

# An Analysis of Normalization Methods for *Drosophila* RNAi Genomic Screens and Development of a Robust Validation Scheme

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Genome-wide RNA interference (RNAi) screening allows investigation of the role of individual genes in a process of choice. Most RNAi screens identify a large number of genes with a continuous gradient in the assessed phenotype. Screeners must decide whether to examine genes with the most robust phenotype or the full gradient of genes that cause an effect and how to identify candidate genes. The authors have used RNAi in *Drosophila* cells to examine viability in a 384-well plate format and compare 2 screens, untreated control and treatment. They compare multiple normalization methods, which take advantage of different features within the data, including quantile normalization, background subtraction, scaling, cellHTS2 (Boutros et al. 2006), and interquartile range measurement. Considering the false-positive potential that arises from RNAi technology, a robust validation method was designed for the purpose of gene selection for future investigations. In a retrospective analysis, the authors describe the use of validation data to evaluate each normalization method. Although no method worked ideally, a combination of 2 methods, background subtraction followed by quantile normalization and cellHTS2, at different thresholds, captures the most dependable and diverse candidate genes. Thresholds are suggested depending on whether a few candidate genes are desired or a more extensive systems-level analysis is sought. The normalization approaches and experimental design to perform validation experiments are likely to apply to those high-throughput screening systems attempting to identify genes for systems-level analysis. (*Journal of Biomolecular Screening* 2008:777-784)

**Key words:** RNAi, high-throughput screen, normalization, validation

## INTRODUCTION

GENOME-WIDE RNA interference (RNAi)-based screening is a powerful technology to assign phenotype to a gene and is facilitating investigations into complex network interactions in a variety of organisms.<sup>1</sup> Its compatibility with high-throughput technology<sup>2</sup> has revolutionized the quest for identification of novel genes in signal transduction pathways, human diseases, and drug targets.<sup>1,3,4</sup> Publications from analysis of these types of screens are constantly being added to the literature, with relatively few publications addressing experimental design and data analysis. Although publications have addressed general protocols and logical approaches for gene selection<sup>5</sup> or have introduced novel methods of data analysis and criteria for assessment of quality,<sup>6</sup> there is a lack of data in the literature to compare and contrast data

analysis methods and a lack of design of rigorous validation methods. Because RNAi-based screening is a labor-intensive technology involving complex experimental setup, because there is a deficiency of appropriate standards and the current analytical tools are relatively limited, we address some of the deficiencies related to appropriate experimental design for *Drosophila* RNAi genomic screening, examine the available analytical methods, and develop a rigorous validation scheme.

## METHODS

### *Tissue culture*

*Kc<sub>167</sub>* cells were grown in Schneider medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; 65 °C, 10 min), 100 U/mL penicillin, and 100 µg/mL streptomycin, at 24 °C in a humidified chamber.

### *Genome-wide viability screen following methyl methanesulfonate exposure*

Methods were adapted from the work of Boutros and others,<sup>3</sup> with  $1.2 \times 10^4$  *Kc<sub>167</sub>* cells suspended in 10 µL of serum-free Schneider media (Invitrogen) added to each well of 63 different 384-well plates. Each well contained, on average,

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0.05  $\mu\text{g}$  (0.01-0.43  $\mu\text{g}$ ) of a double-stranded (ds) RNA, with 22,915 individual dsRNA represented in the whole library (original *Drosophila* RNAi Screening Center [DRSC] library). One hour at 24 °C allowed phagocytic uptake of the dsRNA, then 20  $\mu\text{L}$  of serum containing medium (15% heat-inactivated FBS) was added and the plates incubated for a further 72 h. Medium was exchanged for 40  $\mu\text{L}$  of fresh serum containing medium (10% heat-inactivated FBS), with or without 0.004% (w/v) methyl methanesulfonate (MMS; Sigma-Aldrich, St. Louis, MO). Following an additional 72 h incubation, medium was removed and the number of viable cells assessed using prediluted Celltiter-Glo (Promega, Madison, WI) per the manufacturer's instructions and an Analyst GT Reader (Molecular Devices, Sunnyvale, CA). Screens were performed in duplicate, with the replicate screen initiated on a different day.

### Data analysis of library screen

It was assumed that the majority of the dsRNA on any one plate did not have any significant effect on viability; thus, the median value of each plate was used for all normalization methods. Five different normalization methods were applied to the data, with 2 additional normalizations conducted by combining different methods.

### Background subtraction and scaling

Background luminescence for each plate was calculated based on the median value of the plate and the value obtained for the well containing dsRNA targeting Thread (Th; CG12284), an antiapoptotic gene. This positive control is present in well B02 of each library plate provided by the DRSC and consistently results in a loss of cell viability. For background subtraction, the luminescence value of the well containing Th was subtracted from the value of each other well in the plate. If after normalization the value was negative, the normalized value was set to zero.

Data were scaled as follows. For untreated plates, the median value ( $m_{\text{untrt}}$ ) of each plate was assumed to be equivalent to 100% viability plus background (0% viability), and Thread ( $\text{Th}_{\text{untrt}}$ ) was assumed to be equivalent to 40% viability plus background. The 100% viability ( $100\%_{\text{untrt}}$ ) was then calculated for each plate by

$$100\%_{\text{untrt}} = \frac{m_{\text{untrt}} - \text{Th}_{\text{untrt}}}{0.6}. \quad (1)$$

And 0% viability ( $0\%_{\text{untrt}}$ ) was calculated for each plate by

$$0\%_{\text{untrt}} = m_{\text{untrt}} - 100\%_{\text{untrt}}. \quad (2)$$

Well values for each plate ( $wv_{\text{untrt}}$ ) were then corrected for background and normalized to 100% viability ( $wv_{\text{untrt\_norm}}$ ) by

$$wv_{\text{untrt\_norm}} = \frac{wv_{\text{untrt}} - 0\%_{\text{untrt}}}{100\%_{\text{untrt}}}. \quad (3)$$

Similarly, for the plates treated with MMS, the median value of the plate ( $m_{\text{MMS}}$ ) was assumed to be equivalent to 65% viability of untreated cells and Thread ( $\text{Th}_{\text{MMS}}$ ) was to 30% viability of untreated cells, based on an examination of the actual cell number after treatment in other experiments (data not shown). The calculated 100% viability in the absence of MMS treatment ( $100\%_{\text{MMS}}$ ) was calculated by

$$100\%_{\text{MMS}} = \frac{m_{\text{MMS}} - \text{Th}_{\text{MMS}}}{0.3}. \quad (4)$$

And 0% viability ( $0\%_{\text{MMS}}$ ) was calculated by

$$0\%_{\text{MMS}} = m_{\text{MMS}} - (0.6 \cdot 100\%_{\text{MMS}}). \quad (5)$$

Well values for each MMS exposed plate ( $wv_{\text{MMS}}$ ) were then corrected for background and normalized to the calculated 100% viability ( $wv_{\text{MMS\_norm}}$ ) by

$$wv_{\text{MMS\_norm}} = \frac{wv_{\text{MMS}} - 0\%_{\text{MMS}}}{100\%_{\text{MMS}}}. \quad (6)$$

These data were either examined by themselves or then further normalized to account for interplate variation by quantile normalization.

### cellHTS2

The R package cellHTS2<sup>6</sup> designed for RNAi genome-wide screen analysis was employed with the per-plate normalization function `normalizePlates` set to median and the summary function set to mean. Z scores were obtained, and the difference between z scores of treatment and control were determined in order to rank genes. Only 2 replicates of each plate were used in this analysis.

### Interquartile range normalization

The interquartile range (IQR) is the difference between the 1st and 3rd quartiles of a data set. The IQR was used to measure the difference of each well value from the median for each plate.<sup>7</sup> This method is insensitive to the presence of outliers in

the data set. Genes were ranked by the difference between the IQR measurements of treatment and control.

### Quantile normalization

Quantile normalization<sup>8</sup> is based on comparing quantiles between data sets, with no special allowance for outliers. Each of the 63 plate types was quantile normalized between replicates of untreated and between replicates of MMS treatment, with usually 2 replicates in each group. Following quantile normalization, “M,” the moderated *t* statistic in  $\log_2$  fold change, was calculated for each well by which genes were ranked. This resulted in the moderation of well values across genes and shrunk toward a common value using a Bayesian model. This is therefore a log-odds Bayesian statistic that a gene differentially affects viability.<sup>9</sup>

### Background normalizations combined with quantile normalization

To improve normalization by quantile normalization, background subtraction and scaling were applied to the data before normalization. Two new sets of normalized data were generated: background subtraction followed by quantile normalization (BSQN) and scaling followed by quantile normalization (SQN). Our scaling method followed by quantile normalization is very similar to robust multichip average (RMA) analysis,<sup>8</sup> which is a standard for microarray analysis. Neither background subtraction nor scaling had an effect on IQR measurement. CellHTS2 includes its own background correction method.

### Production of dsRNA

DsRNA was produced similarly to that previously described,<sup>5</sup> with the following exceptions: Primer sequences were obtained from the DRSC for amplicons to be validated, and oligonucleotides were purchased from Invitrogen (Carlsbad, CA). Primers contain the T7 promoter sequence at their 5' end to allow in vitro transcription. RNA from adult *Drosophila* was reverse transcribed using the Impromp-II Reverse Transcriptase system (Promega) to produce cDNA. This cDNA was amplified with amplicon-specific primers and Taq polymerase (Roche, Indianapolis, IN) using conditions previously described.<sup>5</sup> Products were resolved by agarose gel and gel purified using a 96-well gel isolation kit custom made by Invitrogen. A 2nd round of PCR using the same amplicon-specific primers was conducted to increase the amplification. In vitro transcription was then performed using the T7 Ribomax Express RNAi system (Promega), and the dsRNA product was purified using Millipore nucleic acid filter plates and recovered in 10 mM Tris HCl (pH 7.0). The resuspended product was quantified using a Spectramax M5 spectrophotometer (Molecular Devices). The concentration of dsRNA was adjusted to 0.08  $\mu\text{g}/\mu\text{L}$ , the quality of which was verified by agarose gel resolution.

### Validation analysis

For validations, experiments were performed as described,<sup>5</sup> with the following modifications: dsRNA was created in template 96-well plates and aliquoted into 4 distributed wells of a 384-well plate at a final quantity of 0.4  $\mu\text{g}$  using a Precision XS robot (BioTek, Winooski, VT). A distributed pattern was chosen to reduce plate location effects. Control dsRNA was included on each plate, including 4 wells with dsRNA against Th to demonstrate the function of dsRNA knockdown; 40 wells of nontargeting dsRNA against luciferase (Luc) to provide a control for the presence of dsRNA; 4 wells of dsRNA targeting GCLC, a gene required for viability following MMS exposure, as a positive control for treatment; and 4 wells containing a high level of MMS (0.12% w/v) resulting in 99% cell death to determine the background luminescence in the absence of any viable cells. Methyl methanesulfonate exposure and control experiments were performed in separate plates.

Luminescence values for each well were scaled similarly to the screen data, except the value for high MMS was used as 0%. For data analysis, scaled well values were normalized by dividing by the median of scaled Luc values, where  $wv_{\text{norm}}$  is the normalized well value and  $mlv_s$  is the median Luc value:

$$wv_{\text{norm}} = \frac{wv_s}{mlv_s} \quad (7)$$

To determine whether there is a difference between control and treatment, *t*-tests (2 tailed, type 2) were performed between normalized quadruplicates. In RNAi-based screens, however, removal of certain genes can result in decreased viability without any treatment (e.g., Th). To account for reduced cell number under these circumstances and to determine if the reduced viability is significantly different from the expected 65% viability following MMS exposure, a 2nd *t*-test (2 tailed, type 2) was performed on percentage control viability (PCV) for each targeting dsRNA against the PCV of Luc. From this, significant hits were selected using a difference denoting death (i.e., 55% or less viability compared with Luc).

The normalized quadruplicate data set for each dsRNA experiment was used to perform a *t*-test for comparing untreated cells against MMS treatment to determine the significance. To determine PCV, the cell density from the experimental wells was calculated as follows, where ECV is the estimated control viability, EMV is the estimated MMS viability,  $cwv_{\text{norm}}$  is the control well value, and  $mwv_{\text{norm}}$  is the MMS well value:

$$\text{ECV} = 100 \cdot cwv_{\text{norm}} \quad (8)$$

$$\text{EMV} = 65 \cdot mwv_{\text{norm}} \quad (9)$$

$$\text{PCV} = \frac{100}{\text{ECV}} \cdot \text{EMV} \quad (10)$$

Using the PCV for MMS treatment, a 2nd *t*-test was performed to determine the significant difference between the PCV for each dsRNA against PCV with Luc dsRNA. A significant effect was deemed for any dsRNA that, following MMS treatment, resulted in 55% or less viability compared with 65% PCV with Luc dsRNA, with significance being  $p < 0.001$ . High confidence positives were those with significant *p*-values after both *t*-tests, whereas dsRNA with a significant *p*-value by only 1 of the *t*-tests was considered positive with moderate confidence.

### **Comparison of screen normalization methods using validated data**

Receiver operating characteristic (ROC) curve analysis was performed and graphs generated using STATA (StataCorp LP, College Station, TX).

## **RESULTS AND DISCUSSION**

Use of genome-wide RNAi has become an important strategy to investigate functional genomics. The reliability of data generated from such genome-wide screens, however, is suspect because of confounding variables, such as the dynamic range and variation in the data, biological variations in the effectiveness of RNAi, and other physical constraints associated with the experimental setup,<sup>10</sup> which often causes significant problems for the screeners when choosing the appropriate statistical method for analysis. Although several statistical parameters for the analysis of a high-throughput RNAi screen have been published,<sup>6,10-12</sup> the applicability of these statistical methods depends on the experimental setup of the screen, which could vary among screening centers and could depend on the experimental output. Here we describe a strategy using a combination of normalization and analysis methods to improve the selection of a large number of genes as well a robust design for experimental validation of these genes for prospective studies.

We have used a *Drosophila* genomic library of dsRNA to determine the genes required for viability when exposed to MMS. This was done by comparing the data derived from RNAi viability screens performed with MMS treatment to that performed without treatment (see **supplemental Table 1** at <http://jbx.sagepub.com/supplemental/>) or normalized and validated data (**supplemental Table 2**, also available at [www.flyrnai.org](http://www.flyrnai.org) and at [gcri.uthscsa.edu/ABPublished\\_Data.asp](http://gcri.uthscsa.edu/ABPublished_Data.asp)). To determine which of these genes was required after treatment, we devised a validation scheme to confirm the authenticity of 806 dsRNA as conferring reduced viability after treatment, corresponding to 835 wells in the original library. We then compared several normalization strategies to determine the ability of each method to predict those dsRNA that actually validate as having an effect on viability. This article describes novel usage of established normalization methodologies; none of these methods except cellHTS2 has previously been applied to

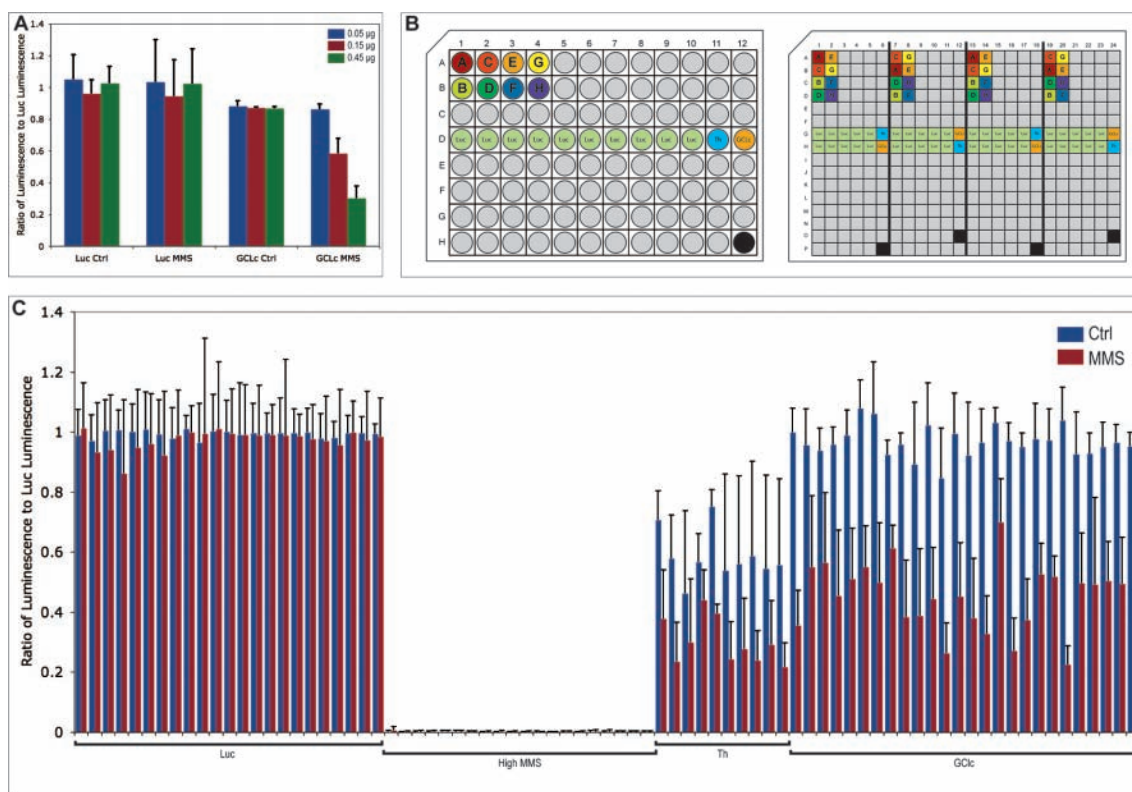
RNAi screens. We have then adapted these methods to conduct an original cross-screen comparison of our continuous data to examine those genes required for viability after a damaging treatment.

RNAi genomic screens are performed in 384-well plates, with each well containing a dsRNA targeting a single gene; with the 1st DRSC dsRNA library, 63 such plates are used to screen the entire genome. The labor-intensive preparative procedures and costs involved in both the library production and the screening procedures limit the number of biological replicates for any particular experiment. With only 2 replicate plates available from the DRSC for any 1 screen, variability in the results obtained is of significant concern. Intraplate variations arise because of physical constraints of the plate; edge effects can result from both evaporation at the edge and differences in fluorescent or luminescent signal bleed through from neighboring wells. Interplate variations tend to occur because of the sheer volume of plates required for the experiments and the time and effort required in their processing. As such, the performance of biological replicates often occurs at different times, especially when conducting cross-screen comparisons—this may cause variability because of the use of different batches of reagent or cells. These problems are further compounded by inconsistencies in the concentration of dsRNA present in each well and a lack of knowledge regarding the requirement for effective concentration of dsRNA to achieve complete knock down of their respective target genes. Irrespective, the data generated from RNAi screens have led to significant advancements in science.<sup>1</sup> To obtain the highest quality data from these genome-wide screens, we have attempted several different normalization methods either designed specifically for RNAi screens or borrowed from genome-wide expression analysis tools. Genes selected from these analyses were subjected to a robust validation methodology both to confirm the selection for future analyses and to examine the quality of the data normalization methods. We have applied several stringent criteria in the validation experiments to reduce the variations observed in the original screen and embedded controls that can be used to normalize experiments and to monitor their quality.

### **Experimental setup of screen and validation**

To evaluate normalization methods, we analyzed the validation experiments to correctly identify true-positives and -negatives. Here, we validated the same dsRNA as was tested in the original screen to analyze screen normalization methods. Correct validation for genes identified as affecting the phenotype of interest, however, should be done with a dsRNA targeting a different site on the mRNA.

Validation experiments were performed using the same dsRNA amplicon present in the original genome-wide screen. Unlike the original DRSC library, which had an average of about 0.05  $\mu\text{g}$  dsRNA per well with a range up to about 0.43  $\mu\text{g}$  (DRSC, personal communication), we used a normalized concentration of dsRNA,



**FIG. 1.** Arrangement and analysis of validation experiments. **(A)** Ratio of viability of cells with double-stranded (ds) RNA targeting GCLc normalized to cells with nontargeting dsRNA against luciferase (*luc*), with and without methyl methanesulfonate (MMS) treatment. Concentrations of dsRNA targeting GCLc were 0.05  $\mu\text{g}$  (blue), 0.15  $\mu\text{g}$  (red), and 0.45  $\mu\text{g}$  (green) per well. Error bars are standard deviation for quadruplicates. **(B)** Layouts of the 96-well stock plate and 384-well assay plate. Unique dsRNA is filled gray, with 8 of these labeled A through H and colored. Controls of dsRNA against luciferase (*Luc*, green), Thread (*Th*, light blue), and GCLc (orange) are also indicated. The black wells are empty. In the stock plate, each dsRNA is represented only once, except dsRNA targeting *Luc*. The assay plate is aliquoted using a robot with an 8-channel pipettor, and it is composed of 4 copies of the stock plate, with a distributed pattern to reduce edge effects (notice the positioning of dsRNA A through H; this pattern is continued for all gray [unique] dsRNA). In the assay plate, a high concentration of MMS is added to the empty well. **(C)** Ratio of viability of embedded controls in assay plates, with (red) and without (blue) MMS treatment, normalized to the median *Luc* value for each plate. Error bars are standard deviation for 40 replicates (*Luc*) or quadruplicates (high MMS, *Th*, and GCLc).

0.4  $\mu\text{g}$  dsRNA per well, to eliminate false-negatives due to incomplete knock down. This concentration was chosen based on that needed to achieve an effect on viability after MMS treatment after knock down of GCLc, a gene required after MMS (Fig. 1A). The concentration of 0.45  $\mu\text{g}$  per well provided the maximum effect on viability, whereas the lower concentrations did not, presumably because of incomplete knock down. With a high level of dsRNA and a consistent concentration in every well, complete knock down is ensured for almost every gene targeted.

The original genome-wide RNAi screen available from the DRSC is provided only in duplicate, with each dsRNA unique on each plate. In our validation method, control and MMS treatment were performed simultaneously with quadruplicates of dsRNA present in the same plate, to lessen effects due to variation between biological replicates and to avoid interplate variation (Fig. 1B). In addition, in an attempt to alleviate variation due to

edge effects, validation experiments were set up with the quadruplicate dsRNA arranged in a distributed pattern within the plate (Fig. 1B). The pattern is limited by the physical constraints of the robotics used but was designed so that a single dsRNA would never be located at the edge in every instance.

Decreased cell growth in wells located on the edges of the plate is due to excessive evaporation of media and can result in edge effects. Despite maintaining 384-well plates in a humidified container, such edge effects are especially severe in plates located on top of a plate stack. These edge effects can be diminished by placing either plates containing water or wet paper towels on top of the stack.

We included several controls in our validation experiments (Fig. 1B). For viability assays, the DRSC screening plates provide the positive control of dsRNA targeting the antiapoptotic gene *Th*, but it is in well B02, near the outside of the plate. As

a negative control, dsRNA against GFP, not present in the *Drosophila* genome, is provided in well A02, on the plate edge. We found that this dsRNA against GFP had an effect after MMS treatment (data not shown) and discovered that the dsRNA had possible off targets (data not shown). We therefore designed dsRNA against another gene not present in the genome, luciferase (Luc), to be used as a nontargeting control. To confidently normalize the plates to a dsRNA standard with truly no effect, dsRNA against Luc was included in 40 wells of each plate. To ensure the presence and functionality of dsRNA knock down, dsRNA targeting Th also was included. It should be noted that we observed that dsRNA against Th did not result in the death of all cells (albeit it was most often the lowest luminescence value on the plate) and that the dsRNA to Th also resulted in increased sensitivity to MMS treatment. Because Th is inconsistent between control and treatment, it is not well suited for correction of background. Therefore, to normalize the plates to true background, a quadruplicate control of a high dose of MMS, sufficient to kill all cells, was embedded in the plate. We also included the positive control for treatment of dsRNA targeting GCLC, a gene that is not required for viability itself but is required after MMS treatment (**Fig. 1A**).

### Quality metrics of validation experiments

In the comparison of Luc dsRNA negative control values with high MMS positive control values from 17 different plates assayed on different days, the Z factor<sup>13</sup> was calculated to be 0.705. In addition, Luc dsRNA used in validation normalization had less than 10% standard deviation in untreated plates, whereas the MMS-exposed plates for the same target dsRNA resulted in less than 20% standard deviation. Employing the described plate layout to decrease interplate variation was successful, as can be seen (**Fig. 1C**). The background luminescence values obtained from the wells with high MMS were minimal and consistent across multiple plates. Wells containing dsRNA against the positive control Th and against the experimental control GCLC were also consistent across plates. Standard deviations are greater following normalization because data were divided by the median of Luc dsRNA values, whereas the observed standard deviation using raw values within any plate were far less (data not shown). By reducing interplate and intraplate variation and normalizing to a large number of negative controls, results are more consistent and reliable.

### Comparison of normalization methods

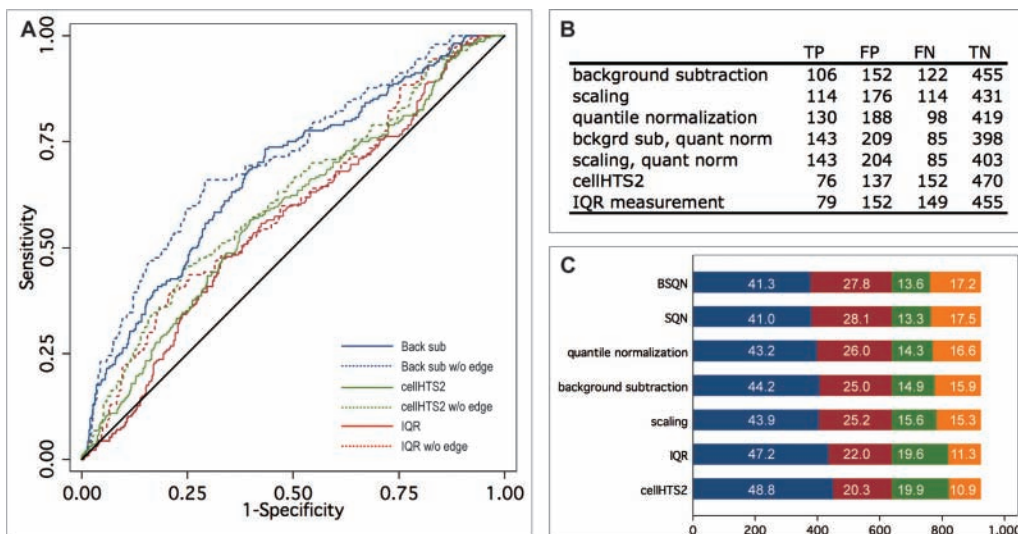
Genes that were confirmed from the validation experiments were used for a retrospective analysis comparing screen analysis methods. Seven methods of normalization or combination of normalizations were employed to normalize for interplate variation:

background subtraction, scaling, cellHTS2, IQR measurement, quantile normalization, BSQN, and SQN. The data were subsequently compared between treatments to provide scores to rank genes required for viability; in each case, the normalized value for untreated control was subtracted from the normalized value following MMS exposure. After quantile normalization, an M score was calculated, which also serves this purpose.

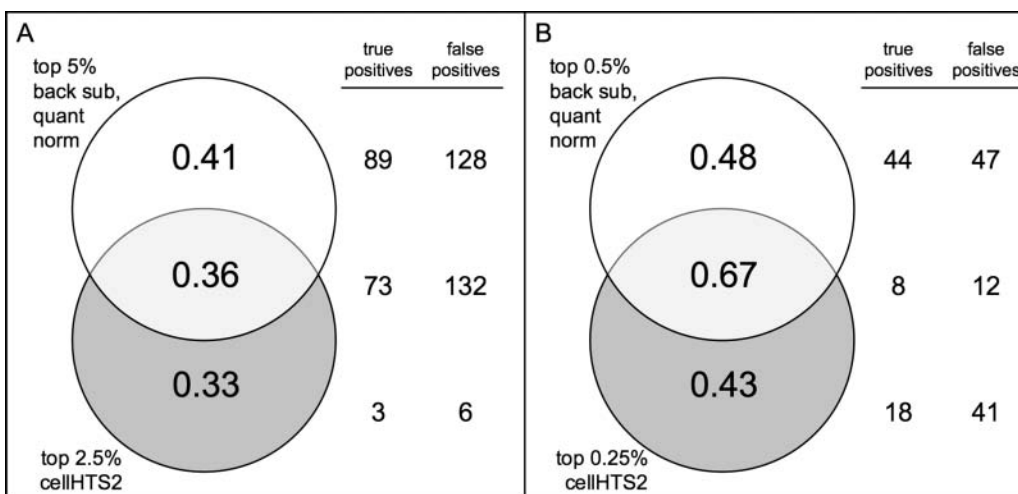
This analysis is meant for analyzing data generated from RNAi screens yielding continuous data, such as a viability output, and not necessarily for other outputs, such as microscopy or reporter assays with internal controls. In particular, our analysis attempts to find the best methods to cross-compare screens, such as a treatment and its untreated control, which inherently cannot be combined within the same well. Toward this end, researchers applying those methods we discuss that require values for percentage viability after treatment or percentage viability of a particular positive control, either treated or untreated, must empirically derive those values for themselves.

Using the data obtained from validation experiments, a retrospective analysis was performed to identify the best normalization method for identifying the most true-positive results. Ranked genes were plotted on an ROC curve using data generated by validation analysis as positives and negatives (**Fig. 2A**) to determine the method yielding the best sensitivity and selectivity. Unfortunately, no single method excelled. BSQN and SQN were the best 2 methods, with areas under the curve of 0.6758 and 0.6746, respectively, whereas IQR measurement and cellHTS2 were the worst 2 methods, with 0.5600 and 0.5826, respectively (**Fig. 2B**). Because the normalizations and subsequent rankings were so poor, we chose the top 5%, 2.5%, 0.5%, and 0.25% ranked genes in the screen as the positive thresholds for each method to examine their individual validation rate. **Figure 2C** shows true- and false-positives and -negatives for each method tested at the 5% threshold.

We wanted to determine the overlap within the top-ranked wells after each normalization method. We expected that cellHTS2 and IQR measurement methods would have a good amount of overlap because the methods are based on a similar concept, and we found this to be true (data not shown). In addition, we expected that the quantile normalization methods would overlap in dsRNA amplicons ranked; we found this to be true (data not shown). We also found that wells ranked after background subtraction or scaling overlapped with amplicons ranked after any of the quantile normalization methods (data not shown). We therefore examined the overlap between dissimilar methods, namely, BSQN and cellHTS2 (**Fig. 3**). We found that to obtain the highest ratio of true-positives to total positives ranked, the combination of the top 5% following BSQN and the top 2.5% following cellHTS2 provided the most true-positive amplicons with the least amount of false-positives (**Fig. 3A**). We currently recommend these cutoffs for examination of any continuous distributed data set generated by RNAi



**FIG. 2.** Analysis of normalization methods. (A) Receiver operating characteristic (ROC) curves for the best (background subtraction, blue) and worst (cellHTS2, green, and interquartile range [IQR] measurement, red) normalization methods, both with (solid lines) and without (dashed lines) double-stranded (ds) RNA located in the outer edge of the screening plate. The reference line is indicated in black. (B) Area under the curves for each method tested, both with and without dsRNA located in the outer edge of the screening plate. (C) The number of true-negatives (TN, blue), false-positives (FP, red), false-negatives (green), and true-positives (orange) for each normalization method at thresholds of the top 5% ranked wells. Percentages of element are given.



**FIG. 3.** Comparisons between normalization methods. Venn diagrams representing the ratio of true-positives to total positives for (A) the top 5% ranked wells after background subtraction followed by quantile normalization and the top 2.5% ranked wells after cellHTS2 and (B) the top 0.5% after background subtraction followed by quantile normalization and the top 0.25% after cellHTS2. Total numbers of true- and false-positives overlapping for each method are given to the right.

screens to encompass the most reliable data set for a systems-level analysis. Alternatively, for a more selective study in which only a few, top-candidate genes are of interest, we advise using a higher stringency, combining the top 0.5% following BSQN and the top 0.25% following cellHTS2 (**Fig. 3B**).

We attempted to improve prediction by these normalization methods by examining the extent to which edge effects were having a negative impact on prediction. Indeed, ROC analysis indicates that by removing those genes represented in the wells at the outer edge of the 384-well plate from analysis, predictions

improve greatly by every method (Figs. 2A and B). Removing both the outermost wells of the plate and the next well in from the edges resulted in no further improvement of any method (data not shown). Amplicons located on edges are therefore more likely to result in false-positives than amplicons located internally. It is therefore critical that when these amplicons are validated, they be distributed in the plate.

Overall, we have demonstrated that our validation method is extremely stringent, with an experimental design including use of consistent and effective quantity of dsRNA, performance of quadruplicates distributed within a single plate, and the embedding of multiple control features such as nontargeting dsRNA in a good portion of the wells to facilitate normalization between treatments (see Figs. 1C for reproducibility between assays). Each of these points was addressed because of limitations of the screen experimental design, and thus, we have been able to establish a stringent method by which to validate potential screen hits.

Unfortunately, we were not able to determine a sensitive or selective normalization method, even though multiple methods were examined. Clearly, additional methods of normalization and ranking need to be developed as they have been for microarrays. An improved screen design would also reduce some of the issues. When the screening library is plated, those amplicons on the edge can be plated internally in the replicate plate to reduce edge effects (swapping quadrants). Additional controls for normalization could be included besides positive controls for the assay along with more replicates, although we acknowledge that this might be cost prohibitive. Finally, a standardized concentration of 0.4  $\mu\text{g}$  of dsRNA per well would ensure that more genes are fully knocked down, thus reducing the number of false-negatives at a given threshold. These points emphasize the importance of rigorous validation to follow up to any hits found by genome-wide RNAi screens of this nature.

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and [http://gccri.uthscsa.edu/ABPublished\\_Data.asp](http://gccri.uthscsa.edu/ABPublished_Data.asp), and normalized data are available from [http://gccri.uthscsa.edu/ABPublished\\_Data.asp](http://gccri.uthscsa.edu/ABPublished_Data.asp).

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