InvivoGen insight

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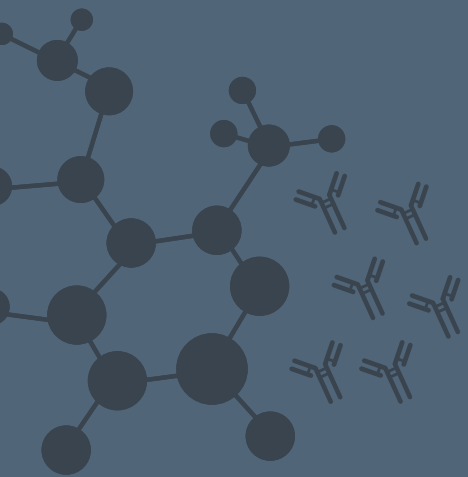


InvivoGen
www.invivogen.com
2023

TLR3: racing for vaccine advantages

Toll-like receptors (TLRs) play a pivotal role in the initiation of prompt innate immune defenses, as well as in the activation of adaptive immune cells for enhanced and memory responses. Thus, TLR agonists are attractive candidates for vaccine adjuvants and cancer therapeutics. Yet, major challenges limit their translation from bench to bedside. As of today, hundreds of TLR ligands have been identified, but only one TLR4 agonist (MPL®, monophosphoryl lipid A) and one TLR9 agonist (CpG 1018®) have been approved for anti-infectious vaccine adjuvantation[1,2]. One TLR7 agonist (Imiquimod) is currently used as a stand-alone anti-viral and anti-cancerous drug[1,2]. Excitingly, recent advances might now bring TLR3 under the spotlight.

TLR3 is expressed by immune cells, mostly conventional dendritic cells (cDCs), and non-immune cells, including transformed cells, as described in several epithelial cancers[3,4]. It senses intracellular double-stranded (ds)RNA which signs the presence of a virus as well as damaged cells[3-5]. TLR3 and dsRNA interaction is independent of the RNA sequence, but its length must be at least 40 bp for TLR3 dimerization and signaling[3,5].
Akin to TLR4, TLR3 triggering induces the simultaneous activation of IRF and NF-κB. In cDCs, it results in the upregulation of co-stimulatory molecules and the production of both type I IFNs and pro-inflammatory cytokines. Of note, IFN-α/β and IL-12 are critical for $CD8^+$ and Th1-biased $CD4^+$ T cell activation, respectively. $CD4^+$ Th1 cells, in turn, potentiate B cell activation and differentiation into plasmocytes[6-8]. Moreover, in tumors, TLR3 is reported to induce immunogenic apoptosis in cancer cells, while sparing normal cells. This allows effective uptake and MHC presentation of tumor-associated antigens by cDCs to specific $CD4^+$ and $CD8^+$ T cells[4].
Altogether, TLR3 expression and signaling pathways in normal and malignant cells make it a one-of-a-kind target for the development of prophylactic anti-infectious and therapeutic anti-tumoral vaccine adjuvants.

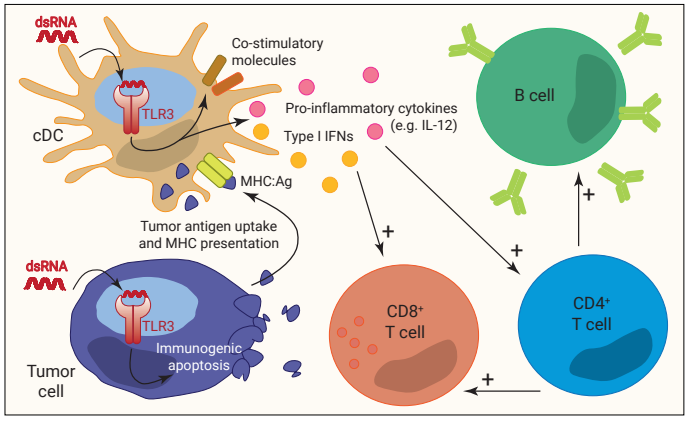
At least four criteria determine the access of TLR3 agonists to the restrictive circle of vaccine adjuvants approved by regulatory agencies: 1) compliance with current Good manufacturing Practices (cGMP), 2) tolerability and absence of major side effects, 3) potency to enhance antibody responses, as well as $CD4^+$ and $CD8^+$ T cells immunity, and 4) contribution to the generation of memory cells.

Poly(I:C) (polyinosinic polycytidylic acid), a mix of imperfectly annealed synthetic 1,5 to 8 kb dsRNAs and ssRNAs, was the first candidate for TLR3-targeted adjuvantation[1].

It was reported to efficiently activate DCs and potentiate antigen-specific T cell and B cell proliferation in preclinical studies[1,9]. However, it was also reported to have toxic side effects in humans, probably conferred by its undefined chemical structure and poor homogeneity[2,7,8]. Therefore, more stable and/or safer derivatives of Poly(I:C) were developed, and entered clinical trials. For example, Poly-ICLC, Poly-$IC_{12}U$, and PIKA were included in vaccination strategies against HIV, Influenza, Rabies lyssavirus, and more recently, SARS-CoV-2[7]. Regarding cancer treatments, Poly-ICLC and Poly-$IC_{12}U$ have been tested in combination with other therapies in a range of solid tumors[2,7,8]. To date, these molecules have reached phase I or phase II, and closed trials are awaiting results[2,7,8]. Importantly, they still present a lack of homogeneity due to technical limitations in the manufacturing process.

Continual efforts to find potent and lot-to-lot reproducible TLR3 agonists have led to the development of two new promising adjuvants.
NexaVant™ is a synthetic, cGMP grade, 424 bp dsRNA generated through *in vitro* transcription of a viral nucleotide segment[10]. It exhibits extreme homogeneity and absence of serious toxicity in preclinical studies[10]. It is a potent inducer of both IRF and NF-κB activation in cellular assays *(see page 3)*[10]. Moreover, it promotes effective DC activation and migration into draining lymph nodes, as well as antigen-specific B, $CD4^+$ Th1 and $CD8^+$ T cell responses in mouse immunization models[10].
TL-532 is another homogenous synthetic dsRNA of 70 bp length[11]. It is safe in non-human primates, and triggers activation of the immune system with anti-cancer effects in mice[11,12].

Different delivery platforms, such as aluminum salts, oil/water emulsions, lipids, or polymer nanoparticles, are available and allow prolonged bioavailability of immunostimulants together with specific antigens[2]. These formulations aim at delivering the lowest possible doses of adjuvant to elicit robust immune responses and avoid systemic side-effects. The next challenge is now to find the best formulations for the new TLR3 agonists, depending on the vaccination intended responses.



1. Ong, G.H., *et al.*, 2021. Front Cell Infect Microbiol, 2021. 11: p. 745016. **2. Zhao, T., *et al.*, 2023.** Signal Transduct Target Ther, 8(1): p. 283. **3. Chen, Y.G. and S. Hur, 2022.** Nat Rev Mol Cell Biol, 23(4): p. 286. **4. Estornes, Y., *et al.*, 2017.** TRAIL, Fas Ligand, TNF and TLR3 in Cancer, Resistance to Targeted Anti-Cancer Therapeutics, p159-185. **5. Kawai, T. and S. Akira, 2008.** Annals of the New York Academy of Sciences, 1143(1): p. 1-20. **6. Komal, A., *et al.*, 2021.** Immunol Res. 69(4): p. 312-322. **7. Yang, J.-X., *et al.*, 2022** Pharmaceutics, 14(2): p. 423. **8. Yang, Y., *et al.*, 2022** Front Immunol,13:1049340. **9. Robinson, R.A., et al., 1976.** JNCI: Journal of the National Cancer Institute, 57(3): 599. **10. Ko, K.H., *et al.*, 2023.** Front Immunol, 14:1075291. **11. Thierry, S., *et al.*, 2023.** Microb Cell. 10(6): p. 117-132. **12. Le Naour, J., *et al.*, 2023.** Oncoimmunology, 12(1): p. 2227510.

TLR3 dual reporter cell line

InvivoGen introduces the first HEK293-derived Dual™ cell lines allowing simultaneous monitoring of the NF-κB and IRF pathways upon Toll-like receptor (TLR) stimulation. The new cell line HEK-Dual™ hTLR3 is designed for the screening of agonists or inhibitors of human TLR3. Overexpression of this important dsRNA sensor ensures detection and quantification of potent as well as weak TLR3 agonists.

- **HEK-Dual™ hTLR3 Cells** NEW
- **HEK-Dual™ (control) Cells** NEW

Key Features

- Dual reporter system: NF-κB-SEAP and IRF-Lucia
- Stable overexpression of TLR3
- Highly responsive to TLR3 ligands

Toll-like receptor 3 (TLR3) is an endosomal sensor of double-stranded (ds)RNA, that signals through the adaptor TRIF. The subsequent activation of the transcription factors IRF3 and NF-κB leads to the production of type I IFNs and proinflammatory cytokines, respectively. It plays a key role in the anti-viral response and is involved in tumor regression. Thus, TLR3 is a target of interest for the development of anti-viral and anti-cancer therapies, as well as preventive or curative vaccines.

HEK-Dual™ hTLR3 cells overexpress human (h)TLR3 and the well-established Dual™ Reporter gene system, consisting of an NF-κB-inducible SEAP (secreted embryonic alkaline phosphatase) and IRF-inducible Lucia (secreted luciferase) reporter constructs. Thus, they allow the simultaneous monitoring of the NF-κB and IRF responses triggered by TLR3 agonists (e.g. Poly(I:C) HMW). In contrast, InvivoGen's cell lines HEK-Blue™ hTLR3 and HEK-Blue-Lucia™ hTLR3 only allow the monitoring of the NF-κB response *(Fig. 1)*. The NF-κB and IRF responses can be readily assessed by measuring the SEAP and Lucia activities in the supernatant of HEK-Dual™ hTLR3 cells, using QUANTI-Blue™ and QUANTI-Luc™ 4 Lucia/Gaussia detection reagents, respectively.

HEK-Dual™ hTLR3 cells are highly responsive to synthetic analogs of dsRNA, in contrast to their parental cell line **HEK-Dual™**. Indeed, HEK293-derived cells do express endogenous low levels of various PRRs, including TLR3. However, weak agonists (e.g. Poly(A:U)) might not trigger a quantifiable response, and thus, remain undiscovered in compound screenings *(Fig. 2)*.

PRODUCTS	QTY	CAT. CODE
HEK-Dual™ hTLR3 Cells	3 - 7 x 10^6	hkd-htlr3
HEK-Dual™ Cells	3 - 7 x 10^6	hkd-nfis
Poly(I:C) HMW	10 mg	tlrl-pic
Poly(A:U)	10 mg	tlrl-pau

RELATED PRODUCTS

- **HEK-Blue™ hTLR3 cells:** NF-κB reporter cell line (hkb-htlr3)
- **HEK-Blue-Lucia™ hTLR3 cells:** Double NF-κB reporter cell line (hkd-htlr3ni)
- **QUANTI-Blue™:** SEAP detection reagent, liquid (rep-qbs)
- **QUANTI-Luc™ 4:** Luciferase detection reagent, liquid (rep-qlc4lg1)

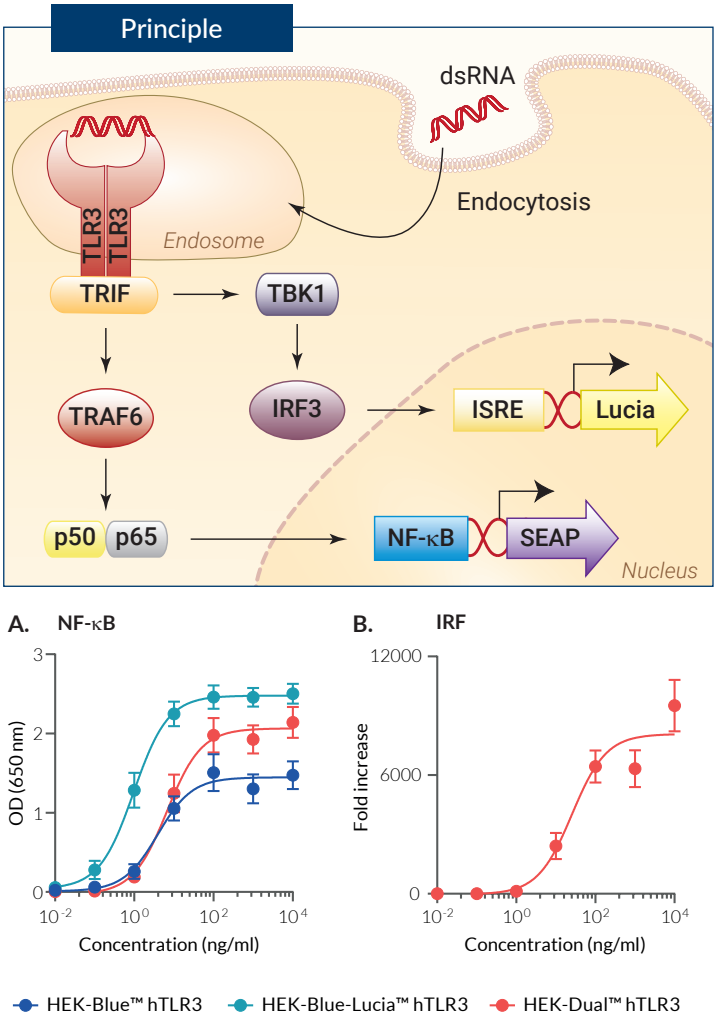


Figure 1. NF-κB and IRF responses in HEK293-derived cell lines upon stimulation with Poly(I:C) HMW. HEK-Blue™ hTLR3, HEK-Blue-Lucia™ hTLR3, and HEK-Dual™ hTLR3 cells were cultured with increasing concentration of Poly(I:C) HMW. After 24h, the TLR3-induced **(A)** NF-κB and **(B)** IRF responses were assessed by measuring SEAP and Lucia activity, respectively. Data are shown as optical density (OD) at 650 nm or in fold response over non-induced cells (mean + SEM).

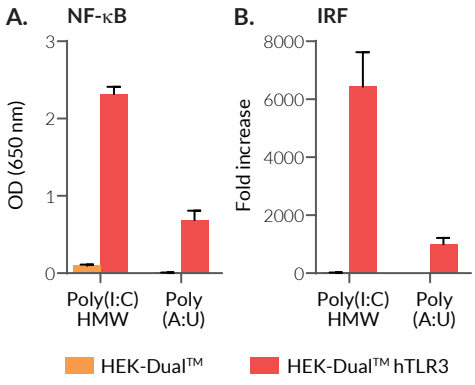


Figure 2. NF-κB and IRF responses in HEK-Dual™-derived cells to strong and weak TLR3 agonists. HEK-Dual™ and HEK-Dual™ hTLR3 cells were incubated with 1 µg/ml of Poly(I:C) HMW and 100 µg/ml of Poly(A:U). The TLR3-induced **(A)** NF-κB and **(B)** IRF responses were assessed and data are shown as described above.

TLR3 agonist and vaccine adjuvant

InvivoGen adds NexaVant™, a novel TLR3 agonist and promising vaccine adjuvant to its extensive collection of pattern recognition receptor ligands. It is available in two grades for *in vitro* and *in vivo* use. All of our high-quality ligands are guaranteed free of bacterial contaminants and functionally tested.

- NexaVant™ **NEW**
- NexaVant™ Vaccigrade™ **NEW**

InvivoGen provides **NexaVant™**, a synthetic dsRNA of 424 base pairs produced by performing PCR-coupled bidirectional *in vitro* transcription of a viral nucleotide segment using T7 RNA polymerase technology.

It displays high purity (≥ 95%) and molecular homogeneity, therefore overcoming various obstacles of currently available dsRNA-based adjuvant candidates[1], including Poly(I:C) *(Fig. 1A)*. **NexaVant™** induces strong NF-κB and IRF responses in HEK-Dual™ hTLR3 cells; although not as potent as Poly(I:C) HMW *(Fig. 1B)*. However, it has been shown to trigger no serious toxicity in preclinical studies and to enhance antigen-specific antibody responses in mice *(Fig. 2)*[1].

NexaVant™ is available in a ready-to-use liquid formulation in two grades, standard for *in vitro* TLR3 studies, and Vaccigrade™ to test its potential as a vaccine adjuvant *in vivo*.

NexaVant™ is a trademark that belongs to NA Vaccine Institute.

1. Ko KH, *et al.*, 2023. Front Immunol. ;14:1075291.

PRODUCTS	QTY	CAT. CODE
NexaVant™	100 µg	tlrl-nvt
NexaVant™ Vaccigrade™	100 µg	vac-nvt

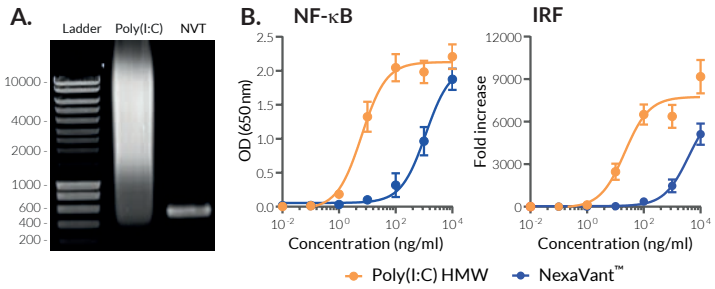


Figure 1. Comparison of NexaVant™ and Poly(I:C) HMW. (A) Gel detection of NexaVant™ (NVT, 100 ng) and Poly(I:C) HMW (10 µg). **(B)** HEK-Dual™ hTLR3 cells were incubated with increasing concentrations of NVT and Poly(I:C) HMW. After 24h, the TLR3-induced NF-κB and IRF responses were assessed by measuring SEAP and Lucia activity. Data are shown as OD or in fold response (mean + SEM).

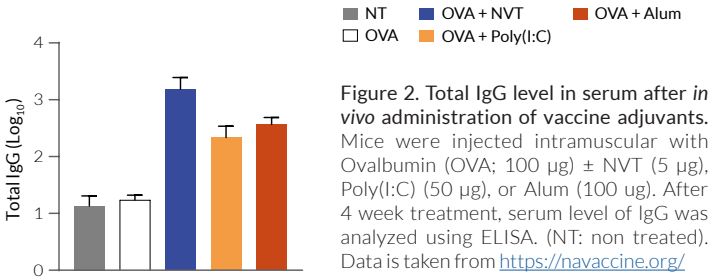


Figure 2. Total IgG level in serum after *in vivo* administration of vaccine adjuvants. Mice were injected intramuscular with Ovalbumin (OVA; 100 µg) ± NVT (5 µg), Poly(I:C) (50 µg), or Alum (100 ug). After 4 week treatment, serum level of IgG was analyzed using ELISA. (NT: non treated). Data is taken from https://navaccine.org/

Type I interferon inhibitor

InvivoGen offers recombinant B18R, a specific inhibitor of type I IFN-mediated signaling, widely used to increase RNA-mediated gene delivery.

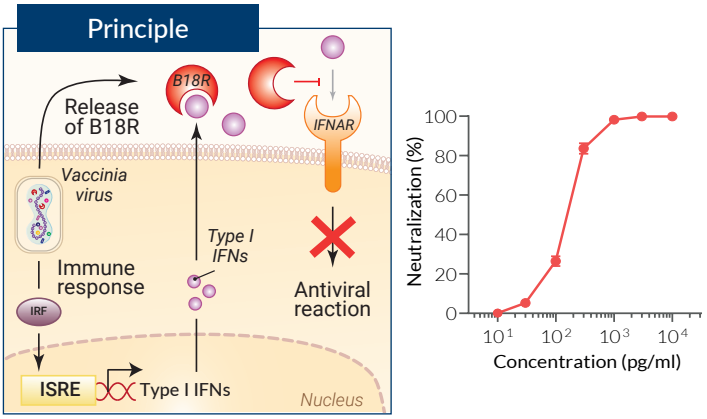
- Recombinant B18R **NEW**

B18R is encoded by the Western Reserve (WR) strain of the vaccinia virus. This soluble protein works as a potent decoy receptor of type I interferons (IFNs). It binds human IFN-α/β and prevents anti-viral responses in infected cells[1]. Thus, recombinant B18R has become an essential tool in cell technologies which use RNA-mediated gene delivery, such as epigenetic reprogramming. It is able to maintain RNA-replicons and increase the viability of cells upon transfection with synthetic mRNA[1].

InvivoGen's **recombinant B18R protein** is produced in CHO cells, provided carrier-free, His-tagged, and functional tested. It successfully blocks type I IFN-mediated signaling in HEK-Blue™ IFN-α/β cells stimulated with recombinant hIFN-α *(Fig.1)*.

1. Warren L, *et al.*, 2010. Cell Stem Cell.; 7(5):618-30.

PRODUCTS	QTY	CAT. CODE
Recombinant B18R	25 µg	inh-b18r



Decoy receptor activity of B18R upon viral infection (principle).

Figure 1. Type I-IFN inhibition by B18R (right). Increasing concentrations of B18R were incubated for 1 hour with 0.1 ng/ml of recombinant hIFN-α2b prior to the addition of HEK-Blue™ IFN-α/β cells. After 24h, SEAP activity was assessed using QUANTI-Blue™. Data are shown in percentage of neutralization (mean + SEM).

RELATED PRODUCTS

- **HEK-Blue™ IFN-α/β:** Type I Interferon reporter cells (hkb-ifnabv2)
- **Recombinant human IFN-α2b:** IFN with HSA (rcyc-hifna2b)

Dual inducible reporter plasmids

InvivoGen provides the pNiFty plasmid collection to monitor pattern recognition receptor (PRR) and cytokine signaling through the inducible expression of reporter genes. These NF-κB- or IRF-inducible reporter plasmids were designed to help you develop exclusive Dual™ reporter cells. They are available with various antibiotic resistances and reporter genes to ensure maximum flexibility and customization to your needs.

- pNiFty2 - NF-κB reporter plasmids **NEW**
- pNiFty3 - IRF reporter plasmids **NEW**

Key Features

- Dual pathway monitoring: NF-κB and IRF
- Choice of reporter: SEAP, Lucia, Firefly, Renilla
- Flexible: Blasticidin, Puromycin, Zeocin® resistances

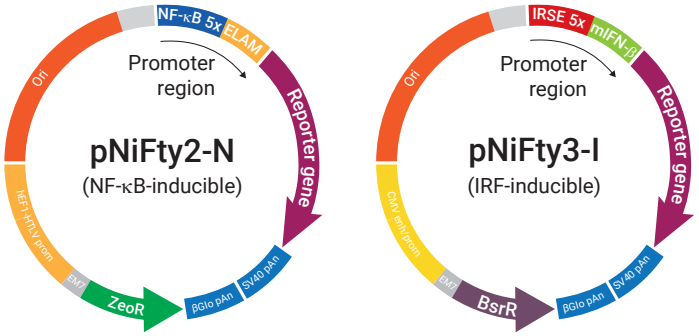


Figure 1. Plasmid maps of pNiFty2-N and pNiFty3-I. All pNiFty plasmids are composed of the following key elements: a proximal promoter region, an inducible reporter gene, and an antibiotic resistance.

Although InvivoGen already provides a number of ready-to-use Dual™ reporter cell lines including THP1-Dual™, Jurkat-Dual™, and the novel HEK-Dual™, everyone's research is unique and requires flexible tools.

The pNiFty reporter plasmids **pNiFty2-N and pNiFty3-I** were designed to monitor pattern recognition receptor and cytokine signaling upon NF-κB and IRF activation, respectively. They are available with different antibiotic resistances including Blasticidin, Puromycin, or Zeocin® and reporter genes, such as SEAP, Lucia, Firefly, or Renilla luciferases.

These **inducible plasmids** allow you to create ideal **Do-it-yourself Dual™ reporter cells** in the species/tissue background of choice and develop the cell-based assay for your application of interest.

Create your own dual reporter cell lines

Gain an in-depth understanding of how your therapeutic molecule simultaneously affects the NF-κB and IRF signaling pathways. Generate your own **Dual™** reporter cells by following three simple steps:

- **Choose a parental cell type/line**

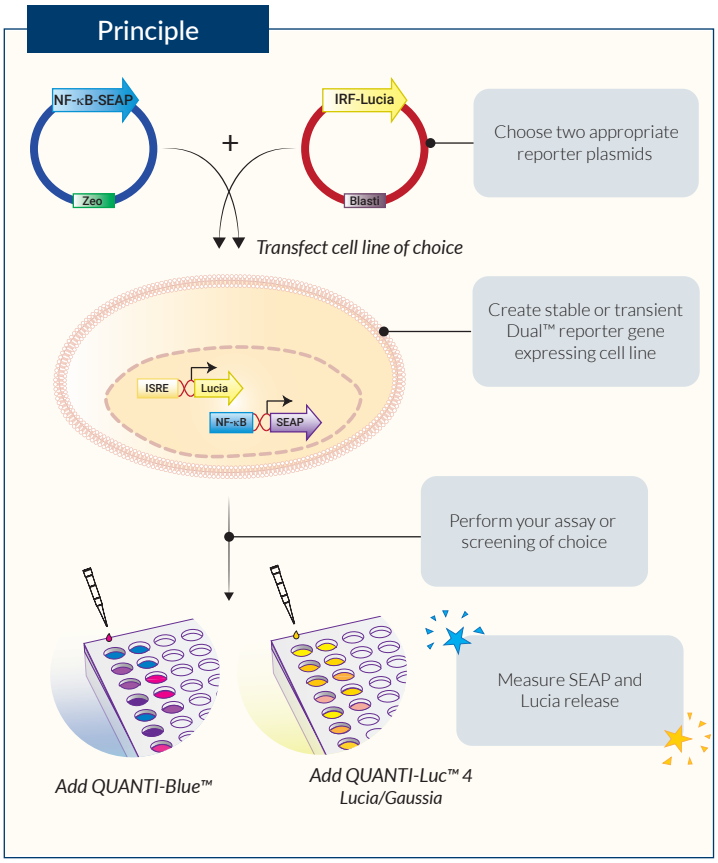
Choose a parental cell type that suits your assay setup.

- **Co-transfect two inducible reporter plasmids**

Select two inducible reporter plasmids. They are available comprising different reporter genes and antibiotic resistances.

- **Assess the NF-κB and IRF-mediated responses**

Perform your assay of interest and use InvivoGen's fine-tuned detection reagents to quantify the modulatory effects of your compound.



PRODUCTS	DESCRIPTION	CAT. CODE	
pNiFty2-N family Zeocin® resistant	NF-κB-inducible reporter plasmids	pnf2-sp1 pnf2-fluc	pnf2-lc pnf2-rluc
pNiFty3-I family Blasticidin resistant	IRF-inducible reporter plasmids	pnf3b-sp4 pnf3b-fluc4	pnf3b-lc4 pnf3b-rluc4

Learn more here www.invivogen.com/create-dual-cells

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