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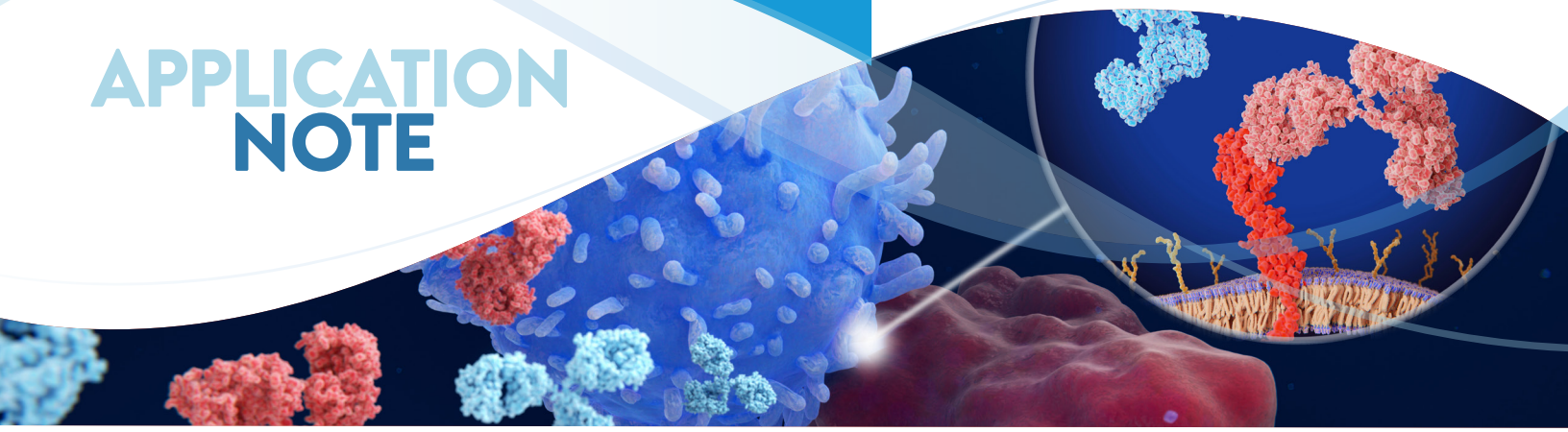
APPLICATION NOTE



# A PD-L1 CELL LINE WITH GAS-LUC2 REPORTER FOR CHECKPOINT INHIBITOR SCREENING

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**This study demonstrates a robust, responsive, and reproducible reporter system for the assessment of T cell and other immune/tumor microenvironmental cell-mediated immune responses triggered by PD-1/PD-L1 checkpoint inhibitors.**

## ABSTRACT

Immune checkpoint inhibitors have shown recent success in the treatment of lung, liver, breast, renal, and skin cancers; however, the built-in complexity of immunological models and the variable drug responses among different cancer types have challenged the development and application of these novel immunotherapies. To facilitate large-scale drug discovery for this growing class of immunomodulators, we conducted a comprehensive protein profiling of ATCC's vast portfolio of human tumor and immune cell lines for established and novel immune checkpoint molecules. Based on this protein profiling data, we generated HCC827-GAS-Luc2, an immune checkpoint reporter cancer cell line with high expression of endogenous programmed death-ligand 1 (PD-L1). This reporter cell line contains a gamma interferon activation site (GAS)–response element upstream of a luciferase gene. The luciferase expression is suppressed when PD-L1 on the cancer cells binds to programmed death-1 (PD-1) on the T cells. In the presence of a PD-1/PD-L1 inhibitor, HCC827-GAS-Luc2 cells produce a luciferase expression–based bioluminescent signal, which can be readily detected and quantified to evaluate the efficacy, potency, and dynamics of the inhibitor.

## INTRODUCTION

The success of immune checkpoint inhibitors in the treatment of various types of cancers and their continued growth in the market have driven burgeoning interests in developing more immuno-oncology drugs in this category.[1] However, the intrinsic complexity of immunological models and the variable drug responses among different cancer types have challenged this endeavor.[2] While immuno-oncology studies have made significant progress by using syngeneic mouse models or humanized mouse models, the inevitable differences between the mouse immune system, or a hybrid human/mouse immune system, and the human immune system remain a concern.[3] Hence, interests regarding in vitro fully human immuno-oncology assays have rapidly increased in recent years, especially for co-culture assays utilizing human CD8+ cytotoxic T cells that kill human cancer cells.[4] Nonetheless, the prevailing assays in this category largely employ an artificial system with engineered ectopic overexpression of immune checkpoint molecules and a T cell activation signaling reporter gene in non-cytotoxic T cells, such as an engineered Jurkat T cell line (CD4+, non-cytotoxic) with stable expression of human PD-1 and a nuclear factor of activated T cells (NFAT)–driven luciferase reporter.[5] More physiologically relevant immuno-oncology reporter assays are urgently needed for better modeling of the in vivo CD8+ cytotoxic T cell–mediated cancer killing events and for immuno-oncology drug discovery and development.

Cancer immunotherapy has been an effective therapeutic approach in many hematopoietic malignancies, especially for the PD1/PD-L1 and CTLA-4 blockade therapies.[6] However, the success rate is significantly lower in solid tumors.[7] Recent studies have demonstrated—both in vitro and in vivo—that the interferon-γ receptor (IFN-γR) JAK-STAT signaling pathway is required for T cells to kill cancer cells in solid tumors but not in liquid cancers.[8] Meanwhile, the cell signaling reporter system containing a

GAS-response element upstream of the luciferase gene (eg, Firefly luciferase; Luc2) has been widely used for detecting inter-cell IFN-γ signaling.[9] Hence, we utilized the demonstrated IFN-γ-IFN-γR JAK-STAT GAS-Luc2 reporter system for engineering a solid tumor cell line that is capable of robustly reporting the activity of IFN-γ from activated CD8+ cytotoxic T cells during their process of killing solid tumor cells (Figure 1).

To construct this robust reporter assay system of solid tumor cells and cytotoxic T cells to facilitate cancer immunotherapy drug screening, we performed flow cytometry–based checkpoint protein profiling in over 50 tumor cell lines available at ATCC (data not shown). Based on the proteomics results, the HCC827 cell line (ATCC® CRL-2868™) was selected for its high endogenous expression of PD-L1 immune checkpoint inhibitor.

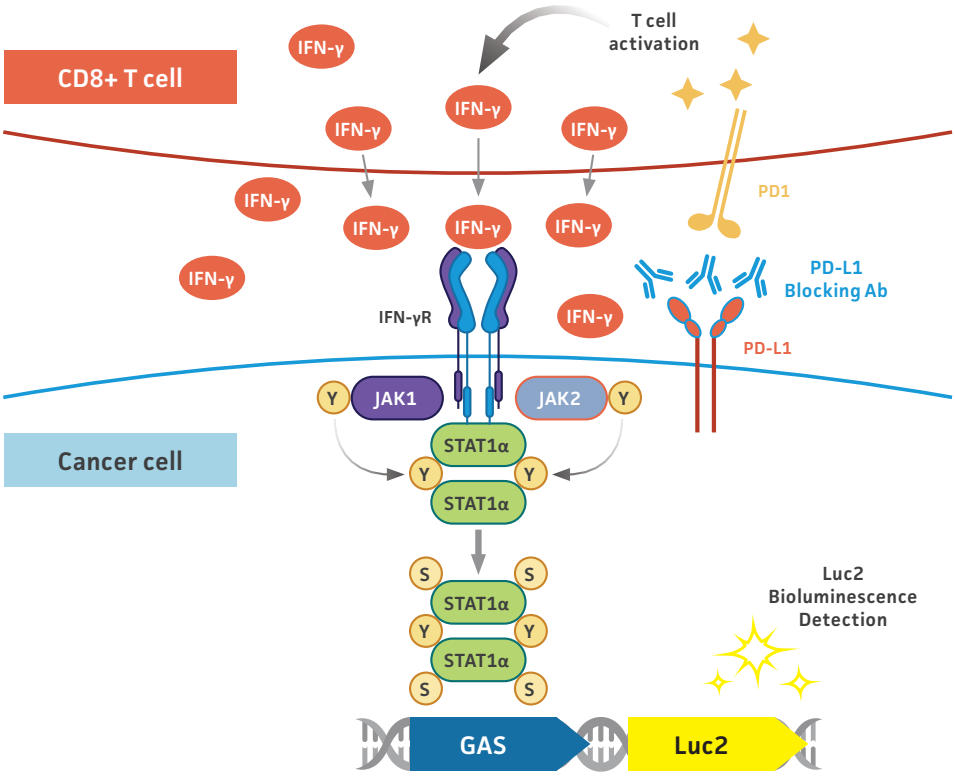


**Figure 1: Mechanism of action.** Luciferase signal generated by HCC827-GAS-Luc2 cells upon T cell activation through the PD-L1 blockade. Created with BioRender.com.

## RESULTS WITH MATERIAL & METHODS

To establish the HCC827-GAS-Luc2 (ATCC® CRL-2868-GAS-LUC2™) cell line, the parental cells were seeded at 1 x $10^5$ cells/well into a 12-well culture plate and transduced with lentiviral-GAS-Luc2 plasmids in the presence of 50 µg/ mL protamine sulfate (Sigma) for 24 hours. Transduction was stopped by replacing the medium with fresh culture medium. The lentiviral-GAS-Luc2 plasmids were generated by replacing the EF-1 alpha promoter with a GAS response element in a lentiviral-luciferase plasmid. The transfected cells were enriched by puromycin selection and evaluated by IFN-γ stimulation as described in the next paragraph. Following the luciferase expression assessment, single cells were isolated by automated cell sorting (Sony SH800) into 96-well plates and expanded for approximately 10-14 days until their confluency reached 70%. The growing single cell clones were then subcultured and stimulated with IFN-γ again. The clone that yielded the highest luciferase signal upon IFN-γ stimulation was chosen for the T cell–conditioned-media stimulation and T cell co-culture experiments.

After single-cell sorting and single-cell clonal expansion, the luciferase signal intensity upon stimulation (Figure 2A) was re-evaluated by adding IFN-γ (R&D Systems) to HCC827-GAS-Luc2 cells. We then seeded 50 µL of cells (5.0 x $10^3$ cells/well) into a 96-well plate (Corning). Final concentrations of 0, 0.01, 0.1, 1, 10, 100, and 1,000 ng/mL IFN-γ (50 µL) were added to the cells followed by overnight incubation at 37°C in 5% $CO_2$; experimental controls included media only or untreated cells. The next day, an equal volume of Bright-Glo™ Luciferase Assay Reagent (Promega) was added to the cells and the plate was incubated on a shaker for 8 minutes. The assay mixture was transferred to an opaque 96-well plate (Corning) for luminescence detection. The luminescence signals were measured by a SpectraMax® i3x (Molecular Devices). Cells were evaluated based on the average fold increase of relative luminescence (RLU) of IFN-γ-stimulated cells relative to average RLUs from untreated controls. The selected monoclonal HCC827-GAS-Luc2 cell line demonstrated high sensitivity to IFN-γ administration, exhibiting an average 15-fold increase in RLU values relative to untreated controls following 0.01 ng/mL IFN-γ administration. Increased luminescence was observed with increasing IFN-γ administration, with 1,000 ng/mL IFN-γ administration resulting in a 250-fold increase in RLU values as compared to untreated controls.

For T cell–conditioned-media stimulation (Figure 2B), primary human CD8+ cytotoxic T cells (ATCC® PCS-800-017™) were cultured in RPMI-1640 (ATCC® 30-2001™) with 10% heat-inactivated FBS (Gibco) and 50 U/mL interleukin-2 (Miltenyi Biotec). On day 0, half of the CD8+ cytotoxic T cells were activated using T Cell Activation/Expansion Kit (Miltenyi Biotec) following the manufacturer's instructions. The remaining cells were centrifuged at 400 x g for 5 minutes to remove old medium and were then resuspended in fresh culture medium at a seeding density of 2.0 x $10^6$ cells/mL. On day 3, the activated and non-activated T cells were harvested by centrifugation, and the supernatants were collected as activated and non-activated CD8+ cytotoxic T cell–conditioned media, respectively. Upon the preparation of

the T cell–conditioned media, HCC827-GAS-Luc2 cells were harvested and seeded in 100 µL culture media on a 96-well plate at the seeding density of 2.0 x $10^4$ cells/well. Cells were incubated at 37°C in 5% $CO_2$ for 1 hour to attach to the plates. After the cells attached, an equal volume of the T cell–conditioned media was added to the cells. Cells were then incubated overnight before proceeding to the luciferase assay as described above. The T cell–conditioned-media-stimulated HCC827-GAS-Luc2 cells were shown to express a 50- and 100-fold increase in RLUs relative to untreated controls from non-activated or activated media, respectively.

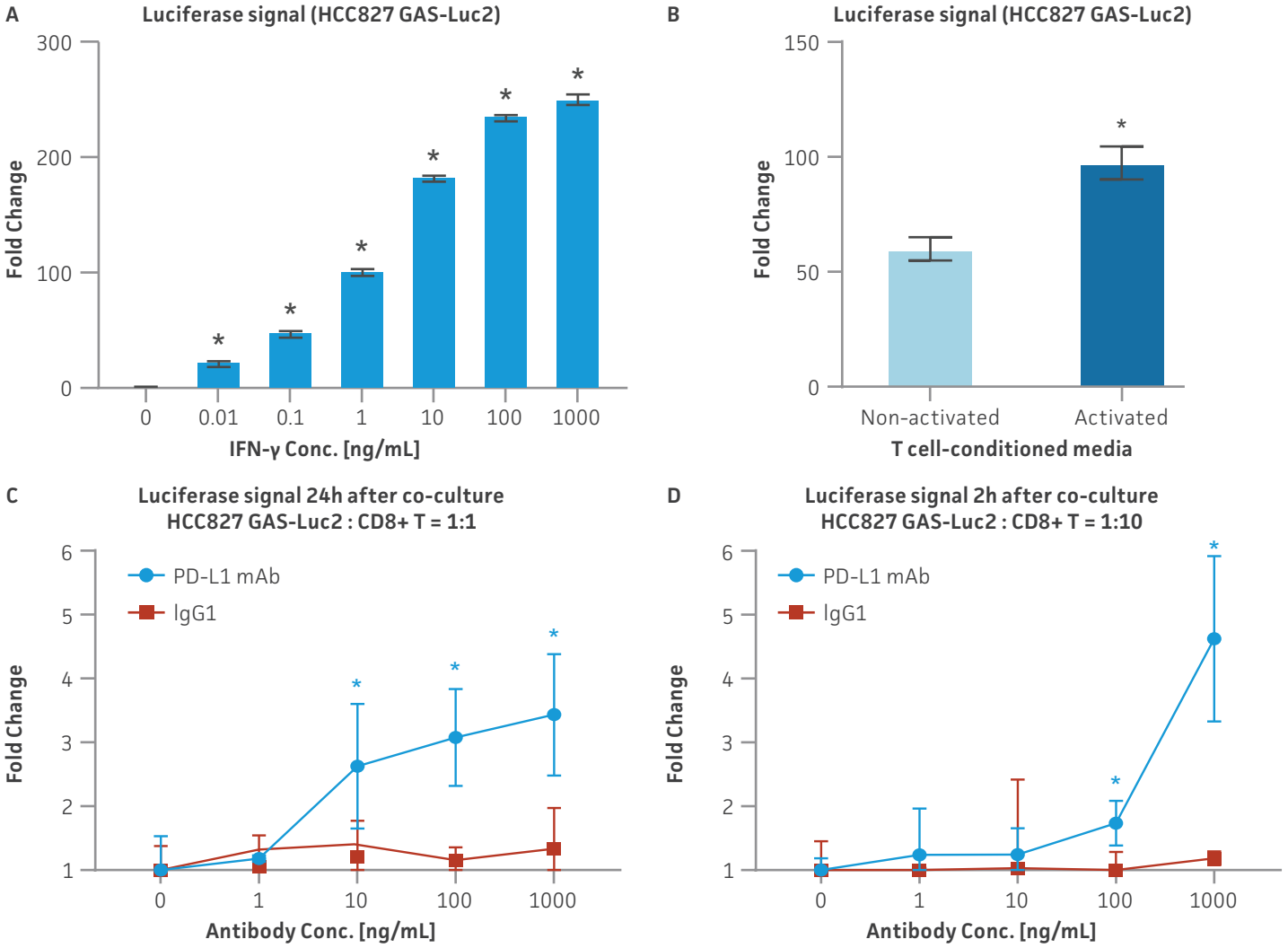


**Figure 2: Evaluation of HCC827-GAS-Luc2 cell line.** Luciferase expression from HCC827-GAS-Luc2 cells upon signaling activation by (A) IFN-γ stimulation (0.01 – 1,000 ng/mL), (B) conditioned-media stimulation from checkpoint matched non-activated and activated primary CD8+ cytotoxic T cells, and (C, D) co-culture with primary human CD8+ cytotoxic T cells in the presence of PD-L1 blocking antibody or isotype control IgG1 (1-1,000 ng/mL). N=3 in all experiments. *, $P < 0.05$.

Next, the ability of HCC827-GAS-Luc2 cells to serve as a checkpoint inhibitor reporter cell line was assessed by primary human CD8+ cytotoxic T cell co-culture (Figure 2C, 2D). HCC827-GAS-Luc2 cells were added to a 96-well plate at 50 µL (1.0 x $10^4$ cells/well). Five different concentrations (0, 1, 10, 100, or 1000 ng/ mL) of PD-L1 blocking antibody (Invivogen, hpdl1-mab1) or isotype control (Invivogen, bgal-mab1) were prepared and added to the cells. After 15 minutes of incubation, primary human CD8+ cytotoxic T cells were added to the cancer cells at the designated ratios (1:1 or 1:10). Co-cultured cells were then incubated at 37°C in 5% $CO_2$ for 24 or 2 hours, respectively, followed by evaluation via the luciferase assay as described above.

PD-L1 mAb administration served as a blocker of the checkpoint inhibitor, preventing the cancer cells in silencing the immune activity of CD8+ cytotoxic T cells via their PD-L1 to PD-1 binding. Increasing the PD-L1 mAb concentrations in the co-culture models resulted in a dose-dependent increase in the HCC827-GAS-Luc2 cell luciferase expression, exhibiting a 3- and 5-fold increase of luciferase expression relative to untreated controls under the conditions of 24- and 2-hour co-culture incubation period with 1:1 and 1:10 ratios of HCC827-GAS-Luc2 to CD8+ cytotoxic T cells, respectively. In contrast, increasing isotype control IgG1 concentrations failed to induce any luciferase expression from HCC827-GAS-Luc2 cancer reporter cell lines relative to untreated controls.

Additional co-culture studies were conducted testing the effects of increased CD8+ T cell populations as well as increasing co-culture

incubation time. Altering the HCC827-GAS-Luc2 to CD8+ T cell ratio was found to greatly affect maximal luciferase expression under high PD-L1 mAb administration. Increasing the proportion of CD8+ T cells demonstrated decreased luciferase expression, with 1:5 and 1:10 HCC827-GAS-Luc2:CD8+ T cell ratios exhibiting no detectable luminescence signaling under maximum PD-L1 mAb administration (data not shown). Moreover, increasing co-culturing incubation period was found to decrease luciferase signaling under maximum PD-L1 mAb administration. Here, 2-, 4-, 6-, and 24-hour co-culture incubation with maximal PD-L1 mAb administration demonstrated 5-, 1-, 0.7-, and 0-fold increase in luciferase expression relative to untreated controls, respectively (data not shown). These phenomena were attributed to the cell death of HCC827-GAS-Luc2 cells, killed by CD8+ cytotoxic T cells over time, or via elevated cytotoxicity with increased cell number of CD8+ cytotoxic T cells. Therefore, when designing cell-based assays using luciferase reporter systems it is critical to consider the effect of cytotoxicity on bioluminescent signal and optimize the assay based on time and target to effector cell ratio.

## CONCLUSIONS

Based on our comprehensive proteomics data, we selected HCC827—a solid tumor cell line with naturally high expression of PD-L1—to develop an IFN-γ-IFN-γR JAK-STAT GAS-Luc2 reporter system (HCC827-GAS-Luc2 cell line) that is capable of reliably reporting the activity of IFN-γ from activated primary human CD8+ cytotoxic T cells when killing these cancer cells. Such a robust full human in vitro reporting system for immune checkpoint blockade drug testing is time-dependent and cell number–restricted as over time the CD8+ cytotoxic T cells gradually kill all these cancer cells and can do so at a faster rate if more cytotoxic T cells are present. As compared to other immune checkpoint assays on the market that use engineered cancer cell lines with ectopic overexpression of certain immune checkpoint molecules, our novel immune checkpoint assay reporting system is much more physiologically relevant as the cancer cells that we selected have naturally high expression of relevant immune checkpoint molecules.[5]

To maximize assay versatility, we incorporated the IFN-γ-IFN-γR JAK-STAT GAS-Luc2 reporting system in the cancer cells so users can employ multiple kinds of human immune cells for the counterpart of the cancer cells in the full human co-culture assay. For example, we can use T cells that were newly defined by single cell sequencing (scRNAseq), CAR-T cells, B cells, and myeloid cells. Additionally, these cells could be used in combination with or without other human tumor microenvironmental cells, such as primary human fibroblasts and endothelial cells, for better modeling of the in vivo T cell-mediated cancer–killing events in the solid tumor microenvironment. All of these permutations are improvements over other immune checkpoint assays on the market whose signaling reporting system is restricted to a non-cytotoxic T cell line such as the CD4+ Jurkat T cell line with an NFAT-Luc2 reporter.[5]

Lastly, considering the recent studies that have demonstrated the importance of IFN-γ-IFN-γR JAK-STAT signaling for T cell–mediated killing of cancer cells specifically in solid tumors,[8] our full human cancer GAS-Luc2 reporting system better supports the immune checkpoint drug discovery and development in solid tumors where the success rate of immune checkpoint blockade therapy has been markedly lower. Therefore, because of its high endogenous target marker expression, robust bioluminescent signal, and assay versatility, the ATCC HCC827-GAS-Luc2 reporter cell line is ideal for assaying candidate PD-1/PD-L1 signaling checkpoint blockers.

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**AP-012023-v01**