CHAPTER

7

OPTIMIZING HIGH THROUGHPUT RNAi-BASED ASSAYS USING TRANSIENT TRANSFECTION OF SYNTHETIC siRNAs IN CULTURED MAMMALIAN CELLS

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7.1 Introduction

The sequencing of the human, mouse and other experimentally tractable genomes in themselves represent important milestones for biomedical research, but more importantly, they also offer crucial new resources, gold mines of data that hold huge promise as new starting points for advancing our understanding of gene function. To this end, the first generation of so-called "functional genomics technologies", including high throughput (HT) expression profiling methods and a wide range of bio-informatics database mining tools have offered impressive, though often imperfect, first glimpses into the vast potential of these new resources. Imperfect because too many of these HT methods yield data that is, at best, only indirectly relevant to the actual functions of the genes being studied. Indeed, comparative profiling of gene expression in diseased versus normal tissues can only offer correlational data, without addressing whether a gene's relative over- or under-expression represents part of the cause or rather the consequence of the disease state. It also tells us little about that gene's normal functions in the healthy individual. Although proteomics methods have more recently sought to go further in this direction by exploring the complex networks of protein-protein interactions, the *in vitro* nature of these analyses and their propensity to generate high rates of false positives have often clouded their physiological relevance, thereby also limiting their probative value. Thus, while these technologies are clearly contributing major new advances in their own right, their "signal-to-noise" ratio has been less than stellar, leaving behind a fast-growing plethora of so-called "disease-related genes", a term that is potentially misleading since their direct physiological and disease relevance most often remains unclear.

The search for better second-generation functional genomics technologies has therefore focused on the need for a scalable methodology that can test the function of many human or mammalian genes at HT by creating direct causative links between their expression and a particular biological process or disease state of interest. The best way to achieve this is to selectively silence the desired gene and to characterize the resulting loss of function phenotype with respect to the process of interest. Not only can silencing experiments yield direct new insights into gene function, but they also offer inexpensive, early-stage predictors of the phenotypic effects that one might expect from an eventual pharmaceutical inhibitor. Until recently however, this proved fiendishly difficult to achieve in mammalian cells, despite the somewhat uneven, low throughput successes of antisense and ribozyme technologies in recent years. Instead many have settled for the technically easier though scientifically less direct over-expression experiments, which, despite some notable successes, are all too often plagued by difficulties in interpreting their results.

Since its discovery in 1998[1], RNA-mediated interference (RNAi) has quickly become the new method of choice for carrying out targeted gene silencing in a wide range of experimental systems from *C. elegans* to human cells[2]. Importantly, the genome-scale HT application of RNAi has been proven to be feasible both by our group[3 and unpublished data], and others[4,5,6,7]. Combined with the advent of siRNA technology enabling the application of RNAi in mammalian cells[8], this opens major new opportunities for accelerating the identification and functional characterisation of novel genes by exploiting genomic sequence data to drive HT-RNAi based assays in a wide range of biomedical fields.

Here, we will focus on methods for the HT characterization of gene functions using siRNAs in mammalian cell cultures, to enable the analysis of a few dozen, hundred or thousand gene targets through a panel of primary screening assays, and to retain the promising ones for more in-depth secondary assays.

7.2 Design and Interpretation of HT-RNAi Experiments: Dealing with Common Caveats

7.2.1 Caveat 1: Partial silencing

RNAi experiments should be recognized as "knock-downs" rather than "knock-outs". Although we have found RNAi to be generally very potent and very reproducible, in many cases, complete silencing of target gene expression will not be achievable under the desired assay conditions. This is an inherent truth of the method, which, since it relies on destruction of mRNA, can be thwarted by a particularly stable protein, for example, or one whose residual expression, even when reduced to very low levels, may still be sufficient to fulfil the cellular functions being tested. This limitation can also relate to technical issues such as difficulties in transfecting the cells of interest, or lack of sensitivity of the assay readout being used, and must be considered when interpreting the results. On the other hand, the ability to "titrate" the silencing effect by RNAi is a significant strength of the technology, as it can be readily applied to generate "allelic series" or different levels of partial loss of function, which can often yield further important insights into gene functions, particularly in the case of multi-functional or essential gene products.

Because of this "caveat", it is often invaluable to monitor RNAi-induced silencing potency to be able to accurately interpret resulting phenotypic datasets. Ideally, one would seek to apply quantitative, well-normalised and internally controlled analyses of target protein levels, such as quantitative Western blotting or ELISA-based methods, and target mRNA levels, such as quantitative RT-PCR or branched DNA-based analyses. We have found that the two, protein and mRNA, most often mirror each other, and have come across

and then this function is restored in the silenced background using a suitable expression vector. If the siRNA used is directed against the target's coding sequence, the expression construct must be engineered to contain appropriate silent mutations making it insensitive to the siRNA. Alternately, siRNAs targeting the endogenous 3'-UTR may be convenient, as this portion of a message can easily be left out of expression constructs. However, it is recognized that such rescue experiments are technically often quite challenging, and while positive results are always compelling, negative ones may be due to a wide variety of technical reasons, which inevitably make them difficult to interpret.

An excellent second choice of specificity control that is technically more widely feasible is the use of multiple siRNAs directed against the same target gene. The sequences of these siRNAs must be carefully designed such that they share essentially no sequence homologies over any significant stretches with each other, thus insuring that any unexpected sequence-specific off-target events they may trigger should be completely distinct ones. Titration of the siRNA can serve as a quantitative control to demonstrate across which concentration range the down-regulation and eventually the phenotypic alteration(s) occur. Lower concentrations might avoid unwanted off-target effects.

As for off-target effects which are sequence-independent, this risk must be controlled for using equally well-designed "nonsense" controls: siRNAs carrying sequences that bear no close homologues in the targeted genome. When new series of experiments are initiated involving new assays and biological processes, it is advisable to test several such control sequences to convince oneself of their validity.

For a further discussion of specificity issues in RNAi, and choice of controls, see the editorial in Nature Cell Biology[10].

7.2.3 Some notes on optimising experimental design to suit scale and scope of the project

Large-scale screens almost always require multiple rounds of analysis, and are therefore exercises in minimizing costs and complexity of the study. Combining high throughput and high content is often achieved by keeping the number and technical complexity of analyses relatively low for the first and broadest round of screening, such that more detailed secondary and tertiary rounds of analysis can then be applied to smaller groups of gene candidates. This leaves room for some careful compromises to maximise efficiency in the first round, as long as these are well compensated for thereafter, such that in the end, there is no sacrifice in quality, depth or scientific integrity of the overall analysis. Much of the challenge resides in designing the "simplified" first

screening rounds such that they can efficiently filter out most of the "irrelevant" genes, while not losing any.

In the case of HT primary rounds of RNAi screens, we favour applying technically simple, robust and preferably inexpensive assays, but which offer maximal content, such as those based on automated microscopy analyses (see last section of this chapter). For secondary assays, more complex assays following independent assay principles are generally applied so as to offer an independent confirmation of the relevance of the first result. Confirmation of specificity can be applied at this stage, using distinct siRNAs targeting the same candidate gene of interest. Each experimental round should also include sufficient replicates to address intra-experimental variability and insure statistical interpretability of the results. The multiple rounds of screening will usually inherently address the issue of inter-experimental reproducibility. Finally, in all rounds of screening, control samples should always be included on each assay plate to insure that plate-wide effects can be accounted for.

Thus, with these caveats and recommendations in mind, we will focus the rest of this chapter on large scale, HT applications of RNAi using siRNAs in mammalian cells. First, a few practical issues should be approached:

- What is the right cell for my assay?
- How do I deliver siRNAs into these cells?
- How can I show that my siRNA actually is efficient in down-regulating the target mRNA?

Therefore, we will begin with optimising transfection efficiencies and the tools that can be applied to measure the siRNA transfer in order to assess success. Further, we will discuss how to show functionality of your designed siRNAs against your specific targets and finally we will introduce a few examples for functional assays that can be performed applying RNAi in order to validate targets. All aspects will be viewed from the perspective to allow higher throughput applications.

7.3 Optimisation of siRNA Transfer

The principle of transfecting mammalian cells using liposomal vectors is reviewed elsewhere[11,12]. Crucial steps include the efficient packaging of the nucleotides, attachment of the complex to the cellular membrane and its internalisation, protection from endosomal degradation and the quantitative release into the cytosol. Contrary to plasmid-based constructs, which require uptake

into the nucleus for their activity, siRNAs carry out their "work" in the cytosol. This most likely explains our repeated observations that much higher transfection efficiencies can be achieved with siRNAs as compared to plasmids within the same cell lines. Cells that demonstrated low transfection efficiency determined by lacZ-reporter plasmid transfection and subsequent X-gal staining, showed excellent results for siRNA transfer examined by target mRNA down-regulation and in functional assays. In the following paragraphs we will explain various strategies to determine efficient transfection protocols.

7.3.1 Selection of a transfection reagent and considerations for optimisation

A number of commercially available transfection reagents are claimed to be suitable for siRNA transfection into mammalian cells, including some said to be specifically designed for siRNA transfer, and others which were originally developed for DNA oligonucleotides, DNA plasmids or mRNA. ***Table 7-1*** shows a partial listing of some commonly used reagents (more extensive and updated listings can be found at www.biocompare.com).

The key factors for transfection optimisation are the ratio of nucleic acid to transfection reagent and cell density. Further, antibiotics, the presence of serum and timing issues (e.g. for complexing of nucleotides with reagent, or for exposure of cells to the complexes) are often important, and therefore should be examined. In the case of RNAi experiments, the concentration of siRNA should be carefully optimised so as to achieve maximal silencing potency while minimizing the risk of unspecific "off-target" effects. Indeed, recent publications using expression profiling methods have documented the increased risk of off-target effects associated with the use of higher concentrations of siRNAs[13]. Thus, starting with a standard protocol and keeping the amount of transfection reagent and total concentration of siRNAs constant, the effector siRNA -directed against the intended target mRNA- is titrated against a negative control siRNA -designed to have no complementary sequences in the chosen experimental organism. A concentration of effector siRNA is first defined that is sufficient to achieve maximal down-regulation, as judged preferentially by quantitative RT-PCR. At this desired concentration the transfection optimisation starts: The transfection reagent is titrated to screen for the best ratio of siRNA to reagent. For some reagents toxicity will be observed at higher reagent concentrations. There is a balance between efficient down-regulation and cytotoxicity. Observation by eye and an additional toxicity assay will enable to find the right conditions.

Cell density is the next critical factor. On the one hand, you may want to run assays over a period exceeding 48 hours and therefore need to seed the cells

at lower density. On the other hand below a certain density the transfection efficiency will drop dramatically. The right window for optimal transfection between cell density and assay is very cell-type specific, and therefore must be determined empirically. To make cell counting more accurate, we recommend strongly to use an automatic cell counter, particularly in view of routine application for example for screening.

Although many claim that antibiotics might affect transfection efficiency, we never observed any decline when antibiotics were added along with serum after the initial incubation time of the complex on the cells.

Incubation times within the transfection protocols can be varied, especially the incubation of the complex on the cells, which is often critical. The exposure time will result from a balance between transfection efficiency and toxicity. Although in some cases, the incubation period can be concluded by simply adding serum-containing growth medium, strong toxicity that is sometimes associated with the transfection reagents may require removal of the complex, or additional careful washing steps, before fresh growth medium is added.

To increase the transfection efficiency or to elongate the duration of the down-regulation over a longer period one might consider multiple transfections. We have indeed used this approach successfully, implementing a second transfection of the same wells after 3 days to run a total assay time of 7 days.

Panel 7-1A shows a sample transfection protocol, which was established as a baseline method for delivering siRNAs into several commonly used human cancer cell lines such as A549, DU145, MCF-7, and PC-3. Typical exposure times for assays at the indicated densities are 48 to 72 hours.

Panel 7-1A: Transfection Protocol for siRNAs using OligofectAMINE

All amounts are given for one well of a 96-well plates. Master mixes should be done for triplicates and for several cell lines in parallel if appropriate.

1) Seed the cells 20-24 hours prior transfection: A549 (8000cells/well), DU145 (10000/well), MCF-7 (13000/well) or PC-3 (8000/well).

2) Dilute the siRNA duplex to a 10µM working stock in annealing buffer (100mM potassium acetate, 30mM HEPES-KOH at pH 7.4, 2mM magnesium acetate). Considering a molecular weight of about 13000 a concentration of 0.13µg/µl is achieved. Thereof 1µl/well will be applied to the cells in a final volume of 100µl during the incubation on the cells without medium achieving a final concentration of 100nM during transfection.

3) Prepare mixtures for each siRNA. This may be done best in deep-well 96-well plates.

	siRNA duplex	Opti-MEM	total volume
1x mix (0.13µg siRNA)	1µl (of 10µM stock)	16µl	17µl

4) Prepare a (master) mix for OligofectAMINE and incubate for 5-10min at room temperature.

OligofectAMINE/siRNA ratio	OligofectAMINE	Opti-MEM
1x mix (0.4µl/0.13µg)	0.4µl	2.6µl

5) Combine the solutions of (3) and (4): Add by a manual multichannel pipette 3µl of OligofectAMINE in Opti-MEM of (4) to each tube or well containing the 17µl siRNA solution of (3) and gently mix by pipetting up and down; do NOT vortex!

6) Incubate for 15-20min at room temperature.

7) Remove the culture medium from the cells.

8) Optionally, cells may be washed once with 200µl serum-free medium.

9) Serve the 40-50% confluent cells first with 80µl DMEM (w/o phenol red, serum or antibiotics) per 96well.

10) Then carefully overlay by adding 20µl transfection mix to the centre of each well. Take an automatic multichannel-multistep pipette and add sequentially each 20µl transfection mix to all replicates, the error by pipetting is minimal. After completion shake the plate slightly.

11) 4 hours after transfection overlay carefully with 50µl/well of medium containing three times the amount of fetal calf serum and antibiotics to achieve normal growth medium at a total culture volume of 150µl per 96-well.

12) Incubate for 2 days.

7.3.2 Quantifying transfection efficiency applying reporter gene technology

The general optimisation of a transfection protocol is most easily and efficiently achieved using siRNAs targeting reporter molecules such as luciferase, β-galactosidase, green fluorescent protein or others that are readily detected and quantified. Most often, the quantification makes use of homogenous assays (i.e. plate reader-based) for their speed and ease of use to analyze supernatants or cell lysates. Ideally, reporter genes should be stably expressed in the cell line of interest, but failing this, the next best alternative is the use of a transiently transfected reporter. The transfection can be carried out with plasmids and siRNAs combined, using the same transfection reagent as a one-step protocol. Alternately, some assays may favor or even require a two-step approach. For example (see Panel 7-1B), it is often desirable to pre-transfect a reporter plasmid one day ahead of siRNA delivery, to allow enough time for the reporter to accumulate in the cells before the RNAi effect is triggered. In these cases, one can prepare a large batch of pre-transfected cells that are allowed to grow in larger dishes for ~24h, then harvested, pooled and re-seeded in multiwell plates. The siRNA transfection can be performed once the cells have properly re-attached to the growth surface. This approach has the advantage that all wells will exhibit minimal variability in their reporter expression levels, and therefore, normalization against transfection efficiency may not be necessary. We have found FuGENE to be particularly well adapted for this use, as it never yielded any detectible cytotoxicity or anti-proliferative effects under these conditions in our hands.

Panel 7-1B: Protocol to co-transfect plasmid DNA and siRNAs in two separate experiments

1) 20-24 hours before transfection, seed 700000 MCF-7 cells into each well of a 6-well plate.

2) Next morning, start transfection on 50-80% confluent cells applying 2µg plasmid vector with 7µl FuGENE [Roche Life Science, Germany] per well.

3) Mix per well 7µl FuGENE with 93µl serum-free basal medium such as DMEM, RPMi or Opti-MEM and incubate for 5min at room temperature. Make master mixes if desired for multiple reactions.

4) Dilute the plasmid vector to 0.2µg/µl in sterile water and place into fresh tubes each 10µl plasmid.

5) Add dropwise the FuGENE diluted in medium of (3) and gently tap the tube to mix.

6) Incubate for 15min at room temperature.

7) Remove the old growth medium from the cells, overlay with 2ml fresh serum-containing growth medium.

8) Add dropwise the transfection mix onto the cells and swirl the wells. A removal of the transfection complex is not required.

9) Incubate for about 6 hours then harvest the transfected cells and pool them from all harvested 6-wells. Seed into each well of a 96-well assay plate 13000 transfected MCF-7 cells in the evening. Two wells of a 6-well plate are normally sufficient to load all wells of a one 96-well plate.

10) Incubate over night in a 5% CO_2 incubator at 37°C.

11) Next morning continue with the siRNA transfection protocol as outlined in Panel 7-1A.

All such experiments must also integrate adequate internal standards for normalizing all readings, against possible well-to-well variations in cell numbers that are not due to the siRNA effects themselves, but rather, related to pipeting inaccuracies and toxicities that may appear due to the transfection protocol itself. A relatively convenient option here is the measurement of endogenous mitochondrial reductase activity. This can be done by overlaying the cells with reagents containing tetrazolium salts, such as the Cell Proliferation Reagent WST-1 [Roche Life Science, Germany], before the cells are lysed for reporter protein quantification. A dual assay (described in detail in Panel 7-1C) can thus be carried out, whereby mitochondrial reductase is first monitored, followed by luciferase activity[14]. An example of a transfection optimisation performed in a 96-well plate delivering siRNAs targeting luciferase mRNA into the lung cancer cell line MCF-7 is shown in Figure 7-1.

Panel 7-1C: Dual reductase/luciferase assay

I Cell viability assay

1) Freshly prepare medium without serum and phenol red containing 10% Cell Proliferation Reagent WST-1.

2) Remove the old medium from the wells.

3) Add freshly prepared 100µl/96-well 1x concentrated WST-1/medium with a multistep-multichannel-pipette; If possible, also pipette a triplicate of medium/WST-1 controls without cells to determine the background staining.

4) Incubate the cells for 15min at 37°C (The colour development is linear at least up to an OD of 1.5 at 450nm).

5) Shake thoroughly for 1min on a shaker.

6) Measure the absorbance against a medium/WST-1 control on no cells in a plate reader at 450nm (420-480nm; reference wavelength >600nm) after 15min.

7) Withdraw reagent/medium, wash once with 250µl serum-free medium & add 50µl basal medium w/o phenol red, FCS & antibiotics.

II Luciferase assay

8) Load the 96-well plate by adding 50µl luciferase assay reagent [Bright-Glo™ Assay System, Promega] to the 50µl basal medium of each well using an automatic multistep-multichannel pipette, which provides also good mixing of the two volumes.

9) Immediately afterwards, measure the relative light units produced by the luciferase in an appropriate multiwell plate reader.

III Evaluation

10) For both luciferase and WST-1, subtract the background from all values.

11) For normalisation, divide all individual luciferase values [RLU] by the corresponding mitochondrial reductase values [A_{450nm}].

12) Then combine the normalised values of the triplicates for statistical analysis.

7.3.3 Determining cytotoxicity separately

One important shortcoming of the tetrazolium salt-based assay is that it only measures "live cells", and therefore, cannot easily distinguish between variations in cell number that are due to seeding differences from those due to

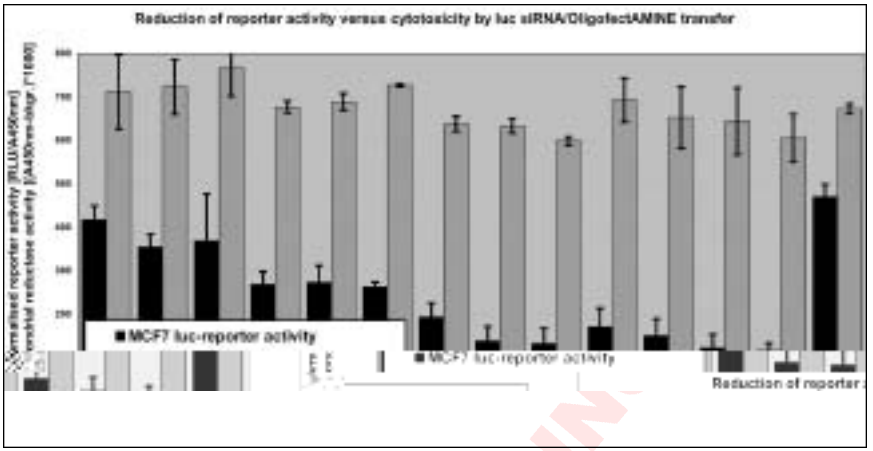

Figure 7-1. Transfection optimization in co-transfected MCF-7 cells. The dual reductase/luciferase assay was performed 48 hours after transfer of the siRNA according to the protocol of Panel 7-1C. Normalised reporter activity means relative light units produced by luciferase protein divided by absorption of the reductase product at 450nm. Background levels were subtracted. Cytotoxicity was measured from mitochondrial reductase activity using WST-1 substrate. The data were integrated into the graph after subtracting the background from the measured values and then by multiplying by a factor of 1000.

cytotoxicity. Thus, when a correct analysis of toxicity is desired, one must additionally get a measure of "dying cells" in each well. This can be achieved by monitoring for evidence of membrane damage in the culture, namely, through the presence of enzymatic activities in the growth medium that are normally restricted to intracellular compartments. There are indeed various assays available commercially for this purpose, including the Cytotoxicity Detection Kit [Roche Life Science, Germany] or the CytoTox-ONE™ Homogeneous Membrane Integrity Assay [Promega, WI] both measuring the lactate dehydrogenase (LDH) release, or the ToxiLight BioAssay Kit [BioWhittaker, Belgium] quantifying adenylate kinase (AK) release. These assays are particularly convenient as they simply require an aliquot of growth medium to be set aside (which can even be frozen for later analysis) before other assays are conducted.

7.3.3 Transfection optimisation applying a phenotypic assay

The microtubule-dependent kinesin-like protein Eg5 is involved in the assembly of the mitotic spindle [15, 16]. This is a convenient test target for many siRNA experiments and especially for functional assays. The protein half-

life is very short, allowing siRNA-induced phenotypes to become clearly visible within 24 hours post-transfection. The key phenotype in this case is the accumulation of HeLa cells arrested in mitosis with a rounded morphology and much weakened attachment to the growth substrate. This arrest represents activation of the spindle checkpoint, and corresponds to that seen also after treatments with microtubule-depolymerizing drugs such as nocodazole or colchicine. Exploiting this easily visible phenotype resulted in the proposal to target Eg5 for transfection optimisation of siRNAs and an approved siRNA sequence is published[17]. The given sequences (sense strand: 5'-CUGAAGACCUGAAGACAAUdTdT-3' and antisense strand: 5'-AUUGUCUUCAGGUCUUCAG-dTdT-3') target human as well as mouse Eg5 mRNA. Also, a control sequence that does target the mRNA about 1000 nucleotides upstream is given[17].

Normally, about 2-3% of asynchronously growing HeLa cell populations are in mitosis and show a rounded phenotype. In experiments for transfection optimisation using OligofectAMINE for siRNA transfer we routinely achieved over 90% mitotic arrests after 24 or 48 hours. Results for transfection optimisation are shown in Figure 7-2. Further, images demonstrate the round phenotype that is induced by siRNAs targeting Eg5. More applications of Eg5 siRNAs in other assays are shown in examples later in this article.

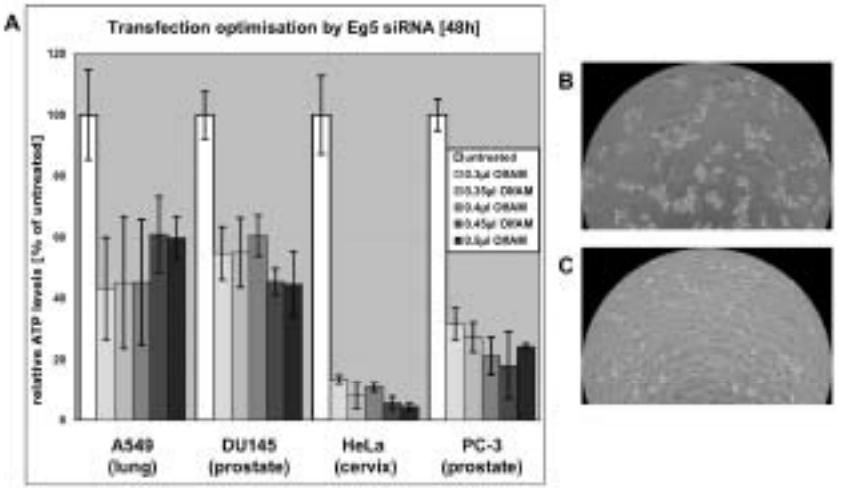

Figure 7-2. Transfection optimization for four cell lines applying Eg5 siRNAs and OligofectAMINE delivery reagent. Phenotypic changes were evaluated using phase contrast microscopy. A cell proliferation assay was then performed measuring ATP content as described later in this chapter. The images show HeLa cells treated either with Eg5 siRNA (B) or luciferase control siRNA (C) for 48h. A very high percentage of rounded cells is clearly visible after Eg5 siRNA treatment, consistent with mitotic arrest due to activation of the spindle checkpoint.

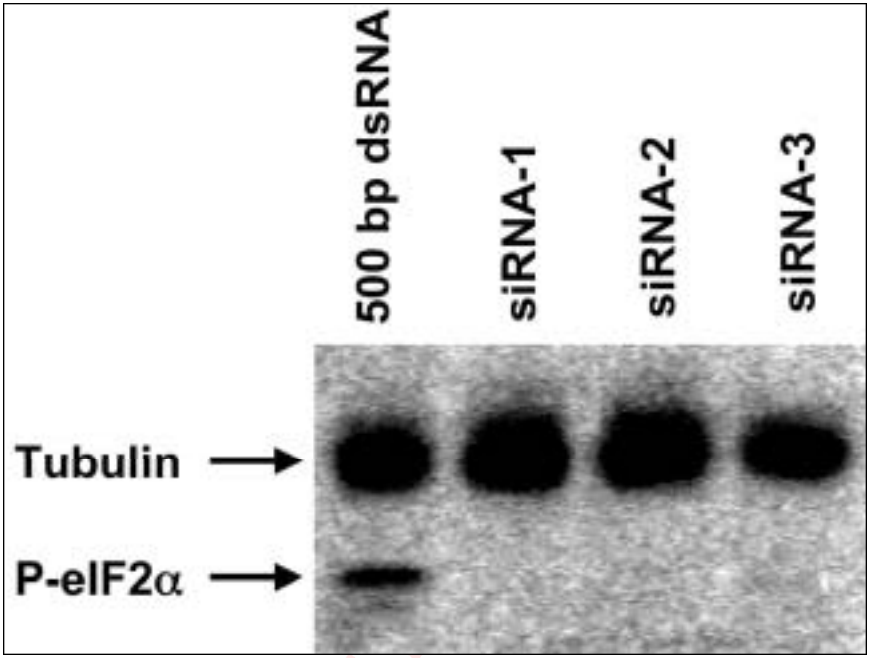

Figure 7-3. Western blot experiment to detect interferon response: Phospho-eIF2alpha was detected in extracts from cells treated with different dsRNAs. Whereas no response was observed for the three siRNAs (measured 20 h after transfection), a 500 bp long dsRNA (4 h after transfection) served as positive control.

being documented to support results from functional assays, then it is highly desirable, in principle, to also document the knockdown at the target protein level, using quantitative Western blotting. This is indeed feasible for low-throughput studies where appropriate antibodies are available, although one must be careful to consider possible differences in specificities between siRNAs and antibodies when interpreting this type of analysis. Westerns and Northerns are less feasible for high throughput applications, however, where technical limitations on up-scalability and antibody availability become key problems.

7.4.1 Measuring RNAi knockdown: when is the right time?

RNA interference is an active enzymatic process to degrade specifically mRNA. The kinetics of RNAi is illustrated in Figure 7-4. As shown, the RNAi effect is stable over a couple of days, which gives the researcher convenient flexibility for both monitoring and assay development. However, there are dif-

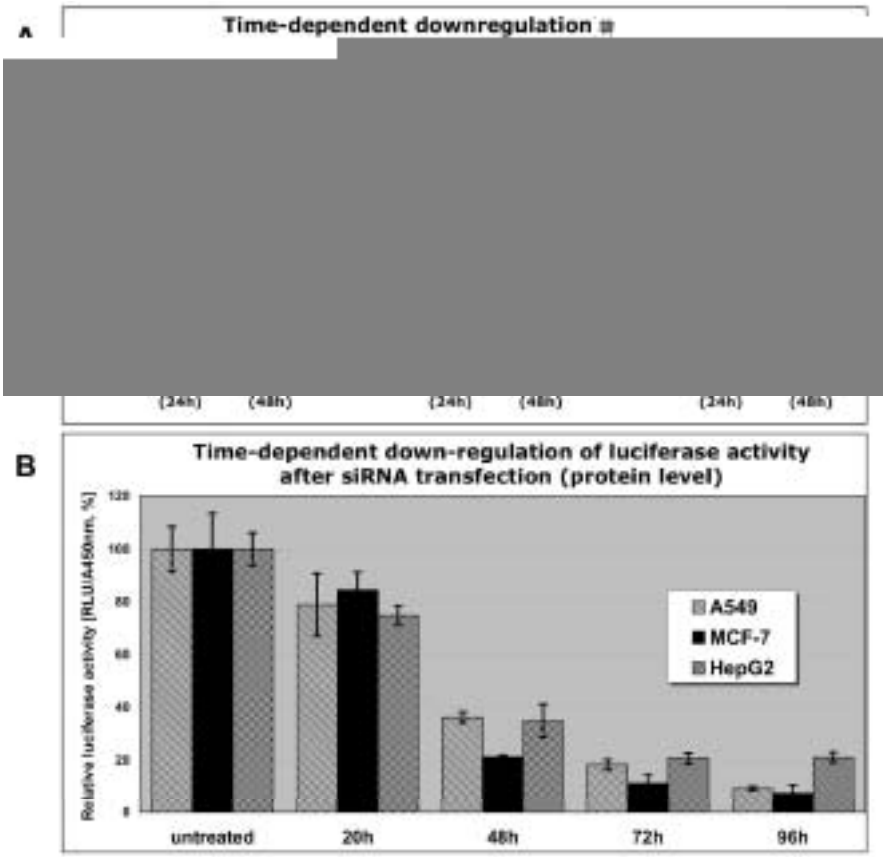

Figure 7-4. Time dependency of the RNAi effect. **Upper panel:** mRNA knockdown for three target genes in HeLa as measured by Real-Time PCR at two given time points. **Lower panel:** time course of luciferase knockdown in three transiently transfected cell lines determining the relative light units produced by enzymatic luciferase activity.

ferences between the kinetics of RNAi-mediated knockdown on mRNA and protein level. In contrast to the RNAi-mediated mRNA degradation, which is generally believed to be very quick and independent of the target gene's mRNA, the resulting protein knockdown is fully dependent on the half-life of the particular protein. Especially for the set-up of functional assays, it has to be taken into account that there might be a considerable time difference between mRNA degradation and actual protein depletion which will then be responsible for phenotypic alterations.

As seen in Figure 7-4, the time point for getting the best knockdown is generally between 2-4 days after transfection. But at the mRNA level, monitoring as early as 24h after transfection shows a reasonably pronounced knockdown. Taking into consideration the fact that the cells should ideally be in the phase of linear growth, detection of the mRNA degradation at 48h after transfection is a good compromise for many cell lines, including HeLa. In contrast, the ideal time point for measuring protein knockdown can vary in the range of several days. For luciferase, we observed maximal down-regulation between 48 and 96 hours. After 7 days, an increase in luciferase activity was noted (data not shown).

7.4.2 Monitoring silencing potency at the mRNA level: Quantitative RT-PCR (qRT-PCR)

7.4.2.1 General aspects

For measuring the knockdown of mRNA in RNAi experiments, Northern blot analysis, real-time RT-PCR (or qRT-PCR) and branched-DNA-based methods are all valid approaches. For reasons of scalability and flexibility, qRT-PCR has emerged as perhaps the most commonly used standard for these types of studies. The achievable throughput will depend heavily on the instrument and reagents sets used. Whereas the term "high-throughput" is typically used to describe methods where dozens or hundreds of 96-well or 384-well plates are being assayed in a day, "high-throughput qRT-PCR" is most often limited to handling four to six 96-well plates a day mainly due to the duration of the PCR process (approximately 2.5 hours). Other limiting factors are the duration of RNA extraction (~45min per plate, depending on the system), cDNA generation (~1h), (~2.5h per plate), the time that is needed to check the quality of total RNA and cDNA preparations on gels, and finally the evaluation time. The restrictions of "conventional RT-PCR", namely the low sensitivity, the need to test the linear range for every new target mRNA, and the limited up-scalability, have largely been overcome through the advent of real-time PCR technology. This method, combined with the excellent instruments now available, enables quantification of mRNA levels with an excellent sensitivity and good reproducibility at high throughputs.

7.4.2.2 Comparison of different approaches in Real-Time qPCR

In general, target mRNA levels of RNAi treated samples are measured relative to their respective (untreated) control template. For quantitative expression analysis, the results are normalized to reference genes. As a reference, various so-called housekeeping genes (HKG), e.g. 18S rRNA, 28S rRNA, GAPDH, tubulins and cyclophilin, can be used. These genes are mostly implicated in

basal cell metabolism and are constitutively expressed in all cell types. However, different treatments could also affect the expression of housekeeping genes. One should carefully choose the reference gene(s) and control the influence of the treatment of the expression level. In the literature, there are no universal internal standards described. The safest, but not cheapest, solution is to run the RT-PCR experiment with more than one HKG, at least in the process of method validation.

The three most popular ways of RT-PCR quantification are SYBR Green, TaqMan probes and molecular beacons. The latter two methods both rely on fluorescence resonance energy transfer (FRET) for quantification. Specific probes have to be designed for every gene of interest, which for hundreds of genes is not feasible in a manual approach, thus requiring special automated bioinformatics software solutions. Alternatively, "Assay-on-Demand" pre-designed probes against a wide range of human genes are now available from ABI. The advantage of pre-designed TaqMan assays is clearly that there is little or no optimization work needed. However, the cost factor for such probes remains significant if one needs to screen for a large number of different genes. In these cases, the SYBR Green approach offers a compelling alternative.

SYBR Green binds double-stranded DNA, and emits light upon excitation. The accumulation of PCR product can be detected as an increasing fluorescence signal. The advantages of SYBR Green are that it is inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers, non-specific reaction products and secondary amplicons caused by alternative splicing (see Figure 7-5), which would ultimately result in an overestimation of the target concentration. Thus, to rule out these artifacts and insure quality control here, standard RT-PCR programs for SYBR Green always include a final temperature gradient to profile the "melting curve" (also called "dissociation curve") of the PCR product (see Figure 7-5 for details). This determines whether or not one specific product (i.e. one melting temperature) was amplified, therefore making this test an essential requirement of every RT-PCR reaction. Also, it has been noted by some that overlaps between the PCR amplicon and the position at which the siRNA targets the mRNA can also yield significant qRT-PCR artifacts. Taken together, avoidance of an overlap between siRNA and PCR amplicon, avoidance of homologies between the primers, and a proper BLAST search against possible secondary products and alternative splice variants, as well as the usual rules of PCR primer design are recommended to get an optimal SYBR Green Real-Time qPCR assay. To include all these criteria in a high-throughput approach (i.e. design of PCR primers for hundreds or thousands of target genes), it is essential to have automated bioinformatics resources available. In reality, the actual proof of a PCR primer set's specificity in a PCR reaction is an important factor that is required for establishing a SYBR Green-based RT-PCR.

ourselves. If the number of target genes is lower, or if cost is not an issue, pre-designed TaqMan assays are also an excellent option.

7.4.2.3 RNA extraction methods

For extraction of total RNA from 96-well plates, kits are available from a variety of providers. They are based on the extraction of total RNA from lysed cells through column-plates (either using vacuum or centrifugation) or via magnetic bead separation. Some commonly used platforms for total RNA are listed in ***Table 7-2***. When comparing different kits for this purpose, one should consider the following issues:

1. How many cells per well can be extracted? (typically 10 to 10^5 or 10^6 per well),

2. What is the total RNA recovery rate / the well-to-well variability?

3. Is there an additional DNase digestion step involved / is the total RNA free of DNA / can the total RNA be used for Real-Time PCR?

4. How long does is take per plate? (range 20-70min per plate)

5. How much does it cost? (range 150 to $300 list price per plate)

Over the past few years, kits for RNA extraction from 96-well plates have become available also as automated solutions, using liquid-handling robots such as Packard's Multiprobe, Tecan's Genesis or Beckman's Biomek platforms. In our experience, both RNA extraction kit vendors and robot companies are very eager and helpful assisting in the set-up of RNA extraction protocols via particular robots. The extracted RNA should preferably be used immediately, but can also be stored non-precipitated at -80°C without extra addition of RNase inhibitor. An aliquot of total RNA extract is run on a 96well agarose gel to allow quality control.

7.4.2.4 cDNA synthesis

As for RNA extraction, a variety of excellent Reverse Transcriptase and Real-Time PCR kits are available from several suppliers, including ABI, Ambion, Eurogentec, Invitrogen, Qiagen, Stratagene and others. Random hexamers or dT oligomers are usually taken as primers and RNase inhibitor is added during the enzymatic reaction. The total RNA preparation should be re-stored at -80°C without precipitation immediately after the appropriate amount was taken out for RT reaction. After the RT reaction, the resulting cDNA can be stored at -20°C

or immediately processed further. An aliquot is run again on a 96-well agarose gel to allow quality control.

The Reverse Transcription step is critical for sensitive and accurate quantification and the amount of cDNA produced by reverse transcriptase must accurately represent the RNA input amount. The method (and supplier) should be carefully established and maintained unchanged throughout any large scale study.

7.4.2.5 Set-up of the Real-Time PCR (qPCR) reaction

It is generally advisable to run the qPCR reactions in a "protected space", either in a laminar flow hood or a separate, dedicated room, such that the running reactions cannot be affected by airborne contaminants from other molecular biology applications in the lab, including pipeting of PCR reactions, RNA extraction setup, general cloning, etc..

One step that is crucial for a reliable PCR setup is the dispensing of reagents. For a trained person (preferably equipped with electronic hand-held pipetors), it is feasible to manually set-up the PCR run with reasonably low variations even in a 384-well plate. However, for high throughput applications, automated mechanization of the pipeting steps is strongly recommended, to minimize error, variability and to maintain the user's long-term sanity. Several companies including Perkin Elmer, Tecan, MWG and Hamilton, offer robotic liquid handling systems with sufficiently accurate pipeting capabilities for the small volumes used in such reactions (typically less than 5µl). For automation, the PCR setup needs to fit a standard plate template. For each sample, this template should comprise the following reactions:

1. One well of treated sample + specific gene primers.

2. One well of treated sample + housekeeping gene primers.

3. One well of untreated control sample + specific gene primers.

4. One well of untreated control sample + housekeeping gene primers (if the HKG is the same over the entire plate, it is not required to repeat this reaction for each gene).

5. One well of non-template control + specific gene primers.

6. One well of non-template control + housekeeping gene primers (if the HKG is the same over the entire plate, it is not required to repeat this reaction for each gene).

It is recommended to measure each sample at least in triplicate. In this case, RNAi knockdown can be monitored in 25 to 30 samples per qRT-PCR run, when working with a 384-well plate. In ***Table 7-3,*** a brief overview is given of some of the established providers of qRT-PCR machines with a comparison of their capacities.

RT-PCR reagent providers give optimised protocols for setting up qRT-PCR with their kits, and normally, there is not much flexibility in varying the composition of an qRT-PCR mix. When working with 96-well plates, the PCR mix can be scaled down to 20 - 25µl per well, whereas for 384-well PCR plates, the use of as little as 10 - 15µl per well can considerably help saving on reagent costs. The following could be an example of an 11µl PCR mix:

5.5 µl	2x SybrGreen PCR mastermix
3.0 µl	cDNA
2.5 µl	Forward/reverse primer mix (2 µM)
11 µl	Total

For reproducibility of a high throughput experiment, it is recommended to use a commercial master mix including all the PCR reagents and the SYBR Green dye. The preparative manual work is then minimized to cDNA preparation in a 96-well scale and, if needed, adjusting of primer concentrations and their rearraying in a 96-well plate.

For an automated PCR setup, an appropriate pipetting program has to be created and validated. Figure 7-6 shows a possible PCR setup using a MultiProbe II Automised Liquid Handling System (Packard, now Perkin Elmer).

A typical Real-time PCR amplification blot is shown in Figure 7-7. More manual work is then required for the evaluation of the results. Beside the quality control of melting curves for each run (when working with SYBR Green), a manual adjustment of the Background Area and the Threshold Values is needed. The Real-time PCR software is providing three hundred and eighty four (384) measured Ct-values (threshold cycle values) per plate that can be exported and processed in Excel or other appropriate software applications.

7.4.2.5 Analysis of results from Real-time Q-PCR

To calculate the level of a gene's mRNA, the most practical method, without the need of a standard curve, is the Ct method (ΔΔCt method). The first

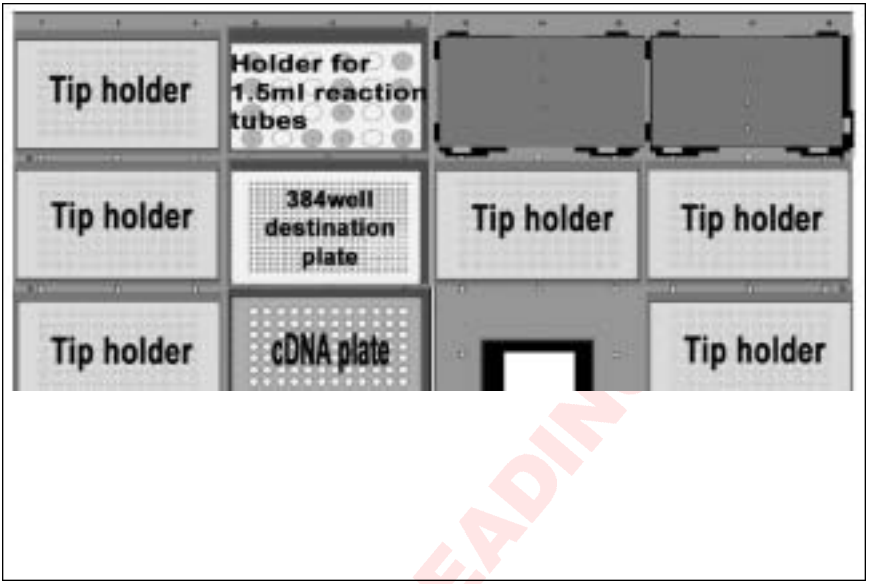

Figure 7-6. Worktable for Real-time PCR setup, on a Packard MultiProbe II Automated Liquid Handling System. Positions for mixed primer plates, cDNA, 1.5 reaction tubes and 384-well destination plate are all set on a cooling block (4(C). SYBR Green master mix, HKG primer mix and water for NTCs are distributed out of 1.5 ml reactions tubes. For accuracy reasons and to avoid cross-contaminations between single wells, tips need to be changed after each pipetting step. This makes the whole process more expensive and more time consuming. However, the time limiting step is the Real-Time PCR run itself. With a runtime of over 2 hours, the throughput on a normal working day is limited to 5 runs per instrument.

calculation step is normalization against the reference gene, i.e. subtraction of the Ct of the housekeeping gene from the Ct of the target gene, for both RNAi treated sample (ΔCt_T) and control sample (ΔCt_C).

I.) $\Delta Ct_T = Ct_T$ (TG) - Ct_T (HKG)

$\Delta Ct_C = Ct_C$ (TG) - Ct_C (HKG)

The comparative $\Delta\Delta$Ct method involves the calculation of the difference between each sample ΔCt_T to the control ΔCt_C. Depending on up- or down-regulation of the target, the $\Delta\Delta$Ct value is negative or positive.

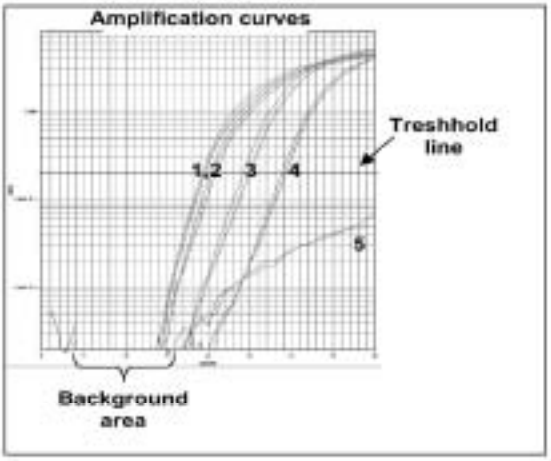

Figure 7-7. Amplification curves of a typical RT-PCR run. Amplification curves 1 and 2 are for the housekeeping gene, 3 and 4 are for the specific gene, without and with RNAi treatment, respectively. The quantification of target mRNA in the case shown above is depicted in Figure 7-8.

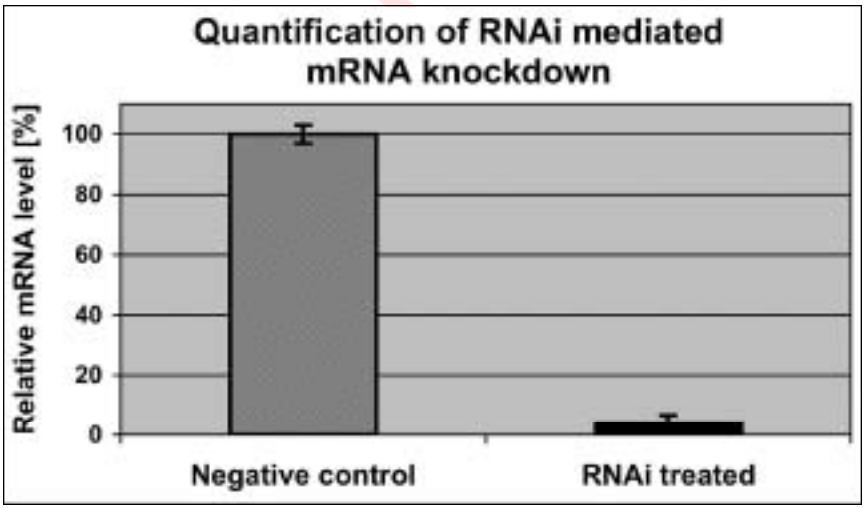

Figure 7-8. The amplification data shown in Figure 7-7 were subjected to a calculation as described in the text. As a result, the quantification of target mRNA (human PCNA gene) in the present case indicates 3.7% remaining mRNA level (HeLa, 48 h after transfection).

$$Z' = \frac{\Delta \text{ signal} - \Sigma \text{ 3xSD}}{\Delta \text{ signal}} = 1 - \frac{3\text{xSD}_{\text{treated}} + 3\text{xSD}_{\text{control}}}{\text{mean}_{\text{treated}} - \text{mean}_{\text{control}}}$$

Zhang and coworkers define also how to interpret the resulting numbers. The value 1 would define the ideal assay with either no variation or an unlimited distant signal. $1 > Z' \geq 0.5$ is termed an excellent assay with a large separation band and $0.5 > Z' \geq 0$ a double assay with a small separation band. At $Z' = 0$ there is no separation band means sample signal variation touch background signal variation, this is called a "yes/no" type of assay. At $Z' < 0$ the signals overlap and screening is not advisable.

We determined the Z'-factor for the dual reductase/luciferase assay described in Panel 7-1C with a siRNA targeting the luciferase versus a control sequence targeting bacterial kanamycin resistance gene mRNA which is not present in HeLa cells. We transfected all 96 wells of one plate with the luciferase siRNA and one separate plate with the kana control siRNA. Two days later the dual reductase/luciferase assay [see Panel 7-1C] was performed. All data points were plotted in a graph and various parameters were determined.

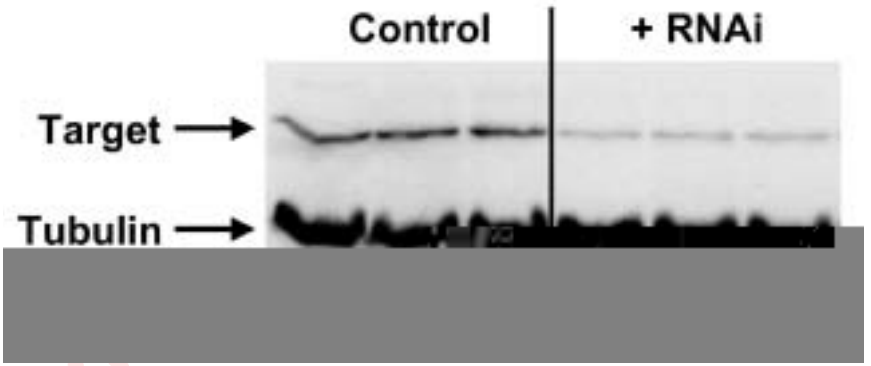

Figure 7-9. RNAi experiment monitored by a Western blot. First, the level of target protein is normalized against the level of the "housekeeper" tubulin. Then, dividing the target/housekeeper ratios of the RNAi experiment and the control gives the normalized remaining protein level after knockdown, which is 25% in the case shown above. (Protein extracts were derived from HeLa cells grown on 24-well plates 72h after transfection. Chemiluminescence detection quantification was done using an enhanced ECL reader).

The average remaining luciferase activity in the luciferasesiRNA treated plate was at 670 ± 118 normalised relative light units [RLU/A450nm] compared to 4123 ± 494 RLU/A450nm of the control treated plate. The remaining luciferase activity is at 16.25% of control, which is equivalent to a 6.15-fold reduction by the luciferasesiRNA. Standard deviations (SD) were determined and thereof the coefficient of variation (CV) was derived, 12.0% CV for the kana control and 17.7% CV for the luciferase siRNA treatment. The resulting Z'-factor for this assay was calculated according to the formula

$$Z' = 1 - \frac{3xSD_{treated} + 3xSD_{control}}{mean_{treated} - mean_{control}} = 1 - \frac{3x118 + 3x494}{|\ 670 - 4123\ |} = 0.47$$

According to the definition by Zhang and coworkers, this is a good assay suitable for screening.

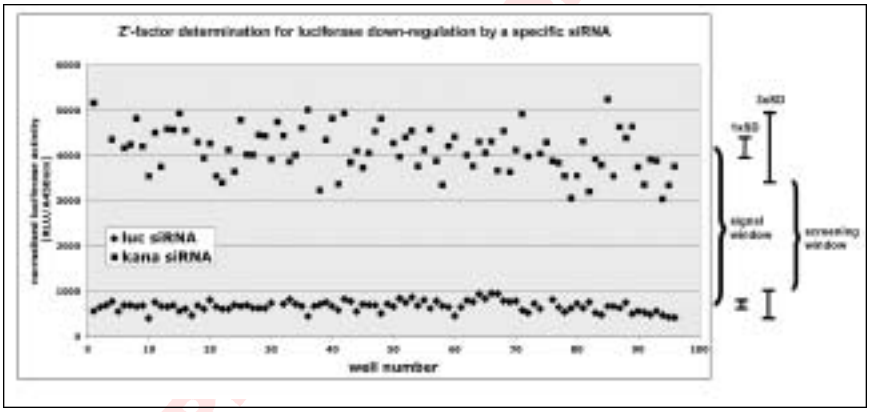

Figure 7-10. Z'-factor determination for luciferase down-regulation after luciferase siRNA treatment. One-fold standard deviation (1xSD) and three-fold SDs (3xSD) are indicated. Further the signal window that indicates the difference between the means of control and treatment and the screening window that is calculated from the difference of the two means subtracted by the two correlating 3xSDs are illustrated.

7.5.2 High throughput cell proliferation assays

The monitoring of cell proliferation throughout an assay period represents a baseline dataset that is fundamental to a wide range of functional investigations. The accuracy of such datasets varies widely according to the method used, but is generally based on the principle of only counting living cells. Therefore, simply counting cells that remain attached to the growth surface -if

any- is inadequate since many such cells can be dead or dying and therefore skew the counts unpredictably.

Selection criteria for choosing the right assay should be:

- Complexity (simple principle; limited number of pipetting steps, best a 'add-mix-and-read' format)
- Linearity of the signal over several magnitudes of cell number (50-50000cells/well)
- Signal stability, intensity and detection limit (sensitivity)
- Signal-to-noise ratio and size of the screening window
- Costs per well

A number of proliferation assays applicable for higher throughput in multi-well plate formats are commercially available based on several assay principles, all of which focus on quantifying living cells. A multi-well plate reader usually allows detection of colorimetric, luminescent and fluorescent intensities. Many assays do not allow distinctions between reduction in cell number for example by clean growth arrest, loss by apoptosis or general cytotoxicity. As with transfection reagents, we have found the website www.biocompare.com a good starting point for getting an overview of currently available kits (see chapter on "Cell Viability/Proliferation Assay"', as this table is regularly updated and commercial sources are linked). Depending on experimental needs and priorities, one can either opt for a homogenous assay (measurements in cell lysates) or an *in situ* analysis method (microscopy based).

Among available kits, a popular one already mentioned above makes use of tetrazolium salts to quantify cell numbers based on mitochondrial reductase activity in living cells. Although such assays using MTT or XTT were widely used in the past, their products were not water-soluble and the colorimetric readings required additional steps to solubilise the coloured reaction products that precipitate immediately after the metabolic reaction. More recently, water-soluble reagents such as WST-1 have become available from various suppliers, but we have found the linearity of this system to remain rather limited overall.

More complex assays (involving more pipetting steps) include ELISA-based assays, for example to quantify incorporation of BrdU into the chromosomal DNA during S-phase, which are also available for chemiluminescent read-out [Cell Proliferation ELISA, Roche].

An alternative assay principle consists in measuring ATP levels in cell lysates. Addition of luciferase and a defined substrate yield luminescence levels proportional to the total ATP content of the lysate, achieving linearity that can go over several orders of magnitude. Several such kits are commercially available, which, as they depend on luciferase, typically strike a balance between high sensitivity and signal half-life. Highly sensitive systems emit the signal as a short burst of luminescence, which unfortunately very often result in artefactual signal gradients as the reader progresses across a multi-well plate. For this reason, systems that emit a more sustained, relatively stable light signal over a longer time period a generally better adapted for higher throughput applications.

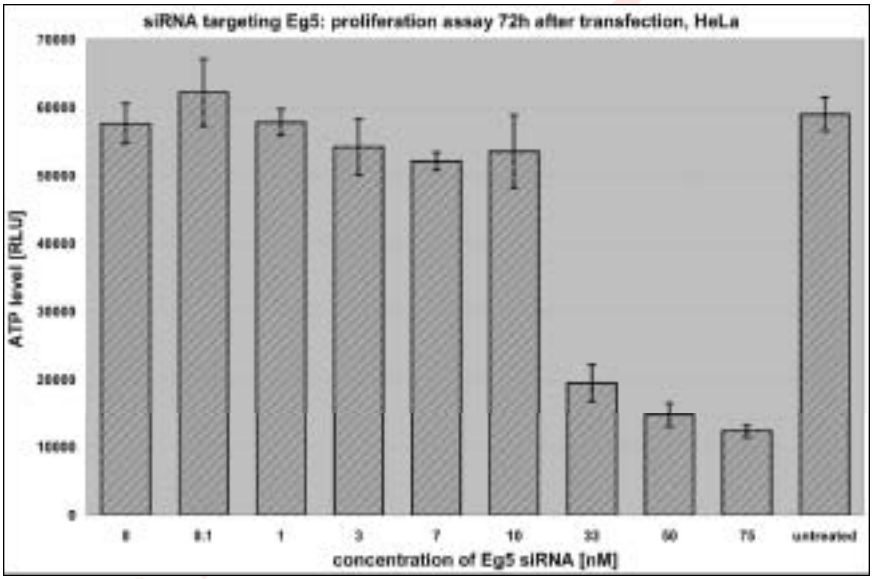

Figure 7-11. Proliferation assay 72 hours after transfection with siRNA targeting endogenous Eg5 in HeLa cells. All plates were transfected at once with a total of 100nM siRNAs composed of a titration of Eg5 siRNA versus luciferase control siRNA, each concentration in triplicate. ATP levels were measured using the ATPlite Luminescent ATP Detection Assay System [PerkinElmer, MA] according to the manufacturer's instructions and the Wallac Victor2 1420 Multilabel Counter [PerkinElmer].

As an example of applying this ATP method in a HT-RNAi experiment, we have analyzed the effects of siRNA treatments targeting Eg5, a known inhibitor of progression through mitosis. We have titrated the Eg5 siRNA between 1nM and 100nM against an "irrelevant control" siRNA having no target sequence in the cell shown in Figure 7-11. Transfections were done in triplicates for each

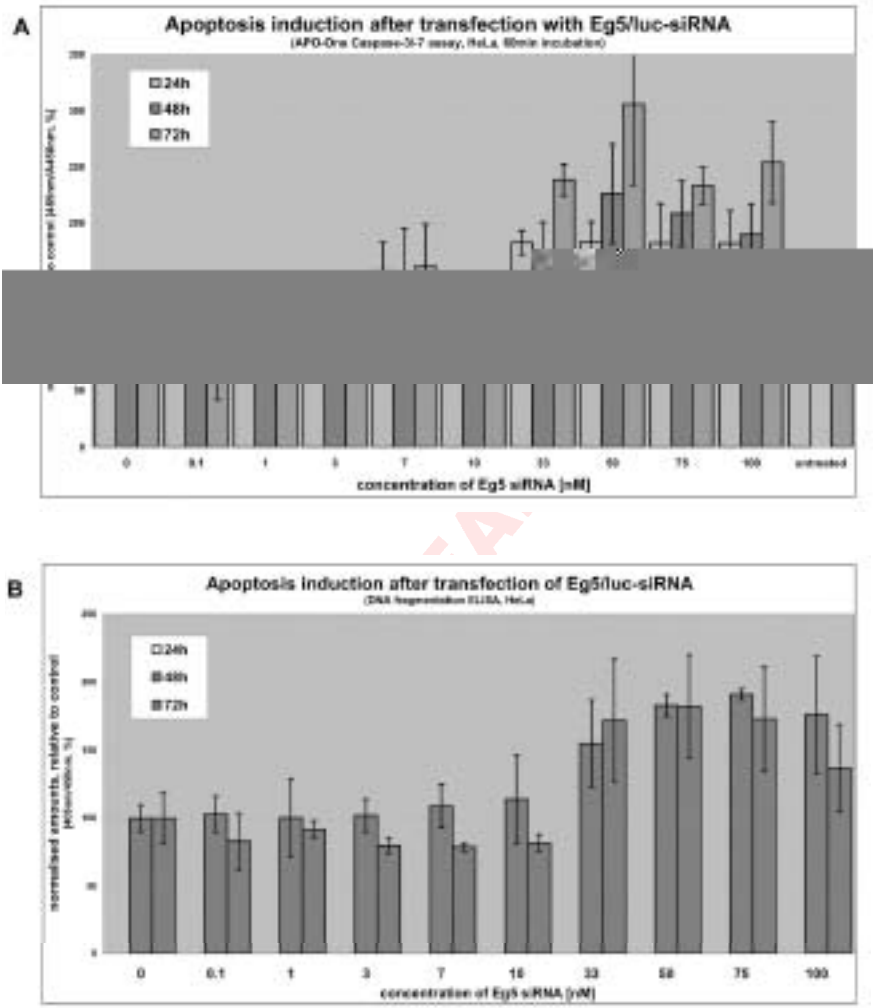

Figure 7-12. Determination of apoptosis by two independent assay principles after knockdown of Eg5 in HeLa cells. All plates were transfected at once with a total of 100nM siRNAs composed of a titration of Eg5 siRNA versus luciferase control siRNA. The Apo-ONE Homogeneous Caspase-3/7 Assay (A) [Promega, WI] and the Cell Death Detection ELISAPLUS (B) [Roche Life Science, Germany] measuring DNA fragmentation were run in parallel at 24, 48 and 72 hours according to the manufacturers' protocols. Caspase activity was measured after 60min incubation of the substrate.

The most commonly available and typically quite robust apoptosis assay kits are based on the detection of caspase activity, through the use of specialized peptide substrates. Cleavage of these peptides by specific caspases results in the release of highly fluorescent dyes. Other dyes are also available to detect changes in the mitochondrial membrane potential [JC-1 of various suppliers], thereby monitoring the apoptotic collapse of the gradient that normally exists across the mitochondrial membrane. ELISA-based assays for *Cytochrom c* release or DNA fragmentation are also excellent but, in view of their higher complexity and costs, their applicability tends to be restricted to lower throughput experiments.

As a good primary screen, we have come to favor a caspase-3/-7 assay, as it offers a convenient "add-mix-and-read" format, requiring a minimal number of pipeting steps, and exhibiting a relatively low cost per well. To illustrate the performance of this assay in comparison to the ELLISA-based DNA fragmentation assay, we again titrated the siRNA targeting Eg5 versus the "irrelevant control" siRNA between 1nM and 100nM for Eg5 siRNA. Transfections were carried out in a 96-well format, in triplicates for each concentration. In certain cell types, apoptosis is triggered after prolonged mitotic arrests, such as that which is induced by Eg5. We were indeed able to observe clear morphological changes by phase contrast microscopy, suggestive of ongoing apoptosis in cells treated with Eg5 siRNA concentrations above 10nM. This conclusion was readily confirmed by both independent apoptosis assays at 3 subsequent time points: plates were analyzed in parallel using the caspase-3/-7 assay (Figure 7-12A) as well as the ELISA-based DNA fragmentation (Figure 7-12B).

7.6 Outlook: Combining HT-RNAi with Microscopy-Based High-Content Assays

As noted above, we are finding homogeneous assay to be more and more limited because the response of cells to a treatment with either siRNAs is a complex and dynamic process, which is difficult to adequately assess with reader-based methods. Very often a few cells at a time show the biological response and the signal cannot be detected within the background noise.

The rapid evolution of automated microscopy systems in recent years, is now giving hope for overcoming traditional limitations of homogenous assays, namely lack of sensitivity and the inability to distinguish between individual cell behaviors within the population in a well. Indeed, HT cell-based assays using microscopy readouts are now a reality that not only offers much greater depth of data, but in our experience, also much improved signal windows for many commonly used assays. The vastly increased depth of data comes first from the *in situ* nature of the analysis, and second, from the associated ability

to monitor multiple sample parameters in parallel. This can either come from detailed morphological analyses achievable even with relatively simple optical methods such as Phase or Differential Interference Contrast, or from the use of multiple fluorescent markers in living or fixed samples[19,20]. With the relative ease of expressing multi-colored fluorescent (BFP, CFP, GFP, DsRed) fusion proteins and the ever-growing abundance of antibody probes and dyes, the possibilities for monitoring the fate of multiple sub-cellular components or even molecules in parallel are nearly endless.

The price paid for this advance resides mainly in the bigger challenges inherent to managing, storing and analyzing these much larger datasets. In particular, it is crucial to insure that the analysis criteria applied to such complex datasets be very tightly standardized and applied with minimal user bias or variability of any kind. In many cases, this will require the development of new, more sophisticated automated image analysis tools, which can be run quickly over large datasets, and offer ease of customization for different assays. This is an important and very active area of development now. If these data analysis challenges are well met, the resulting databases will offer users a treasure trove of information, whose value will increase exponentially with each new screen completed.

Table 7-1. Suppliers of Transfection Reagents for siRNA transfer.

Company	Reagent Name	Web Address
Amaxa Biosystems	Nucleofector	www.amaxa.com
Ambion	siPORT Amine, siPORT Lipid	www.ambion.com
Biontex Laboratories	METAFECTENE	www.biontex.com
Gene Therapy Systems	Gene Silencer	www.genetherapysystems.com
Invitrogen	OligofectAMINE	www.invitrogen.com
Mirus	TransIT-TKO	www.genetransfer.com
Novagen	RiboJuice	www.novagen.com
Q-BIOgene	JetSI	www.qbiogene.com
QIAGEN	TransMessanger	www.qiagen.com
STRATAGENE	GeneEraser	www.stratagene.com

Ambion	RNAqueous (tm)-96	Centr./Vac./Autom.
Invitek	Invisorb™ RNA Cell HTS 96	Centr./Vac./Autom.
Machery-Nagel	N	

Table 7-3. List of providers for RT-PCR machines and their products.

Company	RT-PCR Machine	Sample Capacity
Applied Biosystems	ABI PRISM 7900 Sequence Detection System	Interchangeable 96-well and 384-well microtiter plates,384-well Microfluidic Card Option
Bio-Rad Laboratories	iCycler iQ Real Time PCR System	96-well microtiter plates
MJ Research	Opticon 2	96-well microtiter plates
Roche Applied Science	LightCycler Instrument	32 Light Cycler Capillaries: Glass capillaries, each holding 20 µl
STRATAGENE	Mx4000 Multiplex Quantitative PCR System	96-well microtiter plates

References

1. ***Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC:*** Potent and specific interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998, 391:806-811.

2. ***Hannon GJ:*** RNA interference. *Nature* 2002, 418:244-251.

3. ***Gönczy P, Echeverri C, Oegema K, Coulson A, Jones SJ, Copley RR, Duperon J, Oegema J, Brehm M, Cassin E, Hannak E, Kirkham M, Pichler S, Flohrs K, Goessen A, Leidel S, Alleaume AM, Martin C, Özlü N, Bork P, Hyman AA:*** Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature* 2000, 408:331-336.

4. ***Fraser AG, Kamath RS, Zipperlen P, Martinez-Campos M, Sohrmann M, Ahringer J:*** Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* 2000, 408:325-330.

5. ***Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J:*** Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 2003, 421:231-237.

6. ***Ashrafi K, Chang FY, Watts JL, Fraser AG, Kamath RS, Ahringer J, Ruvkun G:*** Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. *Nature* 2003, 421:268-272.

7. ***Lum L, Yao S, Mozer B, Rovescalli A, von Kessler D, Nirenberg M, Beachy PA:*** Identification of Hedgehog pathway components by RNAi in *Drosophila* cultured cells. *Science* 2003, 299:2039-2045.

8. ***Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T:*** Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001, 411:494-498.

9. ***Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS:*** Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* 2003, 21:635-637

10. ***Editorial:*** Whither RNAi? *Nat Cell Biol* 2003, 5:489-490

11. ***Gao X, Huang L:*** Cationic liposome-mediated gene transfer. *Gene Ther* 1995, 2:710-722.

12. ***Pedroso de Lima MC, Simoes S, Pires P, Faneca H, Duzgunes N:*** Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. *Adv Drug Deliv Rev* 2001, 47:277-94.