BioTechniques

For reprint orders, please contact: reprints@future-science-group.com

An improved shotgun antisense method for mutagenesis and gene identification

Yu Tang[1] & Richard H Gomer*,[1]

ABSTRACT

Shotgun expression of antisense cDNA, where each transformed cell expresses a different antisense cDNA, has been used for mutagenesis and gene identification in *Dictyostelium discoideum*. However, the method has two limitations. First, there were too few clones in the shotgun antisense cDNA library to have an antisense cDNA for every gene in the genome. Second, the unequal transcription level of genes resulted in many antisense cDNAs in the library for some genes but relatively few antisense cDNAs for other genes. Here we report an improved method for generating a larger antisense cDNA library with a reduced percentage of cDNA clones from highly prevalent mRNAs and demonstrate its utility by screening for signal transduction pathway components in *D. discoideum*.

METHOD SUMMARY

We present an improved shotgun antisense method for generating gene expression knockdown mutants. This method incorporates a cDNA-normalization step to equalize the transcript number of each gene in the antisense cDNA library.

KEYWORDS

cDNA normalization • genetic screen • shotgun antisense

[1]Department of Biology, Texas A&M University, College Station, TX 77843, USA; *Author for correspondence: rgomer@tamu.edu

BioTechniques 68: 163-165 (March 2020)
10.2144/btn-2019-0123

Genetic screens are broadly used to generate and identify mutants with a desired phenotype. Techniques for genetic screens include chemical and radiation mutagenesis [1,2], insertional mutagenesis [3,4], CRISPR libraries [5,6], siRNA libraries [7,8] and shotgun antisense [9]. The basis of shotgun antisense is antisense repression. For antisense repression of a single gene, the corresponding cDNA is cloned into an expression vector plasmid in a backward orientation and then transformed into cells. The expression vector will generate an antisense RNA that hybridizes to, and effectively neutralizes, the selected RNA sequence (typically an mRNA). This hybridization and neutralization process reduces, but does not eliminate, levels of the selected RNA. Using a transformation vector or plasmid where cells typically take up only one copy of the vector and an antisense construct with an entire cDNA library rather than a selected cDNA allows a mutagenesis referred to as shotgun antisense [9].

Among the mutagenesis techniques, shotgun antisense has several benefits. First, the library is relatively easy to construct and the mutant pool is easy to generate. It only requires RNA isolation and cDNA synthesis, followed by cloning the cDNA into an expression vector in a backward orientation. CRISPR and siRNA libraries require careful design and CRISPR mutagenesis requires the expression of CAS9 protein in the target cells [5,6]. Second, shotgun antisense can be directed to target genes expressed in a specific tissue or developmental stage by using RNA isolated from a specific tissue or developmental stage. Third, as a gene knockdown technique, shotgun antisense is able to identify genes where complete disruption is lethal. Fourth, identification of the gene associated with an interesting phenotype from shotgun antisense requires only a PCR reaction on whole cells to amplify and sequence the antisense cDNA in the shotgun antisense plasmid. Fifth, antisense can repress the expression of multiple genes whose transcripts share closely related sequences, such as the three-member Discoidin I gene family in the model eukaryote *Dictyostelium discoideum* [10].

Shotgun antisense screens have been used for mutagenesis and gene identification in *D. discoideum* [9], but the original protocol had two disadvantages. First, there are 12,257 protein-coding genes in *D. discoideum* [11], but the size of the shotgun antisense library from each ligation was only approximately 15,000 individual clones [9], so that by Poisson statistics many genes will not have a corresponding cDNA in the library [12]. Second, the unequal transcription level of genes causes the levels of some RNAs and corresponding cDNAs in the library to be much lower than those of other genes. In a eukaryotic cell, 20–40% of genes only have one to several dozen transcripts, but as few as five to ten genes have several thousand transcripts [13]. Thus, if a conventional cDNA library is used for shotgun antisense, genes with rare transcripts have a low chance of generating a corresponding mutant and being identified.

To overcome these disadvantages, we developed an improved shotgun antisense technique with a cDNA normalization step that minimizes the biased repression of highly prevalent mRNAs and high-efficiency electrocompetent bacteria were used to increase the size of the library from each ligation. We checked the utility of this improved technique in a genetic screen in *D. discoideum*.

Total RNA of proliferating *D. discoideum* cells was isolated using a RNA prep kit (Zymo Research, CA, USA) and 3 μg of this RNA was used for directional double-strand cDNA library synthesis using a Mint-2 cDNA synthesis kit (Evrogen, Moscow, Russia), as detailed in the supplementary file. This generates cDNAs with adapters added at the ends, with a SpeI site on the end corresponding to the 5′ end of the mRNA and a *BglII* site on the other. We then normalized 1.2 μg

 © 2020 Yu Tang and Richard Gomer

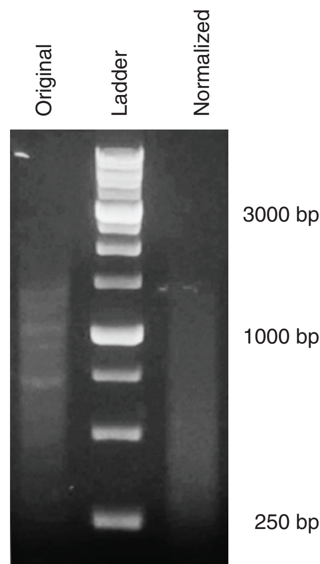

Figure 1. Normalization of cDNA removes bands corresponding to highly prevalent mRNAs. Original cDNAs (left) and the normalized cDNAs (right) were electrophoresed on a 1.5% agarose gel in TAE buffer (Tris 40mM, Acetic Acid 20 mM and EDTA 50 mM) with a DNA ladder in the middle. Molecular masses (in bp) are at right.

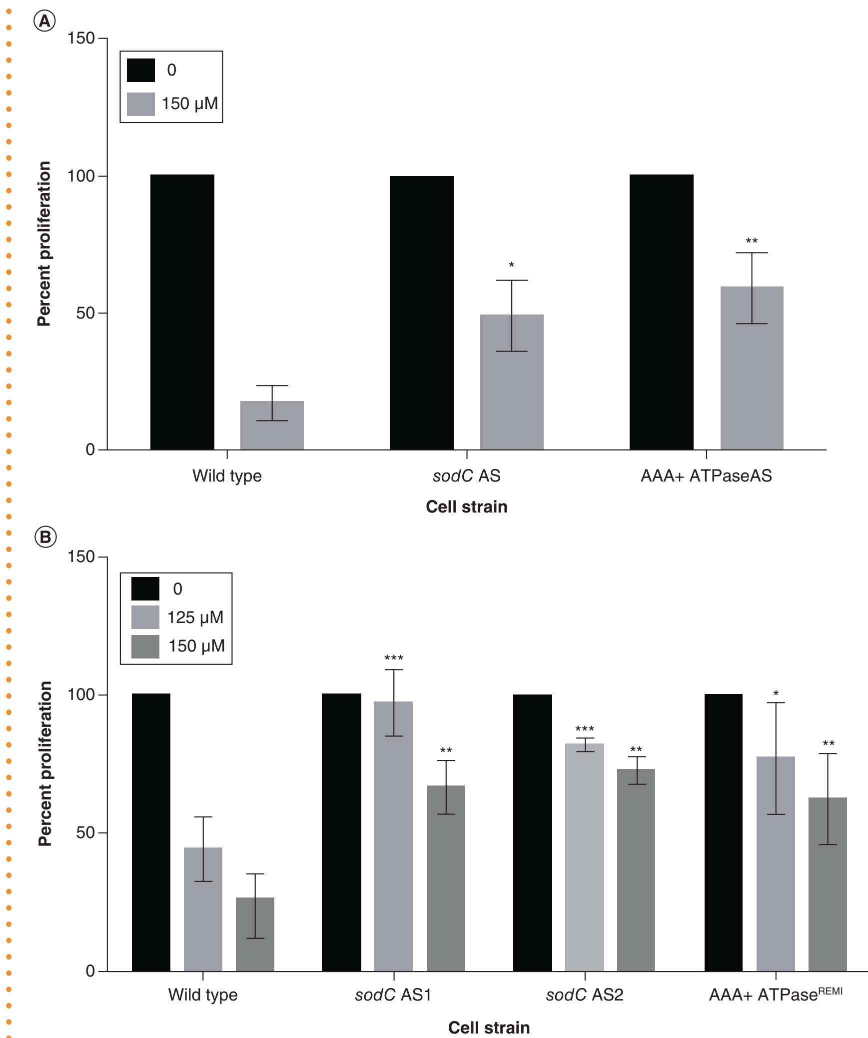

Figure 2. Identification of polyphosphate-resistant mutants from a shotgun antisense screen. (A) Wild-type cells and two clones from the shotgun antisense screen were tested for proliferation in the presence or absence of 150-µM polyphosphate. (B) Wild-type cells, two SodC antisense transformants generated by constructing specific antisense transformants and a mutant with an insertion in the AAA-ATPase gene were tested for proliferation in the presence of 0-, 125- or 150-µM polyphosphate. For (A & B), percent proliferation is the cell density at 24 h as a percent of the cell density with no added polyphosphate. All values are mean ± standard deviation, n ≥ 3. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with wild-type cells at the same polyphosphate concentration (unpaired t-test, correcting for multiple comparisons using the Holm–Sidak method).

of this dscDNA library using a Trimmer-2 kit (Evrogen) by melting the double-stranded DNA, allowing the abundant cDNAs to partially rehybridize, digesting the hybridized DNA and then amplifying the remaining DNA by PCR. Whereas the unnormalized cDNA pool showed bands after electrophoresis on agarose gels, indicating the presence of high levels of some cDNA species, there were no obvious bands after normalization, indicating that the normalization step reduced levels of these prominent cDNAs (Figure 1).

The normalized dscDNA was then amplified by PCR with primers matching the adapters as detailed in the supplementary file. The enriched PCR product and the vector plasmid pDM326 containing the blasticidin resistance cassette [14] were digested with the restriction enzymes BglII and SpeI, ligated with T4 DNA Ligase (New England Biolabs, MA, USA) and then concentrated with a DNA Clean & Concentrator™-5 Kit (Zymo Research) to a volume of 10 µl, as detailed in the supplementary file. The ligation was then used to transform 5-α electrocompetent *Escherichia coli* cells (New England Biolabs) with 1 µl purified ligation product and 25 µl competent cells per transformation. A tenth of the total transformed cells from one transformation were plated on a Luria-Bertani (LB)/ampicillin agar plate (100 µg/ml ampicillin) to determine the library size. There were approximately 800 colonies after overnight incubation at 37°C. Thus, the library from each ligation contained approximately 80,000 independent clones (800 × 10 × 10) [12]. The total transformed cells from 10 µl ligation product were plated on 35 LB/ampicillin agar plates and incubated at 37°C overnight. The next day, we picked 16 colonies for plasmid minipreps and then did a BglII and SpeI double digestion of the plasmids. Electrophoresis showed that all of the plasmids had approximately 250–1000 bp insertions. The remaining colonies were collected by a plate scraper with 3 ml LB per plate. The collected cells were grown in a 500-ml culture to an OD_{600} of approximately 3; 25 ml of this culture was mixed with 25 ml of 50% glycerol in H_2O and aliquots were stored at -80°C. The remaining culture was used for a plasmid DNA maxi-prep using a ZymoPURE™ II Maxiprep Kit and the plasmid DNA was

used to transform *D. discoideum* cells by electroporation [15].

Electroporated cells were then transferred into 10 ml HL5 medium/100 μg/ml ampicillin in a 100-mm tissue culture petri dish and 10 μg/ml blasticidin (GoldBio, MO, USA) was added 16–20 h later for selection of transformed cells. Colonies normally appeared after 5–7 days and were transferred to shaking culture for screen assays. Each plate typically contained 800 colonies.

Polyphosphate inhibits the proliferation of *Dictyostelium* cells through a signal transduction pathway involving the receptor GrlD [16]. As a check of the utility of the new shotgun antisense library, we screened for mutants resistant to polyphosphate. Each transformation generated three pools with approximately 800 individual clones per pool. These pools were cultured in 150-μM polyphosphate for 2 days and allowed to recover for 2 days in the absence of polyphosphate and the cycle was then repeated. After four cycles, we observed that cells in three of the 20 pools screened, representing approximately 16,000 clones screened, proliferated more quickly than control untransformed cells in the presence of polyphosphate. In this screening, most transformed cells showed normal sensitivity to polyphosphate compared with untransformed cells. These transformed cells (they have a vector with other inserts) served as controls to rule out the effect of vector transformation on cells.

Cells from these three pools were cloned and tested for abnormally fast proliferation in the presence of polyphosphate. Antisense cDNA plasmids were extracted from the clones that passed the verification and the antisense cDNA inserts were sequenced. The lengths of the antisense cDNAs were 244–406 bp. We found four antisense cDNAs (two from one pool and one from each of the other two pools). One cDNA sequence matched the *Dictyostelium* gene *ai2a* (DDB_G0294421), one matched *ddcB* (DDB_G0276067), one matched *sodC* (DDB_G0282993) and one matched DDB_G0273573 (AAA-ATPase, core domain-containing protein).

To evaluate the utility of the new shotgun antisense technique, we checked the identification of SodC and the AAA-ATPase as proteins affecting polyphosphate inhibition of proliferation. For SodC, we generated two *sodC* knockdown strains by antisense repression, one with full-length antisense mRNA (*sodC* AS1) and the other one with a truncated antisense mRNA (*sodC* AS2). The detailed construction procedure is in the supplementary file. For the AAA-ATPase, we obtained a mutant (GWDI_488_A_5, henceforth AAA-ATPase^{REMI}) with an insertion of a plasmid at bp 13,710 in the 16,893-bp long coding sequence. Compared to wild-type cells, these three mutants and the corresponding original clones from the shotgun antisense screen had reduced sensitivity to proliferation inhibition by polyphosphate (Figure 2). This indicates that the modified shotgun antisense mutagenesis described here can successfully generate and identify mutants.

A limitation of this study is that there is no straightforward way to perform a comparative study between this modified method and the 1996 shotgun antisense method. A comparison would require multiple genetic screens to saturation using the two protocols and then identification of the resulting mutants. Despite this limitation, the shotgun antisense protocol presented here has two theoretical improvements. First, the modified shotgun antisense cDNA library has a larger size (~80,000 individual clones) than that made before (~15,000 individual clones), so that by Poisson statistics many more genes will have a corresponding cDNA in the library [12]. Second, the addition of cDNA normalization for the shotgun antisense cDNA library reduces bias of gene repression and thus helps increase the chance of generating mutants corresponding to genes with low transcription levels. Together, these modifications should increase the utility of shotgun antisense for mutagenesis and gene identification.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2019-0123

AUTHOR CONTRIBUTIONS

Y Tang and R Gomer designed the project and wrote the manuscript. Y Tang performed the experiments.

ACKNOWLEDGMENTS

The authors thank S Kirolos and K Consalvo for helpful comments on the manuscript.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

This work was supported by NIH/NINDS R21NS102600. The authors have no other relevant affiliations of financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

OPEN ACCESS

This work is licensed under the Attribution-NonCommercial-NoDerivatives 4.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/4.0/

REFERENCES

1. Singer B, Kusmierek JT. Chemical mutagenesis. *Annu. Rev. Biochem.* 51, 655–693 (1982).
2. Frankenbergschwager M. Induction, repair and biological relevance of radiation-induced DNA lesions in eukaryotic cells. *Radiat. Environ. Biophys.* 29(4), 273–292 (1990).
3. Adachi H, Hasebe T, Yoshinaga K, Ohta T, Sutoh K. Isolation of ***Dictyostelium discoideum*** cytokinesis mutants by restriction enzyme-mediated integration of the blasticidin S resistance marker. *Biochem. Biophys. Res. Commun.* 205(3), 1808–1814 (1994).
4. Carlson CM, Largaespada DA. Insertional mutagenesis in mice: new perspectives and tools. *Nat. Rev. Genet.* 6(7), 568–580 (2005).
5. Shalem O, Sanjana NE, Hartenian E *et al.* Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 343(6166), 84–87 (2014).
6. Wang T, Wei JJ, Sabatini DM, Lander ES. Genetic screens in human cells using the CRISPR-Cas9 system. *Science* 343(6166), 80–84 (2014).
7. Echeverri CJ, Perrimon N. High-throughput RNAi screening in cultured cells: a user's guide. *Nat. Rev. Genet.* 7(5), 373–384 (2006).
8. Gonczy P, Echeverri C, Oegema K *et al.* Functional genomic analysis of cell division in ***C. elegans*** using RNAi of genes on chromosome III. *Nature* 408(6810), 331–336 (2000).
9. Spann TP, Brock DA, Lindsey DF, Wood SA, Gomer RH. Mutagenesis and gene identification in ***Dictyostelium*** by shotgun antisense. *Proc. Natl Acad. Sci. USA* 93(10), 5003–5007 (1996).
10. Crowley TE, Nellen W, Gomer RH, Firtel RA. Phenocopy of discoidin I-minus mutants by antisense transformation in ***Dictyostelium***. *Cell* 43(3), 633–641 (1985).
11. Eichinger L, Pachebat JA, Glockner G *et al.* The genome of the social amoeba ***Dictyostelium discoideum***. *Nature* 435(7038), 43–57 (2005).
12. Clarke L, Carbon J. A colony bank containing synthetic Col El hybrid plasmids representative of entire ***E. coli*** genome. *Cell* 9(1), 91–99 (1976).
13. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson J. *Molecular Biology of the Cell (4th Edition).* Garland Science, NY, USA, 18–19 (1994).
14. Veltman DM, Akar G, Bosgraaf L, Van Haastert PJ. A new set of small, extrachromosomal expression vectors for ***Dictyostelium discoideum***. *Plasmid* 61(2), 110–118 (2009).
15. Gaudet P, Pilcher KE, Fey P, Chisholm RL. Transformation of ***Dictyostelium discoideum*** with plasmid DNA. *Nat. Protoc.* 2(6), 1317 (2007).
16. Suess PM, Tang Y, Gomer RH. The putative G protein-coupled receptor GrlD mediates extracellular polyphosphate sensing in ***Dictyostelium discoideum***. *Mol. Biol. Cell* 30(9), 1118–1128 (2019).