APPLICATION NOTE

# Quantification of bi-specific anti-CD3-CD19 activity using *iLite*® CD3 Effector Assay Ready Cells together with *iLite*® CD19 Target Assay Ready Cells

***For research and professional use only. Not for use in diagnostic procedures.***

*This application note contains a suggested protocol and performance data.*
*Each individual laboratory must set up their own method and perform relevant validations.*

## Background

In diseases such as cancer and autoimmunity, a variety of immunotherapy strategies are used where T cell responses play a central role. CD3 bispecific antibodies (bsAbs) are able to bridge CD3+ T cells to the cancer target and by doing so activating the CD3+ T cells resulting in increased cytolytic activity and elimination of the cancer cell (1).

US FDA grouped bsAbs into two classes based on their mode of action, cell-bridging bsAbs and antigen-cross-linking bsAbs (2). Most of the cell-bridging bsAbs are used in cancer treatment where the bsAb recognize a tumor associated antigen together with either T cells via CD3 or NK cells via CD16 (3). The anti-CD3 x anti-CD19 bsAb T cell engager, Blinatumomab, which binds simultaneously to CD3 on T cells and CD19 on malignant B cells was approved in 2014 by FDA for treatment of relapsed or refractory B-cell acute lymphoblastic leukemia (4).

To quantify bispecific anti-CD3-anti-CD19 activity and potency, *iLite*® CD3 Effector Assay Ready Cells can be used together with and *iLite*® CD19 Target Assay Ready Cells in therapeutic antibody discovery and development of novel CD3+ T cell engaging bsAbs.

## Principle of the assay

CD3/CD19 bi-specific antibodies bind to CD3 on the surface of the *iLite*® CD3 Effector Assay Ready Cells and to CD19 on the *iLite*® CD19 Target Assay Ready Cells. Cross-linking of effector and target cell leads to activation of the firefly luciferase (FL) reporter gene construct in the effector cells through the NFAT pathway. *iLite*® CD3 Effector Assay Ready Cells also contain the Renilla Luciferase (RL) reporter gene, under the control of a constitutive promoter, that allows drug-induced FL activity to be normalized with respect to the constitutive expression of RL.

This render assay results independent of variations in cell number, serum matrix effects, or lysis of the effector cells by the target cells. In addition, we also describe the use of a negative control in the form of a target cell line depleted of CD19 expression (*iLite*® CD19 (-) Target Assay Ready Cells). The Firefly luciferase signal can be measured in a luminometer following addition and incubation of luciferase substrate. The Firefly luciferase signal is proportional to the functional activity of Blinatumomab in the sample (Fig.1).

## Specimen collection

Quantification of bi-specific anti-CD3-CD19 activity using *iLite*® CD3 Effector Assay Ready Cells together with *iLite*® CD19 Target Assay Ready Cells can be performed in test samples including human serum.

## Material and equipment needed

| Material and equipment | Suggested supplier | Reference |
|---|---|---|
| *iLite*® CD3 Effector Assay Ready Cells | Svar Life Science | BM5005 |
| *iLite*® CD19 (+) Target Assay Ready Cells | Svar Life Science | BM5025 |
| *iLite*® CD19 (-) Target Assay Ready Cells | Svar Life Science | BM5026 |
| Diluent (RPMI 1640 + 9% heat inactivated FBS + 1% Penicillin Streptomycin) | Gibco | 61870 (RPMI) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin) |
| Blinatumomab or analogues | Amgen | NA |
| Firefly/Renilla luciferase substrate | Promega | E2940, Dual-Glo® Luciferase Assay System |
| Plate; White walled micro well plate suitable for luminescence | PerkinElmer | 6005680 |
| Microplate Luminometer with appropriate reading software – no filter on luminometer | Contact Svar Life Science for list of recommended suppliers | NA |
| Incubator, 37°C with 5% CO2 | NA | NA |
| Water bath, 37°C | NA | NA |
| Single-channel and multi-channel pipettes with polypropylene disposable tips | NA | NA |
| Polypropylene tubes or plate for dilution | NA | NA |
| Single-use polypropylene reservoir | NA | NA |
| Plate shaker | NA | NA |
| Timer | NA | NA |

## Preparation of calibrators (Blinatumomab)

The bi-specific CD3/CD19 effect of Blinatumomab from Amgen has successfully been measured in combination with a mix of CD3 Effector Assay Ready Cells and CD19 (+) Target Assay Ready Cells. As a negative control, a combination of CD3 Effector Assay Ready Cells and CD19 (-) Target Assay Ready Cells was used. In the present assay an Effector: Target ratio of 4:1 has been used. The optimal ratio is dependent on the antibody and target cells used and should be determined each time a new assay is set up. The table below shows recommended dilutions of Blinatumomab when making an 8-point calibration curve.

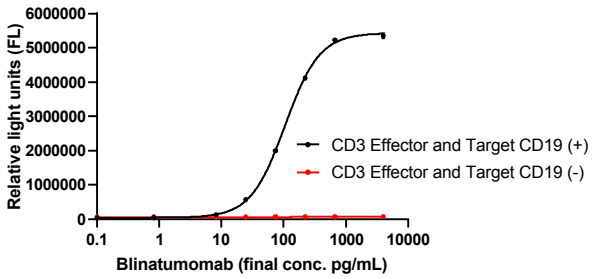


**Figure 1**. Example of Blinatumomab calibration curve using Firefly Luciferase substrate Dual-Glo® Luciferase Reagent. Values are shown as mean of triplicate ± SD and values on x-axis are given as **final concentration** in the wells before addition of Dual-Glo® Luciferase Reagent.

| Calibrator | Blinatumomab |
|---|---|
| | Calibrator **solution conc.** (pg/mL) |
| **1** | 8 000 |
| **2** | 1 333 |
| **3** | 444 |
| **4** | 148 |
| **5** | 49 |
| **6** | 16 |
| **7** | 1.6 |
| **8** | 0 |

**Table 1.** Suggested calibrator **solution concentrations** for Blinatumomab.

## Protocol

### Assay preparation and incubation

1. Design a plate layout.
2. Dilute calibrators, controls and samples to fall within the expected **in assay values (= final concentration)** of 0-4000 pg/mL.
3. Add 40 µL calibrators, controls and samples in duplicate to assigned wells.
4. Thaw the vial of CD3 Effector Assay Ready Cells and the vials of CD19 (+) Target Assay Ready Cells and CD19 (-) Target Assay Ready Cells in a 37 °C water bath with gentle agitation.
5. Mix the cell suspensions very carefully **at least 10 times with a pipette** in order to ensure a homogeneous distribution of cells.
6. Dilute 200 µL of the CD3 Effector Assay Ready Cells and 200 µL of the CD19 (+) Target Assay Ready Cells with 3.44 mL Diluent. The total volume of the diluted CD3 Effector /Target CD19 (+) Assay Ready Cells mixture is 3.84 mL.
7. In a separate tube, dilute 50 µL of the CD3 Effector Assay Ready Cells with 50 µL of the CD19 (-) Target Assay Ready Cells with 860 µL Diluent. The total volume of the diluted CD3 Effector/Target CD19 (-) Assay Ready Cells mixture is 960 µL.
8. Add 40 µL of the diluted cells to each well to be tested.
9. Place the lid on the plate and mix on a plate shaker at **minimum of 750** rpm for 10 sec. Alternatively, mix the cell suspensions very carefully in the wells by pipette. Insufficient mixing can cause reduced assay sensitivity.
10. Incubate for 5 hours at 37°C with 5% $CO_2$.

### Adding substrate solutions

11. Equilibrate the plate and the substrate solutions to room temperature.
12. Prepare the **Firefly luciferase substrate** in accordance with the supplier's instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer.
13. If appropriate, prepare the **Renilla luciferase substrate** in accordance with the supplier's instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer.

## Normalization

The reporter gene used for normalization, Renilla luciferase, is under the control of a tyrosine kinase promoter, and is thus constitutively expressed. Unspecific effects such as serum matrix effects or differences in cell number can be obviated by relating the specific Firefly signal with the Renilla normalization signal through simple division.

**A**

**B**

**C**

**Figure 2.**

**A:** Measurement of the specific Firefly (FL) signal. Cells were stimulated with increasing concentrations of Blinatumomab.

**B:** Measurement of the Renilla (RL) signal for normalization, from the identical assay as in A .

**C:** Dose response curve after normalization of the specific Firefly signal with the according Renilla signal.

## Precautions

- This application note is intended for professional laboratory research use only. The data and results originating from following the Application Note should not be used either in diagnostic procedures or in human therapeutic applications.
- Use and handle the material and instruments referenced according to the suppliers'/manufacturers' instructions or product specifications accompanying the individual material and instruments.
- Dispose of all sample specimens, infected or potentially infected material in accordance with good microbiological practice. All such materials should be handled and disposed as though potentially infectious.
- Residues of chemicals and preparations are generally considered as biohazardous waste and should be inactivated prior to disposal by autoclaving or using bleach. All such materials should be disposed of in accordance with established safety procedures.

## Proprietary Information

In accepting delivery of *iLite*® Assay Ready Cells the recipient agrees not to sub-culture these cells, attempt to sub-culture them or to give them to a third-party recipient, and only to use them directly in assays. *iLite*® cell-based products are covered by patents which are the property of Svar Life Science AB and any attempt to reproduce the delivered *iLite*® Assay Ready Cells is an infringement of these patents.

# QUICK GUIDE

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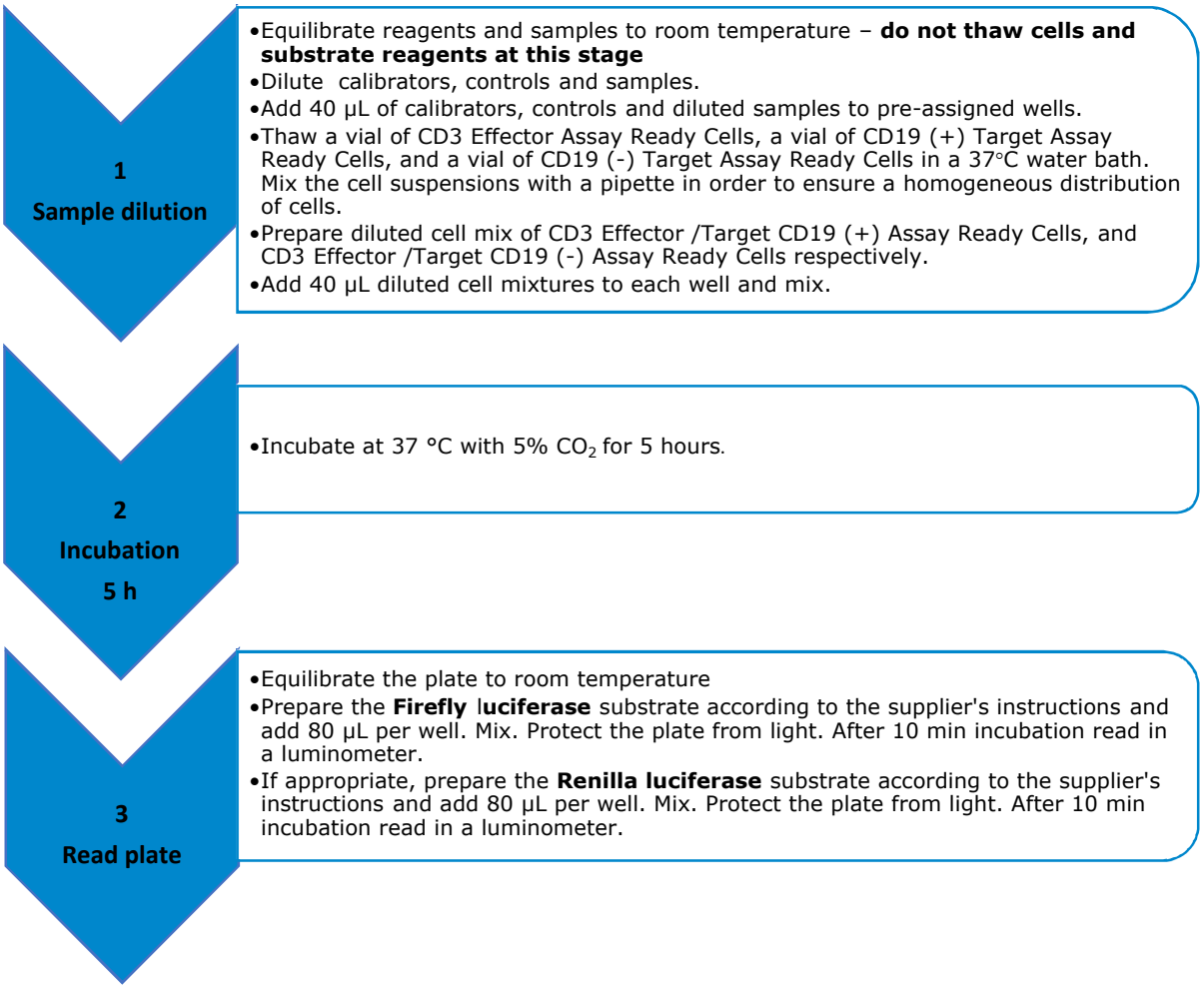


## Troubleshooting and FAQ

Please consult the Svar Life Science website www.svarlifescience.com

## References

1. Singh A, Dees S, and Grewal S. *Overcoming the challenges assocated with CD3+ T-cell redirection in cancer*. Br J of Cancer 124:1037-1048 (2021)
2. Labrijn AF et al., *Bispecific antibodies: a mechanistic review of the pipeline*. Nat Rev Drug Discov 18:585-608 (2019)
3. Oberg et al., Tribody [(HER2)2xCD16] is more effective that Transtuzumab in enhancing gamma delta T cell and natural killer cell cytotoxicity against HER2 expressing cancer cells. Front Immunol 9:814 (2018)
4. Przepiorka et al., *FDA approval: blinatumomab*. Clin Cancer Res 21: 4035-4039 (2015)