siTOOLs BIOTECH

The **Pack Hunter** Approach for Superior Gene Silencing

siPOOLs™

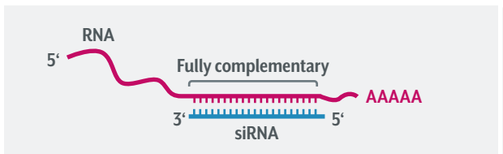
Clean, Reliable & Hassle-Free RNAi

Dealing with
RNA interference

Scientists have been using RNA interference (RNAi) as a rapid and efficient tool to establish gene function. Yet the off-target effects and variable performance of short interfering RNAs (siRNAs) remain a troubling drawback, consuming precious time and resources in validation efforts.

Key Problem - Off-target effects of siRNAs

Leading cause: miRNA-like transcript downregulation



siRNA-based RNAi

RNA cleavage

miRNA-based RNAi

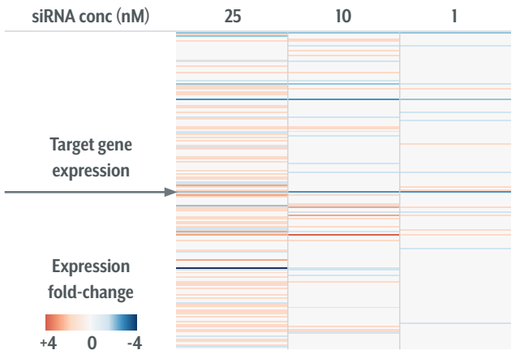
RNA degradation / translation inhibition

siRNAs typically bind with full complementarity to target RNA transcripts, guiding their degradation by the RNAi machinery.

Off-target effects are largely caused by siRNAs mimicking endogenous gene regulators, micro RNAs (miRNAs). As miRNAs require only a 6 base seed match to 3' untranslated regions (UTR) to trigger transcript downregulation, siRNAs can alter the expression of numerous unintended targets when processed via this mechanism.

The consequences:

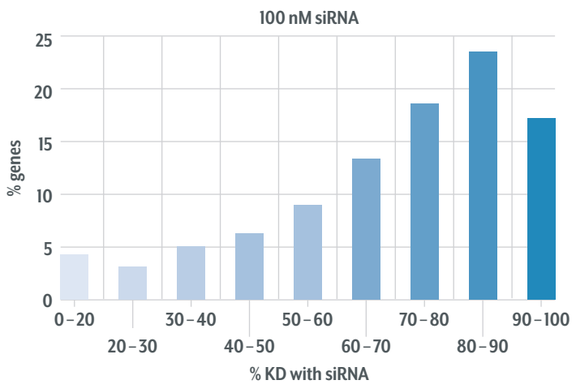
Wide-spread off-target gene deregulation



Gene expression changes after STAT3 siRNA treatment by microarray analysis. Arrow shows STAT3 expression. Data reconstructed from Caffrey et al., 2011[1]

Multiple expression studies like this one show wide-spread off-target gene deregulation by siRNAs[2,3]. High siRNA concentrations produce more off-targeteffects. Off-target genes are enriched for 3' UTR seed-se-quence matches, indicating significant miRNA-like activity.

Variable on-target gene knockdown

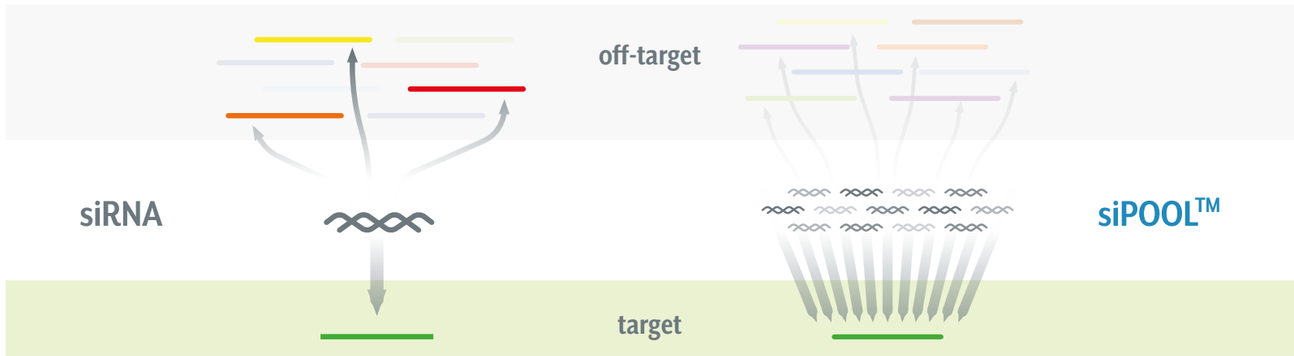


Gene knockdown efficiency by branched DNA assay. Data reconstructed from Simpson et al. 2008[4]

Off-target effects reduce the efficiency of on-target knockdown (KD). At **100 nM**, nearly half of commercial siRNAs (220 of 541 tested, 41%) had knockdown efficiency of < 70% with 4% of siRNAs showing little to no activity.

How siPOOLs improve specificity
and reliability of gene silencing

The siPOOL concept



siPOOLs are **high complexity, defined siRNA pools** containing **30 distinct siRNAs**. **siPOOLs** have a significantly reduced off-target profile and more robust on-target gene knockdown. This is attributed to three key features:

Feature 1
High complexity pooling

Reduced concentration of individual siRNAs

High siRNA concentrations produce more off-target effects[1,2,3]. A high complexity pool allows individual siRNAs to be administered at very low concentrations. This substantially dilutes siRNA off-target signatures.

Increased diversity of siRNA sequences

Single siRNAs dominate performance of low complexity pools. The high complexity of **siPOOLs** reduces off-target effects more efficiently than low complexity siRNA pools (see Box 1, pg 4).

Effect

Reduced off-target effects

Benefits

Less false results

Clean, reliable phenotypes

How siPOOLs improve specificity
and reliability of gene silencing

Feature 2

Detailed Bioinformatics-Based Design

siRNAs	Transcript	Length	Mapping image	siRNAs	Transcript	Length	Mapping image
30/30	NM_001123066.3	6816		30/30	XM_005257364.4	6767	
30/30	NM_001123067.3	5724		30/30	XM_005257365.4	6761	
30/30	NM_001203251.1	5631		29/30	XM_005257366.3	6722	
30/30	NM_001203252.1	5718		30/30	XM_005257367.4	6656	
30/30	NM_005910.5	5811		30/30	XM_005257368.4	6563	
29/30	NM_016834.4	5637		30/30	XM_005257369.4	5789	
30/30	NM_016835.4	6762		30/30	XM_005257370.4	5766	
29/30	NM_016841.4	5544		29/30	XM_005257371.4	5615	
30/30	XM_005257362.4	6854					

siPOOL: MAPT Symbol: MAPT Gene ID 4137 siRNA CDS boundaries

Complete transcript coverage

Multiple siRNAs allow for more robust and complete transcript isoform coverage. **siPOOLs** target XM as well as NM transcripts

Optimal siRNA thermodynamics

Proprietary design algorithms select most potent siRNAs based on thermodynamic properties that favour guide strand loading into the RNA-induced silencing complex (RISC).

Avoidance of paralogues

Genes sharing highly similar sequences (paralogues) are avoided by **siPOOLs**. Paralogue filtering is applied genome-wide.

Effects

Co-operative knockdown | Maximal potency | High specificity

Benefits

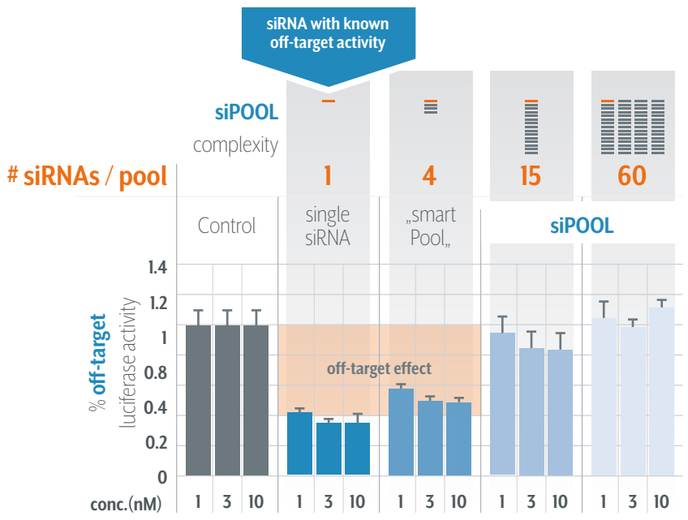
Robust, efficient and specific on-target knockdown

Box 1

Why 30? Because 4 are not enough!

Low complexity pools of 4 siRNAs are commonly used and marketed as "smart pools". We show here that an siRNA with known off-target activity against MAD2 gene required high complexity pools of > 15 siRNAs to sufficiently reduce off-target effects.

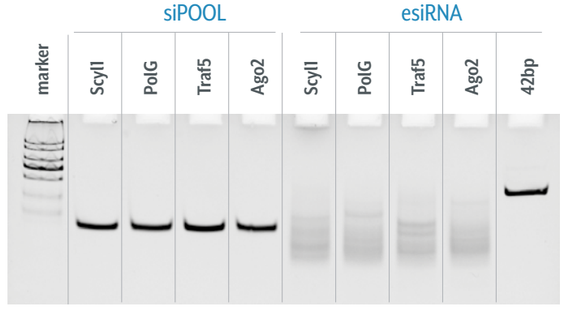
Similar results were obtained when off-target activity was assayed by a MAD2 3'UTR-linked luciferase reporter, MAD2 protein expression and MAD2 functional assay (mitotic escape).



How siPOOLs improve specificity
and reliability of gene silencing

Feature 3

Quality Production Process



siPOOLs/esiRNAs loaded on a 20% polyacrylamide gel and visualized by EtBr staining[5].

Defined, equimolar siRNAs

The patented **siPOOL** production process ensures every siRNA within a **siPOOL** is present in equimolar amounts.

Highest purity levels

siPOOLs undergo polyacrylamide gel electrophoresis (PAGE) purification to remove impurities and extract full-length siRNAs. This results in the highest achievable purity levels of siRNAs.

Effects

Defined, highly pure siRNA that evade immune stimulation (Box 2)

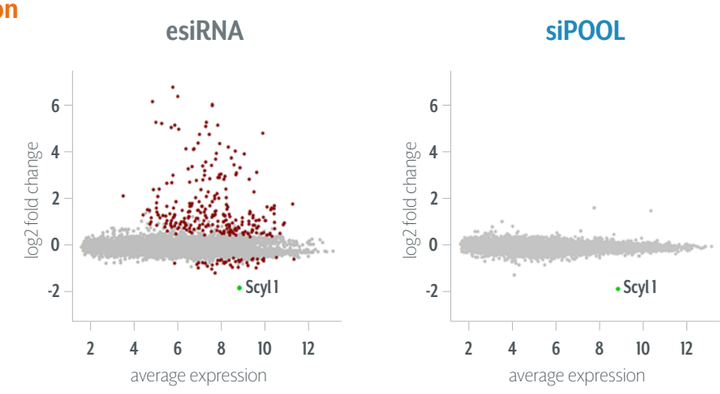
Benefits

Avoid toxicity and off-target effects from immune stimulation

Box 2

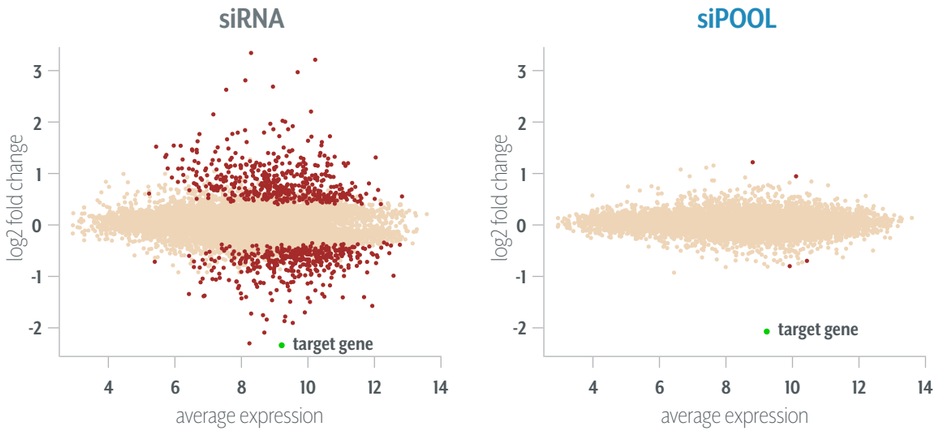
Off-targets from immune stimulation

Long double-stranded RNA fragments tend to stimulate the immune response as seen in this transcriptome-wide expression profile of Scyl1 esiRNA-treated MCF7 cells (left). Upregulated genes in esiRNA-treated cells were largely interferon response genes which were not induced in **siPOOL**-treated cells (right). The immune response can also be stimulated by impurities in siRNA preparations, producing toxicity and off-target effects that interfere with functional read-outs.



Data with siPOOLs –
Proof of concept

Reduced off-target effects with siPOOLs



HeLa cells

3 nM

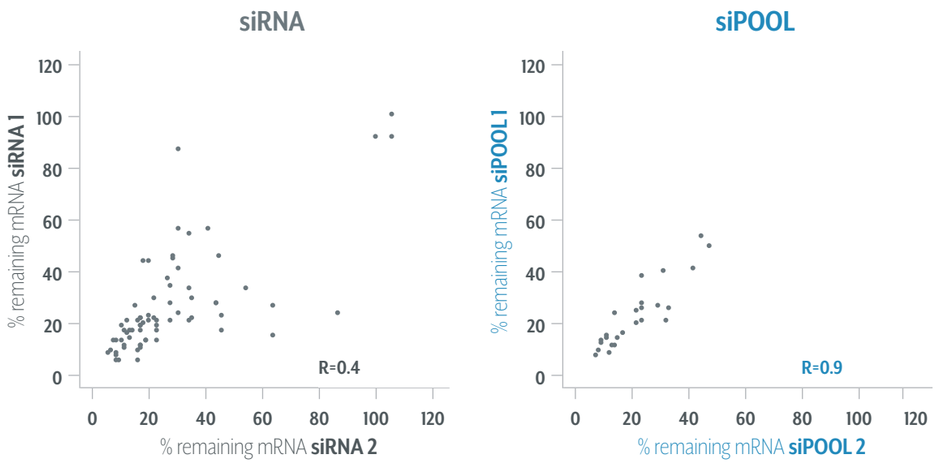
48 h

Human Gene 1.0 ST microarray (Affymetrix)

Specific reagents should only affect their target.

Transcriptome-wide profiling revealed a single siRNA can induce numerous off-target genes (red dots) while a **siPOOL** against the same target gene (green dot), and **containing the non-specific siRNA**, had greatly reduced off-target effects.

Robust on-target knockdown with siPOOLs



A549 cells

1 nM

24 h

Real-time quantitative PCR (rtqPCR)

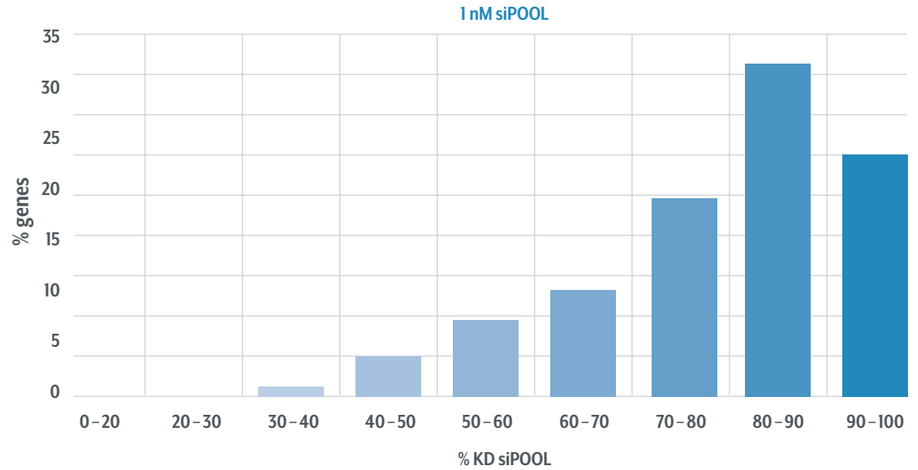
siRNAs vary strongly in their knockdown efficiency.

Two **siPOOLs** against the same gene gave similar knockdown efficiencies (good correlation, R=0.9) while knockdown with single siRNAs was far more variable (poor correlation, R=0.4).

This shows greater robustness and reproducibility of **siPOOL**-mediated knockdown.

Data with siPOOLs –
Proof of concept

Efficient on-target knockdown with siPOOLs



Various standard cell lines

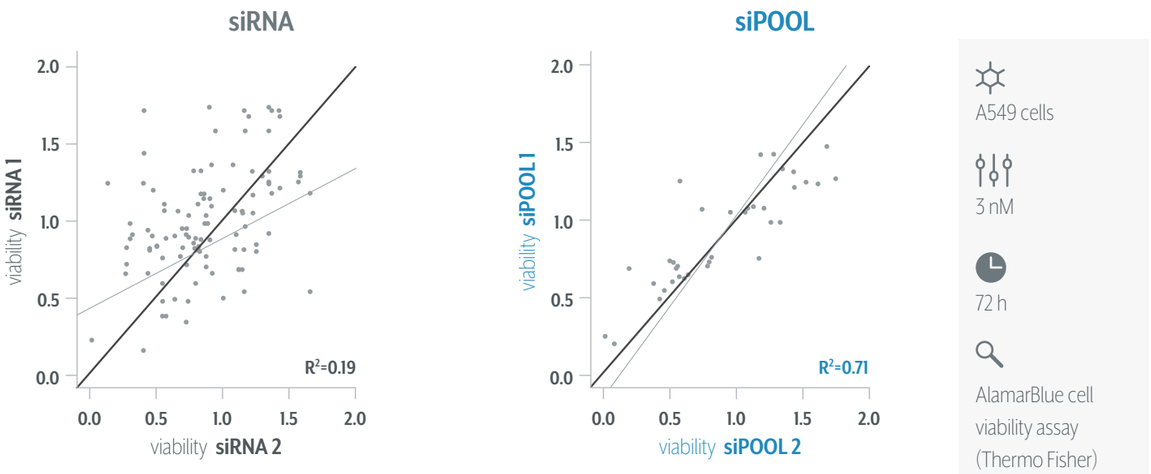
1 nM

24 h

Real-time quantitative PCR (rtqPCR)

siPOOLs exhibit potent knockdown efficiencies at low nanomolar concentrations. **At 1 nM**, a large proportion of **siPOOLs** (178 of 233 tested, 76%) had > 70% gene knockdown efficiency[1] (for indirect comparison, refer pg 2).

Phenotypes you can trust with siPOOLs



A549 cells

3 nM

72 h

AlamarBlue cell viability assay (Thermo Fisher)

If reliable and specific, two reagents that target the same gene should produce similar phenotypes[6].

We screened 36 genes using two **siPOOLs** per gene and three siRNAs per gene from a commercially available library and measured their effect on cell viability. Only **siPOOLs** produced consistent phenotypes.

Further benefits of using siPOOLs

- **One gene – one siPOOL.** Unlike other siRNA reagents that require testing of multiple reagents, a single **siPOOL** is sufficient to reliably knockdown the gene of interest. **siPOOLs** have been cited in various publications - refer to website: **Resources > Publications**. Our landmark paper, Hannus et al., 2014, can be referenced when using **siPOOLs** for gene function validation. For further validation, **siPOOL**-resistant rescue constructs are also available (see pg 11).
- **Easy transfection.** A **siPOOL** is applied the same way as other siRNA reagents, meaning wide-ranging compatibility with transfection reagents at similar conditions previously established with cell lines of choice. A **standard transfection protocol** is available on our website under **Resources > Protocols.** Please contact us if you have questions regarding siRNA transfection.
- **Custom siPOOL design with sequence information available.** All **siPOOL** design is undertaken by us and custom design requests can be incorporated e.g. custom species, isoforms or transcript regions. Sequence information of the siRNAs within the **siPOOL** is available upon request. A **siPOOL**-transcript map to visualize siRNA binding sites can also be provided.
- **Support guaranteed.** Gene knockdown efficiency can vary with the characteristics of the gene as well as transfection efficiency. A high level of support is provided after purchase of **siPOOLs** to make sure that **siPOOLs** are performing to your satisfaction. If **siPOOLs** fail to meet your expectations, we endeavor to fully evaluate the reasons why and generate a re-design when possible. Our support ranges from in-house **siPOOL** knockdown validation, bioinformatics analysis and transfection optimization.

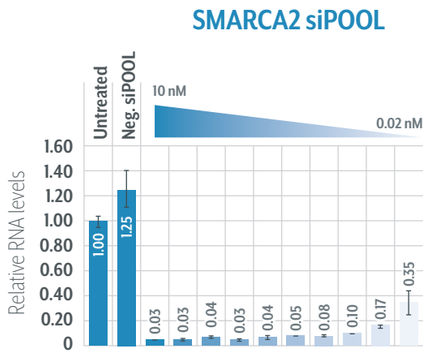
Various applications of siPOOLs:

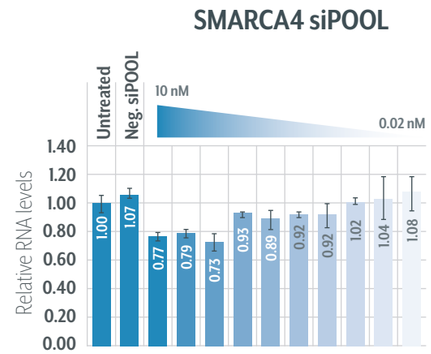
Target validation (further validation available with siPOOL-resistant rescue constructs)	Case study 1 and 4
Combinatorial gene knockdown	Case Study 2
Selective paralogue/isoform knockdown	Case Study 1 and 3
Efficient targeting of long non-coding RNAs	Case Study 3
Target identification with RNAi screening (siPOOL human kinase library and custom libraries available)	
Disease-associated investigations (enquire about siPOOL toolboxes)	

Customer Data with siPOOLs

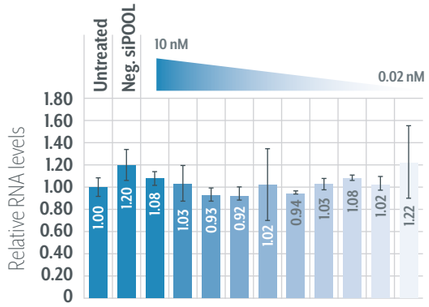
Case study 1: Highly efficient and paralogue-specific knockdown

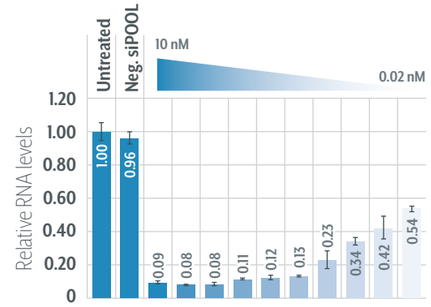
rtq-PCR
SMARCA2





rtq-PCR
SMARCA4



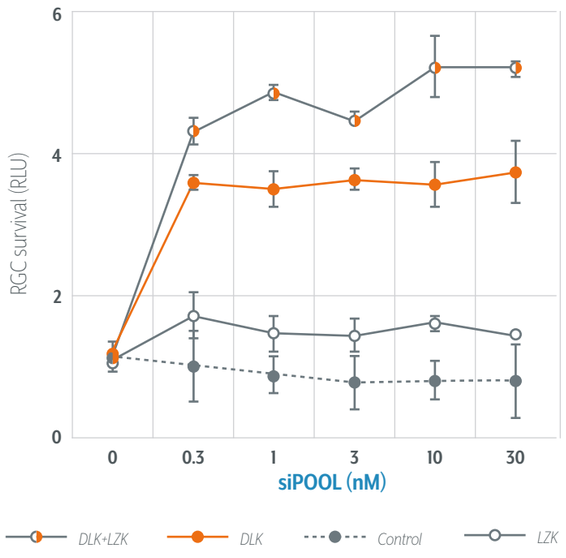


At 0.3 nM, **siPOOLs** produced 95% knockdown of SMARCA2 and 87% knockdown of SMARCA4 in HT1080 fibrosarcoma cell line as quantified by real-time quantitative PCR. No cross-reactivity was observed between the paralogue-specific **siPOOLs**.

„Our Cancer Drug Discovery group uses siRNA technology on a routine basis for target validation experiments. To guarantee robustness of target validation processes we use several independent siRNAs or siRNA pools for the same target of interest. We often observe however, a discrepancy between these reagents in produced cancer phenotypes due to off-target effects, which frequently delivered false-positive results. By using **siPOOLs**, we were able to overcome this discrepancy. **siPOOLs** are highly target-specific and show an efficient knockdown of the target already in the range of low nanomolar concentrations. Another advantage of **siPOOLs** is that they are able to distinguish very specifically between highly homologous transcript sequences. We are very happy with those **siPOOLs**, which helped to avoid waste of time and costs within the drug discovery process by producing reliable data."

Dr. Mona Malz, PhD
Senior Scientist
Cancer Drug Discovery
German Cancer Research Center (DKFZ)
Heidelberg, Germany

Case study 2: Combinatorial gene knockdown to study synergistic effects

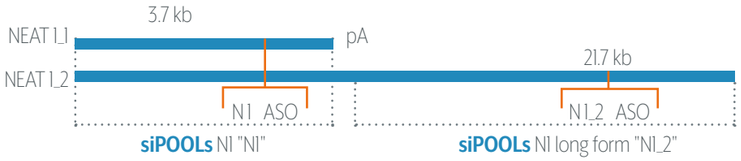


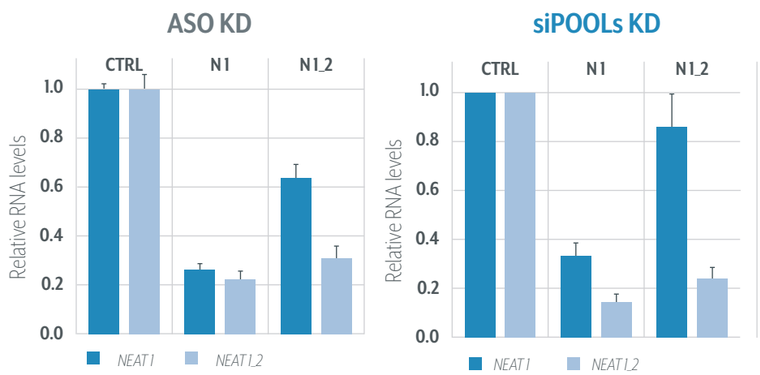
The high efficiency of **siPOOLs** at low concentrations encourages its use for combinatorial gene knockdown.
A synergistic effect on survival of primary retinal gangliocytes was observed on combinatorial knockdown of dual leucine zipper kinase (DLK) and leucine zipper kinase (LZK) by Welsbie et al. (published in Neuron, 2017)[7].

„Our lab uses arrayed, high-throughput functional genomic screening in primary neurons to identify potential neuroprotective drug targets. Having tested over 75,000 siRNA sequences, it is quite apparent that off-target effects dominate siRNA-mediated phenotypes. In contrast, in our hands, **siPOOLs** have much greater predictive power in that phenotypes we see with these (and we have tested approximately 15) can be reproduced using cells containing conventional knockouts for the same genes. We now routinely use **siPOOLs** and are moving away from single siRNAs."

Dr. Derek S Welsbie, MD, PhD
Assistant Professor of Opthalmology
University of California, San Diego, USA

Case study 3: Knockdown of long non-coding RNAs





siPOOLs were used to knockdown long non-coding RNA, NEAT1, in MCF7 cells. An isoform-specific **siPOOL** (N1_2) was also generated that targets only the long form of NEAT1 (NEAT 1_2). Both **siPOOLs** performed comparably with antisense oligos (ASO) and induced measurable phenotypic changes.

Data as published in Adriaens et. al, Nature Medicine, 2016[8]

Customer Data with siPOOLs

Ms. Jasmine Barra
PhD Student
Lab for Molecular Cancer Biology
Prof. Dr. Chris Marine Group
VIB Center for the Biology of Disease
KU Leuven, Belgium

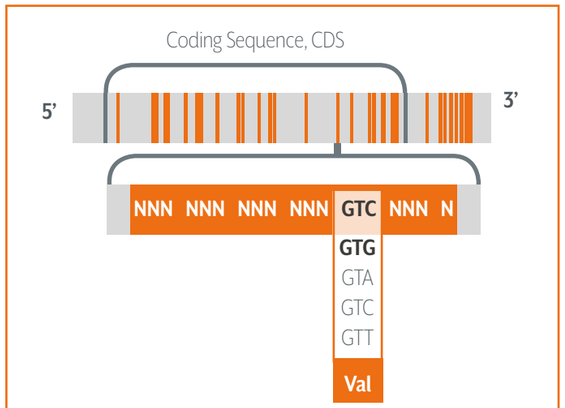
„For our research purpose the use of **siPOOLs** proved to be a key choice. We could overcome the major issues of both cell toxicity and off target effects we observed using GAPMERs.

In our hands the **siPOOLs** performed always with high reproducibility, allowing us to increase the efficiency of knock down of our target of interest, despite the poor results given by standard siRNA approaches.

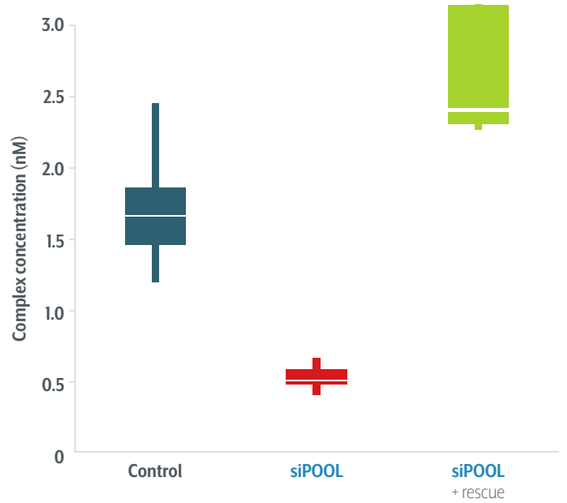
Moreover siTOOLs could custom design for us isoform-specific **siPOOLs** that allowed us to address in a more specific way our biological questions."

Case study 4: Further validation with siPOOL-resistant rescue constructs

Design of siPOOL-resistant rescue constructs



siPOOL-resistant rescue sequences are designed by wobbling single bases at siRNA recognition sites (orange) till sufficient mismatch is reached to confer **siPOOL** resistance. The codon-optimized sequence when expressed via a DNA vector thereby 'rescues' the loss-of-function phenotype induced by the co-administered **siPOOL**. Rescue constructs are available as **sequence data files** or in **sequence-verified standard/custom vectors.**



Complex formation measured by fluorescence cross-correlation spectroscopy was decreased on **siPOOL**-mediated knockdown of one labelled binding partner. Complex formation was restored upon expression of **siPOOL**-resistant rescue construct.

Data kindly provided by

Intana Bioscience GmbH

How to order

Via Webshop:

1. Register an account
2. Enter gene name/NCBI ID and select species
3. Select gene with option to verify on NCBI website
4. Choose product
5. Select payment method - Invoice, Paypal, Visa/Mastercard or direct bank transfer
6. Receive confirmation via Email

Direct requests:

A quotation can also be obtained by contacting us directly with your requests. Please contact us and we will respond within 24 h:

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About Us

siTOOLs Biotech is an agile, science-driven start-up providing innovative gene function analysis tools and services. Based in the dynamic Munich-Martinsried biotech cluster in Germany, siTOOLs was founded in 2013 by experts in the RNA field: Dr. Michael Hannus (previously RNAi Screening Lead from Cenix Bioscience) and Prof. Dr. Gunter Meister (Head of RNA Biochemistry at the University of Regensburg).

With initial funding from the German EXIST start-up grant, siTOOLs has grown from a local start-up to a world-wide supplier. siTOOL's reagents are routinely being used in target validation for drug discovery in pharma/biotech and in multiple research projects by leading academic labs. We strive towards easing scientists' workflows with robust tools and rigorous, scientific support.

Dr. Michael Hannus

Prof. Dr. Gunter Meister

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Learn more:

Website **www.sitoolsbiotech.com**

Blog **blog.sitoolsbiotech.com**

References

1 Caffrey, D. R., Zhao, J., Song, Z., Schaffer, M. E., Haney, S. A., Subramanian, R. R., ... Hughes, J. D. (2011). Sirna off-target effects can be reduced at concentrations that match their individual potency. PLoS ONE, 6(7), e21503. **2** Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V, Burchard, J., Mao, M., ... Linsley, P. S. (2003). Expression profiling reveals off-target gene regulation by RNAi. Nature Biotechnology, 21(6), 635-637. **3** Birmingham, A., Anderson, E. M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., ... Khvorova, A. (2006). 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nat Meth, 3(3), 199-204. **4** Simpson, K. J., Selfors, L. M., Bui, J., Reynolds, A., Leake, D., Khvorova, A., & Brugge, J. S. (2008). Identification of genes that regulate epithelial cell migration using an siRNA screening approach. Nat Cell Biol, 10(9), 1027-1038. **5** Hannus, M., Beitzinger, M., Engelmann, J. C., Weickert, M.-T., Spang, R., Hannus, S., & Meister, G. (2014). siPOOLs: highly complex but accurately defined siRNA pools eliminate off-target effects. Nucleic Acids Research, 42(12), 8049-61. **6** Marine, S., Bahl, A., Ferrer, M., & Buehler, E. (2012). Common seed analysis to identify off-target effects in siRNA screens. Journal of Biomolecular Screening, 17(3), 370-8. **7** Welsbie, D. S., Mitchell, K. L., Jaskula-Ranga, V., Sluch, V. M., Yang, Z., Kim, J., ... Zack, D. J. (2017). Enhanced Functional Genomic Screening Identifies Novel Mediators of Dual Leucine Zipper Kinase-Dependent Injury Signaling in Neurons. Neuron, 94(6), 1142-1154.e6. **8** Adriaens, C., Standaert, L., Barra, J., Latil, M., Verfaillie, A., Kalev, P., ... Marine, J.-C. (2016). p53 induces formation of NEAT1 lncRNA-containing paraspeckles that modulate replication stress response and chemosensitivity. Nature Medicine, 22(8), 861-868.