Human Primary T Cells: A Practical Guide

Bulent Arman Aksoy*, Pınar Aksoy*, Megan Wyatt*, Chrystal M. Paulos*, Jeff Hammerbacher*
*: *Microbiology and Immunology Department at Medical University of South Carolina*

Abstract

Human primary T cells are invaluable and feasible model systems to study the characteristics of the immune cells in various contexts, including but not limited to cancer immunotherapy. Following isolation of T cells from fresh human blood samples, it is possible to culture, expand, and manipulate these cells, which allows extensive investigation for research purposes. Techniques for isolation and handling of T cells are well-established but parts of the protocols can highly vary across different labs. These differences in the protocols are there due to historical reasons and are often only supported by anecdotal evidence. We systematically modified basic components of the T cell culturing protocols and collected data on how they altered the final yield. Here, based on these data, we provide practical hints and tips on basic cellular and molecular techniques for handling primary human T cells. We hope that this guide will serve as a reference point to allow evaluate, discuss, and improve current practices in T cell culturing and manipulation. The live version of this guide is available at https://bit.ly/tcellguide.
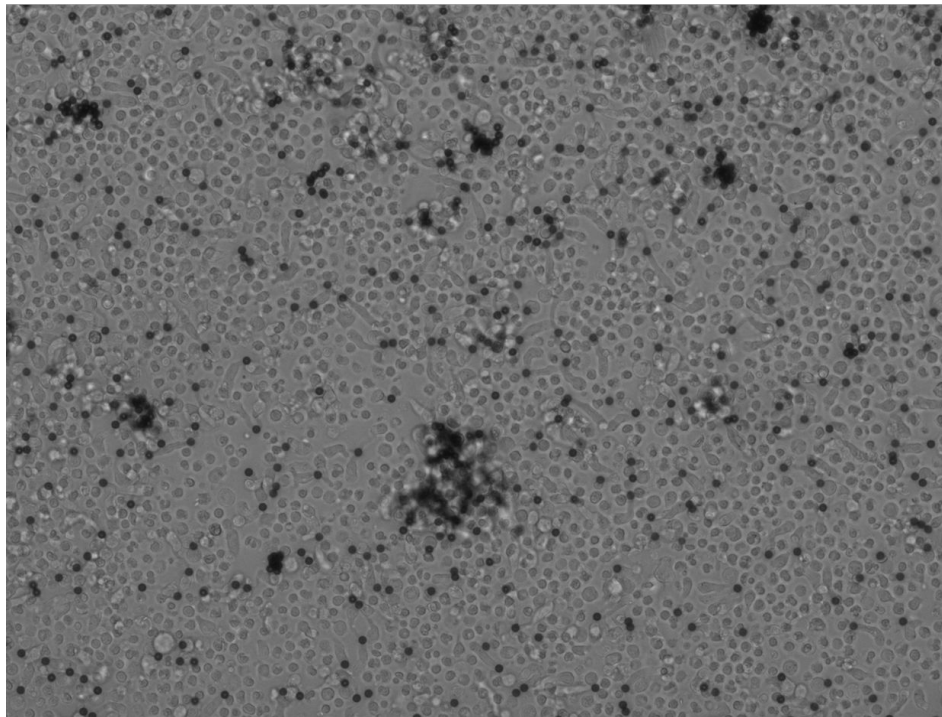
T cells (transparent spheres) activated with Dynabeads Human T-Activator CD3/CD28 (dark-colored spheres). 20X magnification.

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# Practical considerations for handling human primary T cells

Hundreds of millions of T cells can be isolated from a buffy coat sample

The majority of the research that utilizes primary human T cells rely on blood samples from anonymous donors. Although there are commercial options for obtaining fresh or frozen primary human T cells, isolating T cells from buffy coats or leukopaks is considerably cheaper and is a well-established technique that is adopted by most researchers. Furthermore, being able to isolate T cells from blood samples allows flexibility for studying immune cells in different contexts — for example, healthy subjects or cancer patients.

The initial step for isolating T cells from a buffy coat is the isolation of peripheral blood mononuclear cells (PBMCs). This can typically and efficiently be achieved with the help of a solution that forms a density gradient to trap PMBCs in a specific layer with the help of a standard wet-lab centrifuge that can spin 50-mL conical tubes. On average, one 50 mL buffy coat will yield ~800 million PBMCs. PBMC isolation from a buffy coat is relatively easy and cheap (< $100) compared to T cell isolation from PBMC cells.

Starting with PBMC cells, T cells can be isolated by either positive or negative selection strategies. Both selections rely on antibody-based capturing. Negative selection strategies, where immune cells other than T lymphocytes are captured with the help of magnetic beads coated with different antibody cocktails, are more preferable as these will help enrich the population for T cells that are "untouched" — that is, none of the capturing antibodies will bind to the target population and the chances of antibody-based stimulation or perturbation are minimal.

Starting with ~800 million PBMCs, T cell isolation using [Dynabeads™ Untouched™ Human T Cells Kit](#), will yield, on average, ~300 million T cells (i.e. ~37%). Depending on the downstream use cases, the untouched kit could be replaced by other kits that positively or negatively enrich for a particular subset of T cells (for example, CD8 positive T cells).
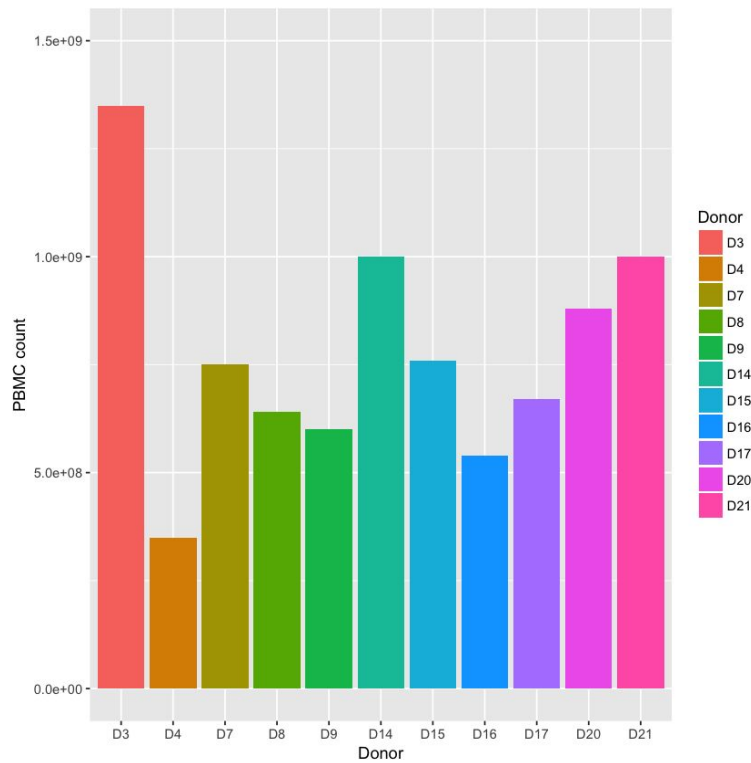
(a) One 50-ml buffy coat from a healthy donor yields 800 million PBMCs via T-lymphocyte-separation-media-based protocol.
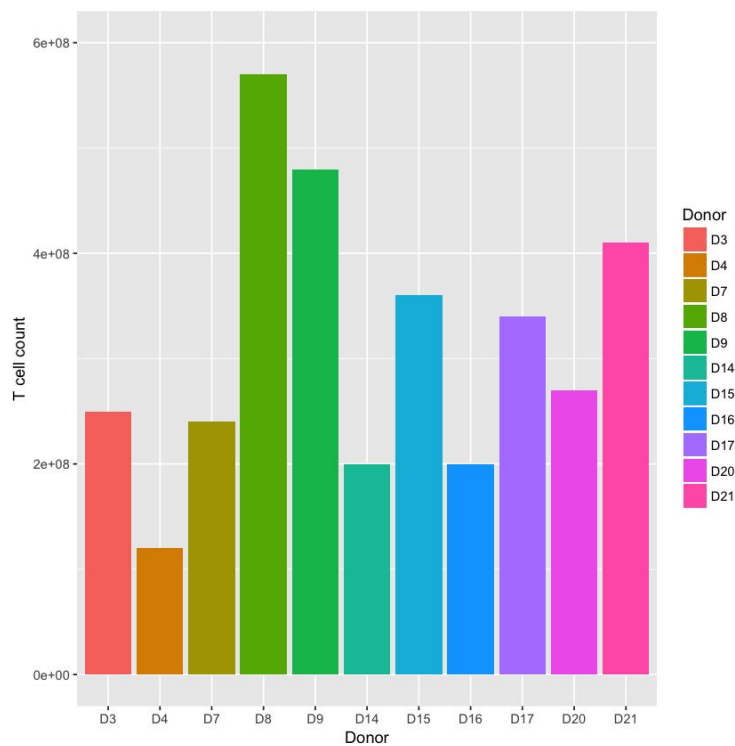
(b) One 50-ml buffy coat yields ~300 million pan T cells via untouched T cell isolation kit.

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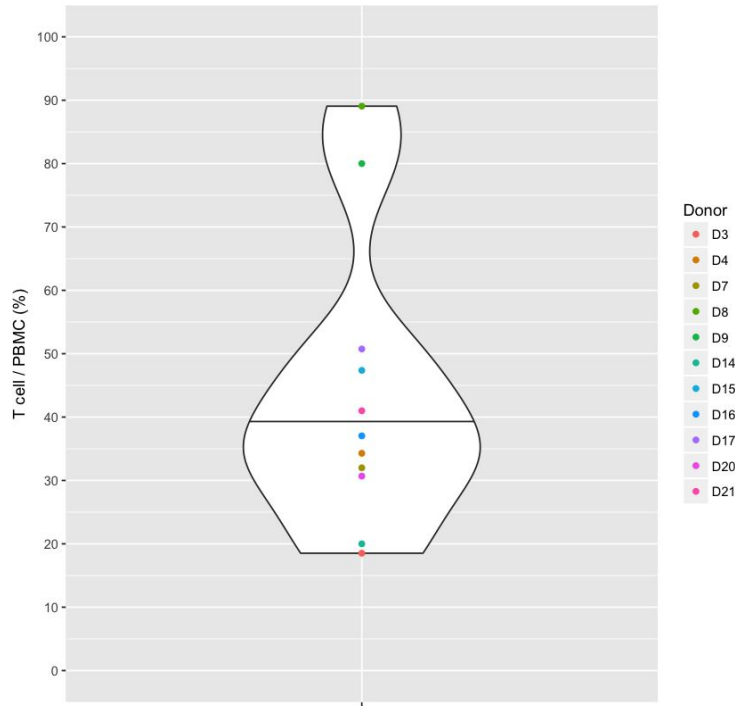
(c) On average, 37% of the PBMCs are pan-T cells as estimated by the median percent (horizontal line) across 11 healthy donors.

Data: Buffy - PBMC - T cell counts.ipynb

The relative abundance of T subsets vary across donors

Based on the age, ethnicity, and prior exposure to pathogens, the fraction of CD4+ and CD8+ T cells can highly vary across donors. After isolation of T cells from buffy coats or PBMCs, any downstream experimentation and quantification on particular T subsets should account for biological variation due to different donor characteristics. Using straight-forward antibody-based staining techniques, these differences can easily be captured for future reference as part of a standardized quality-check and exploration step. We have found that profiling each sample for well-known T subsets (CD4 or CD8 positive, naive, central memory, ...) made it easier to interpret the results of various enrichment, polarization, or manipulation experiments.

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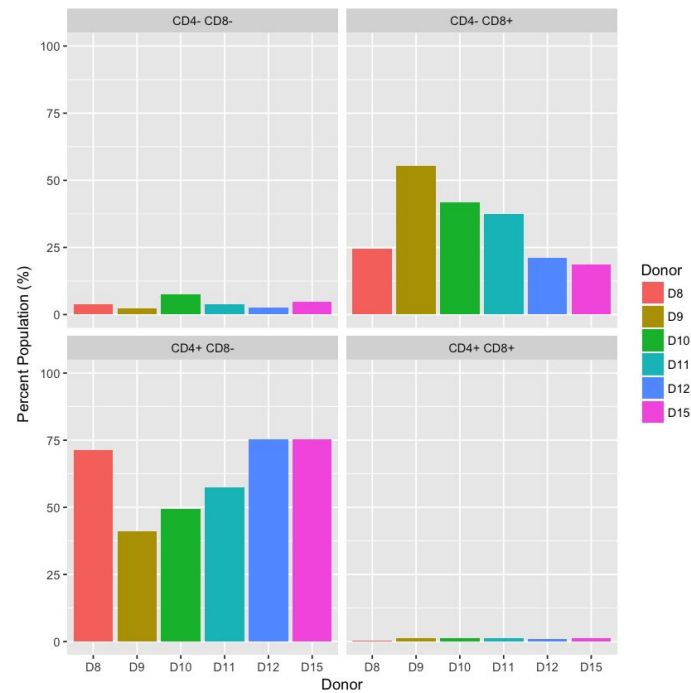
(a) CD4 and/or CD8 positive T cell prevalence can vary across donors as estimated from unactivated pan T cell populations.
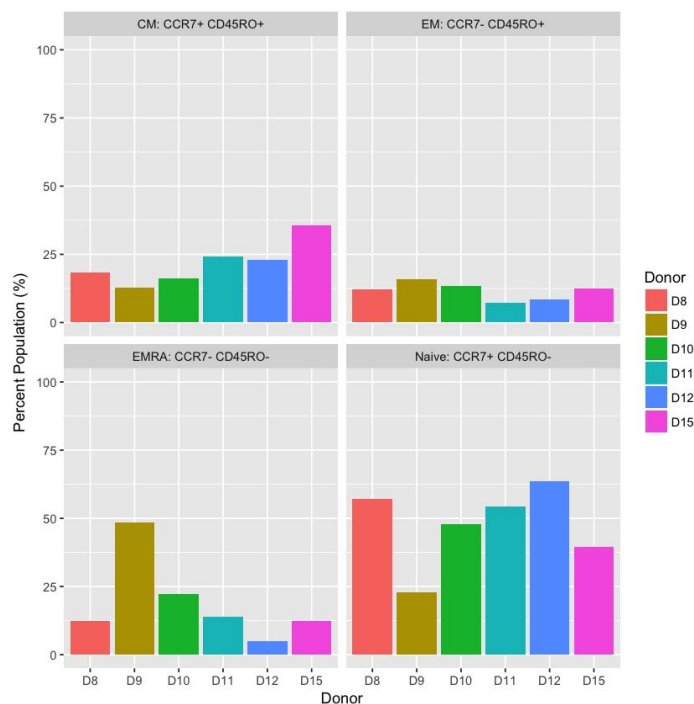
(b) Central memory (CM), effective memory (EM), effector memory RA (EMRA), and naive cell populations also vary across different donors.

Data: T cell subset variance across donors.ipynb

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Activation is required to rapidly expand T cells in culture but cell counts barely increase throughout the activation process

Short- and long-term culturing of the T cells for research or clinical purposes rely on continuous expansion of these cells. Without activation, T cells do not proliferate efficiently and on top of that, their inactive profile makes them harder to manipulate and profile. Commonly used activation techniques are similar to each other in principle but the specific method used for T cell activation can enrich certain T subsets. Anti-CD3- and anti-CD28-based activation is the most commonly used technique in both research and clinical settings. Since this method is not antigen-specific, it does not lead to rapid clonal expansion but instead helps maintain the proliferation of a diverse set of T cells.

T cells are commonly activated for 1-3 days and following this, activation reagents are often removed from the media to not continuously stimulate cells. Following activation, an activated pan T cell population double in number of cells about every 24 hours in RPMI-1640-based complete growth media. However, commercially available specialized ex vivo T cell expansion media could help reduce this doubling time and can therefore help with growing cells faster.

Depending on the time points to be used in an assay or the cell density required for the specific experimentation, the number of T cells seeded in the beginning of the experiment can highly vary. Surprisingly, we have observed that throughout the T cell activation, there is no apparent increase in the number of cells -- that is, we have consistently obtained fewer or about the same number of cells compared to what we have started with (pre-activation). This can be explained by the activation-induced cell death (AICD), where a fraction of the population is lost upon activation. When setting up an experiment, it is important to account for the 24-hour doubling time and also for this steady growth phase throughout the activation to have at least the minimum number of cells required for the assay of interest.
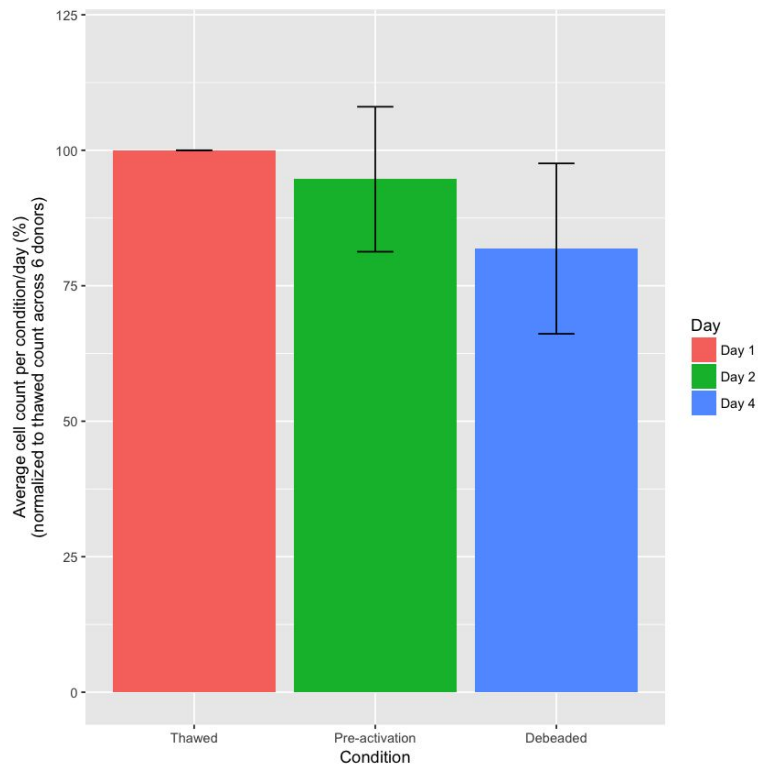
(a) The number of T cells do not immediately increase upon activation via beads coated with anti-CD3 and anti-CD28. On average, T cells that are thawed on the first day and activated on the second for two days do not increase in numbers.

Data: Cell counts pre- and post- activation.ipynb

Freezing T cells allows flexibility in experimental setup

As mentioned earlier, the majority of the T cell manipulation techniques start with activation of the T cells and this limits the flexibility of an experimental setup. Specifically, a typical major blood center and hospitals are closed on the weekends and because of this, buffy coats are usually shipped overnight on the weekdays (Mon-Fri). The isolation of the T cells is preferably done within the same day and assuming that these isolated cells are directly activated after isolation for at least two days, receiving a buffy coat on Thursday or Friday is not that ideal as this will require running experiments on the weekend. Furthermore, blood centers typically require at least a few days to be able to process a buffy coat request, find an anonymous donor, and complete the tests before sending the sample out.

Because a typical buffy coat will yield hundreds of millions of T cells, which is plentiful for common practices, unused cells can be cyropreserved and can later be thawed for further experimentation without compromising their characteristics. This not only enables flexibility with

respect to the experimental setup but also allows spontaneous explorations or verification experiments.

Given that T cell isolation kits have minimum cell count and volume requirements, if the planned experiments involve unactivated T cells, it is more feasible to isolate the T cells upon PBMC isolation and then freeze T cells. This allows thawing small batches of T cells and experiment on them without any further complication. When PBMCs are frozen, it is inefficient and time consuming to thaw small batches and isolate T cells before each experimental procedure. If the experimental procedure starts with activation of T cells, isolation of T cells from PBMCs before freezing can be considered optional *(see cost optimization section for more details)*.

The optimal concentration to be used when freezing cells depends on the specific details of the experimental assay. However, we have found that using a concentration of 10-15 million cells per milliliter to be practical in terms of the logistics of the freezing and thawing processes -- specifically the amount of freezing media to be used when freezing the cells and the number of vials to be handled while freezing or thawing.

Electroporation is a feasible method for genetically manipulating T cells

Genetic and epigenetic manipulation of cells enable efficient ways of studying phenomena at a cellular level. Unlike pharmaceutical inhibition of particular targets, genetic and epigenetic manipulation require delivery of either DNA, RNA, or protein into the cells. However, common delivery techniques, such as transfecting cells with [Lipofectamine Transfection Reagent](#), [have very low efficiency in human primary T cells](#). Due to uncharacterized cellular properties, human primary T cells had been notoriously hard to manipulate. Although lenti- and retro-viral material delivery has been the de facto technique to transduce human primary T cells, the consequences of genomic integration has always been a source for concern, especially for clinical applications. Because of this, electroporation has been widely used as a feasible approach to deliver DNA, RNA, or protein into human primary T cells.

Due to practical reasons, we have optimized our protocols using [Thermo Fisher's Neon Electroporation System](#). Others have explored various electroporation options and we have mainly compared two settings for their efficiency, initially in the plasmid electroporation efficiency: **1600 V 10 ms 3 pulses** versus **2200 V 20 ms 1 pulse**. In our hands, cell viability and electroporation efficiency were both better with the **1600 V 10 ms 3 pulses** setting. We have also observed favorable cell growth and viability when the cells were grown in the same exact media conditions with the conditions used to expand them pre-electroporation without the use of any activation material. Neon electroporation system supports 10 uL or 100 uL electroporation volumes where the recommended maximum cell density is 20 million cells per mL and the material to be electroporated into the cells without exceeding the 10% of the final volume. That is, ideally, for the 10-uL setup: we recommend suspending 200,000 cells in 9 uL of electroporation buffer and mixing it with 1 uL of the material (e.g. ~1 ug plasmid) that will be electroporated into the cells.

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To reduce variation per electroporation sample and to account for practical issues of electroporation (e.g. sparks), we have also found that a 24-well plate setup where we seeded cells from three independent electroporation reactions (within 10-ul tip) into a single well with 500 ul to 1 ml culture media in it had been the most feasible approach. This setup is especially helpful during initial optimization experiments where the goal is to screen many conditions. Pooling three independent reactions into a single sample provides a virtual buffer against variation across electroporation samples and yields enough treated cells for common assaying approaches, such 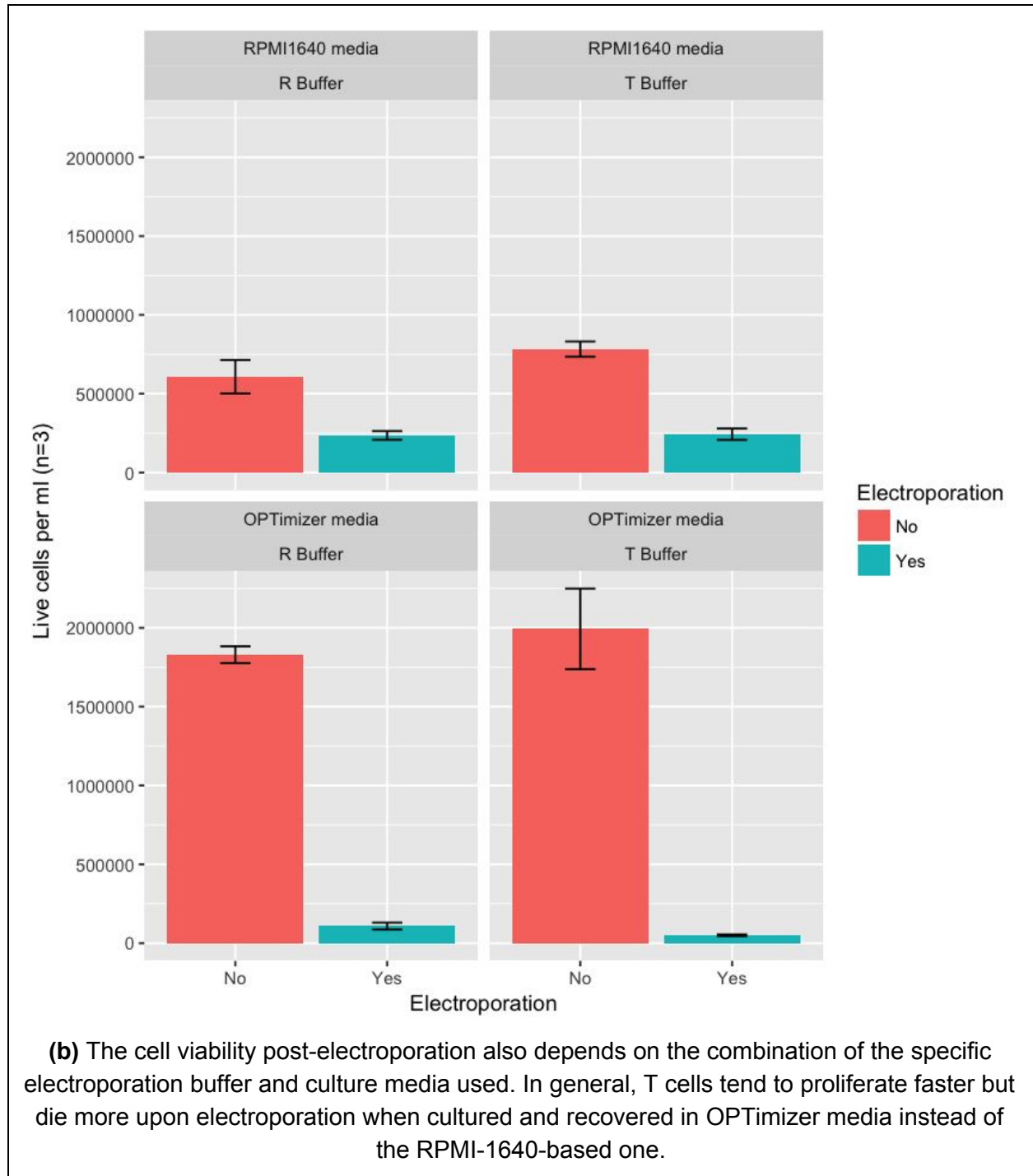as Western Blotting. We always recommend optimizing or screening using 10-ul tip setup and then scaling the reactions up to 100-ul once the optimal settings are known.
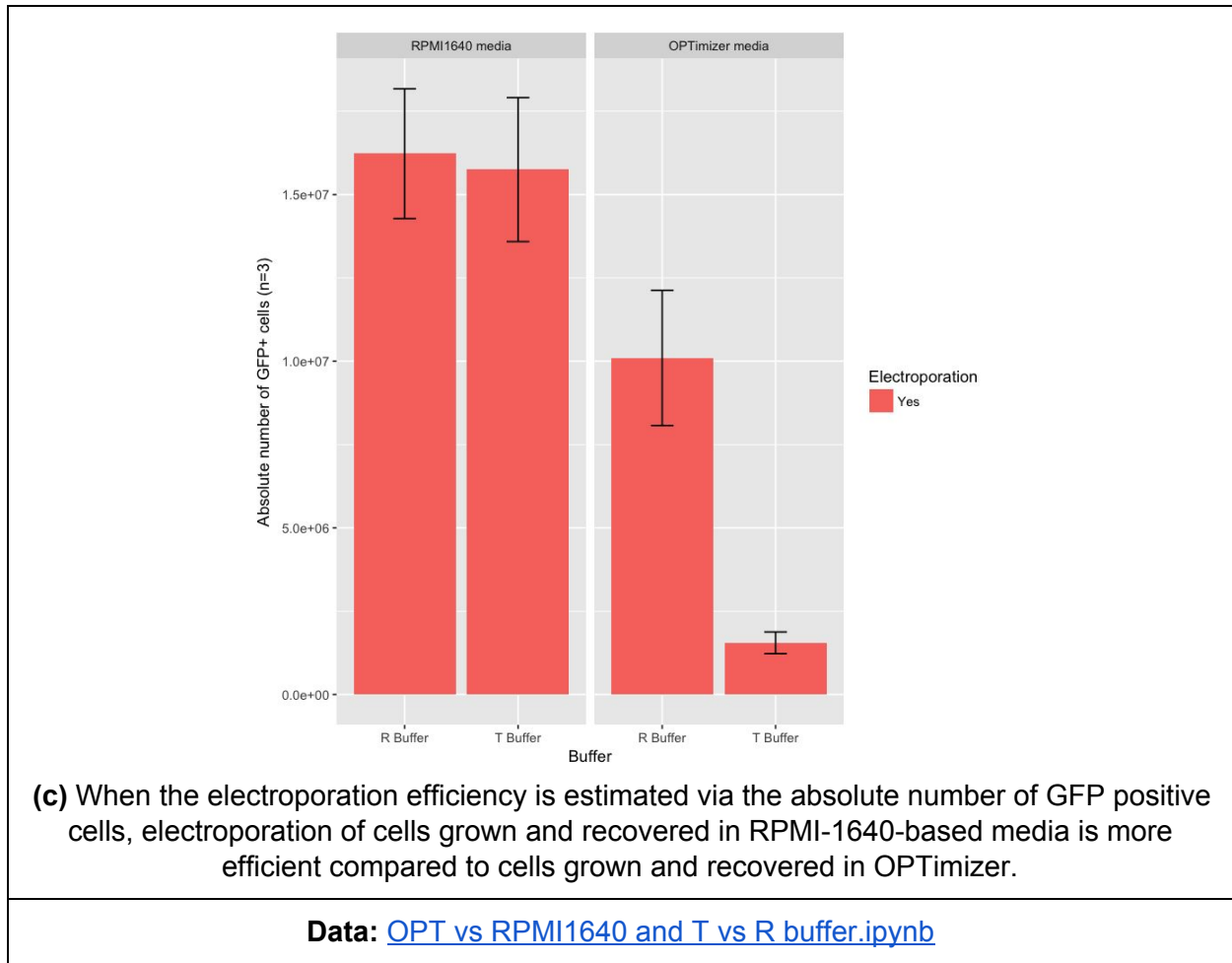
In general, we have found that electroporation with the 1600 V 10 ms 3 pulses setting kills on average 33% (±19.77%; n=5) of the initial population regardless of the material that is being electroporated. Although this means the majority of the cells die upon electroporation, the cells recover after an overnight incubation and continue proliferating in an activated state 1-day-post-electroporation.

Plasmid electroporation is inefficient and causes major cell death

The common way to estimate the efficiency for plasmid electroporation is to electroporate a GFP expression plasmid for mammalian cells and then check for GFP positive cell frequency via flow cytometry. By the time we have started optimizing electroporation for plasmid delivery, Thermo Fisher's official Neon guide was suggesting use of T buffer against R buffer for suspension and hard-to-transfect cells (e.g. human primary T cells). A recent report from the company has reported 90% efficiency for plasmid electroporation in human primary T cells grown in OPTimizer media with human-serum supplement that were electroporated in R buffer. To confirm this report, we have explored expanding activated T cells in RPMI-1640 with FBS supplement or OPTimizer with human serum supplement as described in the report and then electroporating each of these cells in either R or T buffer. We have used 1 ug Lonza's pMAX-GFP plasmid to assess the efficiency of electroporation in cells that have been activated for three days. The cells were then assayed for GFP positivity using flow cytometry after a minimum of overnight incubation. We found that human primary T cells expanded much faster in OPTimizer media with human-serum supplement compared to RPMI-1640-based media and the fraction of GFP positive cells were much higher (~95%) when cells were electroporated in the R buffer. However, the absolute number of GFP positive cells were higher when cells were expanded in RPMI-1640-based media and electroporated in T or R buffer. Therefore, although the efficiency reported by the company is similar to what we have observed, in practice, the absolute number of cells that were successfully transfected with the GFP plasmid were much higher when the traditional RPMI-1640 media was used. Considering the costs associated with the use of the OPTimizer media, we concluded that using this media for only electroporation purposes was not that feasible or favorable.

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(a) Plasmid electroporation efficiency depends on the type electroporation buffer and the culturing media used. In general, efficiency (as measured by the number of GFP+ cells in the final population) was higher when the cells were electroporated in R buffer instead of T buffer in both media conditions. Cell grown and recovered in OPTimizer media and electroporated in R buffer had the highest electroporation efficiency (mean: 93%).

(b) The cell viability post-electroporation also depends on the combination of the specific electroporation buffer and culture media used. In general, T cells tend to proliferate faster but die more upon electroporation when cultured and recovered in OPTimizer media instead of the RPMI-1640-based one.

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(c) When the electroporation efficiency is estimated via the absolute number of GFP positive cells, electroporation of cells grown and recovered in RPMI-1640-based media is more efficient compared to cells grown and recovered in OPTimizer.

**Data:** OPT vs RPMI1640 and T vs R buffer.ipynb

mRNA electroporation is convenient for transiently expressing exogenous proteins

Even though we were able to go up to around 60% efficiency for the GFP-plasmid electroporation, others have reported decreased efficiency of electroporation in correlation to increased size of the plasmid that was being electroporated. This means that in an experimental setup where more than one plasmid will be electroporated into the T cells, one has to take into the plasmid-specific efficiency into account and in an ideal setup, one has to estimate the efficiency of electroporation for each plasmid to be used in the assay to be able to better assess the final yield based on the phenotype to be observed. Using plasmids that allow selection on the cells is an option but the selection process might complicate the experimental setup especially, when the sampling time is an important component.

Given these concerns with the plasmids, we also wanted explore the use of in vitro transcribed mRNA electroporation to transform cells by inducing the expression of an exogenous material via electroporation. We have first explored the use of in vitro transcribed GFP with a nuclear destabilization signal (pcDNA3.3-NDG was a gift from Derrick Rossi; Addgene plasmid #26820) using HiScribe™ T7 High Yield RNA Synthesis Kit and assayed the electroporated cells on

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multiple days (days 1-6) after electroporation. We found that even though there was some donor-specific variation in the mRNA electroporation efficiency, the mRNA electroporation was relatively more efficient compared to plasmid delivery and the percent of GFP positive cells were correlated to the amount of GFP mRNA electroporated into the cells. This suggests that in vitro transcribed mRNA electroporation is a feasible way of inducing exogenous protein expression in the activated T cells where one can fine-tune the abundance of the target protein via changing the amount of RNA to be electroporated into the cells. Moreover, we have found that mixing GFP mRNA into a mix of RNAs to be electroporated into the cells enabled a quick and easy way to estimate the efficiency of the protocol and to isolate the cells that were highly likely to be transformed.
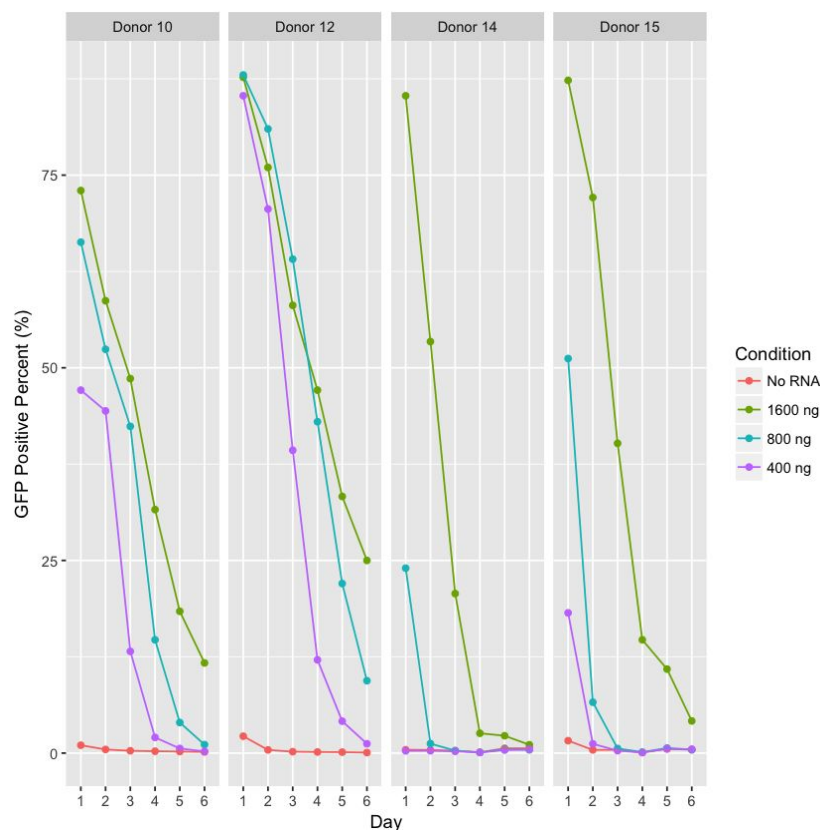
(a) When electroporating RNA into human primary T cells, adding in vitro transcribed GFP (with a nuclear degradation signal) mRNA into the RNA mixture allows estimating electroporation efficiency via flow cytometry after 24 hours after electroporation (Day 1). Furthermore, RNA amount (per 2 million cells; "Condition") can be adjusted to fine-tune the final yield.
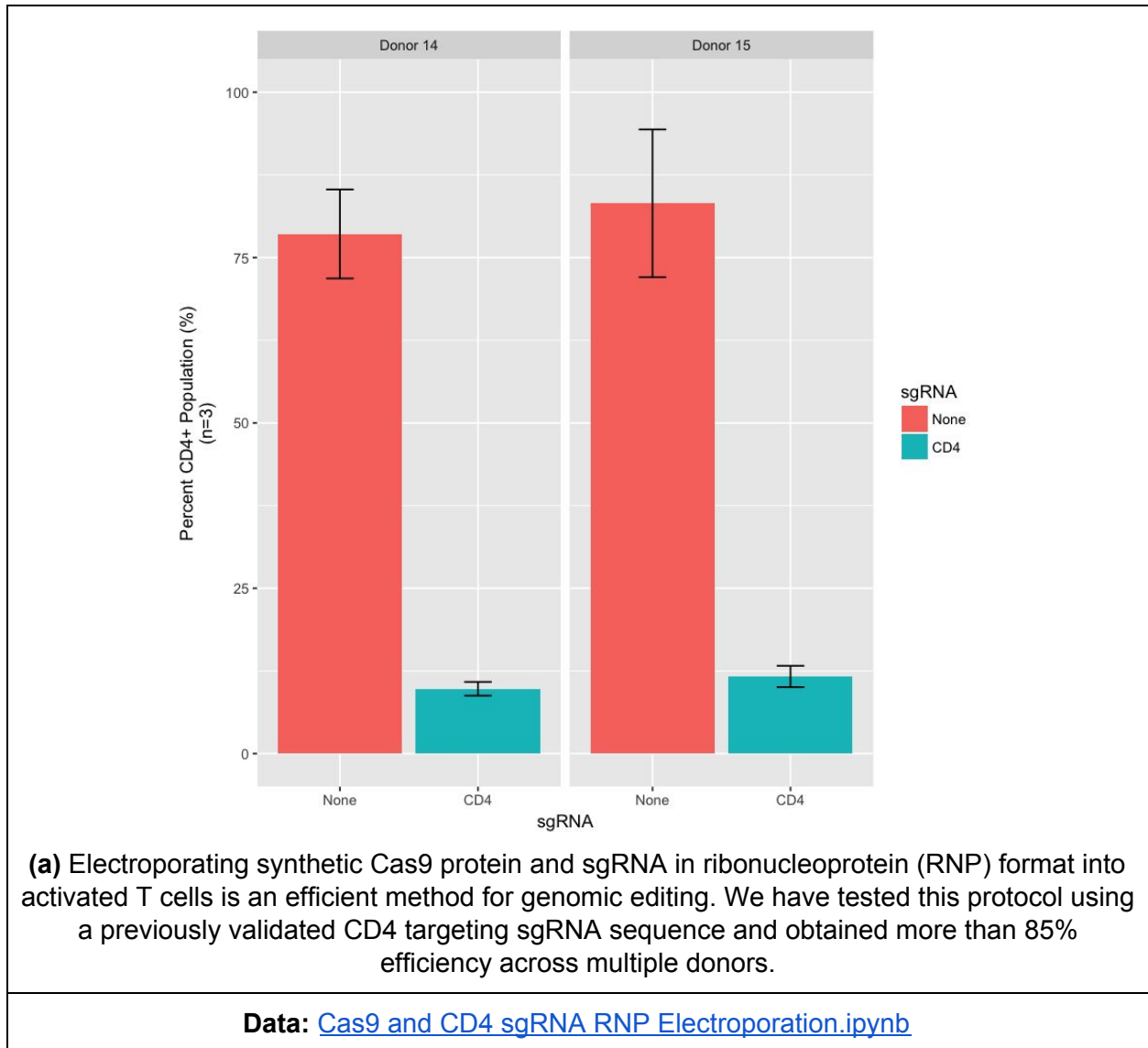
Data: GFP mRNA electroporation.ipynb

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Cas9 RNP electroporation is a feasible technique to knockout a gene

Given the relatively low efficiency of plasmid electroporation in human primary T cells and the concerns with the lenti- or retro-viral delivery techniques, the options to knockout particular target genes in a human primary T cell are limited. Others have recently reported highly efficient gene knockout protocols that rely on electroporation of the single-stranded guide RNA and the synthetically synthesized Cas9 protein complex into human primary T cells. Based on the previously reported successful CD4 targeting sgRNA sequence, we were able to replicate these findings and have achieved more than 85% efficiency of CD4 knockout in activated human primary T cells.

Despite being a highly efficiency technique, the major cost associated with this protocol is the use of synthetic Cas9 protein, which is commercially available but significantly more expensive compared to other use cases, for example plasmid-based protocols. Another difference between the plasmid- and RNP-mediated approaches is that the plasmids enable selection of the cells that bear the plasmid (hence are highly likely to have the successful knockout event) but with the RNP-based approach, selection is only possible when a surface protein is targeted (via flow-cytometry-based sorting approaches).

For targets that do not have previously reported or validated sgRNA sequences, we have found that conducting an initial screen using at least 6 sgRNAs (based on scored computational predictions from GPP Web Portals sgRNA design) on a smaller scale was necessary to be able to assess the feasibility of a particular gene target and before conducting large-scale knockout experiments for further cellular profiling. For intracellular targets, the confidence in estimating the knockout efficiency is limited to the availability of specific primary antibodies for Western Blotting. Mismatch cleavage assay for detecting small insertions and deletions is an alternative; however if the candidate sgRNA target regions are diverse across a gene, validating a genomic editing through this method could become cumbersome.

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(a) Electroporating synthetic Cas9 protein and sgRNA in ribonucleoprotein (RNP) format into activated T cells is an efficient method for genomic editing. We have tested this protocol using a previously validated CD4 targeting sgRNA sequence and obtained more than 85% efficiency across multiple donors.

Data: Cas9 and CD4 sgRNA RNP Electroporation.ipynb

Common T cell techniques can be optimized for cost

Untouched T cell isolation kit contents can be titrated down without major efficiency loss

T cell isolation kits allow obtaining highly pure T cell subsets for immediate experimentation and therefore are important for protocols that focus on characterization of particular T cell types (e.g. CD4 positive T cells). These isolation kits are relatively expensive (~$500-1000) especially in the context of mid- to large-scale experiments with multiple donors. We asked whether we could titrate Dynabeads™ Untouched™ Human T Cells Kit and use them at a lower concentration than the recommended ones without compromising the final T cell yield and the T subset prevalence. We found that when used as recommended, the kit highly enriches for CD3 positive

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cells, however using beads at half or even the quarter of the recommended dose did not compromise the enrichment in a significant manner. When working in larger scales (> 100 million cells), the difference in the yield of CD3 positive population across different bead concentrations can be considered negligible. Moreover, using lower number of beads did not affect the CD4- and CD8-positive cell fractions in the final isolated T cell populations.

(a) Untouched T cell isolation from PBMC highly enriches T (CD3 positive) cells but it does not alter CD4 and CD8 positive T cell frequencies in the final population. Furthermore, the material used for isolation can significantly be titrated down (1:2 or 1:4) without compromising the enrichment to a great extent.

**Data:** Isolation bead titration.ipynb
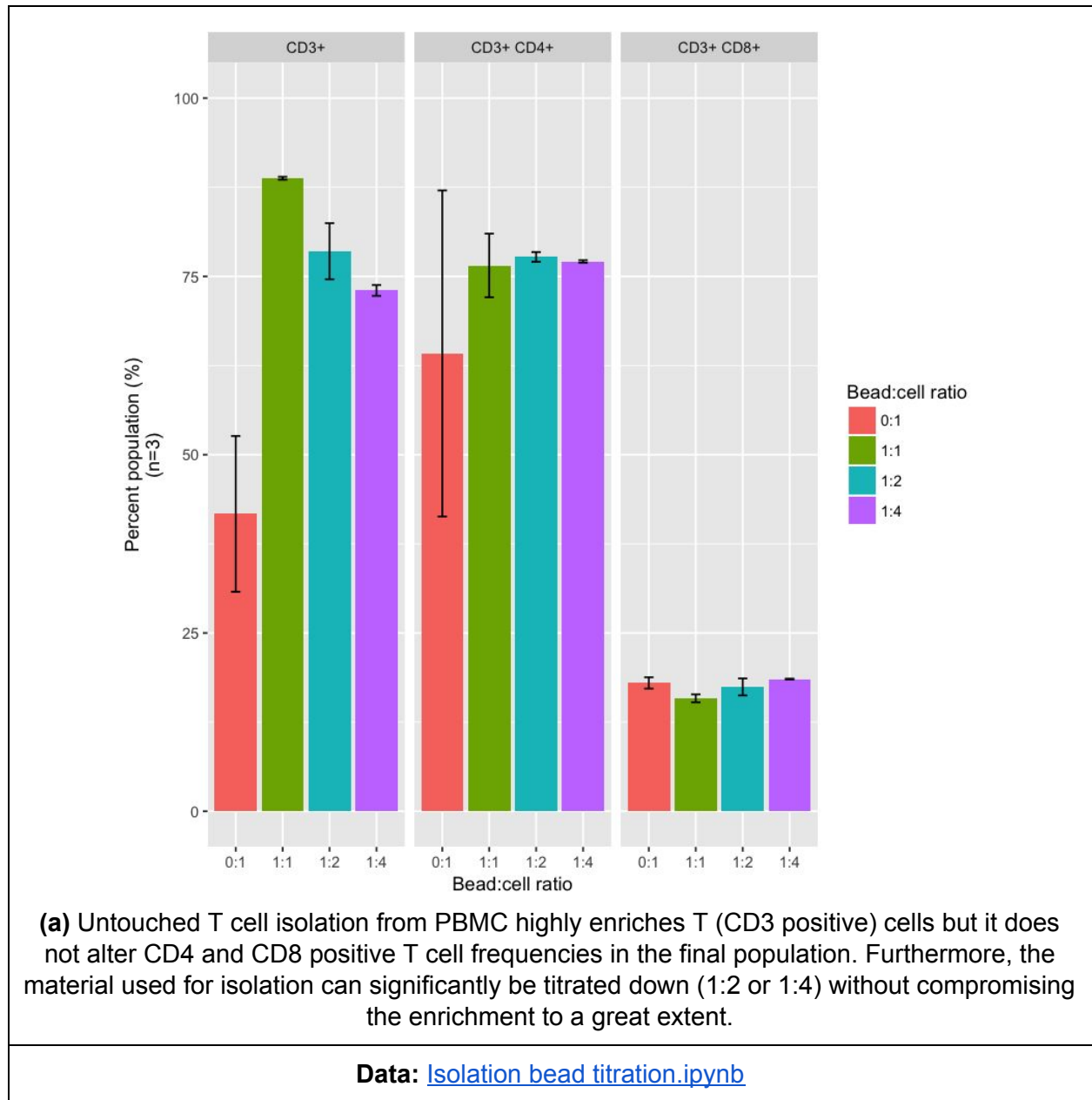
Using fewer anti-CD3 and -CD28 beads for activation doesn't immediately compromise activated cell yield

Similar to untouched T cell isolation bead kits, activation beads are also relatively expensive and can be considered one of the major expenses for T cell culturing. Because the most common T cell manipulation techniques require T cell activation, we asked whether using fewer than recommended beads for activation would compromise activation (as measured by CD69 expression) or bias either of the CD4 or CD8 positive T cells. For this, we initially conducted a 2-fold serial dilution of activation beads to activate pan T cell population from two different donors in the presence of 200 IU/mL IL-2 supplement in the media. We then measured the cell viability via resazurin assay after two days of activation and estimated the total number of cells using the normalized absorbance metric (absorbance at 570 nm normalized against absorbance at 590 nm). As expected, the yield from the recommended bead-to-cell ratio of 1:1 was the highest but surprisingly use of half of recommended beads (that is at 1:2 bead-to-cell ratio) did not compromise the final yield. 1:4 or lower bead-to-cell ratios monotonically decreased the overall yield where at 1:2048 ratio, we observed the minimal cell growth. When we checked the CD4 and CD8 positive population frequencies upon activating the cells with the recommended (1:1) or lower (1:2-1:8) concentration of beads, we did not detect any significant changes. However, when lower concentration of activation beads were used, the early activation marker CD69 level (MFI) detected by flow cytometry was also lower.

These data show that minor deviations (for example due to cell counting errors) from the recommended activation-bead-to-cell ratio should not significantly affect the overall T cell yield upon activation and moreover, using 1:2 bead-to-cell ratio could be considered as a feasible and economical option for optimizing the cost of T cell activation for short-term T cell expansion.
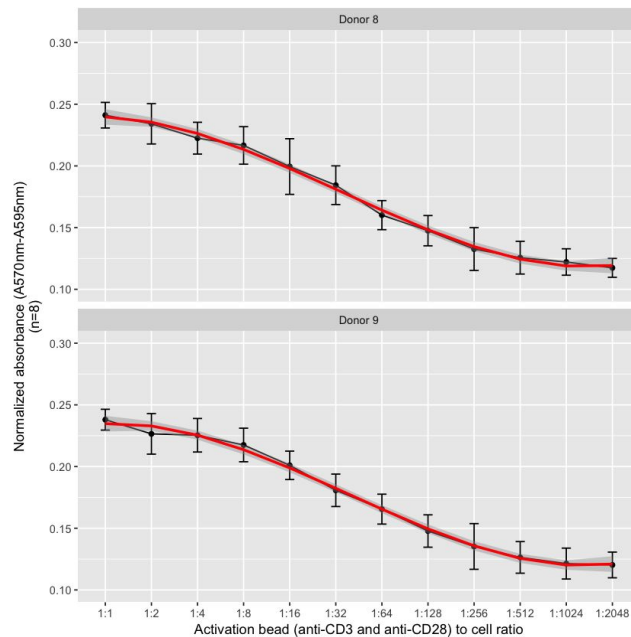
(a) Two-fold titrations of activation beads, starting from the recommended dose, showed that lower than 1:4 bead-to-cell ratios compromised proliferation and yielded relatively fewer T cells as measured by resazurin-based viability assay 2 days after the activation.
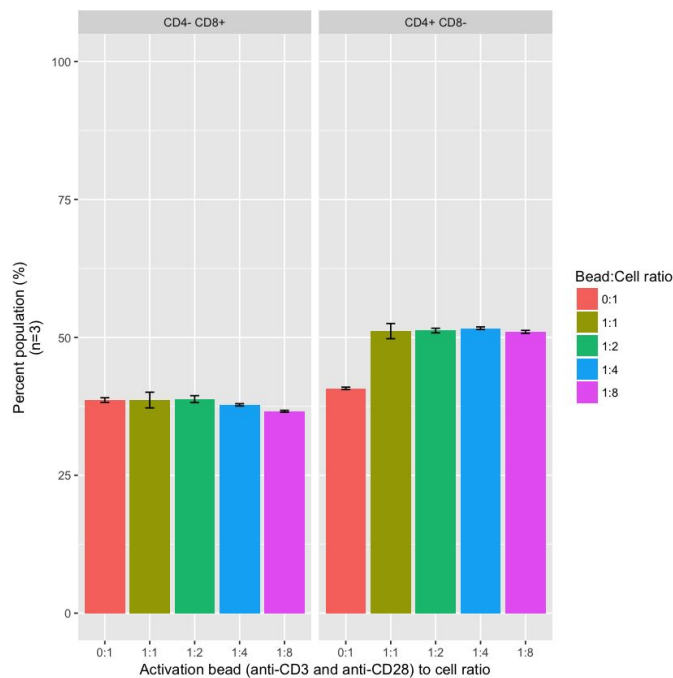
(b) Using fewer than recommended number of beads do not affect the percent of CD8 or CD4 positive T cells in the final T cell population as measured by flow cytometry on the second day of activation.

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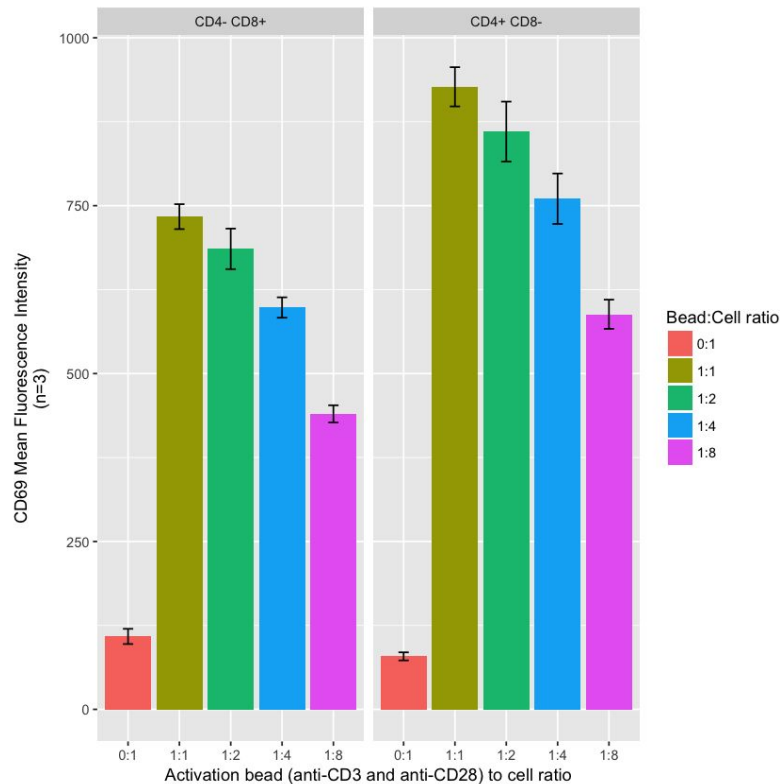
(c) Use of lower than recommended activation beads result in lower expression of early activation marker, CD69 in both CD4 and CD8 positive cell populations as measured by flow cytometry on the second day of activation.

Data: Activation bead titration results via flow and count.ipynb

IL2 supplement is not necessary for short-term T cell cultures during and after CD3/CD28-based activation

IL2 concentration used for T cell culturing highly varies across studies, where the working concentration can vary from as low as 25 IU/ml to as high as 20,000 IU/ml. Because of this varying recommended concentration reported in the literature, we wanted to test the effect of different IL2 concentration within the context of our experimental setup. For this, we first conducted a two-fold titration of IL2 (within the range of 1.5 to 3200 IU/mL) when activating the pan T cells with the anti-CD3 and anti-CD28 beads (1:1 bead-to-cell ratio) and then profiled cells post-activation. To our surprise, we did not see increased cell proliferation 2 days after activation as measured via resazurin-based viability assay. We then asked whether IL2 presence (200 IU/ml) or absence favored either of the CD4 or CD8 positive cells within the pan population when T cells were cultured for a longer term. At both 5 and 8 days after activation, we did not see any difference in CD4 or CD8 positive cell frequencies or CD25 positivity within these two populations across no IL2 and 200 IU/ml IL2 conditions.

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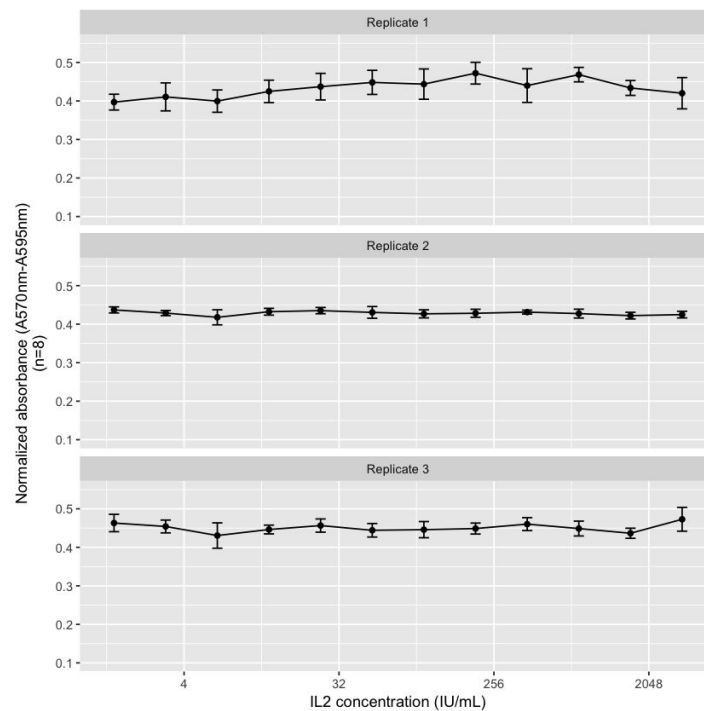
(a) Two-fold IL2 titration in the presence of (1:1) activation beads showed that IL2 supplement within the range of 1.5 to 3200 IU/ml did not affect the final yield as measured via resazurin-based viability assay on the third day of the activation.
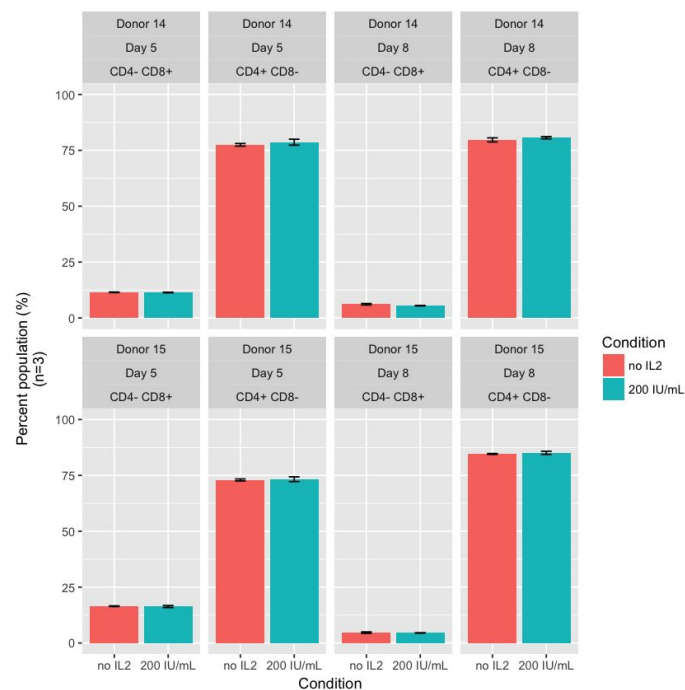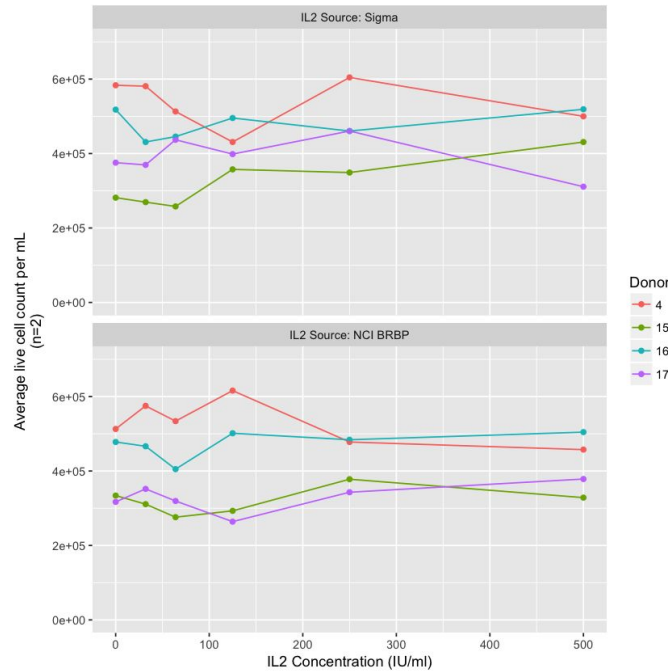

(b) Culturing activated cells in media with (200 IU/ml) or without IL2 did not favor CD4 or CD8 positive cell proliferation in a relatively longer term (5 and 8 days after activation).

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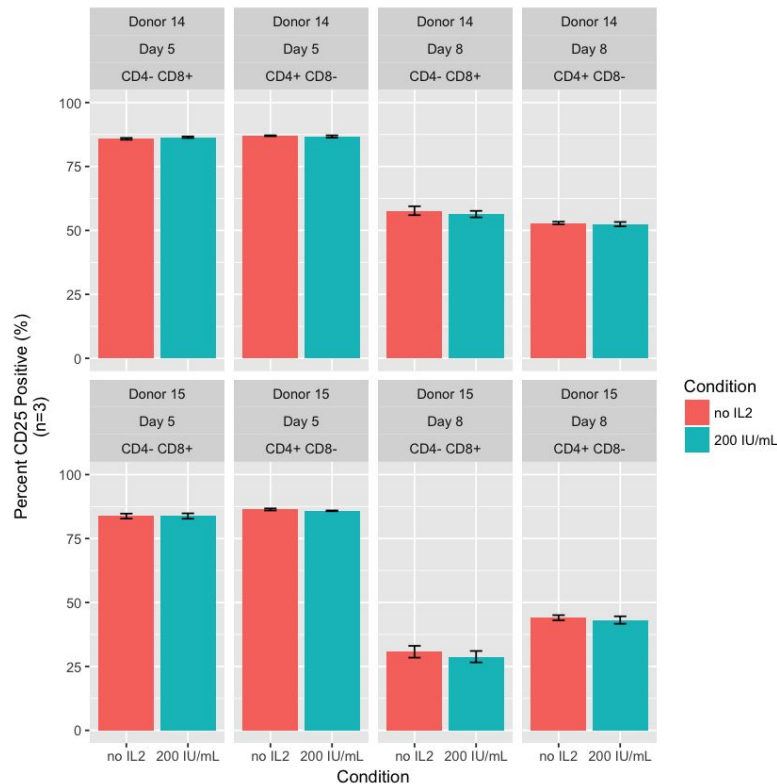
(c) Similar to (b), culturing activated cells in media with (200 IU/ml) or without IL2 did not alter CD25 positive cell frequency 5 and 8 days after the activation as measured via flow cytometry.

Data: IL2 titration results from multiple assays.ipynb

One explanation as to why we did not see different proliferation profiles with different concentrations of IL2 in the media could be attributed to the source of the IL2 and the batch of the IL2 that we prepared. To rule this out, we prepared a new batch of IL2 supplement and also used a commercially available option. We then repeated the titration experiments and tested the effect of IL2 concentrations (from 0 to 500 IU/ml) on the proliferation along with the CD4 and CD8 positive cell frequencies in the final population. We, again, did not see any change in any of the profiles across IL2 reagents from two different sources and batches after five days of culturing.

(d) Two-fold IL2 titration in the presence of (1:1) activation beads showed that IL2 supplement within the range of 0 to 500 IU/ml throughout 2 days of activation and 3 days of culturing did not affect the final yield regardless of the IL2 source as measured via trypan-blue based cell counting on Countess™ II Automated Cell Counter.
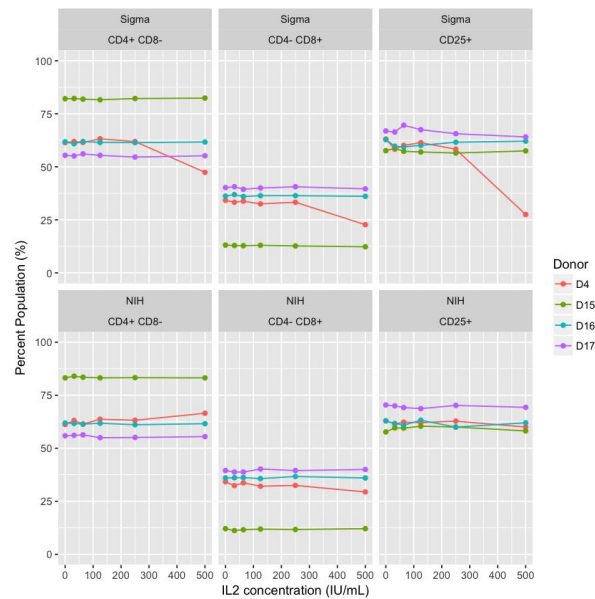
(e) Culturing activated cells in media with (200 IU/ml) or without IL2 for 5 days did not favor CD4 or CD8 cell proliferation regardless of the IL2 source used.

Data: IL2 titration results from multiple assays.ipynb

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Based on these data, when activated through CD3 and CD28 axes, in a pan T cell population, CD4 positive cells produce enough IL2 to sustain population-level proliferation for at least a week. This suggests that there is no need to supplement the media with additional IL2 throughout the the activation.

## NIH's reagent service provides access to highly standardized and cost-effective recombinant IL2

Given IL2 supplement is ubiquitously used in human primary T cell and tumor infiltrating lymphocytes culturing, it can easily become a major operating expense for an immunology research lab. Although recombinant IL2 in either solution or powder format is available through commercial options (e.g. Sigma #I2644-10UG), the costs are infeasibly high to support long-term and large-scale experimententation.

As an alternative, National Cancer Institute's Biological Resources Branch Program (NCI BRBP) offers free (with a signed Material Transfer Agreement) recombinant IL2, IL7, and IL15 for research purposes and the yearly allowance for each of these cytokines are enough to support immunology experiments of different scale up to multiple years. For example, researchers are allowed to request 10 vials of IL2 at a time with a limit of 99 vials per year, where each vial contains 1 million IU of IL2 and the researcher is only responsible for covering the shipping costs. Furthermore, recombinant IL2 from NCI BRBP is highly standardized and it, therefore, eases experimental reproducibility and consistency.

T cell isolation can be circumvented by culturing PBMCs with activation beads

Although isolation kits enable enriching a population for a particular type of T cell or pan T cells, if the goal is to expand T cells for longer than 2-3 days, directly culturing PBMC together with the activation beads allows by-passing the T cell isolation step. Assuming that approximately 30% of the PBMCs were T cells, we cultured PBMCs with (1:3 bead-to-PBMC ratio; de-beaded on the second day) or without activation beads and then profiled the final population after 5 days of culturing in the presence of 200 IU/ml IL2. As expected, there were fewer cells for PBMCs cultured without activation beads compared to PBMCs cultured with the beads -- that is, we saw ~7 fold increase in the presence of beads. This means that T cell proliferation without activation was trivial. Furthermore, the PBMCs cultured with activation beads were highly enriched for T cells (CD3 positive), meaning that the final T cell yield was much higher when the cells were activated. We saw that this culturing condition slightly favored CD4 cells compared to CD8 cells for a single donor.

Overall, if the goal is to activate and expand T cells for at least 5 days, the pan T cell enrichment step can be considered optional to reduce the costs of the ex vivo T cell expansion. However, we highly recommend estimating the number of T cells in a PBMC population via flow cytometry to better calculate the number of beads needed for the T cell activation to maximize the yield.

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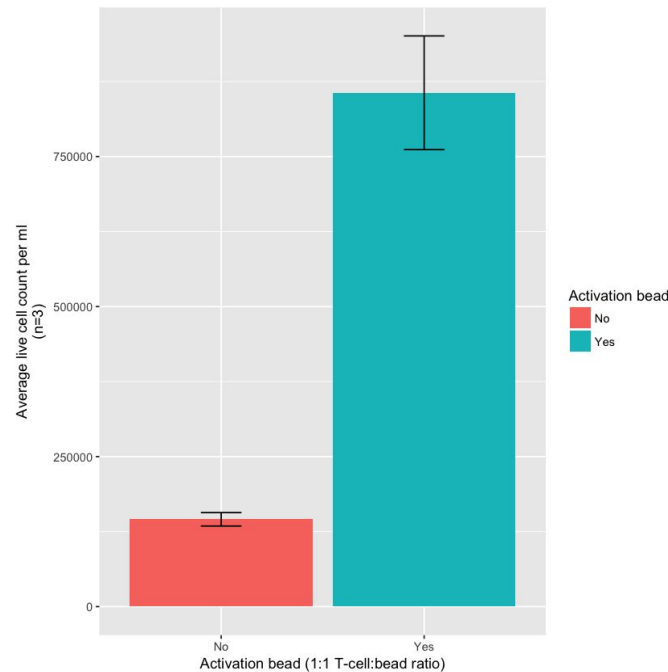
(a) In the absence of activation beads, when PBMC were cultured in IL2 (200 IU/ml) supplemented media for 5 days, cells did not proliferate; whereas, cells cultured with the activation beads increased in numbers.
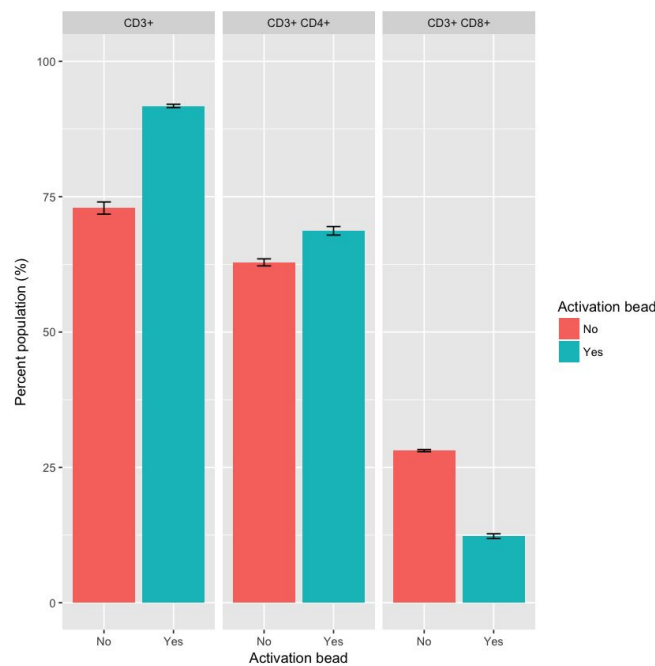
(b) Culturing PBMC cells with the T cell activation beads in the presence of IL2 (200 IU/ml) for five days were enough to enrich the population for T cells (CD3+) without compromising any of the CD4 or CD8 positive cell populations.

Data: PBMC direct activation flow and count.ipynb

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3D-printed magnetic racks are affordable and effective alternatives to commercially available ones

Magnetic racks are necessary components of bead-based cell isolation methodologies. We have found that 3D-printed racks (e.g. Thingiverse #319772 together with K&J Magnetics #BC42) are as efficient as the commercial options (e.g. DynaMag™-2 Magnet) but they cost significantly less (~$5 vs ~$600) to produce with a relatively cheap 3D printer (e.g. Monoprice Select Mini 3D Printer).

Supplementary materials

Data and analysis methods

Data presented in this guide and notebooks that provide analysis details are available at https://github.com/hammerlab/t-cell-guide (v1: zenodo.1286368). The most recent version of this guide is available at http://bit.ly/tcellguide as a live and collaborative document. We highly encourage feedback in the form of comments on the live document or pull requests to the code repository.

Protocols (10.17504/protocols.io.quvdww6)

Unless otherwise noted, we have followed official product manuals throughout this guide. All live cell counts were assessed based on the trypan-blue based cell counting approach using Countess™ II Automated Cell Counter.

Resazurin viability assay in 96-well format (10.17504/protocols.io.quwdwxe)

- Always make sure you have at least 4 replicates
- Always make sure to have blank media control (ideally on the same plate but having another plate with only media in it is OK, too)
- Plate readers are relatively less accurate for the most outer wells so do not use them specifically for a condition alone (it might bias your results). Having a replicate for each of your conditions in either of the most outer wells is OK.
- It is much easier to work in batches via multichannel pipettors and make use of the reagent cuvette
- The dye is sensitive to light/temperature. No need to go crazy about this but make sure you don't leave the plate out for more than 10 minutes before measuring
- Do not go over 200 ul in total when preparing your cultures
- Plan carefully and remember to add the dye a day (24 hours) before your endpoint assay

- The assay is not that accurate > 300,000 cells per well or < 1,000 cells per well so make sure you seed the cells in such a way that you will hit the reliable cell counts on the day of your measurement

Measuring cell viability via absorbance

- Add 10X dye for each well using a multichannel pipettor (so, for 200 ul you will be adding 20 ul of the 10X solution)
- Continue culturing the cells the same way for 24 hours
- Use a plate reader to measure the absorbance at both 570 nm and 595 nm
    - Subtract your 595 nm measurement from the 570 nm for each well
    - Then (optionally) subtract your blank media measurement from your treatment measurements
    - The number you end up is directly proportional to the number of cells in the well.

PBMC isolation from buffy coat (10.17504/protocols.io.qu2dwye)

Before starting, take the isolation buffer out and keep it at room temperature (RT):

- Fill 4x 50-ml conical tubes with 15 ml isolation buffer.
- Cut the buffy coat open and empty it into another 50 ml tube.
- Split the 50 ml blood between the 4X 50-ml tubes containing isolation buffer.
- All tubes should have ~30 ml blood/buffer mix at this point.
- Using a 10 ml pipet, gently underlay 14 ml of Corning™ cellgro™ Lymphocyte Separation Medium below the blood.
- Centrifuge at 800 X g for 25 min at RT with soft deceleration.
- In the meantime, get 4 new 50 ml tubes.
- After the centrifuge, use a 10 ml pipet to transfer the cloudy buffy layer to a fresh tube.
- Wait for 1-2 mins for more of the buffy layer to reform and transfer that to the fresh tube as well.
- Add cold isolation buffer to each tube so that the final volume is now 40 ml.
- Mix by gently inverting tubes for a few times and centrifuge at 500 X g for 10 min at 4°C.
- Discard the supernatant (leave ~5ml liquid) into a bleach filled container.
- Combine the pellet from all tubes into one tube and increase the volume to 40 ml by adding isolation buffer.
- Count the cells (1:10 dilution)
    - Take 10 ul from the sample and mix with 90 ul of PBS (1:10)
    - Mix with 10 ul of Trypan Blue with 10 ul of the sample and load 10 ul to the cell counter chamber for counting (Thermo Fisher, Countess II).
- Continue with the desired T cell isolation kit or freeze PBMC cells.

Freezing and thawing T cells

For cryopreservation, we use Recovery™ Cell Culture Freezing Medium and follow manufacturer's guides. For thawing:

- Prepare same number of 15 ml conical tubes for the number of vials you will be thawing:
  - Add 5 ml of T cell media
- Take the vials (10^7 cells/ml) from the -80°C freezer and quickly thaw the cells (< 1 minute) by gently swirling the vial in the 37°C water bath until there is just a small bit of ice left in the vial. (do not submerge the vials, leave the cap part out of the water).
- Transfer the cells (dropwise) into the 15 ml tube that has the T cell media.
- Spin at 200 x g for 6 mins at RT.
- Aspirate the supernatant.
- Gently add 5 ml (if there are 10 million cells in the vial) of fresh T cell media to the pellet.
- Transfer the cells to a T25 flask or 6-well dish.
- Keep at 37°C incubator and activate the cells the next day.

CRISPR/Cas9-based knock-out (24-well setup - 10.17504/protocols.io.quxdwxn)

This protocol has been optimized for Thermo Fisher's Neon Electroporation System and is based on the report Reprogramming human T cell function and specificity with non-viral genome targeting. We have used TrueCut™ Cas9 Protein v2 and reached very comparable KO efficiencies to those previously shown by others.

Cas9 RNP preparation

- RNase Zap everything before you start
- Resuspend the crRNA (2 nmol) in 100 ul (making 20 uM) and tracRNA (20 nmol) in 1 ml RNA storage buffer (making 20 uM). Aliquot and keep at -80°C.
- Need 0.375ul crRNA and 0.375 ul tracRNA per well (this way, we'll use 7.5pmol sgRNA per 200K cells as Neon protocol suggests).
- Mix 1.5 ul crRNA and 1.5 ul tracRNA (for 4 rxns) in a PCR tube. Keep at 95°C for 5 minutes and then at 37°C for 25 mins.
- For Cas9, Neon suggests 1250 ng Cas9 protein per 200K cells. Our Cas9 is at 5 mg/ml concentration. So, we will need 0.25 ul Cas9/200K cells.
- After incubation of the sgRNA, slowly add 1ul Cas9 (for 4 reactions) in the PCR tube, mix and incubate at 37°C for 15 mins.

Cell preparation and electroporation

- Need 200K cells for one electroporation event. The cells should be in 9 ul T buffer (9 ul T buffer reaction), so that the total volume (9 ul cells + 1 ul RNP mix) will be 10ul for the Neon 10 tip.
- Debead and count the activated cells.
- We need 200,000*3*24 (14.4 million cells).
- To be safe, assume 4 reactions well so we will need 200,000*4*24 (19 million) cells.

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- 19 million cells are actually good for 96 wells.
- So, we need 96*9 = 864 ul T buffer
- After debeading and counting the cells, spin them down at 200 x g for 7 mins.
- Aspirate the media as much as possible.
- Resuspend the pellet in 864 ul T buffer.
- Add 36 ul of cell mix to each PCR tube and mix well.
- The Cas9 RNP and cells are ready for electroporation
- Electroporation at 1600 V 10 ms 3 pulses
- Seed the electroporated cells on the prepared 24-well-plate with warm T cell media.

Profiling

The time to profile CRISPR/Cas9 treated cell depends on the particular assay of interest but in general, cells can be profiled via flow cytometer for surface proteins and via Western Blot for internal proteins 3 days after electroporation.

Sample preparation for flow cytometry

Aim for 200,000 cells per tube at minimum and 10 million cells per ml concentration at maximum. The following protocol can be multiplexed by using a 96-well plate, plate adaptor (swinging bucket) and multi-channel pipette:

- Collect cells and spin them down at 200 (unstimulated) or 350 (activated) g for 5 minutes
- Discard the supernatant (in case of left-over media, wash them once again)
- Resuspend cells in flow buffer (PBS with %20 FBS) at desired concentration. A good rule-of-thumb is to resuspend the cells in the same amount buffer to their original volume.
- Add labeled antibody at recommended concentration (e.g. 1:500). Cover the tubes with aluminum foil and incubate at RT for 15 minutes or at +4°C for 30 minutes.
- Once the incubation is over, spin the cells down at 200 (unstimulated) or 350 (activated) g for 5 minutes
- Discard the supernatant and resuspend the cells in at least 400 ul of PBS
  - If not using flow tube inserts 400 ul is minimum because the flow's sample collection tube goes down to near 200 ul level. You always want to make sure you have more sample volume than that otherwise you will start collecting random noise in the flow. Note: this may vary depending on which flow cytometer instrument you are using.
  - If there are lots of samples, it is fine to dispense PBS on them and resuspend later right before the flow using the vortex next to the machine
- Transfer the sample into a flow tube and run flow

Reagents

Flow antibodies

Name	Vendor	Catalog #	Laser (nm)	Color	Dilution
CD4	Biolegend	317418	635	APC/Cy7	1:500
CD8	Biolegend	301032	488	PerCP/Cy5.5	1:500
CD45RO	Biolegend	304210	635	APC	1:500
CD3	Biolegend	300306	488	FITC	1:500
CD25	Biolegend	302627	405	Pacific Blue	1:500
CD69	BD Sciences	555531	488	PE	1:500

Oligonucleotides

- CD4 crRNA: 5'-GGCAAGGCCACAATGAACCG-3' (source)

Buffers and Solutions

Cell culture media (10.17504/protocols.io.qu5dwy6)

Mix all in a 500 ml 0.22 um filter bottle in the hood:

- 500 ml RPMI-1640 (Corning™ cellgro™ RPMI 1640 Medium (Mod.) 1X with L-Glutamine)
- 25 ul of EMD Millipore™ Calbiochem™ β-Mercaptoethanol, Molecular Biology Grade (50 uM)
- 12.5 ml of HyClone™ HEPES Solution (25 mM)
- 50 ml of FBS (Fetal Plus®)
- 5 ml of Penicillin-Streptomycin (1%)
- 5 ml of HyClone™ 100mM Sodium Pyruvate Solution (1X)
- 5 ml of HyClone™ Non Essential Amino Acids NEAA 100x solution (1X)

Sterile filter (0.22um) and aliquot in 50 ml conical tubes. Keep the media in the fridge, protect from light.

Isolation buffer

- 500 ml PBS w/o Mg and Ca (Fisher Bioreagents, BP2944-100)
- 0.5 g BSA (Fisher Scientific, BP9703-100)
- 2 ml 0.5 M EDTA (Fisher Scientific, BP2482500)

Mix them all and sterile filter the buffer (EMD Millipore™ Stericup™ Sterile Vacuum Filter Units, 0.22 um). Keep the buffer in the fridge.

Resazurin solution

Pre-made Alamar Blue (Resazurin) solutions from companies (e.g. alamarBlue™ Cell Viability Reagent) are relatively expensive for long-term and common use. Therefore, ordering it in powder format is the cheapest and the most feasible way to go for extended use cases. 1 g powder (e.g. Resazurin sodium salt #R7017) should last for a year or two even if it is frequently used.

The final working concentration for Resazurin is 44 uM and our goal is to prepare a 10X solution for use in cell culture (e.g. for 200 ul of media/cells, we will add 20 ul of the dye). Directly preparing this is not feasible (due to the amount of PBS required) so we will first prepare a 1000X stock solution which we will further dilute as needed.

For 1000X Stock (44 mM):
- Weigh 5 gram of Resazurin powder
- Resolve the powder in 500 ml PBS (this will require vigorous shaking/mixing for a few times so make sure you don't have any residues in the solution)
- Sterile filter the solution and prepare 10 aliquots (each 50 ml)
- Store the 1000X stocks at -20°C (either wrap each one with aluminum foil or use a cardboard box to limit light exposure for long term)

For 10X Stock (440 uM):
- Add 500 ul of 1000X stock onto 50 ml of PBS in a conical tube (usually makes sense to prepare 4-5 of these)
- Wrap the tube with aluminum foil so that it is minimally exposed to sunlight
- Store 10X working solutions at 4°C (they should be good for up to 6 months)
- Can also store one 1000X stock at 4°C for convenience (if planning to assay frequently)

Acknowledgements