human CRAC (STIM1/ORAI1) Ion Channel Cell Line

Product No.: AX-013-PCL

Lot No.: P1

Material Provided

Cells:	2 x 1 mL frozen aliquot (AX-013-PCLV)
Format:	~2.5 x 10^6 cells /mL in freezing medium

Product Information

Cellular Background:	HEK-293
Cell Line Development:	HEK cells stably expressing mitochondrially targeted Photina® were transfected with the pBudCE4.1 expression vector (Invitrogen) containing the human ORAI1 coding sequence under the control of the CMV promoter and the human STIM1 coding sequence under the control of the EF-1α promoter. Resistant clones were obtained by limiting dilution and compared for their extracellular calcium entry in response to intracellular calcium depletion using the PhotoScreen® assay. The selected clone was also functionally validated by electrophysiology experiments.

DNA sequence and corresponding protein sequence:

Human STIM1:	Identical to coding sequence of NM_003156.3 (DNA) and NP_003147.2 (protein)
Human ORAI1:	Identical to coding sequence of NM_032790.3 (DNA), with conservative C33T, C546T and T798C variations (numbering according to ATG translation initiation codon); and identical to NP_116179.2 (protein)
Receptor expression level (B_{max})	Not determined for this cell line.
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} value for a reference agonist was determined in a PhotoScreen® assay performed with adherent cells on a LumiLux® instrument. A mycoplasma test was performed using MycoAlert® Mycoplasma (Lonza) detection kit. We certify that these results meet our quality release criteria.

$CaCl_2$ (EC_{50}):	2431 µM
Stability:	Cells were kept in continuous culture for 20 passages (~ 60 days) and showed no decrease in functional response in the PhotoScreen® assay (EC_{50}, E_{max}).
Mycoplasma:	This cell line tested negative for Mycoplasma.

Recommended Cell Culture Conditions

Complete Medium: MEM/EBSS, 10% fetal bovine serum (FBS), 50 µg/mL Zeocin™ (STIM1 and ORAI1expression selection), 0.2 µg/mL Puromycin (mito-Photina® expression selection).

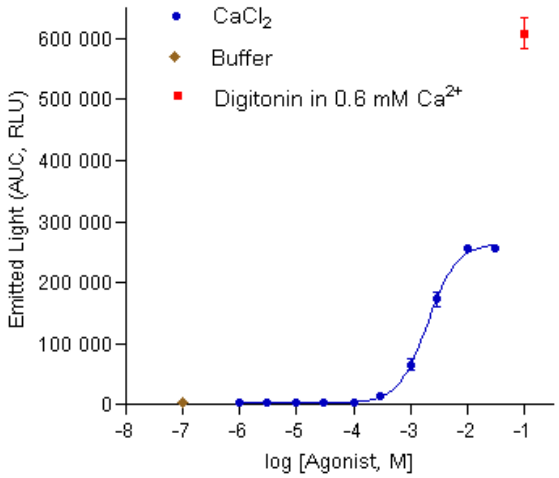
Freezing Medium: MEM/EBSS, 10% fetal bovine serum (FBS) with 10% DMSO, without selection agents.

Thawing Cells: Using appropriate personal protective equipment, 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 with a towel. Under aseptic conditions using a pipette, transfer content to 10 mL complete medium and centrifuge (150 *x g*, 5 min). Resuspend cell pellet in 10 mL of complete medium and transfer to an appropriate culture flask (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: 41,000 – 45,000 cells/cm^2

Cell Culture Protocol: Typically, for regular cell culture maintenance, these cells are grown to 80% confluence and trypsinized (0.05% trypsin / 0.5 mM EDTA in calcium and magnesium free HBSS). Under these conditions, cell passages should be carried out every 3-5 days.

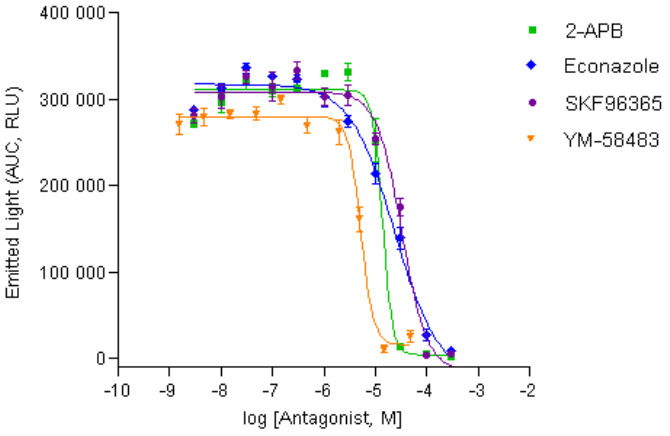
Typical Product Data – PhotoScreen® Assay



Agonist	EC_{50} (M)
$CaCl_2$	1.8×10^{-3}

Z′ (Buffer *vs.* EC_{80}) = 0.77 ± 0.07

Figure 1. PhotoScreen® Agonist Dose-Response.
Cells (20,000 cells/well) were seeded into the wells of a PDL-coated, black, clear bottom 384-well plate. After 24 h, cells were loaded with 20 µL/well of 10 µM native coelenterazine in Ca^{2+}-free HBSS + 15 mM HEPES for 4 h at room temperature, in the dark. $CaCl_2$ dilutions prepared in Ca^{2+}-free HBSS were dispensed on the cells (20 µL/well) and signal was measured from seconds 0 to 60 following agonist addition using the LumiLux® system. Buffer (Ca^{2+}-free HBSS) was used as a negative control, and 200 µM Digitonin in 1.26 mM Ca^{2+} HBSS (final 100 µM and 0.63 mM respectively) was used as a positive control. Data from a representative experiment are shown. As a control of response specificity, mock-transfected cells (i.e. parental cells transfected with the empty pBudCE4.1 vector) were also tested in the PhotoScreen® assay. When using a "passive calcium depletion" protocol, these mock-transfected cells did not show any response to 10 mM $CaCl_2$ compared to the CRAC cells (see Fig 3).



Antagonist	IC_{50} (M)
2-APB	14×10^{-6}
Econazole	24×10^{-6}
SKF-96365	33×10^{-6}
YM58483	5.2×10^{-6}

Figure 2. PhotoScreen® Antagonist Dose Response.
Cells (20,000 cells/well) were seeded into the wells of a PDL-coated, black, clear bottom 384-well plate. After 24 h, cells were loaded with 20 µL/well of 10 µM native coelenterazine in Ca^{2+}-free HBSS + 15 mM HEPES for 4 h at room temperature, in the dark. Antagonist dilutions, prepared in Ca^{2+}-free HBSS + 15 mM HEPES + 0.5% DMSO (final), were dispensed on the cells (20 µL/well) and incubated for 10 min at room temperature. A 9 mM $CaCl_2$ solution prepared in Ca^{2+}-free HBSS (final 3 mM) was dispensed (20 µL/well) on the mixture of cells and antagonists, and signal was measured from seconds 0 to 60 following agonist addition using the LumiLux® system. Data from a representative experiment are shown.

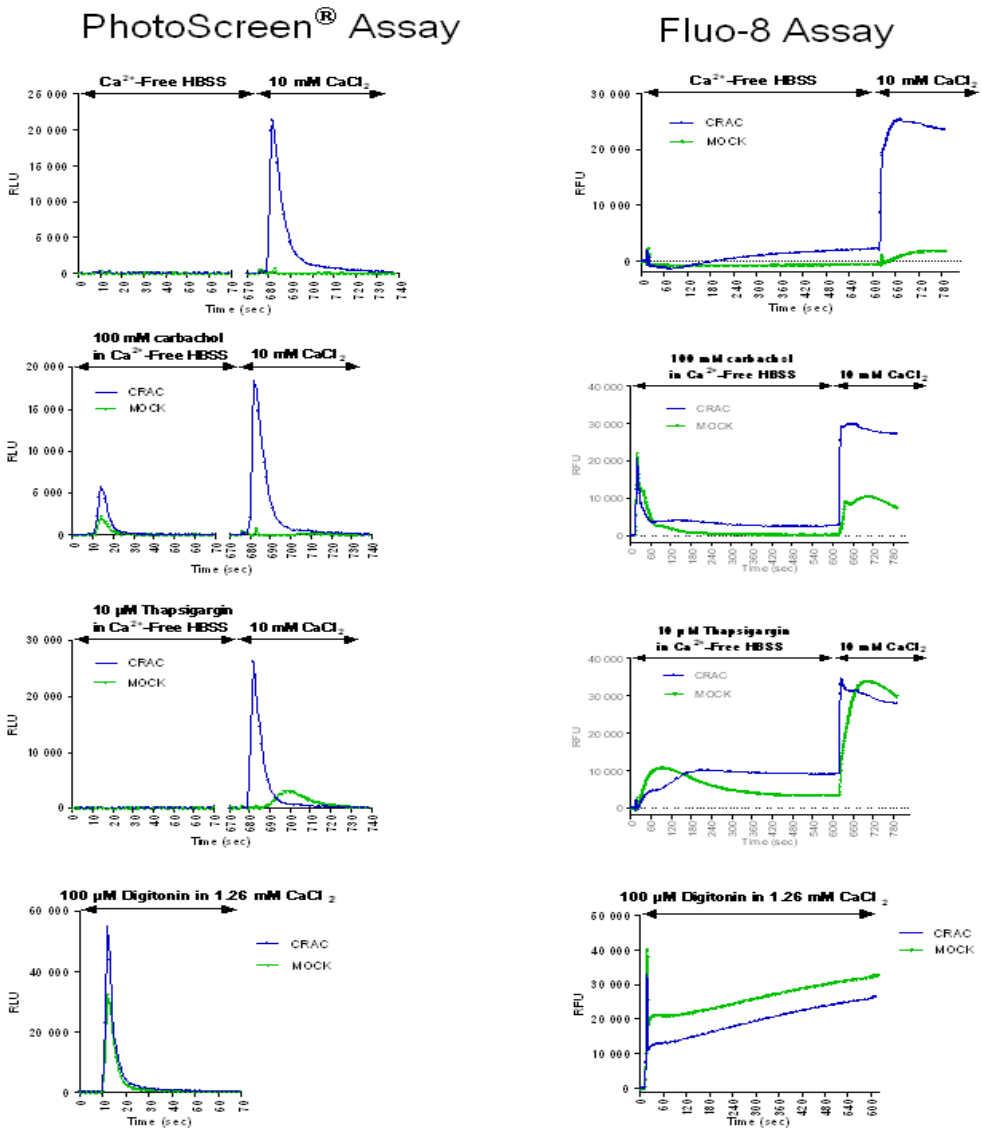


Figure 3. kinetics of the CRAC response in PhotoScreen® and Fluo-8 assays.
Cells (10,000 cells/well) were seeded into PDL-coated black (LumiLux) or white (FLIPR$^{TETRA®}$), clear bottom 384-well plate. After 24 h, cells were loaded with 10 µM native coelenterazine in Ca^{2+}-free HBSS + 15 mM HEPES for 4 h at room temperature, in the dark (PhotoScreen® assay) or with Fluo-8 NW for 1h at room temperature (Fluorescence assay). A dual dispensing protocol was used to dispense (first dispensing) Ca^{2+}-free HBSS Buffer, 100 µM carbachol (final conc.) or 10 µM thapsigargin (final conc.) in Ca^{2+}-free HBSS, or 100 µM Digitonin (final conc.) in 1.26 mM Ca^{2+} HBSS and then (second dispensing) 10 mM $CaCl_2$ (final conc.) in HBSS. These results show that the "passive calcium depletion" protocol using Ca^{2+}-free HBSS elicits a specific activation of the CRAC channel upon $CaCl_2$ dispensing, while the use of carbachol or thapsigargin in the same Ca^{2+}-free HBSS represents an alternative protocol. In addition, the difference of response of the CRAC cells *vs.* the mock-transfected cells was more clear-cut in the PhotoScreen® assay than in the fluorescent calcium assay; which may be in relation to the different intracellular localization of the calcium-sensing probes in these two types of assays: In PhotoScreen® assays, the Photina protein is expressed mitochondrially, while the calcium sensor dye Fluo-8 NW is cytoplasmic. The longer time cells spent in Ca^{2+}-free HBSS in the PhotoScreen Assay (4h) compared to the Fluo-8 assay (1h) may also contribute to explain the difference in signals observed.
Each curve shown here is the averaged kinetics data from 3 wells.

Typical Product Data – Electrophysiology

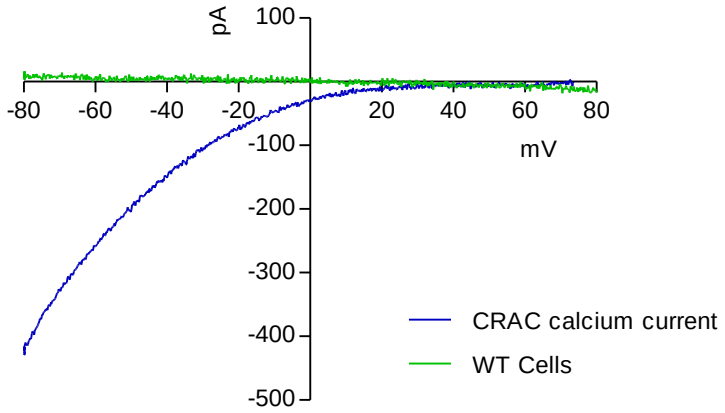


Figure 4. IP_3-induced current measured by whole cell voltage clamp.
Leak current was measured first with two to three ramp measurements after whole-cell clamp establishment. Then after the addition of 30 µM IP_3 in the intracellular solution, 100 ms voltage ramp spanning from –80 to +80 mV were applied at 1 s intervals. Leak current was subtracted from the current measured after IP_3 addition, and the resulting "leak subtracted current" is shown here. For the HEK-CRAC cells, the mean current density induced by IP_3 diffusion measured at -70 mV was 9.0 ± 1.8 pA/pF (n=15 cells, data not shown). WT HEK cells did not show any IP_3-induced current.

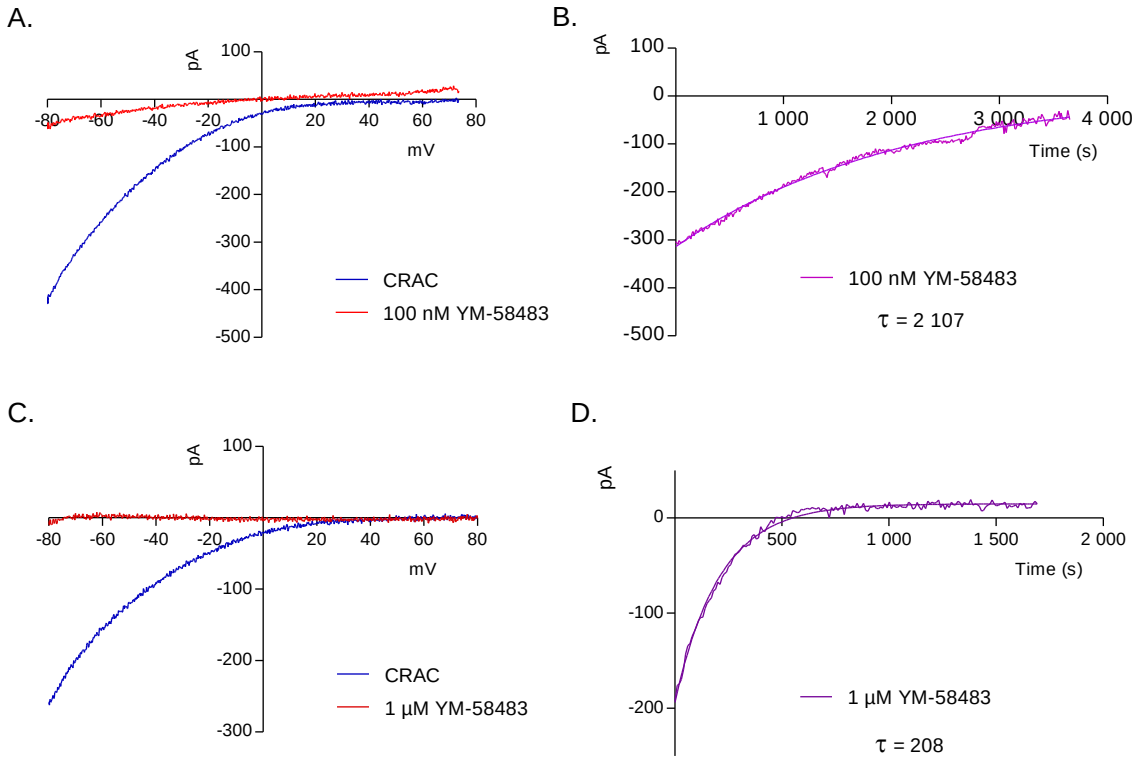


Figure 5. Inhibition of the CRAC current by YM-58483 measured by whole cell voltage clamp.
100 nM (A, C) or 1 µM (B, D) YM-58483 addition in the extracellular solution was used to inhibit the IP_3-induced CRAC current in the HEK-CRAC cells. The current was measured by 100 ms voltage ramp spanning from –80 to +80 mV applied at 1 s intervals. IP_3 was diffused in the intracellular solution, and the blue curves represent the stable currents obtained after IP_3 addition (A, C: "CRAC" control traces). YM-58483 was then added in the extracellular solution, and the current was continuously measured by 100 ms voltage ramp spanning from –80 to +80 mV applied at 1 s intervals. The red curves indicate the current after 1h (A: 100 nM YM-58483 trace) or 30 min (C: 1 µM YM-58483 trace) of incubation of the cells with the indicated YM-58483 concentration (A, C). The kinetics of the blockage of CRAC by YM-58483 is shown for currents monitored at -70 mV (C, D). This blockage was slow, similarly to what was described by Zitt *et al*. (2004) in Jurkat cells. τ is the calculated time constant of the blockage time-courses, when fitted using a single exponential function.

We also observed that 10 µM YM-58483 inhibited as well the CRAC channel, and when using 10 nM or 100 nM YM-58483 we could not observe steady-state block, due to the very long time needed to reach a steady state. No washout protocols were applied when using YM-58483, and traces shown in A/B and in C/D were recorded from two different cells (which explains the difference of current intensities observed in A/B compared to C/D).

PhotoScreen® Assay Procedure

Experimental Procedure for CRAC (Adhesion Mode):

1. Cells grown till mid-log phase (85-90% confluency) are detached using trypsin and are seeded at a concentration of 20,000 cells/well in poly-d-lysine-coated, black, clear bottom assay plates in culture medium without antibiotics and with 10% FBS. Cells are left to adhere in a 37°C, 5% CO_2 incubator for 24 h.
2. Medium is removed by plate overthrow and gentle tapping on a paper towel, then 20 µL/well of Ca^{2+}-free HBSS +15 mM HEPES containing 10 µM native coelenterazine are added to the cells and plates are incubated for 4 h at room temperature, in the dark.
3. For the agonist assay, using the reader's automatic injection system, dispense in triplicate on the coelenterazine-loaded cells 20 µL/well of $CaCl_2$ solution made in Ca^{2+}-free HBSS + 15 mM HEPES at the desired concentrations, and record the relative light emission for the desired time interval. Digitonin at a final concentration of 100 µM in 1.26 mM Ca^{2+} HBSS diluted in assay medium is used to measure the ion channel independent cellular calcium response.
4. For the antagonist assay, dispense per well in triplicate on the coelenterazine-loaded cells 20 µL of antagonist at the desired concentrations, diluted in Ca^{2+}-free HBSS + 15 mM HEPES. After 10 min of incubation, using the reader's automatic injection system, inject 20 µL of $CaCl_2$ at a final concentration equivalent to the EC_{80}, prepared in Ca^{2+}-free HBSS + 15 mM HEPES, and record the relative light emission for the desired time interval.

Electrophysiology – Whole Cell Voltage Clamp

Intracellular solution: 136 mM N-Methyl-D-Glucamine Chloride (NMDGCl), 10 mM EGTA, 10 mM HEPES, 6 mM $MgCl_2$, pH 7.2 with HCl.

Extracellular solution: 125 mM NaCl, 20 mM $CaCl_2$, 10 mM HEPES, 10 mM Glucose, pH 7.4 adjusted with NaOH.

Protocol:
Sixteen hours before experiments, cells were seeded onto poly-D-lysine coated coverslips (200,000 cells/coverslip) and placed in six well plates in antibiotic-free medium. Immediately before experiments, coverslips were washed five times with patch clamp extracellular solution and then put into the recording chamber. Currents were recorded in response to IP_3 addition in the intracellular solution by a pulse protocol: 100 ms ramps from -80 to +80 mV (0.1 Hz). After membrane breaking and before IP_3 addition, the first 2-3 ramps were used for leak subtraction. The whole cell patch clamp success rate was 93% (n = 15 cells).

Data Acquisition:
Standard whole-cell voltage-clamp experiments were performed at room temperature. For data acquisition and further analysis, an EPC10 digitally-controlled amplifier was used in combination with PATCHMASTER software (HEKA Electronics, Lambrecht, Germany). Capacitative currents were automatically subtracted by HEKA EPC10, data were filtered at 2.9 KHz (-3dB, 4-pole Bessel low-pass) and digitized at 100 µs per point.

- Liquid junction potential: No correction.
- Series resistance: Series resistance errors were carefully compensated before each experiment in order to reduce voltage errors to less than 5 mV.
- Pipette resistance and cell capacitance: The input resistance of the patch pipettes was 2-5 MΩ and the mean capacitance of the cells was 22.4 ± 2.1 pF (n = 15).
- Current density: The mean current density induced by IP_3 diffusion measured at -70 mV was 9.0 ± 1.8 pA/pF (n = 15 cells).

References

1. Bovolenta S, Foti M, Lohmer S, Corazza S. (2007) Development of a Ca^{2+}-activated photoprotein, Photina®, and its application to high-throughput screening. J Biomol Screen. 12: 694-704.

2. Button D, Brownstein M. (1993) Aequorin-expressing mammalian cell lines used to report Ca^{2+} mobilization. Cell Calcium. 14: 663-671.

3. Feske S, Prakriya M, Rao A, Lewis RS. (2005) A severe defect in CRAC Ca^{2+} channel activation and altered K^+ channel gating in T cells from immunodeficient patients. J Exp Med. 202:651-662. *Note*: in fig 3 of this paper, authors used a calcium depletion protocol to activate the CRAC channel. Although the incubation time, concentrations used, and measurement methods are different, this is a reference to a protocol similar to the calcium depletion protocol used here.

4. Yonetoku Y, Kubota H, Miyazaki Y, Okamoto Y, Funatsu M, Yoshimura-Ishikawa N, Ishikawa J, Yoshino T, Takeuchi M, Ohta M. (2008) Novel potent and selective Ca^{2+} release-activated Ca^{2+} (CRAC) channel inhibitors. Part 3: synthesis and CRAC channel inhibitory activity of 4'-[(trifluoromethyl)pyrazol-1-yl]carboxanilides. Bioorg Med Chem. 16:9457-9466.

5. Zitt C, Strauss B, Schwarz EC, Spaeth N, Rast G, Hatzelmann A, Hoth M. (2004) Potent inhibition of Ca^{2+} release-activated Ca^{2+} channels and T-lymphocyte activation by the pyrazole derivative BTP2. J Biol Chem. 279:12427-12437

Materials and Instrumentation

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

Table 1. References of compounds used for functional characterization assays

Name	Provider	Cat no	Working Stock Solution
2-APB (2-Aminoethyl diphenyl borate)	Sigma	D9754	500 mM in DMSO
Econazole nitrate salt	Sigma	E4632	100 mM in DMSO
SKF-96365 hydrochloride	TOCRIS	1147	100 mM in DMSO
YM58483	Sigma	Y4895	50 mM in DMSO
Digitonin	Sigma	37006	50 mM in DMSO
Native coelenterazine	Promega	S2001	1 mM in methanol
Fluo-8-NW Ca^{2+} sensitive dye	ABD Bioquest,	36316	dissolve 1 vial of Fluo8NW with 100µL of DMSO
IP_3 (Inositol 1,4,5-triphosphate)	Calbiochem	10008205	2 mM in water, to be diluted in the intracellular clamp solution just before use.

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

Name	Provider	Cat no
Standard HBSS (1.26 mM Ca^{2+})	Invitrogen	14025
Ca^{2+}, Mg^{2+}-free HBSS	Invitrogen	14175
MEM/EBSS	HyClone	SH30024.02
Zeocin™	Invitrogen	R250-01
Puromycin	Sigma	P7255
HEPES	Sigma	H4034
BSA, protease free	Sigma	A3059

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