



# Using Real-Time RT-PCR to Measure Gene Silencing by RNAi

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## Abstract

We have applied the recently discovered techniques for RNAi in mammalian cells (Elbashir et al, Nature 411:494) to generate knockdown reagents for a set of 50 genes. In order to identify effective siRNAs (>80% knockdown), we used Applied Biosystems Assays-on-Demand Gene-Expression products to quantify mRNA levels. We evaluated these pre-designed TaqMan reagents using a dilution series of cell line RNA and found that their sensitivity and reproducibility allowed reliable measurement of low transcript levels following knockdown. Using Assays-On-Demand to measure knockdown, we tested candidates siRNA sequences in batches of three per gene until a successful siRNA was found. For a subset of genes, a 2<sup>nd</sup> potent siRNA was generated to allow confirmation of phenotypic effects using two independent knockdown reagents.

## Methods

One-tube TaqMan RT-PCR was performed using Ampliqa Gold and standard conditions in 25ul reactions on the SDS 7700 (Applied Biosystems). Manually designed TaqMan reagent sequences were chosen using a combination of Primer Express software and manual review to select at least one intron-spanning primer. Standard curves for TaqMan validation were performed in duplicate using a 5-point dilution series from 50ng to 0.2ng of total RNA from PC3 cells. TaqMan standard curve "pass" required R<sup>2</sup> > 0.98 and a slope in log vs. Ct plot between 0.9 and 1.2.

siRNA sequences were selected based on standard criteria (Tuschl lab web site). Some sequences were tested using in vitro transcribed siRNAs (Ambion), but all data shown here are from chemically synthesized siRNAs (Dharmacon and Xeragon). siRNAs were transfected into PC3 cells at 100nM in serum containing media using siRNA optimized transfection lipids (Ambion, Gene Therapy Systems, Invitrogen, Mirus, Novagen, Qbiogene, or Stratagene). Transfections were performed in duplicate in 96-well plates and cells were harvested 48hrs after transfection. One tenth of the RNA from each well was analyzed by TaqMan and mRNA levels for each gene were normalized to the amount of total RNA as measured by Ribogreen (Molecular Probes). The amount of total RNA in each TaqMan reaction was typically 10-40ng. Treated cells were normalized relative to untreated control cells processed in parallel.

## Conclusions

Gene function experiments using RNAi demand careful control of a wide range of variables affecting transfection efficiency and subsequent phenotypic assays. Having a rapid and quantitative measure for target gene mRNA levels in each experiment allows successful optimization of a large number of experimental parameters. Assays-on-Demand offer an easily accessible solution to the problem of finding high quality TaqMan reagents for measuring gene knockdown.

## Results

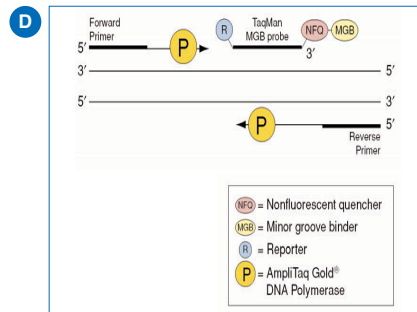
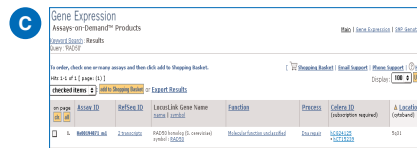
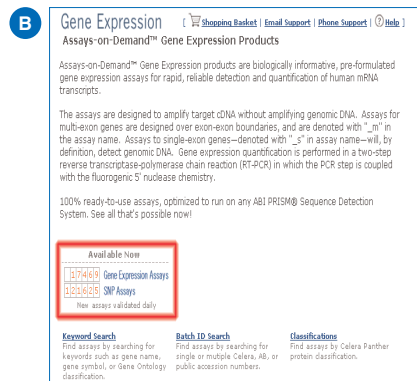
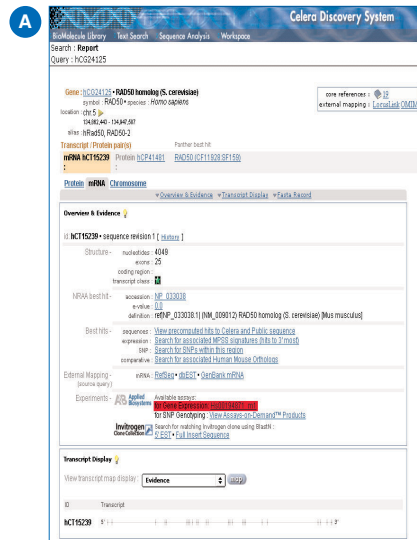


Figure 1: Off-the-shelf Assays-on-Demand Gene Expression TaqMan reagents are available for thousands of genes.

- A Sample web page showing availability of Assays-on-Demand using link from Celera Discovery System.
- B Sample web page showing availability of Assays-on-Demand from the Applied Biosystems Online Store.
- C Current status of Assays-on-Demand gene set.
- D Assays-on-Demand reagents use a quenched-fluorescence probe with Minor Groove Binder. MGB increases T<sub>m</sub> and allows use of shorter probes.

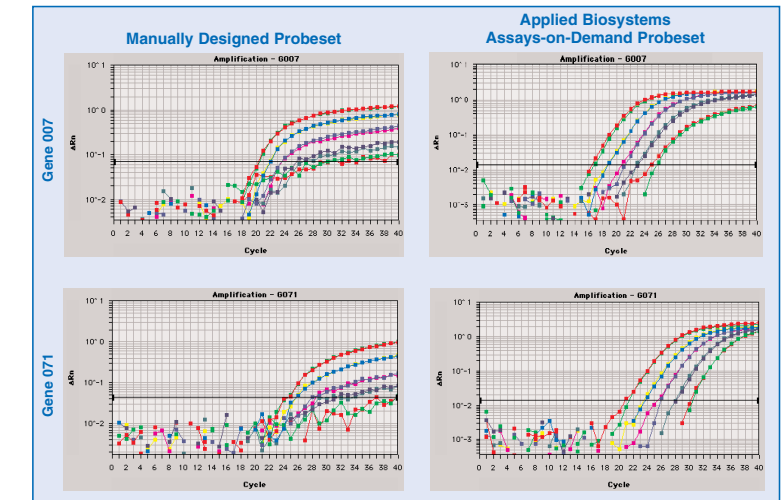


Figure 2: Comparing manually designed TaqMan reagents with Assays-On-Demand TaqMan reagents. Real-time RT-PCR amplification plots are shown for two genes using a dilution series ranging from 50ng to 0.2 ng of total RNA. Assays-on-Demand reagents yield greater absolute changes in fluorescence following amplification and produce more reproducible signals at later PCR cycles.

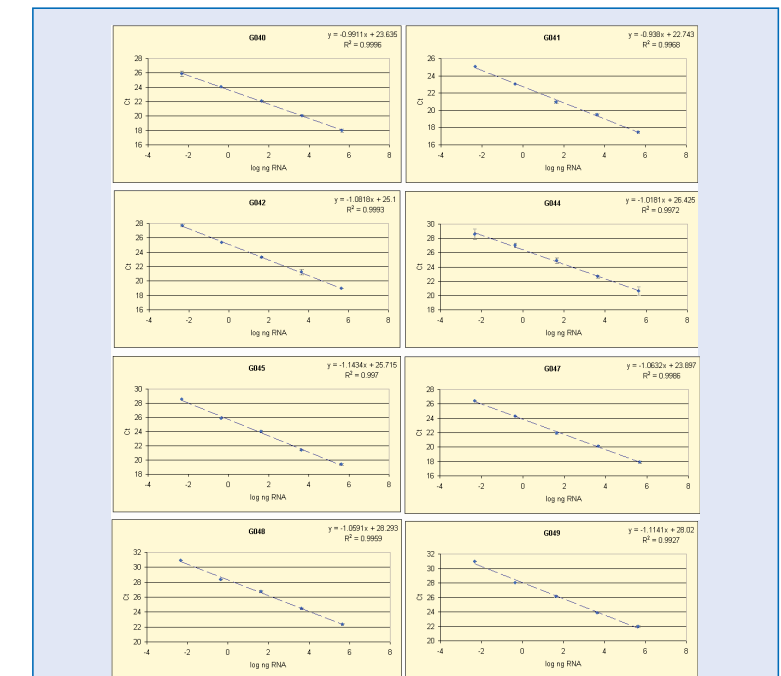


Figure 3: Sample standard curves for 8 Assays-on-Demand TaqMan reagents. Ct is the PCR cycle at which amplification signal is detected above threshold. In total, 65/67(97%) Assays-on-Demand reagents passed standard curve tests.

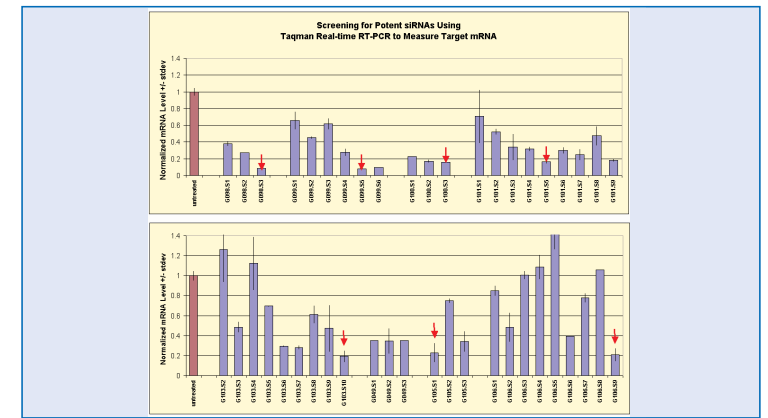


Figure 4: Screening siRNAs for knockdown using Assays-on-Demand to quantitate mRNA levels. For each gene, siRNAs were tested in batches of three until a potent siRNA was identified (indicated by red arrow). Sample data for 8 genes is shown. In screening 356 siRNAs against 64 genes, the average number of siRNAs screened in order to find one that induced >80% knockdown was 4.2.

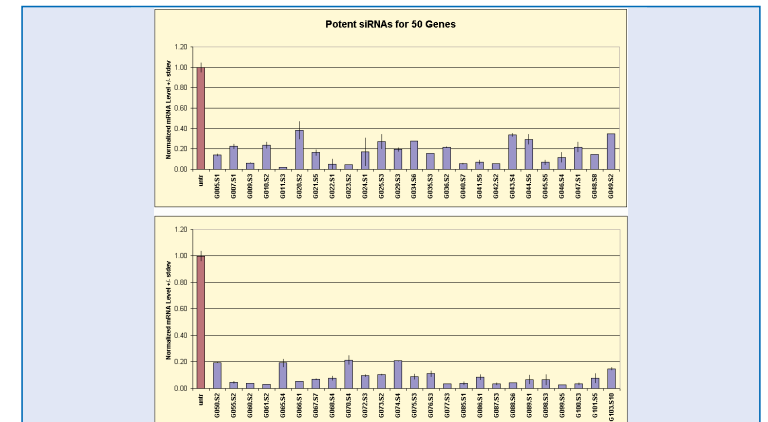


Figure 5: Successful siRNAs identified for a set of 50 genes. Each column shows TaqMan quantitation for knockdown of a single gene following transfection with the siRNA identified during screening. Negative control siRNAs (scramble) did not induce significant knockdown.

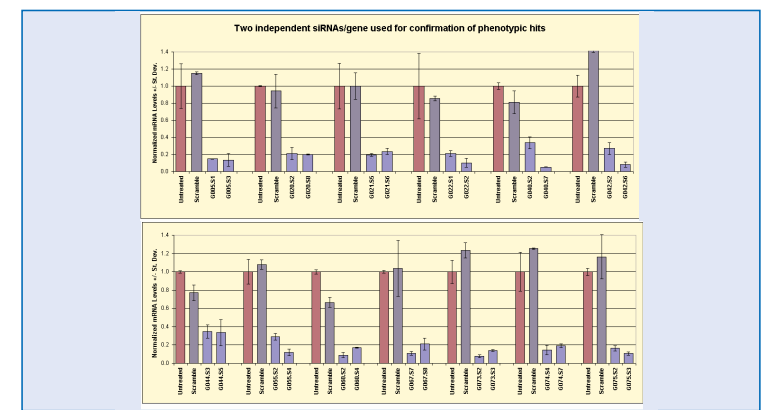


Figure 6: Identification of a 2nd siRNA for confirmation. For siRNAs that induced a particular cellular phenotype (not shown), more siRNA sequences were screened to find a second potent siRNA. Sample data showing knockdown with two independent siRNAs is shown for 13 genes. Phenotypic assays were then tested for confirmation using these siRNA pairs.