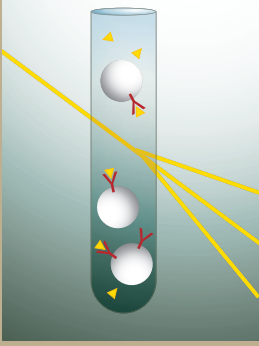




# IMM Immunoturbidimetric Automated Latex Assays





# Introduction

*These past years there was a tremendous development in laboratory automated instruments which allowed automating most of the laboratory assays, and more especially immunoassays. This breakthrough is expanding to all the diagnostic domains, including specialized testing (1). Complete dedicated systems are available for specific immuno-technologies, but many immunoassays have been designed for being used on any photometric automated instrument (2, 3, 4). Latex based immuno-turbidimetric assays are meeting increasing interest and concern today a wide range of applications, as they can be adapted to many laboratory instruments (5, 6, 1), and they only require a reagent pipetting step, in addition to specimen distribution, and measurement of absorbance. The reaction can be read at variable wave lengths. The high popularity of these reagents comes from their suitability for any photometric equipment, their high flexibility and the ease to use of reagents, which offer usually a great stability and are in the liquid presentation.*

*In this application note, our goal is to analyse the various factors which can affect the reagent performances and the assay characteristics, including the assay design and development and adjustment of sensitivity threshold and dynamic range. Assay performance characteristics are highly dependent on the latex used for this application (polymer type, size, chemical functions and their density, coupling method for antibodies or antigens...) the immunological reactant profile (affinity, specificity and avidity of the antibody used for example), and the assay conditions (wave length used, reaction buffer, ...). This technology can be applied to a wide range of tested analyte concentrations, from below 1 µg/ml to > 1 mg/ml. Latex particles can be coated by simple adsorption or through covalent coupling and therefore present a high reagent stability.*

# Assay principle

Latex turbidimetric assays are based on the use of latex particles for enhancing the antigen-antibody reaction and quantization of formed immun-complexes thanks to a photometric measurement. This reaction is then quantitated by the changes in the scattering/diffusion of a monochromatic light beam (7, 8), which is dependent on the aggregates concentration and size. Latex particles offer an important contact surface between antibody and antigen, and they allow increasing dramatically the immun-complexes' size, rendering them measurable by photometry, even at low concentrations. Usually, unreacted latex particles have a small size (diameter range from 0.05 to 0.30 µm) and do not adsorb, or only adsorb slightly light, when the wave length is higher enough than the particle size (measurement wave lengths used are frequently in the range of 405 nm to > 900 nm). Large latex aggregates (induced by the antigen-antibody reaction) are formed during the immunological reaction, and they have a much larger sizes than the unreacted particles (> 1,000 nm), higher than the measurement wave length used. Light is then adsorbed in a dose dependent concentration, and this absorbance is dependent on the aggregates size and amount. There is a relationship between the measured absorbance and the tested antigen (or antibody if antigen is bound onto the latex particles' surface) concentration. When a light beam irradiates a particle suspension, if particles have a smaller size than the wave length, light is homogeneously scattered in the different directions, but most of the light is nevertheless transmitted and measured. Particles are then "almost transparent". This is the "Rayleigh" light scattering model, and light scattering is dependent on the wave length used. Nephelometric measurements are possible by using this principle of light scattering by very small particles. By contrast, when particles size is higher than the wave length used, light is more adsorbed but poorly scattered and mainly transmitted through the beam front. In this case, there is a significative light absorption, dependent on the particle concentration. This is the "Mie" light scattering model, and it is rather independent on the wave length used. This is this latter model which applies to latex immunoassays.

# Designing the Latex automated assays

## Materials and methods : Latex particles, antibodies and chemicals

Various types of Latex particles can be used for coupling antibodies through a covalent bond or by direct adsorption. Latex particles used for automated latex assays should have preferentially a size ranging from 0.05  $\mu\text{m}$  to 0.30  $\mu\text{m}$  for an optimized reactivity. Higher sizes generate a too high blank value, and sensitivity is then decreased when the change in absorbance corresponding to the antigen-antibody reaction is measured. Figure 1 presents Absorbances measured at various wave lengths for 0.1 % unreacted latex particles, of different sizes and made of different polymers. Working sizes below the optimal size range reported is possible, but as the reactive surface is then increased, more antibodies (or antigen) are required for covering it, which will increase the assay cost. Furthermore, using too small particles will also require more aggregates for producing a measurable reaction.

Various latex polymers can be used for designing the assays, such as polystyrene or styrene-acrylate. When a covalent coupling is used, chemical functions must be available on the latex surface, at a concentration enough for allowing a complete and stable binding of proteins or antibodies (surface density of these chemical functions defines the "parking area", and higher is the chemical functions density lower is the "parking area" value).

Many functional groups are available such as  $-\text{COOH}$ ,  $-\text{NH}_2$ ,  $-\text{SH}$ ,  $-\text{CH}_2\text{Cl}$ ,  $-\text{OH}$ ,  $-\text{SO}_3$ , etc. Very convenient particles are those with  $-\text{COOH}$  groups, which can be activated with carbodiimide for reacting with  $\text{NH}_2$  present on proteins or antibodies, or with  $-\text{CH}_2\text{Cl}$  groups, which can react directly with  $\text{NH}_2$ .

In our applications, we usually work with polystyrene particles with sizes from 0.05 to 0.30  $\mu\text{m}$ , which can be coated directly with antibodies (or antigens) using a simple adsorption step, at pH between 7.00 and 9.60. However, we preferentially work with carboxylic particles ( $-\text{COOH}$  groups available), made of polystyrene or styrene-acrylate polymers, at a sufficient density (parking area below 100  $\text{A}^2$  per functional group, which corresponds usually to 50–500  $\mu\text{Eq}/\text{gram}$ , for particles size ranging from 0.05 to 0.30  $\mu\text{m}$ ). This concentration of functional groups, for a same "parking area", varies in an indirect relationship of the particle size. Therefore, when latex particles with lower or higher sizes are used functional group densities can be outside this range.

Another critical factor is the choice of the immunological reactants, and usually that of antibodies, which must have the required specificity, but also an optimized affinity and avidity. Polyclonal or monoclonal antibodies can be used, depending on the analyte to measure. When monoclonal antibodies are used the target antigen must expose the corresponding epitope repetitively on the molecule in order to allow aggregation of particles, and light adsorption. Alternatively, a mixture of several monoclonal antibodies targeted to different epitopes exposed on the target protein can be used (polyclonal antibody). For obtaining a good reactivity, antibodies used must have a high affinity and avidity for the antigen. When polyclonal antibodies are used, it is better to work with affinity purified antibodies, in order to get the greatest immuno-reactive surface on latex beads, and also for avoiding binding of irrelevant antibodies (present in global immunoglobulin fractions extracted from polyclonal antisera), which could result in a decreased reactivity. Concerning chemicals, carbodiimide, especially EDAC [1-Ethyl-3-(3-Diethylaminopropyl)-Carbodiimide], is frequently used for activating  $-\text{COOH}$  groups and reacting them with  $-\text{NH}_2$  groups. Following adsorption or coupling of antibodies, latex particles need to be stabilized by incubating them with the appropriate buffer (usually phosphate, borate or glycine containing 0.15 M Sodium Chloride) in presence of blocking agents such as bovine serum albumin, gelatine (hydrolysed or not), polymers (such as poly-ethylene-glycol), and if necessary substances blocking heterophilic antibodies or rheumatoid factor, which can induce false positive reactions when present. For this latter goal, irrelevant monoclonal antibodies, or human or animal normal immunoglobulins (gamma fractions), native or heath aggregated, can be used.

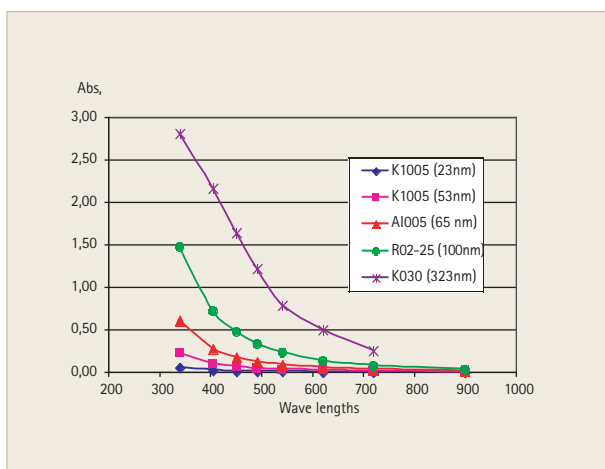


Figure 1: Basic latex microparticle adsorption (0,1 % concentration) at variable wave lengths for different latex beads.

## Preparing the latex immunological reagent

Usually, latex particles are stabilised with surfactants, required for obtaining the expected homogeneity of the colloidal suspension. These surfactants can interfere in the antibody binding onto the latex particles, and then reduce their binding efficacy. They need to be removed by successive centrifugations and washings of the particles, or by dialysis. The washed particles, mainly surfactant free, can then be used directly for binding the antibodies, through a simple adsorption step or through a covalent coupling procedure. Following each step, particles must be resuspended and homogenized. Shaking them thoroughly, rolling them or using sonification is necessary. This is sometimes difficult to achieve, and latex aggregates can remain. Washed latex suspension is then ready for the chemical activation, when required, or for the direct adsorption. With most of activation procedures, latex particles need to be washed again, before addition of the ligand, for removing the excess of coupling chemical. The particles can then be reacted with the antibodies or the required ligand. The optimal antibody concentration should be enough for covering the available latex surface. Washing is again required when the ligand (antibody, or antigen or any other biologically active molecule) has been reacted with particles, usually at an excess concentration, and in order to remove that excess. The required ligand concentration for covering the latex bead surface is an indirect relationship of the latex particle size.

## Performing the assay

Automated latex immunoassays are performed in a very simple way. The assay principle is presented on figure 2. Tested specimen (50 to 250  $\mu$ l), undiluted or already diluted in a reaction buffer, is introduced in a reactive cuvette, and additional reactive buffer is eventually added (0 to 250  $\mu$ l); latex reagent (0.1 % final latex concentration, i.e. 1 mg/ml) is introduced (usually 100 to 250  $\mu$ l) and mixed. This starts the reaction, and the absorbance is recorded (at a wave length which can be from  $< 400$  nm to  $> 900$  nm) from 20 seconds (for allowing the assay mixture to homogenise) up to 5 to 20 minutes. The change in absorbance is measured. A calibration curve can be established with the appropriate calibrator. There is then a direct relationship between the analyte concentration and the change in absorbance. The assay can be adapted to any laboratory analyser, or also used with the microplate format.

For the current sizes used, from 0.05 to 0.30  $\mu$ m, the antibody concentrations range usually from 15 mg (small particles) to 2 mg (larger particles) per 100 mg latex particles (i.e. 1 ml of a 10 % latex suspension). Finally, the final latex suspension is diluted in a stabilization milieu, containing buffer, frequently a chemical with amino groups (for neutralizing unreacted activated chemical residues), such as glycine or ethanolamine, sodium chloride, a protein such as Bovine Serum Albumin or a polymer such as Poly-Ethylene-Glycol, and a preservative such as Sodium Azide (NaN<sub>3</sub>). Alternatively, latex particles can be dialyzed following the chemical activation step, in order to remove surfactants and reactive chemicals in excess. In our experience, we prefer avoiding the time consuming and technically difficult centrifugation steps, and to restrict use of dialysis only when necessary. We privilege calculating the exact amount of reactants required for activating and coupling the latex particles. Then, reactants and ligands are no more in excess. The coated particles are then treated in a homogeneous phase. The stabilization buffer is critical in order to allow long term preservation of the reagent characteristics and for avoiding particles aggregation. For photometric (turbidimetric) latex immunoassays, the working latex suspension is usually at a concentration between 0.1 and 0.5 %.

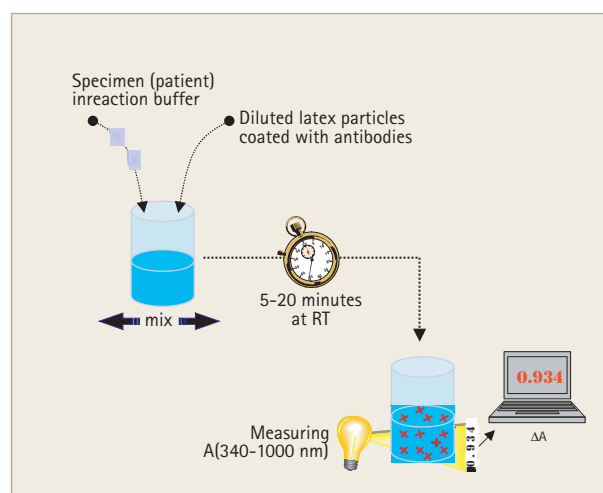


Figure 2: Principle of Automated Latex Immuno-Assays

# Examples of Applications

We present here after some applications developed with Estapor® latex particles coated with antibodies (polyclonal or monoclonal) for the assay of some plasma proteins.

## Automated measurement of hs-CRP (high sensitivity) or of Fibrinogen in plasma

We use 10 % carboxylated styrene-acrylate latex particles (A1-005), with a size of 0.05 to 0.10  $\mu\text{m}$  and with at least 150  $\mu\text{Eq}$  of  $-\text{COOH}$  functions per gram. These particles are activated with carbodiimide and then extensively dialysed against distilled water. Antibodies, at a concentration of about 7.5 mg per ml of 10 % latex particles solution, are then added in a 0.05 M Phosphate, 0.15 M Sodium Chloride buffer at pH 7.50 and let to react for 2 to 24 hours. The reaction is terminated by the addition of glycine buffer, containing PEG, Bovine Serum Albumin and Sodium Azide as preservative and thoroughly shaken for several hours. The solution is then brought to 0.1 % latex concentration and is ready to use.

CRP is measured in plasma or serum with the following protocol: in a reactive cuvette 100  $\mu\text{l}$  of plasma or serum and 150  $\mu\text{l}$  of reaction buffer are introduced; the reaction is started by the introduction of 250  $\mu\text{l}$  of the 0.1 % latex reagent, and mixed. The change in absorbance is recorded at 620 nm from 20 seconds to 10 minutes. The assay has a dynamic range from 0.2 to 10  $\mu\text{g/ml}$  of CRP. Calibration can be performed by reference to the NIBSC International Standard for CRP. If necessary other wave lengths, such as 405 nm, 450 nm, or 540 nm can be used. Figure 3 shows the dose-response curves obtained at various wave lengths, for that assay. Lower is the wave length and higher are the Absorbances measured. We currently chose to work at 620 nm as the plasma interference is low at this wave length. A similar assay was developed for fibrinogen, and the dose response curve is presented on figure 4.

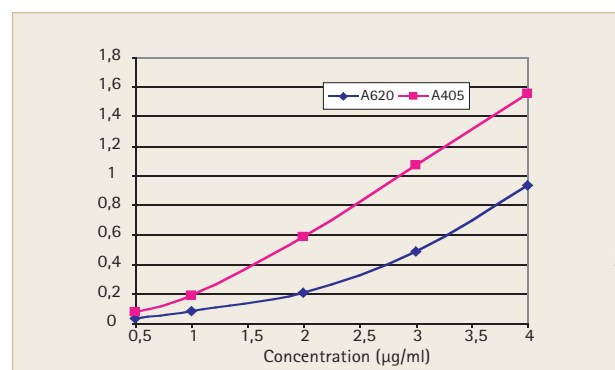


Figure 3: Calibration curve for the Assay of hs-CRP with latex microparticle, at two lengths (405 and 620 nm)

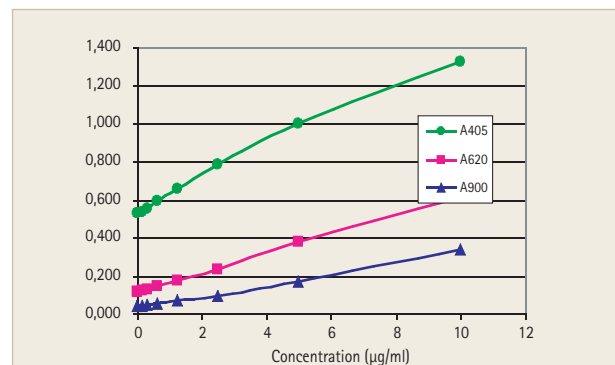


Figure 4: Calibration curve for the Assay of Fibrinogen with latex microparticles (405, 620 or 900 nm)

## Automated measurement of Coagulation Free Protein S in plasma

Another application was developed with the chloro-methyl polystyrene latex particles with a size of 0.100  $\mu\text{m}$ , and with at least 100  $\mu\text{Eq}$  of  $-\text{CH}_2\text{Cl}$  functions per gram. To a mouse monoclonal antibody specific for Protein S, dialysed in a 0.05 M phosphate, 0.15 M Sodium Chloride buffer at pH 7.50, the latex particles at 10 % concentration were added under vigorous shaking. We currently use 10 to 15 mg of antibodies per 100 mg of latex particles of the 0.100  $\mu\text{m}$  size, i.e. per 1 ml of the 10 % latex suspension. The mixture is let to react for 4 - 24 hours at room temperature, under continuous shaking, and the reaction is stopped by the addition of the stopping solution, as here above.

The latex particles are used at 0.1 % concentration for the photometric assay. The assay is performed by introducing in the reactive cuvette 100  $\mu\text{l}$  of tested plasma, undiluted or diluted, 100  $\mu\text{l}$  of reaction buffer and 200  $\mu\text{l}$  of 0.1 % latex particles (only half these volumes are used for micro plates). The change in absorbance is recorded between 20 seconds and 5 minutes at 620 nm (as for CRP, other wave lengths are possible). Assay calibration is established by reference to the NIBSC International Standard for plasma Protein S. The dynamic range is from 0.5  $\mu\text{g/ml}$  to 10  $\mu\text{g/ml}$ . The change in absorbance is of 0.600 to 0.900 for the 10  $\mu\text{g/ml}$  concentration.

# Conclusions

Automated latex immuno assays have been applied to many different analytes, such as blood coagulation proteins (our examples), DDimer (2), PAI-1 (8), Fibrin Complexes (9), CRP (10, 11), Lipo-Protein (a) [Lp(a)] (3), Ferritin (11, 5, 6), Adiponectin (12), Bence Jones proteins (13), 1-microglobulin (14), Theophylline (1), etc. They present the great advantage to being applicable to most of the automatic laboratory analysers, as they require only 2 to 3 pipetting steps for performing the assay. Reagents are in the liquid presentation and offer a good stability (12 months and more). Protein concentrations measured can range from as low as  $< 0.10 \mu\text{g/ml}$  up to  $> 1.00 \text{ mg/ml}$ . When using competitive methods for testing haptens, which are usually linked to a carrier protein, higher sensitivities can be obtained. Although designing and running the latex based turbidimetric assay is easy and simple, preparing the reagent requires much care, skill and experience. Well established and described methods are available for preparing the latex reagent, which is the critical factor for ensuring the right assay performances. But they need to be accurately adjusted for each application, and according to the antibody characteristics used. The small size Estapo<sup>®</sup> latex particles are very convenient for developing those assays.

For these applications the carboxylated styrene-acrylate (A1 series) or the chloro-methyl (R02 series) latex particles were found the most useful, and easy to optimise and to handle. There is an increasing interest for these latter chloro-methyl styrene latex particles, as they offer pre-activated groups allowing a direct binding of any ligand with amino groups, such as proteins.

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Merck Chimie SAS - France  
Estapor® Microspheres,  
Division PLS - Performance & Life Science Chemicals  
201, rue Carnot  
F-94 126 Fontenay-sous-Bois Cedex  
Tel: 33 1 43 94 54 92  
Fax: 33 1 43 94 54 96  
E-mail: [cecile.guignard@merck.fr](mailto:cecile.guignard@merck.fr)  
Web-site: [www.estapor.com](http://www.estapor.com)

