

For life science research only. Not for use in diagnostic procedures. FOR *IN VITRO* USE ONLY.



Mycoplasma Detection Kit

Version May 2009

Enzyme immunoassay for the detection of mycoplasmas/acholeplasmas in cell culture (*M. arginini*, *M. hyorhinis*, *A. laidlawii*, *M. orale*)

Cat. No. 11 296 744 001

For 25 tests

Store the kit at +2 to +8°C

Table of Contents

1.	What this Product Does	3
	Application	3
	Test Principle	3
	Kit Contents	4
	Sample Material	5
	Assay Time	5
	Number of Tests	5
	Kit Storage/Stability	5
	Sensitivity	5
	Advantages	5
2.	How to Use this Product	6
2.1	Before You Begin	6
	Sample Material	6
	Sample Preparation	6
	Positive Mycoplasma Control	6
	Negative Mycoplasma Control	6
	Microplates	6
	Assay Procedure	6
	Assay Capacity	7
	Pipetting	7
2.2	Preparation of Kit Working Solutions	8
	Additional Equipment and Reagents Required	8
	Preparation of Kit Working Solutions	8
2.3	Protocol	9
	Procedure	9
3.	Results	11
	Evaluation	11
	False Positive Signals	11
4.	Troubleshooting	11
5.	Additional Information on this Product	12
5.1	Background Information	12
	Preparation of Antibodies	12
	Mycoplasma	12
	Origins of Mycoplasma Contamination	12
	Detection Techniques	12
5.2	References	13
6.	Supplementary Information	14
6.1	Conventions	14
	Text Conventions	14
	Symbols	14
6.2	Changes to Previous Version	14
6.3	Ordering Information	14

1. What this Product Does

Application

The kit is used for the detection of the most common mycoplasma/acholeplasma species contaminating mammalian cell culture (*M. arginini*, *M. hyorhinis*, *A. laidlawii*, *M. orale*).

The Mycoplasma Detection Kit combines specificity and high sensitivity with short assay time and is easy to evaluate. The kit is based on the ELISA technique (14) and contains polyclonal antibodies for the detection of the most common mycoplasma/acholeplasma species contaminating mammalian cell culture (*M. arginini*, *M. hyorhinis*, *A. laidlawii*, *M. orale*).

Test Principle

Determination of each species is done separately.

- 1 Fixation of coating antibody to the microplate wells.
Blocking of unspecific binding sites.
- 2
 - Incubation of the test sample (*e.g.*, cell culture supernatant, cell suspension, media)
 - Binding of antigen (mycoplasmas or mycoplasma fragments) to the corresponding coating antibody.
- 3 Detection of antigen with biotin-labeled antibody.
- 4 Binding of streptavidin-AP conjugate to the biotin-labeled detection antibody.
- 5 Visualization by enzymatic reaction with 4-nitrophenylphosphate as substrate.

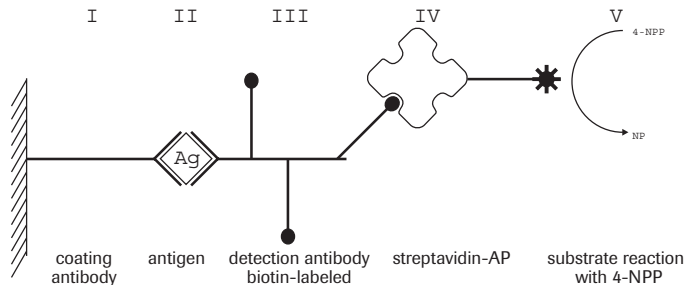


Fig. 1: Test Principle

Kit Contents

Bottle/ Cap Label		Content
1a-d (blue cap)	Coating antibody	<ul style="list-style-type: none"> • One vial each with coating antibody: <ul style="list-style-type: none"> 1a. Anti-<i>M. arginini</i> 1b. Anti-<i>M. hyorhinis</i> 1c. Anti-<i>A. laidlawii</i> 1d. Anti-<i>M. orale</i> • lyophilizate; • after reconstitution ready-to-use solution
2 (blue cap)	Blocking reagent	<ul style="list-style-type: none"> • 120 ml • ready-to-use solution Ⓢ It is possible that the solution shows a slight turbidity or precipitate, respectively. This does not influence the test. Mix the content prior to use.
3 (green cap)	Washing buffer	<ul style="list-style-type: none"> • 2× 100 ml Washing buffer (10× conc.) • after dilution ready-to-use solution Ⓢ It is possible that the solution shows a slight turbidity or precipitate, respectively. This does not influence the test. Mix the content prior to use.
4 (green cap)	Sample buffer	<ul style="list-style-type: none"> • 22 ml • ready-to-use solution Ⓢ It is possible that the solution shows a slight turbidity or precipitate, respectively. This does not influence the test. Mix the content prior to use.
5 (white cap)	Control solution	<ul style="list-style-type: none"> • One vial control solution, mycoplasma-positive; • inactivated • lyophilizate • after reconstitution ready-to-use solution
6a-d (red cap)	Detection antibody	<ul style="list-style-type: none"> • One vial each with Detection antibody, biotin-conjugate: <ul style="list-style-type: none"> 6a. Anti-<i>M. arginini</i>-biotin 6b. Anti-<i>M. hyorhinis</i>-biotin 6c. Anti-<i>A. laidlawii</i>-biotin 6d. Anti-<i>M. orale</i>-biotin • lyophilizate • after reconstitution ready-to-use solution
7 (red cap)	Streptavidin-alkaline phosphatase	<ul style="list-style-type: none"> • 85 ml • ready-to-use solution
8 (colorless cap)	Substrate buffer	<ul style="list-style-type: none"> • 100 ml • ready-to-use solution
(white cap)	Substrate tablets	One vial substrate tablets

Sample Material	<ul style="list-style-type: none"> • Cell suspensions • supernatant from adherent cells • media • sera • detergent-treated or frozen samples 										
Assay Time	The assay needs one day with an overnight incubation period.										
Number of Tests	The kit is designed for 25 tests.										
Kit Storage/ Stability	<p>The unopened kit is stable at +2 to +8°C until the expiration date printed on the label.</p> <p>The reconstituted solutions are stable at +2 to +8°C for 2 months. These solutions as well as Streptavidin-AP (vial 7) can also be stored in aliquots at -15 to -25°C for at least 12 month.</p> <p>⚠ Repeated thawing and freezing should be avoided.</p> <p>⚠ Prepare the Substrate solution always fresh!</p>										
Sensitivity	<p>According to the procedure described the assay shows detection limits as given in the following table. The sensitivity and the time of the assay procedure can be adapted to the particular requirements of the user by varying the quantities used, the incubation periods and temperatures. It is recommended to perform always double determinations as described to guarantee reliability of the results. The assay has to be done always for all four organism species.</p> <p>With the method given in this pack insert the following detection limit has been reached:</p>										
	<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="text-align: left;">Type of mycoplasma</th> <th style="text-align: left;">Detection Limit</th> </tr> </thead> <tbody> <tr> <td><i>M. arginini</i></td> <td>10⁶ – 10⁷ CFU/ml</td> </tr> <tr> <td><i>M. hyorhinis</i></td> <td>10⁶ – 10⁷ CFU/ml</td> </tr> <tr> <td><i>A. laidlawii</i></td> <td>10⁴ – 10⁵ CFU/ml</td> </tr> <tr> <td><i>M. orale</i></td> <td>10⁵ – 10⁶ CFU/ml</td> </tr> </tbody> </table>	Type of mycoplasma	Detection Limit	<i>M. arginini</i>	10 ⁶ – 10 ⁷ CFU/ml	<i>M. hyorhinis</i>	10 ⁶ – 10 ⁷ CFU/ml	<i>A. laidlawii</i>	10 ⁴ – 10 ⁵ CFU/ml	<i>M. orale</i>	10 ⁵ – 10 ⁶ CFU/ml
Type of mycoplasma	Detection Limit										
<i>M. arginini</i>	10 ⁶ – 10 ⁷ CFU/ml										
<i>M. hyorhinis</i>	10 ⁶ – 10 ⁷ CFU/ml										
<i>A. laidlawii</i>	10 ⁴ – 10 ⁵ CFU/ml										
<i>M. orale</i>	10 ⁵ – 10 ⁶ CFU/ml										
Advantages	The easy handling and evaluation (macroscopic or with a microplate reader) and the high specificity and sensitivity makes the Mycoplasma Detection Kit a convenient tool for the routine examination of cell cultures and media for mycoplasma contamination.										

2. How to Use this Product

2.1 Before You Begin

Sample Material Cell suspensions, supernatant from adherent cells, media, sera and also detergent-treated or frozen samples can be used.

The presence of antibiotics within the sample does not interfere with the assay results. However, since the number of mycoplasmas can be reduced the cells may be cultivated free of antibiotics for at least two passages to increase the sensitivity of the assay.

So far it is not necessary to spin samples with cells in suspension down before performing the assay.

To concentrate mycoplasmas a centrifugation step can be done though a number of mycoplasmas will be lysed due to the centrifugation.

The assay results will not be influenced by contamination from bacteria or yeast.

Sample Preparation

Dilute 2 ml of a sample with 0.5 ml Sample buffer (vial 4).

Positive Mycoplasma Control

Positive mycoplasma control should be tested in parallel using the Control solution, mycoplasma-positive (vial 5).

Mycoplasmas in this solution have been inactivated by W-propiolactone treatment.

Negative Mycoplasma Control

Sterile media analogous to the sample should be used.

If there is none available Washing buffer (vial 3) can be used.

Negative controls are also diluted with Sample buffer:

0.25 ml Sample buffer (vial 4) is added to 1 ml media or Washing buffer.

⊕ If a medium containing serum is used, we recommend to use sera of the same lot in the medium used as a negative control.

Microplates

It is recommended to use only commercially available microplates (96 wells each of approx. 300 μ l) with high homogeneity and high binding capacity for peptides/proteins, even in the presence of detergents (*e.g.*, "immunoplate" microplates from Nunc GmbH, Denmark).

Assay Procedure

Determination of each species has to be performed separately, *i.e.*, coating antibody and detection antibody have to correspond. For each sample double determinations should be done for each species.

Neither coating antibodies nor detection antibodies should be mixed. Avoid cross-contamination of the wells!

Assay Capacity

To perform a successful assay it is not necessary that the mycoplasmas/acholplasmas are present as viable organisms. Therefore the handling of the samples, *e.g.*, sample collection, storage, transport or the use of antibiotics in routine biochemical work is not critical.

The Mycoplasma Detection Kit has been developed for routine testing of cell cultures and media for mycoplasma contamination. Since mycoplasmas can show a great variety in subspecies the assay could not have been tested for all variants. Therefore detection of all variants of the four species to be tested in the assay can not be guaranteed. It has been shown that 94% of all tested contaminated routine cell cultures were detected with the Mycoplasma Detection Kit is, based on the ELISA principle.

These results are based on a blind study with about 200 examined cell cultures of which more than 30% were found to be mycoplasma-positive.

In this study the Mycoplasma Detection Kit has been compared with the DAPI method and the culture method. Correspondence between enzyme immunoassay and culture method is approx. 80%, but especially some *M. hyorhinis*-contaminated cell cultures were not detected with the culture method.

Correspondence between enzyme immunoassay and the DAPI method is approx. 75%, but with the DAPI method the detection of contaminated cell cultures was significantly lower than with the enzyme immunoassay.

Pipetting

In case of varying the described pipetting procedure take care that the volume of the detection antibody and the conjugate solution applied is not larger than the volume of the Blocking solution in order to avoid contact with uncoated surfaces.

2.2 Preparation of Kit Working Solutions

Additional Equipment and Reagents Required

Sodium azide

Preparation of Kit Working Solutions

The following table shows the preparation of kit working solutions.

Solution	Composition/ preparation	Storage and Stability	Use
Coating antibody solution	Reconstitute coating antibody (vials 1a-d) with 30 ml double dist. water at +15 to +25°C for 20 min and mix.	At +2 to +8°C for 2 month or in aliquots at –15 to –25°C for at least 12 months	Protocol step 1
Control solution, mycoplasma-positive	Reconstitute Control solution (vial 5) with 25 ml Washing buffer solution at +15 to +25°C for 20 min and mix.	At +2 to +8°C for 2 month or in aliquots at –15 to –25°C for at least 12 months	Protocol step 4
Detection antibody solution	Reconstitute Detection antibody (vials 6a - d) with 25 ml Washing buffer solution at +15 to +25°C for 20 min and mix.	At +2 to +8°C for 2 month or in aliquots at –15 to –25°C for at least 12 months	Protocol step 6
Washing buffer	To reconstitute one bottle, fill 100 ml 10× concentrate in an autoclaved 1 liter bottle and add 900 ml double distilled water. Add 1 g/l sodium azide to avoid microbial contamination, if not used instantly. Stirr with an autoclaved magnetic stirrer.	Recommended to consume soon, reconstitute only as much as needed, take routine measures to avoid microbial contamination.	Protocol step 3, step 5, step 7, step 9
Substrate solution	Dissolve one substrate tablet (vial 9) in 4 ml Substrate buffer (vial 8) (for one determination).	Always prepare fresh!	Protocol step 10

2.3 Protocol

- Before performing the assay all reagents should have reached +15 to +25°C. The assay should be performed at the temperatures indicated below.
- For checking one cell culture sample 16 wells are required:
- 2 estimations for each of four mycoplasma species = 8 wells
- 4 negative controls
- 4 positive controls

Procedure

Please refer to the following table.

1 Coating with coating antibody

- Pipette 0.25 ml of **Coating antibody** (vials 1a–d) each into 4 wells of a microplate (see pipetting scheme).

x	x	x	x	anti- <i>M. arginini</i> coating antibody (x) (vial 1a)
#	#	#	#	anti- <i>M. hyorhinis</i> coating antibody (#) (vial 1b)
=	=	=	=	anti- <i>A. laidlawii</i> coating antibody (=) (vial 1c)
⊗	⊗	⊗	⊗	anti- <i>M. orale</i> coating antibody (⊗) (vial 1d)

- Cover microplate tightly and incubate 2 h at 37°C.

2 Blocking

- Remove the solutions from the wells by suction and tapping on a clean and dry cloth (e.g., cellulose)
- Pipette 0.25 ml **Blocking solution** (vial 2) into all antibody-coated wells.
- Incubate 30 min at 37°C.

3 Washing

- Remove the solutions from the wells by suction and then by tapping on a clean and dry cloth.
- Wash wells 3 × with Washing buffer solution and finally remove Washing buffer thoroughly.

4 Application of sample and controls

- Pipette 0.2 ml of the prepared sample into each of the **sample** wells (s), pipette 0.2 ml of the **negative control** (medium with Sample buffer, vial 4) and positive control – **Control solution** into each of the control wells (+/-) (see pipetting scheme);

S	S	n.c.	p.c.	S : = sample
S	S	n.c.	p.c.	n.c.: = negative control
S	S	n.c.	p.c.	p.c.: = positive control
S	S	n.c.	p.c.	

- Cover microplate tightly and incubate overnight at +2 to +8°C.

5 Washing

As described under step 3.

6 Application of detection antibody

- Pipette 0.2 ml **Detection antibody-solution** into the 4 corresponding wells of each antibody (see pipetting scheme).

x	x	x	x	anti- <i>M. arginini</i> detection antibody (x) (vial 6a)
#	#	#	#	anti- <i>M. hyorhinae</i> detection antibody (#) (vial 6b)
=	=	=	=	anti- <i>A. laidlawii</i> detection antibody (=) (vial 6c)
⊗	⊗	⊗	⊗	anti- <i>M. orale</i> detection antibody (⊗) (vial 6d)

- Cover microplate tightly and incubate 2 h at 37°C.

7 Washing

- Remove the solutions from the wells by suction and then by tapping on a clean and dry cloth.
- Wash wells 4× with **Washing buffer** solution and finally remove Washing buffer thoroughly.

8 Application of Streptavidin-AP

- Pipette 0.2 ml **Streptavidin-AP-solution** (vial 7) into each of the 16 wells (see pipetting scheme).

Δ	Δ	Δ	Δ	Δ = Streptavidin-AP-solution
Δ	Δ	Δ	Δ	
Δ	Δ	Δ	Δ	
Δ	Δ	Δ	Δ	

- Cover microplate tightly and incubate 1 h at 37°C.

9 Washing

- Remove the solutions from the wells by suction and tapping on a clean and dry cloth.
- Wash wells 3 × with **Washing buffer solution** and finally remove Washing buffer thoroughly.

10 Substrate reaction

- Pipette 0.2 ml **Substrate solution** (freshly prepared) into each of the 16 wells (see pipetting scheme).

□	□	□	□	□ = Substrate solution (freshly prepared)
□	□	□	□	
□	□	□	□	
□	□	□	□	

- Incubate 30 min at 18 – 25°C.

11 Evaluation

Evaluate visually or measure samples against Substrate solution in a microplate reader at 405 nm.

3. Results

Evaluation

Evaluation is carried out visually ("positive" = yellow, "negative" = practically colorless) or with the aid of a microplate reader at a wavelength of $\lambda = 405$ nm against substrate solution. The cut off limit "mycoplasma-positive, -negative" must be determined by the user. Following exactly the protocol, no color development can be observed in the absence of mycoplasmas in the sample. In the presence of mycoplasmas above the sensitivity limit an $OD > 0.2$ is measured. Wells containing only medium and sample buffer are used as a negative control.

False Positive Signals

Using media containing serum from swine increase the probability that the assay will show false positive signals. In this case the determination of mycoplasma-contamination cannot be performed.

In extremely rare cases the presence of an unspecific cross reactivity was observed in horse sera, causing false positive results. In that case the test should be redone after culturing the cells in sera of a different serum lot. Usually horse sera do not produce an elevated background level in the assay.

4. Troubleshooting

Please refer to the following table.

Problem	Possible Cause	Recommendation
Some of the negative control values are too high	Negative control solution has been contaminated with positive control.	Repeat assay without positive control.
All values of the negative controls and samples are similar high	The blocking solution has not been pipetted. Possible interference of the medium.	<ul style="list-style-type: none"> • Repeat the assay as described. • Repeat the assay using a different medium. (eventually use a different serum lot).
No or low signal in the positive control	Incorrect pipetting.	Repeat the assay as described.
	The reagents have not reached the correct temperature.	Make sure that all reagents have reached +15 to 25°C before performing the assay.

5. Additional Information on this Product

5.1 Background Information

Preparation of Antibodies

For the preparation of the polyclonal antibodies contained in the kit mycoplasma/acholeplasmas were cultivated and were purified by repeated centrifugation and washing before immunization procedures. After immunization IgG was purified from serum by precipitation and anionexchange-chromatography followed by an affinity-chromatography step to remove crossreacting antibodies. Detection antibodies were conjugated with D-biotinoyl- ϵ -aminocaproic acid-N-hydroxysuccinimide ester*.

Mycoplasma

Mycoplasmas are common and serious contaminants of cell cultures and this remains one of the major problems encountered in biological research using cultured cells. It has been shown that cell cultures are frequently contaminated up to 30% (1–4) with mycoplasmas, the main contaminants being the species *M. orale*, *A. laidlawii*, *M. arginini* and *M. hyorhinis*. These organisms produce a variety of effects on the infected cells in culture (*e.g.*, changes in metabolism, growth, viability, morphology etc.) leading to experiments in cell culture with results not always reliable (1–5).

Origins of Mycoplasma Contamination

The origins of mycoplasmal infection of cell cultures are often bovine serum (*M. arginini*, *M. hyorhinis*, *A. laidlawii*), laboratory personnel (*M. orale*) and mycoplasma-infected cultures. Mycoplasmal infection cannot be detected with the naked eye other than by signs of deterioration in the culture. It is important to keep in mind that mycoplasmas do not always reveal their presence with macroscopic alterations of the cells or media. Many mycoplasma contaminants, particularly in continuous cell lines grow slowly and do not destroy host cells but are even though able to affect various parameters as mentioned above. Therefore there is an absolute requirement for routine, periodic assays for possible covert contamination of all cell cultures, particularly continuous or established cell lines.

Detection Techniques

A variety of techniques have been developed for the detection of cell culture mycoplasmas, *e.g.*, DNA staining with dyes like 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI)* or bisbenzimidazole (H 33258), biochemical detection methods, *e.g.*, with 6-methyl-purine deoxyriboside (6-MPDR), mycoplasma culture method, electron microscopy (2, 3, 6–13). However, each method shows certain disadvantages. Detection via DNA staining requires a fluorescence microscope and much experience in evaluating the results. The 6-MPDR assay is dependent on a indicator cell line and time-consuming, which is also true for electron microscopy. The culture method being also time-consuming, does not detect all relevant mycoplasma species, *e.g.*, *M. hyorhinis*, which is not easy to cultivate in vitro.

5.2 References

- Barile, M. F., Hopps, H. E. & Grabowski, M. W. (1978) In: *Mycoplasma Infection of Cell Cultures* (McGarrity, G. J., Murphy, D. G. & Nichols, W. W., eds.) Plenum Press, New York, pp. 35-45.
- McGarrity, G. J. (1982) In: *Advances in Cell Culture*, Vol. 2 (Maramorosch, K., ed.) Academic Press, New York, pp. 99-131.
- McGarrity, G. J. & Kotani, H. (1985) In: *The Mycoplasmas*, Vol. IV (Razin, S. & Barile, M. F., eds.) Academic Press, New York, pp. 353-390.
- McGarrity, G. J., Vanaman, V. & Sarama, J. (1984) *In Vitro* **20**, 1-18.
- Bölske, G. (1988) *Zbl. Bakt. Hyg. A* **269**, 331-340.
- Russel, W. C., Newman, C. & Williamson, D. H. (1975) *Nature* **253**, 461-462.
- Chen, T. R. (1977) *Exp. Cell Res.* **104**, 255-262.
- Hessling, J. J., Miller, S. E. & Levy, N. L. (1980) *J. Immunol. Methods* **38**, 315-324.
- McGarrity, G. J. & Carson, D. A. (1982) *Exp. Cell Res.* **139**, 199-205.
- Hatanaka, M., DelGuidice, R. & Loug, C. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1401-1405.
- Whitaker, A. M. et al. (1987) *Develop. Biol. Stand.* **66**, 503-509.
- Ishiguro, K. et al. (1988) *J. Immunol. Methods* **108**, 39-43.
- McGarrity, G. J., Kotani, H. & Carson, D. A. (1986) *In Vitro* **22**, 301-304.
- Poutiers, F. et al. (1986) In: *Methods of Enzymatic Analysis*, Vol. IX (Bergmeyer, H. U., ed.) VCH Verlagsgesellschaft mbH, Weinheim, pp. 198-212.

6. Supplementary Information



6.1 Conventions

Text Conventions To make information consistent and memorable, the following text conventions are used in this Instruction Manual:

Text Convention	Usage
Numbered instructions labeled ① , ② etc.	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Applied Science.

Symbols

In this Instruction Manual, the following symbols are used to highlight important information:

Symbol	Description
	Information Note: Additional information about the current topic or procedure.
	Important Note: Information critical to the success of the procedure or use of the product.

6.2 Changes to Previous Version

- New Layout
- Editorial changes
- Information Note added for bottle 3

6.3 Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our homepage:
<http://www.roche-applied-science.com>.

Product	Pack Size	Cat. No
BM-Cyclin Antibiotic combination for the elimination of mycoplasma from cell cultures	275 mg	10 799 050 001
4',6-Diamidino-2- phenylindole dihydrochloride (DAPI)	10 mg	10 236 276 001
Uracil-DNA glycosylase (UNG)	25 U (25 µl) 100 U (100 µl)	11 269 062 001 11 444 646 001
Mycoplasma PCR ELISA	1 kit (96 reactions)	11 663 925 910

Contact and Support

If you have questions or experience problems with this or any Roche Applied Science (RAS) product, please contact our Technical Support staff. Our scientists commit themselves to providing rapid and effective help.

We also want you to contact us if you have suggestions for enhancing RAS product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to RAS and the worldwide research community.

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site at:**

www.roche-applied-science.com/support

To call, write, fax, or email us, visit the Roche Applied Science home page, www.roche-applied-science.com, and select your home country.

Country-specific contact information will be displayed.

On the Roche Applied Science home page select **Printed Materials** to find:

- in-depth Technical Manuals
- Lab FAQs: Protocols and references for life science research
- our quarterly Biochemica Newsletter
- Material Safety Data Sheets
- Pack Inserts and Product Instructions

or to request hard copies of printed materials.

