Strategies for Improving RNAi Screening Success: Using a Ubiquitin-EGFP Assay to Identify Druggable Genes Required for Proteasome Function

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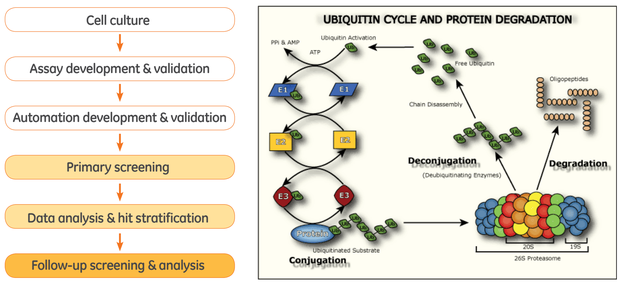
Abstract

RNA interference (RNAi) screens represent an effective method to identify novel therapeutic targets and to elucidate mechanisms of action or sensitivity to drugs. In spite of its immense potential, RNAi screening presents unique challenges that must be addressed to ensure success. We will describe the strategies employed to provide meaningful screening results. As a case study, we will discuss a cell-based assay using a ubiquitin-EGFP cell line to screen for genes that when silenced inhibit proteasome function and/or affect cell viability. We will describe the approaches used to ensure effective delivery of siRNA molecules and to identify robust siRNA controls. We will also provide strategies used to validate compatibility of the RNAi automation platform with the phenotype being analyzed. Finally, we will discuss the data analysis and hit identification methods commonly used along with approaches for follow-up analysis of potential hits from primary screening.

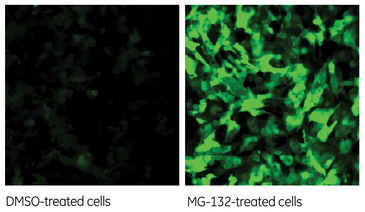
Background

The workflow that is required for effective high throughput (HT) whole genome RNAi screening and identification of meaningful hits is outlined below. We applied this screening workflow to perform an RNAi screen focusing on the ubiquitin-proteasome pathway. Central to this pathway is the 26S proteasome, a multicatalytic proteinase complex with a highly ordered structure composed of a 20S core proteolytic complex and a 19S regulator complex. This figure (from http://www.bostonbiochem.com/products/ubiquitin), illustrates how proteins are normally targeted for degradation by ATP-dependent covalent attachment of ubiquitin moieties to substrate proteins.



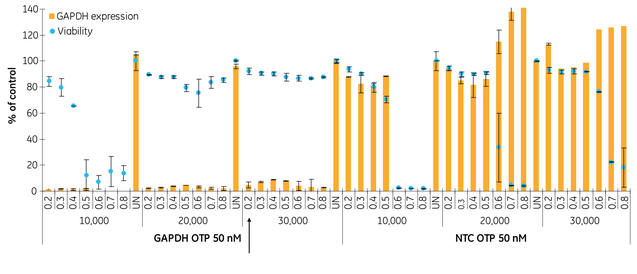
Assay for proteasome function

In this study, we used a recombinant U2OS cell line stably expressing a mutant human Ubiquitin fused to enhanced green flurorescent protein (Ubi[G76V]-EGFP). In untreated cells the expressed fusion protein is constitutively degraded leaving only background fluorescence, whereas cells with inhibited proteasome function display an accumulation of EGFP. We multiplexed the EGFP assay with a second assay (Promega, CellTiter-Glo™) to measure cell viability.



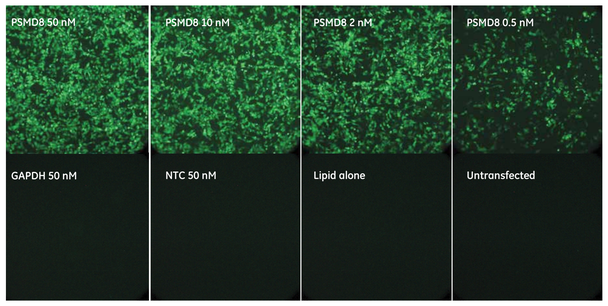
DMSO-treated cells MG-132-treated cells

Transfection optimization



In order to select the best transfection conditions for the RNAi screen in the Proteasome Assay, we tested delivery of a SMARTpool siRNA reagent targeting the human GAPDH gene with a wide range of DharmaFECT 3 lipid concentrations at different cell densities (10,000-30,000 cells/well at plating). The optimal transfection condition (indicated with an arrow) was chosen that shows effective silencing of the target gene with minimal effect on cell viability.

Positive control selection



We chose the PSMD8 gene which codes for the 26S proteasome non-ATPase regulatory subunit 8 as a positive control for proteasome inhibition. Silencing PSMD8 expression results in robust inhibition of the proteasome that is readily detectable by EGFP fluorescence 72 hours after transfection. Fluorescent signal is low or undetectable when cells are transfected with a unrelated housekeeping siRNA (GAPDH), a Non-targeting siRNA (NTC), lipid alone, and in untransfected cells.

Challenges and solutions during automated screen

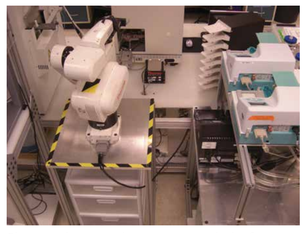
The following are recognized challenges associated with HT RNAi screening:

- Sterility, particularly in cell-based screens with prolonged incubation times
- Timing of key steps associated with RNAi-mediated knockdown
- Throughput of system, and coordination between cell culture and detection systems
- Consistency of inter-plate and intra-plate measurements

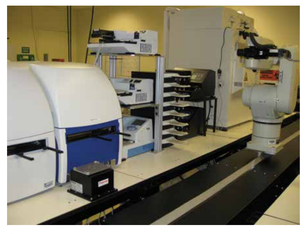
To address these challenges, we implement the following processes:

- Thorough decontamination of instruments as well as monitoring for contamination by collecting origin culture, media only, pre-screen, post-screen, and air quality samples
- Use of Polara™ scheduling software (Thermo Scientific, Waltham MA) to tightly control duration of steps and ensure uniformity of timing
- Perform transfections and assays on distinct systems and use Polara scheduling software to maximize system throughput by staggering start times for groups of plates
- Prior to screening, perform uniformity tests using plates with positive and negative siRNA controls in each well

RNAi screening automation

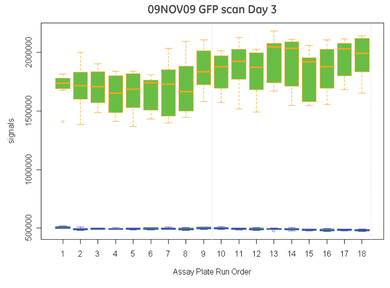


Shown above is the automated platform optimized for siRNA transfection of cells in 96-well or 384-well cell culture plates. The system consists of a Cytomat incubator, ambient storage unit, delid station, two Multidrop Combi reagent dispensers, accessed by a CRS F3 arm.



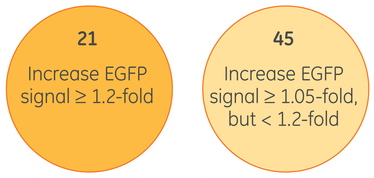
Shown above is the automated assay platform optimized for detection of EGFP fluorescence and CTG Luminescence. The system consists of two PerkinElmer EnVision plate readers, Multidrop Combi reagent dispenser, delid station, Cytomat incubator, Ambient storage unit (not shown), accessed by CRS F3 arm on a 3 m rail.

Primary screen data analysis



Using the automated systems, we screened an siRNA Library (Dharmacon siGENOME Human Druggable Genome siRNA Library) containing ~ 7,588 unique genes in triplicate at 50 nM concentration. Each row of each plate contained one positive control well and one negative control well to monitor transfection efficiency and positional effects. Screening data was visualized using boxplots. Shown are six sets of triplicate assay plates. Boxes with blue borders represent the negative control values, while green boxes represent the positive control values.

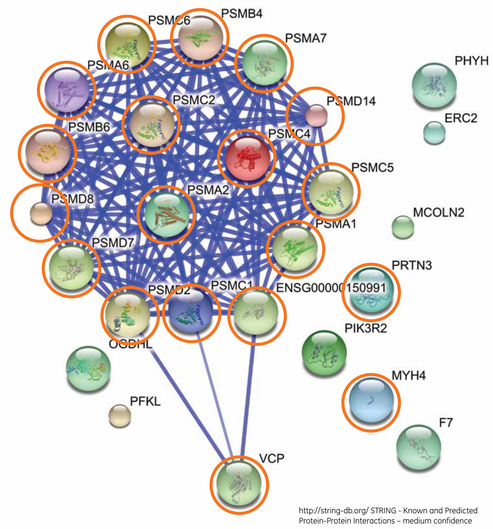
Hit identification



EGFP assay hits (inhibition of proteasome function)

Follow-up study and hit analysis

Hits from the primary screen, both for EGFP fluorescence and cell viability, were re-screened in triplicate using siGENOME and ON-TARGET*plus* SMARTpool siRNA reagents. ON-TARGET*plus* SMARTpool reagents have chemical modifications to both sense and antisense strands of the siRNA that further reduce the number of off-target effects, minimizing false positives and providing higher confidence in potential hits from a primary RNAi screen.



http://string-db.org/ STRING - Known and Predicted Protein-Protein Interactions – medium confidence

The hits that increase EGFP fluorescence and inhibit proteasome activity include many subunits of the proteasome, as expected. We also identified as potential hits other genes that are not subunits of the proteasome. Shown is the network association among the genes identified as hits from our RNAi screen; those outlined in green were confirmed with both siRNA SMARTpool reagents while the others were confirmed with only one reagent.

Future plans

- Reconfirmed hits will be assessed with four individual siRNAs that make up the SMARTpool reagent.
- Follow-up studies will analyze the correlation between phenotype and mRNA knockdown using RT-qPCR.
- Hits will be further analyzed using secondary assays, including high-content assays.

Conclusions

- We have performed a screen to identify genes that when silenced inhibit proteasome function using a cell-based protein assay.
- The high-throughput RNAi screening scheme described here addresses the challenges of RNAi screening and successfully detected RNAi-mediated changes in proteasome function. This scheme is achievable using common laboratory instruments and is also adaptable to 384-well format.

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