Bio Research

Lonza

Nucleofector™ Technology
Guideline for Easy Set Up of RNAi Screening Experiments

Content

1 Introduction

Cell-based screening approaches have become powerful in vitro tools to identify drug targets that play a role in disease development and progression. Classical screens involve the treatment of cells with libraries of small molecules (e.g., chemical compounds). The aim of these treatments is to inhibit protein functions, such as enzymatic activity or receptor functionality. However, as this approach is limited to druggable enzymes and receptors, not every potentially interesting target can be addressed or identified[1]. Therefore, approaches involving either overexpression or RNAi-mediated downregulation of genes have become a potent tool in screening for identifying drug targets. Such screens require transfection of cells with the substrates of interest, for example plasmids (expressing cDNA, shRNA or pre-miRNA) or oligonucleotides (siRNA, miRNA, esiRNA, shRNA or miRNA inhibitors).

This guideline aims to help researchers in setting up a successful RNAi screening experiment using the Nucleofector™ 96-well Shuttle™ System. The recommendations are based on our experience gained in using the Nucleofector™ 96-well Shuttle™ System for screens with Thermo Scientific siRNA libraries in difficult-to-transfect Jurkat T cells and primary HUVEC cells[2], combined with recommendations collected from the literature. It highlights important parameters that may influence the quality of cell-based screening results. We believe that most of the parameters and rules discussed here, contributing to the success of a screening experiment and allowing for the identification of relevant targets, apply for any type of genetic screen, independent of the substrate (e.g., shRNA, cDNA, or miRNA). Nevertheless, on the background of the diversity of possible screening approaches, the guideline is by no means exhaustive.

References

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1.1 RNAi Screening Strategies

Several RNAi screening strategies described in the literature have been used to answer a variety of biological questions (reviewed by Echeverri *et al.,* 2006[3]):

- **Loss/Gain of Function Screens**
  Downregulation of targets by RNAi directly induces a certain phenotype (e.g., proliferation or cell death)[4].

- **Modifier/Sensitizer Screens**
  Downregulation of targets by RNAi only induces a phenotype in combination with cell treatment (pathway induction, e.g., cell stress or apoptosis)[5].

- **Synthetic Lethality Screens**
  Downregulation of targets by RNAi only induces a phenotype in the presence of otherwise sub-effective doses of a toxic drug[6] or in combination with a certain genetic background (e.g., comparison of an isogenic cell model with a wild-type or mutated Ras)[7].

- **Pathway-specific Screens Using a Reporter Gene**
  Downregulation of targets by RNAi modulates signaling that is measured by a pathway-specific reporter gene (e.g., stable reporter cell lines)[8].

1.2 Pre-requisites for Successful RNAi Screens

Successful RNAi Screening Experiments, i.e., Identification of Meaningful Hits, Depend On:

- Selection of an appropriate cell type[9] (see Chapter 1.2.1)
- Highly specific and functional RNAi substrates (see Chapter 1.2.2)
- Efficient delivery of the RNAi substrate into the selected cell type9 (see Chapter 1.2.3)
- Well-established and robust read-out assay (see Chapters 3 and 4)
- Proof of specificity, i.e., exclusion of unspecific effects (see Chapters 1.3 and 6)

1.2.1 Choice of Cell Type

Ideally, the decision as to which cell type is used for a screen is driven by the experimental question. The higher the physiological relevance of the cell type for the pathway of interest, the more relevant targets can be identified. On this background, primary cells freshly isolated from native tissues are gaining more and more interest, as data obtained with these cells are considered to be of high physiological relevance.

However, due to bottlenecks in cell supply and culturing, or limitations of traditional transfection methods for efficient delivery of RNA oligonucleotides or DNA vectors into suspension or primary cells, most screens have so far been performed in easy-to-transfect adherent cell lines, such as HeLa[10, 11, 12]. However, data obtained with immortalized cell lines, that often have accumulated phenotypic and genetic anomalies due to culturing for extensive periods, remain of questionable relevance. Thus, primary cell models should be preferred for cell-based screening whenever possible (as for other *in vitro* experimentation as well). On the other hand, e.g., for studying cancer mechanisms, using a cancer cell line for functional screening can be a valid approach.

References

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11. Bartz SR et al. (2006) Mol Cell Biol 26(24): 9377–9386
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1.2.2 RNAi Libraries

Different substrate types can be used for RNAi-mediated gene silencing: siRNA oligonucleotides, shRNA vectors, esiRNA[13] or D-siRNA (DICER substrates)[14]. So far, only for siRNAs or shRNA vectors, libraries are available from different suppliers, targeting mouse and human gene families (focused libraries) or whole genomes. Features of siRNA oligonucleotides and shRNA vectors are summarized in Table 1.

siRNA Oligonucleotide Libraries

siRNAs provided by different suppliers vary in the specific algorithm used for sequence design and also in chemical modifications. Such chemical modifications (e.g., Thermo Scientific ON-TARGET*plus* siRNA) aim to reduce the risk of "false positives" by minimizing off-target effects generated by the sense and antisense strands[15]. Furthermore, one can select between single or pooled siRNAs (see Table 1). This pooling principle is also employed by D-siRNA and esiRNA strategies which rely on pools of multiple siRNA sequences for one gene target[16, 17].

shRNA Vector Libraries

The potency of shRNA vectors is determined by both the design of the shRNA sequence (siRNA strands plus hairpin loop) and the vector backbone. As with siRNA, different suppliers use different design algorithms and rules. The structure of newer shRNA generations is modeled on naturally occurring pre-miRNAs[18]. Most vector backbones are either retroviral, lentiviral, or plasmid-based, and use different RNA polymerase III promoters (e.g., U6 or H1). Currently available shRNA libraries are provided as retro or lentiviral libraries to enable viral transduction. Alternatively, Nucleofection™ can be used as an efficient non-viral method for transfecting these vectors into a broad range of cells (see www.lonza.com/nucleofection-citations).

	Benefits	Weaknesses
siRNA		
	– Highly evolved design algorithms → high potency and specificity – Easy delivery into cells	– Short-term knockdown
single siRNAs (3 – 4 per target)	– Direct confirmation of the specificity of the resulting phenotype (redundancy, see Chapter 6.2).	– 3 – 4x increased sample number → larger screens
siRNA pools[19, 20] (of 4 individual siRNAs)	– Decreased abundance of an individual siRNA sequence kminimized off-target effects – Maintains overall potency – Number of samples is decreased (smaller screens)	– No direct confirmation of the specificity of the resulting phenotype (redundancy, see Chapter 6.2)
shRNA		
	– Longer-term knockdown – Option for stable integration – Option for inducible knockdown	– Not as easy to transfect as siRNA – Less evolved algorithms → capability of a large proportion of available shRNA sequences in providing a strong knockdown seems to be low[21] → several shRNAs might have to be tested to find one working efficiently

References

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1.2.3 Efficient Delivery of RNAi Substrates

While **lipid-mediated transfection** is a common approach for siRNA delivery into easy-to-transfect cells, many cell types — including suspension cell lines and primary cells — are not compatible with this technology[22]. Using lipid-based reagents, delivery of shRNA vectors or co-transfection approaches are even more challenging. In addition, several lipid delivery reagents can cause cytotoxicity and are capable of inducing a potent interferon response and/or altering gene expression profiles (see Chapter 1.3). These unintended phenotypes can significantly affect experimental outcomes and drastically interfere with the process of elucidating a gene's function.

Viral delivery systems allow efficient transduction of a wide range of cell types and stable integration, and can be used for in vivo applications. However, production of viral particles requires a lot of experience and effort (especially for library formats) and obtaining reproducible, high viral titers with low variation within the library is often challenging. Purchase of ready-to-use particles can be expensive when a high multiplicity of infection (MOI) is necessary for a particular cell type. Furthermore, working with viruses requires BSL 2 laboratory processes.

The **Nucleofector™ Technology** overcomes the limitations of these typical delivery methods. With over 700 RNAi related publications, Nucleofection™ has proven to be the delivery method of choice for any RNAi substrate. The unique versatility of the technology enables a wide range of research applications, from basic research studies — such as analyzing the mechanisms of microRNA — to functional studies via RNAi-mediated gene silencing. Its recent expansion to 96-well format, the **96-well Shuttle™ System**, now offers the possibility of performing screening experiments[23, 24] like RNAi library screens using siRNA or shRNA, in more relevant cell types such as primary cells (e.g., neurons) and difficult-to-transfect cell lines (e.g., Jurkat cells; Figure 1).

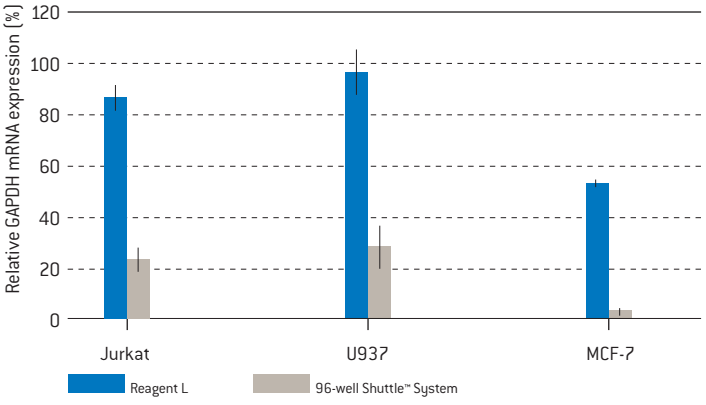

Figure 1: Nucleofection™ out-performs lipofection for effective -GAPDH mRNA knockdown in difficult-to-transfect cell types. Cells were transfected with 5 pmol Thermo Scientific siGENOME SMARTpool reagent targeting GAPDH using the 96-well Shuttle™ System (according to the respective Optimized Protocol) or reagent L (after titration of optimal reagent amount). Negative control samples were transfected with 5 pmol Thermo Scientific siGENOME Non-targeting siRNA #1. 24 hours post transfection, cells were analyzed for mRNA expression by Quanti-Gen® branched DNA assay (Affymetrix).
(Data generated in collaboration with Thermo Fisher Scientific.)

Nucleofection™ for Screening – Benefits at a Glance

96-well Shuttle™ System for High-throughput Nucleofection™

- RNAi library screens in biologically relevant cell types, e.g., primary cells and difficult-to-transfect cell lines
- Easy integration into automation platforms

Highly Reproducible Transfection

- Meaningful hit identification due to low intra-, inter-plate and inter-day variances
- No lipid-mediated off-target effects, e.g., interferon respons

Same Protocol for Different Substrates

- Freedom in selecting siRNA or shRNA libraries for screening
- Easy switch of substrates during hit validation process
- Co-transfection of siRNA oligonucleotides or DNA vectors for rescue experiments

For more details about Nucleofection™ of RNAi libraries see Chapter 2.

References

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Furthermore, Nucleofection™ can be used to prepare cells for a screen, for example by transient or stable transfection of the cells with a reporter construct, a cDNA expressing a target protein (enzyme, receptor, etc.) or a mutant pathway member. This may be advantageous for cells that are not amenable to lipofection of DNA vectors, or in screens that do not involve transfection at all (compound screens).

1.3 Off-Target or Unspecific Effects

Interpretation of RNAi data can be complicated by false positive results caused via so-called "off-target effects". In general, such effects comprise all detectable phenotypic consequences arising from unintended interactions. They can be split into substrate-dependent (siRNA or shRNA) and delivery method-dependent effects. Substrate-dependent off-target effects[25, 26] can result from processes mediated by the specific siRNA or shRNA sequence or can be sequence-independent (see Table 2).

Off-target effects related to activation of interferon responses (see Table 2) depend – at least partially – on the method of substrate delivery. They can be triggered as soon as the endosomal pathway is involved as with lipid-based delivery[27, 28, 29]. Thus, some substrate-dependent effects may be reduced by changing the method of transfer.

In addition, results from Fedorov *et al.* (2005)[30] suggest that lipid-based transfection results in a substantial alteration of gene expression profiles per se (i.e., without siRNA), the effects being much stronger than those observed in electroporation-based transfection, which does not involve the endosomal pathway. This observation is supported by results from Calvin *et al.* (2006)[31] who analyzed alterations of expression profiles after transfection of plasmids with different lipofection reagents.

	Potential Effects	Countermeasures
Sequence-specific Effects		
	– Sequence complementarity to non-targeted mRNAs resulting in RNAi-mediated downregulation of these genes (classical off-target effect)	– Use of high quality siRNA/shRNA designs [32, 33] – Using chemically modified siRNA (e.g., ON-TARGET*plus*® siRNA) – Using low amounts of siRNA (see note below) – Using siRNA pools
	– Activation of immune response by interaction of certain sequence motifs with endosomal toll-like receptors (TLR7 and/or 8) – Interferons trigger global degradation of mRNA by inducing 2'-5' oligo-adenylate synthase (OAS) which activates RNAse L	– Use of high quality siRNA/shRNA designs (avoiding stimulatory elements) – Using low amounts of siRNA (see note below) – Change of delivery method (e.g., Nucleofection™)
Sequence-independent Effects		
	– Activation of immune responses by stretches of dsRNA within the siRNA/shRNA via cytoplasmic protein kinase R (PKR) or endosomal Toll-like receptor 3 (TLR3); PKR phosphorylates eIF2a leading to global inhibition of mRNA translation	– Using low amounts of siRNA (see note below) – Use of negative siRNA/shRNA controls (see Chapter 3.4) – Change of delivery method (e.g., Nucleofection™)
	– Saturation of the RNAi machinery, thereby inhibiting endogenous miRNA pathways	– Using low amounts of siRNA (see note below)

Table 2: Overview about potential substrate-dependent off-target effects

Note:
Based on experience with lipofection reagents, siRNA concentrations lower than 100 nM are usually recommended[34]. However, due to different delivery modes this recommendation cannot be directly extrapolated to Nucleofection™ (see Chapter 3.5.3).

References

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33. Anderson EM et al.(2008) RNA 14(5):853-861
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For Nucleofection™, it has been shown that a described trigger of interferon responses (i.e., 29-mer siRNA targeting DBI 29 (Thermo Fisher Scientific)) does not induce typical responses (Figure 2). In contrast, when using a lipid-reagent for delivery an up-regulation of the interferon-sensitive OAS-1 gene, an increase in IL-6 release and a decreased viability was observed with increasing amounts of DBI-29. Generally, the use of negative siRNA/shRNA controls (see Chapter 3.4) and an appropriate hit validation process (see Chapter 6) allows the determination of specificity of an RNAi effect (i.e., exclusion of off-target effects).

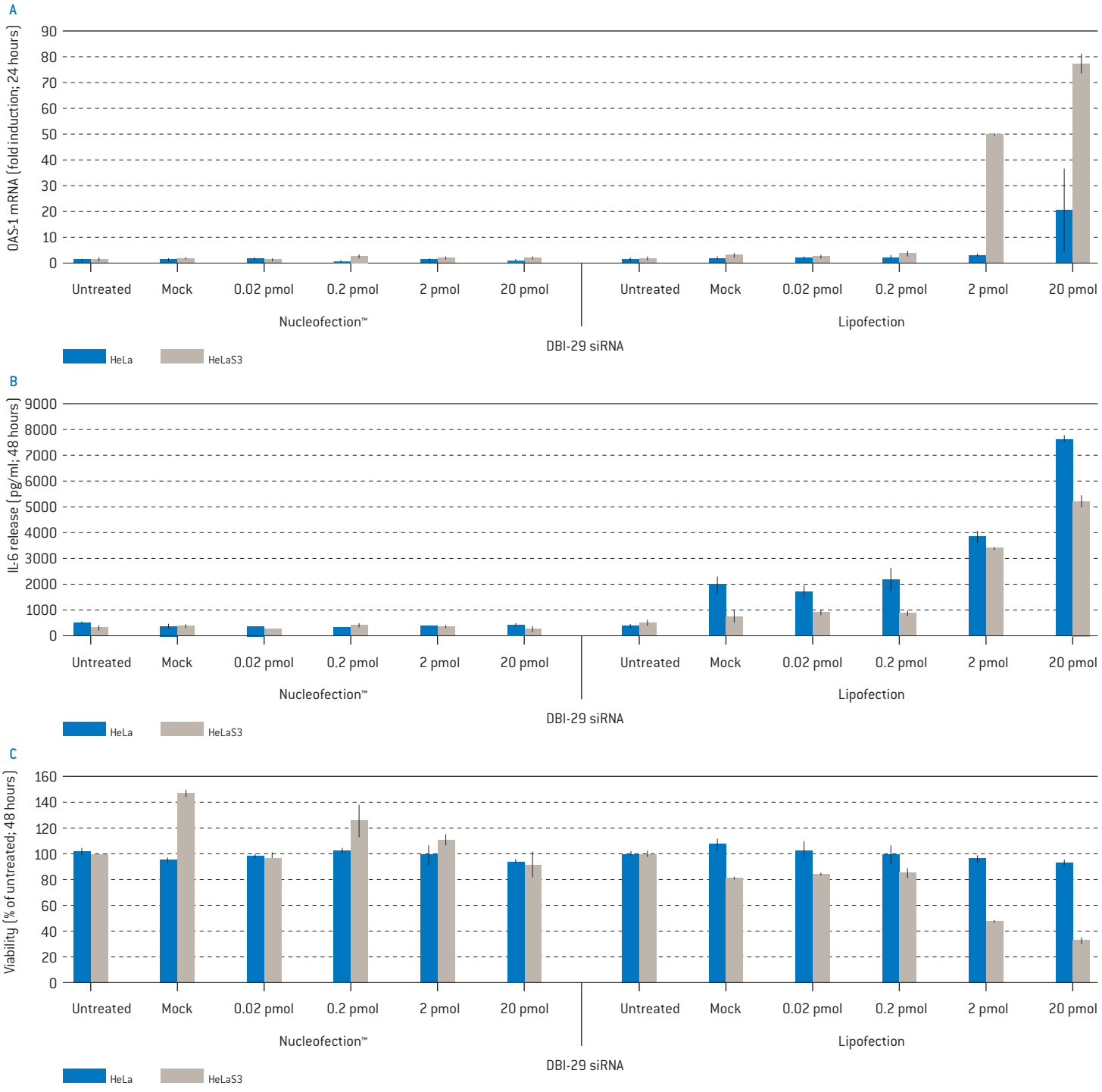


Figure 1: Nucleofection™ out-performs lipofection for effective -GAPDH mRNA knockdown in difficult-to-transfect cell types. Cells were transfected with 5 pmol siGENOME SMARTpool® reagent targeting GAPDH using the 96-well Shuttle™ System (according to the respective Optimized Protocol) or reagent L (after titration of optimal reagent amount). Negative control samples were transfected with 5 pmol siGENOME® Non-targeting siRNA #1 (Thermo Fisher Scientific). 24 hours post transfection, cells were analyzed for mRNA expression by Quanti-Gene® branched DNA assay (Affymetrix). (Data generated in collaboration with Thermo Fisher Scientific.)

2 Nucleofector™ Technology for RNAi Library Transfection

2.1 96-well Nucleofection™ of siRNA Oligonucleotides or shRNA Vectors

For the transfection of siRNA oligonucleotides or shRNA vectors into your specific cell-type, we recommend using the respective cell-type specific 96-well Nucleofector™ Kit and following the Optimized Protocol (for more information about available Kits please see our Cell Database at www.lonza.com/celldatabase).

Optimal Nucleofection™ Conditions for a particular cell type are identical whether you are transfecting DNA or RNA and we recommend performing the following steps:

1. First, perform a preliminary experiment with our pmaxGFP™ Vector positive control plasmid (included in every kit) in order to verify (or in case of using the Cell Line Optimization Nucleofector™ Kit, establish) the optimal Nucleofection™ Conditions for your cells.
2. Next, use the identical conditions for your RNAi experiments, but replace pmaxGFP™ Vector with your control siRNA oligonucleotides or shRNA vectors. You may wish to include a sample with pmaxGFP™ Vector (or even co-transfect pmaxGFP™ Vector) in your RNAi experiments in order to measure the success of Nucleofection™. However, if you are using this plasmid as a means of estimating transfection efficiency for your siRNA, please keep in mind that the transfection efficiency for siRNA duplexes is even higher than for plasmid DNA.

2.2 96-well Nucleofection™ at Higher Throughput

In a screening experiment using a larger number of plates, plate-handling times might require prolonged storage of suspended cells in 96-well Nucleofector™ Solution prior to Nucleofection™. Depending on the duration of this "pre-incubation" step, cell properties may be changed, potentially influencing transfection efficiency and/or cell viability. This can be disadvantageous, as assay robustness relies on a high level of process standardization, including cell input quality (see Chapter 4).

Testing the effects of prolonged storage on several cell types[35] showed that for some cells storage in standard 96-well Nucleofector™ Solution for up to 4 hours does not affect transfection efficiency or viability (e.g., unstimulated/stimulated human T cells and HUVEC; see Figure 3). In other cells (Jurkat, HeLaS3 and Neuro2a), an influence on transfection efficiency or viability due to prolonged storage in 96-well Nucleofector™ Solution was observed. To counter this effect, special Protocols and Kits ("Automation Kits") have been developed that extend the storage time for such sensitive cells to at least 4 hours (see Figure 4). If longer storage times are required (e.g., for very large screens), use of multiple cell batches is recommended.

For other cells and further information please contact our Scientific Support Teams.

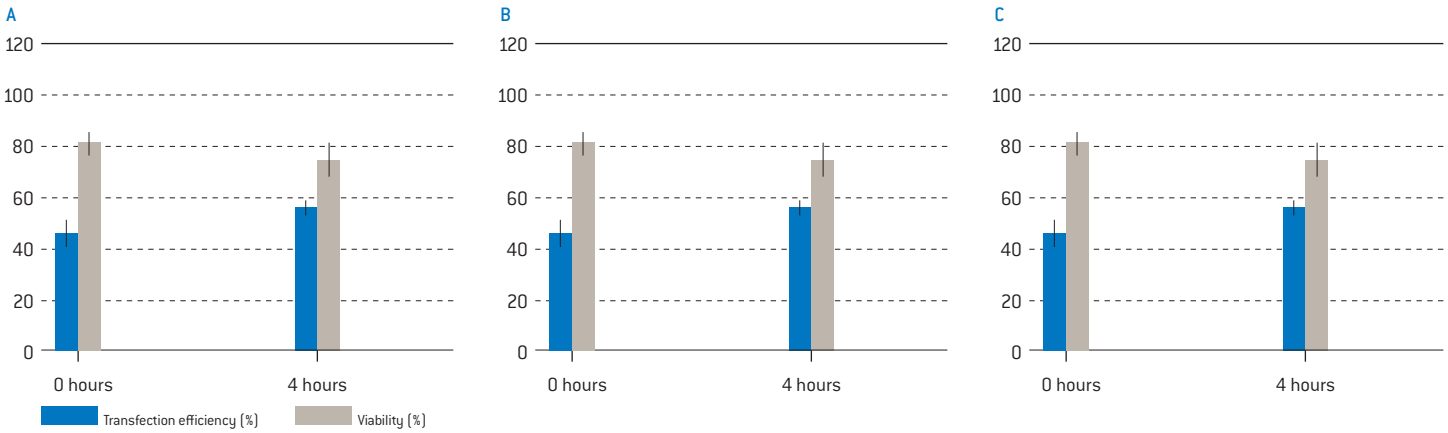


Figure 3: Influence of pre-incubation using standard 96-well Nucleofection™ Protocols. Human unstimulated T cells (A), stimulated T cells (B) or HUVEC cells (Lonza; C) were pre-incubated for 0 or 4 hours in 96-well Nucleofector™ Solution and then transfected with 1 µg pmaxGFP™ Vector according to their standard 96-well Nucleofection™ Protocol. 48 hours post Nucleofection™, the cells were analyzed on a BD FACSCalibur™ (BD Biosciences) with HTS option. Cell viability was determined using PI-staining.

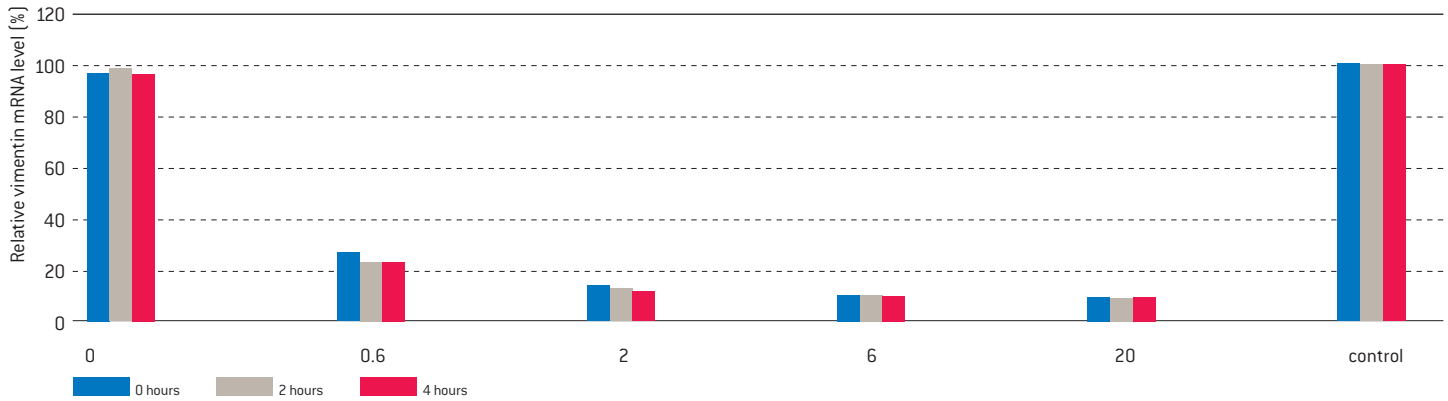


Figure 4: siRNA-mediated knockdown of vimentin in Jurkat cells after different pre-incubation times. After different pre-incubation times (0, 2 and 4 hours) in Automation 96-well Nucleofector™ Solution, Jurkat cells (2.5 x 105 cells per sample) were transfected with siGENOME SMARTpool siRNA reagent directed against endogenous vimentin. 24 hours post transfection, vimentin mRNA levels were analyzed by the QuantiGene® branched DNA assay Affymetrix. Data are plotted as relative expression levels compared to samples with control siRNA. (Data generated in collaboration with Thermo Fisher Scientific.)

References

35. Lonza Tech Note (WTA-1005)

3 Establishing Assays

Besides the pre-requisites related to cells, substrate and delivery (see Chapter 1.2), the selection and establishment of the read-out assay for the RNAi screen is of high importance for hit identification. Before beginning a screening experiment a number of theoretical and experimental questions need to be addressed:

- What is the best way to measure the response (read-out) to the experimental question?
- Is a single assay sufficient or is a multi-parametric analysis necessary?
- Which reagents (e.g., inducers) are required?
- What instrumentation is needed?
- Is the assay compatible with the selected cell type and RNAi substrate?
- What are suitable negative and positive controls?
- Are appropriate negative and positive control(s) available for optimization?
- What are the parameters affecting reaction kinetics and strength of the phenotypic output?

Some of these questions will be further discussed in the following chapters.

3.1 Assay Selection

Read-out assays used for screening experiments can range from simple assay systems (e.g., viability or apoptosis) to very complex assays (e.g., high content analysis [HCA]; see Table 3 and Figure 5). Simple assays are usually commercially available standard assays that are easy to optimize and automate, and which can be performed using standard lab instrumentation. As such, they are the most often selected type of assay for primary screens involving high sample throughput. More specific assays that require high optimization effort, special lab instrumentation as well as possibly complex data analysis tools are more suited for small-scale secondary screens or hit validation. However, with the advent of highly sophisticated robotics, even very complex assays are becoming amenable to use in larger screens. If a multi-parametric analysis is desired, Nucleofection™ Workflows allow for plating transfected cells from one well onto a number of replica culture plates. In contrast, lipofection would require a separate set of transfected cells (increasing the transfection sample number and possibly causing a higher variance).

	Simple	→	Complex
Complexity			
	– Commercially available		– High optimization effort
	– Easy to optimize		– Special instrumentation
	– Easy to automate		– More specific biological questions may be addressed
	– Standard instrumentation		
Examples			
	– Viability	– Calcium assays	– High content analysis (HCA)
	– Proliferation	– ELISA	– Protein-protein interaction (e.g., FRET)
	– Apoptosis	– Flash luminescence	– Pathway specific assays
	– Metabolism	– Reporter gene assays	

Table 3: Assay Types

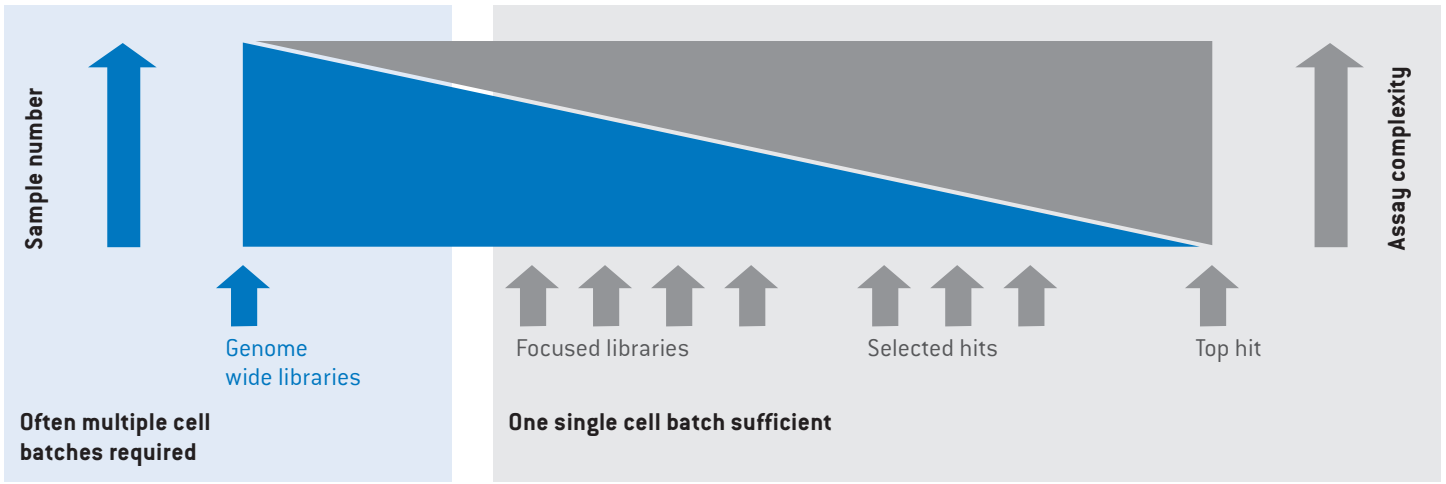


Figure 5: Schematic representation of assay types in relation to the screening process. A screening process usually starts with a high sample number that decreases during process progression. In parallel, the complexity of used read-out assays may increase.

3.2 Compatibility of Assay with Cell Type

First, it should be determined if the biological pathway is active in the selected cell (e.g., not every pathway is active in cell lines or may alter from the cascade found in primary cells). Furthermore, one has to consider if cells and assay requirements fit together, e.g., measuring a secretory protein in a suspension cell would complicate the workflow.

3.3 Compatibility of Assay with RNAi Effect

Different read-out assays may require different levels and/or durations of target gene downregulation to generate a significant assay signal. The strength and duration of knockdown can depend on the RNAi substrate used (see Chapter 1.2.2). Therefore, it is usually necessary to test different RNAi substrates (siRNA vs. shRNA, single siRNAs vs. pools, different modifications) by using appropriate positive and negative controls (see Chapter 3.4) to validate substrate suitability for a specific screening approach.

3.4 Appropriate Experimental Controls

To help ensuring that the conclusions drawn from RNAi experiments are accurate and valid, it is vital to include appropriate experimental controls. For assay establishment, optimization and analysis of parameters that may influence the read-out (see Chapter 3.5) it is recommended that the following controls are always included:

a) **Untreated "Culture-only" Control:** The untreated control sample comprises cells that have neither been treated with siRNA or shRNA nor subjected to the Nucleofection™ Process. This control serves as an indicator of baseline cellular activity to which all other conditions can be compared. However, the specific effect of a given siRNA/shRNA is more appropriately represented by comparison to the mock-treated or negative siRNA/shRNA control sample.

b) **Mock-treated Control:** The mock-treated control sample is one in which the cells are subjected to the Nucleofection™ Procedure in the absence of siRNA/shRNA. The analysis of mock-treated cells will indicate whether the transfection process itself results in cytotoxicity or other non-specific effects.

c) **Negative siRNA/shRNA Control:** Negative siRNA/shRNA control reagents are bioinformatically designed, validated reagents that have no known target in the cell type of choice. These reagents are important for distinguishing sequence-specific silencing from sequence-independent effects that are associated with the delivery of siRNA into the cell. Such sequence-independent effects can include toxicity resulting from the process of transfection in conjunction with nucleic acid delivery or hyper-sensitivity to introduction of double-stranded RNA (see Chapter 1.3). Investigators are encouraged to test multiple candidates in their own experimental systems to empirically confirm that the negative controls do not result in any observable and unintended off-target effect.

d) **Positive siRNA/shRNA Control:**

– For proof of transfection efficiency, checking functionality of RNAi machinery or optimization of Nucleofection™ Conditions for the cell type of choice: This should be a validated siRNA/shRNA targeting a well-characterized housekeeping gene, such as cyclophilin B (also known as PPIB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or lamin. A good positive control reagent targeting a highly-expressed but non-essential gene is useful for establishing experimental parameters without affecting cellular viability.

– For establishment of a phenotypic read-out assay: In order to determine the range and reproducibility of the screening window for detection of hits on the primary screen, ideally at least 2 siRNAs/shRNAs directed against known members of the addressed pathway should be used as positive controls. As shown in Figure 6, when using PLK-1 and CHEK-1 as positive controls for kinases, the strength and kinetics of phenotypic effects can differ for each target, demonstrating the potential differences expected for "strong" and "weak" targets in a kinase library.

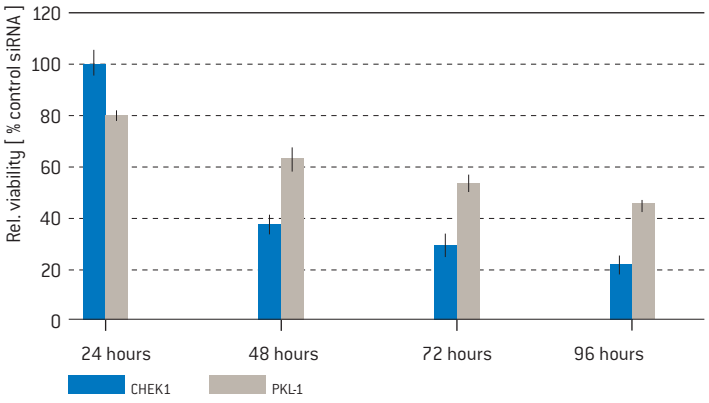


Figure 6: Strength of phenotype for 2 different kinases in HUVEC cells. HUVEC cells were transfected with 20 pmol siGENOME SMARTpool siRNA reagent targeting PLK-1 or CHEK-1 and siGENOME Non-targeting siRNA #1. Cell viability was analyzed using CellTiter-Blue® Cell Viability Assay (Promega) at different time points post transfection (24, 48, 72 and 96 hours). Values were normalized to the negative control sample.
(Data generated in collaboration with Thermo Fisher Scientific.)

3.5 Parameters Affecting Phenotypic Read-Out

Overview:
Factors which may influence signal strength and/or kinetics and thus define the signal window of the assay (signal-to-noise or signal-to-background ratio) include:

- Condition of cell batch (passage number, cell density before harvesting, donor variance)
- Type of RNAi substrate (single siRNA, siRNA pool, shRNA)
- siRNA amount
- Sensitivity of the assay itself
- Cell density in assay
- Concentration of inducers
- Schedule of treatments
- Time point of analysis
- Time course fit of RNA interference and assay
- Variation (standard deviation [SD], coefficient of variation [CV]) of the readout for the normal phenotype
- Influence of transfection method on normal phenotype variation

During assay optimization, the influence of each parameter should be checked using positive and negative controls (see Chapter 3.4). In general, any simplification of the assay protocol (e.g., limiting the number of steps, optimizing pipetting conditions, synchronizing reagent addition, avoiding washing or centrifugation steps) helps to reduce the number of potential influencing factors and thus increases assay robustness (see Chapter 4).

3.5.1 Cell Status

Untreated cells may already display some degree of phenotypic variance. Therefore, it is recommended that untreated cells are always included in each experiment (see Chapter 3.4). The overall physiological status of the cells prior to transfection (e.g., passage number, cell splitting rhythm, donor quality, isolation procedure) can influence transfection efficiency, viability and/or general physiological status/functionality after transfection. This may have an impact on the phenotype of siRNA-treated samples. These factors should be evaluated using the aforementioned positive and negative controls (see Chapter 3.4). In case of obvious variances induced by such factors, cell handling before transfection should be standardized as far as possible. For example, for consistent results it may be necessary to always use cells from the same passage number. For that purpose, large cell batches of one given quality can be frozen and thawed before each experiment. If the phenotypic variance still is too high after standardizing the factors mentioned above, one should consider a different type of assay and/or cell type for the screen.

3.5.2 Cell Numbers

A certain minimum and maximum cell number is required to keep the assay output data within a linear range and allow for detection of significant sample differences. The optimal numbers of cells must be determined empirically for both un-treated and treated samples, as the treatment can also have an influence on cell numbers (e.g., as a result of mortality due to treatment). For instance, to measure viability 72 hours after siRNA transfection one needs to consider the proliferation rate of the cells. If un-treated and treated cells are plated too densely after transfection, the control sample may be confluent and growth-inhibited after 48 hours, while the treated sample continues to proliferate but may also reach confluence before the time of analysis. This may result in no detectable differences between the treated and control samples at the time point of analysis. For example, reduction of HUVEC cell plating densities post Nucleofection™ enabled a more significant discrimination between positive and negative control samples on the phenotypic level (Figure 7). Cell density and proliferation rate not only affect cell titer measurements, but are also intimately connected with the metabolic activity of cells and thus can influence other types of assays as well.

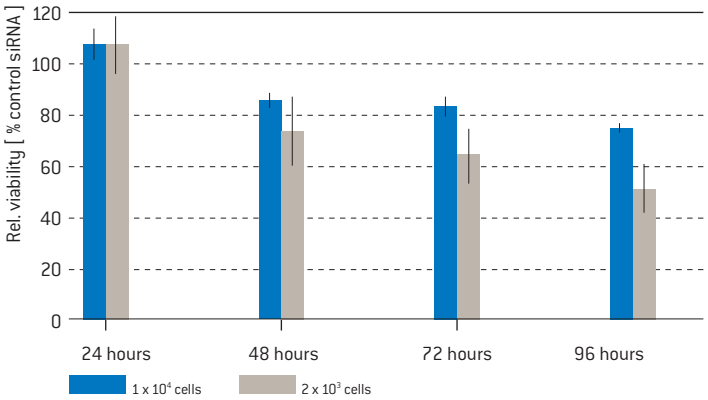


Figure 7: Optimal plating density of HUVEC cells after Nucleofection™ with PLK-1 siRNA HUVEC cells were transfected with 20 pmol siGENOME SMARTpool siRNA reagent targeting PLK-1 and siGENOME Non-targeting siRNA #1. Post Nucleofection™, cells were plated at 2 different densities and analyzed by CellTiter-Blue® Cell Viability Assay (Promega) at different time points. Values were normalized to the negative control sample.
(Data generated in collaboration with Thermo Fisher Scientific.)

3.5.3 Optimal siRNA Concentration

When performing siRNA-mediated knockdown experiments it is advisable to conduct a siRNA dose-response (concentration) analysis to determine the minimum concentration necessary for sufficient target knockdown on the mRNA, protein or functional level (Figure 8A).

For Nucleofection™, the optimal siRNA concentration can range from less than 2 nM up to 2 µM (96-well Shuttle™ System: 0.04 – 40 pmol in 20 µl), depending on multiple factors, such as the cell type (Figure 8B) and the properties of the target itself (e.g., half-life of the mRNA and/or protein) (Figure 8C). When comparing these values for Nucleofection™ with those typically described for lipid-based methods, total siRNA amounts used are in a similar range (e.g., 1 – 100 nM with 96-well lipofection = 0.1 – 10 pmol in 100 µl lipofection sample). However, due to the approximately 5x lower reaction volume used in 96-well Nucleofection™ (20 µl with 96-well Shuttle™ System) the resulting final concentration is higher than with lipofection. Generally, as the extracellular concentration or total amount of siRNA used gives no clue as to the final endogenously effective amount, optimal siRNA amounts should always be determined experimentally (recommended starting points: 30 and 300 nM). When working with shRNA vectors a titration of DNA amounts is also recommended. It is important to maintain a balance between efficient knockdown and minimizing potential sequence-dependent off-target effects (see Chapter 1.3).

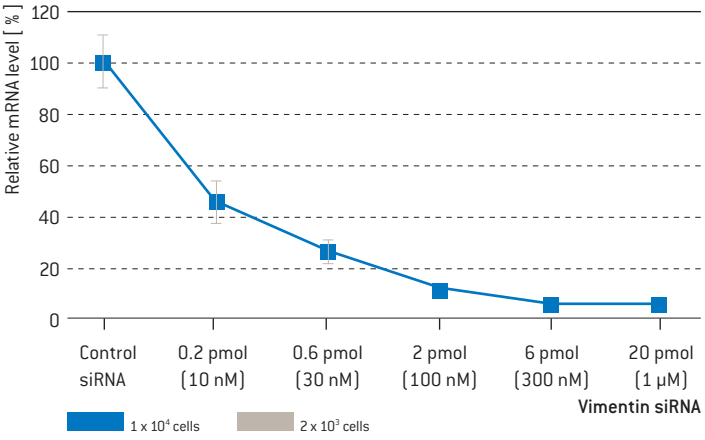


Figure 8A: Target gene knockdown is dose-dependent and reaches saturation. HUVEC cells (Lonza; 2 x 10^5 cells/-sample) were transfected with different amounts of siGENOME SMARTpool siRNA reagent targeting vimentin using the 96-well Shuttle™ System. siGENOME Non-targeting siRNA #1 was used as negative control. 24 hours post trans-fection, mRNA levels were determined by QuantiGene® branched DNA assay Affymetrix and normalized to endogenous GAPDH mRNA. Sample values were further normalized to control siRNA (=100%). siRNA amounts < 1 pmol already achieve 75% mRNA knockdown and the knockdown reaches saturation at 6 pmol.

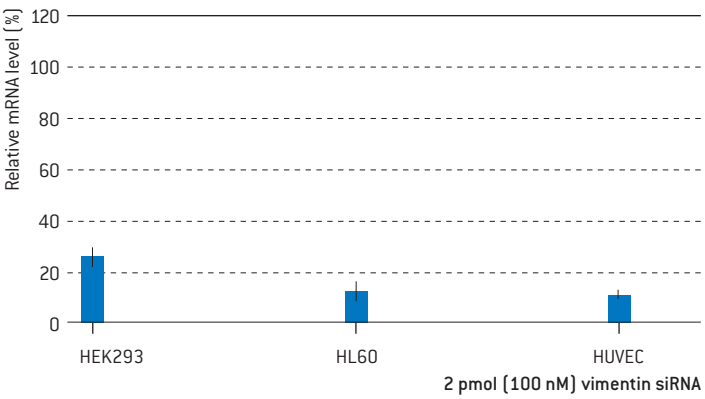


Figure 8B: Strength of target gene knockdown at a given siRNA amount depends on cell type. Different cell types were transfected with 2 pmol (100 nM) of siGENOME SMARTpool siRNA reagent (Thermo Fisher Scientific) targeting vimentin using the 96-well Shuttle™ System. siGENOME non-targeting siRNA #1 was used as negative control. 24 hours post transfection mRNA levels were determined by QuantiGene® branched DNA assay Affymetrix and normalized to GAPDH mRNA. Sample values were further normalized to control siRNA.

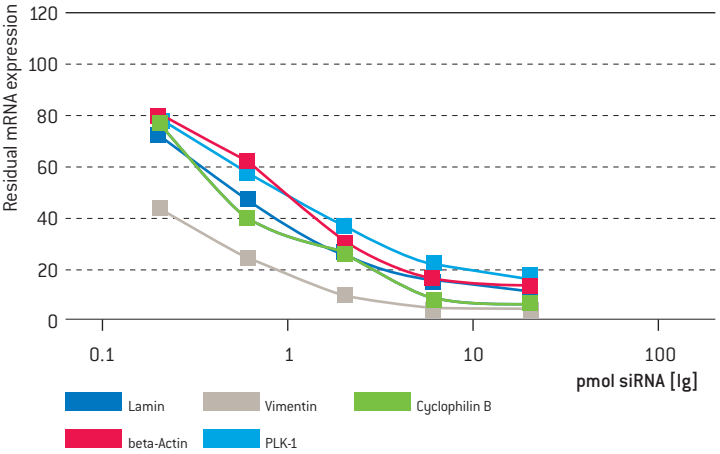


Figure 8C: Strength of knockdown at a given siRNA amount differs for different targets in the same cell type. HUVEC cells (Lonza; 2 x 10^5 cells/sample) were transfected with 2 pmol (100 nM) of siGENOME SMARTpool siRNA reagent targeting various genes using the 96-well Shuttle™ System. siGENOME Non-targeting siRNA #1 was used as negative control. 24 hours post transfection, mRNA levels were determined by QuantiGene® branched DNA assay Affymetrix and normalized to endogenous GAPDH mRNA. Values were further normalized to control siRNA.
(Data generated in collaboration with Thermo Fisher Scientific.)

When using higher amounts of siRNA one will get more primary hits but some may be "false positives". However, when using lower amounts that are insufficient to induce a significant and reproducible phenotype for all targets of interest, some potential targets may be missed ("false negatives"). In our screens[36] we aimed to avoid missing potential hits due to "false negatives"and thus used 20 pmol (1 µM) of siGENOME SMARTpool siRNA reagent (Figure 9; Thermo Fisher Scientific)). Potential "false positives" can be unmasked during the subsequent validation steps (see Chapter 6).

3.5.4 Determination of Optimal Analysis Time Point

As the stability and half-life of different mRNAs and their protein products vary, it is important to empirically determine the best time point(s) for assessing target knockdown, on mRNA, protein or phenotype level (Figure 10). For example, it has been documented that in mammalian cells, mRNA half-life (t1/2) can range from minutes to days while t1/2 of proteins can range from less than a few minutes to several days. Taking this into consideration, the experimental design should allow for a sufficient time span for the RNAi substrate to reduce mRNA/protein concentrations to desired levels. In general, for Nucleofection™, the recommended time course ranges are 5 to 72 hours to deplete target mRNA and 24 to 96 hours to adequately knockdown target proteins and assess phenotypic outcomes.

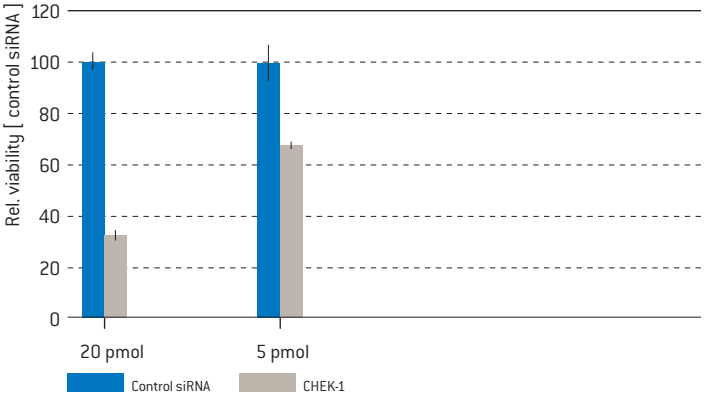


Figure 9: Determination of optimal siRNA amount. HUVEC cells (Lonza; 2 x 10^4 cells/sample) were transfected with 5 or 20 pmol (250 nM or 1 µM) of Dharmacon SMARTpool siRNA targeting CHEK-1 using the 96-well Shuttle™ System. siGENOME Non-targeting siRNA #1 (Thermo Fisher Scientific) was used as negative control. 72 hours post transfection, viability was determined by CellTiter-Blue® Cell Viability Assay (Promega) and normalized to control siRNA.
(Data generated in collaboration with Thermo Fisher Scientific.)

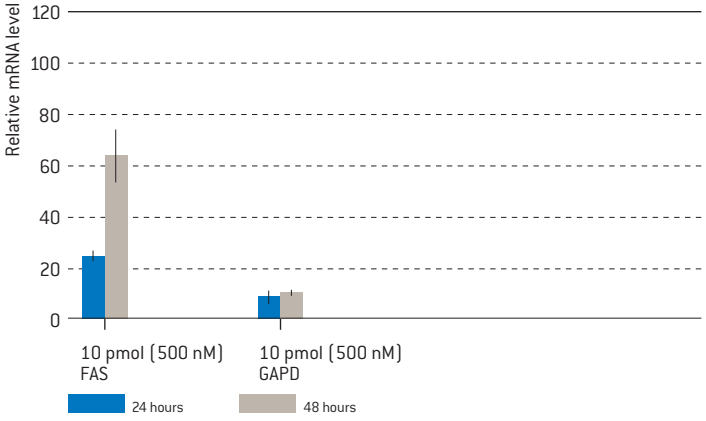


Figure 10A: Kinetic of mRNA knockdown differs for different target genes. Jurkat E6-1 were transfected with 10 pmol (500 nM) SMARTpool siRNA against FAS or GAPDH and siGENOME Non-targeting siRNA #1 (Thermo Fisher Scientific) using the 96-well Shuttle™ System. mRNA levels were analyzed 24 hours post Nucleofection™ by QuantiGene® branched DNA assay (Affymetrix) and normalized to control siRNA.

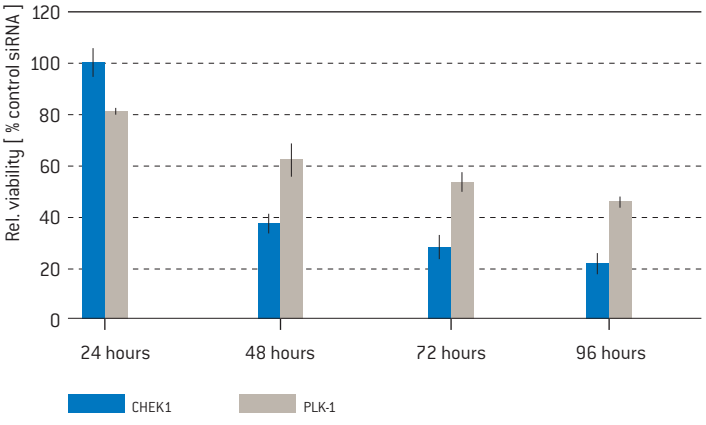


Figure 10B: Determination of suitable time point for different targets. HUVEC cells (Lonza; 2 x 10^4 cells/sample) were transfected with 20 pmol (1 µM) of SMARTpool siRNA reagent targeting PLK-1 or CHEK-1 using the 96-well Shuttle™ System. siGENOME Non-targeting siRNA #1 (Thermo Fisher Scientific) was used as negative control. At different time points post transfection viability was determined by CellTiter-Blue® Cell Viability Assay (Promega) and normalized to control siRNA. (Data generated in collaboration with Thermo Fisher Scientific.)

References

36. Lonza Application Note WTB-1011 (2008)

4 Transferring Assays to Screening Conditions

The increased number of samples that has to be handled within a screening experiment may add another source of variation to assays due to prolonged handling times or variations within or between plates. Thus, the transfer of established assays to screening conditions should include standardization of handling steps and determination of such variances.

4.1 Size of Screen

The size of the screening experiment depends on the size of the library itself (see also Chapter 1.2.2, Table 1) and the number of replicates. Using a library with 3 – 4 single siRNAs per target increases the sample size but also adds a first level of validation (redundancy, see Chapter 6.2). In contrast, a library with siRNA pools reduces the screen size and provides a strong knockdown combined with a minimized off-target effect of the individual siRNA sequence in the pool[37, 38]. Repeating a screen allows for a higher degree of confidence in the selected primary hits (Figure 11 and 18). Selection of hits resulting from only one screening experiment may include a higher number of "false positives" due to accidental "outliers". To account for possible experimental variations we recommend that three independent screens are performed (when n=1 per experiment) rather than performing one screen with triplicate substrate samples. This also covers a pre-validation against possible factors of relevance to variation as discussed above (see Chapter 3).

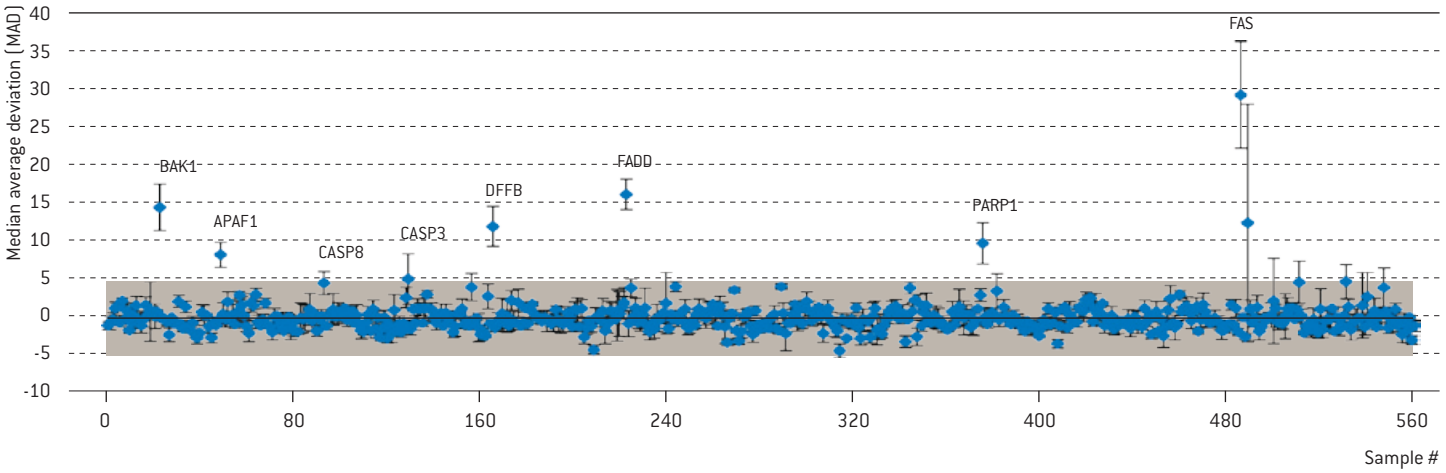


Figure 11: Reproducibility of top primary hits in three independent screening experiments. Jurkat cells were transfected in three independent experiments with the Thermo Scientific Human ON-TARGET*plus* siRNA Library - Apoptosis (targeting 558 genes ; Thermo Fisher Scientific). Apoptosis was induced by adding 10 ng FAS-L to the cells 48 hours post Nucleofection™. Cell viability was analyzed after 2 hours. The mean of robust Z-scores of cell viability measures was calculated for three independent experiments. Targets with an |MAD| of at least 5 are marked as potential hits.
(Data generated in collaboration with Thermo Fisher Scientific.)

References

37. Hsieh AC et al. (2004) NAR 32(3) :893-901
38. Thermo Fisher Scientific Tech Note (2008, Code 00191-08-C-01-U)

4.2 Pipetting Steps/Workflow

A screening experiment involves a number of handling steps resulting in a defined workflow. Each step may need to be optimized considering the following questions:

- What are the time-critical steps?
- How many persons are available to handle the plates in standardized time frames?
- For example, if more than one person is available, processing of the plates can be organized in an assembly line-like fashion for highest speed and constant incubation times.
- What is the optimal equipment for each step, e.g., with regard to the properties (speed, accuracy) of different types of pipette (8-channel, 96-channel, automatic batch dispenser)?

Figure 12 gives an exemplary overview of the steps for a screening experiment using the 96-well Shuttle™ System.

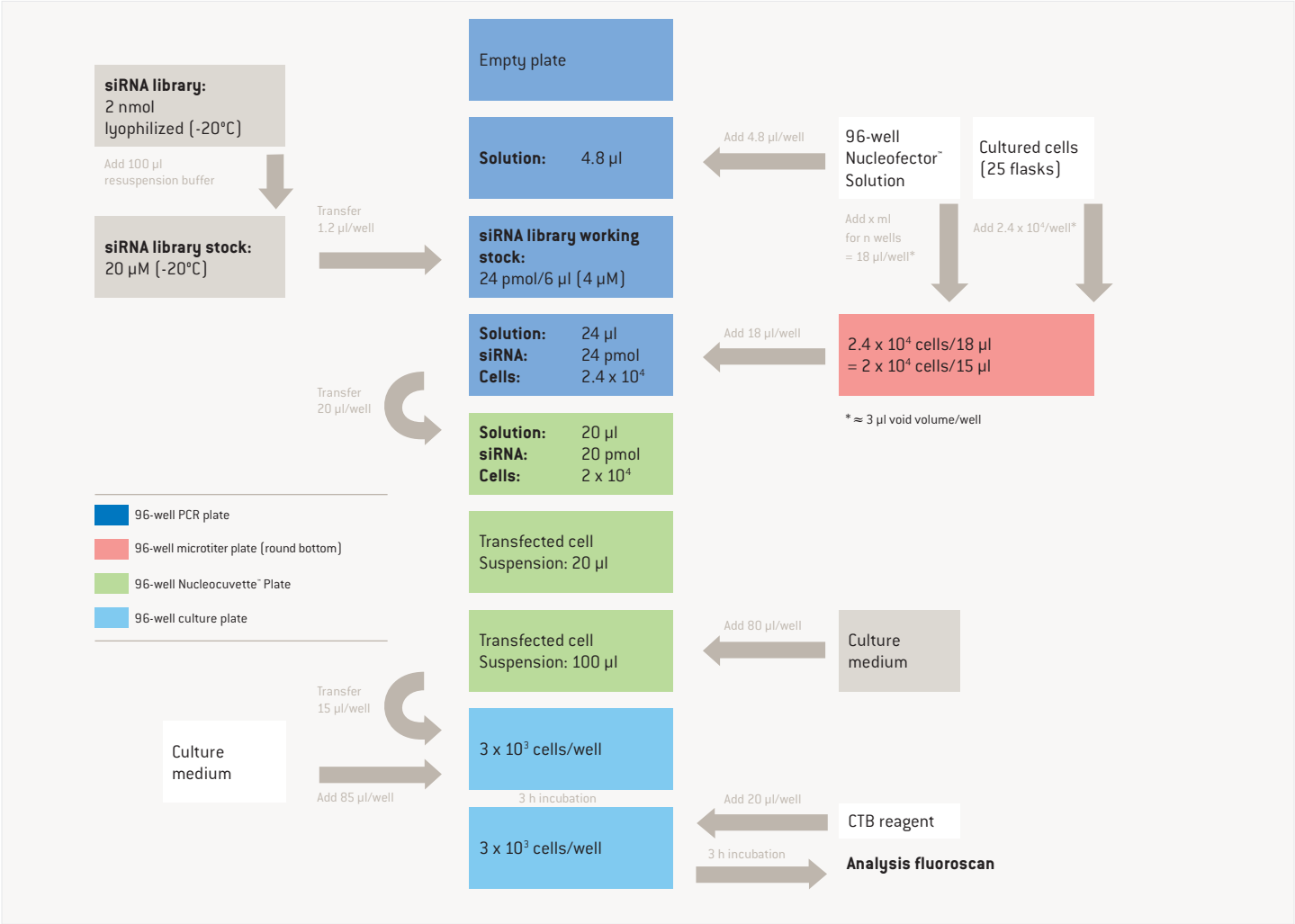


Figure 12: Exemplary schematic workflow. The scheme depicts the pipetting steps that were used for a kinome screen in HUVEC cells[39] (CTB = CellTiter-Blue® Cell Viability Assay (Promega)). Indicated volumes and amounts reflect values per well.

References

39. Lonza Application Note WTB-1011 (2008)

4.3 Automation

Generally, if the screening experiment exceeds a size that can be easily handled manually, an automated screen using a liquid hand- ling system can be an alternative. For an automated screen, one should consider further parameters, such as degree of automation and required workflow optimization (single vs. multiple cell batches, speed, accuracy). For workflows that require prolonged incubation of the cell batch in suspension before Nucleofection™, specific automation compatible protocols may be required (see Chapter 2.2). In cases where pre-incubation times exceed 4 – 6 hours, working with multiple cell batches is recommended.
When automation is preferable to handle the number of samples or improve workflow in terms of speed and/or smaller volumes, it still should be considered that 100% automation does not guarantee highest speeds and can require very expensive equipment. Therefore, one should carefully evaluate whether automation is advantageous for each step, e.g.:

- Perform as many steps as possible before starting with cells.
  - Dispensing of medium into culture plates (e.g., using batch dispenser).
  - Distribution of substrates (best done on the day before performing the screen).
- Calculate void volumes.
- Use fast equipment for time-critical steps.
- Optimize worktable layout and order of steps for short travels of robotic arms.
- Optimize travel heights (fast transport of labware/plates).
- Consider the possibility of semi-automation: e.g., substrate handling or assaying only.
- Consider manual loading/removal of plates into/from plate readers.

For more details about automation of the 96-well Shuttle™ System please refer to our Automation Guideline.

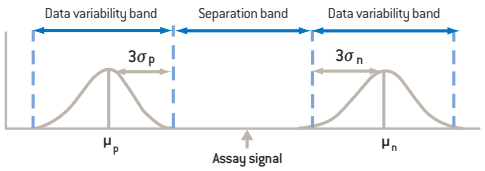
4.4 Determination of Assay Robustness

High assay robustness is key to reproducibility of screening data and meaningful hit identification. It is primarily based on consistent assay performance across a plate, between plates and between experiments (minimal intra-plate, inter-plate, day-to-day, donor-to-donor variation, edge effects, gradients, patterns with regard to well position, etc.).

4.4.1 Reproducibility and Screening Window

For evaluation of assay robustness and screening window we recommend to use the same positive and negative controls as employed for assay establishment. Random distribution of the control samples across entire plates or arrangement in Z, N or H-like pattern (see Appendix) helps ensuring independence from positional artifacts for the determination of:

Intra-plate variation	Variation (SD, CV) within a plate
Inter-plate variation	Variation (SD, CV) between several plates of one run
Inter-run variation	Variation (SD, CV) between plates of independent runs
Z' Factor	The Z' factor[40] was suggested as a useful measure of the quality of a high-throughput screening assay. By considering the variation of both negative and positive controls, it allows to determine the window available for hit distribution (screening window; see also Figure 15) and thus gives a prediction of assay usefulness in a high-throughput setting. Four parameters are needed to calculate the Z' factor: the mean (μ) and standard deviation (j) of both the positive (p) and negative (n) controls (μp, jp, μn, jn, respectively)



$$Z' = 1 - \frac{3\sigma_p + 3\sigma_n}{|\mu_p - \mu_n|}$$

1	= Ideal assay	μ = 0 or unlimited dynamic range
1 - 0.5	= Excellent assay	Separation band is large
< 0.5	= "Yes & No"- type assay	Separation band is small
< 0	= Screening impossible	No separation band, overlap of 3 μ_p and 3 μ_n

In contrast to a compound screen, which often requires an assay with a high Z' factor (Z' 1 0.5) a "Yes-No" type assay might be sufficient for hit selection in an RNAi screen.

References

40. Zhang JH et al. (1999) J Biomol Screening 4(2):67–73

4.4.2 Artifact Effects with a Negative Impact on Hit Identification

Depending on the assay and the time point of analysis, artifact effects such as "edge effects" or positional effects can occur. In screening experiments, such effects can have a negative impact on hit identification and must therefore be reduced or normalized. There are several potential causes of strong artifact effects in the outermost wells (edge effects), but most often they develop during incubation of cell culture plates after transfection due to suboptimal growth conditions (e.g., evaporation of culture medium, ventilation or temperature gradients). Edge effects were also observed during setup of screening workflows for the HUVEC kinome screen[41] using a cell viability read-out (Figure 13A). We have shown that these effects developed during the culturing post transfection and were independent of and not induced by the Nucleofection™ Process itself (Figure 13B).

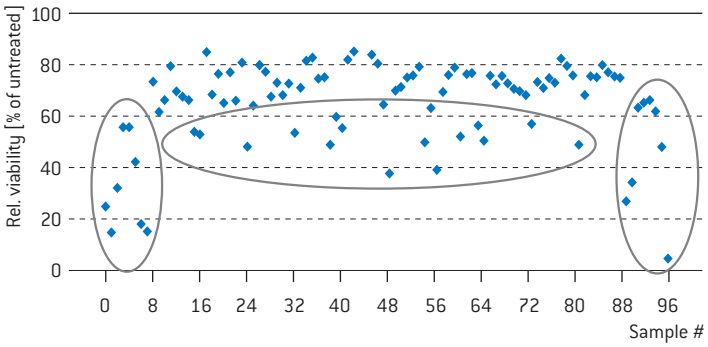
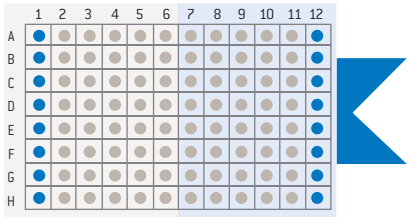


Figure 13A: Occurrence of edge effects in cell viability assay. HUVEC cells (Lonza) were transfected with 20 pmol siGENOME Non-targeting siRNA #1. Cell viability was analyzed 72 hours post Nucleofection™ by CellTiter-Blue® Cell Viability Assay (Promega) and sample values were normalized to untreated cells. Circles indicate edge effects observed in outer rows (A and H) and columns (1 and 12) of a 96-well culture plate.



96-well Nucleocuvette™ Plate

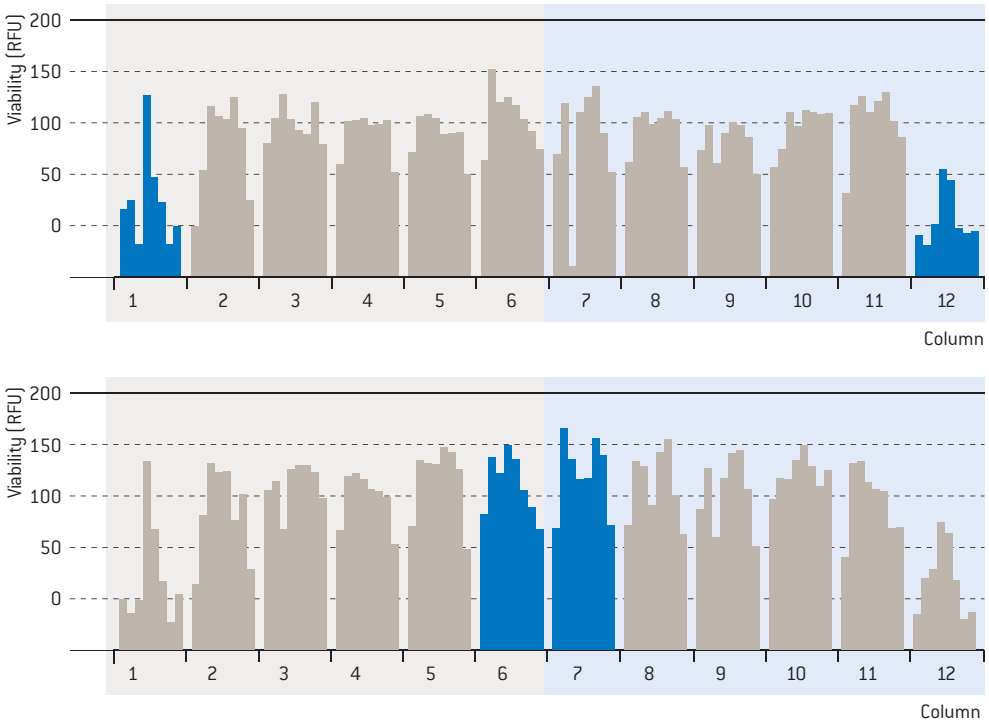


Figure 13B: Edge effects are culture method-dependent and Nucleofection™ independent. HUVEC cells (Lonza) were transfected with 20 pmol siGENOME Non-targeting siRNA #1 (Thermo Fisher Scientific). After transfection in one 96-well Nucleocuvette™ Plate, samples were distributed onto 2 culture plates in different orientations: Culture plate 1 reflects the same position as during transfection, in culture plate 2 transfected samples A1-H6 were plated in area A7-H12 and vice versa. Cell viability was analyzed 72 hours post Nucleofection™ by CellTiter-Blue® Cell Viability Assay (Promega) and sample values were normalized to untreated cells. Edge effect always occurred in outer wells of culture plate independent of well positions during Nucleofection™. (Data generated in collaboration with Thermo Fisher Scientific.)

References

41. Lonza Application Note WTB-1011 (2008)

4.4.3 Strategies to Reduce or Normalize Positional Effects

One way of potentially reducing edge effects is sealing plates with a gas-permeable foil and placing the plates in an incubator without stacking. If this does not help to reduce edge effects, we recommend omitting the outer wells of the culture plates and filling these wells only with medium (see Figure 14). For this purpose, samples from one 96-well transfection plate (i.e., 96-well Nucleocuvette™ Plate) can be distributed onto two 96-well culture plates. This may in addition require a reformatting of the library control sample positions before transfection to enable a distribution that allows for inclusion of the appropriate controls on each culture plate (see Figure 14).

Employing such a reformatting strategy during assay establishment for our HUVEC kinome screen41 allowed for setting up a robust assay: the Z' factors of both positive controls used for assay establishment (CHEK-1: 0.55; PLK-1: 0.22) reflected a suitable window for discrimination of potential hits with different phenotypic strengths from background (Figure 15).

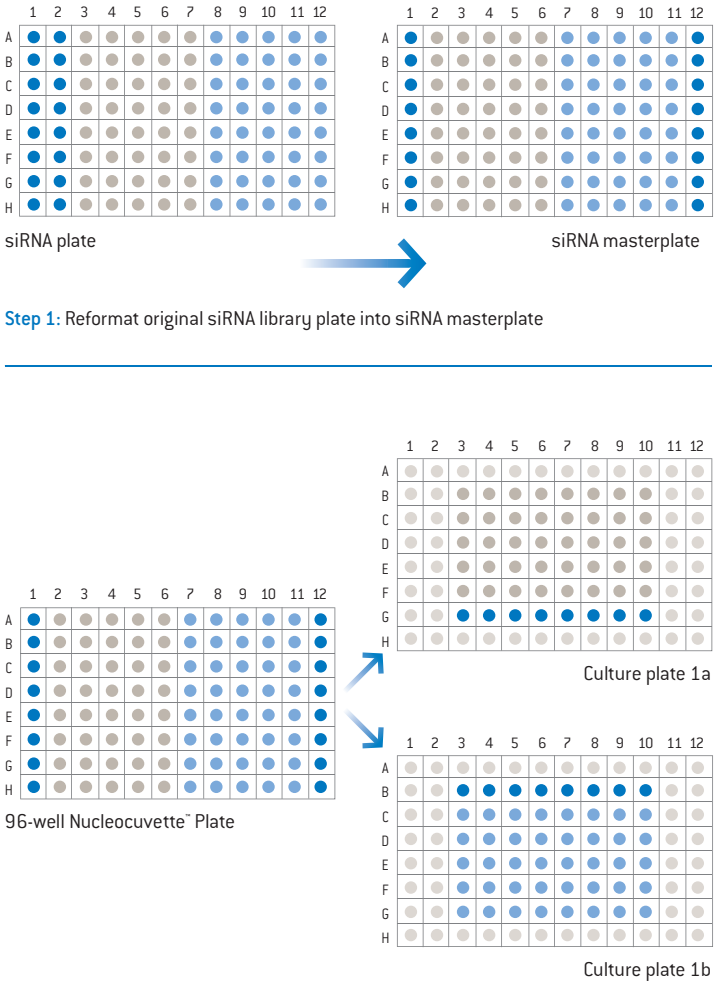


Figure 14: Workflow example for reduction of edge effects. Omitting the outer wells of the 96-well culture plates and filling these wells with medium only can reduce edge effects. Depending on the format of the pre-equipped siRNA library master plate this may require a reformatting of the library to assure inclusion of appropriate control samples on the resulting 2 culture plates (Step 1). After transfection in a 96-well Nucleocuvette™ Plate (Step 2), samples are distributed onto 2 culture plates (Step 3; outer wells filled with medium only).

If plate effects cannot be eliminated completely by library reformatting or workflow optimization, a number of statistical methods are available to normalize such effects (for details see Chapter 5.1). However, as edge effects can have a strong influence on the ability of the cells to display the phenotype, the benefit of such calculations is limited.

4.4.4 Pilot Screens for Exclusion of Technical Obstacles

Pilot screens including all steps and timeframes of the later assays should be performed to assess the reproducibility of identifying positive controls (Figure 16) and to calculate Z' factors for the assays under real conditions (proof of assay robustness; see Figure 15). If necessary, additional fine-tuning of the protocol can be applied to further minimize intra- and inter-plate variation. In order to most appropriately simulate the subsequent screen, we recommend processing a few plates with the final layout and a random distribution of positive and negative controls substituting for the library samples. By including waiting times between the plates, the stability of the overall schedule and the probability of hit identification are best approximated.

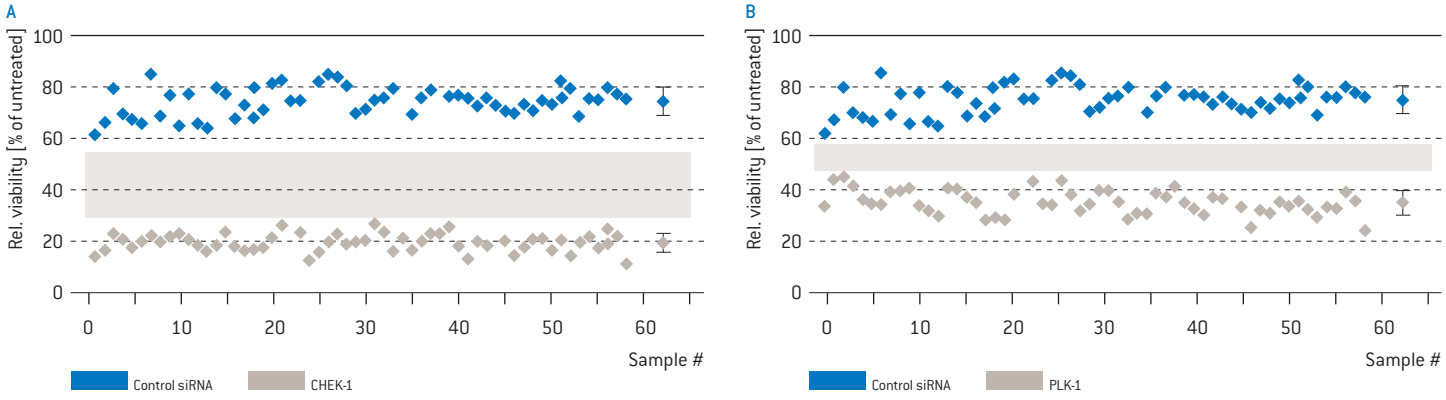


Figure 15: Determination of screening windows. HUVEC cells (Lonza) were transfected with 20 pmol SMARTpool siRNA targeting PLK-1 (A) or CHEK-1 (B) and siGENOM Non-targeting siRNA #1 (Thermo Fisher Scientific). Cell viability was analyzed 72 hours post Nucleofection™ by CellTiter-Blue® Cell Viability Assay (Promega) and sample values were normalized to untreated cells. The rightmost dots represent the average of the 60 individual values. The shaded area represents the screening window calculated via Z' factor (Z' factor for PLK-1 = 0.2 and for CHEK-1 = 0.5). (Data generated in collaboration with Thermo Fisher Scientific.)

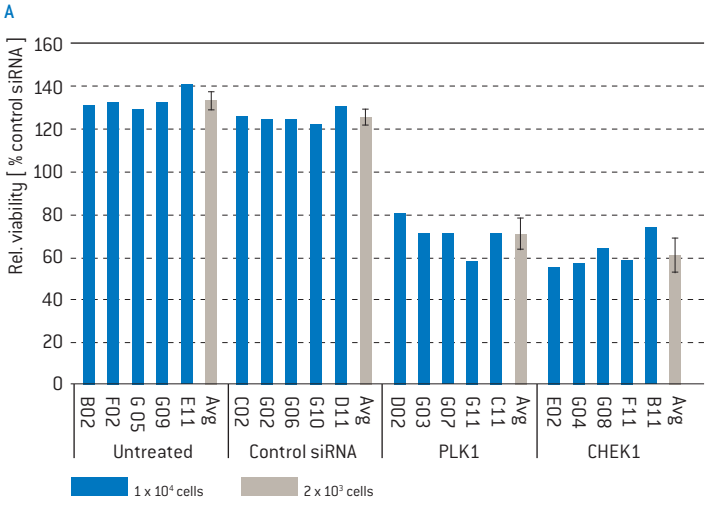


Figure 16A: Reproducibility of controls plated on different well positions. HUVEC cells (Lonza) were transfected with 20 pmol siGENOME Non-targeting siRNA #1 or siGENOME SMARTpool siRNA reagent targeting PLK-1 or CHEK-1 (Thermo Fisher Scientific). Five samples of each control (including untreated cells) were distributed randomly (U-pattern) over the 96-well Nucleocuvette™ Plate. Cell viability was analyzed 72 hours post Nucleofection™ using CellTiter-Blue® Cell Viability Assay (Promega). The grey bars represent the mean of 5 samples each.

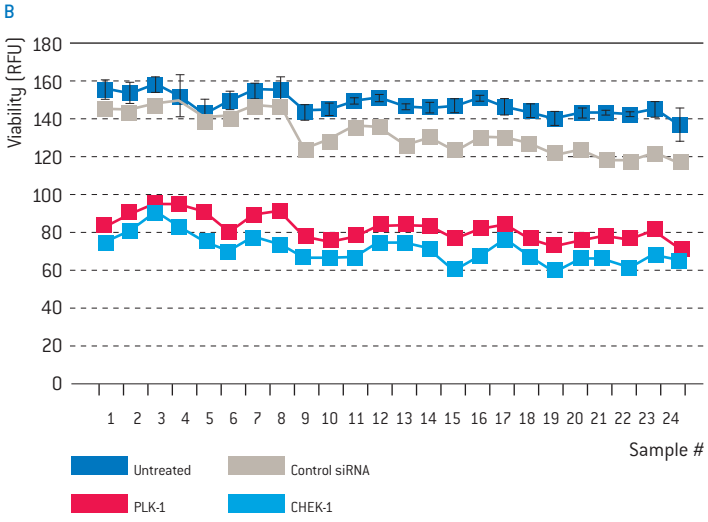


Figure 16B: Reproducibility of controls on all plates was also confirmed in the primary screen. For the screen, control samples were included on each of the 24 plates (untreated cells, siGENOME Non-targeting siRNA #1 or siGENOME SMARTpool siRNA reagent targeting PLK-1 or CHEK-1). HUVEC cells (Lonza) were transfected with 20 pmol siRNA per sample. Cell viability was analyzed 72 hours post Nucleofection™ using CellTiter-Blue® Cell Viability Assay (Promega). The values represent the mean of each control per plate. There is a minor drift over the 24 plates, however the screening window was still sufficient in the last plate. (Data generated in collaboration with Thermo Fisher Scientific.)

5 Screening – Hit Identification

In most cases, the raw data of a screen allow for hit identification only after further processing. To compare results of all plates, raw values of each plate (see Figure 17A) can first be normalized to the controls of the respective plate, i.e., untreated (Figure 17B), mock-transfected or negative control siRNA. Alternatively, raw data can be normalized to mean or median data of each plate. Using the median for normalization is less sensitive to the presence of outliers. For comparison of data from different plates, various statistical methods are available (see Chapter 5.1).

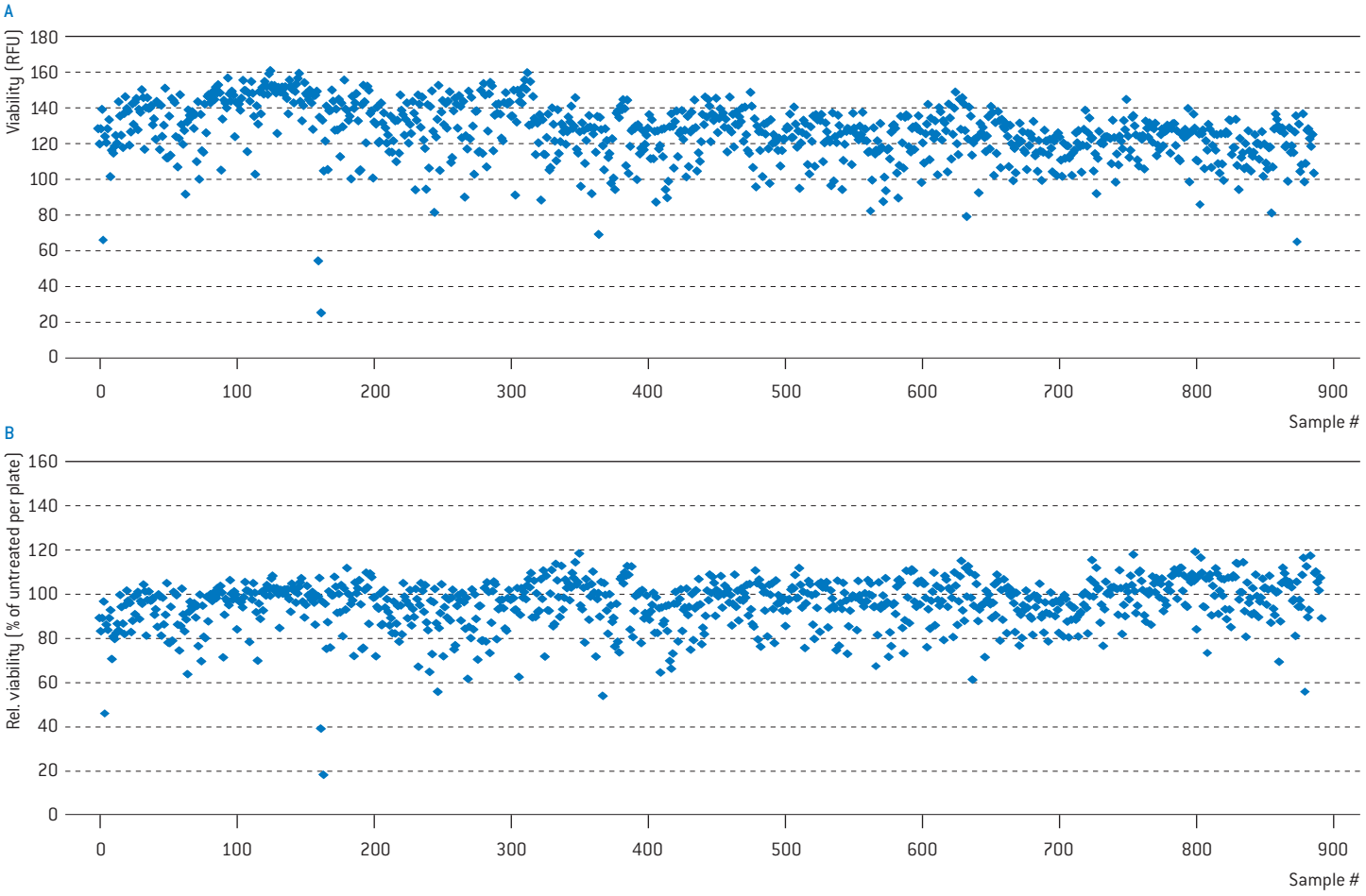


Figure 17: Analysis of primary screen in HUVEC cells. HUVEC cells (Lonza) were transfected with 20 pmol of the combined Thermo Scientific siGENOME Human Protein Kinase siRNA Library (targeting 779 genes and Thermo Scientific siGENOME Human Cell Cycle Regulation siRNA Library (targeting 111 genes). Cell viability was analyzed 72 hours post Nucleofection™ using CellTiter-Blue® Cell Viability Assay (Promega). (A) Raw data including control samples. (B) Raw data of (A) were normalized to untreated controls of the respective plate (% of untreated). (Data generated in collaboration with Thermo Fisher Scientific.)

5.1 Statistical Analysis

There are a number of statistical methods that are used to normalize cell-based screening data for hit identification (an overview is given by Malo *et al.*, 2006[42]). The scoring methods discussed here assume that most of the samples have phenotypic expression levels similar to those in negative controls. To apply these methods a certain minimum library size is required to allow calculation of mean or median from library samples. Thus, for very small libraries (< 50 members) plate mean or median have to be calculated from negative controls.

5.1.1 Z-Score

The Z-score is used to assess plate-to-plate variability (assuming that data display normal distribution). It is a measure of the distance of every individual data point from the plate mean in units of the standard deviation (SD). A sample with a Z-score of 0 has the same value as the plate mean. A sample with a Z-score of 1.0 is exactly one SD above the plate mean, and a Z-score of –0.5 is half a SD below the plate mean.

$$Z_{\text{sample A on plate 1}} = \frac{\text{signal}_{\text{sample A on plate 1}} - \text{mean}_{\text{all samples on plate 1}}}{\text{SD}_{\text{all samples on plate 1}}}$$

5.1.2 Robust Z-Score

As with the Z-score, the robust Z-score can be used to assess plate-to-plate variability, but is less affected by outliers than the Z-score. To calculate the robust Z-score the mean and the SD are replaced by the median and the MAD (median absolute deviation). Chung *et al.*[43] suggested calculations based on the MAD as the method of choice for hit selection in RNAi screens. We also applied the robust Z-score for hit selection in our screens (Figure 18)[44].

$$\text{Robust } Z_{\text{sample A on plate 1}} = \frac{\text{signal}_{\text{sample A on plate 1}} - \text{median}_{\text{all samples on plate 1}}}{\text{MAD}_{\text{all samples on plate 1}}}$$

5.1.3 B-Score

The B (Brideau) score[45] accounts for row and column variations (such as, edge-effects) to normalize each well individually. Generally, it tends to be less precise than other measurements and is very complicated to compute. However, it can be effective if systematic row, column or other positional effects cannot be eliminated by workflow, culture or assay optimization as described in Chapters 3 and 4.

References

42. Malo N et al. (2006) Nat Biotech 24(2):167–175
43. Chung N et al. (2008) J Biomol Screening 13(2):149–158
44. Lonza Application Note (WTB-1011_2008-04-18
45. Brideau C et al. (2003) J Biomol Screen 8:634–637

5.2 Threshold for Hit Selection

The threshold for hit selection can be set on an individual basis for each screen. It depends on the signal-to-noise and signal-to-background ratios. Generally, the lower the threshold, the higher the risk of selecting "false positives". The higher the threshold, the higher the risk of losing potential targets as "false negatives". If the robust Z-score is used for raw data analysis, a typical threshold found in the literature is a |MAD| greater than 2.

When we screened HUVEC cells with a library targeting protein kinases and genes associated with the cell-cycle[46], the robust Z-score for cell viability was calculated for each of the 890 targets in three independent experiments. As an example, the robust Z-scores of one screening experiment are depicted in Figure 18A. A substantial proportion of targets displayed a MAD (median absolute deviation) below –2 or above 2 (|MAD| > 2) including our positive controls PLK-1 and CHEK-1, which are members of both libraries. 37 targets had a mean |MAD| greater than 2 in the three screens and thus were considered as potential hits (Figure 18B).

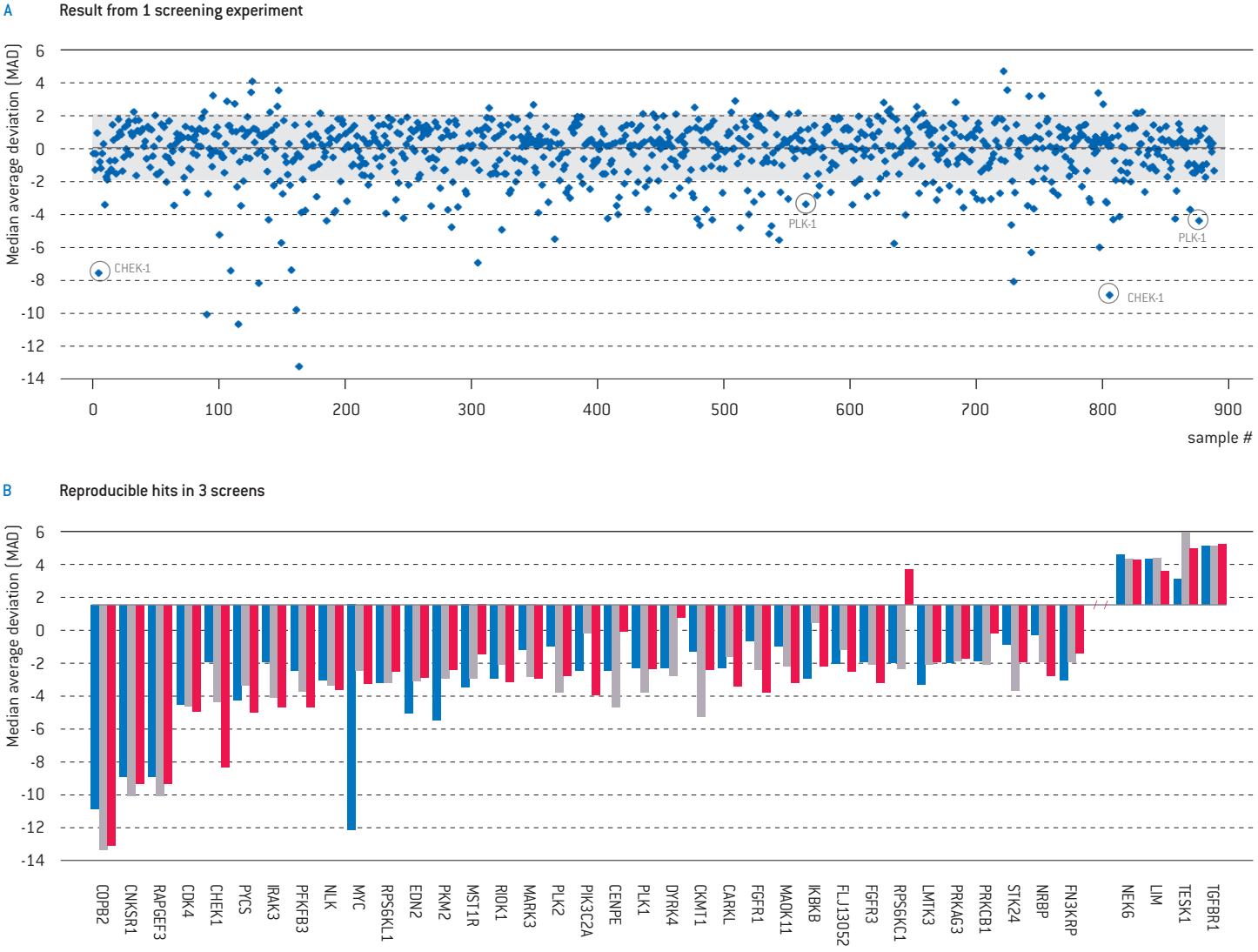


Figure 18: Primary Screen. HUVEC cells were transfected with 20 pmol of the combined Human siGENOME siRNA Libraries for Protein Kinases (targeting 779 genes) and Cell Cycle Regulation (targeting 111 genes). Cell viability was analyzed 72 hours post Nucleofection™. (A) Representation of robust Z-scores of cell viability measures from 1 screening experiment. (B) Robust Z-scores of the top 37 primary hits (with an |MAD| > 2) from 3 independent experiments (displayed in differently shaded blue columns).
(Data generated in collaboration with Thermo Fisher Scientific.)

References

46. Lonza Application Note (WTB-1011_2008-04-18)

6 Screening – Hit Validation

Hits identified in a screen are usually subject to a subsequent validation process confirming the specificity of the RNAi effect, i.e., excluding the observed phenotype from being an off-target effect (see Chapter 1.3). The process of hit validation is normally performed in a number of steps starting with a re-evaluation of primary hits with a higher sample number using the same siRNA substrate that was used in the screen (confirmation, see Chapter 6.1). Subsequently, the specificity of phenotypic effects is confirmed by 2 different strategies, also called the 2 "R's"[47]. Specificity can be verified either via "redundancy", i.e., proving that the same effect is induced by a siRNA sequence targeting a different stretch of the corresponding mRNA (see Chapter 6.2), or by "rescue", i.e., rescue of the normal phenotype by overexpression of a functional protein for which the corresponding mRNA is not targeted by the siRNA (see Chapter 6.3).

6.1 Hit Confirmation

In a first step, for each selected hit, the phenotypic effect observed in the primary screen should be confirmed using a higher sample number with the same RNAi substrate (Figure 19). Random rearrangement of the samples across the processed plate helps ensuring independence of the phenotype from particular well positions.

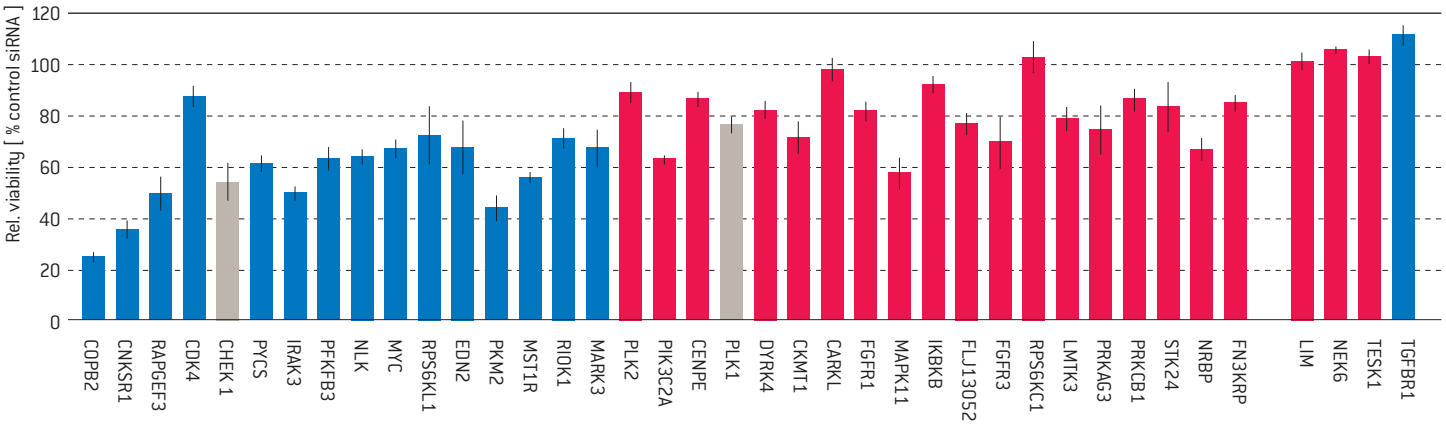


Figure 19: First confirmation of 37 selected primary hits with higher sample number. HUVEC cells (Lonza) were transfected with 20 pmol siGENOME SMARTpool siRNA reagent targeting the 37 selected hits from the primary screen. Four samples per target were distributed randomly over a screening plate. Cell viability was analyzed 72 hours post Nucleofection™ using CellTiter-Blue® Cell Viability Assay assay (Promega) and values were normalized to siGENOME Non-targeting siRNA #1.
(Data generated in collaboration with Thermo Fisher Scientific.)

References

47. Echeverri CJ et al. (2006) Nat Meth 3(10):777–779#

6.2 Redundancy and Knockdown Phenotype Correlation

A good way to enhance confidence in RNAi data and exclude the phenotype of being an off-target effect is to demonstrate a similar phenotypic effect with:

- Different siRNA sequences: siRNAs targeting different stretches of the corresponding mRNA (e.g., single siRNAs from the disassembled pool) or differently modified siRNA type (e.g., ON-TARGET*plus* instead of siGENOME siRNA reagents) (Figure 20A).
- Different substrates (e.g., shRNA, dominant negative cDNA).

In order to further prove specificity of the RNAi-induced phenotype it is important to correlate the phenotypic effect with a downregulation of the targeted gene on mRNA (and ideally also protein) level (Figure 20 A, B). This determines whether a particular siRNA/shRNA sequence is acting through the "classical" RNAi pathway rather than as a microRNA (which — at least in part — inhibits translation of target mRNA, rather than inducing its destruction). A good correlation of mRNA knockdown and phenotype induced by different siRNA sequences gives a strong indication for an on-target effect rather than an off-target effect. Determination of a dose dependence on mRNA and phenotypic level (Figure 20C) can add another piece of confidence but does not per se exclude a sequence or substrate-dependent off-target effect (see Chapter 1.3).

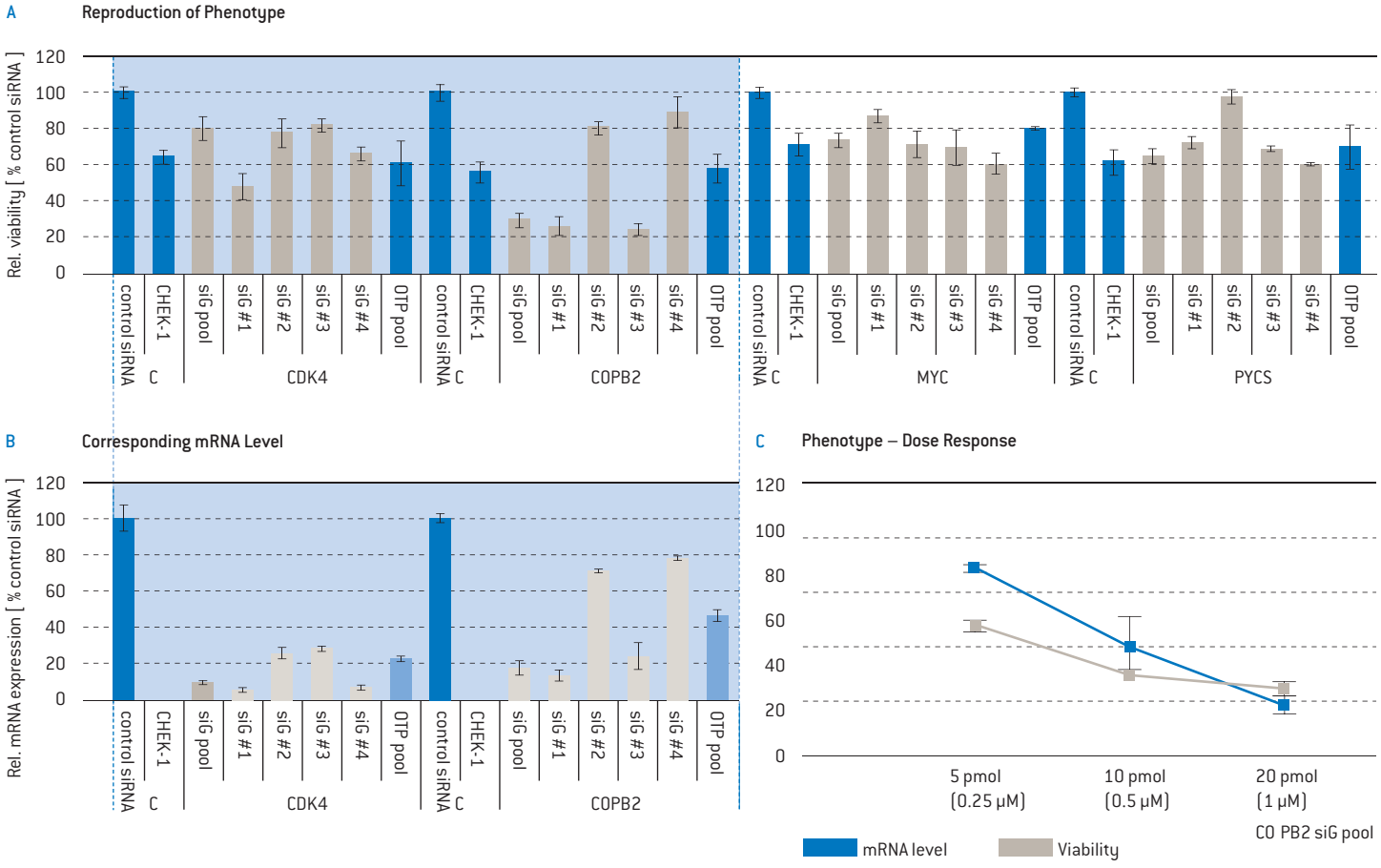


Figure 20: Hit validation via redundancy and knockdown-phenotype correlation. HUVEC cells were transfected with 20 pmol (if not indicated differently) siGENOME (siG) SMARTpool siRNA reagent or single siRNA #1 - 4 (Thermo Fisher Scientific from the de-convoluted pool) targeting CDK4, COPB2, MYC or PYCS. CHEK-1 and siGENOME Non-targeting siRNA #1 served as controls. 72 hours post Nucleofection™, cell viability was analyzed by CellTiter-Blue® Cell Viability Assay (Promega) and normalized to control siRNA (A, C) and mRNA levels were determined for CDK4 (B) and COPB2 (B, C) and normalized to cyclophilin B mRNA and control siRNA.
(Data generated in collaboration with Thermo Fisher Scientific.)

In our HUVEC screen[48], 4 of 16 selected targets (COPB2, PYCS, CDK4 and MYC) were validated by demonstrating that the phenotype could be reproduced with ≥ 3 of 4 single siGENOME siRNA sequences from the original SMARTpool and with the ON-TARGET*plus* SMARTpool siRNA reagent. (Figure 20A). The phenotypes could be well correlated to the knockdown on the mRNA level (Figure 20B) and to the amount of transfected siRNA (Figure 20C; only COPB2 shown). Both results — redundant effects with independent sequences and proven mRNA knockdown — essentially exclude the possibility of the hits being the result of off-target effects.
Six of the 16 selected primary hits were confirmed with 2 of 4 single siGENOME siRNAs but not with the ON-TARGET*plus* pool, suggesting that they still are potential hits but require further validation efforts.
6 of the 16 selected primary hits are considered "false positives" because neither the ON-TARGET*plus* pool nor more than 1 of 4 single siGENOME siRNAs reproduced the phenotype seen with the original siGENOME SMARTpool. These are most likely the result of off-target effects of individual siRNA sequences.

6.3 Rescue Experiments

A rescue experiment is considered as being the ultimate control for specificity of an RNAi experiment. Such rescue is achieved via downregulation of the endogenous gene by siRNA and parallel overexpression of the target gene in a form that is functional but refractory to the siRNA. This refractory target is generated by introducing mutations in the sequences targeted by the respective siRNA molecule (translationally silent point mutations or a deletion in the untranslated region). Alternatively, an orthologous gene from another species can be expressed[49]. For example, a large proportion of mouse genes express a normal functionality in human cells while displaying a low sequence homology.

In experimental terms this means cells are either co-transfected with the siRNA duplex (or shRNA vector) and a plasmid expressing the siRNA-resistant form of the target gene, or using an shRNA-expression vector which co-expresses the shRNA-resistant target gene. Fortunately, the ability of Nucleofector™ Technology to transfect DNA and RNA using identical Nucleofection™ Conditions means that both of these types of experiment are quite straightforward and easy to perform.

6.4 Further Validation

There are several options for further validation of hit specificity, e.g., by using alternative assays, different cell types or pharmacological treatments (e.g., enzyme inhibitors, antibodies) or by performing pathway mapping. These strategies are not treated in more detail in this guide as they largely depend on the background of the biological system and specific questions relevant in the respective experimental direction.

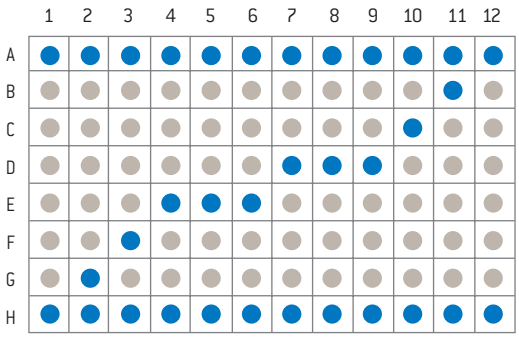
References

48. Lonza Application Note (WTB-1011_2008-04-18)
49. Kittler R et al. (2005) PNAS 102(7):2396-23401

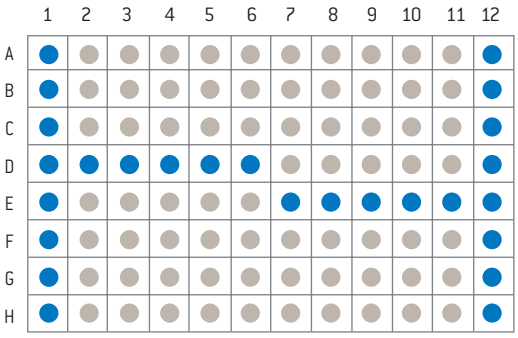
7 Appendix

7.1 Examples for Distribution Patterns of Control Samples

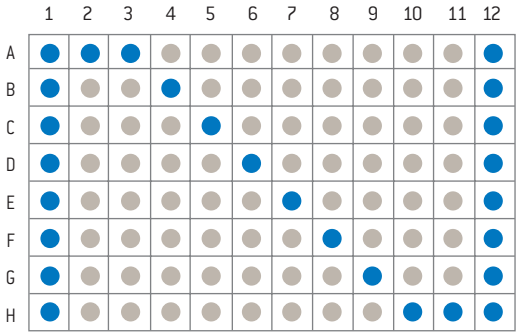
Z-pattern



H-pattern



N-pattern



7.2 Calculation of mRNA Knockdown

a) Logarithmic Value (e.g., RT-PCR)[50]

Sample	$Lg\ Value_{Target} - Lg\ Value_{Reference} = \Delta Lg_{Sample}$
Control	$Lg\ Value_{Target} - Lg\ Value_{Reference} = \Delta Lg_{Control}$
Normalization	$\Delta Lg_{Sample} - \Delta Lg_{Control} = x$
	Residual target mRNA expression $2^{-x} = y$
	Residual target mRNA expression in %: $y * 100 = z$ %
	Knockdown of target mRNA: $(1 - y) * 100 = a$ %

b) Non-logarithmic Values (e.g., QuantiGene® Branched DNA Assay (Affymetrix))

Sample	$Value_{Target} / Value_{Reference} = Ratio_{Sample}$
Control	$Value_{Target} / Value_{Reference} = Ratio_{Control}$
Normalization	$Ratio_{Sample} / Ratio_{Control} = x$
	Residual target mRNA expression $2^{-x} = y$
	Residual target mRNA expression in %: $x * 100 = y$ %
	Knockdown of target mRNA: $(1 - x) * 100 = z$ %

References

50. Applied Biosystems, Application Note (No. 127AP07-02)

7.3 Further Literature Recommendations

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Sachse C *et al.* (2005) High-Throughput RNA Interference Strategies for Target Discovery and Validation by Using Synthetic Short Interfering RNAs: Functional Genomics Investigations of Biological Pathways. Meth Enzymol 392:242-277

Sections II, VII and VIII of Assay Guidance Manual Version 5.0, 2008, Eli Lilly and Company and NIH Chemical Genomics Center. Available online at: http://ncgcweb.nih.gov/guidance/manual_toc.html

Abbreviations

CHEK-1	Cell cycle checkpoint kinase 1 (also CHK-1)
CV	Coefficient of variation
esiRNA	Endoribonuclease-prepared siRNA
HCA	High content analysis
miRNA	microRNA
PLK-1	Polo-like kinase 1
RNAi	RNA interference
SD	Standard deviation
shRNA	Short hairpin RNA
siRNA	Short interfering RNA

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Notes

Notes

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