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Technote 2

Ways to Reduce siRNA Off-target Effects

This technote describes ways to reduce siRNA off-target effects:

1. Lower siRNA concentrations
2. Pooling siRNAs
3. Chemical modification
4. siRNA design
5. The siPOOL approach

Off-target effects by siRNAs occur widely and unpredictably *(refer Technote 1: Causes, extent and impact of siRNA off-targets)*. This technote covers established techniques to reduce them and evaluates the effectiveness of each approach.

1. Lower siRNA concentrations

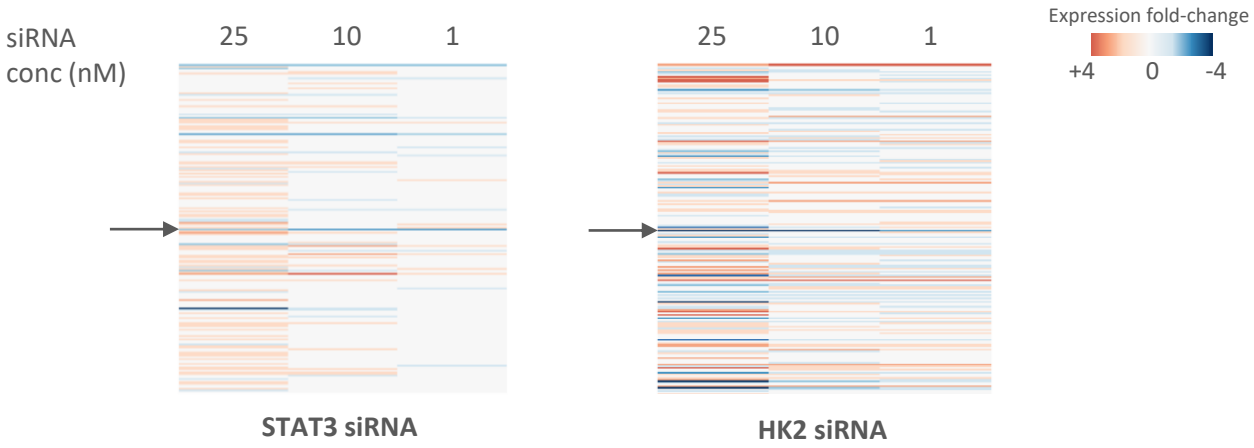

Figure 1. Concentration dependence of siRNA off-targeting. Transcriptome-wide expression assayed by Affymetrix microarray in MCF-7 cells treated with STAT3 or HK2 siRNAs at stated concentrations. Arrow indicates target gene expression. Blue indicates downregulation and orange upregulation (expression scale bar: top right)

The first report of siRNA off-targeting by Jackson et al. (2003) saw vast transcriptome-wide deregulation after siRNA treatment that could not be attributed to target gene knock-down. Multiple reports show lowering siRNA concentrations reduces off-target activity of siRNAs (see fig. 1, adapted from Caffrey et al., 2011). However, strong off-target effects may persist at low concentrations, as seen for HK2 siRNA.

A key drawback of reducing siRNA concentration is the lowered efficiency of on-target gene knock-down. Lowering siRNA concentrations therefore can only be applied to siRNAs with high on-target activity.

Effective?
Effective but limited to highly active siRNAs with weak off-target effects.

2. Pooling siRNAs

Pooling multiple siRNAs can reduce off-targets but complexity (i.e. number of siRNAs pooled) matters. Kittler et al. (2007) compared off-target expression profiles of an siRNA administered singly or in a pool of increasing complexity. It was observed that complexity, independent of siRNA concentration, altered the off-target profile of siRNAs.

Despite the common use of low complexity pools of 3-4 siRNAs, our studies show a pool complexity of 15 or more siRNAs was required to eliminate strong off-target effects (Fig. 2, Hannus et al., 2014). Furthermore, low complexity pooling did not remove seed dominance as seen from large RNAi screens (Marine et al., 2016, refer Technote 1, section 2b).

Having multiple siRNAs against a target also increases efficiency of gene silencing. As single siRNAs are known to perform variably, having multiple siRNAs per gene increases the chances of knock-down success.

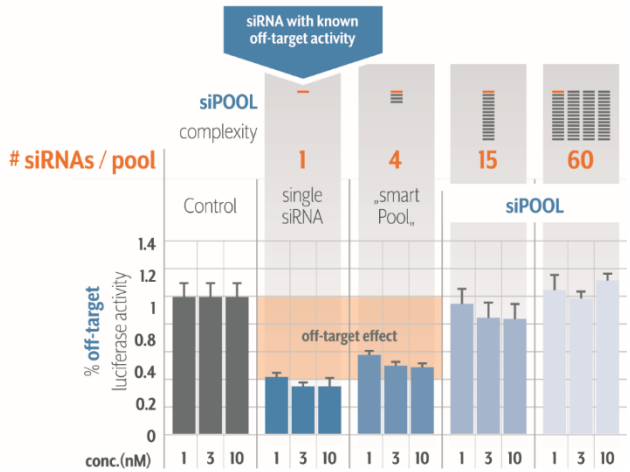

Figure 2. Effect of siRNA pool complexity on siRNA off-targeting. The off-targeting activity of an siRNA was monitored with a luciferase reporter linked to the 3' UTR of known off-target gene (MAD2). The siRNA was administered together with 3, 14 or 59 other sequence-independent siRNAs to the same target gene (PolG). Pools of 15 or more siRNAs were required to eliminate off-targeting to MAD2. Off-targeting was also observed in MAD2 protein expression and MAD2 functional assays (mitotic escape).

Effective?
Effective only with high complexity pools of greater than 15 siRNAs.

3. Chemical modification

Chemical modifications, specifically in the seed region of siRNAs, can reduce off-targeting. This is proposed to work by destabilizing interactions between the siRNA seed region and off-target RNAs *(refer Technote 1 to learn about the mechanism of siRNA off-targeting).*

Various types of modifications can be made including the use of unlocked nucleic acids (UNA), 2'-O-methyl modifications and substituting RNA with DNA at the seed region (Bramsen et al., 2010; Jackson et al., 2006; Ui-Tei et al., 2008). However, effectiveness may vary, working well for off-target transcripts with a weaker seed pairing energy, while failing to affect targets with a strong pairing energy.

This is illustrated in figure 3 from Rasmussen et al. (2013) which demonstrated that chemical modification failed to eliminate seed-based off-targeting for highly active seeds.

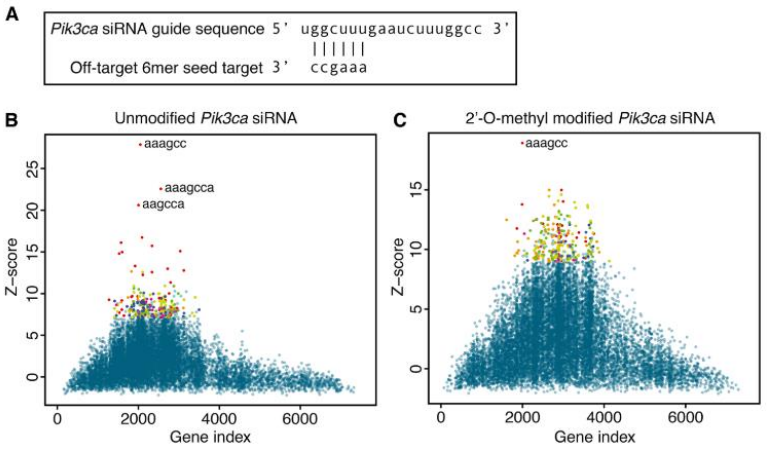

Figure 3. Chemical modification reduces but does not eliminate all seed-based off-target effects A, seed region of Pik3ca siRNA. B, cluster plots showing seeds enriched in 3' UTRs of downregulated genes after transfection with unmodified (B) or 2'-O-methyl modified (C) Pik3ca siRNA. The "aaagcc" seed sequence remains enriched after chemical modification. *(Source: Rasmussen et al., 2013)*

Furthermore, chemically-modified siRNAs still exhibit seed-based dominance in large RNAi screens. A phenotypic correlation analysis between chemically-modified siRNA libraries and non-modified siRNA libraries in an RNAi screen for bacterial infectivity factors showed that both types of siRNAs exhibited extensive off-target correlation and little on-target correlation *(refer Technote 1, Section 2b to understand how correlation data represents penetrance of siRNA off-target effects)*.

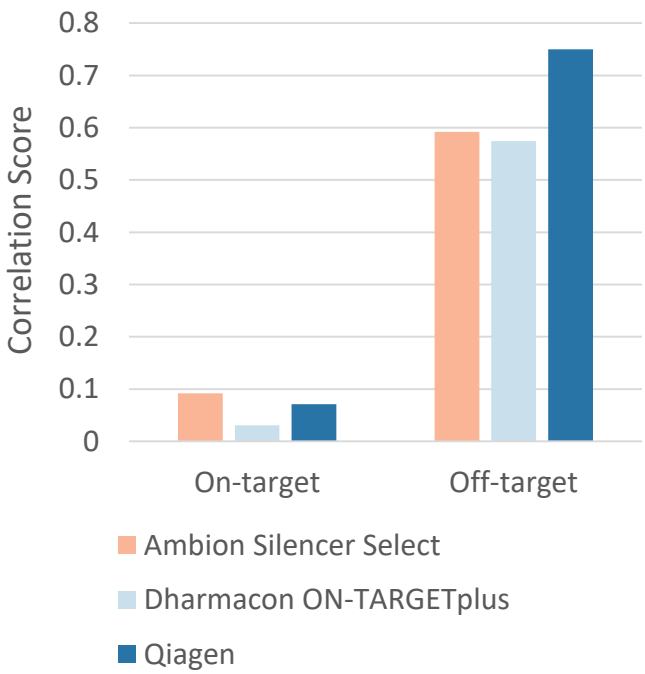

Figure 4. Chemically-modified siRNAs performed similarly to non-modified siRNAs. In a screen for infection factors, Brucella infectivity was monitored after treatment with siRNAs from three commercial vendors. On-target correlation compares two sequence-independent siRNAs targeting the same gene. Off-target correlation compares two target-distinct siRNAs with the same seed sequence. A correlation score closer to 1 indicates highly similar phenotypes between the two siRNAs compared. All commercial siRNAs including chemically-modified siRNAs (Ambion/Dharmacon) showed high off-target correlation scores and little on-target correlation, indicating a dominance of seed-based off-targeting. Similar trends were seen for other phenotypic read-outs such as cell count *(Data source: Franceschini et al., 2014)*

Effective?
Effective but limited to siRNAs with weak seed pairing energies. Used alone is not sufficient to counter seed-based off-target dominance.

4. siRNA design

Various attempts to design siRNAs with a reduced likelihood of off-targeting have been made. We offer a summary of the most commonly applied rules and evaluate their effectiveness.

a. BLAST alignment to filter off-targets

The Basic Local Alignment Search Tool by NCBI (BLAST) aligns the siRNA sequence to the genome of the target organism to avoid choosing siRNAs with high sequence similarity to off-target genes.

As BLAST is only programmed to look for large contiguous matches, the alignment by Smith and Waterman (1981) has been recommended to improve off-target prediction as it is more suited for short non-contiguous sequences. However, an experimental validation of siRNAs designed with the SW alignment revealed that it was not more effective at predicting off-targets (Birmingham et al., 2005).

BLAST is applied for nearly all siRNA design algorithms to minimize the more obvious off-targets but comes at a cost of losing functional siRNA candidates as prediction success is not 100%.

Effective?

Yes, but not 100% predictive and limited by word size (i.e. length of sequence used in BLAST). A shorter word size limits the number of available siRNA candidates. A longer word size does not exclude seed effects or tolerated mismatches.

b. Improve siRNA strand-loading bias

Introducing more thermal instability at one end of the siRNA duplex can bias antisense strand loading into RISC (Fig.5, Khvorova et al., 2003; Schwarz et al., 2003). This can be done by designing siRNAs with a higher G/C content near the 3' end and lower G/C near the 5' end of the antisense strand.

By increasing antisense strand loading into RISC, one decreases off-target effects derived from the sense strand, and increases gene silencing efficiency by having greater numbers of correctly assembled RISC complexes.

Experimental validation of the algorithm (developed and tested by Cenix Bioscience scientists, two of whom now work at siTOOLs) is reported in the Thermo Fisher technote: "siRNA Design It's All in the Algorithm". Chemical modifications have also been used to bias strand loading.

Effective?

Yes, however off-targets associated with the guide strand would still exist and may even increase due to its increased loading into RISC.

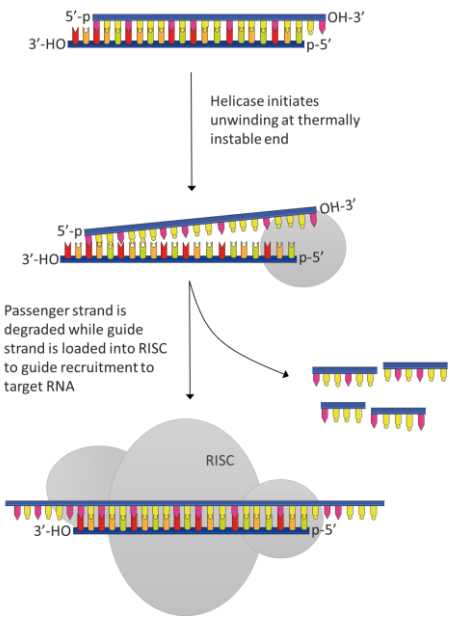

Figure 5. Mechanism of strand loading into RISC. Thermodynamic asymmetry decides strand loading into RISC (figure adapted from Schwarz et. al, 2003)

c. Avoid microRNA seeds

Given that microRNA-like seed-based recognition is the main source of RNAi off-target effects, researchers have proposed avoiding native microRNA sites during siRNA design.

But how effective is this approach? We performed a phenotypic correlation analysis of screening results from Hasson et al. 2013 where 12% (~7800) of all screened siRNAs (~65,000) contained a 7-mer seed shared by a microRNA. *(refer Technote 1, Section 2b to understand how correlation data represents penetrance of siRNA off-target effects).*

Expectedly, off-target effects dominated the readout (off-target correlation: ~0.5, on-target correlation ~0.07). Excluding siRNAs containing native microRNA seeds however, failed to improve the on-target correlation score, nor did it reduce the off-target correlation score. Similar findings for another screen (Panda et al., 2017) show avoiding microRNA seeds in siRNA design does not reduce siRNA off-targeting.

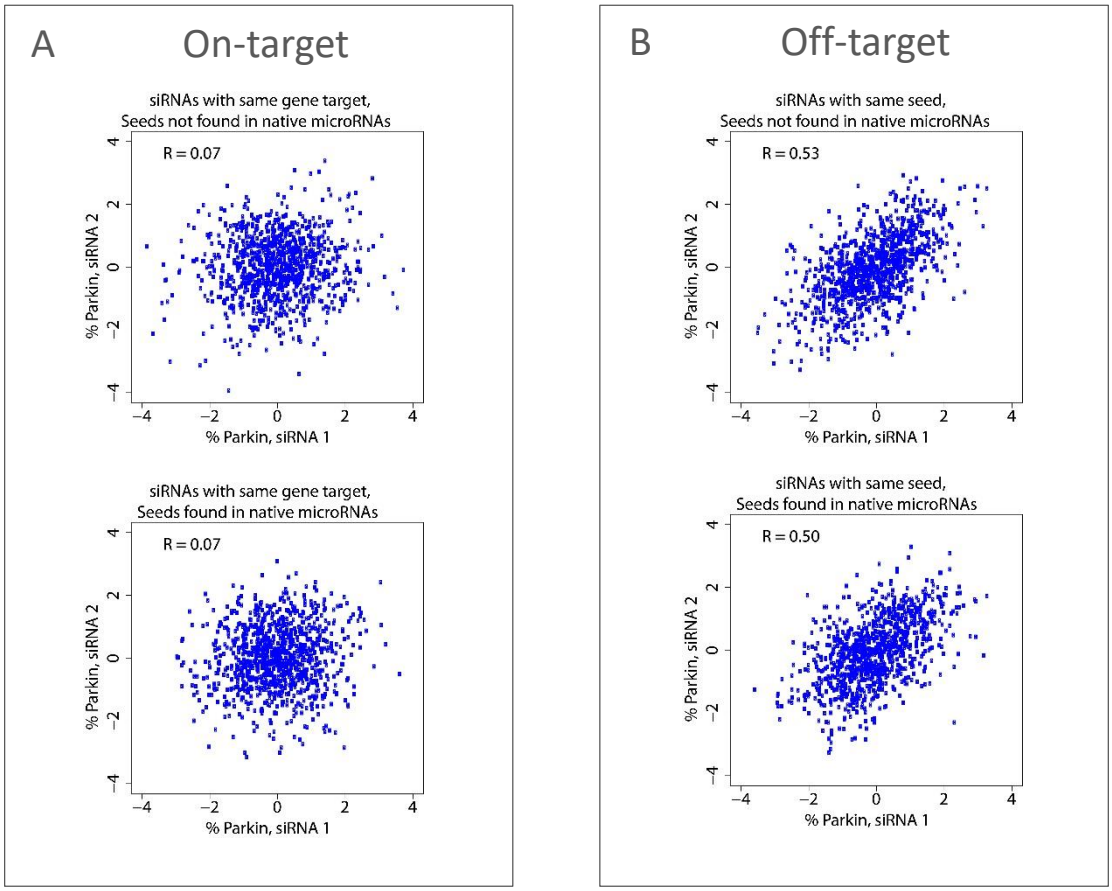

Figure 6. Avoidance of microRNA seeds does not affect on-target or off-target phenotypic correlation. An RNAi screening dataset for factors affecting % Parkin translocation screened 65 000 siRNAs, 7800 of which contained microRNA seeds (12%). When these siRNAs were removed and phenotypic correlation analysis performed, no change was seen for on-target correlation (A) and off-target correlation (B) *(Data source: Hasson et al., 2013)*

Effective?
No.

For the purpose of brevity, we summarize some further design rules and their associated papers which have been incorporated to some extent towards the design of commercial siRNAs:

- Avoidance of immuno-stimulatory motifs (Judge and MacLachlann, 2008).
- Having certain bases at select positions based on a large functional study of 2432 siRNAs. Dominant rule being A/U at position 1 of antisense strand which reflects the strand-loading bias (Huesken et al., 2005).
- Low to medium G/C content and avoidance of internal repeats or palindromic sequences. This regulates siRNA duplex stability and secondary structure that can affect targeting efficiency (Reynolds et al., 2004; Patzel et. al, 2005).
- Not targeting regions with reported single nucleotide polymorphisms (SNPs) as single mismatches may reduce functionality of the siRNA (Du et al., 2005; Birmingham et al., 2006, 2007).
- Avoidance of seed complementarity to 3' UTRs (Birmingham et al., 2006). Due to the short seed sequence length (6 bases), applying this rule would severely limit the number of siRNA candidates. Furthermore, it is not 100% predictive, i.e. not all siRNAs with a seed match to a 3'UTR would downregulate that gene. Hence, this rule is usually applied using a longer stretch of sequence similarity or in the case of siPOOLs, applied mainly towards paralogue filtering.

Another key point with siRNA design is the use of **latest transcript annotations**. With the discovery of non-coding RNAs and increasing large-scale genomic studies, new annotations are constantly being added to genomic/transcriptomic databases. It is therefore imperative that siRNAs designed based on older database versions are consistently updated to avoid off-target effects to these new entities and potential paralogous sequences. Furthermore, updating siRNA designs also ensure newly annotated splicing transcripts are efficiently targeted.

Despite the numerous siRNA design rules, off-target effects persist and on-target silencing remains variable. It is clear that a combination of physical and design-based approaches is needed to efficiently counter the current challenges in RNAi.

Effective?
Optimizing siRNA design alone is not sufficient to counter siRNA off-target effects.

Which approaches do siPOOLs incorporate?

A siPOOL consists of ~30 optimally-designed siRNAs. The high complexity of siPOOLs in addition to its effective low working concentrations (1-3 nM in standard cell lines) greatly reduces off-target effects. siPOOLs are also designed with proprietary algorithms that aim to maximize transcript coverage, avoid paralogues and enhance knock-down efficiency. Therefore, siPOOLs combine low concentration, high complexity siRNA pooling and optimized design of siRNAs in a holistic approach towards managing siRNA off-target effects.

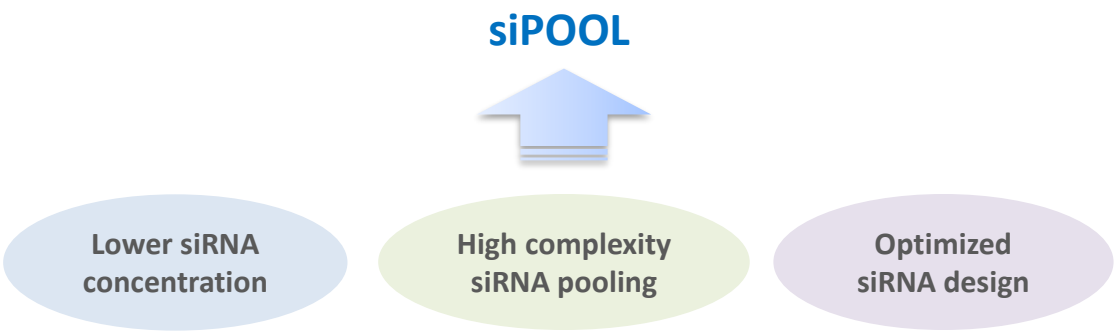

Figure 7. The siPOOL approach to increase specificity and efficiency of gene silencing.

siPOOLs were demonstrated to greatly reduce the off-target effects of single siRNAs (Fig. 8). Compared to low complexity pools of 3-4 siRNAs, siPOOLs produced more specific gene silencing, eliminating strong off-target effects attributed to single siRNAs in off-target spiking experiments (Hannus et al. 2014).

With greater specificity, gene silencing becomes more efficient and reproducible. Correlation analysis of the on-target effect as measured by qPCR of target RNA levels and phenotypic read-outs (cell viability) was notably higher for siPOOLs as compared to single siRNAs (Fig. 9).

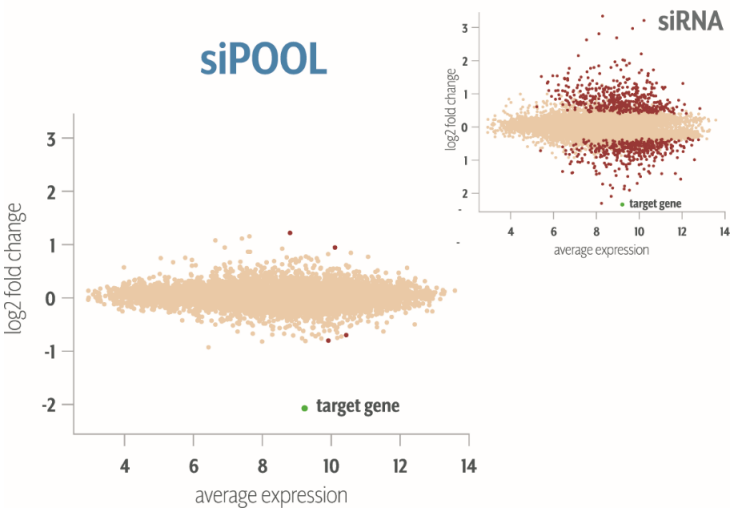

Figure 8. siPOOLs reduce off-target effects. Whole transcriptome profiling of HeLa cells treated with 3 nM siRNA or a siPOOL, *containing the same siRNA*, was carried out after 48 h. siPOOLs efficiently reduced off-target effects (red dots) of siRNA while maintaining on-target (green dot) knock-down.

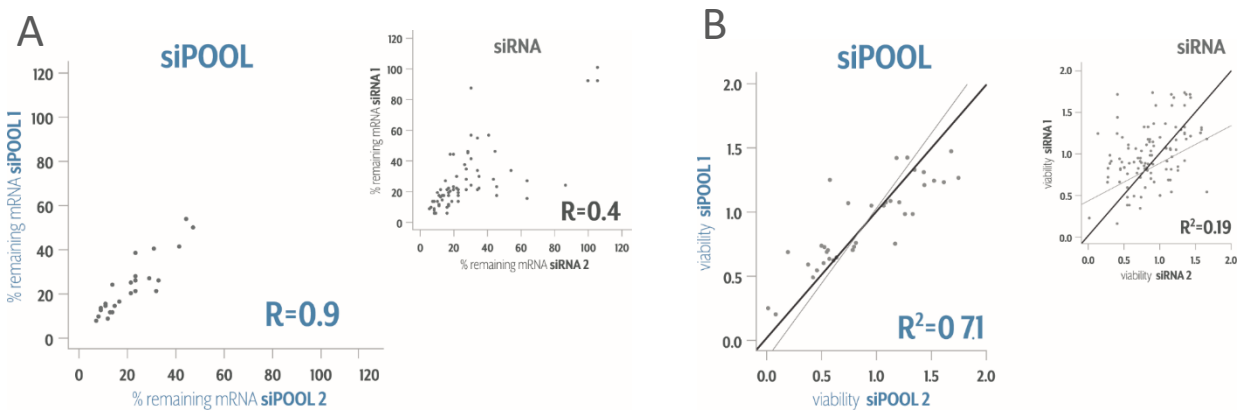

Figure 9. siPOOLs increase on-target correlation in terms of gene knock-down and resultant phenotypes. (A) Two sequence-independent siPOOLs against the same gene produce similar gene knock-down efficiencies with a higher correlation score (R= 0.9) as opposed to single siRNAs (R= 0.4). (B) Similar effect was observed phenotypically as depicted by effects on cell viability. A549 cells were treated at 3 nM siPOOL/siRNA and RNA quantified by real-time qPCR after 24 h. Cell viability was measured with AlamarBlue assay after 72 h.

The advantages of siPOOL design

1. Maximal transcript coverage

A gene may have multiple functional transcripts due to alternative splicing. A siPOOL is designed for maximal transcript coverage (fig. 10). Given the variable knock-down performance of single siRNAs, having multiple siRNAs per transcript increases the likelihood of successful gene silencing.

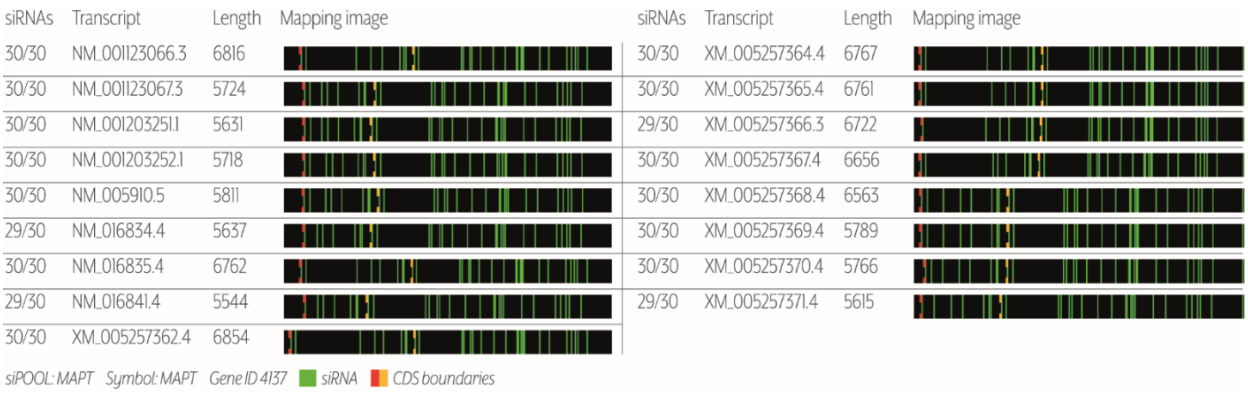

Figure 10. siPOOLs are designed for maximal transcript coverage. The gene MAPT has 17 predicted transcripts. The MAPT siPOOL-transcript mapping is shown with siRNA binding sites in green and CDS boundaries shown in dotted red and orange lines. All siRNAs within a siPOOL are designed to maximally cover every transcript subject to available sequence.

2. Paralogue filtering

siPOOLs undergo stringent paralogue filtering. Paralogues are genes that share highly similar sequences to the targeted gene. They are proposed to arise via historical gene duplication events and may perform functions related to or non-related to the target gene (Espinosa Cantu et al., 2015).

To avoid off-target effects to paralogues, regions of high sequence similarity to other genes are avoided in siPOOL design. If available sequence is limited, a seed-based Smith-Waterman alignment is carried out. This looks for 6-mer seed matches within regions shared by paralogues. siRNAs with high alignment scores are removed to avoid knock-down of these off-target paralogues through microRNA-based mechanisms. siRNAs with 7-mer seed matches to 3' UTRs of paralogues are also avoided.

As some paralogues may share overlapping functions, it may be beneficial to target certain closely-related paralogues to avoid functional redundancy. siTOOLs Biotech alerts customers when closely-related paralogues are encountered and provides customers the choice to target them as well with the siPOOL.

3. Empirical siRNA design based on latest annotations

siTOOLs Biotech incorporates various siRNA design rules well-established to provide advantages in terms of specificity or gene silencing efficiency. These are derived from multiple experimental observations (published or in-house) and large RNAi screening datasets (Huesken et al., 2005).

The proprietary siPOOL design algorithm ensures siRNAs within a siPOOL have optimized siRNA thermodynamics that bias antisense strand loading into RISC. In addition, it incorporates the use of historical RNAi screening data (curated in an internally-hosted database called the Phenovault) to avoid motifs or seeds shown to give strong off-targets or toxic effects.

Importantly, siPOOL design is consistently being updated with changes to annotations in the NCBI RefSeq database to ensure target gene silencing is specific and efficient.

The siRNA sequence information of siPOOLs are made available upon request.

Effective?
Yes. As long as the targeted gene and its transcripts have sufficient unique sequence to accommodate ~30 siRNAs.

Summary Table: Ways to manage off-target effects of siRNAs and their pros and cons

Method	Mechanism	Pros	Cons
Lower siRNA concentration	Dilutes sequence-dependent off-target effects of siRNA	Easy to implement without significant effort or resources	• Does not work when off-target effect is strong • Weakens on-target knock-down
Pooling siRNAs	Reduces siRNA concentration and alters off-target profile	• High pool complexity can eliminate strong off-target effects • Increases efficiency of on-target silencing	• High complexity required demands greater resources for siRNA production and design • Target sequence must be sufficiently long and unique to accommodate multiple siRNAs
Chemical modification	Lowers binding energy of siRNA to off-target site	Reduces off-target effects at low energy pairing sites	• Does not apply to all off-target effects • Does not improve phenotypic correlation when implemented alone • May reduce on-target knock-down efficiency
Optimize siRNA design	• Avoids alignment to obvious off-target genes • Enhances antisense strand RISC loading • Increases siRNA duplex stability • Avoids toxic motifs	Easy to implement with the help of computational algorithms	• Not fully predictive • Limits selection of available siRNA candidates • Does not improve phenotypic correlation when implemented alone
siPOOL approach	• Lower siRNA concentrations • High complexity siRNA pooling • Optimized siRNA design with paralogue filtering based on latest annotations	• Reduces strong and weak off-target effects • Increases efficiency of on-target silencing • Improves phenotypic correlation	• Requires more resource and effort for siRNA production and design • Target sequence must be sufficiently long (> 300 bases) and unique to design multiple siRNAs

Read previous technote:

Technote 1: siRNA Off-target Effects - Causes, Extent & Impact

Read the siPOOL guide for data and applications of siPOOLs:

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