White Paper

Flow Cytometry reagent for the detection of CAR T-Cells

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Background

Last years, many advances have been made in cancer medicine, highlighting the immunotherapy of CAR T-Cells (Chimeric Antigen Receptor T-Cells).

This targeted therapy is based on the extraction of patient T-Cells, the introduction in them of a chimeric receptor specific to a protein (antigen) present on the cancer cells (e.g. CD19) and the consequently reinfusion of these modified cells, now CAR T-Cells, into the patient to recognize the target cells and eliminate them. [1,2]

T-Cells are a leukocyte type involved in immune response and they are capable to eliminate and/or help to eliminate the targets they recognize. However, cancer cells escape to their recognition, that provokes the disease propagation (Fig. 1A).

Therefore, is necessary to equip these cells with a receptor (CAR) that recognizes the malign cells and destroys them. This is made by in vitro genetic engineering with the extracted cells that, once modified and expanded, are return to the patient to begin the recognition-elimination process. (Fig. 1B-F)

This therapy has gained relevance due to its efficacy and to have available detection CAR T cells tools has become a critical need,, both to check that they have been correctly modified and to monitoring them after reinfusion, since they have a limited life span. [3]

Flow cytometry is a well-established technique in the immunology field, both in research and diagnostics, capable of providing the necessary sensitivity to detect the CAR T-Cells using highly specific reagents.

These reagents are based on the binding of a protein, in this case CD19 (which must be recognized by CAR T-Cells), with a fluorescent dye (fluorochrome) that the cytometer can detect. Thus, the cell will recognize the CD19 protein with the fluorescent label and the cytometer will detect that fluorescence, being able to identify the cells with the receptor.

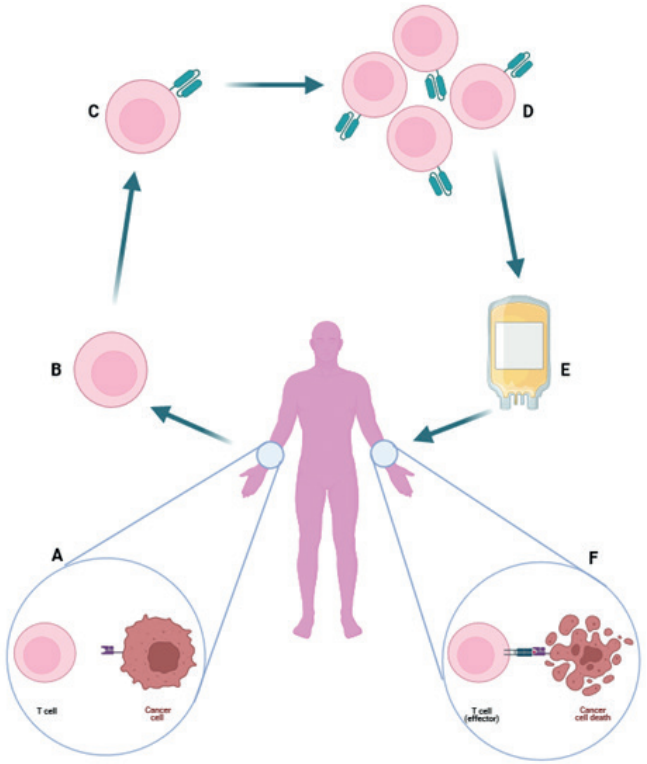


Figure 1. Basic scheme of CAR T-Cell therapy. (A) Own T-Cell does not recognize cancer cell. (B) T-Cell extraction. (C) Modification of T-Cell with CAR. (D) CAR T-Cell cultivation and harvesting. (E) Harvested CAR T-Cells are reinfused into the patient. (F) CAR T-Cell recognizes and eliminates cancer cell.

CD19-Targeted CAR T-Cell therapies

Since 2017, some therapies with CAR T-Cells, targeted to CD19 antigen, have been approved in many countries. These therapies are prescribed for a specific oncologic disease and patient population. (Table 1) [2]

Brand Name	Generic Name	Targeted Disease
Kymriah®	Tisagenlecleucel	ALL
		DLBCL
Yescarta®	Axicabtagene ciloleucel	DLBCL
		FL
Tecartus®	Brexucabtagene autoleucel	MCL
		ALL
Breyanzi®	Lisocabtagene maraleucel	DLBCL

Table 1. Current therapies (CD19 antigen-based) and the disease to be treated: ALL (B-Cell Acute Lymphoblastic Leukemia), DLBCL (Diffuse Large B-Cell Lymphoma), FL (Follicular Lymphoma), MCL (Mantle Cell Lymphoma).

There are countries where these therapies are being studied for approval, so it is very important to accelerate this process through improved technology that allows researchers to advance and makes it easier for medical experts to perform better treatment.

Sometimes, the requirements to apply an approved commercial therapy are not met and specialized medical centers, under accreditation, decide to create their own CAR for treatment, these CAR T-Cell are known as academic CAR T-Cell.

Limitations of current reagents

There are some reagents for identifying and quantifying CAR T cells, based on fluorescent labeled (PE or FITC) recombinant CD19.

However, current reagents have limitations in separating the desired populations with sensitivity, making it more difficult for the analysts to provide an accurate and reliable result.

Those reagents that overcome this problem have the disadvantage of involving more complex and time-consuming protocols, using an amplification reagent in addition to the protein.

It is therefore necessary to introduce new simple, straightforward and easy to use reagents that are able to clearly discriminate the CAR T-Cells from the rest of populations for a more reliable analysis.

Detection of CAR T-Cells

To meet these needs, we have developed a single reagent for direct and specific recognition of CAR T-Cells with high resolution.

This reagent allows the specialists to confirm the successful modification of T-Cells in a quality control prior infusion into the patient and to monitor them after this step. (Fig. 2) [4]

Technology

Recombinant Fc-Tag CD19 protein (CD19p) has been labeled, by a proprietary process, with R-Phycoerythrin fluorochrome (R-PE or PE), obtaining a conjugate that is able to be recognized by CAR T-Cells and separates them efficiently without any secondary reagent (**Immunostep, ref. CD19-0212**).

Other manufacturers have similar products based on this fluorochrome-protein complex (e.g. ACRO Biosystems, ref. CD9-HP2H3 | ref. CD9-HF2H2 | ref. CD9-HF251) but have limitations in the separation due to bioconjugation techniques and/or the fluorochrome used.

Methodology and analysis

The reagent is supplied in aqueous solution to direct labeling of cells with 5 µl of it. This, with the use of an antibody panel to discriminate T-Cells, is sufficient to separate those which have CAR in just one 30 min incubation at room temperature (RT) spending around 1 hr of complete protocol, whereas other protocols spend same time just to label the specific population.

Other commercially reagents, such as CD19 CAR Detection Reagent (Miltenyi Biotec, ref. 130-129-550), are based on an indirect staining, with a biotinylated protein that must be

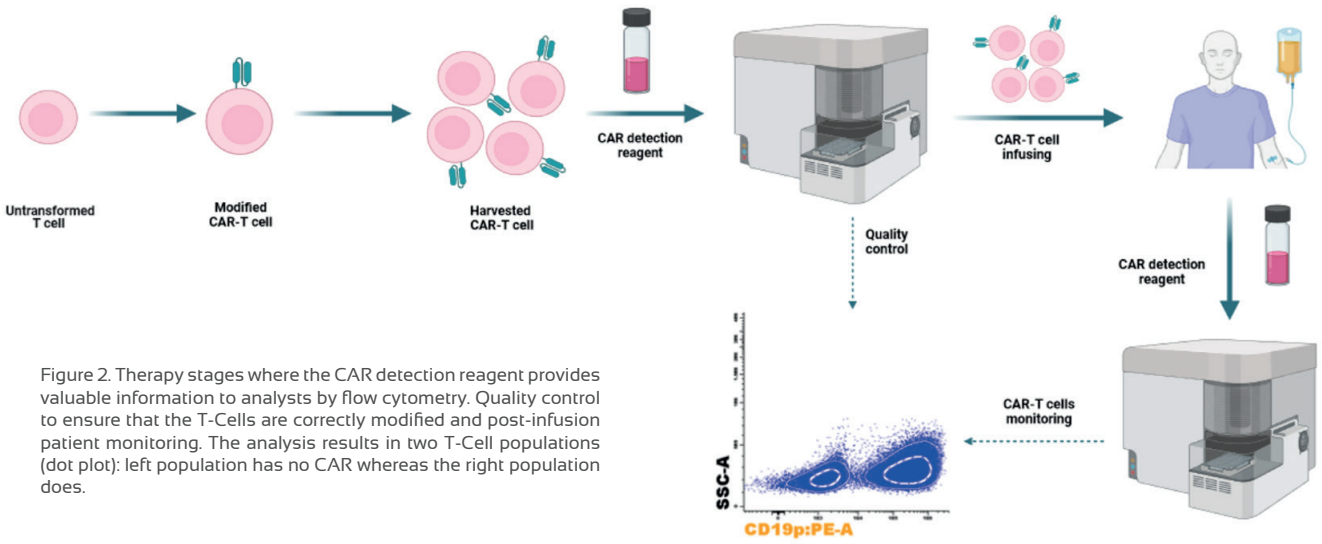


Figure 2. Therapy stages where the CAR detection reagent provides valuable information to analysts by flow cytometry. Quality control to ensure that the T-Cells are correctly modified and post-infusion patient monitoring. The analysis results in two T-Cell populations (dot plot): left population has no CAR whereas the right population does.

revelated by a fluorescent secondary reagent. This strategy has good results, but a high cost and a more complex protocol.

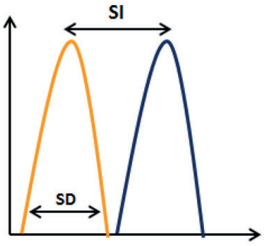
The fundament of flow cytometry is binding the reagent to a specific population and discriminating it by the reagent fluorescence [5]. The reagent targets the CAR in the engineered T cell, so the separation depends on the level of expression of the CAR and the discriminatory ability of the reagent.

The analysis consists of the use of an antibody panel (selected by the analysts) to identify total T-Cells plus the CAR detection reagent to discriminate between normal and modified (CAR) T-Cells.

The antigen expression level depends on the modification, so different CAR T-Cells from each manufacturer or academic modifications may express different amount of CAR and that affects the number of antigens they can recognize; the less CD19 antigen they recognize, the worse fluorescence signal and the more difficult it is to discriminate the population. Thus, less CAR means that each T-Cell will recognize cancer cells worse.

The reagent identify the specific population by his fluorescence, but it is not so important the intensity of that fluorescence, measured as MFI (Median Fluorescence Intensity), but the ability to discriminate between the positive population (CAR T-Cells) and the negative populations (rest of the cells).

It means that it is necessary the greatest difference between both populations MFI; this value is known as Stain Index (SI), the greater SI the better discrimination. (Fig. 4)



$$\text{Stain Index} = \frac{\text{(Median of Positive - Median of Negative)}}{\text{(SD of Negative * 2)}}$$

Figure 4. Stain Index, value to estimate the level of discrimination between positive and negative populations.

A highly fluorescent reagent is needed when the amount of target molecule is low but sometimes also increases the negative MFI, getting worse the SI. Best reagents have high SI with a moderate or high fluorescence to identify CAR T-Cells, even with low CAR expression.

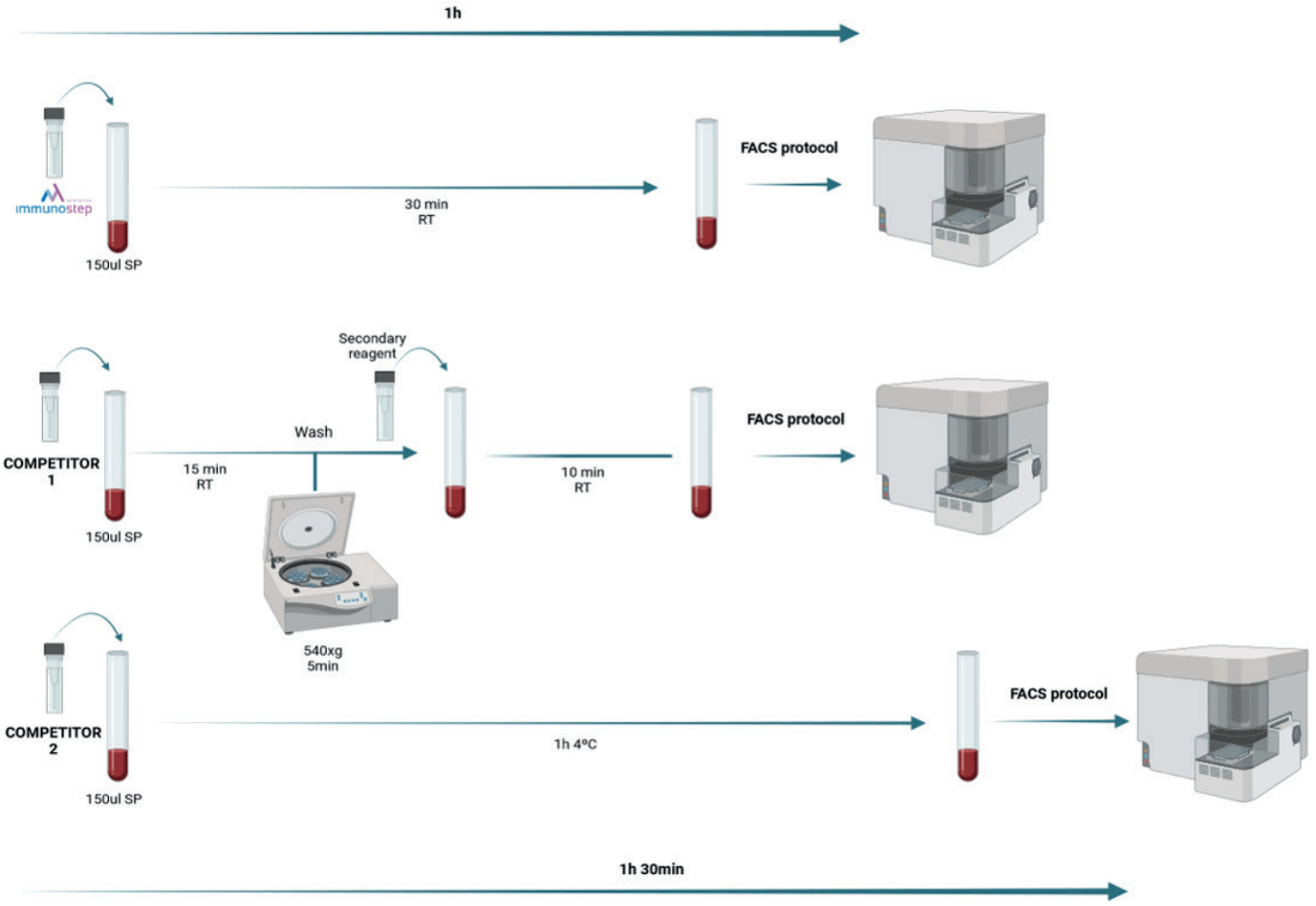


Figure 3. FACS (Fluorescent-Activated Cell Sorting) protocol. Immunostep vs competitors. Other competitors require more steps and reagents (competitor 1) or longer protocols (competitor 2). Immunostep combines the simplicity with the speed, providing analysts with an easier protocol.

To determine the efficiency of the reagent, it has been tested with multiple clinic samples from different commercial therapies (e.g. Yescarta) and academic CAR T-Cells, and different cancer diseases associated to these therapies (e.g. DLBCL). (Fig. 5)

It has been compared with commercial reagents from two different competitors. Depending on the sample, CAR expression may be variable, sometimes is good enough to be easily detected and even the worst reagents can separate the CAR T-Cells efficiently (Fig. 5A). But other samples have low expression, and this is when a good reagent that discriminates the negative and positive populations with confidence is needed. (Fig. 5C)

Immunostep shows the best results in all the samples. The more difficult sample to separate the greater the difference with commercial reagents, so it can discriminate population when others cannot. (Fig. 6)

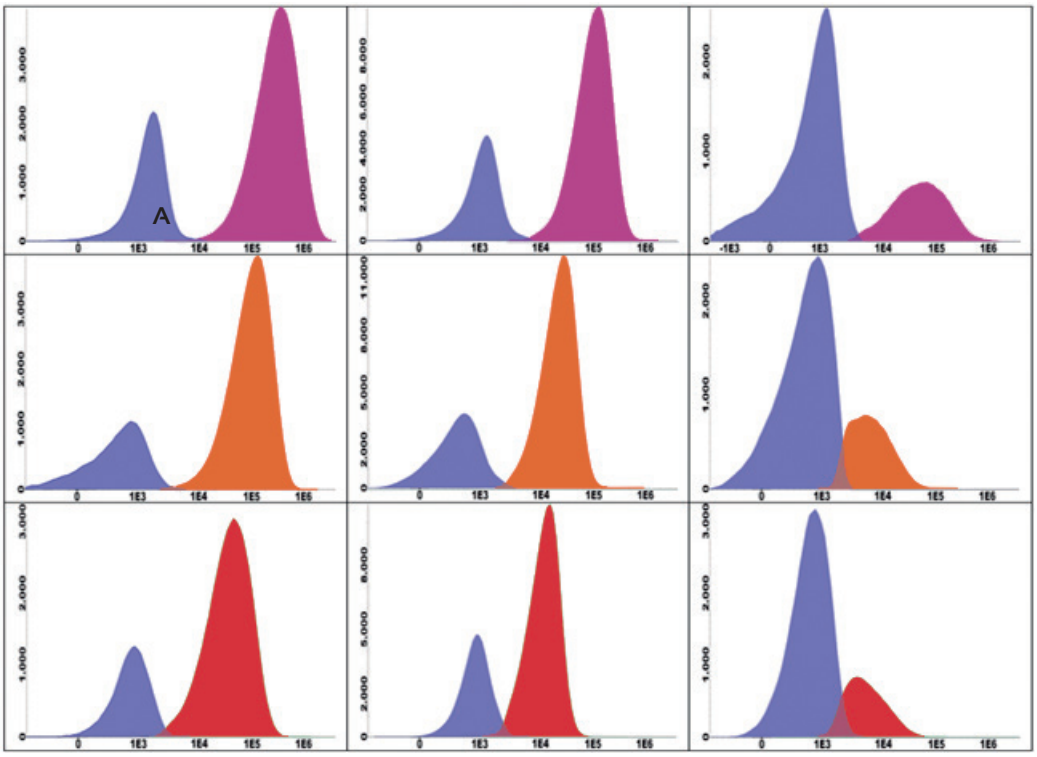


Figure 5. Histogram analysis. Immunostep (pink) vs competitors (1 – red and 2 – green) with different samples:

(A) Yescarta® – DLBCL
(B) Yescarta® – FL
(C) Academic CAR T-Cells – DLBCL

Immunostep shows good separation in all samples, even when the CAR expression is low and it is difficult to discriminate CAR T-Cells.

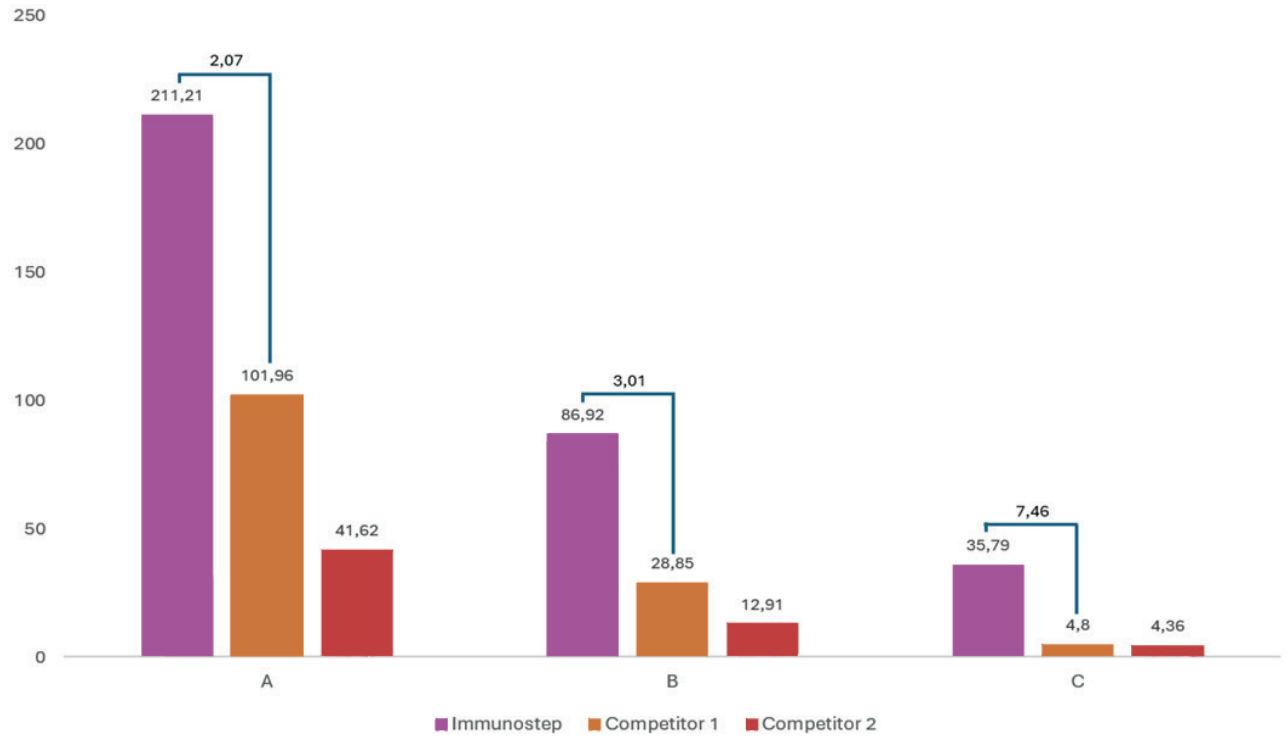


Figure 6. A bar graph representing the SI data obtained from Figure 5. The ratio between Immunostep and the best competitor (1) has been determined. In Sample C, the most difficult discrimination, Immunostep shows highest difference, providing reliable result.

Advantages of Immunostep's reagent

We have developed a reagent capable of specifically identifying CAR T-Cells from other populations with high resolution, even with difficult samples. No secondary reagents or complex protocols with multiple steps are required. Furthermore, R-PE is a fluorochrome compatible with any flow cytometer.

The high performance and convenient protocol of Immunostep PE CD19p for CAR detection makes it the best choice to the current needs of diagnostic and developmental in the field of CAR T-Cell therapies.

Upcoming developments

We are working on other proteins for CAR detection, such as BCMA, describe for Multiple Myeloma CAR T-Cell therapy, and on labeling these proteins with other fluorescent tags to facilitate their incorporation into the already constructed cytometry panels.

Immunostep product references

REF		Σ	
19-0212-25T	human PE-CD19	25 test	RUO
19-0212-100T	human PE-CD19	100 test	

References

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