# DELFIA® Sm-Labelling kit 1244-303

## INTENDED USE

This DELFIA® Sm-Labelling kit is intended for labelling of proteins with samarium ($Sm^{3+}$), for use in dissociation-enhanced time-resolved fluoroimmunoassays. $Sm^{3+}$ can be used together with europium ($Eu^{3+}$) in dual label assays.

## INTRODUCTION

Each DELFIA Sm-Labelling kit contains:

- 0.2 mg Labelling reagent
- Sm-Standard and Enhancement Solution for measuring $Sm^{3+}$
- Stabilizer, purified BSA for increasing the stability of labelled proteins
- An uncoated microtitration plate, DELFIA Assay Buffer and Wash Concentrate for testing of labelling results.

Samarium forms a highly fluorescent chelate with ligands present in the DELFIA Enhancement Solution. The long fluorescence life-time enables the use of the chelate in time-resolved fluorometry. The time-resolved principle is applied in fluoroimmunoassays to eliminate background interferences (1,2,3).

The Labelling reagent is the Sm-chelate of $N^1$-(p-isothiocyanatobenzyl)-diethylenetriamine-$N^1$,$N^2$,$N^3$,$N^3$-tetraacetic acid (DTTA) (Figure 1). The DTTA group forms a stable complex with $Sm^{3+}$ and the isothiocyanate-group reacts with a free amino group on the protein to form a stable, covalent thiourea bond (2,4) (Figure 2). The high water solubility and the stability of the chelate, in addition to the mild coupling conditions of the isothiocyanate reaction, enable easy labelling of e.g. antibodies with up to 10-20 $Sm^{3+}$/IgG.

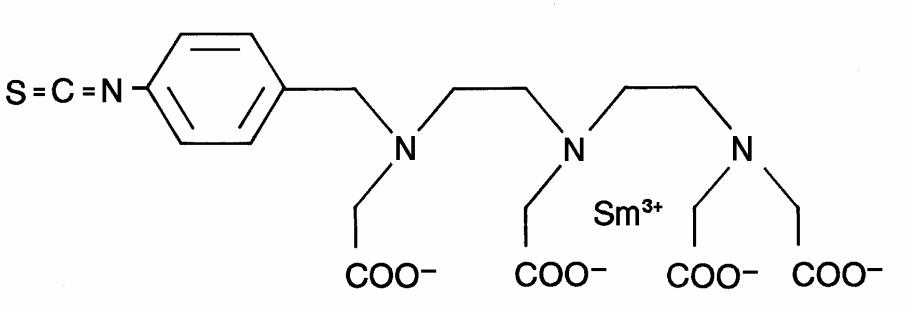


**Figure 1:** Chemical structure of the Sm-Labelling reagent, $N^1$-(p-isothiocyanatobenzyl)-diethylene-triamine-$N^1$,$N^2$,$N^3$,$N^3$-tetraacetic acid chelated with $Sm^{3+}$.

DELFIA is a registered trademark of Wallac Oy.

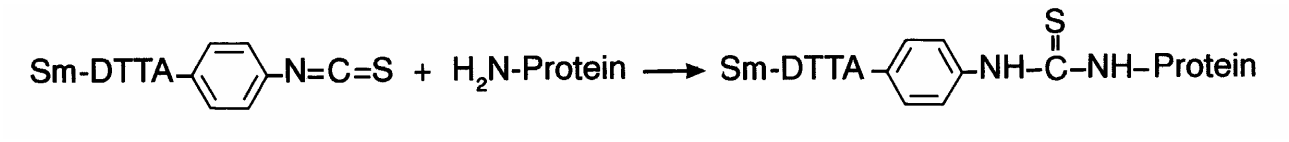


**Figure 2:** The conjugation reaction between the aromatic isothiocyanate group of the labelling reagent and an amino group of a protein.

In order to use a simple gel filtration for fractionation of labelled proteins and separation of proteins from free Sm-chelates, the molecular weight of the protein needs to be at least 5000. For smaller peptides or other amine-containing compounds to be labelled, separate purification systems need to be specifically developed.

The thermodynamic stability of the chelate allows long-term storage of labelled proteins and the kinetic stability allows use of the labelled reagents in assays in contact with e.g. serum samples.

The labelled protein as such is practically non-fluorescent. Consecutively to the immuno-reactions and appropriate washing steps, however, $Sm^{3+}$ is efficiently released from the chelate within a few minutes by the low pH of the Enhancement Solution. Free $Sm^{3+}$ rapidly forms a new highly fluorescent chelate with the components of the Enhancement Solution (2,4). The fluorescence is then measured with the time-resolved fluorometer.

The same Enhancement Solution used for the measurement of $Eu^{3+}$ is also optimal for $Sm^{3+}$, and this enables a simultaneous determination of the two lanthanides. The different main emission wavelengths of the lanthanides (613 nm for $Eu^{3+}$ and 643 nm for $Sm^{3+}$) and their different emission life-times (730 µs for $Eu^{3+}$ and 50 µs for $Sm^{3+}$) serve to minimize the spillover between the respective signals, when measured with separate filters and time-windows (3, 5).

If the Eu-concentration exceeds 0.1 nmol/L the minor peak at 650 nm of the Eu-emission can cause signal spillover into the Sm-channel (643 nm). This Eu-interference, due to its longer decay-time, can be automatically corrected for in the DELFIA Research Fluorometer. This signal overlapping can be calculated also manually by measuring standards with the relevant instrument programs (correction-factors obtained are specific for delay- and window-times used).

## KIT CONTENTS

The expiry date of the complete package is stated on the outer label. Store at +2 - +8°C.

**Reagents**

| Component | Quantity | Shelf life and storage |
|---|---|---|
| Sm-Labelling Reagent | 1 vial, 0.2 mg | < +8°C until expiry date stated on the vial label. |
| 0.2 mg (300 nmol) of $N^1$-(p-isothiocyanatobenzyl)-diethylenetriamine-$N^1$,$N^2$,$N^3$,$N^3$-tetraacetic acid chelated with $Sm^{3+}$, lyophilized. | | |
| Sm-Standard | 1 vial, 0.5 mL | +2 - +8°C until expiry date stated on the vial label. |
| 1 µmol/L of $Sm^{3+}$ in 0.1 mol/L acetic acid. | | |
| Stabilizer | 1 vial, 0.5 mL | +2 - +8°C until expiry date stated on the bottle label. |
| Bovine serum albumin (BSA), 7.5 %, in Tris-HCl buffered salt solution (pH 7.8), containing < 0.1 % sodium azide as preservative. BSA is highly purified from heavy metal contaminants. | | |
| Enhancement Solution | 1 bottle, 50 mL | +2 - +8°C until expiry date stated on the bottle label. (+20 - +25°C: see the bottle label). Avoid direct sunlight. |
| Ready for use Enhancement Solution with Triton X-100[1], acetic acid and chelators. | | |
| Assay Buffer | 1 bottle, 50 mL | +2 - +8°C until expiry date stated on the bottle label. |
| Ready for use Tris-HCl buffered (pH 7.8) salt solution with bovine serum albumin, bovine gammaglobulin, Tween 40, diethylenetriaminepentaacetic acid (DTPA), an inert red dye, and < 0.1 % sodium azide as preservative. | | |
| Wash Concentrate | 1 bottle, 40 mL | +2 - +8°C until expiry date stated on the bottle label. |
| A 25-fold concentration of Tris-HCl buffered (pH 7.8) salt solution with Tween 20. Contains Germall II[2] as preservative. | | |

[1] Triton is a registered trademark of Rohm and Haas Co.
[2] Germall is a registered trademark of Sutton Laboratories Inc.

| | |
|---|---|
| Microtitration Plate | 1 plate |

One uncoated plate of microtitration strips, 8 x 12 wells.

## MATERIALS REQUIRED BUT NOT SUPPLIED WITH THE KIT

1. **Labelling buffer**: Carbonate buffer, 50 mmol/L (pH 9.0 - 9.3), containing 0.9 % NaCl. pH of the buffer can be in the range of 8.5 to 9.8. A high pH, however, may damage sensitive proteins, e.g. some monoclonal antibodies cannot withstand overnight incubation at a high pH and room temperature.

2. **Elution buffer**: 50 mmol/L Tris-HCl (pH 7.8), containing 0.9% NaCl and < 0.1 % $NaN_3$.

3. **Column decontamination buffer**: 10 mmol/L potassium hydrogen phthalate (pH 4.0), containing 0.001 % **d**iethylene**t**riamine**p**enta**a**cetic acid (DTPA), and 0.1 % Germall II or < 0.1 % $NaN_3$ as preservative.

4. **Precision pipettes**: range 10 µL - 500 µL.

5. **Chromatographic system**: Gel filtration columns for changing buffers for proteins prior to labelling and for purification and fractionation of labelled proteins.

   PD-10 columns or NAP™-10[3] columns (Pharmacia Biotechnology) are recommended for removing interfering substances (e.g. $NaN_3$ and primary amines) from the protein solution prior to labelling. PD-10 columns can also be used for crude separation of labelled proteins from unreacted chelates. The use of such short columns may, however, impose a risk of unsatisfactory separation of the protein from free chelates and from possible aggregates.

   Alternatively, Sephadex G-25 or -50 (Pharmacia Biotechnology) with suitable column sizes (e.g. 1.5 x 30 cm) can be used for separation of labelled proteins from free chelates.

   Sepharose 6B (Pharmacia Biotechnology) (e.g. 1.5 x 40 cm) is recommended for fractionation and purification of labelled antibodies from unreacted chelates and aggregated proteins. By combining Sepharose 6B with Sephadex G-50 (e.g. 10 cm of Sephadex on the top of the Sepharose column) the resolution between free Eu-chelates and proteins can be improved.

   Fraction collector, peristaltic pump, UV-detector and tubings.

6. **Spectrophotometer** for measurement of protein concentrations.

7. **Automatic shaker.**

[3] NAP-10 is a trademark of Pharmacia Biotechnology.

8. **1420 VICTOR multilabel counter or alternatively 1234 DELFIA Research Fluorometer with Multi-Calc® [4] software. Additionally laboratory computer and printer.**

## WARNINGS AND PRECAUTIONS

This DELFIA Sm-Labelling kit is intended for research use only.

The handling of concentrated $Sm^{3+}$ solutions constitutes a contamination risk which may cause elevated backgrounds in time-resolved fluoroimmunoassays. Keep the labelling reagents away from the place where the assay is performed. Also ensure that accessories used for the labelling procedure are kept separate from those needed for the assay. Avoid contaminating Sm-reagents with $Eu^{3+}$. Use separate accessories and columns for $Sm^{3+}$ and $Eu^{3+}$.

Reagents contain sodium azide ($NaN_3$) as a preservative. Sodium azide may react with lead or copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.

Disposal of all waste should be in accordance with local regulations.

## PROTEIN LABELLING

### Conditions for labelling

The labelling depends upon the nature and concentration of the protein to be labelled, the temperature and pH of the reaction and the intended final labelling yield. The proteins to be labelled must be in a buffer that does not contain any amines or sodium azide (carbonate buffer, pH 9.0 - 9.3, is recommended). If there is any doubt about the protein stability at higher temperature, labelling at +4°C is recommended.

## LABELLING YIELD

When labelling antibodies for IFMA, generally about 5 - 15 $Sm^{3+}$/IgG is an optimal yield giving high sensitivity with low background. For many assays even a lower labelling yield gives acceptable results. Labelling of antibodies with over 20 $Sm^{3+}$/IgG may occasionally cause aggregation and an elevated background, especially after storage.

The labelling yield needs to be optimized separately for each particular protein and the assay requirements. Especially monoclonal antibodies may behave individually. Table 1 gives examples of labelling yields obtained with different proteins, when labelled according to the protocol described below. The reactivity of different proteins in labelling depends on the number of available amino-groups on the protein surfaces and the isoelectric point. For example avidin is a protein with a high isoelectric point, and accordingly it reacts easily with the reagent. The incorporation of negatively charged chelates on avidin decreases its non-specific binding properties, and thus a relatively high chelate density is optimal (8 - 15

[4] MultiCalc is a registered trademark of Wallac Oy.

$Sm^{3+}$/Protein). In contrast, streptavidin has a lower isoelectric point resulting in lower incorporation, but it has also a lower labelling yield optimum (4 - 10 $Sm^{3+}$/Protein).

| Protein | $Sm^{3+}$/Protein |
|---|---|
| Monoclonal Anti-AFP | 8.5 |
| Monoclonal Anti-TSH | 10.9 |
| Monoclonal Anti-CEA | 8.2 |
| Monoclonal Anti-FSH | 9.8 |
| Bovine gammaglobulin | 10.0 |
| Avidin | 11.8 |
| Streptavidin | 5.5 |

Table 1. Examples of labelling yields for immunoglobulins and avidins.
Labelling was performed according to recommended conditions with 0.5 mg of proteins.

The kinetics of the conjugation reaction depend upon the pH and temperature used (Figure 3). When higher labelling yields are desired, the labelling reaction can be extended to 2-3 days at room temperature, or higher temperatures (up to +37°C) may be used. Similarly, higher incorporation rates are achieved at higher pH's (up to 9.8). On the other hand, if a lower labelling yield is desired a lower pH, lower temperature or shorter reaction time is recommended (Figure 3). The labelling yield is not dependent on the protein amount up to 1 mg, thereafter increasing protein amounts give a slightly decreasing labelling yield (Figure 4).

Figure 3a

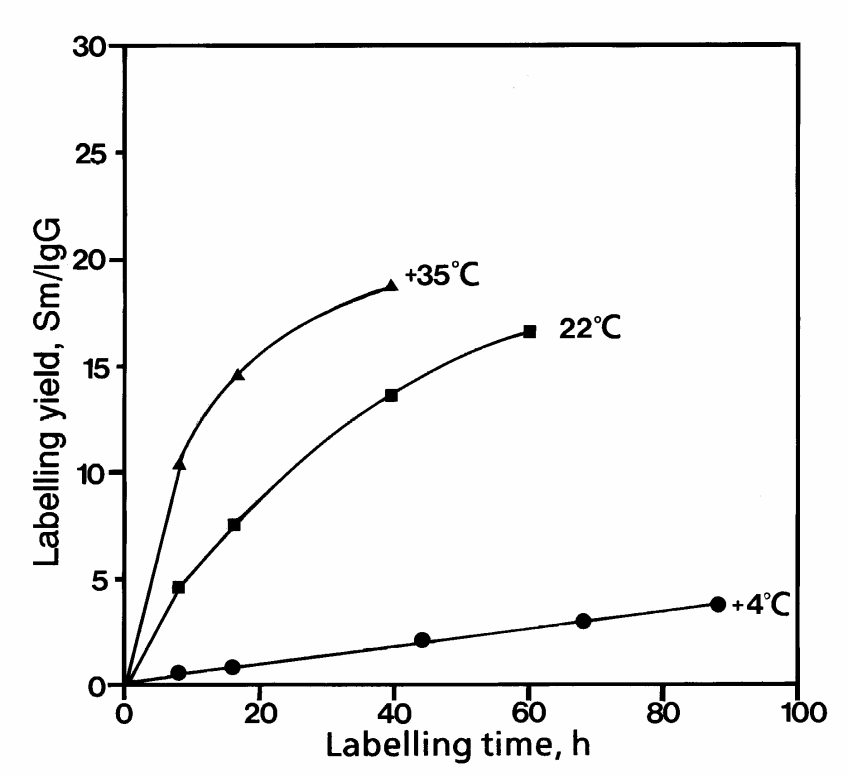

Figure 3b

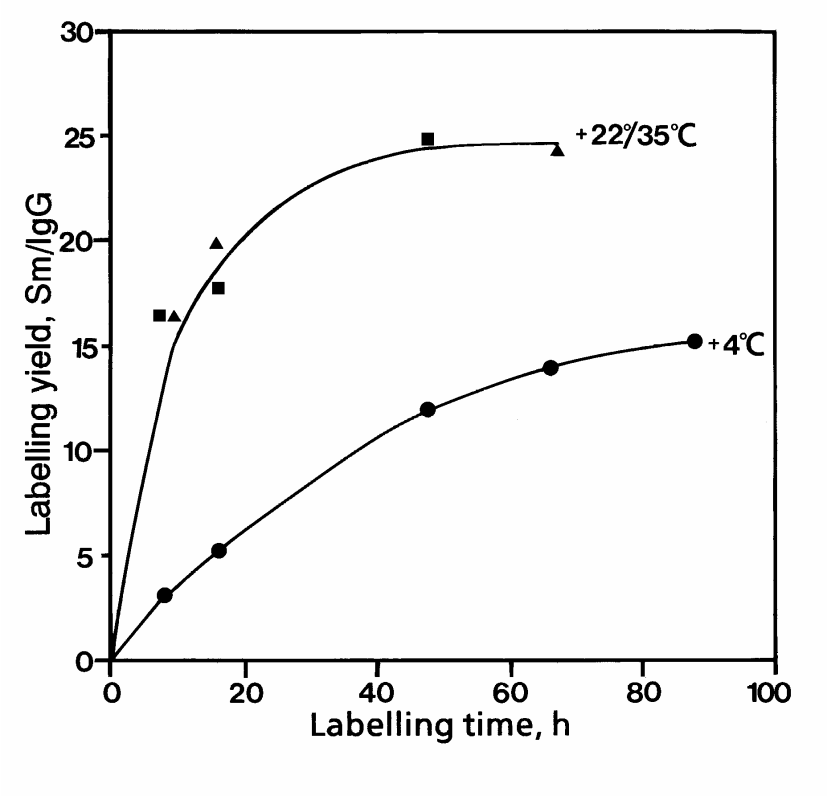


**Figure 3**[5]: Effect of temperature on labelling kinetics at different pHs. One mg of IgG was labelled in 500 µL buffer at indicated temperatures at pH 8.5 (Figure 3a) or pH 9.8 (Figure 3b).

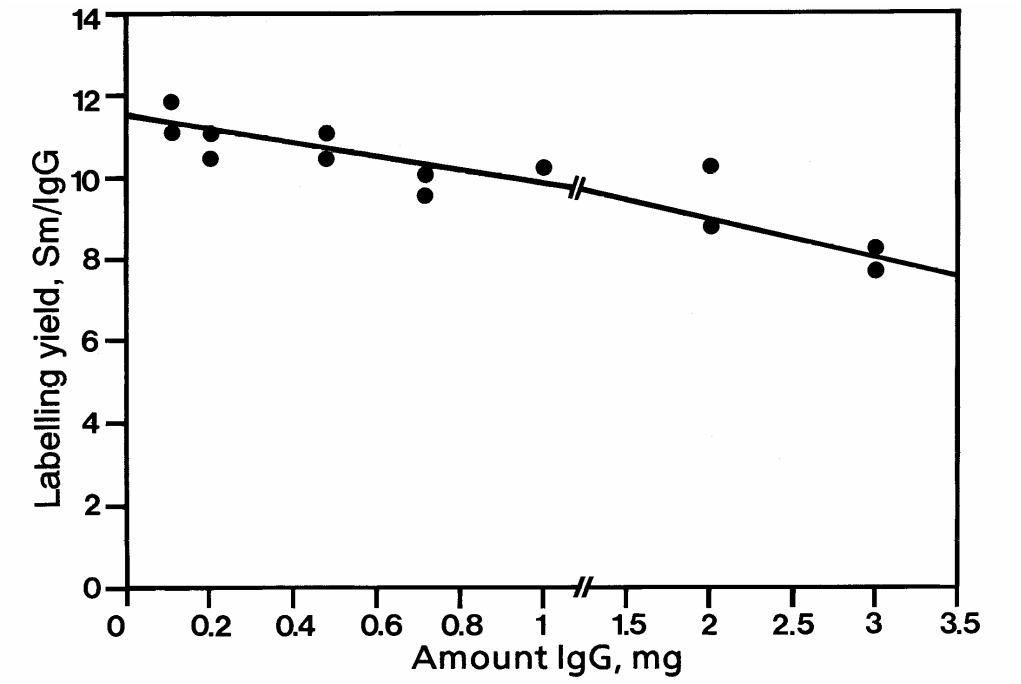


**Figure 4**[6]: The labelling yields obtained when various amounts of bovine gammaglobulin are labelled with the 0.2 mg Labelling reagent. Labelling was performed according to the kit insert.

[5] Study performed at Wallac Oy, Turku, Finland.
[6] as above

## PROCEDURAL NOTES

1. A thorough understanding of this kit insert is necessary for succesful use of the Sm-Labelling kit. The procedure described in this insert is intended for the labelling of an 'average IgG' to a final labelling yield of 5 - 15 $Sm^{3+}$/IgG. It must be taken into consideration that individual antibodies (e.g. monoclonal antibodies) may behave differently with respect to reactivity (labelling yield), and dependence on pH, temperature and reaction times. When working with monoclonal antibodies with unknown characteristics, it may be worth labelling them initially at a low pH (e.g. at 8.3) to a relatively low $Sm^{3+}$/IgG level (2-5 $Sm^{3+}$/IgG).

2. For the labelling, do not use buffers which contain free amines or bacteriostatic agents (e.g. $NaN_3$ interferes with the reaction). Buffers containing even trace amounts of primary amines (e.g. Tris or glycine) or secondary amines (HEPES, MOPS, BICINE etc.) cannot be used.

3. **Do not store labelled proteins in Assay Buffer (prod. nos. 1244-106 and 1244-111) or phosphate buffer.** If during storage of labelled antibodies the background level of the assay tends to increase due to aggregation formation, the labelled antibodies should be filtered through a 0.2 µm membrane.

4. Free $Sm^{3+}$ and $Eu^{3+}$ contaminate the gel filtration column material. Therefore separate columns should be used for purification of Sm- and Eu-labelled proteins. Columns should be decontaminated between purifications by washing the column with decontamination buffer (use a volume of approximately 1/3 of the volume of the column). Re-equilibrate the column carefully with elution buffer before adding sample, since the decontamination buffer contains DTPA.

5. To avoid $Eu^{3+}$ and $Sm^{3+}$ contaminations which can result in a high fluorescence background in assays, high standard pipetting and washing techniques are required. Avoid contaminating pipettes with $Eu^{3+}$ or $Sm^{3+}$ reagents or labelled proteins.

## LABELLING PROCEDURE

### 1. Pre-treatment of proteins to be labelled (buffer exchange):

It is recommended that the buffer be changed to the labelling buffer prior to labelling in order to avoid possible interferences and additional pH adjustment. PD-10 or NAP™-10 columns (Pharmacia Biotechnology) are recommended. When small amounts or diluted solutions of proteins are to be labelled, concentration with an appropriate method may be necessary, or buffer exchange may, alternatively, be done e.g. by dialysis.

**1.1.** Equilibrate the PD-10 column with 25 mL of labelling buffer.

**1.2.** Add the protein solution to the column and rinse with labelling buffer (do not exceed a total volume of 2.5 mL).

**1.3.** Collect the protein fraction after the 2.5 mL void volume. Because the labelling procedure is for 500 µL volume of pre-treated protein, it may be worth collecting the protein in as small a volume as possible. The maximum volume of protein for buffer

exchange (2.5 mL for PD-10) produces 3.5 mL of eluted protein solution. (Respective volumes for NAP™-10 columns are 1 and 1.5 mL). Smaller protein volumes can be eluted with smaller buffer volumes provided that the protein elution is monitored e.g. by absorption measurement.

**2. Labelling:**

**2.1.** Open the Sm-Labelling reagent vial carefully.

**2.2.** Add 500 µL of the protein in the labelling buffer (max. ~1 mg) to the reagent vial.

**2.3.** Mix gently to dissolve the reagent and incubate overnight at room temperature (14-18h, +20 - +25°C).

**3. Purification:**

**3.1.** Equilibrate a chromatography column with 3 x void volume of elution buffer. If the column has been used earlier with Eu-labelled proteins, it is recommended to wash it with decontamination buffer prior to the equilibration. Add the reaction mixture to the re-equilibrated column and rinse the labelling vial with a small volume of elution buffer and elute with the same buffer.

**3.2.** Monitor the eluate by UV-absorbance at 280 nm, collect fractions of 1 - 2 mL.

**3.3.** Measure $Sm^{3+}$ concentrations in the fractions by measuring their fluorescence after appropriate dilution with Enhancement Solution (1:1000 - 1:10 000, e.g. by serial dilution of 10 µL with 990 µL Enhancement Solution twice). Use a clean (rinsed with Enhancement Solution) plastic pipette tip to dispense 200 µL of diluted samples into the microtiterstrip wells. Mix gently using a plateshaker for about 5 minutes before measuring with the time-resolved fluorometer.

**3.4.** Pool fractions containing the labelled proteins (Figure 5). Avoid pooling aggregated proteins.

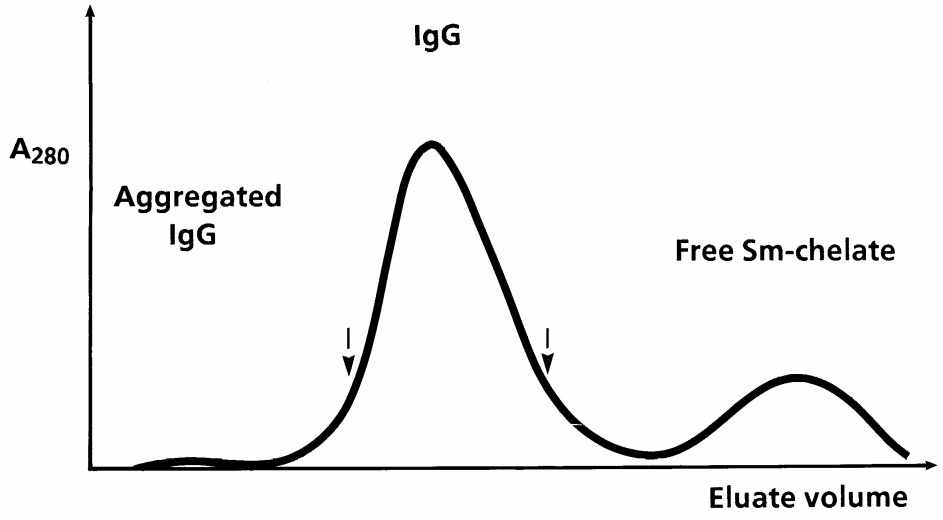


**Figure 5**: The elution profile of labelled IgG from a column of Sephadex G-50 and Sepharose 6B. The fractions recommended to be pooled (monomeric IgG) are indicated with arrows.

## 4. Characterization of the labelled proteins:

**4.1. $Sm^{3+}$ Content:** The exact concentration of $Sm^{3+}$ after dilution with Enhancement Solution (1:10 00 - 1:10 000), is calculated by measuring the fluorescence in microtiterstrip wells (200 µL/well; in duplicates) and comparing to the fluorescence of 10 nmol/L Sm-standard (stock standard diluted 1:100 in Enhancement Solution).

**4.2. Protein Content:** Protein concentration in the pooled fractions can be measured with appropriate methods, e.g. Lowry's method, or it can be calculated from the protein absorbance at 280 nm after subtracting the absorbance of the formed aromatic thiourea-bonds (0.008/µmol/L).

**4.3. Eu-Contamination:** The original Eu-contamination level of Sm-Labelling reagent is below 0.02%. A relatively small additional amount of Eu-contamination can cause a signal in the Eu-channel in dual label assays. Possible contamination of the labelled protein can be checked by measuring the fluorescence in both Sm- and Eu-channels. The spillover can be calculated from these signals and is used for correction of the results.

**4.4 Calculations:** For IgG the following equations can be used. They are valid when the labelling yield is < 20 $Sm^{3+}$/IgG. 1.34 is used for absorptivity value (for 1 mg/mL) of IgG, and 160 000 for MW.

$$Sm^{3+}\ (\mu mol/L) = \frac{\text{Sm-counts x dilution factor}}{\text{100 x counts of 10 nmol/L } Sm^{3+}}$$

$$\text{Protein (mg/mL)} = \frac{\text{Abs(280) - 0.008 x } Sm^{3+}(\mu mol/L)}{1.34}$$

$$\text{Protein (µmol/L)} = \frac{\text{Protein (mg/mL) x 1 000 000}}{\text{160 000 (g/L)}}$$

$$\text{Yield } (Sm^{3+}/IgG) = \frac{Sm^{3+}\ (\mu mol/L)}{\text{Protein (µmol/L)}}$$

$$\text{Recovery (\%)} = \frac{\text{100 x Protein (mg/mL) x volume of pooled fractions (mL)}}{\text{Protein added (mg)}}$$

## 5. Storage:

**5.1.** To increase the stability of labelled proteins, the Stabilizer (purified BSA) can be used as a carrier protein at a final concentration of 0.1 %. Store labelled proteins at +4°C. Repeated freezing and thawing should be avoided.

In cases where bovine albumin cannot be used as a carrier protein, the labelled proteins could be stored as such, if the protein concentration is over 50 µg/mL. If other proteins are used as carriers, these **need to be purified from any heavy**

**metal contaminations** prior to addition. The carrier used also needs to be free from chelating agents.

## USE OF LABELLED ANTIBODIES

Sm-labelled immunoreagents can be applied in different types of immunoassays based on solid-phase separation (e.g. competitive or non-competitive assays). The design of an immunoassay depends on the analyte, the antibodies, the possibility of using a sandwich-type assay or the need to employ a competitive assay-design, the required sensitivity and dynamic range etc.

As a general rule, about 25 - 100 ng of labelled antibodies per well is enough for non-competitive sandwich-type assays, but the actual optimal level depends on the purity and affinity of the antibodies and the desired signal levels. For competitive assays no general rules can be given and the assay always has to be separately optimized.

DELFIA Assay Buffer (prod. nos. 1244-106 and 1244-111) is optimal for most assays. In some assays, additional components might be needed to overcome cross-reactivity problems.

Sm-labelled immunoreagents are suitable for use in dual-label assays together with $Eu^{3+}$ as the second label because the same DELFIA Enhancement Solution is optimal for their measurement. $Eu^{3+}$ gives higher fluorescence ($Q_{Eu}$ = 70 %), and is recommended for use in assays requiring higher sensitivity. Additionally, assays should be optimized to give relatively high signal levels of $Sm^{3+}$ in order to compensate for the lower fluorescence intensity of $Sm^{3+}$-chelates ($Q_{Sm}$ = 2 %).

The minor spillover from Eu-emission to the Sm-channel can be corrected, where necessary, automatically or manually. If the Sm-standard dilution gives a signal in the normal Eu-counting channel, it implies Eu-contamination in reagents. Calculated theoretical signal spillover is about 0.01 %, which corresponds to a Eu-contamination level of 2 ppm in the Sm-reagent. Generally Eu-contamination in Sm-Labelling reagents is about 100 ppm, but may be increased during labelling and purification. In double-label assays this contamination spillover may be subtracted by using a correction factor measured for each particular labelled protein (see 4.3).

## WARRANTY

The performance data presented here is obtained using the labelling procedure indicated and antibody solutions without interfering compounds. In the indicated conditions the labelling reagent is able to react with available free aminogroups of proteins. Change of buffers or variations in protein characteristics can cause alterations in the labelling reaction.

The reagent is covered by patents on both the chemical structure (6) and use in DELFIA-type assays (7). Purchase of this reagent gives the purchaser the right to use this material in his own research. Further distribution of this reagent or products resulting from its use is expressly prohibited. Purchase of this product implies agreement with these conditions of sale.

**REFERENCES**

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