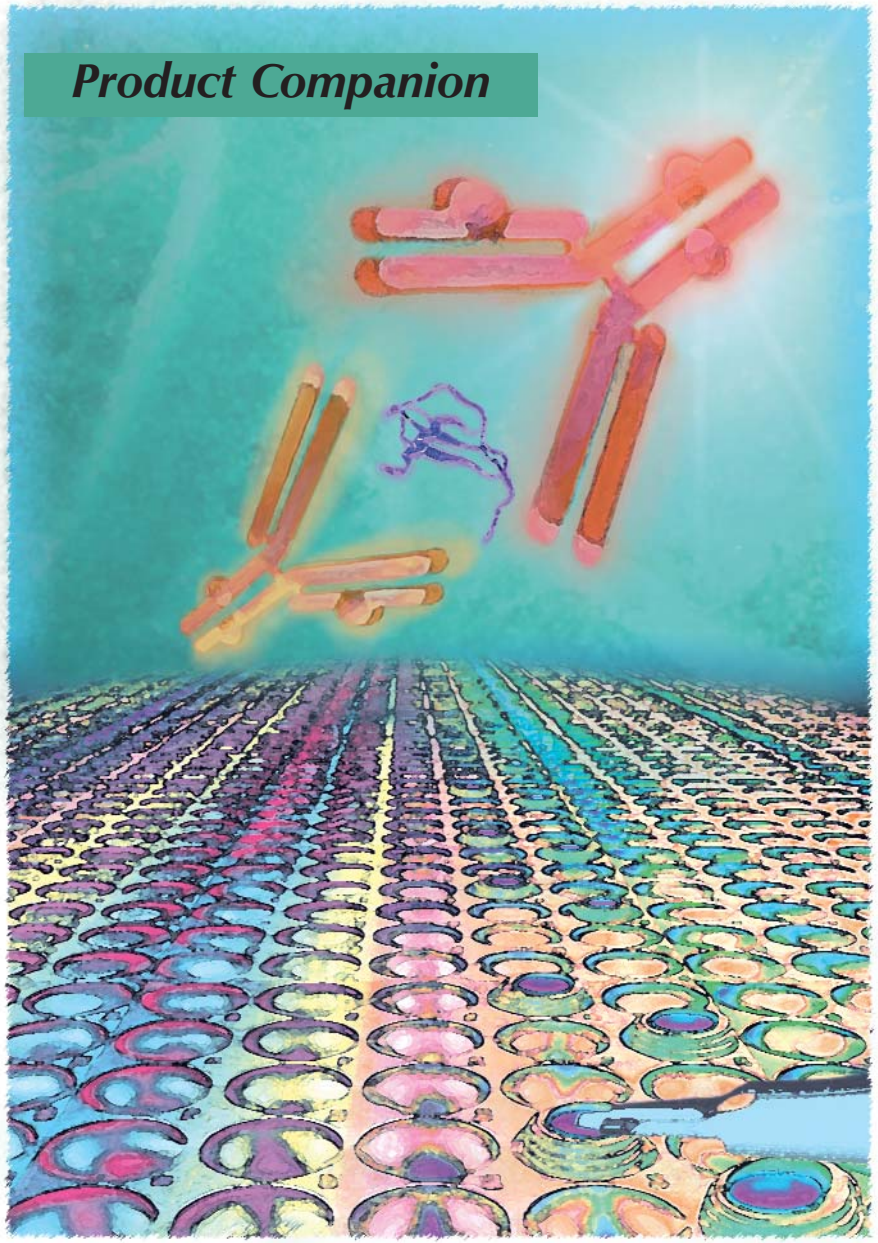
Product Companion



Antibody Pairs for ELISA

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Antibody Pairs for ELISA

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Technical/Customer Services 1-800-242-0607

Editor's Note

The goal of this manual is to provide experienced ELISA users with easy to follow written methods with which to obtain results using antibody pairs. The research and development staff of BioSource International, Inc. has documented their protocols for using paired antibodies for ELISA and devoted much thought to detailing the clues which will aid in obtaining meaningful data. When appropriate, buffer formulations and specific recipes for preparing samples are also provided. Specific data generated at BioSource International is given to illustrate typical results when following these protocols. Suggested product part numbers are also given to guide you in your selection of appropriate reagents. BioSource International's antibody pairs are designed to provide researchers experienced in ELISA development with the flexibility to create assays optimized specifically for their needs. Antibody pair kits are not recommended for those inexperienced with ELISA technique. Our ready-to-use Cytoscreen™ and EASIA™ ELISA kits are fully developed assay systems and are the best choice for researchers less experienced in ELISA development.

Introduction

The method that is described in this publication is the Enzyme-Linked-ImmunoSorbent Assay (ELISA). This method is a benchmark for the quantitation of proteins and there are indeed many variations to this method.

Briefly, there are four general types of ELISAs. (1) Direct ELISAs involve attachment of the antigen to the solid phase, followed by an enzyme-labeled antibody. This type of assay generally makes measurement of crude samples difficult since contaminating proteins compete for plastic binding sites. (2) Indirect ELISAs also involve attachment of the antigen to the solid phase, but in this case, the primary antibody is not labeled. An enzyme-conjugated second antibody, directed at the first antibody, is then added. This format is used most often for the detection of specific antibodies in sera. (3) A third type of ELISA is the Competition Assay, which involves the simultaneous addition of 'competing' antibodies or proteins. The decrease in signal in samples where the second antibody or protein is added gives a highly specific result. (4) The last type of assay is the Sandwich ELISA. Sandwich ELISAs involve attachment of a capture antibody to a solid phase support. Samples containing known or unknown antigen are then added in a matrix or buffer that will minimize attachment to the solid phase. An enzyme-labeled antibody is then added for detection. The Sandwich ELISA is the method which gives the most specific and sensitive results for cytokine antigens found in very low concentrations. For a more detailed description of ELISA theory, refer to *ELISA: Theory and Practice* by J.R. Crowther, 1995, Humana Press.

BioSource International offers two options for the development of sandwich ELISAs to measure natural and recombinant cytokines using matched antibody pairs. Each option will be discussed, methods described and optimization guidelines given. One method combines a Biotin-labeled detection antibody with our proprietary Horseradish Peroxidase- conjugated Streptavidin. The resulting signal provides data that is very sensitive and highly specific. A second method, Flexia™, offers a shorter protocol through the use of avidin-coated plates and a directly conjugated HRP antibody. This method is particularly suited for measurement of biological samples like serum and plasma. BioSource International offers a complete line of antibodies for the quantitation of human, mouse, rat and swine cytokines utilizing ELISA methodology.

As always, your feedback has contributed to the initiative for this publication and will continue to effect improvements in the materials which are supplied with our products. Please contact our Technical Service Staff with any inquiries regarding this manual. We welcome your feedback on the methods described. Please contact us with your comments.

Disclaimer

The procedures presented in this manual are accurate to the best of our knowledge. BioSource International, Inc. makes no warranties either express or implied as to any matter whatsoever, including, without limitations, the condition of the products, their merchantability, or fitness for any particular use. BioSource International, Inc., shall not be held liable for any direct, indirect, incidental, or consequential damages, including without limitation, loss of profit, loss of business, or other loss which may be based directly or indirectly upon the sale, use of products, or inadequacy of product for any purpose or by any defect or deficiency therein, even if BioSource International, Inc., knew or should have known of the possibility of such loss.

The products listed in this booklet are for research use only, and are not intended for use in diagnostic procedures.

Chapter 1: Cytosets™

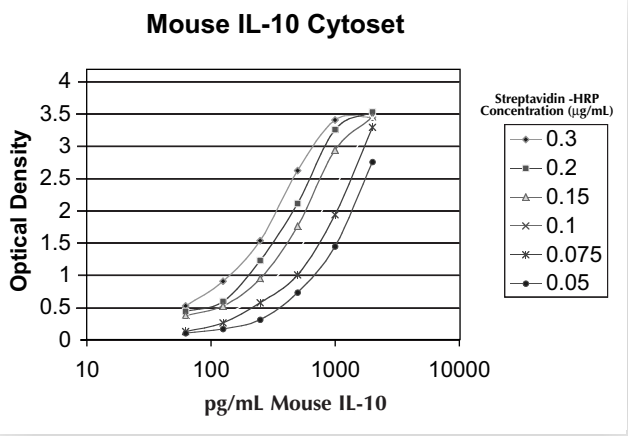
Cytosets™ are lot-matched, pretitered antibody pairs designed for measuring human, mouse, rat and swine cytokine levels in cell culture supernatants. The detection antibody is conjugated to biotin. Each Cytosets™ kit includes:

-Monoclonal or oligoclonal capture antibody, 0.5 mg, sufficient for 40 plates
-Biotin-conjugated monoclonal detecting antibody, 0.1 mg, sufficient for 40 plates
-10 vials of single-use recombinant standards
-Streptavidin-HRP conjugate and easy-to-follow titration chart utilizing actual lot-specific data, sufficient for 40 plates
-General ELISA protocol and lot-specific procedure
-Lot-specific example standard curve

The sensitivities of Cytosets™ will be generally comparable to the corresponding EASIA™ or Cytoscreen™ parameters and can be estimated from the standard curve included in the package insert.

Note: For lot-specific information, refer to the Cytosets™ Information Sheet. The lot-specific Streptavidin-HRP titration chart is as follows:

Figure 1:



A complete listing of Cytosets™ products is shown on page 9.

Cytosets™ General Procedure:

Materials and Equipment

Coating antibody
Blocking solution
Detection antibody
Standards
Diluents
Streptavidin-Horseradish Peroxidase
Chromogen (TMB)
Stop Solution
Wash Buffer
Microwells (96 per plate)
Precision pipettors
Refrigerator (4°C)
Incubator (37°C)
Microplate washing device
Plate Covers
Microplate reader
Shaker

Note: All buffers are shown in bold type, formulations are given in Table 1, page 8.

Plate Coating

1. Dilute Coating Antibody in **Coating Buffer** to the concentration recommended on the accompanying Cytosets™ information sheet. Refer to vial label for concentration of Coating Antibody. Avoid polystyrene containers when storing or diluting antibody. If the standard curve signals are not as high as desired, higher concentrations of Coating Antibody may be tried.
2. Add 100 µL of diluted Coating Antibody per well to polystyrene microplates. (e.g., Dynex Immulon 2 HB or Nunc Maxisorp). Other polystyrene plates may also be suitable.
3. Cover the plates and incubate overnight (12 to 18 hours) at 2 - 8°C. Incubating for longer periods of time such as over the weekend should not cause any problems.
4. Aspirate the Coating Antibody from the wells and tap on absorbent paper to remove excess liquid.
5. Add 400 µL per well of **Wash Buffer**, incubate for 15-30 seconds, and aspirate.
6. Add 300 µL of **Blocking Solution** to each well. Cover the plates and incubate for 1 to 2 hours at room temperature. Excessive blocking should be avoided. If backgrounds are higher than desired, try substituting either casein or calf serum in place of the BSA found in the **Blocking Solution**.
7. Aspirate the **Blocking Solution** from the wells and tap on absorbent paper to remove excess liquid. Plates can now be used. Alternatively, if the plates are not to be used immediately, allow to dry overnight at room temperature and store at 2 - 8°C in a plastic bag with desiccant for up to a week.
8. Prior to starting the assay, wash the microplate 3 to 6x with 400 µL per well of **Wash Buffer** and tap on absorbent paper to remove all excess liquid after the final wash. Do not allow wells to dry completely at any time.

ELISA Method:

1. Dilute standards and samples in **Standard Diluent/Assay Buffer** or in the assay matrix most relevant to your samples (e.g., cell culture medium containing 10% fetal calf serum, pooled serum, urine, etc.). Please note that use of differing standard diluents will dramatically change the slope, dilution limits and recovery of analyte. The guidelines presented here are cited for the **Standard Diluent/Assay Buffer** formulation here. Use of other diluent formulations will require reoptimization of assay time/temperature/concentrations.
2. Add 100 µL of standards, samples and controls to appropriate wells in duplicate. Controls may include a reagent blank (zero standard control) and a substrate blank.

Note: Some assays may require separate incubation of standards, samples and controls prior to addition of the biotinylated detection antibody (Step #4). Refer to the accompanying Cytosets™ information sheet for specific instructions.

3. Dilute biotinylated Detection Antibody in **Standard Diluent/Assay Buffer** to the concentration recommended on the accompanying Cytosets™ information sheet.
4. Add 50 or 100 µL of diluted Detection Antibody (as recommended on Cytosets™ information sheet) to each well except chromogen blank, cover the plate and incubate for the time and temperature recommended on the accompanying Cytosets™ information sheet.
5. Aspirate solution from wells.
6. Wash the microplate 3 to 6x with 400 µL per well of **Wash Buffer** and tap on absorbent paper to remove all excess liquid after the final wash.
7. Dilute Streptavidin-HRP conjugate according to the instructions. Streptavidin-HRP may be diluted in **Standard Diluent/Assay Buffer** if not otherwise specified by the package insert.
8. Add 100 µL of diluted Streptavidin-HRP per well, cover the microplate and incubate at room temperature for 15 to 45 minutes. (Note: Dilution and incubation time will vary depending on the lot).
9. Aspirate solution from wells.
10. Wash the microplate 3 to 6x with 400 µL per well of **Wash Buffer** and tap on absorbent paper to remove all excess liquid after the final wash.
11. Prepare TMB (tetramethylbenzidine) according to manufacturer's instructions.
12. Add 100 µL of TMB to each well and incubate in the dark at room temperature for 10 to 60 minutes (generally 30 minutes) according to the manufacturer's instructions. (Note: Incubation time will vary depending on manufacturer and lot).
13. Stop the reaction by addition of 50 or 100 µL of **Stop Solution** (Catalog #SS01) to each well according to manufacturer's instructions.
14. Read microplate at 450 nm within 30 minutes of adding **Stop Solution**. Prior to stopping color development, plate can be read at 650 nm to determine if desired O.D.'s have been obtained.
15. Calculate the average optical density at 450 nm for all standards, controls and samples. Construct a standard curve by plotting each standard optical density (ordinate) vs. the standard concentration (abscissa) on semi-log graph paper. For plate readers with automated standard curve calculation capability, a log-log or four parameter curve fit algorithm may provide the best curve fit.
16. Determine the concentration of each unknown sample from the standard curve.

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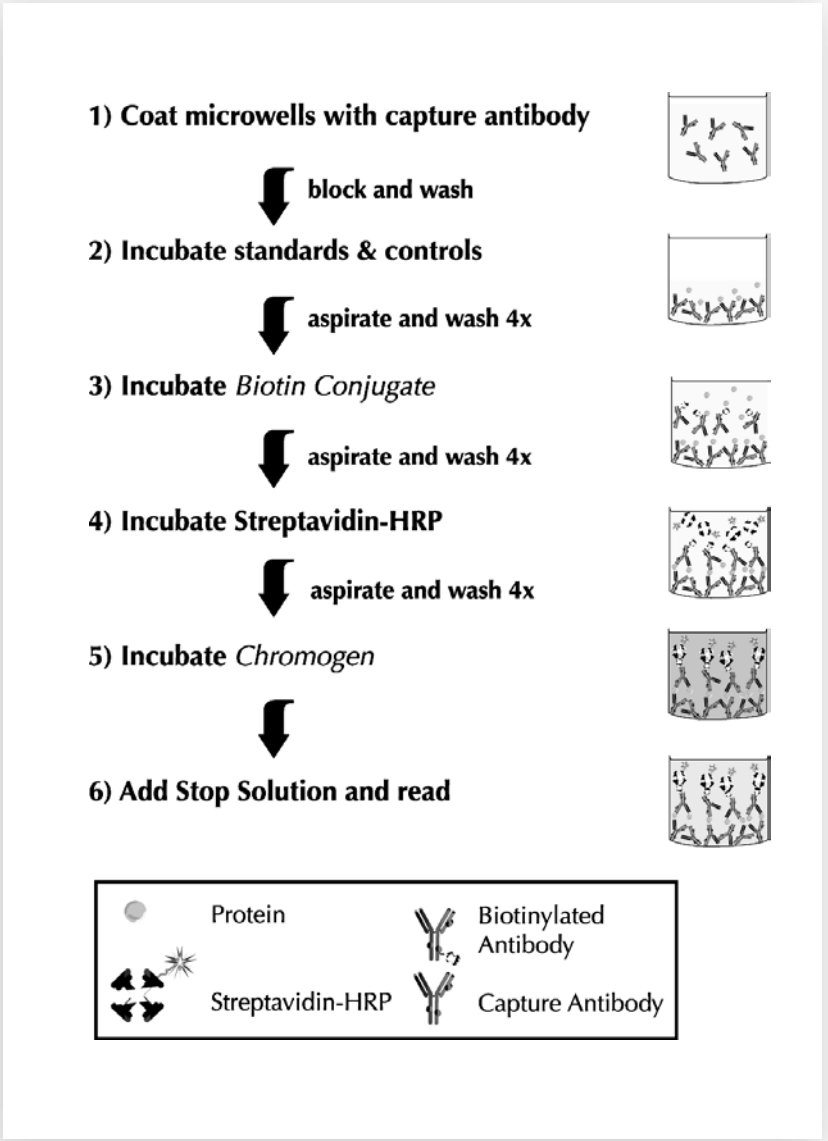

Table 1: Cytosets™ Buffers

Buffer	Formulation
Coating Buffer A	8.0 g NaCl 1.42 g $Na_2HPO_4 \cdot H_2O$ 0.2 g KH_2PO_4 0.2 g KCl q.s. to 1 liter with distilled H_2O, pH 7.4
Coating Buffer B	4.3 g $NaHCO_3$ 5.3 g Na_2CO_3 q.s. to 1 liter with distilled H_2O, pH 9.4
Blocking Solution	8.0 g NaCl 1.42 g $Na_2HPO_4 \cdot 2H_2O$ 0.2 g KH_2PO_4 0.2 g KCl 5.0 g bovine serum albumin (fraction V) q.s. to 1 liter with distilled H_2O, pH 7.4
Standard Diluent/Assay Buffer	8.0 g NaCl 1.42 g $Na_2HPO_4 \cdot 2H_2O$ 0.2 g KH_2PO_4 0.2 g KCl 5.0 g bovine serum albumin (fraction V) 1 mL Tween 20 q.s. to 1 liter with distilled H_2O, pH 7.4
Wash Buffer	9.0 g NaCl 1 mL Tween 20 q.s. to 1 liter with distilled H_2O, pH 7.4
Stop Solution	1.8 N H_2SO_4

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Human Cytosets™: All Cytosets™ contain enough reagents for 40 plates.

Product	Catalog #	Price
GM-CSF	CHC0904	$950
IFN-γ	CHC1234	$950
IL-1α	CHC1194	$950
IL-1β	CHC1214	$950
IL-1ra	CHC1184	$950
IL-2	CHC1244	$950
IL-3	CHC1274	$950
IL-4	CHC1284	$950
IL-5	CHC1254	$950
IL-6	CHC1264	$950
IL-7	CHC1334	$950
IL-8	CHC1304	$950
IL-10	CHC1324	$950
IL-12p40	CHC1564	$950
TNF-α	CHC1754	$950
TNF RI	CHC1764	$950
TNF RII	CHC1774	$950

Mouse Cytosets™: All Cytosets™ contain enough reagents for 40 plates.

Product	Catalog #	Price
IFN-γ	CMC4034	$950
IL-1β	CMC0814	$950
IL-2	CMC0024	$950
IL-4	CMC0044	$950
IL-6	CMC0064	$950
IL-10	CMC0104	$950
IL-12p40	CMC0124	$950
TNF-α	CMC3014	$950

Rat Cytosets™: All Cytosets™ contain enough reagents for 40 plates.

Product	Catalog #	Price
IL-2	CRC0024	$950
IL-4	CRC0044	$950
IL-10	CRC0104	$950

Swine Cytosets™: All Cytosets™ contain enough reagents for 40 plates.

Product	Catalog #	Price
IL-10	CSC0104	$950

Chapter 2: Flexia™

Flexia™ is the next generation of human antibody pairs, bridging the gap between ready-to-use kits and antibody pairs. Flexia™ sets are specifically designed for measuring human cytokines in serum and plasma in the clinical setting. However, Flexia™ can also be used for cell culture supernatant measurements.

- -Flexia™ offers the option of using avidin-coated plates which eliminates the plate coating step. Our avidin-coated plates are manufactured and quality controlled in our ISO 9001-certified manufacturing facility, for superior lot-to-lot consistency.
- -Flexia™ standards are calibrated to NIBSC/WHO reference preparations.
- -Flexia™ utilizes a directly conjugated detecting antibody which eliminates the SAV-HRP titration step.
- -Flexia™ utilizes $F(ab')_2$ antibodies to reduce interference by rheumatoid factors.

For serum and plasma measurements, the simple 1-step procedure utilizes avidin-coated plates. For cell culture supernatant measurements, the 2-step procedure is required using either the avidin-coated plates or the virgin polystyrene plates for coating. In either case, the streptavidin-HRP step is eliminated.

Flexia™ antibodies are $F(ab')_2$ fragments, which eliminate false positive values obtained in the presence of Rheumatoid factor (Rf) and other heterophilic antibodies often found in biological fluids such as serum and plasma. Rfs are antibodies (mostly IgM) which bind to the Fc portion of IgG antibody. They are produced in response to many types of infection, but are also found in normal individuals. Rheumatoid factor interferes with immunoassays by reacting with the Fc portion of the capture and detection antibodies used in the assay and causes a false positive. Elimination of Rheumatoid factor interference can be achieved by removing the Fc portion from the antibodies used in the assay.

Each Flexia™ set includes:

- -Lot-matched biotin conjugated capture antibody, sufficient for 10 plates
- -Lot-matched HRP conjugated detecting antibody, sufficient for 10 plates
- -3 vials of single-use standard
- -General ELISA protocol and lot-specific 1-step (serum & plasma) or 2-step (culture supernatant) procedure
- -Lot-specific example standard curve

Flexia™ sensitivity may vary slightly depending on whether the 1-step or 2-step procedure is employed. Example curves for both procedures are included on the package insert, generated from lot-specific data (see Figure 2, page 13).

A complete listing of Flexia™ products is shown on page 14.

Method:

Materials Required

Coating antibody-biotin	Stop Solution
Detection antibody-HRP	Refrigerator (4°C)
Avidin-coated plate (one step)	Incubator (37°C)
Polystyrene microtiter plate (two step)	Microplate washing device
Antibody diluent	Precision pipettor
Standards	Shaker
Chromogen (TMB)	Microplate reader
Plate covers	

Note: All buffers are shown in bold type, formulations are given in Table 2, page 13.

One Step Procedure (for serum/plasma samples)

Note: Must use avidin-coated plates

1. Dilute Biotin-labeled Coating Antibody in **Antibody Diluent Buffer** to the concentration recommended on the accompanying Flexia™ technical information sheet.
2. Add 50 µL of diluted Coating Antibody to each well.
3. Dilute the standards and samples in the assay matrix most relevant to samples (e.g., normal human or animal serum).
4. Add 100 µL of standards, samples and controls to appropriate wells in duplicate. Controls should include a reagent blank (zero standard control) and a substrate blank.
5. Dilute Detection Antibody-HRP conjugate 11-fold in **Antibody Diluent Buffer**.
6. Add 50 µL of diluted Detection Antibody-HRP per well, except chromogen blank.
7. Incubate the plate for the time and temperature recommended on the accompanying Flexia™ technical information sheet.
8. Aspirate solution from wells.
9. Wash the microplate 3 to 6x with 400 µL per well of **Wash Buffer** and tap on absorbent paper to remove all excess liquid after the final wash.
10. Add 100 µL of TMB to each well and incubate in the dark at room temperature for 30 minutes according to manufacturer's instructions. (Note: Incubation time will vary depending on manufacturer and lot).
11. Stop the reaction by addition of 100 µL of **Stop Solution** (Catalog #SS01) to each well.
12. Read the microplate at 450 nm within 30 minutes of adding **Stop Solution** (reference filter: 630 or 650 nm).
13. Calculate the average optical density at 450 nm for all standards, controls and samples. Construct a standard curve by plotting each standard optical density (ordinate) vs. the standard concentration (abscissa) on semi-log graph paper. For plate readers with automated standard curve calculation capability, a log-log or four-parameter curve fit algorithm will provide the best curve fit.
14. Determine the concentration of each unknown sample from the standard curve.

Two-Step Procedure (for cell culture samples or serum/plasma)

Plate coating

Note: Avidin-coated plates used in the one-step procedure cannot be used with culture media which contains biotin. Biotin in culture media would compete with the Biotin-labeled capture antibody and result in inaccurate data.

1. Dilute Coating Antibody in **Coating Buffer A** to the concentration recommended on the accompanying Flexia™ information sheet. Refer to vial label for concentration of coating antibody.
2. Add 100 µL of diluted Coating Antibody per well to polystyrene microplates coated with avidin. It is also possible to coat virgin polystyrene with the biotin-labeled coating antibody as well (e.g. Dynex Immulon 2 HB or Nunc Maxisorp).
3. Cover the plates and incubate overnight (12 to 18 hours) at 2 - 8°C.
4. Aspirate the Coating Antibody from the wells and tap on absorbent paper to remove excess liquid.
5. Add 200 µL of **Blocking Solution** to each well. Cover the plates and incubate for at least 2 hours at room temperature.
6. Aspirate the **Blocking Solution** from the wells and tap on absorbent paper to remove excess liquid.
7. Wash the microplate 3 to 6x with 400 µL per well of **Wash Buffer** and tap on absorbent paper to remove all excess liquid after the final wash. Proceed with the addition of standards and samples. Do not allow wells to dry completely at any time.

ELISA method

1. Dilute standards and samples in the assay matrix most relevant to your samples (e.g., normal human or animal serum for serum/plasma samples and RPMI containing 10% Fetal Calf Serum).
2. Add 100 µL of standards, samples and controls to appropriate wells in duplicate. Controls can include a reagent blank (zero standard control) and a substrate blank.
3. Dilute Detection Antibody-HRP conjugate 11- fold in **Antibody Diluent Buffer**.
4. Add 50 µL of diluted Detection Antibody-HRP per well, except chromogen blank and incubate for the time and temperature recommended on the accompanying Flexia™ technical information sheet.
5. Aspirate solution from wells.
6. Wash the microplate 3 to 6x with 400 µL per well of **Wash Buffer** and tap on absorbent paper to remove all excess liquid after the final wash.
7. Add 100 µL of TMB to each well and incubate in the dark at room temperature for 30 minutes according to manufacturer's instructions.
8. Stop the reaction by addition of 100 µL of **Stop Solution** (Catalog #SS01) to each well .
9. Read the microplate at 450 nm within 30 minutes of adding **Stop Solution** (reference filter: 630 or 650 nm).
10. Calculate the average optical density at 450 nm for all standards, controls and samples. Construct a standard curve by plotting each standard optical density (ordinate) vs. the standard concentration (abscissa) on semi-log graph paper. For plate readers with automated standard curve calculation capability, a log-log or four-parameter curve fit algorithm will provide the best curve fit.
11. Determine the concentration of each unknown sample from the standard curve.

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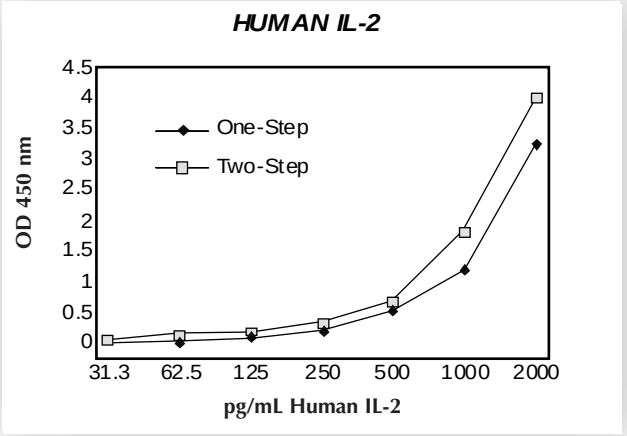
Table 2: Flexia™ Suggested Solution Formulations
Note: All solutions must be prepared prior to performing the assay.

Solution	Formulation
Coating Buffer A	8.0 g NaCl 1.42 g $Na_2HPO_4 \cdot H_2O$ 0.2 g KH_2PO_4 0.2 g KCl q.s. to 1 liter with distilled H_2O, pH 7.4
Blocking Solution	8.0 g NaCl 1.42 g $Na_2HPO_4 \cdot 2H_2O$ 0.2 g KH_2PO_4 0.2 g KCl 5.0 g bovine serum albumin (fraction V) q.s. to 1 liter with distilled H_2O, pH 7.4
Antibody Diluent Buffer	8.0 g NaCl 1.42 g $Na_2HPO_4 \cdot 2H_2O$ 0.2 g KH_2PO_4 0.2 g KCl 10.0 g bovine serum albumin (fraction V) q.s. to 1 liter with distilled H_2O, pH 7.4
Wash Buffer	9.0 g NaCl 1 mL Tween 20 q.s. to 1 liter with distilled H_2O, pH 7.4
Stop Solution	1.8 N H_2SO_4

Figure 2: Human IL-2 Flexia™
The example standard curve was generated using the procedure described in the Flexia™ protocol:



Calibration: All Flexia™ kits are calibrated to NIBSC standard reference material. See Chapter 3 for calibration information.

Human Flexia™ Kits: All Flexia™ kits contain enough reagents for 10 plates		
Product	**Catalog #**	**Price**
IFN-γ	CHC1231	$275
IL-1ra	CHC1181	$275
IL-2	CHC1241	$275
IL-6	CHC1261	$275
IL-8	CHC1301	$275
IL-10	CHC1321	$275
IL-12p40	CHC1561	$275
TNF-α	CHC1751	$275

Additional Reagents:			
Product	**Catalog #**	**Size**	**Price**
Streptavidin-HRP, lyophilized	41.000.03	0.9 µg/vial	$25
Streptavidin-HRP ELISA Grade, liquid	SNN2004	1 mg/1 mL	$121
TMB Liquid, Ready-to-use	SB01	25 mL	$50
Stop Solution	SS01	25 mL	$40
Avidin microtiter plates (96 wells)	CNN0001	10 plates	$225
	CNN0004	40 plates	$950

Chapter 3: Optimization of Cytosets™ and Flexia™ Antibody Pairs for ELISA

Preparation

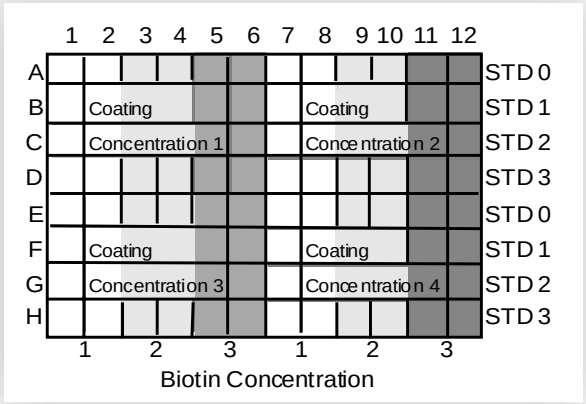
1. Read both the general procedure and the technical information sheet. Use the general procedure for guidelines to prepare all reagents. The information sheet describes the kit components in detail and the specific procedure to be followed in order to obtain the example standard curve illustrated.
2. Visually inspect all materials and check expiration date.
3. Determine the number of plates to be run. It is recommended to coat only the number of plates needed for each sample run.
4. Collect all materials required for the assay. The quality of the microtiter plates will influence the performance of the assay. Good results have been obtained with the following microplates: Nunc Maxisorp (Catalog # 468667) and Dynex Immulon 2 HB (Catalog #6506).
5. Prepare fresh buffers. For Cytosets™, use of NaN_3 in the streptavidin dilution buffer must be avoided. For Flexia™, use of NaN_3 in the antibody diluent buffer must be avoided.
6. Check pipette calibration and if an automated washing device is used, check that it is working properly. A squirt bottle will suffice for washing if automated equipment is not available.
7. Check the microplate reader for the appropriate filter. The substrate recommended requires capacity to read at a wavelength of 450 nm with a reference filter of 650 nm.

Antibodies

Certainly one of the most important characteristics of a good assay is the selection of the antibodies and a suitable antigen. Once they have been selected, the appropriate titers and volumes must be chosen. A standard curve range of 0-2 ng/mL is generally acceptable for measurement of cytokines in most sample types. Both the capture and detecting antibodies must be independently titered and working volumes chosen. A titration scheme using the standard as the indicator is given in Figure 3.

1. The capture antibody can be tested in concentrations ranging from 0.5-10 µg/mL. Serial dilutions should be prepared starting with 10 µg/mL and the coating volume should be 100-200 µL.
2. The biotinylated detection antibody should be tested in concentrations ranging from 0.05-2.0 µg/mL. The volume of detection antibody can be 50 to 100 µL.

Figure 3: Titration of Antibodies



Example of a matrix plate for antibody titration. Divide the plate in 4 parts according to 4 different coating concentrations to be tested. Each coating concentration will be tested together with 3 different detecting antibody concentrations and 4 standard points (ranging from zero = standard 0 and increasing to standard 3 = highest standard value). The SAV-HRP concentration is maintained at a fixed concentration during this experiment.

The best working conditions will satisfy the following criteria:

-standard 0:	< 0.2 O.D.
-standard 3:	1.5-2.5 O.D.
-$\frac{\text{standard 1}}{\text{standard 0}}$:	≥ 2

Incubations: Time and Temperature

Incubation times and temperatures for all steps should be empirically determined. In general, the temperature of incubation will contribute to determination of the incubation time. For example, if the standard/sample incubation is performed at 4°C, then usually an overnight incubation will be required to obtain a desirable signal. When incubations are performed at 37°C, then much shorter incubation times are necessary.

1. Coating of the microwells may be performed at room temperature or at 4°C. Optimal saturation of the plastic should occur over 4-48 hours. Note, however, that various plastics from different vendors must also be tested for optimal performance.
2. The temperature of the detection antibody incubation can also be varied from 4-37°C. The incubation time is dependent on sample recovery and desired signal for both a separate and combined assay. Generally, 30 to 120 minutes is enough time for the detection antibody incubation step.

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Signal Intensity

With the protocols given, the non-specific signal (zero value) should be less than 0.2 O.D. units. To lower the non-specific signal:

1. Decrease the incubation times or volumes of either the sample and/or the biotinylated detection antibody.
2. Improve the washing procedure. Use the wash buffer recommended in the package insert. When washing, fill the wells by delivering a forceful stream of buffer into the wells. Allow the wells to soak for 15-30 seconds. Aspirate the wash buffer completely. Repeat 2-5 times. Following the final aspiration, tap the plate forcefully on absorbent paper until all of the residual buffer is removed. Proceed immediately to the next step. Omitting Tween-20 from the wash buffer has also been observed to reduce the non-specific signal.
3. Increase the incubation time and concentration of the blocking solution. The use of casein as a blocking agent may reduce non-specific signal.
4. Optimize the Standard Diluent/Assay Buffer by decreasing or increasing the amount of animal serum or BSA. (Refer to Buffers section.)

Buffers

1. The coating buffer most commonly used is carbonate buffer (**Coating Buffer B**). Phosphate buffers have also worked well in our labs. The ionic strength (200-400 nM) and pH (3-10 pH units) of these buffers can be altered to achieve more efficient capture antibody binding.
2. Assay diluent buffers usually contain a serum protein which helps to mimic the proteins contained in sample types. In both the Cytosets™ and Flexia™ assay systems, bovine serum albumin is the protein recommended. In some cases where recovery is poor (e.g., less than 80%), increasing the concentration of BSA or addition of animal serum will increase recovery.
3. Streptavidin diluents affect not only the stability of the enzyme, but also non-specific binding and the rate of the color change. Horseradish Peroxidase, the enzyme used in Cytosets™ and used in Flexia™, is active over a range of pH, but if the pH is too low, the HRP may become unstable. Detergent, temperature and molarity of the buffers are all variables which, when altered, can affect the performance of HRP.

Sensitivity

Sensitivity, or Minimum Detectable Concentration (MDC), is confirmed by assaying a minimum of 20 replicates of the zero standard in a single assay. The concentration extrapolated from the standard curve of the average O.D.s for the standard 0 replicates + 2 SD is the MDC. This value represents the lowest value read from the standard curve that can be statistically differentiated from zero.

Assay sensitivity may be estimated from the example standard curve provided on the information sheet. If the specific procedure is closely followed, the example standard curve can be duplicated.

The desired level of sensitivity and range of the curve may affect the concentrations of reagents used. For example, if the range of the curve is too narrow, a higher concentration of streptavidin may be required. The optical limitations of the plate reader must also be considered.

Assay precision and background affect the assay sensitivity. Precision can be improved with the use of calibrated pipettes, careful technique and vigorous washing. Choose assay conditions which show the highest signal to noise ratio.

Standards

The standards provided in the Human Cytosets™ are calibrated against NIBSC/WHO standard reference preparations. For best accuracy, the use of these standards is recommended for calibrating the assay. The use of proteins other than those included with the Cytosets™ to calibrate the assay may produce different recovery values. Expressing results in NIBSC/WHO units will minimize confusion when comparing inter-laboratory data. To avoid any discrepancy, compare weight expressed standard preparations for other sources of protein by converting from mass in nanograms (ng) to activity (in Units or International Units) as follows:

Human:			
1 ng	TNF-α Cytosets™ standard =	24 IU	NIBSC 87/650
1 ng	IFN-γ Cytosets™ standard =	20 IU	NIH: Gg 23/901/530
1 ng	IL-1β Cytosets™ standard =	150 IU	NIBSC 86/553
1 ng	IL-1ra Cytosets™ standard =	1 µIU	NIBSC 92/644
1 ng	IL-2 Cytosets™ standard =	11.9 IU	NIBSC 86/504
1 ng	IL-4 Cytosets™ standard =	11.6 IU	NIBSC 88/656
1 ng	IL-6 Cytosets™ standard =	100 IU	NIBSC 89/548
1 ng	IL-8 Cytosets™ standard =	7.1 IU	NIBSC 89/520
1 ng	IL-10 Cytosets™ standard =	7 IU	NIBSC 92/516
1 ng	GM-CSF Cytosets™ standard =	7.3 IU	NIBSC 88/646
1 ng	LIF/HILDA Cytosets™ standard =	20 IU	NIBSC 91/602
1 ng	RANTES Cytosets™ standard =	10 IU	NIBSC 92/520

Mouse:			
1 ng	IL-1β Cytosets™ standard =	450 U	NIBSC 93/668
1 ng	IL-2 Cytosets™ standard =	30 U	NIBSC 93/566
1 ng	IL-3 Cytosets™ standard =	71 U	NIBSC 91/662
1 ng	IL-4 Cytosets™ standard =	3.8 U	NIBSC 91/656
1 ng	IL-6 Cytosets™ standard =	36 U	NIBSC 93/730
1 ng	TNF-α Cytosets™ standard =	180 U	NIBSC 88/532

Note: the mouse reference preparations have no official status at this time and are cited in Units rather than International Units.

Appendix-Troubleshooting Guide

Problem:	**Cause**:	**Solution**:
Poor Standard Curve	Improper preparation of standard stock solution	Dilute lyophilized standard as directed with the suggested diluent buffer.
	Improper dilutions from standard stock solution	Check your calculation for the dilution.
	Freezing/thawing of standard	Use fresh and single use standard.
	Reagents used beyond expiration date	Check the expiry date listed in the information sheet.
	Use of non-calibrated external recombinant protein preparation as standard	Calibrate against NIBSC/WHO reference preparation standard.
High Background	Wrong saturation component	Use a saturation buffer with a higher protein content (such as BSA or casein).
	Incubation time is too long	Reduce incubation time.
	Incubation temperature is too high	Reduce incubation temperature.
	Evaporation of fluid during incubation (37°C)	Cover the plates during incubation.
	Concentration of biotinylated detection antibody and/or streptavidin HRP is too high	Block titrate biotin and SAV-HRP conjugates.
	Inadequate washing after the streptavidin-HRP step	Verify function of automated plate washer.
	Contamination of substrate with metal ions or oxidizing agents	Always use distilled water.
	Substrate exposed to light	Check for unusual appearance in all components.
	Degraded streptavidin-HRP	Check for unusual appearance in all components.

Problem:	Cause:	Solution:
Weak/no color development	Use reagents beyond expiration date	Use reagents which are not expired.
	Plate allowed to dry out between incubations	Keep plate covered during all incubations; perform pipetting steps in a timely manner to avoid excess exposure.
	Incorrect storage of components	See product insert for storage recommendations.
	Reagents not at RT (18-25°C) at start of assay	Allow all reagents to warm to RT prior starting the assay.
	Wrong coating concentration (too low)	Titrate coating antibody concentration.
	Incorrect volume of reagents dispensed	Review assay steps to avoid error.
	Omission of any incubation step	Review assay steps to avoid error.
	Contamination of one of the reagents	Check for unusual appearance.
	SAV-HRP incubation step at 37°C when RT is directed	Follow instructions for SAV-HRP step incubation temperature recommended in the protocol.
	Incorrect chromogen/stop solution used	Use chromogen/stop solutions which are recommended in the protocol.
	Buffer contains azide which is not compatible with HRP	The use of azide in the assay should be avoided.
	Plate read at incorrect wavelength	The correct wavelength for TMB after acid stop is 450 nm.

BIOSOURCE

Problem:	Cause:	Solution:
Weak/no color development	Plate read after a half-hour of stopping the reaction	Read the assay within 30 minutes of adding stop.
	Wells scratched with pipette or washing tips	Restart assay using new wells.
	Improper titer of SAV-HRP	Refer to information sheet for proper titration of SAV-HRP or retiter.
	Mixing of reagents from different Cytosets™/Flexia™	Do not mix reagents from different kits.
Poor precision	Incorrect volume of reagents dispensed	Follow protocol for reagent dispensing volumes.
	Errors in pipetting the standards, samples or subsequent steps	Check pipette for calibration and leaking.
	Bottom of microplate scratched with pipette tip or washing tips	Repeat assay using new wells.
	Particulates or precipitates found in the samples prior to dispensing into the assay	Remove any particulates/ precipitates by centrifugation.
	Improper washing	Verify proper function of washing device.
	Transfer of liquid from one well to the other by shaking too vigorously when shaking required	Check for correct rotator RPM.
	Unequal evaporation of fluids	Cover the plate during incubation.
	Repetitive use of tips for several samples or different reagents	Use fresh tips for each sample or reagent transfer.

Frequently Asked Questions:

Q. How long are the antibodies stable after dilution?
A. All diluted antibody reagents should be used as soon as possible after diluting.

Q. How long are the assay buffers stable after preparation?
A. It is recommended that investigators prepare fresh dilutions.

Q. What is the limit of sensitivity that can be attained by Cytosets ™& Flexia™?
A. The sensitivity limit can be easily estimated from the standard curve presented on the information sheet. Lot performance will vary slightly. However, buffer formulations employed by the Investigator will alter the slope and the standard curve which ultimately affects sensitivity.

Q. Are components available separately?
A Yes, components may be ordered by the individual part number listed on the technical data sheet.

Q. Why is the Flexia™ 2-step procedure preferred for use with culture supernatant samples?
A. Culture media contains biotin, which will compete with the biotin on the coating antibody. Avidin-coated microplates cannot be used.

References:

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Technical Support:

Our expert Technical Support Staff is available to assist you in answering your questions and resolving issues to ensure complete customer satisfaction.
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