human Adenosine A_1 Receptor Cell Line

Product No.: ES-010-C

Lot No.: 3080452

Material Provided

Cells:	2 x 1 mL frozen aliquot (ES-010-CV)
Format:	~2.5 x 10^6 cells /mL in freezing medium

Product Information

Cellular Background:	CHO-K1
Cell Line Development:	Our proprietary bicistronic expression plasmid containing the sequence coding for the human Adenosine A_1 receptor was transfected in CHO-K1 cells. Geneticin-resistant clones were obtained by limit dilution and compared for receptor expression levels using a radioligand binding assay. The clone with the highest receptor expression level was selected for characterization in binding and functional assays.
DNA Sequence:	Identical to coding sequence of GenBank AY136746.1
Corresponding Protein Sequence:	Identical to GenBank NP_000665.1
Receptor expression level (B_{max}):	Estimated to be 4.2 ± 2.5 pmol/mg protein, using [^{3}H]-DPCPX
K_d for the above radioligand:	2.9 nM
Shipping Conditions:	Shipped on dry ice. Please ensure dry ice is still present in the package upon receipt or contact customer support.
Storage Conditions:	Store in liquid nitrogen (vapor phase) immediately upon receipt.

Quality Control

The EC_{50} for a reference agonist was determined in a LANCE® *Ultra* cAMP assay performed on an EnVision® instrument. A mycoplasma test was performed using MycoAlert® (Lonza) mycoplasma detection kit. We certify that these results meet our quality release criteria.

N^6-Cyclopentyladenosine (CPA) (EC_{50}):	0.32 nM
Stability:	Cells were kept in continuous culture for at least 60 days and showed no decrease of receptor expression level in a saturation binding assay (stable B_{max} and K_d).
Mycoplasma:	This cell line tested negative for mycoplasma.

Assay Procedures

We have shown for many of our GPCR cell lines that freshly thawed cells respond with the same pharmacology as cultured cells. All of our products validated in this way are available as frozen ready-to-use cells in our catalogue. This demonstrates that cells can be prepared and frozen in advance of a screening campaign simplifying assay logistics.

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Recommended Cell Culture Conditions (CHO-K1)

- The recommended media catalogue number and supplier reference information are listed in this Product Technical Data Sheet (last page). Media composition is specifically defined for each cell type and receptor expression selection. The use of incorrect media or component substitutions can lead to reduced cell viability, growth issues and/or altered receptor expression.
- Cells undergo major stress upon thawing, and need to adapt to their new environment which may initially affect cell adherence and growth rates. The initial recovery of the cells, and initial doubling time, will vary from laboratory to laboratory, reflecting differences in the origin of culture media and serum, and differences in methodology used within each laboratory.
- For the initial period of cell growth (i.e. until cells have reached Log-phase, typically 4-10 days), we strongly recommend removal of the antibiotics (G418, Zeocin™, Puromycin, Blasticidin, Hygromycin, Penicillin and Streptomycin) from the culture media. Immediately after thawing, cells may be more permeable to antibiotics, and a higher intracellular antibiotic concentration may result as a consequence. Antibiotics should be re-introduced when cells have recovered from the thawing stress.

Growth Medium:	Ham's F-12, 10% FBS, 0.4 mg/ml G418 (receptor expression selection).
Freezing Medium:	Ham's F-12, 10% FBS with 10% DMSO, without selection agents.

Thawing Cells: Using appropriate personal protective equipment, rapidly place the frozen aliquot in a 37°C water bath (do not submerge) and agitate until its content is thawed completely. Immediately remove from water bath, spray aliquot with 70% ethanol and wipe excess. Under aseptic conditions using a sterile pipette, transfer content to a sterile centrifuge tube containing 10 mL growth medium without antibiotics, pre-warmed at 37°C, and centrifuge (150 x g, 5 min). Discard supernatant using a sterile pipette. Resuspend cell pellet in 10 mL of pre-warmed growth medium without antibiotics by pipetting up and down to break up any clumps, and transfer to an appropriate culture flask (e.g. T-25, T-75 or T-175, see recommended seeding density below). Cells are cultured as a monolayer at 37°C in a humidified atmosphere with 5% CO_2.

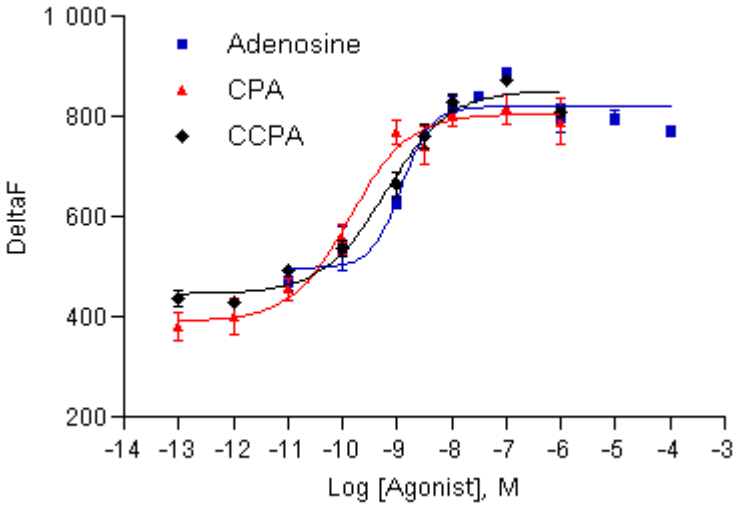
Recommended Seeding Density:	Thawing:	15,000 – 33,000 cells/cm^2
	Log-phase:	11,000 – 15,000 cells/cm^2

Troubleshooting: Initial doubling time can vary between 18 and 96 hours (Average = 25 hours). If cells are still not adhering after 48 hours or grow very slowly, we recommend maintaining the cells in culture and not replacing the media before 5-6 days (cells secrete factors that can help with adherence and growth). If confluence is still <50% after 5-6 days, it is recommended that you replace the media with fresh media (without antibiotics). Do not passage the cells until they reach 80-90% confluence (Log-phase). If cells have not recovered after 10-12 days, please contact our Technical Support.

Culture Protocol: Under aseptic conditions, cells are grown to 80% confluence (Log-phase) and trypsinized (0.05% trypsin/0.5 mM EDTA in calcium and magnesium-free PBS). See recommended seeding density for Log-phase above.

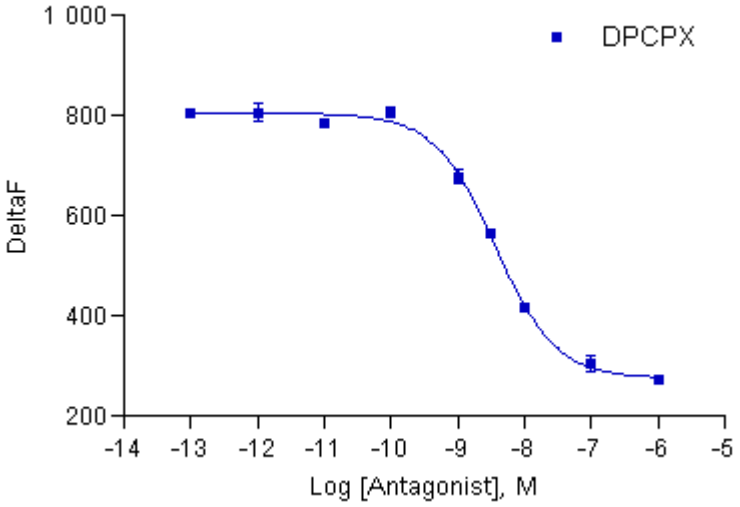
Banking Protocol: Cells are grown to 70-80% confluence (Log-phase). Under aseptic conditions, remove medium and rinse the flask with an appropriate volume of calcium and magnesium-free PBS (example 10 mL for T-175). Trypsinize (0.05% trypsin/0.5 mM EDTA in calcium and magnesium-free PBS) to detach cells (example 5 mL for T-175), let stand 5-10 min at 37°C. Add fresh, room temperature growth medium (without antibiotics) to stop trypsinization and dilute EDTA (example 10 mL for T-175). Transfer cells to a sterile centrifuge tube and centrifuge (150 x g, 5 min). Discard supernatant using a sterile pipette. Resuspend cell pellet in ice-cold freezing medium by pipetting up and down to break up any clumps. Count cells and rapidly aliquot at the selected cell density (e.g. 2.5 x 10^6 cells/mL) in sterile polypropylene cryovials. Use appropriate material to ensure slow cooling (about -1°C/min) until -70°C. Transfer vials into a liquid nitrogen tank (vapor phase) for storage.

Typical Product Data – cAMP Assay (TR-FRET)



Agonist	EC_{50} (M)
Adenosine	1.2×10^{-9}
CPA	1.4×10^{-10}
CCPA	6.2×10^{-10}

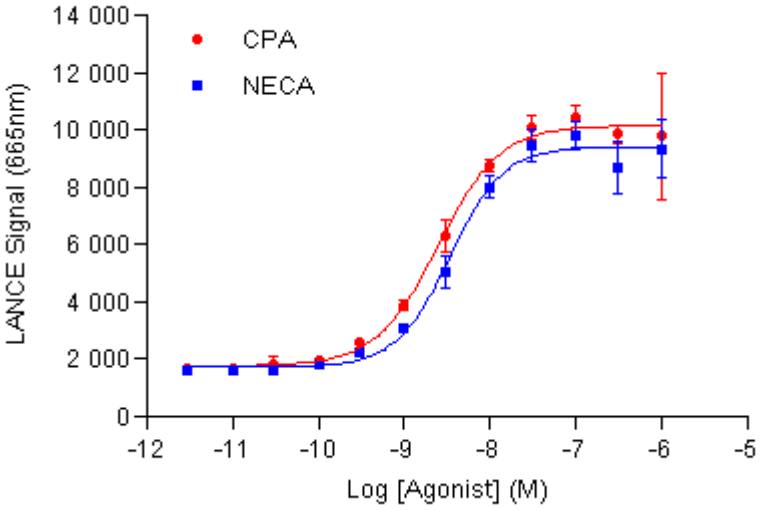
Figure 1. Agonist Response in TR-FRET cAMP assay
An agonist dose-response experiment was performed in 96-well format using 5000 cells/well. Forskolin was used at a final concentration of 10 µM. Time-resolved fluorescence was measured on a RUBYstar (BMG Labtech) instrument. Data from a representative experiment are shown.



Antagonist	IC_{50} (M)
DPCPX	3.6×10^{-9}

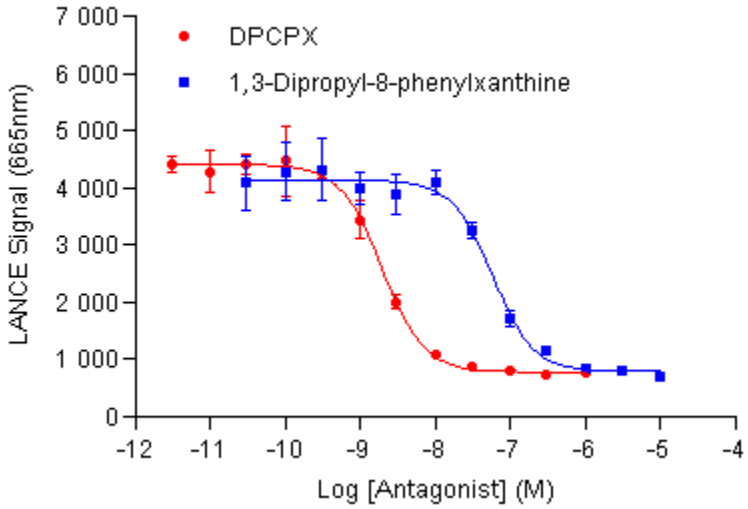
Figure 2. Antagonist Response in TR-FRET cAMP assay
An antagonist dose-response experiment was performed in 96-well format using 5000 cells/well. Forskolin (final concentration of 10 µM) mixed with the reference agonist (CPA at a final concentration of 3 nM, corresponding to the EC_{80}) was used to stimulate the cells. Time-resolved fluorescence was measured on a RUBYstar (BMG Labtech) instrument. Data from a representative experiment are shown.

Typical Product Data –LANCE® cAMP Assay (ES-010-CF frozen cells)



Agonist	EC_{50} (M)
CPA	2.5×10^{-9}
NECA	3.4×10^{-9}

Figure 3. Agonist Response in LANCE® cAMP assay
An agonist dose-response experiment was performed in 384-well format using 2500 cells/well. Frozen cells (ES-010-CF) prepared from ES-010-C cells were incubated for 30-min with 10 µM Forskolin (FK) and the indicated agonist concentrations. Time-resolved fluorescence was measured on an EnVision®. Data from a representative experiment are shown.



Antagonist	IC_{50} (M)
DPCPX	2.0×10^{-9}
1,3-Dipropyl-8-phenylxanthine	5.8×10^{-8}

Figure 4. Antagonist Response in LANCE® cAMP assay
An antagonist dose-response experiment was performed in 384-well format using 2500 cells/well. Frozen cells (ES-010-CF) prepared from ES-010-C cells were incubated for 30-min in the presence of 10 µM Forskolin (FK), a final concentration of 4.5 nM CPA, corresponding to the EC_{80} concentration, and the indicated antagonist concentrations. Time-resolved fluorescence was measured on an EnVision®. Data from a representative experiment are shown.

Typical Product Data –Radioligand Binding Assay (Filtration)

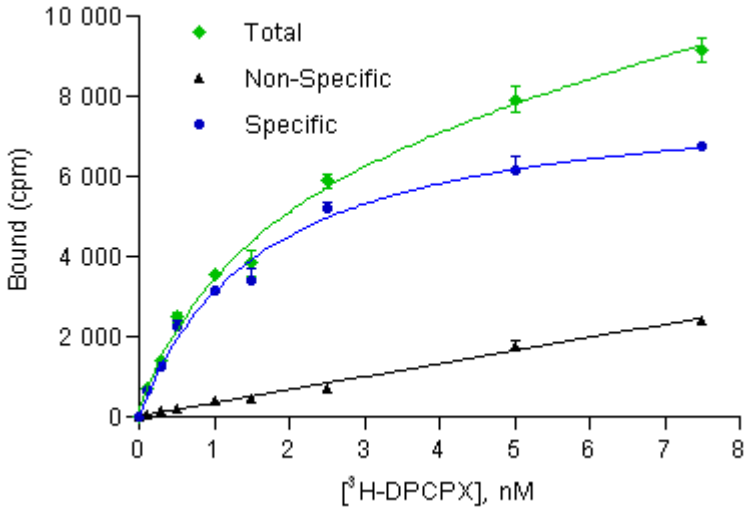
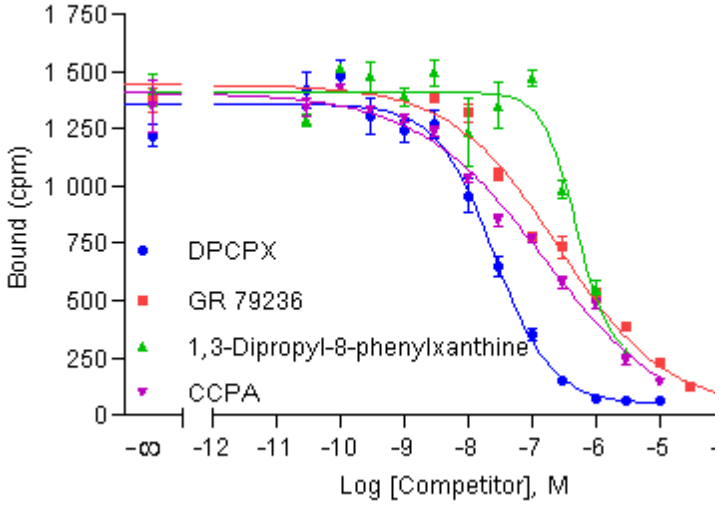


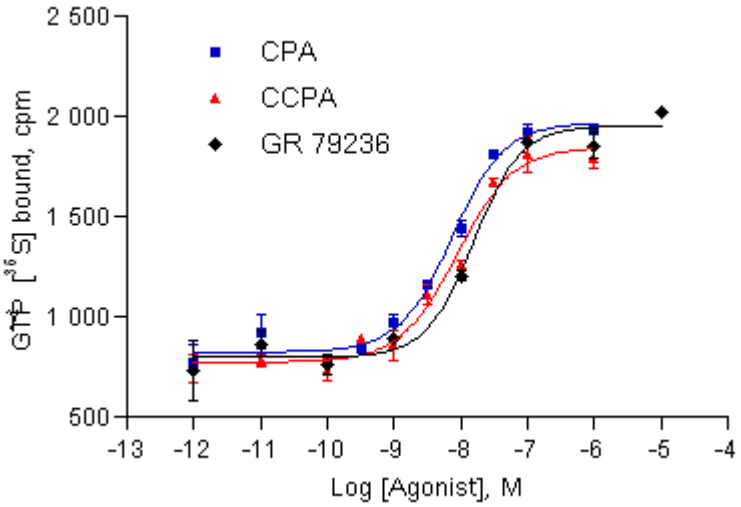
Figure 5: Saturation Binding Assay Curve (Filtration)
A saturation binding assay was performed in 96-well format using 20 µg membranes/well. Counts per minute (cpm) were measured on a TopCount®. Data from a representative experiment are shown.



Agonist / Antagonist	IC_{50} (M)
DPCPX	2.5×10^{-8}
GR 79236	2.3×10^{-7}
1,3-Dipropyl-8-phenylxanthine	6.7×10^{-7}
CCPA	1.1×10^{-7}

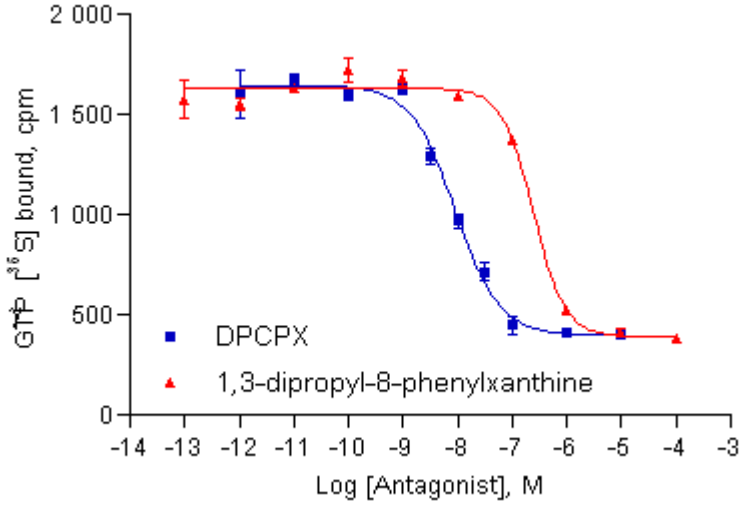
Figure 6: Competition Binding Assay Curve (Filtration)
A competition binding assay was performed in 96-well format using 10 µg membranes/well. Displacement of 1.7 nM [³H]-DPCPX was used. Counts per minute (cpm) were measured on a TopCount®. Data from a representative experiment are shown.

Typical Product Data – GTPγS - SPA® Assay



Agonist	EC_{50} (M)
CPA	7.4×10^{-9}
CCPA	8.7×10^{-9}
GR 79236	1.6×10^{-8}

Figure 7. Agonist Response in GTPγS - SPA® assay
An agonist dose-response scintillation proximity assay (SPA) was performed in 96-well format using 2.5 µg membranes/well. Counts per minute (cpm) were measured on a TopCount® instrument. Data from a representative experiment are shown.



Antagonist	IC_{50} (M)
DPCPX	9.1×10^{-9}
1,3-dipropyl-8-phenylxanthine	2.4×10^{-7}

Figure 8. Antagonist Response in GTPγS - SPA® assay
An antagonist dose-response scintillation proximity assay (SPA) was performed in 96-well format using 2.5 µg membranes/well. The reference agonist (CPA) at a concentration corresponding to the EC_{80} (9 nM) was used. Counts per minute (cpm) were measured on a TopCount® instrument. Data from a representative experiment are shown.

LANCE® *Ultra* cAMP Assay Procedure

- As IBMX has antagonist activity to some adenosine receptors (Jockers *et al*. 1994), rolipram was used here instead for the inhibition of cAMP degradation by phosphodiesterases.

Stimulation Buffer: HBSS, 5 mM HEPES, 0.1 % Protease-free BSA, 0.025 mM Rolipram, pH 7.4.

Cells/well: For compounds not tested herein we recommend titrating the cells for optimal performance, i.e. 500-3000 cells per assay point.

cAMP measurements can be performed with the LANCE® *Ultra* cAMP 384 Kit (Revvity # TRF0262), according to the manufacturer instructions. Briefly:

Protocols for a 384-well white Optiplate (total assay volume of 20 µL):

cAMP Standard curve	G_s Agonist	G_s Antagonist	G_i Forskolin titration	G_i Agonist	G_i Antagonist
5 µL cAMP Standard	5 µL cell suspension	5 µL cell suspension	5 µL cell suspension	5 µL cell suspension	5 µL cell suspension
5 µL Stimulation Buffer	5 µL Agonist	2.5 µL Antagonist	5 µL Forskolin	2.5 µL Agonist	2.5 µL Antagonist
-	-	2.5 µL Agonist	-	2.5 µL Forskolin	2.5 µL Forskolin/Agonist
Incubate 30 min at room temperature (optional step for cAMP Standard curve)					
5 µL 4X Eu-cAMP Tracer Working Solution					
5 µL 4X U*Light*-anti-cAMP Working Solution					
Incubate 1 h at room temperature					
Read on an EnVision® instrument. Remove microplate seal prior to reading					

1. Cells in mid-log phase, grown in media without antibiotics for 18 hours prior to the experiment, are detached by gentle flushing with PBS-EDTA, recovered by centrifugation and resuspended in stimulation buffer e.g. at the concentration of 6.0 x 10^5 cells/mL (for 3000 cells/well).

2. Prepare the 4X Tracer Working Solution by making a 1/50 dilution of the Eu-cAMP stock solution in the cAMP Detection Buffer.

3. Prepare an U*Light*-anti-cAMP Intermediate Solution by making a 1/10 dilution of the U*Light*-anti-cAMP stock solution in cAMP Detection Buffer. Prepare the 4X U*Light*-anti-cAMP Working Solution by making a 1/30 dilution of the U*Light*-anti-cAMP intermediate solution in the cAMP Detection Buffer.

Notes:

For 96- and 1536-well formats, adjust proportionally the volume of each assay component in order to maintain the volume ratios for the 384-well format. Do not modify the Eu-cAMP and/or the U*Light*-anti-cAMP concentrations.

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LANCE® cAMP Assay Procedure

Precautions and Recommendations:

- Do not vigorously vortex solutions containing cAMP antibody.
- When preparing the Detection Mix, always dilute the Eu-SA component first, and then add the Biotin-cAMP component to the Eu-SA solution.
- As IBMX has antagonist activity to the A_1 receptor (Jockers *et al*. 1994), rolipram is used here instead for the inhibition of cAMP degradation by phosphodiesterases.

Stimulation Buffer: HBSS, 5 mM HEPES, 0.1 % Protease-free BSA, 0.025 mM Rolipram, pH 7.4.

Cells/well: 2500. For compounds not tested herein we recommend titrating the cells for optimal performance, i.e. 1000-10,000 cells per assay point.

Antagonist Pre-incubation: Simultaneous addition of antagonists with reference agonist.

Agonist Stimulation: 30 min at room temperature (22°C).

cAMP measurements were performed with the LANCE® cAMP 384 Kit (Revvity # AD0262), according to the manufacturer instructions. Briefly:

1. Compounds (6 µL/well) were dispensed into a 384-well white Optiplate:

	$G_{\alpha s}$ and $G_{\alpha i}$ assay modes		$G_{\alpha s}$ assay mode		$G_{\alpha i}$ assay mode	
	Basal	Forskolin	Agonist Assay	Antagonist Assay	Agonist Assay	Antagonist Assay
Buffer	6 µL	-	-	-	-	-
Antagonist	-	-	-	3 µL of 4x final conc.	-	3 µL of 4x final conc.
Agonist	-	-	6 µL of 2x final conc.	3 µL of 4x final conc.	6 µL of 2x final conc. in 2x final FK conc.	3 µL of 4x final conc. in 4x final FK conc.
Forskolin	-	6 µL of 2x final conc.	-	-		

2. Cells in mid-log phase, grown in media without antibiotics for 18 hours prior to the experiment, were detached by gentle flushing with PBS-EDTA, recovered by centrifugation and resuspended in stimulation buffer at the concentration of 4.2 x 10^5 cells/mL.

3. The Alexa Fluor® 647-anti cAMP antibody was added 1/100 (vol/vol) to the cell suspension.

4. 6 µL/well of cell and antibody suspension (2500 cells/well) were dispensed on top of the compounds prepared in the 384 well Optiplate.

5. After incubation for 30 min at room temperature the reaction was stopped by addition of 12 µL of Detection Mix.

6. The plate was incubated for 60 min at room temperature and read on an EnVision®.

   Note: Assays can also be miniaturized into 1536-well format.

cAMP Assay Procedure (TR-FRET)

KRH Buffer:	5 mM KCl, 1.25 mM $MgSO_4$, 124 mM NaCl, 25 mM HEPES, 13.3 mM Glucose, 1.25 mM KH_2PO_4, 1.45 mM $CaCl_2$, 0.5 mg/mL protease free BSA and 10 µg/mL Phenol red, pH 7.4 (can be stored at -20°C).
Assay Buffer:	25 µM Rolipram in KRH (freshly made), agitate during 15-20 minutes at room temperature. Note: As IBMX has antagonist activity to the A1 receptor (Jockers et al. 1994), rolipram is used here instead for the inhibition of cAMP degradation by phosphodiesterases.
Cells/well:	5000 cells/well. For compounds not tested herein we recommend titrating the cells for optimal performance, i.e. 1000-10000 cells per assay point.
Antagonist Pre-incubation:	Simultaneous addition of antagonists with reference agonist.
Agonist Stimulation:	30 min at room temperature (22°C)

cAMP measurements were performed with the Cisbio International cAMP Kit (# 62AM2PEB), according to the manufacturer instructions. Briefly:

1. Compounds were dispensed into a 96-well plate.

2. Cells in mid-log phase, grown in media without antibiotics for 18 hours prior to the experiment, were detached by gentle flushing with PBS-EDTA, recovered by centrifugation and resuspended in assay buffer.

3. Cells and antibody suspension (5000 cells/well) were dispensed on top of the compounds prepared in the 96-well plate.

4. After incubation for 30 min at room temperature the reaction was stopped by addition of the lysis Assay buffer and read with a RUBYstar (BMG Labtech). cAMP concentrations were estimated, according to the manufacturer's specification.

Membrane Radioligand Binding Assay Procedure (Filtration)

Note: The following are recommended assay conditions and may differ from the conditions used to generate the typical data shown in the above section.

Assay Buffer: 25 mM HEPES pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM NaCl

Wash Buffer: 25 mM HEPES pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM NaCl

Radioligand: [3H]-DPCPX (Revvity # NET974)

Filters: Unifilter 96 GF/C (Revvity #6055690)

Membrane Binding Protocol:

Binding assays were performed in 550 µL total volume according to the following conditions. All dilutions are performed in assay buffer:

1. Membrane dilution:	10 µg of membranes per well, diluted in order to dispense 500 µL/well. Keep on ice.
2. Assembly on ice (in 96 Deep well plate) Saturation Binding:	• 25 µL of assay buffer or of unlabeled ligand (8-Cyclopentyl-1,3-dipropylxanthine, 250 µM final) for determination of non specific binding • 25 µL of radioligand at increasing concentrations (see figure 5) • 500 µL of diluted membranes
Competition Binding:	• 25 µL competitor ligand at increasing concentrations (see figure 6) • 25 µL of radioligand (1.7 nM final) • 500 µL of diluted membranes
3. Incubation:	60 min at 27°C.
4. Filters preparation:	GF/C filters were presoaked in 0.5 % PEI at room temperature for at least 30 min.
5. Filtration:	Aspirate and wash 9 x 500 µL with ice cold wash buffer using a FilterMate Harvester.
6. Counting:	Add 30 µL/well of MicroScint™-O (Revvity # 6013611), cover filter with a TopSeal-A PLUS (Revvity # 6050185) and read on a TopCount®.

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GTPγS - SPA® Assay Procedure

Assay Buffer:	20 mM HEPES pH 7.4; 100 mM NaCl, 10 µg/ml saponine, 1 mM $MgCl_2$
GDP concentration:	10 µM GDP (final)
SPA Beads:	PVT-WGA (Revvity # RPNQ0001), 0.5 mg/well
Radioligand:	GTPγS, [^{35}S] - (Revvity # NEG030H)
Membranes:	2.5 µg/well
Format:	96-well
Final volume:	100 µL/well

GTPγS-SPA assays were performed in 100 µL total volume according to the following conditions. All dilutions are performed in assay buffer:

1. Membrane Dilution:	2.5 µg of membranes per well, diluted in order to dispense 10 µL/well. Keep on ice.
2. GDP saturation:	Mix a 10-fold concentrated GDP solution (i.e. 100 µM) with the membranes dilution. Incubate "*membranes : GDP mix*" on ice for 15 min.
3. GTPγS, [^{35}S] - dilution:	Dilute GTPγS, [^{35}S] - to give ~25.000 dpm/10µL
4. Beads:	Dilute beads to 50mg/mL (0.5 mg/10 µL). Premix beads with the GTPγS, [^{35}S] - dilution just before starting the reaction ("*GTPγS, [^{35}S] - : Beads mix*").
5. Assembly (in Optiplate™), Agonist Assay:	• 50 µL of 2x agonist dilution at increasing concentrations • 20 µL of the "*membranes : GDP mix*" • 10 µL of assay buffer • 20 µL of the "*GTPγS, [^{35}S] - : Beads mix*"
Antagonist Assay:	• 50 µL of 2x antagonist dilution at increasing concentrations • 20 µL of the "*membranes : GDP mix*" • 10 µL of 10x reference agonist dilution to reach a final concentration corresponding to its EC_{80} • 20 µL of the "*GTPγS, [^{35}S] - : Beads mix*"
6. Incubation:	• Cover plate with a TopSeal • Shake on an orbital shaker for 2 min • Incubate for 1h at RT • Centrifuge the plate for 10 min at 2000 rpm • Incubate for 1h at RT
7. Counting	Count for 1 min on a TopCount®

References

1. Libert F, Van Sande J, Lefort A, Czernilofsky A, Dumont JE, Vassart G, Ensinger HA, Mendla KD. (1992) Cloning and functional characterization of a human A1 adenosine receptor. BBRC, 187:919-926

2. Jockers R, Linder ME, Hohenegger M, Nanoff C, Bertin B, Strosberg AD, Marullo S, Freissmuth M. (1994) Species difference in the G protein selectivity of the human and bovine A1-adenosine receptor. J Biol Chem 269:32077-32084.

Materials and Instrumentation

The following tables provide the references of compounds and reagents used or recommended for the characterization of the human Adenosine A_1 cell line, as well as some advice on how to use these compounds:

Table 1. References of compounds used for functional characterization and binding assays

Name	Provider	Cat no	Working Stock Solution
CPA (N^6-Cyclopentyladenosine)	Sigma	C8031	10 mM in Ethanol
CCPA (2-Chloro-N^6-cyclopentyladenosine)	Tocris	1705	5 mM in DMSO
DPCPX (8-Cyclopentyl-1,3-dipropylxanthine)	Sigma	C101	10 mM in Ethanol
NECA (5′-(N-Ethylcarboxamido)adenosine)	Sigma	E2387	10 mM in DMSO
GR 79236	Tocris	1957	100 mM in dH_2O
1,3-Dipropyl-8-phenylxanthine	Tocris	0486	10 mM in DMSO
Adenosine	Sigma	A9251	10 mM in dH_2O
Rolipram	Tocris	0905	50 mM in DMSO
[3H]-DPCPX	Revvity	NET974	N/A

Table 2. References of cell culture media and assay buffers

Name	Provider	Cat no
HAM's F-12	Hyclone	SH30026.02
DMEM	Hyclone	SH30022.02
Advanced DMEM/F12 (serotonin receptors)	Invitrogen	12634-010
EMEM	BioWitthaker	06-174G
EX-CELL DHFR$^-$ media (DHFR deficient cell lines)	Sigma	C8862
FBS	Wisent	80150
FBS dialyzed	Wisent	80950
G418 (geneticin)	Wisent	400-130-IG
Zeocin	Invitrogen	R25005
Blasticidin	invitrogen	R210-01
Puromycin	Wisent	400-160-EM
Standard HBSS (with $CaCl_2$ and $MgCl_2$)	GIBCO	14025
HEPES	MP Biomedicals, LLC	101926
BSA, Protease-free	Sigma	A-3059
PEI	Sigma	P3143
Trypsin-EDTA	Hyclone	SH30236.02
Sodium Pyruvate	GIBCO	11360
L-Glutamine	GIBCO	25030
NEAA (non-essential amino acids)	GIBCO	11140
Forskolin	Sigma	F6886

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