InvivoGen insight

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REVIEW

RIG-I and cancer immunotherapy

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- THP1-Dual™ KO-IRF3 Cells

Nucleic acid-signaling inhibitors

- BX795
- MRT67307
- RU.521
- H-151

NATE™ Nucleic acid transfection enhancer

InvivoGen
USA
10515 Vista Sorrento Pkwy
San Diego,
CA, 92121

T : +1 888 457 5873
F : +1 858 457 5843

info@invivogen.com
www.invivogen.com

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RIG-I & cancer immunotherapy

The development of immune checkpoint inhibitors (ICIs) has revolutionized cancer immunotherapy, although complete remission remains limited to a small panel of cancers and patients. ICIs act by relieving checkpoint restraints on antitumor T cell responses. They work best against immunogenic, T-cell inflamed or « hot » tumors. In contrast, ICIs are poorly efficient in «cold» tumor microenvironments (TMEs) that are largely devoid of T cells and infiltrated by immunosuppressive cells. In «hot» TMEs, increased expression of type I interferons (IFN-I) and IFN-stimulated genes (ISGs), such as apoptosis-inducing molecules and T-cell attracting chemokines, contribute to potent antitumor responses. Many therapeutic strategies are actively being explored to transform «cold» TMEs into «hot» ones. One emerging strategy exploits the adjuvancity of pattern recognition receptor (PRR) agonists. Indeed, combinations of ICIs with agonists of Toll-like receptor 9 (TLR9) or stimulator of interferon genes (STING) have reached clinical evaluation but have so far yielded disappointing preliminary results[1].

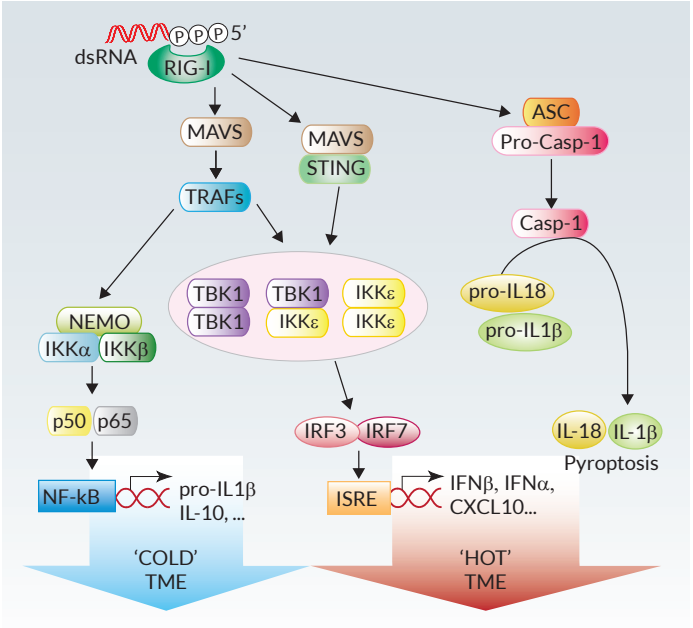
Retinoic acid-inducible gene I (RIG-I), a viral RNA sensor, is a promising alternative to enhance ICI efficacy[2,3]. RIG-I is the best-known member of the RIG-I-like helicase receptor (RLR) family. Unlike TLR9 and STING, RIG-I is expressed in virtually all cell types, including tumor cells. Preclinical studies have shown that systemic delivery of a synthetic RIG-I agonist inhibits tumor growth through mechanisms similar to those triggered for elimination of virally-infected cells[3]. RIG-I engagement leads to preferential tumor cell death (via intrinsic or extrinsic apoptosis, and inflammasome-induced pyroptosis), and to IFN-I-mediated activation of the innate and adaptive immune systems[4]. RGT100, a specific RIG-I agonist, is currently in phase I/II clinical trials for treatment of advanced solid tumors and lymphomas (NCT03065023)[4].

A growing number of studies have revealed multiple levels of complexity in the activation of the RIG-I pathway, starting from the agonist characteristics. RIG-I discriminates between viral and host RNA by recognizing the terminus of cytosolic short double-stranded RNA (dsRNA) containing 5' di- or tri-phosphates (2p- or 3pRNA). Additional features, such as higher-order structure, sequence, and RNA modifications, confer better affinity for RIG-I, although their impact *in vivo* remains largely unknown[4]. RIG-I signaling cascade is often presented in a simplified manner. Activated RIG-I interacts with the mitochondrial antiviral signaling (MAVS) adaptor, promoting the coordinated activation of TBK1 (TANK-binding kinase 1)/IKKε kinases and IKKα/IKKβ kinases. In turn, these kinases induce activation of IRF (interferon regulatory factor)-3 and -7, and of NF-κB, leading to the production of IFN-I and pro-inflammatory cytokines, respectively[4]. However, RIG-I signaling engages many other proteins, including TRIM25 and Riplet ubiquitinases, TRAF adaptors, and the inflammasome ASC protein.

Recently, elaborate regulation of RIG-I signaling has been revealed, providing hints for future clinical applications. RIG-I ligands, such as self 5' mono-phosphate dsRNA or viral-induced long non-coding RNA, exert antagonistic functions[4,5]. It has also been suggested that distinct TBK1/TBK1, TBK1/IKKε, or IKKε/IKKε complexes are at play depending on the cellular and stimulus context[8]. Additional complexity may also arise from preferential cellular expression of individual TRAFs, which mediate TBK1/IKKε activation differently[9]. Finally, other PRRs contribute to a transregulation of RIG-I signaling. While the NLR (NOD-like receptors) member NLRP12 controls TRIM25-mediated RIG-I activation and RNF125-mediated RIG-I degradation[6], RIG-I responses are potentiated through cross-talk with STING signaling[7].

RIG-I is a promising target for cancer immunotherapy, either as a single agent, or in combination with ICIs. Its major features are its ubiquitous expression and signaling outcomes, notably IFN-I production and preferential tumor cell death, which are two keys factors in potent T cell responses. However, RIG-I-based therapeutic strategies face multiple challenges, such as designing highly specific and stable agonists, and developing efficient agonist delivery modes while avoiding uncontrolled release of pro-inflammatory cytokines. Finally, a deeper understanding of RIG-I signaling in different TME cell types will be required to reach therapeutic success.

1. Iurescia S. *et al.*, 2018. Nucleic acid sensing machinery: targeting innate immune system for cancer therapy. Recent Pat. Anticancer Drug Discov. 13:2. **2. Heidegger S. *et al.*, 2019.** RIG-I activating immunostimulatory RNA boosts the efficacy of anticancer vaccines and synergizes with immune checkpoint blockade. EBioMedicine. 41:146. **3. Poeck H., *et al.* 2008.** 5'-triphosphate-siRNA: turning gene silencing and Rig-I activation against melanoma. Nat. Med. 14:1256. **4. Elion DL., *et al.* 2018.** Harnessing RIG-I and intrinsic immunity in the tumor microenvironment for therapeutic cancer treatment. Oncotarget. 9:29007. **5. Ren X. *et al.*, 2019.** RIG-I selectively discriminates against 5'-monophosphate RNA. Cell Reports. 26:2019. **6. Chen ST., *et al.*, 2019.** NLRP12 regulates anti-viral RIG-I activation via interaction with TRIM25. Cell Host Microbe. 25:602. **7. Zevini A. *et al.*, 2017.** Crosstalk between cytoplasmic RIG-I and STING sensing pathways. Trends Immunol. 38:194. **8. Perry AK. *et al.*, 2004.** Differential requirement for TANK-binding kinase-1 in type I interferon responses to Toll-like receptor activation and viral infection. J. Exp. Med. 199:1651. **9. Fang R. *et al.*, 2017.** MAVS activates TBK1 and IKKe through TRAFs in NEMO dependent and independent manner. PLoS Pathog. 13(11):e1006720.



Nucleic acid signaling reporter cell lines

InvivoGen offers an expanding collection of human (THP-1-derived) and murine (RAW 264.7-derived) reporter cells for the study of nucleic acid-induced signaling pathways. These cells are knockout (KO) for essential genes, such as the DNA sensor cGAS, the RNA sensor RIG-I, or the CDN (cyclic dinucleotide) sensor STING. Cells generated from the THP1-Dual™ cell line harbor two reporter systems: an IRF (interferon regulatory factor)-inducible Lucia luciferase, and an NF-κB-inducible SEAP (secreted embryonic alkaline phosphatase). Cells generated from the RAW-Lucia™ ISG cell line feature an IRF-inducible Lucia luciferase reporter gene. Two new cell lines have been added to this comprehensive collection.

- THP-1 Dual™ KO-TBK1 Cells NEW
- THP-1 Dual™ KO-IRF3 Cells NEW

THP1-Dual™ KO-TBK1 and THP1-Dual™ KO-IRF3 cell lines are KO for TBK1 (TANK-binding kinase 1) and IRF3 genes, respectively. The KO has been confirmed by PCR, sequencing, western blot, and functional assays. Upon stimulation with the STING agonist 2'3'-cGAMP, both cell lines show a significantly reduced IRF response compared to the parental THP1-Dual™ cell line (Fig. 1). However, we observe unexpected divergent responses when using different RIG-I agonists and transfection means (Fig. 2, below).

Validation of KO-TBK1 and KO-IRF3 Cells

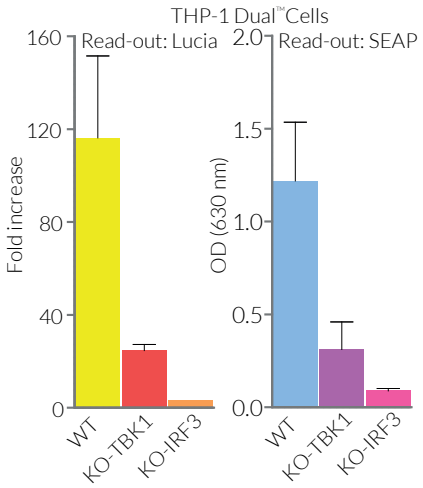


Figure 1: 2'3'-cGAMP-induced IRF or NF-κB responses in THP-1 Dual™-derived cells. $2x10^5$ THP1-Dual™ (WT), KO-TBK1 or KO-IRF3 cells were incubated with 30 µg/ml 2'3'-cGAMP (STING agonist). After overnight incubation, ISG and NF-κB induction were assessed by measuring the activity of Lucia luciferase and SEAP in the cell supernatant using QUANTI-Luc™ and QUANTI-Blue™ Solution, respectively. Activity fold increase over non-induced cells (Lucia luciferase readout) or reading of optical density (OD) at 630 nm (SEAP readout) are shown.

Nucleic acid signaling reporter cell collection

- RAW-Lucia ISG™ KO-cGAS
- RAW-Lucia ISG™ KO-IFI16
- RAW-Lucia ISG™ KO-IRF3
- RAW-Lucia ISG™ KO-IRF5
- RAW-Lucia ISG™ KO-IRF7
- RAW-Lucia ISG™ KO-MAVS
- RAW-Lucia ISG™ KO-MDA5
- RAW-Lucia ISG™ KO-RIG-I
- RAW-Lucia ISG™ KO-TBK1
- RAW-Lucia ISG™ KO-TREX1
- THP-1 Dual™ KO-cGAS
- THP-1 Dual™ KO-IFI16
- THP-1 Dual™ KO-MyD
- THP-1 Dual™ KO-STING
- THP-1 Dual™ KO-TREX1

The Lucia luciferase and SEAP reporter activities can be readily measured in the cell supernatant using the detection reagents, QUANTI-Luc™ and QUANTI-Blue™ Solution, respectively.
RAW-Lucia ISG™ KO cells are resistant to Zeocin™. THP1-Dual™ KO cells are resistant to Zeocin™ and Blasticidin.

For more information, visit our website

PRODUCT	QUANTITY	CAT. CODE
THP-1 Dual™ KO-IRF3 cells	3-7 x 10^6 cells	thpd-koirf3
THP-1 Dual™ KO-TBK1 cells	3-7 x 10^6 cells	thpd-kotbk

RELATED PRODUCTS

PRODUCT	DESCRIPTION	CAT. CODE
2'3'-cGAMP	mammalian cyclic dinucleotide	tlrl-nacga23-02
3p-hpRNA	5' triphosphate hairpin RNA	tlrl-hprna
5'ppp-dsRNA	5' triphosphate double stranded RNA	tlrl-3prna

www.invivogen.com/ko-cell-lines

Differential responses depending on the RIG-I agonist and transfection reagent

Impact of RIG-I agonist on the IRF response

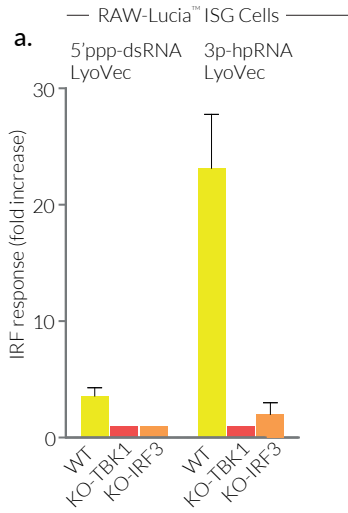
3p-hpRNA and 5'ppp-dsRNA are structurally different RIG-I agonists. 3p-hpRNA, which contains a panhandle secondary structure, is a much more potent RIG-I agonist than 5'ppp-dsRNA, as shown by the magnitude of the IRF response in RAW-Lucia™ ISG and THP-1 Dual™ cells (Fig. 2.a-d).

Impact of transfection reagent on the IRF response

5'ppp-dsRNA and 3p-hpRNA must be complexed with a transfection reagent to enter the cytoplasm and activate RIG-I. As expected, 5'ppp-dsRNA significantly loses its ability to induce Lucia luciferase in TBK1-KO or IRF3-KO cell lines when complexed with LyoVec™ (InvivoGen) or LTX (Fig. 2a-c). Similar results are observed when using 3p-hpRNA complexed to LyoVec™ (Fig. 2a) but not when using 3p-hpRNA complexed with LTX (Fig. 2b, c). Rather than being reduced, the IRF (Lucia) response is unexpectedly increased in TBK1-KO and IRF3-KO cells. Surprisingly, the NF-κB response is also barely affected, if not slightly increased (Fig. 2d). These data suggest that the transfection reagent impacts the intracellular availability and/or localization of the agonist and affects downstream signaling.

Interestingly, the use of different agonists and transfection reagents could highlight overlapping RNA-sensing or regulatory mechanisms[1,2].

1. Perry AK. *et al.*, 2004. Differential requirement for TANK-binding kinase-1 in type I interferon responses to Toll-like receptor activation and viral infection. J. Exp. Med. 199:1651.
2. Deng W. *et al.*, 2008. Negative regulation of virus-triggered IFN-B signaling pathway by alternative splicing of TBK1. J. Biol. Chem. 283:35590.



Nucleic acid signaling inhibitors

InvivoGen offers an extensive collection of high quality synthetic inhibitors, including molecules targeting the nucleic acid-induced signaling. Our inhibitors are functionally validated using cellular assays.

- BX795
- MRT67307 NEW
- RU.521
- H-151 NEW

TBK1/IKKε inhibitors

BX795 is a potent inhibitor of the IKK-related kinases TBK1 and IKKε, preventing further IRF activation and ISG (interferon stimulated genes) expression[1] (Fig. 3). Importantly, BX795 also indirectly inhibits the activity of canonical IKKα/IKKβ kinases , thereby precluding activation of the NF-κB transcription factor[2] (Fig. 3).

MRT67307 is a potent and reversible inhibitor of the IKK-related kinases TBK1 and IKKε, preventing further IRF activation and ISG expression (Fig. 3). Unlike BX795 from which it is derived, MRT67307 has no effect on the canonical IKKα/IKKβ kinases, and thus on NF-κB activation[2] (Fig. 3).

cGAS/STING inhibitors

RU.521 is a small molecule inhibitor of the cytosolic DNA sensor cGAS. This drug targets the catalytic pocket of cGAS, and reduces its affinity for ATP and GTP, thereby preventing the synthesis of the second messenger 2'3'-cGAMP, a STING agonist[3] (Fig. 4). RU.521 displays a higher inhibitory potency towards murine cGAS compared to human cGAS[4].

H-151 is a selective inhibitor of human and murine STING activity (Fig. 4). This synthetic indole-derivative blocks STING palmitoylation and clustering, which are two essential steps for STING signaling[5]. H-151 treatment was shown to reduce the production of pro-inflammatory cytokines in human cells and *in vivo*.

1. Clark K. *et al.*, 2009. Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and IκB kinase ε. J. Biol. Chem. 284:21. **2. Clark K. *et al.*, 2011.** Novel cross-talk within the IKK family controls innate immunity. Biochem. J. 434:93. **3. Vincent J. *et al.*, 2017.** Small molecule inhibition of cGAS reduces interferon expression in primary macrophages from autoimmune mice. Nat. Commun. 28:750. **4. Zhou W. *et al.*, 2018.** Structure of the human cGAS-DNA complex reveals enhanced control of immune surveillance. Cell. 174:300. **5. Haag S.M. *et al.*, 2018.** Targeting STING with covalent small-molecule inhibitors. Nature. 559:269.

BX795 and MRT67307 inhibition of TBK1/IKKε signaling

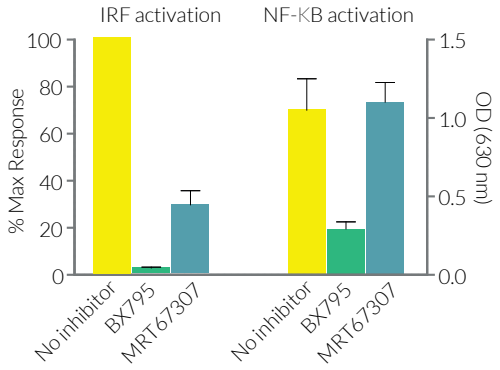


Figure 3: Inhibition of 3p-hpRNA-induced responses in THP1-Dual™ Cells: 2x10^5 THP1-Dual™ cells were transfected with 1 µg/ml 3p-hpRNA/Lyovec™ with or without 1 µg/ml BX795 or 3 µg/ml MRT-67307. After overnight incubation, activation of IRF or NF-κB was assessed by measuring Lucia luciferase or SEAP activity in the supernatant.

RU.521 and H-151 inhibition of cGAS and STING signaling

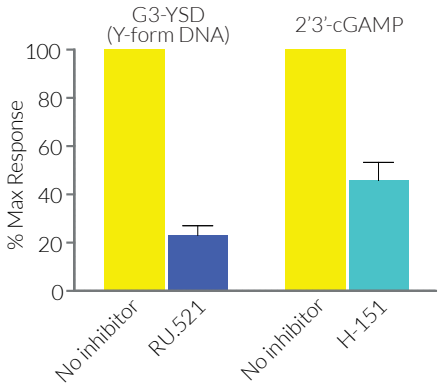


Figure 4: Inhibition of DNA-induced responses in RAW Lucia™ ISG Cells: 10^5 RAW-Lucia™ ISG cells were transfected with 1 µg/ml G3-YSD/LTX or incubated with 20 µg/ml 2'3'-cGAMP in the presence or absence of 2.5 µg/ml RU.521 or 130 ng/ml H-151, respectively. After overnight incubation, IRF activation was assessed by measuring Lucia luciferase activity in the supernatant.

PRODUCT	TARGET	QUANTITY	CAT. CODE
BX795	TBK1/IKKε	5 mg	tlrl-bx7
H-151	STING	10 mg	inh-h151
MRT67307	TBK1/IKKε	10 mg	inh-mrt
RU.521	cGAS	2 mg	inh-ru521

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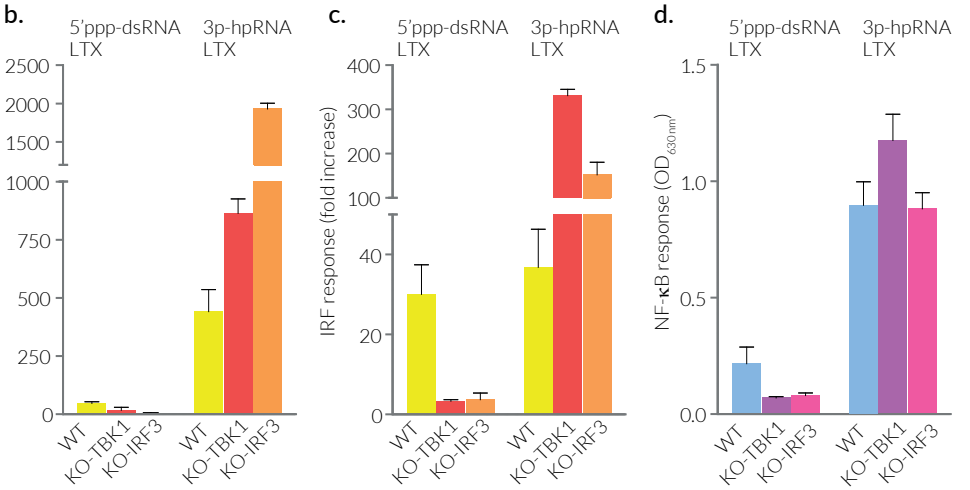


Figure 2: RNA induction of ISG or NF-κB in RAW-Lucia™ ISG or THP1-Dual™-derived cells. 10^5 RAW-Lucia™ ISG, KO-TBK1 or KO-IRF3 cells **(a, b)** or 2x10^5 THP1-Dual™, KO-TBK1 or KO-IRF3 cells **(c-d)** were transfected with 1 µg/ml 3p-hpRNA or 5'ppp-dsRNA complexed with Lyovec™ **(a)** or LTX **(b-d)**. After overnight incubation, ISG induction was assessed by measuring bioluminescent activity of the Lucia luciferase in the supernatant using QUANTI-Luc™. Activity fold increase over non-transfected cells is shown **(a-c)**. The NF-κB activity in THP1-Dual™-derived cells was assessed by measuring the SEAP activity in the supernanant using QUANTI-Blue™ Solution. Reading of optical density (OD) at 630 nm is shown **(d)**.

More data available on our website

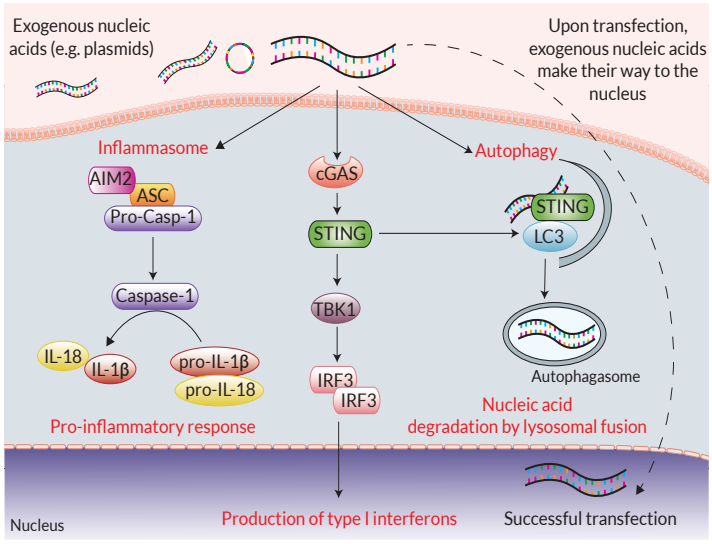
NATE™ Nucleic Acid Transfection Enhancer NEW

InvivoGen now offers the NATE™ reagent, a nucleic acid transfection enhancer, to boost both transient and stable transfection efficiencies in hard-to-transfect cell lines such as monocytes and macrophages. As trusted experts in engineering THP-1 and RAW 264.7 reporter cell lines, do you want to know our little secret? The NATE™ reagent!

- **Flexible:** Can be used with all common transfection methods
- **Easy to use:** Simply add to the cells prior to transfection
- **Efficient:** Increases transfection yield in hard-to-transfect cells

Obstacles to successful nucleic acid transfection

During eukaryotic cell transfection, exogenous nucleic acids are detected by cytosolic sensors such as cGAS/STING, AIM2 inflammasome, and LC3-mediated autophagy. The activation of these defensive signaling cascades frequently leads to low transfection yield, reduced cell viability, and inconsistent results especially in hard-to-transfect cells (e.g. immune cells).



What is NATE™ ?

NATE™ is a ***nucleic acid transfection enhancer*** specifically designed by InvivoGen to improve transfections in immune cells. The NATE™ reagent inhibits a number of nucleic acid sensing pathways, thereby protecting exogenous DNA and facilitating its expression. Using the NATE™ reagent allows an increase in both DNA expression and rate of transfected cells.

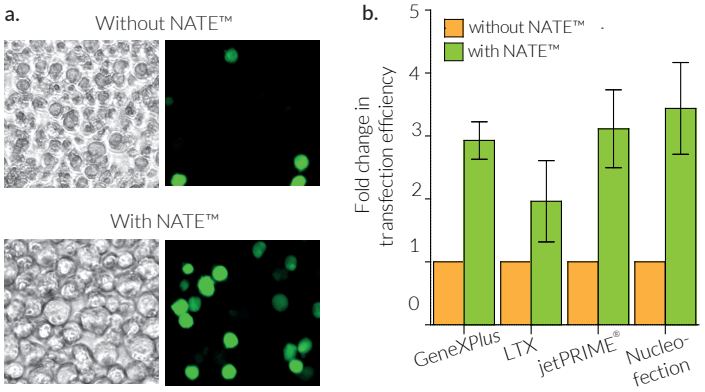
Key features of NATE™ reagent:

- **Compatibility** with commonly used transfection reagents (e.g. Gene*XPlus*, Lipofectamine® LTX, and jetPRIME®) and physical methods (nucleofection)
- **Higher transfection yield**, even with large plasmids (>10 kB)
- **Increased cellular viability** with all tested transfection protocols

PRODUCT	QUANTITY	CAT. CODE
NATE™	1 ml (~100 reactions)	lyec-nate

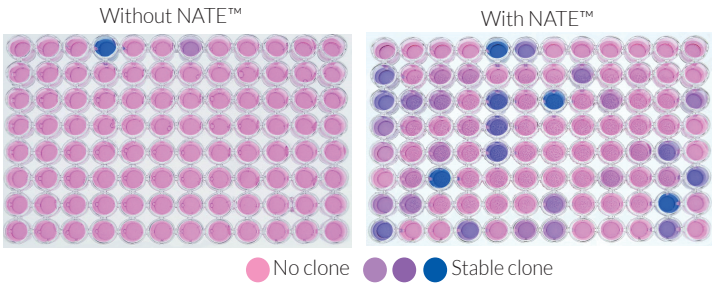
www.invivogen.com/nate

Increased DNA expression in NATE™ treated cells



NATE™ enhances transient DNA expression in human THP-1 monocytes: Transfection of a ~3 kb GFP-expressing plasmid into THP-1 cells was performed using GeneXPlus without (**a**:top; **b**:yellow) or with (**a**:bottom; **b**:green) the NATE.™ reagent. After 48 hours, cells were visualized by fluorescence microscopy (**a**), and transfection efficiency was measured using flow cytometry (**b**). Data are presented as a fold change normalized to the transfection efficiency without NATE™ reagent.

Increased rate of stably transfected cells with NATE™



NATE™ increases the number of stable SEAP-expressing clones in murine RAW 264.7 macrophages: Transfection of a ~10 kb SEAP-expressing plasmid into RAW 264.7 cells was performed using Lipofectamine® LTX, without (left) or with (right) the NATE™ reagent. After 10 days in selection with Blasticidin, the number of stables clones expressing SEAP (blue wells) was readily visualized using QUANTI-Blue™ Solution detection reagent.

Examples of transfections with NATE™

	THP-1	RAW 264.7
Plate size	12-well	24-well
Seeding Cell Density	5 x 10^5 c/w/ml	2 x 10^5 c/w/ml
Transfection reagent used	Gene*XPlus*	Lipofectamine® LTX
Volume of 100x NATE™	10 µl	10 µl

Note: Use of the NATE™ reagent will vary slightly depending on the cell-culture plate size (6-, 12-, 24-well plates) and the transfection method.

InvivoGen

Europe Tel: +33 562 71 69 39 Fax: +33 562 71 69 30 info.eu@invivogen.com
USA Tel: +1 888 457 5873 Fax: +1 858 457 5843 info@invivogen.com
Asia Tel: +852 3622 3480 Fax: +852 3622 3483 info.hk@invivogen.com