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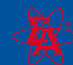
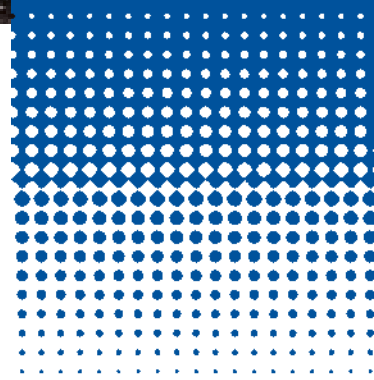
Advances in Analytical Chemistry





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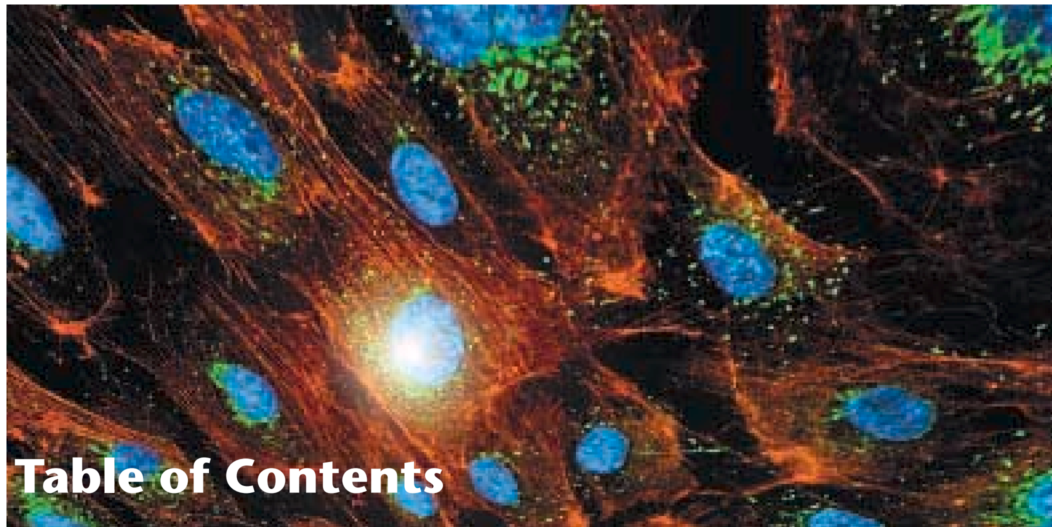

Table of Contents

Protein Analysis

High Resolution Ampholytes for IEF 1
- IEF for Analytical Purposes
- High Resolution Ampholytes
- IEF in 2D-Electrophoresis

Top-Block™ – a Superior Blocking Agent 2

Mass Spectrometry in Bioanalysis 3
- MALDI Matrices – Properties and Requirements
- Proteomics and other Applications
- PSD-MALDI and Protein Sequencing
- Quality of Matrices
- New LC-MS Grade Solvents

Highest Quality Reagents for Edman Sequencing 4

Crystallography Screening Kits 6
- Basic and Extension Kits
- Cryo Kit
- Low Ionic Kit
- Membrane Protein Kit

Cellulose Acetate Electrophoresis 8
- Dry Cellulose Acetate Strips
- Humid Cellulose Acetate Strips
- Non-toxic Reagent System

CBind™ L-Matrices for Antibody Purification 9
- Applications
- Benefits

Stabilization of Biomolecules 10
- Biostab Products

Fluorescent Probes

Atto Fluorescent Labels – New Functionalization 10
- Atto Labels
- Maleimides
- Biotin- and Streptavidin Conjugates

New Cy3 and Cy5 Alternatives 12

Fluorescent DNA/RNA Stains 13

Additional Long Wavelength Fluorescent Labels 14

Long Lifetime Fluorophores 15

Fluorescence based Kits 16
- Fluorescent Protein Assay
- Fluorescent Cell counting Assay
- Nitrite/Nitrate Assay Kit
- Albumin Fluorescence Assay Kit

Micro- and Nanoparticles

Your Choice – the most promising Polymers 17
- Monodispersity and Size Standards
- Modified and labeled Particles
- Fluorescent Nanoparticles
- Base Materials
- Customized Particles

Your Sigma-Aldrich Service Partners 19

High Resolution Ampholytes for IEF

Isoelectric focusing (IEF) is an electrophoretic separation based on the isoelectric points of proteins. It is used as an alternative electrophoresis format complementing the widely used SDS-PAGE electrophoresis, which is based on the size of proteins. Specific separation problems, such as the differentiation of protein isoforms or enantiomeres, have been successfully resolved by isoelectric focusing [1]. That makes it a very useful technique, especially for purposes like food analysis, species determination, or seed testing. Compared to alternative methods (e.g., PCR based), IEF is:

- efficient
- expressive
- economic
- fast and easy

IEF for Analytical Purposes

Isoelectric focusing as a stand-alone analytical or micropreparative technique requires ampholytes, complex mixtures of bifunctional amphoteric (both acidic and basic) buffer molecules which form a pH gradient in the medium during electrophoresis. Within that gradient, a protein will migrate towards the anode or cathode until it arrives at the point equal to its pI value. At this point it will have a net charge of zero and is said to be focused. Carrier ampholytes have to be added to the separation medium (polyacrylamide, agarose, or sephadex). Carrier ampholytes supplied as 40% solutions are typically diluted to a final concentration of 2% in the IEF matrix.
Our broad range of well-established carrier ampholytes provides you the convenience of various formulations covering the range from pH 2.5 to 10.5 in fractions of different width. Fluka's extensive experience in the production of carrier ampholytes provides reliable high quality in these well-established ampholytes (table 1).

High Resolution Ampholytes

A new series of carrier ampholytes (table 2) is based on a specific method to produce mixtures of numerous polyamino-polycarboxylic acids. These new carrier ampholytes have been developed for use in isoelectric focusing in quality control applications, especially in food and seed analysis.

Figure 1:
IEF of maize seeds. From left to right side: female, male, and F1 generation seed.

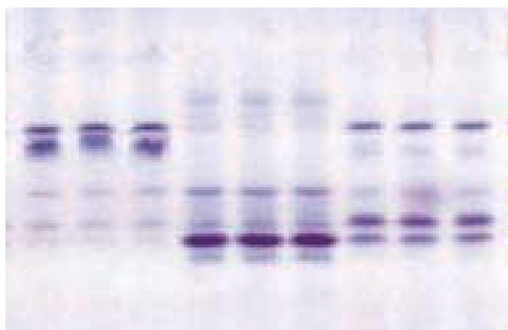

Figure 2:
2-D Electrophoresis of E. coli proteome. First dimension was performed on pH 3–10 gradient strip. Staining was performed using Ruthenium II tris (bathophenanthroline disulfonate). Courtesy of Andreas Lamanda, University Bern

Advantages:

- High buffer capacity: Compared to established ampholytes they offer buffering capacities 1.5-2.5 times higher (up to pH 8, equal above pH 8)
- Good even conductivity
- Linear and reproducible pH gradient
- High resolution

IEF in 2D-Electrophoresis

Isoelectric focusing has gained importance as the first dimension of 2D-electrophoresis. For these purposes, the first dimension is typically applied on strips carrying immobilized pH gradients. These are prepared prior to electrophoresis by pouring the pH gradient with the acrylamide-derivatized buffer that becomes polymerized in the gel. These strips may be purchased in ready-to-use format (please ask for our electrophoresis

Table 1

Broad and narrow range ampholytes of Fluka

Cat. No.	Product
17102	Ampholyte pH 2-11
10036	Ampholyte pH 3-4.5
10037	Ampholyte pH 3.5-4.5
11923	Ampholyte pH 3-6
10043	Ampholyte pH 3-10
17099	Ampholyte pH 4-5
10038	Ampholyte pH 4-6
04638	Ampholyte pH 4-7
17101	Ampholyte pH 4-9
10048	Ampholyte pH 5-7
17103	Ampholyte pH 6.6-7.6
10051	Ampholyte pH 6-8
10053	Ampholyte pH 8-9.5
10054	Ampholyte pH 9-10.5
10052	Ampholyte pH 7-9

Table 2

New high resolution ampholytes of Fluka

Cat. No.	Product
41965	Ampholyte high resolution pH 2-4
79858	Ampholyte high resolution pH 3-5
51304	Ampholyte high resolution pH 3-6
39878	Ampholyte high resolution pH 3-10
68653	Ampholyte high resolution pH 4-6
15752	Ampholyte high resolution pH 5-7
89096	Ampholyte high resolution pH 5-8
89191	Ampholyte high resolution pH 6-8
95632	Ampholyte high resolution pH 7-9
08689	Ampholyte high resolution pH 8-10

Fluka Acrylamido buffers for preparation of IEF strips

Cat. No.	Product
01712	Acrylamido buffer pK 1, 0.2 M in water
01714	Acrylamido buffer pK 3.1, 0.2 M in water
01713	Acrylamido buffer pK 3.1
01716	Acrylamido buffer pK 3.6, 0.2 M in water
01715	Acrylamido buffer pK 3.6
01718	Acrylamido buffer pK 4.6, 0.2 M in water
01717	Acrylamido buffer pK 4.6
01721	Acrylamido buffer pK 6.2, 0.2 M in n-propanol
01719	Acrylamido buffer pK 6.2
01723	Acrylamido buffer pK 6.6, 0.2 M in n-propanol
01722	Acrylamido buffer pK 6.6
01726	Acrylamido buffer pK 6.85, 0.2 M in n-propanol
01724	Acrylamido buffer pK 6.85
01729	Acrylamido buffer pK 7.0, 0.2 M in n-propanol
01727	Acrylamido buffer pK 7.0
01732	Acrylamido buffer pK 7.4, 0.2 M in n-propanol
01731	Acrylamido buffer pK 7.4
01736	Acrylamido buffer pK 8.5, 0.2 M in n-propanol
01735	Acrylamido buffer pK 8.5
01738	Acrylamido buffer pK 9.3, 0.2 M in n-propanol
01741	Acrylamido buffer pK 10.3, 0.2 M in n-propanol
01739	Acrylamido buffer pK 10.3
01743	Acrylamido buffer pK >12, 0.2 M in water

Table 3

brochure listing a range of those products) or set up individually using Fluka's well-established Acrylamido buffers (table 3). Recipes for typical gradients are available from our technical service department. A special software is also available (Fluka Cat. No. 79303, as simple DOS version). Ampholytes play another role - for rehydration of dry strips before use. Rehydration solutions typically consist of 0.2% Ampholyte, 8 M urea, 0.5% CHAPS, and 15 mM DTT.

Reviews

[1] P. Glukhovskiy, G. Vigh, Analytical- and Preparative-Scale Isoelectric Focusing Separation of Enantiomers, Anal. Chem., 71 (17), **1999**, 3814 –3820.

[2] Corthals, G.L. et. al., in Rabilloud, T. (ed.): Proteome Research: Two-Dimensional Electrophoresis and Identification Methods, Springer, Berlin Heidelberg New York, **2000**, 197-231.

[3] Link, A. (Ed.), Methods in Molecular Biology, 112: 2-D Proteome Analysis Protocols, Humana Press, Totowa, NJ, USA 1999.

Top-Block™ – a Superior Blocking Agent

Unspecific adsorption is a major problem in many bioanalytical techniques, like enzyme linked immunoassays (ELISA), western blotting, and biochips (carrying e.g. DNA or antibodies). It can also affect the preparation of purified proteins such as antibodies. Especially if small quantities have to be purified from related proteins, such as labeled protein from unlabeled protein. If chromatography is used for this purpose, unspecific adsorption decreases the yield significantly.
For blocking unspecific adsorption, BSA (Bovine serum albumin) in various preparations and modifications (especially acetylated BSA) is the most frequently used tool. We are now introducing Top-Block, a new product which can be used as an alternative to BSA with several significant advantages. Top-Block was developed by Swiss researchers and is distributed by Fluka worldwide.
The superior results achieved with Top-Block are based on:

- Better blocking capacity than BSA
- Virtually no background in Western Blotting
- Increased signal-to-background ratio in ELISA
- Perfect solubility (50% (w/v) in water is guaranteed)
- No precipitation at 4°C in aqueous solution

Top-Block is available in reliable high quality, free of fat and carbohydrates. Although it was just recently introduced, Top-Block has already proven to be an excellent alternative to BSA with real advantages in some applications. It can be used as a direct replacement in many procedures established for BSA. It has already been used in a wide range of applications including western blots, ELISA, coating of chromatographic matrices and others [1–4]. It has been extensively used in western blot applications where visualization was based on chemiluminescence. Top-Block is suitable for western blot applications with specific phosphorylation antibodies, as well as antibodies against lipo- and glycoproteins. In such applications it could also replace acetylated BSA. Protocols for standard western blots, or specifically for western blots with phosphotyrosine antibodies, are available on request.
Please ask for a sample to convince yourself (Fluka, Cat. No. 92818, 10 g). Top-Block is available in pack sizes of 100 g, 500 g, 1 kg (Fluka, Cat. No. 37766). For local prices please look in our catalog or on our web site. Top-Block saves more than 50% of your costs compared to BSA.

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[1] Fritsche, J., et. al., *Mol. Cell. Neuroscience* (MCN), 14(4), **1999**, 398-418.

[2] used in Chen, M.S., et. al., *Nature,* 403, **2000**, 434-438.

[3] Schaer, M., et. al., Cerebral Vasculature is the major target of oxidative protein alterations in bacterial meningitis, Journal of Neuropathology and exp. *Neurology,* 61, no. 7, **2002**, 605-613.

[4] Girault, I. et. al., Immunodetection of 3-Nitrotyrosine in the liver of..., *Free radical biology and medicine,* 31, no 11, **2001**, 1375-1387.

Mass Spectrometry in Bioanalysis

Several different methods of mass spectrometry have been successfully established for analysis of biomolecules. MALDI became the most important for those applications. This is because MALDI (matrix-assisted laser desorption/ionization), a laser based soft ionization method, has proven to be one of the most successful ionization methods for mass spectrometric investigation of large molecules. Developed in the late 1980s [1, 2], its distinguishing feature is that the sample is embedded in a chemical matrix (ca. 1000 x molar excess) that greatly facilitates the production of intact gas-phase ions from large, non-volatile, and thermally labile compounds such as proteins, oligonucleotides, synthetic polymers and large inorganic compounds. A laser beam (UV- or IR pulsed laser) serves as the desorption and ionization source. The matrix plays a key role in this technique by absorbing the laser light energy and causing a small part of the target substrate to vaporize. Once the sample molecules are vaporized and ionized they are transferred electrostatically into a mass spectrometer where they are separated from the matrix ions and individually detected, usually by TOF (time-of-flight) mass spectrometry. Although there have been numerous approaches to replace chemical solid matrices by direct MS from solid supports like silicon surfaces or directly from 2-D-gels, still the typical method is to use a matrix substance. A typical matrix substance is an aromatic acid with a chromophore that strongly absorbs the laser wavelength. Figure 1 shows the homogeneity of a protein labeling reaction (1.4-fold molar excess; matrix: sinapinic acid) being easily monitored by MALDI-MS. The label ratio is indicated over the peaks of the different conjugates [3].

MALDI Matrices: Properties and Requirements

The MALDI matrix must meet a number of requirements simultaneously:

- be able to embed and isolate analytes (e.g. by co-crystallization)
- be soluble in solvents compatible with analyte
- be vacuum stable
- absorb the laser wavelength
- cause co-desorption of the analyte upon laser irradiation
- promote analyte ionization

Proteomics and other Applications

The most important applications of MALDI mass spectrometry are (in decreasing order of importance): peptides and proteins, synthetic polymers, oligonucleotides, oligosaccharides, lipids, inorganics. One of the applications which gained crucial importance recently is the analysis of tryptic digests from proteins. Those digests result in typical peptide size patterns (so called "footprints") which can be used for determination of the investigated protein by comparison with large protein data bases. That is a fast method which ideally fits the needs of high throughput proteomics. Although electrospray ionization (ESI) is somewhat competitive and certainly complementary, MALDI remains the method of choice in several key areas, particularly proteomics. ESI mass spectra include many peaks of multiply charged ions which can complicate the interpretation of the spectra of complex samples. Also, the ion current is distributed over a range of m/z values, sometimes compromising sensitivity. The sensitivity of ESI is also severely reduced by the presence of salts, impurities, and organic buffers which are more easily tolerated by MALDI.
Numerous MALDI matrices have been found for above mentioned classes of compounds; a summary is given in table 1.

Guide for selection of matrix substances

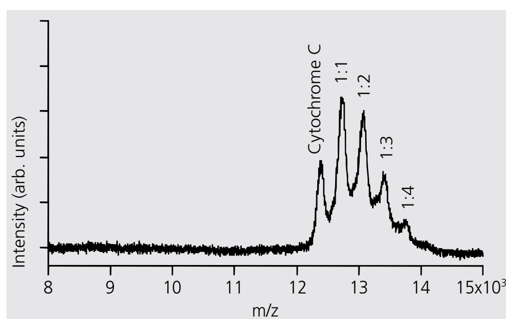
Preconditions	Abbrev.	Product	Fluka Cat. No.
Peptide/Protein			
Mass < 10 kDa	CHCA	α-Cyano-4-hydroxy-cinnamic acid	70990
Mass > 10 kDa	SA	Sinapinic acid	85429, 78867
	HABA	2-(4-Hydroxyphenylazo) benzoic acid	54793
IR-Laser		Succinic acid	14078, 69612
UV-Laser		2,6-Dihydroxyaceto-phenone	37468, 05757
UV-Laser		Ferulic acid	46278, 18077
UV-Laser		Caffeic acid	60018, 06773
Liquid matrix		Glyerol	49771
Liquid matrix		4-Nitroaniline	72681, 39497
Oligonucleotide			
Mass < 3.5 kDa	THAP	2,4,6-Trihydroxyacetophenone	91928, 47144
Mass > 3.5 kDa	HPA	3-Hydroxypicolinic acid	56197
		Anthranilic acid	10678
		Nicotinic acid	72311, 38372
		Salicylamide	84228, 06789
Carbohydrates			
	DHB	2,5-Dihydroxybenzoic acid	85707, 05757
	CHCA	α-Cyano-4-hydroxy-cinnamic acid	70990
		3-Aminoquinoline	07336
Acidic	THAP	2,4,6-Trihydroxyaceto-phenone	91928, 47144
Lipids	DIT	Dithranol	10608
Oligosaccharide		1-Isoquinolinol	55433

Table 1

PSD-MALDI and Protein Sequencing

As MALDI is known to be a soft ionization technique, one has expected nearly only intact molecular ions. But it has been found, that molecular ions may be fragmented later on, through acceleration or even while drifting through the mass spectrometer [4]. Special adaptions of mass spectrometers have enabled the detection of those post-source decay (PSD) fragments. As for proteins, fragmentation mostly appears at the peptide bonds, one can detect series of fragments

Figure 1:
MALDI TOF mass spectra of Cytochrome C covalently labeled by Biotin-NHS (Biotinamidocaproate N-hydroxy-succinimide ester)



LC-MS CHROMASOLV® Grade Solvents developed by Riedel-de Haën			
Cat. No.	**Brand**	**Product**	**Pack Size**
34967	Riedel-de Haën	LC-MS CHROMASOLV® Acetonitrile	1.0; 2.5 l
34972	Riedel-de Haën	LC-MS CHROMASOLV® Ethyl Acetate	1.0; 2.5 l
34966	Riedel-de Haën	LC-MS CHROMASOLV® Methanol	1.0; 2.5 l
34965	Riedel-de Haën	LC-MS CHROMASOLV® 2-Propanol	1.0; 2.5 l

Table 2

Maldi Mass Spectrometry

with mass differences reflecting the peptide sequence. Thus PSD-MALDI could be established for de-novo-sequencing issues. The speed of this method makes it somewhat favorable compared with traditional Edman sequencing. On the other hand some remaining limitations, like the indistinguishability of L and I, still underline the usefulness of Edman chemistry as complementing technology (see next chapter).

Quality of Matrices

Due to the extraordinary requirements for absence of organic impurities as well as ions, many users have been repurifying commercially available products. Several years ago, Fluka defined a strict application-based specification for specific matrices to be offered for MALDI. Through the following years, we have developed a broad range of matrices, which are extensively purified to meet those specifications – and provide sufficient quality even for the strictest requirements.

Additional Information

For a more detailed description of MALDI including the different types of sample preparation and ionization sources, please take a look at our former AnalytiX newsletter MALDI-Mass Spectometry at www.sigma-aldrich.com/analytix.

New LC-MS Grade Solvents

Even if MALDI plays an extraordinary role in bioanalysis, the whole broad range of differing MS methods is of importance to cover different requirements. Over the past few years, analytical chemists and researchers have placed increasing emphasis on the use of liquid chromatography (LC) coupled with a mass spectrometer (MS). This technique, called LC-MS (liquid chromatography-mass spectrometry), combines the advantages of a chromatographic separation with mass detection by MS. Low detection limits and qualified analysis of molecular structures brought this application new and growing popularity. To better cater to this new technology, Riedel-de Haën has developed new LC-MS CHROMASOLV® grade solvents (table 2), specially tailored to meet the needs of modern LC-MS instruments. These LC-MS solvents are designed specifically with low contents (max. 100 ppb) of alkaline impurities, such as calcium, magnesium, potassium and sodium, which can cause interferences by creating artifacts with the analyte. The LC-MS CHROMASOLV® solvents are also run through specific UV-spectroscopic quality control tests to guarantee the traditional Riedel-de Haën CHROMASOLV® specifications.
To obtain a Certificate of Analysis or the local prices of these products, please contact your local Sigma-Aldrich partners listed on page 19 or visit our web site.

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[2] Tanaka, K. et. al., *Rapid Comm. Mass Spectrom.* **1988**, 2, 151–153.
[3] Lu, J.; Zenobi, R. Fries., *J. Anal. Chem.* **2000**, 366.
[4] Kaufmann, et. al., *Int. J. Mass Spectrometry Ion Proc.* 131 **1994**, 355.

Highest Quality Reagents for Edman Sequencing

Edman sequencing has been the standard technology for protein sequencing and identification over the years. Even since MS technologies have gained ground and are widely used for identification of proteins, Edman sequencing is still of true value in several aspects. First it is a proven method for de-novo-sequencing, but also important for double checking results of MS identification or sequencing. Although it takes more time than MS, results are easy and clear to interpret.
Due to the progress of instrument manufacturing, the traditional Edman chemistry has gained extremely high sensitivity, allowing reliable sequencing of proteins in the lower pico- or upper femtomole level. An important precondition for successful protein sequencing of such low sample sizes is also the use of ultra-pure high quality reagents. It is obvious that a contamination of solvents or reagents with just some picomoles of reactive impurities significantly lowers the quality of Edman sequencing.
For example the yield of PTH-amino acid is highly dependent on the quality of solvents and reagents used. Especially lysine and tryptophan are extremely sensitive and may be degraded even by the smallest amounts of oxidizing agents. This is demonstrated for lysine in figure 1, which shows two sequencing cycles representing the sequence –M-K-of a standard peptide. Sequencing was performed with a series of improved Fluka reagents specially designed to meet current quality requirements of protein sequencing. These chromatograms are characterized by a very low background and especially a strong signal of lysine (figure 1b). In comparison figure 2 reveals the sequencing of the same peptide using ethyl acetate for protein sequencing as it was offered several years ago. Although this ethyl acetate contained DTT as a protecting agent (recognizable by the PITC-DTT adduct peak (*)) the yield of PTH-lysine is significantly reduced. Fluka offers the complete range of reagents used for automated sequencing. All these reagents are extremely pure, use tested, and packaged in vials which can be directly placed into the sequencer (table 1). This range includes also a new formulation of the methylpiperidine solution. This new formulation does not exhibit the typical «base peak» in the chromatogram.
The range of high quality reagents required for automated sequencing reactions is accomplished by ultra pure acetonitrile (with DTT) suitable for the preparation of PTH amino acid standards. Only specifically purified acetonitrile ensures the stability of the standard for a sufficient time. This is demonstrated in figure 3, whereas figure 3a shows a standard prepared with ultra pure acetonitrile after 1 month storage. Figure 3b shows a standard prepared in acetonitrile, HPLC-quality with addition of DDT after 2 days storage. In the case of HPLC-quality, after two days His, Arg, Met and Lys have already decreased and several additional peaks have appeared.
Besides those reagents we also offer the eluents required for HPLC separation/identification of the PTH amino acids. Usage of these products results in an extremely low background, thus enabling a

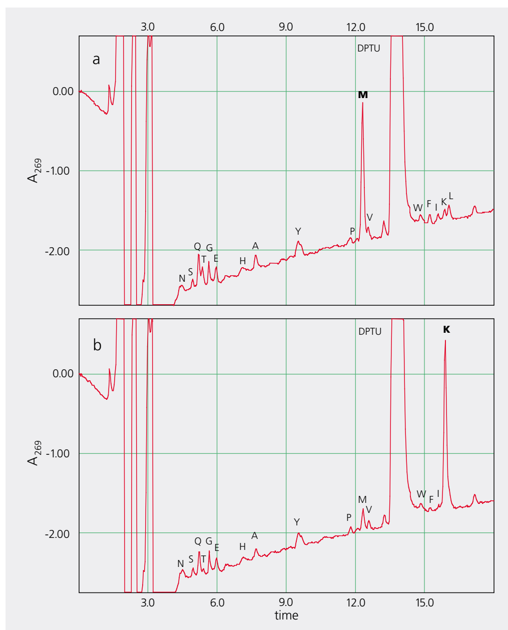


Figure 1: Edman determination of sequence –M-K- using current Fluka reagents. Yield: M: 5.1 pmol; K: 5.5 pmol

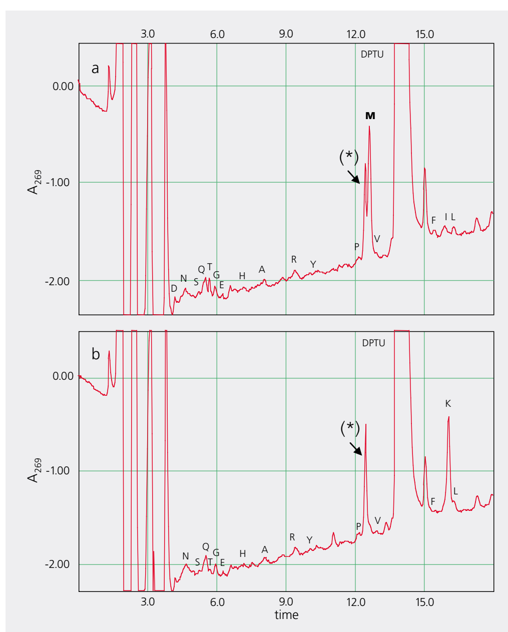
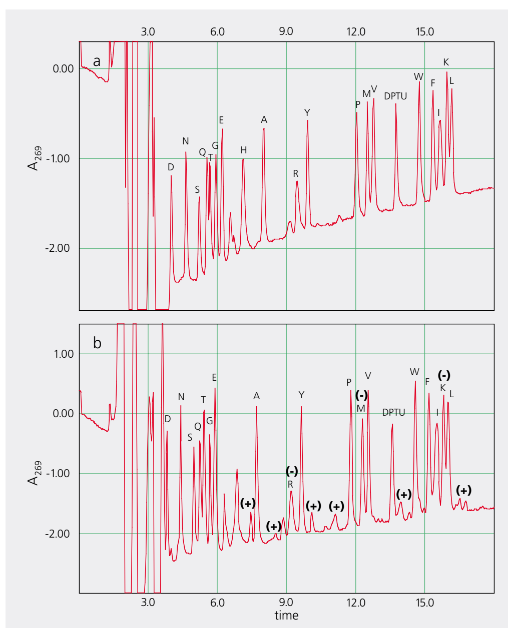


Figure 2: Edman determination of sequence –M-K- with ethyl acetate as offered earlier. Yield: M: 4.5 pmol; K: 3.0 pmol (*) = PITC-DTT adduct peak



Reagents for automated sequencing and Endoproteinases for protein fragmentation

Cat. No.	Brand	Product	Pack Size
09653	Fluka	TFA	40 ml
91709	Fluka	25% TFA solution	40 ml
87859	Fluka	10% Acetonitrile	40 ml
00712	Fluka	20% Acetonitrile	200 ml
00682	Fluka	Acetonitrile (with DTT)	40 ml
09642	Fluka	Heptane	100 ml
09652	Fluka	Ethyl acetate	450 ml
09651	Fluka	Butyl chloride	200 ml
78787	Fluka	5% PITC solution	40 ml
68835	Fluka	Methylpiperidine solution improved base preparation	40 ml
72827	Fluka	Eluent A	500 ml; 1 l; 2.5 l
85278	Fluka	Eluent B	500 ml; 1 l; 2.5 l
79923	Fluka	Premix	100 ml
45172	Fluka	Endoproteinase Glu C	1 mg, 5 mg
45168	Fluka	Endoproteinase Asp N	2 µg
45173	Fluka	Endoproteinase Arg C	100 U
45175	Fluka	Endoproteinase Lys C	0.1 mg
45167	Fluka	Endoproteinsase Pro-C	1 mg
27207	Fluka	Chymotrypsin	25 µg
T8658	Sigma	Trypsin	100 µg

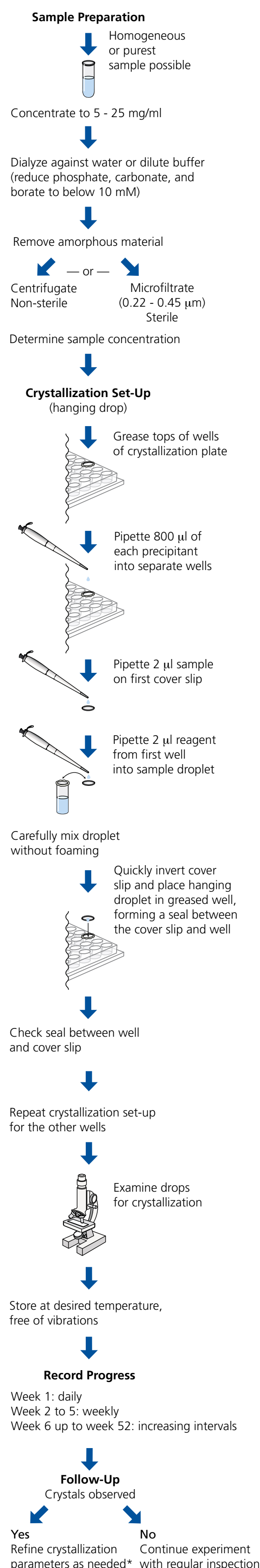
Table 1

maximum of sensitivity. Due to a different composition our premix is much more efficient than what has previously been on the market.
The above mentioned examples demonstrate the requirement for an extraordinary high quality of reagents to meet current needs for protein sequencing. Fluka's reagents for protein sequencing have proven to fulfil even the strictest requirements. These reagents are prepared, specified and tested to meet even low picomolar sample sizes. The packaging enables direct use in current ABI sequencers.
Besides these core products we also offer many special reagents for less widely used sequencing applications.
Please take the opportunity to evaluate our convenient high quality products. Call your local representative or send an e-mail to pnording@sial.com to receive a free sample of your choice or to discuss our attractive conditions.

Figure 3: PTH amino acid standard (a) prepared with specified Fluka acetonitrile, 1 month storage (b) prepared with HPLC acetonitrile and DDT, following 2 days storage; quantity of PTH amino acid standard: 4 pmol

Crystallography Screening Kits

Procedure for crystallization screening



* Optimization may include varying pH, buffer or salt type, precipitant concentration, sample concentration, temperature, additives (ligands, inhibitors, stabilizing ions) or other variables. **Note:** Non X-ray grade crystals may be used in seeding

Separation, as well as the identification, of large numbers of proteins is becoming state of the art. But real understanding of protein function and interactions, as well as directed drug design, often requires more than just the primary structure of proteins. The more knowledge is generated regarding abundance of proteins in different tissues, cell types, and cell states, the more interest is directed into the investigation of three-dimensional structures. Structure determination is typically achieved by X-ray diffraction technology, which requires pure crystals of proteins. Due to the extremely high variability of properties of proteins, achieving crystals is still a very difficult, highly empirical task, which typically includes at least two steps:

1. Screening a wide range of conditions to find suitable crystallization conditions
2. Refinement of those conditions to generate optimal crystals

For initial gross screening of conditions, various principles have been established, of which sparse matrix screening seems still to be the most efficient to find suitable conditions in a limited time and with a limited amount of sample. Sigma-Aldrich provides a series of new kits which are designed and qualified to help researchers determine the optimal conditions for growing protein crystals reliably and reproducibly. Using efficient sparse matrix screening, crystallography kits are compatible with key crystallographic techniques such as hanging, sitting, or sandwiched drops in vapor diffusion, and with free-layer or membrane-bound diffusion. Each kit offers a combination of high-purity buffers, salts, and precipitants to allow the exploration of salting-out, salting-in, cryo resistance, or micelle-mediated crystallization of proteins. Optimized protocols are included to simplify and refine the initial screening and growing of macromolecular crystals. So researchers can now achieve reliable results with a minimum investment of time, effort, and macromolecular samples. The kits are offered by Sigma brand. They are based on the line of Fluka Biochemika ultra products (former Microselect products) which have already proven to offer unsurpassed quality for crystallization processes.

Basic and Extension Kits

The Basic Crystallography Kit (**Cat. No. 82009**), is a rapid, empirical, and efficient screening method

Figure 1: Basic Crystallography Kit

for determining the solubility and optimal starting conditions for the crystallization of biological macromolecules. Based on known and published data for various proteins, the Basic Kit uses a sparse matrix method and a minimal amount of protein sample to explore a broad range of buffers, pH, and precipitants.
The Extension Kit (**Cat. No. 70437**) substantially increases the screening range of the Basic Crystallography Kit by providing a broader range of organic solvents, PEGs, and additives (divalent ions and stabilizers). Both the Basic and Extension Kit are based on the «salting-out» principle: that increasing the concentration of the precipitating agent depletes the macromolecules of bound water and leads to their precipitation and crystallization.

Cryo Kit

The Crystallization Cryo Kit (**Cat. No. 75403**) is a rapid, empirical, and efficient screening method to determine the solubility and optimal starting conditions for the crystallization of biological macromolecules in the presence of a cryoprotectant (primarily glycerol). Based on known or published data for various proteins, the Cryo Kit uses a sparse matrix method and minimal amount of protein sample to explore a broad range of buffers, pH, and precipitants. The screening range and probing ability of the Cryo Kit are comparable to those of the Basic Kit. A cryoprotectant is added to freeze crystals in an amorphous glass at –173 °C. The Cryo Kit is also based on the «salting-out» principle: that increasing the concentration of precipitating agent and cryoprotectant depletes the macromolecules of bound water and leads to their precipitation or crystallization.

Low Ionic Kit

The Crystallization Low Ionic Kit (**Cat. No. 86684**) for proteins is based on a screening protocol for monoclonal antibodies, which has proven to be an effective screening method for soluble pro-

Crystallization Kits from Sigma-Aldrich offer:

Feature	Benefit
Variety of proven precipitants	Allows rapid and simplified screening across a broad range.
High-purity reagents	Ensures reliable, reproducible results. Avoids waste of precious samples.
Sterile solutions	Avoids waste of precious samples.
Conveniently-packaged combination of reagents and solutions	Reduces waste, allows easy handling.
Individual solutions and reagents are available	Simplifies scale-up of crystallization following determination of optimal conditions.

Table 1

Parameters covered by Basic and Extension Protein Crystallization Kits		
Screening Parameters	**Basic Kit – Fluka Cat. No. 82009**	**Extension Kit - Fluka Cat. No. 70437**
pH	4.6 to 8.5	4.6 to 9.0
Buffers	acetate, tartrate, phosphate, Tris, citrate, HEPES, imidazole, formate, and cacodylate	tartrate; phosphate; magnesium and sodium chloride; sodium acetate; sodium citrate; ammonium formate; lithium and ammonium sulfate; imidazole; CTAB
Precipitating Salts*	tartrate; phosphate; ammonium and lithium sulfate; magnesium and calcium chloride; magnesium, ammonium, sodium, zinc and calcium acetate; sodium citrate; sodium and magnesium formate	MPD, 2-propanol, ethylene glycol, dioxane, ethanol, 1,6-hexanediol, t-butanol, glycerol
Precipitating Organic Solvents*	MPD, 2-propanol	PEG 400, 6000, 1000, 8000, 10000, and 20000, PEG MME 550, 2000, 5000, and 2000, Jeffamine M-600
PEGs*	PEG 400, 1500, 4000, and 8000	PEG 400, 6000, 1000, 8000, 10000, and 20000, PEG MME 550, 2000, 5000, and 2000, Jeffamine M-600
Additives	magnesium	Co^{2+}, Cd^{2+}, Fe^{3+}, Ni^{2+}, and Zn^{2+} ions, dioxane, ethylene glycol, polyethyl-eneimine

Table 2

Parameters covered by Crystallization Cryo Kit	
pH Range	4.6 to 8.5
Buffer	Tris, citrate, HEPES, tartrate, phosphate, cacodylate, acetate, imidazole, and formate
Precipitating Salts*	tartrate; sodium, potassium, and ammonium phosphate; magnesium and calcium chloride; sodium, magnesium, zinc, calcium, and ammonium acetate; sodium citrate; sodium formate; lithium and ammonium sulfate; calcium and sodium chloride
Precipitating Organic Solvents*	MPD, 2-propanol, glycerol
PEGs*	PEG 400, 4000, and 8000

* As sole precipitant and/or as combinations

Table 3

Parameters covered by low ionic Crystallization Kit	
pH Range	3.0 to 10.0 (steps of 0.5 pH units)
Buffers (0.05 M)	citrate, MES, Tris, imidazole, HEPES, glycine, Bis-tris
PEGs*	PEG 3350 (4 to 28 %)

*As sole precipitants

Table 4

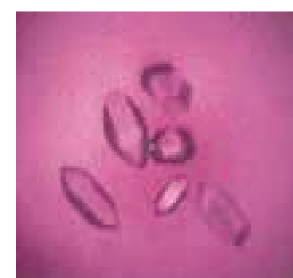
Parameters covered by Membrane Protein Crystallization Kit	
pH Range	4.6 to 8.5
Buffers	Tris, citrate, ADA, HEPES, acetate
Precipitating Salts*	sodium and potassium tartrate; sodium, potassium and ammonium phosphate; sodium and magnesium chloride; sodium and zinc acetate; sodium citrate; lithium, magnesium and ammonium sulfate
Precipitating Organic Solvents*	MPD, 2-propanol
PEGs*	PEG 400, 4000, and 6000
Detergent**	stock solution of choice

* As sole precipitant and/or as combinations
** Proceed from detergents that form small micelles to those with larger micelle sizes.

teins. The high-efficiency of this kit can be further improved by pre-determining the isoelectric point (pI) of the subject macromolecule followed by screening within a range at or near that value (within 2–3 pH units of the pI). The Low Ionic Kit is based on the «salting-in» principle: that decreasing ion concentrations leads to a loss of electrostatic screening of charges on the macromolecules by low molecular weight ions. This loss of screening is compensated by the mutual screening of opposite charges on neighboring macromolecules. This leads to decreased macromolecular solubility and subsequent precipitation or crystallization.

Membrane Protein Kit

The Crystallization Basic Kit for Membrane Proteins (**Cat. No. 73513**) is a rapid, empirical, and efficient screening method to determine the best conditions for crystallization of hydrophobic macromolecules (primarily membrane proteins). Based on known or published data for various proteins, broad ranges of buffers, pH, and precipitants in combination with a micelle-forming detergent are explored using the sparse matrix method with a minimal amount of protein. The crystallization principle of the kit is based on the screening of the hydrophilic, charged parts of the macromolecules by the binding of the detergent



to hydrophobic areas of the macromolecules and subsequent reduction of their solubility. Detergent mediated contacts then lead to precipitation or crystallization of the screened molecules.

Individual Compounds, Stock Solutions, customized Products

All the solutions which are part of above described kits, are available separately. Routine package size is 100 ml, other package sizes are available on request.
For further optimization we are introducing a wide range of single component stock solutions for easy preparation of your individual optimization screening conditions. We already offer a range of such solutions listed in table 5. You may also find the individual pure substances. Based on our extensive range of high-purity reagents as

Table 5

Stock solutions for preparation of individual crystallization conditions

Cat. No.	Product
88163	Ammonium chloride solution 5 M
83263	BICINE solution 1 M
83264	HEPES solution 1 M
83265	Zinc sulfate solution 2 M
83269	Sodium sulfate solution 1 M
83268	Lithium chloride solution 10 M
95843	Potassium acetate solution 5 M
68475	Magnesium chloride solution 2 M
83266	Magnesium sulfate solution 2.5 M
83267	Ammonium sulfate solution 3.2 M
68268	Imidazole solution 1 M
83270	Magnesium nitrate 3 M
83273	Trisodium citrate solution 1 M
83272	Polyethylene glycol 3350-solution 50%
83271	Polyethylene glycol 8000 solution 50%

well as our long experience in the preparation of formulations, we are ready to supply any specific formulation you may need. Please contact us under pnording@sial.com.

Further Information

For helpful notes, more flow charts, or literature please ask for our crystallization screens brochure. This brochure can be downloaded from our web site. For detailed description of individual kits please ask for the manuals.

Table 1

Fluka Cellulose Acetate Sheets

Cat. No.	Description	Pack Size
38378	25 mm x 130 mm	200 sheets
05008	25 mm x 145 mm	200 sheets
08604	25 mm x 150 mm	200 sheets
68875	57 mm x 130 mm	100 sheets
42518	57 mm x 145 mm	100 sheets
15938	60 mm x 135 mm	100 sheets
44994	66 mm x 150 mm	100 sheets
44993	76 mm x 135 mm	50 sheets
41776	145 mm x 192 mm	50 sheets

Table 2

Fluka humid Cellulose Acetate Sheets

Cat. No.	Description	Pack Size
03263	humid, perforated, 5.7 mm x 14 mm	25 sheets
03262	humid, unperforated, 5.7 mm x 14 mm	25 sheets
03261	humid, 7.8 mm x 15 mm	25 sheets
16391	humid, perforated, 17 mm x 17 mm	10 sheets

Cellulose Acetate Electrophoresis

Electrophoresis on cellulose acetate sheets was introduced by Kohn in 1957, two years before polyacrylamide electrophoresis and four years before agarose gel electrophoresis. Nowadays it plays an important role in clinical diagnostics procedures, but has also helped to investigate a broad range of subjects in life science research. Analysis of hemoglobin is a typical example where cellulose acetate electrophoresis is superior to other methods [1]. Other examples are the separation of enzymes (e.g. creatinine phosphokinase, GOT, acidic erythrocyte phosphatase, phosphoglucomutase, etc.), muco-polysaccharides, plasma, serum, cerebrospinal fluid, urine and other body fluids. Another field of applications is the quality control of biological compounds. Cellulose acetate electrophoresis has been shown to be an accurate, but simple, method for protein quantification [2].
Cellulose acetate electrophoresis separates proteins primarily by charge. Protein migration takes place on the buffer film on the surface of the cellulose acetate plate. The buffer used depends on the enzyme being screened. Cellulose acetate plates require less sample and stain volume and allow shorter gel running and staining time than other media.

Dry Cellulose Acetate Strips

With our dry cellulose acetate strips (table 1) you can separate nearly all sample materials that migrate in an electrical field. You can use substance mixtures of 0.06 to 0.15 mg per cm application line. The starting concentration of the mixture to be separated should be more than 1 mg/ml. Weaker solutions should be enriched or concentrated. Advantages of these strips include:

- High porosity of the strip (approx. 80% free pore volume)
- Isotropic, integral matrix for ideal flow conditions
- Minimal diffusion effect and low adsorption capacity
- Perfect transparency because of the pore system
- Ideal for the non-toxic ATX reagent system
- Virtually unlimited stability at room temperature
- Two identical sides, no need to search for the «right» side
- Available in different sizes for all current chamber types

Besides standard cellulose acetate electrophoresis, these strips can also be used for high-voltage electrophoresis, chromatography and immunodiffusion.

Humid Cellulose Acetate Strips

The humid state ensures that strips are rapidly buffered and that gel properties are maintained. Owing to a unique structure (conical pores) these strips (table 2) are an ideal supporting medium for both analytical and micro-analytical electrophoresis and for all immunological techniques.

Thus they are a genuine alternative to agarose gels providing results at least as good as with agarose gels. Compared with dry cellulose acetate strips, the wet strips have the following advantages:

- The samples do not run into each other even during multiple application.
- These strips reliably absorb the samples and guarantee optimal application, even of a large sample volume.
- Even with low-voltage electrophoresis, wet strips guarantee sharp bands. There is no overlapping.
- Owing to their special structure, there is no streaking. Endosmotic effects are safely avoided, especially for important immunological techniques. Thus you will receive better and more reproducible results in immunological techniques but it costs less and takes less time.
- They are extremely resistant to strong alkalis, which means, for example, that staining with conventional oil-soluble sudan dye in NaOH poses no problem.

Non-toxic Reagent System

With conventional electrophoresis reagents, there is always a risk to your health. By contrast, the reagents in the **ATX reagent system** are non-toxic and environmentally friendly.

Fluka ATX Reagent System

Cat. No.	Product	Pack Size
09187	ATX clear, ready-to-use solution	6 x 1 l
09194	ATX decolorant	6 x 1 l
05578	ATX decolorant, ready-to-use solution	6 x 1 l
09276	ATX Ponceau S red concentrate	12 x 250 ml
09189	ATX Ponceau S red staining solution	6 x 1 l
53331	ATX Tris buffer concentrate	6 x 1 l
21685	ATX Tris buffer, ready-to-use solution	6 x 1 l

Conventional reagents contain various toxic substances: buffer solutions contain barbituric acid, classified as a narcotic according to the Dangerous Drugs Act; staining solutions contain methanol and acetic acid which irritate the skin and airways; clarifying solutions contain methanol and dioxan isobutanol, a carcinogenic, inflammable substance which is classified as hazardous waste. Stop using these toxic substances. For your electrophoresis, start using the non-toxic ATX reagent system and protect yourself and the environment against permanent damage.

Advantages include:

- non-toxic and non-irritant to skin and airways.
- environmentally friendly
- pose no disposal problems
- odorless
- non-inflammable
- stable at room temperature
- ATX-reagents are specially coordinated with ATX micro-solid strips
- available as ready-to-use solutions or as concentrates
- guarantee excellent, reproducible results

CBind™ L-Matrices for Antibody Purification

Antibodies are the workhorses for a huge range of bioanalytical techniques and assays. Thus purification of antibodies is of great importance. Protein A and protein G have been widely used for such purposes for a long time. Protein L is another protein which may be used. Protein L originates from Peptostreptococcus Magnus. The protein contains four immunoglobulin (Ig) binding sites and it binds primarily through the κ light chain without interfering with the antigen binding site. This specific binding capacity enables it to be bound to a wider range of Ig classes and subclasses from a wider range of species than any other commercially available Ig binding protein.
CBind™ L is an immobilized CBD-rProtein L matrix, designed for quick and efficient purification of immunoglobulins (Ig) and Ig fragments (Fab and scFv). It is composed of regenerated cellulose beads stabilized by hydrogen bonds and is stable over a broad pH range (1-14) and with most chromatographic buffers, detergents, chaotropic agents and organic solvents. We also offer the pure CBD-Protein L fusion protein, which we use for coupling to cellulose. CBDs constitute a family of independently folding domains found in polysaccharidase enzymes and scaffoldins of cellulolytic microogranisms. CBDs bind to cellulose with very high affinity through hydrophobic interactions. They provide a simple and efficient means of coupling a broad range of bioactive entities to cellulose.

Applications

- Purification of IgG, IgA, IgE, IgD containing kappa light chains, especially human κ light chains I, III and IV and mouse κ light chain I

Table 1

Properties		
Ligand density	approx. 2.5 mg rCBD-Protein L /ml cellulose	
Bead size range	50-80 µm	
pH stability	2.0 – 10	
Storage buffer	20% ethanol in PBS	
Static binding capacity* for IgG:		
	Human	approx. 17 mg/ml
	Mouse	approx. 16 mg/ml
	Rat	approx. 15 mg/ml
	Rabbit	approx. 2 mg/ml

Reviews:

[1] Goldbloom R.B., Screening for hemoglobinopathies in Canada, in Canadian Task Force on the Periodic Health Examination. Canadian Guide to Clinical Preventive Health Care. Ottawa: Health Canada **1994**, 206 - 218.

[2] Barufaldi, M.; Pappano, N. B.; Debattista, Nora B., Quantitative Protein Determination from Cellulose Acetate Strip Electrophoresis, *J. Chem. Educ.*, 76 **1999**, 965

- Purification of scFv fragments containing kappa light chains as listed above
- Purification of human or mouse antibodies directly from cow, goat or sheep

Benefits

- Protein L from Peptostreptococcus magnus binds immunoglobulins (Ig) primarily through kappa light chain interactions without interfering with the antigen-binding site of Igs[2].
- Protein L binds to a wider range of Ig classes and subclasses from a wider variety of species than any other commercially available Ig binding protein.
- CBind™ L (CBD-rProtein L-cellulose) has improved binding capacity but similar binding properties as Protein L-agarose
- very hydrophilic, and thus non-specific binding of proteins is minimal
- designed to have enhanced flow properties

Stabilization of Biomolecules

Figure 1: Chemical Chaperones are prepared from Red Algae, which express those compounds as natural protein protecting agents.

During long-term storage many biomolecules are subject to activity loss. Even through a limited time for certain investigations, stability of proteins in solution may become a problem. The BioStab Products are used to preserve the activity of biomolecules like enzymes, antibodies, DNA, whole cells or cell membranes, during long-term storage in solution.
Fluka offers Ectoine and Hydroxyectoine, which are small, cyclic amino acid derivatives produced from halophile eubacteria or streptomyces (figure1). As chemical chaperones their natural role is the protection of proteins, nucleic acids or whole cells.

BioStab Products

These products are ready-to-use solutions, which are applied by simply mixing with protein solutions in a certain volume relation. They are optimized for various application fields and come with documentation, which makes their use very easy.
We will add new products in this range to even better serve your needs. For additional information please download product information sheets from our web site www.sigma-aldrich.com/biostabilization or contact the technical service at your local Sigma-Aldrich office (see page 19).

Fluka BioStab Products

Cat. No.	Product	Application
92889	BioStab Biomolecule Storage Solution	The biomolecule storage solution is used to preserve the activity of biomolecules like enzymes, antibodies, DNA, whole cells or cell membranes during storage over a long time.
95576	BioStab Enzyme Stabilizer	The enzyme stabilizer is used to protect biomolecules like enzymes from denaturation by thermal stress, proteolysis, pH changes or salt concentration.
93895	BioStab Immunoassay Stabilizer	Immunoassays, like an ELISA-test, can be protected from external degradation using BioStab Immunoassay Stabilizer. The addition of BioStab Immunoassay Stabilizer helps to increase the ability of stocking and the coating rates of the antibodies.
92832	BioStab PCR Optimizer	The PCR optimizer stabilizes biomolecules like enzymes and is used to increase the thermostability and the half-life period of Taq Polymerase, so it is possible to realize more cycles.

Atto Fluorescent Labels – New Functionalization and Conjugates

Atto Labels

Last year Fluka introduced a series of fluorophores for labeling of biomolecules that are designed for the most sensitive applications. A unique combination of advantages makes them highly favorable tools for many labeling applications. All our new labels are characterized by high molar extinction coefficients (around 120.000 $M^{-1} cm^{-1}$) and high quantum yields, which results in a strong fluorescence. The Stokes' Shift is between 20 and 30 nm. Especially long wavelength labels have proven to be more photostable than other commonly used dyes. Atto labels are based on rigid structures and do not show any cis-trans-isomerization, as is typically observed with cyanin based fluorophores. The absence of cis-trans-isomerization circumvents potential problems in Fluorescent Resonant Energy Transfer (FRET) or single molecule detection.

For more details please see AnalytiX 8/2001 at www.sigma-aldrich.com/analytix.

Analytix

Atto Labels – A Challenging Range of New Labels

Table 1

Properties of Atto dyes measured in water (ethanol)								
Cat. No.	Product	Cat. No. corresponding NHS ester	Pack Size	ε [$M^{-1}cm^{-1}$]	λ_{max} abs [nm]	λ_{max} em [nm]	η_{fl} [%]	τ_{fl} [ns]
77810	Atto 520	70706	1 mg	110.000	520 (525)	542 (547)	90 (95)	3.6 (3.7)
75784	Atto 565	72464	1 mg	120.000	561 (566)	585 (590)	92 (97)	3.4 (3.6)
70425	Atto 590	79636	1 mg	120.000	598 (598)	634 (634)	80 (90)	3.7 (4.0)
78493	Atto 610	93259	1 mg	110.000	605 (616)	646 (630)	70 (70)	3.3 (3.3)
93711	Atto 655	76245	1 mg	110.000	665 (655)	690 (680)	30 (50)	1.9 (3.6)
94875	Atto 680	75888	1 mg	120.000	680 (675)	702 (699)	30 (40)	1.8 (3.4)

Our new range of labels have excitation and emission maxima spanning the range from 520 to 680 nm (figure 1). This allows the use of various excitation sources and commonly used emission filter sets. It also allows a variation of multicolor visualization. The small size of our labels (MW between 400 and 600) minimizes the risk of unwanted steric effects and the interaction of labeled molecules with other targets. Furthermore Atto labels are quite insensitive to pH fluctuation – most of them do not show any effects between pH 2 and 10, and feature low non-specific adsorption.

Maleimides

Atto Labels are now also available as maleimides, which offer an alternative method to amino labeling. Maleimides are suitable for coupling to thiol-containing groups, such as those contained in cysteine residues, and for thiol groups introduced as modifiers during automated synthesis (e.g., oligonucleotides).

Biotin- and Streptavidin Conjugates

For convenient use of fluorescent labels in common assay formats we now also offer Atto labels conjugated to specific proteins. Biotin- and Streptavidin conjugates are the most commonly used. The high affinity of streptavidin (like avidin) to biotin has been the basis for wide-spread use of streptavidin conjugates as secondary detection reagents in microscopy, flow cytometry, immunoassays, blot analysis and nucleic acid hybridization methods. Streptavidin was chosen as it shows much lower non-specific binding than avidin.

Our streptavidin conjugates basically show the same spectral pattern as the unbound label. An interesting feature is that several of our streptavidin conjugates show low fluorescence, but strong enhancement of fluorescence after binding to biotin. This phenomenon helps to improve sensitivity in case a conjugate might be left adsorbed unspecifically.

We are ready to prepare additional conjugates on your demand. Please contact Pierre Nording at pnording@sial.com or send a fax +41 81 755 2848.

Table 2

Fluka Maleimide of Atto Labels	
Cat. No.	Product
49349	Atto 425 maleimide
16590	Atto 520 maleimide
39887	Atto 590 maleimide
41061	Atto 610 maleimide
80661	Atto 655 maleimide
04971	Atto 680 maleimide

Table 3

Fluka Biotin and Streptavidin conjugates of Atto Labels	
Cat. No.	Product
09260	Atto 425-Streptavidin
41469	Atto 520-Streptavidin
56304	Atto 565-Streptavidin
40709	Atto 590-Streptavidin
56767	Atto 610-Streptavidin
02744	Atto 650-Streptavidin
16630	Atto 680-Streptavidin
28616	Atto 425-Biotin
01632	Atto 520-Biotin
92637	Atto 565-Biotin
43208	Atto 590-Biotin
43292	Atto 610-Biotin
06966	Atto 650-Biotin
55819	Atto 680-Biotin

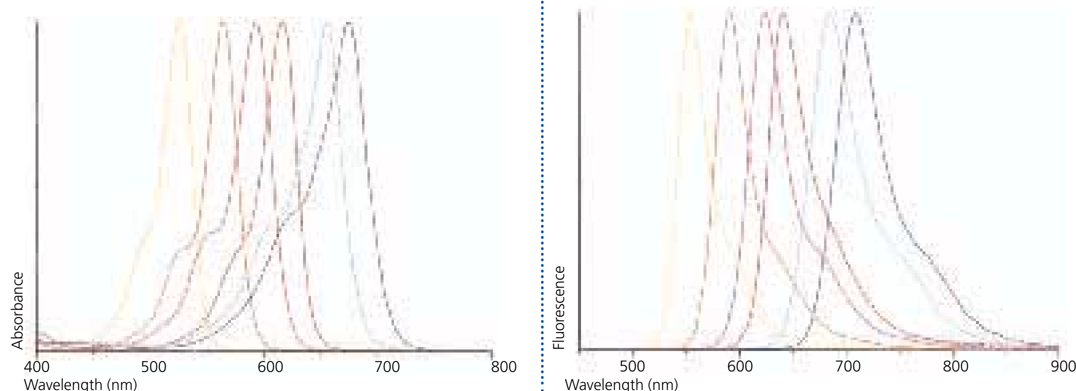


Figure 1: Absorption and fluorescence spectra of Atto labels measured in ethanol

Given the extensive use of these two Cy dyes, equipment and procedures have often been set up to match their well-known properties. Thus changes, even to superior labels, might be unlikely. We are adding two labels, Fluorescent Orange 548 and Fluorescent Red 646, which feature very similar spectral and handling properties. Excitation and emission maxima closely match and the molar extinctions are very strong (table 1). Binding of dye (activated as NHS esters) may be handled by similar procedures and the resulting dye/protein ratios are in the same range. Our new labels are also highly water soluble.

These labels together are ideally suited for energy transfer studies (figure 1), but each of them can also be used together with other dyes, e.g. the long lifetime ruthenium labels described below. Fluorescent Resonant Energy Transfer (FRET) has been exemplified for an immunoassay, a competitive immunoassay (figure 2), a hybridization assay (figure 3) and a competitive hybridization assay. The use of Fluorescent Red antibody conjugates in flow cytometry has also been demonstrated (figure 3). Both labels are available as carboxylic acid as well as NHS ester packaged both as 1 mg solid or as 5 times the quantity needed for labeling 1.5 mg.

For your convenience we also offer labeling kits including the reactive labels (5 vials sufficient for labeling at least 1.5 mg protein each) and ready made buffers for coupling to proteins and purification of conjugates.

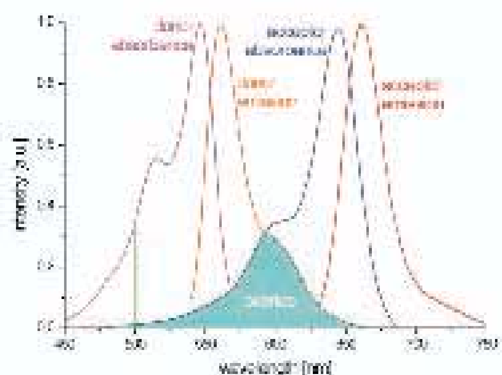


Figure 1:
Fluorescence energy transfer applications: following excitation of Fluorescence Orange at 500 nm radiationless energy transfer occurs to the acceptor dye Fluorescent Red 646. The acceptor fluoresces without being directly excited. Fluorescence of both donor and acceptor can be measured which enables a radiometric determination of the analyte.

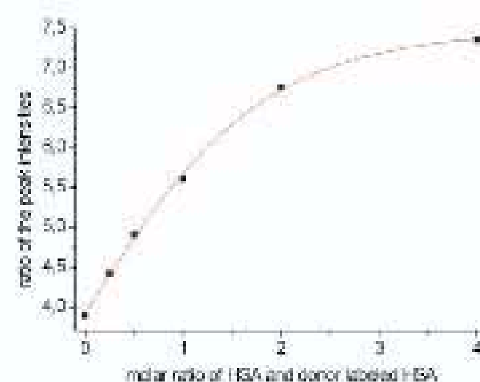


Figure 2:
Competitive fluorescence energy transfer immunoassay: relation between fluorescence emission intensities of donor (Fluorescent Orange 548) and acceptor (Fluorescent Red 646) mirrors the balance between donor labeled antigen and antigen present in the sample.

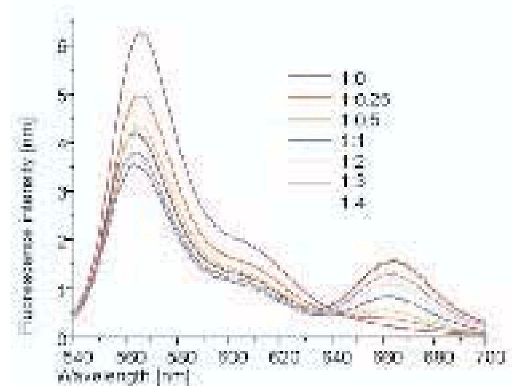


Figure 3:
Hybridization assay for oligonucleotides: 15mers coupled with Fluorescent Orange 548 resp. Fluorescent Red 646 have been combined in various ratios (fixed amount of Fluorescent Orange 548-oligo, varying amounts of Fluorescent Red 646 oligos).

Table 1

Spectral properties of Fluorescent Orange and Red (aqueous solutions)			
Product	**ε [M^{-1}cm^{-1}]**	**λ_{max} abs [nm]**	**λ_{max} em [nm]**
Fluorescent Orange 548	150.000	548	568
Fluorescent Red 646	250.000	647	661

Table 2

Fluka Fluorescent Orange 548 and Red 646 products		
Cat. No.	**Product**	**Pack Size**
40468	Fluorescent Orange 548	1 mg
73223	Fluorescent Orange 548 reactive	1 mg
42024	Fluorescent Orange 548 reactive	5 vials for labeling 1.5 mg protein each
92813	Fluorescence Marker Kit 550	1 kit for labeling 5 x 1.5 mg
30894	Fluorescent Red 646	1 mg
95043	Fluorescent Red 646 reactive	1 mg
62164	Fluorescent Red 646 reactive	5 vials for labeling 1.5 mg protein each
92821	Fluorescence Marker Kit 650	1 kit for labeling 5 x 1.5 mg

Fluorescent DNA/RNA Stains

Similar to well-known nucleic acid stains (ethidium bromide, ethidium homodimer etc.) our new dyes are nucleic acid intercalators. They do not show any fluorescent properties in absence of nucleic acids. In the presence of DNA or oligonucleotides they intercalate into the DNA immediately. In that state they are heavily fluorescent [1-3]. Our new nucleic acid stains provide strong molar absorption and quantum yields over 0.7 (table 1). We offer a range of stains that covers emission wavelengths from 570 to 610 nm.
DNA/RNA intercalators are valuable tools for visualization and quantification of nucleic acids and may be used for various applications as

- Detection and Quantification of nucleic acids in solutions
- Detection of nucleic acids in cells
- Staining for nucleic acid electrophoresis

Using the strong emission of Atto-Dino 2-4 dyes, detection of nucleic acids may be achieved far below 100 ng/ml as shown for Atto-Dino 4 (figure 1). Atto-Dino 4 was successfully applied for staining in gel electrophoresis. Atto-Dino 1 has a comparably low quantum yield, but this dye may be interesting as its intercalation is site specific [1]. Atto-Mono 1 and Atto-Mono 2 are cell permeable and may be used in many cell biology applications [4].
All our intercalators are offered as 1 mM solutions in DMSO. For dilutions in aqueous solutions please do not use vials from glass, as free stains tend to adsorb at glass surfaces.

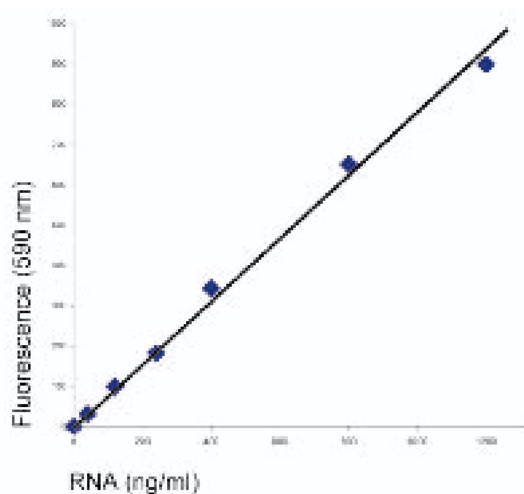


Figure 1: Response curve of Atto-Dino 4 with different concentrations of RNA

References:

[1] J. Bunkenborg et. al., Concerted intercalation and minor groove recognition of DNA by a homodimeric thiazole orange dye, *Bioconjugate Chem.* 11 (2000), p. 861–867.
[2] T.G. Deligeorgiev et. al., Synthesis of homodimeric monomethine cyanine dyes as noncovalent nucleic acid labels and their absorption and fluorescence spectral characteristics, *Dyes and Pigments* 44 (2000), p. 131–136.
[3] I. Timtcheva et al., Homodimeric monomethine cyanine dyes as fluorescent probes of biopolymers, *J. Photochem. Photobiol.* 58 (2000), p. 130–135.
[4] I.I. Timcheva et al., Fluorescence spectral characteristics of novel asymmetric monomethine cyanine dyes in nucleic acid solutions, *FEBS Letters* 405 (1997), p. 141–144.

Table 1

Fluka Fluorescent DNA/RNA stains

Cat. No.	Product	ε (water) $[M^{-1}cm^{-1}]$	Abs. Max. of free stain [nm]	Abs. Max. of bound stain [nm]	Fluor. Max. of bound stain [nm]	QY bound to DNA
51796	Atto-Mono 1	68.500	515	538	610	> 0.25
67888	Atto-Mono 2	57.200	516	540	600	> 0.25
53925	Atto-Dino 1	179.000	506	531	582	> 0.7
83399	Atto-Dino 2	162.000	506	529	576	> 0.7
97166	Atto-Dino 3	107.000	480	520	570	> 0.7
83392	Atto-Dino 4	140.000	485	523	572	> 0.7

Additional Long Wavelength Fluorescent Labels – up to 800 nm

As most of the auto fluorescence in biological samples appears in the range of below 600 nm, labels with longer wavelength emission are always of great interest. Besides that, additional products in that range also extend the possibilities of multicolor analysis. Up to now only a few NIR labels have been available. One example is the IRD 700 and IRD 800 labels used for DNA sequencing applications, which showed some limitations in terms of stability. Also some available dyes are not suitable if used on solid supports. This was reflected in numerous inquiries for long wavelength dyes and labels. Thus we have introduced a range of additional fluorescent labels covering the whole range up to absorption/ emission values near 900 nm. All these products are available as reactive esters which may be coupled to proteins or other biomolecules via their amino groups.
Fluorescent Orange 550 and Fluorescent Red 630 may be additional options for replacement of Cy3 and Cy5. These two new dyes show a superior solid state emission, making them ideal for labeling cDNA to be hybridized to DNA glass arrays. They are cost-effective, and possess high sensitivity and reproducibility.

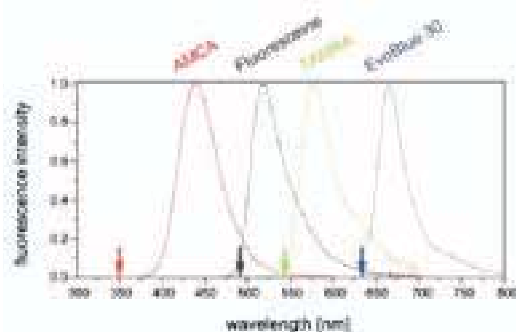


Figure 1: Evoblue – an ideal complement to common labels for multicolor analysis.

Fluorescent Red 700 and Fluorescent Red 780 are fluorescent labels designed for excitation by infrared diode lasers and may be used for labeling primers for DNA sequencing (using a Li-Cor reader). For Fluorescent Red 780 we also offer the phosphoramidite, which can be used for covalent coupling to the 5'-end of DNA-oligomers during automated synthesis. The chromophore responsible for absorption and emission properties is based on a bridged hemicyanine and was optimized with respect to base stability and solubility to allow standard coupling, deprotection and cleavage conditions during oligo-synthesis. Read lengths up to 600 bases could be easily achieved with Fluorescent Red 780 coupled primers. DY-780-OH is a hydroxy-modified dye which is ready for activation to phosphoramidite. Evoblue was originally developed by Evotec for screening assays. Due to the broad range of assays to be performed, the label had to match a multitude of requirements. Excitable by He-Ne lasers (Excitation maximum at 635 nm) and trackable by common filter sets (Emission maximum at 670 nm) it has many advantageous properties for this purpose. Due to these spectral properties it is an ideal complement for well established labels (figure 1). It reveals a high chemical stability, which makes it applicable for organic chemistry (solution or solid phase). High water solubility minimizes unspecific binding. It is stable against organic solvents and not affected by pH changes. Neutral over all charge avoids electrochemical effects on labeled target molecules.

Table 1

New fluorescent labels with long wavelength emission

Cat. No.	Product	Excitation [nm]	Emission maximum [nm]
90283	Fluorescent Orange 550	550	590
92592	Fluorescent Orange 550 reactive	550	590
83756	Fluorescent Red 610	610	645
70543	Fluorescent Red 610 reactive	610	645
78361	Fluorescent Red 630 reactive	620	650
72572	Fluorescent Red NIR 680	680	715
93662	Fluorescent Red NIR 700	672 or 700	735
61059	Fluorescent Red NIR 700 reactive	672 or 700	735
78581	Fluorescent Red NIR 730	680 or 730	755
92315	Fluorescent Red NIR 730 reactive	680 or 730	755
91517	Fluorescent Red NIR 782	635 or 782	800
93873	Fluorescent Red NIR 782 reactive	635 or 782	800
08478	DY780-Phosphonate	635 or 782	800
49328	Evoblue	635	666

Long Lifetime Fluorophores

We have extended our range of fluorescent labels based on ruthenium complexes. Fluorescent ruthenium complexes have found certain applications, as they overcome some limitations of traditional organic fluorophores. First they show a very long decay time of 400–2000 ns. The fluorescence signal of long-lived fluorophores can be gated to eliminate the emission from short-lifetime fluorophores and autofluorescence from cells and biomolecules to further improve sensitivity. This means that measurement takes place with a certain delay after flash excitation. Since it can be used in investigations based on decay time or frequency, gated measurement has become a widely used technique.
Second these labels can be used for polarization based antigen-antibody assays, even if the antigen is quite big (figure 1). Dyes with short lifetimes can be used in such fluorescence polarization assays if the molecular weight of labeled antigen is relatively low (Mr < 1,000). On binding to an antibody, the change in the effective molecular weight of the complex is great enough to be reflected as a change in anisotropy. If the molecular weight of the antigen is large (Mr > 20,000), changes in rotational correlation time on Ag:Ab complex formation are small. In this case, fluorophores with short (<10 ns) lifetimes are no longer useful. Dyes with lifetimes in the range of 400 to 500 ns range are better matched to the rotational correlation times of very high molecular weight complexes. This was first demonstrated in a model fluorescence polarization immunoassay for polyclonal anti-HSA [1].
Other advantages include:

- Large Stokes' Shift
- No quenching at high labeling ratios
- Solubility in water or organic solvent
- Easy handling
- High thermal and chemical stability
- High photostability

Ruthenium complex fluorophores can very well be used for FRET (Fluorescence Resonance Energy Transfer) assays together with traditional fluorophores [2].
We do offer various ruthenium complexes, which are functionalized to enable easy coupling of proteins and other biomolecules via amine groups.
Fluorescent ruthenium complexes also play a crucial role as sensor dyes, especially for detection and quantification of oxygen. You will find suitable new sensor dyes and more details in our AnalytiX newsletter 8/2001 on our web site www.sigma-aldrich.com/analytix.

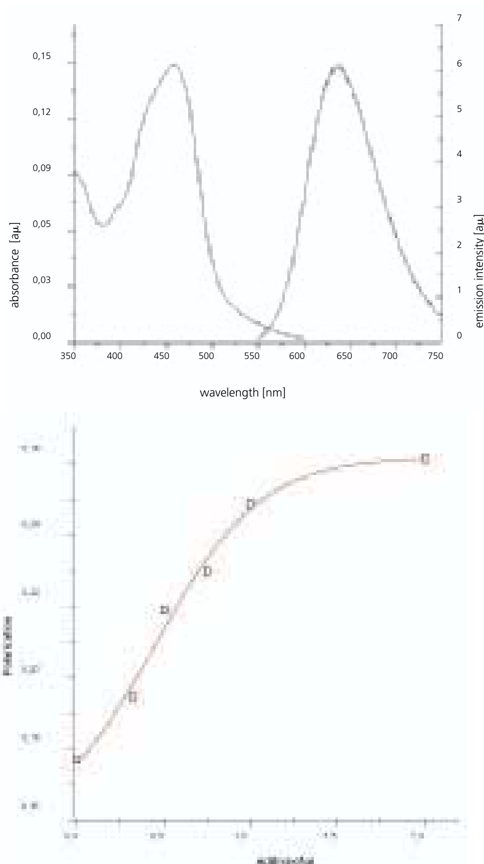


Figure 1: Polarization assay using various ratios of labeled antigen and antibody

References:

[1] Terpetschnig, E., Szmacinski, H. and Lakowicz J., *Anal. Biochem.,* Fluorescence Polarization Immunoassay of a High Molecular-Weight Antigen based on a Long-Lifetime Ru-ligand Complex, 227, (1995), p. 140–147.

[2] Augustin, Ch., Ruthenium-Ligand Complexes as Bioanalytical Luminescent Probes for Polarization and Energy Transfer Systems, dissertation, University of Regensburg (2001).

Atto Labels – A Challenging Range of New Labels

Table 1

Fluka Ruthenium complex fluorophores

Cat. No.	Product	Excitation Wavelength [nm]	Emission Maximum [nm]
96631	Bis-(bipyridine)-4'-methyl-4-carboxy-bipyridine-Ru-N-succinimidyl ester $(PF_6)_2$	458	628
96632	Bis-(bipyridine)-4,4'-dicarboxybipyridine-Ru-di-N-succinimidyl ester $(PF_6)_2$	630	658
71603	Bis-(bipyridine)-5-(isothiocyanato-phenanthrolin)-Ru$(PF_6)_2$	445	615
04188	Bis(2,2-bipyridin)-4-Me-4-carboxy-bi-pyridin- Ru$(PF_6)_2$	450	626
90819	Bis-(2,2'-bipyridin)-(5-amino-phenanthrolin)- Ru$(PF_6)_2$	458	592

Specific applications not only require well-suited fluorescent probes, but also an optimized assay procedure and adapted supplements. Thus we are introducing several specific reagents or kits dedicated to such applications.

Fluorescent Protein Assay

The Advanced Protein Assay Reagent is designed to optimize the speed and accuracy of protein measurement. The reagent combines the useful properties of low protein to protein variance and a strong signal for a sensitive assay. A simple one step procedure results in a green to blue color change which can be recognized by measuring absorbance at 570 to 615 nm. It may be used for protein determination in buffers, biological fluids, or tissue culture media. The linear range for this assay is from 1 to 40 µg protein per ml reagent. Detailed procedures for different samples and sample concentrations are provided.

Fluorescent Cell counting Assay

Fluorescent Cell Counting Reagent provides an optimized tool and procedure for the fluorometric detection of living cell numbers. The amount of a fluorescent dye, calcein, produced from Calcein-AM by esterases in cells is directly proportional to the number of viable cells in a culture medium. Since Calcein-AM is highly lipophilic because of the acetoxymethyl groups in the molecule, it can rapidly permeate into the cytoplasm through the cell membrane (figure 1). The assay does not require any radioisotopes (such as in the [3H]-thymidine incorporated assay) or a solubilization procedure (such as in the MTT assay). Therefore, it allows the users to obtain highly reproducible and accurate cell proliferation assay results.

Nitrite/Nitrate Assay Kit

Nitric oxide (NO), a gaseous paramagnetic radical, is a very important and versatile messenger in biological systems. It has been identified as an endothelial derived relaxation factor (EDRF) and antiplatelet substance. It serves as a neurotransmitter derived from a neutrophile and a cytotoxic substance from an activated macro-phage. Although NO's molecular action in the biological system is very versatile, the most important role of NO is the activation of guanylate cyclase.

The most popular and simplest methods used to detect the NO concentration is the Griess assay. However, it is sometimes not sufficient to determine µM levels of NO_2. Our assay is based on 2,3-Diaminonaphthalene (DAN), which reacts with NO_2 and generates a fluorescent product. The NO_2/NO_3 Assay Kit (Cat. No. 04508) contains DAN, nitrate reductase, enzyme cofactor, buffer solution and NO_2, NO_3 solutions as standard. Therefore, NO metabolites, NO_2 and NO_3 are easily detectable by this kit. The suitable detection range is from 1 to 10 µM.

Albumin Fluorescence Assay Kit

The specific and sensitive determination of albumin in biological fluids is required in many areas of biomedical sciences. Assays suitable for the determination of low concentrations (<100 mg/l) of albumin in natural matrices are either nonspecific for albumin and test total protein content (dye binding methods), or use complicated and costly procedures (immunoassays). The new dye albumin blue 580 (AB 580) provides an easy, robust, sensitive and specific assay for albumin [1]. The kit (Cat. No. 09753) enables quantification of albumin in the range of 0.4 – 200 mg/ml with high accuracy. The interference by proteins other than albumin is less than 1% compared to the response of albumin.
The kit contains Albumin blue reagent, suitable buffer solutions, calibrators, standards and the manual. It provides sufficient reagents for 200 determinations.

References:

[1] M.A. Kessler, A. Meinitzer and O.S. Wolfbeis, *Anal.Biochem.* 248 (1997), p. 180.

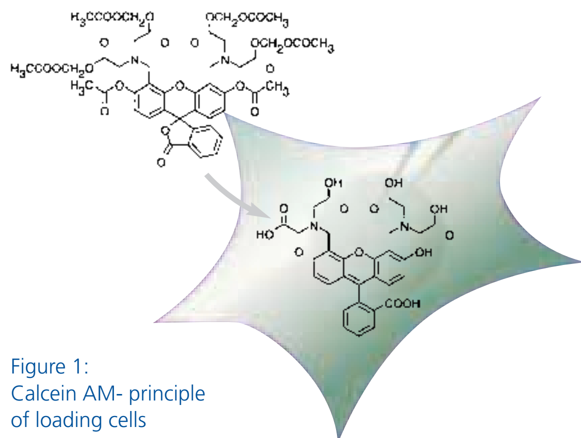


Figure 1:
Calcein AM- principle of loading cells

Microparticles have found widespread applications ranging from model systems in medicine to biochemistry, colloid chemistry, and aerosol research. They are also suitable as separation phases for chromatography, as support for immobilized enzymes, and as spacers in liquid crystal displays (LCD).
Functionalized surfaces allow multiple modifications, e.g. labeling with dyes. Fluorescent labeled particles can be used as standards (e.g. in Flow Cytometry, Confocal Laser Scanning Microscopy, Light Scattering Instruments) as well as tracers in environmental science, flow measurements in gases and liquids like Laser Doppler Anemometry (LDA), Particle Dynamics Analysis (PDA), and Particle Image Velocimetry (PIV).

Your Choice - the most promising Polymer

Fluka offers you microparticles based on various polymers. As most of the current applications are still based on well known polystyrene (latex) particles, newer technology based on polymethacrylate or melamine resin lead to advantages, which will help you for certain applications. The table 1 gives a short overview about the most significant advantages of the different matrices.

Monodispersity and Size Standards

Microparticles from Fluka are always homogeneous in size and shape. Typically the particle size variation coefficient is below 2%, and the particle size standard derivation for the most typical sizes is below 0.1 µm. The typical uniformity is visualized in figure 1 and 2.
Increasing need for validation and quality assurance demands valuable tools for calibration. In the range of 80 nm - 15 µm our calibration standard grade polystyrene particles are among the best-characterized particle standards currently available. The diameter of these particles is determined by methods described by the National Institute of Standards and Technology (NIST, USA), like Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), optical microscopy, and COULTER COUNTER® particle size measurement. Devices for particle characterization are calibrated using BCR® (Bureau Communautaire de Référence) and NIST-traceable standards. The certificate of analysis of each batch includes particle diameter and standard deviation as well as data like solids content, particle number per ml, specific weight, refractive index.

Modified and labeled Particles

Extensive know-how in manufacturing of microparticles enables us to offer a wide range of modified particles. This includes functionalized particles (carboxylated particles are widely used for coupling specific molecules), fluorescence labeled particles (e.g. with fluorescein, nile red or rhodamine B, figure 3) and even magnetic particles. Although all particles are routinely offered as 2% or 10% aqueous suspensions we can also provide them as white powders. Melamine particles can be handled well as powders also. That is in contrast to polystyrene particles which are difficult to handle due to electrostatic effects.

Additional Information, customized Products

For a complete overview about our offering as well as local prices please have a look into our main catalog or visit our web site at www.sigma-aldrich.com/flukanew (see biochemistry;

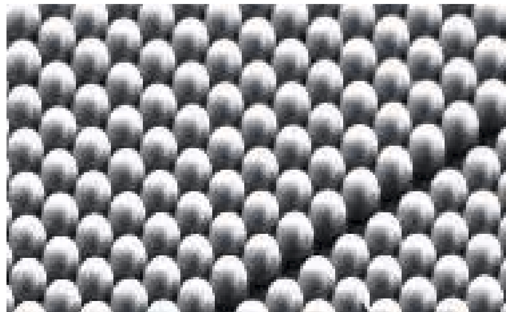


Figure 1: Scanning electron microscopy image of 1.6 µm PS particles

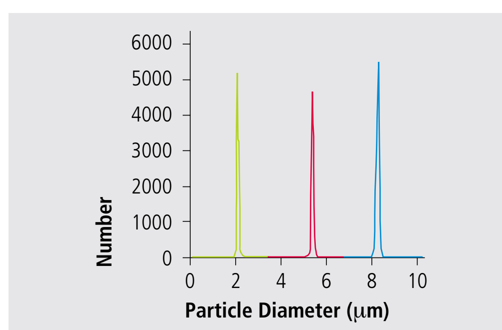


Figure 2: Size distribution curves of three different calibration standard particles measured on the COULTER COUNTER™, 2.0 µm (green); 5.5 µm (red); 8.1 µm (blue).

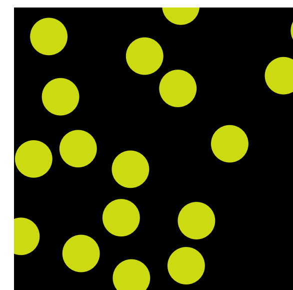


Figure 3:
Fluorescence microscopy image of FITC-labeled MF particles

Table 1

Specific advantages or properties	
Polymer, monodisperse Particles Size Range	**Specific Advantages or Properties**
Polystyrene (latex) 0.1 – 10 µm	• Most referenced material, extensive experience available • Hydrophilic surface • Non-specific adsorption of proteins
Melamine 1-55 µm	• High crosslinking density • High temperature stability up to 300 °C • Superior mechanical strength • Stable and insoluble in acids and bases • Extremely high stability in organic solvents, no swelling or shrinking upon contact with organic solvents • Outstanding long-term stability in dispersions, no additives or stabilizers required • Freeze - thaw stability in water • Particles can be dried directly from their aqueous dispersions • Free flowing powders of dried particles can be redispersed in any dispersing agent without agglomeration • Hydrophilic surface
Polymethacrylate 1-100 µm	• Excellent biocompatibility • Soluble in organic solvents like acetone, benzene, or halogenated hydrocarbons • Reduced non-specific protein binding activity • Monodisperse particle sizes up to 100 micrometers available • Hydrophilic anionic surface

microbeads). Detailed description of different product types, advantages and protocols for further usage may be found in our manual, which is available as an electronic file. For additional information ask the technical service at your Sigma-Aldrich office listed on page 19. Although we already offer a large range of products from stock, you might have special needs regarding size, functionalization or other criteria. We welcome the opportunity to design and develop particles tailored to your needs.

Fluorescent Nanoparticles

The physics and chemistry of nanoscale systems has advanced rapidly over the last few years and there are real prospects of translating these exciting scientific developments into a new generation of high technology products and processes. Since nanotechnology is a generic technology, it also has the potential to impact on a wide range of industrial sectors, from chemicals to electronics, from sensors to advanced materials.

Figure 4:
Fluorescent nanoparticles can be prepared in various colors

One major aspect of nanotechnology is the development of fluorescent nanoparticles, which might find applications in optical data storage, as well as in biochemistry, bioanalyses, and medicine. Current medical and biological fluorescent imaging techniques are mainly based on dye markers, which are limited in light emission per molecule, as well as photostability. Nanoparticles overcome these problems offering strong and stable fluorescence. Fluorescence labeled nanoparticles have been successfully used for various types of immunoassays [1, 2]. In latex agglutination assays some of the limiting factors can be avoided by using very small particles, which reduces the blank absorbance and provides a better colloidal stability [3, 4]. Nanoparticles are also used for more specific purposes, e.g., drug carriers [5–7].

Base Materials

Fluka offers fluorescent nanoparticles based on different materials, each of which provides certain advantages. PAN (Polyacrylnitrile) Particles are ideally suited for FRET applications. They are highly fluorescent and extremely small (less than 30 nm in diameter). They have low interference from interfering substances and are available with carboxylated or avidin functionalized surfaces. All particles are offered as a 0.5% (w/w) aqueous suspension in buffer (10 mM MES, pH 7).
PD is a novel polymer offering properties similar to PS, but has a much lower oxygen permeability which results in a higher photostability for most dyes. The size is about 40 nm, the surface can be either carboxylated or additionally modified. All particles are offered in a 0.5% (w/w) aqueous suspension in buffer (10 mM MES, pH 7).

Table 2

Fluka Fluorescent Nanoparticles	Abs. 545 nm/ Em. 565 nm	Abs. 642 nm/ Em. 662 nm
PAN Beads	65779	83393
PD Particles	66392	83394

Catalog Products and customized Particles

In our catalog you find a sample of products which are available from stock. We offer a selection of labeled particles for the above polymers. These are suitable for fluorescence resonance energy transfer studies. We are ready to offer particles based on both polymers, as well as the well-established polystyrene. These particles may be prepared unlabeled or fluorescence labeled, whereby excitation maximum can be chosen in the range from 480 to 780 nm, emission maximum between 560 and 800 nm.
For different applications and specific adaptation you may also ask for various modifications as
– Carboxylated surface (for amine coupling)
– Avidin modified (for Biotin-Avidin based assays)
– Blocked surface (hydroxy-groups)
For additional information about our current range of products or inquiries for customized products please contact Pierre Nording at pnording@sial.com or send a fax +41 81 755 2848.

Reviews:

[1] Härmä, H., Particle-based Bioaffinity Assay Systems - Sensitive Miniature Single-particle Immunoassay, Annales Universitatis Turkuensis, Sarja - Ser. AI Osa - Tom. (2001), p. 273.

[2] Bangs, L.B., New Developments in Particle-Based Immunoassays: Introduction, *Pure and Applied Chemistry* 68, No. 10 (1996), p. 1873-1879.

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[4] Medcalf, E.A. et. al., Robust Method for Measuring Low Concentrations if Albumin in Urine, *Clinical Chemistry* 36, No. 3 (1990), p. 446 – 449.

[5] Couvreur, P, Polyalkylcyanoacrylates as colloidal drug carriers, Crit.Rev.Ther.Drug Carrier.Syst., 5 (1988), p. 1-20.

[6] Chavany, C., et. al., Polyalkylcyanoacrylate nanoparticles as polymeric carriers for antisense oligonucleotides, *Pharm.Res.,* 9, (1992), p. 441-449.

[7] Fattal, E., et. al., Biodegradable polyalkylcyanoacrylate nanoparticles for the delivery of oligonucleotides, *Journal of Controlled Release,* 53, (1998), p. 137-143.

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Atto Labels

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