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ddTRAP: A Method for Sensitive and Precise Quantification of Telomerase Activity

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Abstract

Telomerase is a cellular RNA template-dependent reverse transcriptase that adds telomere repeats to the 3' ends of chromosomes. Telomerase is expressed almost universally in tumor cells (>85%) to maintain telomere length, thus providing the ability of tumor cells to avoid senescence and to have unlimited replication ability, one of the key hallmarks of cancer. ddTRAP (droplet digital Telomere Repeat Amplification Protocol) is a two-step assay with whole cell lysates that utilizes a telomerase-mediated primer extension followed by droplet digital PCR (ddPCR) detection of extended products. The adoption of the TRAP assay to ddPCR has resulted in improved throughput, increased sensitivity and better repeatability of the TRAP assay. The protocol described below details our procedures for ddTRAP.

Keywords

Telomerase activity; Droplet digital PCR

1 Introduction

Telomere maintenance and telomerase enzyme activity are almost universal features of transformed cancer cells [1–4]. Telomeres are repeat DNA sequences (5'-TTAGGG_n-3') found at the ends of chromosomes [5, 6]. Telomeres function the in completion DNA replication each cell cycle and also provide a “capping” function by preventing recombination, end-to-end fusions, and degradation at the chromosome ends [7, 8].

Telomeres, therefore prevent unwanted DNA damage signaling at chromosome ends and may be a potent initial tumor suppressor mechanism when telomeres become critically short and cells enter senescence [9,10]. In normal diploid cells telomeres shorten with each cell division due to the end replication problem (i.e., due to the placement of the terminal RNA primer, the inability of DNA polymerase to fully replicate the lagging strand of DNA, and the lack of the enzyme telomerase there is a loss of about 60 nucleotides per cell division/DNA replication cycle). Almost all tumor cells adapt a telomere maintenance mechanism that utilizes telomerase to maintain telomeres [1]. Telomerase is a ribonucleoprotein complex that utilizes a protein component with reverse transcriptase activity (hTERT) and a RNA template (*hTR* or *hTERC*) to add telomere repeats to

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chromosome ends [11]. The two major molecular properties that are commonly assayed are telomerase activity (ability of the enzyme to extend a substrate by adding nucleotides) and repeat addition processivity (the number of TTAGGG repeats added to a substrate)[12] Since the vast majority of cancer cells utilize telomerase to maintain telomeres, telomerase activity an important transformation biomarker, and thus accurate detection is critical. The discovery that telomerase synthesizes telomeric repeats onto 3' ends of chromosomes led to the development of an assay for the detection and measurement of its activity in cells and tissues and was established over 20 years ago [1].

To measure the activity of telomerase the most common assay is the telomere-repeat amplification procedure or TRAP, a two-step procedure involving telomerase mediated primer extension and PCR-based detection of extended products. Briefly, cells are lysed in a buffer containing a detergent, then a portion of the lysate is mixed with an extension reaction containing the telomerase substrate (a DNA oligonucleotide) and dNTPs which are used by telomerase (if present in the sample) to add hexameric telomere repeats. Finally, the extended substrates are PCR amplified and detected (See Fig. 1 for a graphical representation of the ddTRAP assay). We have recently adapted the original TRAP assay that provided only relative quantification (quantitated to an internal standard DNA) to be detected with intercalating DNA dye (i.e., Evagreen®) on the Bio-Rad droplet digital PCR QX150/200 Eva-green® compatible droplet reader, we call this assay the “ddTRAP” [13]. The adaptation of the TRAP assay to droplet digital PCR improved the sensitivity, repeatability, and throughput of the TRAP assay [13]. These properties of ddTRAP have enabled single cell telomerase assays and the screening of genes and small molecules aimed at manipulating telomerase activity. The detailed procedure below describes how to perform a ddTRAP to assay and quantitate telomerase activity in adherent or suspension transformed cells and primary human cells in culture.

2 Materials

Prepare all solutions and set up all reactions using typical PCR precautions *see* **Note 1**.

2.1 Whole Cell Lysate Preparation

1. Use of a well-characterized telomerase positive cell line such as HeLa, HEK293, H1299, MDA-231, HCT116, or HT1080 as a positive control.
2. A cell line lacking telomerase activity such as U2OS (human osteosarcoma) or primary (nontransformed) BJ fibroblasts should be used as a negative control.
3. Scale up the cell line you plan to use as positive and negative controls and freeze aliquots for future use to avoid unintentional changes that could occur in long term cultured cell lines.
4. NP-40 lysis buffer (RNase/DNase-free): 10 mM Tris-HCl, pH 8.0; 1 mM MgCl₂; 1 mM EDTA; 1% (vol/vol) NP-40; 25 mM sodium deoxycholate; 10% (vol/vol) glycerol; 150 mM NaCl; 5 mM β-mercaptoethanol; 0.1 mM AEBSF (4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride). (*See* **Note 2** for cell extract preparation.)

5. Ca^{2+} - and Mg^{2+} -free PBS: 137 mM sodium chloride, 1.5 mM potassium phosphate, 7.2 mM sodium phosphate, 2.7 mM potassium chloride, pH 7.4.
6. RNase/DNase-free DEPC-treated dH_2O (Ambion).
7. Cell counting device such as a hemacytometer, Coulter® counter, or automated cell counter (e.g., TC20, Bio-Rad). Alternatively, protein quantification can be determined prior to extension reaction with a BCA protein assay for cell extracts (*see Note 2*).
8. Bicinchoninic Acid protein assay kit—such as BCA protein assay kit (23225, ThermoFisher).
9. Spectrophotometer that can measure absorbance at 562 nm (for BCA assay).

2.2 Telomerase Substrate Primer Extension Reaction

1. Ultrapure BSA (50 mg/mL, Ambion).
2. $50 \times$ dNTP mix (2.5 mM of each dATP, dCTP, dGTP, dTTP).
3. 10 μM telomerase substrate, “TS” primer (telomerase substrate/primer for extension; 5'-AATCCGTCGAGCAGAGTT HPLC purified).
4. $10 \times$ TRAP buffer (RNase/DNase-free): 200 mM Tris-HCl, pH 8.3; 15 mM MgCl_2 ; 630 mM KCl; 0.5% Tween 20; 10 mM EGTA.
5. Thin walled (250 μL volume) PCR grade tubes/strips/plates.
6. Thermocycler.
7. RNase/DNase-free DEPC-treated dH_2O (Ambion).

2.3 ddPCR Detection of Telomerase Extended Substrates

1. 10 μM ACX primer (reverse amplification primer for detection—5'-GCGCGGCTTACCCTTACCCTTACCCTAACC HPLC purified—*see Note 9* about primers).
2. 10 μM telomerase substrate, “TS” primer (telomerase substrate for extension, also forward primer for detection—5'-AATCCGTCGAGCAGAGTT HPLC purified).
3. $2 \times$ Evagreen® super mix for ddPCR (Bio-Rad).
4. RNase/DNase-free DEPC-treated dH_2O (Ambion).
5. Twin-Tec 96-well plate—Eppendorf 951020362 (Fisher).
6. Pierceable foil heat seal—(Bio-Rad #1814040).
7. Droplet generator cartridges (DG8) (Bio-Rad 186–3008).
8. Droplet generator oil (Bio-Rad 186–3005).
9. Droplet cartridge gaskets (DG8) (Bio-Rad 186–3009).

10. QX200 evagreen ddPCR supermix (Bio-Rad).
11. Thermocycler capable of fitting the 96-well skirted plates and adjusting the temperature ramp rate (i.e., Bio-Rad T100).
12. Droplet reader oil.
13. Droplet reader (Bio-Rad QX150/200) capable of reading Eva- green[®] DNA binding dye.
14. PX1 PCR plate sealer (Bio-Rad).

3 Method/Procedures

3.1 Preparation of Tissue Culture Cells for ddTRAP Assay

1. Culture cells to a desired density, typically to a density of 90% confluence (one needs to be consistent from experiment to experiment since in some cases cell density can affect telomerase activity). We typically grow cells on 10 cm dishes for TRAP assay analysis.
2. Trypsinize and count cells *see Note 1*.
3. Pellet cells. Centrifuge cells at $2000 \times g$ at room temperature and aspirate media (*see Note 2*). Cells can be washed with cold $1 \times$ PBS following initial pelleting to remove carry over tissue culture media but this is not necessary.
4. If cell counting is not performed, ddTRAP assays can be performed using protein concentration (*see Note 13*).
5. Pellets can be used immediately by placing cells on ice in lysis buffer.
6. Pellets can be also be stored for later use by flash freezing in liquid nitrogen and stored at -80°C (*see Note 2*).

3.2 Lysis of Cells

1. Generate a whole cell lysate by adding 40 μL of NP40 lysis buffer to pelleted cells (per 1 million cells) and incubate on ice for 40 min (*see Notes 12 and 14*).
2. During the lysis period periodically vortex or mix vigorously at least twice (e.g., at 15 min and 30 min of lysis). This is essential to ensure the cells are lysed and homogenous (*see Note 14*).

3.3 Telomerase Primer Extension Reaction Setup

1. While the cells are in lysis buffer, set up the extension reaction mix as shown below in Table 1 and store on ice.
2. Set up tubes for dilution of the lysates on ice.
3. Lysates need to be diluted to a cell equivalent of 1250 cells per microliter (2 μL of lysate in 18 μL of NP40 lysis buffer) on ice.

4. Prepare all diluted lysates before mixing with the extension reaction. Please *see* **Note 3** below for details on extension reactions.
5. In an ice cold tube containing 49 μL of extension reaction mix add 1 μL of diluted lysate to thin walled (250 μL) PCR tubes. Reactions should be mixed by pipetting.
6. When all lysates are added to the extension reaction mix, the PCR plate/tubes should be immediately moved to a thermocycler set with the following reaction conditions: 25 $^{\circ}\text{C}$ for 40 min, 95 $^{\circ}\text{C}$ for 5 min, and 12 $^{\circ}\text{C}$ hold. Please *see* **Notes 15–17** for further information on the lysis step and storage of lysates.

3.4 Digital PCR Setup

1. Using the PCR precautions outlined in *see* **Notes 4–11** prepare a master mix containing a final concentration of; 1 $\times$ Evagreen ddPCR Super Mix v2.0 (Bio-Rad), 50 nM TS primer, 50 nM ACX primer, 50 cell equivalents or less of extension product and dH_2O to 20 μL per sample, with 10% extra. See Table 2 for example volumes.
2. Allow reaction mix to reach room temperature (*see* **Notes 5–8**).
3. Set up the droplet generation (DG) cartridge. Load 20 μL of PCR mixture into the sample well in the cartridge. Then add 70 μL of droplet generation oil for Evagreen® into the oil well. Secure a gasket on the DG cartridge.
4. Place assembled droplet cartridge into the DG machine.
5. Remove cartridge from the DG machine once droplet generation cycle is completed (about 90s).
6. Transfer droplets to PCR plate. Remove gasket gently. Using an eight-channel pipet remove ~42 μL of emulsion (droplets) from the droplet wells in the cartridge and place into a 96-well plate (*see* **Note 9**).
7. Seal plate. Once all samples are loaded into the 96-well PCR plate, the plate must be foil sealed to prevent evaporation and light exposure of the emulsion (droplets).
8. Run PCR. Load the 96-well plate into the thermocycler and close the lid. In these experiments, we use a Bio-Rad T100. PCR reaction conditions—All ramp rates between temperature steps must be set to 2.5 $^{\circ}\text{C}/\text{sec}$ in order to achieve even heating of the reaction mixture.

Thermocycling:

95 $^{\circ}\text{C}$ for 5 min (activation of hot-start polymerase).

40 cycles of:

95 $^{\circ}\text{C}$ for 30 s.

54 $^{\circ}\text{C}$ for 30 s.

72 °C for 30 s.

22 °C hold.

Timing = 1 h 45 min.

3.5 Detection of the Telomerase Extended PCR Amplified Substrates

1. Read the droplets. Load the 96-well plate into the droplet reader plate holder matching well “A1” in the proper orientation.
2. Set up the reader. Open[®] software on the desktop of the laptop computer synchronized with the droplet reader. Double click well “A1” in the plate template. This will change the upper part of the screen to display “sample name,” “experiment,” “assay name,” and assay channels (fluorescence dyes fam or Vic[®]/hex. *See Note 10.*
3. Define the wells. Click “Experiment” and a pull-down menu with choices for experiment types will be displayed. Choose “absolute quantification” for the experiment type. Select wells (highlight wells) and click apply or press enter. With the same wells highlighted, Select assay channels as unknown channel 1 (6FAM/Evagreen[®]). Click “apply” or press enter. Name the assay—As an example for ddTRAP put the word ddTRAP and indicate the extension time used (i.e., ddTRAP 40 min ext.) and press apply. Name the samples.
4. Run the plate. Click run. A screen will prompt you to save the template. Give your plate a logical name (record this in your lab note book or experiment log in Excel[®] etc.). Click “save.” A screen will prompt you to pick the dye types (Fam/Vic or Fam/Hex) for ddTRAP pick FAM/Vic and if you want to read the wells in columns or rows.

3.6 Data Analysis

1. Determine if the sample is valid for further analysis. The data generated from the droplet reader is given in several formats. The most important component that should be addressed when analyzing ddTRAP data is the number of droplets generated. This can be queried several ways in the QuantaSoft[®] software. By clicking “events,” the total number of droplets per well can be visualized in a histogram. Alternatively under the “table” display the column titled “accepted droplets” will give the investigator the same information. We typically consider a sample valid for further analysis with more than 10,000 accepted droplets; more stringent criteria of 12,000 accepted droplets can also be applied, but the same criteria should be applied across all samples in a given experiment and all experiments in a particular manuscript unless noted in the figure legend.
2. Set the threshold. Setting the thresholds between positive and negative droplets can be a subjective task when analyzing digital PCR data and thus certain standard criteria should be applied for each new assay. For the ddTRAP assay, positive droplets typically fall between 6000 and 10,000 fluorescent units,

however since telomerase generates amplicons of various sizes and is a “GC”-rich template, longer molecules (i.e., those with more repeats added) appear lower on the amplification plot. We use the following guides to set thresholds for ddTRAP assays: (1) A “no-template lysis buffer control” (NTC-LB) sample should be analyzed first, set threshold at 2000 fluorescence units above population of negative droplets (typically around 4000 fluorescence units see Fig. 1). There may be some background in this sample, however given the quantitative nature of ddTRAP, this background can and should be subtracted from all positive wells. See Fig. 1 for an example of background in NTC-LB controls. (2) Set thresholds for all wells. We typically use the same threshold for all wells as was set for the NTC-LB. Occasionally, the population of negative droplets will vary between wells and in this case a well specific background needs to be set to analyze the data. We recommend in this case that the threshold be set at a minimum of 2000 fluorescent units above the “negative population.” (3) Dealing with “rain.” “Rain” or intermediate droplets between the negative and positive populations are common with the ddTRAP assay and can have an impact on the quantitative results. Users must be cautious when setting thresholds as to not skew data one way or another. To avoid potential problematic “rain” we recommend using as few cell equivalents as possible and repeating analysis with samples that produce a large amount of “rain.” When this is not possible the user must set thresholds equivalently for all samples to be compared. Once the thresholds are set quantitative information is now available (see **Notes 18 and 19**).

3. Extracting quantitative data. In terms of the digital TRAP assay we refer to the data as “the number of telomerase extended substrates/products per number of cell equivalents analyzed.” This information is obtained after correction for background signal and number of cell equivalents analyzed. From the QuantaSoft® software refer to the “concentration in molecules per microliter” column in the tabular view of the software. This table can be exported as a .csv file and analyzed in Excel®. First we generate background corrected data by subtracting the NTC-LB control concentration (molecules per microliter) from each unknown sample and positive control. This background corrected value is then converted to total telomerase- extended products by multiplying by 20 (20 µL PCR volume), this number is then divided by the number of cell equivalents added to the extension reaction (typically 50). See example data below.
4. Determining telomerase-extended products per cell. If 1,000,000 cells are harvested, lysed in 40 µL, the lysate diluted 1:20 to give 1250 cells per microliter, 1 µL of the diluted lysate added to a 50 µL telomerase extension (25 cell equivalents per microliter) and then 2 µL of the extension (50 cell equivalents) added to a 20 µL PCR the For example calculation see **Note 20** (Table 3a and 3b).

4 Notes

1. Cell counts are critical to determine cell equivalents added to the extension reactions and for calculating the number of telomerase-extended products per cell.
2. Pellets are typically stable under ideal conditions for 1 year at -80°C . If you freeze cells it is best to remove all extra liquid before placing in the -80°C .
3. Following the extension reaction small white particulate matter may be observed in the extension reaction, to avoid carrying this material over to the ddPCR setup briefly centrifuge the PCR tubes and avoid pipetting from the bottom of the extension reactions. The amount of detergent from the lysate/ extension can negatively impact droplet formation. We found that 0.008% of NP40 did not negatively impact droplet formation. We suggest testing concentrations of detergent higher than 0.008% on droplet formation prior to performing assays if higher concentration lysates are used.
4. Ten percent (10%) extra volume of each reagent should be used so that the final reaction volume is 22 μL to help prevent volume shortage when pipetting into the droplet generator cartridge.
5. You must make samples in intervals of 8.
6. The polymerase in the $2 \times$ ddPCR Evagreen[®] Super Mix is hot-start so all steps should be performed at RT.
7. Performing PCR set up on ice may increase solution viscosity ($2 \times$ PCR master mix) which will affect droplet formation and is not recommended.
8. Order of loading the droplet generation cartridge essential. The oil is “heavy” which if loaded first causes the oil to flood the microfluidics and create a situation of poor droplet formation. Thus it is essential that you load your PCR sample first then the oil for proper droplet formation. After the PCR and oil are added to the cartridge wells attach a droplet generator cartridge gasket. The droplet generation machine works only with a full cartridge of 8 wells and a gasket properly attached to the cartridge. The machine stops when air (i.e., an empty well) is encountered or when a gasket is misplaced on the cartridge. If you only have 12 samples you must still set up 16 reactions, just four of the reactions will be blanks (mixing 11 μL $2 \times$ ddPCR supermix Evagreen[®] control with 11 μL of water per open well) or no template controls (NTC).
9. Transferring the droplets to the PCR plate is critical. Move slowly and consistently. Do not repeat pipet or completely aspirate the droplets from the pipet, as this will enhance droplet breaking/blending.
10. Evagreen[®] is read on the same channel as 6-fam.
11. Due to the sensitivity of the PCR-based TRAP assay, avoiding contamination is of utmost importance for assay success. We suggest setting up a dedicated set of pipettes and an area for ddTRAP assays only. Always using barrier tips. Do not

use ART tips. Since telomerase is a reverse transcriptase with an RNA component, special precaution must be taken to ensure an RNase-free environment. All solutions should be made with DEPC-treated water, and benches, pipettes and labware decontaminated with an RNase inactivator such as RNaseZap[®].

12. An alternative lysis buffer using CHAPS (0.5–1.0%) can be used in place of NP40 to generate cell extracts instead of whole cell lysates. Whole cell lysates give maximal activity detection, which is important for comparing telomerase-modulating compounds quantitatively [14, 15]. If desired, cell extracts can be made (lyse as above but spin down debris at $10,000 \times g$ for 10 min at 4 °C), and supernatant removed for analysis. Cell extracts will underestimate the telomerase activity but may be useful in analyzing primary samples that may contain PCR inhibitors (such as primary tumor samples, [14]). CHAPS lysis buffer and cell extracts should be used when analyzing tissue samples. Mechanical homogenization (avoid heat treatment) or a mortar and pestle can be used to help disrupt the tissue. Centrifuge as above and determination of protein concentration as in note.
13. Determination of equivalent loading input for telomerase assays can be done in two ways: Cell counting or protein concentration. We prefer cell counting when using whole cell lysates. We advise pelleting large numbers of cells so that the impact of loss of cells due to aspiration techniques is minimal. We typically use greater than 300,000 cell pellets and prefer 1×10^6 cell pellets for optimal data. Protein concentration should be determined when cell extracts are used in ddTRAP. For ddTRAP 1–6 µg of protein is sufficient to detect telomerase activity from HeLa cells. Also, protein concentrations are needed for tissue samples but caution should be noted that telomerase positive cells will be comixed with telomerase silent stromal cells.
14. Cell pellets must be thoroughly lysed in NP40 buffer for a minimum of 40 min and a maximum of 1 h on ice. For pellets up to 1 million cells, typically 40 µL of NP40 lysis buffer is sufficient for lysis (1 million cells lysed in 40 µL of buffer results in 25,000 cell equivalents per microliter of lysate). We do not recommend using large volumes of lysis buffer to avoid dilutions; this may cause loss of telomerase activity or data that is not quantitative and repeatable. Do not lyse more than 45 min.
15. Lysates can also be stored at -80 °C but telomerase enzyme activity decreases overtime in the freezer, thus we recommend the use of fresh lysates and that lysates are aliquoted to avoid freeze-thaw cycles.
16. We have found that adding 1250 cell equivalents to a 50 µL extension reaction is the most reproducible in the ddTRAP assay (this results in a final extension reaction cell equivalent of 25 cells per microliter). Since we typically use 1 million cell count pellets, a 1:20 dilution in NP40 lysis buffer is necessary (2 µL of lysate in 18 µL of NP40 lysis buffer) and will generate a diluted lysate with 1250 cell equivalents per microliter. Once the lysates are diluted, 2 µL are added

to the extension reaction mixture in thin wall PCR tubes/plates on ice. Tip—make sure that all lysates and dilutions are homogenous prior to pipetting. Important control reactions should be set up to confirm assay integrity. Controls such as treatment with RNase A to digest the RNA component of telomerase and or heat treatment (95 °C for 10 min) prior to extension can be performed to ensure specific detection of telomerase in the ddTRAP assay (Fig. 2). These controls are important for analysis of new tumor lines (i.e., lines with unknown telomere maintenance strategies) and for laboratories unfamiliar with the telomerase and TRAP assays (Fig. 3).

17. An extension time of 40 min is satisfactory for quantitative detection of telomerase (see Fig. 4), longer extension times (up to 2 h) can be used to detect maximal telomerase activity however longer extension times risk degradation of the enzyme at 25 °C and thus have not been tested currently in the TRAP assay. Linear dilution analysis should be performed by inputting different amounts of cell equivalents into the extension reactions. Two types of linearity can be performed here: (1) Inputting different numbers of cell equivalents into the extension reaction (Fig. 2) or 2. Making an extension reaction and then diluting it prior to PCR to test the PCR linearity [13]. The extension products are single stranded DNA and should be used as soon as possible in the digital PCR reaction. We have found that storage of extension products at 4–12 °C overnight does not result in loss of detectable extension products (Fig. 5)
18. Setting digital PCR thresholds with “rainy” data. Since telomerase uses whole cell lysates and generates a “GC”-rich amplicon of various sizes there can be samples that produce “rain” or droplets of intermediate fluorescence intensity between the major populations of negative and positive droplets (see Fig. 4). This can cause problems in the analysis. For this reason controls are essential in the ddTRAP. We also always try to use 50 cell equivalents when possible. If samples seem to produce a lot of “rain” at this cell input we dilute the sample until better separation is achieved. Alternatively, the extension can be diluted and corrected for cell equivalents in the analysis.
19. We found that purchasing HPLC purified primers was necessary for the success of this assay, it will not work otherwise. We suggest diluting stock primers at a concentration of 100 µM and making working concentration aliquots of no more than 100 µL and avoiding freeze-thaw cycles.
20. Example calculation of cell equivalents: $1,000,000/40 \mu\text{L lysis buffer} = 25,000 \text{ cells}\mu\text{L lysate}$; diluted 1:20 = $1250 \text{ cells}\mu\text{L lysate}$; $1 \mu\text{L of } 1250 \text{ cell equivalents}/50 \mu\text{L extension reaction} = 25 \text{ cell equivalents}\mu\text{L extension reaction}$ *2 µL into 20 µL ddPCR = 50 cell equivalents analyzed.

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Fig. 1. TRAP assay theory.

Tissue culture cells are plated on a 10 cm culture dish and grown to about 90% confluence. Cells are then counted and pelleted in 1 million cell aliquots. Whole cell lysates are prepared and then added to the extension mix containing the telomerase substrate primer (TS primer) and dNTPs. The lysate containing active telomerase enzymes will extend the TS primer using the RNA template of the enzyme. The extension reaction is heat-inactivated, and the products are then PCR amplified in the presence of the reverse primer ACX and the forward primer TS to amplify the telomerase-extended substrates. The PCR products are double stranded and can be detected using Evagreen[®] dsDNA binding dye in the QX100/200 droplet digital PCR reader. Note—the bolded nucleotides in the ACX primer are mismatches with the telomerase-extended products

Fig. 2.

Workflow and optimization of droplet digital TRAP. **(a)** The ddTRAP workflow. Cells are lysed and then diluted to a concentration of 1250 cells per μl , telomerase extension products generated at a concentration of 25 cells/ μl , then telomerase is heat inactivated and extension products dispersed into droplets. PCR thermocycling is done for 40 cycles and droplets analyzed for the presence or absence of fluorescence by the droplet reader (QX150/200 Evagreen[®] compatible machine). **(b)** ddTRAP output showing BJ fibroblasts (input of 100 cell equivalents, telomerase negative), H1299 cells (input of 100 cell equivalents, telomerase positive), a lysis buffer only control, and a control with no primers and input of 100 cell equivalents of H1299 lysate to test for specificity of amplification. Only very low background signals are seen in these controls. Each well or sample of the ddPCR analyzes about 17,000 droplets. Event number at the bottom of the output represents the number of droplets counted in the wells overtime. Each dot on the ddPCR output represents a unique droplet that is either positive or negative for fluorescent signal. Fluorescence amplitude is a measure of the fluorescence detected for each droplet in the assay. Fluorescence amplitude is used to separate the positive and negative droplets. Since the droplets are detected with EvaGreen[®] double stranded DNA binding dye there will be inherent background fluorescence of DNA molecules not amplified during PCR. The heat map scale represents the density of droplets at given fluorescent amplitudes. NTC-LB = no-template control-lysis buffer ([13]; Figure is reproduced with permissions and slight modification from NAR and Oxford press - Ludlow, A.T., Robin, J.D., Sayed, M., Litterst, C.M., Shelton, D.N., Shay, J.W. and Wright, W.E. (2014) Quantitative telomerase enzyme activity determination using droplet digital PCR with single cell resolution. *Nucleic Acids Res*, 42, e104)

Fig. 3. ddTRAP control reactions and linearity. **(a)** Heat and RNase A inactivation of telomerase resulted in virtually no ddTRAP signal. This permitted a small background correction to be included, and established the specific measurement of telomerase activity from lysates. Background signal is highlighted by the red box. **(b)** HeLa cell dilution series from 50 to 1 cell equivalents produced a linear relationship ($R^2 = 0.99$) between input and detection of telomerase enzyme activity as indicated by total product generated in the ddPCR. Error bars are the standard deviation of the replicates. Although the extract was not made from a single cell, ddTRAP was able to reproducibly detect telomerase activity above background at the dilution equivalent to one cell input [13]

Fig. 4.

Comparison and correlation of ddTRAP to gel based TRAP. ddTRAP quantification is less variable than gel based TRAP and allowed accurate determination of the IC₅₀ of Imetelstat in HeLa (0.2 μM). HeLa cells were incubated with Imetelstat (0, 0.125, 0.25, 0.5, 1, and 3 μM) for 72 h. Cells were pelleted and triplicate extracts prepared from three separate tissue culture experiments (nine total extracts and extensions per dose). (a) ddTRAP quantification with 50 cell equivalents added to the PCR. (b) Gel quantification (representative gel image of two experiments in Fig. 3c). (c) Gel based TRAP was performed with 125 cell equivalents. Data are expressed as relative telomerase activity compared to control (untreated HeLa) and standard error of the mean. (d) Correlation analysis of gel-based TRAP to ddTRAP. The $P < 0.0001$ positive relationship indicates that the methods are measuring the same phenomenon. IC = Internal competitive telomerase activity substrate (also known as ITAS) [13]

Fig. 5. Dealing with “rainy” data. ddTRAP fluorescent droplet outputs are shown on the right, and quantified outputs graphed on the left.

Lysates were incubated with TS substrate for various amounts of time prior to heat inactivation, using 100 cell equivalents from H1299 cells. Data are presented as means of background corrected total telomerase products generated $\pm$ standard error of the mean. The horizontal black line represents the threshold used in these samples. It is essential when setting threshold for “rainy” data that controls are used as a guide and the same threshold is used for all samples [13]

Table 1

Extension reaction setup

Reagent	X1 (volumes in μL)	X10 (volumes in μL)
10 $\times$ TRAP buffer	5	50
50 mg/mL BSA	0.4	4
10 μM TS primer	1	10
2.5 mM dNTPs	1	10
1250 cell equivalents lysate	1	-
Water (dH_2O)	40.6	406

Table 2

PCR setup

Reagent	Volume (μL) 1×	Volume (μL) 10×	Volume (μL) 20×
2× Evagreen ddPCR super mix	11	110	220
10 μM TS	0.11	1.1	2.2
10 μM ACX	0.11	1.1	2.2
25 cells/μL extension product	2	-	
dH ₂ O	8.8	88	176

Table 3a

Data from select columns from a QuantaSoft spreadsheet

Sample type	Experiment	Accepted droplets	Concentration (molecules per microliter)
NTC-LB	Abs quant	14,267	1.64
Positive control cancer line	Abs quant	12,587	43.8

Table 3b

Example calculations for telomerase-extended products per cell

Data quantification	NTC-LB background correction'	Total telomerase extension products	Telomerase extension products per cell
Description of step	Subtract "NTC-LB" concentration from unknowns to generate background corrected data	Multiply background corrected concentration by 20	Divide total telomerase extension products by the number of cell equivalents added to the extension reaction. In this example 50 cell equivalents.
Positive control cancer line	42.16	843.2	16.864