APPLICATION NOTE

Monitoring degraders and inhibitors of K-Ras protein expression with a cell-based HTRF immunoassay as an alternative to Western Blot.

Authors

Ludivine Vossier
Karine Baudet
Elodie Dupuis
Stéphane Martinez
Fabienne Charrier-Savournin

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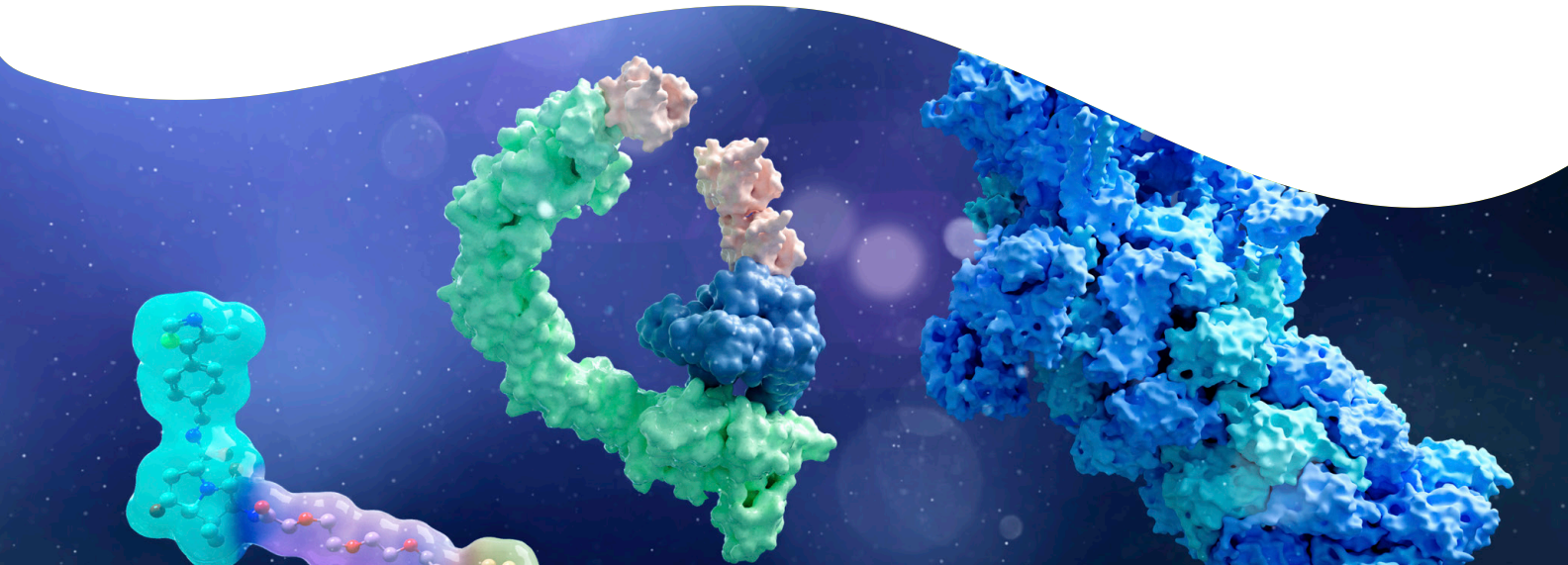
Introduction

The RAS family of genes comprises three primary isoforms: H-Ras, N-Ras, and K-Ras. Each isoform encodes a distinct protein that plays a pivotal role in regulating cell growth, survival, and differentiation through signal transduction pathways. Despite their structural similarities and overlapping functions, these isoforms exhibit unique patterns of expression and mutation frequencies in different cancers. H-Ras is often associated with bladder, thyroid, and skin cancers. Mutations in H-Ras are less common compared to the other isoforms but are significant in certain tumor types. N-Ras is frequently mutated in melanoma, hepatocellular carcinoma, and hematological malignancies such as acute myeloid leukemia. K-Ras is the most mutated isoform in human cancers. K-Ras mutations, especially at codons 12, 13, and 61, lead to constitutive activation and persistent cell signaling, promoting uncontrolled cell proliferation, and contributing to oncogenesis. These mutations are prevalent in various cancers, including pancreatic, colorectal, and lung cancers, making K-Ras a critical therapeutic target (Prior et al., 2020; Cox et al., 2014).

Targeting K-Ras has been historically difficult due to its high affinity for GTP/GDP and lack of suitable binding pockets for small molecules. Recent drug discovery advancements have led to innovative approaches to inhibit K-Ras activity (see Figure 1). Small molecule inhibitors bind directly to K-Ras, usually at allosteric sites or mutant-specific pockets. Covalent inhibitors targeting the G12C mutant, such as sotorasib (AMG 510) and adagrasib (MRTX849), irreversibly bind to the cysteine residue, marking a significant breakthrough (Canon et al., 2019). Targeting downstream signaling pathways activated by K-Ras, like RAF-MEK-ERK and PI3K-AKT-mTOR, offers an alternative strategy. Synthetic lethality targets genes crucial



for K-Ras mutant cells, sparing normal cells and selectively eliminating cancer cells with K-Ras mutations (Shen et al., 2021). Antisense oligonucleotides and RNA interference-based therapies aim to reduce K-Ras protein levels by targeting its mRNA, though delivery and stability challenges persist (Ng et al., 2017). Immunotherapy leverages the immune system to target K-Ras mutant cells, including vaccines and T-cell therapies that recognize mutant K-Ras epitopes (Tran et al., 2016). Disrupting the K-Ras/SOS interaction is another approach. SOS activates K-Ras by facilitating GDP for GTP exchange. Inhibitors targeting this interaction can prevent K-Ras activation by allosterically inhibiting the SOS-mediated nucleotide exchange process (Winters et al., 2018). PROTAC® molecules (proteolysis-targeting chimeras) are a promising strategy to induce the degradation of K-Ras by recruiting it to the cellular proteasome degradation machinery (Burslem et al., 2018; Bond et al., 2020).

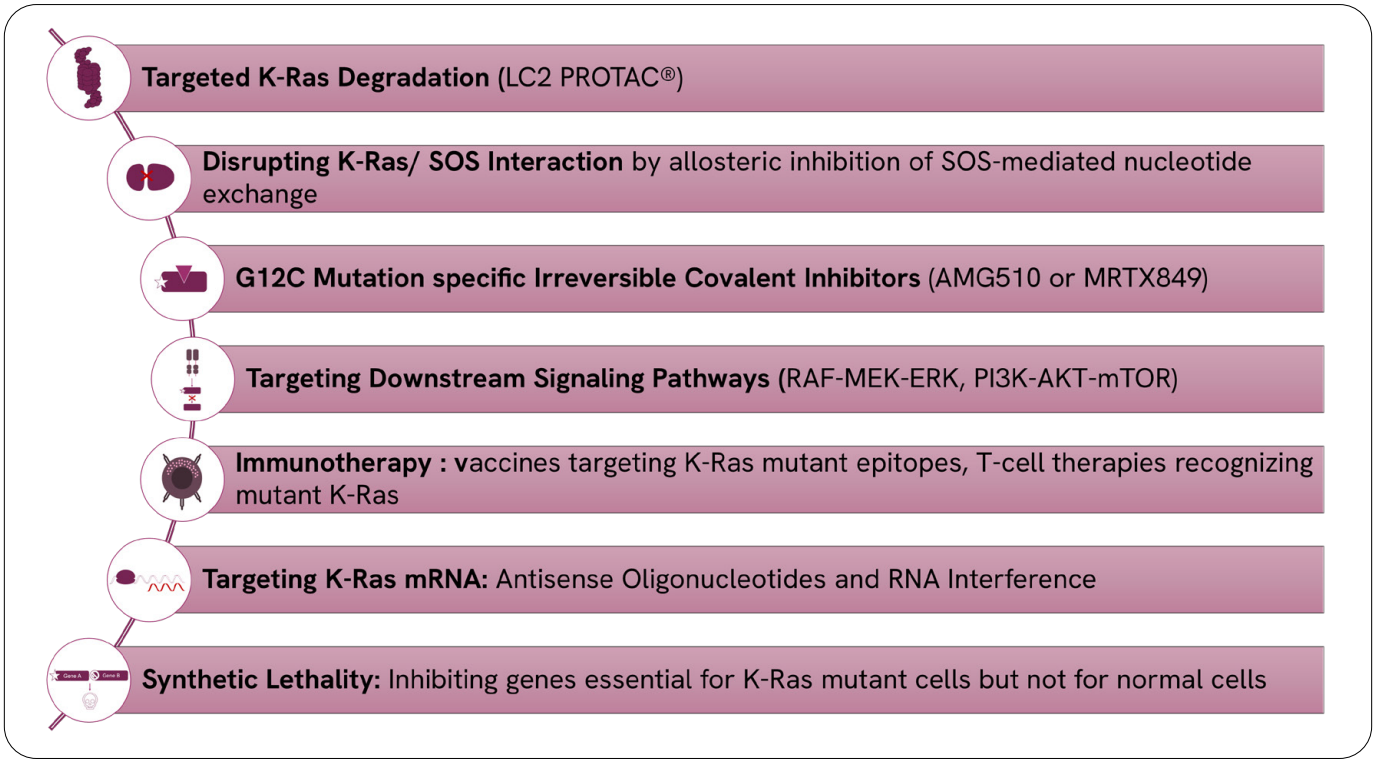


Figure 1: Recent drug discovery advancements to inhibit K-Ras activity.

Combining these diverse strategies highlights the multifaceted efforts to overcome challenges in targeting K-Ras, and accurately monitoring K-Ras protein expression levels is crucial in cancer research and such therapeutics development.

Popular techniques for assessing K-Ras expression include Western Blot, ELISA, Mass Spectrometry, Flow Cytometry, and Immunofluorescence/Immunohistochemistry. Each method presents technical limitations, including complexity, time consumption, antibody cross-reactivity, the need for sophisticated equipment and expertise, subjective interpretation, and low throughput. These constraints often require integrating various methods to achieve a precise and thorough assessment of K-Ras protein expression.

Our study relies on the use of both wild-type (WT) and mutated K-Ras cells to demonstrate the high specificity and sensitivity of the no-wash HTRF™ K-Ras immunoassay. Notably, our findings highlight the assay's ability to distinguish between small molecule inhibitors and degraders, including the PROTAC LC2. This underscores the reliability and precision of the method in studying the K-Ras/MAPK pathway. Additionally, in a pharmacological experiment where cells were treated with increasing concentrations of LC2 PROTAC, K-Ras protein degradation was evaluated using both HTRF and Western Blot. Our results demonstrate a strong correlation between HTRF and Western Blot. Overall, the HTRF K-Ras immunoassay proves to be an effective tool, offering advantages in terms of speed, throughput, and precision compared to traditional methods.

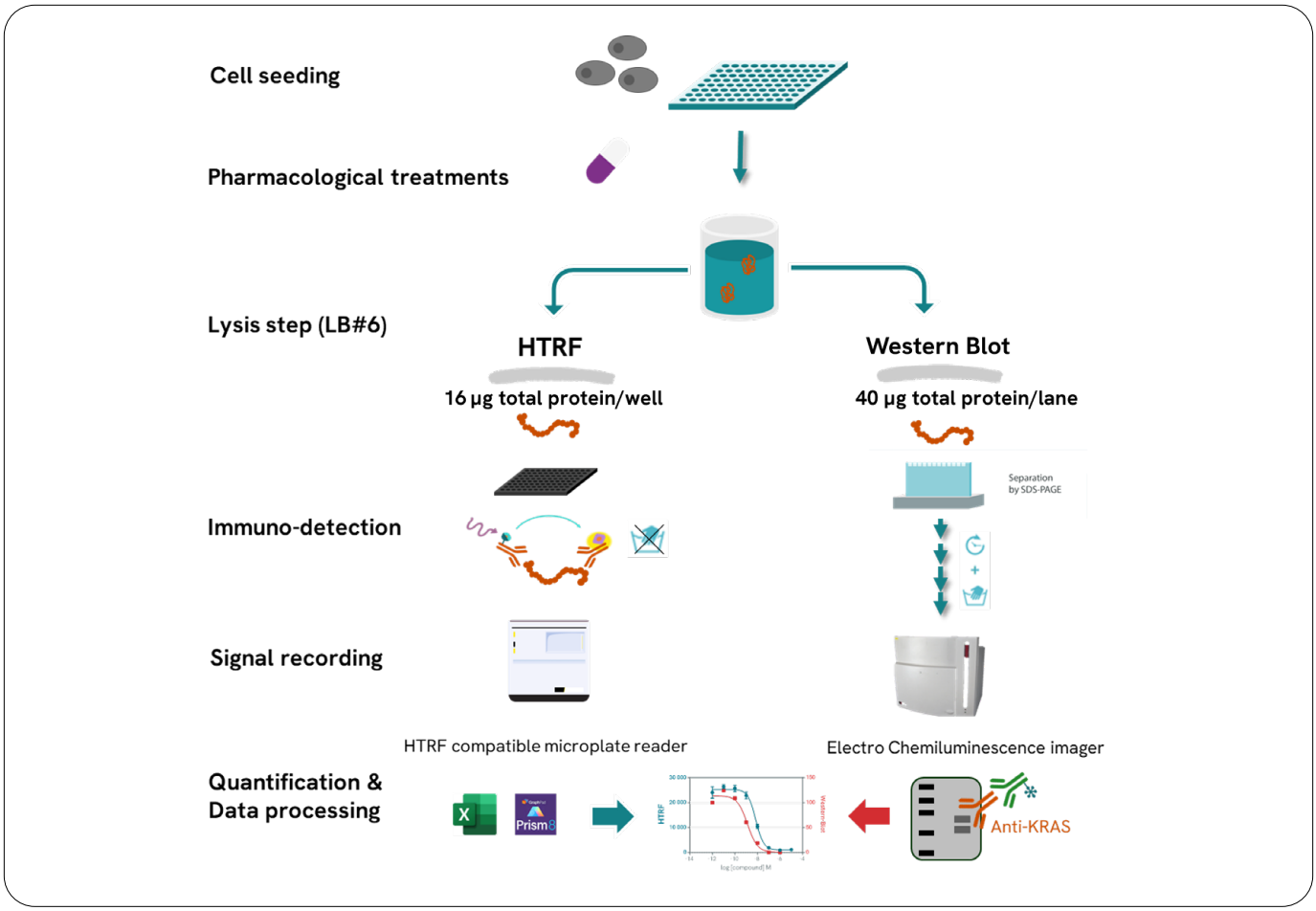


Figure 2: Workflow of the study design.

Results

Selective degradation of K-Ras G12C using PROTAC across multiple cell lines

Mia-Paca-2, SW1573, and Calu-1 cells, all homozygous K-Ras G12C, were seeded in a 96-well culture-treated plate at a density of 100,000 cells per well in complete culture medium and incubated overnight at 37°C with 5% CO_2. The following day, cells were treated for 24 hours with increasing concentrations of LC2 PROTAC, which is designed to induce degradation of the K-Ras G12C mutant, as well as MRTX-849, a covalent inhibitor of K-Ras G12C, used in LC2 PROTAC as the warhead. For control conditions, cells were pre-incubated with the proteasome inhibitor Epoxomicin (1 µM) for 1 hour at 37°C prior to the addition of the PROTAC. Cell lysates were then analyzed using the HTRF K-Ras kit.

As depicted in Figure 3, the LC2 PROTAC triggered a dose-dependent decrease in K-Ras protein levels in all three cell lines with a potency in the micromolar range, and a degradation rate ranging from 60% to 95%, which are consistent with the publication from Bond et al., 2020. The lack of K-Ras decrease observed in the presence of epoxomicin confirmed that the degradation was driven by the ubiquitin-proteasome system, as also described in the literature. Importantly unlike the LC2 PROTAC, the specific K-Ras G12C covalent inhibitor MRTX-849 did not modulate K-Ras expression level, as expected.

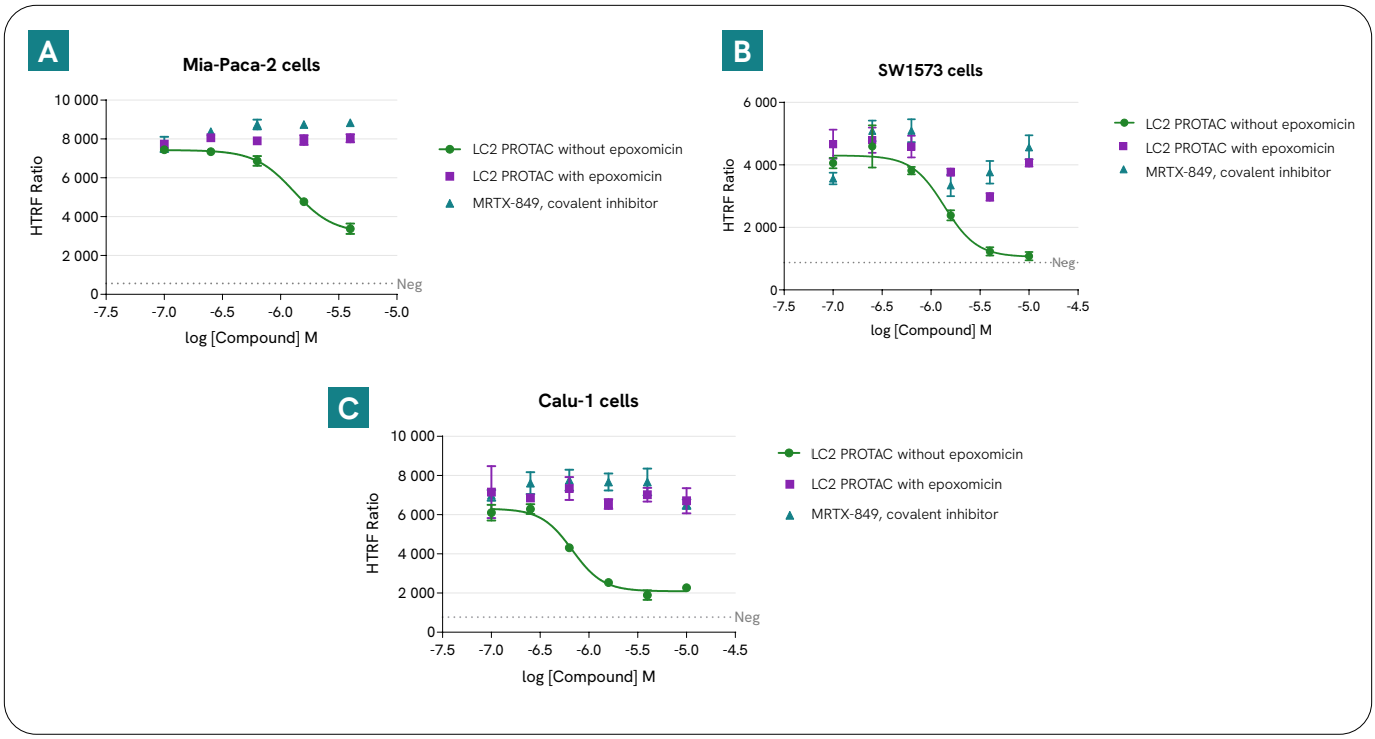


Figure 3: K-Ras detection in Mia-Paca-2 cells (panel A), SW1573 cells (panel B), and Calu-1 cells treated with LC2 PROTAC in the absence or presence of epoxomicin, as well as MRTX-849, a covalent inhibitor of K-Ras G12C (warhead in the LC2 PROTAC), utilizing the HTRF K-Ras Total Kit.

Table 1: LC2 PROTAC potency (DC50 in µM) and efficacy (degradation rate in %) determined in the three different cell lines.

LC2 PROTAC®	Potency (DC50)	Efficacy (Bmax or degradation rate)
Mia-Paca-2 cells	1.3 µM	60%
SW1573 cells	1.36 µM	95%
Calu-1 cells	0.67 µM	80%

Comparison of HTRF K-Ras total detection kit and Western Blot analysis

Mia-Paca-2 cells were seeded at a density of 100,000 cells per well in a 96-well culture-treated plate and treated with increasing concentrations of LC2 PROTAC for 24 hours. As a control, cells were pre-incubated with the proteasome inhibitor Epoxomicin (1 µM) for 1 hour at 37°C prior to the addition of the PROTAC. Following cell lysis using HTRF Lysis Buffer 6, lysates were analyzed by both HTRF and Western Blot techniques. The HTRF K-Ras kit, which is more sensitive than the Western Blot method, used 16 µg of total protein, while 40 µg were required for Western Blot analysis.

To ensure consistency, both HTRF and chemiluminescent signals were normalized against the GAPDH housekeeping protein, using the HTRF Housekeeping GAPDH kit and GAPDH immunoblotting, respectively.

As illustrated in Figure 4, the results from HTRF K-Ras and Western Blot analyses show LC2 induced K-Ras degradation. As anticipated, inhibition of the proteasome activity with epoxomicin prevents LC2 PROTAC induced K-Ras downregulation. Overall, HTRF and Western Blot technique exhibit a high degree of correlation, indicating the comparability of these two methods and the consistency of HTRF results.

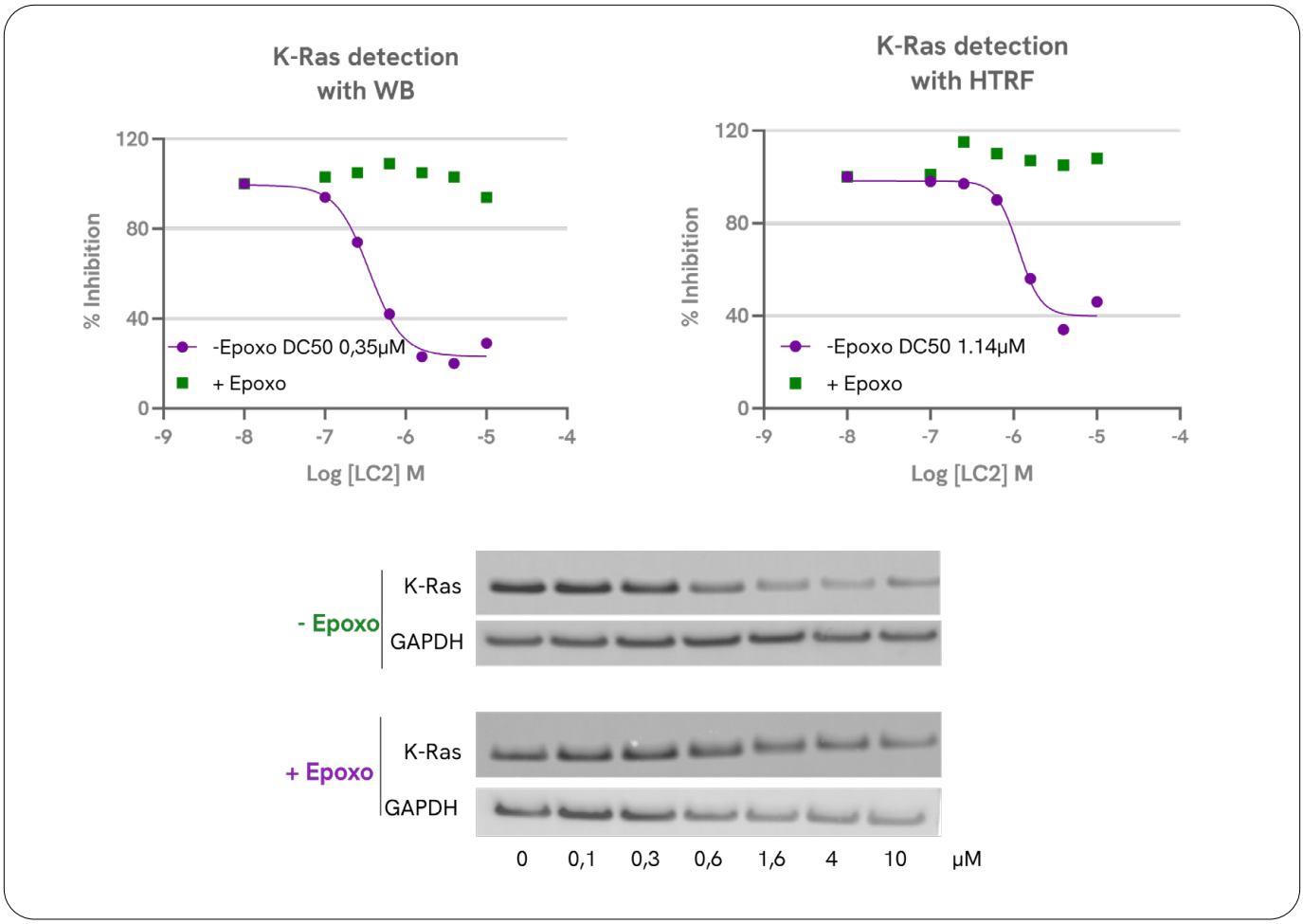


Figure 4: Correlation between HTRF K-Ras Total kit and the Western Blot detection methods.

HTRF total K-Ras assay specificity using HAP-1 K-Ras KO cell line

RNA expression levels of K-Ras, H-Ras, and N-Ras genes were quantified in HAP-1 cells. As reported in Figure 5A, significant RNA expression for all three genes was found in HAP-1 cells (catalog reference Horizon Discovery: HAP1 WT #C631).

To ensure the detection specificity of K-Ras without cross-reactivity to N-Ras and H-Ras, cell lysates from HAP1-WT and HAP1-K-Ras KO cells (catalog reference Horizon Discovery: HAP1 KO K-Ras #HZGHC005816c001) were prepared and tested using the HTRF Total K-Ras Kit.

In Figure 5B, the HTRF signal was absent in HAP-1 K-Ras KO cells, which express N-Ras and H-Ras. This finding confirmed the specificity of the HTRF K-Ras Total Kit for detecting K-Ras exclusively.

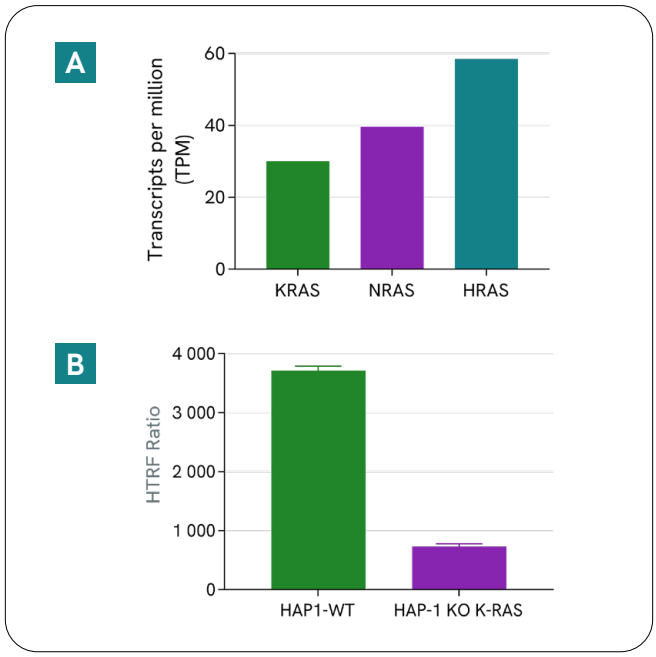


Figure 5: K-Ras, H-Ras, and N-Ras mRNA transcripts in HAP1 cells (panel A), K-Ras protein expression in in HAP-1 K-Ras WT and KO cells determined with HTRF K-Ras Total Kit (panel B).

Quantification of total K-Ras expression level in various cell lines

Cell lysates from various commercially available cell lines harboring different K-Ras genotype profiles at codon 12 were prepared and tested using the HTRF Total K-Ras Kit (table 2).

K-Ras levels were monitored using the HTRF Total K-Ras Kit in three homozygous cell models expressing K-Ras G12C and one model expressing both WT and G12C alleles.

As depicted in Figure 6, significant detection of K-Ras expression was observed in all cell lines tested demonstrating the HTRF K-Ras Total Kit enables the detection of WT as well as K-Ras mutated proteins.

Table 2: Status of K-Ras gene at codon 12 in different cell lines.

Cell lines	K-Ras gene status codan 12
SW1573	Homozygous G12C
Mia-Paca-2	Homozygous G12C
Calu-1	Homozygous G12C
NCI-H358	Heterozygous G12C/WT
HeLa	Heterozygous WT/WT

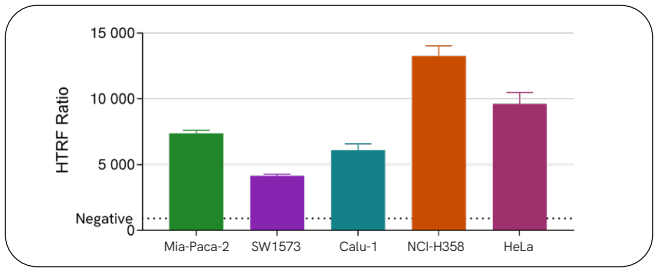


Figure 6: K-Ras protein expression detected in multiple K-Ras WT and K-Ras G12C cells with HTRF K-Ras Total Kit.

Conclusion

This study demonstrates that the HTRF K-Ras Total Kit enables the distinguishing between K-Ras small molecule inhibitors and degraders highlighting its utility in characterizing diverse therapeutic modalities. Furthermore, the assay's effectiveness is confirmed across various cell line models, including those harboring K-Ras mutations, further establishing it as a valuable tool for cancer research and therapeutic development. Overall, the development and validation of the no-wash HTRF K-Ras immunoassay represents a significant advancement in K-Ras-targeted therapeutic strategies, offering a rapid, precise, and reliable method for detecting both wild-type (WT) and mutated K-Ras protein levels with high specificity, even in the presence of other RAS isoforms.

Materials and reagents

	Reagent	Supplier	Part number
Microplates	SpectraPlate 96-well	Revvity	6005650
	ProxiPlate 384-shallow well Plus, white	Revvity	6008280/9
HTRF kits	HTRF Total K-Ras	Revvity	64K-RasTPEG/H
	HTRF Alpha-tubulin Housekeeping Cellular Kit	Revvity	64ATUBPEG/H
Cell lines	SW1573	ATCC	CRL-2170
	Mia-Paca-2	Cytion	300438
	Calu-1	ATCC	HTB-54
	NCI-H358	ATCC	CRL-5807
	HeLa	Cytion	300194
	HAP-1 WT	Horizon discovery	C631
	HAP-1 K-Ras KO	Horizon discovery	HZGHC005816c001
Compounds	LC2	TOCRIS	7420
	MRTX-849	MedChemExpress	HY-130149
	Epoxomicin	MedChemExpress	HY-13821
Antibodies	Anti-K-Ras Ab for WB	ThermoFisher	703345
	Anti-rabbit Ab (HRP)	ThermoFisher	G21324
	Anti-GAPDH AB (HRP)	CST	8884

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