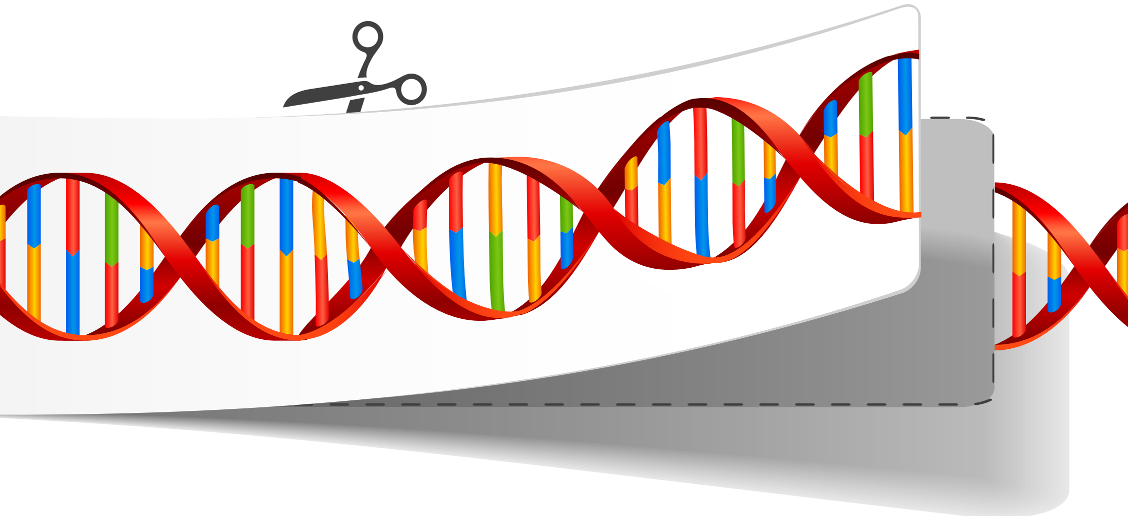
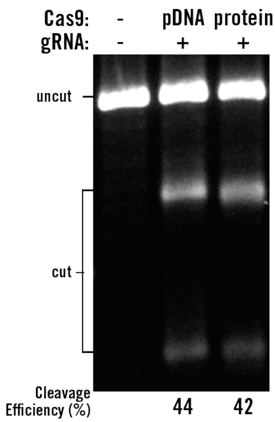


CRISPR/Cas9 Genome Editing: Transfection Methods

For over 20 years Mirus Bio has developed and manufactured high performance transfection products and technologies. That expertise is now being applied to the cutting edge of genome editing techniques, providing researchers delivery solutions for multiple CRISPR/Cas9 formats:

- **Plasmid DNA** – Simple, low cost approach
- **Messenger RNA** – Rapid gene expression, eliminates the risk of insertional mutagenesis
- **Cas9/gRNA RNP** – Rapid pulse of genome activity, reduces off-target cleavage



T7E1 cleavage efficiency in 293T/17 cells transfected with Cas9 pDNA/gRNA or Cas9 protein/gRNA (RNP) using *Trans*IT-X2® Dynamic Delivery System.

Delivery by Mirus

Delivery by Mirus

What is CRISPR/Cas9 Genome Editing?

The CRISPR/Cas9 system is a powerful tool for genome editing in mammalian cells that allows researchers to generate genetic variants at lower cost with higher throughput than alternative methods like zinc finger nuclease (ZFN) or transcription activator-like effector nuclease (TALEN) genome editing.

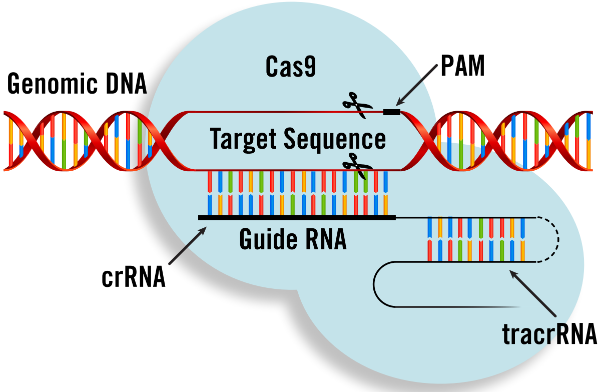


FIGURE 1. The CRISPR/Cas9 RNP Complex. The CRISPR associated protein 9 (Cas9) endonuclease (blue) is targeted to DNA by a guide RNA (gRNA), which can be supplied as a two-part system consisting of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) or as a single guide RNA (sgRNA), where the crRNA and tracrRNA are connected by a linker (dotted line). Target recognition is facilitated by the protospacer-adjacent motif (PAM). A double strand break (DSB) occurs 3 bp upstream of the PAM.

CRISPR Handles Multiple Types of Genome Modification

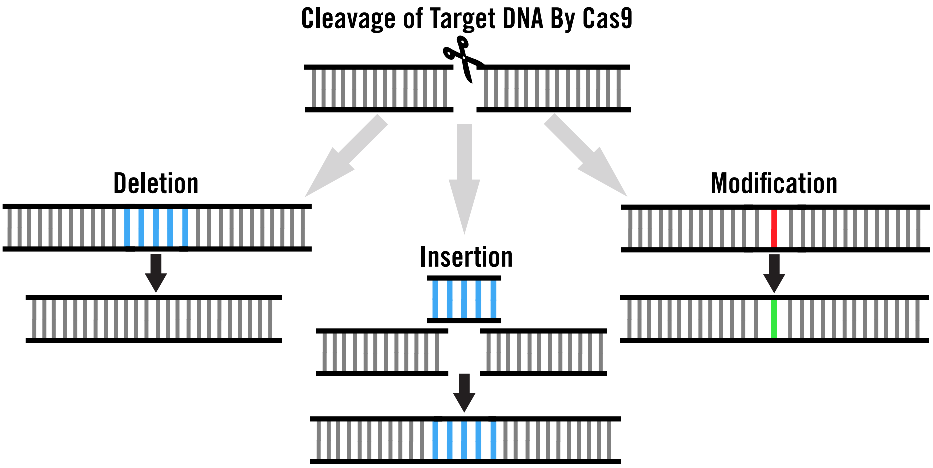


FIGURE 2. Multiple Genomic Alterations are Possible Following Cleavage of Target DNA by Cas9. Variable length insertions and/or deletions (indels) can result near the DNA break due to mistakes in DNA repair by the endogenous non-homologous end joining (NHEJ) pathway. These indels frequently result in disruption of gene function. Alternatively, by supplying a DNA repair template, researchers can leverage the homology-directed repair (HDR) pathway to create defined deletions, insertions or other modifications.

Glossary of CRISPR Terms

Term	Definition
Cas9	**C**RISPR **As**sociated Protein **9** - Cas9 is an RNA-guided DNA endonuclease from the type II CRISPR system of *Streptococcus pyogenes* that has been adapted for use in genome editing applications.
CRISPR	**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats - CRISPR refers to prokaryotic DNA elements involved in adaptive immunity which are characterized by clusters of identical repeats interspaced with non-identical segments called spacers. CRISPR has evolved to refer more generally to the use of Cas9 for genome editing.
crRNA	**CR**ISPR **RNA** - One of two RNAs required to form a functional gRNA. The crRNA contains the sequence complementary to the DNA target and a segment of RNA that base pairs with the tracrRNA.
DSB	**D**ouble **S**trand **B**reaks result from endonucleolytic cleavage of both strands of DNA. This can be achieved through the use of wild type Cas9 or by employing two Cas9 nickases targeting opposite DNA strands.
gRNA	**G**uide **RNA**s bind to Cas9 and direct the complex to a specific genomic location. Naturally occurring guide RNAs consist of two parts: a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA). Alternatively, the crRNA and tracrRNA can be combined into a single chimeric oligonucleotide called a single guide RNA (sgRNA).
HDR	**H**omology **D**irected **R**epair is a mechanism of DNA repair that uses a homologous DNA template to rebuild sites of genomic damage. HDR can be leveraged in genome editing experiments to make precise genomic alterations by supplying the desired sequence for insertion flanked by segments of DNA that are homologous to the sequence surrounding the Cas9-induced DSB.
indel	(**In**sertion/**Del**etion) Following creation of a DSB by Cas9, the cell initiates DNA damage repair. Repair by the error-prone NHEJ pathway can result in small insertions and/or deletions at the site of cleavage. These indels can cause frameshift mutations or premature stop codons resulting in a genetic knock-out.
Mismatch Assay	A method for detection of indel mutations following Cas9 cleavage. Targeted genomic DNA is amplified by PCR. The PCR products are melted and reannealed to allow heteroduplexes to form between wild-type and mutant DNA. The hybridized products are then incubated with an enzyme that cleaves heteroduplexes but not perfectly matched DNA. The resulting DNA fragments are analyzed by electrophoresis to determine the percentage of cleavage events that results from indel formation.
NHEJ	**N**on-**H**omologous **E**nd-**J**oining is the predominant DNA DSB repair mechanism in mammalian cells. Unlike HDR, NHEJ directly ligates the ends of the DSB and does not require a homologous repair template. Researchers capitalize on the error-prone nature of NHEJ to create indels following targeted cleavage with Cas9.
PAM	**P**rotospacer-**A**djacent **M**otif - In the naturally occurring prokaryotic CRISPR/Cas system, the DNA sequences recognized by gRNA are called protospacers. The PAM is a short sequence next to the target site that is required for Cas9 targeting both in prokaryotic adaptive immunity and in mammalian genome editing experiments.
sgRNA	**S**ingle **G**uide **RNA**, a chimeric RNA composed of crRNA and tracrRNA, connected by a short RNA linker.
Target Sequence	A 20 nucleotide genomic DNA sequence which base-pairs with gRNA and is cleaved by Cas9.
tracrRNA	**Tr**ans-**A**ctivating **crRNA** One of two RNAs required to form a functional gRNA. The tracrRNA forms base pairs with the crRNA and is required for Cas9-mediated target cleavage.

Comparison of Cas9 Formats: DNA, RNA and Protein

	pDNA	mRNA	Protein
High Efficiency	+	++	++++
Low Cost	++++	++	+
Specificity	+	++++	++++

FIGURE 3. Pros and Cons of DNA, RNA and Protein Formats for Genomic Editing. Cas9 can be delivered as plasmid DNA for a simple, low-cost approach. Cas9 mRNA enables rapid gene expression and eliminates the risk of insertional mutagenesis. Cas9/guide RNA ribonucleoprotein (RNP) complexes exhibit the most rapid pulse of genome editing activity and reduce the possibility of off-target cleavage events. Cas9 mRNA and RNP formats can also be efficiently delivered to cell types that are resistant to transfection with plasmid DNA.

CRISPR Gene Editing Workflow

DESIGN

- Identify target site
- Design gRNA

TRANSFECT

- Deliver Cas9 + gRNA

ANALYZE

- Enrich
- Screen

Mirus® Bio Transfection Solutions

***Trans*IT-X2® Dynamic Delivery System** - A novel, polymeric system for delivery of multiple nucleic acids to mammalian cells. Ideal for delivery of CRISPR/Cas9 components in DNA, gRNA and RNP formats.

***Trans*IT®-mRNA** - A high efficiency, low toxicity transfection reagent for mammalian cells. Designed to deliver CRISPR/Cas9 components in mRNA and gRNA formats.

Ingenio® Electroporation Solution - A high efficiency electroporation solution compatible with most conventional electroporation devices including Lonza-Amaxa®, Bio-Rad® or Harvard BTX®. Delivers CRISPR/Cas9 components in DNA, mRNA, gRNA, and RNP formats.

Plasmid DNA and Guide RNA Oligonucleotide Transfection

Cas9 protein and guide RNA can both be encoded by plasmid DNA for transfection. Alternatively, Cas9 can be delivered as plasmid DNA, and guide RNA can be supplied as an RNA oligonucleotide. Benefits of these approaches include:

- **Low Cost** - Plasmid DNA is a renewable, cost-effective format
- **Flexibility** - Cas9 and guide RNA plasmids are suitable for stable or transient transfection
- **Ease-of-use** - Guide RNA oligonucleotide format enables simple retargeting of Cas9 to different loci

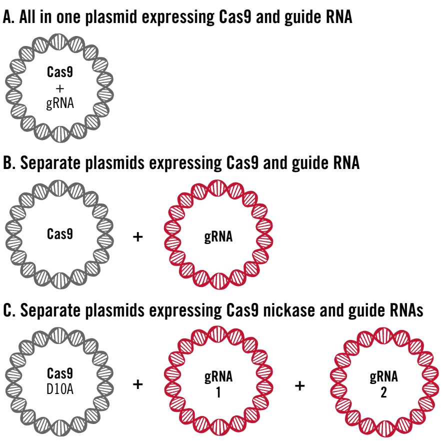
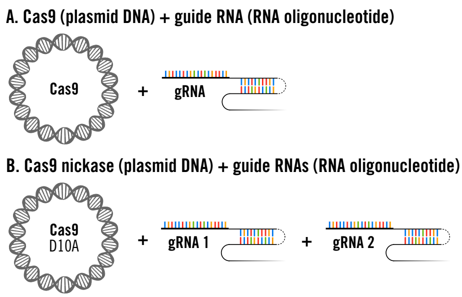


FIGURE 4. Cas9 + Guide RNA Plasmids. (A) Cas9 and guide RNA are encoded on the same plasmid. (B,C) Cas9 and guide RNA(s) are encoded on separate plasmids. (A,B) The wild-type Cas9 enzyme contains two endonuclease domains which cleave the target DNA on both strands when programmed with a guide RNA. (C) The D10A mutation converts Cas9 to a nickase that generates single-stranded breaks in the target DNA. For improved target specificity, Cas9 D10A can be used with paired guide RNAs targeting opposite strands to create staggered double-stranded breaks.

FIGURE 5. Cas9 Plasmid + Guide RNA Oligonucleotides. Cas9 is supplied as plasmid DNA, and guide RNA(s) are supplied as either synthetic or *in vitro* transcribed RNA oligonucleotides. (A) The wild-type Cas9 enzyme contains two endonuclease domains which cleave the target DNA on both strands when programmed with a guide RNA. (B) The D10A mutation converts Cas9 to a nickase that generates single-stranded breaks in the target DNA. For improved target specificity, Cas9 D10A can be used with paired guide RNAs targeting opposite strands to create staggered double-stranded breaks.



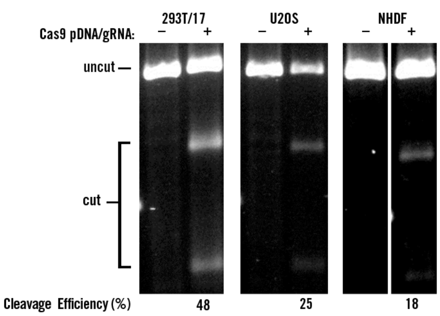


FIGURE 6. Efficient Genome Editing with Cas9 Plasmid DNA + Guide RNA Oligonucleotides. HEK293T/17, U2OS and NHDF cells were co-transfected with 0.5 µg of Cas9 encoding pDNA (MilliporeSigma) and 50nM PPIB targeting two-part gRNA (Dharmacon) using *Trans*IT-X2® Dynamic Delivery System (2 µl/well of a 24-well plate, Mirus Bio). A T7E1 mismatch detection assay was used to measure cleavage efficiency at 48 hours post-transfection.

mRNA and Guide RNA Oligonucleotide Transfection

In order to avoid off-target cleavage and unwanted genomic integration of plasmid DNA, Cas9-encoding mRNA can be co-transfected with guide RNA oligonucleotides. Benefits of RNA-based genome editing include:

- **High Specificity** - Rapid gene expression generates a transient pulse of genome editing activity
- **Ease-of-use** - Deliver mRNA and guide RNA with a single reagent
- **DNA Free** - No risk of insertional mutagenesis

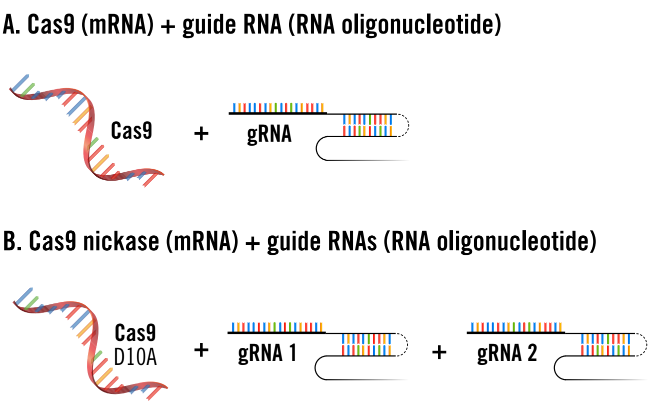


FIGURE 7. Cas9 mRNA + Guide RNA Oligonucleotides. Cas9 is supplied as messenger RNA, and guide RNAs are supplied as either synthetic or *in vitro* transcribed RNA oligonucleotides. (A) The wild-type Cas9 enzyme contains two endonuclease domains which cleave the target DNA on both strands when programmed with a guide RNA. (B) The D10A mutation converts Cas9 to a nickase that generates single-stranded breaks in the target DNA. For improved target specificity, Cas9 D10A can be used with paired guide RNAs targeting opposite strands to create staggered double-stranded breaks.

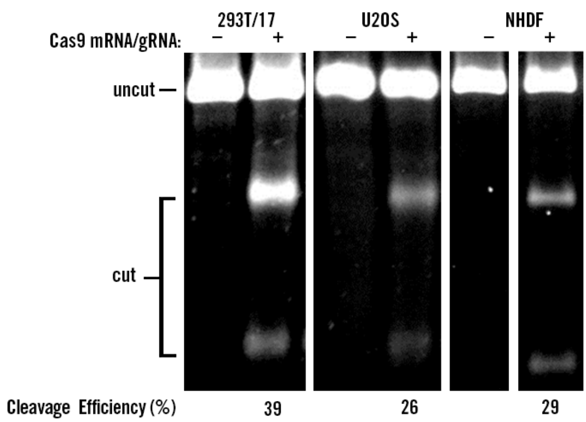


FIGURE 8. Efficient Genome Editing with Cas9 mRNA + Guide RNA Oligonucleotides. HEK293T/17, U2OS and NHDF cells were co-transfected with 0.5 µg of Cas9 encoding mRNA, 5meC, (Trilink Biotechnologies) and 25nM of PPIB targeting two-part gRNA (Dharmacon) using *Trans*IT®-mRNA Transfection Kit (0.5 µl/well of 24-well plate of both mRNA Reagent and Boost, Mirus Bio). A T7E1 mismatch detection assay was used to measure cleavage efficiency at 48 hours post-transfection.

Cas9/gRNA Ribonucleoprotein (RNP) Transfection

Purified Cas9 protein can be combined with guide RNA to form an RNP complex to be delivered to cells for rapid and highly efficient genome editing. Benefits of RNP-based genome editing include:

- **High Efficiency Delivery** - Deliver Cas9/gRNA complexes to multiple cell types, including hard to transfect cells such as immune and stem cells
- **High Specificity** - Pre-formed RNP complexes provide a rapid pulse of genome editing activity
- **DNA Free** - No risk of insertional mutagenesis

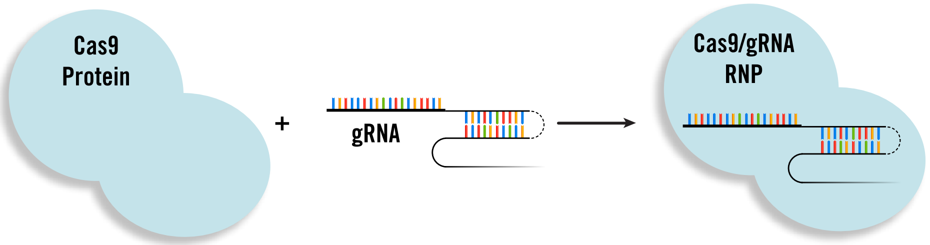


FIGURE 9. Cas9 RNP. Purified Cas9 protein and guide RNA oligonucleotides are combined to form a ribonucleoprotein (RNP) complex.

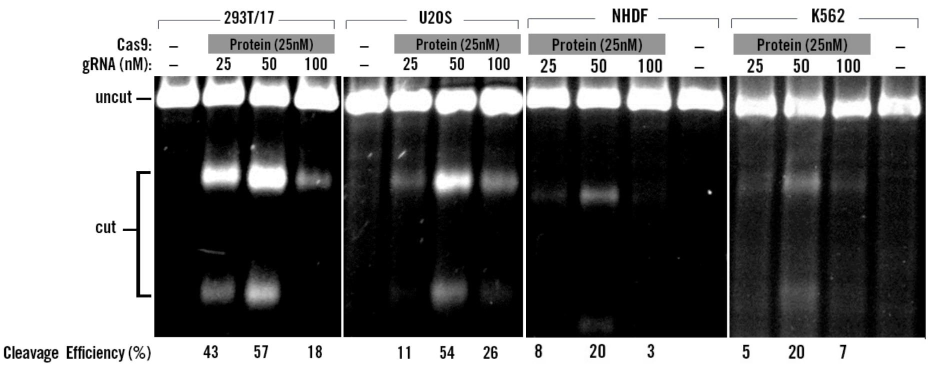


FIGURE 10. Genome Editing with Cas9 + Guide RNA Ribonucleoprotein Complexes. The RNP complex of PPIB targeting two-part gRNA (Dharmacon) and Cas9 protein (PNA Bio) was delivered into HEK293T/17, U2OS, NHDF and K562 cells using *Trans*IT-X2® Dynamic Delivery System (1 µl/well of a 24-well plate, Mirus Bio). A T7E1 mismatch detection assay was used to measure cleavage efficiency at 48 hours post-transfection. High levels of gene editing can be achieved in cells that were transfected with an RNP complex comprised of 50nM of gRNA and 25nM of Cas9 protein.

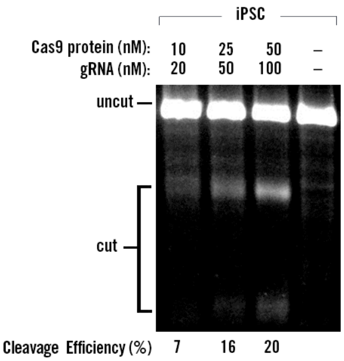


FIGURE 11. Genome Editing in IPS Cells with Cas9 + Guide RNA Ribonucleoprotein Complexes. *Trans*IT-X2® Dynamic Delivery System was used to deliver Cas9 protein/guide RNA ribonucleoprotein (RNP) complexes in human induced pluripotent stem cells (iPSCs). A T7E1 mismatch assay was used to measure cleavage efficiency at 48 hours post-transfection.

Delivery by Mirus

PRODUCT	DESCRIPTION	PRODUCT NO.	QUANTITY
*Trans*IT-X2® Dynamic Delivery System 	A novel, polymeric system for delivery of multiple nucleic acids to mammalian cells. Delivers CRISPR/Cas9 components in the following formats: • **DNA** -Deliver plasmid DNA expressing Cas9 or guide RNA • **Guide RNA** -Deliver gRNA oligonucleotides targeting your gene of interest • **RNP** -Deliver Cas/gRNA ribonucleoprotein complexes	MIR6003	0.3 ml
		MIR6004	0.75 ml
		MIR6000	1.5 ml
		MIR6005	5 x 1.5 ml
		MIR6006	10 x 1.5 ml
*Trans*IT®-mRNA Transfection Kit 	A high efficiency, low toxicity transfection reagent for mammalian cells. Delivers CRISPR/Cas9 components in the following formats: • **mRNA** -Deliver messenger RNA expressing Cas9 • **Guide RNA** -Deliver gRNA oligonucleotides targeting your gene of interest	MIR2225	0.4 ml
		MIR2250	1 ml
		MIR2255	5 x 1 ml
		MIR2256	10 x 1 ml
Ingenio® Electroporation Solution 	A high efficiency electroporation solution compatible with most conventional electroporation devices including Lonza-Amaxa®, Bio-Rad® or Harvard BTX®. Delivers CRISPR/Cas9 components in the following formats: • **DNA** -Deliver plasmid DNA expressing Cas9 or guide RNA • **mRNA** -Deliver messenger RNA expressing Cas9 • **Guide RNA** -Deliver gRNA oligonucleotides targeting your gene of interest • **RNP** -Deliver Cas9/gRNA ribonucleoptrotein complexes	Ingenio® Electroporation Kits for Amaxa Nucleofactor® II/2b Device (solution, 0.2 cm cuvettes, cell droppers)	
		MIR50112	25 RXN
		MIR50115	50 RXN
		MIR50118	100 RXN
		Ingenio® Electroporation Kits for other devices, such as Bio-Rad® or Harvard-BTX® (solution, 0.4 cm cuvettes, cell droppers)	
		MIR50113	25 RXN
		MIR50116	50 RXN
		MIR50119	100 RXN
		Ingenio® Electroporation Solution	
		MIR50111	25 RXN (6.25 ml)
		MIR50114	50 RXN (12.5 ml)
		MIR50117	100 RXN (25 ml)
Selection Antibiotics for CRISPR Applications 	High quality antibiotics for selection of CRISPR-modified cell lines • **Ease-of-use**-Sterile filtered ready-to-use stock solutions for stable cell line generation • **High Purity** -Greater than 90% purity determined by HPLC • **High Potency** -In combination with high purity ensures better selection at optimal concentrations	G418 Sulfate Solution	
		MIR5920	10 ml (50 mg/ml in DI water)
		Hygromycin B Solution	
		MIR5930	20 ml (50 mg/ml in PBS)
		Puromycin Dihydrochloride Solution	
		MIR5940	5 x 2 ml (10 mg/ml in DI water)

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