

# Journal of Visualized Experiments

## Generation of cancer cell clones to visualize TERRA molecules expressed from a single telomere in living cells --Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58790R2
Full Title:	Generation of cancer cell clones to visualize TERRA molecules expressed from a single telomere in living cells
Keywords:	Telomeres; TERRA; long noncoding RNA; MS2-GFP system; CRISPR/Cas9 system; cancer cells; live-cell imaging
Corresponding Author:	Emilio Cusanelli University of Trento Trento, Trentino Alto Adige ITALY
Corresponding Author's Institution:	University of Trento
Corresponding Author E-Mail:	emilio.cusanelli@unitn.it
Order of Authors:	Laura Avogaro Claudio Oss Pegorar Nicole Bettin Emilio Cusanelli
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the <b>city, state/province, and country</b> where this article will be <b>filmed</b> . Please do not use abbreviations.	Trento, Trentino Alto Adige, Italy



UNIVERSITÀ DEGLI STUDI  
DI TRENTO



CIBIO – Centre for Integrative Biology

Trento, September 5<sup>th</sup>, 2018

To Dr. Phillip Steindel,  
Review Editor  
of *Journal of Visualized Experiments*

Dear Dr. Steindel,

We would like to thank you for the opportunity of revising our manuscript entitled “Generation of cancer cell clones to visualize TERRA molecules expressed from a single telomere in living cells” that we were invited to submit to the *Journal of Visualized Experiments*. We have revised the protocol according to the editors’ comments. All changes in the manuscript are indicated with a red color.

We hope our work is ready to be shared with the readership audience of the *Journal of Visualized experiments*.

We are looking forward to hearing from you,

With kind regards,



Emilio Cusanelli  
Group leader  
Centre for Integrative Biology (CIBIO)  
University of Trento,  
Italy

**TITLE:**

**Generation of Cancer Cell Clones to Visualize Telomeric Repeat-containing RNA Expressed from a Single Telomere in Living Cells**

**AUTHORS & AFFILIATIONS:**

Laura Avogaro<sup>1</sup>, Claudio Oss Pegorar<sup>1</sup>, Nicole Bettin<sup>1,2</sup>, Emilio Cusanelli<sup>1</sup>

<sup>1</sup>Laboratory of Cell Biology and Molecular Genetics, Centre for Integrative Biology (CIBIO)  
University of Trento, Trento, Italy

<sup>2</sup>Department of Life Sciences, University of Trieste, Trieste, Italy

**Corresponding Author:**

Emilio Cusanelli (emilio.cusanelli@unitn.it)

Tel: (+39) 0461 285357

**Email Addresses of Co-Authors:**

Laura Avogaro (laura.avogaro@gmail.com)

Claudio Oss Pegorar (claudio.osspegorar@studenti.unitn.it)

Nicole Bettin (nicole.bettin@studenti.units.it)

**Keywords**

Long noncoding RNA, TERRA, telomere, cancer, CRISPR/Cas9, MS2-GFP, live-cell imaging.

**SUMMARY:**

Here, we present a protocol to generate cancer cell clones containing a MS2 sequence tag at a single subtelomere. This approach, relying on the MS2-GFP system, enables visualization of the endogenous transcripts of telomeric repeat-containing RNA (TERRA) expressed from a single telomere in living cells.

**ABSTRACT:**

Telomeres are transcribed, giving rise to telomeric repeat-containing long noncoding RNAs (TERRA), which have been proposed to play important roles in telomere biology, including heterochromatin formation and telomere length homeostasis. Recent findings revealed that TERRA molecules also interact with internal chromosomal regions to regulate gene expression in mouse embryonic stem (ES) cells. In line with this evidence, RNA fluorescence *in situ* hybridization (RNA-FISH) analyses have shown that only a subset of TERRA transcripts localize at chromosome ends. A better understanding of the dynamics of TERRA molecules will help define their function and mechanisms of action. Here, we describe a method to label and visualize single-telomere TERRA transcripts in cancer cells using the MS2-GFP system. To this aim, we present a protocol to generate stable clones, using the AGS human stomach cancer cell line, containing MS2 sequences integrated at a single subtelomere. Transcription of TERRA from the MS2-tagged telomere results in the expression of MS2-tagged TERRA molecules that are visualized by live-cell fluorescence microscopy upon co-expression of a MS2 RNA-binding protein fused to GFP (MS2-

GFP). This approach enables researchers to study the dynamics of single-telomere TERRA molecules in cancer cells, and it can be applied to other cell lines.

## INTRODUCTION:

The long noncoding RNA TERRA is transcribed from the subtelomeric region of chromosomes and its transcription proceeds towards the chromosome ends, terminating within the telomeric repeat tract<sup>1,2</sup>. For this reason, TERRA transcripts consist of subtelomeric-derived sequences at their 5' end and terminate with telomeric repeats (UUAGGG in vertebrates)<sup>3</sup>. Important roles have been proposed for TERRA, including heterochromatin formation at telomeres<sup>4,5</sup>, DNA replication<sup>6</sup>, promoting homologous recombination among chromosome ends<sup>7-9</sup>, regulating telomere structure<sup>10</sup> and telomere length homeostasis<sup>2,11-13</sup>. Furthermore, TERRA transcripts interact with numerous extratelomeric sites to regulate widespread gene expression in mouse embryonic stem (ES) cells<sup>14</sup>. In line with these evidence, RNA fluorescence *in situ* hybridization (RNA-FISH) analyses have shown that only a subset of TERRA transcripts localize at telomeres<sup>1,2,15</sup>. In addition, TERRA has been reported to form nuclear aggregates localizing at the X and Y chromosomes in mouse cells<sup>2,16</sup>. These findings indicate that TERRA transcripts undergo complex dynamics within the nucleus. Understanding the dynamics of TERRA molecules will help define their function and mechanisms of action.

The MS2-GFP system has been widely used to visualize RNA molecules in living cells from various organisms<sup>17,18</sup>. This system has been previously used to tag and visualize single-telomere TERRA molecules in *S. cerevisiae*<sup>12,19</sup>. Using this system, it was recently shown that yeast TERRA transcripts localize within the cytoplasm during the post-diauxic shift phase, suggesting that TERRA may exert extranuclear functions<sup>20</sup>. We have recently used the MS2-GFP system to study single-telomere TERRA transcripts in cancer cells<sup>21</sup>. To this aim, we employed the CRISPR/Cas9 genome editing tool to integrate MS2 sequences at a single telomere (telomere 15q, hereafter Tel15q) and obtained clones expressing MS2-tagged endogenous Tel15q TERRA (TERRA-MS2 clones). Co-expression of a GFP-fused MS2 RNA-binding protein (MS2-GFP) that recognizes and binds MS2 RNA sequences enables visualization of single-telomere TERRA transcripts in living cells<sup>21</sup>. The purpose of the protocol illustrated here is to describe in detail the steps required for the generation of TERRA-MS2 clones.

To generate TERRA-MS2 clones, a MS2 cassette is integrated within the subtelomeric region of telomere 15q, downstream of the TERRA promoter region and transcription start site. The MS2 cassette contains a neomycin resistance gene flanked by lox-p sites, and its integration at subtelomere 15q is performed using the CRISPR/Cas9 system<sup>22</sup>. After transfection of the MS2 cassette, single clones are selected and subtelomeric integration of the cassette is verified by PCR, DNA sequencing and Southern blot. Positive clones are infected with a Cre-expressing adenovirus in order to remove the selection marker in the cassette, leaving only MS2 sequences and a single lox-p site at the subtelomere 15q. Expression of MS2-tagged TERRA transcripts from Tel15q is verified by RT-qPCR. Finally, the MS2-GFP fusion protein is expressed in TERRA-MS2 clones via retroviral infection in order to visualize MS2-TERRA transcripts by fluorescence microscopy. TERRA transcripts can be readily detected by RNA-FISH and live-cell imaging using telomeric repeat-specific probes<sup>1,2,15,23</sup>. These approaches provide important information on the

localization of the total population of TERRA molecules at single cell resolution. The generation of clones containing MS2 sequences at a single subtelomere will enable researchers to study the dynamics of single-telomere TERRA transcripts in living cells, which will help define the function and mechanisms of action of TERRA.

## PROTOCOL:

### 1. Selection of Neomycin Resistant Clones

1.1. Grow AGS cells in Ham's F-12K (Kaighn's) medium supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, penicillin (0.5 units per mL of medium), and streptomycin (0.2 µg per mL of medium) at 37 °C and 5% CO<sub>2</sub>. Transfect the cells at a 50-60% confluence with the sgRNA/Cas9 expressing vector and the MS2 cassette at a 1:10 molar ratio<sup>21</sup>.

NOTE: In a parallel experiment, verify transfection efficiency by transfecting a GFP-expressing vector (*i.e.*, Cas9-GFP vector). At least 60-70% transfection efficiency should be achieved.

1.2. The following day, replace the culturing medium with medium containing neomycin at 0.7 µg/mL final concentration (selective medium).

NOTE: Splitting the cells and seeding them in selective medium the day after transfection will speed up the selection process. All non-transfected cells will immediately die. If transfection is performed in 6 well plates, in which case each well at a 60% confluence would contain approximately  $0.7 \times 10^6$  cells, on the following day the cells can be split from a single well to a 10 cm dish containing selective medium.

1.3. Keep the cells in selective medium for 7-10 days, changing medium every one or two days, until single clones are visible.

### 1.4. Picking of cell clones

1.4.1. Prepare a 96 well plate containing 10 µL of 0.25% trypsin in each well.

NOTE: Prepare two 96 well plates in case more than 96 clones are expected to be picked.

1.4.2. With the use of a microscope, mark the position of each clone visible in the 10 cm dish by making a dot at the bottom of the dish using a marker. Each dot will correspond to a colony to be picked.

1.4.3. Replace the culturing medium with just enough Phosphate Buffered Saline (PBS) to form a thin film of liquid on the clones and not let the cells dry during the clone picking.

1.4.4. Pick single colonies using a 10 µL pipette. Attach the tip containing 5 µL of trypsin to the colony and slowly release the trypsin which will remain localized on the colony. Allow trypsin to detach the cells for 1 min, then scrape the colony with the tip and suck it up into the tip.

NOTE: During this procedure, flipping the dish a bit on one side so to decrease the volume of PBS around the colony being picked will help the picking process by avoiding diluting the trypsin around the colony. Using a clone ring may also help picking up single clones.

1.4.5. Place the cells from the colony into a well of the 96 well plate containing 10  $\mu\text{L}$  of 0.25% trypsin.

1.4.6. Incubate 5 min at room temperature, then fill the well with 150  $\mu\text{L}$  of selective medium (Ham's F-12K (Kaighn's) medium supplemented with 10% FBS, 2 mM L-glutamine, penicillin (0.5 units per mL of medium), streptomycin (0.2  $\mu\text{g}$  per mL of medium) and containing neomycin at a concentration of 0.7  $\mu\text{g}/\text{mL}$ ).

NOTE: During the incubation time other clones can be picked. It is recommended to pick as many clones as possible. The more clones are picked the higher the chances will be of identifying positive ones.

1.4.7. Once all the clones are picked and transferred in the 96 well plate, allow the cells to grow for a few days in selective medium (Ham's F-12K (Kaighn's) medium supplemented with 10% FBS, 2 mM L-glutamine, penicillin (0.5 units per mL of medium), streptomycin (0.2  $\mu\text{g}$  per mL of medium) and containing neomycin at a concentration of 0.7  $\mu\text{g}/\text{mL}$ ) at 37  $^{\circ}\text{C}$  and 5%  $\text{CO}_2$ , until reaching 90% confluence.

## 1.5. Splitting of clones

1.5.1. Prepare three 96 well plates coated with gelatin by adding 100  $\mu\text{L}$  of gelatin per well, incubate for 30 min at room temperature, then wash two times with PBS. These plates will be used for DNA extraction (DNA plate) and for clone freezing (freezing plates).

NOTE: Gelatin will promote the attachment of the cells and of the DNA to the wells. In particular, the gelatin coating will allow the DNA to stick at the bottom of the wells during the DNA extraction and wash procedures (discussed below). During the clone-splitting procedure, it is advisable to use a multichannel pipette.

1.5.2. Once clones reach 90% confluence, aspirate medium from each well of the 96 well plate, wash with PBS, add 30  $\mu\text{L}$  of 0.25% trypsin per well, and incubate for 5 min at 37  $^{\circ}\text{C}$ .

NOTE: Clones will grow at different rates, which will also depend on the number of cells picked per clone. Thus, this step will be performed during the several days when the different clones reach 90% confluence.

1.5.3. Add 70  $\mu\text{L}$  of selective medium per well, disrupt cell clumps by pipetting up and down inside the wells, then transfer 30  $\mu\text{L}$  of the 100  $\mu\text{L}$  to the gelatinized DNA plate prefilled with 120

175  $\mu$ L of selective medium per well and 30  $\mu$ L to the gelatinized freezing plates prefilled with 50  $\mu$ L  
176 medium (without selection).

178 1.5.4. Place the DNA plate in the incubator and allow the cells to grow at 37 °C and 5% CO<sub>2</sub> until  
179 90% confluence.

181 1.5.5. Add 80  $\mu$ L of ice-cold freshly made 2x freezing medium (80% FBS and 20% dimethyl  
182 sulfoxide (DMSO)) to each well of the freezing plate, add parafilm (sprayed with 70% ethanol) on  
183 top of the plate so to seal each well, place the lid on top and wrap the plate with aluminum foil.  
184 Place the freezing plates at -80 °C.

186 1.5.6. Add 90  $\mu$ L of selective medium per well to the original 96 well plate containing the clones  
187 that have been split and keep it in culture until PCR screening results. This plate will be used as  
188 backup plate.

## 190 2. Screening of Neomycin Resistant Clones

### 192 2.1. DNA extraction from the 96 well DNA plate.

194 2.1.1. Once the clones cultured in the DNA plate reach 90% confluence, wash 2 times with PBS,  
195 then lyse with 50  $\mu$ L lysis buffer (10 mM Tris pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% SDS, and 1  
196 mg/mL proteinase K). Cover the plate with parafilm, sealing each well, put the lid on, cover with  
197 saran wrap, and place at 37 °C overnight.

199 2.1.2. Add 100  $\mu$ L of cold ethanol (Et-OH)/NaCl solution (0.75 M NaCl in 100% ethanol) to each  
200 well and precipitate for 6 h or overnight at room temperature.

202 NOTE: The protocol can be paused here.

204 2.1.3. Remove the Et-OH/NaCl solution by inverting the plate and wash 3 times with 200  $\mu$ L of  
205 70% ethanol per well.

207 2.1.4. Add 25  $\mu$ L of RNase A solution in distilled water and incubate at 37 °C for 1 h.

209 2.2. Use 3  $\mu$ L of genomic DNA for PCR amplification. Perform PCR screening of the selected  
210 clones using primers annealing within the neomycin resistance gene and subtelomere 15q. PCR  
211 amplification is performed using standard polymerase enzymes and PCR protocols<sup>21</sup>.

213 NOTE: PCR conditions for MS2 primers (MS2-subtel15q-primer-S and MS2 primer AS) and CTR  
214 primers (CTR prime S and CTR primer AS) are the following: 98 °C for 20 s as denaturation step  
215 and then 34 cycles at 98 °C 10 s, 58°C 20 s, 72 °C 15 s, using polymerase enzyme in a 25  $\mu$ L reaction  
216 mix (see **Table of Materials**). Primer sequences are indicated in **Table 1**.

2.3. Run PCR reactions on agarose gel and extract PCR bands obtained from positive clones using standard gel extraction procedures (see **Table of Materials** for gel extraction reagents).

2.4. Perform DNA sequencing analyses of the gel-extracted PCR product for confirmation of the presence of the MS2 sequences<sup>21</sup>.

NOTE: The sequencing analyses can be performed using the primers used for the PCR screening.

## 2.5. Southern blot screening of PCR positive clones

2.5.1. Grow the clones positive at PCR and sequencing screening from the original 96 well plate (the backup plate) to 6 well plates in Ham's F-12K (Kaighn's) medium supplemented with 10% FBS, 2 mM L-glutamine, penicillin (0.5 units per mL of medium), streptomycin (0.2 µg per mL of medium) and containing neomycin at a concentration of 0.7 µg/mL) at 37 °C 5% CO<sub>2</sub>.

NOTE: Alternatively, if some of these clones have been lost, thaw them from one of the freezing plates (see protocol 2.6).

2.5.2. Once the clones are at 90% confluence in the 6 well plate, wash the cells with PBS and add 250 µL of lysis buffer containing 0.5 µg proteinase K per well.

2.5.3. Scrape the cells using a cell scraper and transfer the lysate in a 1.5 mL tube.

2.5.4. Incubate at 37 °C for 16 h.

2.5.5. Add 1 mL of 100% ethanol, shake vigorously, and allow the DNA to precipitate at least 2 h or overnight at -20 °C.

NOTE: The protocol can be paused here.

2.5.6. Spin at 13,400 x g at 4 °C for 10 min, discard the supernatant, and wash the pellets with 70% ethanol. Let the pellets air dry at room temperature. Alternatively, use a vacuum concentrator.

2.5.7. Resuspend the DNA pellets in 50 µL of distilled water containing RNase A and incubate for 1 h at 37 °C.

2.5.8. Digest 5-10 µg of genomic DNA using NcoI and BamHI restriction enzymes (two independent digestions) in 100 µL reaction volume by incubating the digestion reactions at 37 °C overnight.

2.5.9. Run 4 µL of the digestion on agarose gel (0.8% agarose) for complete digestion confirmation.



2.5.10. Add 1/10 volume of sodium acetate 3 M solution pH 5.2 and 2 volumes of 100% ethanol to the restriction digestion reactions and incubate at least 2 h or overnight at -20°C to precipitate DNA.

NOTE: The protocol can be paused here.

2.5.11. Centrifuge at 13,400 x g at 4 °C for 20 min, discard the supernatants, and wash the pellets with 70% ethanol. Allow the pellets to air dry at room temperature. Alternatively, use a vacuum concentrator.

2.5.12. Resuspend pellets in 20 µL of distilled water and load the digested DNA on a 0.8% agarose gel.

NOTE: For a better resolution of the digested DNA, prepare a gel at least 15 cm long and run overnight at low voltage (~30 volts). The electrophoresis set up should be optimized.

2.5.13. The following day, stain the gel with a DNA labelling agent, such as ethidium bromide at 1 µL/10 mL final concentration, for 30 min at room temperature and take a picture with a ruler close to the gel on a gel imaging instrument.

2.5.14. Set the transfer of DNA to a nylon membrane and perform membrane hybridization with a MS2 sequence-specific probe using standard procedures<sup>21</sup>.

2.5.15. Thaw the clones that are positive at PCR, DNA sequencing and Southern blot from one of the two freezing plates (see next step).

## 2.6. Thawing of clones

2.6.1. Prepare one 15 mL tube containing 5 mL of pre-warmed Ham's F-12K (Kaighn's) medium supplemented with 10% FBS, 2 mM L-glutamine, penicillin (0.5 units per mL of medium), and streptomycin (0.2 µg per mL of medium) for each clone to be thawed.

2.6.2. Remove one of the freezing plates from -80° and add 100 µL of pre-warmed F12K complete medium to the well containing the positive clone to be thawed.

NOTE: This procedure should be performed quickly and the 96 well plate should be placed on dry ice after each clone is thawed, in order to allow the other clones to remain frozen. This is particularly important if multiple clones need to be thawed from the same 96 well plate.

2.6.3. Transfer the cells to the 15 mL tube containing 5 mL of medium and centrifuge at 800 x g for 5 min at room temperature.

2.6.4. Aspirate the medium, resuspend the cells in 500 µL of pre-warmed complete F12K medium, and transfer each clone to a single well of a 12 well plate.

2.7. Elimination of the neomycin resistance gene from the MS2 cassette integrated at subtelomere 15q.

2.7.1. Allow the clones to grow from a 12 well plate to a 10 cm dish in complete F12K medium.

NOTE: Neomycin should not be included in the medium unless otherwise indicated.

2.7.2. Add the Cre-expressing adenovirus to the cells cultured in a 10 cm dish at 70% confluence in 10 mL of complete F12K medium.

NOTE: Using a Cre-GFP expressing adenovirus will allow evaluation of the efficiency of infection, which should approach 100%. The replication-defective adenovirus will be lost after few passages in culture.

2.7.3. 48 hours after infection, split the cells in three 10 cm dishes. Two dishes will be used for verification of the neomycin gene removal by negative selection, growing the cells in neomycin-containing medium (first dish), and by Southern blot (second dish).

2.7.4. Culture the third dish containing the clone for cell freezing and RNA extraction.

### 3. Verification of TERRA-MS2 Transcript Expression by RT-qPCR

3.1. Upon verification of neomycin gene removal, perform total RNA extraction from TERRA-MS2 clones using organic solvents (phenol and guanidine isothiocyanate solution)<sup>21</sup>.

3.1.1. Resuspend the RNA extracted from a 10 cm dish in 100  $\mu$ L of diethyl pyrocarbonate (DEPC) water.

3.1.2. Run 3  $\mu$ L of RNA on a denaturing (1% formaldehyde-containing) 1x 3-(N-Morpholino) propanesulfonic acid (MOPS) gel in order to verify concentration and integrity of the RNA. Also analyse RNA concentration using a spectrophotometer.

3.1.3. Treat 3  $\mu$ g of RNA with DNase I using 1 unit of DNase I enzyme in 60  $\mu$ L final reaction volume.

3.1.4. Incubate the reaction for 1 h at 37 °C.

3.2. Reverse transcription reaction and qPCR analyses

3.2.1. Add the following components to a nuclease-free microcentrifuge tube: 2  $\mu$ L of a 1  $\mu$ M TERRA specific primer, 1  $\mu$ L of dNTPs mix (10 mM each), 6  $\mu$ L of DNase I-treated RNA (corresponding to ~300ng RNA). Adjust the volume to 13  $\mu$ L with 4  $\mu$ L of DEPC water.

NOTE: For each RNA to be analysed, a second tube containing the same reagents but a reference specific primer, instead of TERRA specific primer, should be prepared.

3.2.2. Heat the mixture to 65 °C for 5 min and incubate in ice for at least 1 min.

3.2.3. Collect the content of the tubes by brief centrifugation and add 4 µL of 5X RT enzyme Buffer, 1 µL 0.1 M dithiothreitol (DTT), 1 µL (4 units) of RNase inhibitor, and 1 µL of reverse transcriptase (see **Table of Materials**).

3.2.4. Incubate the samples at 42 °C for 60 min, then use 2 µL of the RT reaction for qPCR analyses.

3.3. Prepare qPCR reaction mix in a final volume of 20 µL consisting of 10 µL of 2x qPCR master mix, 2 µL of cDNA template, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), and 6 µL of water.

3.4. Perform qPCR reaction in a thermocycler using standard protocols<sup>21</sup>.

#### **4. Production of a MS2-GFP Expressing Retrovirus**

4.1. To generate MS2-GFP expressing retrovirus, transfect 80% confluent phoenix packaging cells with a MS2-GFP fusion protein expressing retrovirus vector (pBabe-MS2-GFP PURO) and an *env* gene expressing vector (such as pCMV-VSVG) using a molar ratio 4:1 of the two vectors.

4.2. On the following day, replace the culturing medium (DMEM supplemented with 10% FBS, 2 mM L-glutamine and Pen/Strep) with fresh medium containing 10mM sodium butyrate.

4.3. Incubate for 8 h at 37 °C, 5% CO<sub>2</sub>, then replace the medium with fresh culturing medium from which the virus will be collected.

4.4. After 48 h, remove the retrovirus-containing medium from the phoenix cells. This medium can be directly used for infection of TERRA-MS2 clones, in which case filter the medium through a 0.45 µm filter, add polybrene (30 µg/mL final concentration) and add it to the cells (in this case the protocol continues at step 5). Alternatively, retrovirus can be precipitated in a 50 mL falcon tube by adding 1/5<sup>th</sup> volume of 50% PEG-8000/900 mM NaCl solution and incubating overnight at 4 °C on a rotator wheel.

4.5. On the following day, pellet retrovirus particles by centrifugation at 2000 x g for 30 min, remove supernatant, and resuspend the pellet in F12K medium without serum.

NOTE: The virus particles can be resuspended in 1/100<sup>th</sup> of the original supernatant volume. An infection test should be performed in order to verify the minimum volume of retrovirus required to efficiently infect the cells.

## 5. Visualization of TERRA-MS2 Transcripts in Living Cells

5.1. Plate TERRA-MS2 clones and WT AGS cells in glass-bottomed dishes. On the day of infection, add polybrene to the medium (30 µg/mL final concentration) and the MS2-GFP expressing retrovirus.

5.2. After 24 hours, discard the virus-containing medium and add fresh medium without phenol-red.

5.3. Analyze the cells at an inverted microscope using the appropriate microscope setting. Image the cells with a 100X or 60X objective with large numerical aperture (1.4X) and using a sensitive camera (EMCCD). Use an environmental control system to maintain the samples at 37 °C and 5% CO<sub>2</sub> during imaging.

### REPRESENTATIVE RESULTS:

**Figure 1** represents an overview of the experimental strategy. The main steps of the protocol and an indicative timeline for the generation of TERRA-MS2 clones in AGS cells are shown (**Figure 1A**). At day 1, multiple wells of a 6 well plate are transfected with the MS2 cassette and sgRNA/Cas9 expressing vectors (shown in **Figure 1B**). Two different subtelomere 15q-specific guide RNA sequences are cloned in the Cas9 nickase-expressing pX335 vector, generating two sgRNA-pX335 vectors that are co-transfected with the MS2 cassette. One well of the plate can be transfected with a GFP expressing vector to verify the transfection efficiency. At day 2, the cells are trypsinized and transferred from a single well to a 10 cm dish containing selective medium. At day 3, medium should be changed as most untransfected cells will be dead. Cells are grown in selection until clones are visible and ready to be picked. During this time, cells should not be trypsinized and culturing medium should be changed every 1-2 days for the first week and then every 2-3 days afterwards. Once single clones are visible, they are picked and transferred in a 96 well plate where they are allowed to grow until reaching 80-90% of confluence. At this point, clones are split in 4 different 96 well plates, which are marked in **Figure 1** with a color code. Once the clones cultured in the DNA plate (green) reach 80% confluence, they are lysed and genomic DNA extracted for PCR screening; the clones in the backup plate (blue) will be kept in culture until the results of the PCR screening, while the red freezing plates are frozen at -80°C. **Figure 2B** shows representative results of a PCR screening of neomycin-resistant clones. For this screening, two sets of primers are used: one primer pair (MS2 primers) annealing within the neomycin gene and subtelomere 15q sequence is used to verify the presence of the MS2 cassette (a representative image of the primers localization is shown in **Figure 2A**). A second primer pair (CTR primers) annealing at an internal chromosomal region is used to verify the presence of false negative clones (primer sequences are indicated in **Table 1**). Negative clones for the integration of the cassette should be negative to the MS2 primers' amplification and positive to the CTR primers' amplification. Technical problems in genomic DNA extraction resulting in the absence of genomic DNA or its contamination would preclude PCR amplification from MS2 and CTR primer reactions. In these cases, the clones involved can be re-screened by PCR upon extraction of genomic DNA from the backup plate. Bands obtained from MS2 primers amplification of positive clones can be

gel-extracted and sequenced using the MS2 primers, in order to confirm the presence of ten MS2 sequences.

The clones that are positive at PCR screening are cultured from the 96 well backup plate (the blue plate in **Figure 1**) to a 6 well plate, then lysed for genomic DNA extraction and Southern blot analyses. **Figure 2D** shows representative images of a gel (left) and membrane hybridized with a radioactively labelled MS2 sequence specific probe (right) of a Southern blot screening of PCR positive clones. For confirmation of the MS2 sequences integration at subtelomere 15q, genomic DNA extracted from each clone should be digested with two different restriction enzymes (NcoI and BamHI) in two separate digestion reactions. The NcoI and BamHI restriction enzymes are suitable for verification of the MS2 cassette integration at subtelomere 15q. Other enzymes can also be used. The gel shows a complete digestion of genomic DNA using the BamHI restriction enzyme, as indicated by the presence of a smear. The results from Southern blot indicate that one clone is positive for the integration of the MS2 cassette at subtelomere 15q while several clones show multiple integration events of the cassette, as indicated by the presence of multiple bands. A positive clone should be analyzed by a second Southern blot upon NcoI digestion of genomic DNA<sup>21</sup>. A representative image of a subtelomere 15q containing the MS2 cassette and the position of BamHI and NcoI restriction enzyme sites is shown in **Figure 2C**.

The clones positive to PCR and Southern blot analyses are infected with a Cre-GFP expressing adenovirus in order to remove the neomycin gene present in the MS2 cassette. **Figure 3A** depicts a MS2-tagged subtelomere 15q before and after Cre expression. An image of a clone positive for the MS2 cassette integration at subtelomere 15q infected with a Cre-GFP expressing adenovirus is shown in **Figure 3B**. For a complete removal of the neomycin gene in all cells, the infection efficiency should reach approximately 100%. As shown in **Figure 3C**, in order to verify the elimination of the neomycin gene, Cre-GFP infected cells are split in three plates after 48 hours from the infection. One plate will be cultured in the presence of neomycin. All cells in this plate should die within 6-7-days. In a second plate, cells will be allowed to grow in complete medium without selection up to 80-90% confluence. At this point, genomic DNA is extracted and analyzed by Southern blot. The third plate is cultured in complete medium to allow the preparation of frozen stocks of the clone and for RNA extraction and RT-qPCR analyses of TERRA-MS2 transcript expression. **Figure 3D** shows representative images of Southern blot analyses of a positive clone before and after Cre-GFP infection. DNA agarose gel (image on the left) confirms the complete digestion of the genomic DNA using the NcoI restriction enzyme. Southern blot analysis was performed using a radioactively-labelled MS2-sequence specific probe (image on the right). This analysis confirms the removal of the neomycin gene. The presence of a smear in the Cre-GFP infected sample confirms the telomeric integration of the MS2 sequences. The position of the NcoI restriction sites within the subtelomere 15q is shown in **Figure 3A**.

Once the elimination of the neomycin resistance gene is confirmed, the clones can be tested for the expression of TERRA-MS2 transcripts. To this aim, total RNA is extracted from each clone and run on a denaturing MOPS gel to confirm its integrity (**Figure 4A**). Ribosomal RNA bands should be visible at 4,700 bases (rRNA 28S) and 1,900 bases (rRNA 18S). Retrotranscription reaction is performed using a telomeric repeat-specific primer and reference gene-specific primer (**Figure**

481 **4B**, top) while qPCR analyses of TERRA expression are performed using two sets of primers: one  
482 primer pair annealing within the MS2 sequence and the subtelomere 15q sequence; a second  
483 pair of primers annealing within the subtelomere 15q. Primer sequences are indicated in **Table**  
484 **1**. The graph presented in **Figure 4B** shows RT-qPCR analyses of TERRA expression from AGS WT  
485 cells and two different TERRA-MS2 clones. These analyses confirm the expression of TERRA-MS2  
486 transcripts in the two clones at levels that are comparable to TERRA transcripts expressed from  
487 subtelomere 15q in WT cells. These data indicate that the two TERRA-MS2 clones selected are  
488 suitable for the analyses of TERRA-MS2 transcripts by live cell imaging.

489  
490 In order to visualize TERRA-MS2 transcripts in living cells, the selected clones are infected with a  
491 retrovirus expressing the MS2-GFP fusion protein. **Figure 5A** shows the procedure to generate  
492 MS2-GFP expressing retrovirus, as described in protocol step 4. AGS WT cells and TERRA-MS2  
493 clones are infected in glass-bottomed dishes. After 24 h from the infection, cells are analyzed by  
494 fluorescence microscopy. The specificity of the signal is confirmed by the presence of TERRA-  
495 MS2-GFP foci detected in the nucleus of TERRA-MS2 clones and not in AGS WT cells (**Figure 5B**).  
496 In a population of MS2-GFP expressing cells, heterogeneity in terms of MS2-GFP levels among  
497 cells is expected and cells expressing low levels should be chosen for the imaging analyses.  
498 Alternatively, the MS2-GFP expressing cells can be sorted by FACS prior microscopy analyses in  
499 order to collect the subpopulation of cells expressing low levels of GFP. We have previously  
500 detected TERRA-MS2-GFP foci in 40% to 60% of cells of AGS TERRA-MS2 clones<sup>21</sup>. In these cells,  
501 one to four TERRA-MS2-GFP foci were imaged per cell. TERRA-MS2-GFP foci showing distinct sizes  
502 and dynamics can be detected<sup>21</sup>. TERRA-MS2 clones can be used to study the dynamics of single-  
503 telomere TERRA transcripts in living cells. As an example of this application, **Figure 5C** shows  
504 representative images from microscopy analyses of a TERRA-MS2 clone expressing the MS2-GFP  
505 fusion protein and the telomere-binding protein TRF2 fused to mCherry in order to visualize MS2-  
506 tagged TERRA transcripts and telomeres in living cells. Using this approach, we have previously  
507 observed that TERRA-MS2-GFP foci co-localize with telomeres in 44% of the cells, indicating that  
508 TERRA transcripts only transiently co-localize with chromosome ends in AGS cells<sup>21</sup>.

#### 509 510 **FIGURE AND TABLE LEGENDS:**

511 **Figure 1. Overview of the experimental strategy and indicative timeline for the generation of**  
512 **TERRA-MS2 clones in AGS cells. A)** The steps described in the protocol and an indicative time  
513 line for the selection of TERRA-MS2 clones are shown. Cells are transfected in a 6 well plate with  
514 the linearized MS2 cassette and sgRNA/Cas9 expressing vectors. A color code is used to  
515 distinguish the DNA plate, the backup plate and the freezing plates indicated in the protocol  
516 section. **B)** The MS2 cassette consists of an 800 nt long subtelomere 15q sequence followed by  
517 10 repetitions of the MS2 sequences and a neomycin resistance gene flanked by lox-p sites and  
518 terminates with a 300 nt long telomeric repeat tract at its 3' end. sgRNA/Cas9 expressing vectors  
519 were generated by cloning subtelomere 15q-specific guide RNA sequences in pX335 vector using  
520 the BbsI restriction site<sup>21,22</sup>. Two different sgRNA-pX335 vectors were generated and a 1:1 mix of  
521 the two vectors was used for the transfection. The sequences of the sgRNAs are indicated in  
522 **Table 1**.

**Figure 2. Representative images of PCR and Southern blot screening of neomycin resistant clones.** **A)** Representative image of subtelomere 15q containing the MS2 cassette. The position of the MS2 primers (MS2-subtel15q-primer-S and MS2 primer AS) used for PCR screening is indicated. The loxP sites present within the cassette are shown in red. **B)** Representative image of PCR screening of neomycin resistant clones using two set of primers, MS2 primers and CTR primers (CTR prime S and CTR primer AS). **C)** Representative image of subtelomere 15q containing the MS2 cassette. BamHI and NcoI restriction sites and the MS2 probe used for the Southern blot screening are shown. **D)** Left: 10 µg of genomic DNA per clone were digested with BamHI and run on an agarose gel. The image was acquired after an overnight run at 30 volts. Right: genomic DNA digested with BamHI restriction enzyme was transferred to a positively charged nylon membrane and hybridized with a radioactively-labelled MS2 sequence-specific probe. The red box indicates the expected size of the positive band. The red arrow indicates one positive clone.

**Figure 3. Elimination of the neomycin resistance gene and validation experiments.** **A)** Representative image of subtelomere 15q containing the MS2 cassette before and after Cre expression. The MS2 specific probe used for Southern blot analyses and NcoI restriction enzyme sites are shown. The loxP sites present within the cassette are shown in red. **B)** Fluorescence microscopy analysis of a TERRA-MS2 clone infected with Cre-GFP expressing adenovirus. Representative image acquired at a single focal plane is shown. MOI, multiplicity of infection, is the ratio between the number of viruses used for the infection and the number of host cells. Scale bar: 30 µm **C)** Overview of the experimental strategy used to verify the elimination of the neomycin gene. Cre-GFP infected cells are split in three plates after 48 hours from the infection for i) negative selection, ii) Southern blot analyses and iii) preparation of frozen cell stocks and RNA extraction. **D)** Southern blot analyses of a TERRA-MS2 clone before and after Cre-GFP adenovirus infection. 10 µg of genomic DNA were digested with the restriction enzyme NcoI. Agarose gel confirms that complete digestion is achieved in both clones (left). The digested genomic DNA was transferred to a positively charged nylon membrane and hybridized using a MS2 sequence-specific probe. Complete elimination of the neomycin gene is confirmed by the absence of the specific band.

**Figure 4. RT-qPCR analyses of Tel15q-TERRA and Tel15q-TERRA-MS2 transcripts expression.** **A)** Representative image of a MOPS gel showing total RNA extracted from AGS WT cells and TERRA-MS2 clones. Bands corresponding to the ribosomal RNAs 28S and 18S are indicated. **B)** Top: A schematic of the MS2-tagged subtelomere 15q expressing TERRA transcripts. Primers used for TERRA retrotranscription (yellow) and qPCR analyses of Tel15q-TERRA (blue) and Tel15q-MS2 TERRA (red) are shown. The loxP site is shown in red. Bottom: RT-qPCR analyses of Tel15q-TERRA and Tel15q-MS2 TERRA transcripts expression in AGS WT cells and TERRA-MS2 clones. \* $p < 0.05$ , unpaired  $t$ -test.

**Figure 5. Live cell imaging analyses of TERRA-MS2 clones.** **A)** An overview of the procedure to produce a MS2-GFP expressing retrovirus, as described in protocol step 4. A schematic of the MS2-tagged subtelomere 15q and TERRA transcripts recognized by the MS2-GFP fusion protein is shown on the right. **B)** Representative fluorescence microscopy image of AGS WT and two TERRA-MS2 clones expressing MS2-GFP. TERRA-MS2-GFP foci are indicated by arrows. Images

were acquired using a spinning disc confocal microscope equipped with an EMCCD camera. The images were captured using a 100X/1.46 apochromat objective in an imaging chamber maintained at 37 °C with 5% CO<sub>2</sub>. A 488 laser was used as light source. Scale bar: 5 µm. **C)** Live cell imaging analyses of TERRA-MS2-GFP foci and TRF2-mCherry labeled telomeres. A co-localization event between a TERRA-MS2-GFP focus and a single telomere is shown. Images were acquired as in **B**, using 488 and 520 lasers as light source. A maximal projection of a z stack experiment performed at a single time point of time-lapse imaging experiment is shown. Scale bar: 5 µm.

**Table 1: List of the primers used in this study.** A list of the primers and the sequences of the sgRNAs used in this protocol are provided. Sequences are 5' to 3'.

## DISCUSSION:

In this article we present a method to generate human cancer cell clones containing MS2 sequences integrated within subtelomere 15q. Using these clones, the MS2-tagged TERRA molecules transcribed from the subtelomere 15q are detected by fluorescence microscopy by co-expression of a MS2-GFP fusion protein. This approach enables researchers to study the dynamics of TERRA expressed from a single telomere in living cells<sup>21</sup>. In this protocol, TERRA-MS2 clones are selected in the AGS cell line, which represents an interesting model system to study TERRA, since TERRA expression is upregulated in human stomach cancer samples<sup>24</sup>. However, the protocol described here can be adapted for the selection of TERRA-MS2 clones in other cell lines. The integration of the MS2 sequences can in principle be promoted at a subtelomere different from telomere 15q by using a specific MS2 cassette and CRISPR strategy. In the method presented here, a Cas9 nickase enzyme and double guide RNAs are employed in order to increase the specificity of integration<sup>22</sup>. Using this strategy, an overall 2% of positive clones are expected to be identified in AGS cells. Other versions of the Cas9 enzyme can be tested in the attempt to increase the success rate of the clones selection<sup>25</sup>. In addition, the following steps of the protocol are critical ones to take into account to maximize the efficiency of the clone selection:

I) In order to increase the chances of selecting the TERRA-MS2 clones, it is important to achieve a high transfection efficiency of the MS2 cassette and the CRISPR vectors. Poorly transfected cell lines will most likely require several rounds of transfection, clone selection and screening in order to select positive clones.

II) It is important to determine the precise concentration of the neomycin to use for the selection of the clones. Indeed, using a low concentration of the drug will result in false positive clones while a high concentration may preclude the identification of positive clones. The neomycin concentration indicated in this protocol is lethal to the AGS cells within 6-7 days from the addition to the culturing medium.

III) Clone picking is a critical step. Indeed, during this procedure it is required that only single clones are picked. For this reason, colonies that are too close to each other should be avoided in order to prevent mixing cells from different clones, resulting in the selection of a mixed



population of clones which may contain MS2 sequences integrated at multiple genomic sites. These events should be identified during the Southern blot screening.

IV) The amount of genomic DNA extracted from a confluent 96 well DNA plate (protocol 2) is estimated to be around 5 µg and should suffice to screen every clone by PCR and Southern blotting with a single restriction enzyme digestion. However, in order to confirm the integration of the cassette at the expected telomere, it will be important to screen each clone by Southern blot by digesting the genomic DNA with two different restriction enzymes. To this aim, as indicated in the protocol, the clones positive at PCR screening should be cultured in 6 well plates. The amount of genomic DNA extracted from a well of a 6 well plate will be sufficient for at least two restriction digestions required for the Southern blot analyses.

In the protocol described here, the MS2 sequences are integrated within subtelomere 15q for a number of reasons: i) TERRA promoter region and TERRA transcription start sites have been identified on this subtelomere<sup>26,27</sup>; ii) TERRA expression from subtelomere 15q has been validated by using *in vitro* techniques<sup>4,27-30</sup>; iii) the subtelomeric region of the chromosome 15q has been sequenced and it contains a unique region adjacent to the telomeric repeat tract that can be targeted for the integration of the MS2-cassette<sup>21</sup>. PCR and Southern blot approaches were developed in order to confirm the single integration of the MS2 sequences within subtelomere 15q in the TERRA-MS2 clones. It would be interesting to also perform DNA-FISH experiments on chromosome spreads in order to visualize the chromosomal localization of the MS2 sequences in fixed metaphase chromosomes. However, the length of the 10xMS2 sequences (450 bp) imposes the use of very short probes as compared to the probes generally used in DNA-FISH experiments on chromosome spreads (Bacterial artificial chromosomes (Bac), cosmids or plasmids)<sup>31</sup>. This technical challenge has prevented us from visualizing the MS2 sequences on chromosome spreads which represents a limitation of the protocol. One further limitation of the method is the time and the effort required for selecting the TERRA-MS2 clones. In addition, specific laboratory set up and equipment for the use of viruses, radioactive material and microscopy analyses are required.

The method described here can be implemented for the generation of TERRA-MS2 clones in different cell lines in order to define the dynamics of TERRA in various biological contexts, such as during telomere dysfunction or cellular senescence, helping us to understand the function of TERRA in these processes. An interesting development of this approach will be the generation of TERRA-MS2 clones containing TetO repeats integrated at a specific subtelomere, including the MS2-tagged telomere. The expression of a TetR protein fused to a fluorescent protein (*i.e.*, mCherry) will allow visualization of this particular telomere in living cells<sup>32</sup>. This approach will enable investigation of whether human TERRA transcripts localize and act *in cis* at the TERRA transcribing telomere and chromosome, or *in trans* by relocating to other chromosomes. This question in the biology of TERRA remains to be clarified.

## ACKNOWLEDGMENT

We are grateful to the staff of the advanced imaging facility of CIBIO at the University of Trento and the BioOptics Light Microscopy facility at the Max F. Perutz Laboratories (MFPL) in Vienna.

The research leading to these results has received funding from the Mahlke-Obermann Stiftung and the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 609431 to EC. EC is supported by a Rita Levi Montalcini fellowship from the Italian Ministry of Education University and Research (MIUR).

## DISCLOSURE

The authors declare no competing financial interests

## REFERENCES

- 1 Azzalin, C. M., Reichenbach, P., Khoriauli, L., Giulotto, E. & Lingner, J. Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. *Science*. **318** (5851), 798-801, (2007).
- 2 Schoeftner, S. & Blasco, M. A. Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. *Nature Cell Biology*. **10** (2), 228-236, (2008).
- 3 Diman, A. & Decottignies, A. Genomic origin and nuclear localization of TERRA telomeric repeat-containing RNA: from Darkness to Dawn. *FEBS Journal*. 10.1111/febs.14363, (2017).
- 4 Arnoult, N., Van Beneden, A. & Decottignies, A. Telomere length regulates TERRA levels through increased trimethylation of telomeric H3K9 and HP1alpha. *Nature Structural & Molecular Biology*. **19** (9), 948-956, (2012).
- 5 Montero, J. J. *et al.* TERRA recruitment of polycomb to telomeres is essential for histone trimethylation marks at telomeric heterochromatin. *Nature Communications*. **9** (1), 1548, (2018).
- 6 Beishline, K. *et al.* CTCF driven TERRA transcription facilitates completion of telomere DNA replication. *Nature Communications*. **8** (1), 2114, (2017).
- 7 Arora, R. *et al.* RNaseH1 regulates TERRA-telomeric DNA hybrids and telomere maintenance in ALT tumour cells. *Nature Communications*. **5** 5220, (2014).
- 8 Balk, B. *et al.* Telomeric RNA-DNA hybrids affect telomere-length dynamics and senescence. *Nature Structural & Molecular Biology*. **20** (10), 1199-1205, (2013).
- 9 Graf, M. *et al.* Telomere Length Determines TERRA and R-Loop Regulation through the Cell Cycle. *Cell*. **170** (1), 72-85 e14, (2017).
- 10 Lee, Y. W., Arora, R., Wischnewski, H. & Azzalin, C. M. TRF1 participates in chromosome end protection by averting TRF2-dependent telomeric R loops. *Nature Structural & Molecular Biology*. 10.1038/s41594-017-0021-5, (2018).
- 11 Moravec, M. *et al.* TERRA promotes telomerase-mediated telomere elongation in *Schizosaccharomyces pombe*. *EMBO Reports*. **17** (7), 999-1012, (2016).
- 12 Cusanelli, E., Romero, C. A. & Chartrand, P. Telomeric noncoding RNA TERRA is induced by telomere shortening to nucleate telomerase molecules at short telomeres. *Molecular Cell*. **51** (6), 780-791, (2013).
- 13 Moradi-Fard, S. *et al.* Smc5/6 Is a Telomere-Associated Complex that Regulates Sir4 Binding and TPE. *PLoS Genetics*. **12** (8), e1006268, (2016).

698 14 Chu, H. P. *et al.* TERRA RNA Antagonizes ATRX and Protects Telomeres. *Cell*. **170** (1), 86-  
699 101 e116, (2017).

700 15 Lai, L. T., Lee, P. J. & Zhang, L. F. Immunofluorescence protects RNA signals in  
701 simultaneous RNA-DNA FISH. *Experimental Cell Research*. **319** (3), 46-55, (2013).

702 16 Zhang, L. F. *et al.* Telomeric RNAs mark sex chromosomes in stem cells. *Genetics*. **182** (3),  
703 685-698, (2009).

704 17 Gallardo, F. & Chartrand, P. Visualizing mRNAs in fixed and living yeast cells. *Methods in*  
705 *Molecular Biology*. **714** 203-219, (2011).

706 18 Querido, E. & Chartrand, P. Using fluorescent proteins to study mRNA trafficking in living  
707 cells. *Methods in Cell Biology*. **85** 273-292, (2008).

708 19 Cusanelli, E. & Chartrand, P. Telomeric noncoding RNA: telomeric repeat-containing RNA  
709 in telomere biology. *Wiley Interdisciplinary Reviews: RNA*. **5** (3), 407-419, (2014).

710 20 Perez-Romero, C. A., Lalonde, M., Chartrand, P. & Cusanelli, E. Induction and  
711 relocalization of telomeric repeat-containing RNAs during diauxic shift in budding yeast.  
712 *Current Genetics*. 10.1007/s00294-018-0829-5, (2018).

713 21 Avogaro, L. *et al.* Live-cell imaging reveals the dynamics and function of single-telomere  
714 TERRA molecules in cancer cells. *RNA Biology*. 10.1080/15476286.2018.1456300 1-10,  
715 (2018).

716 22 Ran, F. A. *et al.* Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing  
717 specificity. *Cell*. **154** (6), 1380-1389, (2013).

718 23 Yamada, T. *et al.* Spatiotemporal analysis with a genetically encoded fluorescent RNA  
719 probe reveals TERRA function around telomeres. *Scientific Reports*. **6** 38910, (2016).

720 24 Deng, Z. *et al.* Formation of telomeric repeat-containing RNA (TERRA) foci in highly  
721 proliferating mouse cerebellar neuronal progenitors and medulloblastoma. *Journal of Cell*  
722 *Science*. **125** (Pt 18), 4383-4394, (2012).

723 25 Casini, A. *et al.* A highly specific SpCas9 variant is identified by *in vivo* screening in yeast.  
724 *Nature Biotechnology*. **36** (3), 265-271, (2018).

725 26 Nergadze, S. G. *et al.* CpG-island promoters drive transcription of human telomeres. *RNA*.  
726 **15** (12), 2186-2194, (2009).

727 27 Porro, A. *et al.* Functional characterization of the TERRA transcriptome at damaged  
728 telomeres. *Nature Communications*. **5** 5379, (2014).

729 28 Farnung, B. O., Brun, C. M., Arora, R., Lorenzi, L. E. & Azzalin, C. M. Telomerase efficiently  
730 elongates highly transcribing telomeres in human cancer cells. *PLoS One*. **7** (4), e35714,  
731 (2012).

732 29 Flynn, R. L. *et al.* Alternative lengthening of telomeres renders cancer cells hypersensitive  
733 to ATR inhibitors. *Science*. **347** (6219), 273-277, (2015).

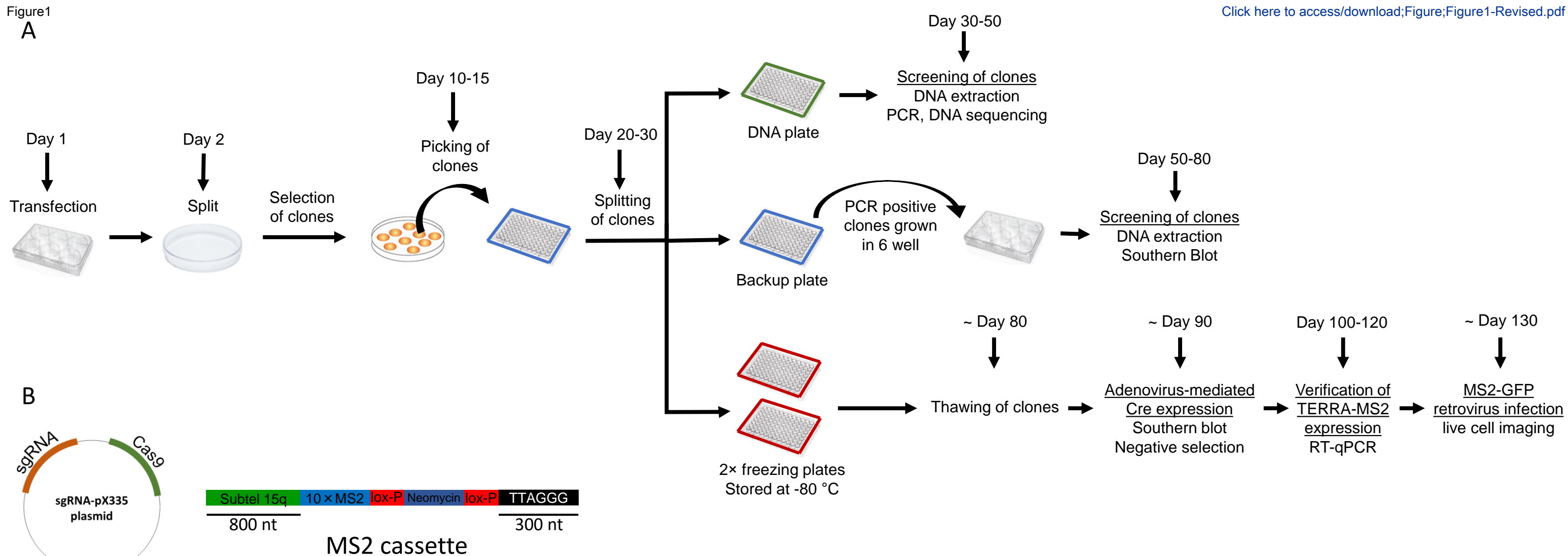
734 30 Scheibe, M. *et al.* Quantitative interaction screen of telomeric repeat-containing RNA  
735 reveals novel TERRA regulators. *Genome Research*. **23** (12), 2149-2157, (2013).

736 31 Bolland, D. J., King, M. R., Reik, W., Corcoran, A. E. & Krueger, C. Robust 3D DNA FISH using  
737 directly labeled probes. *Journal of Visualized Experiments*. 10.3791/50587 (78), (2013).

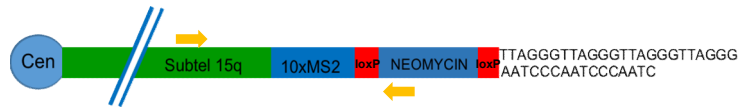
738 32 Masui, O. *et al.* Live-cell chromosome dynamics and outcome of X chromosome pairing  
739 events during ES cell differentiation. *Cell*. **145** (3), 447-458, (2011).

740

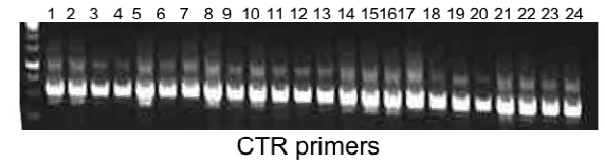
A



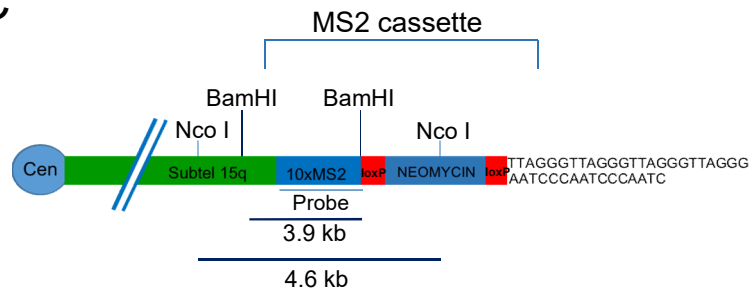
A



B



C



D

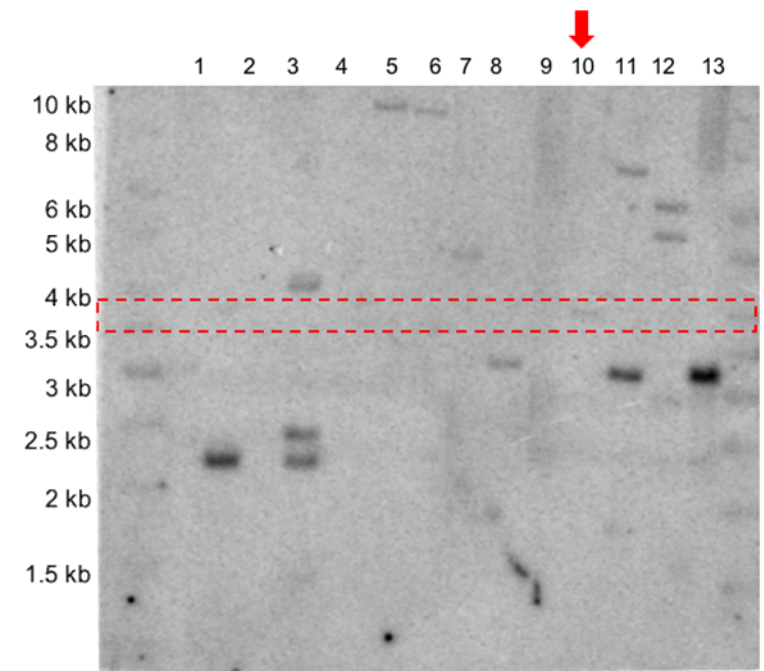
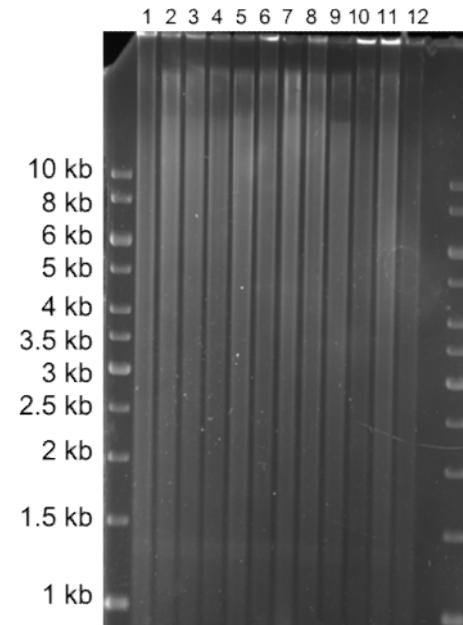


Figure3

[Click here to access/download;Figure;Figure3-Revised.pdf](#)

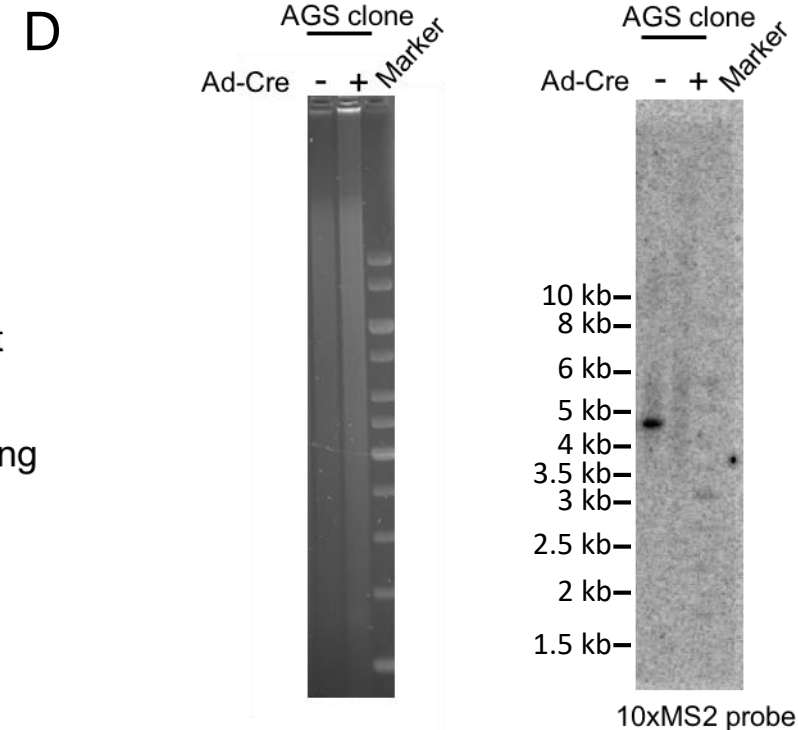
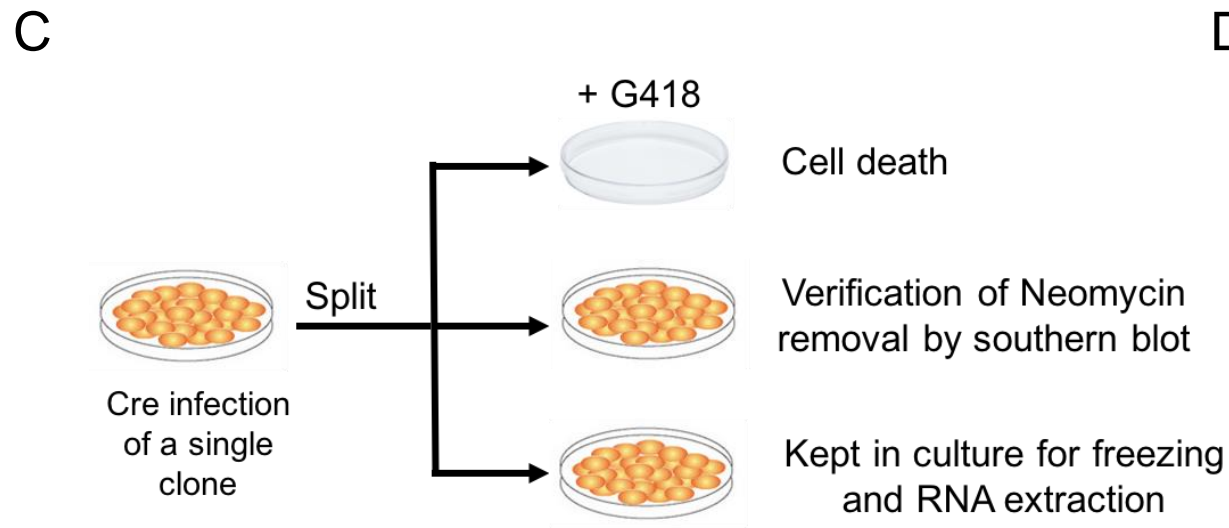
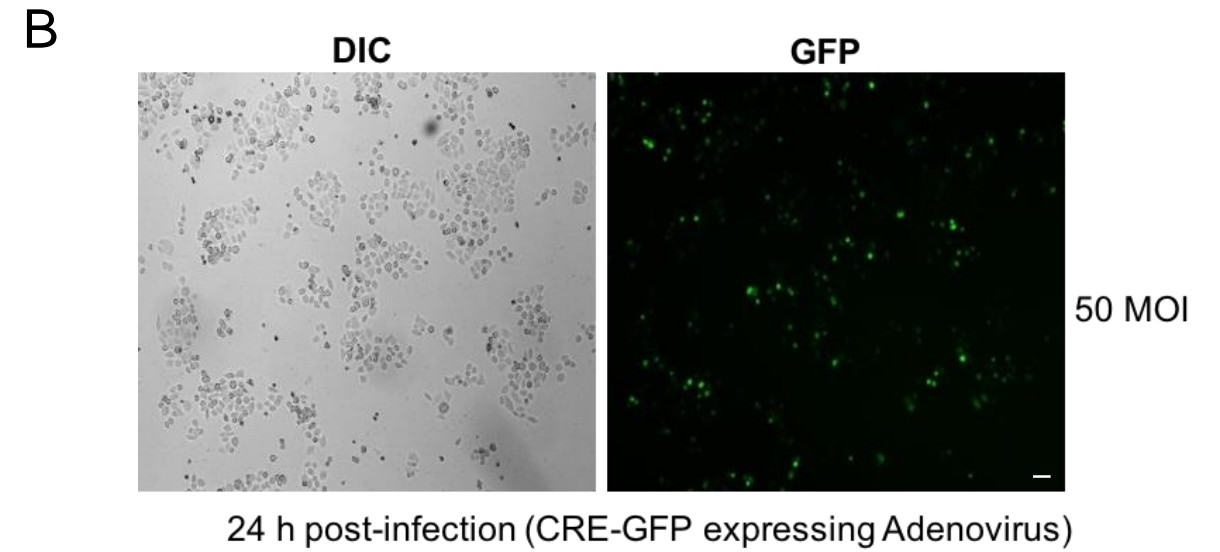
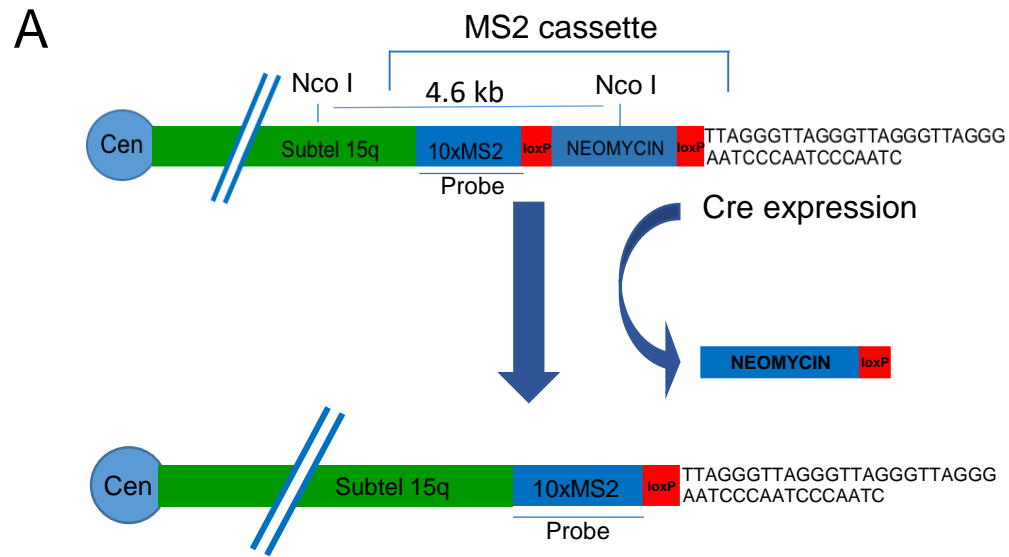


Figure 4

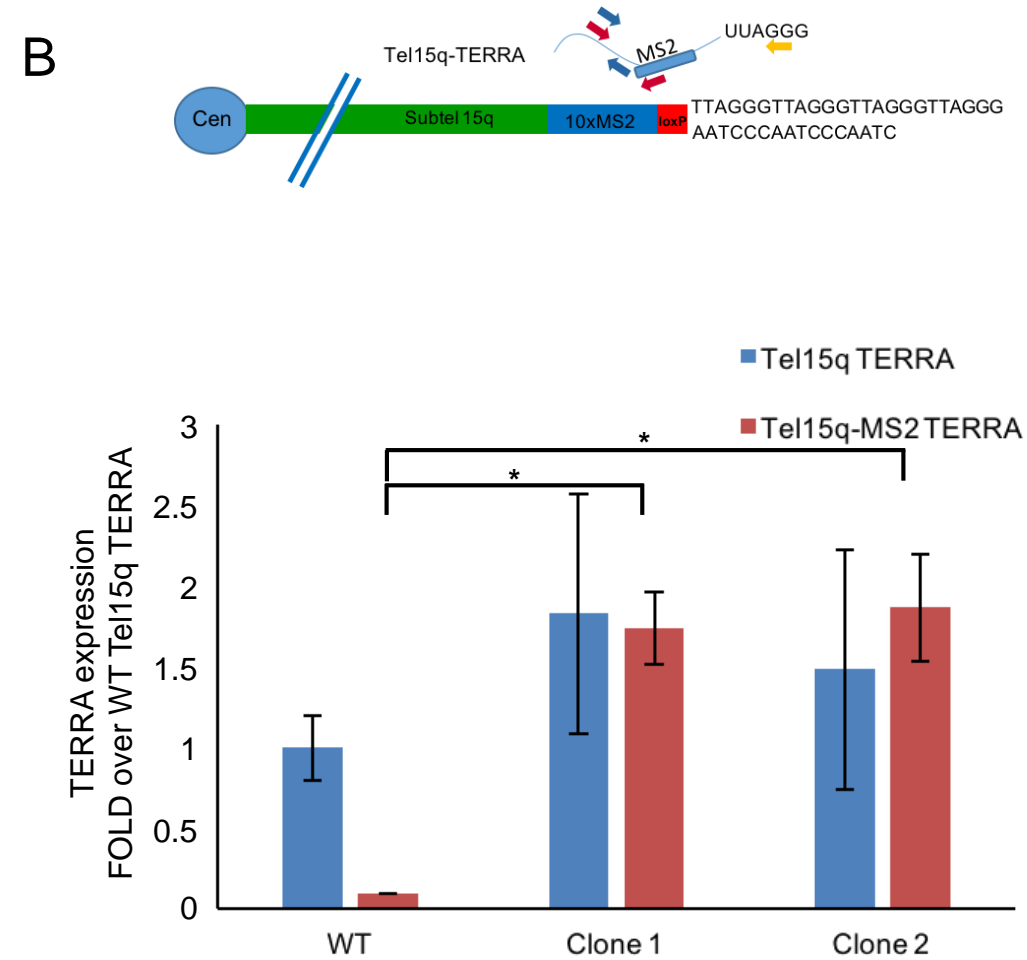
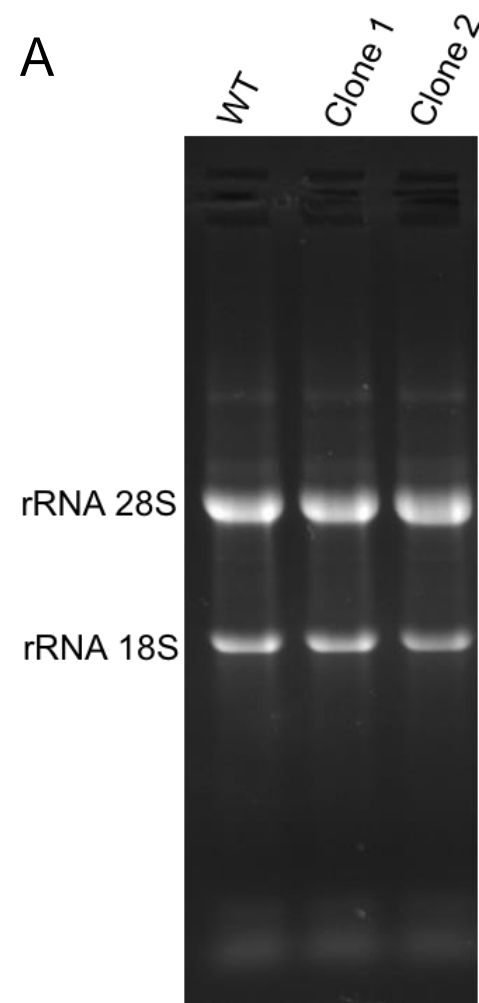
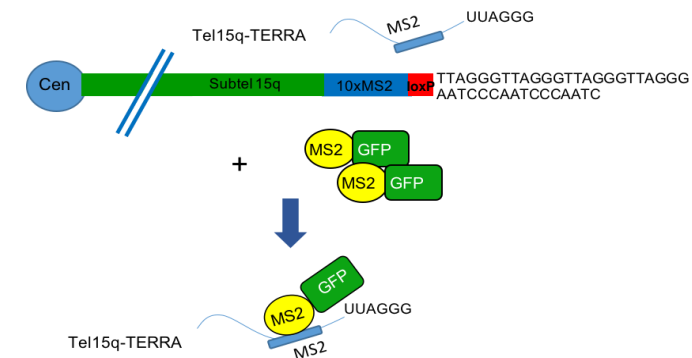
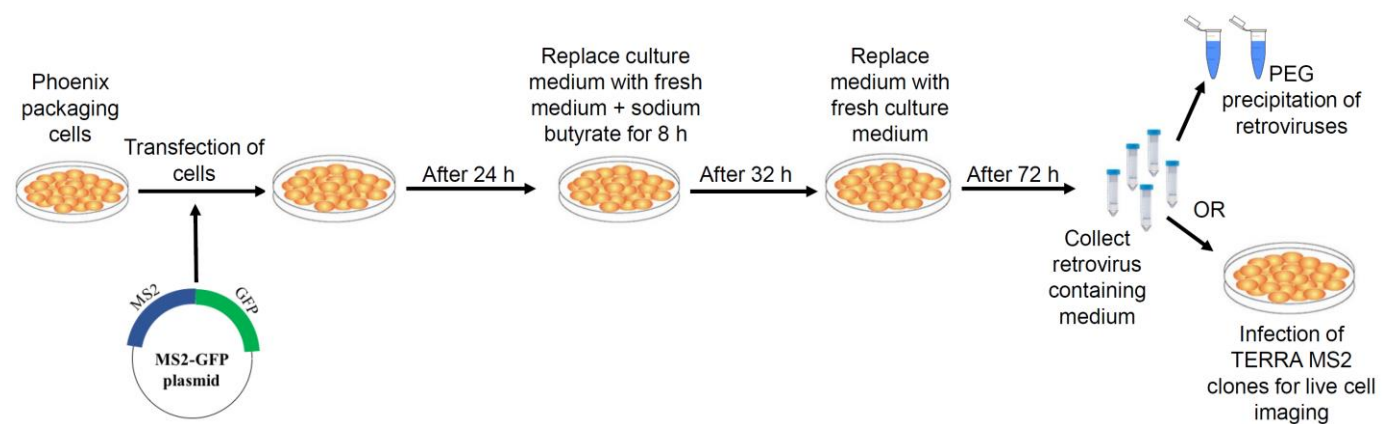


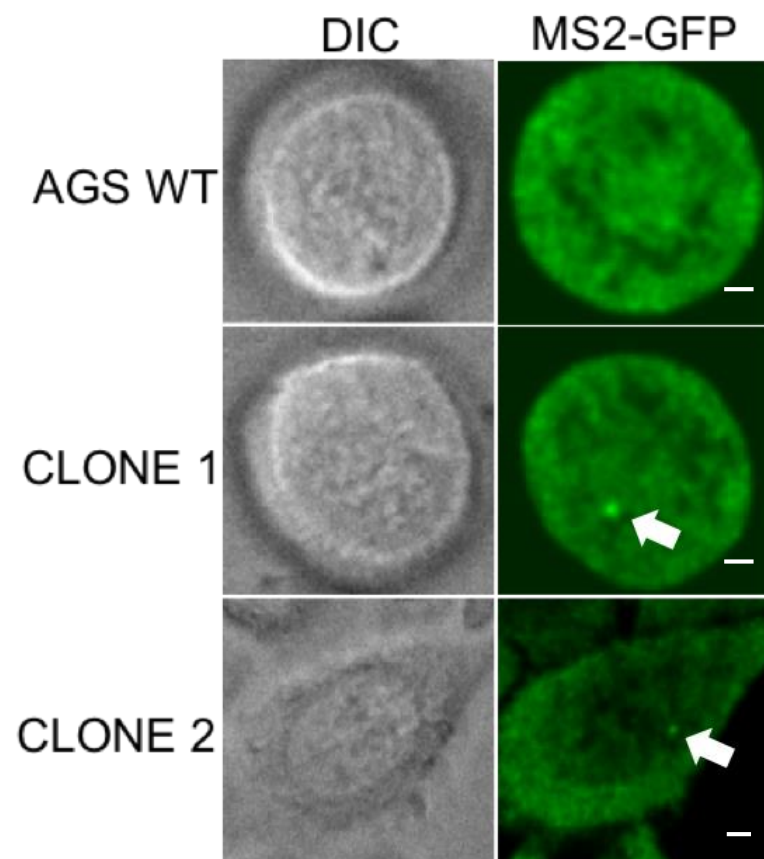
Figure 5

[Click here to access/download;Figure;Figure5-Revised2.pdf](#)

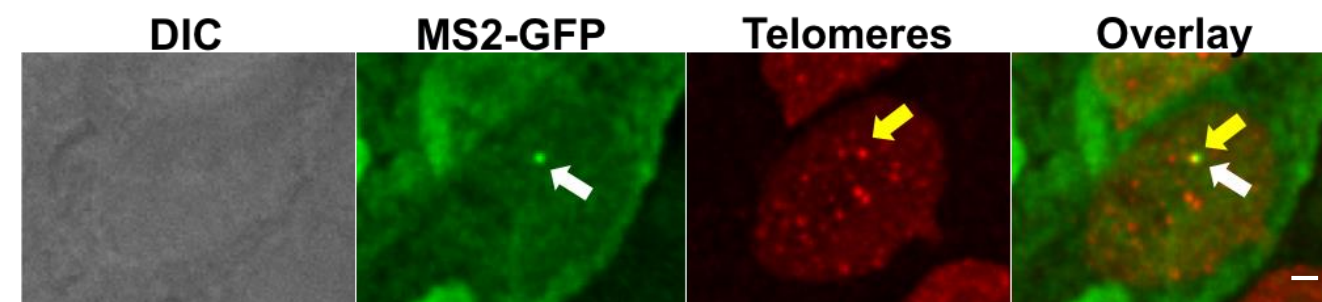
A



B



C





Primer name	Primer sequence	Application
MS2-subtel15q-primer-S	TGCATTAAAGGGTCCAGTTG	MS2 primer forward used for PCR screening of neomycin resistant clones
MS2 primer AS	CCTAACTGACACACATTCCACAGA	MS2 primer reverse used for PCR screening of neomycin resistant clones
CTR primer S	TGT ACG CCA ACA CAG TGC TG	CTR primer forward used in PCR screening of neomycin resistant clones
CTR primer AS	GCT GGA AGG TGG ACA GCG A	CTR primer reverse used in PCR screening of neomycin resistant clones
Tel15q-S1	GCAGCGAGATTCTCCAAGC	Sense primer used to detect Tel15q and Tel15q-MS2 TERRA expression by qPCR
hTel15q-AS	TAACCACATGAGCAATGTGGGTG	Antisense primer used to detect Tel15q and Tel15q-MS2 TERRA expression by qPCR
Tel15q-MS2-AS	ATGTTTCTGCATCGAAGGCATTAG G	Antisense primer used to detect Tel15q-MS2 TERRA expression by qPCR
TERRA-RT-primer	CCCTAACCCTAACCCTAACCCTAA CCCTAA	Primer used for retrotranscription of TERRA transcripts
sgRNA1	TTGGGAGAATCTCGCTGGCC	Sequence of the short guide RNA 1 cloned in pX335 vector and used to direct Cas9 nickase enzymatic activity to subtelomere 15q
sgRNA2	TGCATTAAAGGGTCCAGTTG	Sequence of the short guide RNA 2 cloned in pX335 vector and used to direct Cas9 nickase enzymatic activity to subtelomere 15q

Name of Material/ Equipment	Company	Catalog Number
AGS cells	-	-
F12K Nut Mix 1X	GIBCO	21127022
L-Glutamine	CORNING	MT25005CI
Penicillin Streptomycin Solution	CORNING	30-002-CI
Fetal Bovine Serum	Sigma Aldrich	F2442
DMEM 1X	GIBCO	21068028
CaCl <sub>2</sub>	Sigma Aldrich	C1016
HEPES	Sigma Aldrich	H3375
KCl	Sigma Aldrich	P9333
Dextrose	Sigma Aldrich	D9434
NaCl	Sigma Aldrich	S7653
Na <sub>2</sub> HPO <sub>4</sub>	Sigma Aldrich	S3264
TRYPsin EDTA SOLUTION 1X	CORNING	59430C
DPBS 1X	GIBCO	14190250
DMSO	Sigma Aldrich	D8418
G-418 Disulphate	Formedium	G4185
Gelatin solution Bioreagent	Sigma Aldrich	G1393
Tris-base	Fisher BioReagents	10376743
EDTA	Sigma Aldrich	E6758
SDS	Sigma Aldrich	71729
Proteinase K	Thermo Fisher	AM2546
RNAse A	Thermo Fisher	12091021
Agarose	Sigma Aldrich	A5304
Atlas ClearSight	Bioatlas	BH40501
ethanol	Fisher BioReagents	BP28184
Sodium Acetate	Sigma Aldrich	71196
Wizard SV Gel and PCR clean-Up system	Promega	A9282
Trizol	AMBION	15596018
Dnase I	THERMO SCIENTIFIC	89836
dNTPs mix	Invitrogen	10297018
DTT	Invitrogen	707265ML
diethyl pyrocarbonate	Sigma Aldrich	D5758
Ribolock	Thermo Fisher	EO0381
MOPS	Sigma Aldrich	M9381
Paraformaldehyde	Electron Microscopy Sciences	15710
Superscript III Reverse transcriptase	Invitrogen	18080-093
Pfu DNA polymerase (recombinant)	Thermo Scientific	EP0501
2X qPCR BIO SyGreen Mix Separate-ROX	PCR BIOSYSTEMS	PB 20.14
Cre-GFP adenovirus	<a href="https://medicine.uiowa.edu/vectorcore">https://medicine.uiowa.edu/vectorcore</a>	1174-HT
Sodium Butyrate	Sigma Aldrich	B5887
PEG8000	Sigma Aldrich	89510
35µ-Dish Glass Bottom	Ibidi	81158

Comments/Description
Gift from Christian Baron (Université de Montréal).
Culturing medium for AGS cells
Component of cell culturing medium
Component of cell culturing medium
Component of cell culturing medium
culturing medium for phoenix cell
used in phoenix cell transfection
used in phoenix cell transfection (HBS solution)
used in phoenix cell transfection (HBS solution)
used in phoenix cell transfection (HBS solution)
used in phoenix cell transfection (HBS solution) and retrovirus precipitation
used in phoenix cell transfection (HBS solution)
used in cell split
Dulbecco's Phosphate Buffered Saline
Component of cell freezing medium (80% FBB and 20% DMSO)
selection drug for
cotaing of 96 well DNA plate and freezing plate
Component of Cell lysis buffer for genomic DNA extraction
Component of Cell lysis buffer for genomic DNA extraction
Component of Cell lysis buffer for genomic DNA extraction
Component of Cell lysis buffer for genomic DNA extraction
RNA degradation during DNA extraction
DNA gel preparation
Stain reagent used for detecting DNA and RNA samples in agarose gel
DNA precipitation
Used for DNA precipitation at a 3M concentration pH5.2
Extraction of PCR fragments from agarose gel during PCR screening of neomycin positive clones
Organic solvent used for RNA extraction
degradation of genomic DNA from RNA
used in RT and PCR reactions
used in RT reactions
used to inactivate RNases in water (1:1000 dilution)
RNase inhibitor
preparation of RNA gel
preparation of denaturing RNA gel (1% PFA in 1x MOPS)
Retrotranscription reaction
PCR reaction
qPCR reaction
used to infect TERRA-MS2 clones in order to remove the neomycn gene
used to promote retrovirus particles production in phoenix cells
Precipitation of retrovirus partcles
used in live cell imaging analyses of TERRA-MS2 clones



1 Alewife Center #200  
Cambridge, MA 02140  
tel. 617.945.9051  
www.jove.com

## ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: GENERATION OF CANCER CELL CLONES TO VISUALIZE  
Author(s): TERNIA MOLECULES EXPRESSED FROM A SINGLE TSCOMORE W LIVING CELL  
LAURA AUGANO, CLAUDIO OSS PEGONAK, NICOLE BERTIN,  
ERICO CUSANECU

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

☒ Standard Access

☐ Open Access

Item 2: Please select one of the following items:

☒ 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



## 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

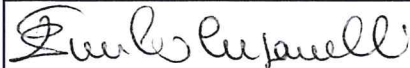
the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's 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 of 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 be 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:	EMILIO COSANSCU	
Department:	CENTRE FOR INTEGRATIVE BIOLOGY (CIBIO)	
Institution:	UNIVERSITY OF TORONTO	
Title:	PRINCIPAL INVESTIGATOR	
Signature:		Date: 16.7.2018

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

First, we would like to thank the editor for the opportunity of revising our manuscript. We have addressed all the comments indicated by the editor as explained below:

**Editorial comments:**

1. 1.1: Please explain how transfection is done, either with more detail or citations.

We now include a citation of our article recently published in *RNA Biology* (Avogaro *et al.*, 2018) in which we describe the methodology of cell transfection used in the protocol.

2. Figure 2D: There are two red arrows, as well as a red box that is not explained; please clarify in the figure legend.

In the new Figure 2D we have removed one red arrow. We now explain the red arrow and the red box in the Figure legend.

3. Figure 3B: Please use periods instead of commas for decimals (0.1, 1.5, etc.). Also, what statistical test did you use to obtain p values?

The editor might be referring to Figure 4B as there are no periods in Figure 3B. We have thus modified the graph shown in Figure 4B using periods instead of commas for the y-axis label. We indicate the statistical test used for p-value calculations in the figure legend.

4. Figure 4A: You have 'hour' and 'hr' here; please change both to 'h'.

We have now changed the text accordingly.

Sincerely,

Emilio Cusanelli