High-throughput RNAi screening in cultured cells: a user's guide

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Abstract | RNA interference has re-energized the field of functional genomics by enabling genome-scale loss-of-function screens in cultured cells. Looking back on the lessons that have been learned from the first wave of technology developments and applications in this exciting field, we provide both a user's guide for newcomers to the field and a detailed examination of some more complex issues, particularly concerning optimization and quality control, for more advanced users. From a discussion of cell lines, screening paradigms, reagent types and read-out methodologies, we explore in particular the complexities of designing optimal controls and normalization strategies for these challenging but extremely powerful studies.

Ribozyme
An RNA molecule with catalytic activity.

RNAi
RNA interference refers to the process by which dsRNA molecules silence a target gene through the specific destruction of its mRNA.

dsRNA
Long dsRNAs (usually referring, in this context, to those that are >200 bp in length) that are made from cDNA or genomic DNA templates.

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Tissue culture cells have provided a powerful system for studying many fundamental problems in signal transduction, cell differentiation and physiology. However, functional studies in cultured cells were hampered in the past by the lack of a powerful method for perturbing gene activities. Several technologies designed to knock down gene function, such as those based on ribozymes and antisense approaches, showed initial promise but ultimately failed to deliver robust protocols.

A turning point came with the discovery of RNAi (REF. 1) and its rapid rise from small-scale experimentation to genome-scale screening in *Caenorhabditis elegans* using dsRNAs[2,3]. Hopes were raised that this method might also be applicable in mammalian cells, providing a direct causal link between gene sequence and functional data in the form of targeted loss-of-function (LOF) phenotypes. The use of long dsRNAs to trigger RNAi was initially hindered in mammals by the fact that these molecules simultaneously activate the interferon response[4]; however, it quickly proved successful in cultured *Drosophila melanogaster* cells[5]. Subsequently, short dsRNAs designed to mimic small interfering RNAs (siRNAs), which were initially identified in plants[6], were shown to elicit a potent and specific RNAi response in cultured human cells, without interferon activation[7]. Several strategies have now been devised to trigger the RNAi pathway, each of which is adapted and optimized for different cell systems. Today, the most commonly used approaches are based on long dsRNA for *D. melanogaster* cells, and either synthetic siRNAs or vector-expressed short hairpin RNAs (shRNAs) for mammalian cells. The fast development of these RNAi tools has been driven by advances in the molecular understanding of the RNAi pathway (BOX 1).

RNAi has accelerated a wide range of small-scale gene characterization studies, but arguably the most important way in which it has transformed biological research is by enabling genome-scale screens in cell culture systems. Driven by genome sequence data, RNAi is now widely used in high-throughput (HT) screens in both basic and applied biology[8]. It is a powerful method for addressing many questions in cell biology, and its amenability for use in modifier screens in addition to direct LOF screening has made it particularly useful for the analysis of signal transduction pathways (BOX 2). RNAi has also become a method of choice for key steps in the development of therapeutic agents, from target discovery and validation to the analysis of the mechanisms of action of small molecules[9]. Although several HT screens have already been carried out in both *D. melanogaster* and mammalian cells[10–32] this is still an area of huge opportunity, especially as new technical advances arise.

Here we provide a guide to carrying out HT RNAi screens in cell systems, focusing on *D. melanogaster* and mammalian cells — the systems in which such HT screens are mainly carried out. Most HT RNAi screens are complex and expensive undertakings, requiring significant automation and computing infrastructures, and a combination of disparate skills, ranging from informatics to cell-culture expertise and HT assay development (BOX 3). In addition to these infrastructure requirements, designing a cell-based HT RNAi screen

Box 1 | **RNAi biology**

The experimental use of RNAi represents the harnessing of endogenous cellular pathways that are present in species ranging from plants to humans. These pathways use two types of small RNA — siRNAs and miRNAs — to direct the sequence-specific downregulation of endogenous or exogenous target genes. In *Drosophila melanogaster* and *Caenorhabditis elegans*, long dsRNAs of a few hundred base pairs are commonly used in RNAi experiments, and silencing is ultimately induced by siRNAs, the key pathway intermediate. In mammalian cells, shorter dsRNAs that closely mimic siRNAs are commonly used to elicit an RNAi response without triggering the interferon pathway, sometimes through a short hairpin (shRNA) construct. As understanding of the miRNA pathway deepens, some efforts have also sought to make further RNAi reagent design improvements, either by directly mimicking miRNA biogenesis, or by learning from their targeting principles.

The siRNA pathway

Long dsRNAs and shRNAs, either ectopically introduced into cells or endogenously generated, are processed by Dicer, a dsRNA-specific RNase III, to form siRNAs. These siRNAs, which are actively maintained in the cytosol by exportin[60], are then loaded into argonaute 2-containing RNA-induced silencing complexes (RISCs). This process imposes a selection, which is based on the relative thermodynamic lability of the two ends of the siRNA, whereby one siRNA strand becomes the 'guide', or targeting co-factor, and the other becomes a temporary 'passenger', which is quickly degraded as a pseudotarget. The guide strand is then used by RISC to direct repeated rounds of target mRNA recognition, cleavage and release, in a powerful processive cycle. A search for clear 'rules' that define target mRNA recognition by the guide strand, which are important for optimizing the specificity of silencing reagents, has proved difficult. Most focus is on the so-called 'seed region' of bases 2–8, which is defined as the primary targeting region for miRNA action and is the region that is least tolerant of mismatches. Nonetheless, siRNA targeting specificity remains incompletely understood.

The miRNA pathway

miRNAs are initially produced as long transcripts (pri-miRNAs) that include hairpin structures and contain one or more miRNAs. Pri-miRNAs are processed in the nucleus by a microprocessor complex that contains the RNase III endonuclease Drosha and an RNA-binding protein Pasha or DGCR8 (DiGeorge syndrome critical region gene 8) (REFS 61–64), which produces 60–70 nt stem-loop intermediates (pre-miRNA). Pre-miRNAs are exported from the nucleus in a process that is dependent on exportin 5 and RAN[65,66], and are processed in the cytoplasm by a complex that contains the enzyme Dicer and RNA-binding protein loquacious or TRBP (TAR RNA binding protein)[67–69], producing an imperfect RNA duplex of the miRNA, the future 'guide strand' and its complement, the so-called 'miRNA*' strand. The miRNA strand is preferentially loaded into the RISC complex, whereas the miRNA* strand is degraded. The miRNA containing RISC complex then associates with target mRNA, leading to cleavage or to translational repression[70,71].

involves many levels of decision-making, including the choice of species and cell line, screening paradigm and format, reagent type and read-out methodology used in phenotypic assays. We discuss each of these considerations, and provide an overview of the necessary controls and optimization procedures for the successful implementation of a cell-based HT RNAi screen.

Choice of cell type

***Drosophila melanogaster* cells.** With a relatively modest but fast-growing list of available cell types, *D. melanogaster* cells are excellent for RNAi screens. They typically grow at or near room temperature under ambient CO_2 levels[33] and several *D. melanogaster* cell lines efficiently take up dsRNA from the medium without the need for transfection reagents[5]. In addition, *D. melanogaster* cells, like mammalian cells, allow high-resolution spatio-temporal observations to be made by microscopy[10].

S2 and Kc cells are the most commonly used lines for *D. melanogaster* RNAi screens (TABLE 1), and both take up dsRNA efficiently by bathing cells in a serum-free medium (for detailed protocols see REFS 5,34). Another popular cell line, clone 8, shows poor uptake with the bathing method, but has been successfully implemented in RNAi screens that use standard lipid-based dsRNA transfection methods[14]. Many other *D. melanogaster* cell lines of various origins[35] can be used for RNAi applications, and are available from the *Drosophila* Genome Resource Center.

RNAi can also be carried out effectively in primary cells that are isolated from *D. melanogaster* embryos. This approach can provide advantages over cell lines, as the differentiation programmes of primary cells follow *in vivo* differentiation patterns more closely. For example, screens for axonal outgrowth and muscle integrity have been completed by simply deriving cells from embryos that express a GFP marker in the cells of interest (K. Sepp, J. Bai and N.P., unpublished observations), and the primary cells tested so far elicit a robust RNAi response after bathing with dsRNAs.

Mammalian cells. The vast compendium of publicly available human and rodent cell lines offers a wide range of genotypes, cellular characteristics and tissue derivations, and therefore provides a broad potential for accurately modelling many biological processes. Although RNAi silencing reagents are available for targeting virtually any human, mouse or rat gene, most mammalian cell-based RNAi studies so far have used human cells of various origins. Adherent lines such as HeLa and U2OS offer easy, efficient delivery and fast, robust growth in the well-ordered monolayers that are most desirable for microscopy read-outs. For many of these, the transient transfection of synthetic RNAi-based silencing reagents (for example, siRNAs) has proved highly efficient (>95%) using standard lipid-based transfection reagents, although often not without significant optimization (see the later section on this topic). In such experiments, the doubling time of the cell line directly affects the duration of silencing, which usually does not exceed 5–6 days for most lines[36].

Importantly, using the transfection and culture conditions required for adequate silencing efficiency sometimes comes at the price of increased toxicity or other significant alterations to cell physiology, such that the processes under study might no longer be well represented. The robustness of cell lines varies widely in this respect: certain commonly used lines, such as HeLa, have higher tolerance to conditions that will prove markedly toxic to many others (such as the MCF-7 line) (REF. 37). This highlights the importance of careful optimization of RNAi conditions for each individual cell line, not just for maximal silencing but also to achieve optimal silencing in healthy cells. Beyond these toxicity issues, many cell lines also show genetic instability, which leads to loss of clonality and intra-line heterogeneities in karyotype and physiology. Although too often overlooked, these factors can underlie significant variability in HT RNAi screening results, and might warrant subcloning of the cell line.

Interferon response
A primitive antiviral mechanism that triggers sequence-nonspecific degradation of mRNA and downregulation of cellular protein synthesis.

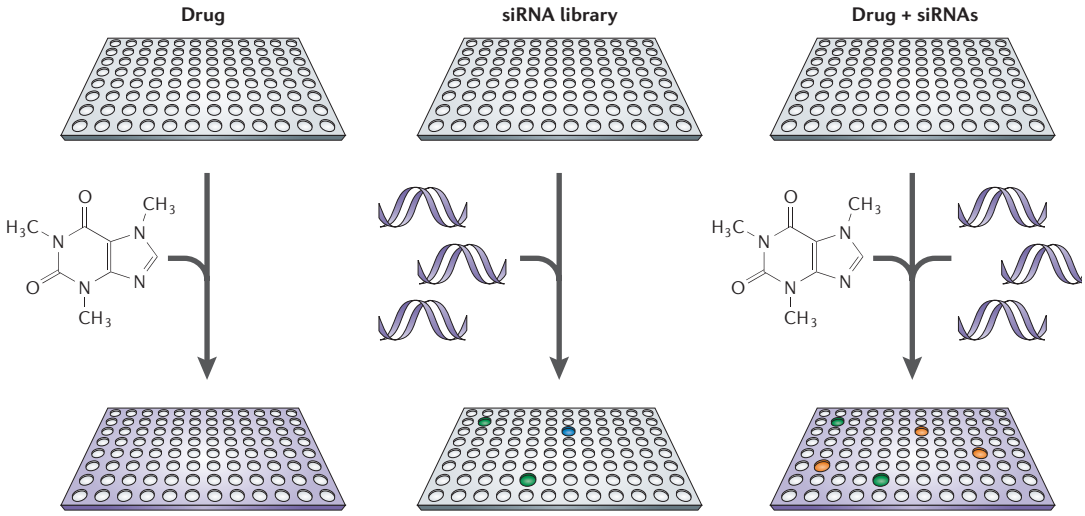
Small interfering RNA
Small RNAs of 21–23 nucleotides in length that engage the complementary mRNA into the RISC complex for degradation.

Short hairpin RNA
Small dsRNA constructs that are usually 22–29 nucleotides long and form a hairpin-like secondary structure.

The accurate modelling of certain biological processes, such as immunological and neurological pathways, remains contentious in transformed cell lines, leading many researchers to preferentially study these in primary cells. With few exceptions (for example, HUVEC cells), primary cells have presented serious obstacles to HT RNAi screening applications, primarily because most are refractory to standard lipid- or peptide-based transfection methods[38]. In some cases, such as B cells and CD34+ haematopoietic progenitors, advanced electroporation-based methods have yielded effective protocols for small-scale work, but these are not yet fully optimized for HT RNAi screening. Most other primary cells have only been accessible to HT RNAi through the use of virally delivered shRNA vectors (see below). This approach has yielded significant successes[24,25,27] despite the generally sub-optimal level of silencing that was observed with early shRNA libraries and the risk that certain viruses might alter key aspects of cellular physiology.

Another important factor to take into account for primary cell screens is the need for a constant supply of biologically homogeneous cells to support a large study

Box 2 | **Direct loss-of-function versus modifier screens**



Loss-of-function screens

The most obvious application of RNAi screening, direct loss-of-function (LOF) screening, involves identifying and functionally characterizing genes of interest on the basis of their LOF phenotypes. Such studies offer the broadest discovery potential, as they simply analyse single-gene LOF phenotypes in otherwise untreated cells. This approach has proved effective for many types of gene, including those that encode structural components, cell-surface receptors, transcription factors and enzymes. It is nonetheless important to remember that RNAi is a method for gene knock down and not knock out. Therefore, the high activity and/or long protein half-life and/or high endogenous expression of some genes might make it difficult to generate detectable LOF phenotypes, especially in the case of certain enzymes, as residual activity might be sufficient to fulfil their cellular roles.

Modifier and synthetic lethal screens

RNAi screens can also be refined through many of the same screening strategies that have been developed and perfected for decades in classic genetic screens. Particularly powerful are modifier screens, whereby RNAi is used to identify genes and pathways that, when silenced, can either enhance or suppress a given phenotype of interest. The phenotype to be modified can be the result of an initial drug treatment (see figure; change in array colour indicates the phenotypic effect of the drug that is to be modified; wells of different colours indicate the effects of siRNAs alone (middle panel) or the combination of drug treatment plus siRNAs (right panel)), in which case the screen will potentially yield insights into both the mechanism of drug action and the drug-targeted molecular pathway(s). The initial phenotype can also be generated by an initial genetic modification, through gene overexpression or even RNAi-mediated pre-silencing. In this case, the screen can potentially shed light on cellular pathways that are relevant to the function of this gene. This principle was recently applied in the context of *in vitro* neoplastic transformation assays to identify novel tumour suppressors[26,27].

In the broader context of developing novel therapeutic agents, these methods are of particular value not only for analysing a compound's mechanism of action and understanding unwanted side-effects, but also for identifying potential gene targets for developing sensitizing agents for existing drugs[72]. By focusing on silencing events that suppress the drug's action, the same approach can also identify and/or validate novel biomarkers to predict non-responsiveness to the compound, an increasingly important tool for optimizing the design of clinical trials. Among the most compelling examples of this approach are synthetic lethal screens, whereby lethal combinations of multiple non-lethal modifications are sought. Here RNAi screening is conducted in cells that are pre-treated to duplicate or mimic naturally occurring genetic lesions that are known to underlie disease states such as cancers. In such studies, the desired RNAi-modified phenotype is cell death, thereby offering a way of specifically killing cancerous cells while preserving healthy ones.

miRNA
Endogenously expressed small dsRNA (21–24 nucleotides), which can either interfere with translation of partially complementary mRNAs (usually through their 3′ end UTRs) or cause small interfering RNA-like degradation of perfectly complementary mRNAs.

Dicer
Refers to members of a highly conserved family of RNase III endonucleases that mediate dsRNA cleavage. This produces the small interfering RNAs or mature miRNAs that direct target silencing in RNAi and miRNA pathways, respectively.