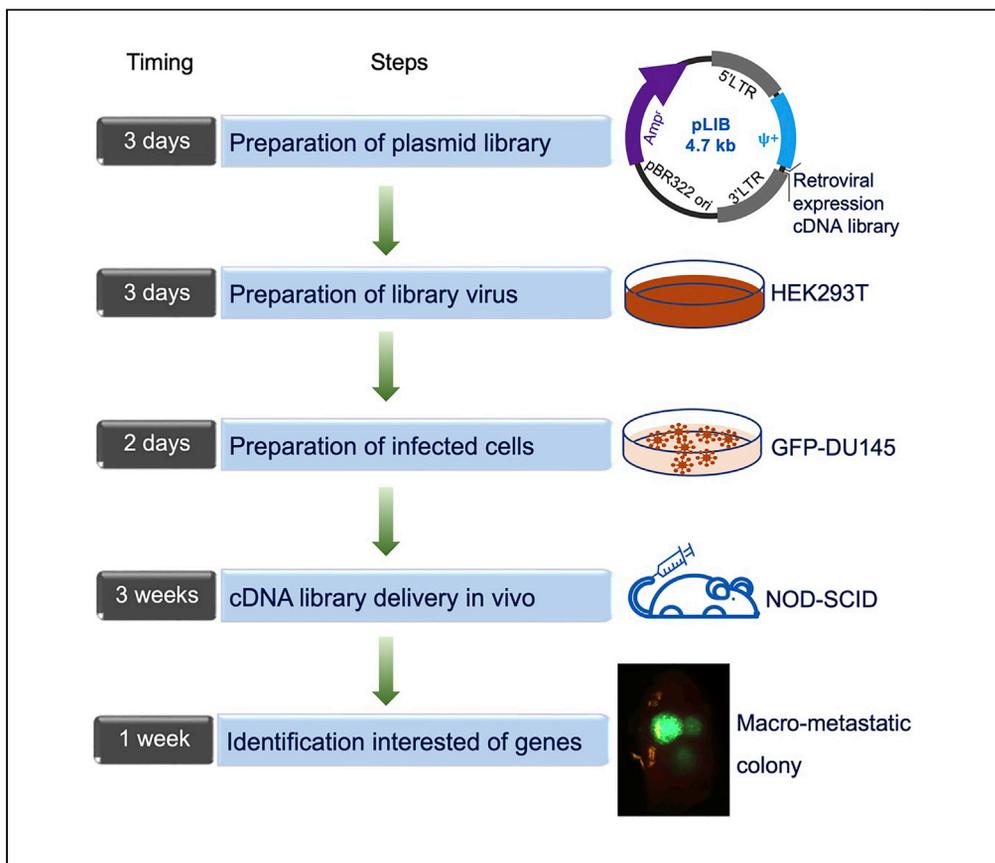


## Protocol

# *In vivo* gain-of-function cDNA library screening for colonization genes in a mouse model of pulmonary metastasis



We provide a protocol for gain-of-function (GOF) cDNA screen of genes that foster cancer cell colonization of secondary tissues, the last and most lethal step of the metastasis cascade. We present techniques for cDNA viral library preparation and delivery leading up to the recovery of colonization-promoting sequences in a proof-of-concept DU145-based mouse model of pulmonary metastasis. Adapted to other cDNA libraries and cancer models, this approach would prove widely useful in enumerating intrinsic genetic determinants underlying metastatic colonization.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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### Highlights

Colonization is the last and most lethal bottleneck step of the metastasis cascade

Gain-of-function (GOF) cDNA screen was adapted to probe cancer colonization of lung

DU145 cells harboring GOF prostate cDNA library generated pulmonary macro-metastases

Genomic PCR of macro-colonies exposed inserted cDNAs of potential colonization genes

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## Protocol

# *In vivo* gain-of-function cDNA library screening for colonization genes in a mouse model of pulmonary metastasis

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<https://doi.org/10.1016/j.xpro.2022.101413>

## SUMMARY

We provide a protocol for gain-of-function (GOF) cDNA screen of genes that foster cancer cell colonization of secondary tissues, the last and most lethal step of the metastasis cascade. We present techniques for cDNA viral library preparation and delivery leading up to the recovery of colonization-promoting sequences in a proof-of-concept DU145-based mouse model of pulmonary metastasis. Adapted to other cDNA libraries and cancer models, this approach would prove widely useful in enumerating intrinsic genetic determinants underlying metastatic colonization.

For complete details on the use and execution of this protocol, please refer to Tu et al. (2021).

## BEFORE YOU BEGIN

### Institutional permissions

In vitro and animal experiments were performed in accordance with and following approval by Harvard Committee on Microbiological Safety (COMS) and Institutional Animal Care and Use Committee (IACUC) of Beth Israel Deaconess Medical Center. Investigators following this protocol need to first seek approval from their own institutional regulatory agencies for the use of microbiological tools and animals in experimental research.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Bacterial and virus strains</b>		
DH10B Competent Cells	Thermo Fisher Scientific	Cat# EC0113
<b>Chemicals, peptides, and recombinant proteins</b>		
Dulbecco's modified Eagle's medium	Corning	Cat# 10-017-CV
Fetal bovine serum	Gibco	Cat# 26400044
Phosphate-Buffered Saline (PBS) 10×	Multicell	Cat# 311-012-CL
Glycerol	Thermo Fisher Scientific	Cat# 158920025
Agar	Fisher Bioreagents	Cat# BP1423-500
Agarose	KSE Scientific	Cat# BMK-A1705
LB broth	Fisher Bioreagents	Cat# BP1426-2

(Continued on next page)



**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ampicillin	Corning	Cat# 69-52-3
Polybrene	Santa Cruz	Cat# sc-134220
<b>Critical commercial assays</b>		
Fugene HD	Roche	Cat# 04709705001
Lipofectamine 2000	Invitrogen	Cat# 11668019
Qiagen DNeasy Blood & Tissue Kits	QIAGEN	Cat# 69506
Qiagen Maxi Prep	QIAGEN	Cat# 12162
DNA ladder 1 kb+	Invitrogen	Cat# 10787026
PicoMaxx High Fidelity PCR System	Agilent	Cat# 600424
Advantage™-GC 2 PCR Kit	Clontech	Cat# 639119
<b>Experimental models: Cell lines</b>		
HEK293T (Passage <10)	ATCC	Cat# ACS-4500
DU145 (Passage <10)	ATCC	Cat# HTB-81
<b>Experimental models: Organisms/strains</b>		
NOD/SCID (mouse) (8–10 weeks males)	Charles River	Strain code 394
<b>Oligonucleotides</b>		
pLIB-3' primer	Integrated DNA Technologies; <a href="#">Tu et al. (2021)</a>	5'-ACC TAC AGG TGG GGT CTT TCA TTC CC-3'
pLIB-5' primer	Integrated DNA Technologies; <a href="#">Tu et al. (2021)</a>	5'-AGC CCT CAC TCC TTC TCT AG-3'
<b>Recombinant DNA</b>		
Human prostate retroviral library	Clontech	Cat# 634204
pCMV-VSV-G	<a href="#">Stewart et al. (2003)</a>	Addgene Cat# 8454
pUMVC	<a href="#">Stewart et al. (2003)</a>	Addgene Cat# 8449
pCMV-dR8.2 dvpr	<a href="#">Stewart et al. (2003)</a>	Addgene Cat# 8455
pRRL3-GFP	Whitehead Institute	N/A
<b>Software and algorithms</b>		
GraphPad Prism 8	GraphPad Software	<a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a>
<b>Other</b>		
C1000 Thermal Cycler	Bio-Rad	C1000 Touch
SteREO stereomicroscope	Zeiss	Discovery. V8

**MATERIALS AND EQUIPMENT**

**LB/Amp agar plate**

Reagent	Final concentration	Amount
LB broth	n/a	25 g
Agar	n/a	15 g
ddH <sub>2</sub> O	n/a	up to 1 L
Ampicillin	100 µg/mL	1 mL
Total		1 L

Autoclave (121°C, 30 min) LB broth mixture and allow to cool until 50°C before adding ampicillin. Pour mixture into 15-cm dishes (15–20 mL/plate) and allow solidification at room temperature (25°C). Store LB/Amp plates at 4°C (maximum storage time < 1 month).

**LB/Amp medium**

Reagent	Final concentration	Amount
LB broth	n/a	25 g
ddH <sub>2</sub> O	n/a	up to 1 L
Ampicillin	100 µg/mL	1 mL
Total		1 L

Autoclave (121°C, 30 min) LB mixture and allow to cool until 50°C before adding ampicillin. Store medium at room temperature (25°C) (maximum storage time < 1 week).

Cell culture medium		
Reagent	Final concentration	Amount
Dulbecco's modified Eagle's medium	n/a	500 mL
Fetal bovine serum	10%	50 mL
Total		500 mL

Store media at 4°C (maximum storage time < 2–3 weeks). Warm the medium to 37°C before using.

## STEP-BY-STEP METHOD DETAILS

### Setting up the plasmid library

⌚ Timing: 3 days

This section describes determination of the titer and amplification of the plasmid library.

1. Determining plasmid library titer.
  - a. Prepare LB/Amp agar plates. Measure 25 g broth powder and 15 g bacteriological agar and swirl to mix in 1 L ultrapure water. Autoclave on liquid cycle (121 degree, 30 min) then cool to around 50°C in a temperature-controlled water bath. Add Ampicillin (final concentration 100 µg/mL) then pour the mixture into 18–20 15 cm plates (15–20 mL per plate) and allow so solidify at room temperature (25°C).
  - b. Dilute 1 µL of the bacterial plasmid library into 1 mL of LB broth and mix by gentle vortexing as Dilution A.
  - c. Dilute 1 µL of Dilution A into 1 mL of LB broth and mix by gentle vortexing as Dilution B.
  - d. Remove 50 µL aliquots from Dilution B and spread onto each prewarmed LB/Amp agar plate.
  - e. Leave the plate at room temperature (25°C) for 20 min and then incubate it inverted at 37°C overnight.
  - f. Count the total number of colonies and calculate the titer by following this formula: (colony# from Dilution B/Plating volume) × 10<sup>3</sup> × 10<sup>3</sup> × 10<sup>3</sup> = colony-forming unit (cfu) per mL.

**Note:** The titer should be at least 10-times higher than the number of independent clones present in the library. In this case, since our library size was  $\sim 8.0 \times 10^6$ , our cfu was  $> 10^8$ .

2. Amplification of plasmid library.
  - a. Prepare enough of the cDNA library for transfection. Typically, this represents about 1 µL of 1,000-fold-diluted cDNA library culture (in E.coli DH10B) diluted in 150 µL of LB broth before spreading. It takes about 20 plates (150-mm) to amplify the whole cDNA library.
  - b. Incubate plates at 37°C overnight.
  - c. Add 5 mL of LB medium to each plate and scrape off colonies into 2 liters of LB/Amp broth. Repeat to ensure as much as possible was recovered from plates.
  - d. Incubate cultures in a shaking incubator at 37°C to obtain a saturated culture (OD<sub>600</sub> > 2.0).
  - e. Proceed with plasmid isolation using QIAGEN Maxi Prep (Catalog number 12162) (<https://www.qiagen.com/us/resources/download.aspx?id=0bd0c5fb-c271-43e7-af43-32d539374fa9&lang=en>) to obtain highly purified plasmid library.
3. Verification of cDNA representation.
  - a. Use the 5' and 3' LIB primers (sequences in the [key resources table](#)) to amplify cDNA inserts by Advantage™-GC 2 PCR Kit (<https://www.takarabio.com/resourcedocument/x32404>) in C1000 Thermal Cycler.

#### PCR reaction mix

Reagent	Amount
5x GC 2 PCR Buffer	10 $\mu$ L
GC Melt (5 M)	5 $\mu$ L
Primer mix (10 $\mu$ M each)	2 $\mu$ L
Advantage-GC 2 Pol. Mix (50x)	1 $\mu$ L
50x dNTP mix (10 mM each)	1 $\mu$ L
DNA template	~100 ng/ $\mu$ L
PCR-grade H <sub>2</sub> O	up to 50 $\mu$ L

#### PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	3 min	1
Denaturation	94°C	30 s	35
Annealing/Extension	68°C	1.5 min	
Final extension	68°C	3 min	1

- b. Conduct gel electrophoresis on the reactions to see a smear pattern consistent with the library size intervals. For the pLIB library we used here, that pattern should be between 0.5 kb to 5 kb size (Figure 1A).

**Note:** The library used here was commercially procured, but cDNA libraries can be prepared in-house from any RNA pool. Flanking linkers can be added to the 5' and 3' insert ends using sequences compatible with 5' and 3' LIB primers or any other linker sequences determined fitting by the investigator.

**△ CRITICAL:** If the need arises, the amplified bacterial library can be stored frozen in 33% glycerol at  $-80^{\circ}\text{C}$  for later use.

#### Preparation of viral library

⌚ **Timing:** 3 days

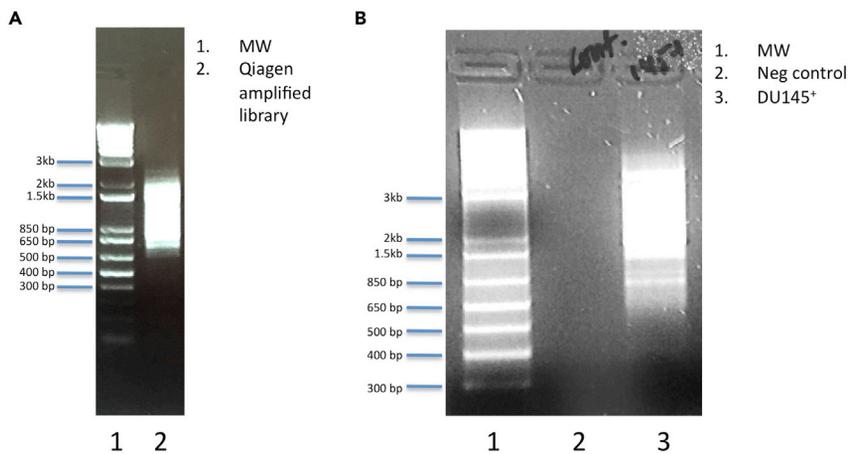
This section describes packaging plasmid library into viral particles.

4. Culture of packaging cells.
  - a. Plate HEK293T cells at a density of  $6.0 \times 10^6$  cells per 100-mm plate 12 h before transfection.

**△ CRITICAL:** Replace old medium with fresh medium before transfection (1–2 h).

**Note:** The number of packaging cell plates should be calculated and scaled based on library size. For example, the pLIB-human prostate retroviral library clone size we used here was  $\sim 10 \times 10^6$ , so 10-times more packaging cells were needed, i.e.,  $100 \times 10^6$  cells.

5. Transfection of packaging cells.
  - a. Transfect 10  $\mu$ g of plasmid DNA along with 1  $\mu$ g pCMV-VSV and 10  $\mu$ g pUMVC using 63  $\mu$ L Lipofectamine 2000 per 100-mm plate. Adapt viral envelope and packaging plasmid based on the nature of the plasmid (e.g., retroviral or lentiviral plasmid) carrying library inserts.



**Figure 1. Library representation**

(A) PCR of pooled bacterial library DNA after expansion.

(B) PCR of genomic DNA isolated from library-transduced DU145 cells. Lane 2, negative control without primers.

**Note:** Gently swirl plates to make sure the transfection solution is distributed uniformly throughout the culture surface.

6. Virus recovery.
  - a. Aspirate old medium at 10–12 h post-transfection, then wash the cells twice with 1 × PBS and add 5–6 mL of fresh medium.
  - b. Aspirate medium 32–38 h after transfection and pass through 0.45 μm filters.
  - c. Media containing undiluted viral particles can be immediately used, stored at 4°C for use within 24 h, or frozen at –80°C for later use.

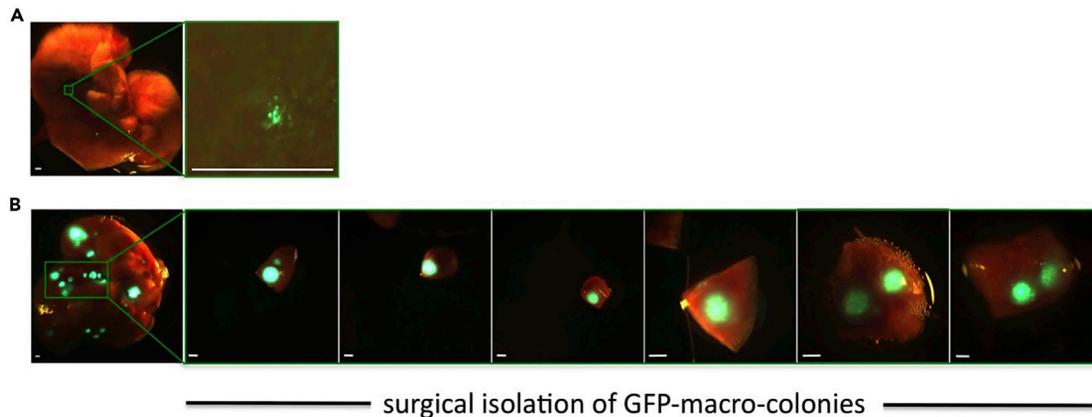
### Cancer cell infection

⌚ Timing: 2 days

This section describes viral library transduction of cancer cells.

7. Preparation of GFP-DU145 cells.
  - a. Infect DU145 cells with pRRL3 lentivirus to generate GFP-DU145 cells.
  - b. Plate GFP-DU145 cells at a density of  $2.0 \times 10^6$  cells per 100-mm plate 12 h before infection.
  - c. Generate 1:10 dilution of filtered library virus (preferably fresh) and administer to the GFP-DU145 cells in the presence of 5 μg/mL polybrene for 18 h.
  - d. To ensure the library is well represented, conduct genomic PCR of infected cells after 48–72 h using 5' and 3' LIB primers. A smear between 0.5–5 kb should be obtained, indicating adequate transcript representation (Figure 1B). Of note, comparative library sequencing could also be conducted to ensure maintenance of library complexity.

⚠ **CRITICAL:** First, dilute the virus-containing media at least two-fold with fresh media. Second, it is very important to maintain a virion number that is substantially lower than the number of cells to be infected in order to reduce the risk of more than one cDNA insertion per cell. In our case here, and as an example, we used a multiplicity of infection (MOI) of 0.2, which we determined using pLAPSN, an expression plasmid containing the sequence of bacterial alkaline phosphatase. Assessment of alkaline phosphatase activity in the infected population allowed for a calculation of percentage of



**Figure 2. Library screening in vivo**

(A and B) Pictures of GFP-positive cancer cell clusters or macro-colonies in lungs excised from mice 3 weeks after tail vein injection with control GFP-DU145 cells (A) or GFP-DU145 cells expressing library cDNAs (B) imaged under fluorescence microscopy. Bars shown indicate 1 mm.

cells transduced, which, when combined with the dilution factor used and the number of cells plated provided us with a surrogate measure of viral titer and virion numbers to utilize.

### Cellular library delivery in vivo and recovery of candidate colonization genes

⌚ Timing: 3–4 weeks

This section describes generation of metastases and verification of candidate colonization genes.

8. Generation of lung metastases.
  - a. Mix  $1.5 \times 10^5$  of the infected GFP-DU145 cells in 100  $\mu$ L PBS supplemented with 1% FBS and intravenously inject via tail vein into recipient NOD/SCID mice (8–10 weeks old).
  - b. After 18 days, sacrifice these mice and excise and wash lungs in ice cold PBS. Lay down extracted lungs flat on a clean TC plate and examine the metastatic colonies using fluorescence-equipped dissecting SteREO stereomicroscope (Zeiss, Axio). Surgically cut pieces of lungs harboring distinct visible ( $\sim$ 1 mm in diameter) GFP-positive colonies, minimizing the carryover of peripheral lung tissues (Figure 2). (troubleshooting 1).
9. Colonization gene recovery.
  - a. Isolate genomic DNA from GFP-positive colonies using Qiagen DNeasy ([https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&ved=2ahUKEwj1xebG86\\_3AhUkhIkEHdJkAFMQFnoECAUQAQ&url=https%3A%2F%2Fwww.qiagen.com%2Fbr%2Fresources%2Fdownload.aspx%3Fid%3D63e22fd7-6eed-4bcb-8097-7ec77bcd4de6%26lang%3Den&usg=AOvVa w345qmlTImF31ptxHmQKeF](https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&ved=2ahUKEwj1xebG86_3AhUkhIkEHdJkAFMQFnoECAUQAQ&url=https%3A%2F%2Fwww.qiagen.com%2Fbr%2Fresources%2Fdownload.aspx%3Fid%3D63e22fd7-6eed-4bcb-8097-7ec77bcd4de6%26lang%3Den&usg=AOvVa w345qmlTImF31ptxHmQKeF)).
  - b. To identify pLIB-library-derived integrated sequences, amplify DNA using manufacturer-supplied 5' and 3' linker primers following PicoMaxx High Fidelity PCR system guidelines. (troubleshooting 2).

#### PCR reaction mix

Reagent	Amount
10 $\times$ PicoMaxx reaction buffer	5 $\mu$ L
100 mM dNTP mix (25 mM of each dNTP)	0.4 $\mu$ L
Primer #1 (100 ng/ $\mu$ L)	1 $\mu$ L

(Continued on next page)

### Continued

Reagent	Amount
Primer #2 (100 ng/ $\mu$ L)	1 $\mu$ L
50 $\times$ dNTP mix (10 mM each)	1 $\mu$ L
PicoMaxx high fidelity PCR system	1 $\mu$ L
PCR-grade H <sub>2</sub> O	up to 50 $\mu$ L

### PCR cycling conditions

Segment	Temperature	Time	Cycles
1	95°C	2 min	1
2	95°C	40 s	35
	Primer T <sub>m</sub> -5°C	30 s	
	72°C	1 min/kb	
3	72°C	10 min	1

- c. Resolve amplification reactions using 1% agarose electrophoresis (Figure 3).
- d. Cut out resolved sequences and send out for DNA sequencing, then BLAST for gene identification. (troubleshooting 3).

### EXPECTED OUTCOMES

Inserted sequences isolated from genomic DNA derived from macro-colonies are expected to represent cDNA sequences that are postulated, when re-expressed on their own, to be instigators of cancer cell metastatic growth in mouse lungs (e.g., Aldolase A; Figure 3). Also, it is expected for visible >1 mm macro-colonies to be clonal and derived from one cell harboring integrated cDNA sequence(s).

### LIMITATIONS

It is our experience with the pLIB library used here that many of the sequences recovered by PCR from mouse lungs represented in fact pieces of cDNAs rather than full-length gene sequences. However, this did not preclude the identification of real modifiers of the colonization phenotype, as we describe in our paper of Tu et al. (2021). Since the pLIB platform was commercially generated, we could not optimize to purge such partial sequences from the library. This aspect limited the number of full-length sequences recovered from our otherwise proof-of-concept screen. Of note, cDNA libraries can be generated in-house from any tissue, and flanking linker sequences can be added as well to facilitate sequence recovery using PCR.

### TROUBLESHOOTING

#### Problem 1

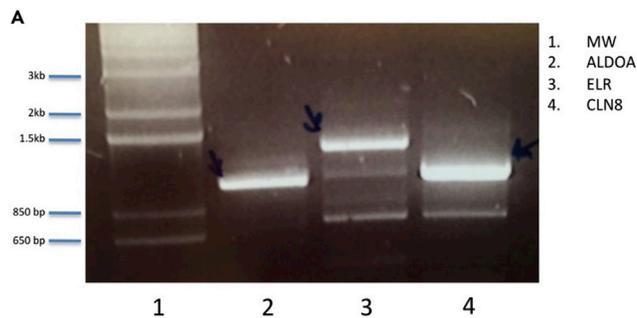
Insufficient recovery of genomic DNA quantities in step 9a.

#### Potential solution

For rather smaller nodules, recovering sufficient genomic DNA presented a significant challenge. To circumvent this step, we resorted to harvesting the nodules, digesting them with collagenase (Type I) under tumbling overnight at 37°C and plating the cells for expansion in tissue culture. Within 7–14 days, recovered cancer cells (which were GFP-labeled, hence their growth was followed using fluorescence microscopy) were then harvested for genomic DNA isolation and PCR. This approach has consistently led to better outcomes for subsequent reactions, although the fact that cancer cell populations drift in culture and may vary in their heterogeneity is a prominent drawback/concern with this expansion.

#### Problem 2

Failed amplification reactions from macro-colonies in step 9b.



**Figure 3. Identification of colonization genes**

(A) Gel electrophoresis of PCR products amplified from genomic DNA derived from isolated macrocolonies. Fulllength amplified products subsequently identified by sequencing as ALDOA (Aldolase A), p67 elastin-laminin receptor (ELR), and Ceroid lipofuscinoses gene 8 (CLN8) are shown.

### Potential solution

We have experienced a higher than expected ratio of failed PCR reactions compared to the successful ones in which bands were easily identifiable. This could be due to impurities that are inadvertent when surgically removing metastatic nodules (in this case, from the lungs), suboptimal PCR conditions for genomic DNA integration sites of the library sequences in the specific nodule isolated, or insufficient quantities of genomic DNA recovered. We found that using aseptic techniques in lung excision, minimizing surgical handling on ice, extensive washing of the lung pieces with cold 1× PBS or 1× HBSS, and immediate processing of tissues for genomic DNA isolation can help with minimizing impurities and in avoiding genomic DNA degradation. Regarding genomic PCR, we have experimented with several genomic DNA extraction and PCR kits and have rested on PicoMaxx High Fidelity PCR system as a successful system to amplify picogram quantities of recovered DNA. We also found that inclusion of 2%–5% DMSO in the reactions improved yields.

### Problem 3

Amplification of multiple sequences from a single macro-colony in step 9c.

### Potential solution

We have experienced the appearance of several bands when amplifying genomic DNA from isolated colonies. These occurrences could derive from (1) the fact that the macro-colony was derived in effect from multiple clones that coexisted/cooperated to generate the macro-metastasis, (2) the insertion of multiple library cDNA sequences into the genome of cancer cells that generated the macro-colony, and (3) non-specific priming during the amplification step. While we cannot control the first possibility, and the MOI of 0.2 minimized (but did not absolutely preclude) the occurrence that more than one cDNA was inserted per infected cell, we were able however, to improve PCR by modifying the cycling/temperature parameters and by increasing DMSO concentrations in the amplification reactions.

### Problem 4

Failed sequencing reactions in step 9d.

### Potential solution

Many of our submitted sequencing reactions failed because of poly-T's and slippage. Although there is nothing that we could do experimentally to avoid such outcomes, we found that submitting >100–200 ng of PCR product decreased the chances of this happening. We have also found that replacing the 3' library primer with Oligo-dT primer in the PCR assays lessened such occurrences.

### Problem 5

Use of retroviral library that is only amplified in dividing cells.

### Potential solution

We used here a commercial retroviral library for proof-of-principle purposes. We recognize, however, that retroviruses carrying certain cDNAs may be lost in subsequent steps if they infect non-dividing cells, and are hence unable to integrate into genomic DNA, thus leading to loss of representation. Although we used here highly proliferative HEK293T and DU145 cells, this remains a possibility considering the heterogeneity of these populations and the dynamics of quiescence and division among pre-existing clones. To circumvent this problem and transduction of all cells, use of lentiviral vectors for library cloning would be recommended.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Antoine E. Karnoub ([akarnoub@bidmc.harvard.edu](mailto:akarnoub@bidmc.harvard.edu)).

#### Materials availability

This study did not generate new unique reagents.

#### Data and code availability

This study did not generate/analyze [datasets/code].

### ACKNOWLEDGMENTS

We acknowledge institutional support from Beth Israel Deaconess Medical Center (to A.E.K.), Department of Defense BCRP grants BC160702 and BC142139 (to A.E.K.), and NCI grants CA207322 and CA249223 (to A.E.K.).

### AUTHOR CONTRIBUTIONS

Z.T. and A.E.K. wrote the manuscript. Both authors contributed to the manuscript and approved it for publication.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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