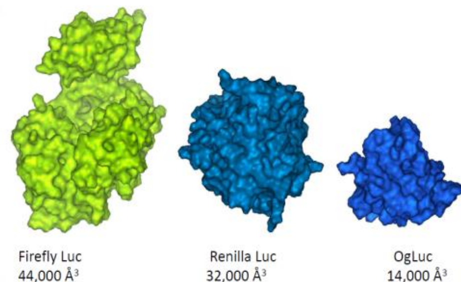
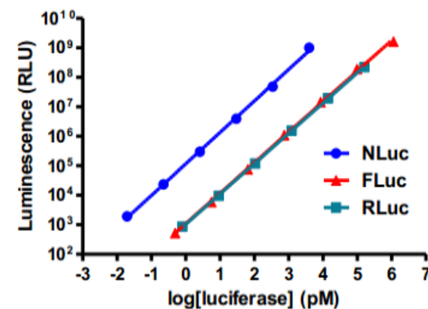
NanoLuc® Reporter Lines: Technology Background

- NanoLuc® derived from a new luciferase from Deep Ocean Shrimp (Oplophorus)
- Serially mutated to create a monomeric & 150-fold brighter luciferase vs FF and Renilla
- Secreted or intracellular; ATP independent light emission with novel substrates
- **Able to detect gene/protein levels at very low endogenous expression levels**
- Endogenous high-throughput screening now possible





150 fold increased specific activity
compared with firefly or Renilla luciferase

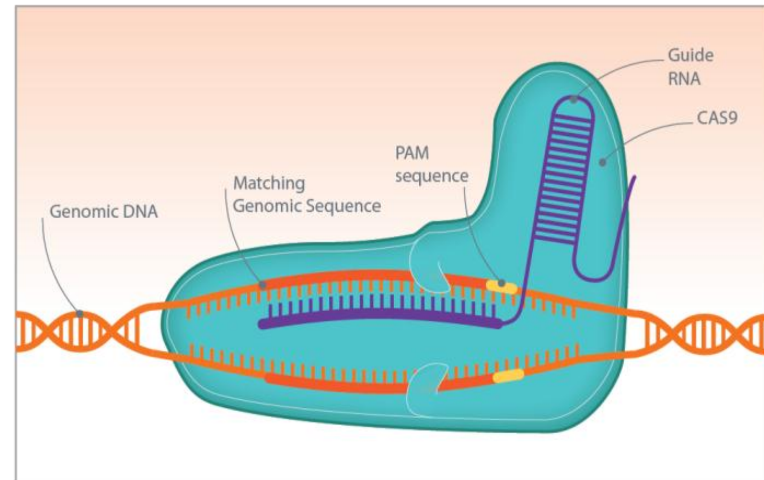
Image of live cells expressing NLuc

Captured with a handheld iPhone

Better biology ... Endogenous HTS ... Biologically relevant results

CRISPR/Cas9 system

- RNA-guided platform to introduce either a double strand DNA break or a single strand nick at a specified location in the genome.
- 2 Principal Components
- Cas 9 protein
  - Cas9wt = double-strand break
  - Cas9n = single strand “nickase”
- Guide RNA (gRNA)
  - crRNA + trRNA



Targeting CRISPR

- Target choice only requires a PAM sequence (NGG) to design a gRNA

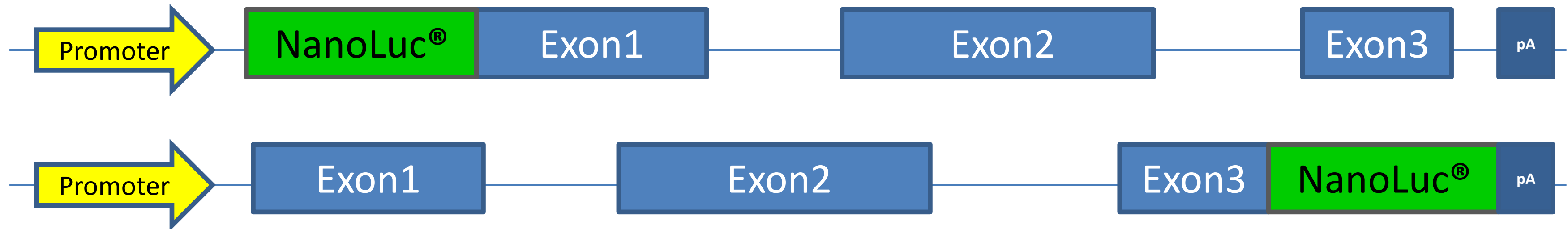
gRNA target sequence	PAM
AGCTGGGATCAACTATAGCG	**CGG**

This 23bp sequence must be present in the genome, but the gRNA to be used only contains the 20bp "target sequence"

The target sequence is appended to a fixed "scaffold" that together comprise a complete gRNA.

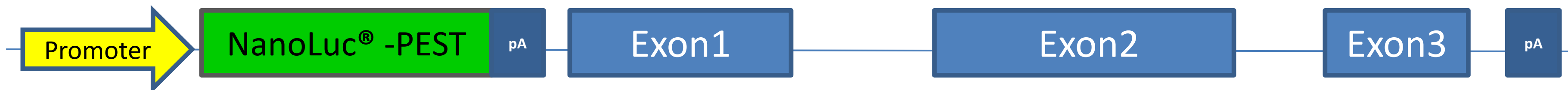
NanoLuc® Tagging to Generate a Reporter

➢ Protein expression reporter – NanoLuc® fused to a gene of interest



- Engineered to generate N- or C-terminal fusion of NanoLuc® to the endogenous protein of interest
- Ideal for use in protein expression reporter assays
- Choice of 5’ or 3’ should be based on prediction of least disruption to activity

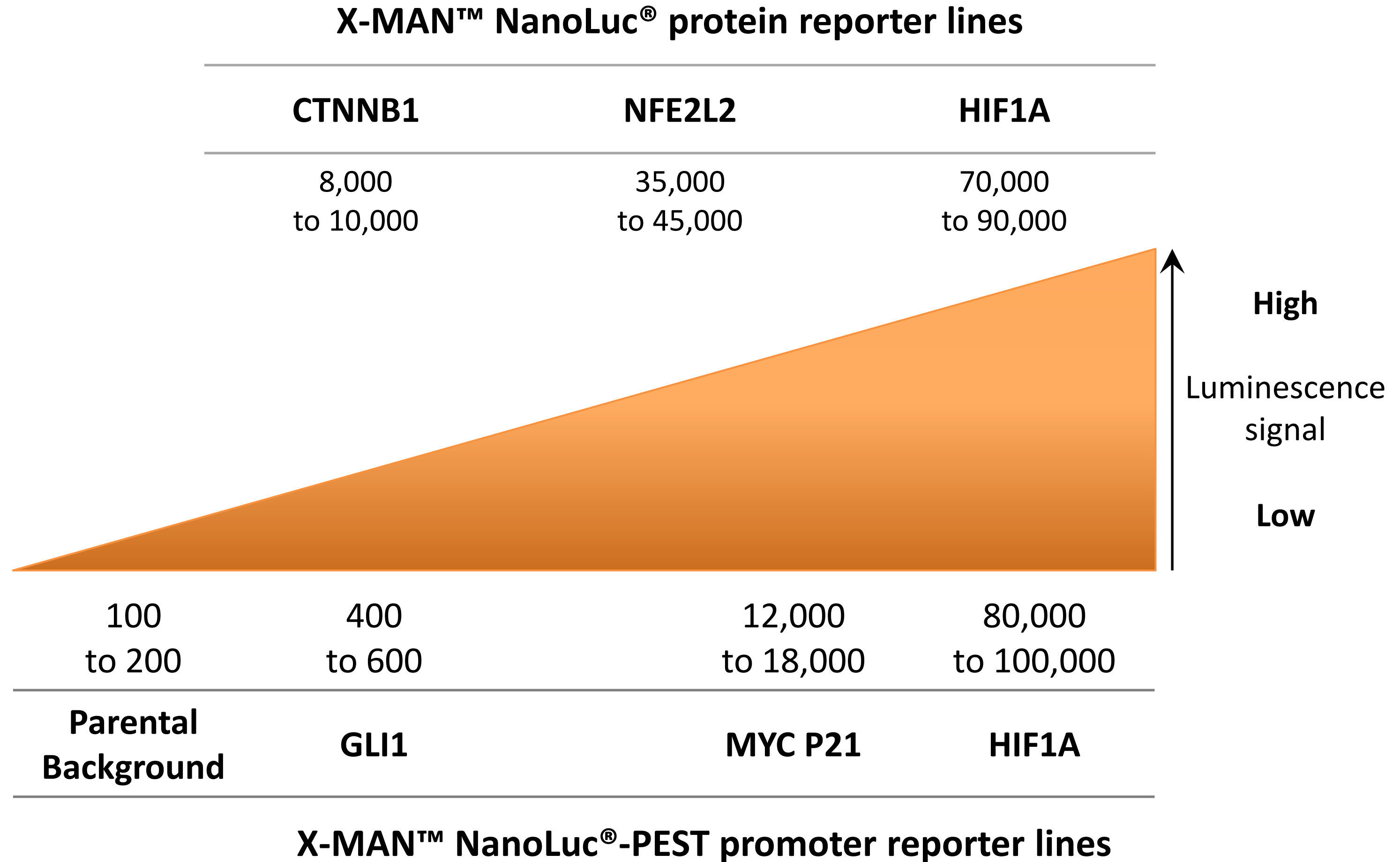
➢ Promoter reporter – NanoLuc® linked directly to a gene’s promoter



- Promoter fusions with PEST-NanoLuc® for rapid turnover and thus dynamic signal kinetics
- Short intracellular lifetime

NanoLuc® : Signal Range

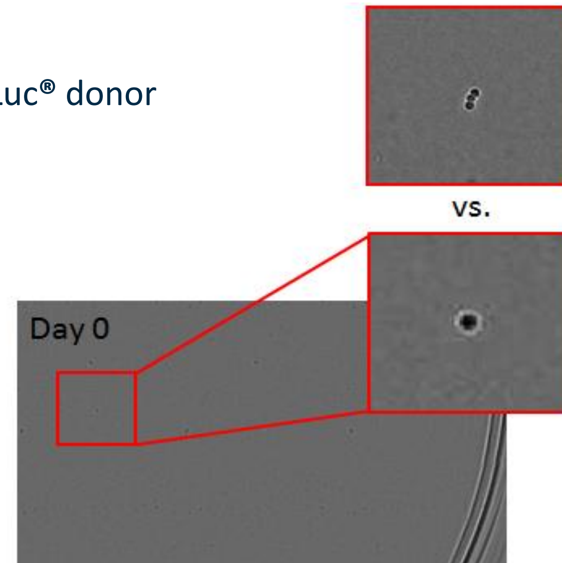
- Baseline luminescence signal will be specific for each NanoLuc® reporter line, dependent on gene or protein expression levels



Cell Line Considerations – Suitability of Your Cell Line

- Does it transfect/electroporate well?
  - Need to deliver plasmids encoding Cas9+gRNA and the NanoLuc® donor
  - Viral delivery possible, but can be complicating

- Can the cells be single-cell diluted and recover?



- What is the doubling time for single-cell clone recovery?

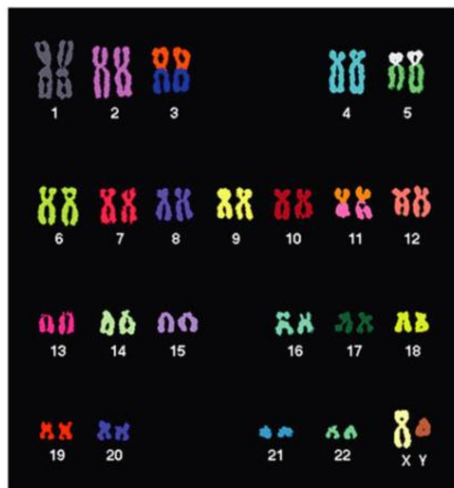
	4000	2000	1000	500	250	125	63	31	16	8	4	2	1	0.5	
RPMI	1.333	1.333	1.111	1.083	0.867	0.667	0.667	0.375	0.208	0.208	0.042	0	0	0	x3
SensiCell (SC)	2	2	2	2	1.933	1.722	1.476	1.25	0.917	0.708	0.542	0.125	0.048	0	x3
plus hESCS (hS)	1.333	1.667	1.333	0.917	0.8	0.722	0.238	0.25	0.125	0.125	0.042	0	0.048	0	x3
30% CoMed	1.5	2	1.5	0.875	0.7	0.5	0.214	0.188	0	0.063	0	0	0	0	x2
50% CoMed	1	1	1	1	0.8	0.333	0.143	0	0	0.125	0	0	0	0	
SenCell + hESCS	0	0.5	0.667	0.75	0	0	0	0	0	0	0	0	0	0	
25%CM + hESCS	0	0.5	1	1.25	1.2	0.667	0.286	0.125	0.125	0	0	0	0	0	
SC ,30% CM + hS	0	0.5	0.667	0.75	0.6	0.167	0	0	0	0	0	0	0	0	
SuperMed1	2	2	2	2	2	1.833	1.714	1.5	1.375	1.25	0.375	0.375	0	0	
plus NEAA	2	2	1.667	1.75	1.6	1	0.714	0.125	0	0	0	0	0	0	
2x GlutaMax	2	2	2	1.75	1.4	0.833	0.429	0	0	0	0	0	0	0	
Transferrin	2	2	2	2	2	2	1.571	1.5	1.5	0.75	0.375	0	0.714	0.167	
Hydrocortisone	2	2	2	2	1.8	1.667	1.222	1.5	1.125	0.375	0.75	0.25	0	0	
hEGF	2	2	2	2	2	1.833	1.857	1.625	1.375	1.125	0.75	0.75	0	0.167	
Insulin	2	2	2	2	2	2	1.857	1.25	1	0.375	0.25	0	0.143	0	
Liothyronine	2	2	2	2	2	1.833	1.571	1.375	1.25	1	0.5	0.25	0.286	0	

Range	Rating
2 – 1.5	Good
1.5 – 0.5	Poor
0.5 – 0	No growth

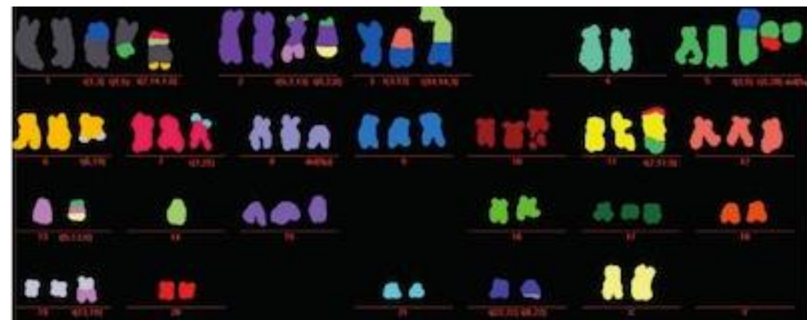
gRNA Design Considerations

- What sequence source are you using?
  - A single basepair mismatch can drastically affect efficiency
- What is the best guide sequence?
  - Guides have a range of cutting activity
- How close is the guide to the desired mutation?
  - Distance of the cut from the site of insertion affects efficiency
- What are the potential off-target considerations?
  - Does the guide have the potential to disrupt important regions?

Normal human karyotype

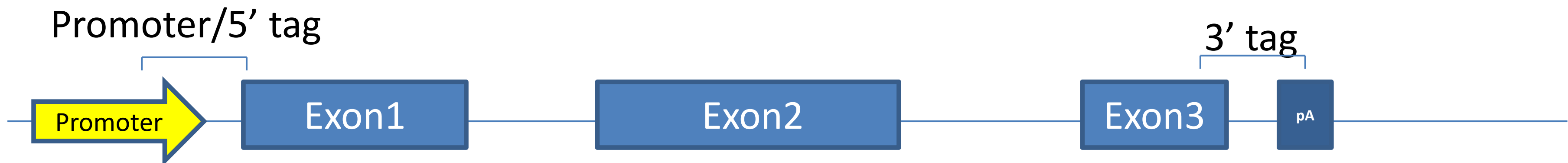


Hela cell karyotype



Targeting a Cut Near the Insertion Site

- Search a region of approximately 250bp surrounding the intended insertion site for suitable gRNA designs



- Keep in mind the gRNA site itself will need to be modified in the final cell line, so avoid essential elements (coding or regulatory)
  - Downstream of the 5' target is best
  - Downstream <u>or</u> upstream of the 3' target works equally well

gRNA Design

- Several sources of guide RNA design software tools are available
  - Horizon uses its own design tool developed in collaboration with Desktop Genetics
  - http://crispr.mit.edu/ is a publicly available site for gRNA design

Candidates

	No	Score	Strand	Sequence	PAM
☑	4	98	Top	AGCCACCGGAGCACTCCATA	AGG
☑	1	97	Top	GGGTGCATAAGTTCTCTAGT	AGG
☑	5	96	Bottom	TGCCTTATGGAGTGCTCCGG	TGG
☑	2	94	Top	GGTGCATAAGTTCTCTAGTA	GGG
☑	11	91	Top	GCCAGGAAGAAACCACCGGA	AGG

Mismatches

Sequence	PAM	Score	Mism.	Locus	Gene
AGCCACCGGAGCACTCCATA	AGG	100	None	chr4:+74606261	Yes
TGCCACCTGAGCACACCATA	CAG	1	6,13,20	chr3:+41589597	No
TGCCACGGGAGCACTCCAAA	AGG	1	2,14,20	chr15:+78095143	No
AGCAACCGGAGAACTCCAAA	AAG	1	2,9,17	chr20:+45150512	No
AGCCACCTGAGAACTCCCTA	AAG	0	3,9,13	chr3:-183906869	No

Cas9 wild-type or Cas9 nickase?

➢ Cas9 wild-type

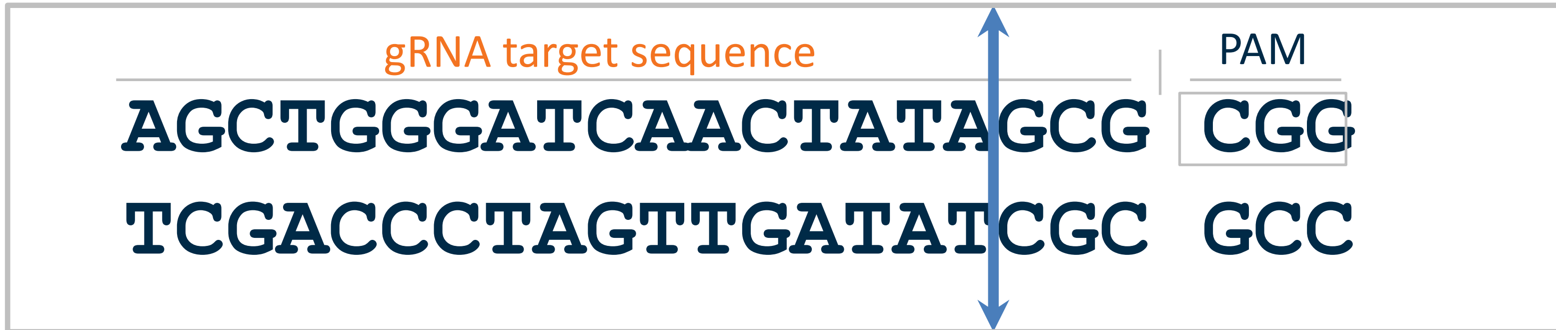
- Induces a double-strand break
- Only requires a single gRNA
- High efficiency of cleavage
- Especially good for random indels (= KO)
- Concerns about off-target specificity

➢ Cas9 nickase (D10A)

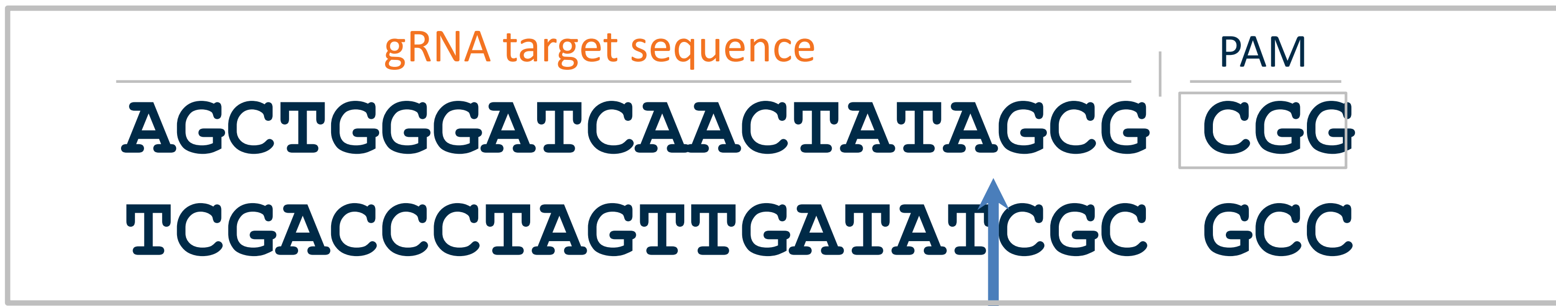
- Only “nicks” one strand
- Requires two guide RNAs for reasonable activity
- Guide efficiency dictated by efficiency of the weakest gRNA
- Reduced likelihood of off-target events

Designing a guide RNA

- Cas9 wild-type: The cut site occurs 3 bp 5’ of the PAM sequence



- Cas9 nickase: the single strand nick occurs on the opposite strand (with D10A)



- Data suggests that nicks that provide a 5’ overhang are most efficient at being modified

Designing a guide RNA

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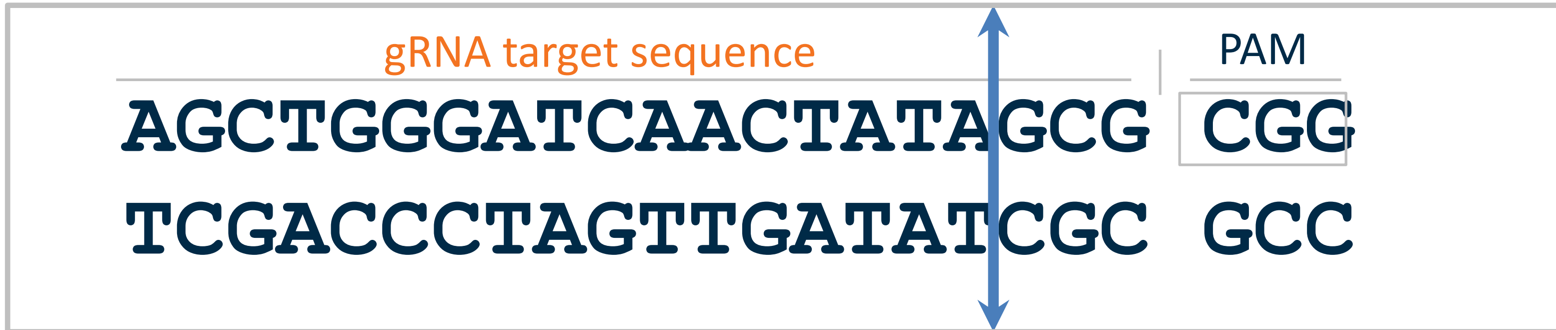

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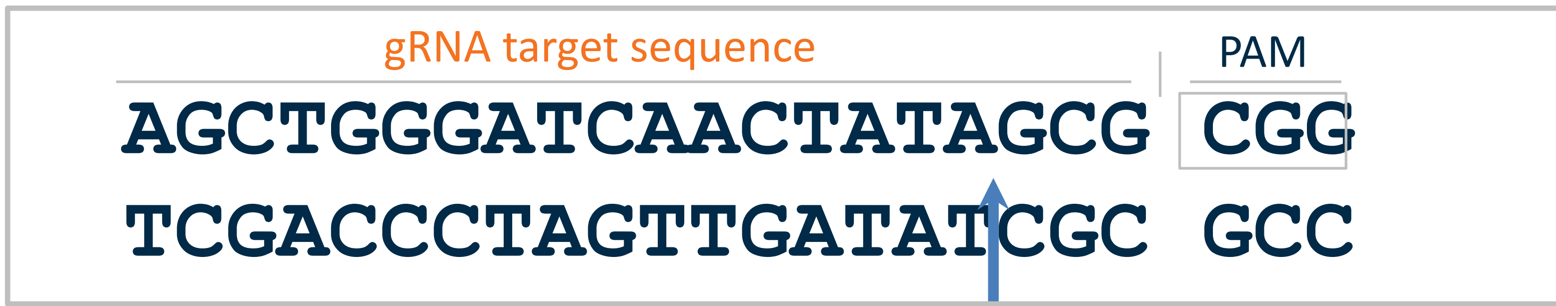

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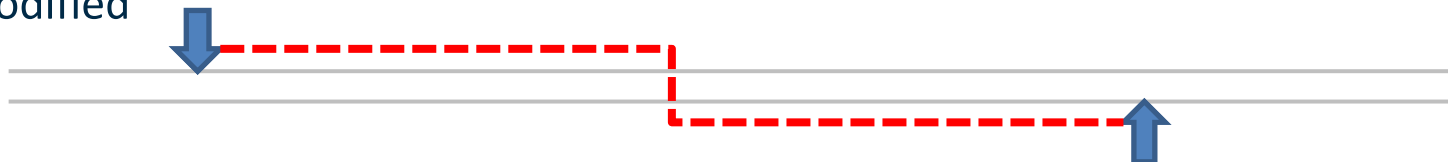
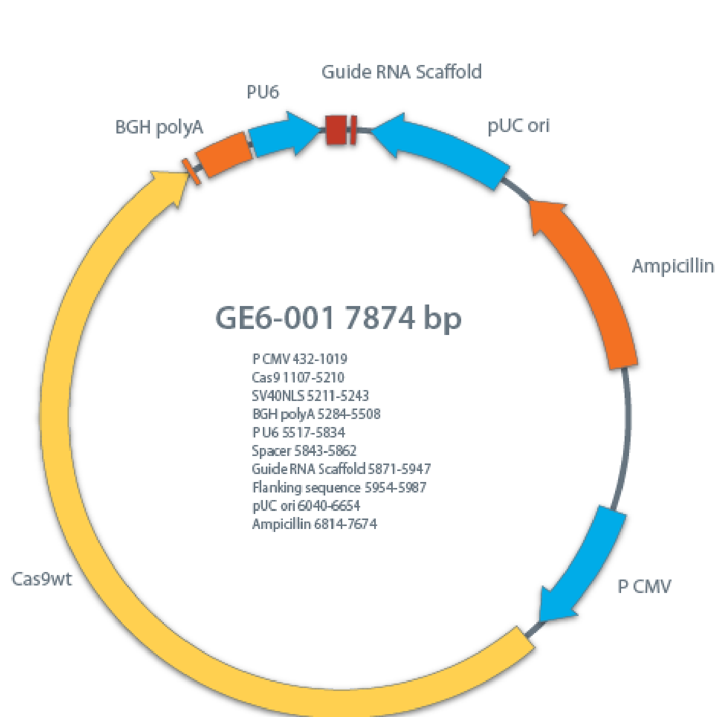
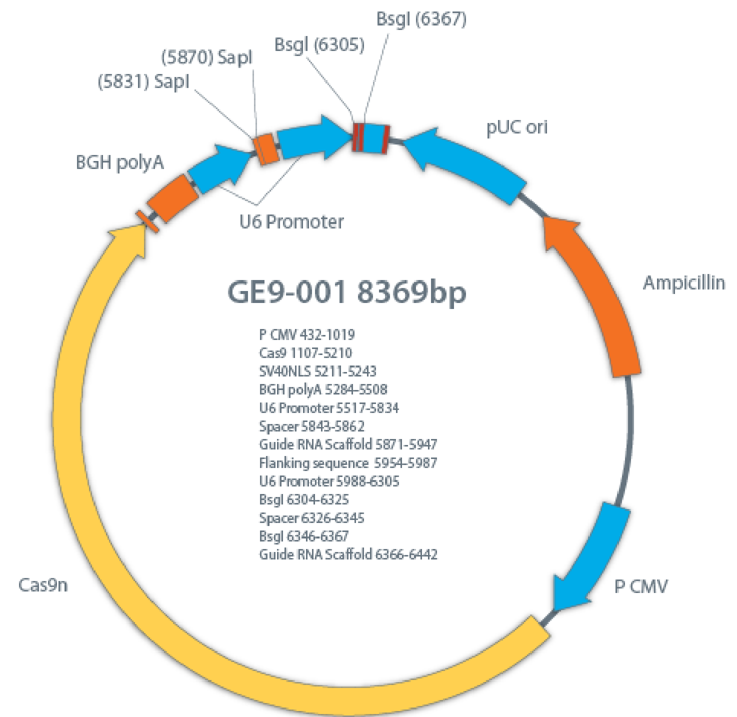


Cloning gRNA(s) into Vectors

- Use an appropriate vector for your guide choice (Cas9wt or Cas9n)
  - U6 drives RNA expression of the guides
  - CMV drives protein expression of Cas9



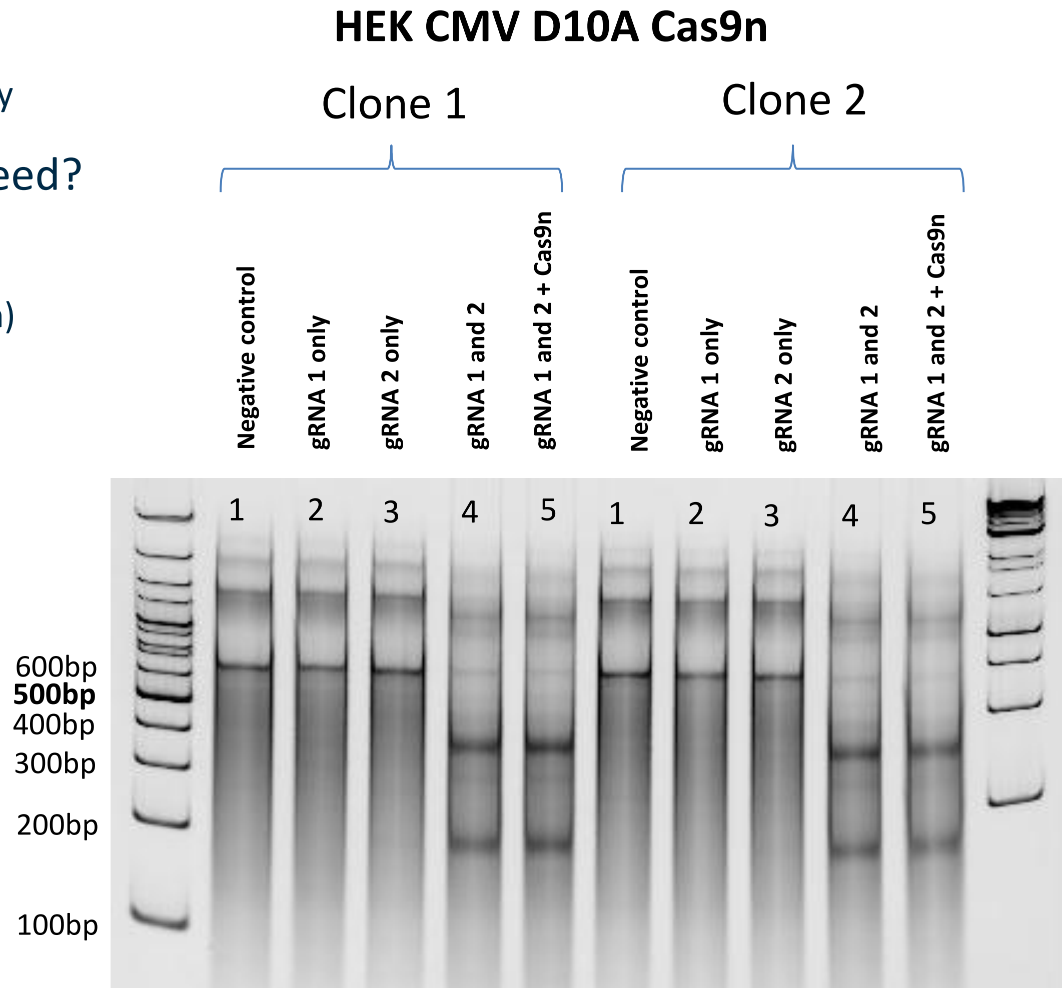


gRNA Activity

➢ How many gRNAs need to be tested?
- Test at least 5

➢ How will you assess activity?
- Surveyor Assay
- T7E1 Assay
- Restriction Enzyme (dropout) assay

➢ What activity level do you need?
- The highest possible, but...
- Distance matters
- Location matters (for donor design)



Donor Design

➢ Effective donor design is crucial

NanoLuc®	516 basepairs in length
NanoLuc® -PEST	639 basepairs in length

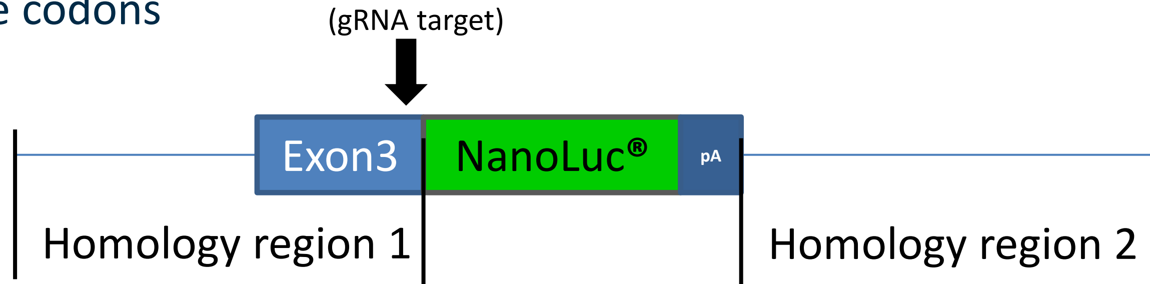
Starting material (target)

Ending material (goal)

Donor: (Not to scale)

Limiting re-cutting by the gRNA can improve the odds (… greatly)

- If your gRNA target is in a coding region (e.g., in exon 3 below) you will want to make sure you alter the target sequence in your donor AND maintain coding frame AND use appropriate codons



Candidates

	No	Score	Strand	Sequence	PAM
☑	12	99	Bottom	ACCCATTGGTGAGGAAGCAG	GGG
☑	4	99	Top	TGCTTTGCAACCCCTCAGCA	TGG
☑	5	99	Top	TTGCAACCCCTCAGCATGGC	AGG
☑	16	100	Top	TCACCAATGGGTCCTTTCTT	TGG
☑	17	100	Bottom	AGTCCAAAGAAAGGACCCAT	TGG
☑	24	100	Top	TGGAGCATTCTACACTAAAA	TGG
☑	8	99	Bottom	GCTGCAGGCCTGCCATGCTG	AGG

Silent Substitutions

gRNA: T GCT TTG CAA CCC CTC AGC ATG G (#4, PAM)

Amino Acid	A	L	Q	P	L	S	M
Position in gRNA	19..17	16..14	13..11	10..8	7..5	4..2	1..-2
Codon	GCT	TTG	CAA	CCC	CTC	AGC	ATG
Frequency	0.28	0.12	0.27	0.33	0.20	0.25	1.00
Alt. 1 - Codon	GCa	cTt	CAg	CCt	CTt	tcC	None
Alt. 1 - Frequency	0.22	0.12	0.73	0.29	0.12	0.23	
Alt. 2 - Codon	GCc	cTa		CCa	tTg	tct	
Alt. 2 - Frequency	0.4	0.07		0.27	0.12	0.18	
Alt. 3 - Codon	GCg	Tta		CCg	tTa	tca	
Alt. 3 - Frequency	0.1	0.06		0.11	0.06	0.15	
Alt. 4 - Codon		cTc			CTa	Agt	
Alt. 4 - Frequency		0.2			0.07	0.14	
Alt. 5 - Codon		cTG			CTg	tcg	
Alt. 5 - Frequency		0.43			0.43	0.06	

Donor Types

- Plasmid
  - No size constraints
  - Requires transfection/electroporation
  - Super-coiled appears better than linear
  - Double-stranded format

- rAAV
  - ~5KB size limit
  - Highly efficient transduction of many cell types
  - Efficient delivery to the nucleus
  - Naturally recombigenic
  - Single-stranded format

- Adenovirus
  - ~10KB size limit
  - Good transduction of many cell types
  - Double-stranded format
  - Long intracellular residency

- Lentivirus
  - Not recommended due to high probability of random integration

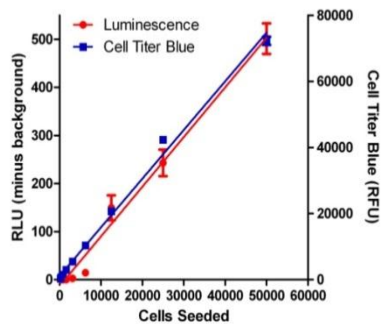
Screening & Validation

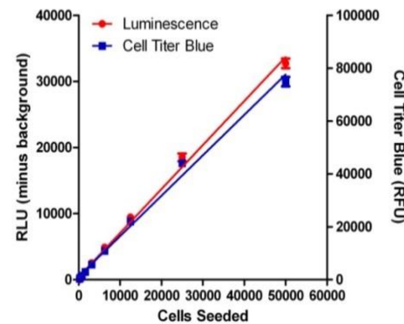
- Screen transfected/transduced pool(s) after 3-5 days
- Confirm gRNA activity
- Confirm presence of modified alleles in transfected/transduced pool(s)
  - Design PCR primers which straddle the insertion

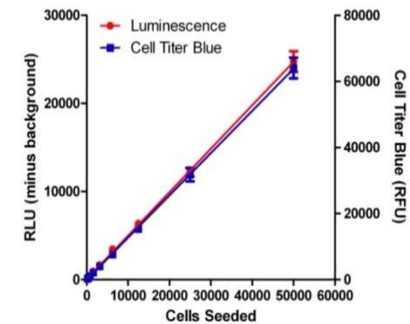
- Single-cell clone, expand, and screen for modified allele
  - May be able to use NanoLuc® if endogenous expression is expected at sufficient levels
  - Confirm proper fusion using PCR screening primers
  - Final validation by Sanger sequencing
    - May need to pay particular attention to non-targeted alleles to see if they have been disrupted
- Recommendations (optional)
  - Freeze early passages (in case fusion destabilizes cell line)
  - STR assay to verify cell line origin
  - Mycoplasma testing

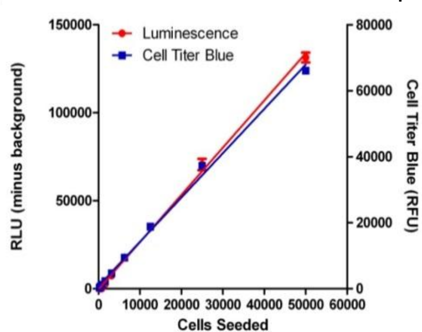
Signal Linearity: NanoLuc® reporter line signal consistent with cell number

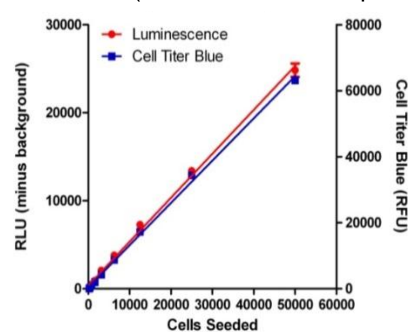
- A range of X-MAN™ NanoLuc® reporter cell lines were created using HCT116 cells
- Good signal linearity with increasing cell number
- Minimal background luciferase signal in parental cells, even at high cell densities

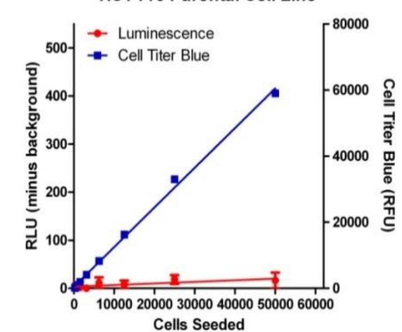




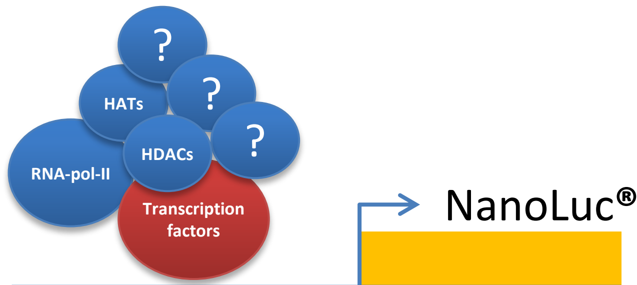


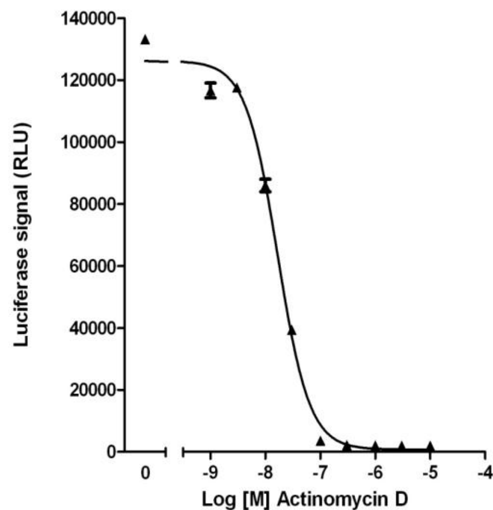




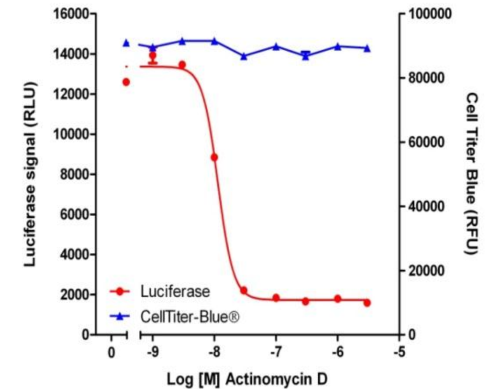


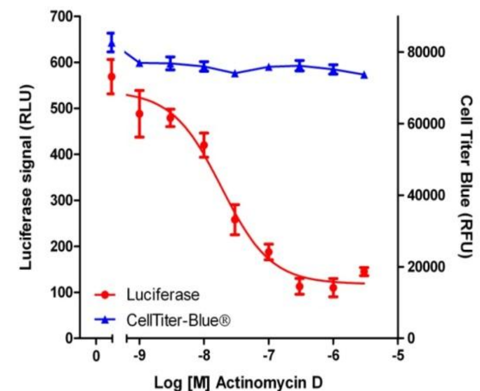
Excellent signal dynamics: NanoLuc® Promoter reporter lines





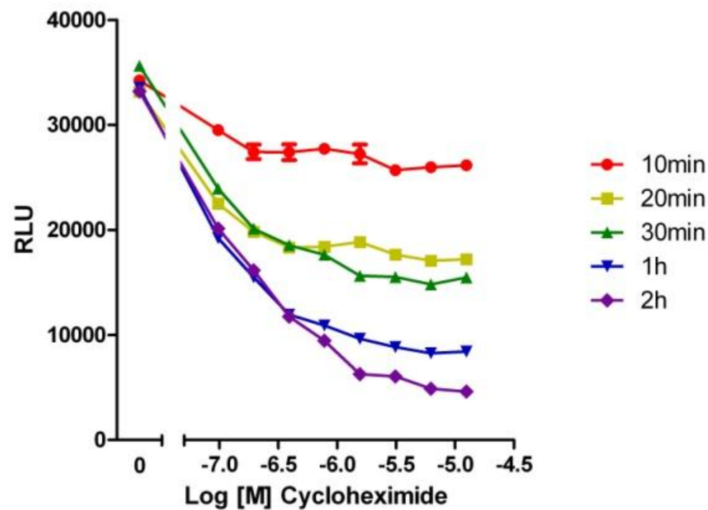
Good signal to noise ratio for transcription inhibition at the endogenous level using Nano-luciferase





NanoLuc® assays can be multiplexed with CellTiter-Blue® to control for effects on cell viability

Excellent signal dynamics: NanoLuc® Protein reporter lines



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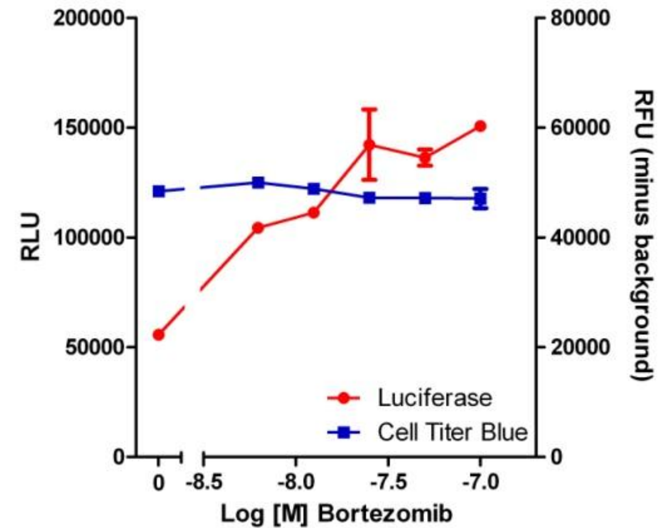

Good dynamics seen for stabilisation of endogenous protein levels with nano-luciferase

NanoLuc® and HaloTag® Donors

- If you need some help...

Horizon and Promega have partnered to enable development of endogenous pathway tag and reporter cell lines for application in functional genomics research and drug discovery and development. You can now access donor vectors optimized to insert the best-in-class reporter tags HaloTag® and Nanoluc® into the gene of interest for pathway monitoring, *in vitro* imaging, and protein purification. Horizon will design the homology arms so that the reporters are integrated into precisely the right spot in the genome.