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Application Note

Development of a chemiluminescent immunoassay (CLIA) using Estapor® magnetic microspheres

Introduction

Many factors must be considered before the development of an immunoassay. One critical consideration is 'sensitivity'. What level of analyte do you need to detect? If your analyte of interest is <10 ng/mL, you may need to consider a CLIA (chemiluminescence immunoassay) over the typical ELISA as it offers increased sensitivity and full automation for high-throughput screening.

For the last 25 years, Estapor® magnetic microspheres have proven functionality in CLIA assays. The following protocol outlines a step-by-step guide in setting up a CLIA-based assay in a 96-well format using Estapor® magnetic microspheres. The described assay design is potentially transferable to other platforms, such as automatic immunoanalyzers.[1]

Product Description

	General specifications	Lot specifications (M9023/2)
Cat. No.	23710087	
Trade name	Estapor®	
Description	Encapsulated Magnetic Microspheres – EM1 100/40	
Functional group	COOH	
Solid concentration	10%	
Media color	Brown	
Polymer	Polystyrene	
Charge content	10–100 µEq/g	58 µEq/g
Ferrite content	36–50%	46.80%
Diameter	0.9–1.8 µm	0.91 µm

Microsphere type can be adapted to suit customer applications. The range of commercially available Estapor® magnetic microspheres are outlined in reference 2.

For research use only. Not for diagnostic purposes.

Required Materials

Encapsulated Carboxylated Microspheres (-COOH)

- EM1-100/40 (MilliporeSigma, Cat. No. 23710087)

Equipment/plastics

- Magnetic plate DynaMag™-96 Side Skirted (Thermo Fisher Scientific, Cat. No. 12027; **Figure 1**) facilitates manual washes of the 96-well plate. If assay automation is necessary, BioTek 405™ LS Microplate Washer (BioTek Instruments) will suffice.
- White walled 96-well plate. Use breakaway strips if performing manual washes (performing manual washes with breakaway strips will only allow you to test 48 wells instead of 96 wells; see **Figure 2**)
- Magnetic stand (PureProteome™ magnetic stand, MilliporeSigma, Cat. No. LSKMAGS08)
- Incubator at 37°C
- 2 mL low protein binding tubes (VWR, ref. 022431102)
- Rotary wheel (Stuart® Rotator SB3)
- Vortex
- UP50H compact lab homogenizer (Hielscher Ultrasonics); recommended for bead monodispersity

Reagents

- Matched pair antibodies for your analyte of interest
  - e.g. Anti-Myoglobin Capture antibody: MABX7611 (purified via affinity chromatography)
  - e.g. Anti-Myoglobin Detector antibody: MABX7612 (purified via protein A affinity)

MilliporeSigma

- CLIA positive and negative controls; e.g. human serum spiked with known concentration of target analyte, analyte-free serum.
- Abcam alkaline phosphatase conjugation kit (Cat. No. ab102850). Alternatively, alkaline phosphatase conjugation can be performed without a kit.[3]
- Alkaline phosphatase substrate (1,2-dioxetane chemiluminescent substrate) (e.g. MilliporeSigma C0712 or Thermo Fisher T1026)
- Chemiluminescent plate reader (e.g. BioTek Synergy™ HT)
- MES (MilliporeSigma M8250)
- PBS (MilliporeSigma P3813)
- Tris (MilliporeSigma T1378)
- Tween-20 (MilliporeSigma P7949)
- EDC (Thermo Fisher Cat No. 24510)
- Sulfo NHS (Thermo Fisher Cat No. 22980)
- Ethanolamine (MilliporeSigma 1008452500)
- Casein (MilliporeSigma C7078)

Table 1. Recommended buffers and solutions

Recommended buffers and solutions	
Activation/ Coupling buffer	50 mM MES, pH 6.0
Blocking buffer	50 mM Tris, pH 8.0, 0.5% (w/v) casein
EDC solution	200 mM EDC Add 19.2 mg of EDC at room temperature to 500 µL Milli-Q® ultrapure water
Sulfo-NHS solution	200 mM Sulfo-NHS Add 21.7 mg Sulfo-NHS to 500 µL activation/ coupling buffer
CLIA wash buffer	PBS + 0.5% Tween 20
Enhancer wash buffer	200 mM Tris (pH 9.8), 10 mM $MgCl_2$. Dilute 1:10 with water for use.

Health and safety

Ear protection must be worn when sonicating samples. All hazardous chemicals (e.g. ethanolamine) should be used in a fume hood with the appropriate safety precautions.

Protocol

Conjugate primary antibody onto magnetic microspheres.

Day 1

Use the two-step EDC/Sulfo NHS covalent coupling procedure for Estapor® carboxyl-modified microspheres. Coating concentrations may require optimization for each assay. Initial studies should use 60 mg antibody per gram of microspheres.

1. Aliquot 50 µL of Estapor® microspheres EM1-100/40 (at 10% w/v) into a 2 mL low protein binding tube.
2. Add 1 mL activation/coupling buffer and mix thoroughly (**Figure 3**).
3. Place the tube on the magnetic stand for 2 minutes and decant the supernatant (**Figure 4**).
4. Remove the tube from the magnetic stand and add 1 mL activation/coupling buffer. Mix thoroughly by pipetting up and down.
5. Repeat steps 3 and 4.
6. After the final wash, resuspend the microspheres in 1 mL activation/coupling buffer.
7. Sonicate the microspheres briefly to ensure microspheres are monodisperse or not aggregated.

Important: prepare EDC and Sulfo-NHS reagents immediately prior to use:

8. To 1 mL washed microspheres (from step 7), add 12 µL EDC and 120 µL Sulfo-NHS.

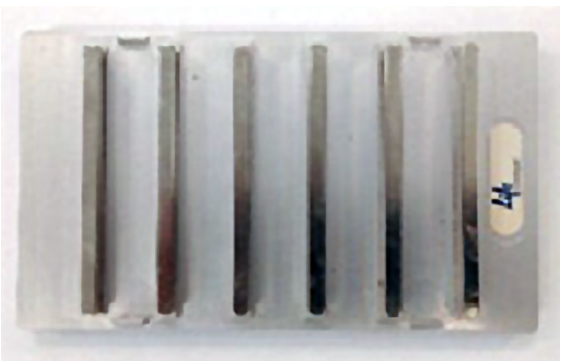

Figure 1. Magnetic plate DynaMag™-96 side skirted (Cat. No. 12027)

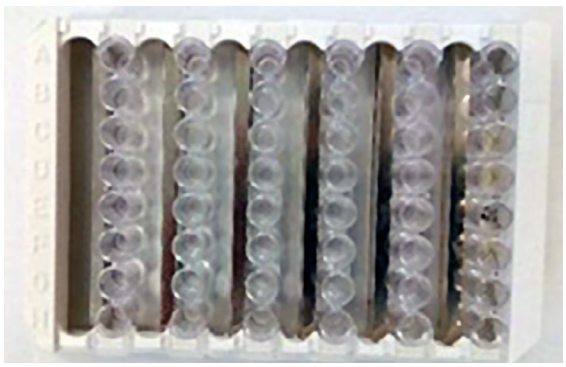

Figure 2. Magnetic plate with breakaway strips

9. Vortex and mix on a rotary wheel for 30 minutes at room temperature.
10. Place the tube on the magnetic stand for 2 minutes and decant the supernatant.
11. Remove the tube from the magnetic stand and add 1 mL activation/coupling buffer. Mix thoroughly by pipetting up and down.
12. Repeat steps 10 and 11.
13. After the final wash, resuspend the microspheres in 850 µL activation/coupling buffer.
14. Sonicate the microspheres briefly to ensure they are monodispersed.
15. Prepare the primary antibody at a concentration of 2 mg/mL in activation/coupling buffer.
16. Based on a coating concentration of 60 mg antibody per gram of microspheres, add 150 µL to the 850 µL microspheres. Mix thoroughly. The total volume will now be 1 mL.
17. Mix on a rotary wheel for 2.5 hours at room temperature.
18. Add 15 µL ethanolamine (in fume hood). Vortex and mix on a rotary wheel for 30 minutes to quench the microspheres.
19. Place the tube on the magnetic stand for 2 minutes and decant the supernatant.
20. Remove the tube from the magnetic stand and add 1mL blocking buffer. Vortex and mix on a rotary wheel at room temperature overnight.

Day 2

21. Place the tube on the magnetic stand for 2 minutes and decant the supernatant.
22. Remove the tube from the magnetic stand and add 1 mL blocking buffer. Mix thoroughly by pipetting up and down.
23. Repeat steps 21 and 22.
24. After the final wash, resuspend the microspheres in 10 mL blocking buffer.

Microspheres are now at a concentration of 0.5 mg/mL or 0.05% and ready for use in the CLIA assay. Store at 4°C until ready to use. Use within 5 days.

Conjugate secondary antibody to Alkaline Phosphatase using the Abcam AP conjugation kit following kit instructions.

Day 2

25. Dilute antibody in PBS so at a concentration of 2 mg/mL. 200 µg antibody will suffice for reaction.
26. Add 10 µL of modifier reagent from kit into 200 µg antibody. Mix well by pipetting.
27. Add 110 µL of Ab/modifier mix to 100 µg lyophilized vial of AP – Keep in the dark.
28. Leave standing for 3 hours in dark.
29. After incubation, add 10 µL of quencher from Abcam kit. Leave for at least 30 minutes before using.
30. Dilute conjugated antibody to desired working concentration – this will have to be optimized to each assay. If you are using the myoglobin antibodies given as an example in this application note, use 10 ng/mL of AP conjugated antibody.

Block plate

Day 2

31. Add 200 µL blocking buffer per well. Leave for 2 hours at room temperature or overnight at 4°C.
32. Wash plate with 200 µL CLIA wash buffer 3X.

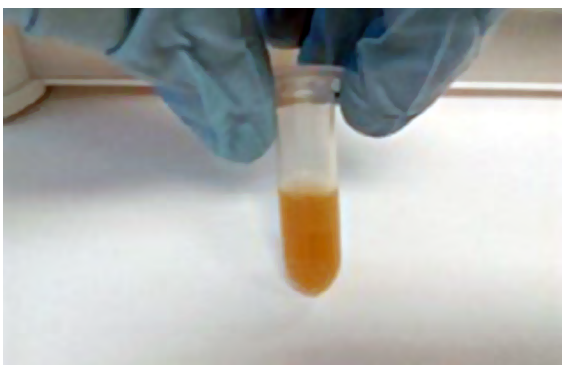

Figure 3. Microspheres resuspended in 1 mL activation/coupling buffer (step 2)

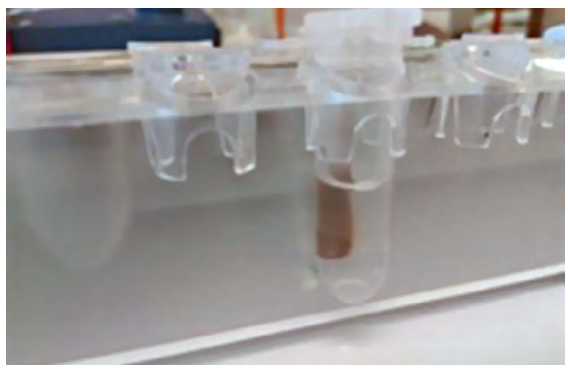

Figure 4. Washing the microspheres (step 3)

Perform CLIA assay

Day 3

33. Add 100 µL of conjugated microspheres to wells (Note: microspheres @ ~0.5 mg/mL).
34. Add 100 µL of analyte sample per well. Mix well by pipetting up and down, ensuring that the microspheres are well mixed with the analyte.
35. Leave plate at 37°C for one hour.
36. Wash wells 3x.
37. Add 100 µL AP conjugated antibody per well and leave in dark for 30 min at room temperature.
38. Wash wells 3x.
39. Wash wells 2x with enhancer wash buffer.
40. Add 100 µL alkaline phosphatase substrate per well. Mix contents of the well so that microspheres are well mixed with the substrate.
41. Read plate on chemiluminescent reader, taking multiple readings every 10-15 minutes until the signals plateau.

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 [2-(N-morpholino)ethanesulfonic acid] 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 be aware that EDC is extremely sensitive to moisture and should be discarded if damp or clumped.

Optimizing the coating procedure

As outlined above, coating concentrations may require optimization for each assay. Numerous coating concentrations may be assessed in an optimization study, ranging from 60 mg antibody per gram of beads down to 20 mg of antibody per gram of beads. The optimal coating concentration should be chosen based on the sensitivity of the assay. If using the myoglobin antibody pair that is given as an example, you may decrease the coating concentration down to 45 mg antibody per gram of beads, but do not decrease beyond 45 mg of antibody per gram of microspheres as this will lead to a decrease in sensitivity in the assay.

Sonication of microspheres

Ensuring the microspheres are monodispersed is important in obtaining a uniformly coated microsphere. However, this monodispersity is not crucial to the CLIA application. Your CLIA assay may work without having access to a 'UP50H compact lab homogenizer'. If you are using sonication, avoid overheating. If required, sonicate the sample with the tube immersed in ice water. Aggregation can be assessed using 400X magnification. Monodispersed microspheres are difficult to see under 400X magnification and will appear as a hazy sea of microspheres. Aggregated microspheres, however, are easily observed under 400X magnification.

Define the expected range of analyte

You should know the approximate concentration range of analyte in your sample. The positive control sample (as outlined in 'Reagents' above) should be titrated in a standard curve with decreasing concentrations of analyte (as illustrated in **Figure 5**). Use this standard curve to quantify the unknown analyte concentration in your sample(s).

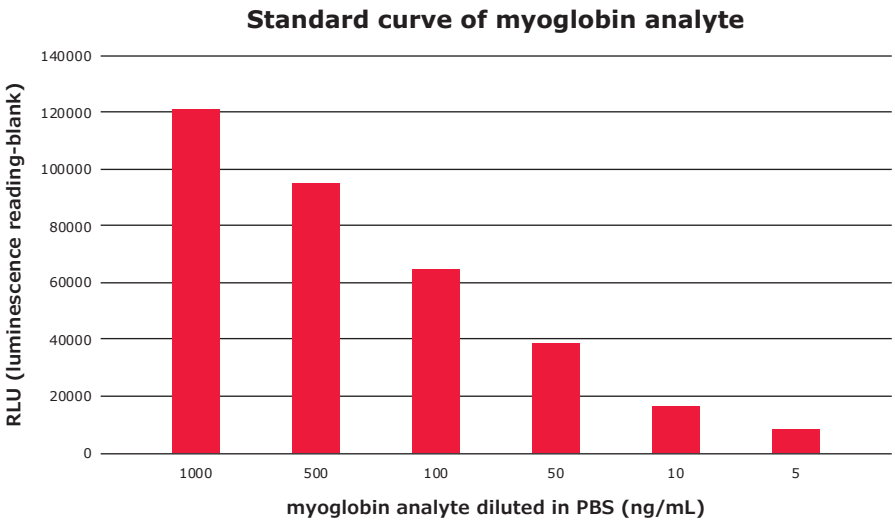

Figure 5. CLIA assay for myoglobin detection using Estapor® microspheres (EM1-100/40)

Select your matched pair antibodies carefully

Your chosen antibodies must be highly specific for your analyte of interest. Monoclonal antibodies offer increased specificity over polyclonal antibodies. Note – if the matched pair of antibodies are both monoclonal, they must recognize separate epitopes. It is important to work with highly purified IgG.[5]

Manual or automatic washes during CLIA protocol

Using an automatic plate washer can enhance consistency in your assay. However, you must ensure that the magnet on the plate washer can retain the microspheres rather than sucking them up. You may need to adjust the protocol on the plate washer to ensure that this does not happen, i.e. allowing a 2-minute interval between each wash during your 3-wash cycle.[6]

Conjugating alkaline phosphatase without a kit

The most popular method to conjugate alkaline phosphatase to proteins is the one-step glutaraldehyde coupling method:[3]

1. Combine 10 mg of antibody and 5 mg of high activity alkaline phosphatase (MilliporeSigma Cat. No. P0114) in 1 ml PBS (20 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl)
2. Dialyze against 0.1 M sodium phosphate pH 6.8 overnight to remove any free amine-containing compounds.
3. While gently stirring in a fume hood, add 0.05 ml of a 1% glutaraldehyde solution. Let stir 5 minutes.
4. Incubate for 3 hours at room temperature. Then add 0.1 ml of 1 M ethanolamine pH 7.0.
5. Incubate for an additional 2 hours at room temperature.
6. Dialyze overnight at 4 °C against three changes of PBS.
7. Centrifuge at 40,000 g for 20 minutes to remove unwanted precipitate.
8. Store clarified supernatant at 4 °C in the presence of 50% glycerol, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, and 0.02% sodium azide.

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2019-20708
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