siRNA screening: development of hit stratification strategies

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Introduction

While synthetic siRNA libraries are powerful tools for functional genomic screens, off-target effects mediated by siRNA seed interactions with the 3' UTR of unintended targets can result in false positives. Given the frequency of off-target effects in some assays, the development of hit validation/stratification strategies is imperative. In the following study we have compared two strategies for identification of high confidence hits: 1) a multiple reagent approach where two or more individual siRNAs induce the same phenotype and 2) a chemical modification approach where hit confirmation is achieved using pools of siRNA that contain specificity enhancing modifications. A comparison of these two strategies (using a collection of primary hits generated from a cell viability screen) reveals significant overlap between the high confidence hits identified. However, for low confidence hits, i.e. where a single siRNA induces a phenotype, the concern is that an important hit will be missed. To determine if the phenotype is due to gene targeting or a seed-mediated off-target effect, a chimeric approach was used whereby a gene-specific seed sequence is introduced into a non-targeting siRNA scaffold. Together, these data provide well-defined approaches for prioritization of hits derived from RNAi screens.

siRNA screening workflow

RNAi screens typically result in a large number of hits. Distinguishing between true hits and false positives due to off targets is a crucial task in first line hit validation.

- Assay development
- Screening
- Hit identification
- Hit validation/Hit stratification
- In-depth target validation

Current Practices

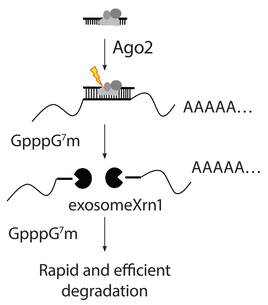
Redundancy: Confirmation of hits with multiple reagents

Rescue: Exogenous target expression reverses the phenotype

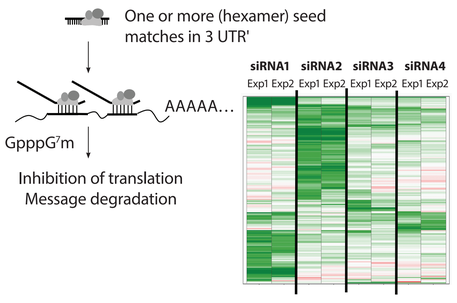
Mechanism of RNAi-mediated effects

RNAi can lead to both specific target mRNA down-regulation and nonspecific off-target effects due to partial complementarity with unintended mRNAs through seed region matches to the 3' UTR. Typical results of microarray expression analysis is shown below.

1. siRNA-mediated message cleavage

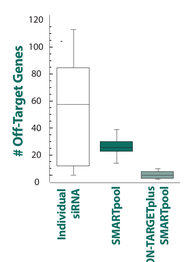


2. miRNA-mediated message down-regulation *(or siRNA off-targeting)*



Strategies for reducing off-targeting

Bioinformatics – Select for potent siRNAs and assess for seed content to promote specificity (1,4,5)
Chemical modifications – Expand sequence space enabling potent siRNA sequence selection and interfere with off-target mechanism (2)
Pooling – Promote competition between potent siRNAs for optimal silencing and dilution of off-targets associated with individual siRNAs (3)
Specificity – enhanced pools are ON-TARGETplus™, SMARTpool™, siRNA reagents, standard siRNA and pools are siGENOME SMARTpool and individual siRNA reagents

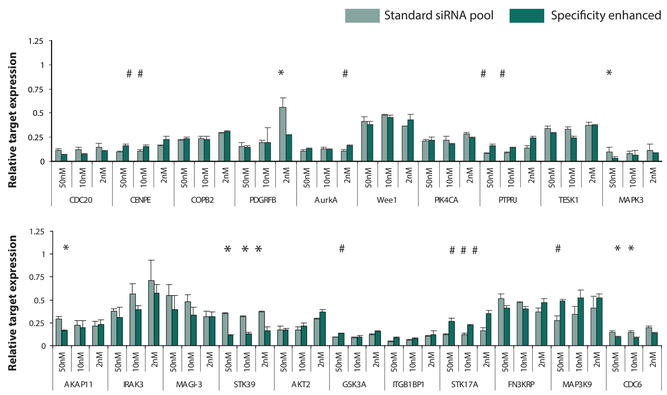


Goal of this study

- siRNA screen by standard pools → Kinome siRNA screen for apoptosis (ApoONE assay) and cell viability (CTB) in HeLa cells (859 targets)
- Primary hits → 55 primary hits were identified in ApoONE and/or CTB assays
- Hit confirmation by standard pools → 28 ApoONE hits and 26 CTB hits were confirmed by the original pool
- Standard pool Deconvolution / Specificity-enhanced pools
- Dose-dependent phenotype/knockdown analysis → compare different reagents to determine the most efficient & effective process for hit stratification
- Prioritize the Hits

Standard pool = siGENOME SMARTpool reagent (pool of 4 unmodified siRNAs)
Standard pool deconvolution = 4 individual siGENOME siRNAs that make up the pool
Specificity-enhanced pool = ON-TARGETplus SMARTpool reagent (pool of 4 modified siRNAs)

Standard and specificity-enhanced pools have comparable silencing

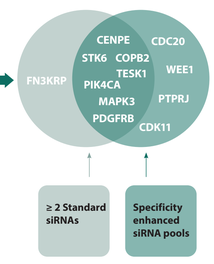


The target mRNA knockdown was compared between the standard and specificity-enhanced siRNA pools. Statistically significant silencing differences, p<0.01
(*Specificity enhanced pool better, # Standard pool better)

Deconvolution of the standard siRNA pool vs. specificity enhanced pool

Standard pool	≥ 2 Standard siRNAs	Specificity enhanced pool
CENPE	CENPE	CEN PE
COPB2	COPB2	COPB2
MAPK3	MAPK3	MAPK3
PDGRFB	PDGRFB	PDGRFB
PIK4CA	PIK4CA	PIK4CA
STK6	STK6	STK6
TESK1	TESK1	TESK1
FN3KRP	FN3KRP	
CDC20		CDC20
CDK11		CDK11
PTPRJ		PTPRJ
Wee1		Wee1
AKAP11		
AKT2		
GSK3A		
IRAK3		
ITGB1BP1		
MAGI3		
STK17A		
STK39		
C6orf199		C6orf199
CSF1R		
ERBB4	ERBB4	ERBB4
ERN1		
FGFR2	FGFR2	
K DR	K DR	K DR
LMTK3	LMTK3	
MAP2K7		
RAPGEF3		

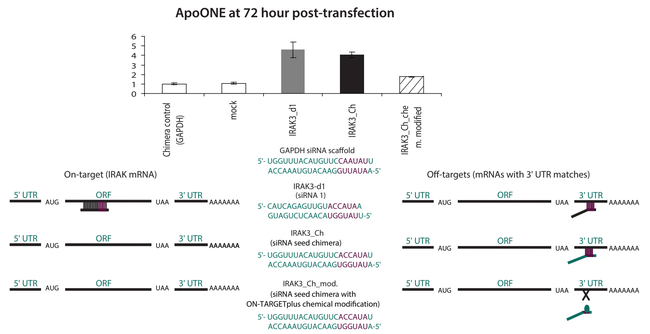
Confirmed by both methods
Confirmed by one method
Confirmation by neither method
Low or not expressed genes



High confidence hits → There is significant overlap between the hits that are confirmed by the two validation methods (≥ 2 siRNA and specificity-enhanced pools).
Low confidence hits potential false positives) → Majority of hits with only one positive standard siRNA are not confirmed by specificity enhanced pools.
Low confidence hits (potential false positives) → A number of hits are either not expressed or are expressed at low levels.
Ambiguous hits (potential false positives/negatives) → Few hits are confirmed by only one method.

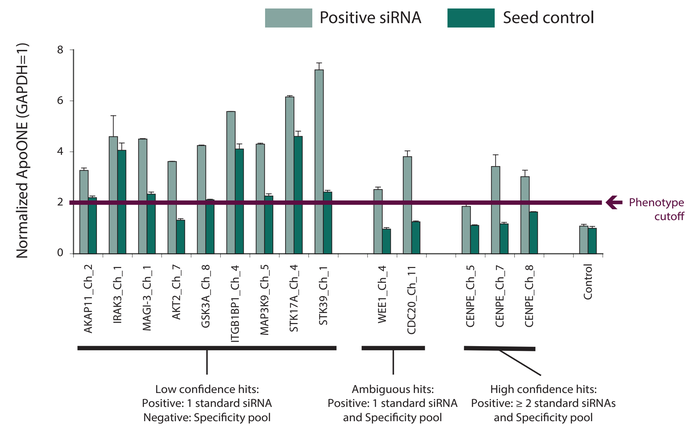
Seed controls for ruling out false negatives

A seed control is a chimeric siRNA that contains the 6 nucleotide seed sequence from the standard siRNA in a GAPDH siRNA scaffold that does not induce the phenotype (5).



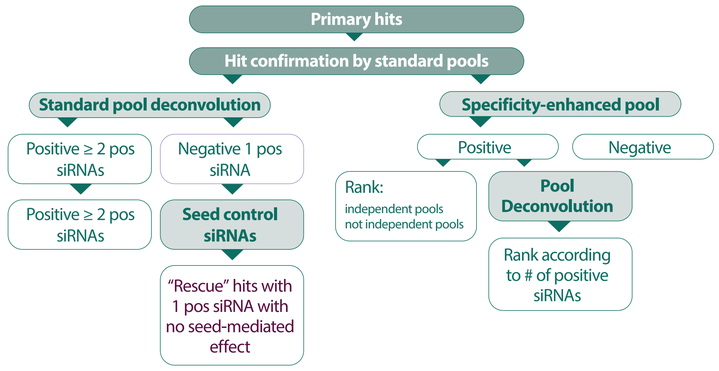
IRAK3: Confirmed only by one standard siRNA; negative with the specificity-enhanced pool.
The seed control is positive in phenotypic assay →the phenotype is due to seed-mediated off-targeting.'
Specificity-enhancing chemical modification on the seed control abolishes the phenotype.

Low-confidence hits are due to seed-mediated off-target effects



Eight out of nine seed controls examined for low confidence hits induce seed-mediated phenotype. There was no seed-mediated effects on phenotype for the tested ambiguous hits. CENPE is a high confidence hit used as a control with no seed-mediated off-targeting.

Proposed hit stratification strategies



Follow up of candidate targets form primary screens with either deconvolution of standard pools or specificity-enhanced pools are equally viable approaches to prioritize potential targets based on confidence level.

High priority hits are identified by both methods
Low priority hits are negative by both methods
A few hits are ambiguous and confirmed by one or the other method

Seed-matched controls reveal that where only one siRNA produces the phenotype, often this is due to seed-mediated off targeting.

Seed-matched controls are important tools for ruling out seed-mediated off-target events during the hit stratification process.

References

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Acknowledgements

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