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## Use of retroviral-mediated gene transfer to deliver and test function of chimeric antigen receptors in human T-cells --Manuscript Draft--

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<b>Abstract:</b>	<p>Chimeric antigen receptors (CARs) are genetically delivered fusion molecules that elicit T-cell activation upon binding of a native cell surface molecule. These molecules can be used to generate a large number of memory and effector T-cells that are capable of recognizing and attacking tumor cells. More recently, it has been shown that expression of CARs in regulatory T-cell populations can be used to harness the immunoregulatory properties of these cells in the experimental treatment of chronic inflammatory diseases or transplant rejection. Most commonly, stable CAR expression is achieved in T-cells using retroviral vectors. In the method described here, retroviral vectors are packaged in a two-step procedure. First, H29D human retroviral packaging cells (a derivative of 293 cells) are transfected with the vector of interest, which is packaged transiently in vesicular stomatitis virus G pseudotyped particles. These particles are used to deliver the vector to PG13 cells, which achieve stable packaging of gibbon ape leukaemia virus-pseudotyped particles that are suitable for infection of human T-cells. To achieve T-cell transduction, cells must first be activated using a non-specific mitogen. Phytohemagglutinin provides an economic and robust stimulus to achieve this. After 48-72 hours, activated T-cells and virus-conditioned medium are mixed in retronectin-coated plasticware, which enhances transduction efficiency. Transduced cells are analyzed for gene transfer efficiency by flow cytometry after a further 48 hours and may then be tested in several assays to evaluate CAR function, including target-dependent cytotoxicity, cytokine production and proliferation.</p>
<b>Author Comments:</b>	I would like to try the sponsored option and would suggest approaching Sigma from whom several of the reagents used in this protocol have been sourced.

Additional Information:	
Question	Response

# Use of retroviral-mediated gene transfer to deliver and test function of chimeric antigen receptors in human T-cells

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**Key Words:** Retroviral; transduction; T-cell; chimeric antigen receptor

**Short Abstract:** A method is described to express and test chimeric antigen receptors in human T-cells.

## Abstract

Chimeric antigen receptors (CARs) are genetically delivered fusion molecules that elicit T-cell activation upon binding of a native cell surface molecule. These molecules can be used to generate a large number of memory and effector T-cells that are capable of recognizing and attacking tumor cells. More recently, it has been shown that expression of CARs in regulatory T-cell populations can be used to harness the immunoregulatory properties of these cells in the experimental treatment of chronic inflammatory diseases or transplant rejection. Most commonly, stable CAR expression is achieved in T-cells using retroviral vectors. In the method described here, retroviral vectors are packaged in a two-step procedure. First, H29D human retroviral packaging cells (a derivative of 293 cells) are transfected with the vector of interest, which is packaged transiently in vesicular stomatitis virus G pseudotyped particles. These particles are used to deliver the vector to PG13 cells, which achieve stable packaging of gibbon ape leukaemia virus-pseudotyped particles that are suitable for infection of human T-cells. To achieve T-cell transduction, cells must first be activated using a non-specific mitogen. Phytohemagglutinin provides an economic and robust stimulus to achieve this. After 48-72 hours, activated T-cells and virus-conditioned medium are mixed in retronectin-coated plasticware, which enhances transduction efficiency. Transduced cells are analyzed for gene transfer efficiency by flow cytometry after a further 48 hours and may then be tested in several assays to evaluate CAR function, including target-dependent cytotoxicity, cytokine production and proliferation.

## Introduction

Chimeric antigen receptors (CARs) are bespoke fusion molecules that couple the binding of a selected cell surface target molecule to the delivery of a tailored T-cell activating signal. Developed over 25 years ago, CAR technology has advanced to the point that several clinical trials are now ongoing in which this technology is being tested in patients with diverse malignancies<sup>1</sup>. Recently, durable complete clinical remissions have been achieved in patients with B-cell malignancy and neuroblastoma using this technology<sup>2-6</sup>. Signaling by CARs is most commonly provided by a fused endodomain in which the CD3 $\zeta$  subunit of the TCR/CD3 complex (signal 1) is combined with one or more co-stimulatory elements, including CD28, OX40 or 4-1BB. Most commonly, the CAR ectodomain consists of a single chain antibody fragment. **Figure 1** shows a typical example of such a composite CAR, named HOX and targeted against the MUC1 mucin<sup>7</sup>. Alternatively, CARs may be targeted using a ligand (eg IL-13 to target IL-13 receptor- $\alpha$ 2 or CD27 to target CD70)<sup>8,9</sup> or chimeric ligand with multiple target specificities (eg the T1E peptide – to target several ErbB dimers)<sup>10</sup>.

Most commonly, CAR expression is achieved in human T-cells using gamma retroviral vectors. These vectors achieve stable transgene expression but have not been linked to clinically significant genotoxicity when expressed over many years in T-cells<sup>11</sup>. Retroviral vectors also provide a convenient system for pre-clinical testing and refinement of CAR-based immunotherapy. Stable retroviral packaging master cell banks based upon the PG13 cell line are widely used to achieve gene transfer in the clinical evaluation of this technology. Here, we describe a practical method to transduce activated human T-cells with CAR-encoding retroviral vectors and illustrate how transgene expression and function may subsequently be evaluated *in-vitro*. The method described here provides an alternative to gene transfer using the *Sleeping Beauty* transposon system<sup>12</sup> and expands upon previously reported methods to achieve retroviral transduction of human<sup>13,14</sup> and murine T-cells<sup>15</sup>.

A two-step method is described for production of virus-like particles, involving the sequential use of H29D and PG13 cells (**Figure 2**). The H29D retroviral packaging cell line is derived from adenovirus 5-transformed 293 cells that have been engineered to express retroviral *gag-pol* polyprotein constitutively<sup>16</sup>. These cells also produce the vesicular stomatitis virus (VSV) G protein in a manner that is suppressed by tetracycline. Since VSV G is toxic, H29D cells are routinely propagated in the presence of tetracycline, which is removed when viral packaging is required. Viral particles derived from H29D cells are used to produce a stable PG13 retroviral packaging cell line. PG13 cells produce retroviral particles with a gibbon ape leukaemia virus pseudotype (GALV), suitable for stable transduction of activated human T-cells<sup>17</sup>. Virus produced by H29D cells is less useful for effecting gene transfer into human T-cells since gene transfer is less efficient and compromised by pseudotransduction<sup>14</sup>.

## Protocol

A schematic representation of the protocol is shown in **Figure 2**. All steps are carried out under sterile conditions, working in a class II laminar flow cabinet wherever possible.

### 1) Transient virus production using H29D cells.

- 1.1) Maintain H29D cells (a gift of Dr Michel Sadelain, Memorial Sloan Kettering Cancer Center, NY) in 6 well plates containing 4ml of DMEM + 10% FBS + antibiotic/antimycotic solution + glutamax ("D10 medium"). Medium is routinely supplemented with 2µg/mL tetracycline (to maintain repression of VSV G expression), 0.3mg/ml G418 (to maintain *gag-pol* expression) and 2µg/ml puromycin (to maintain tetracycline-regulated VSV G expression). Cells are passaged when they reach 90% confluence by trypsinization.
- 1.2) Achieve CAR expression using the SFG gammaretroviral vector<sup>18</sup> (a gift of Dr Michel Sadelain). Transgene expression is driven by the retroviral long terminal repeat.
- 1.3) On the day of transfection (day 1), select a well containing H29D cells that is 80-90% confluent. Remove medium and replace with 4ml DMEM + 10% FBS (eg without additional tetracycline, G418 or puromycin). Return to the incubator (37°C and 5% CO<sub>2</sub>) for at least 3 hours.
- 1.4) Transfect H29D cells with the SFG vector of interest using a calcium phosphate transfection kit as follows. "Solution A" is prepared in 5ml polystyrene tubes using the recipe described below:
  - 5-10µg plasmid DNA
  - 6µl 2.5M calcium chloride
  - sterile molecular biology grade water to 60µl
  - 1.4.1) Incubate solution A at room temperature for 5 minutes.
  - 1.4.2) Add solution A drop-wise to a 5ml polystyrene tube containing 60µl of 2X HEPES buffered saline (HBS), whilst vortexing gently.
  - 1.4.3) Incubate the mixture at room temperature for 20 minutes to ensure the formation of a precipitate. This is generally not macroscopically visible.
  - 1.4.4) Vortex the precipitate briefly (1-2 seconds) and then add drop-wise to the H29D cells using a P200 pipette.
  - 1.4.5) Rock the 6 well plate gently to ensure complete mixing of the transfection mixture.
  - 1.4.6) Incubate the transfected H29D cells at 37°C and 5% CO<sub>2</sub> for 24 hours.

- 1.5) After 24 hours, replace the medium with fresh D10 (e.g. lacking any supplemental G418, puromycin or tetracycline). Inspect the cells and medium daily and replace the medium before day 5 if yellowing is observed.
- 1.6) Harvest supernatants daily from day 5 until day 8, at which point VSV G-mediated syncytialization of H29D cells should be visible using the inverted microscope. Harvested supernatants may be used to infect target cells (see below) or alternatively may be snap frozen in an ethanol bath.
- 1.7) Replace harvested supernatants daily with 4ml fresh D10 media. By day 8, H29D cells generally appear highly syncytialized and cultures deteriorate. H29D cells cannot be propagated for longer periods in the absence of tetracycline.

## 2) Preparation of PG13 retroviral packaging cells

- 2.1) Propagate PG13 cells (obtained from the European Collection of Cell Cultures) in 6 well plates containing 4ml of D10 medium at 37°C and 5% CO<sub>2</sub>.
- 2.2) Ensure that PG13 cells are approximately 30% confluent (eg proliferating maximally) and evenly dispersed throughout the well in order to achieve productive infection by H29D-derived viral particles.
- 2.3) Replace medium with 4ml of H29D-derived viral particles, harvested as described in 1.6. Filtration is not strictly necessary since any transferred H29D cells will not be viable when propagated in the absence of tetracycline.
- 2.4) Add polybrene (8µg/mL). Mix thoroughly to facilitate retroviral gene transfer and incubate cells at 37°C and 5% CO<sub>2</sub>.
- 2.5) After 24 hours, replace medium with 4ml D10 and incubate cells at 37°C and 5% CO<sub>2</sub>.
- 2.6) Assess efficiency of gene transfer after a further 24-48 hours by flow cytometry.
  - 2.6.1) Briefly, stain cells with a specific antibody directed against the CAR. Incubate on ice for 20 minutes followed by two washes using PBS.
  - 2.6.2) If the CAR-specific antibody is not directly conjugated with an appropriate fluorochrome, perform an additional staining step with a conjugated secondary antibody. Incubate samples on ice for 20 minutes and wash twice with PBS.
  - 2.6.3) Alternatively, use a biotinylated ligand or ligand derivative (eg peptide) to detect CAR expression. In that case, use fluorochrome-coupled streptavidin as a secondary detection reagent (**Figure 3**). Perform incubation and wash steps as above.
  - 2.6.4) To determine relative CAR expression in T-cell subsets, incubate cells with antibodies that bind CD4 and/ or CD8 (**Figure 3**). However, if an unconjugated primary antibody followed by conjugated secondary antibody is used to detect CAR expression, detection of other markers may be compromised by incomplete blockade of the secondary reagent. To avoid this:

2.6.4.1) Perform steps 2.6.1-2 as above, adding primary and secondary antibody reagents sequentially and washing as indicated.

2.6.4.2) Add mouse serum (10µl of 1/50 dilution) to block unoccupied antigen combining sites in the secondary fluorochrome-conjugated antibody. Incubate for 20 minutes on ice. There is no need to wash after this step.

2.6.4.3) Add fluorochrome conjugated CD4 and/ or CD8 antibodies. Incubate for 20 minutes on ice. Wash twice using PBS.

2.6.5) Analyze stained cells by flow cytometry. It is necessary to have appropriate compensation controls, as dictated by the flow cytometer in use.

2.6.6) Ensure that the PG13 cell line generated as described above is close to 100% positive for cell surface expression of the CAR of interest. Typically, best results are obtained with supernatants harvested at later time points from H29D cells.

### 3) **Transduction of activated human T-cells**

3.1) Collect blood (45ml) using a 21-gauge butterfly needle into a 50ml syringe.

3.2) Transfer harvested blood immediately after collection to anticoagulant (5ml citrate dextrose solution) in a 50ml Falcon tube. Blood must NOT be cooled. Ensure that anticoagulant has been warmed to room temperature.

3.3) Transfer Ficoll-Paque (15ml) into each of two 50ml conical centrifuge tubes.

3.4) Tilt the Ficoll-containing centrifuge tube as close to horizontal as possible. Draw up anticoagulated blood into a pipette and gently add to the side of the tube, aiming not to disrupt the interface between the blood and ficoll. As blood is added, the tube is gradually brought to the vertical position.

3.5) Repeat this process for both ficoll tubes. Some settling of red cells through the ficoll layer may be observed, particularly in the first tube and is not of concern.

3.6) Centrifuge the tubes at 500g for 25 minutes. Ensure the acceleration and deceleration settings of the centrifuge are at zero. The centrifuge should *not* be refrigerated.

3.7) Transfer the peripheral blood mononuclear cell (PBMC) layer, present at the interface between the Ficoll-Paque and the plasma, into a fresh 50ml tube using a Pasteur pipette. Dilute the cells to a final volume of 50ml in phosphate buffered saline (PBS).

3.7.1) Centrifuge at room temperature for 10 minutes at 370g.

3.8) Aspirate the supernatant and re-suspend the cell pellet in 50ml PBS.

3.8.1) Centrifuge at room temperature for 10 minutes at 270g.



- 3.9) Aspirate the supernatant and re-suspend the cell pellet in RPMI 1640 + 10% human AB serum + antibiotic/antimycotic solution + glutamax ("R10 medium").
- 3.10) Plate the cells at a density of  $3 \times 10^6$  cells/ml in a six well tissue culture treated plate (4ml per well).
- 3.11) Add phytohemagglutinin (PHA; 5 $\mu$ g/ml) to activate T-cells and incubate at 37°C and 5% CO<sub>2</sub>.
- 3.12) Add interleukin-2 (IL-2; 100U/ml) 24 hours prior to performing gene transfer. Gene transfer may be conducted either 48 or 72 hours after T-cell activation with PHA. Lower concentrations of IL-2 may be used for this purpose.
- 3.13) Re-suspend RetroNectin (200 $\mu$ g) in 18ml PBS and transfer in 3ml aliquots to each well of a six well *non-tissue culture treated* plate. RetroNectin is transferred carefully to each well using 1000 $\mu$ l polypropylene pipette tips ("blue tips"; to minimize loss resulting from protein binding). Polystyrene pipettes should be avoided since, although more convenient for this step, they have much higher protein binding capacity.
- 3.14) Ensure coating of RetroNectin is complete by incubation of the plate at room temperature for a minimum of 3 hours or at 4°C for a minimum of 16 hours.
- 3.15) One day prior to performing gene transfer, split confluent PG13 retroviral packaging cells 1 in 2 by trypsinization. Each of the resulting cell aliquots is plated in 4ml fresh D10. After incubation at 37°C and 5% CO<sub>2</sub> for 24 hours, PG13 cells generally reach confluence and medium can be harvested as a source of viral vector for T-cell gene transfer. Prepare one well containing 4ml viral vector conditioned medium for each transduction of  $1 \times 10^6$  PBMC.
- 3.16) On the day of gene transfer, remove unbound RetroNectin from the non-tissue culture treated six well plate. Much of the added RetroNectin remains free in solution. Rather than discarding this material, it may be transferred using a polypropylene Pasteur pipette to a second non-tissue culture treated six well plate. After wrapping in cling film, this "RetroNectin 2" plate should be stored at 4°C and may be used within 2 weeks in a second gene transfer experiment. Prolonged storage (e.g. for 2 or more weeks) is not advised and it is important to check for evaporation to the lid of the plate and drying out of wells.
- 3.17) Do not allow RetroNectin-coated wells to dry out. Following removal of unbound RetroNectin, transfer 4ml of PG13 viral conditioned medium promptly to each well of the RetroNectin-coated plate and leave in the flow cabinet at room temperature during the minutes required to collect activated PBMC. This order is recommended since PBMC should be kept in the incubator whenever possible.
- 3.18) Collect activated PBMC in a 50ml conical tube and count viable cells by trypan exclusion using a hemocytometer. Do *not* centrifuge the cells as conditioned medium is likely to facilitate gene transfer efficiency.

- 3.19) Determine the volume that contains 1 million activated PBMC (mainly T-cells) and, after careful mixing by inversion, transfer this volume to each well of the RetroNectin-coated plate containing viral conditioned medium.
- 3.20) Add IL-2 (100U/ml) to each well. This is most conveniently achieved by adding the appropriate amount of IL-2 to the activated PBMC stock and then distributing the cytokine-supplemented cells to the RetroNectin-coated plate.
- 3.21) Centrifuge plates at 50g for one hour at room temperature. This step is believed to facilitate gene transfer. However, transduction may still be achieved if this step is omitted. Incubate cultures at 37°C and 5% CO<sub>2</sub>.

#### **4) Propagation of retrovirus-transduced T-cells and examination of CAR expression**

- 4.1) Two days after gene transfer, analyze transgene expression by flow cytometry, as explained in step 2.6. A representative example is shown in **Figure 3**.
- 4.2) Propagate transduced T-cells at 37°C and 5% CO<sub>2</sub> in R10 medium with IL-2 (100U/ml). Add fresh medium and cytokine three times per week or more frequently if a change in medium color occurs, consistent with acidification.

#### **5) Testing of CAR functionality in retrovirus-transduced T-cell cultures.**

Test functionality by performing co-culture experiments in which CAR-engineered T-cells are placed on paired adherent target monolayers that are discordant for expression of the antigen of interest. Additional control cultures should include T-cells that are untransduced and/or engineered to express CARs that lack ectodomain or endodomain elements (e.g. the truncated control CAR, HDFTr; **Figure 1**). Quantify T-cell activation by measurement of target cell destruction, cytokine production and/ or T-cell proliferation.

- 5.1) Culture target cell monolayers to confluence in 24 well tissue culture plates containing the appropriate medium (generally D10 is sufficient).
- 5.2) Centrifuge engineered T-cells for 5 minutes at 200g and re-suspend at  $1 \times 10^6$  cells/ml in R10 without exogenous cytokine. Where two CARs are being compared, it may be appropriate to add untransduced T-cells to some cultures in order to equalize the proportion of transduced cells present.
- 5.3) Remove medium from target monolayer cultures. Add 1ml of T-cells ( $1 \times 10^6$  cells).
- 5.4) After 24 hours, remove supernatant for measurement of cytokine content (e.g. IL-2, interferon (IFN)- $\gamma$ ). Cytokine production is a useful and objective method to quantify T-cell activation upon encounter with tumor cells. Supernatants are analyzed according to manufacturers instructions (eBioscience Ready-Set-Go ELISA kits), which may require dilution in order that optical density values lie on the linear portion of the standard curve.

- 5.5) Quantify target cell destruction using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay.
- 5.5.1) Reconstitute MTT in PBS at a concentration of 5mg/ml.
- 5.5.2) Aspirate medium from the co-culture at the desired time-point (generally after 16 – 72 hours). Wash wells with 500µl PBS to remove residual T-cells. Washing gently is essential to avoid disturbing the adherent cell monolayer. Washing is not completely efficient but will remove most of the cells in suspension. Dilute MTT stock solution 1/10 (to a concentration of 500µg/ml) in D10 media and add 500µL (250µg) to each well.
- 5.5.3) Incubate cultures for 2-4 hours at 37°C and 5% CO<sub>2</sub>.
- 5.5.4) Aspirate supernatant and re-suspend formed formazan crystals in 300µL DMSO.
- 5.5.5) Measure absorbance (A) spectrophotometrically at 570nm.
- 5.5.6) Calculate relative tumor cell viability using the following equations:
- $\text{Viability} = \frac{A(\text{Well containing T-cell and tumor monolayer co-culture})}{A(\text{Well containing tumor cell monolayer only})} \times 100\%$
  - $\text{Percentage tumor cell death} = 100 - \text{viability}$
- 5.6) Alternatively, visualize monolayer destruction by crystal violet staining (**Figure 4**). It is essential to prepare a well containing an adherent tumor cell monolayer alone as a control.
- 5.6.1) Aspirate medium carefully after completion of the T-cell monolayer co-cultivation.
- 5.6.2) Wash wells gently with 500 µ L PBS and then fix using 500 µ L of ice-cold methanol, incubated at -20°C for a minimum of ten minutes.
- 5.6.3) Aspirate methanol and submerge each well in crystal violet (0.5% solution in 25% methanol, stored at room temperature). Incubate the plate at room temperature for five minutes.
- 5.6.4) Aspirate crystal violet and wash plates by gentle submersion in water to remove excess dye.
- 5.6.5) Dry plates at room temperature overnight.
- 5.6.6) Capture light microscopy images using an inverted microscope with appropriate software.
- 5.7) Quantify T-cell proliferation as follows:
- 5.7.1) Establish T-cell / target cell co-cultivations as described in 5.1 – 5.3.

- 5.7.2) Add IL-2 (100U/ml) after 24 hours and every 2-3 days thereafter, together with R10 medium, as dictated by the appearance of the cultures.
- 5.7.3) Transfer cultures to a 6 well plate when volume exceeds 1.5ml.
- 5.7.4) Determine cell number by trypan exclusion at the appropriate interval, generally every 7 days.
- 5.7.5) Re-evaluate the proportion of CAR<sup>+</sup> T-cells present in the culture by flow cytometry, generally every 7 days.
- 5.7.6) Re-stimulate cultures if all target cells have been destroyed by the CAR-engineered T-cells. Generally, this is performed after 7 days and is achieved by placing  $1 \times 10^6$  T-cells on a fresh target cell monolayer in a 24 well plate.
- 5.7.7) Conduct periodic re-stimulation in this manner until cultures are no longer capable of expanding.

## Representative Results

HOX is a CAR that can bind several tumor-associated glycoforms of the MUC1 mucin, commonly found in breast carcinomas (**Figure 1**)<sup>7</sup>. HDFT<sup>r</sup> is a matched control CAR in which the endodomain has been truncated, rendering it functionally inactive (**Figure 1**)<sup>7</sup>. **Figure 3** shows a representative example of transduction efficiency achieved when these CARs were expressed using the SFG gammaretroviral vector, delivered to human T-cells using the protocol described above. Note that both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells are transduced at comparable efficiencies, allowing for the relative proportion of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells that were present in these PHA-activated T-cell cultures. **Figure 4** shows an experiment in which monolayer destruction was visualized using crystal violet staining. Note that HOX<sup>+</sup> but not control HDFT<sup>r</sup><sup>+</sup> T-cells cause the selective elimination of MUC1-expressing breast cancer monolayers. When CAR<sup>+</sup> T-cells undergo antigen-driven activation, they also form large clusters within the wells and undergo proliferation (**Figure 5**). Destruction of MUC1-expressing T47D breast tumor cell monolayers by HOX<sup>+</sup> but not HDFT<sup>r</sup><sup>+</sup> control T-cells may also be visualized by time-lapse video recording (**Video 1** and **Video 2** respectively).

## Tables and Figures

**Figure 1.** The MUC1-specific CAR, HOX. In HOX,<sup>7</sup> reactivity with tumor-associated glycoforms of MUC1 is conferred by a single chain antibody fragment (scFv) derived from the HMFG2 (human milk fat globulin 2) hybridoma. To overcome MUC1-imposed steric hindrance, a flexible elongated hinge has been introduced derived from human IgD. CAR expression has been stabilized using IgG1 Fc. In this MUC1-specific CAR signaling is provided by a composite endodomain in which CD28 and OX40 modules are placed upstream of CD3 $\zeta$ . The name HOX arises from the scFv (from HMFG2) and additional co-stimulatory domain (OX40) used to construct the CAR. The HDFTTr CAR is a matched control in which the endodomain has been truncated to render it inactive.<sup>7</sup> The name HDFTTr arises from the scFv (from HMFG2), hinge (from IgD), spacer (from IgG1 Fc) and endodomain (truncated CD28) used to engineer this control CAR.

**Figure 2.** Protocol used to transduce and test CAR function in human T-cells. CAR expression is achieved using the SFG gammaretroviral vector, packaged via a two step-procedure. Since H29D cells are derived from 293 parental cells, they are readily transfectable. In H29D, retroviral *gag-pol* and the vesicular stomatitis virus (VSV) G envelope are also produced, the latter under the control of a “tet-off” system. When transfected with SFG plasmid DNA and cultured without tetracycline, these cells provide a transient source of VSV G pseudotyped viral particles. These are used to infect PG13 cells, thereby generating a stable packing cell line. PG13 cells release viral particles with a GALV pseudotype, suitable for infection of human T-cells. Transduction of T-cells is facilitated by the use of RetroNectin-coated plasticware and can be conveniently quantified by flow cytometry. Several functional studies can then be performed to test the specificity and potency of CAR function.

**Figure 3.** Flow cytometric assessment of gene transfer efficiency. In this case, gene-modified T-cells are incubated on ice with a biotinylated 24mer peptide (produced in house) containing one copy of the MUC1 epitope recognized by the HOX (A) or HDFTTr (B) control CARs. After washing, cells are then incubated with phycoerythrin-coated streptavidin, together with a fluorescein-conjugated CD4 antibody and a Phycoerythrin cyanin 5.1 (PC5)-conjugated CD8 antibody prior to analysis by flow cytometry. Quadrant markers are set using untransduced T-cells. Percentage of events indicate the proportion of transduced CD4<sup>+</sup> and CD8<sup>+</sup> T-cells present, expressed as a percentage of all T-cells present in the culture.

**Figure 4.** Crystal violet monolayer destruction assay. Engineered T-cells ( $1 \times 10^6$  cells) were co-cultivated for 24 hours with confluent monolayers of the indicated MUC1<sup>-</sup> (MDA-MB-435; MDA-MB-231) or MUC1<sup>+</sup> (T47D; BT20) breast tumor cell lines, cultured in 24-well plates. Non-adherent cells were then removed and, after fixation, residual tumor monolayers were stained using crystal violet. “Nil” indicates monolayers that were not co-cultivated with T-cells.

**Figure 5.** Appearance of T-cell monolayer co-cultures after 72 hours. Co-cultures were established as described in Figure 4 in which T-cells were incubated with MUC1<sup>+</sup> T47D cells. “Nil” indicates monolayer alone. Note that the monolayer remains intact when HDFTTr<sup>+</sup> T-cells are added. By contrast, HOX<sup>+</sup> T-cells have destroyed the T47D monolayer and form large clusters of activated T-cells, admixed with dead tumor cells.

## Videos

**Video 1.** Tumor monolayer destruction by HOX<sup>+</sup> T-cells. In this recording,  $1 \times 10^6$  HOX<sup>+</sup> T-cells were co-cultivated overnight with a confluent monolayer of MUC1-expressing T47D cells, cultured in a 24 well plate.

**Video 2.** T-cells engineered to express a signaling-defective CAR do not elicit antigen-specific monolayer destruction. In this recording,  $1 \times 10^6$  HDFT<sup>+</sup> T-cells were co-cultivated overnight with a confluent monolayer of MUC1-expressing T47D cells, cultured in a 24 well plate.

## Discussion

The method presented here provides a reliable approach to transduce human T-cells and to evaluate the function of newly produced chimeric antigen receptors. Several critical steps require attention in order to achieve optimal results. To ensure that both H29D and PG13 packaging cell lines produce high viral titer, it is recommended to use cells that have been recently thawed or that are in early passage. Transfection of H29D is optimally achieved if cells are about 80% confluent. It is generally good practice to prepare these monolayers at least 2-3 days in advance of transfection so that they are established in culture. Also removal of tetracycline tends to result in more rapid growth of these cells and should be performed at least a few hours before undertaking the transfection step. Following transfection, a fine precipitate should be seen to settle on the monolayer within 1-2 hours. To the inexperienced eye, this may be almost invisible and it may also mimic the appearance of contamination. Poorer results tend to occur if coarse precipitates form.

The viral titer produced by H29D cells is not known and may be variable. However, if viral titer is excessive, PG13 cells will grow poorly owing to VSV G-mediated toxicity. In this situation, PG13 cells undergo profound apoptosis and may take weeks to recover. Since viral titer tends to peak about 7 days after transfection (eg on day 8), this is why it is recommended to repeat this procedure using supernatants harvested on days 5 to 8. By this means, it is generally possible to catch the cells at a point where VSV G-pseudotyped viral titer is sufficient to achieve 100% PG13 infection without causing undue toxicity to these cells. If there is any suspicion of PG13 toxicity, it is best to allow the cells to recover before harvesting some cells for FACS analysis to determine CAR expression. To ensure efficient T-cell transduction, PG13 packaging cell lines should be strongly positive for expression of the transgene of interest. However, this does not guarantee adequate viral titer and where necessary, it may be advisable to sub-clone PG13 cells and then test individual clones for viral titer.

A number of alternative systems are available to deliver CAR transgenes into T-cells. For example, transient viral supernatant suitable for transduction of T-cells may be prepared by triple transfection of NIH 293T cells with vector, RDF plasmid (encodes for RD114 feline leukemia virus envelope) and a Moloney leukemia virus gag/pol plasmid (eg pEQ-Pam3-(E))<sup>19</sup>. This has the advantage of rapidly generating sufficient vector to test new CARs but is disadvantaged by lack of stable retroviral packaging cell line and variability of viral titer. Alternatively, transient CAR expression may be achieved by electroporation of mRNA<sup>20</sup>.

The most critical step in the procedure is the transduction of T-cells. To achieve maximum success, T-cells should be in optimum condition at the time of gene transfer. Cooled material should never be used for ficoll separation. Cells should not be left out of the incubator for more than the minimum period required for inspection. The protocol described here applies to PHA and many find that smaller concentrations of this lectin (eg 0.5µg/ml) can achieve sufficient T-cell activation to facilitate gene transfer. Alternatively T-cells may be activated with CD3 + CD28-coated paramagnetic beads (eg Cell Expander Dynabeads, Invitrogen Life Technologies, as indicated by the manufacturers). We generally find that a 1:1 ratio of PBMC to beads is sufficient for this purpose and that beads do not need to be removed prior to undertaking experiments.

Detection of CAR expression generally requires an antibody that recognizes a component of the CAR ectodomain. Commonly, this is achieved using an anti-human IgG antibody to



detect CARs in which a human IgG spacer and/ or hinge has been used. In the example presented here, an alternative system has been used whereby the peptide epitope (from MUC1) was biotinylated and then detected using fluorochrome-coupled streptavidin. Alternatively a myc epitope tag may be included in the hinge and is conveniently detected using the 9e10 antibody <sup>21</sup>. The latter can be produced in house from this hybridoma, which is available from several cell banks (eg the European Collection of Cell Cultures or American Tissue Culture Collection).

Functional studies can generally provide a robust discrimination between CAR-transduced and control T-cell populations. However, with some weakly active CARs, this may be less evident <sup>7</sup>. Monolayer destruction can sometimes be seen in control cultures and may reflect a carry-over effect of PHA activation. Assays based upon MTT reduction and crystal violet staining are useful methods to quantify and visualize T-cell killing respectively. However, both assays are limited to target cells that are adherent. Since the MTT assay is quantitative, it is essential to wash the monolayer carefully in order to remove as many T-cells as possible without disturbing the tumor cells. We have observed that those few residual T-cells that remain do not reduce MTT as efficiently as tumor cells, minimizing the error induced by their presence. Nonetheless, in our experience, cytokine production remains the most useful discriminator of function between CARs under study. When CAR<sup>+</sup> T-cells are activated, they generally produce high levels of IFN- $\gamma$ , which correlates with destruction of the target cells. Additionally, effective second- and third-generation CARs generally enable T-cells to produce greater quantities of IL-2 and to proliferate more robustly. In part, the latter reflects the provision of IL-2 independent proliferative signals by co-stimulatory motifs within the CAR endodomain.

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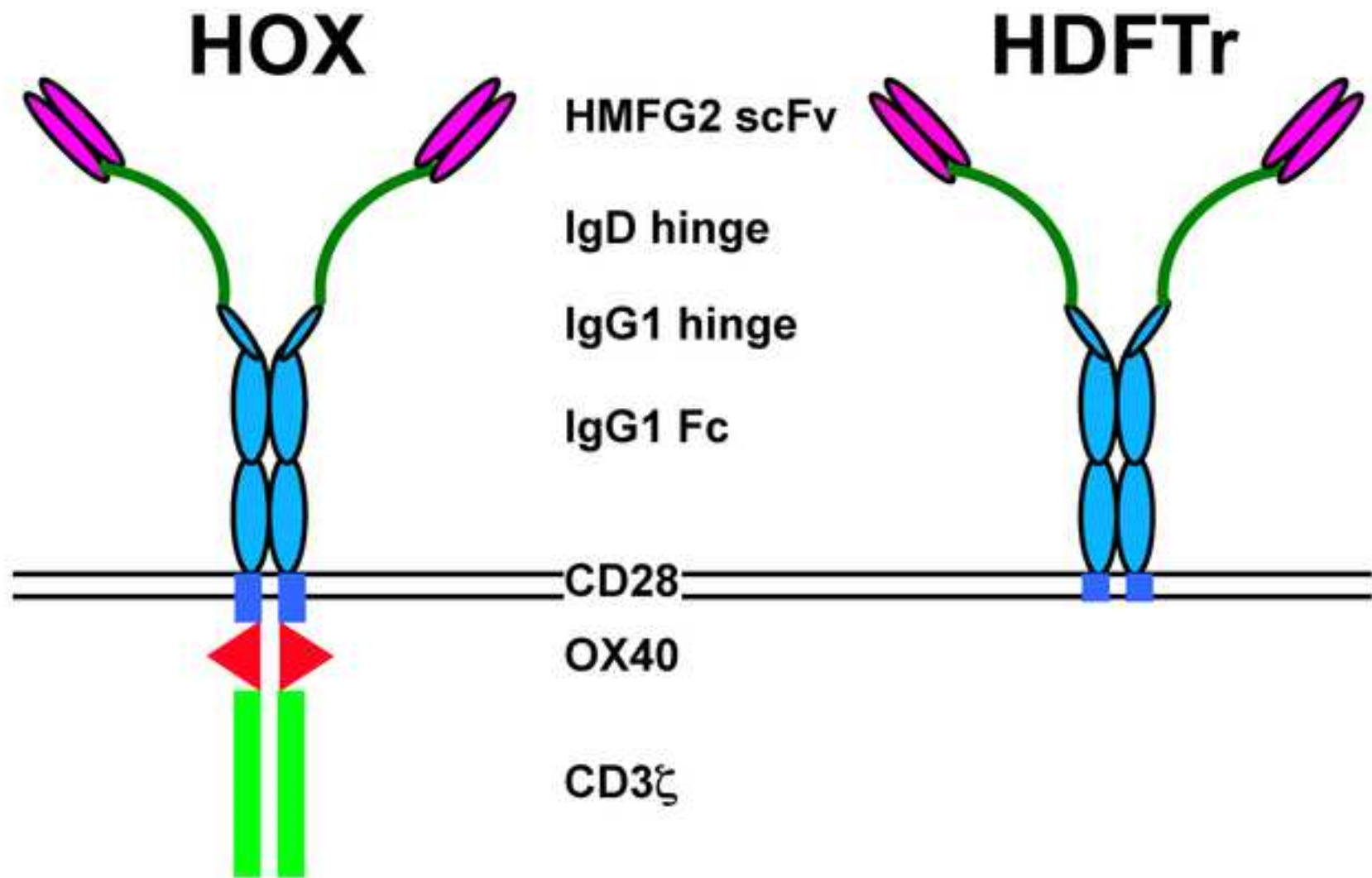
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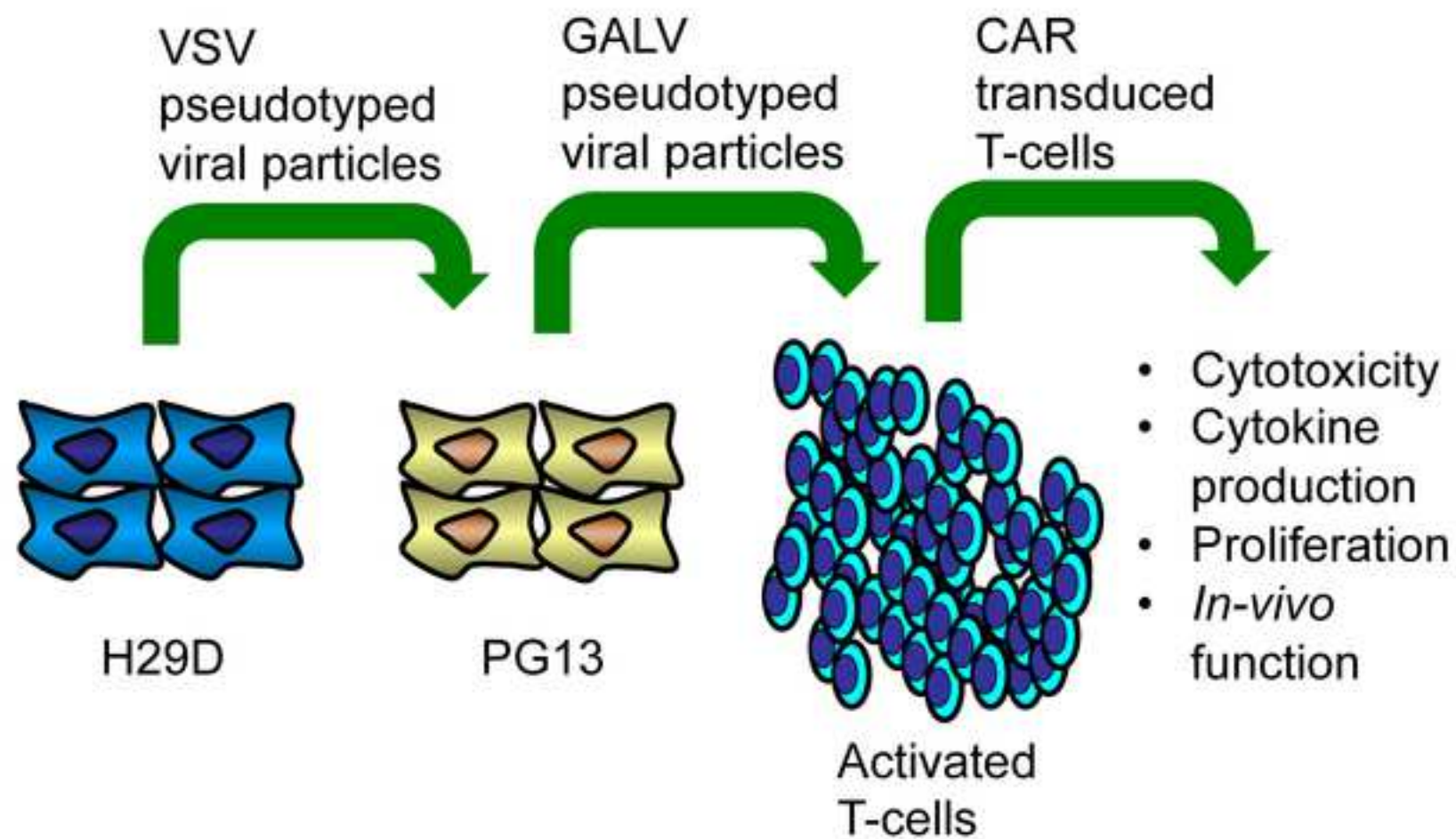
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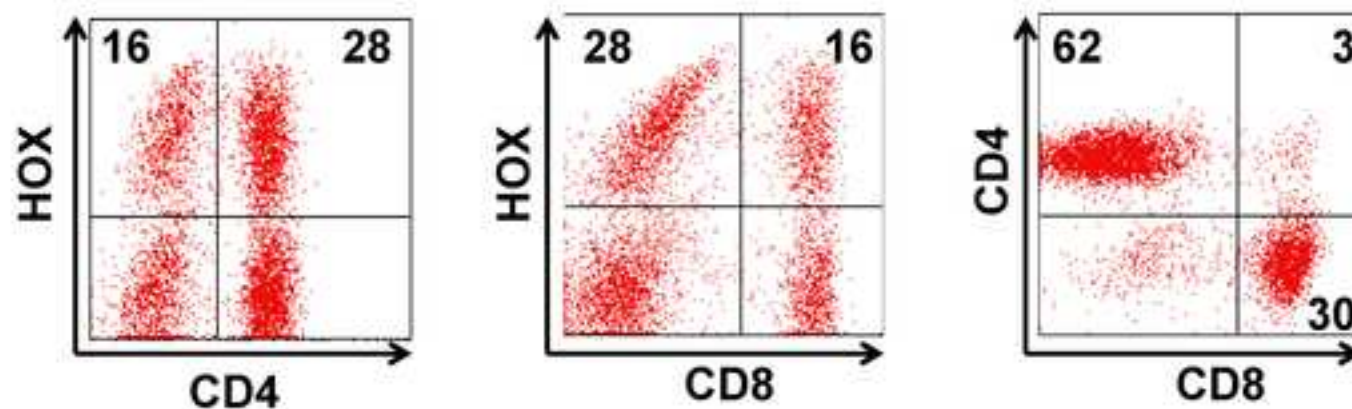
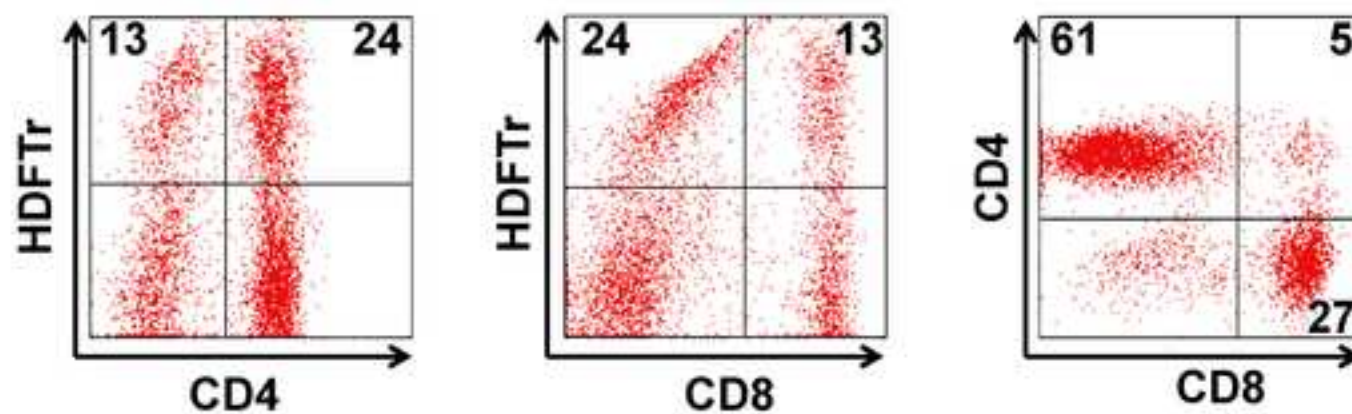
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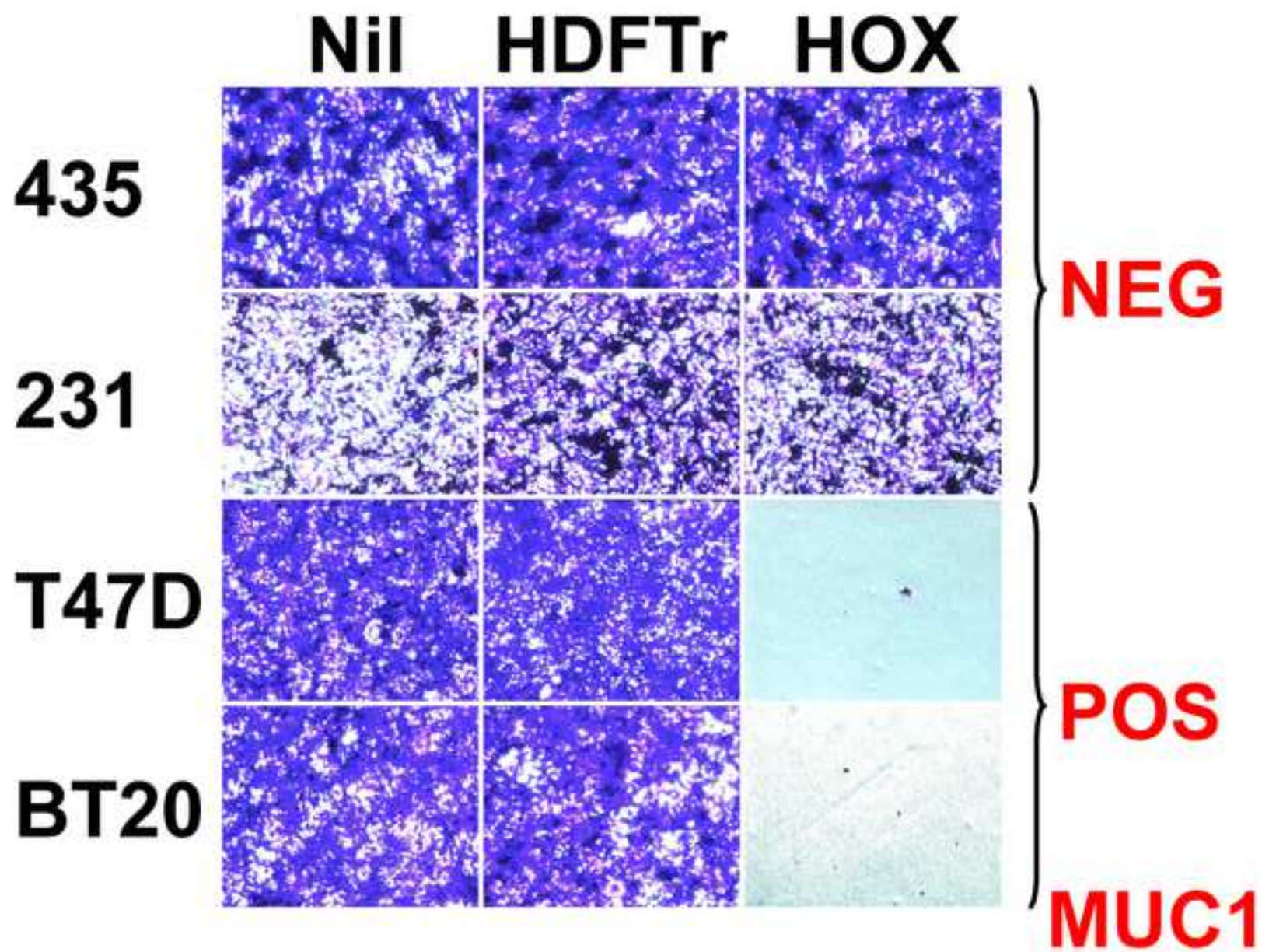
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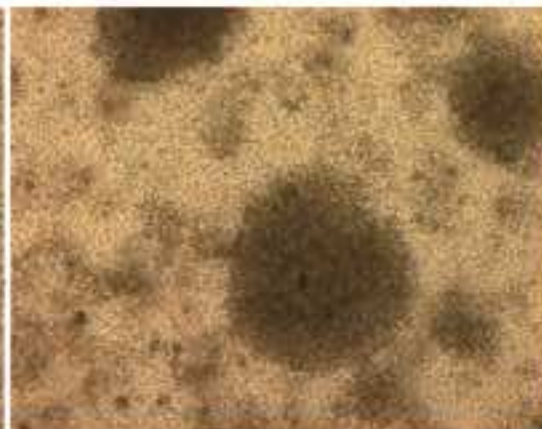
**Nil**



**HDFT<sub>r</sub>**



**HOX**



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Aldesleukin	Novartis	-	Human recombinant interleukin-2
Antibiotic antimycotic solution	Sigma-Aldrich	A5955	
Butterfly needle and Luer adaptor 21G x 0.75 inches	Greiner Bio-one	450066	
Calcium phosphate transfection kit	Sigma-Aldrich	CAPHOS-1KT	
CD4-FITC conjugated	Miltenyi Biotec	130-080-501	
CD8-PC5 conjugated	Beckman Coulter	A07758	
Citrate-dextrose solution	Sigma-Aldrich	C3821	
Crystal violet	Sigma-Aldrich	C3886	
DMEM with glucose	Lonza	Lonz12-614F/12	Supplied by VWR International
DMSO	Sigma-Aldrich	D2650	Dimethylsulfoxide
Fetal bovine serum	Autogen Bioclear	-	
Fibronectin fragment (RetroNectin)	Takara BioEurope Clontech	T100B	
Ficoll-paque PLUS	GE Healthcare	17-1440-02	
Glutamax	Invitrogen	35050038	
Human AB serum (male)	Sigma-Aldrich	H4522-100ml	
Human IL-2 ELISA Ready-Set-Go	eBioscience	88-7026-88	
Human IFN-γ ELISA Ready-Set-Go	eBioscience	88-7316-88	
Methanol	Fisher Scientific	M/4000/17	
PG13 cells	European Collection of Cell Cultures		

Phosphate buffered saline	Gibco	10010-015	
Phytohemagglutinin	Sigma-Aldrich	L 2769	<i>Phaseolus vulgaris</i> Leukoagglutinin PHA-L
Polybrene	Sigma-Aldrich	H9268	Also known as 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide, hexadimethrine bromide or hexadimethrine bromide
Puromycin	Sigma-Aldrich	P9620	
RPMI 1640 without L-glutamine	Lonza	733-1690	
Six well tissue culture-treated plates	Greiner Labortechnik	700-1425	Supplied by VWR International; part number 657160
Six well non tissue culture-treated plates	Becton Dickinson	734-0948	Supplied by VWR International; part number 351146
Streptavidin phycoerythrin conjugated	Invitrogen Life Technologies	S866	
Syringe polypropylene 50ml with Luer Lok	Fisher Scientific	SZR-150-080U	

Tetracycline	Sigma-Aldrich	T7660	Cell culture grade; water soluble at 10mg/ml. Store as frozen aliquots
Trypan blue solution, 0.4% in phosphate buffered saline	Fisher Scientific Ltd	HYC-044-010D	
Thiazolyl blue tetrazolium bromide, 98%	Sigma-Aldrich	M2128	Used in MTT assay
Tubes – conical centrifuge tubes 50ml	PAA Laboratories	PAA50050	
Tubes – polystyrene 12x75mm 5ml with cap	Becton Dickinson	352054	
Twenty four well tissue culture treated plates	PAA Laboratories	PAA32024X	
C412 Centrifuge	Jouan	-	
FLUOstar Omega	BMT Labtech	-	MTT assay
Omega Software (version 1.20)	BMT Labtech	-	MTT assay
MARS data analysis software (version 1.20 R2)	BMT Labtech	-	MTT assay
TMS Inverted microscope	Nikon	-	Crystal violet assay
Axiovision HR camera	Axiovision	-	Crystal violet assay
Axiovert 100 Software	Zeiss	-	Crystal violet assay

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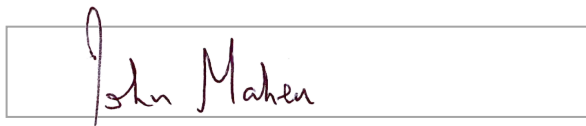
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**To: Dr Larissa Jarzylo, Science Editor, Journal of Visualized Experiments**

Dear Dr Jarzylo,

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The comments to be addressed were as follows:

Thank you for your careful revisions. We feel your manuscript is close to Editorial finalization, however, two issues need to be addressed:

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**Response** - I have re-written the protocol in the imperative tense.

2) Please review the highlighted portion of your protocol. The current highlighted section of your protocol outlines some important steps, but we would like to include each step that is necessary to tell a continuous story. The highlighted portion is what our scriptwriters will use to generate the narration for your video. For example, the highlighted portion begins with step 1.4.2) Adding Solution A to the tube and vortexing gently". Since the highlighting starts here, we think the reader/viewer would benefit from knowing what else is in the tube and therefore, we suggest you include one or more of the previous steps. Also, you may highlight up to 2.75 pages to be filmed and we welcome including more of your interesting and important protocol.

**Response** – I have highlighted some additional areas. Hopefully the movies we have submitted may also be incorporated with these sections to provide a complete story.

Many thanks once again for these helpful comments. I trust that these alterations address the issues raised in a satisfactory manner.

With kind regards,

A handwritten signature in black ink that reads "John Maher". The signature is written in a cursive style with a horizontal line underneath.

Dr John Maher