Functional Genomic Screening with CRISPR technologies

An introduction to CRISPR screening and some of the approaches used at Horizon Discovery

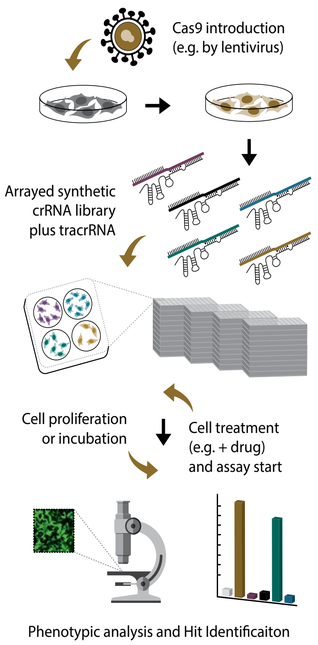
horizon™ INSPIRED CELL SOLUTIONS

Arrayed CRISPR screening

One of the main benefits of functional genomic screening in arrayed format is the possibility to more readily apply complex functional assays or powerful phenotypic readouts that are less compatible with pooled screening approaches. Arrayed loss- or gain-of-function screens using CRISPRko and CRISPRa reagents offer more precise means to perturb gene expression than the traditionally used siRNA or cDNA overexpression reagents. An important distinction of CRISPRko/a to **RNAi**, however, is that cells also require Cas9 (or dCas9) present to perturb gene function. In most cases, this can be achieved by generating a cell line with stable Cas9 expression, e.g. using lentivirus transduction prior to screening[18,19], although this can pose a hurdle for certain primary cell types. Alternatively, Cas9 mRNA or protein[20] can be transiently delivered to cells to enact the gene perturbation.

Following the introduction of Cas9 or dCas9, arrayed CRISPRko/a screening can then be conducted by transfecting cells with synthetic **crRNA** and **tracrRNA** reagents, which mimic the native CRISPR system. These reagents can be conveniently pre-dispensed and stored in microwell assay plates using automated liquid handling similar to siRNA or compound-based screening. In many other technical aspects, arrayed screening with CRISPR is very similar to RNAi. For instance, optimisation steps of reagent delivery, determination of the assay length, treatment conditions or robust phenotype responses all need to be carefully evaluated prior to execution of a high-throughput arrayed genetic screen.

Arrayed CRISPR screening tools allow researchers to conduct a very diverse range of complex assays and readouts including measuring changes in biomarker expression/localization or cell/organelle morphology by high-content microscopy, even at multiple time points. In addition, live cell imaging, migration/invasion, 3D culture, and co-culture assays can be applied. Even readout of mRNA/protein expression level changes by RT-qPCR and FACS are highly compatible with these tools. The possibility of multiplexed assay readouts and complex signature evaluation significantly increases the value of arrayed over pooled approaches.

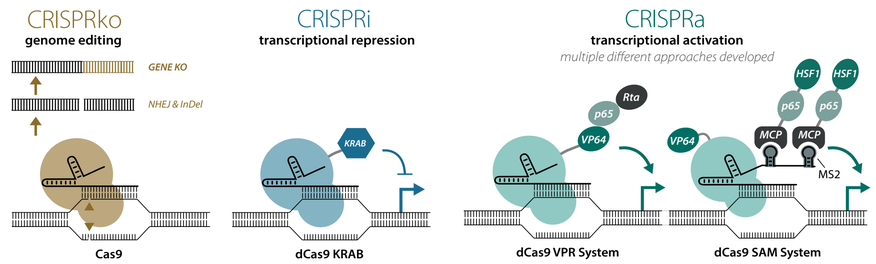


CRISPR screening

Forward genetic screening with **CRISPR** tools has provided a promising new way to interrogate the phenotypic consequences of gene manipulation in high-throughput, unbiased analyses in target ID, target validation, drug **MOA** analysis and **patient stratification**[1,2]. Diseases previously refractory to systematic high-throughput approaches, can now be interrogated with unprecedented precision and statistical power.

Functional genomic screening with CRISPR was first demonstrated with a fully active **Cas9 endonuclease** used to knock-out specific gene loci and evaluate the phenotypic effect of the deletion[3,4], but since these early analyses additional new approaches have also been developed which augment and complement genomic approaches to new discovery[5]. The generation of nuclease deficient versions of Cas9 has enabled the generation of two additional techniques – CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) – that enable the repression or overexpression, respectively, of target genes. CRISPRko uses double-strand breakage by the Cas9 enzyme to drive the introduction of insertions and deletions at genomic loci following repair by non-homologous end joining (**NHEJ**) in the cell[6]. Rather than mutating specific genomic loci, CRISPRi and CRISPRa use dCas9 which allows precise and robust perturbation of gene transcription. In CRISPRi, dCas9 is covalently linked to a **KRAB** transcriptional repressor domain, driving transcriptional silencing[7]. CRISPRa, works by fusing dCas9 to activation domains[8–11]. Effective gene activation with CRISPRa has been accomplished by several approaches: the SunTag array, which uses multiple VP64s recruited onto a peptide array[12]; VPR, a synergistic tripartite activation method using a fusion of VP64, p65 and Rta[13]; and the Synergistic Activation Mediator complex (SAM[14]), which uses a dCas9-VP64 and recruitment of p65 and HSF1 via RNA binding protein components. These adapted CRISPR tools provide remarkable new opportunities to study gain-of-function mutations in genome-wide screens[7,14].

Screening with CRISPR can now be conducted with either pooled or arrayed approaches, each providing a distinct advantage and application to discovery. In general, pooled approaches use the power of **next generation sequencing** (NGS) to drive rapid and high-quality screening all the way up to whole-genome analysis without the need for substantial automation. Arrayed screening approaches use microwell plates and traditional liquid-handing approaches, but offer the possibility to explore multi-parametric or multiplexed readouts with high resolution.

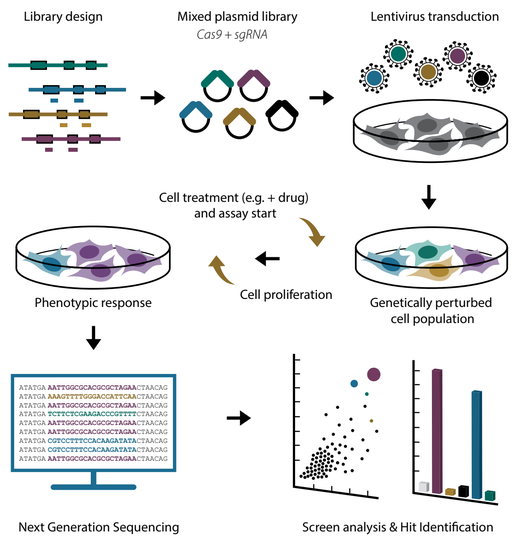


Pooled CRISPR screening

Pooled screening is amongst the most powerful CRISPR screening tools available, offering the opportunity to readily interrogate cell systems at the whole-genome level, and thus delineate a plethora of biological questions including mechanistic insights into the action of drugs, toxins and pathogens[1], or definition of gene essentiality.

Lentiviral sgRNA libraries can be designed to cover subsets of genes at very high **library complexity** (e.g. 10 guides per gene) or a whole genome, often at a slightly lower complexity. Following computational design, these sgRNA sequences are synthesised and the oligonucleotides are cloned into a lentiviral vector using pooled cloning approaches. Lentiviral particles generated from the library are then used to transduce the target cells at low multiplicity of infection, minimising the possibility of multiple knockouts per cell. Pooled screens that investigate the mechanism of drug action can either be tailored to primarily detect genes that convey drug resistance or to detect sensitizing genetic factors. These two paradigms are also known as positive and negative selection screens. In the case of positive selection, a high selection pressure is exerted to favour the survival of treatment-resistant cell populations. For negative selection, the selection pressure is generally low. This results in the loss of cells harbouring genetic perturbations that render them more susceptible to the drug. In both cases, the genomic DNA is extracted and analysed by next generation sequencing. Hits are then identified by comparing **sgRNA** counts from screen end pellets of treated cells with those of controls.

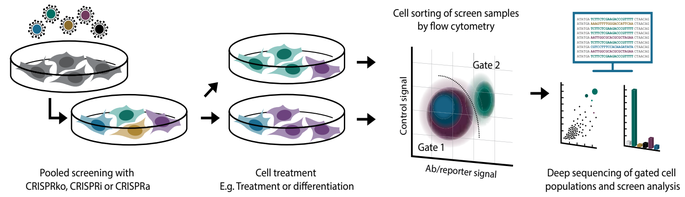
The necessity to investigate diseases such as cancer in an environment that recapitulates human disease has led to the development of pooled CRISPR screens in *vivo*[16]. The in vivo screen can be approached from two different angles, the **xenograft** model[16] and the direct in vivo model[17]. In the xenograft model cancer cells that have been transduced with a pooled lentiviral library are transplanted into immunodeficient (nude) mice. For downstream analysis the primary tumour and/or metastatic sites can be analysed by next generation sequencing to determine genes that favour engraftment, migration and metastasis. Direct *in vivo* screens are more complex as it requires direct injection of pooled lentiviral or pooled adeno-associated viral particles at the anatomical site of interest[17], but both offer novel approaches to understanding functional genomics with pooled CRISPR screening systems.



Pooled phenotypic screening

Pooled phenotypic CRISPR screening is a powerful and highly adaptable method to interrogate the phenotypic consequences of gene loss with a high-throughput genomics-based strategy. One approach uses high-throughput fluorescence-activated cell sorting (FACS) to determine cell phenotypes based on a biomarker signal. This can be done via immunostaining approaches or by using fluorescence-linked reporter cell systems and can be used to explore cell surface, intracellular or even secreted biomarkers. In contrast to proliferation-linked screening (where the analysis is of sgRNA abundance in surviving cell populations), pooled phenotypic CRISPR screening allows for the examination of phenotypes where cellular pathophysiology is uncoupled from cell health. These studies can also be conducted using bead-based approaches for positive or negative selection of defined phenotypically-marked populations[21].

Excitingly, recent advances have seen rapid development of novel platforms that massively increase the phenotypic throughput of CRISPR screening. These new approaches combine CRISPR-based pooled genetic screening with single cell **RNA sequencing** (scRNA-seq). Perturb-seq, CRISP-seq and CROP-seq[22–26] are reverse genetic methods that enable researchers to directly link CRISPR-Cas9 imposed genetic perturbations to gene expression phenotypes at the single cell level. Comparing the transcriptomes of many cells in parallel enables the comprehensive assessment of subpopulations within a heterogeneous population, and provides mechanistic information on which genetic perturbations influence the phenotypes measured.

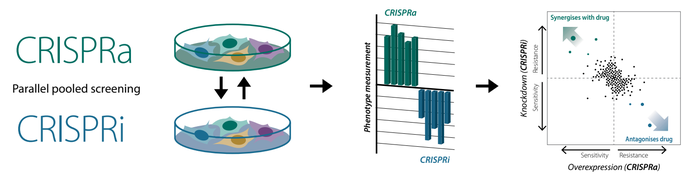


Dual CRISPR Sreening

Combining CRISPR-based screening platforms has the potential to substantially augment the quality and value of data derived from functional screening campaigns, as well as providing novel insights not accessible to one technology used in isolation[27,28]. The Dual Screen uses parallel analysis of loss-of-function screening and gain-of-function screening, by harnessing the power of CRISPRi (or CRISPRko) and CRISPRa gene modulation technologies simultaneously. This provides a paired dataset with additional depth and improves the value and quality of the screening dataset.

One of the major benefits of this approach is the ability to cross-validate hits with two independent data sets. This combination approach is of particular value since with appropriate design, the power of enrichment-based screening (e.g. resistance screening) can be co-opted to identify genes which result in sensitisation by analysing the effect of the opposing function. The effect of depletion of a target gene on loss of cell viability might be hard to study with loss-off-function screening, but with a Dual Screen approach the response of cells to gene hyper-activation driving resistance to cell death can be readily detected. This provides valuable genetic insights into cellular physiology.

An additional approach is provided by a dual loss-of-function screen, which uses CRISPRko, CRISPRi or **shRNA** in tandem and can be used to increase both the sensitivity and confidence of whole-genome drop-out screening. This way, the two datasets can be combined and compared to yield novel hits and biological discoveries.



Glossary

CRISPR: Stands for Clustered Regularly Interspaced Short Palindromic Repeats, but is often used as a short hand to describe Cas9-based gene editing and perturbation technologies.

MOA: Drug mechanism of action (MOA) describes the biological process by which a specific therapeutic agent affects the cells.

Patient stratification: The identification of patient populations that either respond or do not respond to a given drug, often based on genetic factors that result in altered drug interaction.

Next generation sequencing: Or NGS describes the high-throughput approaches to gene sequencing that have been developed to increase the speed of genomic analysis. There are many different approaches, but they all have scale, precision and speed advantages over traditional sequencing methods.

Cas9 endonuclease: CRISPR associated protein 9 is an RNA-guided endonuclease which drives CRISPR using its ability to bind DNA and cut in a site-specific manner. It is endogenously found as part of an adaptive immunity system in Streptococcus pyogenes, among other bacteria.

NHEJ: Non-homologous end joining (NHEJ) is a DNA double-strand break repair pathway. It is referred to as "non-homologous" since the broken ends are re-ligated without the need for a template DNA, which is in contrast to homology directed repair.

KRAB: Krüppel associated box is a transcriptional repression domain found in zinc finger transcription factors.

Library complexity: Describes the number of individual guides (crRNAs or sgRNAs) included in a pooled or arrayed library which target one individual gene.

crRNA: CRISPR-RNA. An RNA molecule that includes the protospacer-complementary sequence.

tracrRNA: Transactivting crRNA, hybridises to the crRNA to form a complex with the Cas9 protein.

sgRNA: Single-guide RNA, is a fusion of the tracrRNA and crRNA in a single molecule.

References

1. Doench, J. G. Am i ready for CRISPR? A user's guide to genetic screens. *Nat. Rev. Genet.* **19**, 67–80 (2018).
2. Moore, J. D. The impact of CRISPR-Cas9 on target identification and validation. *Drug Discov. Today* **20**, 450–457 (2015).
3. Shalem, O., Sanjana, N. E. & Zhang, F. High-throughput functional genomics using CRISPR-Cas9. *Nat. Rev. Genet.* **16**, 299–311 (2015).
4. Wang, T., Sabatini, D. M., Jenny J. Wei & Eric S. Lander. Genetic Screens in Human Cells Using the CRISPR / Cas9 System. *Science* (80-). 1–8 (2013). doi:10.1126/science.1246981
5. Wang, H., La Russa, M. & Qi, L. S. CRISPR/Cas9 in Genome Editing and Beyond. *Annu. Rev. Biochem.* **85**, 227–264 (2016).
6. Cross, B. C. S. *et al.* Increasing the performance of pooled CRISPR–Cas9 drop-out screening. *Sci. Rep.* **6**, 31782 (2016).
7. Gilbert, L. A. *et al.* Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell* **159**, 647–661 (2014).
8. Gilbert, L. A. *et al.* XCRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* **154**, (2013).
9. Perez-Pinera, P. *et al.* RNA-guided gene activation by CRISPR-Cas9–based transcription factors. *Nat. Methods* **10**, 973–976 (2013).
10. Maeder, M. L. *et al.* CRISPR RNA–guided activation of endogenous human genes. *Nat. Methods* **10**, 977–979 (2013).
11. Farzadfard, F., Perli, S. D. & Lu, T. K. Tunable and multifunctional eukaryotic transcription factors based on CRISPR/Cas. *ACS Synth. Biol.* **2**, 604–613 (2013).
12. Tanenbaum, M. E., Gilbert, L. A., Qi, L. S., Weissman, J. S. & Vale, R. D. A protein-tagging system for signal amplification in gene expression and fluorescence imaging. *Cell* **159**, 635–646 (2014).
13. Chavez, A. *et al.* Highly efficient Cas9-mediated transcriptional programming. *Nat. Methods* **12**, 326–328 (2015).
14. Konermann, S. *et al.* Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* **517**, 583–588 (2014).
15. Shalem, O. *et al.* Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells. *Science* (80-). *343*, 84–87 (2014).
16. Chen, S. *et al.* Genome-wide CRISPR screen in a mouse model of tumor growth and metastasis. *Cell* **160**, 1246–1260 (2015).
17. Zhou, H. *et al.* In vivo simultaneous transcriptional activation of multiple genes in the brain using CRISPR-dCas9-activator transgenic mice. *Nat. Neurosci.* **21**, 440–446 (2018).
18. Strezoska, Ž. *et al.* High-content analysis screening for cell cycle regulators using arrayed synthetic crRNA libraries. **J. Biotechnol. 251**, 189–200 (2017).
19. Tan, J. & Martin, S. E. Validation of synthetic CRISPR reagents as a tool for arrayed functional genomic screening. *PLoS One* **11**, 1–14 (2016).
20. Hultquist, J. F. *et al.* A Cas9 Ribonucleoprotein Platform for Functional Genetic Studies of HIV-Host Interactions in Primary Human T Cells. *Cell Rep.* **17**, 1438–1452 (2016).
21. Arroyo, J. D. *et al.* A Genome-wide CRISPR Death Screen Identifies Genes Essential for Oxidative Phosphorylation. *Cell Metab.* **24**, 875–885 (2016).
22. Adamson, B. *et al.* A Multiplexed Single-Cell CRISPR Screening Platform Enables Systematic Dissection of the Unfolded Protein Response. *Cell* **167**, 1867–1882.e21 (2016).
23. Datlinger, P. *et al.* Pooled CRISPR screening with single-cell transcriptome readout. Nat. Methods 14, 297–301 (2017).
24. Hill, A. J. *et al.* On the design of CRISPR-based single-cell molecular screens. *Nat. Methods* **15**, 271–274 (2018).
25. Dixit, A. *et al.* Perturb-Seq: Dissecting Molecular Circuits with Scalable Single-Cell RNA Profiling of Pooled Genetic Screens. *Cell* **167**, 1853–1866.e17 (2016).
26. Jaitin, D. A. *et al.* Dissecting Immune Circuits by Linking CRISPR-Pooled Screens with Single-Cell RNA-Seq. *Cell* **167**, 1883–1896.e15 (2016).
27. Le Sage, C. *et al.* Dual direction CRISPR transcriptional regulation screening uncovers gene networks driving drug resistance. *Sci. Rep.* **7**, (2017).
28. Jost, M. *et al.* Combined CRISPRi/a-Based Chemical Genetic Screens Reveal that Rigosertib Is a Microtubule-Destabilizing Agent. *Mol. Cell* **68**, 210–223.e6 (2017).

horizondiscovery.com