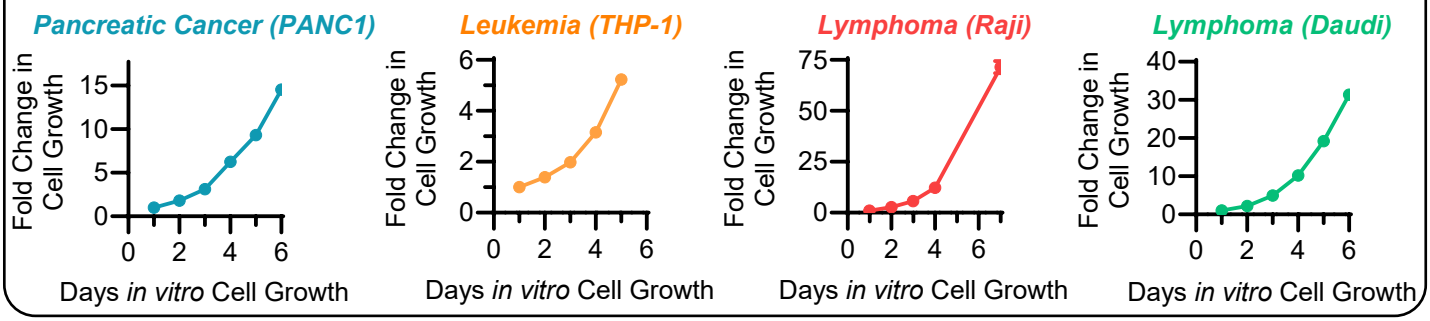
In vitro efficacy of cell therapy products determined using a G-Rex co-culture assay

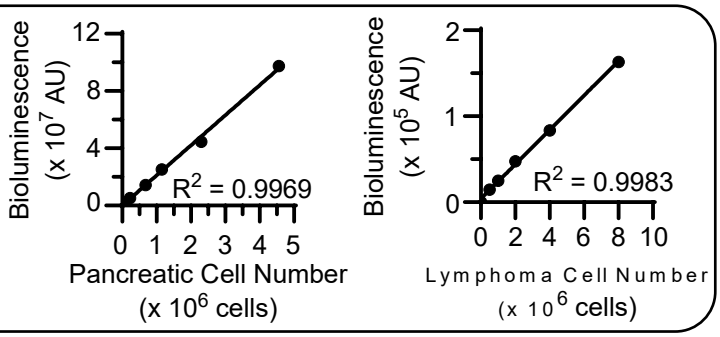
G-Rex®, or Gas-permeable Rapid Expansion device, allows the long-term uninterrupted expansion of large numbers of cells without the need for media exchange (PMID: 31707675, 22421946, 23242592, 26015959, 31707675). This is accomplished by 1) a gas-permeable silicone membrane at the base of the device, which allows efficient gas exchange at the point of cell growth, and 2) a large media volume to growth surface area ratio. The primary purpose of G-Rex® has been the expansion of immune cells for therapeutic administration. G-Rex® is used across academic institutions and the biotechnology industry in pre-clinical research and clinical trials in the testing and manufacture of cell and gene therapies, including several FDA approved cell therapies as well as approvals in Europe and Asia.

The ability of G-Rex® to expand other cell types beyond immune cells, including cancer cells of varying origins, was explored (see **Box 1**). Growth of suspension cell lines, as well as a solid tumor cell line, was tracked over several days in a G-Rex®6 Well Plate without the need for media changes. Real-time tracking of cell growth within a G-Rex®6 was accomplished using bioluminescent imaging of genetically modified cell lines(see **Box 1 Technical Note**).

Box 1. The G-Rex®6 can be used to culture and track the growth suspension and adherent tumor cells over time.



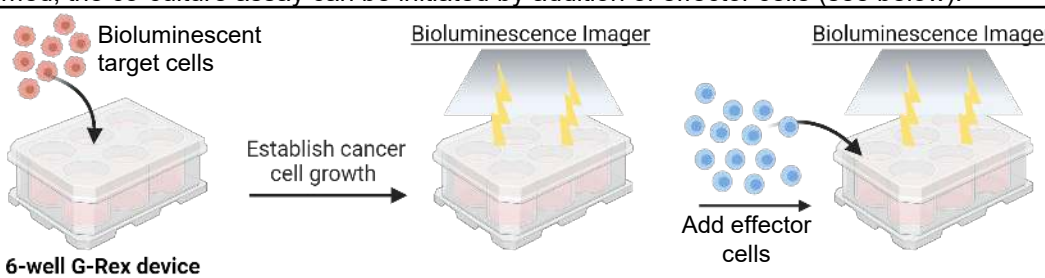
Box 1 Technical Note: Continuous measurement and tracking of ***cell growth kinetics without culture disruption*** is achievable. By genetically engineering cells to express the firefly luciferase enzyme, cells become bioluminescent in the presence of D-Luciferin substrate. In this manner, the bioluminescent light measured linearly correlates to the number of cells in the culture. The data to the right exemplifies the strong correlation (R^2) between the number of cells in a G-Rex®6 well and the amount of measured bioluminescence.



The cell lines above were used to demonstrate a G-Rex®6 is capable of real time bioluminescent monitoring of cancer growth and represent several clinical indications for which cell therapies have either been approved or are under clinical investigation. Traditionally, *in vitro* efficacy of these therapies has been demonstrated using short co-culture assays like chromium release and flow-based assays (PMID: 23019373, 25198528, 33321428, 30617140). As researchers desire deeper characterization of their cell therapy, including persistence, longer co-culture assays that offer additional data on top of anti-tumor activity will be preferred. Because G-Rex® offers long-term monitoring of cancer cell growth without interruption (bioluminescent readout and no media change), it is an ideal platform to monitor suppression of cancer cell growth in the presence of therapeutic agents like cell and gene therapies.

After a cell target is selected and engineered, these cells are seeded into a G-Rex®6 with media and D-luciferin. Once cancer growth is confirmed, the co-culture assay can be initiated by addition of effector cells (see below):

The G-Rex® Killing Assay



- ***Simple*** *set up, reducing experiment-to-experiment variation*
- ***Short*** *5-minute exposure time for* ***acquisition*** *of bioluminescence*
- ***Quantitation*** *of bioluminescent images using instrument software*

Eric Smith, Ph.D. & Andrew Lewis, Ph.D.

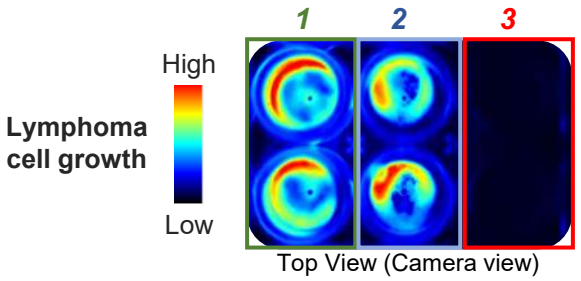
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Typical G-Rex®6 Well Plate co-culture setup:

1) Establish cancer growth (e.g. for Daudi cells, a starting cell number of 1 x 10^6 cells in each well is recommended) in 35 mLs media containing 100 µM D-Luciferin
2) Typical setups allow cancer cells to incubate 24 hours at 37°C prior to addition of effectors.
3) Effector cell addition to assay: *Option 1*: a) overnight rest cryopreserved effector cells, b) count effector cells and add to cancer cells to achieve desired effector:target ratio; if volume added is greater than 1% culture volume, pellet effector cells and resuspend in media from respective well; *Option 2:* thaw cryopreserved cells in 37°C water bath and add a range of volumes directly to the cancer cells (100 µL max suggested but should be optimized based on specific cell product)
4) Bioluminescent images are acquired using BioRad ChemiDoc (chemiluminescent setting, 5 min exposure, 3x3 bin)

Density heatmap of lymphoma cell growth demonstrating specific eradication of lymphoma cells by effective anti-CD19 CAR-T cells in a G-Rex®6. The ability of anti-CD19 CAR-T cells (column 3) to eliminate CD19 antigen positive lymphoma cells compared to negative control anti-CD19 CAR-T cells (no CD3ζ domain) (column 2) was tested in the G-Rex® Killing Assay. Effector:Target ratio of 2.5:1; image is 5 days after co-culture initiated.

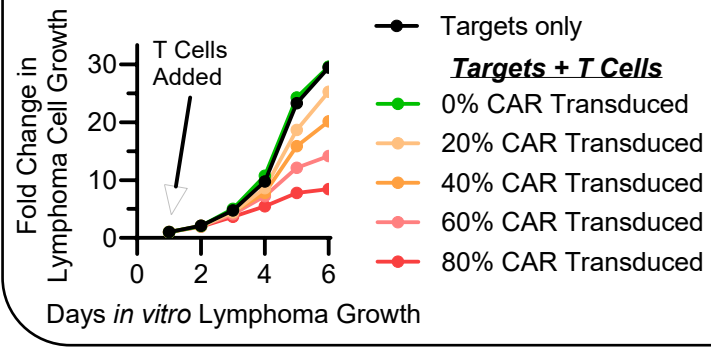
★ **Colorized image of G-Rex® assay from the BioRad instrument**



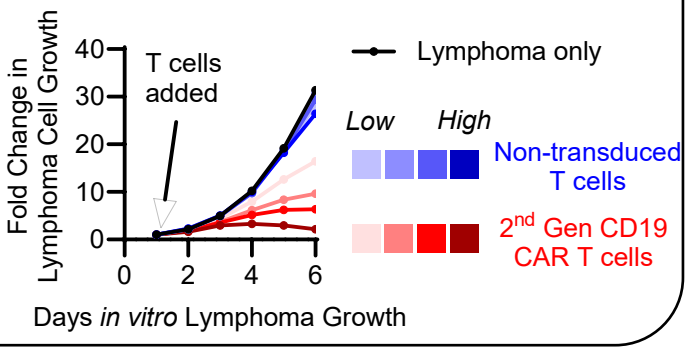
- ***Column 1*** *= lymphoma cells only*
- ***Column 2*** *= lymphoma cells co-cultured with CD19 CAR-T cells lacking CD3ζ domain (**no killing**)*
- ***Column 3*** *= lymphoma cells **killed** by co-culture with CD19 CAR-T cells*

A G-Rex® killing assay can highlight different attributes of cell therapy product. *Left*: anti-CD19 CAR T cells of varied transduction efficiencies were manufactured and compared in the killing assay. The assay readily distinguishes a range of transduction efficiencies. *Right*: The G-Rex® killing assay distinguishes between different doses of CAR T cells compared to similar doses of non-transduced T cells (E:T ratios of 1:25, 1:15, 1:10, 1:5).

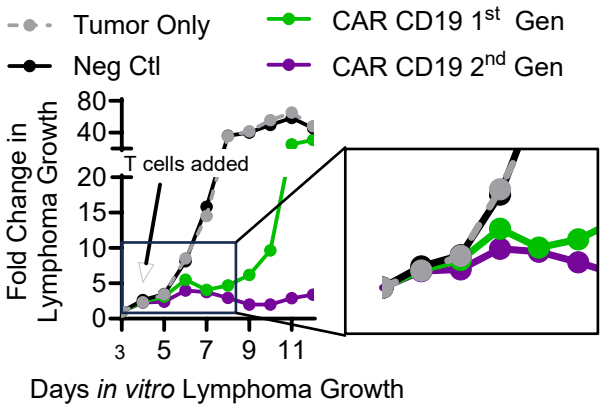
Killing dependent on product purity



Killing dependent on product dose



Longer co-culture timeframes needed to distinguish critical features of CAR-T cells.

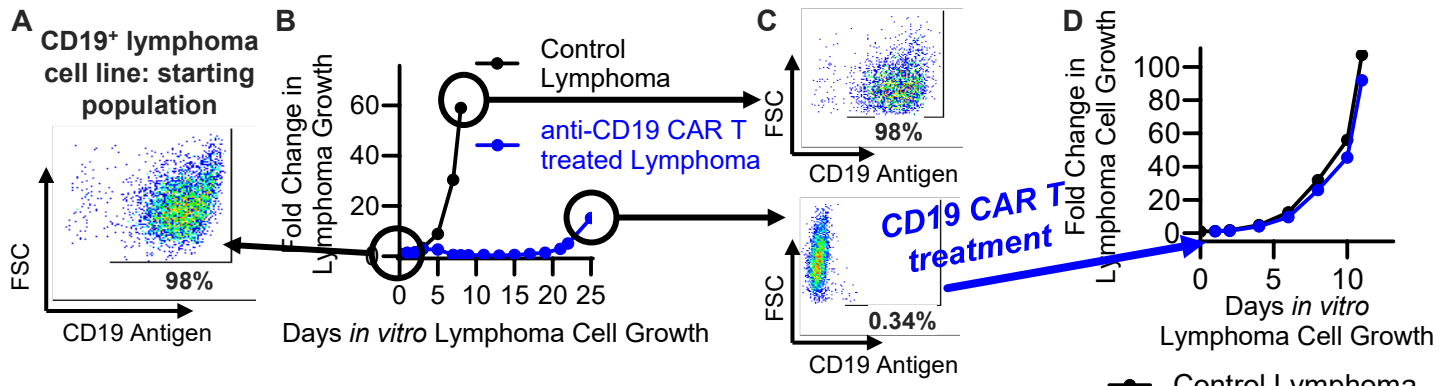


The G-Rex® killing assay is useful for screening new therapeutic candidates: The enhanced cytotoxicity and proliferation of 2nd generation CAR T cells compared to 1st generation CAR T cells have significantly improved clinical outcomes (PMID: 11714771, 9743337). When tested using the G-Rex® killing assay, 1st generation CAR T cells were indistinguishable from 2nd generation CAR T cells in their ability to control lymphoma cell growth through a short, one week assay timeframe (*see zoomed-in inset*). However, when the assay time is extended, the 2nd gen CAR-T is better able to control lymphoma cell growth compared 1st gen CAR-T, where lymphoma cell growth surges.

Eric Smith, Ph.D. & Andrew Lewis, Ph.D.

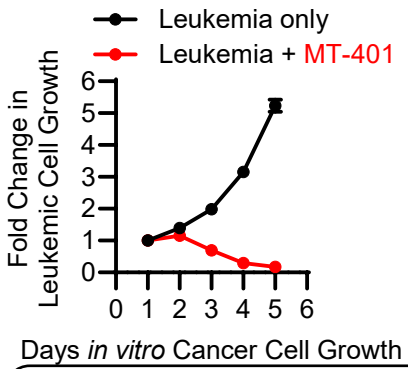
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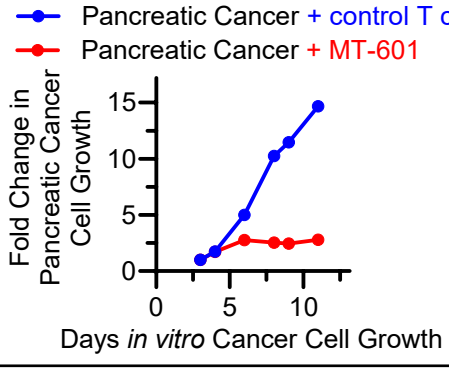
The long-term capability of the G-Rex® killing assay creates *in vitro* CAR refractory model: CD19 CAR T cell infusion characterized by antigen-negative lymphoma cell outgrowth

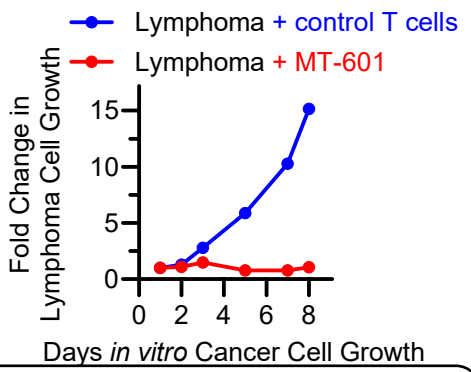


A) Lymphoma cell line is not 100% CD19⁺ **B) & C)** anti-CD19 CAR T significantly controls lymphoma cell growth, but after 3 weeks of undisturbed culture, a CD19⁻ cell population arises. **D)** This CD19⁻ cell population is now refractory to additional CAR T treatment

The G-Rex® killing assay can be applied to other cell therapies in multiple indications, including non-engineered tumor antigen-specific T cells (Marker Therapeutics).







Required instrumentation, consumables, and reagents**

**Quantities listed below represent a suggested T cell experimental design consisting of a negative control (e.g. target cells only) and two different effector:target ratios of positive control (e.g. cell therapy product known to kill tumor cells) and the manufactured cell therapy product to be tested. Quantities for triplicate wells per condition are listed.

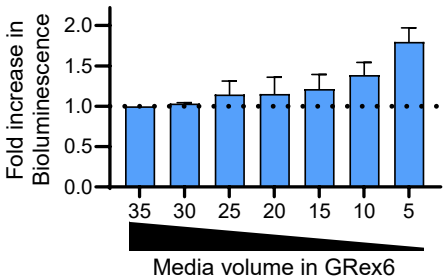
Instrumentation / components	G-Rex®6 Well Plate	Wilson Wolf Manufacturing, LLC	P/N 8024M	Quantity = 3 plates
	ChemiDoc™ Instrument	Bio-Rad Laboratories	Cat. #12003153	
	Laptop computer with free Bio-Rad software (Image Lab) installed	Bio-Rad Laboratories	Free download at website	
	Standard CO_2 Incubator	Various suppliers	Various product numbers	
	Tabletop Centrifuge	Various suppliers	Various product numbers	
	Firefly luciferase engineered target cell line(s)	In-house engineered or purchased (e.g. ATCC)	Various	At 1 x 10^6 target cells per well = 18 x 10^6 cells
	Respective effector cells	In-house manufactured product		
Reagents	Appropriate media for respective effector cells	e.g. Bio-Techne T Cell Media (#CCM-038)	e.g. Bio-Techne T Cell Media (#CCM-038)	Quantity = 650 – 750 mLs
	Appropriate cytokine supplement(s)	Bio-Techne	e.g. IL-7, #BT-007	Quantity = 3.2 µg
	D-Luciferin, luciferase substrate	Bio-Techne (Tocris)	Cat #5427	Quantity = 2.6 mLs of 25 mM stock

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Frequently Asked Questions:

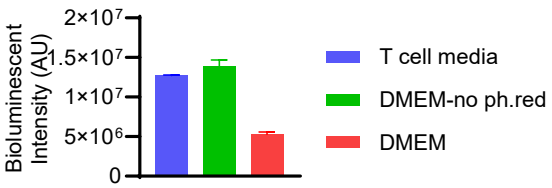
1. *Why is 100µM the recommended concentration of D-Luciferin?*
   - 100 µM D-luciferin provides sufficient signal above noise while limiting any toxicities to cells.
2. *Do I need to add D-luciferin daily (prior to image acquisition)?*
   - No. This is not recommended. The D-Luciferin remains active in the assay through 1 week of co-culture, or longer. For longer co-culture periods, D-Luciferin should be added if there are any media exchanges due to depletion of nutrients, especially glucose. Bioluminescence is dependent on ATP production. As cells run out of nutrients, their ATP production slows, leading to decreased bioluminescence.
3. *How do I determine optimal seeding density for cancer cell targets?*
   - It is recommended to determine growth kinetics for your cancer cell targets prior to use in a G-Rex killing assay. This can be accomplished by seeding a range of up to 6 seeding densities in a G-Rex®6 in the media that will eventually be used for killing assay (+ 100µM D-Luciferin). Monitor growth daily via bioluminescence. Monitor glucose depletion at least every other day. Select the seeding density that provides 1) excellent signal above noise and 2) uninterrupted long-term growth without complete depletion of glucose. Cells use glucose to produce ATP, a required reagent for bioluminescence to occur. Should complete depletion occur, bioluminescence will significantly decrease independent of cell number.
4. *Do I need to use 35 mLs media? Does the height of the media influence bioluminescent measurement?*
   - The recommended volume of media is 35 mLs, intended to provide the maximum volume of media while decreasing the risk of splashing from the well during repeated movement of the G-Rex®6 Well Plate from the incubator into the BioRad ChemiDoc. Less media can be used in extreme circumstances if certain reagents to be added to the media are limiting, but this will directly affect the length of the time the assay can proceed undisturbed. Additionally, there is an inverse relationship between media volume and bioluminescence. As shown in the figure to the right, as the volume of media is decreased, bioluminescence measured increases.



5. *How do I translate bioluminescent measurements into fold change in growth and/or anti-tumor activity?*
   - To calculate fold change in growth, normalize bioluminescence measurements to first measurement.
   - To calculate anti-tumor activity, use the following formula, where 'x' is bioluminescence of experiment condition (i.e. effectors + targets) and 'control' is bioluminescence of control condition (i.e. targets only or non-specific effectors + targets):

$$\%\ \text{anti-tumor activity} = \left(1 - \frac{x}{\text{control}}\right) * 100$$

6. *How do I determine E:T ratio when effectors are added day(s) after cancer cells seeded?*
   - Because cell number is linearly correlated to bioluminescence measured, the formula derived from a linear regression can be used to estimate the numbers of cells for a given bioluminescence measurement.
7. *Does phenol red inhibit acquisition of bioluminescence?*
   - In the figure to the right, T cell media (7.5 mg/L phenol red) shows minimal inhibition of bioluminescence compared to DMEM without phenol red. However, phenol red at concentrations of 15 mg/L (DMEM) reduces the measured bioluminescence by ~50%.



8. *Do I need to acquire bioluminescence immediately after removing the G-Rex6 from the incubator?*
   - Yes. Extended incubation outside of 37°C will adversely affect bioluminescence. The G-Rex®6 should be returned to the incubator for a minimum of 30 minutes prior to re-measuring bioluminescence.
9. *There is condensation on the lid of the G-Rex®6. Will this affect bioluminescence measurement?*
   - Condensation can decrease bioluminescence up to 10%. It is recommended to avoid condensation build-up by not exposing G-Rex®6 to room temperature before placing G-Rex in the BioRad. Setup the BioRad ChemiDoc first. Then, remove the G-Rex®6 from the incubator and place in the BioRad ChemiDoc.