Expanding the Toolbox of Gamma-irradiated Frozen Cells Validated for GPCR-mediated Kinase Signaling

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1 Abstract

The use of frozen cells for cell-based screening has become widely accepted within the drug discovery community. Separating cell production from the actual screening campaign not only increases your flexibility but also improves the data consistency as the cellular material can be controlled and validated before running the functional assay. One of the methods used to deliver frozen cells as a consumable to the final user is to gamma-irradiate the cells, so that cells can not resume growth after thawing.

This material is currently available for over 90 different GPCR targets: 60 AequoZen® cells, validated for calcium flux assays (AequoScreen® or fluorescent assay) and 48 cAMPZen® cells, validated for the LANCE® cAMP assay.

The increasing amount of evidence for biased agonism (collateral efficacy and varying potencies according to the signal transduction pathway observed), and the search for more physiologically relevant recording of the activity of drugs in development results in an increasing demand for assays relating to other signaling pathways activated by GPCRs.

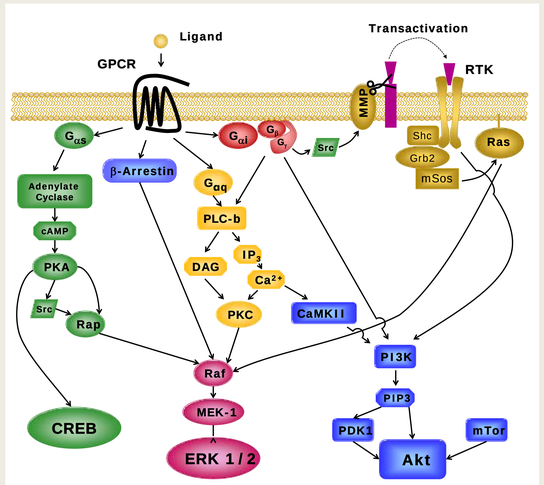
Amongst these GPCR-triggered pathways are numerous kinase pathways, including the ERK MAP Kinase cascade, PI3-Kinase, leading to Akt activation, and PKA, leading to CREB activation. The joint recording of the activation of these kinases upon GPCR activation provides a universal platform for evaluating pathway activation/inhibition in the presence of small molecules at all GPCRs, whatever their coupling to G_{ai}, G_{as} or G_{aq} proteins.

AlphaScreen® *SureFire*® assays provide a homogenous assay format for measuring ERK, Akt and CREB phosphorylation in cells. In this assay system, phosphorylated kinase substrates are captured by a combination of two antibodies, one recognizes the phosphorylate site and the other the total kinase. Only modified proteins that bind both antibodies are detected, using the Alpha technology (PerkinElmer) containing streptavidin coated donor beads and Protein A coated acceptor beads.

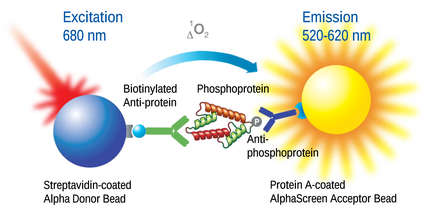
We show here that commercially available frozen, gamma-irradiated cAMPZen and AequoZen cells can be used together with AlphaScreen *SureFire* assays to assay GPCR stimulation of multiple kinase pathways and compare these data with the same assays performed on cells in culture.

2 MAPK, PI3K and CREB signaling

GPCR activation can lead to the activation of Mitogen-activated protein kinase (MAPK) pathways. Amongst these is the Raf/MEK/ERK pathway. Various pathways leading to ERK phosphorylation can be activated from the stimulation of $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ proteins. In addition, ERK phosphorylation can result from β-Arrestin activation, or proceed through the transactivation of receptor tyrosine kinases (RTKs). Several pathways also lead to the activation of PI3-Kinase, resulting in Akt phosphorylation. Activation of PKA by $G_{\alpha s}$-coupled GPCRs can lead to phosphorylation of CREB. Depending on the GPCR and on the cellular background, these activations will proceed through one or several of the indicated pathways, or as well through other pathways not represented here.



3 AlphaScreen *SureFire* Assay Principle

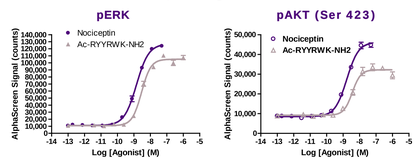


In AlphaScreen *SureFire* assays, following treatment of the cells with an agonist for the GPCR, the endogenous phospho-protein is captured in the cell lysate using an antibody against the phospho-protein and another antibody against the total (phospho- and non-phospho-) protein. An AlphaScreen signal is generated when beads are brought into close proximity via simultaneous capture of the two antibodies respectively by the AlphaScreen Streptavidin Donor beads and the AlphaScreen protein A Acceptor beads.

4 ERK and Akt Assays on Cultured and cAMPZen CHO-NOP (ORL1) Cells

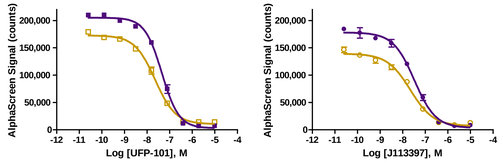
Nociceptin, known as a full agonist of the NOP receptor, induced a full response, while Ac-RYYRWK-NH2, described as a partial agonist in GTP-γ-S assays (McDonald et al. 2003 Br. J. Pharmacol. 140:61–70) behaved as a partial agonist in both ERK and Akt assays. The pharmacology was similar when cultured and frozen cells were used, and assay windows were at least as good when using frozen, γ-irradiated, cells compared to cultured cells.

CHO-NOP (ORL1) cultured cells – pERK and pAkt Assays
Full and partial agonist detection



	pERK		pAkt	
	Nociceptin	Ac-RYYRWK-NH2	Nociceptin	Ac-RYYRWK-NH2
EC_{50} (nM)	1,4	2,9	1,7	3,6
S/B	11,4	9,7	5,4	3,5
Efficacy	100%	82%	100%	73%

CHO-NOP (ORL1) cells – pERK Assay
cAMPZen *vs* Cultured Cells

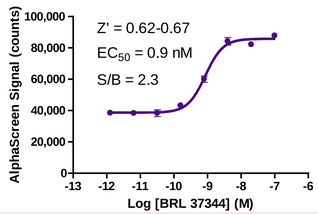


	UFP-101		J113397	
	Cultured	cAMPZen®	Cultured	cAMPZen®
IC_{50} (nM)	23	45	22	33
S/B	17	70	21	53
Z'	0,91	0,95	0,76	0,92

5 CREB Assay on cAMPZen CHO-β3-adrenergic Cells

BRL 37344, a known full agonist of the β3-adrenoceptor, stimulated CREB phosphorylation with a potency similar to the one described for this compound in cAMP assays.

CREB (S133) phosphorylation in CHO-ß3 adr cAMPZen cells



6 Summary of 80 GPCRs Testing using cAMPZen and AequoZen cells

GPCR	coupling[s]	ERK	Akt	CREB *
5-HT_{1A}	$G_{\alpha i}$	+	(-)	n.t.
A_1 (Adenosine)	$G_{\alpha i}$	+	-	n.t.
A_{2A} (Adenosine)	$G_{\alpha s}$	+*	n.t.	+
A_3 (Adenosine)	$G_{\alpha i}$	+	+	n.t.
α_{1A}-adr.	$G_{\alpha q}$	+	-	n.t.
α_{1B}-adr.	$G_{\alpha q}$	+	+	n.t.
α_{2C}-adr.	$G_{\alpha i}$	+	+	n.t.
β_2-adr.	$G_{\alpha s}$	+*	n.t.	+
β_3-adr.	$G_{\alpha s}$	+*	n.t.	+
APJ (Apelin-R)	$G_{\alpha i}$	+	+	n.t.
AM_2 (CRLR + RAMP3)	$G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$	+	(-)	n.t.
CGRP1 (CRLR + RAMP1)	$G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$	+*	n.t.	+
AT_1	$G_{\alpha i}$, $G_{\alpha q}$	+	-	n.t.
C3a	$G_{\alpha i}$	+	+	n.t.
C5a	$G_{\alpha i}$	+	+	n.t.
CB_1	$G_{\alpha i}$	+	+	n.t.
CCR1	$G_{\alpha i}$	+	+	n.t.
CCR2B	$G_{\alpha i}$	+	+	n.t.
CCR3	$G_{\alpha i}$	+	+	n.t.
CCR6	$G_{\alpha i}$	+	+	n.t.
CCR7	$G_{\alpha i}$	+	+	n.t.
CCR8	$G_{\alpha i}$	+	+	n.t.
CCR9a	$G_{\alpha i}$	+	+	n.t.
CCR10	$G_{\alpha i}$	+	+	n.t.
CX3CR1	$G_{\alpha i}$	+	+	n.t.
CXCR2	$G_{\alpha i}$	+	+	n.t.
CXCR3	$G_{\alpha i}$	+	+	n.t.
CXCR6	$G_{\alpha i}$	+	+	n.t.
XCR1	$G_{\alpha i}$	+	+	n.t.
D1 (dopamine)	$G_{\alpha s}$	+	n.t.	+
D2L (dopamine)	$G_{\alpha i}$	+	+	n.t.
DP_2 (CRTH2, PGD2)	$G_{\alpha i}$	+	+	n.t.
EP_2	$G_{\alpha s}$	(+) *	-	+
ET_A	$G_{\alpha q}$	+	+	n.t.
ET_B	$G_{\alpha q}$	+	-	n.t.
FPR2 (FPRL1)	$G_{\alpha i}$	+	+	n.t.
$GABA_{B(1b)(2)}$	$G_{\alpha i}$	+	+	n.t.
GAL_1	$G_{\alpha i}$	+	+	n.t.
GAL_2	$G_{\alpha i}$, $G_{\alpha q}$	+	+	n.t.
GPR1 (chemerin-R)	$G_{\alpha i}$ co-expr.	+	-	n.t.

GPCR	coupling[s]	ERK	Akt	CREB *
GPR40 (docosahexanoic acid)	$G_{\alpha q}$	+	+	n.t.
GPR109 (nicotinic)	$G_{\alpha i}$	+	(+)	n.t.
GPR120 (palmitoleic acid R)	$G_{\alpha q}$	+	+	n.t.
GRS-R (Ghrrelin)	$G_{\alpha q}$	(+)	-	n.t.
GLP-2	$G_{\alpha s}$	+**	n.t.	+
H_1	$G_{\alpha q}$	+	-	n.t.
H_2	$G_{\alpha s}$	+	+	n.t.
H_3	$G_{\alpha i}$	+	+	n.t.
H_4	$G_{\alpha i}$	+	+	n.t.
M_1	$G_{\alpha q}$	+	-	n.t.
M_2	$G_{\alpha i}$	+	+	n.t.
M_3	$G_{\alpha q}$	+	-	n.t.
M_4	$G_{\alpha i}$	+	+	n.t.
M_5	$G_{\alpha q}$	+	-	n.t.
MCH_1	$G_{\alpha i}$, $G_{\alpha q}$	+	+	n.t.
MC_1	$G_{\alpha s}$	+*	n.t.	+
MC_3	$G_{\alpha s}$	+*	n.t.	+
MC_4	$G_{\alpha s}$	+*	n.t.	+
MC_5	$G_{\alpha s}$	(+) *	n.t.	+
MT2	$G_{\alpha i}$	+	+	n.t.
NOP (ORL1)	$G_{\alpha i}$	+	+	n.t.
NPFF1	$G_{\alpha i}$	+	+	n.t.
NPFF2	$G_{\alpha i}$	+	+	n.t.
OP3	$G_{\alpha i}$	+	+	n.t.
PAR4	$G_{\alpha q}$	+	-	n.t.
PZY11	$G_{\alpha i}$, $G_{\alpha q}$	-	n.t.	+
S1P5 (Edg8)	$G_{\alpha i}$	+	+	n.t.
S1P2 (Edg5)	$G_{\alpha i}$, $G_{\alpha q}$	+, + *	+	(+)
S1P4 (Edg6)	$G_{\alpha i}$, $G_{\alpha q}$	-, + *	-	-
Secretin-R	$G_{\alpha s}$, $G_{\alpha q}$	+*	n.t.	+
sst_2	$G_{\alpha i}$	+	+	n.t.
sst_3	$G_{\alpha i}$	+	+	n.t.
sst_4	$G_{\alpha i}$	+	+	n.t.
sst_5	$G_{\alpha i}$	+	+	n.t.
SUCNR1 (GPR91)	$G_{\alpha i}$, $G_{\alpha q}$	+	+	n.t.
TRH1	$G_{\alpha q}$	+	-	n.t.
UT (rat)	$G_{\alpha q}$	+	-	n.t.
V_{1A}	$G_{\alpha q}$	+	-	n.t.
V_2	$G_{\alpha s}$	+**	-	+
Y_1	$G_{\alpha i}$	+	+	n.t.
Y_2	$G_{\alpha i}$	+	+	n.t.

* indicates when ERK was tested for a stimulation time of 20-min
** indicates an inhibitory response
n.t. = not tested

7 Materials and methods

cAMPZen, γ-irradiated Frozen cells (PerkinElmer NOP: Cat no. ES-230-CF; β_3: ES-035-CF) were rapidly thawed, or cultured cells were harvested with trypsin-free cell dissociation solution, and seeded at 40,000 cells/well in 96-well plates, in Ham's F12 medium containing 10% FBS. After 6 hours of adhesion cells were serum-starved overnight. The next day were stimulated with the indicated agonists for 10 min (ERK, Akt) or 20 min (ERK*, CREB), and were lysed. The same cell lysates were used in parallel to assess ERK and Akt or CREB phosphorylation using the corresponding AlphaScreen *SureFire* kits (PerkinElmer Cat no. TGRES, TGRA3S and TGRCBS). For antagonist assays, cells were stimulated with nociceptin after a 15-min pre-incubation time with the indicated antagonists.

8 Summary

- Frozen, γ-irradiated cells, are a well established product, that can be ordered as a consumable, and readily used to perform Aequorin functional assays (AequoZen) or cAMP assays (cAMPZen).
- These cAMPZen and AequoZen cells can be used as well in AlphaScreen *SureFire* assays, as exemplified here for a few receptors, and summarized for 80 GPCRs.
- While ERK is the kinase activated by the broadest range of GPCRs, recording of Akt and CREB phosphorylation provide alternative methods to record GPCR activation.
- These data show that γ-irradiation does not prevent the use of cells for assaying ERK, PI3-kinase and CREB pathways. This will provide additional flexibility for the characterization in multiple assays of drugs in development and allow an easier introduction of the detection of biased agonism (collateral efficacy) in drug discovery programs.

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