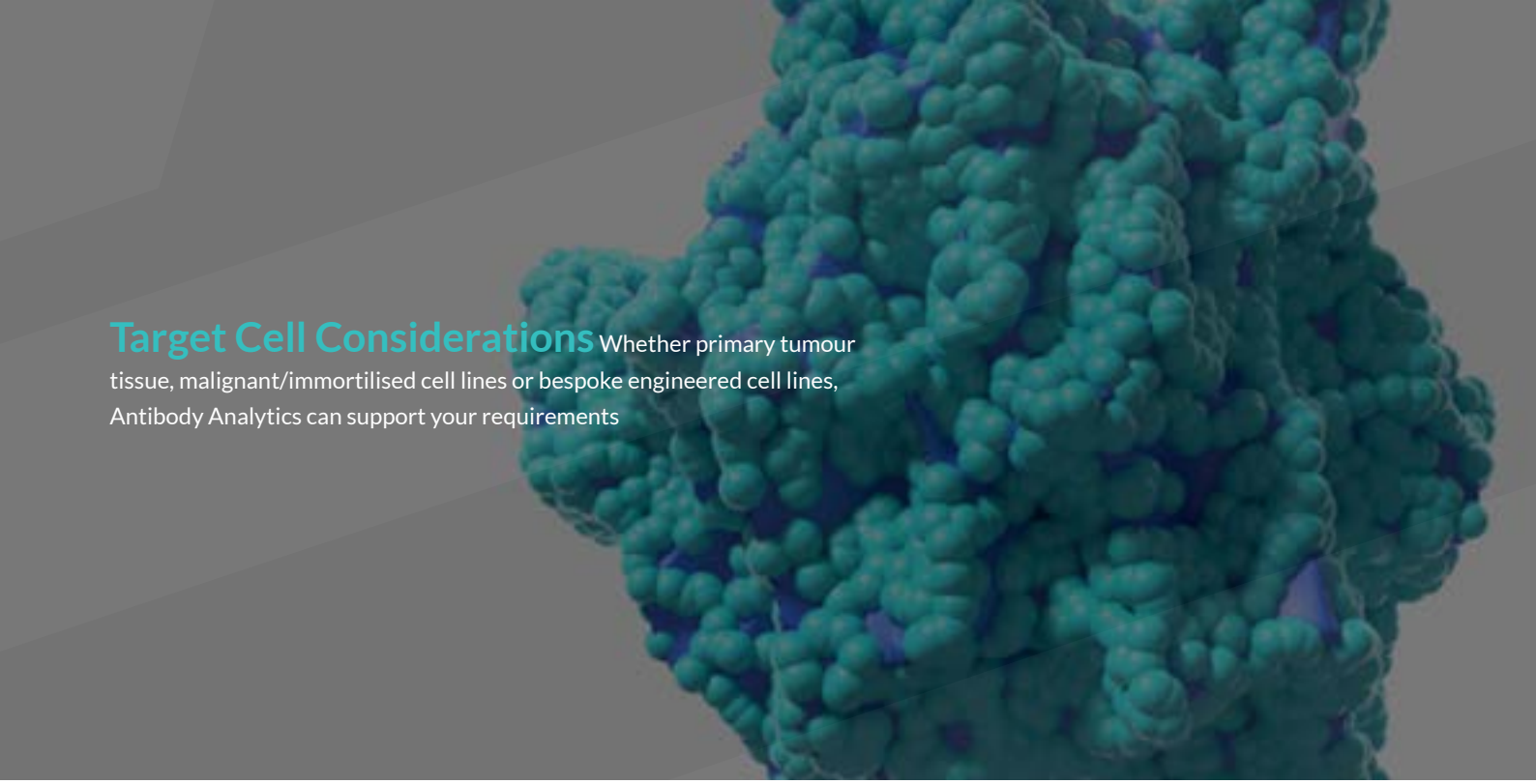
Antibody Analytics

T CELL MEDIATED CYTOXICITY

Combining a range of technologies, access to a wide variety of primary T cells and experts in bioassay design, we can develop industry-leading T cell mediated cytotoxicity assays to support early screening activities through to development of lot release potency methods

TARGET CELL CONSIDERATIONS



Target Cell Considerations

Whether primary tumour tissue, malignant/immortilised cell lines or bespoke engineered cell lines, Antibody Analytics can support your requirements

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Target cells are typically derived from primary tumour tissue, malignant/immortalised cell lines and engineered cell lines. These options represent a gradient of biological relevance and potentially predictive ability of *in vivo* efficacy versus ease of use and reliability when used in *invitro* bioassays.

Malignant/immortalised cell lines are widely available and represent the fundamental model for characterisation of T cell mediated cytotoxicity in early development. However, consideration should be given to the characteristics of the cell line that may limit the translational value of the selected cell line as well as available endpoint options and compatible effector cell populations. For example, real-time cell killing assays, yielding high value kinetic data require adherent cells.

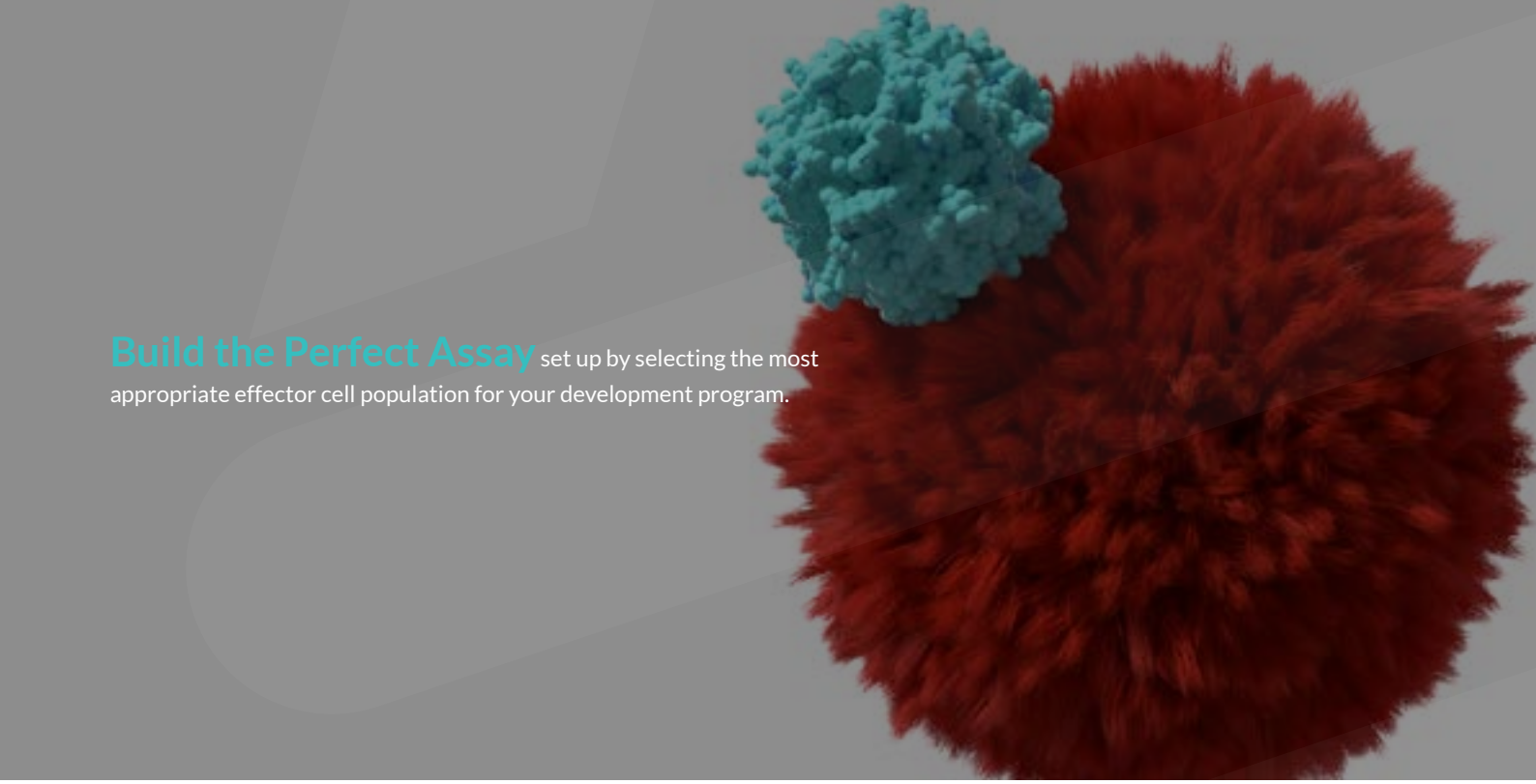
Engineered cell lines may be the preferred option where either a malignant/immortalised cell line is not available that expresses the antigen of interest or requires complicated protocols to induce expression of the antigen for bioassay.

Antibody Analytics have in-house cell line development capabilities and a library of target antigens covering common bispecific T cell engaging targets.

Engineered cell lines may also be the first choice of target cell as they allow novel end point approaches to be used that can offer unparalleled assay sensitivity and signal to noise. A highly reliable system is to constitutively express luciferase that can be used as a direct and specific measure of target cell viability at the end of the assay.

Feature	Why?
Constitutive Expression	Allows a direct measure of target cell viability at the end of the assay
Non-cytotoxic	Luciferase is well tolerated by the cells, meaning there is no restriction on the overall assay duration that can be employed
Specificity	Luminescence signal is specific to the target cell alone and suffers no interference from effector cell viability issues.

EFFECTOR CELL CONSIDERATIONS



Build the Perfect Assay set up by selecting the most appropriate effector cell population for your development program.

The selection of an appropriate effector cell population is critical to the design of T cell mediated cytotoxicity assays. Factors for consideration include the source of the cells (fresh or cryopreserved), pre-activated, expanded, engineered reporter cells, disease status and the type and level of isolation (PBMC, Pan T cells (CD3+ isolated), CD8+ isolated).

KEY FACTS

T cells are not a homogeneous population and can be subdivided much more that the CD4+/CD8+ T cells. There are various effector states in each subpopulation that respond differently to anti-CD3 stimulation. For example, naïve T cells (CD4 or CD8), when stimulated through CD3 crosslinking instead of being activated, can turn into a state of functional anergy due to lack of co-stimulation. The relative proportion of these populations vary in individuals and this potentially may result in assay variability.

Many T cells in tumours have been found to be irrelevant when the T cell receptor repertoire of tumour infiltrating lymphocytes (TILs) was investigated as most were specific for viral antigens (CMV, EBV etc). These were probably attracted to the tumour by inflammation. These cells have effector memory phenotype and can be the target of bispecific T cell engagers. Thus, a good model for bispecific T cell engagers would be to use viral specific T cells to assess cytotoxic activity. These should be less dependent of co-stimulation and could be activated easier. It is possible to expand CMV specific T cells and bank them for bioassays.

Simple donor variability. A theoretically "identical" cell from one donor, does not behave identically as the same cell from another donor. From experience of oligoclonal γδ T cells, that theoretically should have similar effector functions (by the expression of NCR receptors etc), there can be great variability in cytotoxicity between donors. Also, the kinetics of killing can vary, with intra-donor killing half-time variability may be observed.

CD8+ T cells require CD4 for maximum effector functions. CD4+ T cells produce cytokines that promote the function of CD8+ T cells directly and indirectly. Assays that only incorporate CD8+ T cells will not capture the true biological response.

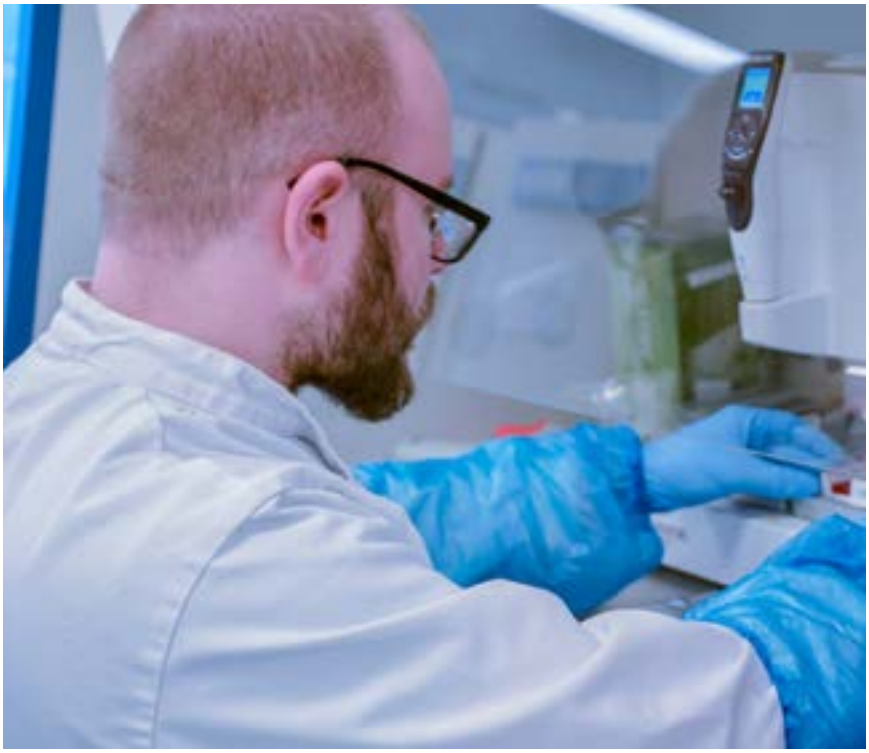
Absence of innate T cells may mask potential effector function of a T cell engaging bispecific. The highest CD3 expressing T cells are considered to be γδ T cells that constitute 1-10% of PBMCs. They have approximately a log scale higher CD3 expression than alpha-beta T cells ("normal" CD4 and CD8 T cells). These are probably the biggest antigen specific T cell population in the blood, recognising stress signals expressed by cancer cells. These cells are primed to kill upon TCR stimulation and don't require as much co-stimulation.

Single read-out. Killing is one way that CD8+ T cells function. These cells also produce cytokines that potentiate the function of other cells. It would be wise to investigate other readouts (especially IFNγ) to ensure that there is not absence

of function. In short assays (often limited to ~4 hours with some endpoints) however, this is not easy to monitor, and a longer assay would be required (>24 hours).

Quality of the T cells. For some assays cryopreserved immune cells do not perform adequately, especially if high metabolic activities are required from them in a short term. For this reason, some assays perform better when a "fresh" source of cells are used. However, secondary variability is likely to be introduced as different donors need to be used, so the day-to-day performance of cells even from a single donor could change.

Common effector cell options are listed in the table below:



T-Cell Format	Description
PBMC	Global measurement of target cell death mediated by T cells, NK cells (if functional Fc domain is present)
Pan T cell (CD3+)	T cell mediated cytotoxicity response of CD4+ and CD8+ and T cells
CD8+	Specific CD8+ T cell mediated cytotoxicity activity
Expanded T-cells	Clonal pools of expanded T cells can be created from a primary donor and subsequently used for T cell mediated cytotoxicity assays. This process can also be used to generate antigen-specific T cells
Healthy and Disease State Donors	Healthy and/or disease state tissue can be used to generate data of improved clinical relevance
Reporter Cells	T cell activation measured using a luciferase reporter gene responsive to CD3+ engagement

ASSAY ENDPOINT CONSIDERATIONS

CYTOTOXICITY

How target cell death will be measured dictates the format of a T cell mediated cytotoxicity assay. Several approaches are available, each with their own benefits and challenges:

Method	Description	Benefits	Limitations
Chromium-51 (51-Cr)	51-Cr is used to label target cells, with dead cells releasing the radioactive label which can be quantified	Direct, specific measurement of target cell death	Safety concerns of radioisotope use
Calcein-AM	Non-fluorescent label is hydrolysed within target cells to fluorescent product which can be used to quantify target cell viability	Direct, specific and non-radioactive measurement of target cell death.	Can leak from labelled cells over time meaning assay duration is limited. Leakage also causes high assay background
LDH Release / Dead Cell Protease Activity	Measurement of enzymes that are released from cells upon death are detected and quantified	Simple add and read format	Measure non-specific death as incapable of discriminating between target and effector cell death during the assay.
Luciferase Labelled Target Cells	Target cells are engineered to endogenously express luciferase which can be used to measure viability	Specific target cell viability measured without effector cell interference allowing a large range of potential assay formats. Low background. Safe to use. Can be used for longer assay durations.	Engineering target cells can be labour intensive.
Real-Time Cell Analysis	Using noninvasive electrical impedance, cell index (which correlates to cell number) can be measured.	Real-time, kinetic measurement of T cell mediated target cell death	Assay development requires significant optimisation and limited throughput. Target cells must be adherent

PROLIFERATION AND ACTIVATION MARKERS

How target cell death will be measured dictates the format of a T cell mediated cytotoxicity assay. Several approaches are available, each with their own benefits and challenges:

Method	Description
Proliferation	During early T cell activation, proliferation occurs and can be measured using common proliferation reagents such as BrdU.
Cell Membrane Markers	T cell status can be monitored using membrane expressed markers of activation such as CD69 or CD25 among others
Cytokine Markers	Cytokines such as IFN-γ and TNFα are upregulated following T cell activation and can be quantified in the cell culture media

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