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- Raji-hPD-L1 Cells

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# Immune Checkpoint Blockade: InestimAble Advances

Over the last decade, the understanding of key steps in the regulation of T cell responses has led to the groundbreaking development of immune checkpoint blocking monoclonal antibodies (mAbs) to fight cancer. The first FDA-approved mAbs have provided unprecedented remissions in melanoma and non-small cell lung cancer, although with considerable variation in response rates (10% to 90%) and significant toxicity<sup>1,2</sup>. This revolution in cancer therapy is now the basis for new IC-based curative strategies.

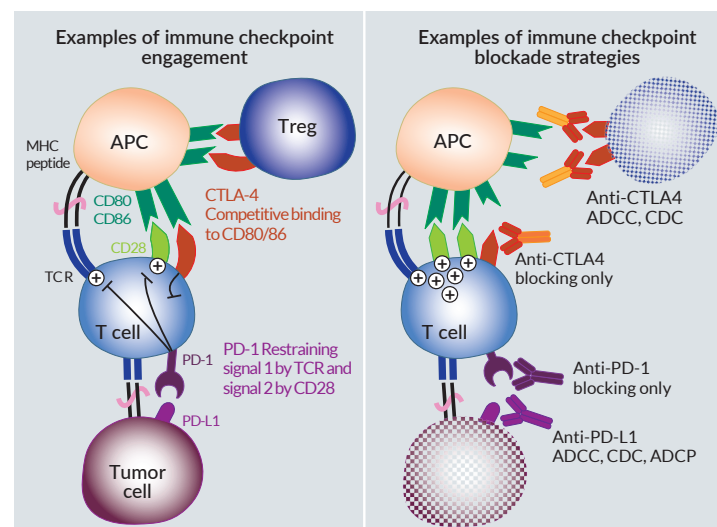
T-cell responses first rely on the T-cell receptor (TCR) recognition of MHC:peptide complexes on antigen presenting cells (APCs). Further engagement of co-stimulatory and co-inhibitory molecules guarantees the onset and the limitation of T-cell activities. These molecules have rightfully been named “immune checkpoints” (ICs). Initial studies focused on relieving the immunosuppressive brake by the co-inhibitory CTLA-4 (cytotoxic T lymphocyte associated protein 4) and PD-1 (programmed cell death 1) receptors<sup>3</sup>. CTLA-4 is expressed by activated and regulatory T cells (Tregs), and exerts competitive binding for stimulatory CD28 ligands (CD80/CD86). PD-1 is expressed by activated and exhausted T cells, and binding to its ligands PD-L1 and PDL-2 directly inhibits TCR signaling through SHP2-mediated dephosphorylation of proximal signaling elements<sup>3</sup>. Of note, recent findings point CD28 as a convergent regulation target for both CTLA-4 and PD-1, and call attention to the regulation of intra-tumoral T-cell trafficking by PD-1<sup>3</sup>.

Therapeutic IC-blocking mAbs have a dual activity inherent to their structure: while the variable regions bind to IC-epitopes, the “fragment crystallizable” (Fc) mediates targeted cell death through selective interaction with the complement molecule C1q (CDC: complement dependent cytotoxicity) and the Fc receptors on innate effector cells (ADCC: antibody dependent cellular cytotoxicity, ADCP: antibody dependent cellular phagocytosis)<sup>4</sup>. To date, approved IC-mAbs are IgG1s and IgG4s depending on the

necessity to protect or kill the target cells<sup>5</sup>. Anti-CTLA4 ipilimumab and anti-PDL1 atezolizumab are IgG1s that are expected to cause preferential Treg and tumor cell depletion, respectively<sup>1,3</sup>. On the contrary, anti-PD1 nivolumab & pembrolizumab are modified IgG4s with low effector functions, mainly operating through blocking PD-1 interaction with its ligands<sup>1,5</sup>. Altogether, these IC-mAbs allow better activation of effector T cells, and the combination of anti-CTLA4 and anti-PD1/PDL1 improves survival<sup>1,3</sup>.

The challenge of IC immunotherapy is to improve the mAb response rates in a larger panel of cancers. A first approach is to modulate mAbs' functionality through Fc engineering<sup>6</sup> (see next page). For example, atezolizumab is a non-glycosylated IgG1 that retains its blocking activity but lacks cytotoxic functions<sup>3</sup>. New anti-CTLA4 mAbs with increased or dampened effector functions have also been developed and are under clinical trials<sup>1</sup>. Other strategies targeting more co-inhibitory molecules on T cells (e.g. LAG3, TIM-3, TIGIT, VISTA) have entered clinical trials, even though their biological roles is not fully understood<sup>3,7</sup>. The next generation of therapeutic mAbs also includes agonist agents targeting co-stimulatory molecules (OX40, ICOS, GITR, 4-1BB, CD40) on T cells to potentiate effector responses<sup>3,7</sup>. Importantly, combination of the above-mentioned approaches hold important promises<sup>7</sup>.

The future of anti-tumor immunotherapy relies on the induction of responses at multiple levels, including harnessing of other effector cells (e.g. natural killers, neutrophils) in addition to T cells. As an example, anti-NKG2A monalizumab blocks inhibitory signaling in natural killer cells and subsets of cytotoxic T cells, and further potentiates other therapeutic mAbs<sup>8</sup>. The identification of reliable predictive biomarkers and the combination of IC-therapies with other immunotherapies (e.g. adoptive T cell-therapy, oncolytic viruses, agonists for pattern recognition receptors), and radio/chemotherapies, may lead to the ultimate maximization of response rates<sup>9</sup>.



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2. Ribas A. and Wolchok J.D. 2018. Cancer immunotherapy using checkpoint blockade. *Science*. 359:1350.
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8. André P. et al. 2018. Anti-NKG2A mAb is a checkpoint inhibitor that promotes anti-tumor immunity by unleashing both T and NK cells. *Cell*. 175:1731.
9. Marshall H.T. and Djamgoz B.A. 2018. Immuno-oncology: emerging targets and combination therapies. *Front. Oncol.* 8:315.

# Immune Checkpoint Antibodies

To meet your needs in the development and study of therapeutic monoclonal antibodies (mAbs) against immune checkpoints (ICs), InvivoGen offers a series of clinically relevant mAbs targeting CTLA-4, PD-1 or PD-L1, either in their original format, or with different/engineered isotypes conferring altered effector functions.

- Anti-hCTLA4 Isotype Family
- Anti-hPD1 Isotype Family
- Anti-hPD-L1 Isotype Family

InvivoGen's IC mAbs feature the Fab (fragment antigen binding) region of approved immune checkpoint inhibitors (ICIs; see table) and the Fc (crystallizable fragment) region of different immunoglobulin isotypes (see below), including the original. ICIs induce variable effector functions: ADCC (antibody dependent cellular cytotoxicity), ADCP (antibody dependent cellular phagocytosis), and CDC (complement dependent cytotoxicity). Depending on the necessity to protect or kill the target cells, ICIs' functions can be modulated through modification of the Fc region.

InvivoGen's IC antibodies are fully human mAbs. They are generated by recombinant DNA technology and produced in CHO cells. Their sequence, isotype, and binding activity are thoroughly verified.

## InvivoGen ICI isotypes

	Native isotypes			Engineered isotypes		
	IgG1	IgG2	IgA2	IgG1Nq	IgG1fut	IgG4 (S228P)
ADCC	++	+/-	+/-	-	+++	+/-
ADCP	+++	+/-	+	-	+++	+
CDC	++	+	-	+/-	++	-

### Potent effector function-inducing isotypes

**IgG1** is the isotype of the majority of approved mAb therapies (e.g. anti-CTLA4 ipilimumab and anti-CD20 rituximab). It induces potent ADCC, ADCP and CDC, and thus can engage both humoral and cellular components of the immune system. IgG1-induced ADCC can be increased by defucosylation of the glycan sequences (**IgG1fut**). This modification, obtained by using a specific CHO cell line, enhances the mAb binding to FcγRIIIa/CD16. The approved anti-CD20 obinutuzumab is an engineered mAb with reduced fucose content. Also, a non-fucosylated variant of ipilimumab is currently under clinical trials.

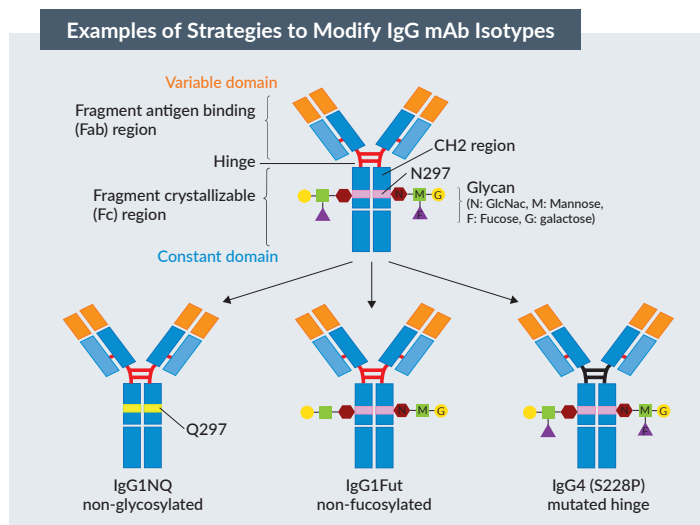
### Reduced effector function-inducing isotypes

**IgG1Nq** and **IgG1 (N298A)** are engineered isotypes with a mutation in glycosylation sites of the CH2 domain, at position 297 (Asparagine (N) to Glutamine (Q)), and 298 (Asparagine (N) to Alanine (A)), respectively. These non-glycosylated mAbs, such as the anti-PD-L1 atezolizumab, mostly act as blocking agents. They induce no ADCC nor ADCP, and only minimal CDC.

**IgG2** induces poor ADCC and ADCP, while retaining some CDC function. Tremelimumab is an IgG2 targeting CTLA4 under clinical trials.

**IgG4 (S228P)** is an IgG4 engineered isotype that displays reduced ADCC, ADCP and no CDC. A Serine to Proline substitution at position 228 (S228P) in the hinge region prevents Fab arm exchanges frequently occurring between IgG4 molecules. IgG4 (S228P) mAbs, such as the anti-PD-1 nivolumab and pembrolizumab, mostly act as blocking agents.

**IgA2** is a native isotype inducing low ADCC and ADCP, and no CDC. Although not yet introduced in clinical trials, IgAs have shown promising results in pre-clinical studies.



PRODUCT	CAT. CODE
<b>Anti-hCTLA4 antibodies, variable regions of ipilimumab</b>	
Anti-hCTLA4-hlgG1	hctla4-mab1
Anti-hCTLA4-hlgG1Nq	hctla4-mab12
Anti-hCTLA4-hlgG1fut	hctla4-mab13
Anti-hCTLA4-hlgG2	hctla4-mab2
Anti-hCTLA4-hlgG4 (S228P)	hctla4-mab14
Anti-hCTLA4-hlgA2	hctla4-mab7
<b>Anti-hPD1 antibodies, variable regions of nivolumab</b>	
Anti-hPD1-Ni-hlgG1	hpd1ni-mab1
Anti-hPD1-Ni-hlgG1Nq	hpd1ni-mab12
Anti-hPD1-Ni-hlgG1fut	hpd1ni-mab13
Anti-hPD1-Ni-hlgG2	hpd1ni-mab2
<b>Anti-hPD1-Ni-hlgG4 (S228P)</b>	hpd1ni-mab114
Anti-hPD1-Ni-hlgA2	hpd1ni-mab7
<b>Anti-hPD1 antibodies, variable regions of pembrolizumab</b>	
Anti-hPD1-Pem-hlgG1	hpd1pe-mab1
Anti-hPD1-Pem-hlgG1Nq	hpd1pe-mab12
Anti-hPD1-Pem-hlgG2	hpd1pe-mab2
<b>Anti-hPD1-Pem-hlgG4 (S228P)</b>	hpd1pe-mab14
Anti-hPD1-Pem-hlgA2	hpd1pe-mab7
<b>Anti-hPD-L1 antibodies, variable regions of atezolizumab</b>	
Anti-hPD-L1-hlgG1	hpd1l-mab1
<b>Anti-hPD-L1-hlgG1 (N298A)</b>	hpd1l-mab12
Anti-hPD-L1-hlgG1fut	hpd1l-mab13
Anti-hPD-L1-hlgG2	hpd1l-mab2

All antibodies are provided as 100 µg units. Larger quantities are available upon request.

Please visit our website for a full list of InvivoGen's engineered antibody isotype families.



# ADCC Reporter Cell-Based Assay

NEW

InvivoGen's antibody dependent cellular cytotoxic (ADCC) reporter cell-based assay is an alternative to classical assays mainly relying on natural killer (NK) cell cytotoxic activity. Our bioassay uses an engineered human T- cell line to monitor NFAT (nuclear factor of activated T-cells) activation, an early signaling event in ADCC induction. The level of ADCC induction is measured as a bioluminescent signal produced by an NFAT-dependent Lucia luciferase reporter protein.

- **Robust and reliable:** highly reproducible results
- **Rapid:** bioassay performed in a single day (7 hours total incubation)
- **Convenient:** - pick and choose among our different target cells  
- optimized effector to target cell ratios
- **Cost-effective:** unlimited use of the cell lines

ADCC is an immune mechanism through which Fc receptor-bearing effector cells, such as NK cells, can recognize and kill antibody-coated target cells expressing antigens on their surface. ADCC is triggered by the cross-linking between antigen-bound antibodies and the Fc receptor CD16A (FcγRIIIA) at the surface of immune effector cells. These interactions induce the increase of intracellular calcium concentrations and the translocation of the NFAT transcription factor to the nucleus, where it can bind to the promoter regions of ADCC relevant genes.

## Reporter effector cell line

### • Jurkat-Lucia™ NFAT-CD16 Cells

InvivoGen's Jurkat-Lucia™ NFAT-CD16 cells have been engineered from the human T-lymphocyte Jurkat cell line. They stably express the Lucia luciferase reporter gene under the control of a minimal promoter and six NFAT response elements, along with the cell surface Fc receptor CD16A (FcγRIIIA; V158 high affinity allotype). Jurkat cells naturally express a functional NFAT pathway.

The Jurkat-Lucia™ NFAT-CD16 cells have been functionally tested with various target cells and specific mAb isotype combinations, with a low EC<sub>50</sub> correlating with higher ADCC potencies.

These cells are resistant to Blasticidin and Zeocin™.

## Immune Checkpoint Inhibitor Target Cells

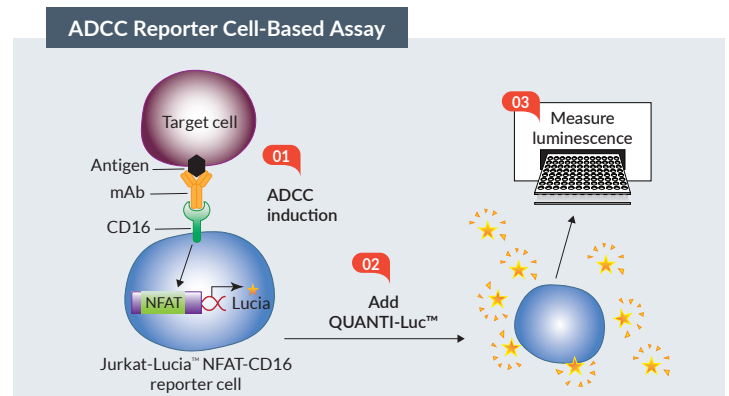
- Raji-hCTLA4 Cells
- Raji-hPD-1 Cells
- Raji-hPD-L1 Cells

These three cell lines belong to an expanding collection of Raji (human B lymphocyte)-derived target cells that stably express not only immune checkpoints, such as human CTLA-4, human PD-1, or human PD-L1, but also tumor antigens\*, such as HER2, EGFR, and CD20 (an antigen naturally expressed by Raji cells).

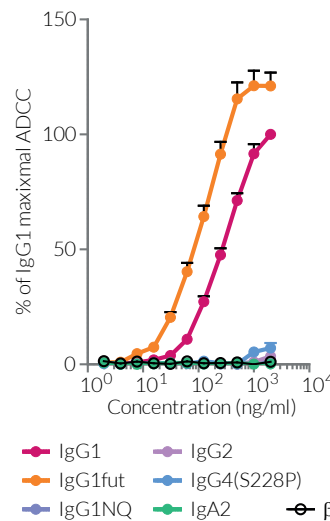
Raji-hCTLA4, Raji-hPD-1 and Raji-hPD-L1 are used as part of InvivoGen's quality control for immune checkpoint anti-human antibodies featuring engineered isotypes.

These cells are resistant to Blasticidin.

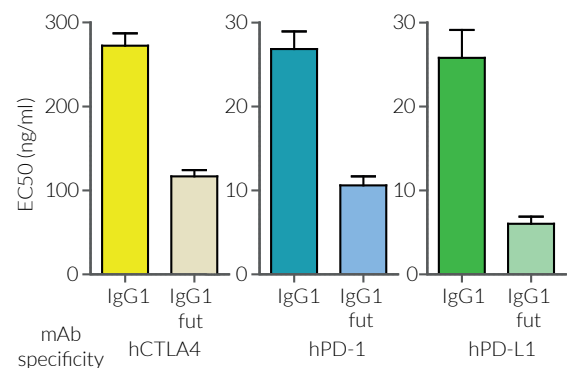
PRODUCT	QUANTITY	CAT. CODE
Jurkat-Lucia™ NFAT-CD16 cells	3-7x10 <sup>6</sup> cells	jktl-nfat-cd16
Raji-hCTLA4 cells	3-7x10 <sup>6</sup> cells	raji-hctla4
Raji-hPD-1 cells	3-7x10 <sup>6</sup> cells	raji-hpd1
Raji-hPD-L1 cells	3-7x10 <sup>6</sup> cells	raji-hpd11



**ADCC reporter cell-based assay procedure.** 1- **ADCC Induction:** the target cells expressing surface antigens are incubated with specific mAbs for 1 hour before the addition of Jurkat-Lucia™ NFAT-CD16 effector cells at a determined target:effector ratio for 6 hours. 2- **Addition of Lucia luciferase detection reagent QUANTI-Luc™:** detection reagent is added to supernatant samples. 3- **ADCC measure:** bioluminescent signal is detected using a luminometer.



Example of a comparison of ADCC potency for native and engineered isotypes of an anti-human CTLA4 using Raji-hCTLA4 target cells. Raji-hCTLA4 cells were incubated with gradient concentrations of Anti-hCTLA4 or Anti-β-galactosidase mAbs for 1 hour. Jurkat-Lucia™ NFAT-CD16 effector cells were then co-incubated with targets cells for 6 hours. NFAT activation, reflecting the induced ADCC response, was assessed by determining Lucia luciferase activity in the supernatant using QUANTI-Luc™. Percentages of the maximal response normalized to the IgG1 isotype are shown. See left page for each isotype description.



**Increased ADCC activity mediated by IgG1 compared to IgG1fut (non-fucosylated):** Raji-hCTLA4, -hPD-1, and -hPD-L1 cells were incubated with Jurkat-Lucia™ NFAT-CD16 effector cells and corresponding IgG1 or IgG1fut specific mAbs. The data represent the EC<sub>50</sub> for each antibody.

# pTRIOZ plasmids NEW

InvivoGen now offers pTRIOZ, next generation plasmids designed for large-scale production of whole monoclonal antibodies (mAbs). pTRIOZ contains distinct cassettes for expression of both heavy (H) and light (L) chains under the control of unique composite promoters in a single plasmid.

- **Reliable:** used in-house to produce InvivoGen's mAb collection
- **Cost-effective:** single plasmid for both the heavy and light chain
- **High Expression:** up to 30x more antibody produced

pTRIOZ is the newest member of InvivoGen's expanding collection of plasmids for the engineering and production of recombinant mAbs. This collection includes our best-selling and exhaustive pFUSE plasmid family designed for the expression of either the H or L chain. pFUSE plasmids are commonly used to change a mAb from one isotype to another, and from one species to another.

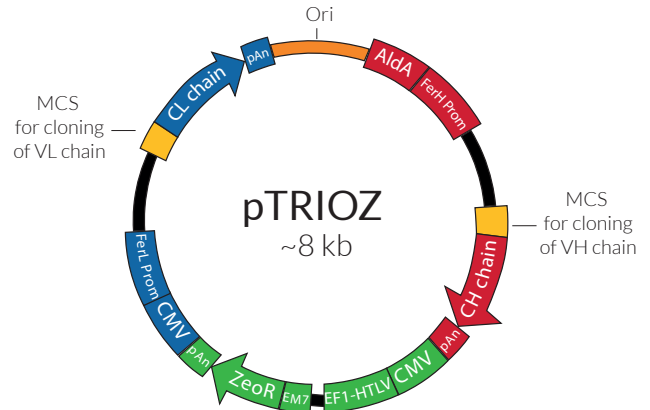
pTRIOZ is a single plasmid for high yield production of whole recombinant mAbs. For optimal mAb production, a precise expression ratio of the heavy to light chain is required. Therefore, similar to the pFUSE plasmids, the pTRIOZ plasmid utilizes a pair of promoters that drive the successful co-expression of the two human ferritin (Fer) subunits. pTRIOZ features:

- **Light chain cassette:** FerL promoter + MCS + constant (C) IgG L chain
- **Heavy chain cassette:** FerH promoter + MCS + constant (C) IgG H chain
- **Zeocin™ selection cassette:** enhancer + composite promoter + *Sh Ble* gene

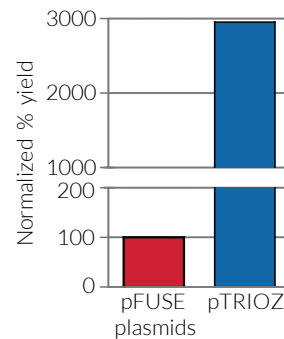
pTRIOZ is utilized in-house for the production of InvivoGen's mAbs. It is ideal for upscaled production of whole mAbs, with yields of up to 30x more antibody when compared to the two-plasmid (pFUSE) method. InvivoGen provides the 4 most popular isotypes used for mAb production. They have been optimized to ensure maximum expression and purification.

PRODUCT	CAT. CODE
pTRIOZ-hIgG1	ptrioz-higg1
pTRIOZ-hIgG4 (S228P)	ptrioz-higg4sp
pTRIOZ-mIgG1e2	ptrioz-migg1e2
pTRIOZ-mIgG2a	ptrioz-migg2a

All plasmids are provided as 20 µg lyophilized DNA.



**pTRIOZ general plasmid map.** pTRIOZ contains three distinct cassettes with composite promoters for the optimized expression of the H and L chains, as well as the *Sh ble* gene conferring resistance to Zeocin™. It contains two unique multiple cloning sites (MCS) for the insertion of the variable H and L chains.



**Comparison between pTRIOZ and pFUSE mAb yield.** For the production of anti-PDL-1, an optimized 1:1 ratio of the pFUSE heavy chain and pFUSE light chain plasmids was tested alongside pTRIOZ. The maximum yield is normalized to the pFUSE ratio.

[www.invivogen.com/pTRIOZ](http://www.invivogen.com/pTRIOZ)

## Zeocin™

### Selective antibiotic

- **Exclusive manufacturer:** InvivoGen's proprietary antibiotic
- **Convenient:** for both prokaryotic and eukaryotic selection
- **High quality:** cell-culture tested, endotoxin level < 1 EU/mg

Zeocin™ is a copper-chelated glyco-peptide selective antibiotic that causes cell death by intercalating with, and cleaving DNA. InvivoGen is the sole manufacturer of Zeocin™ worldwide, thus ensuring the highest quality. Zeocin™ is subject to rigorous in-house testing to guarantee the absence of bacterial contamination and maximum purity. Zeocin™ has minimal cytotoxicity and can be used for a number of applications. Zeocin™ is a popular and effective antibiotic for vector selection in both prokaryotic (e.g. *E. coli*) and eukaryotic (e.g. CHO cells, yeast) cell types.

InvivoGen provides a wide range of products that utilize Zeocin™ for strict selection including our large collection of reporter cell lines as well as many cloning and expression vectors.



PRODUCT	QUANTITY	CAT. CODE
Zeocin™ (solution)	1g (10 x 1ml)	ant-zn-1
Zeocin™ (solution)	5g (50 x 1ml)	ant-zn-5
Zeocin™	Bulk	Available upon enquiry

Please see the website for our full range of selective antibiotics.

[www.invivogen.com/zeocin](http://www.invivogen.com/zeocin)