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Application Note

Development of a Quantitative Lateral Flow Test Using Estapor® Europium Microspheres

Introduction

Lateral flow test strips faciltate user friendly, rapid, low-cost analysis for clinical diagnostics. Advancements in lateral flow tests have largely focused on increasing the sensitivity of analyte detection.[1] Typically, gold nanoparticles or dyed microspheres are used for qualitative analysis. The development of quantitative tests often uses organic fluorophores[2] to achieve increased sensitivity. Pain points associated with fluorophores include 'photobleaching', which can compromise sensitivity. Europium chelate microspheres are an alternative to traditional fluorophores that can bypass this limitation. Europium chelates possess a long Stokes' shift (the difference between the peak excitation and the peak emission wavelengths) and a prolonged decay time.[3] These characteristics, which eliminate the background fluorescence associated with the use of many existing fluorophores, qualify europium microspheres as highly desirable reagents in highly sensitive, quantitative immunoassays. The following protocol outlines a step by step guide to developing a quantitative lateral flow test using Estapor® Europium Microspheres. The protocols for microsphere conjugation and test strip manufacture may be applied to different antibodies for the detection of alternative antigens.

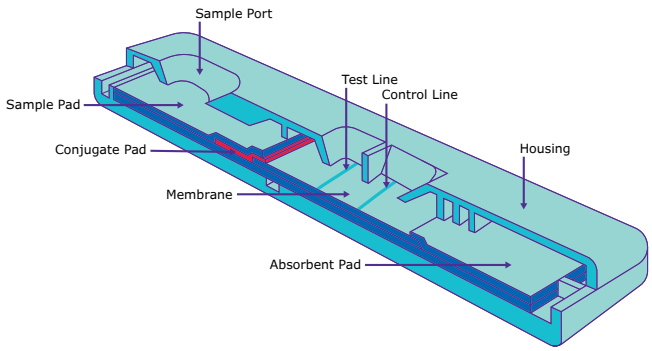

Fig. 1 Lateral flow test strip design

Table 1 Product Description

Product Information	Description
Product name	F1-Eu- 030
Catalog number	80380624
Trade name	Estapor®
Description	Europium Microspheres
Functional group	COOH
COOH content (range)	200 – 500 µeq/g
Solid concentration	1%
Polymer	Polystyrene
Diameter (range)	0.270 - 0.330 µm
Ex (max), Em (max)	365 nm, 610 nm

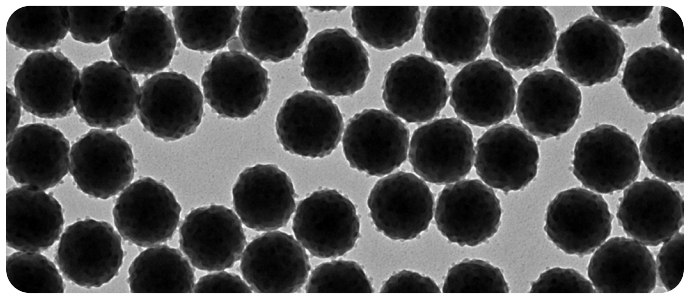

Fig. 2 TEM analysis of Estapor® Europium Microspheres

Europium Microspheres:

- Significantly improve lateral flow assay sensitivity
- Reduce background fluorescence
- Make assays easier to read and quantify
- Exhibit a longer Stokes shift than traditional fluorescent labels
- Exhibit an enhanced fluorescent quantum yield facilitating a low detection limit
- Functionalized with a carboxylated surface for protein conjugation
- Available in three different size options

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Table 2 Summary of key components and reagents

Components	Reagents
Sample	Myoglobin-free diluted human serum spiked with myoglobin (serum at 20% when diluted)
Sample Pad	Cellulose fibre sample pad, treated with 10 mM Tris, 1% Tween® 20, 0.075% w/v BSA, pH 8.2
Conjugate Pad	Glass fibre conjugate pad
Detector Microspheres	Estapor® Europium Microspheres conjugated with myoglobin antibody MABX7612
Membrane	Hi-Flow™ Plus 135
Test Line	Myoglobin antibody MABX7611
Control Line	Anti-mouse IgG
Absorbent Pad	Cellulose fibre pad, untreated

Table 3 Recommended buffers and solutions

Buffer	Solutions
Activation/ Coupling buffer	50 mM MES, pH 6.0.
EDC solution	200 mM EDC. Add 19.2 mg of EDC at room temperature to 500 µL Milli-Q® water.
Sulfo-NHS solution	200 mM Sulfo-NHS Add 21.7mg Sulfo-NHS to 500 µL activation/ coupling buffer.
Blocking buffer	50 mM Tris, pH 8.0, 0.5% (w/v) casein (pH adjusted with 4 M HCl).
Conjugate pad diluent buffer / serum diluent	50 mM Tris, 0.5% casein, 10% sucrose, 2.5% trehalose, 0.5% PVP pH 8.0 (adjusted with HCl).
Sample pad buffer	10 mM Tris, 1% Tween®-20, 0.075% BSA, pH 8.2 (adjusted with HCl).
Capture antibody diluent	10 mM MES, pH 6.0.

Materials

Estapor® Europium Microspheres

- Sigma-Aldrich®, 80380624

Equipment/ Plastics

- Oven or incubator at 37 °C
- Fine wire mesh tray
- Centrifuge (Eppendorf® 5430 with FA-45-24HS rotor) at 11,900 x g
- Kinematic Matrix™ 1600 Reagent Dispensing Module
- Kinematic Matrix™ 2360 Programmable Shear
- Kinematic Matrix™ 1201 Membrane Cutter
- Rotary wheel, Stuart Rotator SB3
- Vortex mixer
- Water bath sonicator, BRANSON 2800
- ESEQuant® LR3 reader (customized to detect europium signals)
- UV viewing cabinet (optional)

Reagents

- MES (Sigma-Aldrich®, M8250)
- D-(+)-Trehalose dihydrate (Sigma-Aldrich®, T5251)
- Sucrose (Sigma-Aldrich®, S7903)
- Polyvinylpyrrolidone (PVP), mol wt 10,000 (Sigma-Aldrich®, PVP10)
- Casein from bovine milk (Sigma-Aldrich®, C7078)
- Sigma 7-9® (Tris base) (Sigma-Aldrich®, T1378)
- Bovine Serum Albumin (Sigma-Aldrich®, A2153)
- Tween® 20 (Sigma-Aldrich®, P7949)
- 37% Hydrochloric Acid (HCI) (Sigma-Aldrich®, 320331) diluted to 4M
- EDC (ThermoFisher, 24510)
- Sulfo-NHS (ThermoFisher, 22980)
- Ethanolamine (Sigma-Aldrich®, 411000-100 mL)
- SureWick® GFDX Glass Fibre Conjugate Pads (Millipore®, GFDX203000)
- SureWick® C083 Cellulose Fibre Sample Pad (Millipore®, CFSP223000)
- Hi-Flow™ Plus 135 membrane, 2-mil backing, (Millipore® HFBB13502S25)
- Adhesive backing cards (Millipore®, HF000MC100)
- Aluminium foil
- Human myoglobin analyte (HyTest (Finland), 8M50 @ 0.1 mg/mL)
- Myoglobin-free human serum (HyTest (Finland), 8MFS)
- Anti-Myoglobin, clone 1 (Sigma-Aldrich®, MABX7611)
- Anti-Myoglobin, clone 2 (Sigma-Aldrich®, MABX7612)
- Goat Anti-Mouse IgG (Sigma-Aldrich®, M8890)

Procedure

Day 1 of 2

Conjugation of detector antibody (MABX7612) onto europium microspheres

Important: Keep europium microspheres in the dark, i.e. wrap microfuge tubes with aluminium foil when possible.

Coat 60 mg antibody per gram of microspheres.

1. Aliquot 500 µL of europium microspheres at 1% w/v into a 2 mL low protein binding tube.
2. Add 1 mL activation/coupling buffer and mix thoroughly.
3. Centrifuge for 7 minutes at 11,900 x g.
4. Decant supernatant and resuspend microspheres in 1 mL activation/ coupling buffer. Mix well by pipetting up and down so that no clumps are visible.
5. Repeat steps 3 and 4.
6. After the final wash resuspend the microspheres in 1 mL activation/ coupling buffer.
7. Sonicate the microspheres for 30 seconds to ensure microspheres are not aggregated. At this time a visual check can be performed to ensure microspheres are not aggregated.

   Note: Single microspheres are difficult to see under 400X magnification and will appear as a hazy sea of particles. Aggregated microspheres, however, are easily observed under 400X magnification.
8. Prepare EDC and Sulfo-NHS reagents immediately prior to use (see Table 2).
9. To 1 mL washed microspheres (from step 7) add 12 µL of 200 mM EDC and 120 µL of 200 mM Sulfo-NHS.
10. Vortex and mix on a rotary wheel for 30 minutes at room temperature
11. Centrifuge for 7 minutes at 11,900 x g.
12. Decant supernatant and resuspend microspheres in 1 mL activation coupling buffer – mix well by pipetting up and down (no clumps should be visible after the microspheres are resuspended).
13. Repeat steps 11 and 12.
14. After the final wash resuspend the microspheres in 850 µL activation/ coupling buffer.
15. Sonicate the microspheres for 30 seconds to ensure microspheres are monodisperse (refer to step 7).
16. Prepare the detector antibody (MABX7612) at a concentration of 2 mg/mL in activation/coupling buffer.
17. Based on a coating concentration of 60 mg antibody per gram of microspheres, add 150 µL of the 2 mg/mL antibody to the 850 µL microspheres. Mix thoroughly. The total volume will now be 1 mL.
18. Mix on a rotary wheel for 2.5 hours at room temperature.
19. In a fume hood, add 15 µL ethanolamine to the microsphere suspension. Vortex and mix on a rotary wheel for 30 minutes to quench any remaining active sites.
20. Centrifuge for 7 minutes at 11,900 x g.
21. Decant supernatant and resuspend microspheres in 1 mL blocking buffer – mix well by pipetting up and down so that no clumps are visible.
22. Sonicate the microspheres for 30 seconds to ensure the microspheres are monodisperse (refer to step 7).
23. Mix on a rotary wheel at room temperature overnight.

Day 2 of 2

24. Centrifuge for 7 minutes at 11,900 x g
25. Decant supernatant and resuspend microspheres in 1 mL blocking buffer and mix thoroughly by pipetting up and down so that no clumps are visible.
26. Repeat steps 24 and 25.
27. After the final wash resuspend the microspheres in 0.5 mL blocking buffer. The microspheres are now at 1% w/v. Store at 4 °C until ready to use.

    Note: Use within 5 days.

28. Preparation of conjugate pad
    - Prepare conjugate pad diluent as per Table 3.
    - Dilute microspheres from 1% to 0.005% using conjugate pad diluent buffer.
    - Using a 1 mL pipettor, carefully apply the diluted microspheres to pre-cut strips of glass fibre pads (1 mL per 5 mm x 30 cm strip).

      Note: During the application, move the pipette along the glass fibre pad (not touching) so that you are evenly dispensing the volume throughout the strip.

    - Dry conjugate pads on fine wire mesh tray overnight at room temperature.
    - Store in sealed foil pouches with desiccant until needed.
29. Preparation of sample pad
    - Prepare sample pad buffer as per Table 3.
    - Soak one cellulose fiber pad with excess sample pad buffer (approximately 100 mL).
    - Dry on fine wire mesh tray overnight. If needed, the sample pad can be further dried at 37 °C for 3 hours.
    - After drying, sample pads are cut into strips 2 cm wide x 30 cm long using the Matrix™ 1201 Membrane Cutter.
    - Stored in sealed foil pouches with desiccant until needed.
30. Preparation of absorbent pad
    - Cut cellulose fiber pad into strips 1.7 cm wide x 30 cm long (this is untreated).
31. Stripe test line and control line reagents on Hi-Flow™ Plus 135 membrane
    - For the control line reagent dilute goat anti-mouse IgG to 1 mg/mL using Milli-Q® water (make up fresh just before use).
    - For the test line, dilute myoglobin antibody (Sigma-Aldrich®, MABX7611) to 1 mg/mL in 10 mM MES buffer, pH 6.0.
    - Set up the Matrix™ 1600 Reagent Dispensing Module (Fig. 3).
    - Stripe test line and control line reagents onto the membrane at 1.0 cm and 1.5 cm from the bottom edge of the membrane, respectively. Both antibodies are dispensed at a rate of 1 µL/cm and a bed speed of 10 cm/sec.
    - Dry the striped membrane at 37 °C for 2 hours. This can be stored under desiccation prior to card assembly if desired.

Fig. 3 Kinematic Matrix™ 1600 Reagent Dispensing Module

32. Assemble master cards
    - Remove the 2.5 cm wide release liner from the adhesive card (Fig. 4A).
    - Place the striped Hi-Flow™ Plus 135 membrane onto the exposed adhesive. The test and control lines should be oriented so that the sample flows across the test line first (Fig. 4B). In this example, the test line is placed closest to the narrow release liner immediately below the membrane.
    - Remove the narrow release liner below the membrane.
    - Place the conjugate pad onto the exposed adhesive. One edge of the conjugate pad strip should be placed so that there is a consistent overlap of 2 mm onto the lower edge of the membrane (Fig. 4C).
    - Remove the bottom release liner and place the sample pad onto the exposed adhesive so that it is flush with the bottom edge of the plastic card. This will give a consistent overlap on the bottom edge of the conjugate pad (Fig. 4D).
    - Remove the release liner along the top of the master card.
    - Place the absorbent pad strip onto the exposed adhesive with overlap of 2 mm on the upper edge of the membrane (Fig. 4D).
    - Cut the cards into test strips 5 mm wide using the Matrix™ 2360 Programmable Shear.
    - Store test strips in foil pouches with desiccant until ready to analyse samples.

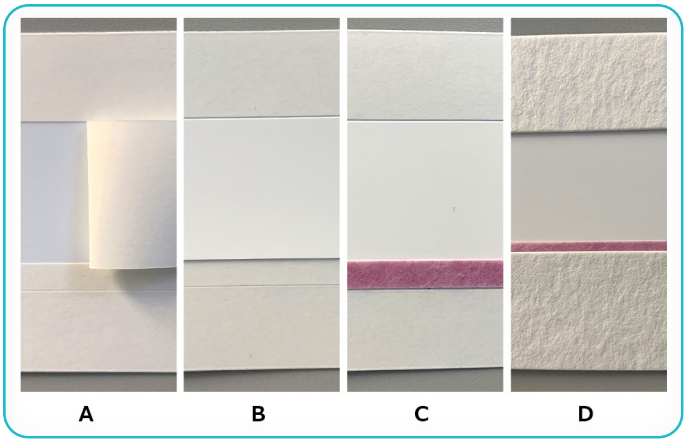

Fig. 4 Assembling master cards

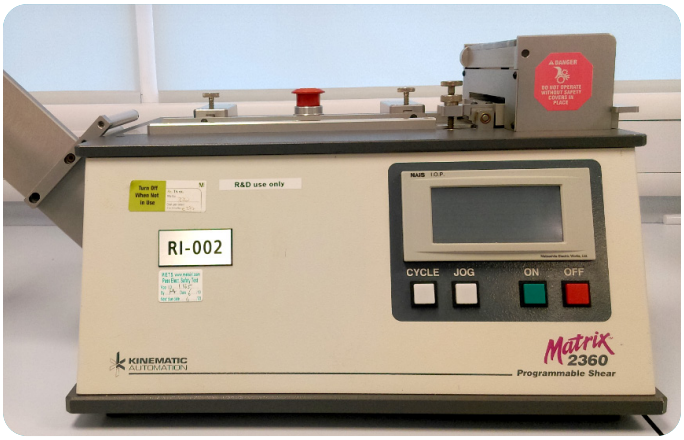

Fig. 5 Kinematic Matrix™ 2360 Programmable Shears

33. Running test strip on quantitative reader
    - Dilute myoglobin-free serum 5-fold in serum diluent.
    - Spike the diluted serum with myoglobin analyte.
    - Add 150 µL serum to the sample pad on the test strip.
    - Place test strip(s) inside the fluorescent reader. Depending on the reader configuration, you may be able to read multiple test strips simultaneously. The ESEQuant® LR3 reader has the capability to read 3 test strips simultaneously (as illustrated in Fig. 6).
    - Read the test strips in the quantitative reader after 10 minutes.
    - Analyse the results.
    - If you are experiencing problems reading the test strips in the quantitative reader it is recommended that you view the test strips under a UV light to provide a qualitative image.

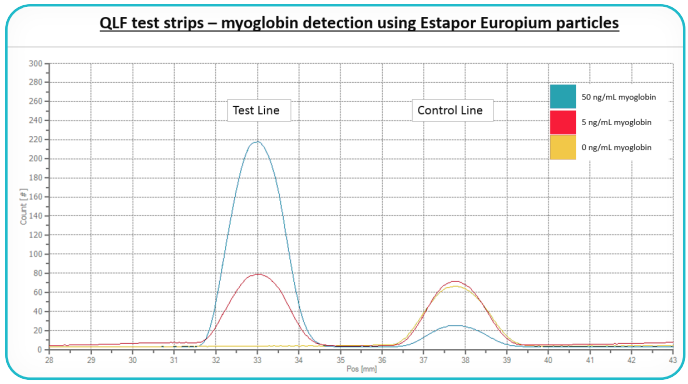

Fig. 6 Myoglobin detection on lateral flow test strips

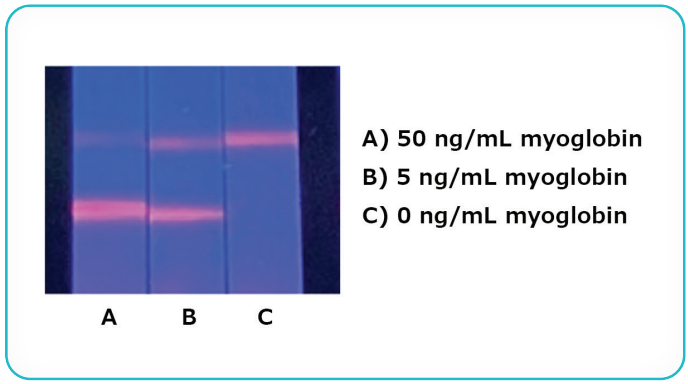

Fig. 7 Qualitative image of myoglobin lateral flow test strips viewed under UV light; representative of results obtained in Fig 6.

Results indicate that the newly developed Estapor® Europium Microspheres combined with lateral flow test strips are suitable for rapid quantitative detection of myoglobin. This represents a useful platform for other biomarkers in point-of-care tests.

General Considerations

Activation and coupling of carboxyl-modified microspheres

Activation using EDC and Sulfo-NHS is most efficient between pH 4.5 and 7.2. Therefore, it is often preferred to use MES buffer at pH 6 for the activation reaction.[4] The activation buffer should not contain any primary amine or carboxyl groups, as they will compete with the activation reaction. Phosphate and acetate buffers may also reduce the reactivity of the EDC. MES works very well as a coupling buffer but can be exchanged for an alternate buffer at a different pH if required for coating optimization. It is important to note that EDC is extremely sensitive to moisture in storage and should be discarded if it appears damp or clumped.

Selection of antibodies

High affinity antibodies should be chosen for the detection of low levels of target analyte.

Note: Even if antibodies paired well in ELISA format, this does not mean they will successfully transfer to lateral flow format. Conducting a pre-screen of numerous antibody pairs in ELISA is recommended.

Size of the microspheres

The microsphere size should be carefully chosen to maximize assay sensitivity. Larger beads (300 – 500 nm diameter) flow through the membrane at a slower rate, thereby offering greater sensitivity, whilst smaller microspheres (100 – 300 nm diameter) may allow a faster run time but with lower sensitivity.

Compatibility of the reader

The reader must be compatible with the excitation and emission properties of the microspheres. In this body of work, the reader was specifically chosen to quantify fluorescent signals from europium microspheres (excitation at 365 nm and emission at 610 nm). The strips must also have dimensions that are compatible with the scanning system inside the reader.

Viscosity of sample liquid

If your target analyte is being analysed in whole blood, then a filter mechanism for the removal of the red blood cells is critical for the running of the assay. This can be manually performed by the user prior to the test being run. Alternatively, there are filter materials available on the market that can be incorporated into the lateral flow test strip (typically used in the sample pad).

Relative amounts of critical reagents

In developing a new quantitative lateral flow test strip, reagents and materials should be optimized including antibody concentrations, antigens and sample and buffer formulations. You will need to optimize the antibody load on the europium microspheres, the concentration of capture antibody on the test line, the membrane being used and the striping rate (often depends on membrane flow rate). It should be noted that europium microspheres achieve a higher level of sensitivity in quantitative lateral flow assays compared to other types of fluorescent microspheres. Therefore, you will likely require significantly less europium microspheres on your test strip.

Estapor® Europium Microspheres

Product Name	Diameter (µm)	Surface COOH (µeg/g)	Cat. No.
F1-Eu- 010	0.130-0.190	>300	80380623
F1-Eu- 030	0.270-0.330	200-500	80380624
F1-Eu- 050	0.450-0.530	1-10	80380625

References:

1. Application Note - Performance of Estapor® Microspheres and Hi-Flow™ Plus Membranes in a Lateral Flow Assay for Human Chorionic Gonadotropin (hCG)
2. Khreich N, Lamourette P, Boutal H, Devilliers K. Detection of Staphylococcus enterotoxin B using fluorescent immunoliposomes as label for immunochromatographic testing. Analytical Biochemistry 2008. 377:182-188
3. Laitala V, Hemmilä I. Homogenous Assay based on anti-Stokes' shift time-resolved fluorescence resonance energy-transfer measurement. Anal. Chem. 2005. 77(5)1483-1487
4. Nakajima N, Ikada Y. Mechanism of amide formation by carbodiimide for bioconjugation in aqueous media. Bioconjug Chem. 1995. 6(1):123-30

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