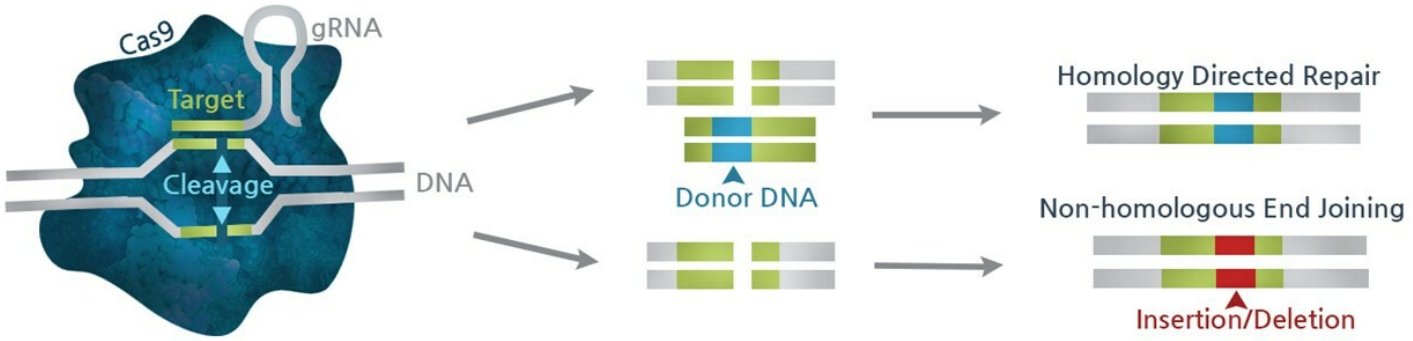
Gene editing using CRISPR technology

The adaptation of the CRISPR-Cas9 system for gene editing in mammalian cells has rapidly evolved to become a mainstream technology. The most important advantages of CRISPR-Cas9 over other gene editing techniques are speed and efficiency. This powerful tool holds great potential in the areas of *in vitro* diagnostics and therapeutic applications.

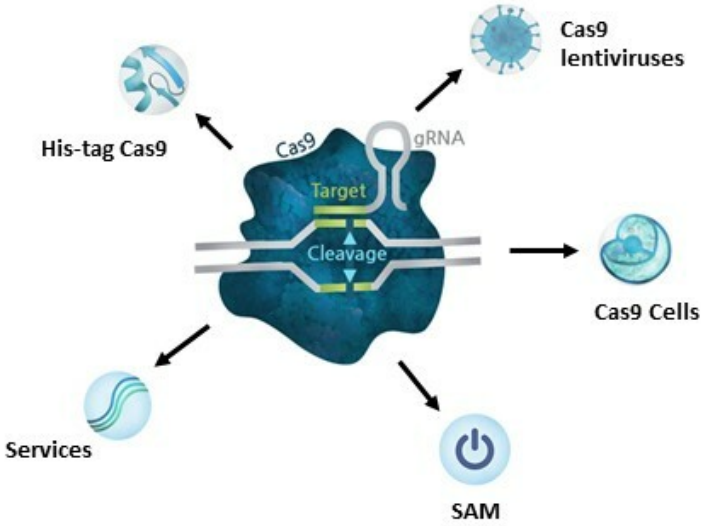


Introduction

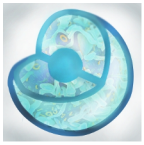
CRISPR-Cas9 technology is adapted from the antiviral innate immune response of bacteria, which capture and store DNA fragments from invading viruses within a region of their genome. These CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) sequences help protect the bacteria from future infections: when the bacteria is infected by a virus with a DNA sequence that is complementary to the CRISPR guides, the Cas9 (CRISPR-associated protein 9) nuclease is recruited to specifically cleave the invading DNA, resulting in its degradation.

Mammalian CRISPR-Cas9 gene editing systems exploit the ability of Cas9 to use CRISPR sequences as a guide to recognize and cleave complementary strands of DNA. By expressing the Cas9 nuclease in mammalian cells and introducing a single guide RNA sequence (sgRNA) specific for a gene of interest, one can force Cas9 recruitment to the target DNA sequence to introduce double-stranded breaks into genomic DNA. In mammalian cells, this double-stranded break is most commonly repaired through Non-Homologous End Joining (NHEJ), which causes the deletion or insertion of several base pairs at the cut site, often resulting in a frameshift and in the functional inactivation of the targeted gene. Alternatively, the Homologous Recombination (HR) system may be engaged in the repair, which can be exploited by providing a template DNA to generate knock-in mutations or introduce tags.

BPS Bioscience offers several off-the-shelf product lines and custom services to help scientific projects from start to finish.



- **Recombinant Cas9: optimize sgRNA**
- **Cas9-expressing cells**
- **Cas9 lentivirus: generate knock-out cell lines and cell pools**
- **Integrating and non-integrating lentivirus pairs: target-optimized**
- **CRISPRa (SAM): engineer robust protein overexpression**
- **Custom services**



Cas9-Expressing Cells

Cas9-expressing cells represent a cost-effective platform to generate knock-outs, knock-ins, or to perform CRISPR screens. The Cas9 recombinant protein contains a nuclear localization signal (NLS) to ensure proper sub-cellular localization and a C-terminal FLAG-tag to facilitate detection. Genome editing in these cells is simplified because the delivery of Cas9 is not necessary, resulting in increased transfection efficiency of sgRNA and/or donor DNA.

The following 10 cell lines or pools are currently available (Table 1).

Cell name	Pool	Cas9 low	Cas9 high
MDA_MB-231	78069	78150-L	78150-H
Jurkat	78070	78136-L	78136-H
A459	78072	78134-L	78134-H
HCT116	78073	78135-L	78135-H
RaJI	78071		78156
HeLa	78161		
Daudi	78089		78157
Neuro-2A	78087	78137-L	78137-H
MCF-7	78179		
HEK293			78166

Cell lines may display high or low Cas9 expression: high expression is expected to increase efficiency and reduce time to results, whereas low expression minimizes the occurrence of off-target effects. Cell lines are generated by limiting dilution of the original Cas9-expressing cell pool followed by isolation of individual clones, which are screened based on Cas9 expression, confirmed by flow cytometry (Figure 2).

Potential applications include fast generation of knock-out or knock-in cells for target validation, drug discovery and development, or CRISPR screening for functional gene studies.

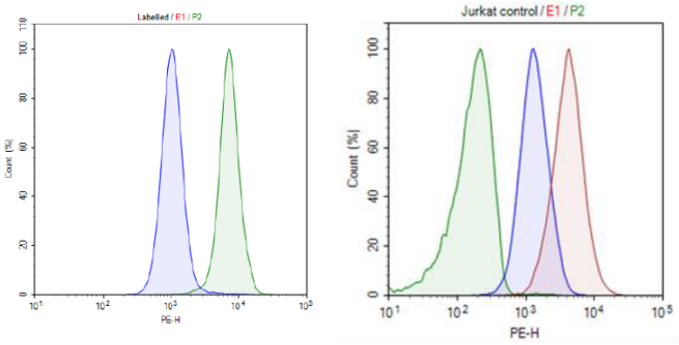


Figure 2: Analysis of Cas9 expression in cell lines or cell pools. Cells were stained with PE-labeled anti-FLAG antibody and analyzed by flow cytometry. The left panel shows Cas9 expression in the Neuro-2A cell pool. Parental cells (blue), are compared to the Cas9 cell pool (green). The right panel shows Cas9 expression in Jurkat cell lines. Parental cells (green), are compared to low-expressing cells (blue) and high-expressing cells (red).

For example, **TCR-knock-out Jurkat cells** were established using Cas9-expressing Jurkat cells, as shown in Figure 3.

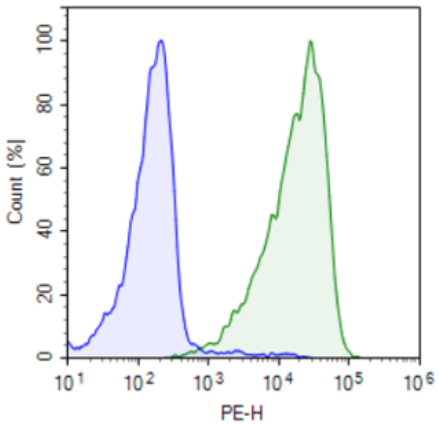
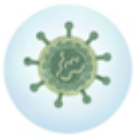


Figure 3: Analysis of TCR expression following knock-out in Cas9-expressing Jurkat cells. Cas9-expressing cells (BPS Bioscience, #78136-H) were electroporated with 0.1 nmol TCR sgRNA, and TCR expression was analyzed by flow cytometry 72 hours later. Cas9 expression is shown in green in the parental cells and in blue in the TCR knock-out cell pool.



Cas9 Lentiviruses

Cas9 Lentiviruses are replication incompetent, VSV-G pseudotyped lentiviral particles that are ready to transduce most types of mammalian cells, including primary and non-dividing cells. None of the HIV replication genes can be expressed in the transduced cells, therefore these lentiviruses require only a Biosafety Level 2 facility. The particles contain a Cas9-NSL-Flag gene driven by an EF1a promoter along with an antibiotic selection marker, typically hygromycin or puromycin.

The integrating Cas9 lentivirus can be used to generate Cas9 expressing cells. Thus, it was used to generate most of the Cas9-expressing cells described in Table 1. Once stable Cas9-overexpressing cells have been generated, they can be transduced or electroporated with sgRNAs targeting a gene of interest to quickly achieve the desired genetic modification.

Pre-validated lentiviruses, currently available to target immune checkpoint regulators, allow for dual expression of Cas9 and target-specific sgRNAs. These integrating lentiviruses contain a Cas9-NLS-Flag gene driven by an EF1a promoter, along with 4 sgRNAs driven by a U6 promoter and a puromycin selection marker (Figure 4). Puromycin selection forces integration of the construct into the genome and results in sustained expression of both Cas9 and the sgRNA, which increases knock-down efficiency. Of note, efficiency also varies depending on the cell type and the gene of interest. Figure 5 illustrates the transient knock-down efficiency in LAG3 overexpressing Jurkat cells transduced with Cas9 and LAG3-targeting sgRNAs.

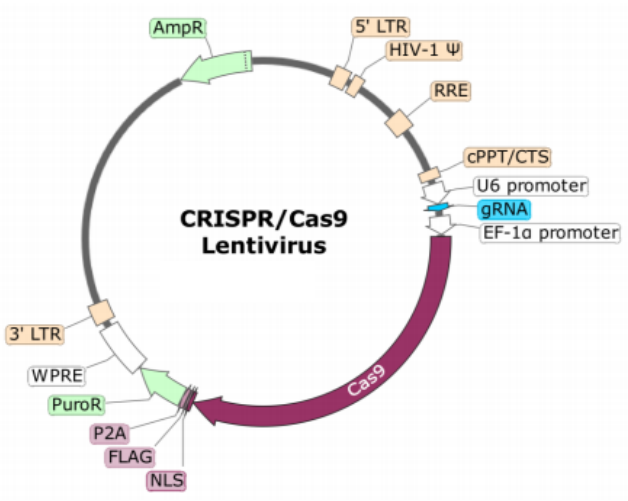


Figure 4: Vector used to generate a pre-validated, target-specific lentivirus.

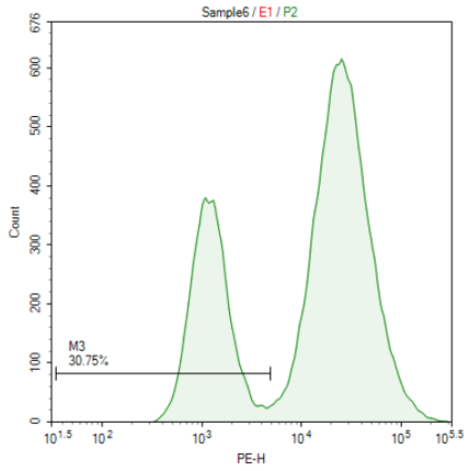
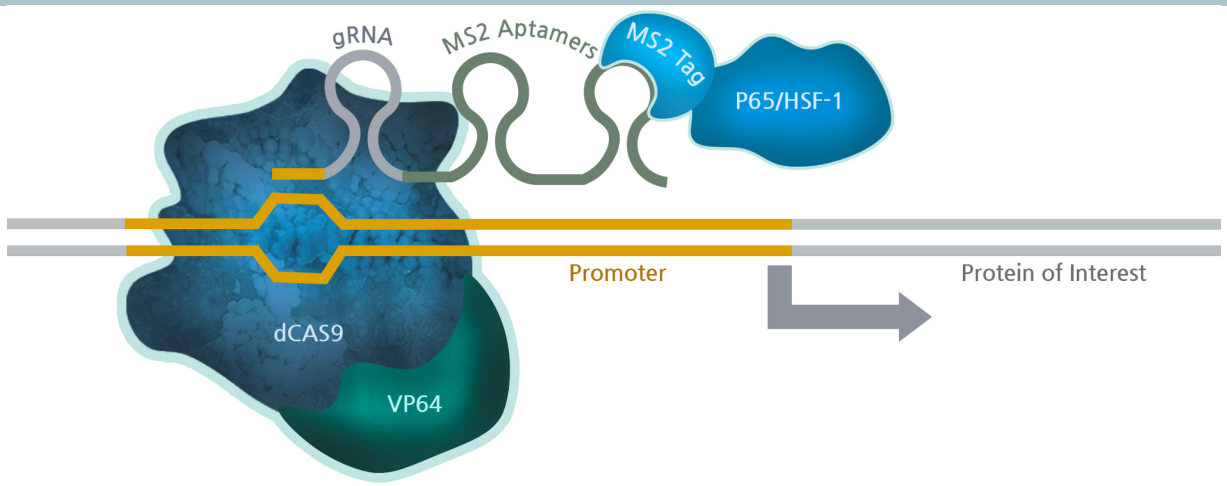


Figure 5: Knock-down of LAG3 in LAG3-expressing Jurkat cells. LAG3 over-expressing Jurkat cells (BPS Bioscience, #79813) were transduced via spinoculation with 5,000,000 TU/well of LAG3 CRISPR/Cas9 lentivirus (BPS Bioscience, #78053). 72 hours after transduction, cells were stained with PE anti-human LAG3 antibody and analyzed by flow cytometry. M3 gates the population of cells in which LAG3 is knocked-down.

A consideration to keep in mind when establishing stable Cas9 cell lines is that integration into the genome occurs randomly and may occasionally disrupt a biologically relevant gene. In addition, sustained expression of Cas9 may lead to the accumulation of off-target cleavage events over time, with unpredictable effects not imputable to the function of the target gene. Our integrating/non-integrating virus pairs were explicitly designed to control for this. The non-integrating virus was engineered with the same plasmid construct as the integrating virus, but with a mutated integrase. The integrase-deficient virus permits transient Cas9 and sgRNA expression in the target cell, but not stable expression. This may result in lower knock-down efficacy compared to an integrating lentivirus; however, it eliminates the risk of random insertion while considerably diminishing the occurrence of off-target cleavage. Pre-validated integrating and non-integrating sgRNA/Cas9 lentiviruses pairs have been designed for TCR (T-cell receptor) and for a growing list of immune checkpoint inhibitors (LAG3; PDL1; PD1; CD47; TIGIT; CTLA4).



CRISPR Synergistic Activation Mediator (SAM)

The CRISPR(SAM) system is an astute combination of Cas9 and molecular biology tools engineered to activate the transcription of any endogenous gene of interest. The system comprises 3 components that form a DNA-binding complex once introduced inside cells. The first component is a mutated dCas9 lacking endonuclease activity, fused to transcriptional activator VP64 typically composed of four tandem copies of VP16 (Herpes Simplex Viral Protein 16, amino acids 437-447) connected with glycine-serine linkers.

The other two components exploit the unique MS2 bacteriophage protein/RNA interaction system in which the coat protein of the bacteriophage binds tightly and specifically to a distinct 19-nucleotide RNA aptamer. Thus, in the second component of SAM, MS2 aptamers forming a characteristic stem loop structure are added to the single guide RNA. The sgRNA-MS2 component forms a complex with dCas9 and directs it to the target DNA sequence next to the promoter region of the gene of interest. The sgRNA-MS2 also recruits the third SAM component consisting of transcriptional activators P65 (Nuclear Factor NF-κ-B p65) and HSF1 (Heat Shock Factor 1) fused with the MS2 coat protein. These synergize with VP64 to robustly activate transcription of the target gene, up to a hundred-fold depending on the gene.

CRISPR Activation (CRISPRa) Cell Lines

CRISPRa(SAM) cells stably express two of the required SAM components: the mutated dCas9 fused to VP64 and the p65/HSF1/MS2 tag construct. When the sgRNA-MS2 aptamer targeting the promoter of a gene of interest is introduced into the cells via electroporation, transfection or transduction, the dCas9-VP64 and MS2-P65-HSF1 are recruited to induce transcription of the desired gene.

For example, the PD1 (Programmed Cell Death 1) plasmid (BPS Bioscience #78091) encodes 5 validated sgRNAs to ensure robust expression of PD1. Transfection of CRISPRa(SAM) Jurkat cells with this plasmid led to high levels of PD1 expression (Figure 6).

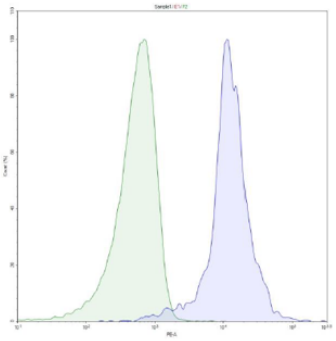


Figure 6: Induction of PD1 in CRISPRa (SAM) Jurkat cells. Cells were electroporated with the sgRNA-MS2 targeting PD1. Cells were stained with PE-labeled anti-PD1 antibody and analyzed by flow cytometry. Parental CRISPRa (SAM) Jurkat cells are shown in green, PD1-transfected cells are shown in blue.

CRISPR Activation (CRISPRa) components

Replication-incompetent, integrating, VSV-G pseudotyped lentiviruses are ready to transduce SAM components in mammalian cells to generate new stable CRISPRa cells. They contain constructs dCas9-VP64 and MS2-P65-HSF1 with blasticidinand hygromycin resistance, respectively. They are used in conjunction with the sgRNA-MS2 Activating Lentiviruses or withsgRNA-MS2 plasmids, which contain a pool of validated sgRNAs targeting the promoter region of a gene of interest, fused to MS2 aptamers and driven by a U6 promoter.

CRISPR Kinase Knockout Library, Array or Pool

Loss of function or CRISPR knock-out screens can be a powerful tool to identify potential new drug targets or signaling partners. The CRISPR system is characterized by high knock-down efficiency, often allowing clearer interpretation of results compared to shRNA or siRNA screens.

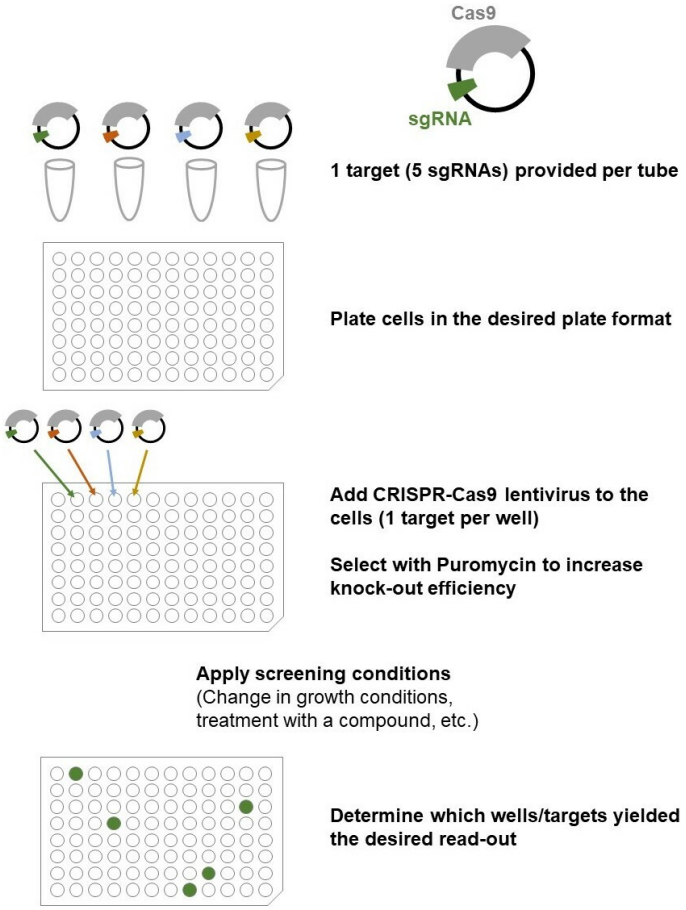
A CRISPR Knockout library targeting all human kinases is available in both array (one gene/well) and pooled (entire library) formats. This library was built using replication incompetent, VSV-G pseudo-typed integrating lentiviruses that are ready-to-use and can transduce a wide range of mammalian cells, including primary and non-dividing cells. The lentiviral particles do not allow for replication and do not contain HIV genes such as rev, gag and pol, therefore they can be used in a biosafety level 2 facility.

Advantages

Each lentivirus particle contains a Cas9 gene driven by an EF1a promoter, an sgRNA driven by a U6 promoter, along with a puromycin selection marker to enable the generation of stable knock-out cells. Since the lentivirus transduces Cas9, there is no requirement for the target cell to already be expressing Cas9. In addition, each sgRNA lentivirus is individually constructed, sequence-verified, individually cultured and titered to ensure high quality and representation across the entire library.

This CRISPR-Cas9 library targets 619 pseudo-kinase or kinase-coding genes, includes a set of 5 sgRNAs for each target gene and 150 control sgRNAs that do not target any gene, for a total of 3,245 sgRNAs.

Since the array format consists of individual genes, scientists may purchase only one gene of interest, or a subset of the library depending on their needs. The pooled format is delivered at a high titer of lentivirus and provides a cheaper alternative than the complete library in the array format, although it requires screening capabilities.



CRISPR array library

Conclusion and custom services

While the CRISPR-Cas9 gene editing system is at the forefront of innovation in molecular biology, it has limitations. Editing the DNA to create desired mutations remains complicated to orchestrate and can be time-consuming. Whether scientists need to build their own CRISPR tool or wish to save time and money using off-the-shelf products, BPS Bioscience can help. Our customized Custom Development Services will support diverse fields of research within a broad range of technologies and tools, from design to final product.

CRISPR Kinase Knockout Library

List of Genes

A	A	C	C	D	E	G	K	M	M
AAK1	AURKA	CAMK2B	CHEK1	DCAMKL2	Erk3ps1	GSK3A	KALRN	MAK	MAPK14
AATK	AURKB	CAMK2D	CHEK2	DCAMKL3	Erk3ps2	GSK3B	KDR	MAP2K1	MAPK15
ABL1	AURKC	CAMK2G	CHK2ps1	DDR1	Erk3ps3	GUCY2C	KIT	MAP2K1ps	MAPK3
ABL2	AXL	CAMK4	CHK2ps2	DDR2	Erk3ps4	GUCY2D	KSGCps	MAP2K2	MAPK4
ACVR1		CAMKK1	CHUK	DMPK	ERN1	GUCY2F	KSR1	MAP2K2ps	MAPK6
ACVR1B	B	CAMKK2	CIT	DYRK1A	ERN2		KSR2	MAP2K3	MAPK7
ACVR1C	BCKDK	CAMKV	CK1aps1	DYRK1B				MAP2K4	MAPK8
ACVR2A	BCR	CASK	CK1aps2	DYRK2		H		MAP2K5	MAPK9
ACVR2B	BLK	CCRK	CK1aps3	DYRK3	F	HCK	L	MAP2K6	MAPKAPK2
ACVRL1	BMP2K	CDC2	CK1g2ps	DYRK4	FASTK	HIPK1	LATS1	MAP2K7	MAPKAPK3
ADCK1	BMPR1A	CDC2L2	CK2a1-rs		FER	HIPK2	LATS2	MAP3K1	MAPKAPK5
ADCK2	BMPR1Aps1	CDC2L5	CLK1		FERps	HIPK3	LCK	MAP3K10	MAPKAPKps1
ADCK4	BMPR1Aps2	CDC2L6	CLK2	E	FES	HIPK4	LIMK1	MAP3K11	MARK1
ADCK5	BMPR1B	CDC42BPA	CLK2ps	EEF2K	FGFR1	HRIps	LIMK2	MAP3K12	MARK2
ADRBK1	BMPR2	CDC42BPB	CLK3	EGFR	FGFR2	HSPB8	LIMK2ps	MAP3K13	MARK3
ADRBK2	BMX	CDC42BPG	CLK3ps	EIF2AK1	FGFR3	HUNK	LMTK2	MAP3K14	MARK4
AKT1	BRAF	CDC7	CLK4	EIF2AK2	FGFR4		LMTK3	MAP3K15	MARKps01
AKT2	BRAFps	CDK10	CRKRS	EIF2AK3	FGR		LRRK1	MAP3K2	MARKps02
AKT3	BRD2	CDK2	CSF1R	EIF2AK4	FLT1	I	LRRK2	MAP3K21	MARKps03
ALK	BRD3	CDK3	CSK	EPHA1	FLT1ps	ICK	LTK	MAP3K3	MARKps04
ALPK1	BRD4	CDK4	CSNK1A1	EPHA10	FLT3	IGF1R	LYK5	MAP3K4	MARKps05
ALPK2	BRDT	CDK4ps	CSNK1A1L	EPHA2	FLT4	IKBKB	LYN	MAP3K5	MARKps07
ALPK3	BRSK1	CDK5	CSNK1D	EPHA3	FRAP1	IKBKE		MAP3K6	MARKps08
ALS2CR2	BRSK2	CDK5ps	CSNK1E	EPHA4	FRK	ILK		MAP3K7	MARKps09
ALS2CR7	BTK	CDK6	CSNK1G1	EPHA5	FYN	INSR		MAP3K8	MARKps10
AMHR2	BUB1	CDK7	CSNK1G2	EPHA6		INSRR		MAP3K9	MARKps11
ANKK1	BUB1B	CDK7ps	CSNK1G3	EPHA7		IRAK1		MAP4K1	MARKps12
ARAF		CDK8	CSNK2A1	EPHA8	G	IRAK2		MAP4K2	MARKps13
ARAFps		CDK8ps	CSNK2A2	EPHB1	GAK	IRAK3		MAP4K3	MARKps15
ATM	C	CDK9		EPHB2	GPRK6ps	IRAK4		MAP4K4	MARKps16
ATR	C9orf96	CDKL1		EPHB3	GRK1	ITK		MAP4K5	MARKps17
AurA	CABC1	CDKL2	D	EPHB4	GRK4			MAPK1	MARKps18
AurAps1	CAMK1	CDKL3	DAPK1	EPHB6	GRK5	J		MAPK10	MARKps19
AurAps2	CAMK1D	CDKL4	DAPK2	ERBB2	GRK6	JAK1		MAPK11	MARKps20
AurB	CAMK1G	CDKL5	DAPK3	ERBB3	GRK7	JAK2		MAPK12	MARKps21
AurBps1	CAMK2A	CGDps	DCAMKL1	ERBB4	GSG2	JAK3		MAPK13	MARKps22

* ps = pseudo-kinase

CRISPR Kinase Knockout Library

List of Genes

M	N	P	P	P	R	S	T	T	Y
MARKps23	NEK1	p70S6Kps1	PKMYT1	PRP4ps	RPS6KA5	SRPK2ps	TAOK3	TSSKps1	YES1
MARKps24	NEK10	p70S6Kps2	PKN1	PRPF4B	RPS6KA6	SRPK3	TBCK	TSSKps2	YESps
MARKps25	NEK11	PAK1	PKN2	PSKH1	RPS6KB1	STK10	TBK1	TTBK1	YSK4
MARKps26	NEK2	PAK2	PKN3	PSKH1ps	RPS6KB2	STK11	TEC	TTBK2	
MARKps27	NEK2ps1	PAK2ps	PLK1	PSKH2	RPS6KC1	STK16	TEK	TTK	
MARKps28	NEK2ps2	PAK3	PLK1ps1	PTK2	RPS6KL1	STK17A	TESK1	TTN	Z
MARKps29	NEK2ps3	PAK4	PLK1ps2	PTK2B	RSKR	STK17B	TESK2	TXK	ZAK
MARKps30	NEK3	PAK6	PLK2	PTK6	RYK	STK19	TEX14	TYK2	ZAP70
MAST1	NEK4	PAK7	PLK3	PTK7	RYKps	STK24	TGFBR1	TYRO3	
MAST2	NEK4ps	PASK	PLK4	PXK		STK25	TGFBR2	TYRO3ps	
MAST3	NEK5	PBK	PNCK			STK26	TIE1		
MAST4	NEK6	PCTK1	POMK		S	STK3	TLK1		
MASTL	NEK7	PCTK2	PRAG1	R	SAKps	STK31	TLK1ps	U	
MATK	NEK8	PCTK3	PRKAA1	RAF1	SBK1	STK32A	TLK2	UHMK1	
MELK	NEK9	PDGFRA	PRKAA2	RAF1ps	SBK2	STK32B	TLK2ps1	ULK1	
MERTK	NIM1K	PDGFRB	PRKACA	RAGE	SCYL1	STK32C	TLK2ps2	ULK2	
MET	NLK	PDIK1L	PRKACB	RET	SCYL2	STK33	TNIK	ULK3	
MINK1	NPR1	PDK1	PRKACG	RIOK1	SCYL2ps	STK33ps	TNK1	ULK4	
MKNK1	NPR2	PDK2	PRKCA	RIOK2	SCYL3	STK35	TNK2		
MKNK2	NRBP1	PDK3	PRKCB1	RIOK3	SGK	STK36	TNNI3K	V	
MLKL	NRBP2	PDK4	PRKCD	RIOK3ps	SgK050ps	STK38	TP53RK	VRK1	
MNK1ps	NRK	PDPK1	PRKCE	RIPK1	SgK110	STK38L	TRIB1	VRK2	
MOS	NTRK1	PEAK1	PRKCG	RIPK2	SGK2	STK39	TRIB2	VRK3	
MPSK1ps	NTRK2	PFTK1	PRKCH	RIPK3	SGK3	STK4	TRIB3	VRK3ps	
MRCKps	NTRK3	PHKG1	PRKCI	RIPK4	SgK384ps	STK40	TRIM24		
MST1R	NUAK1	PHKg1ps1	PRKCQ	RIPK5	SgK424	STLK6ps1	TRIM28		
MST3ps	NUAK2	PHKg1ps2	PRKCZ	RNASEL	SIK3	STLK6-rs	TRIM33	W	
MUSK		PHKg1ps3	PRKD1	ROCK1	SLK	STYK1	TRIO	WEE1	
MYLK		PHKG2	PRKD2	ROCK2	SMG1	SYK	TRPM6	Wee1Bps	
MYLK2	O	PIK3R4	PRKD3	ROR1	SNF1LK		TRPM7	Wee1ps1	
MYLK3	OBSCN	PIM1	PRKDC	ROR2	SNF1LK2		TRRAP	Wee1ps2	
MYLK4	OXSR1	PIM2	PRKG1	ROS1	SNRK	T	TSSK1B	WEE2-AS1	
MYO3A		PIM3	PRKG2	RPS6KA1	SRC	TAF1	TSSK2	WNK1	
MYO3B		PINK1	PRKX	RPS6KA2	SRMS	TAF1L	TSSK3	WNK2	
		PKCips	PRKXps	RPS6KA3	SRPK1	TAOK1	TSSK4	WNK3	
		PKDCC	PRKY	RPS6KA4	SRPK2	TAOK2	TSSK6	WNK4	

* ps = pseudo-kinase