Studying the oncosuppressive functions of *PTENP1* as a ceRNA

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Abstract

PTENP1 is a processed pseudogene of the tumour suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN). It functions post-transcriptionally to regulate *PTEN* by acting as a sponge for microRNAs that target *PTEN*. *PTENP1* therefore functions as a competitive endogenous RNA (ceRNA), competing with *PTEN* for binding of microRNAs (miRNA) and thereby modulating PTEN cellular abundance. Studies of the overexpression of *PTENP1* all confirm its oncosuppressive function to be mediated through the suppression of cell proliferation, induction of apoptosis, and inhibition of cell migration and invasion of cancer cells of differing types. These oncosuppressive functions are a direct consequence of miRNA binding by *PTENP1* and the subsequent liberation of PTEN from miRNA induced suppression. In this chapter, we will focus initially on the description of a high efficiency transient transfection method to introduce and overexpress *PTENP1* in the cell type of interest, followed by accurate methodologies to measure transfection efficiency by flow cytometry. We will then continue to describe two methods to analyse cell proliferation, namely the CCK-8 assay and Click-iT® EdU assay. Due to commonalities in the manifestation of the oncosuppressive effects of *PTENP1*, mediated through its role as a ceRNA, the methods presented in this chapter will have wide applicability to a variety of different cell types.

Keywords: *PTEN*, *PTENP1*, pseudogene, overexpression, cell proliferation, ceRNA

Running title: Methods to study *PTENP1* oncosuppressive functions

1. Introduction

Pseudogenes, once classified as "waste" genomic remnants, have now been reclassified as functional units since the discovery of their capacity to regulate gene expression. Transcribed pseudogenes are a class of long non-coding ribonucleic acid (lncRNAs, >200 nt)) that share sequence similarity with their cognate protein-coding transcripts [1]. However, due to harbouring point mutations and frameshift mutations, which can lead to premature stop codons, there is a lack of protein production, as a consequence. Therefore, pseudogene transcripts lack translational ability [2]. Three categories of pseudogenes have been described on the basis of functional mechanisms. These are (a) unprocessed, (b) unitary and (c) processed pseudogenes [3, 4]. Unprocessed pseudogenes are direct gene replicates of their parent genes, usually with intact intron-exon and promoter structures [4, 5]. Unitary pseudogenes lack functional coding parent genes due to accumulation of mutations, which ultimately results in a lack of functionality [2]. In contrast, processed pseudogenes occur due to retrotransposition events whereby mRNA transcripts have been reverse transcribed into complementary DNA (cDNA) copies prior to genomic integration, and these, therefore, lack introns and other regulatory elements.

The processed pseudogene of the tumour suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is the phosphatase and tensin homolog pseudogene 1 (*PTENP1*, *PTENpg1, PTENp1, PTH2, PTEN* ψ), located on chromosome 9 [6-8]. Bidirectional transcription from the *PTENP1* locus generates three lncRNAs: a sense (*PTENP1-S*) and two anti-sense (*PTENP1-AS*) transcripts, α and β [6, 9]. The *PTENP1-S* transcript has high sequence identity with the *PTEN* transcript, with approximately 95% identity in the 3'UTR [6]. PTEN cellular abundance is tightly regulated at multiple levels including transcriptional, post-transcriptional and post-translational levels, and small changes in cellular levels of PTEN have been shown to lead to tumorigenesis in a variety of tissue types [10-13].

Of most importance and interest to this chapter is the regulation of PTEN at the post-transcriptional level by microRNAs, which are small non-coding RNAs (approximately 18-23 nt in length) that bind to the *PTEN* mRNA at target sites within the 3'UTR, and translationally repress it, leading to a decrease in PTEN abundance and the loss of PTEN functionality as a tumour suppressor [6, 10, 14, 15]. The sense transcript of *PTENP1*, due to its high sequence identity with the PTEN transcript, can act in a competitive endogenous manner to sequester miRNAs that would otherwise target and translationally repress *PTEN*, thereby restoring PTEN abundance and function [6, 9, 29]. The endogenous competitive binding hypothesis states that endogenous RNAs (mRNAs, transcribed pseudogenes, protein-coding genes, long non-coding RNAs and circular RNAs) compete with each other to regulate each other through binding to, or acting as a sponge, referred to as "sponging", for shared miRNAs from the same cellular miRNA pool, and are known as competitive endogenous RNAs (ceRNAs) [14, 16]. The oncosuppressive functions of *PTENP1* as a ceRNA have been shown in a variety of cancer types. Overexpression of *PTENP1* leads to decreased cell proliferation, suppression of cell migration and invasion, and an induction of apoptosis in prostate cancer [6], clear-cell renal carcinoma [17], head and neck squamous cell carcinoma [18], gastric cancer [19], glioma [20], hepatocellular carcinoma [21], bladder cancer [22-24], and breast cancer [25-27].

This methodology chapter will focus on the oncosuppressive functions of *PTENP1* as a ceRNA, bringing about decreases in cell proliferation in cancer cell lines after overexpression of its 3'UTR, followed by an analysis of its anti-proliferative effects using two complementary cell proliferation analytical techniques, namely the Cell Counting Kit-8 (CCK-8) colorimetric method and the 5-ethynyl-2'-deoxyuridine (EdU) staining method. CCK-8 is a colorimetric method that utilises the metabolic activity of dehydrogenases within viable cells to convert a tetrazolium salt into a yellow coloured water-soluble formazan, whereby the intensity of the colour formation is related to cell number and hence measures cell proliferation over time [24]. In the EdU method, a thymidine nucleoside analog (EdU) integrates into DNA during synthesis

in cell culture and can be measured using the Click-iT® EdU kit, by fluorescent imaging [28]. This method provides real-time data of DNA synthesis. An overview of the methodological sequence is illustrated in Figure 1 and listed below.

- Cloning of the 3'-UTR of *PTENP1* into a mammalian expression plasmid (Figure 1A).
- Cell culture and maintenance of cancer cell line(s) of interest (Figure 1B).
- Transfection of *PTENP1* expression plasmid into cancer cells of interest (i.e. overexpression of *PTENP1* in cancer cells) (Figure 1C).
- Determining transfection efficiency of *PTENP1* in cancers of interest using flow cytometry (Figure 1D).
- Analysis of proliferation of cells overexpressing *PTENP1*, using the CCK-8 colorimetric method (Figure 1E).
- Analysis of proliferation of cells overexpressing *PTENP1*, using the EdU staining method (Figure 1F).

[Figure 1 near here]

2. Materials

2.1 Cloning of *PTENP1* into a mammalian expression plasmid

2.1.1 Cloning

1. PureLink™ PCR purification kit (Invitrogen)
2. pcDNA™ 3.3-TOPO® TA mammalian expression plasmid (Thermo Fisher Scientific)
3. One Shot® TOP10 *E. coli* chemically competent cells (Invitrogen)
4. Water bath (42°C)
5. Static and shaking incubators (37°C)

2.1.2 Bacterial growth

1. Luria Bertani medium (LB) containing 5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl
2. LB medium for plate cultures containing 20% (w/v) agar
3. LB medium for plate cultures also containing 100 μg/mL ampicillin (added to autoclaved medium upon cooling to approximately 50$_o$C)
4. Centrifuge (appropriate for 15 mL centrifuge tubes, capable of withstanding centrifugal forces of up to 12,000 g at 4$_o$C)

2.2 Plasmid preparation

1. PureLink™ Quick Plasmid Miniprep system (Invitrogen)
2. 1X TE (10 mM Tris, pH 8.0, 1 mM EDTA) buffer (Invitrogen)
3. NanoDrop spectrophotometer (Thermo Fisher Scientific)
4. Agarose (Bioline)
5. PureLink™ Plasmid Midi or Maxi-prep system (Invitrogen)

2.3 Tissue culture (TC)

1. Plasticware for TC (T25 flasks, T75 flasks, 6-well plates)

2. Cell culture growth medium as specified for the selected cell line(s) (RPMI 1640, or DMEM), supplemented with 10% fetal bovine serum
3. TrypLE Express Enzyme 1X (no phenol red)
4. Cell counter or hemocytometer
5. Water bath (37°C)
6. 37°C/5% CO_2 humidified cell culture incubator
7. Centrifuge (appropriate for 15 mL tubes, capable of centrifugation at a speed of 1200 rpm)
8. Inverted microscope
9. PC2 TC hood

2.4 Transfection

1. Lipofectamine® 2000 reagent kit (Thermo Fisher Scientific)
2. Opti-MEM™ reduced serum medium (GIBCO)
3. *PTENP1* expression construct
4. Microcentrifuge tubes (1.5 mL capacity)
5. Phosphate-buffered saline (PBS, pH 7.4), sterile-filtered (Sigma Aldrich)
6. Falcon tubes with cell strainer caps (BD Biosciences)
7. 6-well TC plates
8. 37°C/5% CO_2 humidified cell culture incubator
9. Flow cytometer (LSRII) (Beckman Coulter, BD)
10. FACSDiva 8.0.1 software (BD Biosciences)

2.5 Cell proliferation assays

2.5.1 CCK-8 assay

1. Cell Counting Kit-8 (Dojindo or Sigma Aldrich)
2. Appropriate cell culture growth medium for the selected cell line(s)

3. Sterile clear 96-well microplate (V-bottomed microplate for non-adherent cell types)
4. Microplate reader with a 450-490 nm filter
5. Pipettes (10 μL and 100-200 μL volume)
6. Multi-channel pipette (8-channel, 10-100 μL volume capacity)
7. 37°C/5% CO_2 humified cell culture incubator
8. Cell counter or hemocytometer
9. Centrifuge (appropriate for 15 mL tubes, capable of centrifugation at a speed of 1200 rpm)

2.5.2 EdU assay

1. Click-iT® EdU Cell Proliferation Kit for Imaging (Thermo Fisher Scientific)
2. Alexa Fluor™ 488 dye (Thermo Fisher Scientific)
3. Hoechst 33342 or 4′,6-diamidino-2-phenylindole (DAPI) fluorescent DNA counterstains
4. Phosphate-buffered saline (PBS), pH 7.4
5. Fixing solution, 3.7% formaldehyde in PBS
6. Permeabilization buffer, 0.5% Triton X-100 in PBS
7. Wash solution, 3% (w/v) bovine serum albumin (BSA) in PBS, pH 7.4
8. Deionised water
9. 18 x 18 mm glass coverslips
10. Microscope slides
11. 6-well plates
12. Aluminium foil
13. DMSO (*see* **Note 1**)
14. Fluoromount™ mounting medium (Sigma Aldrich)
15. Forceps
16. Fluorescence microscope (with 20x magnification)

3. Methods

3.1 Cloning of *PTENP1* into a mammalian expression plasmid

3.1.1 Production, purification and cloning of the *PTENP1* PCR product

1. Amplify the relevant segment(s) of the *PTENP1* sequence (3'-UTR in this case) by PCR from genomic DNA, with the use of specific oligonucleotide primers.
2. Analyse the PCR amplification products by agarose gel electrophoresis to confirm the success of the amplification and length of the desired pseudogene segment(s) (*see* **Note 2**).
3. Purify the *PTENP1* amplicon(s) for cloning using the PureLink™ PCR purification kit (Invitrogen) based on the manufacturer's protocol.
4. Determine the purity and concentration of the purified PCR product(s) by agarose gel electrophoresis and spectrophotometry using the NanoDrop spectrophotometer.
5. The purified *PTENP1* PCR product(s) is/are cloned into the pcDNA™ 3.3-TOPO® TA mammalian expression plasmid according to the manufacturer's instructions (*see* **Note 3**).
6. Carry out transformation of the cloning products into One Shot® TOP10 *E. coli* competent cells according to the manufacturer's instructions (*see* **Note 4**).
7. Pre-warm the LB agar plates in a 37°C incubator. Spread the transformation mixture (containing recombinant plasmid and competent cells) evenly onto the pre-warmed LB agar plates containing 100 μg/mL ampicillin using a sterile L-shaped cell spreader by gently rotating the plate containing the cell suspension. Once the plate surface is dry, incubate the agar plates at 37°C overnight (*see* **Note 5**).
8. After overnight incubation, select individual colonies by touching an individual colony with a sterile pipette tip and placing the pipette tip into 5 mL sterile (LB) medium containing 100 μg/L ampicillin. Grow cultures overnight in a 37°C incubator with shaking (200 rpm).

3.1.2 Preparation and sequencing of plasmid DNA

1. Take 1.5 mL of the bacterial culture and, using the PureLink™ Quick Plasmid Miniprep system (Invitrogen), isolate plasmid DNA according to the manufacturer's instructions. Elute the plasmid DNA into 50 μL 1X TE buffer.
2. Determine the concentration and quality of the isolated plasmid DNA by agarose gel electrophoresis and NanoDrop spectrophotometry.
3. Sequence the purified plasmid DNA using plasmid-specific primers to confirm the sequence of the cloned *PTENP1*.
4. Once the sequence is confirmed, large-scale preparation (midi- or maxi-prep) of the *PTENP1* plasmid construct(s) can be carried out for transient transfection experiments. Plasmid DNA isolation from either 100 mL (for midi-prep) or 200 mL (for maxi-prep) 2X LB medium, containing 100 μg/mL ampicillin, can be carried out after inoculation with a single colony and overnight growth at 37°C, using the PureLink™ midi- or maxi-prep (Invitrogen) system according to the manufacturer's instructions.
5. The isolated plasmid DNA is eluted in either 100 μL or 200 μL of 1X TE buffer for midi- or maxi-prep, respectively.

3.2 Cell culture and maintenance of cell lines

Prepare all tissue culture (TC) reagents and culture cells employing aseptic techniques in a PC2 hood using personal protective equipment (PPE). All plasticware and glassware used in TC protocols must be sterile. Pipette tips with sterile filters are highly recommended for all procedures in TC. An 80% ethanol solution is used to wipe down all surfaces before use (*see* **Note 6**).

To passage cells for routine maintenance, use the following procedure.

1. Select cell lines as appropriate for the study.

2. Warm TC growth medium for the selected cell line (RPMI 1640, or DMEM or any other specific medium) and FBS in a dedicated TC 37°C water bath.
3. In a TC hood, using serological pipettes, prepare culture medium by adding FBS to a final concentration of 10% (v/v).
4. Add 4 mL of the pre-warmed culture medium to a T25 cell culture flask.
5. Retrieve the selected cell line from the -80°C (or liquid nitrogen) store and quickly thaw the vial in a 37°C water bath.
6. Immediately after thawing, add cells to the pre-prepared T25 flask containing the pre-warmed culture medium.
7. Incubate the cells in a 37°C/5% CO_2 humidified cell culture incubator.
8. On the following day, view the culture flask using an inverted microscope and ensure adherent cells have attached to the surface of the flask.
9. If cells have adhered to the flask, remove and discard the culture medium to eliminate any residual DMSO from the cell cryopreservation process.
10. Add 4 mL of fresh pre-warmed culture medium to the cells and incubate in a 37°C/5% CO_2 humidified cell culture incubator.
11. Grow cells until 80% confluent, after which they may be passaged.
12. Pre-warm 1X TrypLE Express enzyme to 37°C.
13. In a TC hood, add the appropriate (approximately 15 mL) pre-warmed culture medium to a T75 flask.
14. Passage cells by discarding the culture medium from the T25 flask, briefly washing the cells with 5 mL sterile PBS to remove excess medium and adding 1 mL 1X TrypLE Express enzyme to detach the adherent cells.
15. Place the flask containing the 1 mL 1X TrypLE Express enzyme into a 37°C/5% CO_2 humidified cell culture incubator for approximately 3-10 minutes (until cells have detached). Check the progress of cell detachment using an inverted microscope (*see* **Note 7**).

16. Add 6-8 mL of culture medium to the detached cells in the flask and gently pipette up and down to inactivate the TrypLE Express enzyme.
17. Pipette the cell suspension (i.e. cells and culture medium) into a sterile 15 mL centrifuge tube and centrifuge at 1200 rpm for 5 minutes (*see* **Note 8**).
18. Using a hemocytometer, count the cells and calculate the number of cells harvested.
19. After centrifugation, discard the supernatant and add an appropriate volume of culture medium to the cell pellet to obtain a cell density of $1x10^6$ cells/mL for re-seeding into T75 flasks and $2.5x10^5$ cell/mL for re-seeding into T25 flasks.
20. Seed 1 mL of the resuspended cells ($1x10^6$ cells/mL cell density) into T75 flasks and incubate cells in a 37°C/5% CO_2 humidified cell culture incubator (*see* **Note 9**).
21. Passage cells when they reach a level of 80% confluence.
22. Culture cells for at least four passages to establish a stable growth rate (i.e. until log phase of the growth cycle is maintained). The doubling times of different cell lines can be found on the website of the American Type Culture Collection (ATCC at https://www.atcc.org).

3.3 Transfection and determining transfection efficiency using flow cytometry

1. Seed 2 $x10^5$ cells/well into 6-well plates containing 2 mL appropriate cell culture medium for the specific cell line and incubate overnight or until cells have reached 70-80% confluence (incubation time may vary depending on cell volume, size and doubling time) (*see* **Note 10**).
2. Optimise transfection efficiency by varying the concentrations of plasmid DNA (using the plasmid into which the *PTENP1* sequence has been cloned but containing a reporter gene, such as GFP, instead of the *PTENP1*) and Lipofectamine® 2000.

   According to the manufacturer's instructions (Thermo Fisher Scientific), Lipofectamine® 2000 based transfection is carried out by preparing two solutions (A and B) whereby solution A contains plasmid DNA (1-10 µg) and Opti-MEM™ (reduced serum medium), in a total volume of 100 µL. Solution B contains lipofectamine® 2000 (2-10 µL) and Opti-MEM™, in a final

volume of 100 μL. These solutions (A and B) are incubated separately for 5 minutes at room temperature before being combined.

3. In a sterile TC hood, combine solution A with solution B and incubate the mixture at room temperature for 20 minutes.
4. Add the DNA-Lipofectamine® 2000 lipid complex (200 μL total volume) drop wise on to the cells in each well of the 6-well plate.
5. After 4-6 hours, change the culture medium and place the plates back into the 37°C/5% CO_2 humidified cell culture incubator.
6. To increase transfection efficiency, repeat the transfection procedure 24 hours after the first transfection (*see* **Note 11**).
7. After transfection (24-40 hours), remove the culture medium and wash cells twice with 5 mL PBS per well each time.
8. Harvest cells by trypsinisation (i.e. use 1X TrypLE Express enzyme) and collect them by centrifugation in Falcon tubes with cell strainer caps. Resuspend the cells in 1 mL PBS.
9. Pass the cells through the cell strainer cap of the Falcon tube (BD) on ice.
10. Analyse by flow cytometry using a flow cytometer (BD LSRII in this case). Acquire a total of 10,000-30,000 events, using FACSDiva 8.0.1 software (BD Biosciences). The use of the GFP expression construct for these experiments allows live cell analysis of protein expression (see Figure 2 for more details).

[Figure 2 near here]

3.4 CCK-8 cell proliferation assay

3.4.1 Producing a standard curve

Generate a standard curve for each selected cell line (untransfected) in order to achieve a cell density that does not saturate the absorbance scale of the microplate reader. The standard curve

is generated also to determine an adequate incubation time with the CCK-8 solution for colour development for the set number of cells.

1. Grow selected cancer cell line(s) in T75 flasks, with the appropriate culture medium until they reach 80% confluence (check on an inverted microscope). Cells are now ready for passaging.
2. Pre-warm fresh cell culture medium to 37°C.
3. Trypsinise cells by adding 1X TrypLE Express enzyme to the untransfected cancer cells for 3-5 minutes until cells become dislodged. Add culture medium to stop the enzyme activity and count cells using a hemocytometer. Resuspend cells to a density of 1.25 $x10_5$ cells/mL in culture medium.
4. Perform serial dilutions of the cells in a 96-well microplate for adherent cells (or a v bottomed plate for non-adherent cell types), as outlined in Figure 3 (*see* **Note 12**).

[Figure 3 near here]

5. Add 10 μL of CCK-8 solution to each well of the 96-well microplate and perform an initial 0 hour reading by measuring absorbance at 450 nm using a microplate reader (*see* **Note 13**).
6. Return the plate to incubate in a 37°C/5% CO_2 humidified cell culture incubator for 1 hour and then read the absorbance of the plate at 450 nm. This set of readings will be the 1-hour time point readings.
7. Repeat step 5 three times to acquire absorbance readings at the 2, 3 and 4 hour incubation time points to determine the optimal incubation time for the CCK-8 reagent to react and produce an orange colour in the selected cell line(s) (*see* **Note 14**).
8. Prepare a standard curve by plotting a graph of the number of cells (x-axis) against the absorbance (y-axis).
9. Use the equation:

   Absorbance at 450 nm (A_{450}) = A_{450} value of the sample - A_{450} value of the negative control.

10. Select an appropriate concentration of cells that does not saturate the microplate reader absorbance scale and an incubation time that is sufficient for colour development for the selected cells (*see* **Note 15**).

3.4.2 Cell coloration

1. Add 100 μL of culture medium to all wells intended for use as described in Figure 4.
2. Prepare the empty plasmid transfected cancer cells by harvesting cells by trypsinisation with 1X TrypLE Express enzyme, adding culture medium to stop the enzyme activity, counting cells and diluting them to the optimised cell density.
3. Repeat step 2 using transfected cancer cells overexpressing *PTENP1*.
4. Add equal numbers of empty plasmid transfected and *PTENP1* transfected cancer cells to a sterile 96-well microplate as shown in Figure 4.
5. Reserve the last set of triplicate wells for the negative controls containing culture medium alone.

[Figure 4 near here]

6. Add 10 μL of CCK-8 solution to the 0 hour wells on the 96-well microplate and, after mixing, incubate the plate in a 37°C/5% CO_2 humidified cell culture incubator for the optimised incubation time and then carry out an initial 0 hours absorbance reading in a microplate reader using the 450 nm filter.
7. Return the plate to the 37°C/5% CO_2 humidified cell culture incubator and allow the cells to proliferate for 24 hours.
8. After 24 hours, add 10 μL of CCK-8 solution to the 24-hour wells, mix well, incubate the plate in a 37°C/5% CO_2 humidified cell culture incubator for the optimised incubation time and measure absorbance at 450 nm.
9. Repeat steps 7 and 8 for cells proliferating for the 48 and 72 hour time points (*see* **Note 16**).

10. Perform this experiment three times to ensure reproducibility of results.

3.4.3 Data analysis

1. To correct for background absorbance readings, subtract the mean absorbance results of the negative control wells (i.e. the wells containing culture medium alone) from those of the transfected cells. Use the following equation:

   A_{450} of sample wells = Mean A_{450} of sample wells – Mean A_{450} of negative control
2. Collate the A_{450} values for all time points (0, 24, 48, 72 hour) and calculate the mean A_{450} ± standard deviation for each time point and each group (i.e. empty plasmid transfected cancer cell lines and corresponding transfected *PTENP1* overexpressing cancer cells).
3. Plot a graph of time (in hours) on the x-axis (independent variable) against A_{450} on the y-axis. In this case, A_{450} corresponds to cell number as metabolic rate is dependent on cell growth.
4. Perform a student's t-test to compare the mean A_{450} values between the two groups (empty plasmid transfected cancer cell line versus corresponding *PTENP1* overexpressing cancer cell line). The obtained p-value indicates statistical significance if $p < 0.05$.

3.5 EdU cell proliferation assay

The EdU method for imaging cell proliferation is based on the Click-iT® EdU Cell Proliferation Kit for Imaging, using the Alexa Fluor™ 488 fluorescent dye (Thermo Fisher Scientific). The protocol below is taken from the manufacturer's protocol.

3.5.1 Pre-preparation of stock solutions

1. Ensure all vials are at room temperature before opening.
2. Prepare a 10 mM stock solution of EdU, by adding 2 mL of DMSO to the EdU vial and mixing well (*see* **Note 17**).
3. Prepare a working solution of the Alexa Fluor™ 488 fluorescent dye, by adding 70 μL of DMSO to the vial and mixing well (*see* **Note 18**).

4. Prepare a working solution of 1X Click-iT® EdU reaction buffer from the 4 mL of 10X Click-iT® EdU reaction buffer, by adding 36 mL of deionised water. Prepare small aliquots of this solution (*see* **Note 19**).
5. Prepare a 10X stock solution of the Click-iT® EdU buffer additive, by adding 2 mL of deionised water to the vial and mixing well (*see* **Note 20**).

3.5.2 Cell labelling

1. Based on the cell proliferation conditions used for the CCK-8 method, select the appropriate cell density for EdU cell labelling.
2. After selecting the desired density, place a coverslip into a 6-well plate and seed cells onto the coverslips (Figure 5). Allow the cells to recover overnight before proceeding.

[Figure 5 near here]

3. Prepare a 2X working solution of EdU in complete medium for the appropriate cell line(s) from the 10 mM stock solution. The manufacturer recommends an EdU concentration of 10 μM in the culture medium (*see* **Note 21**).
4. Pre-warm the 2X EdU working solution and add the appropriate volume to the culture medium to obtain a final concentration of 1X EdU.
5. Incubate the cells in a 37°C/5% CO_2 humidified cell culture incubator for 24 hours to allow cell proliferation. EdU staining time is a direct measure of cellular DNA synthesis, as EdU is incorporated into DNA during DNA synthesis.

3.5.3 Fixation and permeabilization of cells

1. After incubation, proceed immediately to cell fixation. Remove the culture medium by gentle aspiration and add 1 mL of fixing solution each well, then incubate for 15 minutes at room temperature.

2. Remove the fixing solution and wash cells twice with 1 mL of wash solution.

3. Remove the wash solution from each well, add 1 mL permeabilization buffer and incubate for 20 minutes at room temperature.

3.5.4 EdU detection

1. Prepare 1X Click-iT® EdU buffer additive by making a 1 in 10 dilution of the 10X Click-iT® EdU buffer additive with deionised water. This solution should be freshly prepared and used the same day.

2. Prepare the Click-iT® EdU reaction cocktail according to the manufacturer's instructions. For one coverslip, add 430 µL of 1X Click-iT® EdU reaction buffer, 20 µL of 100 mM $CuSO_4$, 1.2 µL of Alexa Fluor™ 488 and 50 µL of 10X Click-iT® EdU buffer additive into a total volume of 500 µL (*see* **Note 22**).

3. Remove the permeabilization buffer, then wash cells twice with 1 mL of was solution. Remove the wash solution.

4. Add 500 µL of Click-iT® EdU reaction cocktail to each well containing cells cultured on a coverslip. Ensure the reaction cocktail is spread evenly over the coverslip by rocking the plate briefly.

5. Use aluminium foil to cover and protect the plate from light and incubate at room temperature for 30 minutes.

6. Remove the reaction cocktail and wash once with 1 mL of wash solution. Remove the wash solution.

3.5.5 DNA/nuclear staining

1. Wash wells with 1 mL PBS and remove the wash solution.

2. Prepare a 2X Hoechst 33342 working solution, by diluting the stock Hoechst 33342 solution in PBS (1:1000) (*see* **Note 23**).

3. Add 1 mL of the prepared 2X Hoechst 33342 solution into each well, protect the plate from light by covering the plate with aluminium foil and incubate for 15 minutes at room temperature. Remove the Hoechst 33342 solution.

4. Wash each well twice using 1 mL of PBS. Remove the wash solution and proceed to the mounting of coverslips.

3.5.6 Mounting of coverslips

1. Place a drop of the Fluoromount™ medium onto a microscope slide. Using forceps, carefully retrieve the coverslip from the 6-well plate and place the coverslip, cells facing downwards, onto the drop of mounting medium on the microscope slide.

3.5.7 Fluorescence imaging

1. Image the cells using a fluorescence microscope with the correct filters set at the appropriate fluorescence excitation/emission maxima. The Alexa Fluor® 488 excitation and emission maxima are 495 nm and 519 nm, respectively. To visualise cells stained with Hoechst 33342 or DAPI, the excitation and emission maxima are 350 nm and 461 nm, respectively (*see* **Note 24**).

2. Repeat for the cells from the 48 and 72 hour time points.

3.5.8 Image analysis

1. Pick at least three fields of view (x20 objective) from each coverslip for each of the different time points (24, 48 and 72 hours).
2. Count EdU stained cells (green fluorescent cells) and the total number of cells (i.e. count the Hoechst (blue) stained nuclei) from at least three fields of view for the empty plasmid transfected cancer cells and the corresponding *PTENP1* overexpressing cancer cells. From these counts, calculate the average number of cells for at least three fields of view, in quadruple replicate coverslips, for each time point (*see* **Note 25**).
3. Use the equation below to calculate the percentage of EdU stained cells in each group, (i.e. the empty plasmid transfected and corresponding *PTENP1* overexpressing cancer cells).

$$\% \textbf{ EdU stained cells} = \frac{\text{Average number of EdU stained cells}}{\text{Average number of nuclei stained with Hoechst 33342 stain}} \times 100$$

4. Plot values as % EdU stained cells (y-axis) versus different time points (x-axis in hours) for the two groups (empty plasmid transfected cancer cells and corresponding *PTENP1* overexpressing cancer cells).
5. Conduct a student t-test and depict significance ($p < 0.05$) on the graph with ± standard errors.

3.6 Additional oncosuppressive functions of *PTENP1*

Additional to the methodologies outlined in this chapter, the oncosuppressive functions of *PTENP1* as a ceRNA can be further extended to include its effects on apoptosis, cell invasion and migration and cell cycle phase distribution analysis. Some suggestions for the analysis of apoptosis include, but are not limited to, techniques such as flow cytometry, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assays or the use of the Annexin-based apoptosis kits. Cell migration and invasion assays include scratch tests and transwell assays, while cell cycle phase distribution analysis can be carried out using flow cytometry.

4. Notes

1. Dispose of DMSO waste in compliance with local regulations.
2. A single PCR product of the expected length should be observed.
3. This system uses topoisomerase mediated cloning.
4. The pUC-19 plasmid is supplied and used as a transformation control.

5. Even distribution of the cells on the agar plate is critical for analysis of the colonies. We recommend spreading 2/3 of the cell suspension on one LB agar plate and 1/3 on another. Care must be taken to not puncture the agar surface.
6. Use serological pipettes once only. Change pipettes for different solutions and discard appropriately after use.
7. The incubation time in this step will vary depending on the cell type being used.
8. Before centrifugation, save a 20 μL aliquot of the cell suspension for cell counting.
9. Check cells the following day to ensure adherence and the absence of any contamination. Change the culture medium when the phenol red indicator dye begins to change colour to a lighter red or orange.
10. Based on experience, transfection efficiency may be increased considerably by carrying out the transfection on the suspended cells ($2.5x10^5$ cells), prior to cell seeding. Cells are then seeded after transfection. The use of suspended cells provides a greater cell surface area for transfection by the plasmid constructs.
11. For each set of transfections, ensure controls are included in order to take into consideration any effects of the transfection procedure and reagents in the data analysis of the different experiments. In all individual experiments, controls should include mock transfected cells (transfected with transfection reagents without plasmid DNA) and plasmid-only transfected cells (empty plasmid without *PTENP1* insert).
12. It is recommended that the pipette tip be placed against the wall of each well when dispensing cells to ensure complete addition.
13. The CCK-8 solution is stable for 12 months at 4°C or for 6 months at ambient temperature. For long-term storage, aliquots can be stored at -20°C for 12 months. To mix any reagent stuck on the plate wall, gently tap the plate to mix the reagent with the medium or use a microplate mixer. Formation of bubbles in the wells will cause errors in reading; thus, remove any bubbles using a pipette tip.

14. The higher the number of cells, and the longer the incubation time, the higher the intensity of the orange colour produced.
15. The incubation time and cell concentration are dependent on the chosen cell line, as larger cells produce weaker colouration and thus, there is a need to either increase the incubation time or increase the number of cells while keeping the incubation time constant. Empty plasmid transfected cancer cells should proliferate at a higher rate compared to *PTENP1* transfected cells, as overexpression of *PTENP1* is known to decrease cell proliferation in a variety of cancer cell types.
16. The growth culture medium needs to be changed once the measurement period (measured from the starting incubation time) is longer than 48 hours. The culture medium can be more easily removed by tilting the plate to avoid the pipette touching the cells in each well. For suspension cultures, a microplate rotor may be used to centrifuge the v bottomed plates and the medium removed after the cells have settled to the bottom of the wells.
17. This 10 mM EdU stock solution can be stored at ≤ -20°C and is stable for up to 12 months.
18. This working solution can be stored at ≤ -20°C and is stable for up to 12 months.
19. These 1X Click-iT® EdU aliquots can be stored at 2-6°C and are stable for 6 months at this temperature.
20. This working solution can be stored at ≤ -20ºC and is stable for up to 12 months. If a colour change is observed (to a brown colour), the solution has degraded and should be discarded.
21. To obtain the optimal EdU fluorescence measurement for a particular cell line, a range of concentrations (e.g. 2, 5, 10 μM) may be tried.
22. Use the Click-iT® reaction cocktail within 15 minutes of preparation.
23. 4′,6-diamidino-2-phenylindole (DAPI) can also be used as an alternative to Hoechst 33342 to stain the nuclei of cells. Staining the nuclei allows for the identification of cells that have not been stained with EdU and this will be the total cell count for the field of view.

24. Cells with EdU incorporated into the DNA and stained nuclei may be visualised using the different excitation and emission wavelengths and images superimposed to visualise the proliferating cells.
25. EdU stained cells are identified as proliferating cells that have EdU incorporated into their DNA.

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Figure Legends

Figure 1. Representation of the methodological steps in the analysis of cell proliferation of *PTENP1* transfected cancer cells.

See text for details.

1) *PTENP1* segments (3'-UTR in this case) produced by PCR are cloned into a mammalian expression plasmid. **2)** Cells are cultured and maintained, and **3)** cancer cells are transiently transfected with the *PTENP1* expression construct. **4)** The transfection efficiency is determined by flow cytometry using an expressed reporter gene, and **5)** cell proliferation is assessed using the CCK-8 method by measuring the absorbance of proliferating empty plasmid transfected and *PTENP1* overexpressing cancer cells at various time intervals. **6)** Data analysis for the CCK-8 assay, where absorbance values of the empty plasmid transfected cancer cells are compared to the *PTENP1* overexpressing cancer cells, is carried out. **7)** Cell proliferation is also analysed using the EdU staining method, and **8)** images of the two groups of cells (empty plasmid transfected and *PTENP1* overexpressing cancer cells) are taken, and EdU stained cells and nuclei stained cells are counted and depicted as % EdU stained cancer cells compared to the nuclei stained cells per group at the various time points.

Figure 2. Optimising transfection efficiency using a GFP expression plasmid.

(A-B) U87MG cells were transiently transfected with a GFP expression plasmid. 40 hours after transfection, cells were harvested and analysed by flow cytometry, gating on the viable cell population (Side Scatter (SSC) vs Forward Scatter (FSC), left panels) and then on cells expressing Green Fluorescent Protein (GFP). In the histograms (right panels), the X axis represents the green fluorescence (GFP) and the Y axis represents the cell count. **(A)** Untransfected U87MG cells; **(B)** GFP transfected U87MG cells. **(C)** Tabulation of cell numbers, and proportions of cells (as a %), in each of the cell populations (untransfected and GFP-transfected) from **(A)** and **(B)**.

Figure 3. Plate setup for the optimisation of cell number and incubation time for colour development using the CCK-8 method.

Plates are set up using serial dilutions: empty plasmid transfected cells are added into each of 3 wells of a 96-well plate containing 200 μL of culture medium to form a row of triplicate wells containing $2.5x10^4$ cells per well. Serial dilutions of these triplicate wells are conducted by taking 100 μL from each well and placing it into a subsequent set of three wells containing 100 μL culture medium. These dilutions are continued 6 more times (i.e. $1.25x10^4$, $6.2x10^4$, $3.1x10^3$, $1.55x10^3$, $7.75x10^2$ and $3.88x10^2$ cells/mL). The final row of wells is reserved for culture medium alone (no cells) and represents the negative controls for the experiment

Figure 4. Plate setup for cell proliferation analysis using the CCK-8 method.

Equal numbers of empty plasmid transfected cancer cells and *PTENP1* overexpressing cancer cells are added to row A and B, respectively. Row C is the negative control and contains cell culture medium alone (i.e. no cells). Cells are grown for a series of time intervals (0, 24, 48 and 72 hours, 3 wells per time point) in a 37°C (with 5 % CO_2) humidified cell culture incubator.

Figure 5. Plate setup for cell proliferation analysis using the EdU staining method.

Equal numbers of empty plasmid transfected cancer cells and *PTENP1* overexpressing cancer cells are seeded on coverslips placed in 6-well plates. Cells are grown for a series of time intervals (24, 48 and 72 hours, 4 well per time point) in a 37^oC (with 6% CO_2) humidified cell culture incubator.

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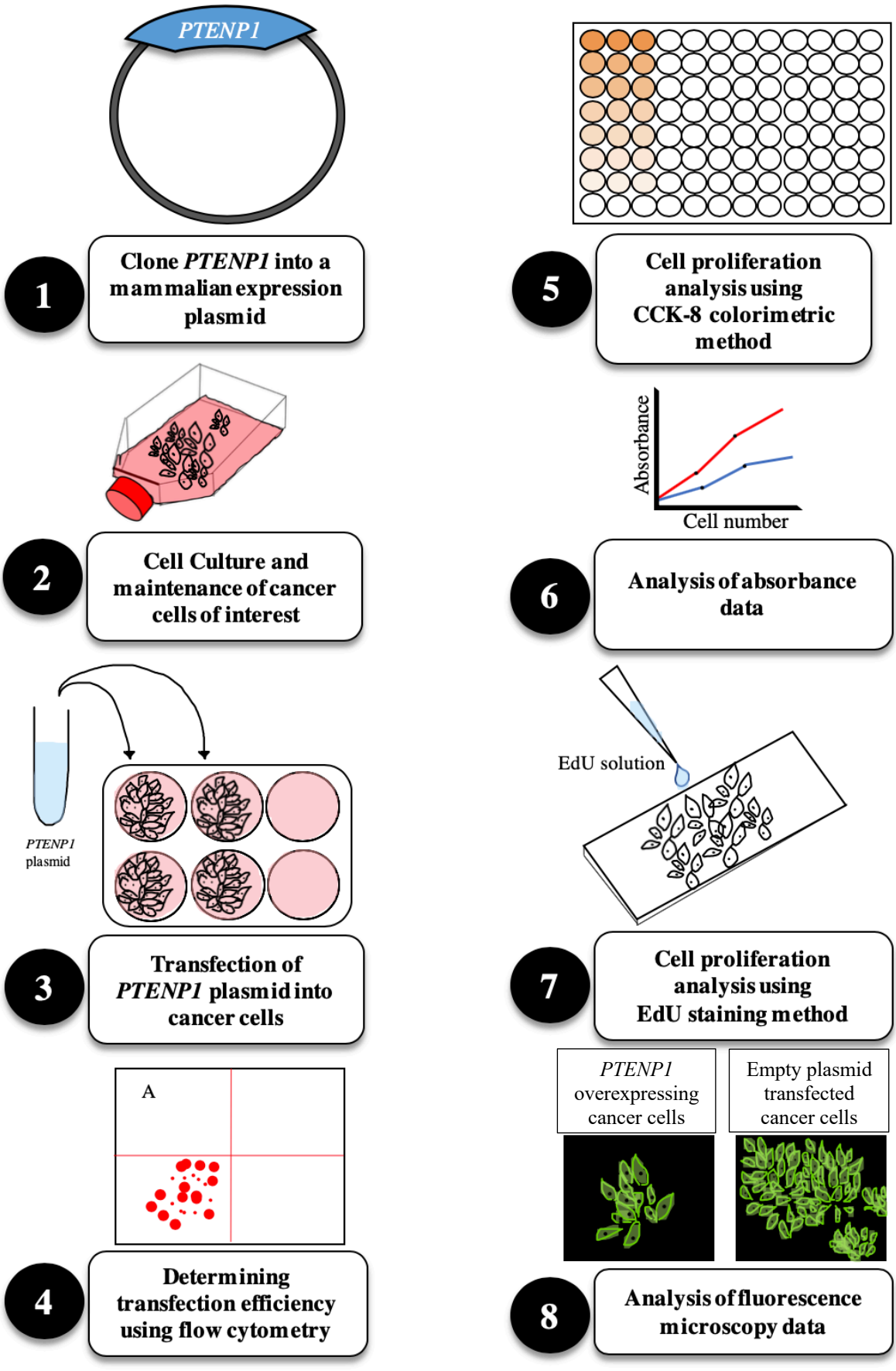
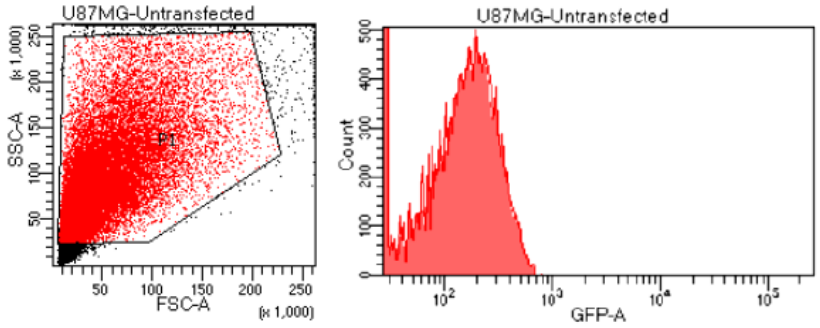
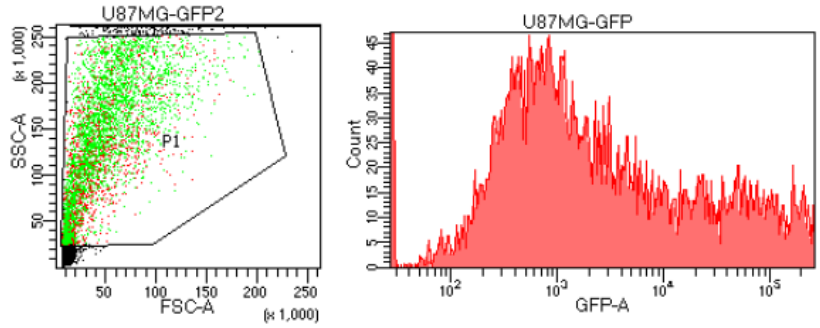

PTENP1
1
Clone PTENP1 into a mammalian expression plasmid
2
Cell Culture and maintenance of cancer cells of interest
PTENP1 plasmid
3
Transfection of PTENP1 plasmid into cancer cells
A
4
Determining transfection efficiency using flow cytometry
5
Cell proliferation analysis using CCK-8 colorimetric method
Absorbance
Cell number
6
Analysis of absorbance data
EdU solution
7
Cell proliferation analysis using EdU staining method
PTENP1 overexpressing cancer cells
Empty plasmid transfected cancer cells
8
Analysis of fluorescence microscopy data

Figure 1

A



B



C

Tube: GFP2

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	4,917	49.2	49.2
P2	3,077	62.6	30.8

Figure 2

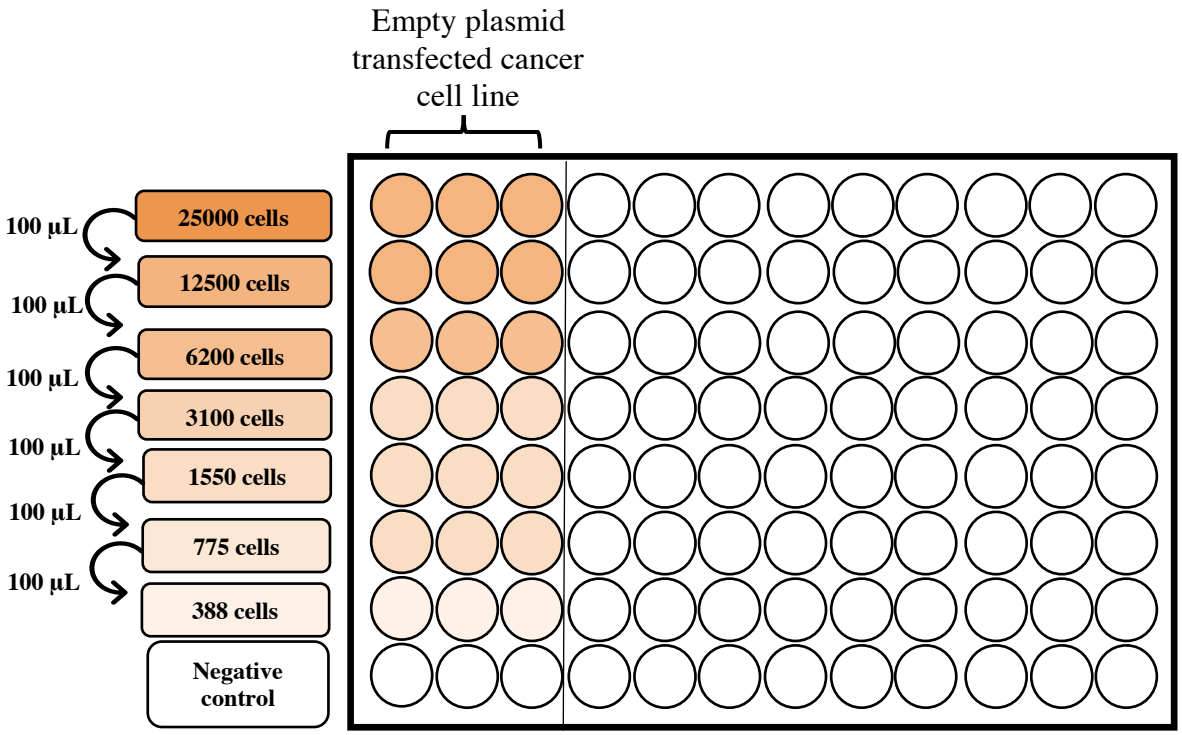

Empty plasmid transfected cancer cell line
100 µL
25000 cells
100 µL
12500 cells
100 µL
6200 cells
100 µL
3100 cells
100 µL
1550 cells
100 µL
775 cells
100 µL
388 cells
Negative control

Figure 3

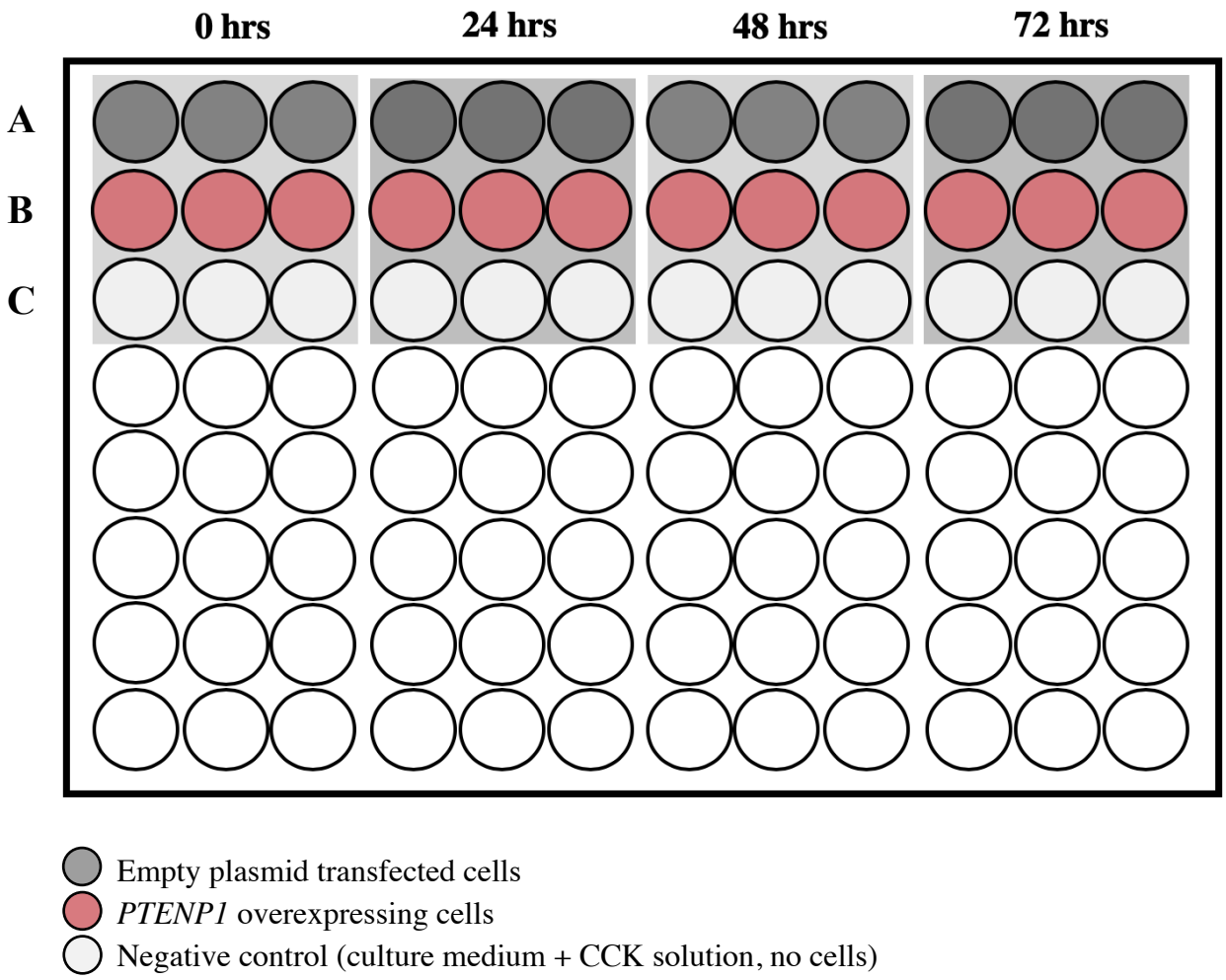

0 hrs
24 hrs
48 hrs
72 hrs
A
B
C
Empty plasmid transfected cells
PTENP1 overexpressing cells
Negative control (culture medium + CCK solution, no cells)

Figure 4

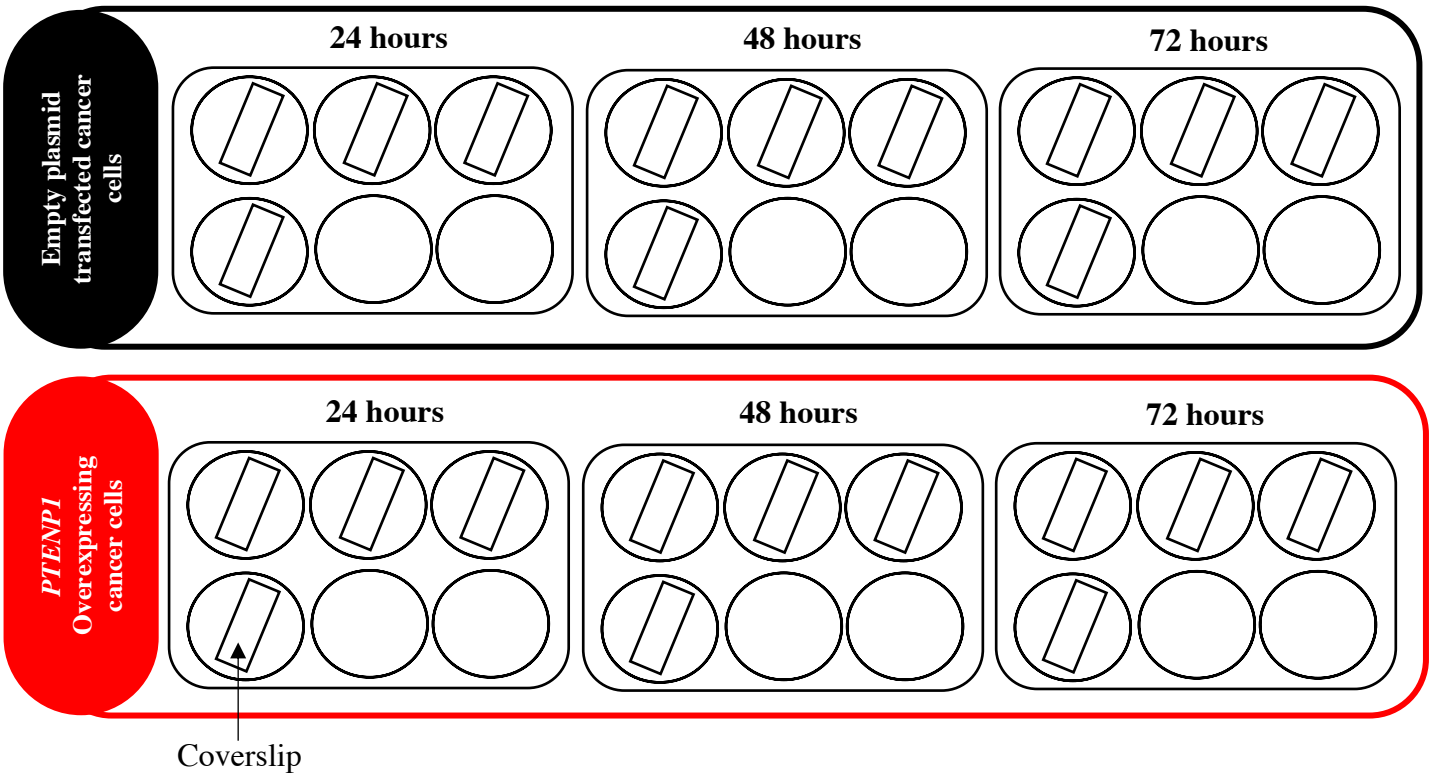

Empty plasmid transfected cancer cells
24 hours
48 hours
72 hours
PTEN/P1 Overexpressing cancer cells
24 hours
48 hours
72 hours
Coverslip

Figure 5