ProteOn™ XPR36 Quantikinetics: Antibody Concentration and Detailed Kinetic Analysis in a Single Experimental Cycle

Gary Ross, Mingde Zhu, Ruben Luo, Bio-Rad Laboratories, Inc., 2000 Alfred Nobel Drive, Hercules, CA 94547

Tech Note

Protein Interactions

Bulletin 6411

Introduction

Monoclonal antibodies are an essential tool in the development of immunoassays used in basic biomedical research. These antibodies are central to biotherapeutic strategies, directed toward the diagnosis and treatment of diseases.

An initial step in the production of specific monoclonal antibodies involves the characterization of dilution clone media samples. This is normally a multistep process: (1) determination of the differential antibody concentration expressed across individual culture wells, (2) antibody ranking based on comparative affinities for the specific antigen, and (3) detailed kinetic analysis, using surface plasmon resonance (SPR), to provide the association (k_a, $M^{-1}s^{-1}$) and dissociation (k_d, s^{-1}) rate constants that characterize each antibody-antigen pair.

Here, we describe a single cycle (<60 minute) workflow called "quantikinetics" using the Bio-Rad SPR biosensor platform, the ProteOn XPR36 system, to implement quantitation of six unknown antibody samples in culture media as well as the detailed kinetic analysis of specific antigen binding to each of the six captured antibodies.

Quantikinetics Workflow

SPR biosensors measure biomolecular interactions in a label-free and real-time manner. The ProteOn XPR36 protein interaction array system is an SPR biosensor platform designed for analyzing label-free biomolecular interactions. It features an array of 6 x 6 biomolecular interactions based on a criss-cross fluidic design, which allows for the immobilization of six ligands into six vertical channels and injects six analytes into six horizontal channels. Thus the ProteOn XPR36 system allows for high efficiency experimental optimization as well as high productivity. The 6 x 6 configuration also enables novel referencing options for high quality data that include interspot reference and real-time injection reference that are inherent to the criss-cross fluidic design as shown in Figure 1. The interspot reference saves interaction spots for referencing and provides immediate proximate correction to the refractive index effect and nonspecific binding in sample injections. The real-time injection reference monitors the real-time change of the ligand surface to precisely correct the exponential baseline drift when using ligand-capture surface chemistry.

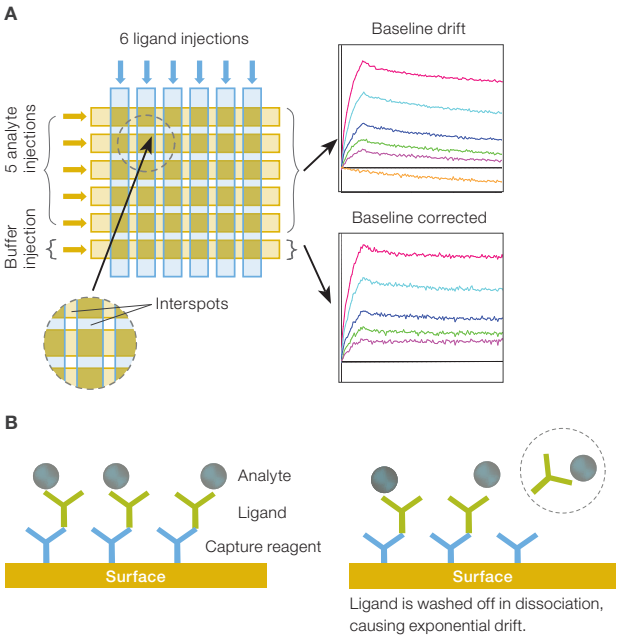
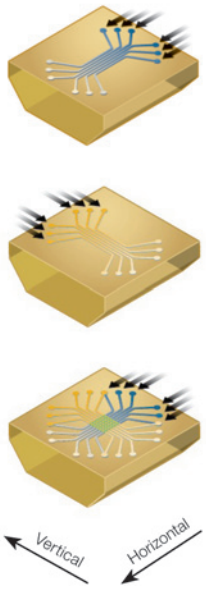


Fig. 1. The ProteOn XPR36 system features a 6 x 6 interaction array, which allows novel referencing options. A, the interspot reference saves interaction spots for referencing and provides immediate proximate correction to refractive index effect and nonspecific binding in sample injections. There are vertical interspots (light blue) and horizontal interspots (light orange) that can be used in ligand and antibody injections for referencing; **B**, the real-time injection reference monitors the real-time change of the ligand surface to precisely correct the exponential baseline drift when using ligand-capture surface chemistry.

BIO-RAD

Based on the 6 x 6 configuration, it is possible to include the quantitation of six unknown antibody samples in culture media as well as the detailed kinetic analysis of specific antigen binding to each of the six captured antibodies. Named quantikinetics, this workflow significantly increases the efficiency and simplifies the process of antibody discovery and development. The basics of this workflow are shown in Figure 2.



Step 1. Couple a capture antibody (capture reagent) on GLM chip surface.
1. Covalently couple capture antibody to the chip surface.
2. Confirm that high density capture antibody surface is uniformly obtained (>7000 RU).

Step 2. Generate standard curve and quantitate antibody samples (ligand) in culture media.
1. Inject antibody samples with known concentrations to generate standard curve.
2. Inject antibody samples in culture media with unknown concentrations.

Step 3. Perform One-shot Kinetics™ analysis of antigen (analyte) to the ligand.
1. Inject dilution series of an analyte sample with real-time injection reference.
2. Correct with baseline drift due to reversible antibody capture using the real-time injection reference.
3. Fit the five parallel interaction sensorgrams for kinetics.
4. Regenerate the chip surface to allow the repeat of steps 2 and 3.

Fig. 2. The quantikinetic workflow of the ProteOn XPR36 system.

Model Experiment

A model experiment was designed and carried out to demonstrate the performance of this novel quantikinetics workflow using the ProteOn XPR36 system. A ProteOn GLM sensor chip was used and the experimental steps are outlined as follows.

Step 1. With the flow channels set to the horizontal direction, the capture reagent, goat anti-mouse IgG antibody (GAM-IgG Ab), was injected and amine-coupled to the chip surface. The flow rate was set at 25 µg/ml with an injection time of 3 min. The sample was diluted in 10 mM sodium acetate, pH 4.5. The purpose of immobilizing the GAM-IgG Ab in the horizontal direction is to cover the horizontal interspots which allows for similar surface properties to the interaction spots. The horizontal interspots can be used for referencing in the kinetic analysis step (step 3), in which the analyte samples were injected in the horizontal direction.

Step 2. Prior to ligand injection, the flow channels were rotated to the vertical direction and the system was equilibrated in PBS + 0.05% Tween 20 + 0.1% BSA (PBST-B). The ligand monoclonal mouse anti-human IL-2 antibody (MAH-IL-2 Ab) in the culture media was diluted (1:5 or 1:10) in PBST-B buffer and injected at 25 µl/min. The binding of the MAH-IL-2 to the GAM-IgG Ab was recorded in the sensorgrams. The sensorgrams were referenced to the vertical interspot positions (devoid of GAM-IgG Ab). The initial slope for each sample is linearly dependent on the ligand concentration. The data analysis using ProteOn Manager™ software is shown in Figure 3. Injections of known MAH-IL-2 Ab concentrations were used to create a standard curve. By adjusting the ligand injection time, we have found the concentration analysis for the antibody samples to be effective across the 10 ng/ml to 100 µg/ml range.

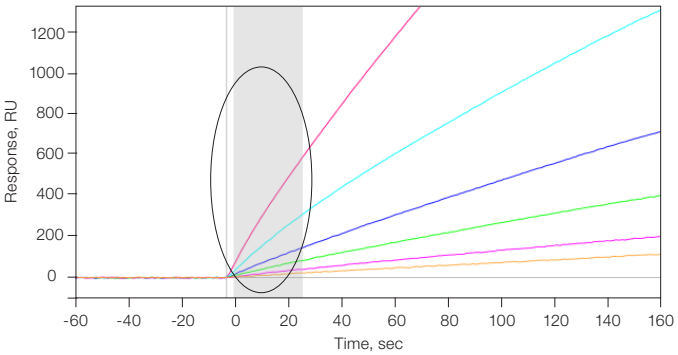


Fig. 3. The initial slope analysis for sample quantitation in ProteOn Manager software.

Step 3. The flow channels were rotated to the horizontal direction, and two PBST-B buffer injections (approximately 12 min) were carried out for surface stabilization. Five concentrations of the analyte, human IL-2, and the blank buffer were injected in parallel in all six horizontal channels. For referencing, the horizontal interspots that have covalently coupled GAM-IgG Ab were used to correct for refractive index effect and nonspecific binding, and the real-time buffer injection was used to correct for negative drift due to the decay of the underlying GAM-IgG Ab-MAH-IL-2 Ab complex.

The ligand, MAH-IL-2 Ab, was prepared in a wide range of concentrations and captured to the GAM-IgG Ab surface, as shown in Figure 4. A 1 min or a 10 min capture period was used. By extending the capture period to 10 min, we could establish a lower limit (approximately 25 ng/ml) for reproducible kinetic analysis upon injecting the analyte, IL-2 (15.4 kD), as shown in Figure 5.

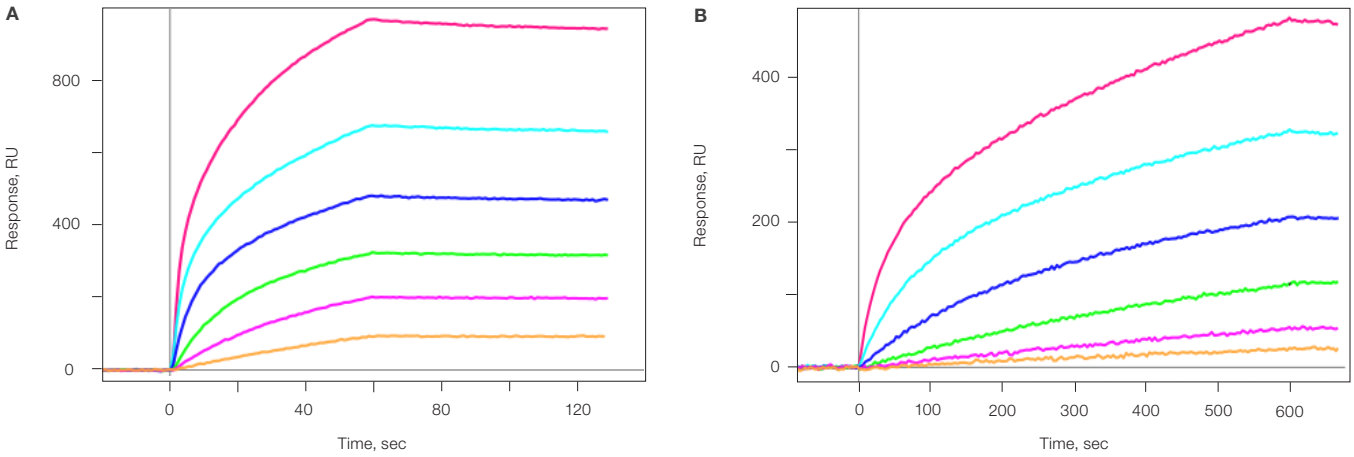


Fig. 4. The sensorgrams of capturing MAH-IL-2 Ab to the GAM-IgG Ab surface. The MAH-IL-2 Ab samples were serially diluted in PBST-B running buffer. The sensograms were referenced to the corresponding vertical interspot positions. **A**, one-minute capture of the antibody in the 1–100 µg/ml range. The ligand concentrations were 188 µg/ml (—), 63 µg/ml (—), 21 µg/ml (—), 6.9 µg/ml (—), 2.3 µg/ml (—), and 0.77 µg/ml (—); **B**, Ten-minute capture of the antibody in the 10 ng/ml to 2 µg/ml range. The ligand concentrations were 2.0 µg/ml (—), 0.67 µg/ml (—), 0.22 µg/ml (—), 74 ng/ml (—), 25 ng/ml (—), and 8.2 ng/ml (—).

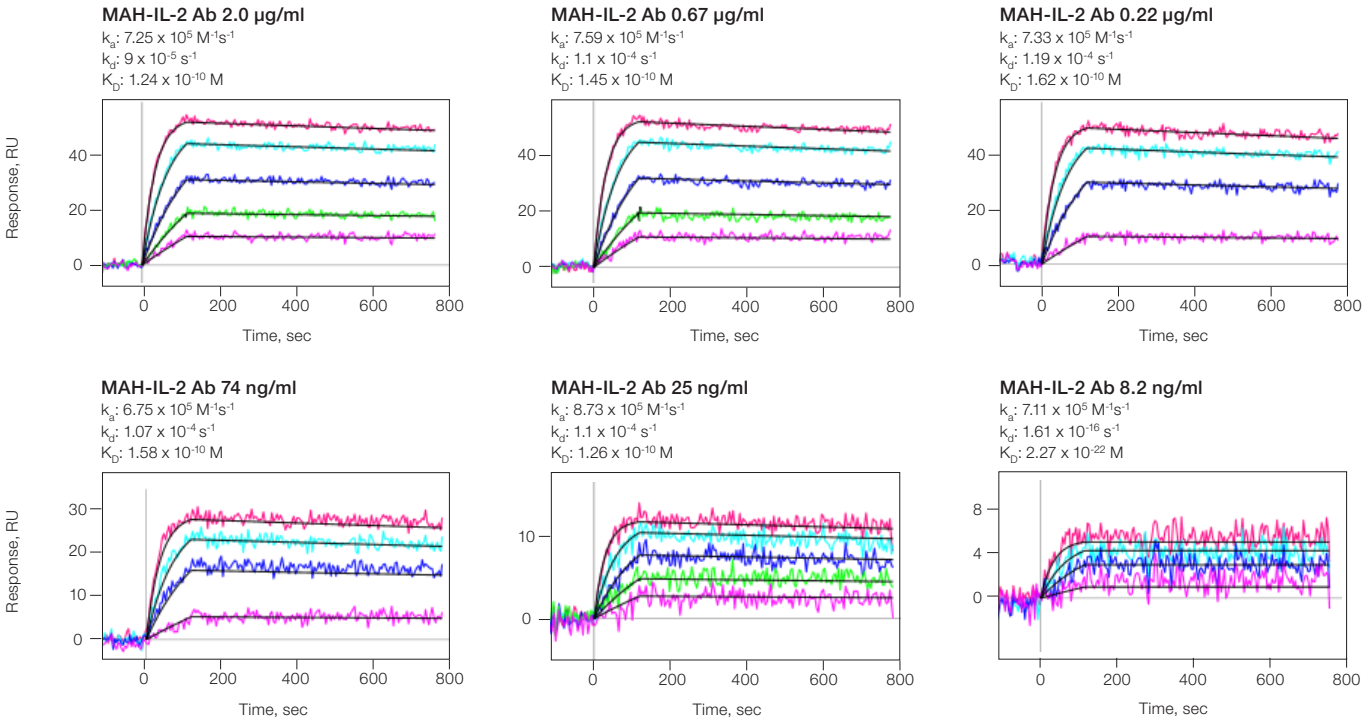


Fig. 5. Kinetic analysis of the interaction between MAH-IL-2 Ab and IL-2. Each graph was obtained in the vertical channel that captured MAH-IL2 Ab samples at a particular concentration. The sensorgrams were referenced to the horizontal interspots and a real-time buffer injection. The fitted curves are marked in black. The kinetic rate constants k_a and k_d, as well as the calculated equilibrium constant K_D ($K_D=k_d/k_a$) obtained from each graph, were labeled. Reproducible k_a and k_d values are obtained when MAH-IL-2 Ab is captured at levels as low as 25 ng/ml. The analyte, IL-2, concentrations were 40 nM (—), 20 nM (—), 10 nM (—), 5.0 nM (—), and 2.5 nM (—).

An additional test was performed to determine the effect of the culture media components on the capture of MAH-IL-2 Ab and the resulting kinetic analysis. Figure 6 shows the substantial bulk shift effects of neat Ex-Cell media (Sigma-Aldrich), containing 2% fetal bovine serum. Sensorgrams of MAH-IL-2 Ab (10 ng/ml to 10 µg/ml) in such culture media can be corrected by using the vertical interspot referencing.

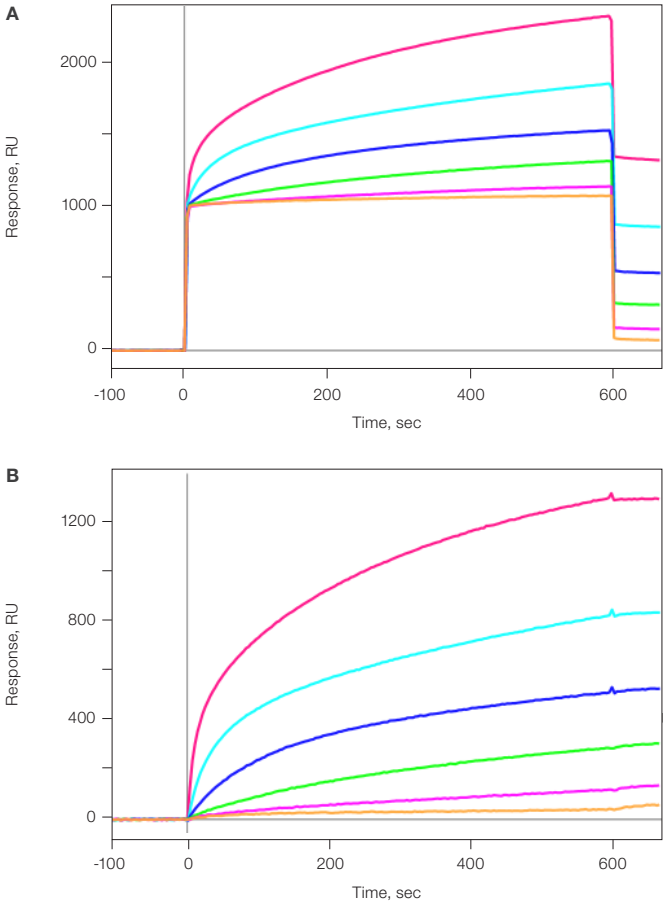


Fig. 6. Effect of culture media in the capture of MAH-IL-2 Ab. The antibody was serially diluted in Ex-Cell media + 2% FBS and the sample is injected for 10 minutes. Sensograms before **(A)** and after **(B)** vertical interspot referencing. The ligand concentrations were 10 µg/ml (—), 2.5 µg/ml (—), 0.63 µg/ml (—), 0.16 µg/ml (—), 39 ng/ml (—), and 9.7 ng/ml (—).

Screening Experiment

Based on the optimized protocols from the model experiment, five monoclonal mouse anti-human cytokine antibody samples in multiple culture media were screened. The five mouse anti-human cytokine antibody samples, specifically against eotaxin, IL-21, GMCSF, IFN-β, and MCP-1, were serially diluted (1:5) in PBST-B buffer and injected for 2 min. The chip surface was immediately regenerated by injecting glycine pH 2 and 1% phosphoric acid in vertical channels. The regeneration resulted in a clean GAM-IgG Ab surface that is available for the next cycle of antibody capture. Table 1 shows the resulting mouse anti-human cytokine antibody concentrations, averaged among the six interaction spots in each vertical channel. The experiment was achieved in a 2 hr run time.

Table 1. Five mouse anti-human cytokine antibody samples in multiple culture media against eotaxin, IL-21, GMCSF, IFN-β, and MCP-1 were quantitated on the GAM-IgG Ab surface, using the first step in the quantikinetics workflow.

Cytokine	Media	µg/ml	% CV
Eotaxin	AE01	5.59	4.0
	AE03	4.52	4.2
	AE04	6.43	7.1
	AE05	9.56	8.8
	AE06	4.11	6.7
IL-21	E01	10.14	4.9
	E02	4.45	3.3
	E03	8.9	5.8
	E04	2.9	5.2
	F02	3.99	5.2
GMCSF	C06	8.8	11.1
	C10	2.09	6.6
	F02	10.18	8.2
	F03	10.53	9.1
	F05	10.26	7.9
IFN-β	F01	3.98	4.9
	RD01	2.67	8.8
	RD02	0.21	28.0
	RD03	1.35	11.5
	RD04	1.91	7.5
MCP-1	AA02	6.47	7.0
	AA03	8.66	7.3
	AA04	4.65	7.0
	AA06	3.76	6.4

Following quantitation, the second step in the quantikinetics workflow, kinetic analysis was carried out with two mouse anti-human eotaxin antibody (MAH-ET Ab) samples, as shown in Figure 7. The two MAH-ET Ab samples were prepared in different culture media, AE04 and AE05. Both were serially diluted (1:5, 1:10, and 1:20) in PBST-B running buffer. After the MAH-ET Ab was captured on the GAM-IgG Ab surface, five eotaxin concentrations were injected for kinetic analysis. Reproducible kinetic analysis was observed in the interaction between MAH-ET Ab and eotaxin. The consistency of the k_a and k_d values, as well as calculated K_D values across different culture media verifies the high performance of this quantikinetics workflow.

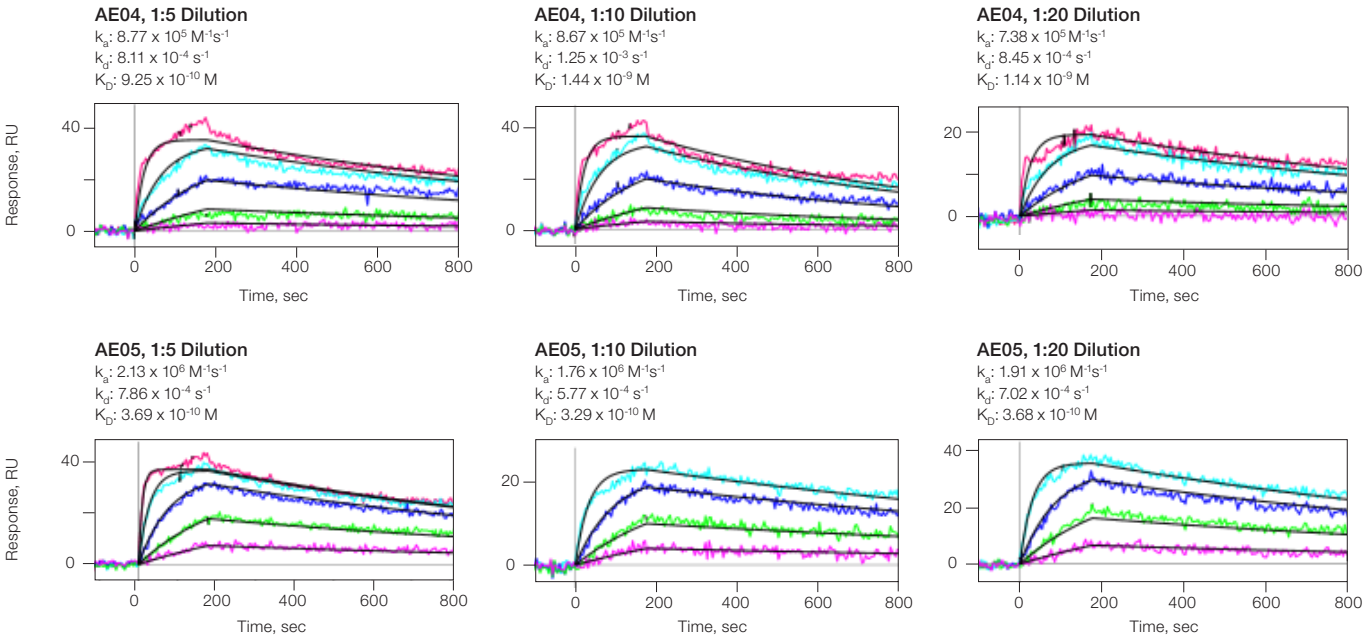


Fig. 7. Kinetic analysis of the interaction between MAH-ET Ab and eotaxin. Each graph was obtained in the vertical channel that captured MAH-ET Ab samples in AE04 or AE05 culture media. The samples were diluted in PBST-B running buffer in the ratio of 1:5, 1:10, or 1:20. The sensorgrams were referenced to the horizontal interspots and a real-time buffer injection. The fitted curves are marked in black. The kinetic rate constants k_a and k_d, as well as the calculated equilibrium constant K_D ($K_D=k_d/k_a$) obtained from each graph, were labeled. Reproducible k_a and k_d values were obtained using MAH-ET Ab samples prepared in different culture media. The analyte, eotaxin, concentrations were 50 nM (—), 17 nM (—), 5.5 nM (—), 1.9 nM (—), and 0.62 nM (—).

Conclusion

The reported experiments show the high performance and efficiency of this novel quantikinetics workflow. Table 2 provides recommendations for media preparation and antibody capture time periods. The quantikinetics workflow, described here, demonstrates the potential to obtain both antibody concentration levels and full kinetic profiling of a 96-well media sample set within a single, unattended overnight run.

Table 2. Recommended antibody concentration and antibody capture time.

Step	Dilution	Capture Time	Linear Period	Effective Range	Cycle Time (6 samples)
Antibody concentration screening	1:5 to 1:20	2 min	20 sec	0.1–20 µg/ml	20 min
Screening + kinetics	1:5 to 1:20	2 min	20 sec	0.1–20 µg/ml	45–50 min
Low concentration antibodies + kinetics	Neat or 1:5	5 min	2 min	10–300 ng/ml	50 min
Very low concentration antibodies + kinetics	Neat or 1:5	10–15 min	2 min	5–50 ng/ml	60 min

Tween is a trademark of ICI Americas Inc.

Ex-Cell is a trademark of Sigma-Aldrich.

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *800 424 6723* ***Australia*** *61 2 9914 2800* ***Austria*** *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 11 5044 5699*
Canada *905 364 3435* ***China*** *86 21 6169 8500* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00*
France *01 47 95 69 65* ***Germany*** *089 31 884 0* ***Greece*** *30 210 9532 220* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 459 6100* ***India*** *91 124 4029300*
Israel *03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *03 6361 7000* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *0318 540666*
New Zealand *64 9 415 2280* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 495 721 14 04*
Singapore *65 6415 3188* ***South Africa*** *27 861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *026 674 55 05*
Taiwan *886 2 2578 7189* ***Thailand*** *800 88 22 88* ***United Kingdom*** *020 8328 2000*

Bulletin 6411 Rev A US/EG *13-1050 0613 Sig 1212*