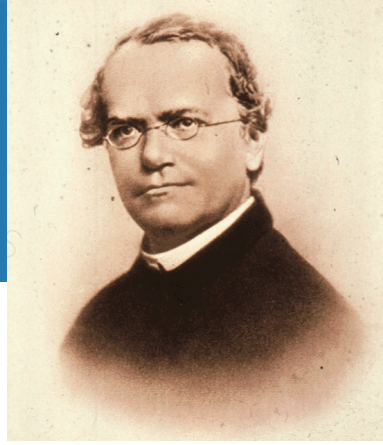
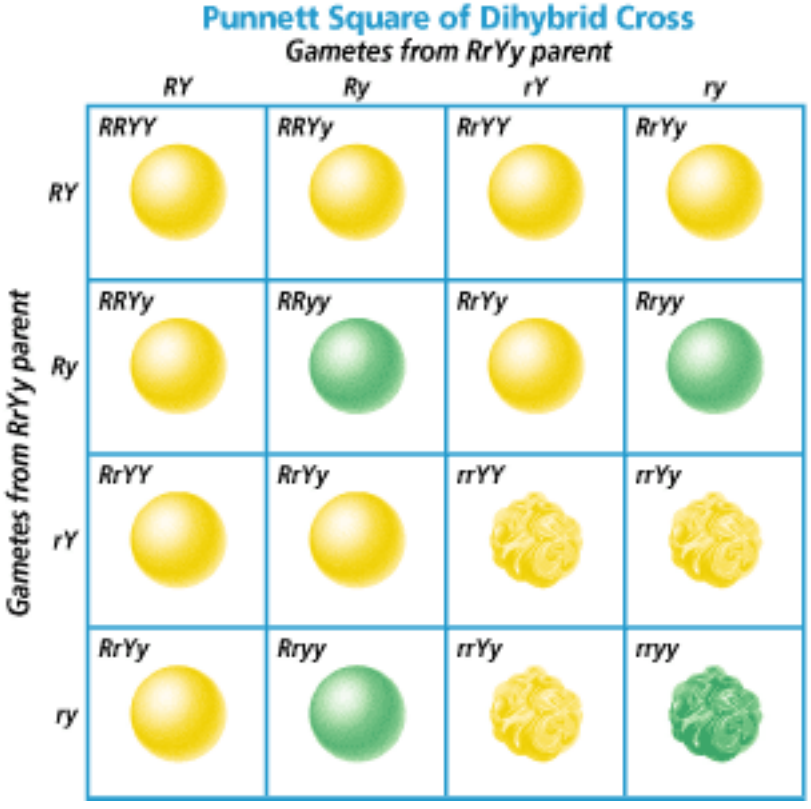
Section 2 Overview

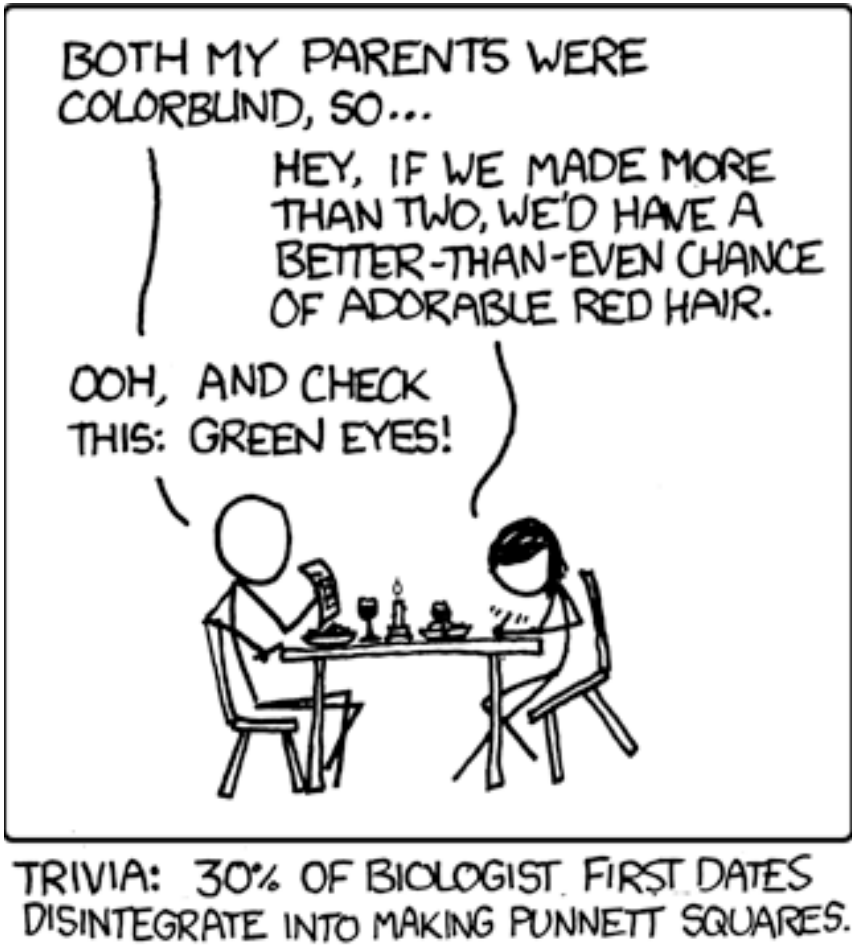
- Discovery of RNAi
- Modes of triggering RNAi
- RNAi reagents in the Platform
- ORF reagents in the Platform
- TALE reagents in the Platform

What is a gene?

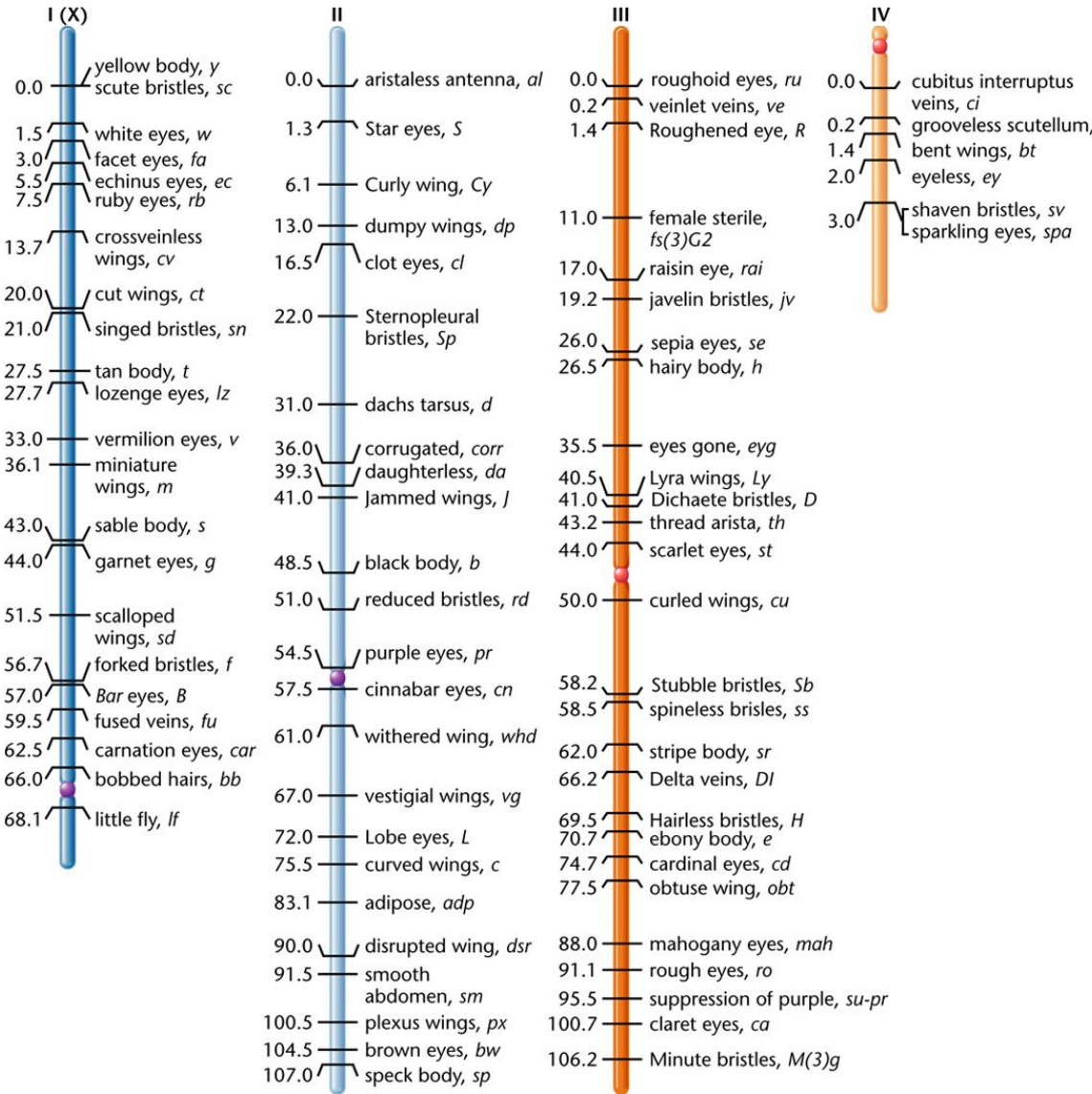


- A molecular unit of heredity

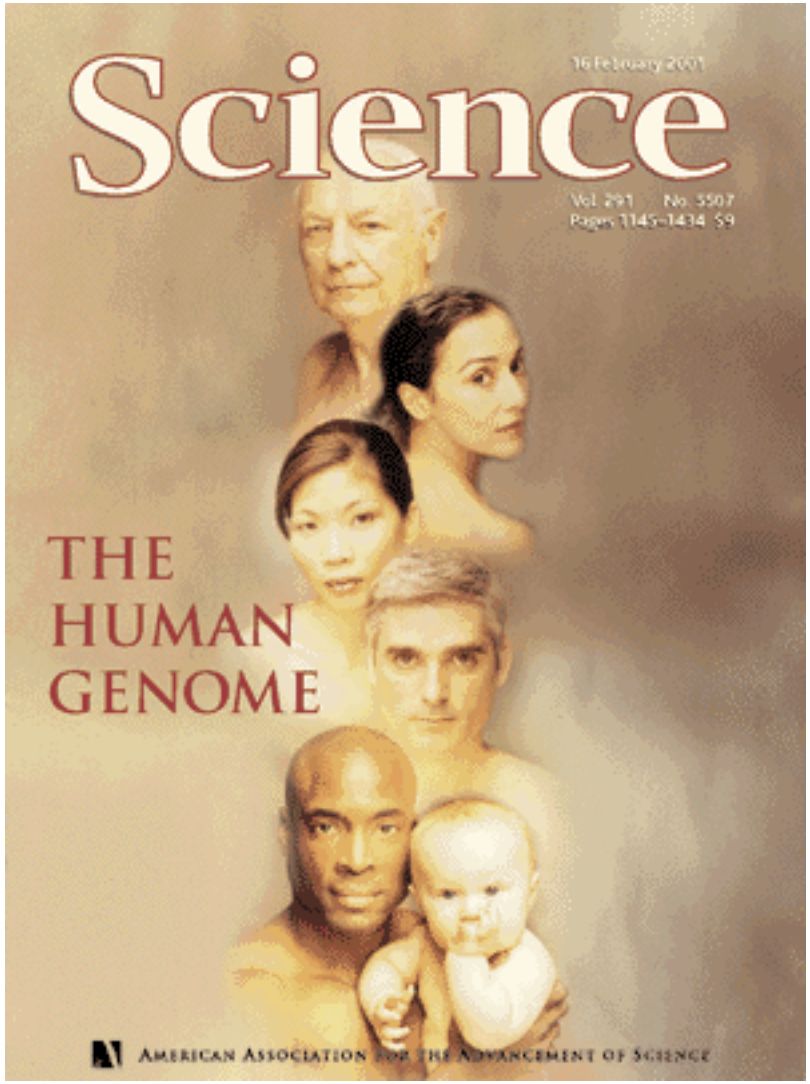


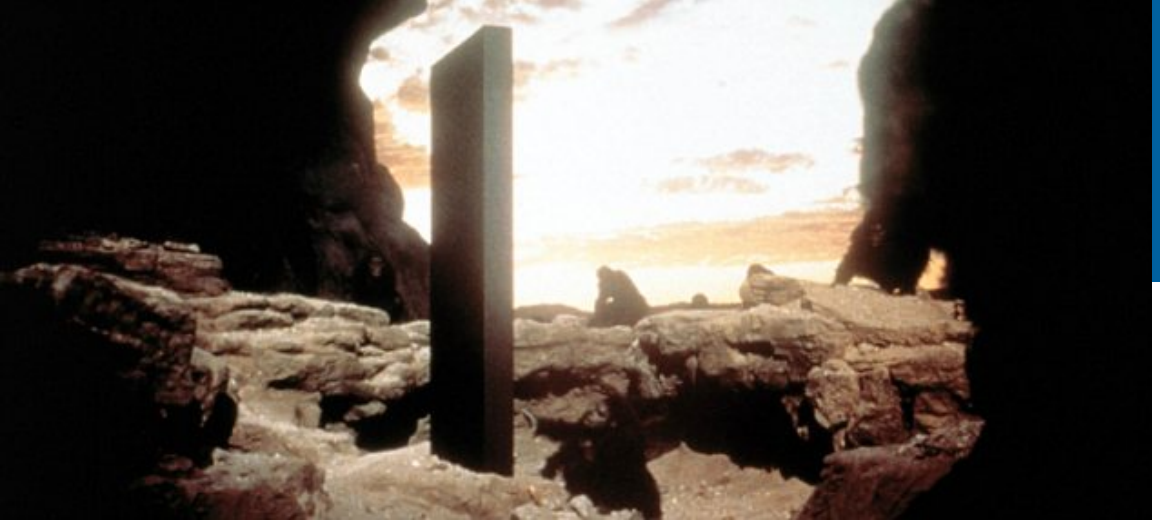


Where are genes?



What are *all* the genes?
Human Genome Project

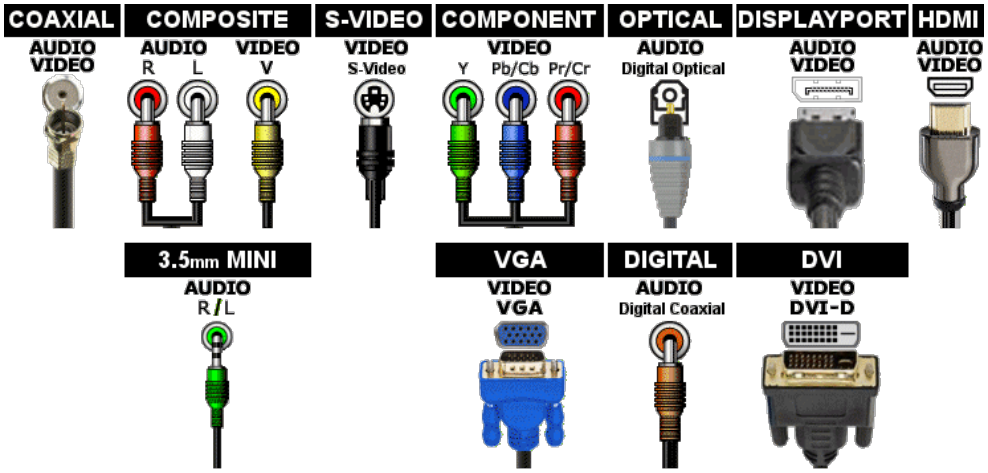




By 2001

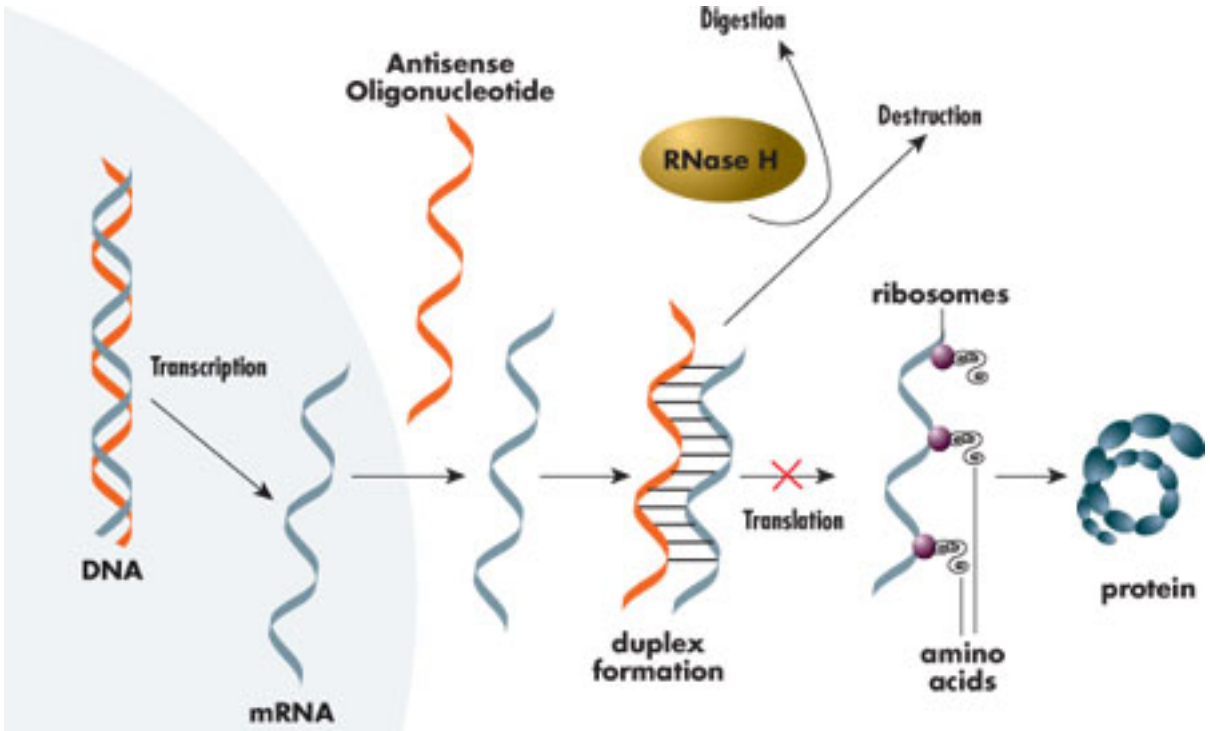
- Sequence of the human genome is completed
- Identify genes computationally
- But what do all these genes *do?*

How to do genetics?



- Determine the function of a gene by breaking it
- Classical approach:
  - Mutagenize a fly/worm/yeast with X-rays or chemicals, look for interesting progeny, and map the gene involved
- Forward genetics:
  - Start with a gene and determine its function by breaking it

Interfere with RNA by antisense



- Simple in theory, and it worked pretty well, but…

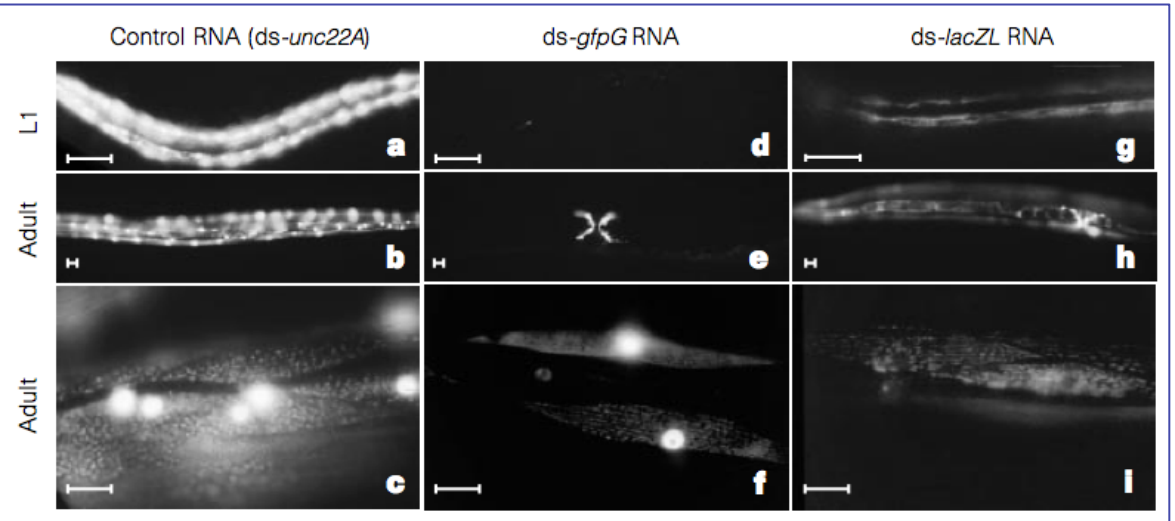
Fire & Mello, *Nature,* 1998

"Despite the usefulness of RNA interference in *C. elegans*… features of the process have been difficult to explain… **sense and antisense RNA preparations are each sufficient to cause interference…** RNA populations to be injected are generally prepared using bacteriophage RNA polymerases. These polymerases, although highly specific, produce some random or ectopic transcripts... **From these facts, we surmised that the interfering RNA populations might include some molecules with double-stranded character.** To test whether double-stranded character might contribute to interference, we further purified single-stranded RNAs and compared interference activities of individual strands with the activity of a deliberately prepared double-stranded hybrid."

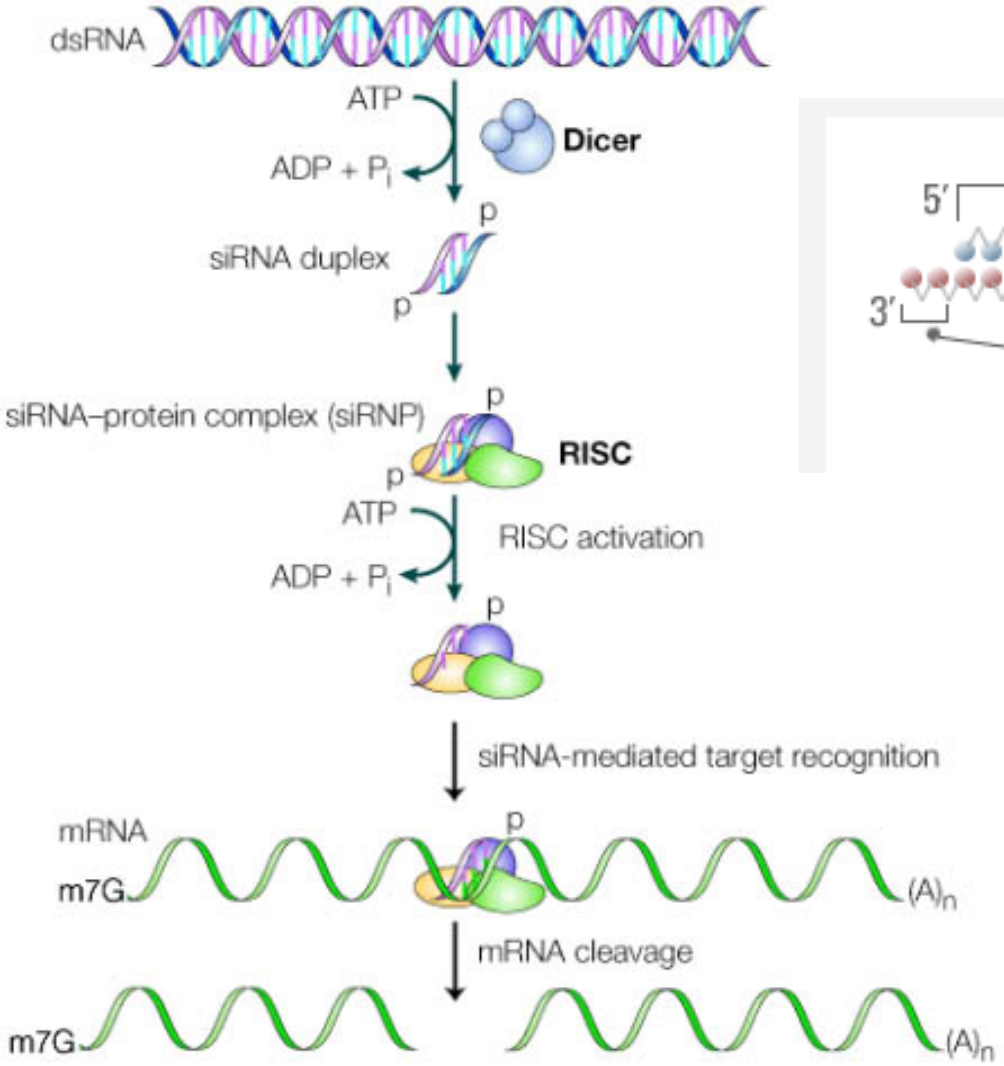
Fire & Mello, 1998

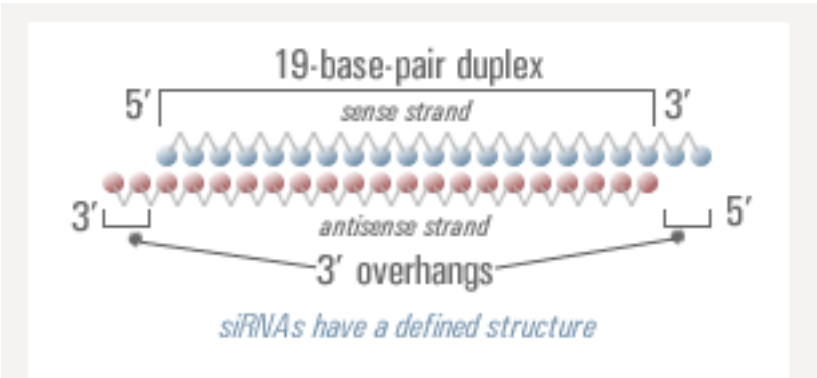
Table 1 Effects of sense, antisense and mixed RNAs on progeny of injected animals

Gene	segment	Size (kilobases)	Injected RNA	F_1 phenotype
unc-22				*unc-22*-null mutants: strong twitchers[7,8]
*unc22A**	Exon 21–22	742	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
unc22B	Exon 27	1,033	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
unc22C	Exon 21–22†	785	Sense + antisense	Strong twitchers (100%)
fem-1				*fem-1*-null mutants: femal (no sperm)[13]
fem1A	Exon 10‡	531	Sense	Hermaphrodite (98%)
			Antisense	Hermaphrodite (>98%)
			Sense + antisense	Female (72%)
fem1B	Intron 8	556	Sense + antisense	Hermaphrodite (>98%)

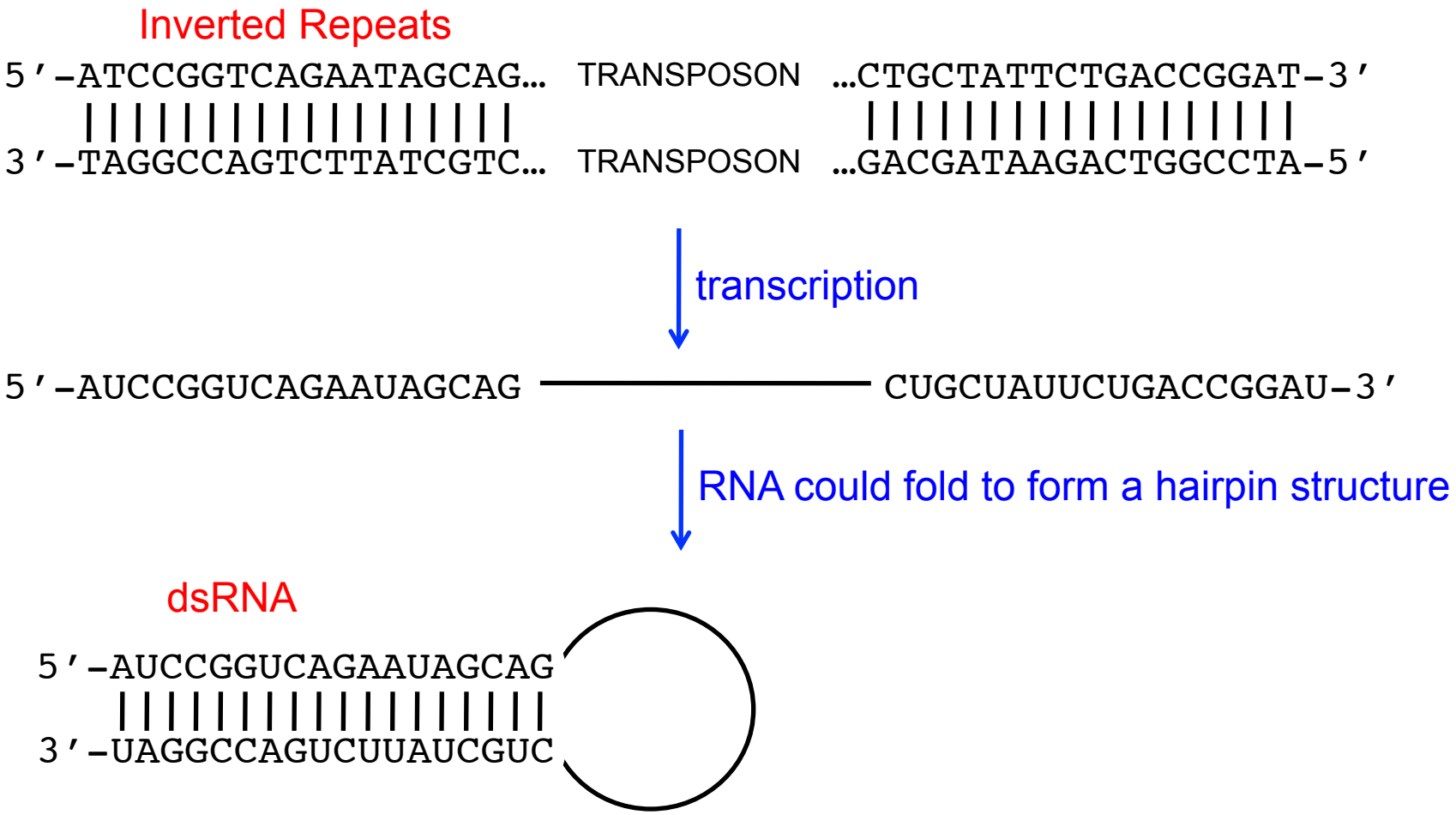


RNAi mechanism





Why do cells do this in the first place?



miRNA discovery: Endogenous small RNAs

- 1993 – Victor Ambros & Gary Ruvkun separately discover that the lin-4 gene is a small RNA that regulates the lin-14 mRNA in *C. elegans*
- 1998 – Fire and Mello discover RNAi triggered by exogenous dsRNA
- 1999 – Baulcombe discovers small RNAs generated during Post Transcriptional Gene Silencing (PTGS) in plants
- 2000 – Ruvkun group discovers that let-7 gene is also a small RNA that regulates developmental timing
- 2000 – Zamore, Tuschl, Bartel, and Sharp discover that RNAi is mediated by small RNAs: siRNAs
- 2001 – Tuschl, Ambros and Bartel groups discover presence of *many* endogenous small RNAs in worms, flies, and mammals

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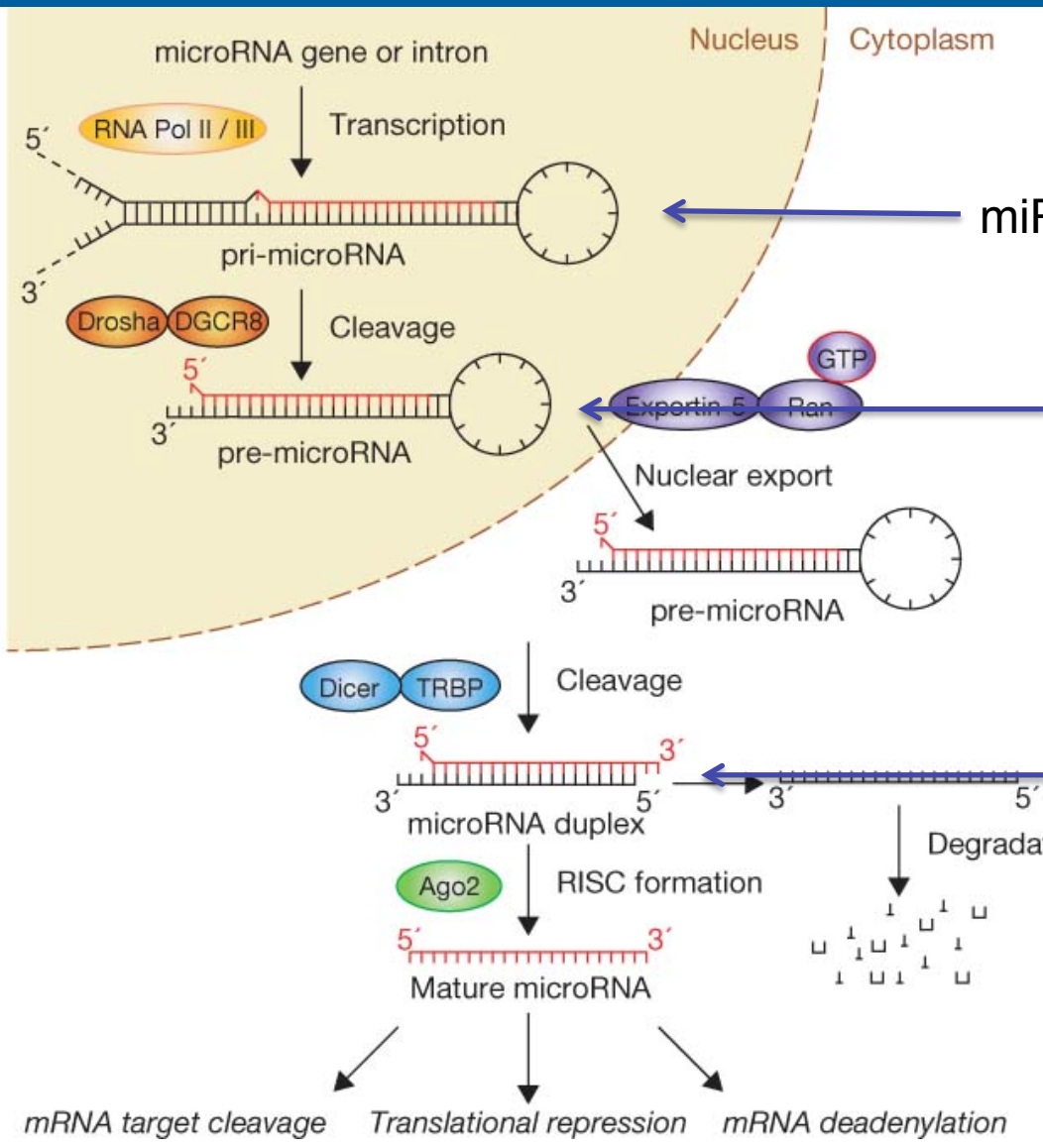
siRNA vs. shRNA vs. miRNA delivery: where you enter the pathway



miRNA-like: pre-Drosha, pre-Dicer

shRNA: post-Drosha, pre-Dicer

siRNA: post-Drosha, post-Dicer

From Nature Cell Biology **11**, 228 - 234 (2009)

Direct comparison

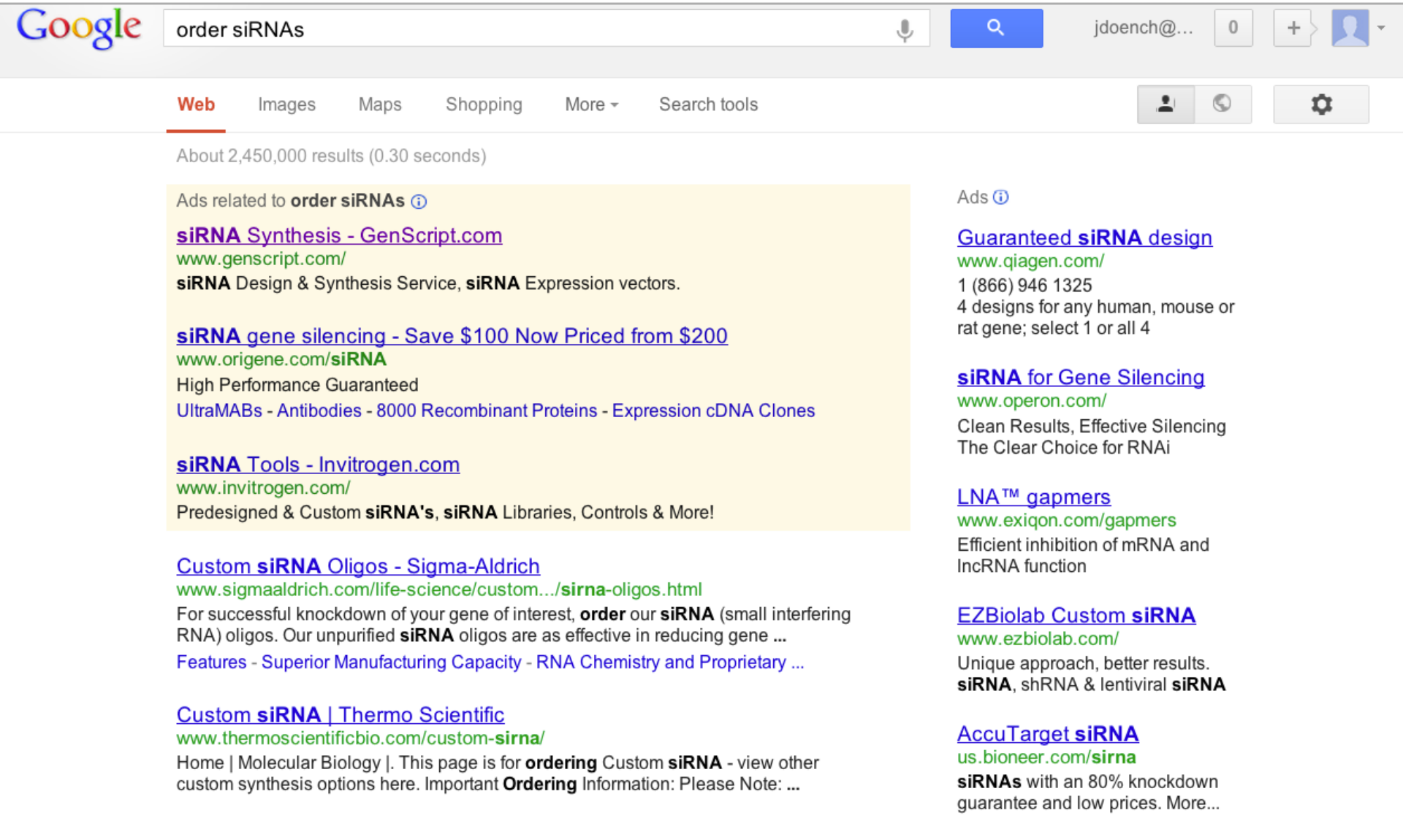
Minimizing variables among hairpin-based RNAi vectors reveals the potency of shRNAs

RYAN L. BOUDREAU,[1,2] ALEX MAS MONTEYS,[1] and BEVERLY L. DAVIDSON[1,2,3]

[1]Department of Internal Medicine, University of Iowa, Iowa City, Iowa 52242, USA
[2]Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, Iowa 52242, USA
[3]Department of Neurology, University of Iowa, Iowa City, Iowa 52242, USA

"In this work, we demonstrate how early generation shRNAs with poor strand biasing confound the comparison of hairpin-based RNAi approaches. Minimizing the variables for comparison reveals that, for three independent target sequences and in different settings (in vitro and in vivo), shRNAs are more potent than artificial miRNAs."

siRNAs can be ordered from pretty much anywhere



Google

order siRNAs

jdoench@...

Web Images Maps Shopping More Search tools

About 2,450,000 results (0.30 seconds)

Ads related to **order siRNAs**

siRNA Synthesis - GenScript.com
www.genscript.com/
siRNA Design & Synthesis Service, **siRNA** Expression vectors.

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Custom **siRNA** Oligos - Sigma-Aldrich
www.sigmaaldrich.com/life-science/custom.../**sirna**-oligos.html
For successful knockdown of your gene of interest, **order** our **siRNA** (small interfering RNA) oligos. Our unpurified **siRNA** oligos are as effective in reducing gene ...
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Home | Molecular Biology |. This page is for **ordering** Custom **siRNA** - view other custom synthesis options here. Important **Ordering** Information: Please Note: ...

Ads

Guaranteed **siRNA** design
www.qiagen.com/
1 (866) 946 1325
4 designs for any human, mouse or rat gene; select 1 or all 4

siRNA for Gene Silencing
www.operon.com/
Clean Results, Effective Silencing
The Clear Choice for RNAi

LNA™ gapmers
www.exiqon.com/gapmers
Efficient inhibition of mRNA and lncRNA function

EZBiolab Custom **siRNA**
www.ezbiolab.com/
Unique approach, better results.
siRNA, shRNA & lentiviral **siRNA**

AccuTarget **siRNA**
us.bioneer.com/**sirna**
siRNAs with an 80% knockdown guarantee and low prices. More...

siRNA (small molecule) vs. shRNA (plasmid-based)

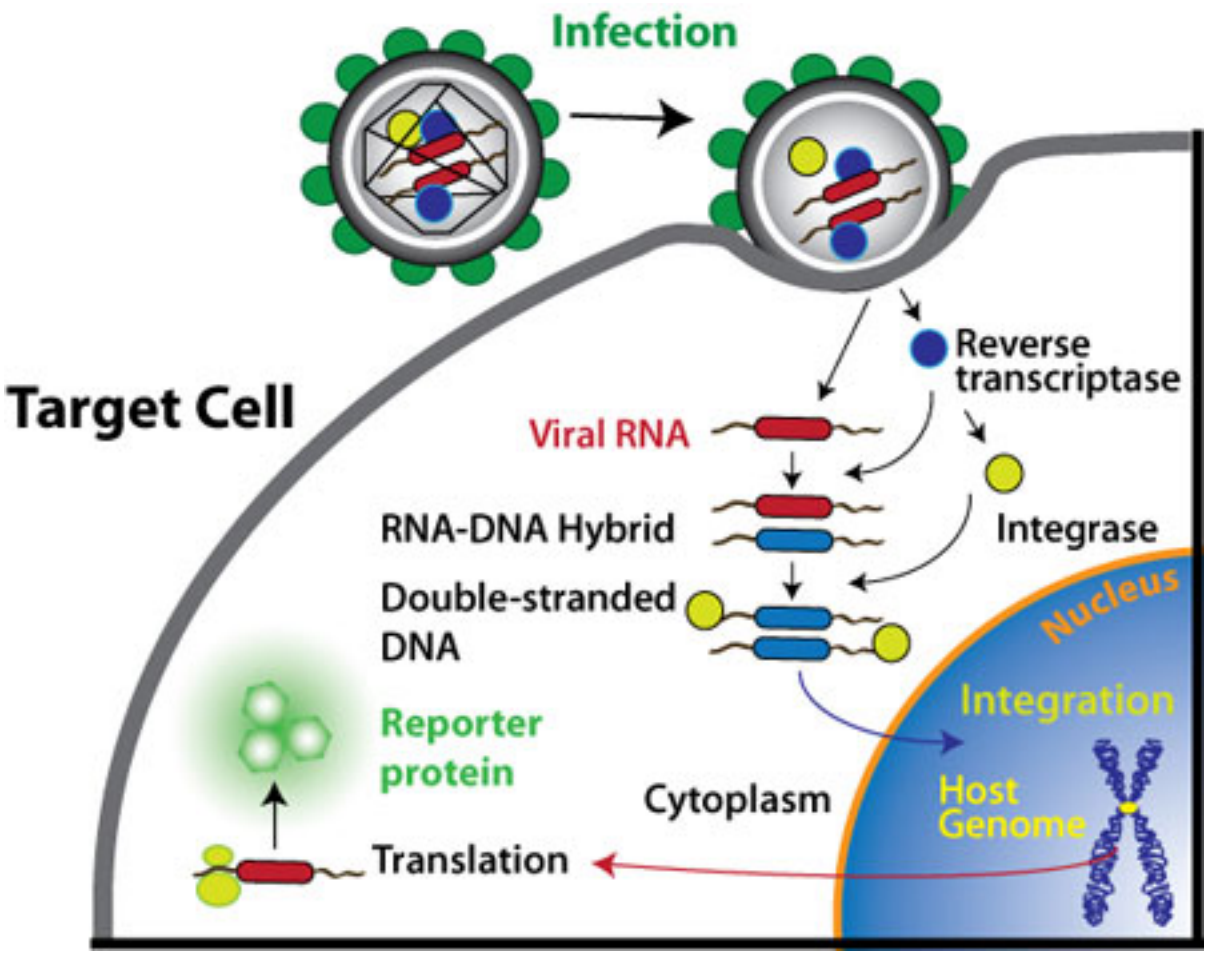
- Delivery
  - siRNA requires transfection; small so comparatively easy
  - shRNA can be done with virus
- Cost
  - siRNA is non-renewable
  - shRNA plasmid essentially limitless
- Temporal
  - siRNA starts working immediately and dilutes out over time
  - shRNA can integrate stably and/or use controllable promoters
- Modification
  - siRNA can have chemical modifications to enhance stability and/or activity

Viral delivery

Virus	Genetic Material	Packaging Capacity	Chromosomal Integration	Comments
Retrovirus	RNA	8 kb	Yes	Only infects dividing cells
Adenovirus	dsDNA	30 kb	No	Extremely high titer achievable
AAV	ssDNA	5 kb	No	Little / no immune stimulation
HSV	dsDNA	40 kb	No	Particularly good for neurons
Lentivirus	RNA	8 kb	Yes	Infects non-dividing cells

TRC library uses lentivirus

Lentiviral delivery



From http://www.systembio.com/support/resources/faqs/lenti

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RNAi Library

TRC3 shRNA library

TRC shRNA Library	Human	Mouse
Target Genes	19,875	21,045
TRC shRNA constructs	163,705	136,232
Avg. # shRNA/target gene	8.2	6.5
Library Performance	**Human**	**Mouse**
Target Genes	9,131	9,248
# of shRNAs evaluated for KD	63,375	55,438
% with ≥70% knockdown	48%	52%

Web Portal

Broad Community TRC Portal

Home | Search by Gene | Search by Clone

Broad Community TRC Portal - Welcome

Full info, Broad login required:
https://iwww.broadinstitute.org/rnai/db/

Limited info, no login required:
http://www.broadinstitute.org/rnai/public/

Search for Genes

Search the TRC shRNA and hORFeome 8.1 ORF libraries

Search by Gene
Search by Clone

Gene Symbol is case-sensitive

Search by Gene

Gene or Transcript IDs:
e.g. '988', 'NM_001253', etc.

Choose File No file chosen

Official Gene Symbols:
Species: Human Mouse

NOTE: This search tool finds EXACT matches to the official symbol (with matching capitalization) for each NCBI mouse and human gene. Search Entrez Gene to find the current official gene symbol.

Reset Search »

Gene query

Gene Search Results Refine Your Search

Genes

	Input	Taxon	Gene ID	Gene Symbol	Gene Description
1	MITF	human	4286	MITF	homolog of mouse microphthalmia

Download CSV

Transcripts

	Input	Taxon	Gene ID	Gene Symbol	Transcript ID	Transcript Description
1	MITF	human	4286	MITF	NM_000248.3	microphthalmia-associated transcription factor ...
2	MITF	human	4286	MITF	NM_001184967.1	microphthalmia-associated transcription factor ...
3	MITF	human	4286	MITF	NM_001184968.1	microphthalmia-associated transcription factor ...
4	MITF	human	4286	MITF	NM_006722.2	microphthalmia-associated transcription factor ...
5	MITF	human	4286	MITF	NM_198158.2	microphthalmia-associated transcription factor ...
6	MITF	human	4286	MITF	NM_198159.2	microphthalmia-associated transcription factor ...
7	MITF	human	4286	MITF	NM_198177.2	microphthalmia-associated transcription factor ...
8	MITF	human	4286	MITF	NM_198178.2	microphthalmia-associated transcription factor ...

Download CSV

Show all clones for these genes

Show validated PCR assays for these genes

Hairpins for a gene

Hairpins designed to target transcripts from requested genes

	Input	Clone ID	Clone Name	Target Taxon [?]	Target Gene [?]	Target Gene Symbol	Vector	Match Position	Match Region	Match % [?]	SDR Match % [?]	Intrinsic Score [?]	Adjusted Score [?]	# KD Tests: high qual (total) [?]	KD: % Expr. [?]	KD Cell Line(s)
1	MITF	TRCN0000019123	NM_000248.2-573s1c1	human	4286	MITF	pLKO.1	573	CDS	100%	100%	5.625	8.505	2 (2)	15%	A549
2	MITF	TRCN0000329869	NM_000248.2-492s21c1	human	4286	MITF	pLKO_TRC005	492	CDS	100%	100%	13.200	7.854	1 (1)	15%	A549
3	MITF	TRCN0000329793	NM_000248.2-573s21c1	human	4286	MITF	pLKO_TRC005	573	CDS	100%	100%	5.625	8.505	2 (2)	15%	A549
4	MITF	TRCN0000329863	NM_000248.2-1392s21c1	human	4286	MITF	pLKO_TRC005	1392	3UTR	100%	100%	10.800	6.350	1 (1)	21%	A549
5	MITF	TRCN0000019119	NM_000248.2-3150s1c1	human	4286	MITF	pLKO.1	3150	3UTR	100%	100%	4.950	1.247	0 (1)	30%	A549
6	MITF	TRCN0000019121	NM_000248.2-789s1c1	human	4286	MITF	pLKO.1	789	CDS	100%	100%	4.950	3.326	1 (1)	41%	A549
7	MITF	TRCN0000019122	NM_000248.2-871s1c1	human	4286	MITF	pLKO.1	871	CDS	100%	100%	5.625	5.625	1 (1)	42%	A549
8	MITF	TRCN0000019120	NM_000248.2-956s1c1	human	4286	MITF	pLKO.1	956	CDS	100%	100%	13.200	1.478	1 (1)	47%	A549
9	MITF	TRCN0000329866	NM_000248.2-669s21c1	human	4286	MITF	pLKO_TRC005	669	CDS	100%	100%	13.200	7.762	1 (1)	59%	A549
10	MITF	TRCN0000329868	NM_000248.2-775s21c1	human	4286	MITF	pLKO_TRC005	775	CDS	100%	100%	13.200	9.240	1 (1)	62%	A549

Download CSV

Description of Features

- CloneID, e.g. TRCN0000000001
  - A physical entity with a freezer location, rack location, well location: 'a thing in a tube'
  - Gremlin: The same 21mer can have different TRCN IDs
- Vector: pLKO.1 vs pLKO_TRC005
  - Two very similar vectors both in sequence and performance
  - pLKO_TRC005 has WPRE and more useful restriction sites
- Intrinsic & Adjusted scores
  - Computational predictors of activity
- KD % Expr
  - qPCR test of activity in a standard cell line
  - Lower % expression is better, i.e. more knockdown

Protocols

TRC Library Production and Performance Protocols

	Protocol Name	Date	Download
1	shRNA Cloning Protocol	13 Feb 2013	pdf
2	shRNA and ORF Glycerol and Plasmid DNA Preparation	5 Dec 2012	pdf
3	shRNA and ORF Viral Production	5 Dec 2012	pdf
4	Lentiviral Infection Protocol	13 Dec 2012	pdf
5	Viral Titering Protocol (alamarBlue)	5 Dec 2012	pdf
6	TRC Knockdown Validation with qPCR	5 Dec 2012	pdf
7	TRC Assay Development Guidelines for ORF clones	4 Feb 2011	pdf
8	shRNA Plasmid DNA/Viral Pool Production Protocol	5 Dec 2012	pdf
9	V5 Tag Staining Protocol	5 Dec 2012	pdf

Pooled Screening Protocols

	Protocol Name	Date	Download
1	Large scale infection and tissue culture protocol	9 Feb 2009	doc
2	Sample and microarray processing	9 Feb 2009	doc
3	dChip preprocessing protocol	9 Feb 2009	doc
4	Illumina Sequencing Nested Barcode PCR Protocol	5 Dec 2012	pdf
5	Illumina Sequencing One Step Barcode PCR	5 Dec 2012	pdf

Order Reagents

Request Reagents

shRNA and ORF Reagents
↳ Information and templates for ordering shRNA and ORF reagents

TALE Reagents
↳ Information and templates for ordering TALE reagents

Target Accelerator Custom Reagents
↳ Information and templates for ordering Target Accelerator Custom Reagents

Rate-limiting step for getting reagents is often you getting permission from your PI to buy them – have that conversation first!

Platforms work on a cost-recovery platform

Alternative Vectors

Vectors	Promoter	Selection markers	Other features
Constitutive shRNA vectors	hU6	Bsd/Hygro/Neo/Puro	tGFP/eGFP
Inducible shRNA vectors	2xTetO 1xLacO 3xLacO	Bsd/Hygro/Neo/Puro	
Constitutive ORF vectors	CMV/EF1a	Bsd/Hygro/Puro	eGFP
Constitutive Gateway destination ORF vectors	CMV PGK EF1a hSyn1	Bsd/Hygro/Puro	C-terminal V5 tag
Inducible Gateway destination ORF vectors	PGK-rtTA	Puro	V5 tag HA tag

Pre-made Arrayed Screening Sets

Focused arrayed shRNA libraries

TRC shRNA set	# of genes	Organisms
Kinases	800	Human, Mouse
Phosphatases	400	Human, Mouse
Transcription Factors	2,000	Human, Mouse
Chromatin	400	Human, Mouse
Vesicle Trafficking	400	Human, Mouse
RNA metabolism	600	Human
Mitochondria	1,000	Human
Metabolism	1,000	Human, Mouse

Pre-made Pooled Screening Sets

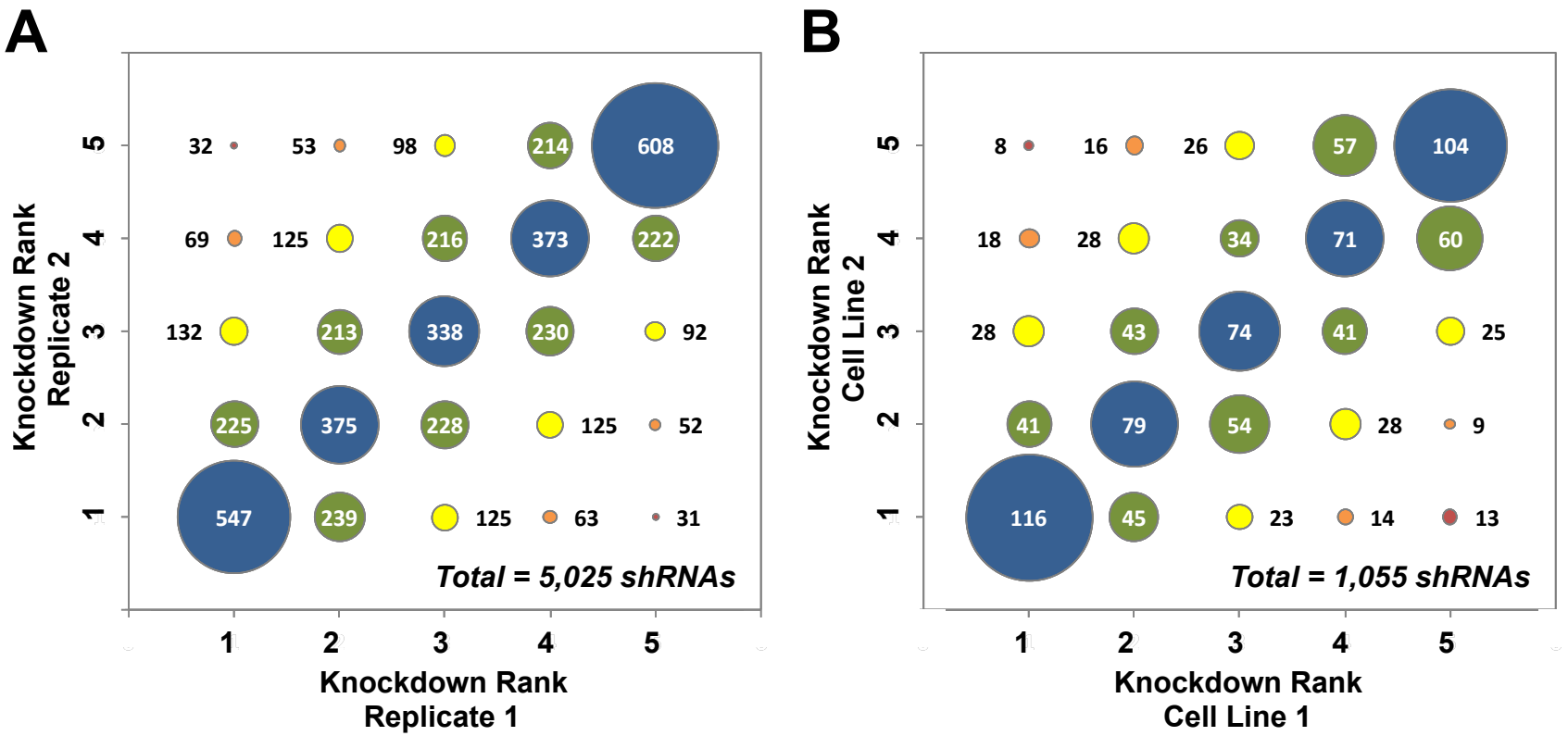
Pooled shRNA libraries

TRC shRNA set	# of genes	# of shRNAs
Human 54k pool	11,000	54,000
Human 98k pool	17,000	98,000
Human kinase pool	700	8,600
Human phosphatase pool	388	3,400
Human epigenetic pool	370	2,900
Mouse 40k pool	8,000	40,000
Mouse 92k pool	16,800	92,000

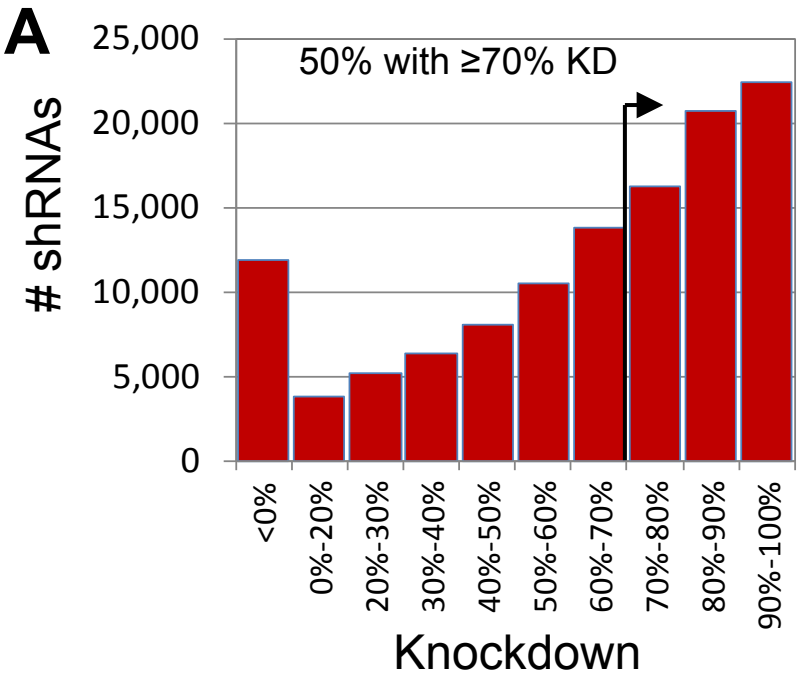
Custom Pools

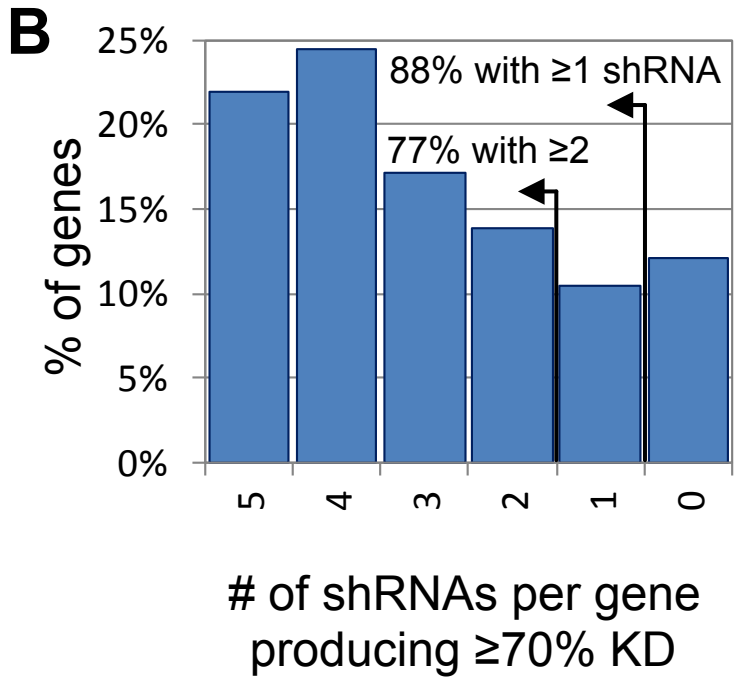
- Begin with arrayed, normalized DNA for set of interest
  - Metabolism genes
  - Genes in 5q deletion
- Pool, amplify in *E. coli*
  - Very little arrayed DNA needed upfront
  - Produce essentially limitless amounts
- Produce virus in pooled format (14cm dishes, T175 flasks)

qPCR assessment of activity: consistency across cell lines



qPCR assessment of overall library performance





A:118,810 targeting 18,379 genes

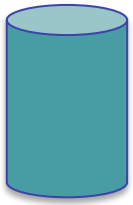
B: Only genes with exactly 5 validated shRNAs. Total = 9,482 genes

Viral Titer

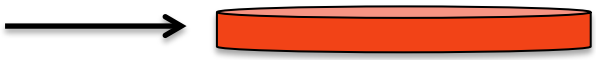
- Titer *attempts* to answer the question of how many infectious particles there are, per unit of volume
  - Historically (i.e. phages on a lawn of bacteria) this was given in Plaque Forming Units (PFUs) per 1 mL
  - With viruses on cultured mammalian cells:

    Titer = Viral Particles/mL
- *Determining* the titer, however, is very much dependent on experimental conditions
- Multiplicity of Infection (MOI) = viral particles ÷ cells
  - BUT, since the number of viral particles is very dependent on experimental conditions, so is the MOI.

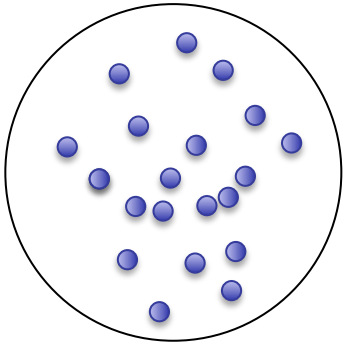
Example of why titer is always relative



Dilute 0.05 uL virus onto to 1 million cells



Add selection, wait for uninfected cells to die and colonies to grow, stain



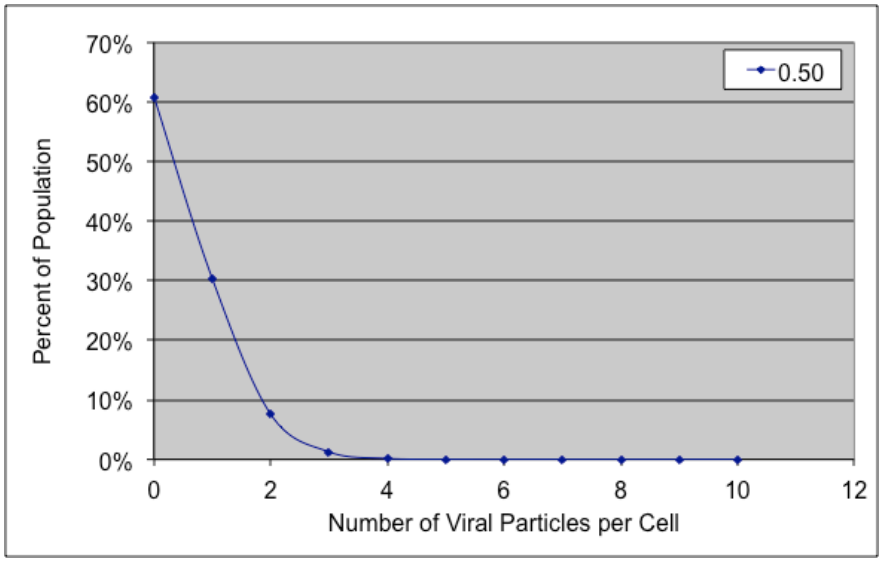
$$\frac{20 \text{ colonies}}{0.05 \text{ uL}} \times \frac{10^3 \text{ uL}}{1 \text{ mL}} = 4 \times 10^5 \text{ infectious particles per mL}$$

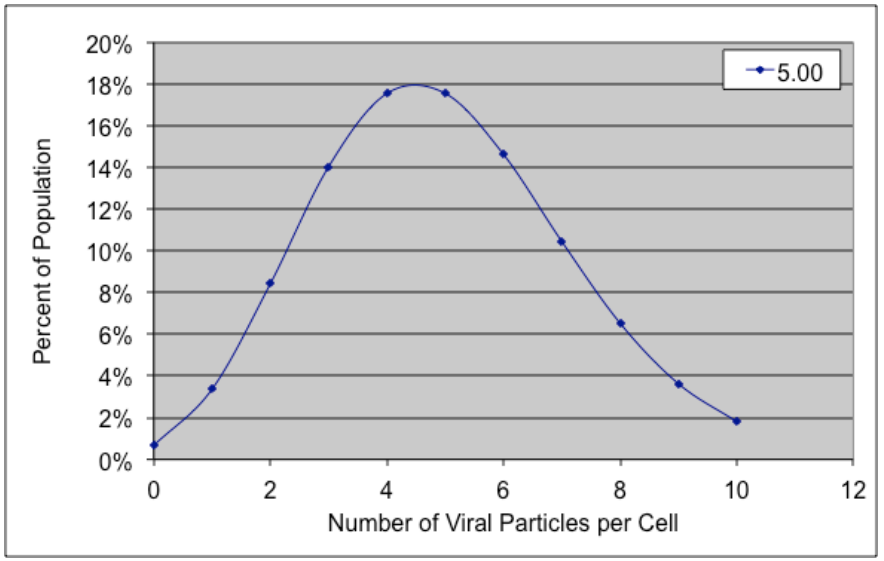
Do the same experiment with the exact same virus on a different cell line, get 400 colonies = 8×10^6 infectious particles per mL

Add polybrene at time of infection and centrifuge the cells for 30 minutes, get 10,000 colonies = 2×10^8 infectious particles per mL

Same virus, three different answers: ***Titer is relative to experimental conditions***

Poisson distribution and MOI





- MOI is a function of the viral titer
- Viral titer depends on experimental conditions
- MOI depends on experimental conditions
- MOI as a function of KD slide

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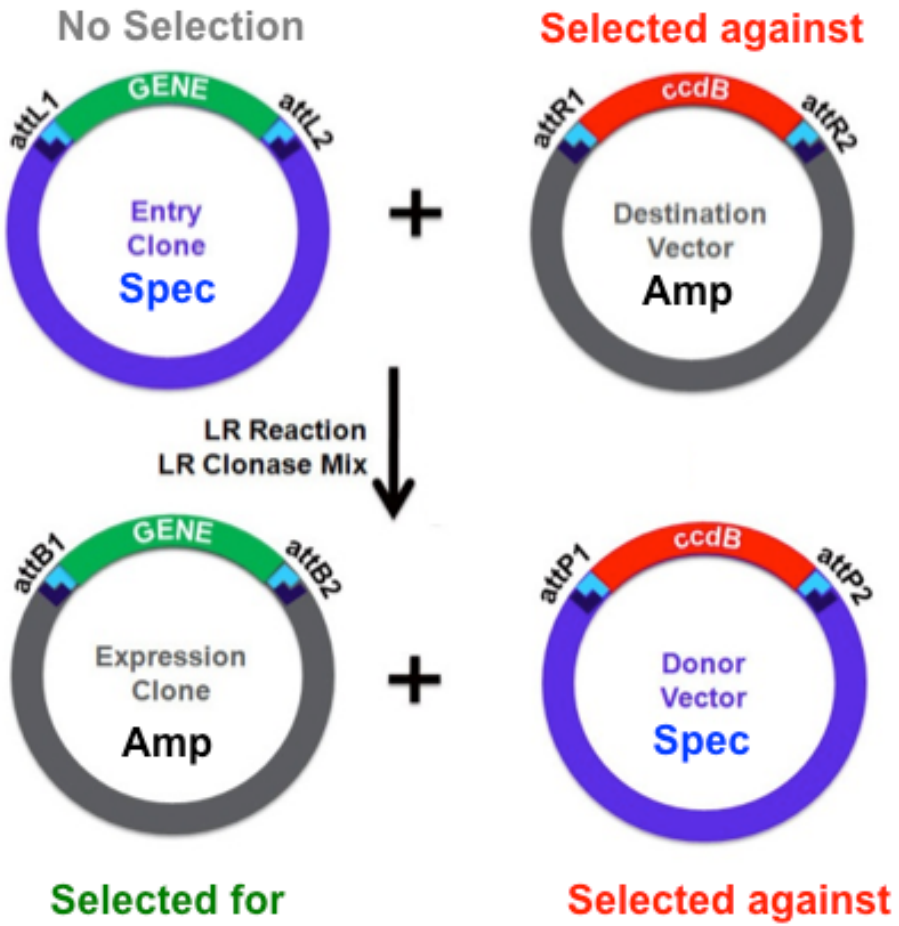
- ORF reagents in the Platform
- TALE reagents in the Platform

Gateway Cloning

- Sold by Life Technologies (Invitrogen)
- Recombination-based transfer of cassettes

PCR product + pDonor + BP enzyme = Entry Clone

pEntry + pDestination + LR enzyme = Expression Clone



Growth Conditions

Vector	Selection	Media	Temp.	*E. coli* strain
pDONR	Kan (-221) or Spec (-223) AND chloramphenicol	TB	30˚	Ccdb-resistant
pEntry	Kan (-221) or Spec (-223)	LB*	37˚	any
pDestination	Carbenicillin AND chloramphenicol	TB	30˚	Ccdb-resistant
pExpression	Carbinicillin	TB or LB	37˚	any

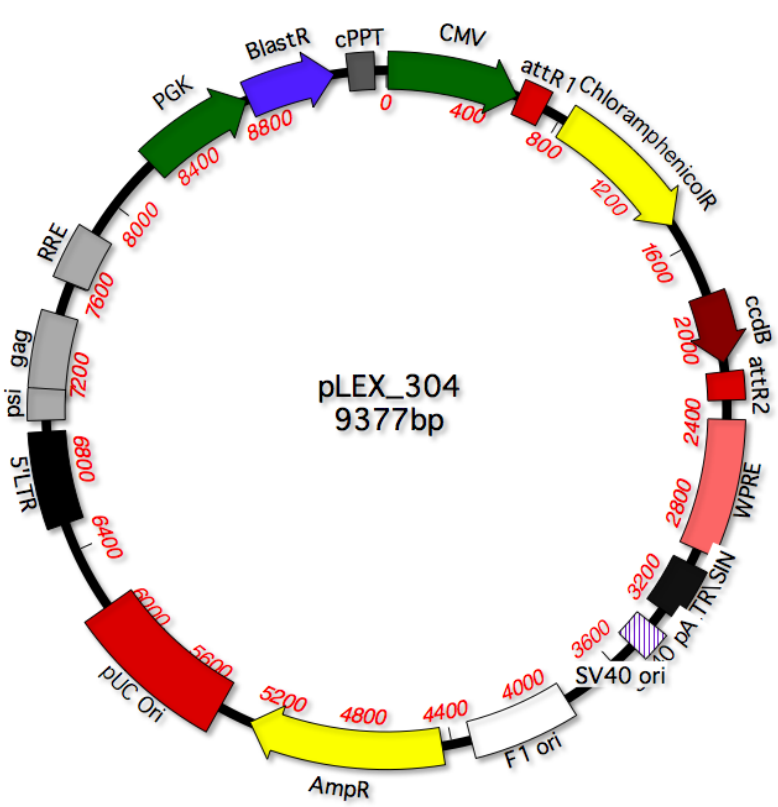
- Entry clones will often overgrow in TB
- Expression clones should be grown in LB if they are ‘uncomplicated’ but TB if they have finicky elements, e.g. LTRs for a lentiviral vector

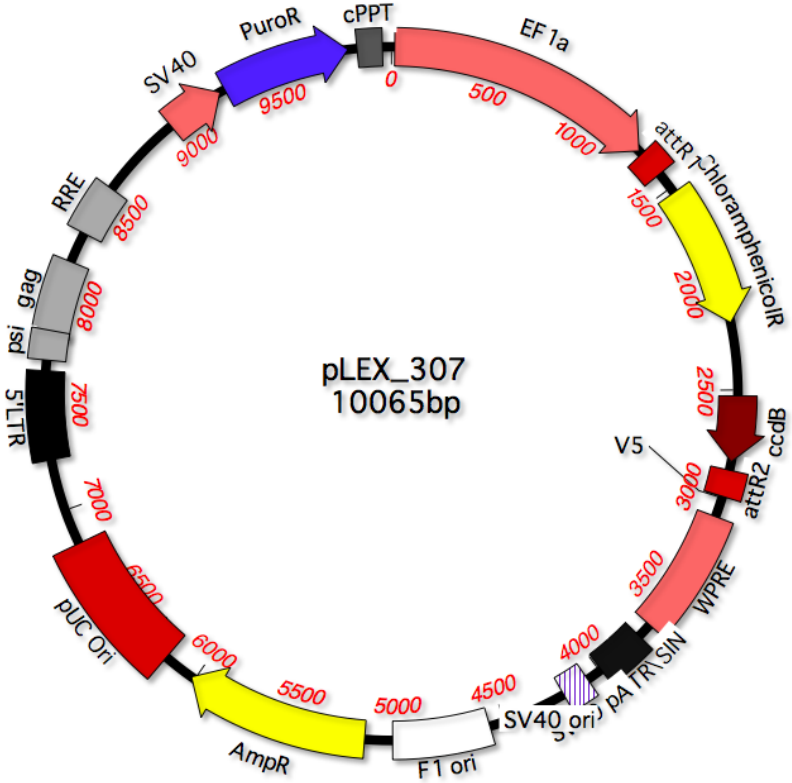
ORF library

ccsb/Broad Human ORF library

TRC ORF Library	Count
Total Clones	16,172 (13,833 genes)
Fully Sequenced Clones	14,524 (12,940 genes)
Full coding transcripts, >99% match to NCBI RefSeq	10,216 genes

Library Vectors



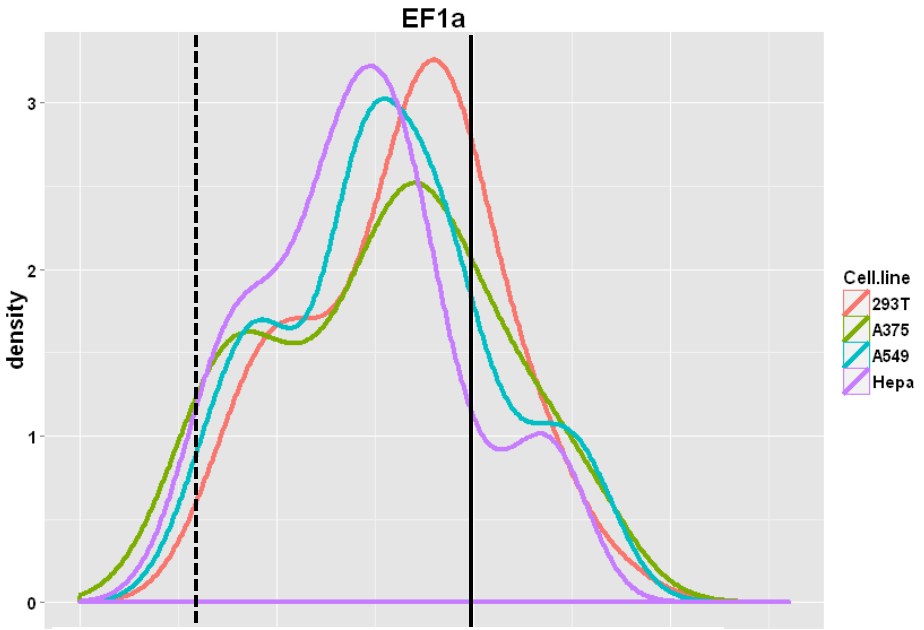


Vector Features

- Promoter of ORF
  - pLEX_304: CMV - very strong, but silenced in some cell types
  - pLEX_307: EF1a promoter - strong, more consistent expression
- V5 tag (both vectors)
  - Will add V5 tag to C-terminus of any ‘open’ clone
- Selection:
  - pLEX_304 uses blasticidin resistance
  - pLEX_307 uses puromycin resistance
- Pooling
  - pLEX_307 has a barcode sequence that allows pooled screening approaches

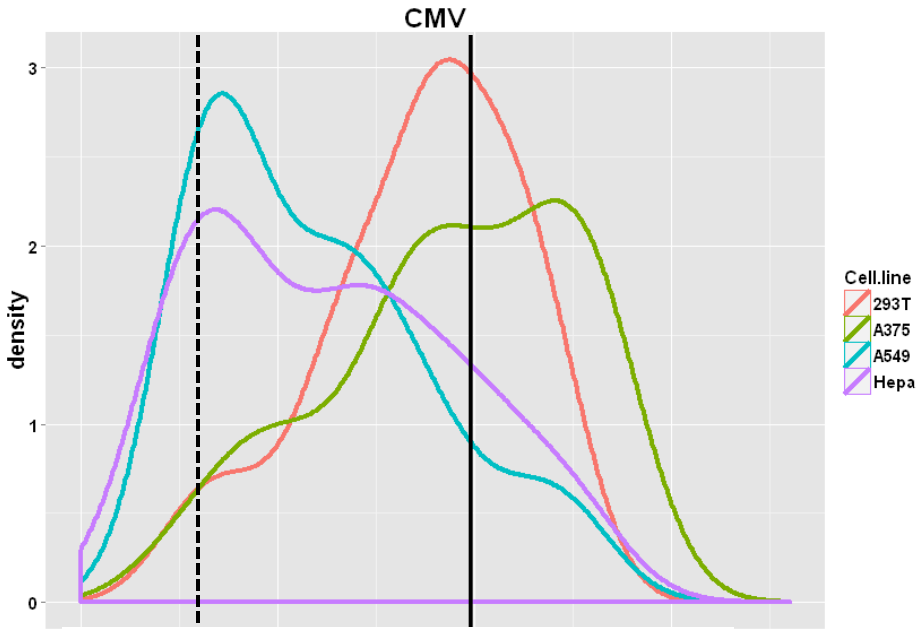
ORF expression across 4 cell lines

pLX307



Relative ORF expression

pLX304

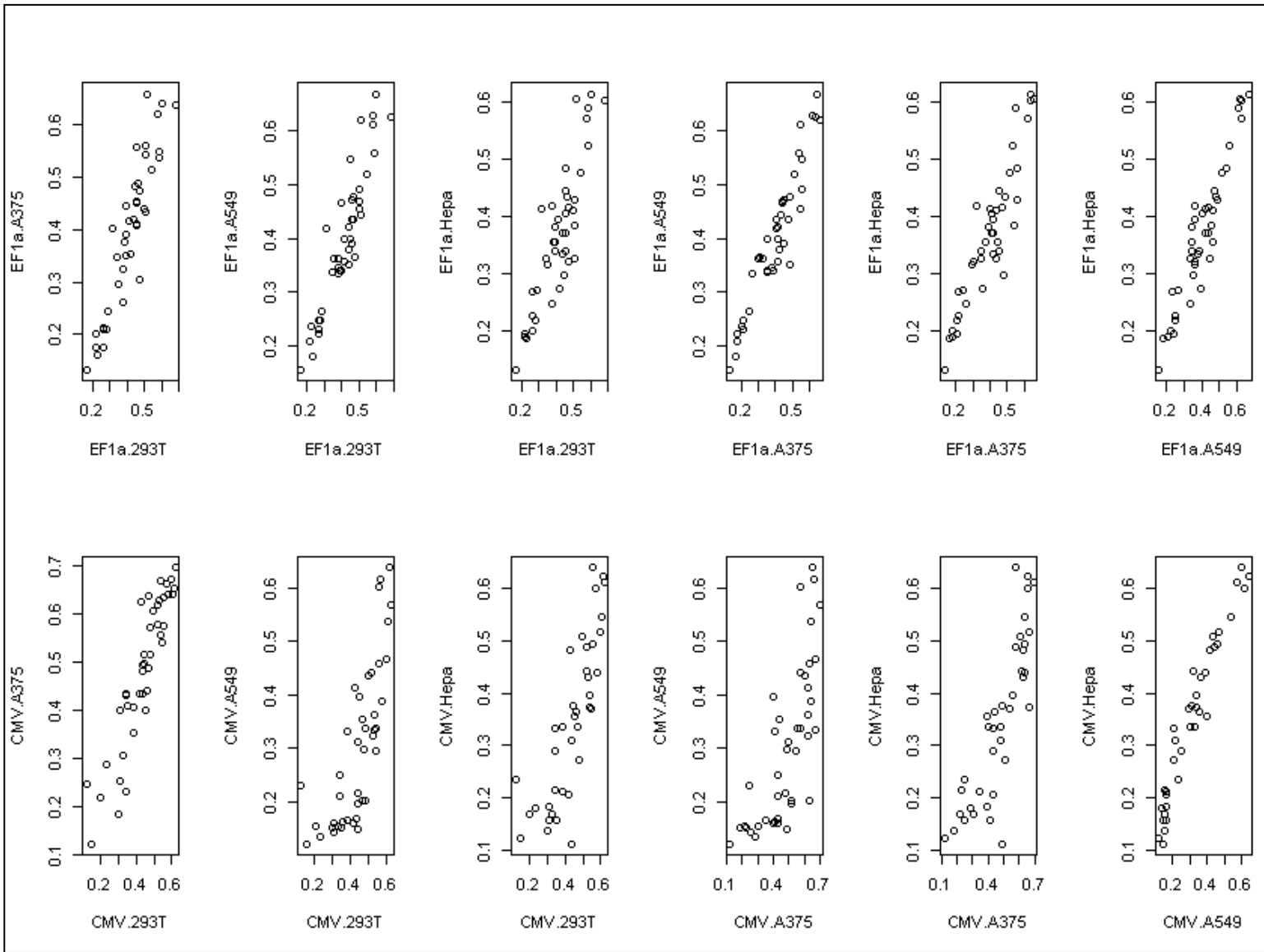


Relative ORF expression

Expression level of ORF is consistent across cell lines

pLX307 (EF1a)

pLX304 (CMV)



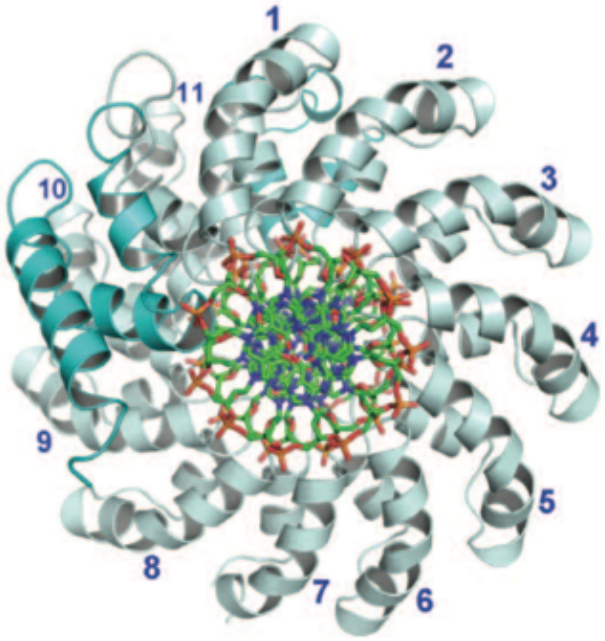
Alternative Vectors

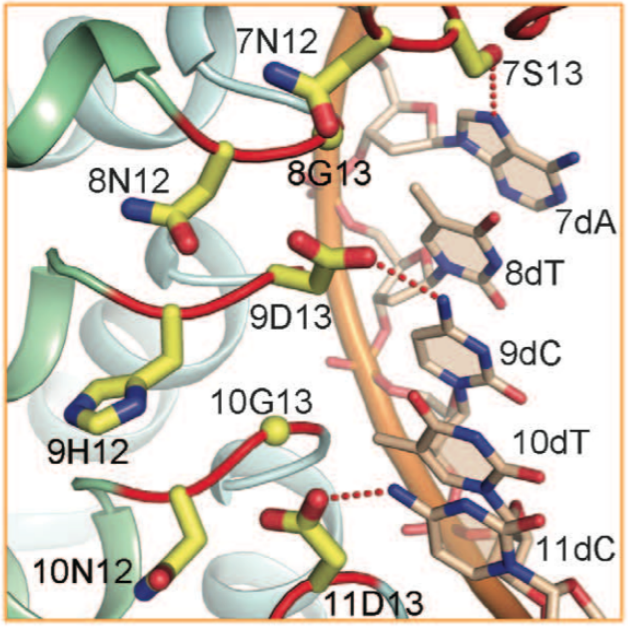
Vectors	Promoter	Selection markers	Other features
Constitutive shRNA vectors	hU6	Bsd/Hygro/Neo/Puro	tGFP/eGFP
Inducible shRNA vectors	2xTetO 1xLacO 3xLacO	Bsd/Hygro/Neo/Puro	
Constitutive ORF vectors	CMV/EF1a	Bsd/Hygro/Puro	eGFP
Constitutive Gateway destination ORF vectors	CMV PGK EF1a hSyn1	Bsd/Hygro/Puro	C-terminal V5 tag
Inducible Gateway destination ORF vectors	PGK-rtTA	Puro	V5 tag HA tag

Section 2 Overview

- Discovery of RNAi
- Modes of triggering RNAi
- RNAi reagents in the Platform
- ORF reagents in the Platform
- TALE reagents in the Platform

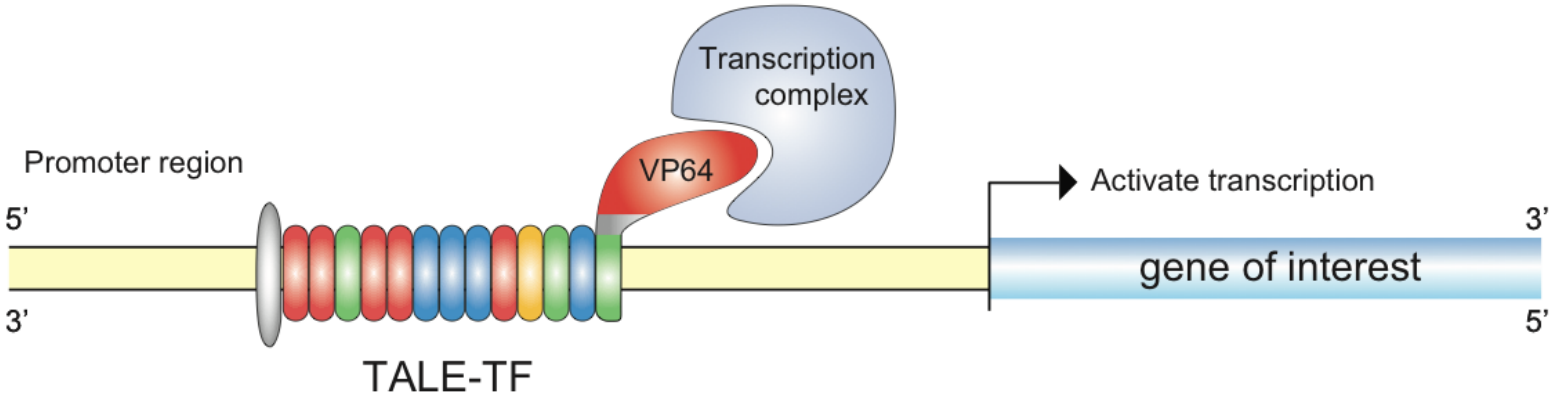
TALEs: Programmable, modular DNA binding domain

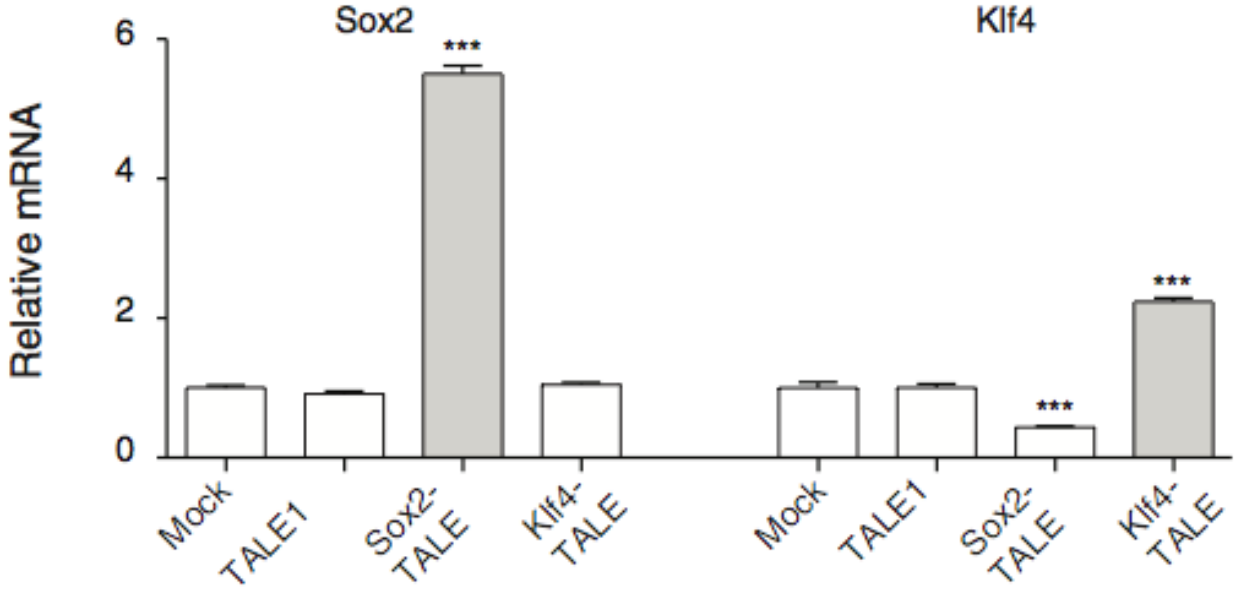




Deng, D. *et al.* Structural basis for sequence-specific recognition of DNA by TAL effectors. *Science* **335**, 720–723 (2012).

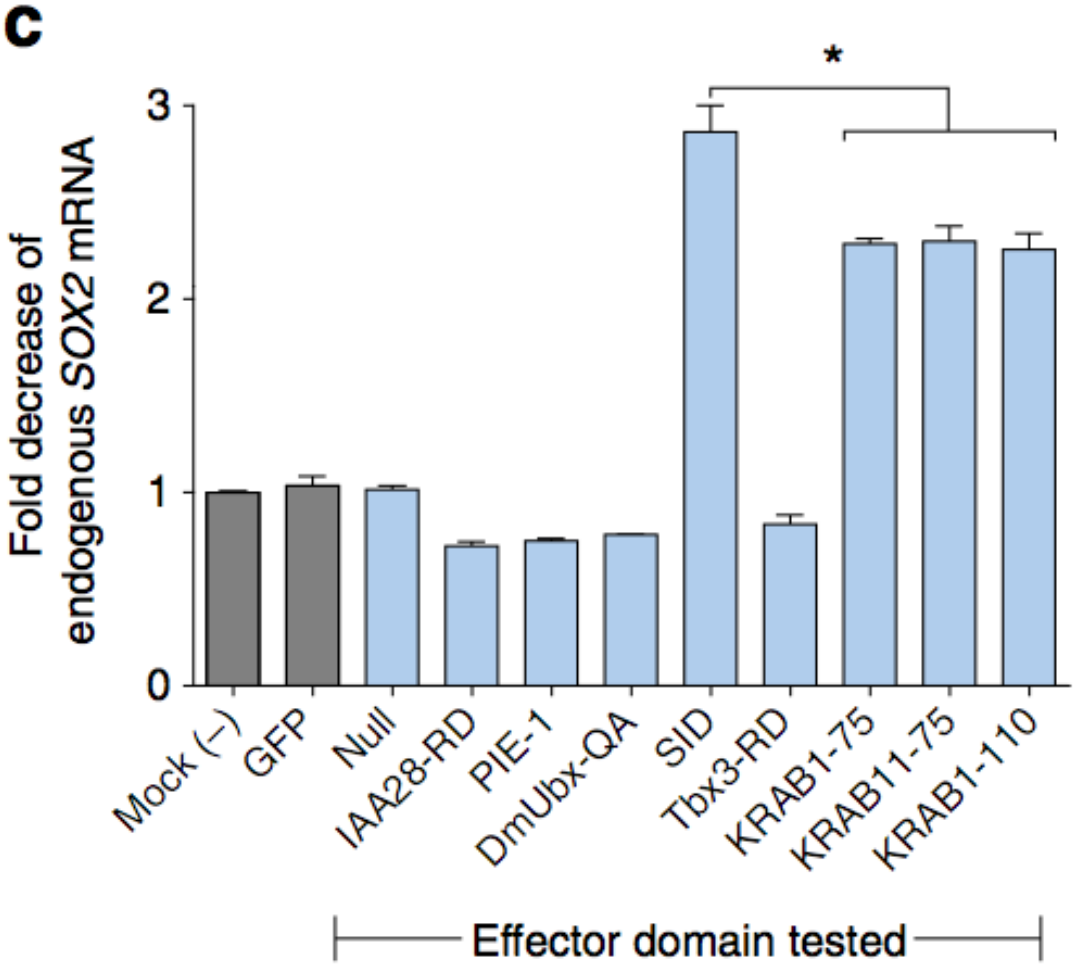
Transcriptional Activation





Zhang F, Cong L, Lodato S, Kosuri S, Church GM, Arlotta P. Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. Nat Biotechnol. 2011 Feb;29(2):149-53.

Transcriptional Repression



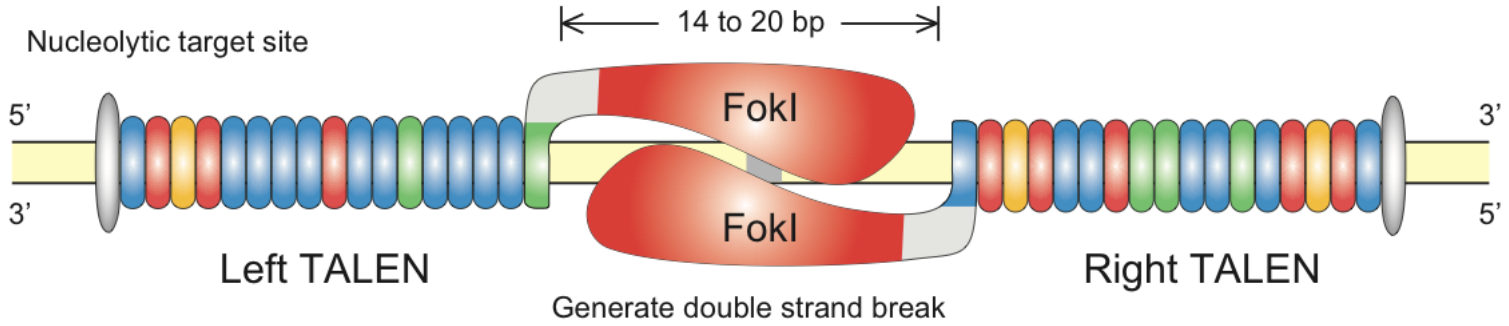
Le Cong, Zhou, R., Kuo, Y.-C., Cunniff, M. & Zhang, F. Comprehensive interrogation of natural TALE DNA-binding modules and transcriptional repressor domains. *Nature Communications* **3**, 968–6 (2012).

Complement existing tools

- ORF library & transcriptional activation
  - Level of expression not nearly as high; might that be better?
  - Not reliant on cloning the ORF (or linc, or miRNA, or…)
  - Capturing diversity of splice isoforms

- shRNAs & transcriptional repression
  - As yet, best shRNAs more potent
  - Different mechanisms for off-target effects

Permanent Genetic Changes



Error-prone NHEJ repair to create LOF mutants

or

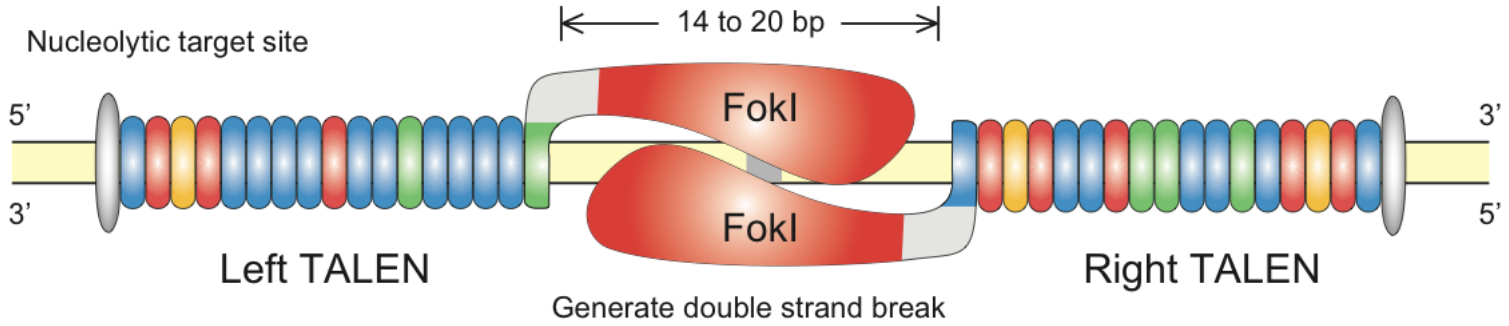
Homologous recombination with provided template

Table 1 TALEN-induced mutation frequencies for 96 endogenous human genes involved in cancer and epigenetic regulation

Gene	Mean indel mutation frequency (%) ± s.e.m.	Gene	Mean indel mutation frequency (%) ± s.e.m.
ABL1	22.5 ± 7.1	*HOXC13*	10.5 ± 0.3
AKT2	14.1 ± 7.3	*HOXD11*	None
ALK	12.7 ± 2.9	*HOXD13*	None
APC	48.8 ± 9.8	*JAK2*	44.9 ± 16.9
ATM	35.5 ± 15.6	*KIT*	None
AXIN2	2.5 ± 0.6	*KRAS*	9.4 ± 0.9
BAX	14.7 ± 11.6	*MAP2K4*	11.9 ± 7.1
BCL6	14.9 ± 5.9	*MDM2*	33.0 ± 20.2
BMPR1A	50.4 ± 16.4	*MET*	40.4 ± 10.7
BRCA1	44.5 ± 15.5	*MLH1*	44.9 ± 6.3
BRCA2	41.6 ± 10.5	*MSH2*	27.5 ± 10.4
CBX3	35.2 ± 22.6	*MUTYH*	24.9 ± 8.4
CBX8	13.5 ± 3.4	*MYCL1*	17.3 ± 0.6
CCND1	40.5 ± 2.2	*MYC*	13.4 ± 4.0
CDC73	36.3 ± 7.7	*MYCN*	16.3 ± 11.6

Reyon, D. *et al.* FLASH assembly of TALENs for high-throughput genome editing. *Nat Biotechnol* **30**, 460–465 (2012).

Permanent Genetic Changes



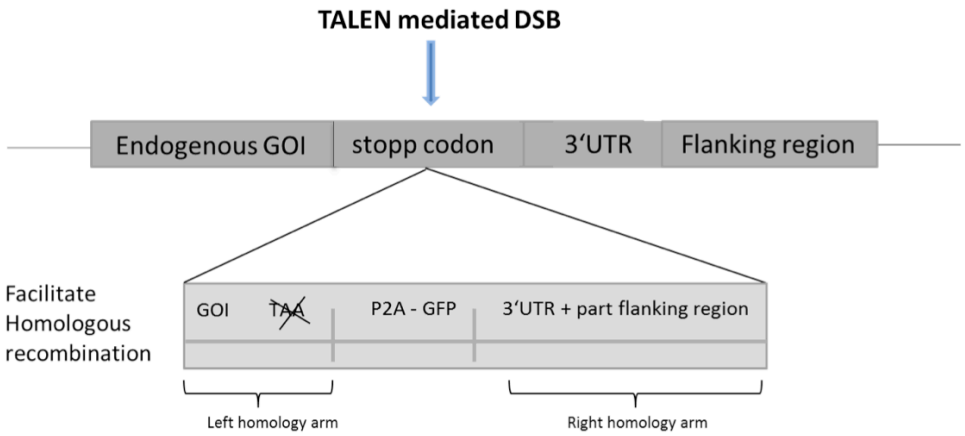
Error-prone NHEJ repair to create LOF mutants

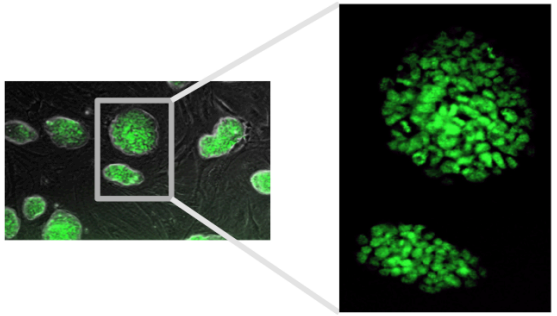
or

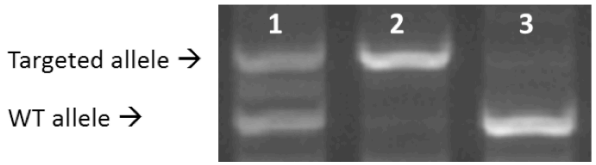
Homologous recombination with provided template

- Introduce small variant
  - GWAS hit, cancer mutation
- Tag endogenous protein
  - ChIP-Seq at endogenous expression level with FLAG tag
- Create reporter cells that maintain endogenous control

Fluorescent reporter lines for genes of interest







- **Make gene casette**
  - homology arms of around 850bp
- **Electroporation**
  - 10 million cells + 35µg of GC + 15µg TALEN
- **FACS**
  - FACS sorting for GFP positive cells, plate in a 6 well dish
- **Pick colonies**
  - Pick individual colonies, expand them for PCR screening
- **PCR screening**
  - Screen clones for allelic integration of the gene cassette

60 GFP positive colonies were picked

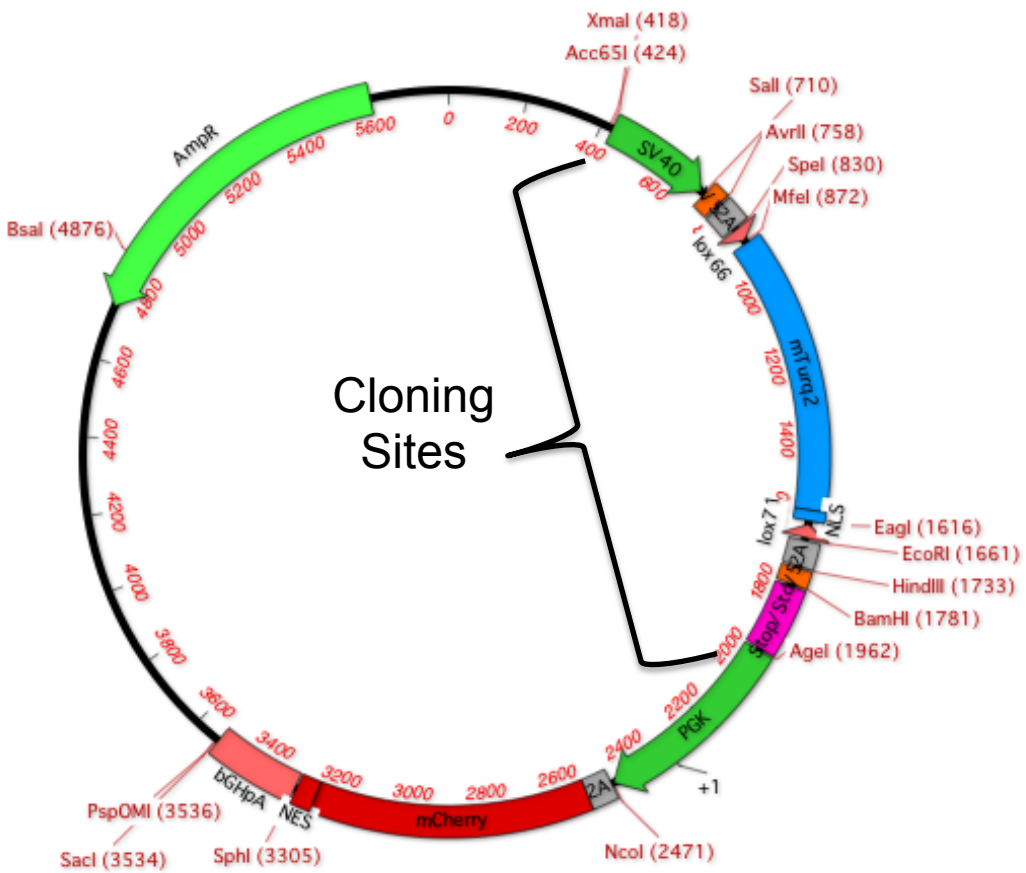
Monoallelic integration of gene cassette: 58 clones

Biallelic integration of gene casette: 2 clones

Random integration: 0

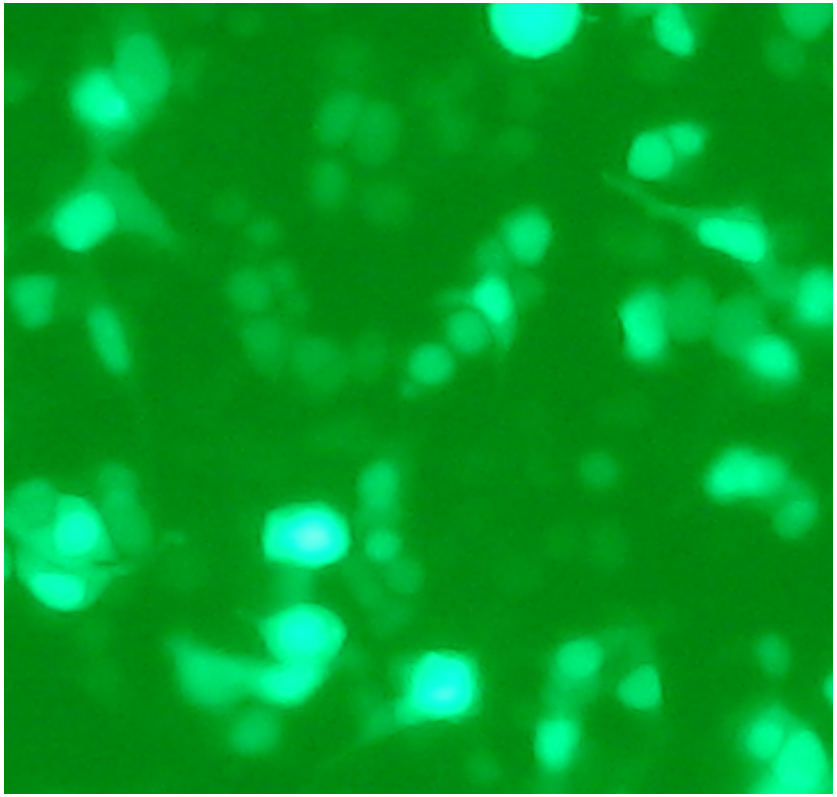
Christina Galonska, Meissner lab

Repair templates

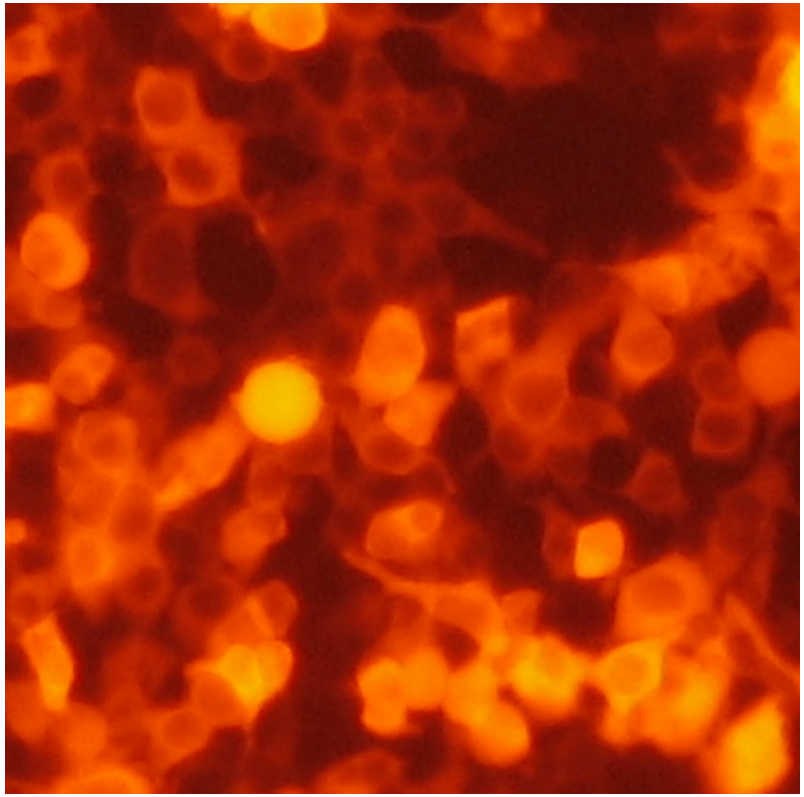


- <u>mTurquoise2</u> has no ATG; relies on proper targeting for expression: **positive selection**
- <u>PGK-mCherry</u> cassette outside of homologous arm cloning sites; cells will only appear red if construct inserted randomly: **negative selection**

Pretty pictures

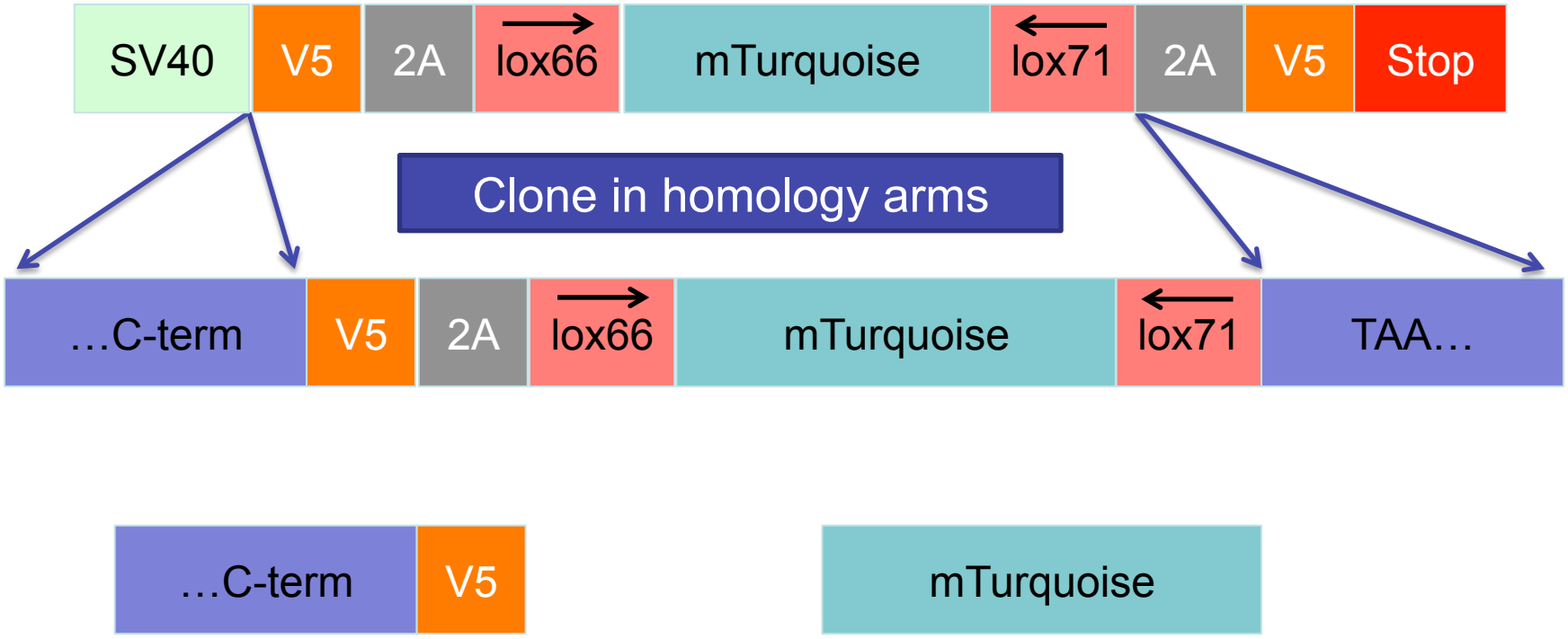


mTurqoise expression (w/ GFP filter)



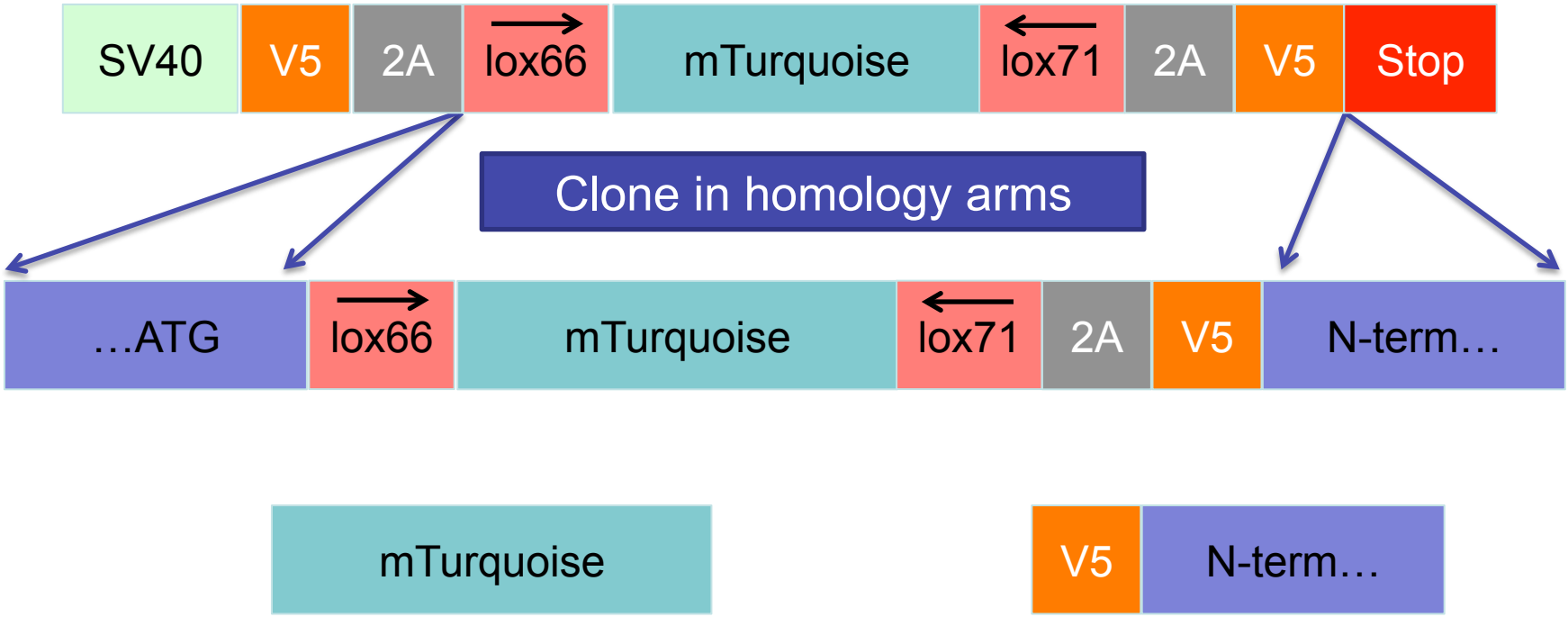
mCherry expression (w/ Rhodamine filter)

C-terminal tagging:
V5 tag and cyan cells



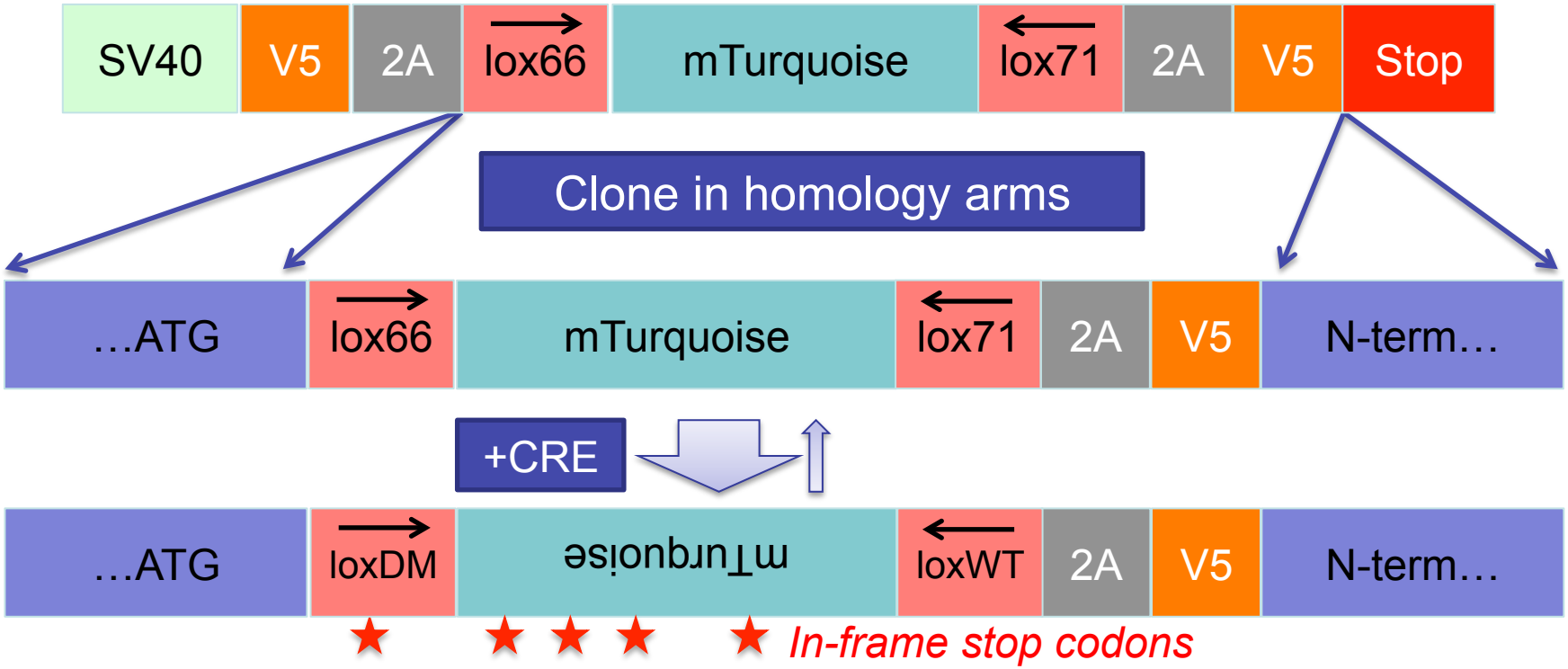
Tagged protein expressed at endogenous levels with endogenous splice isoforms, *and* a reporter for expression

N-terminal tagging: V5 tag and cyan cells



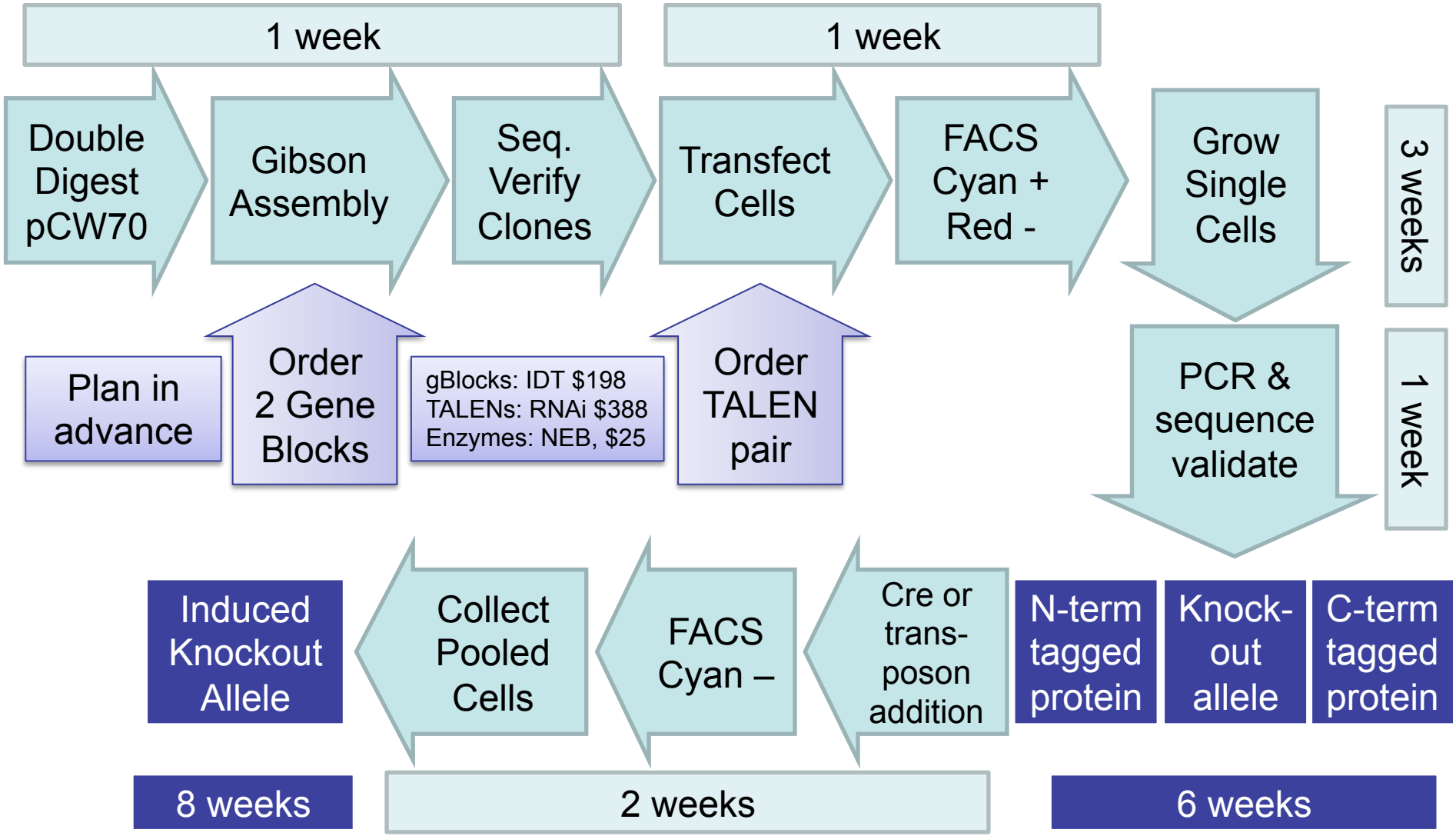
Tagged protein expressed at endogenous levels with endogenous splice isoforms, *and* a reporter for expression

Inducible Knockout:
Add Cre to N-term. tagged



Biallelic targeting possible in one-step (1 – 5% of clones)
Or, TALEN-induced NHEJ-created frame-shift on other allele

Workflow


1 week
1 week
Double Digest pCW70
Gibson Assembly
Seq. Verify Clones
Transfect Cells
FACS Cyan + Red -
Grow Single Cells
3 weeks
Plan in advance
Order 2 Gene Blocks
gBlocks: IDT $198
TALENs: RNAi $388
Enzymes: NEB, $25
Order TALEN pair
PCR & sequence validate
1 week
Induced Knockout Allele
Collect Pooled Cells
FACS Cyan –
Cre or trans-poson addition
N-term tagged protein
Knock-out allele
C-term tagged protein
8 weeks
2 weeks
6 weeks

Variants

- Replace (or add to, with another 2A site) mTurquoise with:
  - Gaussia luciferase, to allow for sensitive, array-based live-cell assay for incorporation and gene expression
  - BlasticidinR (or whatever) to allow for selection with drug
  - Venus, if mTurquoise already in use elsewhere
- Replace mCherry with thymidine kinase, to allow for negative selection with ganciclovir
- Replace inverted lox variants with same-direction wt loxP sites, to remove mTurqoise post-selection
  - Would enable double-targeting with same vector
- SV40 promoter used in cases where target gene is expressed at low levels: still can sort on cyan+/red-, but no longer a reporter for endogenous gene expression

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