CRISPR/Cas9 – transforming gene editing in drug discovery labs

It is evident from a recent market survey on gene editing in drug discovery that CRISPR/Cas9 is now recognised as the superlative method when attempting a gene knockout or when introducing defined mutations, insertions or modifications to the genome. Although use of CRISPR/Cas9 is still mainly confined to the basic research area in the drug discovery process, its potential with respect to the identification and validation of new therapeutic targets, the investigation of mechanism of action and in the creation of screens to identify genes that regulate various cell biological processes are major objectives. The perceived key advantages of CRISPR/Cas9 edits are their efficiency (ie it edits targets sequences at surprisingly high rates), simplicity (ie easy to use and design) and programmability (ie get precision targeting). The main benefits of CRISPR/Cas9 technology that drug discovery labs are most eager to exploit is the ability to make a complete genetic knockout, while minimising off-target effects; the rapid generation of cell lines harbouring desired mutations; and the possibility to develop accurate models of complex human disease in an efficient manner. The delivery of the CRISPR components into some target cells remains sub-optimal and this limits some work on CRISPR/Cas9 gene editing today. Overall, there seems little doubt that CRISPR/Cas9 technology will transform gene editing in drug discovery labs over the coming years.

By Dr John Comley

Genome editing uses engineered nucleases in conjunction with endogenous repair mechanisms to alter the DNA in a cell. Recently, a new genome editing tool based on a bacterial-clustered regularly-interspaced short palindromic repeats (CRISPR) and its associated protein-9 nuclease (Cas9) has generated considerable interest and looks set to transform the field. The CRISPR/Cas 9 system takes advantage of a short guide RNA (gRNA) to target the bacterial Cas9 endonuclease to specific genomic loci. Because the gRNA supplies the specificity, changing the target only requires a change in the design of the sequence encoding the gRNA, as a result editing can be directed to virtually any genomic locus including most eukaryotic cells. The

Figure 1: Current experience of gene editing with CRISPR/Cas 9 technology

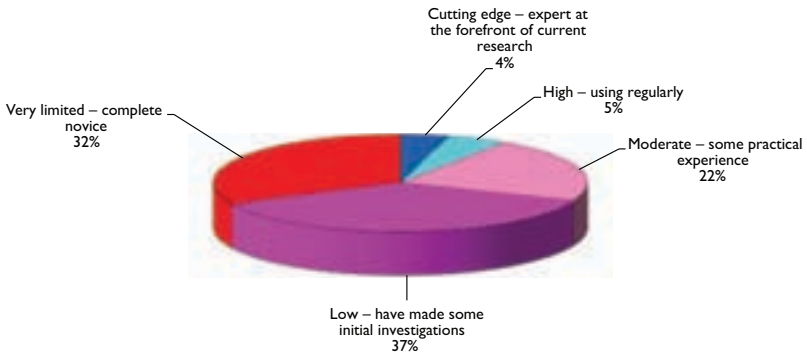

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Figure 2: Time period since first started using CRISPR/Cas9 gene editing technology

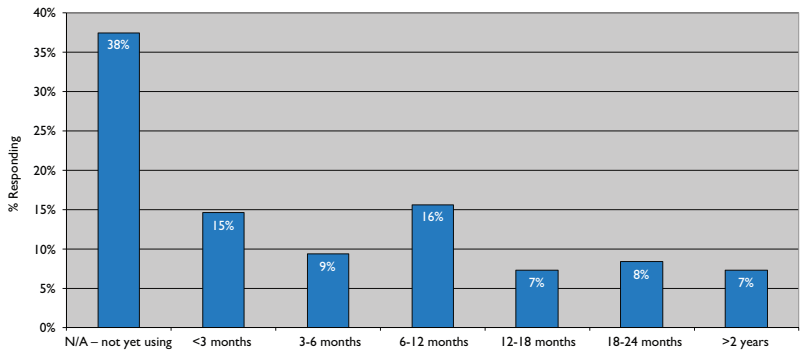

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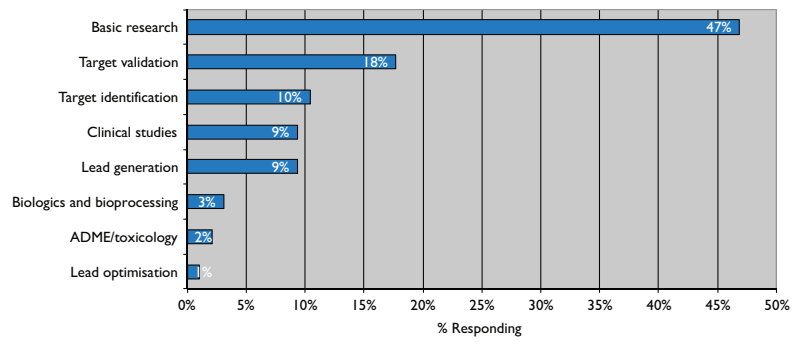
Figure 3: Area of the drug discovery process most applying or intending to apply CRISPR/Cas 9 gene editing



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CRISPR/Cas9 system greatly simplifies genome editing and has great promise in many applications areas such as stem cell engineering, gene therapy, drug development, tissue and animal disease models, agriculture, plant disease resistance, etc. In drug development CRISPR can provide a more effective method to probe the function of specific genes, such that drug discovery and validation can be accelerated. In gene therapy CRISPR offers precise editing or knockout of specific segments of a genome, enabling genetic research of defective genes and their behaviour.

In February 2016, HTStec undertook a market survey on CRISPR/Cas9 gene editing in drug discovery mainly among drug research labs in pharma, biotech and academia[1]. The main objective of this survey was to document the current use of CRISPR/Cas9 in gene editing in drug discovery applications and to understand its future impact. In this article highlights from the market survey are reported and the findings are discussed together with vendor updates on their CRISPR/Cas9 offerings supporting drug discovery research.

Current use of CRISPR/ Cas9 gene editing

Most survey respondents' current experience of gene editing with CRISPR/Cas 9 technology and its potential in drug discovery was low, ie have made some initial investigations. This was followed by 32% very limited, ie complete novice; 22% moderate, ie some practical experience; 5% high, ie using regularly; and then only 4% cutting edge, ie expert at the forefront of current research (**Figure 1**). The median time period since survey respondents had first investigated or started using CRISPR/Cas 9 gene editing technology was 6-12 months ago (**Figure 2**). Most (47%) of survey respondents were applying or intending to apply CRISPR/Cas 9 gene editing to the basic research area of the drug discovery process. This was followed by target validation (18% applying); target identification (10% applying) and then clinical studies and lead generation (both with 9% applying). Other drug discovery areas had in total only 6% applying (**Figure 3**). Oncology/cancer was the key disease or research area most targeted by survey respondents (52% targeting) by CRISPR/Cas 9 gene editing. This was followed by immunology/inflammatory disease/ autoimmune (30% targeting); neurology/CNS/ neurodegeneration/pain (27% targeting); metabolic disease/diabetes (18% targeting); and then cardiovascular disease (17% targeting) (**Figure 4**). Most (49%) of survey respondents answered N/A, ie they have not investigated other gene editing

technologies prior to CRISPR/Cas 9 availability. 29% have previously investigated transcription activator-like effector nucleases (TALENs); 21% have investigated integration via homologous recombination (eg with rAAV); 16% zinc finger nucleases (ZFN); and 11% other approaches (**Figure 5**).

Main rationale for using CRISPR/Cas9 gene editing technology

A gene knockout was what the majority (77%) of survey respondents most want to achieve using CRISPR/Cas 9 gene editing technology. This was followed by introduce defined mutations, insertions or modifications (62% wanting); gene knock-in (52% wanting); and then gene knock down (inducible) (40% wanting).Other aims were wanted by less than a third of survey respondents (**Figure 6**). Identification of new therapeutic targets was the main objective of survey respondents CRISPR/Cas 9 gene editing in drug discovery (61% wanting). This was followed by validation of new therapeutic targets (48% wanting); and then investigation of mechanism of action and screens to identify genes that regulate various cell biological processes (both with 46% wanting). Of least interest was deconvolution and validation of GWAS hits (only 8% targeting) (**Figure 7**). Survey respondents ranked some of the perceived advantages of CRISPR/Cas 9 gene editing technology in order of importance. This analysis revealed that efficient, ie edit targets sequences at surprisingly high rates, was seen as the main advantage of CRISPR/Cas 9 gene editing technology. This was followed by simplicity, ie easy to use and design; programmable, ie get precision targeting; and then fast, ie get tangible results within weeks. Ranked least advantageous was multiplexing, ie can programme multiple guide RNAs and cleave multiple genes simultaneously (**Figure 8**). Survey respondents rated complete genetic knockout, while minimising off-target effects as the potential benefit of CRISPR/Cas 9 in drug discovery they were most interested in exploiting. This was closely followed by enables rapid generation of cell lines harbouring desired mutations; develop accurate models of complex human disease in an efficient manner; and then relative ease with which cellular models can be generated. Rated least interesting was scalable generation of genome-wide CRISPR libraries for HT functional genomics screening (**Figure 9**).

Validating gene edits

The downstream analytical technique most used to validate CRISPR/Cas 9 gene edits was PCR (70%

Figure 4: Key diseases or research areas using CRISPR/Cas 9 gene editing

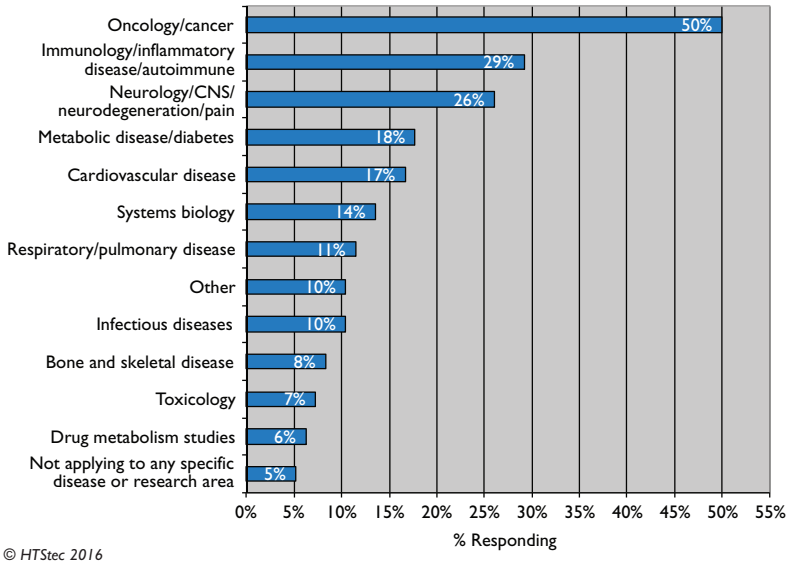


Figure 5: Other gene editing technologies investigated prior to CRISPR/Cas 9

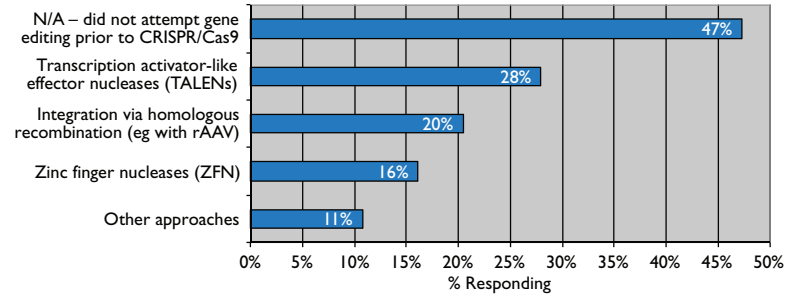


Figure 6: What respondents want to achieve using CRISPR/Cas 9 gene editing

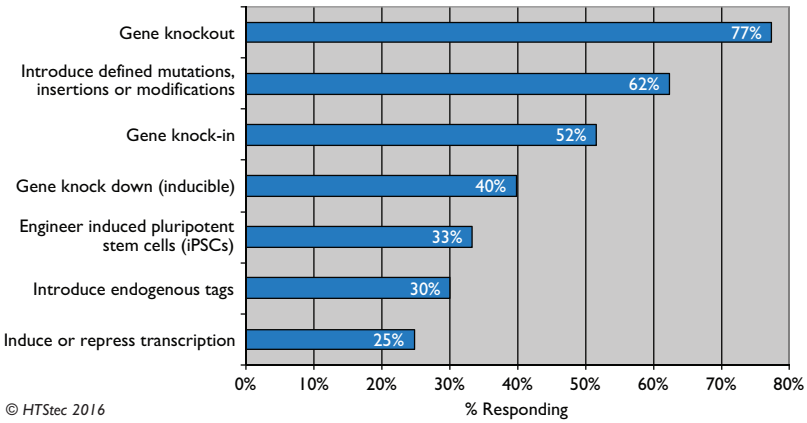


Figure 7: Main objectives of CRISPR/Cas9 gene editing in drug discovery

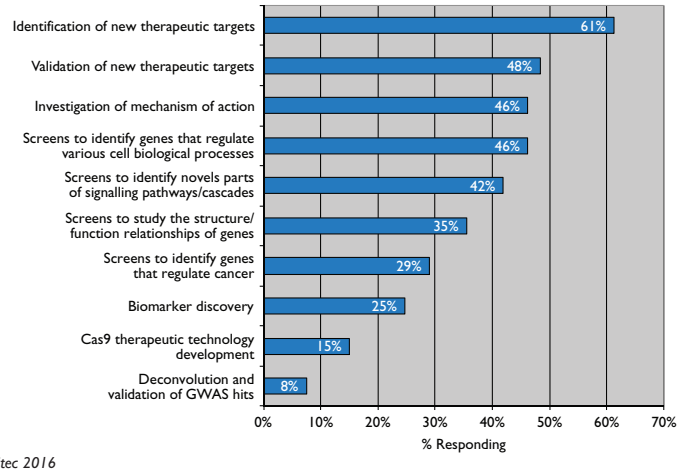


Figure 8: The advantages of CRISPR/Cas 9 gene editing technology

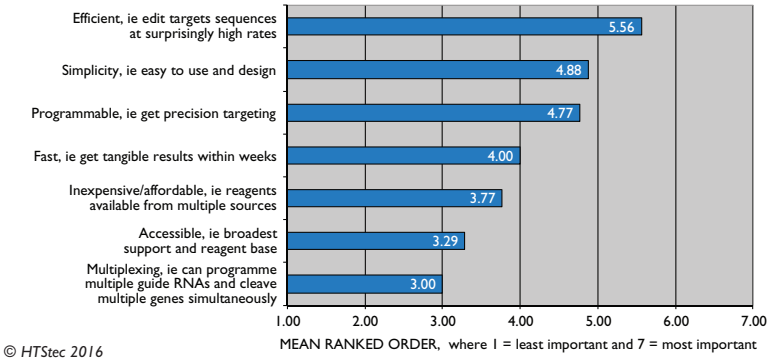
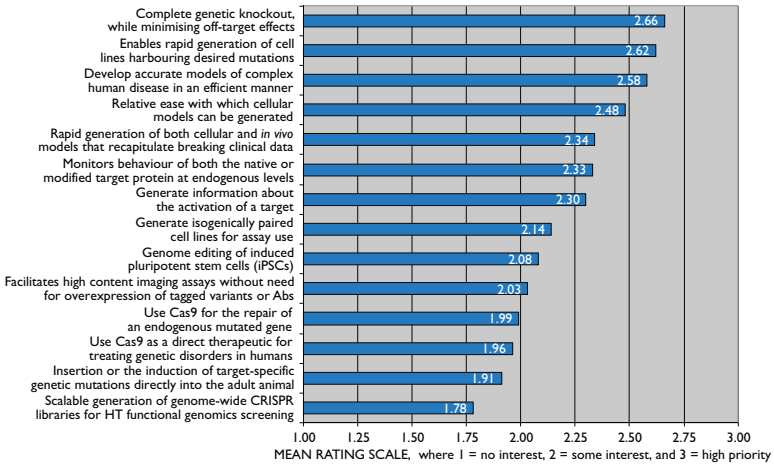


Figure 9: Potential benefits of CRISPR/Cas 9 in drug discovery most respondents want to exploit



using). This was followed by real time PCR (59% using); Western blotting (55% using); FACS (34% using) and then next gen sequencing (miSEQ) (30% using). Least used were capillary electrophoresis, TIDE assay and other DNA mismatch assays (**Figure 10**).

Multiplexing gene edits

For each single target gene survey respondents have attempted to edit they have designed a median of two single guide RNAs (ie 2 sgRNAs). Interest in simultaneously delivering sgRNAs targeting multiple genes to multiplex CRISPR/Cas 9 gene edits is presented in **Figure 11**. This showed that the majority (69%) of survey respondents are not yet undertaking multiplexing, but plan future investigation. This was followed by 25% not undertaking with no plans to investigate, leaving only 5% actually undertaking multiplexing today (**Figure 11**).

CRISPR libraries

The majority (53%) of survey respondents plan to use CRISPR (guide RNA) libraries for target validation or drug mechanism studies. 37% have no plans to use CRISPR libraries and the remaining 10% are currently already using them (**Figure 12**). The preferred CRISPR library type was roughly evenly split between pooled and arrayed guide RNA libraries. Survey respondents ranked reporter assay as the cellular response endpoints (outputs) of greatest value (ie most wanted) in the functional screening of CRISPR libraries. This was followed by cell proliferation, secreted proteins/markers, and then apoptosis. Ranked least needed was cell cycle (**Figure 13**).

Main limitations of CRISPR/ Cas9 gene editing

Survey respondents ranked delivery of CRISPR components into target cell or organism as what most limits their work on CRISPR/Cas 9 gene editing today. This was followed by poor efficiency; post-edit analysis/validation; bioinformatics capability and then assay reproducibility. Ranked least limiting was cytotoxicity (**Figure 14**).

Figure 10: Downstream analytical techniques used to validate CRISPR/Cas 9 gene editing

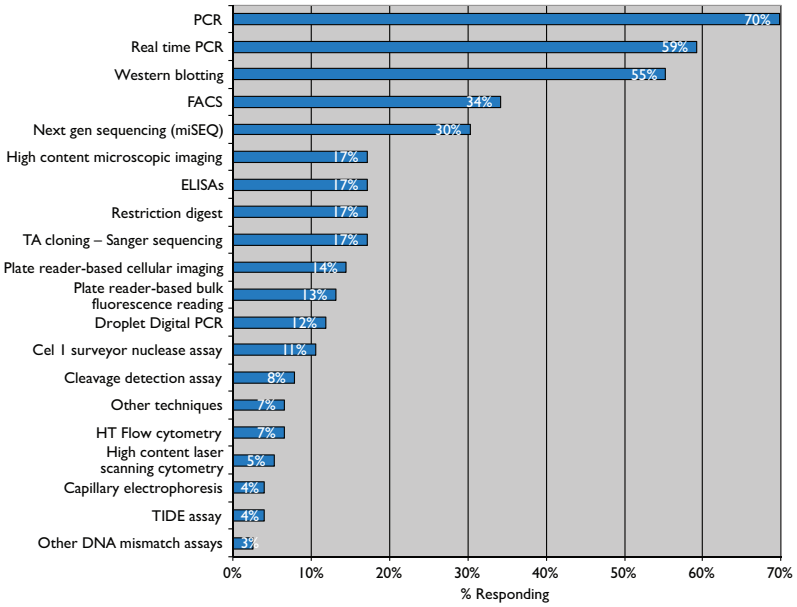


Figure 11: Current interest in multiplexing CRISPR/Cas 9 gene edits

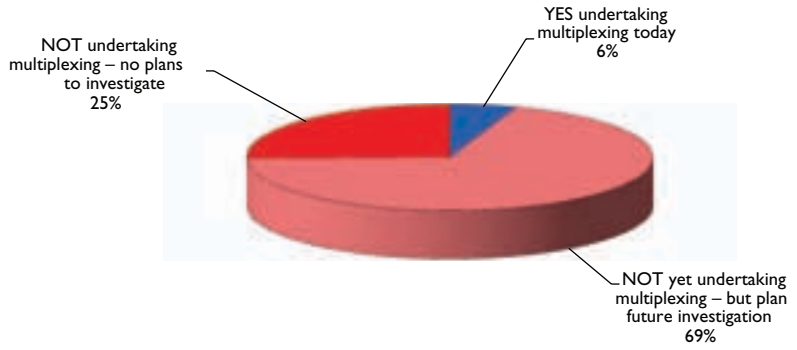


Figure 12: Use of CRISPR libraries

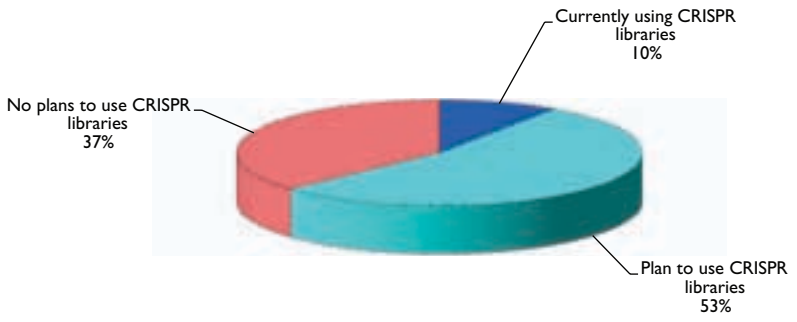


Figure 13: Cellular endpoints of greatest value in the functional screening of CRISPR libraries

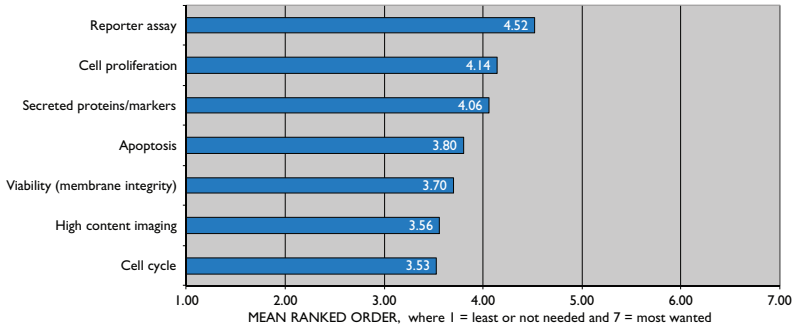
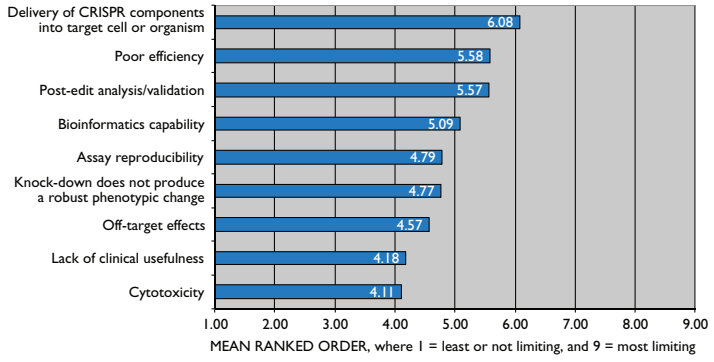


Figure 15: What most limits respondent's work on CRISPR/Cas 9 gene editing today



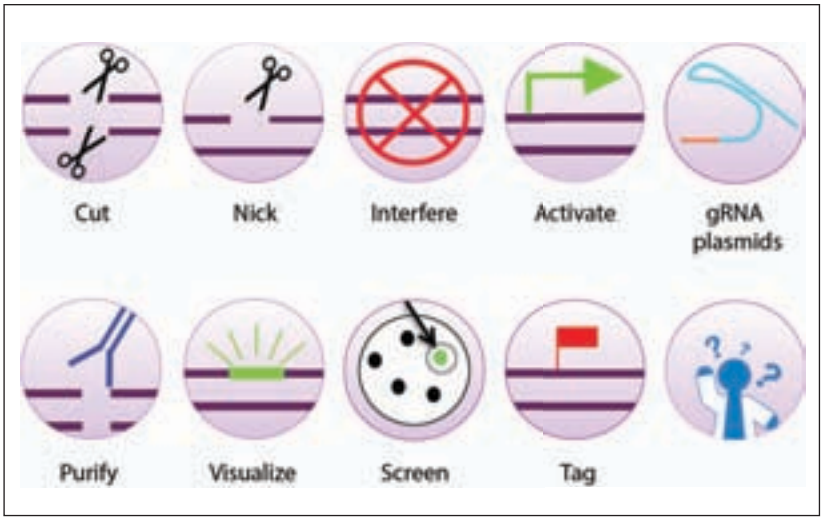


Figure 15: These icons represent applications for CRISPR technology that are already available through Addgene

Latest developments in CRISPR/Cas9 gene editing

The following vendor snapshots provide additional details and describe some of the latest developments in CRISPR/Cas9 technology applicable to the investigation of genome engineering related to drug discovery:

As a non-profit plasmid repository, Addgene has become well-known for making CRISPR genome editing technology widely available to the academic community. The CRISPR/Cas9 system quickly became an important tool for genome engineering, but the technique was initially limited to focused gene knock-outs and direct transfection protocols. CRISPR pooled libraries expand upon the CRISPR toolkit by providing a means for performing large-scale functional screens, such as those for identifying novel drug targets, and use lentiviral vectors in addition to transfection for delivery. CRISPR pooled libraries available to academic researchers through Addgene enable screening for drug targets across both the mouse and human genomes (www.addgene.org/crispr/libraries/). Scientists at the Broad Institute in Cambridge, MA have made the Brie, Brunello (Root and Doench lab) and GeCKO (Zhang lab) CRISPR knock-out pooled libraries available through Addgene for screening in both humans and mice. Additional genome-wide human knock-out libraries from the Sabatini and Lander (Whitehead Institute), Wu (Texas Tech), and Moffat labs (University of Toronto) are also available, along with a mouse knock-out library from the Yusa lab at the Sanger Institute. Addgene also has CRISPR knock-out libraries for specific gene subsets, such as the kinome (mouse and human) and enriched subpools for nuclear, ribosomal and cell cycle genes (human). Finally, CRISPR pooled libraries are also available to activate or inhibit gene expression across the human genome (**Figure 15**).

Agilent (http://www.genomics.agilent.com/campaign.jsp?id=9900001) has been a steady provider of premium nucleic acid synthesis for more than a decade. Using its synthesis technology, which allows for the rapid creation of high-complexity libraries, Agilent has been working with a number of pioneers developing pooled SureGuide CRISPR libraries. These libraries are currently available via an open early access programme and include both licensed, pre-validated GeCKO libraries and fully custom pooled library solutions. In order to provide the highest quality possible, Agilent has been able to refine its synthesis process to provide very

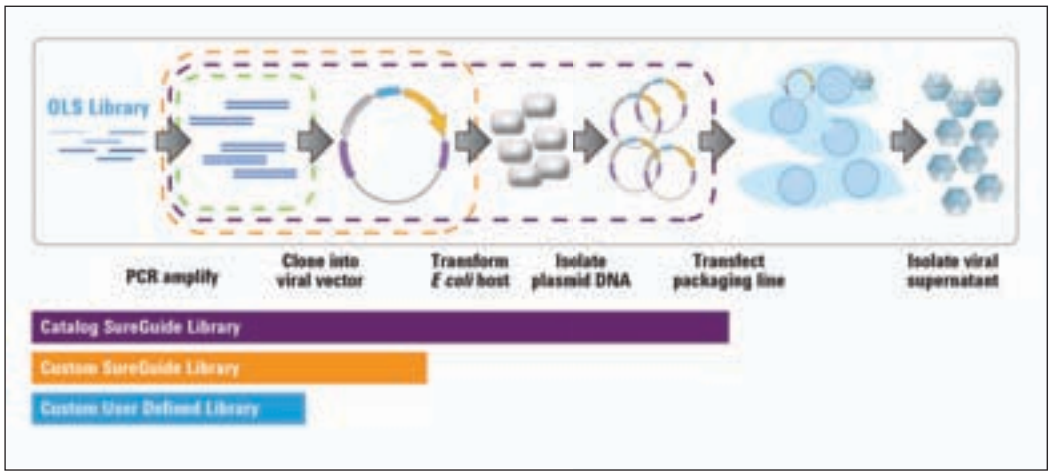


Figure 16
Agilent utilises its array-based oligo library synthesis (OLS) platform to generate CRISPR libraries either with pre-defined content or completely custom. For any pooled screening approach to CRISPR, whether knock-down or CRISPRa/i SureGuide libraries are available to cover workflow from unamplified oligo pools to plasmid libraries

even guide representation throughout the pool while maintaining the high fidelity required for CRISPR applications. Pre-validated GeCKOv2 libraries, fully licensed from the Broad Institute, are available in a plasmid format and are delivered to cells using the included lentiviral vector. Both the mouse and human libraries are available. Agilent offers two tiers of custom libraries. Pre-amplified custom libraries are made using overlaps compatible with Agilent's SureVector library cloning kit and include a U6 promoter which makes these libraries appropriate for human and mouse applications. Any guide sequences, whether a subset of the GeCKO library or an entirely new design, may be specified. Up to 60,000 guides can be accommodated in these libraries. The second tier of custom is supplied as an unamplified library. Here the user can fully design all aspects of their CRISPR library including the promoter system, cloning/expression methodology, delivery and guide sequences. Up to 100,000 guides, each up to 200 nucelotides in length, can be accommodated with this custom solution (**Figure 16**).

The arrival of CRISPR/Cas9 technology and induced pluripotent stem cells (iPSCs) in recent years has expanded the horizons for gene manipulation in human cells. These two technologies have provided a new arena to study human biology and diseases and are paving a sure path toward gene therapy in regenerative medicine. They provide an unlimited resource of *in vitro* models of human genetics and diseases. **Applied StemCell** (www.appliedstemcell.com) is a premier licensee of both CRISPR/Cas9 (The Broad Institute, MIT) and the Yamanaka-iPSC reprogramming (from iPS Academia, Japan) technologies. It uses CRISPR to engineer iPSCs derived from various clinical sources, to generate isogenic control-disease cell line models that provide reliable comparative results without genetic background variability. ASC's CRISPR protocol is well-optimised to: 1) culture and maintain iPSCs without differentiation in a feeder-free culture system; 2) balance transfection of CRISPR elements while maintaining cell viability; 3) uses validated gRNAs to increase Cas9 cutting efficiency and limit off-target modifications; 4) includes a transient expression selection marker to increase the targeting efficiency of CRISPR in iPSCs (**Figure 17**). The optimised CRISPR-iPSC protocol has also reduced project timelines to a competitive 2-3 months for gene knockout, and 3-5 months for gene knock-in and/or point mutation modifications. Applied StemCell's scientists also have adapted improved gRNA selection strategies based on recent research[2], which has greatly increased its efficiency for generating heterozygous and homozygous clones that better mimic human diseases. To

Procedures	Before optimization	After optimization
Transfection efficiency		
Feeder-free culture system	10%	80%
Transfection of CRISPR elements	20%	50%
Targeting efficiency		
Transient selection marker	10%	60%

Figure 17: Improved transfection and targeting efficiency with Applied StemCell's upgraded CRISPR technology to genetically modify human-induced pluripotent stem cells (iPSCs). This table summarises the transfection and targeting efficiencies before and after optimisation of its CRISPR protocols. Optimisation of the feeder-free culture system and concentration CRISPR elements introduced into the cells improved transfection efficiency by 70% and 30%, respectively. By using a selection marker, the gene targeting efficiency of its CRISPR system increased by 50%

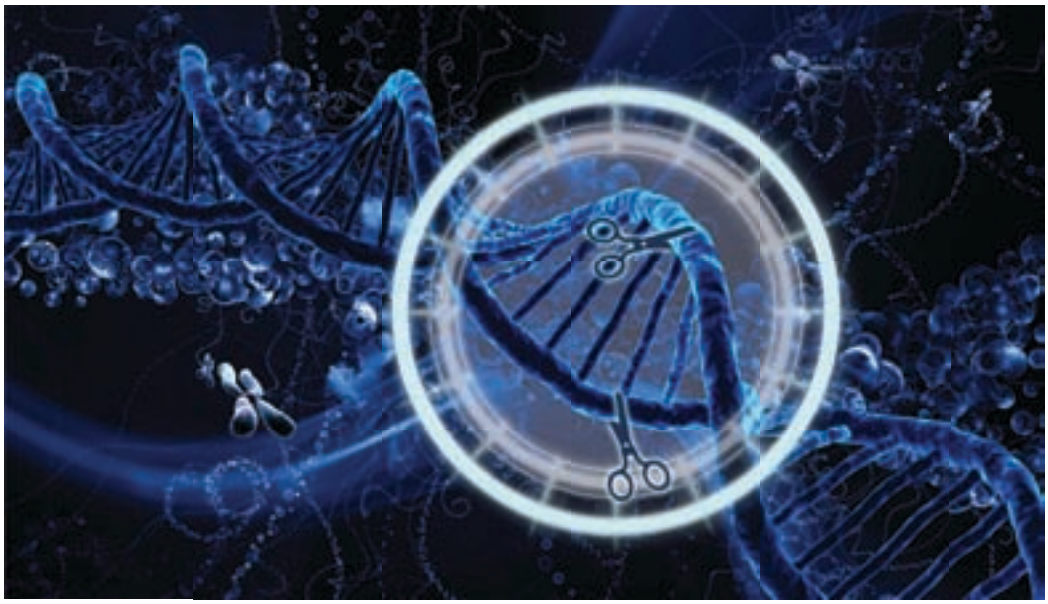


Figure 18
GE Healthcare Dharmacon Edit-R CRISPR-Cas9 genome engineering optimised tools for high-confidence gene editing

date, it has successfully delivered 100% of its CRISPR-iPSC projects to scientists in industry and academia.

GE Healthcare Dharmacon (http://dharmacon.gelifesciences.com/#crispr) specialises in both guide RNA and Cas9 nuclease components of the CRISPR-Cas9 genome engineering system. Its RNA synthesis expertise enables the rapid generation of guide RNAs for gene knockout and knock-in experiments or custom applications. Edit-R™ predesigned human, mouse and rat synthetic dual guide RNAs (crRNA and tracrRNA) and lentiviral sgRNAs are based on an algorithm developed from a large set of functional gene knockout data rather than data that only interrogates genomic DNA for insertions and deletions. High-throughput crRNA synthesis makes large-scale libraries for arrayed screening applications possible, including Edit-R crRNA libraries for human ubiquitin enzymes, kinase proteins and druggable gene families, among others. These libraries can be used in one-gene-per-well genome-scale knockout experiments and enable a wide variety of biological phenotypic assays, such as endpoint assays and high content imaging, as readout to identify relevant gene targets. Edit-R Cas9 nuclease options for cell delivery include transfection or electroporation of an expression plasmid, mRNA, or protein, or through transduction of lentiviral particles. The use of Cas9 mRNA or protein in combination with synthetic guide RNA results in a completely DNA-free gene editing system, which avoids unwanted integration of plasmid DNA and reduced off-target effects by limiting the duration of the Cas9 nuclease and synthetic guide RNA in the cell. In addition, the Dharmacon CRISPR Configurator tool allows researchers to design their own custom guide RNAs for more than 30 different species, as well as guide RNAs for different types of CRISPR-Cas9 systems (**Figure 18**).

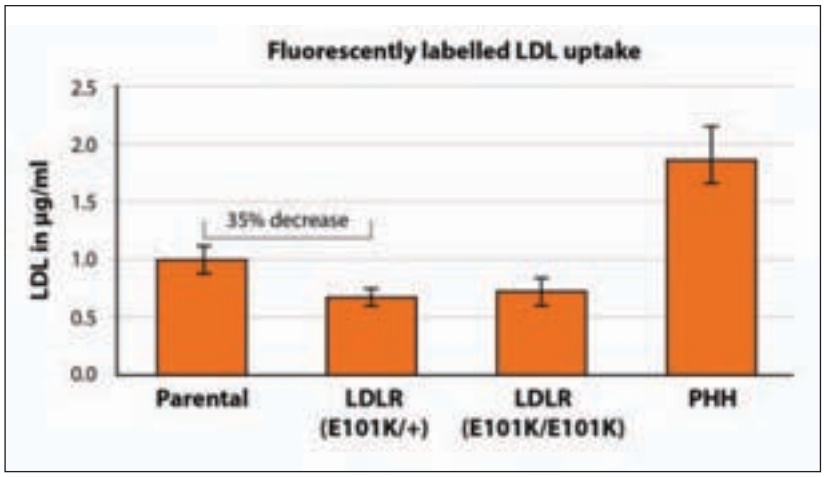


Figure 19: Horizon Discovery engineered IPSCs were differentiated by its partner DefiniGen into hepatocytes for the study of Familial Hypercholesterolemia (FH). Cells in which a FH-associated mutation has been introduced into the LDL Receptor (LDLR) show a reduction in LDL uptake relative to their normal parental cells and Primary Human Hepatocytes (PHH), indicating reduced receptor function in LDLR diseased lines

Horizon Discovery (https://www.horizondiscovery.com/ipsc) is using CRISPR technology to provide solutions at multiple stages of the drug discovery continuum: target identification (genetic screening), target validation (genetically-modified

cell lines), pre-clinical studies (genetically-defined *in vivo* models), and generation of improved biomanufacturing systems (genetically-modified CHO cells). To allow scientists to study the effect of a genetic mutation in a variety of tissues, Horizon is now using CRISPR-Cas9 to engineer pluripotent stem cells (such as iPSCs). Critically these can then be differentiated into a variety of tissues types, such as liver or neural cells, all of which will have the same genetic background and the same introduced mutation. Researchers can then screen and validate compounds in these different but genetically identical backgrounds, making iPSCs a good model for pre-clinical studies (**Figure 19**).

High levels of genome editing can be achieved with Alt-R™ CRISPR-Cas9 System ribonucleoprotein (RNP) complexes from **Integrated DNA Technologies** (www.idtdna.com). Jurkat cell lines, derived from human T-lymphocytic cells, are used to study immune system signalling, immune system diseases, the susceptibility of cancers to treatment and viral infection through chemokine receptors, such as with HIV. However, these cells are notoriously difficult to transfect. This presents a problem for researchers interested in applying CRISPR genome editing technologies in these cells. Scientists at IDT have established that the delivery of RNP complexes, consisting of CRISPR RNAs and Cas9 protein, is the most efficient tool for CRISPR delivery to cell lines and demonstrates the highest potency genome editing. As part of the Alt-R CRISPR-Cas9 System, IDT offers crRNA, tracrRNAs and the S.p. Cas9 Nuclease. The RNAs are length optimised and chemically modified to further enhance genome editing efficiency by protecting the oligos from degradation by nucleases. Optimal electroporation conditions were determined for Jurkat cells using a Neon® optimisation protocol, which tests 24 electroporation settings, including voltage, pulse width and number of pulses. Following optimisation, we observed optimal cell survival with high levels (>75%) of genome modification of the HPRT gene, as measured using the T7EI endonuclease mismatch cleavage assay, which is described in the Alt-R CRISPR-Cas9 System User Guide. These results are promising, and suggest that the combination of the Alt-R CRISPR System and optimised electroporation may facilitate efficient genome editing in other difficult-to-transfect primary cells, such as T-cells, macrophages and adipocytes (**Figure 20**).

When it comes to efficient CRISPR/Cas9 modification in cells, the choice of delivery method can be key to effectively maximising the desired results. If

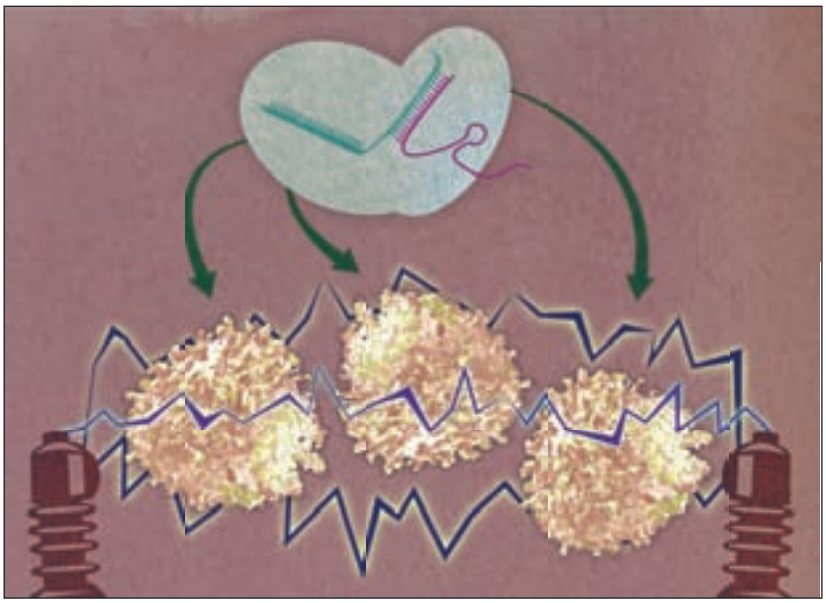


Figure 20: Optimised delivery of Integrated DNA Technologies Alt-R™ CRISPR-Cas9 system ribonucleoprotein facilitates genome editing in difficult-to-transfect cell lines

one is working with primary cells, such as primary human T-cells, iPSCs or CD34+ hematopoietic progenitor cells, then the delivery of genome editing substrates can be more challenging. This is where **Lonza** (www.lonza.com) Nucleofection™ can make a significant difference. Nucleofection™ has long been recognised as a premiere transfection technology for ZFNs, TALENs and CRISPR with the ability to transfect virtually any type of primary cell in

Figure 21
Lonza 4D-Nucleofector™ unit for closed, large-scale transfection of up to one billion cells

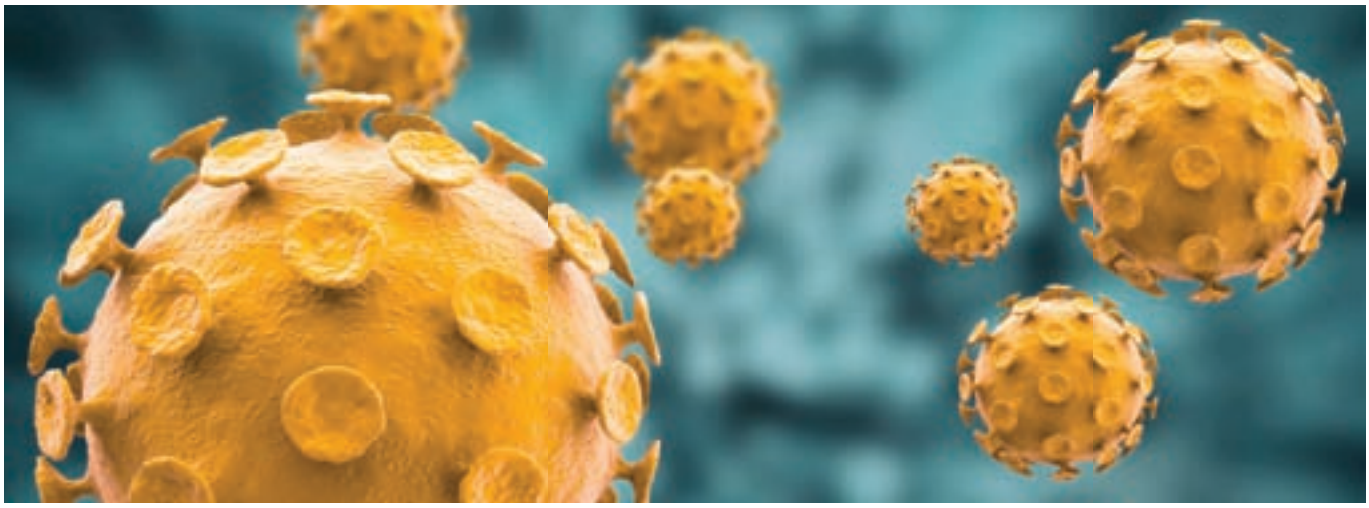


Figure 22
Ready-to-use whole genome arrayed lentiviral CRISPR libraries from MilliporeSigma

addition to any cell line, and with any of the genome editing substrate formats, such as DNA, sgRNA, mRNA or RNP. The Nucleofection™ platform transfection conditions are scalable from the benchtop 4D Nucleofector™ to the 96-well Shuttle™ and 384-well HT Nucleofector™ systems. The latest addition to the Nucleofection™ platform is the new closed system LV Nucleofector™, which can transfect from 10 million to 1 billion cells with comparable performance to the other Nucleofector™ systems, allowing one to optimise transfection conditions with the 4D Nucleofector™, and translate the conditions seamlessly to the large scale device. Currently, the LV Nucleofector™ is for research purposes only, although because of the ability of Nucleofection to transfect primary human cells so well, there will undoubtedly be interest in using this device in clinical applications. Transient transfection with Nucleofection can play a zero-footprint role in innovative personalised cancer therapies, such as CAR-T therapy or other cell-based therapies, which could provide a regulatory advantage over viral-based therapies (**Figure 21**).

MilliporeSigma® (www.milliporesigma.com) and The Wellcome Trust Sanger Institute have collaborated to provide the first arrayed lentiviral CRISPR knockout libraries for human and mouse genomes. Genome-wide loss-of-function screening is an established and powerful approach to discover genes and pathways that underlie biological processes. Early screens used RNA interference (RNAi), which enables gene knockdown, but may miss important gene hits when only total loss of gene expression yields phenotype. The advent of pooled CRISPR libraries brought efficient genome-wide knockout but requires the additional step of deconvolution by deep sequencing for accurate hit identification. By contrast, arrayed CRISPR screening is a major advance, providing complete gene knockout for easy hit identification without the complexity and expense of big data analysis. In addition, the arrayed format enables phenotypic screening by high content assays such as cellular imaging, fluorescence, luminescence and colorimetric experiments. Using a validated approach to CRISPR design, tested at Sanger and published in the literature, more than 36,000 gene-specific gRNA clones per genome were selected to avoid alternative start codons and known single nucleotide polymorphisms (SNPs) while simultaneously reducing potential for off-target effects. Lentiviral delivery of gRNA clones co-expressing both puromycin and unique BFP markers allows screening of cell lines that already express the more common fluorescence cassettes RFP and GFP. Whether for drug-target discovery or basic biology applications, the exclusive whole-genome Sanger arrayed libraries leverage the power of CRISPR gene knockout and the convenience of arrayed lentiviral format to expand high throughput screening capabilities for the entire gene editing community (**Figure 22**).

New England Biolabs (www.neb.com) provides reagents to support a broad variety of CRISPR/Cas9 genome editing approaches. From introduction of Cas9 and single guide RNA (sgRNA) on plasmids, to direct introduction of Cas9 ribonucleoprotein (RNP) and detection of edits using next generation sequencing or enzymatic mutation detection, NEB provides reagents that simplify and shorten genome editing workflows. Generating RNPs for direct introduction requires

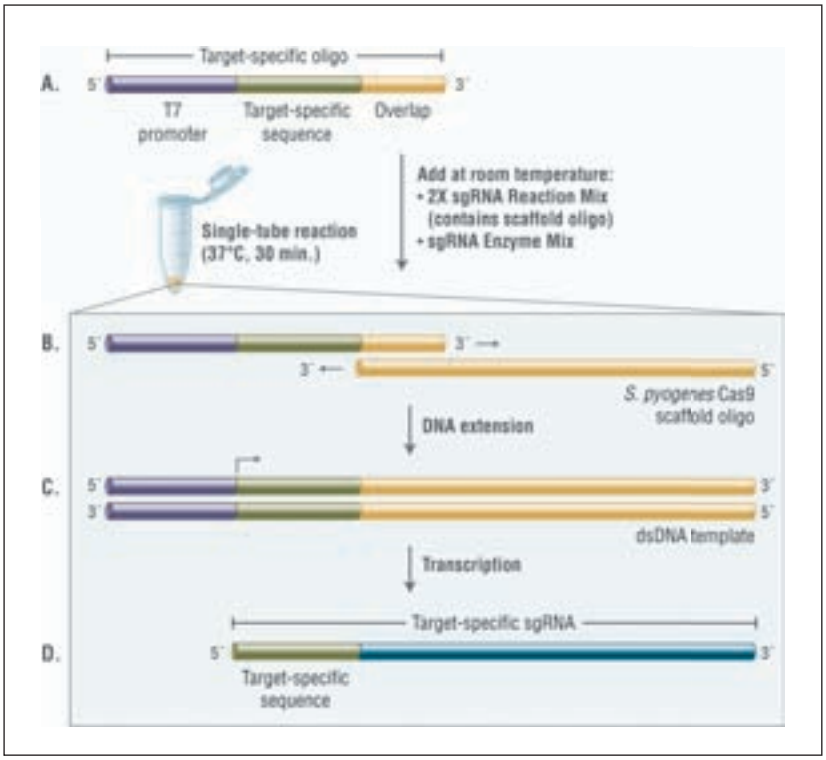
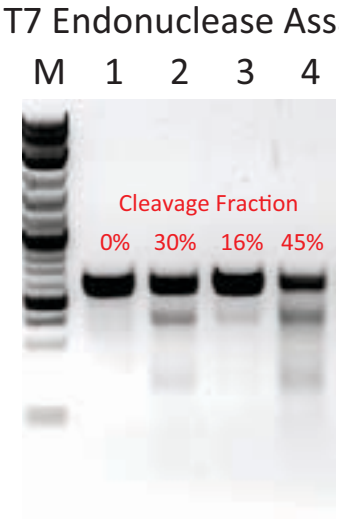


Figure 23: The New England Biolabs EnGen sgRNA synthesis kit (NEB #E3322) enables single-tube synthesis of sgRNA in 30 minutes at 38°C

Figure 24
T7 Endonuclease Assay from Systems Biosciences to detect cleavage efficiency. MicroRNA 21 genomic locus was edited via Cas9 plasmid DNA, or Cas9 protein with regular crRNA:trancrRNA duplex or MS modified crRNA:trancrRNA duplex using lipid mediated transfection of HEK293 cells. Cell samples were taken at one day after transfection and analysed by T7 endonuclease assay to determine the cleavage efficiency



1. Negative transfection control
2. Cas9-mir21 KO vector
3. Cas9 protein+ mir21 KO sgRNA
4. Cas9 protein+ modified mir21 KO sgRNA

Cas9 protein and either sgRNA or separate crRNA and tracrRNA. EnGen® Cas9 NLS, S. pyogenes is engineered for high genome editing efficiency. The EnGen sgRNA Synthesis Kit combines template assembly and *in vitro* transcription for rapid generation of microgram quantities of custom sgRNA, requiring only a user-supplied single ssDNA oligonucleotide. To determine editing efficiency, the EnGen Mutation Detection Kit provides a full workflow from PCR amplification to T7 Endonuclease I-based mutation detection. Alternatively, NEB supplies Cas9 wild type and restriction enzymes, both of which can be used *in vitro* to determine the extent of editing (**Figure 23**).

Studying changes in regulation of genes and proteins is a key need for research and drug discovery. Before gene editing technologies, such as CRISPR/Cas9, it was difficult to address this need in a live-cell context without introducing assay artifacts. **At Promega** (www.promega.com) the use of CRISPR/Cas9 is seen as a way to enable the development of better downstream assays. Using cellular reporter technologies it has developed, such as recombinant luciferase-based and HaloTag, scientists can now tag genes or proteins and study their behaviour under endogenous expression levels from their natural chromatin context. Promega has developed multiple assay systems especially using t he smaller and brighter NanoLuc luciferase to couple with the power of CRIPSR/Cas9. It offers NanoLuc luciferase, NanoBiT (NanoLuc® Binary Technology) and HaloTag technologies for studying gene transcription, protein functions such as protein:protein interactions, translocation, degradation and protein purification. Prior to the availability of CRISPR/Cas9, we collaborated with Horizon Discovery on targeted gene editing using an AAV targeting technology. Now, combining RNA-directed editing for Cas9 with a newer complementation version of our NanoLuc called HiBiT, we will enable researchers to tag their proteins of interest with a short 11 amino acid peptide, which will have high sensitivity to detect and measure quantities and dynamics of the protein of interest, even in a high-throughput applica tions. The ability to perform more targeted genome editing combined with sensitive assay technologies should prove fruitful for basic research and drug discovery.

System Biosciences (SBI) (www.systemnio.com), based in Palo Alto, CA, was the first company to commercialise CRISPR/Cas9 reagents to the research use market with its Cas9 SmartNuclease® technology. Since April 2013,

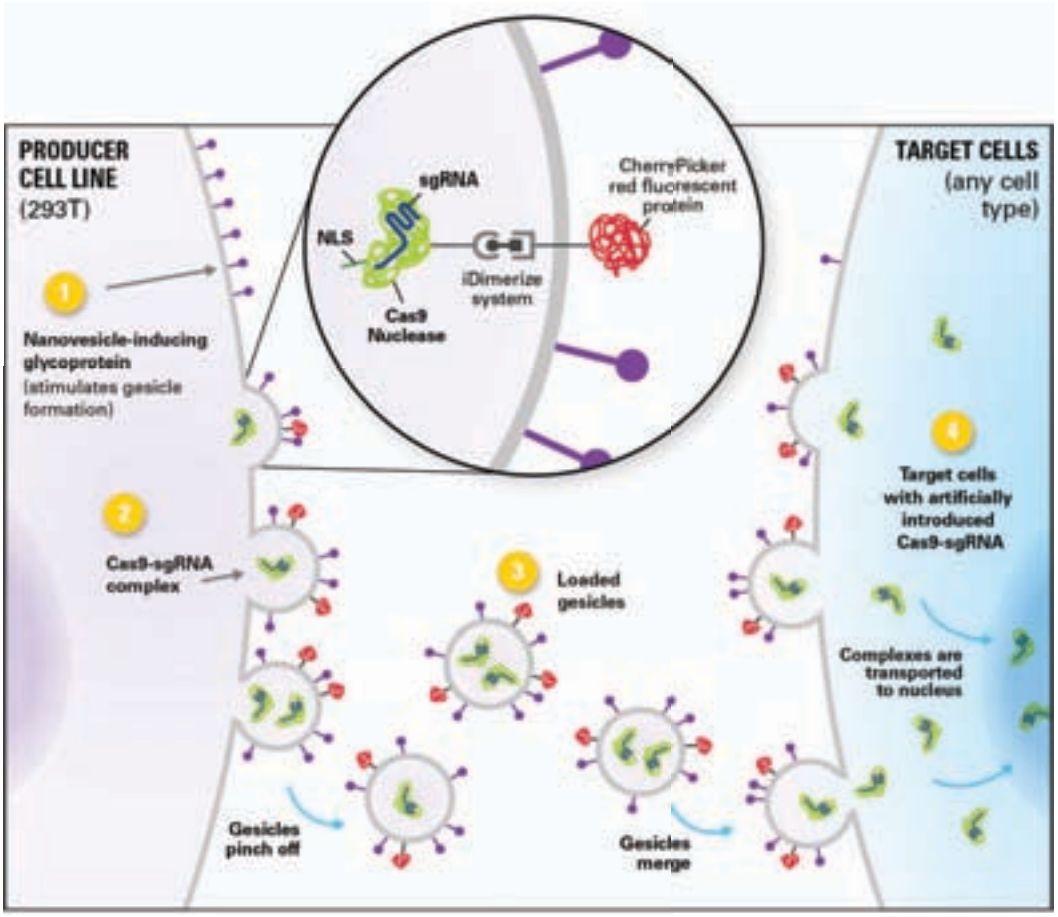


Figure 25
Guide-it CRISPR/Cas9 gesicle production system from Takara Bio. Gesicle formation (step 1) is induced by glycoproteins on the surface of 293T producer cells that have been cotransfected with a gesicle packaging mix and a target-specific guide RNA plasmid. A small ligand is added to load the Cas9-sgRNA ribonucleoprotein complex into the gesicle through interaction with the membrane-bound CherryPicker protein on the gesicle surface (step 2). Loaded gesicles pinch off from the producer cells and are collected from the supernatant, yielding a concentrated stock of Cas9-sgRNA gesicles (step 3). Harvested gesicles can be applied to a broad range of target cell types for CRISPR/Cas9-mediated gene editing (step 4)

SBI has provided CRISPR-based genome engineering solutions to researchers around the world, including plasmid, mRNA and viral-based delivery options (including integrating and non-integrating Lenti-Cas9 formats, and AAV-saCas9 vectors for *in vivo* delivery). Its latest Cas9 technology focuses on the use of synthetic RNA and Cas9 protein to form ribonucleoprotein (RNP) complexes for fast, efficient and accurate genome editing. Synthetic guide RNA is now easier and more affordable than plasmid or IVT methods and offers numerous technical advantages. When combined with Cas9 protein to form an RNP complex, editing can be seen in less than 24 hours. The transient nature of an RNP complex reduces off-target effects and synthetic RNA allows for greater accuracy with precise control over the amount transfected or injected. Researchers report higher survival rates in the production of transgenic models when using synthetic RNA. SBI offers single gene formats, as well as a 96-well plate format for high-throughput, multiplex screening of targets – prepared on demand with superior purity in just a few days. SBI remains focused on driving innovation in CRISPR/Cas9 technology, and there are two other CRISPR-related technologies from SBI to keep an eye out for in late 2016/early 2017: 1) exosome-based delivery of CRISPR/Cas9 (including tissue-specific targeting) and 2) high-throughput, genome-wide screening of off-target effects to ensure model cell lines contain only the desired modifications (**Figure 24**).

CRISPR/Cas9-based gene editing has revolutionised the field of cell biology. However, two significant challenges remain: obtaining efficient delivery of Cas9 to all cell types and achieving fewer off-target effects. It has been previously demonstrated that genome editing via direct delivery of Cas9-sgRNA ribonucleoproteins (RNPs) has the benefit of decreased off-target effects due to the short duration of the RNPs in the cell. Here **Takara Bio** (www.clontech.com) reports on a new RNP delivery method for footprint-free genome editing in a broad range of cell types, including human induced pluripotent stem cells (hiPSC). It is based on the delivery of RNP complexes in cell-derived nanoparticles called 'gesicles'. Gesicles originate at the plasma membrane of a mammalian packaging cell due to the expression of a nanoparticle-inducing glycoprotein. Simultaneously, Cas9 containing a nuclear localisation signal and an sgRNA of interest are also expressed in the producer cell and are actively packaged into the nanoparticles as an RNP complex. In order to enrich RNPs at the plasma membrane where they are incorporated into

the forming gesicles, Takara Bio uses a ligand-dependent dimerisation system (iDimerize™ technology). Once gesicles have formed, they can be harvested from the media supernatant of the packaging cell. When RNP-loaded gesicles are added to target cells, active RNP complexes are delivered to the cells, followed by successful editing of the gene of interest in the target cell nucleus. Overall, gesicles can be considered of high interest for genome editing, providing a direct, rapid and transient method for delivering highly active Cas9-sgRNA RNPs into target cells with a drastically reduced risk for off-target editing (**Figure 25**).

Thermo Fisher Scientific (www.thermofisher.com/crispr) has introduced the Invitrogen™ LentiArray™ CRISPR libraries; a suite of tools that apply the power of CRISPR-Cas9 technology to high-throughput functional genomics screening. CRISPR-Cas9 technology provides an efficient method for specific, complete, and permanent gene knockout, making it a potent tool for making new discoveries about gene function. LentiArray CRISPR libraries harness the power of CRISPR-Cas9 technology, enabling you to utilise this breakthrough technology to rapidly interrogate thousands of genes and determine which are key members of specific biological pathways and whether they are involved in disease development and progression. LentiArray CRISPR libraries are provided in an arrayed format that is compatible with your existing high-throughput screening infrastructure and have been designed and constructed to provide a flexible system that does not impose any limitations on your assay design and research goals. The LentiArray library product line provides all the tools you need to expand your screening capabilities with CRISPR-Cas9 technology. Thermo Fisher Scientific is continually expanding its suite of CRISPR-Cas9 genome editing products from screening and target validation to cell engineering, enabling you to carry out your gene editing or screening research with accuracy, ease and efficiency (**Figure 26**).

Discussion

Table 1 summarises the latest developments in CRISPR/Cas9 genome editing technology applicable to drug discovery reported in the vendor updates. Offerings reported fall into the following categories: 1) Pooled CRISPR libraries; 2) Arrayed CRISPR libraries; 3) CRISPR engineering of human iPSCs; CRISPR toolkit components (eg guide RNAs, Cas9 nucleases, RNP complexes etc); 4) Transfection methodology; and 5) Cellular reporter technologies for tagged genes and proteins.

CRISPR libraries harness this power of the CRISPR/Cas9 system to make precise, permanent gene knockouts and expand its application into the arena of high throughput gene knockout functional screening. Most CRISPR libraries use a lentivirus to deliver the gRNA/Cas9 to target cells.

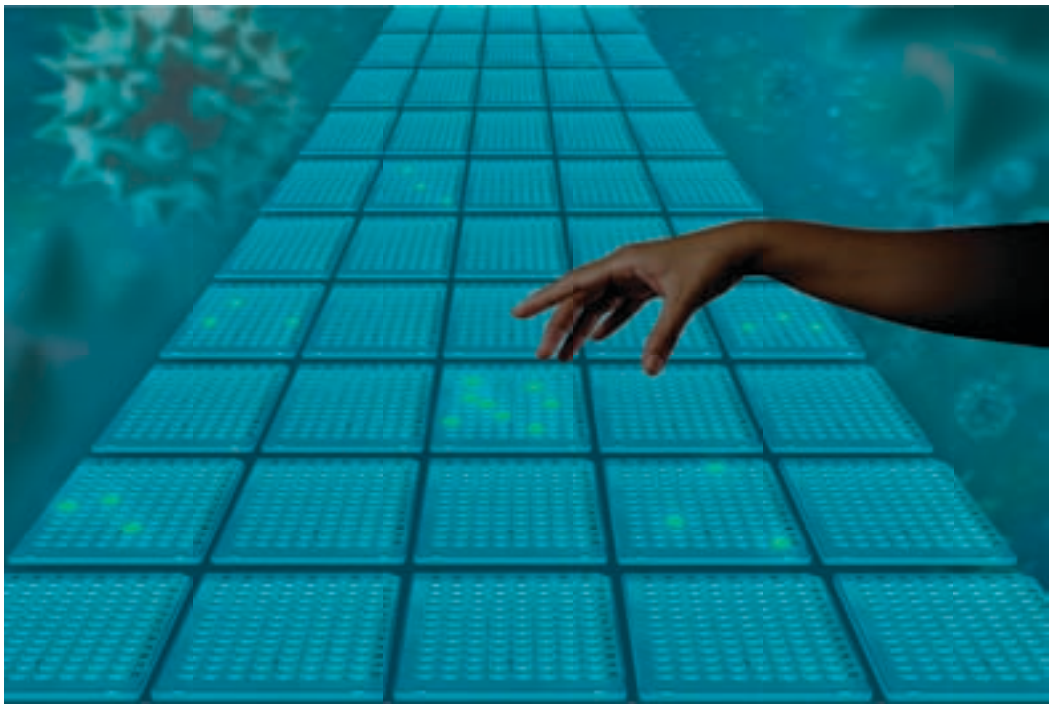


Figure 26
High-throughput functional genomics screening using Invitrogen™ LentiArray™ CRISPR libraries from Thermo Fisher Scientific

Table 1: Summary of latest developments in CRISPR/Cas9 genome editing technology applicable to drug discovery reported in the vendor updates*

PRODUCT OFFERING: / VENDOR	Pooled CRISPR Libraries	Arrayed CRISPR Libraries	CRISPR Engineering of Human iPSCs	CRISPR Toolkit Components (e.g. Guide RNAs, Cas9 Nucleases, RNP Complexes etc.)	Transfection Methodology	Cellular Reporter Technologies For Tagged Genes & Proteins
Addgene	✓					
Agilent	✓					
Applied StemCell			✓			
GE Dharmacon		✓		✓		
Horizon Biodiscovery			✓			
Integrated DNA Technologies				✓	✓	
LONZA					✓	
MilliporeSigma		✓				
New England Biolabs				✓		
Promega						✓
Systems Bioscience				✓	✓	
Takara/Clontech				✓	✓	
Thermo Fisher Scientific		✓				

* PLEASE NOTE - this table is based ONLY on the information provided in the vendor supplied snapshots printed above, vendors may have a wider CRISPR offering, please consult their websites for details.

Pooled libraries usually require NGS for deconvolution of hits. Arrayed libraries, typically with four gRNAs per gene target (pooled in a single well), facilitate the more rapid validation of hits and may utilise existing high-throughput screening infrastructure such as high content analysis or reporter gene assays to validate edited genes. Loss-of-function screening by gene knockout is a powerful tool for systematic genetic analysis in mammalian cells, facilitating gene discovery, genome-scale functional interrogation (eg signal transduction pathways) and drug discovery (eg target identification and drug mechanism studies) (Addgene, Agilent, GE Dharmacon, MilliporeSigma, Thermo Fisher Scientific).

CRISPR engineering of iPSCs allows genetic changes in human cells to be made with much greater efficiency and precision than before. iPSC can then be differentiated into a variety of tissues types with the same genetic background and the same introduced mutation. Researchers involved in drug discovery can then screen and validate compounds in these different but genetically identical backgrounds, making iPSC-derived cells the most relevant and sought after for pre-clinical disease modelling (Applied StemCell, Horizon Biodiscovery).

Innovative investigation with the CRISPR/Cas9 system is driven by the many vendors that now offer a complete suite of tools to facilitate licensed research on genome editing. End-users are presented with an ever-expanding range of guide RNAs, Cas9 nucleases, RNP complexes etc, that aim to simplify and shorten genome editing workflows. Fortunately most vendors have also taken on board the need to educate new investigators with step-by-step guides on how to the exploit their CRISPR/Cas 9 product developments in this extremely fast evolving field (GE Dharmacon, Integrated DNA Technologies, New England Biolabs, Systems Bioscience, Takara/Clontech).

To use the CRISPR system, you need to get both the gRNA and Cas9 expressed in your target cells. The expression system used will depend upon your specific application. Cas9 nuclease options for cell delivery include transfection or electroporation of an expression plasmid, mRNA or protein, or through transduction of lentiviral particles. Certain cell types (eg HEK293 cells) are easier to transfect than others (eg primary cells) and successful transfection typically requires a degree of optimisation. Lonza's Nucleofection™ platform has proved particularly useful in delivering genome editing substrates to a wide variety of cell types. Most recently transfection with RNP complexes, some using synthetic RNA, have enabled genome editing across a broad range of cell types, including hiPSC, with fewer off-target effects (Integrated DNA Technologies, LONZA, Systems Bioscience and Takara/Clontech).

In order to successfully exploit the targeted genome editing now enabled by CRISPR/Cas9 technology, drug discovery efforts require the ability to tag the proteins or genes of interest in live cells. These tags are needed so that high sensitivity assays can detect, quantify and follow the dynamics of these proteins, often in a high-throughput

References

1 CRISPR/Cas9 Gene Editing In Drug Discovery Trends 2016. Published by HTStec Limited, Godalming, UK, March 2016.

2 Paquet, D et al (2016). Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. Nature, 533(7601):125-129.

applications. Cellular reporter technologies are proving particularly useful in studying changes in the regulation of genes and proteins (Promega).

In conclusion, in a very short time span (<2 years), CRISPR/Cas9 gene editing technology has risen to be seen as the superlative method when attempting a gene knockout or when introducing defined mutations, insertions or modifications to the genome. Although use of CRISPR/Cas9 is still mainly confined to the basic research area in the drug discovery process, its potential with respect to the identification and validation of new therapeutic targets, the investigation of mechanism of action and in the creation of screens to identify genes that regulate various cell biological processes are major objectives. The perceived key advantages of CRISPR/Cas9 technology are its efficiency (ie it edits targets sequences at surprisingly high rates), simplicity (ie easy to use and design) and programmability (ie get precision targeting). The main benefits of CRISPR/Cas9 technology that drug discovery labs are most eager to exploit is the ability to make a complete genetic knockout, while minimising off-target effects, the rapid generation of cell lines harbouring desired mutations and the possibility to develop accurate models of complex human disease in an efficient manner. The delivery of the CRISPR components into some target cells is still not optimal and this limits some work on CRISPR/Cas 9 gene editing today. However, there seems little doubt that CRISPR/Cas9 technology will transform gene editing in drug discovery labs over the coming years. DDW

Dr John Comley *is Managing Director of HTStec Limited, an independent market research consultancy whose focus is on assisting clients delivering novel enabling platform technologies (liquid handling, laboratory automation, detection instrumentation; assay methodologies and reagent offerings) to drug discovery and the life sciences. Since its formation 14 years ago, HTStec has published 128 market reports on enabling technologies and Dr Comley has authored 58 review articles in* **Drug Discovery World**. *Please contact info@htstec.com for more information about HTStec reports.*