

Loss-of-Function Genetic Screens to Find Genes Regulating Cell Responses and Identify Potential Drug Targets



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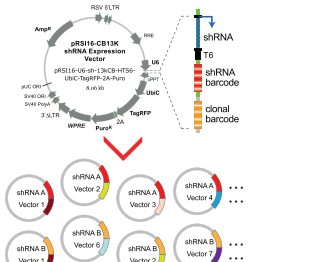


Abstract

To date, several groups have successfully run genome-wide *in vitro* RNAi screening using pooled libraries expressing a complex diversity of shRNA molecules. Integral to obtaining reliable and robust results with this technique has been the use of quantitative next generation sequencing to accurately assay hairpin representation levels in the endpoint populations of cells transduced with an shRNA library. However, there are significant challenges in adapting the current pooled RNAi screening approaches to more sophisticated cell model systems, such as xenograft models. Our studies tracking the fates of thousands of individually barcoded implanted cells found that xenograft growth is characterized by a small subset of cancer cell clones that ultimately produce the bulk of the resulting tumor mass. This phenomenon significantly impacts the ability to reliably detect shRNA-induced growth inhibition in these systems. To address this problem, we have developed a novel approach to constructing pooled shRNA libraries that enables effective viability screening in systems in which external parameters strongly influence cell growth rates, such as xenograft models. We will present data using this approach, as well as data from more conventional *in vitro* "drop-out" lethality screens, to identify genes essential for cell proliferation. The results demonstrate that complex pooled shRNA libraries when combined with clonal tracking technology, offer an efficient and flexible tool for both *in vitro* and *in vivo* screens aimed at discovering potential cancer therapy targets.

shRNA Library with Clonal Tracking Barcodes

Figure 1. The DECIPHER 27K shRNA Library was constructed as described in <http://www.collecta.com/technology/shRNA-library-construction/>. To enable clonal tracking using the pooled shRNA library, an additional vector barcode was introduced just downstream of the shRNA-associated barcode. This was done by first creating a library of barcoded vectors using 13K barcodes. 27K defined shRNA inserts were cloned into this vector library.



Clonal growth profile of tumor xenografts

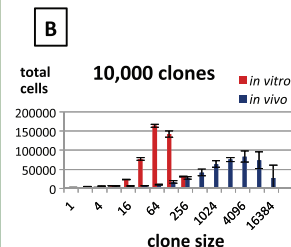
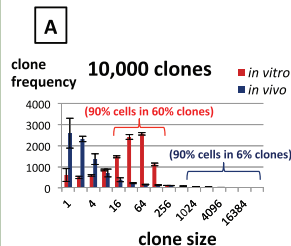


Figure 2. HTC 116 cells were transduced with a lentiviral library containing 27K barcodes but expressing no shRNA. 10K barcoded cell samples were injected into NGS mice, and 10K cell samples were cultured *in vitro*. After 2 wks, barcodes were sequenced. A) *In vitro*, 90% of the total tumor came from 6% of the initial cells. ~90% didn't grow or doubled just a few times. B) *In vitro*, the majority of cells doubled 5-6 times.

Clone size distribution of typical toxic and non-toxic shRNAs

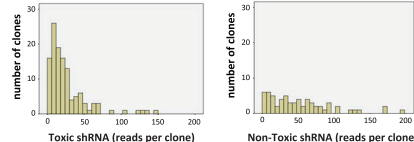


Figure 3. Observed clone size distribution for one toxic and one non-toxic control shRNA from an *in vitro* RNAi screen using clonal barcoded pooled shRNA library. Differences in the distribution of growth between the ca. 75 cells with the toxic shRNA (larger fraction of clones with poor growth) vs. those with the non-toxic shRNA (clones with a wider distribution of growth) are evident.

In vivo (xenograft) clone size distribution of toxic vs. non-toxic shRNAs

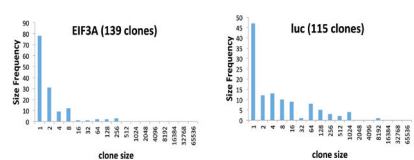


Figure 4. Observed clone size distribution for one toxic and one non-toxic control shRNA from an *in vitro* RNAi screen using clonal barcoded pooled shRNA library. As shown in Fig. 2, a larger fraction of the clones did not grow *in vivo*. As above with the *in vitro* shRNA screen (Fig. 3), differences in the distribution of growth between the ca. 100 cells with the toxic shRNA (EIF3A) vs. those with the non-toxic shRNA (luc) are evident.

Reproducibility of shRNA counts in tumors using standard vs. clonal analysis

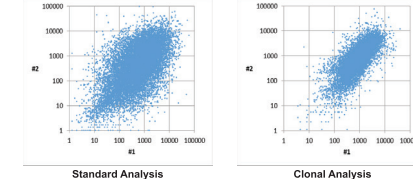


Figure 7. Clonal analysis provides less variation among replicate samples. Left: shRNA counts using Standard analysis in Tumor #1 vs. Tumor #2. Right: shRNA counts using Clonal Analysis in Tumor #1 vs. Tumor #2.

In vivo confirmation rate in xenografts

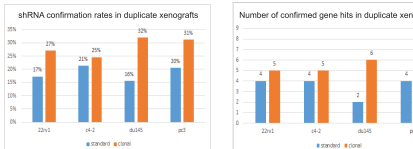


Figure 8. *In vivo* confirmation rate is the percentage of shRNA hits from one tumor which are confirmed in a second tumor. A gene is a hit if it is identified by at least 3 independent shRNA hits. Our results show that clonal analysis gives consistently more reproducible results in any cell line that we tested.

Cell growth results from an in vitro RNAi screen

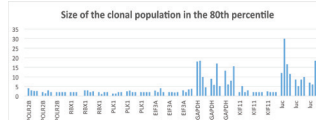


Figure 5. The number of cells in the clonal population found at the 80th percentile in size for each of the shRNA identified. Since injected cells grow into different sized clones in the tumor, assessment of the size of clonal populations above a predefined size percentile better indicates how a specific shRNA influences the growth of cells in which it is expressed.

CV of triplicates in vivo



Figure 6. The data show the coefficient of variation (CV) of the counts for each shRNA in the library from 3 different tumors. CVs are plotted in ascending order. The clonal populations have smaller CVs, indicating there is less variation among the replicates.

Conclusion

Transduction of a pooled shRNA library generates multiple independent transduced cells expressing the same shRNA. As cells are allowed to grow, each originally transduced cell will expand into an independent clonal population. By incorporating a second vector barcode, we can produce complex pooled shRNA libraries that allow assessment of the fate of each one of the multiple clonal populations. The shRNA-induced phenotype can be assessed independently for each clone for every shRNA in the pool.

Clonal analysis is a departure from the traditional method of measuring the overall effect on the total population of cells with a given shRNA. The standard approach averages the effect of the shRNA across all clones in the population, resulting in an inherent bias toward the largest clones. With clonal barcodes, it is possible to assess how the progeny of each originally-transduced cell responds to a given shRNA expression. The higher level of resolution provided by clonal analysis is particularly advantageous in xenograft studies, where clonal level of resolution enables better discernment of the shRNA's versus the environment's effect on growth. Additionally, library complexity does not need to be maintained (difficult for *in vivo* screens). It is the distribution of clone sizes that is used to assess shRNA toxicity. Since all clones start as a single cell, no zero time-point is necessary. Our results show that clonal analysis of *in vivo* screens gives better quality data in terms of reproducibility, which translates into higher confirmation rates both at the shRNA and gene level, and a lower likelihood for each identified hit to be a false positive.