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High throughput, automated and fully contained cytokine release assay using primary human CD4$^+$ T cells

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1. Introduction

CD4$^+$ T cells play a pivotal role in immune responses as the pro-inflammatory cytokines they release drive inflammation and facilitate the recruitment of other immune cells [1]. Altered T cell signaling is characteristic of autoimmune disease and drives a harmful inflammatory immune response. Restricting specific activities such as cytokine secretion in a T cell population is therefore a reasonable strategy to treat autoimmune disease [2].

Current methods for *in vitro* T cell assays include the use of immortalized cell lines, rodent-derived T cells or human T cells under low throughput conditions. Utilization of immortalized cell lines provides advantages such as access to long-term cultures, generation of large amounts of cells, as well as the absence of donor-to-donor variability. However, immortalized cells have multiple drawbacks such as chromosomal abnormalities and phenotypic changes [3–5]. In comparison to immortalized cell lines, *ex vivo* rodent and human cells provide a better understanding of how a test compound might behave in a living organism. Rodent cells have the advantage of being aligned with *in vivo* disease models. However, they have distinct characteristics that might not fully capture the complex interactions present in humans, potentially leading to inaccurate results, and compounds optimized against human proteins might not display similar activity against their rodent orthologs [6–8]. Human primary cells logically offer the most physiologically relevant representation of human biology, resulting in improved translation to the patient [9]. Further, human primary cell-based assays using different donors can assess individual genetic differences and their potential impacts [10]. However, throughput is a major requirement for high-through screening and structure-activity relationship (SAR) campaigns, and this often makes using primary human cells challenging or even unattainable [11].

Although other methods reported in the literature describe the use of T cell assays, to our knowledge none describe a high throughput assay using primary human CD4$^+$ T cells [2,12,13]. To source sufficient cell numbers, we designed an expansion of isolated T cells to yield an approximately 6- to 8-fold expansion in 7 days (Fig. 5). According to the protocol described in this paper, expanding a single vial of human CD4+ T cells containing 1.5E7 CD4$^+$ T cells per vial over seven days yields an adequate quantity of cells to supply approximately 3,000 wells. One vial of CD4$^+$ T cells therefore enables the screening of 3000 single-dose compounds or 125 11-point dose-response curves in duplicate per expansion. By conducting multiple cell expansions each week, it is possible to process up to 15,000 single-dose compounds or 625 11-point dose-response curves per week, classifying this assay as medium- to high-throughput.

While the use of primary human CD4$^+$ T cells is the most physiologically relevant option, a potential limitation of this assay is the choice of stimulus. Some literature reports suggest that bound anti-CD3 and anti-CD28 antibodies provide a more physiologically relevant stimulation due to the cross-linking of receptors on the T cell membrane mimicking the natural interaction between a T cell receptor and an antigen-presenting cell [14]. The two available options for bound antibodies are either antibody-coated beads or antibodies bound to a plate surface. The use of bound beads can add significant cost to a high throughput format. On the other hand, antibody coating of plate surfaces is usually a manual process conducted in 96 well format, leading to a lower throughput assay. The best *in vitro* stimulus would arguably be antigen presenting cells. This form of the assay is called the Mixed Lymphocyte Reaction (MLR) assay which uses CD4$^+$ T cells from one donor and antigen presenting cells from a different donor as the stimulant [15]. Due to its complexity, this assay is usually executed in 96-wells or lower format making it unsuitable for our purposes. Although there is a manuscript describing an MLR assay in 384 well format, no mention was made of the number of compounds screened or the maximum throughput [16]. We selected ImmunoCult^{TM} stimulation, utilizing anti-CD3 and anti-CD28 complexes in solution, to achieve a

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consistent assay format. This approach provides robust T cell activation, facilitating the generation of hundreds of IC_{50} values or tens of thousands of single-dose points weekly to support structure-activity relationship (SAR) and screening initiatives.

Herein, we share the development and data obtained using an automated, high throughput human $CD4^+$ T cell cytokine release assay that takes place in full biological containment (Fig. 4). Briefly, isolated human $CD4^+$ T cells are purchased from Stem Cell Technologies, activated with anti-CD3/CD28 Dynabeads™ for 3 days, and expanded for an additional 4-5 days. The expanded $CD4^+$ T-cells are then added to a 384-well plate containing either compound or control (e.g. DMSO). After a 15-minute compound preincubation, ImmunoCult™ is added for 20 to 24 hours. A sample of the supernatant is removed and combined with IL-2 HTRF antibodies, incubated and read with an EnVision™ plate reader at 665 nm and 615 nm wavelengths. Using the fluorescence intensity ratio data, the percent inhibition of each compound dose is evaluated. An unconstrained sigmoid curve is fitted using a 4-parameter logistic equation and an IC_{50} reported as the midpoint of the curve. Overall, >5,600 IC_{50} and >11,000 single dose datapoints were obtained using this assay.

The use and execution of this protocol is presented in the paper **"Discovery of a Small Molecule ITK and Pan-TRK Kinase Inhibitor for the Potential Topical Treatment of Atopic Dermatitis"** Duffen et al. [18]

2. Materials

2.1. Reagents

Human peripheral blood $CD4^+$ T cells are purchased from Stem Cell Technologies (1.5E7 cells).

T cell expansion reagents

- DMEM, high glucose, pyruvate (Life Technologies/Thermo Fisher, 11995-065).
- RPMI 1640 Medium, with glutamine, with Phenol Red (Life Technologies/Thermo Fisher, 11875093).
- Heat Inactivated FBS (Life Technologies/Thermo Fisher, A5670801).
- Non-Essential Amino Acids (NEAA) Solution, 100x (Life Technologies/Thermo Fisher, 11140050).
- L-Glutamine (200 mM) (Life Technologies/Thermo Fisher, 25030081).
- Penicillin-Streptomycin, 100X (Life Technologies/Thermo Fisher, 15140122).
- Dynabeads Human T-Activator CD3/CD28 (Life Technologies/Thermo Fisher, 11132D).
- 50 ml conical tubes (Falcon, 352070).
- Costar®, 24-well clear, tissue-culture plates (Corning, 3524).
- G-Rex®10 Open System, Sterile Fluid Path Flasks (Wilson Wolf, 80040S).
- 2 ml tubes (Eppendorf, 30123620).
- Falcon® T175 cell culture flasks (Corning, 353112).

[T cell expansion media]

Reagent	Final concentration	Amount
DMEM	50%	215 mL
RPMI with glutamine	50%	215 mL
HEPES (1 M)	10 mM	5 mL
Non Essential Amino Acids (100X)	1X	5 mL
Penicillin Streptomycin (100X)	1X	5 mL
L-Glutamine (200 mM)	2 mM	5 mL
Heat Inactivated Fetal Bovine Serum	10%	50 ml
Total	**n/a**	**500 mL**

[Filter sterilize and store at 4°C; Stable at 4°C for 1 month]

T cell assay reagents

- RPMI 1640 Medium, without glutamine, without phenol red (Life Technologies/Thermo Fisher, 32404-014).
- HEPES Buffer GMP 1M in normal Saline (Lonza, BEBP17-737E).
- Heat Inactivated FBS (Life Technologies/Thermo Fisher, A5670801).
- NEAA, 100X (Life Technologies/Thermo Fisher, 11140050).
- L-Glutamine (200 mM) (Life Technologies/Thermo Fisher, 25030081).
- Penicillin-Streptomycin (100X) (Life Technologies/Thermo Fisher, 15140122).
- ImmunoCult™ Human CD3/CD28 T Cell Activator (Stem Cell Technologies, 10991).
- Dimethyl sulfoxide DriSolv® (EMD, MX1457-7).
- Human IL-2 HTRF assay kit 10,000 tests (Revity, 62HIL02PEH).
- 384-well, polypropylene, v-bottom microplate with lid (Greiner, 781280).
- 384-well, white, tissue-culture, sterile, flat-bottom microplate with lid (Greiner, 781080).
- 384-well, white, tissue-culture, sterile, flat-bottom, HiBase microplate with lid (Greiner, 784080).

[T cell assay media]

Reagent	Final concentration	Amount
RPMI (without glutamine, without phenol red)	95%	473.75 mL
HEPES (1 M)	10 mM	5 mL
Non Essential Amino Acids (100X)	1X	5 mL
Sodium Pyruvate (100 mM)	1 mM	5 ml
Penicillin Streptomycin (100X)	1X	5 mL
L-Glutamine (200 mM)	0.5 mM	1.25 mL
Heat Inactivated Fetal Bovine Serum	1%	5 ml
Total	**n/a**	**500 mL**

[Filter sterilize and store at 4°C; Stable at 4°C for 1 month]

3. Equipment

- DynaMag™-15 Magnet stand (Thermo Scientific, 12301D).
- ViCell XR cell viability analyzer (Beckman Coulter, 731050).
- BioMek i7 automated workstation (Beckman Coulter, BEC-B87586).
- BioMek i-Series 50 μL pipette tips (Beckman Coulter, B85753).
- BioMek i-Series 30 μL pipette tips (Beckman Coulter, B85739).
- MultiDrop Combi (Thermo Scientific, 5840300).
- Small tube plastic tips dispensing cassette (Thermo Scientific, 24073290).
- Standard tube dispensing cassette (Thermo Scientific, 24072670).
- Envision plate reader (Perkin Elmer, 2103 or 2104).
- LANCE dual enhanced mirror (Perkin Elmer, 662).
- 320 nm excitation filter (Perkin Elmer, 111).
- 665 emission Filter (Perkin Elmer, 205).
- 615 emission Filter (Perkin Elmer, 203).
- Bench top containment hoods (Flow Sciences, Inc.).
- Bench top centrifuge with sealed buckets (Thermo Scientific, 75004521).
- ClickSeal biocontainment lids (Thermo Scientific, 75003656).
- Labcyte Echo 555 Liquid Handler (Beckman Coulter).
- Water bath set at 37°C.
- Class II biological safety cabinet.
- Tissue culture incubator set at 37°C and 5% CO2.
- Vortexer.
- Microscope.
- Room-temperature inert chamber for storing DMSO, compounds in vials, compound source plates.
- Standard laboratory pipettes, multichannel pipette.

3.1. Software

ActivityBase-V97-PROD (IDBS).

3.2. Procedure

Expansion of Human CD4$^+$ T cells (Timing: 7 days) – Fig. 1

1. Thaw 1 cryovial of human CD4$^+$ T cells
   a. Warm vial in 37°C water bath for less than one minute. Wipe down the outside of the vial with 70% ethanol. Slowly add 1 mL of warm T cell expansion media with a P1000 pipette to the frozen pellet. Add the thawed cells to warm T cell expansion media in a 50 mL conical tube with a 10 mL total volume of media.

Critical: Thawing of the CD4$^+$ T cells in a 37°C water bath should take place in under one minute leaving ice still in the vial which will dissolve upon addition of T cell expansion media.

1. Centrifuge cells at 300 x g for 5 minutes at room temperature in sealed buckets. Carefully remove supernatant and resuspend cell pellet in T cell expansion media and count 1 mL using the ViCell. Adjust the volume to one million cells per mL.
2. 1 mL of cells are plated per 24 well (one million cells/mL). Anti-CD3/CD28 beads are added to the wells containing cells at a 1:1 bead to cell ratio.
   a. Anti-CD3/CD28 bead prep: vortex stock vial of beads vigorously for 2 to 3 minutes. The amount of beads needed equals the total number of cells divided by the stock concentration of beads per mL (4×10^7 beads per mL). Remove the amount of beads needed from the stock vial, place in a sterile 2 mL tube and bring the volume up to 2 mL with T cell expansion media. Vortex, open cap and place on magnet for about one minute. Using a P1000 pipette, remove the media carefully without touching the sides of the tube. Replace with fresh media equaling the amount originally removed from the source vial of beads. Pipette up and down to resuspend beads. To calculate the amount of beads needed per well divide the volume of beads by the total volume of cells.
3. Add beads to each well and incubate at 37°C and 5% CO_2 for 3 days.
4. After 3 days microscopically inspect cells to confirm the presence of cells clumping around the beads.
5. Critical: After 3 days T cell clustering around the Dynabeads should be microscopically visible to indicate CD4$^+$ T cell activation (Fig. 2A).
6. Use a P1000 pipette up and down approximately 3 times to resuspend the beads.
7. Pool media containing cells and beads (from 24 wells) into 15 mL conical tubes. Evenly distribute total volume over 2-3 tubes.
8. Uncap tubes and put on the magnet for about 2 minutes.
9. Carefully remove media with 5 mL pipette staying away from the sides of the tube. Place media containing cells in a 50 mL conical tube and remove 1 mL for counting on ViCell.
10. Cells are diluted to 5×10^5 cells/mL in T cell expansion media and placed in G-Rex®10 flask. Bring total volume in G-Rex®10 flask to 40 mL with expansion media.
11. Incubate cells an additional 4 days at 37°C and 5% CO_2.

After a total of 7 days in culture the expanded CD4$^+$ T cells are ready for use in the T cell IL-2 assay.

Note: Cells that have been in culture for 8 days can also be used; however, a T cell assay using day 7 and day 8 cells in parallel should be carried out to confirm that there are no major differences in pharmacology based on the target of interest. Nine days and longer cell expansion leads to a decrease in cell viability.

Critical: After 7 days of expansion, a 6 to 8-fold expansion in the number of CD4$^+$ T cells should be noted (Fig. 2B).

Test Compound Preparation (This is completed by an internal compound management group. If completed by hand, timing will depend on number of compounds.)

1. Compounds are solubilized in 100% dimethyl sulfoxide (DMSO) to 8 mM.
2. An 800X compound plate (Greiner 781280) is created with an 11-point 1:4 dilution series in 100% DMSO. Eighty nL of compound is spotted per well using the Echo liquid dispenser yielding a 0.125% DMSO final assay concentration.
3. Just prior to the assay, 80 nL of positive and negative controls are added to the compound plate to define the upper and lower limits for the assay signal. These control wells contain either a standard ITK inhibitor (JTE-051) of IL-2 secretion for the Hundred Percent Effect (HPE) positive control to define the lower signal limit or 100% DMSO (final assay concentration = 0.125%) for the Zero Percent Effect (ZPE) negative control to define the upper signal limit. HPE is added to the wells in column 1 and ZPE is added to the wells in column 24.

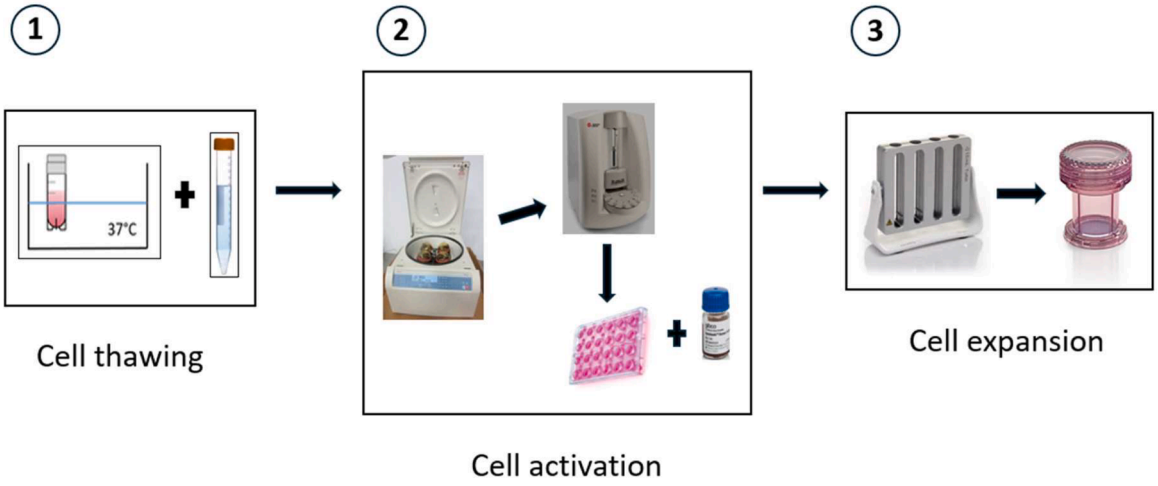

Fig. 1. CD4$^+$ T cell expansion. CD4$^+$ T cells are thawed, counted, plated and activated with anti-CD3 and anti-CD28 Dynabeads. After a 3-day incubation, Dynabeads are removed. Cell count, and viability are assessed, and the cells are placed in a G-Rex flask and allowed to expand for 4 additional days.

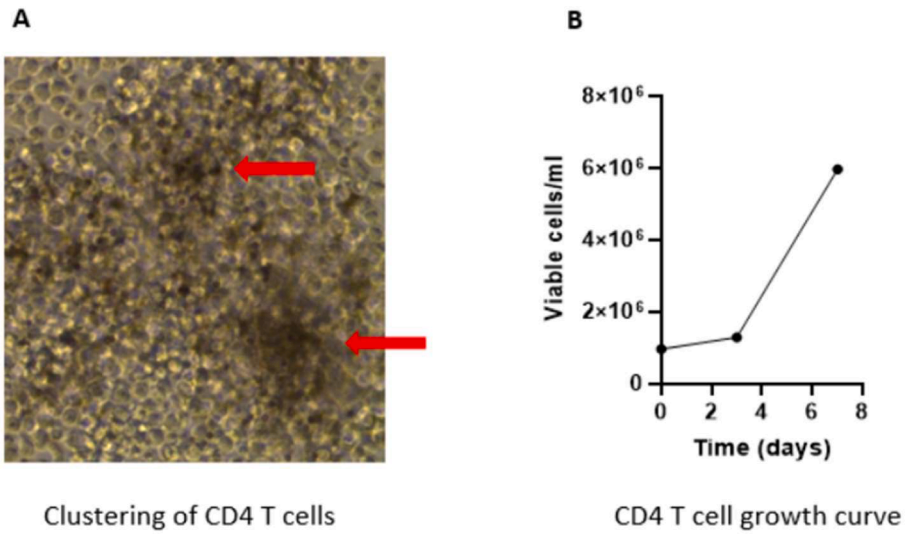

Fig. 2. Activation and expansion of $CD4^+$ T cells. A) Brightfield image of $CD4^+$ T cells clustering (red arrows) around anti-CD3 and anti- CD28 coated beads 3 days post addition. B) $CD4^+$ T cells are counted and assessed for viability at day 0, 3 and 7. At day 7 a 6 to 8-fold expansion of CD4 T cells is expected.

Critical: The final assay concentration of DMSO should be equal or less than 0.125%. Higher assay concentrations of DMSO can cause cell stress and death resulting in poor data quality.

Human CD4+ T cell IL-2 Assay (Timing: 26 hours) – Fig. 3

1. The expanded $CD4^+$ T-cells are centrifuged at 300 x g for 10 minutes at room temperature in sealed buckets and in a tissue culture hood resuspend cells in T cell assay media to 0.5 million cells per mL.
2. In a biocontainment hood using the Combi with a standard cassette, add 60 µL cells per well to a 384 well plate containing 80 nL test compounds (final concentration: 30,000 cells/well).

Critical: Centrifuge the compound plates before the addition of cells to ensure that the compounds are in the bottom of each well. When dispensing cells the use of a standard cassette with the Combi dispenser is important to avoid cell stress and potential cell damage.

1. Incubate the cells with compound at 37°C and 5% CO_2 for 15 minutes.
2. In a biocontainment hood using a Combi and a small cassette, add 20 µL of diluted ImmunoCult per well.
   a. Make fresh before use: ImmunoCult is diluted 1:12.5 in T cell assay media (final assay concentration 1:50)
3. The plates are incubated an additional 20 to 24 hr at 37°C/5% CO_2.

Critical: ImmunoCult should be diluted immediately before use. Cell viability in all steps of the procedure should exceed 80%.

IL-2 HTRF readout (Timing: 4 hours)

1. After 20 to 24 hr the plates are centrifuged at 300 x g for 10 minutes at room temperature in sealed buckets.
   Critical: Cell plates should be centrifuged immediately before transfer step to ensure cells remain at the bottom of the well.
2. Equilibrate assay media and HTRF buffer to room temperature.
3. Place antibodies and standard on ice.
4. Prepare the two HTRF antibody solutions fresh in separate vials.
   a. Dilute anti-IL-2-Cryptate-antibody 1:20 in detection buffer 3.
   b. Dilute anti-IL-2 d2-antibody 1:20 in detection buffer 3.
   c. Immediately before use, combine the two ready-to-use antibody solutions.
5. Using the Combi with a small cassette add 4 µL pre-mixed anti-IL-2 antibodies/well to a 384-well white HiBase plate.

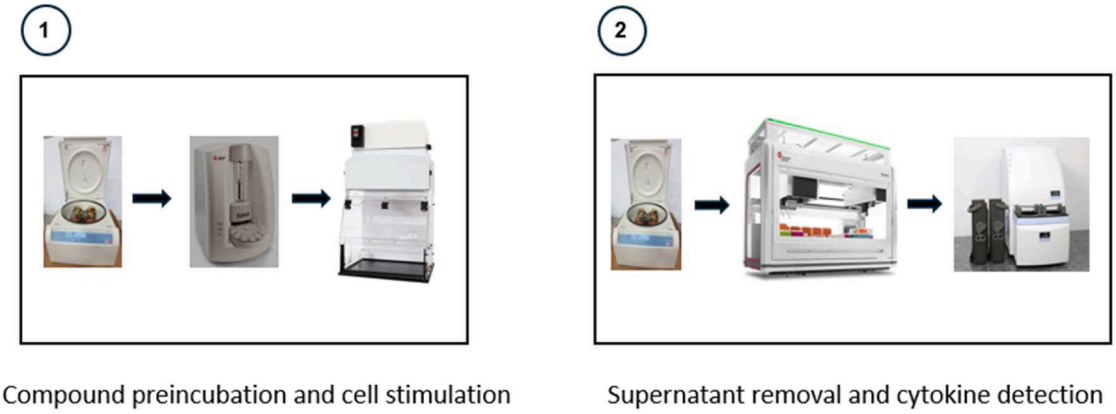

Fig. 3. $CD4^+$ T cell cytokine secretion assay. After 7 days of expansion the cells are again counted, assessed for viability and plated in a 384 well plate containing compounds. After 15-minute preincubation the cells are re-activated with Immunocult and incubated for 24 hours. A sample of the supernatant is removed and added to IL-2 HTRF detection and read on an Envision plate reader.

6. Immediately before use solubilize the standard in T cell assay media and dilute it 1:2 to make the top concentration, dilute 1:1.5, then serially dilute 1:1.8 in T cell assay media.
7. Using the Biomek iSeries 16 μL of supernatant is removed from the cell plates and combined with 4 μL of IL-2 HTRF antibodies in a 384-well white HiBase plate.

   Critical: When transferring supernatant care should be taken to avoid the cells that are at the bottom of the well.
8. Plates are incubated for 3 hr at room temperature and read with an EnVision plate reader at 665 nm and 615 nm wavelengths.

Note: Signal is stable for 24 hr and can be read after an overnight incubation.

Critical: A cytokine standard curve is necessary in every assay to ensure that all values are within the linear range of the assay.

A stepwise time course of $CD4^+$ T cell expansion (Fig. 1) and fully automated cytokine secretion assay (Fig. 3) to enable a robust and reproducible high throughput assay using primary human cells are shown (Fig. 4).

4. Troubleshooting and notes

1. To safely employ this protocol and limit potential exposure to human biofluids and aerosols, equipment that can provide full containment at each step is necessary (i.e. Biomek i-Series automated liquid handler, bench top flow hoods and the use of capped buckets in the centrifugation steps). To monitor assay performance, Z prime should be $\geq$ 0.2 and one or more standard compounds should be run in every assay (e.g. JTE-051) [17]. IC_{50} values for the reference compounds should be within 3-fold of the historical value (Fig. 4).
2. All cell culture should take place in a class 2 biological containment (BSL2) hood to prevent contamination of cultures.
3. Cell viability under 80% at any point in this protocol indicates an issue with cell health and the assay should be abandoned.
4. 3 days post addition of Dynabeads clustering of $CD4^+$ T cells should be noted. If there is no cell clustering proper activation did not occur (Fig. 2A).
5. Cell viability and counts should be assessed throughout the expansion protocol. If consistent expansion is not noted between 3 and 7 days (Fig. 2B) new cells should be thawed.
6. While our protocol relied solely on $CD4^+$ T cells obtained from StemCell Technologies, $CD4^+$ T cells are also available from other suppliers, including Charles River, Lonza, and IQ Biosciences. Each cell source should be validated within the assay by generating a standard curve and incorporating appropriate positive controls or standard compounds relevant to your assay.
7. Following the compound preincubation and overnight stimulation, cell plates should be centrifuged immediately before supernatant is withdrawn. Cells inadvertently transferred with supernatant will increase noise in the data resulting in lower Z primes and assay reproducibility.
8. The IL-2 HTRF assay is developed to operate in the linear portion of the IL-2 standard curve. A standard curve is run in each assay to ensure that the data is in the expected range. If it is determined that the values are outside of the linear range and dilution is not possible, a non-linear regression analysis may be needed to estimate the concentration from your data. Using data which are above the linear range will likely lead to weaker IC_{50} values than expected.
9. The data presented in this paper were obtained using the IL2 HTRF kit from Cisbio/Revity. In other studies, not documented here, Bioauxilium's Thunder Human IL2 TRFRET biomarker assay kit was used as an alternative to the IL2 HTRF kit, yielding comparable results.
10. The ITK clinical candidate is highly reproducible (N of 14) in the $CD4^+$ T cell IL-2 assay. All 14 potency values fall within the upper and lower warning limits (UWL and LWL). The UWL and LWL specify the limits of expected deviation from the mean (mean $\pm$ 2 standard deviations) and can provide warnings of possible process variability.
11. In high-throughput screening, Z-prime evaluates assay quality and reliability. Values >0.5 indicate excellent assays with strong signal separation, high reproducibility, and minimal variability—standards typically applied to biochemical assays under tightly controlled conditions. However, phenotypic screens have greater biological variability, lower signal-to-noise ratios, and more complex readouts. Therefore, acceptable Z-prime thresholds are adjusted accordingly. A Z-prime >0.2 in phenotypic assays indicates sufficient window to distinguish hits from background noise, making it suitable for high-throughput screening. Additionally, this assay was primarily used for SAR support in dose response format. With 22 data points forming a curve (11 doses in duplicate), a Z' > 0.2 is sufficient to produce a reproducible IC_{50} value (Fig. 5).
12. The $CD4^+$ T cell assay is a phenotypic assay. As such there is a possibility for non-specific inhibition of other targets downstream of the T cell receptor in activated T-cells. An orthogonal assay should always be run to confirm specificity for your desired target. For example, in Ref [18]. The inhibitory efficacy of the compounds against the full-length human ITK enzyme was evaluated utilizing a biochemical, cell-free Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) kinase assay (Fig. 6).
13. Automation in biocontainment should be employed at each step. Automation increases throughput as well as data quality and reproducibility (Fig. 3).

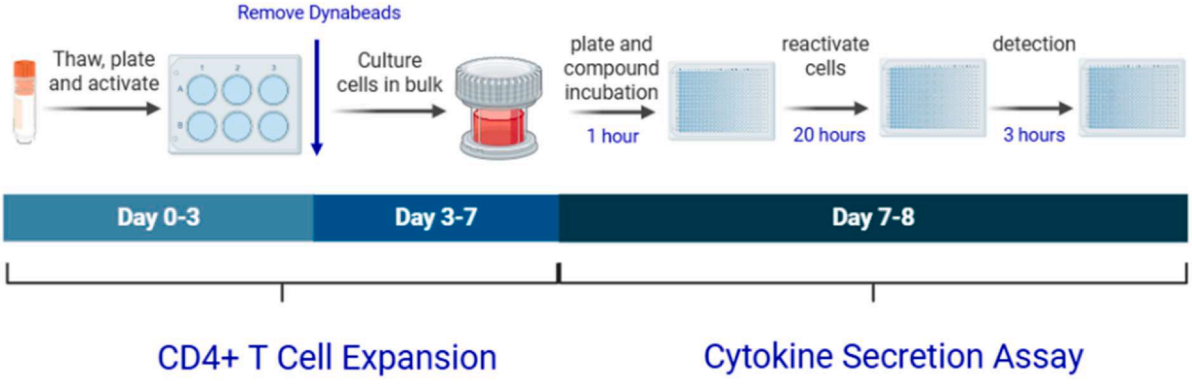

Fig. 4. Flow Diagram.

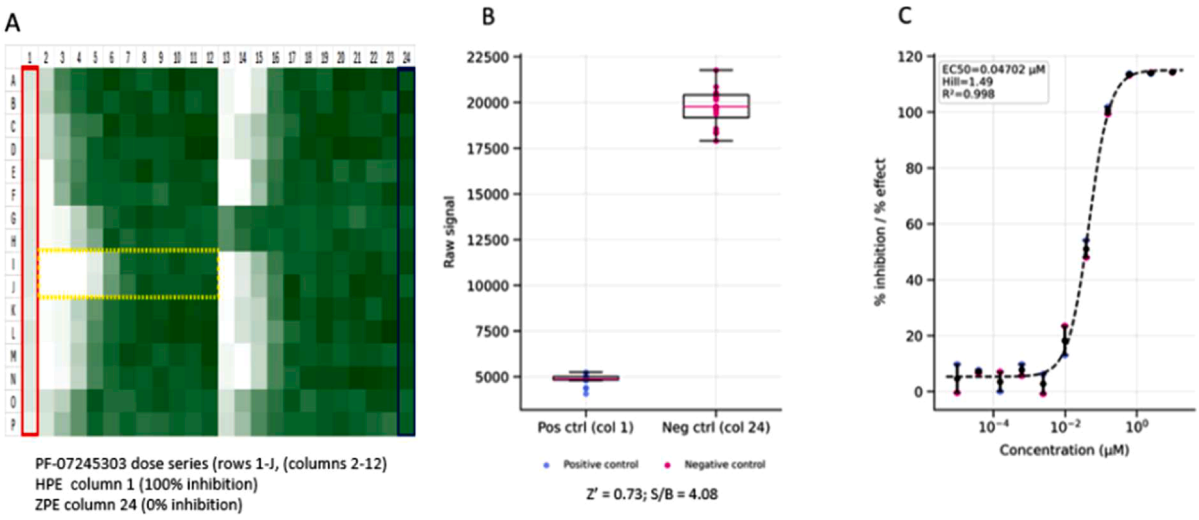

Fig. 5. Representative CD4$^+$ T cell IL-2 HTRF assay results: A) Plate heat map displaying percentage inhibition of 11-point dose response with 16 compounds in duplicate and 32 control wells per plate. Column 1 contained positive control or HPE (e.g. ITK inhibitor) and column 24 contained a negative control or ZPE (e.g. DMSO). The positive control is set at 100% and the negative control is set at 0% inhibition. B) Quality control analysis including Z Prime and assay window. C) The ITK clinical candidate PF-07245303 demonstrates a potency value of 0.047 µM.

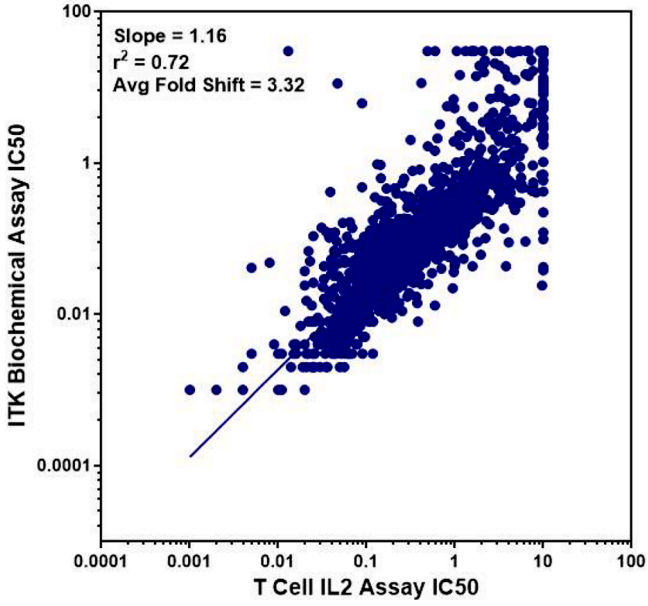

Fig. 6. Correlation of ITK biochemical and CD4$^+$ T cell potencies: Correlation plot of Pfizer proprietary compounds comparing potencies of the ITK biochemical assay versus the CD4$^+$ T cell assay used to identify ITK inhibitors. A strong correlation with a slope of 1.16 is observed between the biochemical assay utilizing purified enzyme and the CD4$^+$ T cell phenotypic screen indicates that the potency values obtained from both assays are generally comparable.

14. The original assay design is for the measurement of a single cytokine; however, multiple cytokines can be measured per well. Under these conditions, a satisfactory assay window was also observed for IFNγ (data not shown).
15. The protocol as described uses 30,000 cells per well of 384 well plate. However, a cell titration is suggested to determine the adequate number of cells per well to give the desired outcome i.e., cytokines with lower abundance may require more cells to generate an acceptable signal to background.
16. Poorly soluble compounds may appear to be inactive or less active. Assess compound solubility and stability before testing in the T cell IL-2 assay.
17. There is a potential for the identification of false negative results from compounds that fluoresce at the wavelengths used. However, this is minimized with a detection system involving a time delay between excitation and emission.
18. With HTRF technology there is a possibility of quenching of the fluorescent signal by fluorescent compounds. Analyzing both the raw donor and acceptor fluorescence intensities will help to identify potential issues with quenching effects.
19. Cytotoxicity could result in false positive results. If cell toxicity is a concern, it is possible to run a Cell Titer Glo assay on the remaining cell plate to determine cytotoxicity.

5. Data analysis

Envision data export:
Ratio = 10,000 x (Fluorescence Intensity 665 nm) / (Fluorescence

Intensity 615 nm)

1. Using Activity Base, an IDBS data analysis software tool, the fluorescence intensity 665 nm/615 nm ratio data is associated to compound, batch and dose information, the data is quality controlled, and the percent inhibition of each compound dose is evaluated.
2. The data from each Max effect/HPE and Min effect/ZPE control wells are assessed, and outliers are excluded from all further calculations.
3. The mean and standard deviation of the HPE and ZPE are then calculated for each plate, along with the Z Prime.
4. The compound data is converted into % effect, using the ZPE and HPE controls as 0% and 100% activity, respectively. The formula used for converting each well reading into % effect is:

$$\frac{\text{(Well activity value – ZPE activity value)}}{\text{(HPE activity value - ZPE activity value)}} \times 100$$

1. Activity Base plots % effect against [log10 compound].
2. An unconstrained sigmoid curve is fitted using a 4-parameter logistic model.
3. Dose response points whose distance from the curve in the y-plane deemed as outliers are automatically excluded. Data points can be manually reincluded or excluded. The curve is then refitted, identifying the excluded points. An IC_{50} is reported as the midpoint of the curve.

Other data analysis software can be used i.e., Genedata Screener or Signals VitroVivo.

QC criteria and IC_{50} analysis guidelines

1. Z prime must be $\geq$ 0.2 to pass QC.
2. No more than 20% of points may be removed from curves or HPE/ZPE.
3. One or more reference compounds should be run in every assay. IC_{50} values for the reference compounds should be within 3-fold of historical value.
4. As a default, IC_{50} curves should be free fit.
5. For incomplete sigmoidal curves, with a max value of <50%, the compound is determined inactive and report IC_{50} as > max dose (e.g. >10 μM).
6. Full sigmoidal curve max asymptote should be 100% (+/ -20%).
7. For incomplete sigmoidal curves, fit the max asymptote to 100% (between 80 and 120%) if the activity at the highest concentration is > 50%.
8. For compounds with a decrease in potency at top doses (often a solubility issue), exclude these doses and refit the curve.
9. Slopes should be $\geq$ 0.5 and $\leq$ 3.

Sample data can be found in the supplementary materials [18].

5.1. Anticipated results

The human $CD4^+$ T cell cytokine secretion assay described in this paper has been used to reliably generate over 5,600 IC_{50} curves and 11,000 single dose screening data points with Z primes higher than 0.2 [18]. This protocol allows for the use of a physiologically relevant human derived cell type in an automated and high throughput manner which supported the discovery of a clinical candidate for the ITK inhibitor program.

CRediT authorship contribution statement

Amy L. Brault: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Theresa A. Dickinson:** Resources, Investigation. **Mary Ellen Banker:** Methodology. **Ruth F. Sommese:** Writing – review & editing, Supervision. **Fabien Vincent:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

Amy Brault, Ruth Sommese and Theresa Dickinson reports a relationship with Pfizer Global Research and Development that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.slasd.2026.100305.

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