Chapter 25

Liposome-Enhanced Lateral-Flow Assays for Clinical Analyses

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Abstract

Clinical and environmental analyses frequently necessitate rapid, simple, and inexpensive point-of-care or field tests. These semiquantitative tests may be later followed up by confirmatory laboratory-based assays, but provide an initial scenario assessment important for resource mobilization and threat confinement. Lateral-flow assays (LFAs) and dip-stick assays, which are typically antibody-based and yield a visually detectable signal, provide an assay format suiting these applications extremely well. Signal generation is commonly obtained through the use of colloidal gold or latex beads, which yield a colored band either directly proportional or inversely proportional to the concentration of the analyte of interest. Here, dye-encapsulating liposomes as a highly visible alternative are discussed. The semiquantitative LFA biosensor described in this chapter relies on a sandwich immunoassay for the detection of myoglobin in whole blood. After an acute myocardial infarction (AMI) event, several cardiac markers are released into the blood, the most common of which are troponin, creatine kinase MB, C-reactive protein, and myoglobin. Due to its early release, myoglobin has value as an indicator of a recent heart attack amongst conditions which present with similar symptoms and its lack of elevation can effectively rule out a heart attack (Brogan et al., Ann Emerg Med 24:665–671, 1994). The assay described within relies on sandwich complex formation between a membrane immobilized capture monoclonal antibody against myoglobin, a detector biotinylated monoclonal antibody against a different epitope on myoglobin, and streptavidin-conjugated visible dye (sulforhodamine B)-encapsulating liposomes to allow for signal generation.

Key words Lateral-flow assay, Liposome, Whole blood, Myoglobin

1 Introduction

Clinical and environmental analyses frequently necessitate rapid, simple, durable, and inexpensive point-of-care or field tests. Lateral-flow assays (LFAs), such as those used in pregnancy tests, often fulfill these requirements. These assays rely on the appearance of a colored band, the intensity of which corresponds to the concentration of the target analyte in the sample. The goal for such assays is typically a qualitative "yes" or "no" answer, though some assays can provide semiquantitative results. LFAs

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are commercially available for the detection of a wide variety of analytes. These targets include pathogenic organisms such as *Legionella pneumophilia* [1, 2], *Leptospira* [3], *Cryptosporidium parvum*, and *Giardia lamblia* [4]; viruses such as HIV [5], influenza [6], and respiratory syncytial virus (RSV) [7, 8]; food allergens [9] and drugs of abuse [10]; health states such as pregnancy, ovulation, or menopause [11, 12]; diseases such as prostate cancer [13, 14]; or biological toxins such as botulinum toxin [15, 16], and verotoxins [17, 18]. Some lateral flow assays have proven to be equivalent or better than enzyme-linked immunosorbent assays (ELISAs), direct fluorescent antibody testing, and viral cultures [5, 6, 8, 19]. Lateral-flow assays used in a clinical setting can obviate the need for patients to return to the clinician's office for results [20] and can provide guidance for the administration of chemoprophylaxis after potential occupational exposure to viruses [21]. In environmental or occupational settings, LFAs provide an initial assessment for mobilization of resources and containment of potential threats. LFAs for research and more fundamental applications such as probe selection, PCR-product identification, genomic sequence search, and identification of single-nucleotide polymorphisms have been suggested [22–24].

Here we discuss in detail the use and preparation of LFAs for analytes present in whole blood, using the 16.7 kDa cardiac marker myoglobin as an example analyte. After an acute myocardial infarction (AMI) event, several cardiac markers are released into the blood, the most common of which are cardiac troponin I (cTnI), cardiac troponin T (cTnT), creatine kinase MB subform (CK-MB), C-reactive protein (CRP), and myoglobin. cTnI, cTnT, and CK-MB are initially elevated 4–6 h after an AMI event; C-reactive protein is initially elevated up to 6 h after the event, and myoglobin is initially elevated after 1–3 h [25, 26]. The relative increase in these markers over time following an incident of chest pain is shown in Fig. 1.

Due to the rapid and reliable release of myoglobin from damaged myocardium, tests that use serum myoglobin levels to determine the risk of a recent myocardial infarction have high sensitivities and high negative predictive values within 90 min of symptom onset [27]. Especially when combined with detection of other cardiac markers like troponin I, the absence of elevated serum myoglobin is useful in quickly ruling out acute myocardial infarction in patients with symptoms such as chest discomfort, dyspnea, and syncope [28, 29]. Since approximately 80% of patients presenting to emergency rooms with AMI-associated symptoms do not develop heart attacks [30], a test that can rapidly rule out AMI may allow hospitals to prevent unnecessary admission of patients to coronary care units. While a serum myoglobin test cannot confirm AMI since myoglobin release is not specific to cardiac muscle damage, detecting elevated levels of myoglobin in the blood

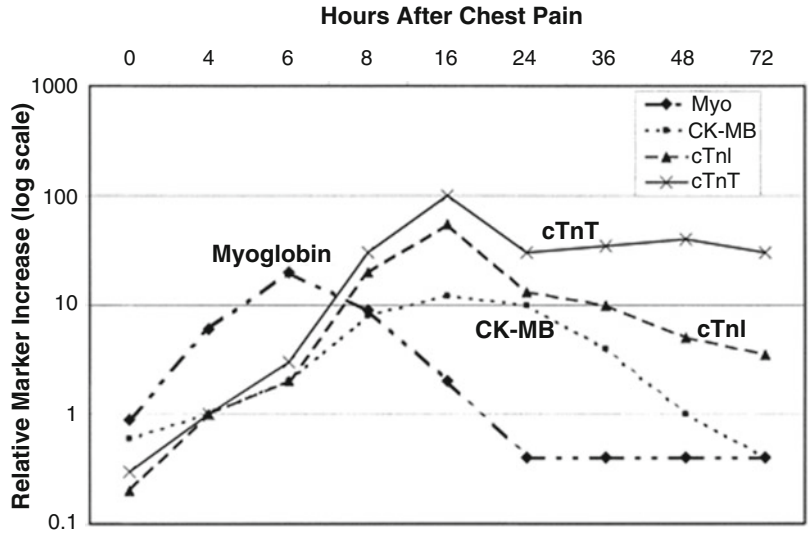


Fig. 1 Temporal release of myoglobin, CK-MB, and cTnT and cTnI. Reprinted with permission from Christenson RH, Azzazy HME. Biomarkers of Myocardial Necrosis: Past, Present and Future. In Morrow DA, ed. Cardiovascular Biomarkers: Pathophysiology and Clinical Management. Totowa, NJ: Humana Press, 2006

regardless of its source is important in preventing kidney damage. In rhabdomyolysis (severe muscle breakdown), serum myoglobin levels may rise to levels that lead to acute renal failure [31]. The approach described within allows for the analysis of myoglobin levels in whole blood using plasma separators incorporated into a LFA device and the use of dye-encapsulating liposomes as a highly visible signaling species in contrast to commonly used latex particles and colloidal gold.

1.1 Sandwich Immunoassay Format

While simple for the end-user to operate, from an engineering standpoint, the sandwich lateral flow immunoassay format is a relatively complicated multicomponent design encompassing a sample pad, conjugate pad, analytical membrane, absorbent pad, signaling species, blocking constituents, and multiple antibodies. When these components are assembled appropriately, the end-user simply needs to add a liquid sample and wait typically 5–15 min before a visible signal is assessed. The flow of the fluid in such an assay is detailed in Fig. 2.

A liquid sample ideally with minimal preparation requirements is applied to the sample pad via an opening in the housing. Depending on the sample matrix, the sample pad can serve as a particulate filter, site to retain interfering substances, and provide pH buffering capacity with the overall aim to prepare the fluid for subsequent analysis and dispense it evenly. Once the fluid passes through the sample pad, it then passes through a conjugate pad where it rehydrates a previously dehydrated signaling species, which is typically

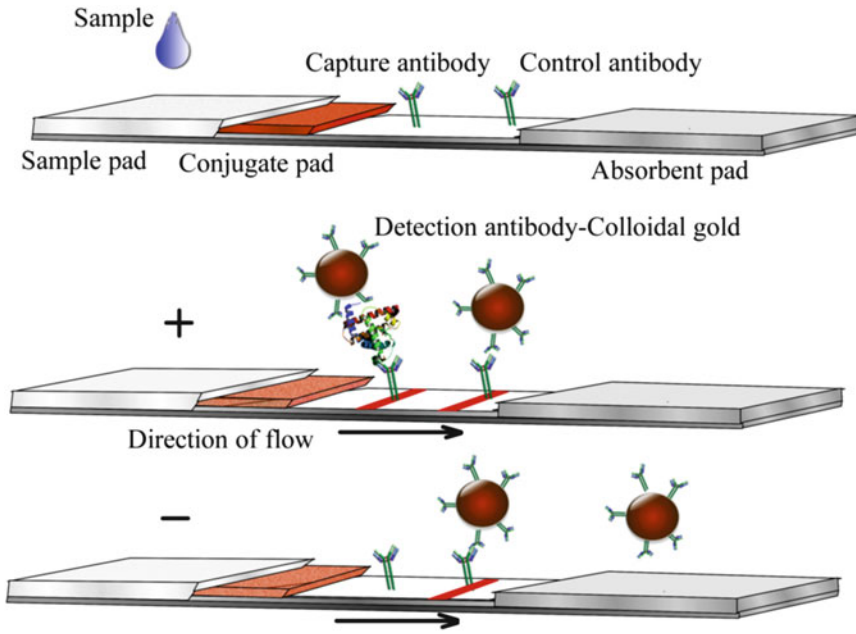


Fig. 2 Lateral flow assay fluid path. (*Top*) A liquid sample is applied to a sample pad through a port in the plastic housing (not shown). The liquid sample then passes through the conjugate pad where the signaling species (most commonly colloidal gold) with an attached detection antibody becomes rehydrated. The solution then passes onto the analytical membrane were capture and control antibodies are immobilized, then lastly is wicked by the absorbent pad at the opposite end of the assembly. (*Middle*) If the analyte is present, it can form sandwich complexes with the detection antibody on the colloidal gold and the capture antibody immobilized on the analytical membrane. A visible signal where the capture antibody is immobilized is observed as well as a visible signal where the control antibody is immobilized to indicate successful fluid flow and conjugate release. (*Bottom*) If the analyte is not present, a signal at only the control line is observed

colloidal gold or visibly dyed latex beads. The surface of the signaling species is functionalized with "detection" antibodies against the analyte of interest and can form antibody-antigen complexes with analyte in the sample solution if it is present. The fluid then passes onto the analytical membrane where a second antibody is immobilized at the test line. This "capture" antibody, most commonly a monoclonal antibody (mAb), can recognize a different epitope on the target analyte than that recognized by the detection antibody and serves to retain the passing analyte-signaling particle complexes. The sandwich complex formed between the immobilized capture antibody, analyte, and detection antibody-tagged particles allows a visible signal to be observed in the presence of the target analyte due to the attached signaling species. When the analyte is not present, no sandwich complexes are formed and thus no signal is generated. The intensity of the signal is proportional to the analyte concentration, which can be used for semiquantitative determinations. At a location downstream of the test line is a control line. This control line typically consists of a secondary antibody, which can bind to the

primary antibody immobilized on the surface of the signaling species (e.g., a goat anti-mouse secondary antibody could be used as a control line if the primary antibody on the colloidal gold particles was a mouse anti-myoglobin antibody). This control line captures passing signaling species regardless of the presence or absence of the target analyte and allows the end user to confirm that sufficient fluid was applied to yield a valid assay and that the conjugate successfully released. At the distal end of the membrane lies an absorbent pad, which serves to draw fluid through the assay and retain excess fluid for the duration of the assay.

The inherent format of the sandwich immunoassay places certain constraints on the types of analytes that can be detected. In order to accommodate the binding of two separate antibodies which are ~150 kDa each, the analyte must be relatively large. As such, it is predominantly used for the quantification of large proteins or whole cells. While this format precludes the detection of small molecules, it has been used for quantification of oligopeptides including, for example, insulin C-peptide (31 amino acids (AA), 3 kDa) [32] provided an appropriate pair of antibodies is available. To satisfy the criteria for appropriate antibodies, either two antibodies that can bind to distinct, nonoverlapping epitopes on the target molecule are required, or if a single antibody type is used, the target molecule must have multiple copies of a single epitope. In practice, the method developer typically needs to determine which antibodies can successfully form a sandwich complex with a given analyte through in-house experimentation. With the price of commercially available primary antibodies typically ranging from ~$350 to $700/mg, this can be a costly trial and error process. Thus, it is a great benefit when "paired" antibodies that are known to work together in sandwich assays are available from antibody vendors. Other considerations to the choice of antibody include its level of purification and concentration. Antibodies used as capture antibodies in LFAs should not contain any stabilizing proteins or other species that may compete for adsorption to the nitrocellulose. Similarly, if the detection antibody is covalently attached to the signaling species, it should not contain stabilizing proteins or buffer constituents that will interfere with the conjugation chemistry. Unless the capture antibody has a very strong affinity, a minimum concentration of 1 mg/mL is recommended. The sensitivity of lateral flow assays stems in part from the basic design where fluid must pass the test line where it has the opportunity to contact the immobilized capture antibody with negligible mass transport limitations. This is in contrast to enzyme-linked immunosorbent assays (ELISAs) where diffusion from the bulk sample volume must occur to the plate surface where capture antibodies are immobilized [33]. While the latter can often achieve low limits of detection using much lower concentrations of antibodies (typically 5–10 μg/mL), extended incubation times (typically 1–2 h) are required. Given that

there is a limited amount of time in which an analyte is in contact with antibody immobilized on the test line which is typically 0.5–1.0 mm wide, binding kinetics are key in LFAs. Aside from high affinity, the association rate is a critical parameter [33, 34].

1.1.1 Membrane Types

Most commonly, nitrocellulose membranes are used as analytical membranes for LFAs due to their high binding capacity, low cost, and wide availability [35]. The mechanism behind protein binding to nitrocellulose is not fully understood, but is believed to be non-covalent through hydrophobic, hydrogen bonding, and electrostatic interactions [36, 37]. The physical adsorption of nucleic acid probes through drying to nitrocellulose is believed to result in attachment through hydrophobic interactions, an effect which may be enhanced through modification with a poly-T tail [38]. We have also had success using polyethersulfone membranes for nucleic acid sandwich hybridization assays [22, 23, 39].

More recently, membranes based on electrospun nanofibers have been applied for LFAs [40]. Electrospun nanofibers are prepared by using a simple set-up consisting of a power voltage supply, a syringe with metal needle, a syringe pump and a ground plate. A polymer solution (dope) is filled into the syringe and pumped out at a steady flow rate. A high voltage (7–15 kV) is applied between the tip of the needle and the ground plate. A taylor cone is formed and a nanofiber in the size range of a few tens of nanometers to a few micrometers are collected on the grounded plate, depending on the spinning dope and electrospinning conditions chosen. Nitrocellulose and nylon via electrospinning have been shown for protein slot blot and Western blot applications [41]. By doping the composite with poly-L-lysine (PLL) or polylactic acid (PLA), specific functional groups including amino and carboxylic acid groups, respectively, may be incorporated [42]. Additionally, through control over polymer dope composition, voltage, distance between needle tip and ground plate control over the mat thickness and porosity can be achieved and offers significant surface area for immobilization.

1.1.2 Membrane Properties

The ideal properties of the membrane include particle and pore size consistency, hydrophilicity, high protein binding, and durability. As nitrocellulose membranes are a fibrous polymeric matrix rather than uniform particles, the membranes are characterized by their capillary rise rate (or capillary flow time), rather than by a specific particle or pore size. This rate is the amount of time required for fluid to flow 4 cm, and based on a survey of current commercial offerings from major manufacturers, is generally between 60 and 280 s. Since fluid flow speed in lateral flow membranes decreases with the distance traveled, the capillary rise rate is a more consistent measure of the speed of flow [43]. This parameter is important to consider during assay development since a fast wicking rate allows for more

rapid results and lower background, but may adversely affect assay sensitivity since less time for interaction between the target and the antibodies immobilized at the test line is available [44].

Commercially available nitrocellulose membranes vary in physical properties based on the effective "pore size," the amount of trapped air, and the thickness of their nitrocellulose layer. These properties affect the surface area available for immobilization of capture reagents. The binding capacity of nitrocellulose itself is inherently high, with estimates ranging from 50 to 200 μg IgG/cm^2 [45]. The thickness of commercially available membranes typically ranges from 100 to 150 μm. Thicker membranes can allow more sample fluid to be accommodated by the membrane during the assay and more capture reagent to be applied. However, the latter does not necessarily yield greater sensitivity in lateral flow assays with optical detection since due to the opacity of nitrocellulose, only the top ~10 μm of membrane is visible to the user [45]. One advantage to thicker membranes is improved tensile strength, however, for ease of handling, durability, and avoidance of adhesive interactions, a supported membrane should be chosen. These membranes are manufactured by directly casting the nitrocellulose membrane material onto a plastic (polyester or cellulose acetate) backing. The backing thickness typically ranges from 50 to 225 μm [43]. Alternatively, non-supported membranes can be laminated manually prior to use. LFAs may be further made more durable and user-friendly by encasing them in an injected molded plastic housing such as that shown in Fig. 3.

Fig. 3 Lateral flow assay membranes assembled in injection molded housings. In the presence of the target analyte, a sandwich complex forms with dye-encapsulating liposomes resulting in a magenta-colored band at the capture zone that is proportional to target concentration (*top*). In the absence of target analyte, no band is visible as no sandwich complex has formed (bottom). Though not necessary, these LFA membranes were housed in more user friendly packaging using a plastic cassette made using injection molding and designed for addition of liposome/target mixture in hole #1 and addition of running buffer in hole #2. Readings can be taken in the "R" hole

In addition, a sample pad, conjugate release pad, and wicking material are often utilized for LFAs to allow for sample pretreatment such as filtration or pH adjustment; storage of dehydrated signaling species; and accommodation of larger fluid volumes, respectively. These are critical components for any assay developed in complicated sample matrices and also those intended for consumer usage where the number of steps needs to be minimized. For the assays described in this chapter, the conjugate pad is omitted since the liposomes used to provide the signal are maintained in the solution phase. The sample pad is selected based on the type of sample matrix that is employed. For the assays described within, a sample pad that is capable of retaining white and red blood cells from whole blood while allowing the plasma to pass is required. The ideal sample pad minimizes hemolysis from red blood cells, which can contribute to an undesired colored background signal on the nitrocellulose membranes (Fig. 4).

The capture antibody can be applied to the membrane either manually using a pipettor, with a thin-layer chromatography (TLC) applicator, or similar approaches. Different binding characteristics, buffers (pH, salt), application rate, and biorecognition element concentration lead to differences in thickness in the capture zone line. Once the biorecognition element is applied, the membrane material is typically immersed in a blocking agent, which is used to

Fig. 4 Whole blood applied to lateral flow assay membranes assembled in injection molded housings to test sample pad types. No test or control lines were immobilized. Significant hemolysis was observed in the left assembly which yielded a *pink colored background* which could potentially obscure weak signals, whereas a clear background was available in the right assembly

reduce nonspecific binding of the assay components. Common agents include proteins such as bovine serum albumin (BSA), casein, and gelatin; non-ionic surfactants such as Tween 20, Triton X-100, or Brij; or synthetic polymers such as polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), and polyethylene glycol (PEG). These components compete for protein binding sites, interfere with hydrophobic interactions, and protein binding, respectively [46, 47]. In addition to serving as a blocking agent, Tween 20 has also been reported to have a renaturing effect on immobilized antigens, which can improve assay sensitivity through enhanced antigen–antibody binding [48, 49]

1.2 Detection Mechanisms

LFAs typically utilize antibody-labeled colloidal gold [5, 19, 50] as a visualization method. Colloidal gold ranges in size 2–250 nm, though 30–80 nm particles are preferred for LFAs [24]. A diameter of 40 nm has been reported to be optimal, allowing for clear visualization, dense packing of these small particles at the capture zone, and reduced steric hindrance for protein binding [51]. These particles have a characteristic red color, allowing for visual detection or semiquantitative measurements with portable reflectometers or scanners [52–54]. Proteins are associated with gold particles ionically through the particle's negative charge with positively charged amino acids such as lysine; through hydrophobic interactions of amino acids such as tyrosine and tryptophan; and through sharing of electrons between gold and sulfur atoms of cysteine [51]. When labeled with anti-biotin or streptavidin, such species allow for recognition of biotinylated biorecognition elements, such as DNA oligonucleotides or antibodies. The sensitivity of LFAs using such particles can be increased with silver enhancement [55]. Alternatively, antibody-tagged latex particles [56, 57], up-converting phosphors [58], superparamagnetic particles [59], or visible dye-encapsulating liposomes [60] have been used as a signal enhancement means. The latter species are the focus of this chapter.

Liposomes are vesicles formed through the association of phospholipid molecules in an aqueous environment, yielding a structure with the hydrocarbon tails forming a lipid bilayer and hydrophilic headgroups directed at both the aqueous core and external aqueous medium. One of the common methods for liposome formation is known as the reverse-phase evaporation method [61, 62]. Here, phospholipids are dissolved in organic solvent; the mixture is introduced to an aqueous medium containing high concentrations of visible dye; the solvent is removed under vacuum; the resulting liposomes are passed through defined pore-size membranes to improve homogeneity and reduce the number of lipid bilayers (lamellarity); then the unencapsulated material is removed through size-exclusion chromatography and dialysis. We commonly use sulforhodamine B (SRB) dye, which is relatively inexpensive, highly

soluble in water, has a high molar extinction coefficient, and has good photostability. However, the most appropriate dye color can be chosen to ensure good visibility against any background signal in the LFA that is not removed by the sample pad. For example, for whole blood, blue liposomes made using Patent Blue dye can improve optical detection against a potentially red or brownish background.

The advantages of dye-encapsulating liposomes as a label for immunoassays include long-term stability of the encapsulated signaling molecules; the substantial signal enhancement afforded by the encapsulation of hundreds of thousands of dye molecules within the large interior liposomal volume; the instantaneous signal provided through either visual detection of intact liposomes or the release of hydrophilic dye molecules from their aqueous cores upon surfactant-induced liposome lysis; and the ease of labeling through the covalent modification of lipid headgroups or the direct incorporation of hydrophobic biorecognition elements into their lipid bilayers (Fig. 5) [60, 63–65].

The stability of the liposomes is a function of their lipid composition, buffer composition, and storage conditions. In the formulation that we most commonly employ (described within), we have found that streptavidin tagged sulforhodamine B-encapsulating liposomes have retained their functionality in LFAs for at least 400 days when stored at either 4 or 21 °C, while loss of encapsulated dye and signal occurs at elevated temperatures [66].

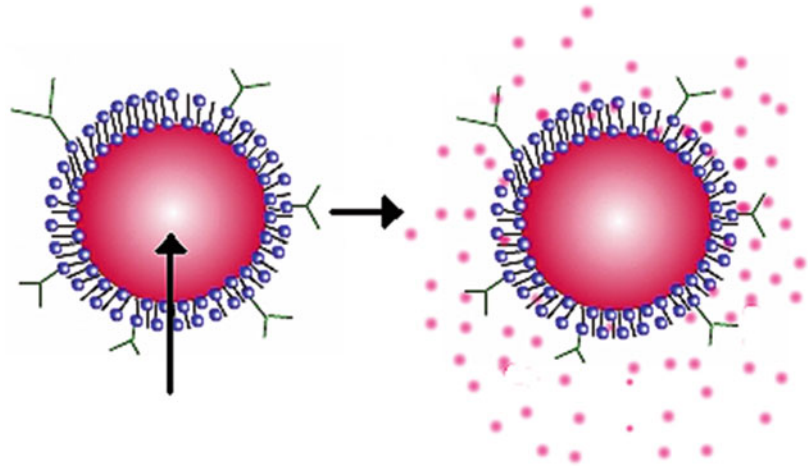


Fig. 5 Liposome structure. (**a**) Biorecognition elements can be covalently conjugated to or inserted into lipid bilayers through hydrophobic interactions (not to scale). (**b**) The large internal volume of unilamellar vesicles can encapsulate hundreds of thousands of hydrophilic signaling molecules and provide for their stability. (**c**) Surfactant introduction can provide for instantaneous signal enhancement through release of encapsulants. Fluorophores encapsulated within liposomes at high concentrations undergo self-quenching with is overcome upon release into the surrounding medium

1.3 Universal Assays

Both immunoassays and nucleic acid based lateral flow assays can also be made in a universal format, obviating the need for the generation of specifically labeled membranes and detection elements. This can be done through membrane immobilization of streptavidin [23] or anti-fluorescein antibodies [22] and subsequent addition of biotinylated or fluorescein-labeled capture antibodies, respectively, with the remaining assay components. Protein A/G [67], streptavidin/avidin [22, 68], or generic oligonucleotide [23] labels can be conjugated to the liposomes to form generic species capable of facile recognition of the Fc′ region of antibodies, biotinylated biorecognition elements, or complementary generic oligonucleotides, respectively. Anti-fluorescein, anti-biotin, or anti-digoxigenin are also options for common universal liposome tags [69]. These assays are of particular interest in research laboratories where a variety of antibodies or probes need to be screened or where different analytes need to be tested. From a commercial and manufacturing standpoint, the universal format is of interest since only one type of membrane and liposome need to be prepared which simplifies packaging of tests for any analyte.

2 Materials

Materials listed here are those that have been used successfully in our laboratory, though substitutions may be made. Unless otherwise specified, reagents are molecular biology grade and are purchased from VWR (Bridgeport, NJ). Table 1 lists the biomolecules used for the detection of myoglobin as an example.

2.1 Liposome Preparation

1. Bath sonicator (Aquasonic Model 150D, VWR, Bridgeport, NJ).
2. Rotary evaporator (Model R-114, Buchi, New Castle, DE).
3. 50-mL round bottom flask (Catalog # 80068-756, VWR).
4. Mini-extruder (Catalog # 610000, Avanti Polar Lipids, Alabaster, AL), including two 1 mL syringes, Teflon supports and o-rings, and extruder holder/heating block. When purchasing initially, the membranes and supports listed in #5 of this section are included with this catalog number as of the date that this chapter was prepared.
5. Extrusion membranes (0.4 and 1.0 μm polycarbonate membranes*, 19 mm) and filter supports (Catalog # 610007, 610010, and 610014, respectively, Avanti Polar Lipids).
6. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)], sodium salt (DPPG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-glutaryl, sodium salt (N-glutaryl DPPE) (Catalog #: 850355, 700000, 840455, and 870245, respectively, Avanti Polar Lipids).

Table 1
Reagents needed for the development of a LFA for human myoglobin

Function	Information	Vendor	Catalog number
Capture antibody	Anti-myoglobin (mouse, mAb, IgG_1)	Meridian Life Science, Inc.	H86703M
Detection antibody	Biotinylated anti-myoglobin (mouse, mAb, IgG_1)	Meridian Life Science, Inc.	H86142B
Control antibody	Anti-streptavidin (goat, pAb)	Vector Labs	SP-4000
Liposome tag	Streptavidin	Thermo Fisher Scientific	S888
Test matrix	Myoglobin-free serum	Meridian Life Science, Inc.	N86580H
Test analyte	Recombinant full-length myoglobin	Meridian Life Science, Inc.	A01428H
Function	Information	Vendor	Catalog Number
Capture antibody	Anti-myoglobin (mouse, mAb, IgG_1)	Meridian Life Science, Inc.	H86703M
Detection antibody	Biotinylated anti-myoglobin (mouse, mAb, IgG_1)	Meridian Life Science, Inc.	H86142B
Control antibody	Anti-streptavidin (goat, pAb)	Vector Labs	SP-4000
Liposome tag	Streptavidin	Thermo Fisher Scientific	S888
Test matrix	Myoglobin-free serum	Meridian Life Science, Inc.	N86580H
Test analyte	Recombinant full-length myoglobin	Meridian Life Science, Inc.	A01428H

7. Chloroform, methanol, and isopropyl ether (HPLC grade).
8. 10×HEPES-saline buffer is composed of 0.1 M HEPES, 2.0 M sodium chloride, and 0.1% (w/v) sodium azide, adjusted to pH 7.5 with NaOH.
9. 1×HEPES–saline–sucrose buffer (1×HSS) is prepared by dissolving 205.4 g sucrose (0.2 M) in 300 mL 10× HEPES-saline and 900 mL water, then bringing the final volume to 3 L with deionized water. However, we usually prepare a 2 M stock solution of sucrose instead of weighing for this solution.
10. 0.419 g sulforhodamine B (SRB, Catalog # S1307, Molecular Probes, Inc., Eugene, OR) is added to 0.5 mL 0.2 M HEPES, pH 7.5 and diluted to a final volume of 5 mL with deionized water to yield a 150 mM solution. For blue liposomes, Patent Blue dye should be substituted.
11. Sephadex G-50 (Catalog # G-50-150, Sigma, St. Louis, MO) and a glass chromatography column (such as VWR catalog #

KT420400-1530). The column is packed by first swelling 1 g of Sephadex in 120 mL deionized water in 3 × 50 mL centrifuge tubes overnight, and decanting off water either after centrifugation or settling by gravity then pouring. The volume is then replaced by 1×HSS and decanting procedure repeated. Overall, three 1×HSS buffer exchanges are typically sufficient. The Sephadex mixture is then poured into the chromatography column to a height of approximately 20 cm and allowed to settle while maintaining a flow of 1×HSS through the column for at least 30 min. The top of the column should be level with no gaps or bubbles throughout the remaining height.

12. Dialysis membranes, Specta/Por 2 (Catalog # 132678, Spectrum Laboratories, Inc., Rancho Dominguez, CA).
13. 15- and 50-mL centrifuge tubes (Catalog #: 21008-216 and 21008-242, respectively, VWR).

2.2 Liposome Conjugation

1. Streptavidin (Catalog # S888, Molecular Probes, Inc., Eugene, OR) is diluted to 200 μM with 50 mM potassium phosphate, pH 7.0 and aliquotted into 50 μL portions prior to storage at −20 °C.
2. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Catalog #22980, Thermo Fisher Scientific).
3. 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 4.65.
4. Sepharose CL-4B (Sigma-Aldrich #CL4B200) packed as the Sephadex G50 column is above in Subheading 2.1. Sepharose CL-4B is provided as a slurry by the manufacturer, which reduces the preparation time to that required for the HSS buffer exchanges.

2.3 Membrane Preparation

1. TLC applicator (Linomat IV, CAMAG Scientific, Wilmington, NC).
2. Vacuum sealer (Foodsaver, San Francisco, CA).
3. Vacuum oven, capable of 23 and 50 °C.
4. Paper cutter.
5. Fine-tip tweezers (Catalog #25729-081, VWR).
6. Kimwipes, 15″ × 17″ (Catalog # 21905-049, VWR).
7. Nitrocellulose membrane cards (HF090 membrane cards, Catalog # HF09004XSS, EMD Millipore, Cheshire, CT) cut into 20 cm sections.
8. Anti-myoglobin capture antibodies diluted to 1.0 mg/mL with a solution of 0.4 M $NaHCO_3/Na_2CO_3$, pH 9.0 containing 5% (v/v) methanol. Anti-streptavidin control antibodies diluted to 1.5 mg/mL with the same buffer.

9. The blocking reagent is prepared by diluting 100 mL 1% (w/v) casein sodium salt and 3.85 mL 2 M sucrose to a final volume of 1000 mL with HPLC grade water.
10. Sample pads (Unbound glass, Catalog #VFE, Whatman International, Ltd., Florham Park, NJ) cut into 1.9 × 20 cm sections.
11. Absorbent pads (Cellulose fiber sample pads, Catalog #CFSP223000, EMD Millipore, Billerica, MA) cut into 1.8 × 20 cm sections.

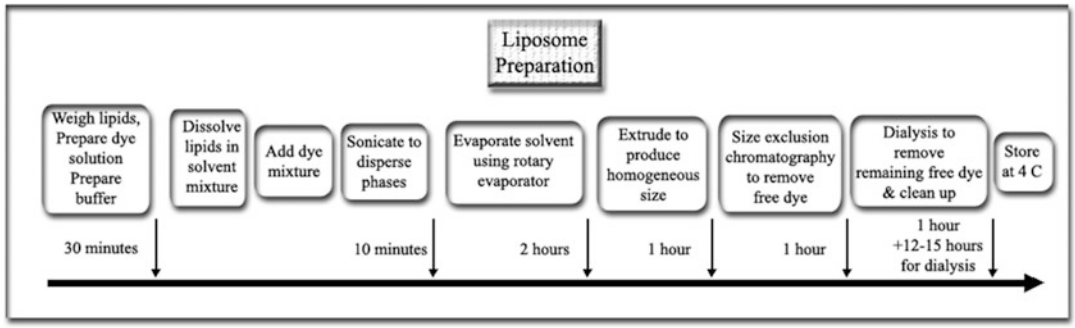
2.4 Assay Optimization Using Recombinant Myoglobin and Serum

1. Flat bottom, non-binding microtiter plates (Corning, #3610, Corning, NY).
2. Prior to running the assay, recombinant full length myoglobin is diluted in TBS with 0.05% (w/v) Tween 20 and 0.1% (w/v) BSA to concentrations of 100 μg/mL, 10 μg/mL, 1 μg/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL, 100 pg/mL, and 0 pg/mL. 30 μL portions are appropriate.
3. Biotinylated anti-myoglobin antibody is diluted to 10 μg/mL with 0.05% (w/v) Tween 20 and 0.1% (w/v) BSA.
4. Streptavidin-tagged liposomes of known phospholipid concentration.
5. Membranes with capture and control antibodies immobilized cut into 4.5 mm wide strips.
6. Reflectometer (λ = 560 nm, ESECO Speedmaster, Cushing, OK) or scanner with image analysis software (Optional if more than qualitative results by eye are desired).

3 Methods

3.1 Liposome Preparation

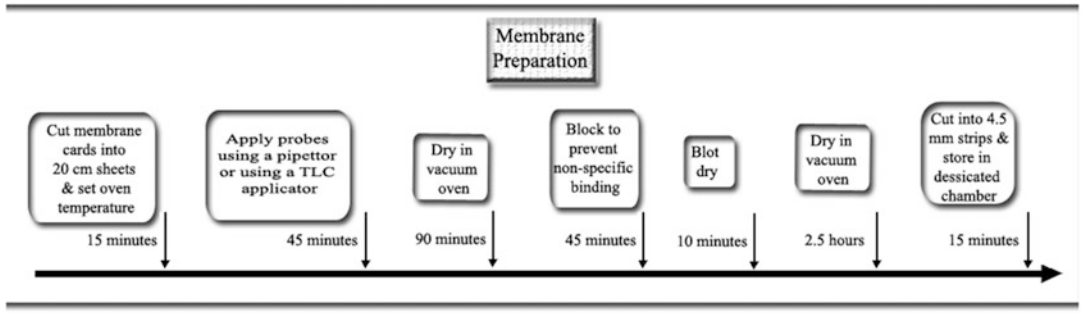
A flowchart of this process is shown in Scheme 1. Note that the times listed in this flowchart, and others in this chapter, reflect overall times for each step, including setup and incubations. For specific times for each step, please refer to the text.



Scheme 1 Flow chart for the preparation of dye-encapsulating liposomes

1. Set rotary evaporator bath and sonicator bath to 45 °C. Fill the condenser on the rotary evaporator with ice and seal condenser top rim to rotary evaporator glass housing with Parafilm.
2. Prepare dye solution as described in materials section using a 15-mL centrifuge tube, cover tube with aluminum foil, and store in the 45 °C sonicator bath.
3. DPPC, DPPG, N-glutaryl DPPE, and cholesterol (40.9:20.1:7.3:51.7 μmol, respectively) are added to a 50-mL round bottom flask (*see* **Note 1**).
4. A solvent mixture containing 3 mL chloroform and 0.5 mL methanol is added and the mixture sonicated for 1 min in a bath sonicator. We use sonication level 6 with the Aquasonic Model 150D bath sonicator.
5. 2 mL of the 45 °C sulforhodamine B dye solution is added to the lipid mixture during the first 30 s while sonicating for a total of 4 min. Swirl the flask manually during sonication (*see* **Note 2**).
6. The mixture is then placed onto a rotary evaporator at the highest rotation speed with the bath at 45 °C. The vacuum should be adjusted such that bubbling or foaming of the contents does not occur. Using the Buchi R-114 rotary evaporator, a vacuum of 500 mBar for 20 min, followed by 400 mbar for 20 min is recommended. During evaporation, return unused portion of the SRB solution to the 45 ° C sonicator bath.
7. The mixture is then transiently vortexed preceding and following an additional introduction of 2 mL 45 °C 150 mM SRB. Using a VWR mini-vortexer, we have found that holding the flask at a 45° angle and vortexing at level 4–5 is appropriate, however, be sure to use a stopper on the flask.
8. The mixture is returned to the rotary evaporator for an additional 30 min under slightly higher vacuum than in **step 6** (350 mBar, in our case.) Generally, the evaporation is considered complete 15 min after no further solvent is observed from the condenser. Removal of nearly all organic solvent is required for successful formation of liposomes.
9. While the liposome mixture is on the rotary evaporator, set the extruder support block of the mini-extruder on a hot plate and adjust temperature such that the temperature of the block does not exceed 65 °C.
10. Set-up the mini-extruder as outlined in the manufacturer's instructions. Label 2–50 mL centrifuge tubes for each extrusion size and clamp them in a 45 °C water bath during the extrusion process and before application to the size-exclusion column.

11. The liposomes are then extruded at 60–65 °C 19 times through 1.0 μm pore membranes, followed by 19 times through 0.4 μm pore membranes. The liposomes must be maintained in the 45 °C water bath during and after extrusion.
12. The level of the 1×HSS buffer volume on the Sephadex G-50 column prepared in **step 12** of Subheading 2.1 is reduced to just below the level of the Sephadex. Do not allow the Sephadex to dry out.
13. The liposomes are pipetted onto the top of the column without disturbing the top of the Sephadex. This addition should be done as carefully, but rapidly, as possible using a glass Pasteur pipet in a circular path along the inside of the glass column just above the Sephadex. *Important:* the tubes containing the liposomes need to be kept in 45 °C at all times and the transfer to the column needs to be efficient. If the liposomes cool to room temperature, they will form clumps with external dye on the column and will not separate.
14. Once all of the liposome volume has entered the column, two ~1 mL aliquots of 1×HSS should be pipetted onto the top of the column allowing each one to enter the column before the next addition. This is done to create a separation between the liposomes/free dye and the remaining 1×HSS used for liposome elution.
15. After carefully pipetting another 1–2 mL of 1×HSS on top of the column, allow 1×HSS to flow freely (typically ~4 mL/min) and collect the eluting liposomes in test tubes. The liposomes will elute from the column first, forming a dark magenta band, followed by the free dye yielding a darker and larger elution volume. If the column is run properly, some separation of liposomes and free dye can usually be seen.
16. The liposome containing fractions are then combined based either visually or on their measured optical density at 532 nm. Optical density measurements are made at 532 nm by diluting 5 μL liposomes with 995 μL 1×HSS in a 1.5 mL spectrometer cell, or, more conveniently, 1 μL liposomes in 199 μL 1×HSS in a clear microtiter plate (Corning #3795). Be selective about which liposomes are pooled together: only the most highly concentrated fractions should be together, the intermediates together, and if needed, the low concentration fractions. This is very important if the liposomes are to be subsequently coupled to proteins where a high liposome concentration is necessary (*see* **Note 3**).
17. The combined fractions are placed into dialysis bags and dialyzed overnight against the sucrose-HEPES-saline buffer. Be sure to transfer liposomes from the test tubes to the dialysis bags over a beaker to reclaim any potential spills. Replace buffer and continue dialysis until external dialysate is no longer notably pink.



Scheme 2 Flow chart for the preparation of lateral flow membranes with immobilized capture antibodies

18. Clean everything thoroughly, including removing the o-rings from the Teflon holders and the needles and plungers from the syringes of the extruder.
19. Store dialyzed liposomes in 15-mL centrifuge tubes at 4 °C.

3.2 Membrane Preparation

A flowchart of this process is shown in Scheme 2.

1. Prepare blocking reagent (0.1% (w/v) casein, sodium salt, 0.25% (w/v) sucrose) or remove previously made blocking reagent from the refrigerator and allow to reach ambient temperature.
2. Cut the HF090 membrane cards into 20 cm sections (*see* **Note 4**).
3. Label each membrane card section with a line drawn ~0.5 cm from its top on the back side using a permanent marker. The top of the HF090 membrane cards has a single covered adhesive section, whereas the bottom has an adhesive section covered by two separate peel strips.
4. For each 20 cm membrane card section, prepare 50 μL of capture antibodies at 1.0 mg/mL in 0.4 M $NaHCO_3$/Na_2CO_3, pH 9.0 containing 5% (v/v) methanol. Prepare also 50 μL of control antibodies at 1.5 mg/mL in the same solution.

3.2.1 Automated Application of Capture Antibodies Using a Linomat IV or Similar TLC Plate Applicator

The following instructions are for operation of the CAMAG Linomat IV, but should be adaptable for similar applicators using the respective manufacturer's directions.

5. Set the Limonat IV parameters as follows:

Plate width:	200	Space:	0
Start position:	0	Rate:	12 s/μL
Band width:	190	Volume:	38

6. Fill the syringe slowly with the capture antibody solution mixture to avoid air bubbles, then insert syringe fully into syringe holder.

7. Gradually lower the plunger mechanism by pressing in the front and rear buttons until the silver lever is just above the syringe plunger.
8. Press the GAS button on the front of the Linomat, then lower the silver lever onto the syringe plunger by holding down the ↓ key until you see liquid consistently bubble from the end of your syringe. You may simultaneously hold down the + and ↓ buttons (faster) if there is a fair distance between the silver lever and syringe plunger.
9. Place one of the 20 cm membrane sections so that is lined up with the bottom and side markers on the black surface. Use the supplied flat magnets to hold down the membrane at its top and right sides. Slide the tower to 3.0 cm from the base of the membrane card using the ruled marks.
10. Press Calc, then Run. Repeat steps for remaining membrane sections.
11. Thoroughly wash the syringe with HPLC grade water, then repeat steps with the anti-streptavidin solution to form a control zone 3.5 cm from the base of the membrane card following the same procedure.

3.2.2 Manual Application of Capture Antibodies Using a Pipettor

If a TLC or similar applicator is not available, the capture antibody solution may be applied manually to the lateral flow membranes. Here, a round spot for the capture and control zones would result, versus the line seen in Fig. 3 generated using the Linomat for antibody application. This procedure is useful for optimization purposes, but is more laborious than the TLC applicator procedure due to the need to apply antibodies and tape individual membranes.

1. Cut the membrane sections into 4.5 mm pieces. We've found it beneficial to tape a laminated piece of paper to the paper cutter with lines every 4.5 mm for a guide. Then, align one end of one section with this paper and advance the section, making perpendicular cuts every 4.5 mm.
2. Using tape with the sticky side exposed placed onto a piece of cardboard, lightly tape the top of each membrane to a piece of clean cardboard using tweezers.
3. Apply 1 μL of the capture antibody solution to each membrane 3.0 cm from the bottom of the strip using a microvolume pipettor. The more consistent the antibody application is, the more consistent the LFA results will be. The antibody solution will rapidly seep into the membrane.
4. Deposit the control zone 3.5 cm from the base of the membrane card following the same procedure.

3.2.3 Membrane Drying and Blocking Steps

5. Place the membranes or membrane cards in the vacuum oven at 40 °C and set vacuum to 15″ Hg for 1.5 h. Alternatively, membranes or membrane cards may be stored overnight in a tightly sealed Tupperware container with the bottom filled to 0.5″ with Drierite and covered with layers of large Kimwipes.
6. Remove membranes from vacuum oven or desiccated storage container.
7. Pour blocking reagent into a plastic Tupperware container so that it is <50% full.
8. Using tweezers, gently remove each membrane or membrane card and place in the blocking reagent laminated side down. Be sure to allow each membrane to gradually sink into your blocking reagent, otherwise blocking will be uneven. You can gently help them along by pressing the ends under the solution.
9. Place container on shaker using slow agitation for 30 min. The speed should be set such that the membranes are being covered by solution and moving freely, but not so much that they become bunched up and solution comes over the sides.
10. Using tweezers, remove each membrane or membrane section and lie flat on three layers of large Kimwipes. Place 2–3 layers of Kimwipes on top of membranes, then add a heavy flat object on top of the membranes, such as a heavy lab supplies catalog.
11. Retape membranes back onto the cardboard.
12. Return the taped membranes to the vacuum oven at 25–30 °C and set vacuum to 15″ Hg for 3–4 h.
13. Remove membrane strips or sheets from cardboard and store in vacuum sealed bags at 4 °C or as above in **step 17** at in sealed Tupperware container at ambient temperature.

LFA assembly steps

14. Cut the absorbent pad into 1.8 × 20.0 cm sections corresponding to the number of membrane cards prepared in **step 2**.
15. Gently peel away the film covering the top section of the HF090 card.
16. Apply the absorbent pad such that it overlaps the nitrocellulose by 2 mm (Fig. 6).

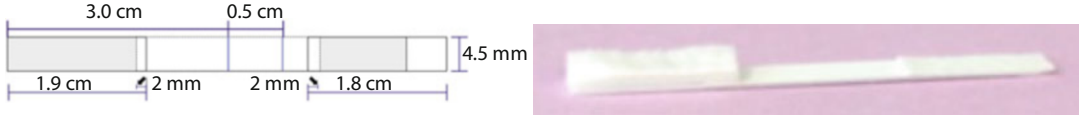


Fig. 6 (*Left*) Layout of assembled LFA membrane showing overlaps and positions of sample and absorbent pads and antibody deposition zones. (*Right*) Image of assembled HF090 membrane with VFE unbound glass sample pad and cellulose fiber absorbent pad before insertion into cassette

17. Apply gentle, but even, pressure along the length of the absorbent pad to secure it to the underlying adhesive.
18. If the membranes are to be used for assay optimization purposes only in the absence of whole blood, the bottoms may be cut off. To cut off the bottoms, align the paper cutter such that the lowest 0.5 mm of nitrocellulose will be removed when the cut is made (*see* **Note 5**).
19. The bottom of the HF090 membrane cards can accommodate both a conjugate and a sample pad. For the assays described within, only a sample pad is used. Prior to assembly, this sample pad is cut into 1.9 × 20 cm sections and blocked with 0.05% (w/v) casein, 0.25% (w/v) sucrose for 30 min and dried in a vacuum oven at 21 °C and 15″ Hg overnight.
20. Gently peel away the film covering the bottom sections of the HF090 card.
21. Apply the sample pad such that it overlaps the nitrocellulose by 2 mm.
22. Apply gentle, but even, pressure along the length of the sample pad to secure it to the underlying adhesive. Excessive pressure may crush the delicate sample pads and impede flow of the samples.
23. The assembled membrane sheets may be cut into strips of desired widths prior to storage as described in **step 12**. Our membranes are typically cut into 4.5 mm widths.

3.3 Analysis of Liposomes

If available, liposome size measurements should be made using dynamic light scattering or alternative sub-micron particle size measurement technique. We make liposome size distribution measurements using a DynaPro LSR (Proterion Corporation, Piscataway, NJ) using the Dynamics (version 6.3.01) software program and the Cumulants method of analysis [70, 71]. The Bartlett assays are necessary for the preparation of protein or antibody-conjugated liposomes (*see* **Notes 6** and **7**).

3.3.1 Bartlett Assays for Phospholipid Content

1. Liposome samples (20 μL) in triplicate are dehydrated at 180 °C for 10 min, then mixed and heated with 1.5 mL of 3.33 N H_2SO_4 for 2 h at the same temperature. Standards prepared from potassium phosphate dibasic in deionized water (16, 32, 64, 128, and 256 nmol phosphate per tube in triplicate) are subjected concurrently to the same procedure. Note that inorganic phosphates will interfere with the Bartlett assay and need to be considered if these are used in alternate buffers or diluents used for liposome preparation.
2. 100 μL of 30% hydrogen peroxide is added and the mixture is returned to the oven for 1.5 h. The tubes are permitted to cool

to ambient temperature prior to, and vortexed vigorously following, each addition.

3. Lastly, 4.6 mL of 0.22% ammonium molybdate and 0.2 mL of the Fiske–Subbarow reagent [72] are added.
4. The Fiske-Subbarow reagent is prepared by mixing 40 mL 15% (w/v) sodium bisulfite, 0.2 g sodium sulfite, and 0.1 g 1-amino-4-naptholsulfonic acid at ambient temperature for 30 min then filtering out undissolved solids. This reagent needs to be prepared fresh at least weekly.
5. The tubes are then heated in a boiling water bath for 7 min, then quickly cooled in an ice water bath. The color of the highest standard should be moderate to dark blue after heating. If it is not sufficiently dark, continue heating for several minutes until it becomes this shade of blue.
6. The absorbance at 830 nm is recorded using a Powerwave XL microtiter plate reader (Bio-Tek Instruments, Winooski, VT).
7. The phospholipid content of the liposomes is determined from a calibration curve prepared from the phosphate standards analyzed in each run. The total lipid concentration is calculated by multiplying the phospholipid concentration by the initial ratio of total lipid to phospholipid.

3.4 Liposome Conjugation

The liposome conjugation procedure assumes that the phospholipid concentration, determined as described in Subheading 3.3 or by other means, is known. The procedure described within is has been optimized for the conjugation of streptavidin to liposomes and may be modified to accommodate other proteins by varying the EDC and protein concentrations. It utilizes EDC to form a zero-length linkage between carboxylic acid groups on the liposomes and protein amine groups. Before beginning this procedure, ensure that the protein to be conjugated is purified and does not contain any stabilizing proteins or buffer components with amine groups.

1. Remove EDC from −20 °C storage and allow to warm to ambient temperature while proceeding with the following steps.
2. Add desired volume of liposomes prepared in Subheading 3.1 to a 1.5-mL microcentrifuge tube.
3. Using the known phospholipid concentration, calculate the nmol of total lipid present. Be sure to account for non-phospholipid constituents such as cholesterol in your calculation.
4. Determine the amount of protein needed for conjugation. Typically, 0.05 mol% of the total lipid content is a reasonable amount.

5. Determine the amount of EDC needed for conjugation. 0.3 molar equivalents EDC based on the total lipid content is optimal. Calculate how much volume of a 100 mg/mL solution of EDC is needed.
6. Add the amount of protein calculated in step 23 to the microcentrifuge tube containing the liposomes and gently vortex until homogeneous.
7. Weigh the requisite amount of EDC into a clean, dry microcentrifuge tube.
8. Dilute EDC to a concentration of 100 mg/mL with 0.1 M MES buffer, pH 4.65 immediately before proceeding with **step 9**.
9. Add desired volume of EDC and immediately vortex the solution for approximately 30 s.
10. Incubate the mixture at room temperature in the dark for 30 min.
11. Purify the conjugated liposomes through a SEC column packed with Sepharose CL-4B using 1× HSS as an elution buffer [73].
12. All fractions may be combined together for subsequent phospholipid determination measurements as described in Subheading 3.3.

3.5 Assay Optimization Using Recombinant Myoglobin

The steps within this section are helpful for determining the best conditions for antibody/antigen interaction and signaling. Here, the buffer composition, biotinylated antibody, and liposome concentrations may be varied to attain the desired level of detection. This information can be utilized to streamline the development of the assay in whole blood. If needed, the capture antibody concentration can also be optimized during membrane preparation which can be done easily if the manual application approach is used.

1. Add 10 μL myoglobin solutions to the bottom of wells of a flat-bottom, non-binding microtiter plate (*see* **Note 7**).
2. Insert a 4.5 mm membrane strip with immobilized capture and control antibodies with the base cut off and allow volume to wick up membrane.
3. Once the solution has completely wicked, the membrane strips are transferred to wells containing 10 μL of MESS with 0.1% (w/v) BSA.
4. Once this solution has wicked, membranes are transferred to wells containing a solution with 6 μg/mL biotinylated anti-myoglobin antibodies and liposomes diluted to 250 μM phospholipid.

5. Once this solution has wicked, membranes are then transferred to wells containing 30 μL of MESS buffer.
6. Remove membrane once all solution has wicked and place on a sheet of paper to dry. A dark colored sheet allows for more contrast when results are scanned. Drying takes ~ 10 min at room temperature.

3.6 Assay Format for the Analysis of Myoglobin in Whole Blood

In our studies, 16 mL of human venous blood was drawn into EDTA vacutainer tubes by venipuncture at the Gannett Health Center at Cornell University. Participants provided informed consent for this study, which was approved by the institutional review board at Cornell University. No participant identifier was associated with each sample.

Important: Human blood should be treated as it is potentially infectious and proper precautions for handling and disposal should be followed, in accordance with your organization's protocols. Obtaining human blood samples also requires informed consent of your subjects, in accordance with your organization's institutional review board.

1. Fully assembled membrane strips with blocked sample pads, immobilized capture and control antibodies, and absorbent pads are inserted into a commercially available cassette. The dimensions of the absorbent pad may be adjusted to ensure that the pressure points of the cassette line up with the sample and absorbent pad overlaps; the sample hole lines up with the sample pad; and the test and control lines are visible.
2. Whole blood is spiked with 0–350 ng/mL myoglobin.
3. 50 μL of the spiked blood is added to 50 μL of an antibody/liposome solution and is gently vortexed for 5 min.
4. 100 μL of the blood mixture is transferred to the sample pad.
5. Allow assay to run for 15 min. A signal proportional to the myoglobin concentration should result (Fig. 7).

3.7 Analysis of Results

The membranes may be read by eye for qualitative results, however, semiquantitative results may be obtained if a scanner, digital camera, or reflectometer is available. After the membranes have fully dried (typically 20–30 min), scan an image of the membranes using a computer and flatbed scanner, then use any available densitometry software to quantify band intensity. Alternatively, if a reflectometer is available, the readings should be taken at this time following the instrument manufacturer's instructions. For optimal reproducibility and sensitivity, the intensity of the bands should be read as soon as the membranes dry as it tends to decrease over time (days to weeks) and exposure to light. While membranes may also be scanned or read using a reflectometer when wet, care must be taken to avoid leaving impressions in the nitrocellulose. Whether read wet or dry, consistency in the timing of measurement is key for optimal reproducibility.

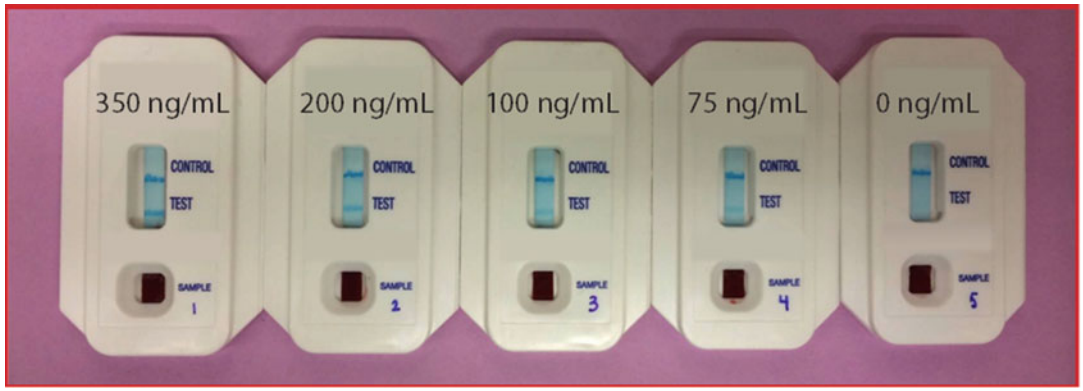


Fig. 7 Whole-blood lateral flow assay at spiked myoglobin concentrations ranging from 350 ng/mL (*left*) to 0 ng/mL. 50 μL blood samples were spiked with various myoglobin concentrations and added to 50 μL of an antibody/liposome solution. This mixture was gently vortexed, and after 5 min, all 100 μL were added to the sample pad. Results were observed after 15 min. A visible signal at the test line could be seen at a spiked myoglobin concentration of 75 ng/mL (*see* **Notes 8** and **9**)

4 Notes

1. For the preparation of liposomes to be tagged to antibodies or other proteins, the N-glutaryl DPPE phospholipid introduces a carboxylic acid group to the lipid bilayer allowing for facile conjugation to primary amine groups using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) [73].
2. Be sure that the solution in the flask is being sonicated moderately vigorously and that the flask is not located in a "dead spot" of the bath. We use sonication level 6 with the Aquasonic Model 150D bath sonicator.
3. If liposomes are instead formed with a cholesterol-modified DNA probe for nucleic acid sequence detection [74], the focus on liposome concentration is less important since no post-formation conjugation needs to take place.
4. Here, HF090 membranes worked out well for this assay as they provided the requisite capillary flow, performance for our analyte, and ability to secure the sample and absorbent pads. However, blank backing cards (#MIBA-010, DCN, Carlsbad, CA) with adhesive for the center portion may be purchased to allow the user to apply their preferred nitrocellulose or other analytical membrane material.
5. By cutting off a small portion of the nitrocellulose, good contact of the nitrocellulose with the fluid during the assay is ensured.

6. For antibody conjugation, the liposomal phospholipid concentration needs to be determined using Bartlett assays [75] (*see* Subheading 3.3). Both antibodies of the matched set should be conjugated to liposomes to determine which serves as the better detection antibody. Note that EDC is very hydroscopic—use care to protect the reagent from moisture.
7. Alternatively, the phospholipid concentration of liposomes can be measured by inductively coupled plasma—atomic emission spectroscopy (ICP-AES). A volume of 20 μL of liposomes is diluted in nitric acid ($c = 0.5$ mol/L) to a total volume of 3 mL. The mixture is vortexed and sonicated thoroughly. Phosphorus standard solutions are prepared with concentrations of 1, 5, 10, 25, 50, and 100 μmol/L phosphate in 0.5 mol/L HNO_3 for calibration. The phosphorus content is then measured via ICP-AES (at the phosphorus specific wavelength of 178.29 nm [76].
8. No signal was observed in this assay with normal levels of circulating myoglobin, but a visible signal was observed if a normal blood sample was spiked with 75 ng/mL myoglobin, which mimics an elevation outside of the normal range. Normal levels of myoglobin in human blood range from 0 to 85 ng/mL, whereas the median level of myoglobin 90 min after an AMI has been reported to be 353 ng/mL [77]. The level at which a signal is observed in a LFA can be altered by optimizing the assay as noted in Subheading 3.5.
9. In any sandwich immunoassay run without a wash step, the risk of a high-dose Hook effect needs to be checked for to avoid underestimating the concentration of the analyte in the sample. This can be done by making dilutions of the sample and repeating the assay to ensure that the signal decreases as expected, rather than increases as would be the case with the Hook Effect.

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