# GAS-Luc2 reporter cell lines to supersede the industrial standard of interferon-gamma ELISA for ex vivo immune activation studies and drug screening

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Abstract 14

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## Abstract

**Background:** Interferon-gamma (IFN-γ) is a cytokine that plays a major role in activating cellular immunity and promoting anti-tumor immune response. Monitoring the level of IFN-γ has become the industrial standard for evaluation of the efficacy of immunotherapy drugs. However, widely used enzyme-linked immunosorbent assay (ELISA) is not only costly and time-consuming but also often fails to detect the low-level IFN-γ in the early-stage immune activation. Moreover, it is difficult to correctly quantify the paracrine signaling of IFN-γ secreted by T cells in recently popularized 3-D co-culture models using ELISA. A novel approach to fulfill the rapidly expanding need of assessing immunotherapy drug candidates and more effective monitoring of immune activation for cancer immunotherapy studies is urgently needed.

**Methods:** We engineered three reporter cancer cell lines that contain a gamma interferon activation site (GAS)-response element upstream of luciferase gene. In response to IFN-γ receptor binding and subsequent signaling activation, these cells express luciferase, which can be readily detected and quantified to evaluate the level of immune activation. The cell lines were selected for their endogenous expression of the immune checkpoint ligands PD-L1, CD155, or B7-H3 based on a comprehensive protein profiling. The established reporter cells were stimulated with various concentrations of IFN-γ, incubated with conditioned media from primary T cells, or co-cultured with IFN-γ-producing immune cells in the presence of immune checkpoint inhibitors followed by a luciferase assay to evaluate the system. Then the reporter cells were 2-D or 3-D co-cultured with primary T cells after which the cells were harvested for luciferase assay and the conditioned media were collected for IFN-γ ELISA for direct comparison of the two assays.

**Results:** Our data showed that the bioluminescence intensity from the reporter cells increased approximately 100-250 fold in a dose-dependent manner in response to IFN-γ stimulation. It increased approximately 50-100 fold in response to primary T cell-conditioned media stimulation. In co-culture assays with primary T cells or NK cells in the presence of corresponding immune checkpoint inhibitors, these reporter cell lines demonstrated 3-12-fold increase in bioluminescence intensity. In comparison to ELISA in 2-D and 3-D co-culture systems, the reporter cells produced robust luminescence signal even when IFN-γ concentrations fell below the assay range of conventional ELISA.

**Conclusions:** These GAS-Luc2 reporter cell lines demonstrated exceptional assay sensitivity and adaptability in detection of IFN-γ in 2-D and 3-D cultures. They provide an excellent tool for early-stage monitoring of immune activation and convenient and sensitive evaluation of immunotherapy drugs.

## Background

### Protein profiling of cancer cell lines for immune checkpoint molecule expression

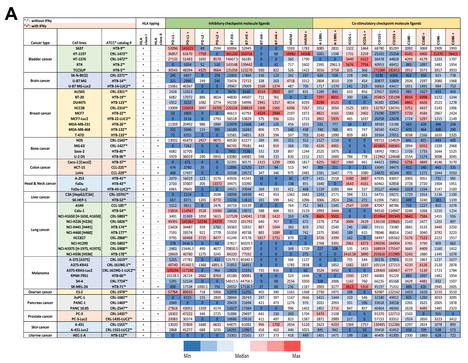


**Figure 1: Heat map based on protein profiling data of selected cancer cell lines for immune checkpoint molecule expression.** (A) Immune checkpoint molecule expression levels in cancer cell lines under basal (-) and 100 ng/mL IFN-γ stimulated (+) conditions were profiled by flow cytometry. Table values represent median fluorescence intensity (MFI) of sample subtracted by isotype control MFI. Each column was color-coded separately to avoid cross comparison. (B) Mechanism of action of the GAS-Luc2 luciferase reporter cell lines for cancer immune checkpoint studies. Activation of JAK/STAT signaling in these cancer reporter cells in response to immune checkpoint inhibition leads to luciferase expression which can be readily quantified and analyzed. Created with BioRender.com.

## Results

### Luciferase expression in GAS-Luc2 tumor cells upon JAK-STAT signaling pathway activation

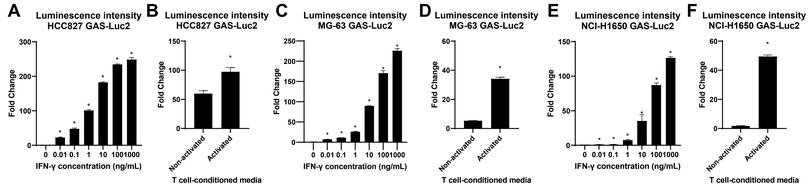


**Figure 2: Evaluation of monoclonal GAS-Luc2 cell lines as immune checkpoint reporters.** (A-B) HCC827 GAS-Luc2 cell line with high endogenous PD-L1 expression was stimulated overnight with (A) IFN-γ or (B) T cell–conditioned media. (C-D) MG-63 GAS-Luc2 cell line with high endogenous CD155 expression was stimulated overnight with (C) IFN-γ or (D) T cell–conditioned media. (E-F) NCI-H1650 GAS-Luc2 cell line with high endogenous B7-H3 expression was stimulated overnight with (E) IFN-γ or (F) T cell–conditioned media. For the conditioned media stimulation, the cells were administered with the conditioned media collected from non-activated or activated human primary CD8+ cytotoxic T cells. The activated conditioned media were harvested 3 days post-activation with anti-CD2/CD3/CD28 beads. Luciferase expression was quantified by Bright-Glo™ Luciferase Assay System (Promega®). Luminescence intensity was measured by SpectraMax® i3x (Molecular Devices®). N=3 in all experiments. *, P < 0.05.

### Luciferase expression in GAS-Luc2 cells upon co-culture with adaptive or innate immune cells

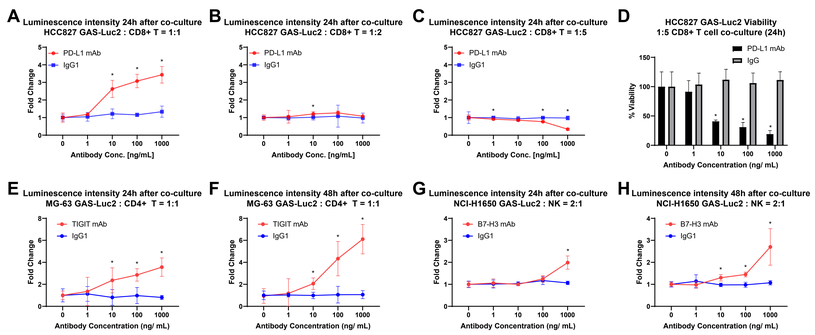


**Figure 3: Co-culture of monoclonal GAS-Luc2 cell lines with primary human immune cells at varying cell ratios and co-culture durations in the presence of a respective blocking antibody.** (A-C) The luminescence intensity from HCC827 GAS-Luc2 after 24-hour co-culture with CD8+ cytotoxic T cells at a (A) 1:1, (B) 1:2, or (C) 1:5 ratio of target cells to effector cells in the presence of a PD-L1 mAb or isotype control IgG (1-1,000 ng/mL). (D) The percent viability of HCC827 GAS-Luc2 after co-culture with CD8+ cytotoxic T cells for 24 hours in the presence of a PD-L1 mAb or isotype control IgG (1-1,000 ng/mL). (E-F) The luminescence intensity from MG-63 GAS-Luc2 after co-culture at a 1:1 ratio with CD4+ helper T cells for (E) 24 hours or (F) 48 hours in the presence of a TIGIT mAb or isotype control IgG (1-1,000 ng/mL). (G-H) The luminescence intensity from NCI-H1650 GAS-Luc2 after co-culture with CD56+ NK cells at a 2:1 ratio of target to effector cells for (G) 24 hours or (H) 48 hours in the presence of a B7-H3 ADCC mAb or isotype control IgG (1-1,000 ng/mL). Luciferase expression was quantified by Bright-Glo™ Luciferase Assay System (Promega®). Luminescence intensity was measured by SpectraMax® i3x (Molecular Devices®). N=3 in all experiments. *, P < 0.05.

## Key References


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- Larson RC, et al. CAR T cell killing requires the IFNγR pathway in solid but not liquid tumours. Nature. 2022 Apr;604(7906):563-570. doi: 10.1038/s41586-022-04585-5. Epub 2022 Apr 13. PMID: 35418687.
- Schmidts A, et al. Cell-based artificial APC resistant to lentiviral transduction for efficient generation of CAR-T cells from various cell sources. J Immunother Cancer. 2020 Sep;8(2):e000990. PubMed: 32900862


### Comparison of luciferase expression in non-aAPC (artificial Antigen Presenting Cells) and aAPC upon co-culture with primary T cells

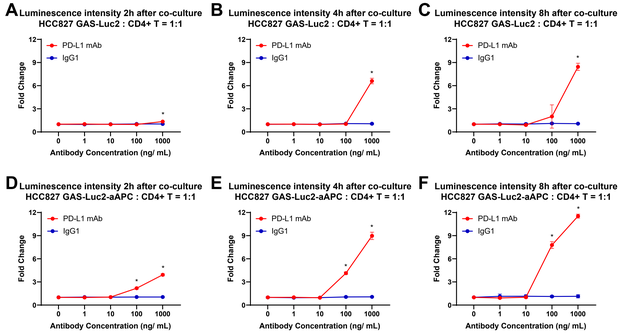
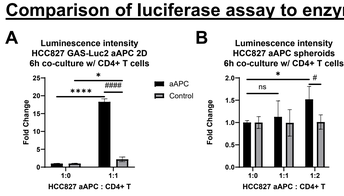



**Figure 4: HCC827 GAS-Luc2 and HCC827 GAS-Luc2-aAPC (multi-clone) in co-culture with CD4+ T cells in the presence of a PD-L1 blocking antibody.** The aAPC multi-clone cell line was generated from HCC827 GAS-Luc2 to express anti-CD3/CD28 to enhance the tumor cell recognition by previously activated and partially exhausted T cells. (A-C) The luminescence intensity from HCC827 GAS-Luc2 after co-culture with a 1:1 ratio of T cells for (A) 2 hours, (B) 4 hours, or (C) 8 hours. (D-F) The luminescence intensity from HCC827 GAS-Luc2-aAPC after co-culture with a 1:1 ratio of T cells for (D) 2 hours, (E) 4 hours, or (F) 8 hours. During the co-culture, the cells were administered with the PD-L1 mAb or isotype control IgG. Luciferase expression was quantified by Bright-Glo™ Luciferase Assay System (Promega®). N=3 in all experiments. *, P < 0.05.

### Comparison of luciferase assay to enzyme-linked immunosorbent assay (ELISA)




| Condition | Cell Type | T:E | OD | IFN-γ conc. (pg/ mL) |
|---|---|---|---|---|
| 2-D / 6h | aAPC | 1:0 | 0.013275 | Below MDD* |
| | | 1:1 | 0.27545 | 59.662791 |
| | aAPC Ctrl | 1:0 | 0.01515 | Below MDD* |
| | | 1:1 | 0.0242 | Below MDD* |
| 3-D / 6h | aAPC | 1:0 | 0.00815 | Below MDD* |
| | | 1:1 | 0.01005 | Below MDD* |
| | | 1:2 | 0.04655 | Below MDD* |
| | aAPC Ctrl | 1:0 | 0.01735 | Below MDD* |
| | | 1:1 | 0.0089 | Below MDD* |
| | | 1:2 | 0.0084 | Below MDD* |
| 3-D / 24h | aAPC | 1:0 | 0.0089 | Below MDD* |
| | | 1:1 | 0.03945 | Below MDD* |
| | | 1:2 | 0.0599 | 1.643478** |
| | aAPC Ctrl | 1:0 | 0.0158 | Below MDD* |
| | | 1:1 | 0.0106 | Below MDD* |
| | | 1:2 | 0.0102 | Below MDD* |

* MDD (minimum detectable dose) = 1.28 pg/ mL
** Below assay range of 15.6 – 1,000 pg/ mL

**Figure 5: Co-culture of 2-D and 3-D HCC827 GAS-Luc2-aAPC (pooled single clone) cells with primary CD4+ T cells evaluated by luciferase assay and ELISA.** (A) HCC827 GAS-Luc2-aAPC cells and aAPC transduction control cells (no anti-CD3/CD28) cultured in 2-D were co-cultured with freshly thawed (no prior in vitro activation) primary CD4+ T cells (T:E = 1:0 and 1:1) for 6 hours. (B-C) HCC827 GAS-Luc2-aAPC cells and control cells cultured as 3-D spheroids were co-cultured with primary CD4+ T cells (T:E = 1:0, 1:1, or 1:2) for (B) 6 hours or (C) 24 hours. After co-culture, the supernatants were collected for ELISA and the cells were harvested for luciferase assay. The luciferase expression was quantified by Bright-Glo™ Luciferase Assay System (Promega®). (D) IFN-γ concentrations in the cell-conditioned media were quantified by Human IFN-γ Quantikine® ELISA Kit (R&D Systems®) N=3 in all experiments. **** or ####, P < 0.0001. * or #, P < 0.05; ns, P ≥ 0.05.

## Conclusion

- Based on the expansive protein profiling data of cancer cell lines for numerous immune checkpoint molecules, we developed three cancer luciferase reporter cell lines, HCC827 GAS-Luc2 (ATCC® CRL-2868-GAS-LUC2™), MG-63 GAS-Luc2 (ATCC® CRL-1427-GAS-LUC2™), and NCI-H1650 GAS-Luc2 (ATCC® CRL-5883-GAS-LUC2™), with a high endogenous expression of PD-L1, CD155, and B7-H3, respectively.
- These immune checkpoint reporter cell lines produce robust, sensitive, and reproducible luciferase expression upon JAK-STAT signaling activation in cancer cells, allowing reliable quantification of various types of immune cell-mediated pro-inflammatory responses triggered by relevant immune checkpoint inhibition while maintaining physiological relevance and stable expression, eliminating the donor variability issue commonly experienced by using primary cell models.
- Artificial expression of anti-CD3/CD28 on HCC827 GAS-Luc2-aAPC to circumvent TCR (T-cell receptor) recognition and trigger TCR signaling elicited more rapid and elevated responses compared to non-aAPC in a co-culture experiment with primary T cells, demonstrating further potential of the reporter cell lines and their broad applications in cancer immunology studies.
- In comparison to the conventional ELISA method, the luciferase reporter cells displayed exceptional assay sensitivity and adaptability, providing an outstanding means of detecting the early-stage immune activation and in 3-D culture models.

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