Journal of Visualized Experiments

Droplet Digital TRAP (ddTRAP): Adaptation of Telomere Repeat Amplification Protocol to droplet digital PCR

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59550R2
Full Title:	Droplet Digital TRAP (ddTRAP): Adaptation of Telomere Repeat Amplification Protocol to droplet digital PCR
Keywords:	Telomerase; TRAP assay; droplet digital PCR; cancer; enzymatic activity detection; Aging; quantitative biology.
Corresponding Author:	Andrew T. Ludlow University of Michigan Ann Arbor, MI UNITED STATES
Corresponding Author's Institution:	University of Michigan
Corresponding Author E-Mail:	atludlow@umich.edu
Order of Authors:	Mohammed E. Sayed
	Aaron L. Slusher
	Andrew T. Ludlow
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Ann Arbor, Michigan, United States of America

TITLE:

Droplet Digital TRAP (ddTRAP): Adaptation of the Telomere Repeat Amplification Protocol to Droplet Digital Polymerase Chain Reaction

AUTHORS AND AFFILIATIONS:

Mohammed E. Sayed[1], Aaron L. Slusher[1], Andrew T. Ludlow[1]

[1]School of Kinesiology, University of Michigan, Ann Arbor, MI, USA

Corresponding author:

Andrew T. Ludlow (atludlow@umich.edu)

KEYWORDS:

telomerase, TRAP assay, droplet digital PCR, cancer, enzymatic activity detection, aging, quantitative biology.

SUMMARY:

We successfully converted the standard telomere repeat amplification protocol (TRAP) assay to be employed in droplet digital polymerase chain reactions. This new assay, called ddTRAP, is more sensitive and quantitative, allowing for better detection and statistical analysis of telomerase activity within various human cells.

ABSTRACT:

The telomere repeat amplification protocol (TRAP) is the most widely used assay to detect telomerase activity within a given a sample. The polymerase chain reaction (PCR)-based method allows for robust measurements of enzyme activity from most cell lysates. The gel-based TRAP with fluorescently labeled primers limits sample throughput, and the ability to detect differences in samples is restricted to two fold or greater changes in enzyme activity. The droplet digital TRAP, ddTRAP, is a highly sensitive approach that has been modified from the traditional TRAP assay, enabling the user to perform a robust analysis on 96 samples per run and obtain absolute quantification of the DNA (telomerase extension products) input within each PCR. Therefore, the newly developed ddTRAP assay overcomes the limitations of the traditional gel-based TRAP assay and provides a more efficient, accurate, and quantitative approach to measuring telomerase activity within laboratory and clinical settings.

INTRODUCTION:

Telomeres are dynamic DNA-protein complexes at the ends of linear chromosomes. Human telomeres are composed of an array of 5'-TTAGGG$_n$ hexameric repeats which vary in length between 12–15 kilobases (kb) at birth[1]. Human telomerase, the ribonucleoprotein enzyme that maintains the telomeres, was first identified in HeLa cell lysates (cancer cell line)[2]. Together, telomeres and telomerase play a major role in a spectrum of biological processes such as genome protection, gene regulation, and cancer cell immortality[3–6].

Human telomerase is comprised primarily of two key components, namely telomerase reverse transcriptase and telomerase RNA (hTERT and hTERC, respectively). The protein subunit, hTERT,

is the catalytically active reverse transcriptase component of the telomerase enzyme. The RNA template, hTERC, provides telomerase with the template to extend and/or maintain telomeres. Most human somatic tissues have no detectable telomerase activity. The inability of DNA polymerase to extend the end of the lagging strand of DNA along with the lack of telomerase leads to the progressive shortening of telomeres after every round of cellular division. These phenomena lead to telomere shortening in most somatic cells until they reach a critically shortened length, whereby cells enter a state of replicative senescence. The maximal number of times a cell can divide is dictated by its telomere length and this block to continued cell division is thought to prevent progression to oncogenesis[7]. Cancer cells are able to overcome telomere-induced replicative senescence and continue to proliferate by utilizing telomerase to maintain their telomeres. Approximately 90% of cancers activate telomerase, making telomerase activity critically important in both cancer detection and treatment.

The development of the TRAP assay in the 1990s was instrumental in the identification of the necessary components of the telomerase enzyme, as well as for the measurement of telomerase in a wide range of cells and tissues, both normal and cancerous. The original gel-based PCR assay used radioactively labeled DNA substrates to detect telomerase activity. In 2006, the assay was adapted into a nonradioactive form using fluorescently labeled substrates[8,9]. By using fluorescently labeled substrates, users were able to visualize the telomerase extension products as bands on a gel by exposing it to the correct excitation wavelength. The sensitivity of the TRAP assay and its ability to detect telomerase activity in crude cell lysates has made this assay the most widely used method for telomerase activity detection. However, the TRAP assay has limitations. The assay is gel-based, making it difficult to perform the necessary replicates in moderate to high-throughput studies, and thus, proper statistical analysis is rarely achieved. Furthermore, the gel-based assay is difficult to quantify reliably due to the inability of detecting less than twofold differences in telomerase activity between samples. Overcoming these two limitations is critical for enzymatic activity assays such as the TRAP to move to clinical or industry settings for the detection of telomerase activity in patient samples or drug design studies.

Digital PCR was initially developed in 1999 as a means to convert the exponential and analog nature of PCR into a linear and digital assay[10]. Droplet digital PCR (ddPCR) is the most recent innovation of the original digital PCR methodology. Droplet digital PCR came about with the advent of advanced microfluidics and oil-in-water emulsion chemistry to reliably generate stable and equally sized droplets. Unlike gel-based and even quantitative PCR (qPCR), ddPCR generates absolute quantification of the input material. The key to ddPCR is the generation of ~20,000 individual reactions by partitioning samples into droplets. Following end-point PCR, the droplet reader scans each droplet in a flow-cytometer-like fashion, counting, sizing, and recording the presence or absence of fluorescence in each individual droplet (i.e., absence or presence of PCR amplicons in each droplet). Then, using Poisson's distribution, input molecules are estimated based on the ratio of positive droplets to the total number of droplets. This number represents an estimate of the number of input molecules in each PCR. Furthermore, ddPCR is performed and analyzed on a 96-well plate which allows the user to run many samples, as well as perform biological and technical replicates for proper statistical analysis. As a result, we have combined the powerful quantification and moderate-throughput nature of ddPCR with the TRAP assay to

develop the ddTRAP assay[11]. This assay is designed for users to study and robustly quantify absolute telomerase activity from biological samples[11,12]. The sensitivity of the ddTRAP allows the quantification of telomerase activity from limited and precious samples, including single-cell measurements. Furthermore, users can also study the effects of telomerase manipulations and/or drugs with absolute quantification of less than twofold changes (~50% differences). The ddTRAP is the natural evolution of the TRAP assay into the digital and higher-throughput nature of modern laboratory experiments and clinical settings.

PROTOCOL:

1. Buffer preparation and storage

1.1. Prepare 50 mL of 1x stock RNase-/DNase-free NP-40 lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% [vol/vol] NP-40, 10% [vol/vol] glycerol, 150 mM NaCl, 5 mM β-mercaptoethanol, and 0.1 mM 4-benzenesulfonyl fluoride hydrochloride (AEBSF)). This buffer can be aliquoted and stored at -20 °C for future use. Avoid freeze/thaw cycles in order to obtain an optimal lysis of cells.

1.2. Prepare 50 mL of 10x stock RNase-/DNase-free TRAP extension buffer (200 mM Tris-HCl [pH 8.0], 15 mM $MgCl_2$, 630 mM KCl, 0.5% [vol/vol] Tween 20, and 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)). This buffer can be aliquoted into 1 mL aliquots and stored at -20 °C for future use.

2. Cell lysis

2.1. Preparation of the cells

2.1.1. Thaw a frozen aliquot of NP-40 lysis buffer. Once thawed, place the lysis buffer on ice. Add phenylmethylsulfonyl fluoride (PMSF protease inhibitor) to the NP-40 lysis buffer to reach a final concentration of 0.2 mM.

NOTE: This should be done immediately prior to lysing the cells.

2.1.2. Remove any excess liquid from the collected cell pellets to ensure a dry cell pellet. Note that cell pellets, whether freshly collected or previously frozen (-80 °C or flash frozen in liquid N_2), must not contain any leftover solutions from previous centrifugations as these solutions may interfere with downstream procedures.

2.1.3. Grow, collect, and freeze both telomerase-positive and telomerase-negative cell lines (BJ fibroblasts, IMR-90 or U2OS) in order to use them as positive and negative controls. Use new control lysates every time the assay is performed to ensure assay reproducibility.

NOTE: It is advised that a large culture of telomerase-negative cells is grown and aliquoted prior to freezing to remove any tissue-culture-related artifacts that may be introduced during the long-

term culture of cell lines to help ensure reproducible results of the negative control sample.

2.1.4. Place the cell pellets on ice. If the cells were frozen, allow them to thaw briefly on ice.

NOTE: Typical cell pellet sizes for the ddTRAP are between 500,000 and 1,000,000 cells. Smaller cell pellets may also be used if necessary, but the cell number must/ideally should be known. The ddTRAP can also be performed based on protein input using a BCA protein assay (input usually is 1 µg).

2.2. Lysing of the cells

2.2.1. Lyse the cells in the NP-40 lysis buffer. Maintain a cell equivalence of 25,000 cells/µL of buffer. For example, lyse a pellet (containing 1,000,000 cells) in 40 µL of NP-40 lysis buffer. Gently pipet the lysate up and down in order to break open the cells. Try to avoid making bubbles.

2.2.2. Allow the cells to lyse on ice for 30 min. Make sure to gently vortex the lysate to prevent clusters of cell debris from forming. This can be done every 10 min and is meant to keep the lysate a homogenized mixture.

2.2.3. Dilute the cell lysate (25,000 cells/µL) 1:20 in NP-40 lysis buffer, making the new cell equivalence 1,250 cells/µL. For example, dilute 5 µL of cell lysate into 95 µL of lysis buffer.

3. Telomerase extension reaction

3.1. Prepare a master mix (**Table 1**) for the telomerase extension reaction (per reaction).

NOTE: It is best to prepare the extension reaction master mix during the cell lysis and store it on ice.

3.1.1. Pipet 48 µL of extension master mix into each PCR tube.

3.1.2. Add 2 µL of the diluted (1,250 cells/µL) lysate to the extension reaction. The total volume should now be 50 µL with a final cell equivalence of 50 cells/µL.

3.2. Perform the telomerase extension reaction (**Table 2**).

3.3. Store the telomerase extension products at 4 °C for up to 3–5 days; however, it is **optimal** to use the extension products within the first 24 h.

4. Droplet digital PCR setup

4.1. Prepare a master mix (**Table 3**) for the ddTRAP (per reaction).

NOTE: Once the reagents are at room temperature, do not place them or the master mix on ice.

Placing the mix on ice may increase viscosity and lead to poor droplet formation. The DNA polymerase in the ddPCR supermix is a hot start and should be stable at room temperature. The ddPCR supermix may be stored at 4 °C after an initial thaw from -20 °C.

4.1.1. Pipet 19.8 µL of ddPCR master mix into each PCR tube.

4.1.2. Add 2.2 µL of the extension reaction to each tube. Note that the total volume should now be 22.2 µL.

NOTE: The total amount of sample needed for a ddTRAP is 20 µL (cell equivalence of 100 cells). The extra volume is a precaution for pipet error or sample loss.

4.2. Set up the droplet generation cartridge.

NOTE: The cartridge contains three different columns of wells that are labeled.

4.2.1 Load 20 µL of the reaction prepared according to step 4.1.2 into the sample well (middle well) in the cartridge. Avoid bubbles when pipetting the sample.

NOTE: If there are bubbles, gently tap the side of the cartridge so that these bubbles come to the top of the solution.

4.2.2 Load 70 µL of droplet generation oil into the oil well (left well).

NOTE: In order to avoid contamination, strictly use a separate set of pipettes and tips for ddPCR droplet generation steps. The order of loading the cartridge is important. The sample must be loaded prior to loading oil as oil is heavier and will fill the microfluidic chambers and lead to poor droplet formation. The minimum number of samples that can be run is eight. All wells of the cartridge must be loaded with the sample as any empty well will lead to a halt in the droplet generation from the droplet generator. If adequate samples are not available to load all eight wells within a cartridge, 20 µL of ddTRAP master mix (step 4.1) can be loaded into the remaining cartridges.

4.2.3. Secure the gasket in place by tethering it to the ends of the cartridge.

NOTE: The lack or improper placement of the gasket will prevent the generator from generating droplets.

4.2.4. Place the loaded and assembled cartridge into the droplet generator.

NOTE: The cartridge is recognized by a magnet in the generator, which will inform the user when the cartridge is placed properly.

4.3. Remove the cartridge once the droplets are generated and the cycle is complete (~60–90 s).

4.3.1. Gently remove the gasket from the cartridge. Pipet the newly generated droplets (right well), using a multichannel pipette, into a 96-well PCR plate.

NOTE: The approximate volume for the newly generated droplet emulsion should be 40–43 µL.

4.3.2. Heat seal the plate with aluminum foil PCR plate seals once all the samples are loaded in the 96-well plate in order to prevent evaporation during the PCR steps.

4.4. Load the 96-well plate into the thermocycler and perform the following PCR reaction (**Table 4**).

NOTE: All ramp rates between the temperature steps must be set to 2.5 °C/s in order to properly heat the reactions.

5. Detection of telomerase extension products

5.1. Load the 96-well plate in the droplet reader.

NOTE: Make sure to orient the plate properly so that the **A1** sample matches with that of the holder.

5.1.1. Open the software associated with the droplet reader. Double-click the first well **A1** to open the sample/well editor screen.

5.1.2. Click **Experiment** and select **ABS** from the drop-down menu.

NOTE: **Experiment** defines the type of assay to be used (i.e., absolute quantification or gene copy number assay). **ABS** stands for absolute quantification.

5.1.3. Select **QX200 ddPCR Evagreen Supermix** to ensure that the correct detection method is employed by the reader. Click **Apply** in the lower right-hand side of the well editor screen to save the user-defined settings to all of the highlighted wells.

NOTE: **SUPERMIX** defines the type of PCR mix and detection chemistry that will be read by the reader.

5.1.4. Click on **TARGET** in the well editor screen of the software in order to define the sample.

5.1.5. Define the **TYPE** of sample by clicking the **TARGET** drop-down menu and selecting either **unknown**, **reference**, or **NTC** (no-template control).

NOTE: **Unknown** would refer to an experimental sample, **reference** could be a control sample of known telomerase activity, and an **NTC** is a critical control for the determination of assay validity

in terms of contamination and background signal.

5.1.6. Label all the samples in the **Sample Name** section and click **Apply** to ensure the highlighted wells are edited appropriately.

5.2. Click **Run** to run/read the plate. Select either **Columns** or **Rows** in the **RUN OPTIONS** screen when prompted to inform the machine about the orientation the plate should be read in.

6. Data analysis

6.1. Determine the number of accepted droplets for each sample by clicking on individual wells. Double-click the individual wells or column/row headers to view and analyze the sample data.

NOTE: The most important criteria on whether or not to proceed with the analysis of a particular sample is the number of "accepted droplets". For the ddTRAP, samples with 10,000 or more accepted droplets are valid for further analysis. Data can be provided to the user in many formats, including a .csv table, .jpg images, histograms, etc. There are many resources available for the in-depth analysis of ddPCR data, including the ddTRAP[11,13]. These resources offer a guide to analyzing ddTRAP data, from selecting thresholds[11,13] to choosing samples for further analysis[11,13], identifying false positives and negatives[13], and general troubleshooting[13].

6.2. Highlight the wells representing sample replicates and NTC samples. Analyze the samples in comparison to either NTC or negative control lines, such as BJ cells (as listed above in step 2.1.2).

6.2.1. Manually set the threshold for the samples by clicking on the icon for setting thresholds on the bottom left of the screen. Set thresholds for each individual well or for multiple wells at a time (recommended).

NOTE: If background is detected in the NTC, it can be subtracted from all of the other samples to 'normalize' the signal and ensure that only the true positive signal is analyzed. NTC values are typically in the 0.2–1 molecule/µL range for the NP-40 lysis buffer system. Other buffer systems and components would need to be tested (for example, CHAPS buffers).

REPRESENTATIVE RESULTS:

Using the ddTRAP, telomerase activity was measured in a cell panel consisting of the following cell lines (**Figure 1**): nonsmall cell lung cancer (H2882, H1299, Calu6, H920, A549, and H2887), small cell lung cancer (H82 and SHP77), and telomerase-negative fibroblasts (BJ). One million cell pellets were lysed in NP-40 buffer, and telomerase extension reactions were performed in biological triplicates. A common and highly recommended negative control is the "NTC", the no-template control. This sample is generated by adding NP-40 lysis buffer (2 µL) to the telomerase extension reaction and proceeding with the extension products in an identical manner to other samples containing actual cell lysate. This sample allows the user to subtract the background signal, if any, to better quantify the telomerase activity. Although not shown in this figure, it is also possible to heat inactivate the lysate at 95° C for 5 min prior to the telomerase extension

reaction as another negative control. This negative control is preferred if cell/sample abundance is not an issue.

By measuring the fluorescence intensity of every single droplet in the droplet emulsion, the droplet reader was able to estimate the concentration of input molecules (molecules/microliter) using the Poisson distribution (**Figure 1A**). In the case of the ddTRAP, these input molecules were telomerase extension products. The mechanism of the ddTRAP assay was as follows: telomerase extended the TS substrate. These extended substrates acted as the PCR templates in the ddPCR. Quantification of the PCR-amplified substrates provided a representation of telomerase enzymatic activity within a given cell line. Every droplet was plotted as shown in **Figure 1A**. Setting the threshold for a ddTRAP may be subjective; however, with the proper negative controls, the user can easily do so. In the example shown in **Figure 1A**, a threshold was set for all three biological replicates for SHP77, H2887, and NTC. Positive droplets had a fluorescence intensity around 6,000 fluorescence amplitude (FA) and formed a clear population at the top and separate from negative droplets around 1,100 EFA. Therefore, the threshold may be set at ~2,000 FA in this experiment.

Once the data was collected and exported, it was possible to calculate the total telomerase extension products per cell equivalent between all the samples (**Figure 1B**). The signal from every well was an absolute concentration (molecules/microliter). By multiplying the concentration by 20 (input volume of the sample into ddPCR cartridges), the user may obtain the total number of molecules. This number can then be divided by the known cell equivalent (in our performance of the ddTRAP, we used 100 cells). This final value is in the units of telomerase extension products per cell equivalent as shown on the *y*-axis.

FIGURE AND TABLE LEGENDS:

Figure 1: Telomerase activity in a lung cancer panel. (**A**) The 1D amplitude of droplet fluorescence intensity (fluorescence amplitude) for SHP77, H2887, and NTC. Wells for SHP77, H2887, and NTC were selected and a manual threshold was set at 2,000 FA. (**B**) Telomerase activity was estimated from the measured concentration of the nucleic acids detected following PCR and plotted in order to compare telomerase activity in lung cancer lines. FA = fluorescence amplitude units.

DISCUSSION:

The measurement of telomerase activity is critical to a plethora of research topics including, but not limited to, cancer, telomere biology, aging, regenerative medicine, and structure-based drug design. Telomerase RNPs are low abundant, even in cancer cells, making the detection and study of this enzyme challenging. In this paper, we described the step-by-step procedures for the newly developed ddTRAP assay to robustly quantify telomerase activity in cells. By combining the traditional telomerase extension reaction with ddPCR, we were able to quantitatively detect telomerase activity (telomerase-extended products) in lung cancer cells.

The ddTRAP assay relies on the same theory as the TRAP assay. Cell lysates are obtained by lysing

cells in a nonionic detergent (NP-40) lysis buffer to maintain enzyme activity and then used to perform a telomerase extension reaction of the "TS" substrate/primer. The novelty of the ddTRAP relies on the formation of droplets prior to PCR. The partitioning of the sample into droplets allows the assay to obtain absolute quantification of telomerase activity per cell.

Telomerase activity was measured in lung cancer cell lines, using the ddTRAP. One of the key limitations of the gel-based TRAP assay is the number of samples that can be processed at a time. Most gel combs can only accommodate up to 20 wells/samples. In contrast, the ddTRAP can run up to 96 samples at a time, significantly increasing the number of samples that can be processed at once. We assayed telomerase activity in eight cell lines. Most importantly, we ran each telomerase extension reaction in biological triplicates for a total of 24 extension reactions. We then were able to run each extension reaction as three technical replicates for PCR for a total of 72 samples on the ddPCR plate. Furthermore, the ddTRAP assay provides reproducible results with an interday CV (coefficient of variation) of 5.1% for 100 cell equivalents and an intraday CV of 8.61% for 100 cell equivalents. Interday and intraday represent biological replicates run on two different plates or the same plate, respectively, on HeLa cell lysates[11]. If 72 samples were processed in the traditional TRAP or any other gel-based assay, it would require much more hands-on time. This leads to higher costs of reagents, more valuable time needed from employees/students/trainees, a higher gel-to-gel variability, and a lack of reproducibility between users and laboratories. The ability to reliably and easily run replicates in the ddTRAP is a dramatic improvement over gel-based assays and allows many more samples to be processed efficiently, which aids in the statistical analysis of the data.

Finally, less variability between the samples in general in the ddTRAP and the possibility to perform the necessary replicates allows users to observe less than twofold changes between samples. The major limitation of most gel-based assays is the lack of proper quantification. Here, using the ddTRAP, we can detect the subtle differences in between the various lung cancer lines. The subtle differences can be critical when it comes to comparing drugs targeting telomerase activity and deciding whether or not to move forward with small molecule compounds from high-throughput screens.

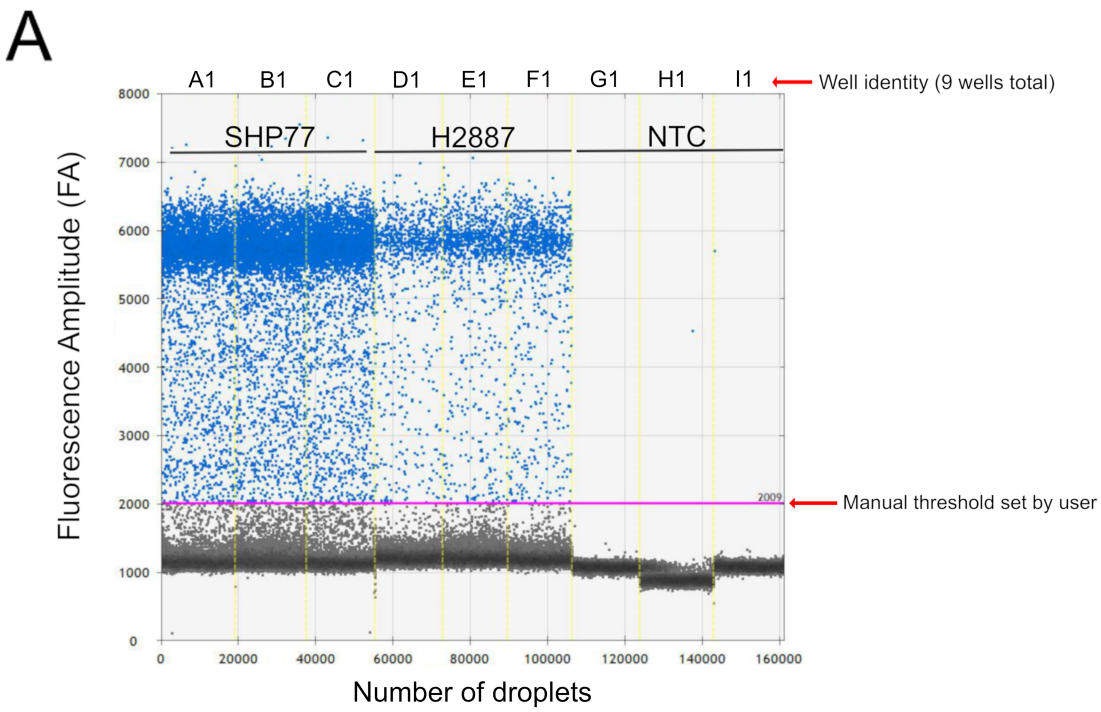
ACKNOWLEDGMENTS:
The authors would like to acknowledge funding sources from the National Institutes of Health (NIH) (NCI-R00-CA197672-01A1). Small cell lung cancer lines (SHP77 and H82) were a generous gift from Drs. John Minna and Adi Gazdar from the UT Southwestern Medical Center.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Frenck Jr., R. W., Blackburn, E. H., Shannon, K. M. The rate of telomere sequence loss in human leukocytes varies with age. *Proceedings of the National Academy of Sciences of the United States of America.* **95** (10), 5607-5610 (1998).
2. Morin, G. B. The human telomere terminal transferase enzyme is a ribonucleoprotein that

synthesizes TTAGGG repeats. *Cell.* **59** (3), 521-529 (1989).
3. de Lange, T. How telomeres solve the end-protection problem. *Science.* **326** (5955), 948-952 (2009).
4. Shay, J. W., Wright, W. E. Role of telomeres and telomerase in cancer. *Seminars in Cancer Biology.* **21** (6), 349-353 (2011).
5. Kim, W., Shay, J. W. Long-range telomere regulation of gene expression: Telomere looping and telomere position effect over long distances (TPE-OLD). *Differentiation.* **99**, 1-9 (2018).
6. Robin, J. D. et al. Telomere position effect: regulation of gene expression with progressive telomere shortening over long distances. *Genes Development.* **28** (22), 2464-2476 (2014).
7. Shay, J. W., Wright, W. E. Senescence and immortalization: role of telomeres and telomerase. *Carcinogenesis.* **26** (5), 867-874 (2005).
8. Norton, J. C., Holt, S. E., Wright, W. E., Shay, J. W. Enhanced detection of human telomerase activity. *DNA Cell Biology.* **17** (3), 217-219 (1998).
9. Herbert, B. S., Hochreiter, A. E., Wright, W. E., Shay, J. W. Nonradioactive detection of telomerase activity using the telomeric repeat amplification protocol. *Nature Protocols.* **1** (3), 1583-1590 (2006).
10. Vogelstein, B., Kinzler, K. W. Digital PCR. *Proceedings of the National Academy of Sciences of the United States of America.* **96** (16), 9236-9241 (1999).
11. Ludlow, A. T. et al. Quantitative telomerase enzyme activity determination using droplet digital PCR with single cell resolution. *Nucleic Acids Research.* **42** (13), e104 (2014).
12. Huang, E. E. et al. The Maintenance of Telomere Length in CD28+ T Cells During T Lymphocyte Stimulation. *Scientific Reports.* **7** (1), 6785 (2017).
13. Ludlow, A. T., Shelton, D., Wright, W. E., Shay, J. W. ddTRAP: A Method for Sensitive and Precise Quantification of Telomerase Activity. *Methods Molecular Biology.* **1768**, 513-529 (2018).

Figure Figure 1



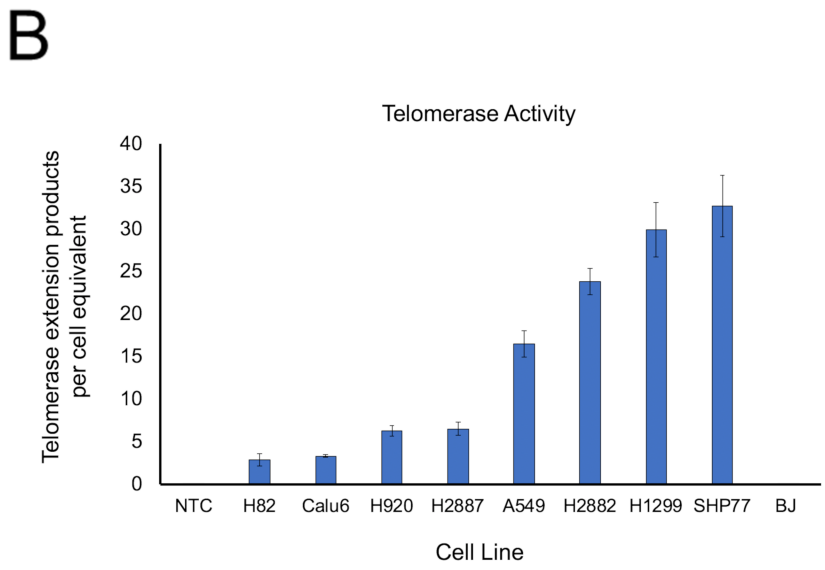


Table 1 (Step 3.1)

Reagent (stock concentration)	Volume (µl)
TRAP extension Buffer (10X)	5
TS Substrate/Primer (10 µM)	1
dNTPs mix (2.5 mM of each)	1
Nuclease Free or DEPC H20	42

Table 2 (Step 3.2)

	Temperature (°C)	Time (min)
Step 1:	40	25
Step 2:	5	95
Step 3:	∝	12

Table 3 (Step 4.1)

Reagent (stock concentration)	Volume (µl)
ddPCR Supermix (2X)	11
TS Substrate/Primer (10 µM)	0.11
ACX Substrate/Primer (10 µM)	0.11
Nuclease Free or DEPC H20	10.78

Table 4 (Step 4.4)

	Time	Temperature (°C)
Step 1:	5 min	95
Step 2:		95
Step 3:	30 sec	54
Step 4:	30 sec	72
40 cycles (**step 2-4**)		
Step 5:	10 min	72
Step 6:	∞	12

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company
1 M Tris-HCl pH 8.0	Ambion
1 M MgCl2	Ambion
0.5 M EDTA pH 8.0	Ambion
Surfact- Amps NP-40	Thermo Scientific
100% Ultrapure Glycerol	Invitrogen
phenylmethylsulfonyl fluoride	Thermo Scientific
2-Mercaptoethanol	SIGMA-ALDRICH
Nuclease Free H20	Ambion
2.5 mM dNTP mix	Thermo Scientific
2 M KCl	Ambion
100% Tween-20	Fisher
0.5 M EGTA pH 8.0	Fisher
Telomerase Substrate (TS) Primer	Integrated DNA Technology (IDT
ACX (Revers) Primer	Integrated DNA Technology (IDT
Thin walled (250 ul) PCR grade tubes	USA Scientific
QX200 ddPCR EvaGreen Supermix	Bio Rad
Twin-Tec 96 Well Plate	Fisher
Piercable foil heat seal	Bio Rad
Droplet generator cartidges (DG8)	Bio Rad
Droplet generator oil	Bio Rad
Droplet generator gasket	Bio Rad

96-well Thermocycler T100	Bio Rad
PX1 PCR Plate Sealer	Bio Rad
QX200 Droplet Reader and Quantasoft Software	Bio Rad
ddPCR Droplet Reader Oil	Bio Rad
Nuclease Free Filtered Pipette Tips	Thermo Scientific

Catalog Number	Comments/Description
AM9855G	RNAse/DNAse free
AM9530G	RnAse/DNAse free
AM9261	RNAse/DNAse free
28324	
15514011	RNAse/DNAse free
36978	Powder
516732	
AM9932	RNAse/DNAse free
R72501	2.5 mM of each dATP, dCTP, dGTP and dTTP
AM9640G	RNAse/DNAse free
9005-64-5	
50-255-956	RNAse/DNAse free
Custom Primer (HPLC Purified)	5'- AATCCGTCGAGCAGAGTT-3'
Custom Primer (HPLC Purified)	5'- GCGCGGCTTACCCTTACCCTTACCCTAACC -3'
1402-2900	strips, plates, tubes etc.
1864034	
Eppendorf 951020362	
1814040	
1863008	
1863005	
1863009	

1861096

1814000

1864001 and 1864003

1863004

10 ul, 20 ul , 200 ul and 1000 ul

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Droplet Digital TRAP (ddTRAP): Adaptation of Telomerase Repeat Amplification Protocol to droplet digital PCR
Author(s):	Mohammed E. Sayed1, Aaron L. Slusher1, Andrew T. Ludlow1

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

[x] Standard Access [ ] Open Access

Item 2: Please select one of the following items:

[x] The Author is **NOT** a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove
JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Andrew T. Ludlow
Department:	School of Kinesiology, Movement Science
Institution:	University of Michigan
Title:	Assistant Professor
Signature:	
Date:	12/12/18

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Dear Dr. Wu,

We thank the editor for the thoughtful comments and suggestions. Below you will find the individual point by point response to each editor comment below (in red font).

Thanks.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
-We proof read the document.

2. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "Evagreen" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.
-We have removed all by one necessary instance to refer to “Evagreen”.

3. Please split some long steps into more sub-steps so that each step contains only 2-3 actions and is less than 4 lines.
-This has been corrected.

4. Lines 154-159: Please make this chart as table and upload the table to your Editorial Manager account in the form of an .xls or .xlsx file.
-This has been corrected.

5. Lines 171-174: Please make this chart as table and upload the table to your Editorial Manager account in the form of an .xls or .xlsx file.
-This has been corrected.

6. Lines 183-188: Please make this chart as table and upload the table to your Editorial Manager account in the form of an .xls or .xlsx file.
-This has been corrected.

7. Lines 243-250: Please make this chart as table and upload the table to your Editorial Manager account in the form of an .xls or .xlsx file.
-This has been corrected.

8. Step 2.1.2: Please ensure that all text is written in the imperative tense.
-This has been corrected.

9. 3.3: Please write this step in the imperative tense.
-This has been corrected.

10. 5.1.2: Please ensure that all text is written in the imperative tense.
-This has been corrected.

11. 5.1.3: Please ensure that all text is written in the imperative tense.

-This has been corrected.

12. 5.2: Please ensure that all text is written in the imperative tense.
-This has been corrected.

13. 6.1.1: Please ensure that all text is written in the imperative tense.
-This has been corrected.

14. 6.1.2: Please ensure that all text is written in the imperative tense.
-This has been corrected.