TECHNICAL DATA SHEET

ValiScreen® GPCR Cell Line

Caution: For Laboratory Use. A research product for research purposes only

human CRF_1 Receptor Cell Line

Product No.: ES-152-C

Lot No.: M2W-C1 / M2W-C2

Material Provided

Cells:	2 x 1 mL frozen aliquot (ES-152-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 CRF_1 receptor was transfected in CHO-K1 cells. Geneticin-resistant clones were obtained by limit dilution and compared for receptor expression levels by radioligand binding assay and for their response in a cAMP assay. Two clones were selected: clone C1, having the highest receptor expression level and clone C2, having a lower receptor expression but a better response in the cAMP assay. (i.e. clone C1 showed dual $G_{\alpha s}$ and $G_{\alpha i}$-coupling, while clone C2 yielded only a $G_{\alpha s}$ response).
DNA Sequence:	Identical to coding sequence of GenBank NM_004382.4.
Corresponding Protein Sequence:	Identical to GenBank NP_004373.2.
Receptor expression level (B_{MAX}):	Clone C1: 179 pmol/mg protein, using [^{125}I]CRF (ovine). Clone C2: N/D.
K_D for the above radioligand:	Clone C1: 9.5 nM. Clone C2: N/D.
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.

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Quality Control

Clone C2: The EC_{50} for a reference agonist was determined in TR-FRET cAMP assay. A mycoplasma test was performed using MycoAlert® (Lonza) mycoplasma detection kit. We certify that these results meet our quality release criteria.

Clone C1: The EC_{50} for a reference agonist was determined in GTPγS assay. A mycoplasma test was performed using MycoAlert® (Lonza) mycoplasma detection kit. We certify that these results meet our quality release criteria.

C2: Urocortin (human) (EC_{50}): 3.2 nM

C1: Sauvagine (frog) (EC50): 25 nM

Stability: Cells were kept in continuous culture for at least 60 days and showed no decrease in receptor expression level for clone C1 in a saturation binding assay (stable B_{max} and K_d) and in functional response for clone C2 (E_{max}, EC_{50} in cAMP assay).

Mycoplasma: These cell lines 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.

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 F12, 10% FBS, 400 µg/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:

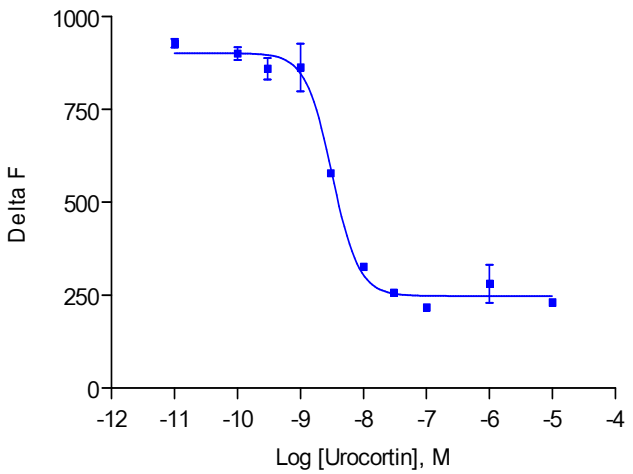
Thawing:	15 000 – 33 000 cells/cm^2
Log-phase:	11 000 – 15 000 cells/cm^2

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 (vapour phase) for storage.

Typical Product Data – cAMP Assay (TR-FRET)



Agonist	EC_{50} (M)
Urocortin	3.2×10^{-9}

Figure 1. Agonist Response in TR-FRET cAMP assay
An agonist dose-response experiment was performed on clone C2 (low receptor expression) in 96-well format using 5 000 cells/well. Time-resolved fluorescence was measured on a RUBYstar (BMG Labtech) instrument. Data from a representative experiment are shown.

Typical Product Data –Radioligand Binding Assay (SPA)

Preliminary results showed this cell line responds positively in SPA binding assays. Please enquire for SPA binding protocol availability.

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Typical Product Data –Radioligand Binding Assay (Filtration)

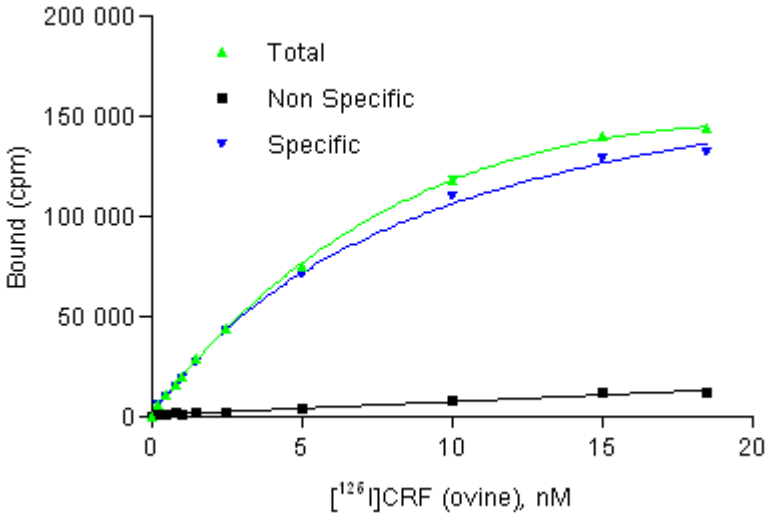
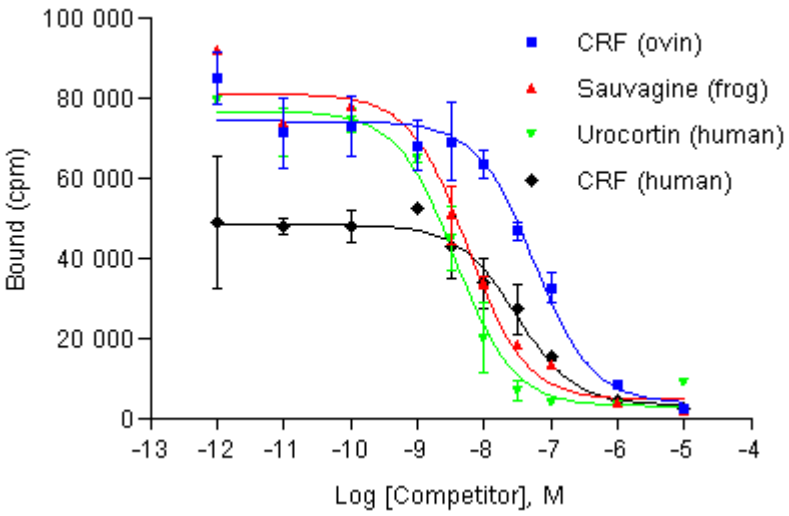


Figure 2: Saturation Binding Assay Curve (Filtration)
A saturation binding assay was performed on clone C1 (high receptor expression) in 96-well format using 2 µg membranes/well. Counts per minute (cpm) were measured on a TopCount® instrument. Data from a representative experiment are shown.



Agonist / Antagonist	IC_{50} (M)
CRF (ovine)	5.8×10^{-8}
CRF (human)	3.1×10^{-8}
Sauvagine (frog)	5.8×10^{-9}
Urocortin (human)	3.8×10^{-9}

Figure 3: Competition Binding Assay Curve (Filtration)
A competition binding assay was performed on clone C1 (high receptor expression) in 96-well format using 2 µg membranes/well. Displacement of 0.75 nM [^{125}I]CRF (ovine) was used. Counts per minute (cpm) were measured on a TopCount® instrument. Data from a representative experiment are shown.

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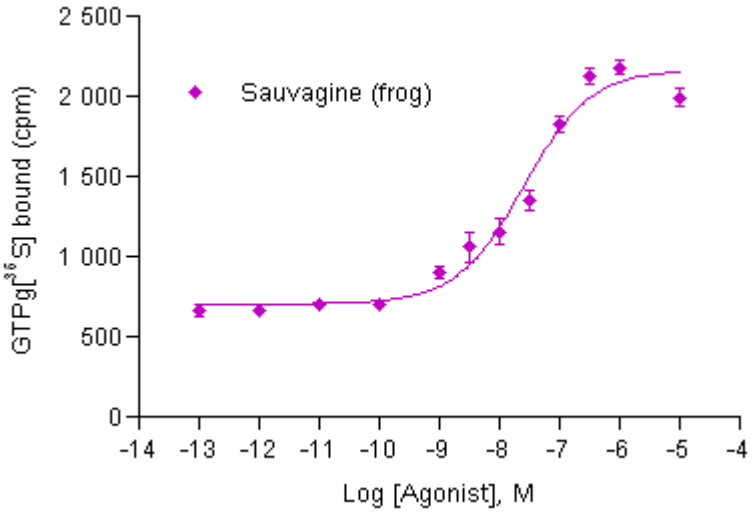
Typical Product Data – GTPγS - SPA® Assay



Agonist	EC_{50} (M)
Sauvagine (frog)	2.5 x 10-8

Figure 4. Agonist Response in GTPγS - SPA® assay
An agonist dose-response scintillation proximity assay (SPA) was performed on clone C1 (high receptor expression) in 96-well format using 10 µg membranes/well. Counts per minute (cpm) were measured on a TopCount® instrument. Data from a representative experiment are shown.

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LANCE® *Ultra* cAMP Assay Procedure

Stimulation Buffer: HBSS, 5 mM HEPES, 0.1 % Protease-free BSA, 0.5 mM IBMX, pH 7.4.

Cells/well: For compounds not tested herein we recommend titrating the cells for optimal performance, i.e. 500-3 000 cells per assay point.

cAMP measurements can be performed with the LANCE® *Ultra* cAMP 384 Kit (PerkinElmer # 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 ULight-anti-cAMP concentrations.

LANCE® cAMP Assay Procedure

Stimulation Buffer: HBSS, 5 mM HEPES, 0.1 % Protease-free BSA, 0.5 mM IBMX, pH 7.4.

Cells/well: 5 000. 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 (PerkinElmer # 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 Assay buffert the concentration of 8.4 x 10^5 cells/mL.

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

4. 6 µL/well of cell and antibody suspension (5 000 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 with 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:	1 mM IBMX in KRH (freshly made), agitate during 15-20 minutes at room temperature.
Cells/well:	5 000. 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 Cisbio International cAMP Kit (Cat n°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 (5 000 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 buffernd 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: 50 mM Tris-HCl pH 7.4, 125 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% Bacitracin, 0.5% BSA

Wash Buffer: 50 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.05% BSA (ice cold)

Radioligand: [^{125}I] CRF (ovine) (PerkinElmer # NEX217)

Filters: Unifilter 96 GF/C (PerkinElmer # 6005174)

Membrane Binding Protocol:

Binding assays were performed in 200 µL total volume according to the following conditions. All dilutions are performed in assay buffer:

1. Membrane dilution:	2 µg of membranes per well, diluted in order to dispense 150µL/well. Keep on ice.
2. Assembly on ice (in 96 Deep well plate) **Saturation Binding:**	• 25 µL of assay buffer or of unlabeled ligand (Urocortin (human), 5 µM final) for determination of non specific binding • 25 µL of radioligand at increasing concentrations (see figure 2) • 150 µL of diluted membranes
Competition Binding:	• 25 µL competitor ligand at increasing concentrations (see figure 3) • 25 µL of radioligand (0.3 nM final) • 150 µL of diluted membranes
3. Incubation:	60 min at 27°C.
4. Filters preparation:	GF/C filters were presoaked in PBS, 0.1 % Tween 20 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 (PerkinElmer).
6. Counting:	Add 30 µL/well of MicroScint™-O (PerkinElmer # 6013611), cover filter with a TopSeal-A (PerkinElmer # 6050195) and read on a TopCount® (PerkinElmer).

GTPγS - SPA® Assay Procedure

Assay Buffer: 20 mM Hepes pH 7.4, 100 mM NaCl, 10 µg/mL Saponin, 3 mM $MgCl_2$, 0.1% BSA

GDP concentration: 3 µM GDP (final)

SPA Beads: PVT-WGA (PerkinElmer # RPNQ0001), 0.5 mg/well

Radioligand: GTPγS, [^{35}S] - (PerkinElmer # NEG030H)

Membranes: 10 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:	10 µg of membranes per well, diluted in order to dispense 20 µL/well. Keep on ice.
2. GDP preparation:	Prepare a 5-fold concentrated GDP solution (i.e. 15 µM).
3. GTPγS, [^{35}S] - dilution:	Dilute GTPγS, [^{35}S] - to give ~25.000 dpm/20µL
4. Beads:	Dilute beads to 25 mg/mL (0.5 mg/20 µL)
5. Assembly (in Optiplate™), **Agonist Assay:**	• 20 µL of 5x GDP dilution • 20 µL of 5x agonist dilutions at increasing concentrations • 20 µL of diluted membranes
Antagonist Assay:	• 20 µL of 5x GDP dilution • 20 µL of a 5x antagonist at increasing concentrations: 5x reference agonist dilution (to reach a final concentration corresponding to its EC_{80}) • 20 µL of diluted membranes
6. Pre-incubation:	Incubate for 15 min at room temperature (RT°)
7. Assemble (continued)	• 20 µL of the GTPγS, [^{35}S] - dilution • 20 µL of the SPA Beads dilution
8. 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 0h to 1h at RT°
9. Counting	Count for 1 min on a TopCount®

References

1. Vita N, Laurent P, Lefort S, Chalon P, Lelias JM, Kaghad M, Le Fur G, Caput D, Ferrara P. (1993) Primary structure and functional expression of mouse pituitary and human brain corticotrophin releasing factor receptors. FEBS Lett. 335:1-5.

Materials and Instrumentation

The following tables provide the references of compounds and reagents used or recommended for the characterization of the human CRF1 receptor ValiScreen® 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 n°	Working Stock Solution
Urocortin (human)	Bachem	H-3722	0.5 mM in acetic acid 10%
Sauvagine (frog)	Bachem	H-4890	1 mM in dH_2O
CRF (human)	Bachem	H-2435	0.5 mM in acetic acid 10%
CRF (ovine)	Bachem	H-2445	0.1 mM in acetic acid 10%
[^{125}I]CRF (ovine)	PerkinElmer	NEX217	N/A

Table 2. References of cell culture media and assay buffers.

Name	Provider	Cat n°
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

Please visit our website: www.perkinelmer.com/CellLines for additional information on materials, microplates and instrumentation.

This product is not for resale or distribution except by authorized distributors.

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