ภาคผนวก ข
วิธีและผลการทดสอบฤทธิ์ทางชีวภาพของ Biotec

93

Bioassay laboratory *PRIMARY TEST REPORT*

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Customer name: ประภาพร ภูริปัญญาคุณ
Customer Address : สาขาวิชาเคมี มหาวิทยาลัยราชภัฏจันทรเกษม

Test: Antifungal Activity against *Candida albicans*
Method: Resazurin Microplate assay (REMA)
Negative control: 0.5% DMSO
IC_{50} of positive control: Amphotericin B = 0.124 µg/ml
Final concentration of samples: 50 µg/ml
Reported by: Komwijit S.
Reported date (dd/mm/yy): 27/12/2012
Total No. of tested sample: 3

Item	Screening code	Sample code	%Inhibition	Activity
1	V8344*	VE ในเฮกเซน	2.08	Inactive
2	V8345	VE ในเอทานอล	5.64	Inactive
3	V8346*	VE ในเอทิลอะซิเตต	11.22	Inactive

Remark: * Partially soluble in 100% DMSO

Assayed by _____ (Somjit Komwijit) (____ /_____ /_____)

Approved by _____ (Tanapong Boonruangprapa) (____ /_____ /_____)

Interpretation

% Inhibition	Activity
< 50%	Inactive
≥ 50%	Active (IC_{50} included)

National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA)
113, Paholyothin Rd, Klong 1, Klong Luang, Pathumthani 12120, Thailand
Tel: 02-5646629, Fax 02-5646707, www.biotec.or.th/bioassay

Bioassay laboratory *PRIMARY TEST REPORT*

BIOTEC a member of NSTDA

94

Customer name: ประภาพร ภูริปัญญาคุณ
Customer Address : สาขาวิชาเคมี มหาวิทยาลัยราชภัฏจันทรเกษม

Test: Anti-Cancer (NCI-H187-Small cell lung cancer)
Method: Resazurin Microplate assay (REMA)
Negative control: 0.5%DMSO
IC_{50} of positive control: Ellipticine = 1.16 µg/ml, Doxorubicin = 0.144 µg/ml
Final concentration of samples: 50 µg/ml
Reported by: Choowong W.
Reported date (dd/mm/yy): 26/12/12
Total No. of tested sample: 3

Item	Screening code	Sample code	% Inhibition	Activity
1	V8344*	VE ในเฮกเซน	91.1	Active
2	V8345	VE ในเอทานอล	8.1	Inactive
3	V8346*	VE ในเอทิลอะซิเตด	74.5	Active

Remark: * Partially soluble in 100% DMSO

Assayed by _____ **Approved by** _____

(Wilunda Choowong) (Kannawat Danwisetkanjana)
(_ _ / _ _ / _ _) (_ _ / _ _ / _ _)

Interpretation

% Inhibition	Activity
< 50%	Inactive
≥ 50%	Active (IC_{50} included)

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95

Bioassay laboratory *PRIMARY TEST REPORT*

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Customer name: ประภาพร ภูริปัญญาคุณ
Customer Address : สาขาวิชาเคมี มหาวิทยาลัยราชภัฏจันทรเกษม

Test: Cytotoxicity against Vero cells (African green monkey kidney) and Anti-HSV-1 (Herpes simplex virus type-1)
Method: Green Fluorescent Protein (GFP)-based assay
Negative control: 0.5% DMSO
IC_{50} of positive control: Ellipticine = 1.19 mg/ml (Cytotoxicity test) ; Acyclovir = 9.62 µg/ml (Anti - HSV-1 test)
Final concentration of samples: 50 µg/ml
Reported by: Srichomthong K.
Reported date (dd/mm/yy): 21/01/2013
Total No. of tested sample: 3

Item	Screening code	Sample code	% Cell growth	Cytotoxicity	% Viral inhibition	Anti-HSV-1 activity
1	V8344*	VE ในเฮกเซน	130.49	Non-cytotoxic	9.62	Inactive
2	V8345	VE ในเอทานอล	118.79	Non-cytotoxic	-3.86	Inactive
3	V8346*	VE ในเอทิลอะซีเตต	91.34	Non-cytotoxic	-4.56	Inactive

Remark: * Partially soluble in 100% DMSO

Assayed by _____ (Kitlada Srichomthong) (__ / __ / __)

Approved by _____ (Kannawat Danwisetkanjana) (__ / __ / __)

Interpretation

Cytotoxicity against Vero cells

% Cell growth	Activity
> 50%	Non-cytotoxic
≤ 50%	Cytotoxic (IC_{50} included)

Anti-HSV-1(>50% cell growth will be evaluated for Anti-HSV-1)

% Viral inhibition	Activity
< 50%	Inactive
≥ 50%	Active (IC_{50} included)

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96

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Bioassay laboratory protocol 05

Assay	Anti-*Candida albicans*
Method	Resazurin microplate assay (REMA)
Positive control	Amphotericin B
Negative control	0.5% DMSO
Maximum test concentration	50 μg/ml
Description	*Candida albicans* (ATCC 90028) is grown on a potato dextrose agar (PDA) plate at 30°C for 3 days. Three to five single colonies are then cultured in a shaking flask containing RPMI-1640 until cell density reaches $5x10^5$ CFU/ml. The yeast cell suspension is added to the 384-well plate; each well containing 45 μl of cell suspension and 5 μl of test sample. The plates are then incubated at 37°C for 4 hrs. Thereafter, 10 μl of 62.5 μg/ml resazurin solution is added to each well and incubated at 37°C for 30 minutes. Fluorescent intensity is measured at the excitation wavelength of 530 nm and the emission wavelength of 590 nm using SpectraMax M5 microplate reader (Molecular Devices, USA). Fluorescence signal from wells containing only medium is used for background subtraction. The percentage of inhibition is determined from the fluorescence units of treated cells (FU_T) and untreated cells (FU_C) using the following equation: $\% \text{ Inhibition} = [1- (FU_T / FU_C)] \times 100$ The 50% inhibitory concentration (IC_{50}) is determined from dose-response curve, using 6 concentrations of 2-fold serially dilution. Amphotericin B and 0.5 %DMSO are used as a positive and a negative control, respectively.
Reference	Brien JO, Wilson I, Orton T, Pognan F. Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem 2000;267: 5421-6.
Latest update	April, 2009

97

Bioassay laboratory protocol 01

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Assay	Cancer cell growth inhibition
Method	Resazurin microplate assay (REMA)
Positive control	Ellipticine, doxorubicin and tamoxifen
Negative control	0.5% DMSO
Maximum final test concentration	50 μg/ml
Description	Three cancerous human-cell lines are available for this assay : 1) KB cell line (epidermoid carcinoma of oral cavity, ATCC CCL-17), 2) MCF-7 cell line (breast adenocarcinoma, ATCC HTB-22) and 3) NCI-H187 (small cell lung carcinoma, ATCC CRL-5804) This assay is performed using the method described by Brien et al. (2000). In brief, cells at a logarithmic growth phase are harvested and diluted to $2.2x10^4$ cells/ml for KB and $3.3x10^4$ cells/ml for MCF-7 and NCI-H187, in fresh medium. Successively, 5 □l of test sample diluted in 5% DMSO, and 45 □l of cell suspension are added to 384-well plates, incubated at 37°C in 5% CO_2 incubator. After the incubation period (3 days for KB and MCF-7, and 5 days for NCI-H187), 12.5 □l of 62.5 □g/ml resazurin solution is added to each well, and the plates are then incubated at 37°C for 4 hours. Fluorescence signal is measured using SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 nm and 590 nm. Percent inhibition of cell growth is calculated by the following equation: $\% \text{ Inhibition} = [1- (FU_T/ FU_C)]*100$ Whereas FU_T and FU_C are the mean fluorescent unit from treated and untreated conditions, respectively. Dose response curves are plotted from 6 concentrations of 3-fold serially diluted test compounds and the sample concentrations that inhibit cell growth by 50% (IC_{50}) can be derived using the SOFTMax Pro software (Molecular Devices, USA). Ellipticine, doxorubicin and tamoxifen are used as a positive control, and 0.5%DMSO and water are used as a negative control.
Reference	Brien JO, Wilson I, Orton T, Pognan F. Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 2000. 267:5421-6.
Latest update	March, 2011

98

Bioassay laboratory protocol 02

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Assay	Cytotoxicity against primate cell line (Vero)
Method	Green fluorescent protein (GFP) detection
Positive control	Ellipticine
Negative control	0.5% DMSO
Maximum test concentration	50 μg/ml
Description	The GFP-expressing Vero cell line was generated in-house by stably transfecting the African green monkey kidney cell line (Vero, ATCC CCL-81), with pEGFP-N1 plasmid (Clontech). The cell line is maintained in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate and 0.8 mg/ml geneticin, at 37℃ in a humidified incubator with 5% CO_2. The assay is carried out by adding 45 μl of cell suspension at $3.3x10^4$cells/ml to each well of 384-well plates containing 5 μl of test compounds previously diluted in 0.5% DMSO, and then incubating for 4 days in 37°C incubator with 5% CO_2. Fluorescence signals are measured by using SpectraMax M5 microplate reader (Molecular Devices, USA) in the bottom-reading mode with excitation and emission wavelengths of 485 and 535 nm. Fluorescence signal at day 4 is subtracted with background fluorescence at day 0. The percentage of cytotoxicity is calculated by the following equation, where FU_T and FU_C represent the fluorescence units of cells treated with test compound and untreated cells, respectively: $\%\ \text{cytotoxicity} = [1-(FU_T / FU_C)] \times 100$ IC_{50} values are derived from dose-response curves, using 6 concentrations of 3-fold serially diluted samples, by the SOFTMax Pro software (Molecular device). Ellipticine and 0.5%DMSO are used as a positive and a negative control, respectively.
Reference	Hunt L, Jordan M, De Jesus M, Wurm FM. GFP-expressing mammalian cells for fast, sensitive, noninvasive cell growth assessment in a kinetic mode. Biotechnol and Bioeng 1999. 65: 201-5.
Latest update	June, 2012