PCSK9 [Biotinylated] : LDL R Inhibitor Screening ELISA Assay Pair

Pack Size: 96 tests / 480 tests

Catalog Number： EP-103

IMPORTANT: Please carefully read this manual before performing your experiment.

For Research Use Only. Not For Use In Diagnostic Or Therapeutic Procedures

PRINCIPLE OF THE ASSAY

PCSK9 is a crucial protein in the regulation of plasma cholesterol homeostasis. It binds low density lipoprotein receptor (LDL R) to enhance the degradation of LDL R. Therefore, inhibition of PCSK9 has been considered a promising strategy to prevent the receptor from being degraded and promote removal of LDL cholesterol from circulation.

This inhibitor screening ELISA pair is designed to facilitate the identification and characterization of new PCSK9 pathway inhibitors. This assay employs a simple colorimetric ELISA platform, which measures the binding between immobilized **human LDL R** and in-house developed **biotinylated PCSK9** protein. This product is uniquely suitable for rapid high-throughput screening of putative **PCSK9** inhibitors. Briefly, we provide you with a **biotinylated human PCSK9** protein, a **human LDL R** protein, an **anti-PCSK9 neutralizing antibody** (*as method verified Reference*), and **Streptavidin-HRP** reagent. Your experiment will include 4 simple steps:

a) Coat the plate with **human LDL R**.

b) Add your molecule of interest to the plates.

c) Add **human PCSK9-Biotin** to the plates.

d) Add **Streptavidin-HRP** followed by TMB or other colorimetric HRP substrate.

Finally, the ability of your compound to inhibit PCSK9: LDL R binding will be determined by comparing OD readings among different experimental groups.

US and Canada: **Tel**: +1 800-810-0816
Asia and Pacific: **Tel**: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

MATERIALS PROVIDED

TABLE 1. MATERIALS PROVIDED

Catalog	Components	Size (96 tests)	Size (480 tests)	Format	Storage
A006-214	Human LDL R	35 μg	160 μg	Powder	-20℃
A007-214	Biotinylated Human PCSK9	10 μg	10 μg	Powder	-20℃
A003-214	Streptavidin-HRP	10 μg	10 μg	Powder	-20℃, avoid light
PC9-NA003	Anti-PCSK9 Neutralizing Antibody	40 μg	200 μg	Powder	-20℃

RECONSTITUTION

Reconstitute the provided lyophilized materials to stock solutions with PBS as recommended in **Table 2.1 and Table 2.2,** Solubilize for 15 to 30 minutes at room temperature with occasional gentle mixing. **Avoid vigorous shaking or vortexing**.

The reconstituted stock solutions should be stored at -70℃. **It is recommended not to freeze thaw more than 3 times.**

To avoid surface adsorption loss and inactivation, the reconstituted protein must NOT be aliquoted to less than 5 μg per vial.

***Note**: Streptavidin-HRP stock solution should be protected from light.*

TABLE 2.1. RECONSTITUTION METHODS FOR 96 TESTS

Catalog	Components	Size	Stock Solution Con.	Reconstitution Buffer and Vol.
A006-214	Human LDL R	35 μg	250 μg/mL	140 μL PBS
A007-214	Biotinylated Human PCSK9	10 μg	100 μg/mL	100 μL PBS
A003-214	Streptavidin-HRP	10 μg	50 μg/mL	200 μL PBS
PC9-NA003	Anti-PCSK9 Neutralizing Antibody	40 μg	250 μg/mL	160 μL PBS

TABLE 2.2. RECONSTITUTION METHODS FOR 480 TESTS

Catalog	Components	Size	Stock Solution Con.	Reconstitution Buffer and Vol.
A006-214	Human LDL R	160 μg	250 μg/mL	640 μL PBS
A007-214	Biotinylated Human PCSK9	10 μg	100 μg/mL	100 μL PBS
A003-214	Streptavidin-HRP	10 μg	50 μg/mL	200 μL PBS
PC9-NA003	Anti-PCSK9 Neutralizing Antibody	200 μg	250 μg/mL	800 μL PBS

US and Canada: **Tel**: +1 800-810-0816
Asia and Pacific: **Tel**: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

SHIPPING AND STORAGE

All components are shipped in lyophilized state at room temperature. This product is stable after storage at:

1) Room temperature (RT) for 1 month in lyophilized state.
2) -20℃ for 1 year in lyophilized state.
3) -70℃ for 1 year under sterile conditions after reconstitution.

MATERIALS OR INSTRUMENTS REQUIRED BUT NOT SUPPLIED (for 96 tests)

Coating Buffer 15 mmol/L sodium carbonate (Na_2CO_3), 35 mmol/L sodium hydrogen carbonate ($NaHCO_3$), 7.7 mmol/L sodium azide (NaN_3), pH 9.6, 12 mL is sufficient for 96 tests.

Wash Buffer 1 PBS with 0.05% (v/v) Tween-20 (pH7.4), 500 mL is sufficient for 96 tests.

Blocking Buffer **Wash Buffer 1** with 2% (w/v) bovine serum albumin (BSA) *(Sigma-Aldrich, Catalog # A4737),* 35 mL is sufficient for 96 tests.

Wash Buffer 2 PBS with 0.05% (v/v) Tween-20, adjust pH to 5.5 with acetic acid, 500 mL is sufficient for 96 tests.

Dilution Buffer **Wash Buffer 2** with 0.5% (w/v) bovine serum albumin (BSA) *(Sigma-Aldrich, Catalog # A4737),* 50 mL is sufficient for 96 tests.

Substrate Dilution Buffer 50 mM disodium hydrogen phosphate (Na_2HPO_4) and 25 mM citric acid, adjust pH to 5.5 with 1 M Sodium hydroxide (NaOH), 25 mL is sufficient for 96 tests.

Substrate Stock Solution 10 mg/mL TMB *(Sigma-Aldrich, Catalog # 860336)* in Dimethyl sulfoxide *(Sigma-Aldrich, Catalog # D8418)*, 1 mL is sufficient for 96 tests. **Protect from light**.

TMB Substrate Working Solution

For **each plate** dilute 250 µL substrate stock solution in 25 mL substrate dilution buffer and add 12 µL 5% H_2O_2 (pipette 10 µL 30% H_2O_2 into 50 µL distilled water), mix well.

Notes:

1) *The TMB Substrate Working Solution should be freshly prepared and used within 15 minutes.*
2) *If you choose to use other commercially available ready-to-use TMB substrate solutions, you should follow the manufacturer's instruction.*

Stop Solution 1 M sulfuric acid (aqueous), 6 mL is sufficient for 96 tests.

High binding surface 96-well microplate, clear flat bottom *(Corning, Catalog # 9018)*

Microplate sealing film *(Sigma-Aldrich, Catalog # Z724742)*

Pipettes and pipette tips

US and Canada: **Tel**: +1 800-810-0816
Asia and Pacific: **Tel**: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

UV/Vis microplate spectrophotometer (absorbance 450 nm, correction wavelength set to 630 nm).

RECOMMENDED PROTOCOL

1. Preparation

Reconstitute and store all reagents as recommended.

2. Coating

1) Dilute **human LDL R** stock solution (250 μg/mL) to 3 μg/mL with **Coating Buffer** to make **human LDL R** working solution.
2) Please leave two wells uncoated for **No-Coating Control (Table 3)**.
3) Add 100 μL of **human LDL R** working solution (3 μg/mL) to each well, seal the plate with microplate sealing film and incubate overnight (or 16 hours) at 4℃.

3. Washing

Remove the remaining solution by aspiration, add 300 μL of **Wash buffer 1** to each well, gently tap the plate for 1 minute, remove any remaining **Wash Buffer 1** by aspirating or decanting, invert the plate and blot it against paper towels. **Repeat the wash step above for three times**.

Note: *For best results, the complete removal of the* ***human LDL R*** *solution is essential. The use of a manifold dispenser or an auto-washer may be necessary.*

4. Blocking

Add 300 μL **Blocking Buffer** to each well, seal the plate with microplate sealing film and incubate at 37℃ for 1.5 hours.

5. Washing

Repeat step 3. At meantime, you can start to prepare your **samples**.

6. Add Samples

1) Make series dilution of the samples as appropriate.
2) If you intend to use the provided **anti-PCSK9 neutralizing antibody** as a reference (Ref.), you may dilute the antibody as recommended in **Figure 3**. And plate layout as recommended in **Figure 4**.
3) For **Positive control** wells, please add 50 μL Dilution Buffer.
4) For all other wells, Add 50 μL of sample solution to each well according to our recommendation (**Figure 1**) or your own plate setup.

7. Binding

1) Dilute **human PCSK9-Biotin** stock solution (100 μg/mL) to 0.04 μg/mL with **Dilution Buffer** to make **human PCSK9-Biotin** working solution.
2) For **No-Binding Control** wells, please add 50 μL **Dilution Buffer**.
3) For all other wells, please add 50 μL **human PCSK9-Biotin** working solution.
4) Seal the plate with microplate sealing film and incubate at 37℃ for 1 hour.

8. Washing

Remove the remaining solution by aspiration, add 300 μL of **Wash buffer 2** to each well, gently tap the plate for 1 minute, remove any remaining **Wash Buffer 2** by aspirating or decanting, invert the plate and blot it against paper towels. **Repeat the wash step above for three times**.

US and Canada: **Tel**: +1 800-810-0816 | **Web**: http://www.acrobiosystems.com
Asia and Pacific: **Tel**: +86 400-682-2521 | **E-mail**: order@acrobiosystems.com

Note: *For best results, the complete removal of the* ***human LDL R*** *solution is essential. The use of a manifold dispenser or an auto-washer may be necessary.*

9. Streptavidin-HRP Labeling

1) Dilute **Streptavidin-HRP** stock solution (50 μg/mL) to 0.1 μg/mL with **Dilution Buffer** to make **Streptavidin-HRP** working solution.
2) For all wells, add 100 μL **Streptavidin-HRP** working solution, seal the plate with microplate sealing film and incubate at 37℃ for 1 hour, **avoid light**.

10. Washing

Repeat step 8.

11. TMB Substrate Reaction

Add 200 μL **TMB Substrate Working Solution** to each well. Seal the plate with microplate sealing film and incubate at 37℃ for 20 minutes, **avoid light**.

12. Termination

Add 50 μL **Stop Solution** to each well, and tap the plate gently for 3 minutes to allow thorough mixing.

Note: *the color in the wells should change from blue to yellow.*

13. Data Recording

Read the absorbance at 450 nm using UV/Vis microplate spectrophotometer.

Note*: the plate may be read at 600 nm without adding 1 M sulfuric acid, but the Signal-to-Background ratio may be reduced.*

US and Canada: **Tel**: +1 800-810-0816
Asia and Pacific: **Tel**: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

FIGURE 1. PREPARATION OF 1:2 SERIAL DILUTIONS OF THE ANTI-PCSK9 NEUTRALIZING ANTIBODY

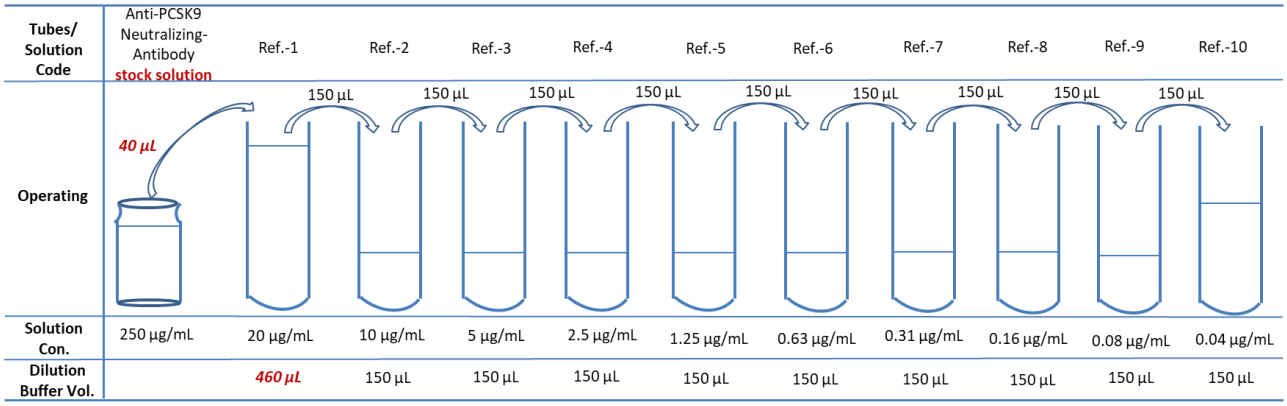
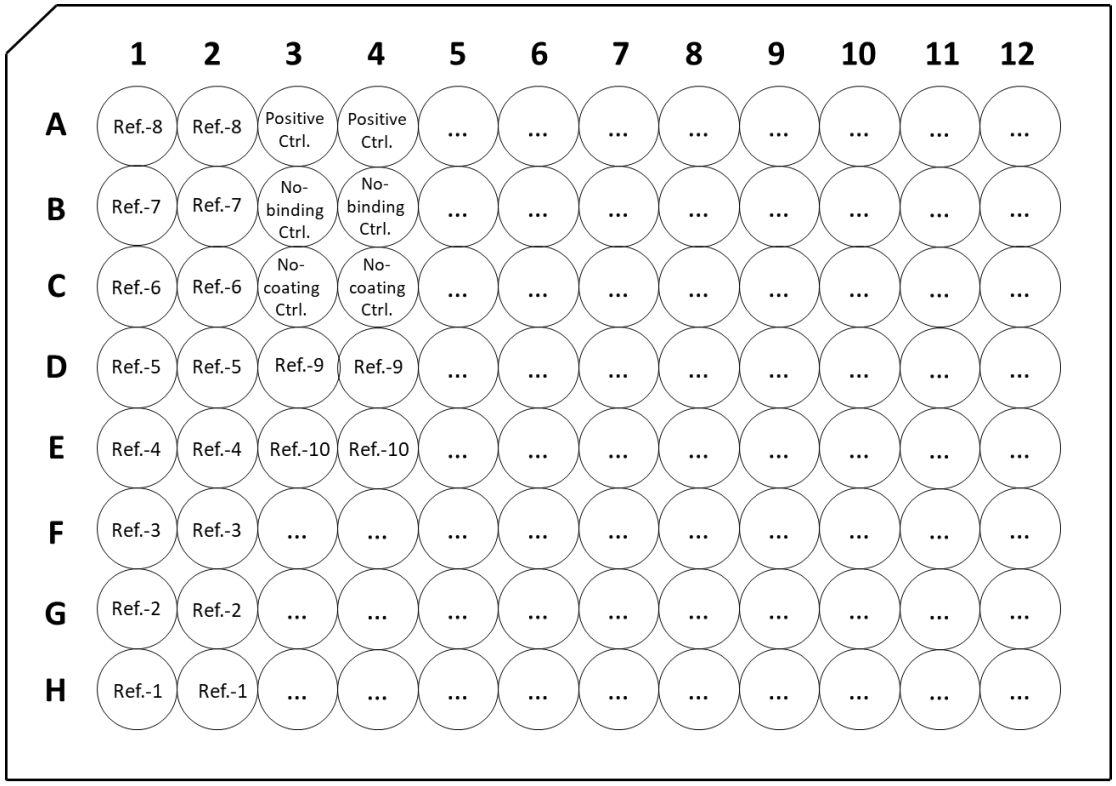


FIGURE 2. PLATE LAYOUT



US and Canada: **Tel**: +1 800-810-0816
Asia and Pacific: **Tel**: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

TABLE 3. ASSAY PROTOCOL

Steps Code	Steps	Reagents & Instruments	Reaction Conditions	Samples	No-binding Control	No-coating Control	Positive Control
1	Preparation	N/A	N/A	N/A	N/A	N/A	N/A
2	Coating	Human LDL R Working Solution	4℃ for overnight	100 µL	100 µL	—	100 µL
3	Washing	Wash Buffer 1	Wash for 3 times	300 µL	300 µL	300 µL	300 µL
4	Blocking	Blocking Buffer	37℃ for 1.5 hours	300 µL	300 µL	300 µL	300 µL
5	Washing	Wash Buffer 1	Wash for 3 times	300 µL	300 µL	300 µL	300 µL
6	Add Samples	Samples	N/A	50 µL	50 µL	50 µL	
		Dilution Buffer		—	—	—	50 µL
7	Binding	Human PCSK9-Biotin Working Solution	Incubate at 37℃ for 1 hour	50 µL	—	50 µL	50 µL
		Dilution Buffer		—	50 µL	—	—
8	Washing	Wash Buffer 2	Wash for 3 times	300 µL	300 µL	300 µL	300 µL
9	Streptavidin-HRP Labeling	Streptavidin-HRP Working Solution	37℃ for 1 hours	100 µL	100 µL	100 µL	100 µL
10	Washing	Wash Buffer 2	Wash for 3 times	300 µL	300 µL	300 µL	300 µL
11	TMB Substrate Reaction	TMB Substrate Working Solution	37℃ for 20 minutes	200 µL	200 µL	200 µL	200 µL
12	Stop the Reaction	Stop Solution	Mix by gentle tapping for 3 minutes	50 µL	50 µL	50 µL	50 µL
13	Data Recording	UV/Vis spectrophotometer	Measure absorbance at 450 nm, with the correction wavelength set at 600 nm				

Note for TABLE 3:

1) ***Samples:*** *Your samples of interest.*
2) ***No-Binding Control:*** *Reaction without* ***biotinylated human PCSK9*** *added. The absorbance should be around 0.05(< 0.1) at 450 nm.*
3) ***No-Coating Control:*** *Reaction without* ***human LDL R*** *coated on the wells. The absorbance should be around 0.05(< 0.1) at 450 nm.*
4) ***Positive Control:*** *Determined the max value in 450 nm absorbance, when out of inhibitors.*
5) *It is recommended that all samples, controls and references should be done in duplicates.*

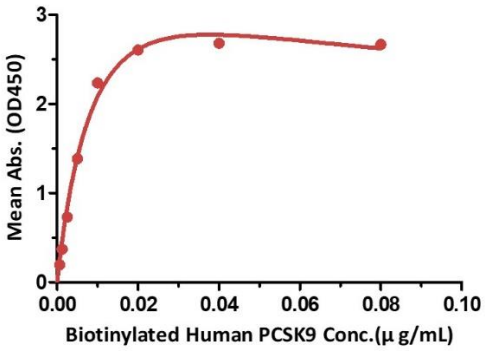
US and Canada: **Tel**: +1 800-810-0816
Asia and Pacific: **Tel**: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

METHOD VERIFICATION

- **PCSK9 [BIOTINYLATED]: LDL R BINDING IN THE ABSENCE OF INHIBITORS**

Immobilized **human LDL R** protein at 3 μg/mL (100 μL/well) can bind **biotinylated human PCSK9** with a linear range of 0.000625-0.02 μg/mL when detected by **Streptavidin-HRP**. Background was subtracted from data points before curve fitting.

FIGURE 3. BINDING OF BIOTINYLATED HUMAN PCSK9 TO IMMOBILIZED HUMAN LDL R IN A FUNCTIONAL ELISA ASSAY

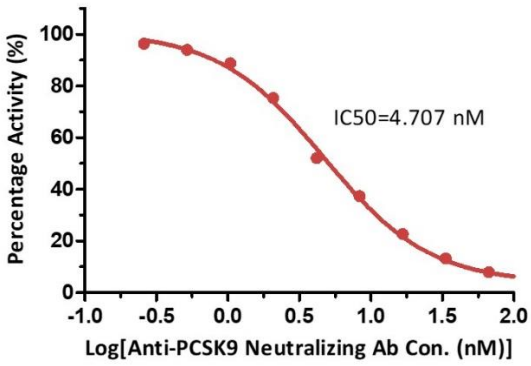


Biotinylated Human PCSK9 (μg/mL)	Mean Abs.(OD450)
0.08	2.669
0.04	2.681
0.02	2.605
0.01	2.236
0.005	1.386
0.0025	0.731
0.00125	0.370
0.000625	0.197

- **INHIBITION OF PCSK9 [BIOTINYLATED]: LDL R BINDING BY ANTI-PCSK9 NEUTRALIZING ANTIBODY**

Serial dilutions of anti-PCSK9 neutralizing antibody (Catalog # PC9-NA003) (1:2 serial dilutions, from 20 μg/mL to 0.04 μg/mL) were added into **Biotinylated PCSK9**: **LDL R** binding reactions. The assay was performed according to the above described protocol. Background was subtracted from data points prior to log transformation and curve fitting.

FIGURE 4. INHIBITION OF PCSK9 [BIOTINYLATED]: LDL R BINDING BY ANTI-PCSK9 NEUTRALIZING ANTIBODY



Anti-PCSK9 Neutralizing Ab Con. (μg/mL)	Anti-PCSK9 Neutralizing Ab Con. (nM)	Log[Anti-PCSK9 Neutralizing Ab Con. (nM)]	Mean Abs. (OD450)	Percent Activity (%)
0	0		2.742	100
0.04	0.26	-0.58	2.640	96.280
0.08	0.52	-0.28	2.575	93.909
0.16	1.04	0.02	2.434	88.766
0.31	2.08	0.32	2.065	75.307
0.63	4.17	0.62	1.427	52.036
1.25	8.33	0.92	1.025	37.374
2.50	16.67	1.22	0.623	22.711
5.00	33.33	1.52	0.364	13.264
10.00	66.67	1.82	0.217	7.903
20.00	133.33	2.12	0.116	4.219

US and Canada: **Tel**: +1 800-810-0816
Asia and Pacific: **Tel**: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

TROUBLESHOOTING GUIDE

Problem	Possible Cause	Solutions
Signal of positive control is weak or abnormal	**The pH of the buffer is incorrect**	✧ **Make sure buffers are prepared and used correctly.**
	Reconstituted protein be aliquoted to less than 5 μg per vial	✧ **Reconstituted protein must NOT be aliquoted to less than 5 μg per vial.**
	The working solution not be prepared immediately before use	✧ **The working solution should be prepared immediately before use and should not be stored.**
	Biotinylated human PCSK9, **human LDL R,** or **Streptavidin - HRP** may have lost activity	✧ Make sure your proteins are aliquoted into single-use aliquots. ✧ Increase the time of reaction or increase the protein concentration may help in case the protein activity is decreased over time.
	Errors in instrument settings	✧ Please check instrument setting.
	Substrate Stock Solution is outdated; Incubation temperature is incorrect; Incubation time is not sufficient; Repeated freeze-thaw cycles;	✧ Make sure the **Substrate Stock Solution** is working. ✧ Use proper incubation time and temperature.
	Pipetting errors	✧ Make sure that the pipette is calibrated and working properly.
High background	Insufficient washing or blocking	✧ Be sure the blocking step is performed. ✧ Increase number of washes and the volume **Wash Buffer** used. ✧ Increase Tween-20 concentration to 0.1% in **Wash Buffer**. ✧ Make sure **Streptavidin-HRP** is diluted in **Blocking Buffer**.
	Sample solvent contains inhibiting factors	✧ Run a negative control assay with the solvent alone. ✧ Maintain DMSO level at <1%. Increase protein incubation time.
	Contamination	✧ Make sure buffers and samples are prepared, used and stored correctly.
	The **TMB Substrate Working Solution** is not fresh	✧ **TMB Substrate Working Solution** must be used within 15 minutes after preparation.
Colorimetric signal is erratic	Inconsistent pipetting or dilution methods	✧ Make sure pipettors are functioning properly and use a multichannel pipettor if possible. ✧ Use master mixes to minimize errors. ✧ Run duplicates for all tests.
	TMB Substrate Working Solution is not completely mixed with the reaction solution	✧ Make sure that **TMB Substrate Working Solution** is adequately mixed with the reaction solution.
	Bubbles in the wells	✧ Tap plate gently to disperse bubbles.
	Signal is too high	✧ The concentration of the samples should be adjusted to achieve optimal reading. ✧ Decrease colorimetric HRP substrate incubation time.
Inadequate color development	Incomplete removal of residual buffers during previous steps	✧ Wells should appear dry after aspiration.
	Problems with conjugate or color reagents	✧ Color should appear immediately after the reagent is added. Make sure no contamination or residual buffers in the wells before you start the color development process.

US and Canada: **Tel**: +1 800-810-0816
Asia and Pacific: **Tel**: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com