Importance of siRNA negative control selection: Evaluation of non-specific protein knockdown by negative control siRNA

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Abstract

Small interfering RNA (siRNA) are double-stranded RNA consisting of 19-22 base-pairs that direct the cleavage of complementary mRNA targets. The utility of siRNA as a research tool depends on the specificity of the gene silencing, as knockdown of unintended gene targets (off-target effects) can potentially complicate the interpretation of siRNA data. To investigate the extent of off-target effects at the protein level, we evaluated RNAi negative control products from three commercial vendors for non-specific protein knockdown utilizing the SuperSignal® siRNA/Antibody Modules and SuperSignal siRNA Chemiluminescent Detection Module. Using Western blotting we found that the expression level of a number of different proteins was reduced when A549 cells were transfected with negative control siRNAs from two different vendors. Minimal non-specific protein knockdown was detected with the ON-TARGET*plus*™ Non-Targeting Pool. These data suggest that the selection of an optimal negative control is critical to correct interpretation of siRNA experiments and that non-specific knockdown of protein should be evaluated.

Introduction

Targeted gene silencing through RNA interference (RNAi) has become a viable and established method to answer important biological questions. In nature, this ancient and evolutionarily conserved viral defense mechanism consists of small interfering RNA enzymatically generated by the Dicer protein from longer RNAs of viral origin. These siRNAs interact with several proteins to form an RNA-induced silencing complex (RISC). The RISC recognizes corresponding sequences in the mRNA and causes its degradation, thereby silencing gene expression. Exploitation of this system as a laboratory reagent employs the use of synthetically generated siRNA consisting of a 19-22 base-pair double-stranded RNA with anti-sense homology to a specific target. Thus, RNAi empowers the researcher with a highly specific tool to ablate the gene of interest and assess the phenotypic and genetic consequences in a biologically relevant setting (1-5).

One of the challenges presented by siRNA technology is the potential for silencing of unintended targets or "off-target effects." Due to promiscuous interactions of the sense and anti-sense strand siRNA, inconsistent phenotypes, false positives and negatives, and cellular toxicity often plague siRNA experiments. To alleviate this problem, Horizon Discovery (formerly Dharmacon) uses the SMARTselection™ algorithm to design its ON-TARGET*plus* SMARTpool siRNAs and makes proprietary chemical modifications to the sense and anti-sense strands (6-8). Further, pooling of four siRNA sequences, which mimics the natural silencing pathway, reduces false negatives by targeting four different mRNA regions at once while reducing the effective concentration of each individual siRNA. These reagents are guaranteed to produce target knockdown of 75% or better at the mRNA level, thus providing researchers with a highly specific and potent tool for gene silencing.

Researchers using siRNA technology are faced with several additional issues. First, since siRNA functions at the mRNA level, researchers often solely monitor mRNA levels of the target gene. However, ultimately it is the resulting reduction in protein level that causes the observed phenotype. Therefore, confirmation of gene silencing by measuring the corresponding protein reduction by Western blot becomes paramount. Unfortunately, the cost and time of screening and optimizing multiple antibodies can be daunting. Second, companies offering siRNA reagents validated at the mRNA level do not necessarily validate silencing at the protein level. In addition, few companies provide application data showing that the reduction in protein levels is sufficient for phenotypic effects.

To address these issues, we have developed a series of validated SuperSignal siRNA/Antibody Modules containing a validated siRNA with a validated antibody. In addition, we have developed a SuperSignal siRNA Chemiluminescent Detection Module optimized for Western blotting. This is a completely validated system that enables accurate determination of siRNA-induced protein knockdown by Western blot using the following reagents:

1) Highly specific ON-TARGET*plus* SMARTpool siRNAs to the gene of interest.
2) ON-TARGET*plus* Non-Targeting pool siRNA shown to have no effect on the expression of the target protein.
3) Validated antibody to determine the protein level through Western blot.
4) Fluorescently-labeled siGLO® Green Transfection Indicator to monitor transfection efficiency.
5) Western blot data to demonstrate the extent of silencing achieved under the recommended conditions.
6) Optimized detection module.

We have used these optimized siRNA/Antibody modules to study signaling downstream of the tumor suppressor protein, p53. p53 has been shown to physically interact with MDM2 (1). In response to growth signals, MDM2 binds p53 and targets it for degradation. However, in the absence of MDM2 the p53 protein becomes stabilized and can affect the transcriptional up-regulation of the p21 gene, leading to increases in p21 protein (2). Similarly, the M-phase promoting protein Polo-like Kinase 1 (PLK1) is negatively regulated by the p53 gene through protein-protein interaction. In the absence of p53, the levels of PLK1 protein have been shown to increase (4, 5). Interestingly, tumors that display overexpression of PLK1 through deletion or misregulation of p53 become highly metastatic, indicating that PLK1 may be a potential anti-cancer target (4). Using our SuperSignal siRNA/Antibody Modules, we show that targeted depletion of p53 protein causes decreased levels of its transcriptional target p21 and increased levels of the M-phase control protein PLK1. In addition, targeted depletion of MDM2 leads to stabilization of p53 as evidenced by an increase in the protein level of p21. Thus, these SuperSignal siRNA/Antibody kits provide a fully validated system for protein knockdown to study complex signaling pathways.

Mechanism of RNAi Induced Gene Silencing

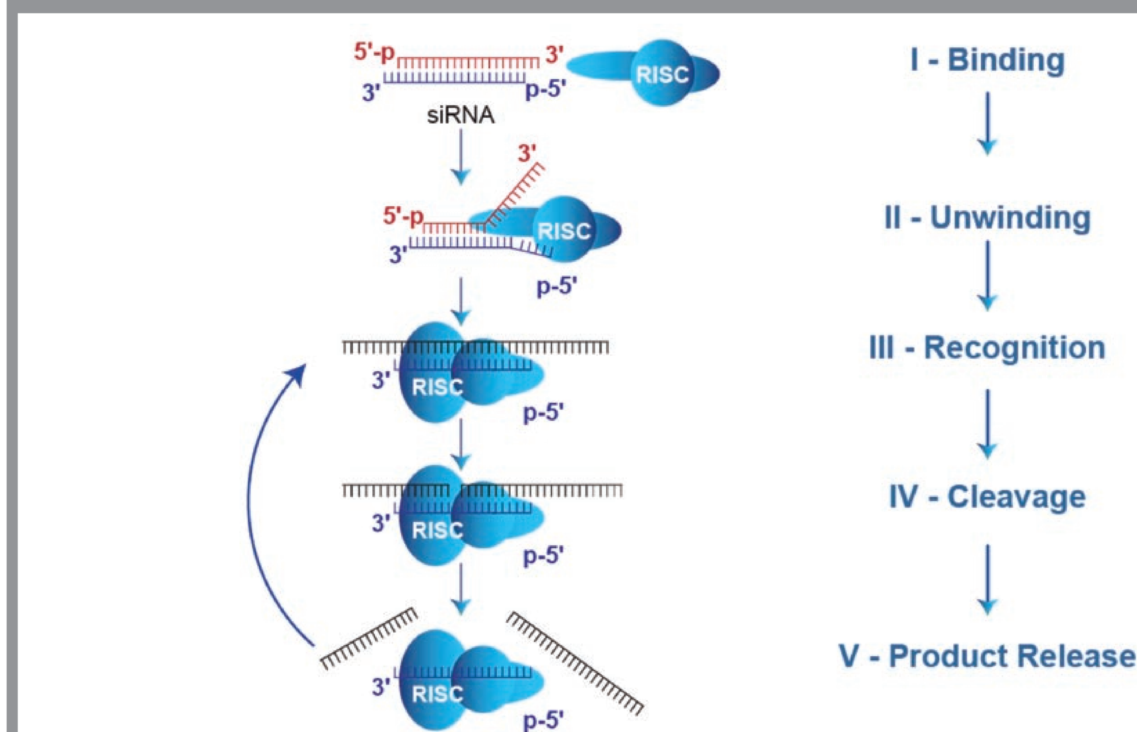

Figure 1: RNAi is the process of mRNA degradation (gene silencing) triggered by double-stranded RNA sharing sequence homology to the "target" mRNA. Mediators of RNA-dependent gene silencing are 19-22 nucleotide "small interfering" RNA duplexes (siRNAs) with dinucleotide 3' overhangs.

Minimizing Off-Target Effects (Non-Specific Knockdown)

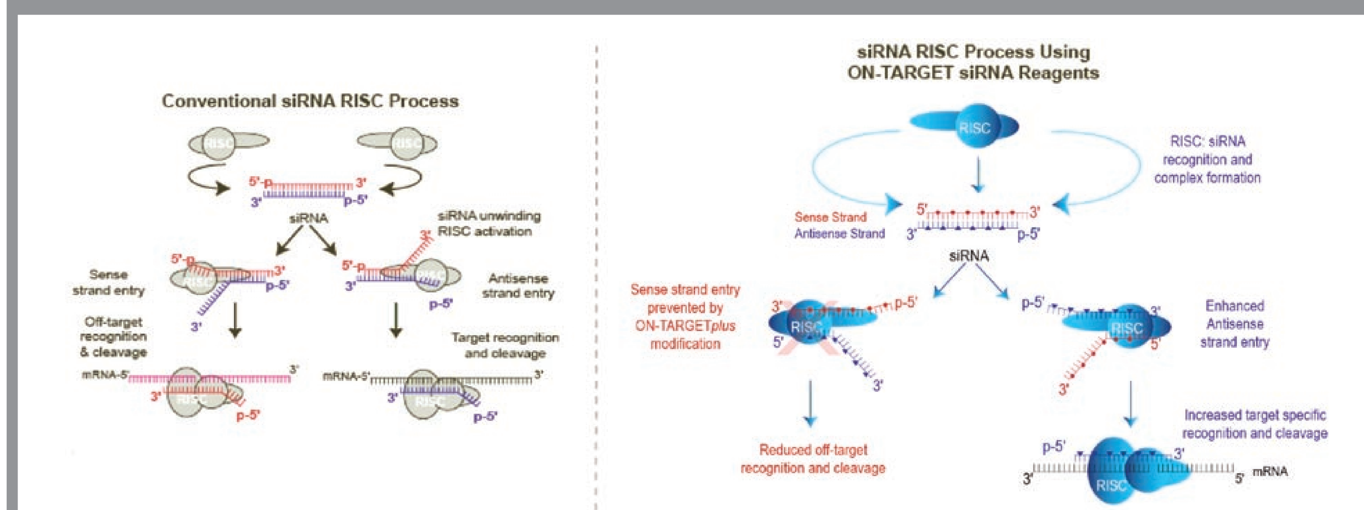

Figure 2: The anti-sense strand of the double-stranded RNA targets the specific gene of interest. The sense and anti-sense strand has the potential to recognize a non-specific target (off-target gene silencing). Horizon Discovery (formerly Dharmacon) has developed proprietary chemical modifications to both strands to eliminate this off-target effect.

No Human Genes are Targeted by ON-TARGET*plus* Negative Control Reagent as Measured at the mRNA Level

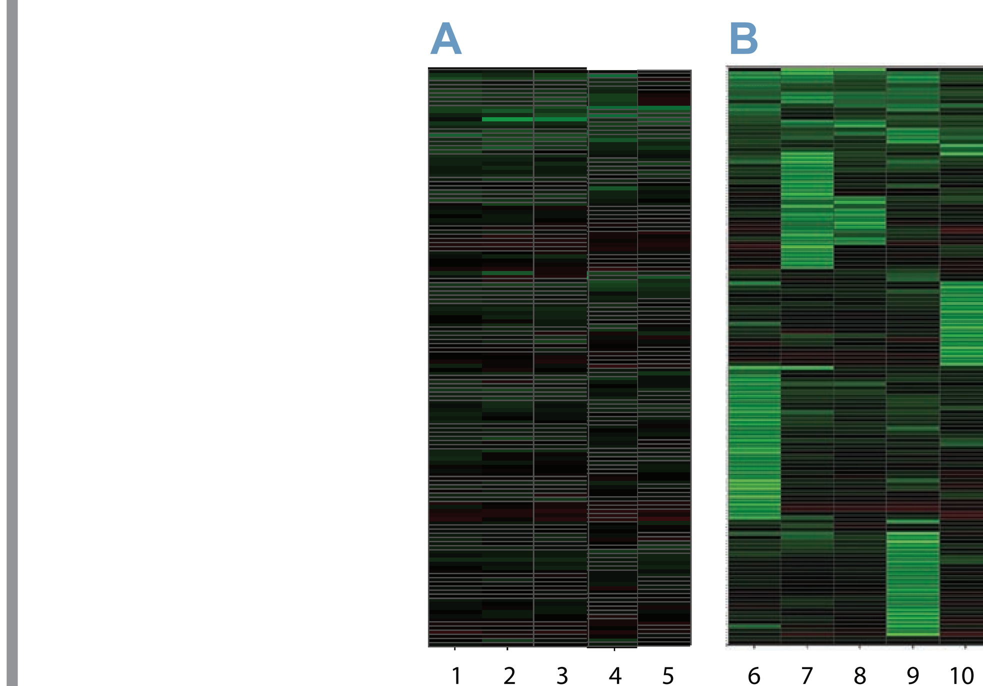

Figure 3: RNA samples from HeLa cells were analyzed by microarray expression profiling (Agilent) 24 hours after transfection with the indicated siRNA reagents. Decreases in mRNA levels are shown in green and increases in red. Panel A (left) shows virtually no off-target effects for ON-TARGET*plus* Non-targeting siRNAs 1-4 (lanes 1-4) or ON-TARGET*plus* Non-targeting pool (lane 5). Panel B (right) shows extensive off-target signatures for "Scramble" control siRNAs. These sequences have no significant sequence homology to known human genes (lanes 6-10).

Comparison of Off-Target Protein Knockdown Using ON-TARGET*plus* Non-Targeting Control Pool siRNA and Negative Control siRNA from Two Other Vendors

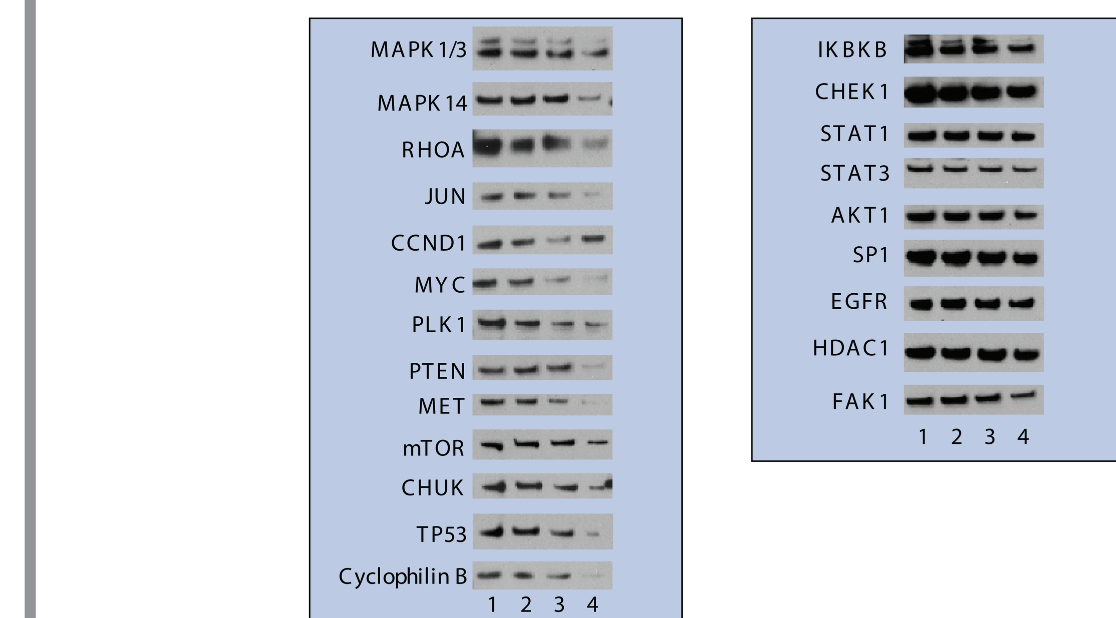

Figure 4: A549 cell were transfected with Transfection reagent alone (Mock) (lane 1), ON-TARGET*plus* Non-Targeting Control Pool siRNA (lane 2), or negative control siRNA from Vendor A and Vendor B (lanes 3 and 4). 72 hours post transfection lysates were collected and analyzed by Western blot. Consistent with the microarray results shown in Figure 3, transfection with ON-TARGET*plus* Non-Targeting Control Pool siRNA resulted in no discernible reduction in the protein levels of the 22 target proteins tested. In contrast, the negative control siRNA from Vendor A and Vendor B showed considerable knockdown of several proteins, indicating potential off-target effects.

Representative Protein Knockdown Data Using SuperSignal siRNA/Antibody Kits

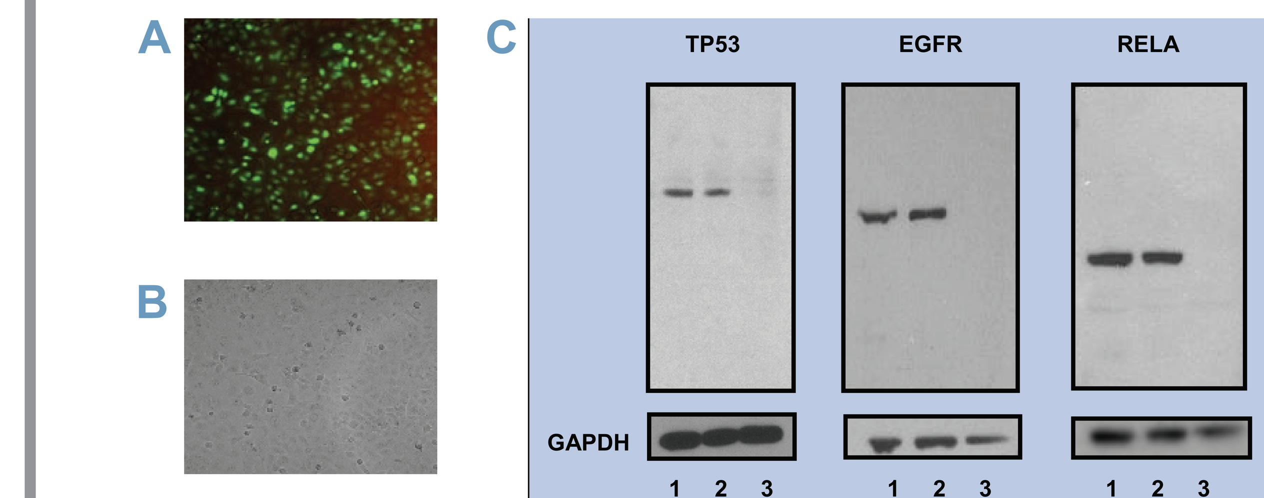

Figure 5: Panel A and B: A549 cells were transfected with siGLO Green Transfection Indicator as described in Methods. The high transfection efficiency was demonstrated using fluorescent microscopy (A). The total cell number is indicated by the bright field image (B). Panel C: HeLa cells were transfected with the indicated siRNA as described in Methods, and protein levels were monitored by Western blot. For all panels, lanes are loaded as follows: Mock transfected (lane 1), Non-Targeting Pool siRNA (lane 2), specific ON-TARGET*plus* siRNA (lane 3).

Efficient mRNA and Protein Silencing is Achieved with CTNNB1 siRNA

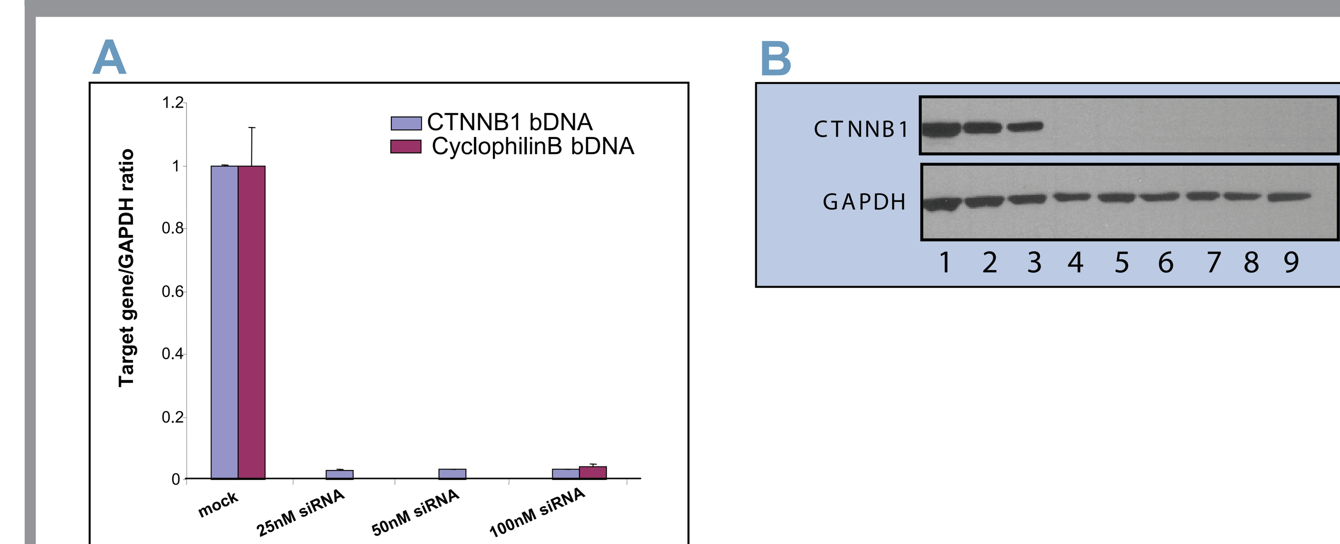

Figure 6: HeLa cells were mock-transfected or transfected with 25, 50 or 100nM of CTNNB1 siRNA or 100nM of Cyclophilin B siRNA (control) in parallel. 72 hrs after transfection, the cells were processed to detect either mRNA levels using branched-DNA probes (A) or protein levels by Western blot analysis (B). All mRNA levels were normalized to GAPDH levels. The mRNA-levels for CTNNB1 and Cyclophilin B in mock-transfected cells were set at 1 and fold reduction in the respective mRNA levels in the presence of corresponding siRNAs were determined. As shown, CTNNB1 siRNA efficiently silences the mRNA levels by greater than 90%. Similarly, greater than 90% reduction in CTNNB1 protein levels were also observed in the presence of 25-100 nM CTNNB1 siRNA (B, lanes 4-9). CTNNB1 protein remained unchanged in the mock treated and control siRNA-treated cells (B, lanes 1-3). GAPDH protein levels were used as a control for protein loading.

Effect of Efficient Knockdown of p53 and MDM2 Proteins on Downstream Signaling Proteins

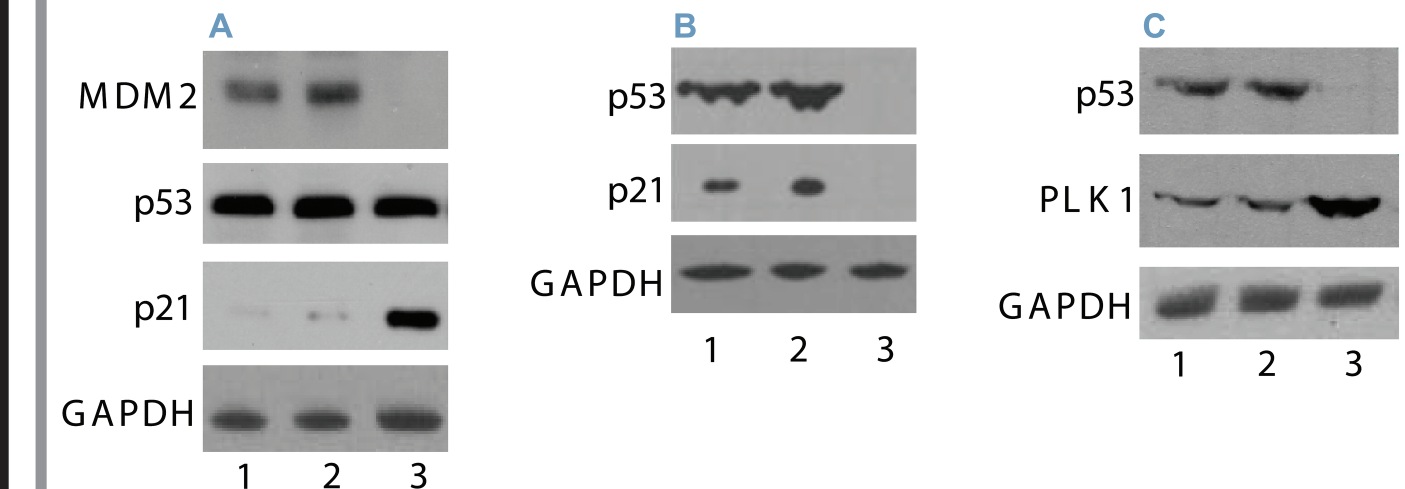

Figure 7: For all panels, lanes are loaded as follows: Mock transfected (lane 1), Non-Targeting Pool siRNA (lane 2), and specific ON-TARGET*plus* siRNA (lane 3). A549 (A and B) or HeLa (C) cells were transfected as with the indicated reagents, and protein levels were monitored by Western blot. These data show that targeted depletion of p53 protein causes decreased levels of p21 and increased levels of PLK1. In addition, targeted depletion of MDM2 leads to stabilization of p53 as evidenced by an increase in the protein level of p21.

27 SuperSignal siRNA/Antibody Kits Currently Available

▪ p53	▪ MAPK1 (p40)
▪ MYC	▪ MAPK3 (p42)
▪ MAPK14 (p38)	▪ SP1
▪ AKT1	▪ CCND1 (cyclin D1)
▪ CTNNB1 (β-catenin)	▪ PLK1
▪ EGFR	▪ HDAC1
▪ IKBKB	▪ PTEN
▪ RELA (NF-κB)	▪ RHOA
▪ STAT3	▪ MAP2K1 (MEK1)
▪ CHUK (IKKα)	▪ ERBB2
▪ MET	▪ CHEK1
▪ CDC2	▪ PTK2 (FAK1)
▪ GSK3B	▪ FRAP1 (mTOR)
	▪ STAT1

Methods

Cell Culture: Cells were maintained in DMEM/High Glucose Media supplemented with 10% FBS (Hyclone) in a 37°C incubator with a 5% CO_2 ambient atmosphere. HeLa or A549 cells were transfected with siRNA from the SuperSignal siRNA/Antibody Module using Dharma*FECT*™ 1 Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Transfected cells were lysed 72 hours post transfection in 1X LDS sample buffer (Invitrogen) supplemented with 100mM DTT (Thermo Scientific). Western blot analysis was performed using the specific antibody from the SuperSignal siRNA/Antibody Module, and Signal detection was performed with the SuperSignal Detection Module (Thermo Fisher Scientific) according to manufacturer's protocol. Analysis of mRNA was performed using the The QuantiGene® Explore Kit and specific probe sets (Panomics) according to manufacturer's protocol.

Conclusions

- **ON-TARGET*plus* Non-Targeting Control Pool siRNA shows minimal off-target effects at the mRNA and protein level as compared to other leading vendors negative control reagents.**
- **Excellent correlation demonstrated between mRNA and protein knockdown with CTNNB1 siRNA.**
- **siRNA mediated silencing of p53 and MDM2 were validated by monitoring genes downstream of the siRNA target.**
- **27 SuperSignal siRNA/Antibody kits provide a fully validated system for protein knockdown to study complex regulatory pathways.**

References

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