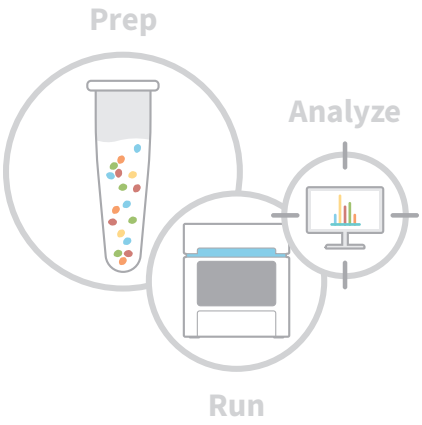


A detailed overview of how to get started with IsoPlexis' sample preparation protocols: CAR-T

Ensure you achieve the maximum benefit from our systems and generate useful data ASAP that relates to in vivo immune activity

In this Project Guide, you will:

- Learn why various steps in Enrichment, Cell stimulation & Cell staining contain recommended elements
- Learn how to guide your protocols and your desired project outcomes to it these recommended guidelines
- See how elements of these recommended protocols relate to past correlative data sets

isoplexis

www.isoplexis.com

Prep, Analyze, Run

Overall Sample Preparation Requirements

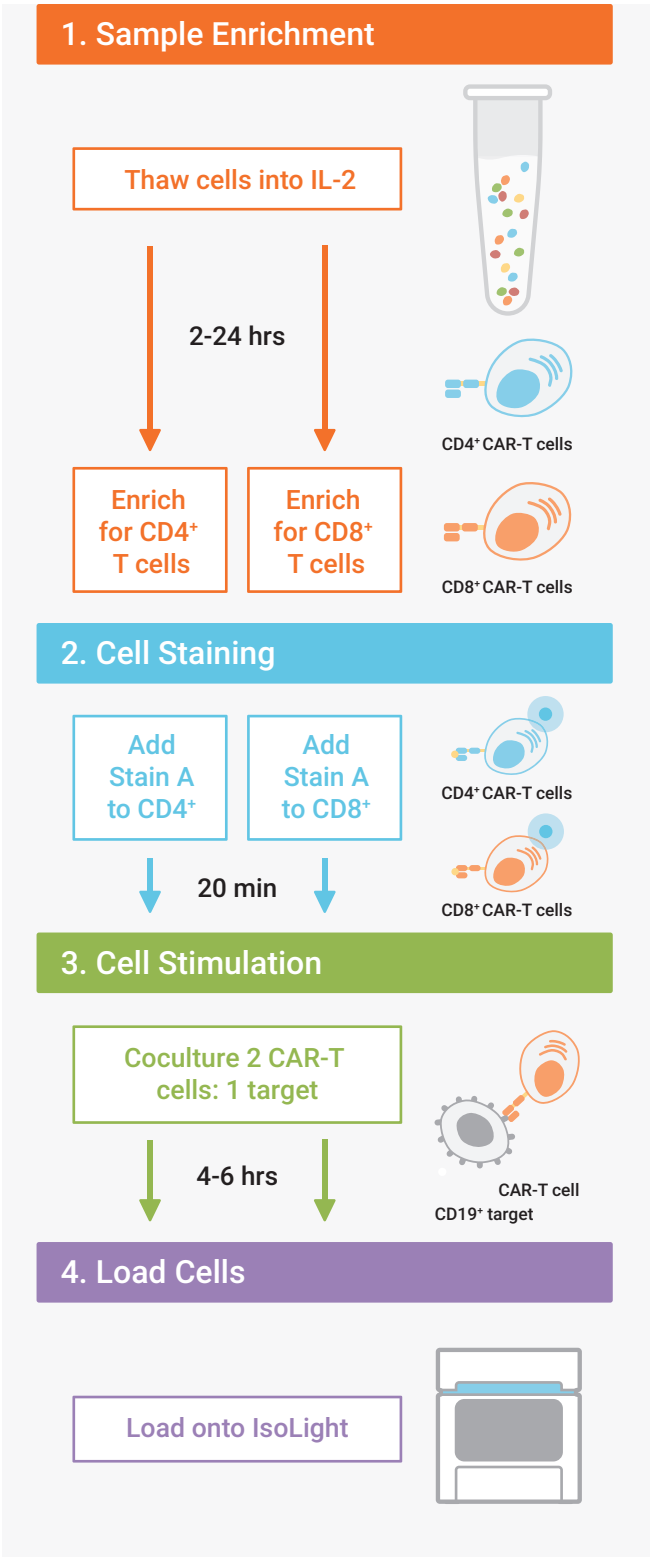
In contrast to technically challenging and time-consuming procedures typical of single cell systems, the IsoLight requires minimal amount of time and experience for successful measurement of true & highly multiplexed secretion from single cells.

Key guidelines should be followed to ensure success in your runs

In the example of analyzing engineered CAR-T cells, three simple steps are required before loading the CAR-T cells onto IsoCode chips and performing automated proteomic analysis on the IsoLight system.

Sample preparation steps with key IsoPlexis requirements:

1. Sample Enrichment
2. Cell Staining
3. Cell Stimulation



Sample Enrichment Requirements

IsoPlexis T-cell enrichment protocols ensure your cells are highly viable, and have enough cells to achieve required sample variabilities across large patient sets.

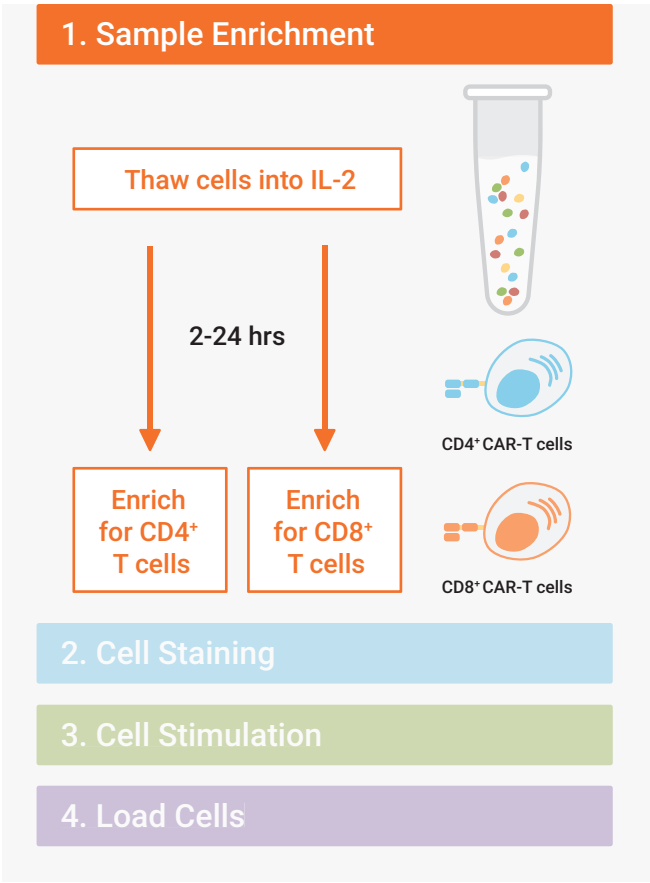
1. Sample Enrichment Requirements

A. **Recover frozen cells in IL-2 for 2-24 hours, if cells are cryopreserved**

B. **Ficoll cells to ensure >80% viability**

C. **Enrich for CD4⁺ CAR-T cells via Miltenyi CD4⁺ T Cell Isolation Kit: see kit protocol**

D. **Enrich for CD8⁺ CAR-T cells via Miltenyi CD8⁺ T Cell Isolation Kit: see kit protocol**

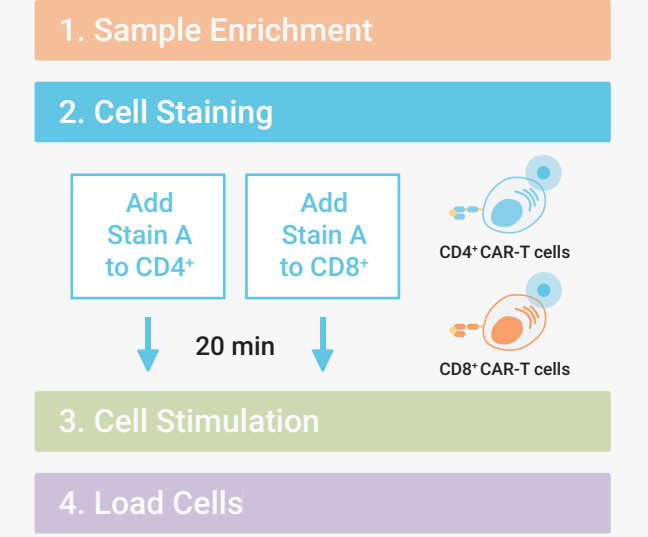


Requirements to achieve goals

- While using fresh samples with any immune cell sample is ideal, high quality data correlating to in vivo activity has also been achieved with frozen samples. Frozen samples, which are the majority of CAR-T product samples, should be thawed and recovered to ensure activity on IsoPlexis' live-cell system, according to previously working protocols
- Ficoll steps will clean up dead cells that may exist once the cells are recovered. It will ensure that the highest percentage of chambers are loaded with live cells, in order to secrete their cytokine products
- Enrichment of both CD4⁺ and CD8⁺ cells are required to gain enough cells of an individual phenotype on chip (roughly 500) **to see the potent cytokine producing cell subsets** (many times between 5 and 25% of the phenotype) that drive the correlates. Even if your CAR-T samples have high CD4 and CD8 cell counts, this step also acts as an additional clean up step and is required

Cell Staining Requirements

IsoPlexis provided cell stains ensure that your cells are labeled with the correct stain that matches the lasers that exist on the IsoLight, to achieve the core requirement of detecting the cells on chip, during the first imaging step after loading. Additionally, these staining requirements have the added benefit of ensuring the required total amounts of cells of a given phenotype are on chip, in order to ensure you have the required amounts of cells to achieve gold standard sample variabilities across chip.



2. Cell Staining Requirements

A. Add Stain A to enriched $CD4^+$ T cells

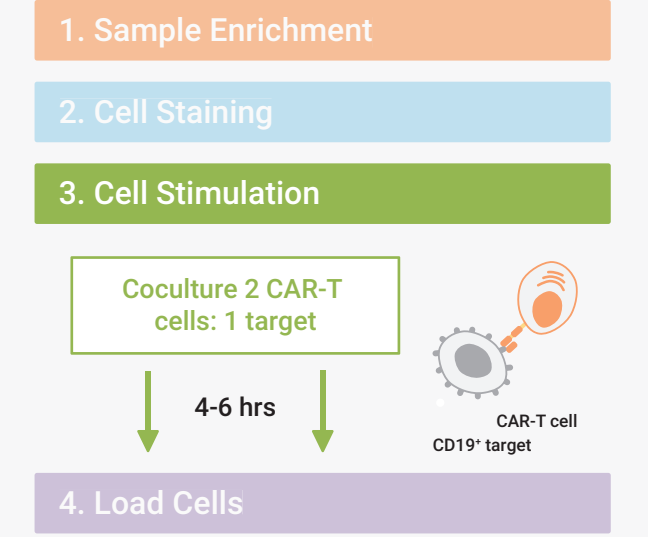
B. Add Stain A to enriched $CD8^+$ T cells

Requirements to achieve goals

- It is critical that users utilize the IsoPlexis stains provided in each kit to ensure that the cells are detected during the first imaging step. If stains from outside of the IsoPlexis kits are used, there is no guarantee of the detection of these cell types, since there is a probability that these non-approved stains to not match the requirements of the IsoLight imaging system.
- Therefore, in order **to achieve cellular data that is in line with the number of cells required of a given phenotype** (at least 500), users must utilize the cell stains required to at baseline detect at least this many cells
- In this case, stains A and B of the single-cell polyfunctional strength kit correspond to the requirements for the CAR-T protocol on the IsoLight. See full 'IsoLight Manual' for stains and for exact volumes and cell concentrations

Cell Stimulation Requirements

IsoPlexis stimulation protocols ensure your cells receive the stimulation required to view the most potent subsets on chip, via the 30+ cytokines that the T-cells use to orchestrate the immune system. These stimulation protocols have been tested and proven to achieve valuable potency information from subsets of CAR-T cells that correlate to in vivo activity. Essentially, these tried and tested stimulations re-awaken key cellular cytokine programs that mirror as closely as possible what is happening in vivo.



3. Cell Stimulation Requirements

A. Coculture 2 CAR-T cells : 1 target for 4-6 hours

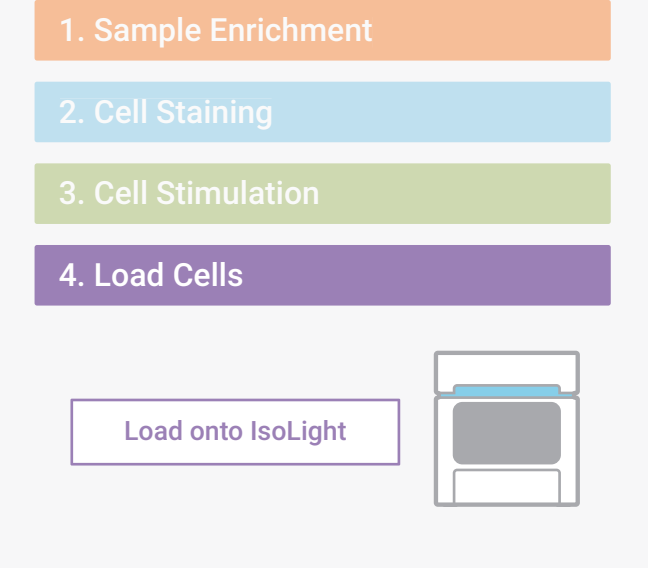
1. **$CD4^+$: CAR-T specific targets**
2. **$CD4^+$: NGFR targets**
3. **$CD8^+$: CAR-T specific targets**
4. **$CD8^+$: NGFR targets**

Requirements to achieve goals

- Choosing the proper stimulation concentration, whether it be with an antigen specific target cell or soluble receptor stimulation (CD3 / CD28) is important. We choose a CAR-T cell to K-562 target ratio which is 2 to 1, in order to get the desired stimulation from the cells themselves, without necessarily overstimulating the cells. This ratio is of course different than typical cell killing assays, but has proven **to achieve correlates that match in vivo activity**
- Further, at this concentration, there is minimal debris from dead target cells that have been killed by the CAR-T, minimizing risk of loading problematic cells on chip

Cell Loading Requirements

Cell Loading, while straightforward, also has required concentrations to load onto the IsoCode consumable chip, in order to make sure the chambers within the consumable chip are not overloaded with cells. The below concentration is required to achieve the type of IsoPlexis readouts required to detect each sample's highly potent cell subsets.



4a. Cell Loading onto the IsoLight

A. **Add cells at 1,000,000 cells/mL to the IsoCode Chip and load into the IsoLight (which equates to 30,000 cells)**

4b. Data Analysis

A. **Analyze data with IsoSpeak after completion of 24 hour run**

Requirements to achieve goals

- It is critical that users follow the 1,000,000 cells/mL concentration in order to not overload the chip with cells.
- If the consumable chip is overloaded with cells, users will not be able to achieve the single-cell count required per given phenotype (at least 500) in order to achieve the sample readouts that allow users **to see the highly potent cell subsets (between 5 and 25%) of cells that drive response**

Conclusion

In this technology note, you have learned to get started quickly with your CAR-T Sample Preparation with the key requirements that will get you to the data. Items learned:

- Why various steps in Enrichment, Cell stimulation & Cell staining contain recommended elements in order to get you the data you need
- How to guide your protocols and your desired project outcomes to fit these recommended guidelines
- How elements of these recommended protocols relate to past correlative data sets

References

1. Rossi et. al '17 KITE Pharma ASCO-SITC Presentation
2. Xue et. Al '17 Journal of ImmunoTherapy of Cancer