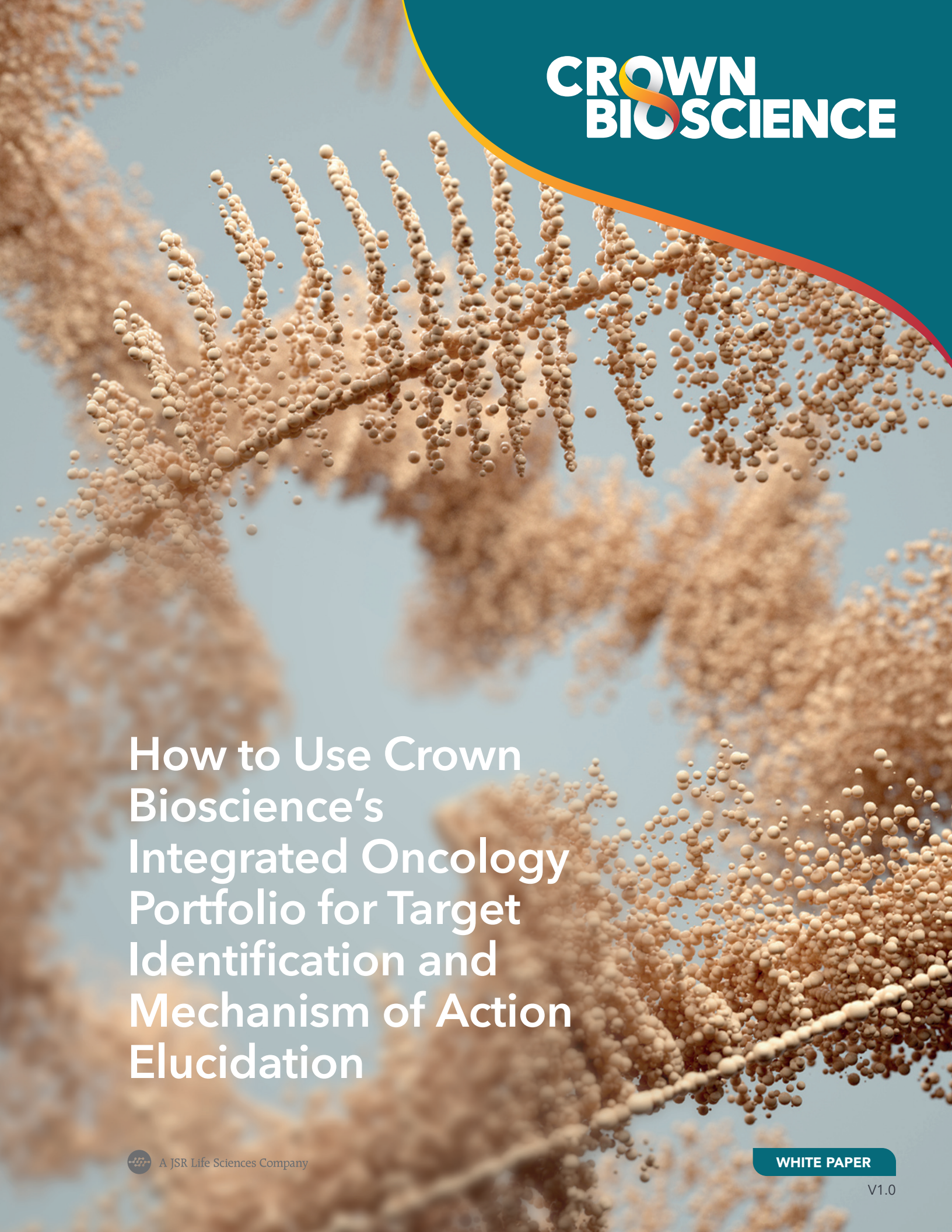

CROWN BIOSCIENCE

How to Use Crown Bioscience's Integrated Oncology Portfolio for Target Identification and Mechanism of Action Elucidation

A JSR Life Sciences Company

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V1.0

Target Identification and Mechanism of Action Elucidation

Target identification and mechanism of action (MOA) studies play a critical role in the early stages of cancer drug discovery and development. Current drug discovery trends focus on understanding disease mechanisms, so targets for drug intervention can be identified and used to discover lead compounds. With the advent of precision medicine, identifying relevant targets across cancer indications and truly understanding how a new agent functions against these targets within diverse patient populations is essential.

Cell-based assays are fundamental for identifying new biologically active compounds. They allow initial tests on potential drug candidates to be performed in an environment more relevant to disease, with later follow-on studies providing more precision on the protein target(s) responsible for the phenotype observed.

Target identification is performed by multiple methods, including:

- direct biochemical and cellular assays
- pharmacogenetic analysis
- *in vivo* pharmacological studies

with combinations of these methods often required to characterize a compound's full effects and to comprehensively elucidate MOA[1,2].

Crown Bioscience provides an integrated oncology portfolio to support our clients in every step of the target identification and MOA assessment process, and then efficiently move lead compounds to the next stage of preclinical drug development.

Step 1: Select the Right Cell Lines

Choosing the right cell lines is an important first step for any oncology drug development project. They need to provide the correct disease biology, genetics (e.g. mutations, fusions), biomarkers, or a varying panel of these factors to fully study both the drug being tested and the possible indications for use. Choosing exactly which cell lines or panel of cell lines to use depends on the indication and the characteristics of new agent targets.

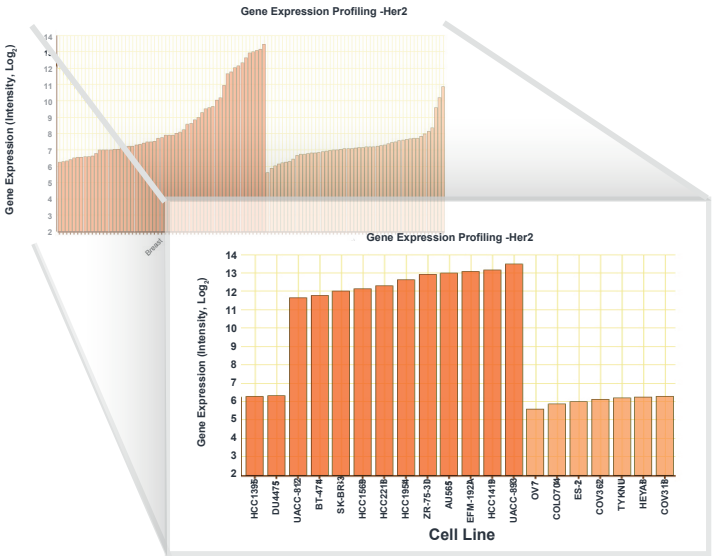
To help you make the right selection at this crucial stage, Crown Bioscience has a large portfolio of models and integrated resources.

Use Crown Bioscience's Proprietary Databases to Make an Informed Decision

One of the best ways to make an informed decision on the right cell lines for your study is to use a collated database containing a wealth of cell line data. Crown Bioscience has a unique collection of human, rat, and mouse cancer cell lines across a wide range of indications, annotated within our easy to use online database, XenoBase®.

Over 600 cell lines can be browsed by cancer type, with basic species and subtype available as well as many including histopathology information, growth curves, and dose response to standard of care agents. This allows cell lines to be chosen dependent on cancer type/subtype and/or treatment response.

Figure 1: Make an Informed Choice using a Collated Cell Line Database



The real power within **XenoBase** comes from searching in house and public profiling data to quickly and easily choose cell lines based on gene expression, copy number, mutational status, gene fusion, or a combination of factors. This enables the rapid stratification of a large cell line collection down to specific cell lines of interest, based on specific genetic features.

Figure 1 exemplifies a **XenoBase** search for breast and ovarian cancer cell lines based on HER2 expression – showing all breast and ovarian cancer cell lines available with HER2 expression level, then stratified by the top and bottom 10% HER2 expressers.

Can't Find the Right Cell Line in Our Database?

If you still can't find the cell line you need within our database, we can generate cell lines specific to your needs including from our PDX models.

PDX Derived Cell Lines

Patient-derived xenografts (PDX) closely recapitulate patient disease histo- and molecular pathology, providing the most predictive model available for preclinical research. As they maintain the genetic profiles of the original patient tumor, they can contain clinically relevant features such as rare fusions or mutations lacking from commercially available immortalized cell lines, or provide rare disease subtypes.

Models of interest can be back translated to provide *in vitro* research resources – including derived cell lines which maintain the unique feature of interest for target identification studies. These **PrimePanel**™ cell lines are derived from mouse stromal cell-depleted cancer cell cultures from our **HuPrime**® PDX tumors[3] (Figure 2).

We have approximately 50 **PrimePanel** cell lines available, with 10 more undergoing validation, searchable through our collated PDX database, **HuBase**™ (with a specific **PrimePanel** filter included). These cell lines cover a variety of cancer types including colorectal, gastric, lung, and pancreatic cancer, and include features such as EGFR and HER2 amplification, BRAF V600E mutation, and RET and RSPO3 fusions.

Figure 2: Derivation of PrimePanel Cell Lines

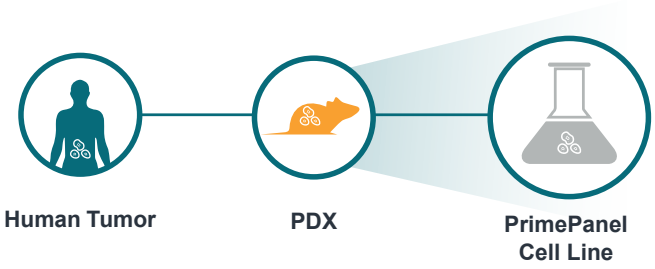


Figure 3: Assess Cell Line Panels to Identify Characteristics of Interest

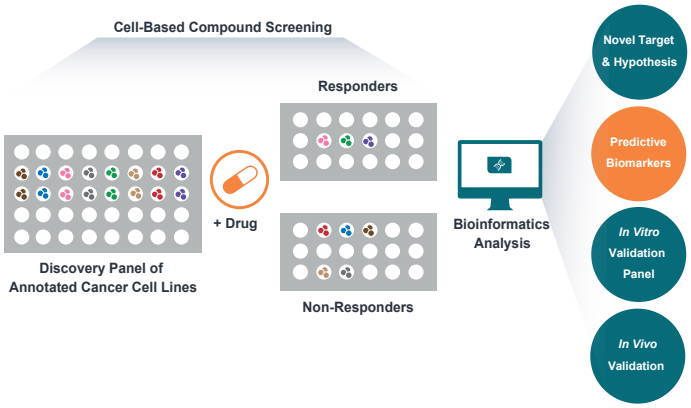
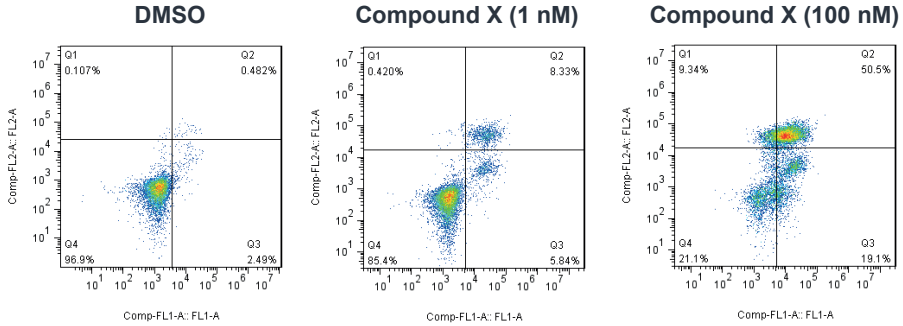


Figure 4: Cell Apoptosis Evaluation by FACS (Annexin V/PI Method)



Cell lines with other clinically relevant features can be created from ~80% of our PDX collection of 2,500 models. Almost 1,800 models are detailed within Hu**Base**, where they can be easily searched by gene expression, copy number, mutation, or gene fusion, or browsed by cancer type and more basic disease subtype and standard of care response filters. Models of interest for cell line creation can also be found from tumor tissue microarrays (TMAs). Crown Bioscience currently offers over 40 PDX TMAs, featuring almost 950 of our models.

Our unrivaled PDX collection offers the highest likelihood of finding your subtype or clinically relevant genetic feature of interest for cell line creation.

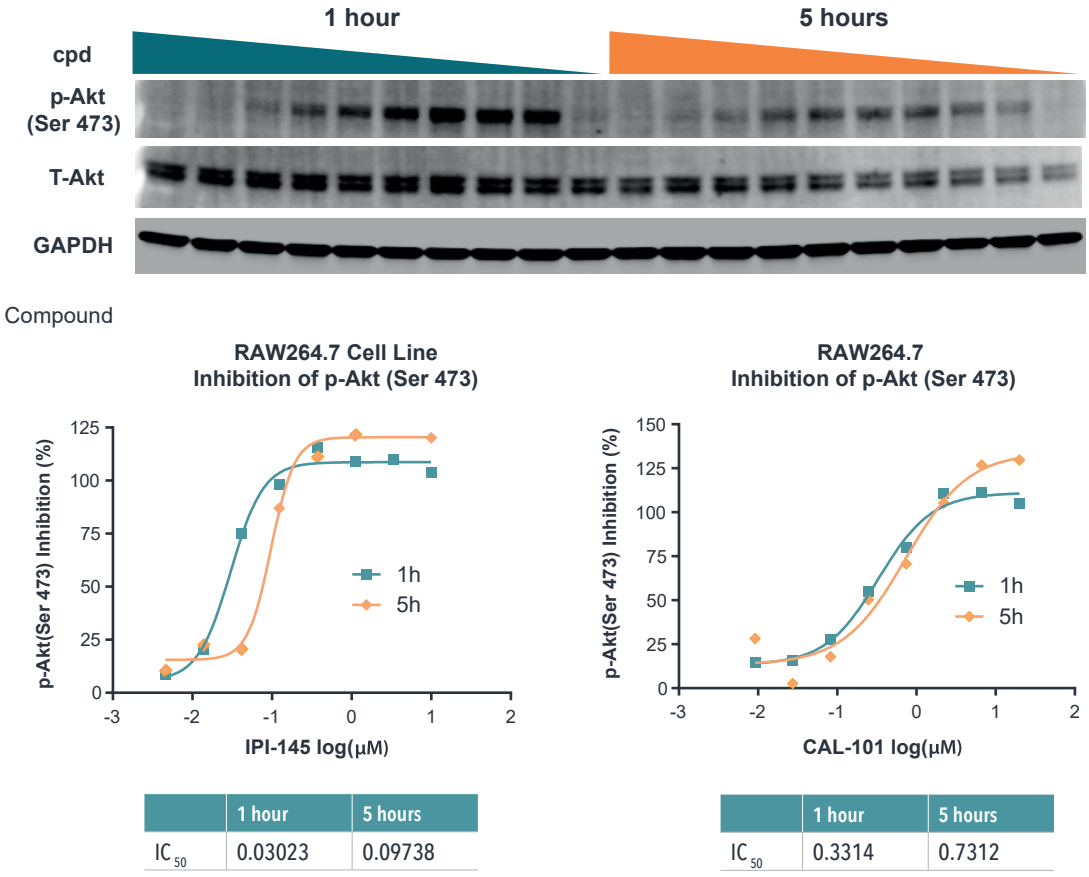
Unsure Which Cell Lines Are Right for You?

If you aren't sure which characteristics would be best for your target identification and MOA studies, don't risk guessing. Select one of our *in vitro* panels of known tumorigenic cell lines to assess multiple cell lines simultaneously (Figure 3). All of our cell line panels are annotated and well-characterized (with searchable data available in Xeno**Base**), STR verified, and mycoplasma tested.

Available cell line panels include:

- **OmniPanel™** - genomically diverse collection of over 500 cancer cell lines, with new cell lines constantly added.
- **XenoSelect™** - an Omni**Panel** subset of over 150 cancer cell lines with corresponding xenograft models for a quick *in vitro* to *in vivo* transition.
- **CCLE Panel** - over 350 cell lines with detailed Cancer Cell Line Encyclopedia gene expression and copy number data available at Crown Bioscience.
- **RNAseqPanel** - 150 cell line panel each with Crown Bioscience's own in house RNAseq data, allowing the correlation of drug response with mutational status, copy number variation, and expression levels.
- **PrimePanel** primary cell lines derived from Hu**Prime** PDX models to recapitulate clinically relevant features of disease.

Figure 5: Molecular Target Inhibition Displayed via Western Blot



	1 hour	5 hours
IC_{50}	0.03023	0.09738

	1 hour	5 hours
IC_{50}	0.3314	0.7312

Step 2: Select Analyses to Further Characterize Your Target

Once you've chosen your cell lines, you then need to select the correct analyses to verify if your target is expressed and assess how your target effects tumor growth.

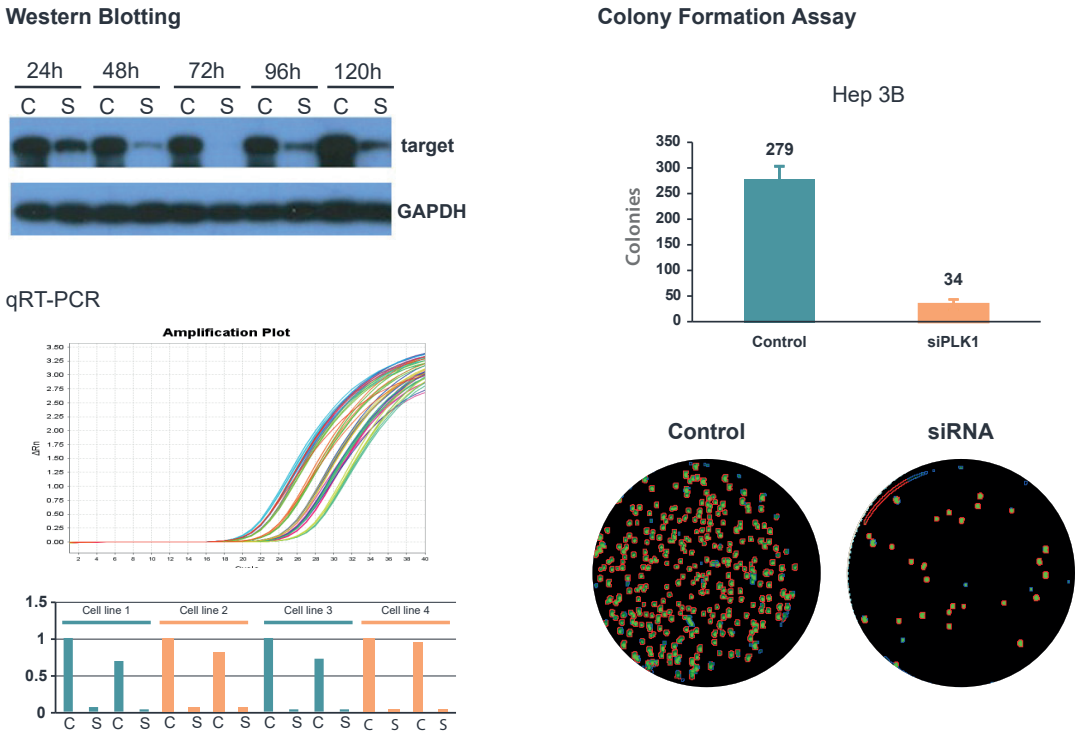
Is your Target Expressed?

Target expression is measured by a range of standard molecular biology techniques e.g. qPCR, western blot, IHC, etc. These techniques plus a variety of cancer biology assays can also verify target inhibition by your novel agent.

A range of our available assays are shown in Figure 4 through Figure 7:

- FACS analysis (Annexin V/PI method) can be used to identify the level of cell apoptosis following drug treatment, with Figure 4 showing the MV-4-11 leukemia cell line 48 hours post treatment with 2 different concentrations of a novel anticancer compound.
- Western blotting can identify molecular target inhibition – Figure 5 shows the decrease in phospho-Akt induced by IPI-145 (duvelisib, a PI3K-δ,γ inhibitor) and CAL-101 (idelalisib, a P110δ specific PI3K inhibitor) treatment, confirming that kinase activity and Akt phosphorylation has been inhibited.
- si/shRNA knockdown can be used to target and silence protein coding genes. Protein knockdown is confirmed by a series of analysis assays including western blot, qRT-PCR, and colony forming assays (Figure 6).
- RNA sequencing identifies genetic features such as gene fusions (can be confirmed by RT-PCR), which can then be targeted in cell viability assays by agents directed at the feature of interest. Figure 7 shows the RET fusion found in PDX model CR1520, and the subsequent testing of RET inhibitors on the derived CR1520 **PrimePanel** cell line.

Figure 6: siRNA/shRNA Protein Knockdown Confirmed by a Range of Assays



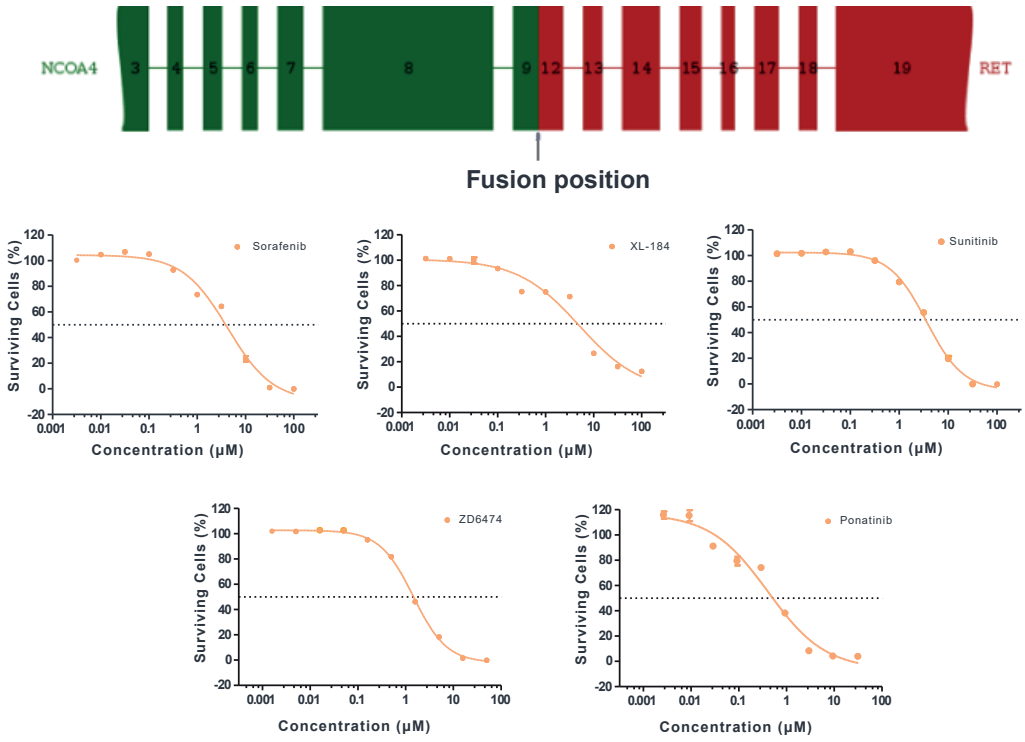
Does your Target Effect Tumor Growth and How?

Once target expression and drug target inhibition are confirmed, Crown Bioscience can also help you find out if your new agent affects tumor growth.

Figure 8 shows treatment of three cell lines with HER2 inhibitors – SK-BR-3 and BT-474 overexpress HER2 and respond well to the novel agents, while cell killing is decreased and surviving cells increased for MCF7 which is a HER2 negative cell line.

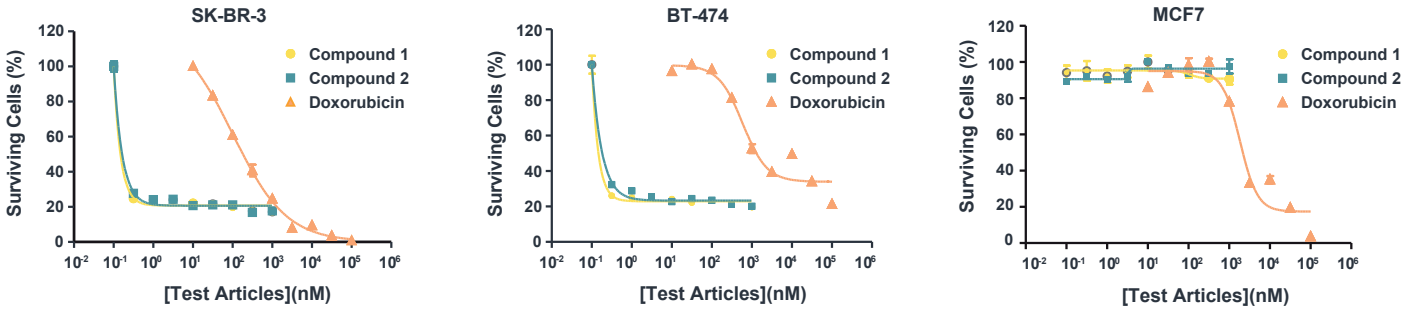
Cancer cell line profiling is shown in Figure 9 – with a blood cancer panel treated with quizartinib (AC220) a second-generation FLT3 inhibitor for FLT3(ITD/WT). Cell lines driven by FLT3 were shown to be highly sensitive to this agent.

Figure 7: RNA Sequencing of RET Gene Fusion and Targeted RET Inhibitor Treatment



Compound	Sorafenib	Sunitinib	XL-184	ZD6474	Ponatinib
IC_{50} (μM)	3.87	3.45	4.58	1.46	0.468

Figure 8: HER2 Inhibitor Evaluation in HER2 Overexpressing and Negative Cell Lines



	Compound 1	Compound 2	Doxorubicin
IC_{50}	~0.01567	~0.005407	101.4

	Compound 1	Compound 2	Doxorubicin
IC_{50}	~0.02491	~0.001984	592.1

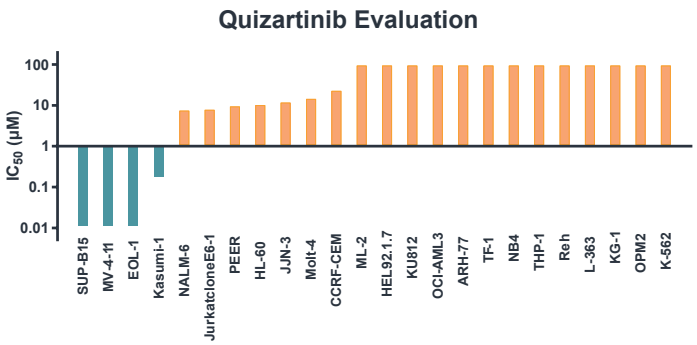
	Compound 1	Compound 2	Doxorubicin
IC_{50}	109.5	~3.345	1849

Not Sure How Your Compound Interacts with Targets of Interest?

If you aren't sure how your compound is interacting with your targets of interest, you can try our compound screening service. Omni**Screen™** is Crown Bioscience's cost-effective, large-scale cell screening platform, based on the panels discussed above or custom built using cell lines selected through Xeno**Base** (Omni**Screen** configuration shown in Figure 3). We offer enhanced cell panel growth data for improved screening quality, including validated seeding densities across multiple time points. Multiple screening platforms for selected cell lines can also be provided on request, including IncuCyte® ZOOM (real time) and Acumen eX3 (multiparameter).

Screening runs every 3 months, and any subpanel of 50 or more cell lines can be scheduled to maximize savings. Omni**Screen** runs on a 384 well plate format, with two compounds per plate and nine test concentrations per compound. We employ rigorous quality controls with chemotherapeutic agents, as well as using medium control, and vehicle control wells to ensure data quality. Internal drug controls and cell line revival costs are covered by Crown Bioscience to enhance cost-effectiveness.
We have experience with a variety of different molecules, such as kinase inhibitors, antibodies, antibody-drug conjugates, epigenetic modulators, and small and large molecules, as well as extensive experience in combination assays and screens. All study data for your agent can be reviewed in real-time via our secure online client account.

Figure 9: Quizartinib Evaluation in Blood Cancer Cell Line Panel



Example study data is shown in Figure 10, profiling a panel of 45 lung cancer cell lines with AZD9291, a third generation EGFR inhibitor, where response can be correlated with known cell line mutations.

Step 3: Translate your Studies to *Ex Vivo* and *In Vivo* Models

Once you have a target identified and MOA established, you need to be able to move to *ex vivo* or *in vivo* studies to progress preclinical development. Crown Bioscience's integrated oncology portfolio contains a range of *in vitro* to *in vivo* linked models and assays to support a swift and seamless transition.

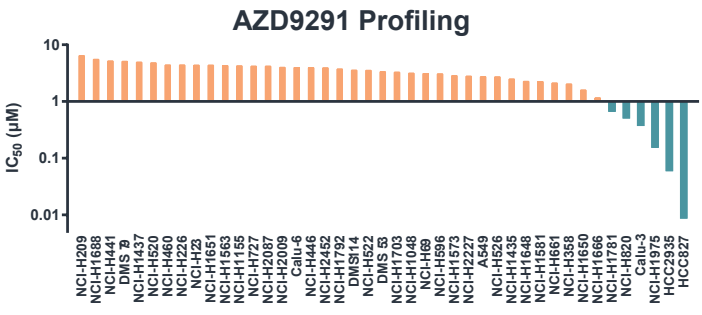
3D *In Vitro* and *Ex Vivo* Platforms

While 2D cell culture is an ideal method for early drug development target identification and rapid drug screening, it lacks many in situ tumor biology characteristics.

To overcome this, Crown Bioscience provides a range of 3D *in vitro* and *ex vivo* assays. 3D assays represent efficient, accurate, and clinically relevant preclinical tools to investigate the activity and understand mechanisms of action of new cancer drugs.

We have validated over 170 cell lines for 3D *in vitro* culture for use in methylcellulose and soft agar assays (shown in Table 1), generating dose response curves for novel agents.

Figure 10: OmniScreen Lung Cancer Cell Panel Profiling



Example IC50 curve for the NCI-H1975 cell line shown in Figure 12.

Gene	Rank in IC_{50}	Cell Line Name	IC_{50} (μM)	Source	Cancer Type	Amino Acid Change
EGFR	1	HCC827	0.009	RNAseq	Lung	746_750del
EGFR	2	HCC2935	0.058	RNAseq	Lung	R521K
EGFR	3	NCI-HI975	0.150	RNAseq	Lung	T790M
EGFR	3	NCI-H1975	0.150	RNAseq	Lung	L858R
EGFR	4	Calu-3	0.366	RNAseq	Lung	R521K

Also available are *ex vivo* models derived from PDX, allowing screening across our vast collection of models. This includes freshly isolated cells (utilized immediately on the day of isolation), or fresh frozen cells which can reduce assay timelines, with no lag time as PDX tumor donors grow out.

Crown Bioscience also provides the 3D Tumor Growth Assay (TGA), a proven platform for oncology drug development utilizing cells derived from PDX models. The 3D TGA provides an assay which more closely represents the human condition than 2D culture[4].

The 3D TGA utilizes a low stiffness laminin rich extracellular matrix (IrBME, Cultrex®) to embed tumor cells. Admixing with hMSCs (e.g. IL-6, HGF) and CAFs provides the paracrine signaling present in the tumor microenvironment (TME) of solid tumors. The addition of hormones (e.g. DHT/E2), restriction of glucose (≤7mM), and maintenance of an acidic pH (6.8) provide a 3D assay that is both "humanized" and TME-aligned for profiling of PDX-derived cells (including Prime**Panel** cell lines) and drug panels[4].

Correlation has been shown between response to anticancer agents in 3D TGA and *in vivo* models, and expected clinical outcome[4]. Figure 11 shows cells derived from the NSCLC LU6422 PDX model. The PDX model harbors a L858R mutation in the intracellular kinase domain, related to hypersensitivity to EGFR targeted therapies such as erlotinib in patients. Cells derived from LU6422 are hypersensitive to erlotinib in 3D TGA, which correlated with complete tumor regression of the PDX model (subcutaneous implantation with MSCs) following erlotinib treatment[4].

Table 1: *In Vitro* Cell Lines Validated in 3D Cell Culture Assays

	Methylcellulose	Soft Agar
Cancer Type	**Cell Line**	
Blood	Daudi, DoHH-2, EHEB, Jurkat (clone E6-1), JVM-13, JVM-2, JVM-3, K-562, Karpas299, Kasumi, MEC-1, MEC-2, MEG-01, Molm-16, MOLT-4, Namalwa, OCI-LY19, REH, SU-DHL-10, SU-DHL-5, SUP-B15, TF-1, THP-1, Toledo, U-937, WSU-DLCL-2	
Bone	CADO-ES1, RD-ES	
Brain & Nerves	A172, H4, IMR-32, LN18, LN229, SF126, SH-SY5Y, SK-N-SH, U-118 MG, U251, U-87 MG	
Breast	BT-549, DU4475	
Colorectum	HT-29, HCT 116	HT-29, HCT-116
Esophageal	KYSE70, KYSE270, KYSE410, TE-1	
Head & Neck	FaDu, SW579	
Liver	HCCLM3, Hep G2, HLF, HUH-7, JHH-5, JHH-7, MHCC97H, PLC/PRF/5, SKHEP- 1, SNU-398, SNU-878, SNU-886	Hep 3B, Hep G2,JHH-7, PLC/PRF/5, SK-HEP-1
Lung	A549, Calu-6, DMS53, DMS79, DMS114, EBC-1, H69AR, HCC4006, NCI-H1155, NCI-H1299, NCI-H1373, NCI-H1395, NCI-H1417, NCI-H1435, NCI-H1437, NCI-H157, NCI-H1581, NCI-H1650, NCI-H1688, NCI-H1703, NCI-H1792, NCI-H1836, NCI-H1975, NCI-H2052, NCI-H209, NCI-2227, NCI-H226, NCI-H23, NCI-H358, NCI-H446, NCI-H460, NCI-H520, NCI-H522, NCI-H526, NCI-H69, NCI-H82, SK-MES-1	NCI-H1299
Ovary	OVCAR-8, SK-OV-3, SW626, SW756	
Pancreas	AsPc-1, Capan-1, MIAPaCa-2, PANC-1, PL45	CFPAC-1, MIAPaCa-2
Prostate	PC-3	
Stomach	A3/KAW, AGS, AZ521, GTL-16, HGC-27, Hs 746T, IM95, IM95m, KATO III, MKN1, MKN45, MKN74, NCI-N87, NUGC-3, NUGC-4, OCUM-1, SCH, SNU-1, SNU-16, SNU-5, SNU-484, SNU601, SNU-620, SNU-638, SNU-668, SNU-719, YCC-2, YCC-7, YCC-10, YCC-11	AGS, AZ-521, GTL-16, HGC-27, MKN1

In Vitro Cell Lines to *In Vivo* Xenograft Models

A traditional step in preclinical development is to move to xenograft studies, allowing further elucidation of individual agent properties and MOA, and to look at a compound's initial efficacy *in vivo*.

Crown Bioscience supports rapid *in vitro* to *in vivo* study turnaround, with many of our *in vitro* cell lines having *in vivo* models available. Specifically our Xeno**Select** collection (including Xeno**Select** Omni**Screen** panel) contains over 150 cell lines ready and waiting for *in vivo* efficacy studies.

All Xeno**Select** models are proven, oncogenic cell lines, validated both *in vitro* and *in vivo*, with full data searchable in Xeno**Base**. Data can be correlated from 2D and 3D *in vitro* to *in vivo* studies, providing confidence in study results. Example data is shown in Figure 12 for the NCI-H1975 NSCLC cell line and xenograft model, which harbor the T790M mutation. Treatment with AZD9291 which is designed to target EGFRm(+) sensitizing and T790M resistance mutants while sparing wild type EGFR, results in IC50s in the very low μM range which corresponds to complete *in vivo* tumor regression.

Figure 11: 3D TGA *Ex Vivo* Response Correlates to *In Vivo* PDX Tumor Growth Inhibition. Adapted from [4]

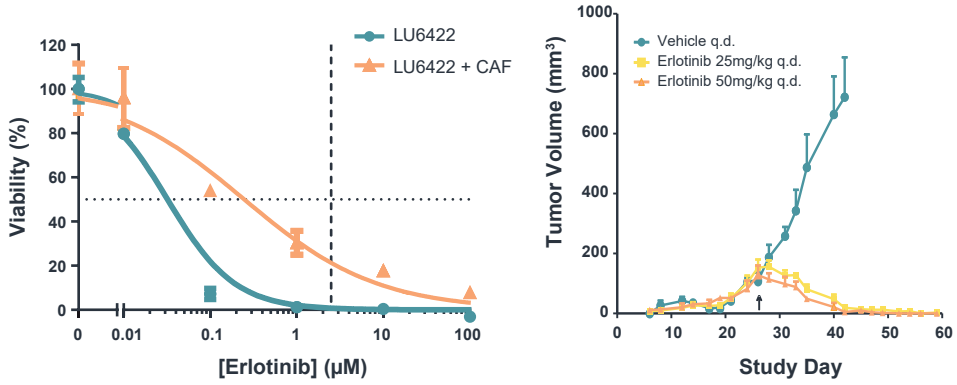
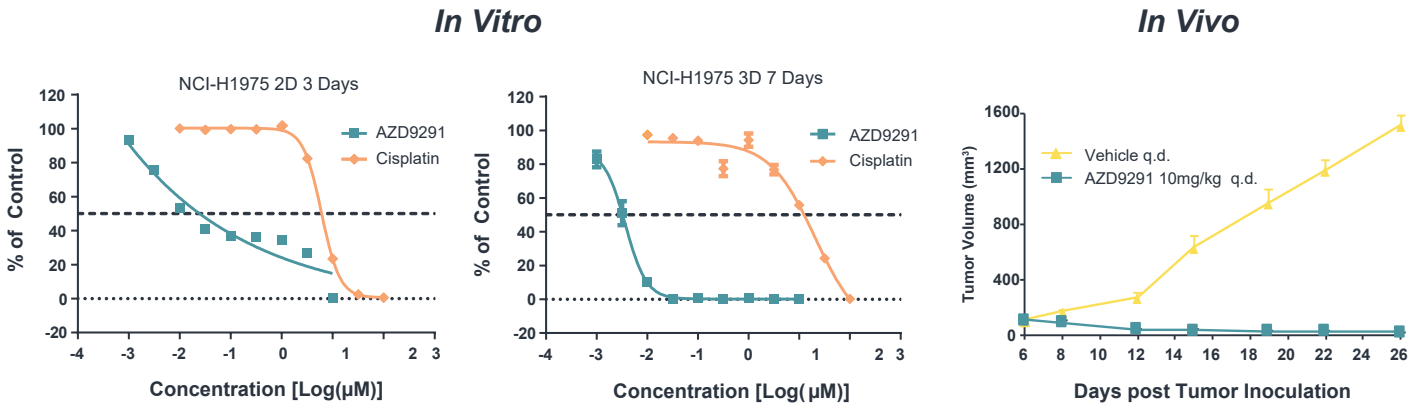


Figure 12: *In Vitro* Cell Line Response Correlates to *In Vivo* Xenograft Tumor Growth Inhibition



Cell Line	AZD9291		Cisplatin	
	IC_{50} (μM)	Max Inhibition (%)	IC_{50} (μM)	Max Inhibition (%)
NCI-H1975 2D	0.024	99.82	6.084	99.43
NCI-H1975 3D	0.003	99.98	12.462	99.75

Treatment	Tumor Volume (mm^3)	T/C Value (%) on Day 26	p Value
Vehicle	1470 ± 68	--	--
AZD9291	18 ± 2	1	<0.001

PrimePanel Cell Lines to PDX Models and PrimeXeno™

If using Prime**Panel** PDX-derived cell lines, then moving to *in vivo* models can mean moving directly back to the parental PDX with the same molecular features of interest. Correlation between cell lines *in vitro* and PDX models *in vivo* has been observed, for example within our lung cancer collection.

Within NSCLC, many factors around the EGFR gene and associated pathways infer tyrosine kinase inhibitor (TKI) and antibody sensitivity and resistance, including:

- EGFR exon 19 activating deletion conferring sensitivity to TKI such as erlotinib (observed in PDX model LU1235)
- EGFR exon 21 L858R mutation usually conferring sensitivity to TKI, sometimes accompanied by MET amplification which instead drives TKI resistance (PDX model LU0858 harbors both genetic events)

Cell lines derived from these models show varying levels of *in vitro* response to erlotinib, which can be directly translated to low or high levels of tumor growth inhibition in the parental PDX model (Figure 13).

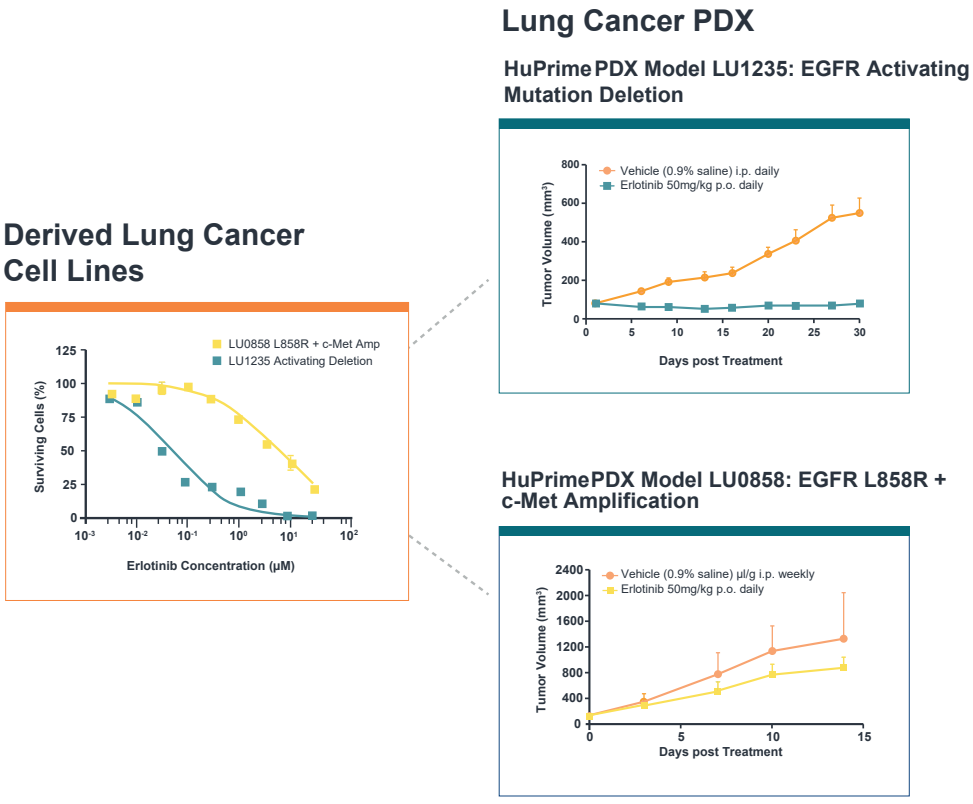
For researchers looking to perform *in vivo* compound screening or PK/PD studies where traditional cell line derived xenografts would normally be used, Prime**Panel** cell lines can also be established as "conventional" Prime**Xeno** xenograft models, again providing the genetic feature of interest in a robust system for early stage drug discovery.

Summary

Target identification and MOA studies are an important early stage in cancer drug discovery and development, requiring disease-relevant cell-based platforms, with linked later stage models to accelerate lead compounds.

Crown Bioscience's integrated oncology portfolio helps preclinical researchers advance their development programs via a comprehensive menu of *in vitro*, *in vivo*, and *ex vivo* products and services. Our portfolio is designed to provide assistance along every step of the preclinical drug development process, including target identification and MOA studies, so that go/no-go decisions can be made with confidence, using a single, uniquely positioned provider.

Figure 13: PrimePanel *In Vitro* Response Correlates to *In Vivo* PDX Tumor Growth Inhibition



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