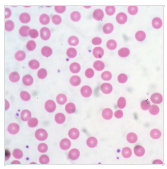

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Eosin-5'-maleimide (EMA) binding test with fluorecent beads

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DISCLAIMER

ABSTRACT

The flow cytometry-based eosin-5'-maleimide (EMA) binding test is used for reliable diagnostics of hereditary spherocytosis, a relatively common hereditary anemia.
In this modified version of the EMA-binding test, we utilize commercially available rainbow beads to overcome the the need for three to six healthy controls. However, two healthy controls are still used in this protocol as an extra safety measure.

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PROTOCOL CITATION

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KEYWORDS

membranopathy, hereditary spherocytosis, EMA, eosin-5-maleimide, hemolysis, anemia

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GUIDELINES

The eosin-5'-maleimide (EMA) binding test was performed on blood within 48 hours of sampling. We recommend using EDTA-stabilized blood and store the samples at 4^{O}C until analysis.

All EMA binding tests were performed on a FACS Canto II (BD Biosciences, Franklin Lakes, NJ, United States) using standard filter options (530/30). Other flow cytometers should do as well.

This protocol deviates from other EMA-binding tests by utilizing commercially available fluorescent beads instead of three to six healthy control samples.

As rainbow bead MFI is slightly higher than the average fluorescence level of healhty controls, one can utilize a constant calibration factor (CF) to make the results comparable to the traditional EMA-binding test.
The reason for this CF is solely to ensure that the EMA value obtained can be compared with the EMA values stated in the literature.

New lots of rainbow beads should be calibrated towards the previous to ensure consistency of the EMA values calculated. Otherwise, a new CF should be calculated.
We rutinely use two healthy controls with each sample as an extra safety measure. We do not age-match these to the patient. If possible, a travel control should be analyzed along with the patient sample.

MATERIALS TEXT

MATERIALS

Sodium chloride **Contributed by users**

5-Maleimido-eosin for fluorescence ≥93% (HPLC) **Sigma-aldrich Catalog #63184** Step 1

Rainbow Fluorescent Particles 3.0-3.4 µm (mid-range FL1 fluorescence) **BD Biosciences Catalog #556298**

Dulbecco's Phosphate Buffered Saline **Merck Catalog #D8537**

FluoroSpheres Calibration beads **Agilent Technologies Catalog #K011011-2**

Bovine Serum Albumin **Sigma Aldrich Catalog #A4503**

SAFETY WARNINGS

Treat all human blood as potentially infectious.
Laboratory coats or appropriate gowns must be worn in the laboratory and fastened properly.
Gloves must be worn when handling human blood.
All open cuts and abrasions must be covered.
Use disposable equipment wherever possible.

DISCLAIMER:

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BEFORE STARTING

If normalization to normal controls is desired, the constant calibration factor (CF) should be calculated in advance.

Preparation of EMA Reagent batches 13m

1 Preparation of stock of EMA-reagent:
5-Maleimido-eosin for fluorescence ≥93% (HPLC) **Sigma-aldrich Catalog #63184**

1.1 2m
1. Dissolve **10 mg** EMA in **20 mL** PBS.
2. Mix well.

1.2 10m
1. Prepare 80 new 500 µl PCR tubes by labeling them (MM.YY)
2. Transfer aliquots of **250 µl** EMA solution to each tube.

1.3 1m
Store in freezer.
Aliquots of frozen dissolved EMA dye should be stable for at least six months.
Aliquots should be discarded after use (i.e. *not* frozen agin for later use).

Prepare samples 2h 16m

2 1m
Remove a tube of stock EMA ([M] **0.5 mg/ml**) aliquot from the freezer to thaw in a dark place @ **Room temperature** .

3 **Prepare samples** 30m

3.1 7m
1. Label a 14 ml tube ***for each sample***
2. **10 mL** 0.9% NaCl is added to each tube
3. **100 µl** of mixed blood is added to its corresponding tube
4. Mix/Vortex tubes
5. **1500 x g, Room temperature , 00:05:00**
6. Discard supernatant

3.2 12m
Wash twice:
1. Add **10 mL** 0.9% NaCl
2. Mix/Vortex
3. **1500 x g, Room temperature , 00:05:00**
4. Discard supernatant

3.3 2m
Transfer **5 µl** of washed packed red cells to a new (labelled) 1.5 mL eppendorf tube

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3.4 1h
- Add **25 µl** of EMA stock solution
- Incubate for **01:00:00** @ **Room temperature** **in a dark place**
- Mix every **00:15:00**

3.5 2m
1. **1500 x g, Room temperature , 00:00:30**
2. Carefully discard supernatant

3.6 2m
1. Add **500 µl** PBS
2. Mix/Vortex

3.7 1m
1. Prepare FACS tubes by adding labels
2. Transfer **25 µl** of labelled red cells to the new tube

3.8 18m **Wash the labelled RBC's 3 times:**
1. Add **3 mL** PBS with 0.5% BSA
2. Mix/Vortex
3. **1500 x g, Room temperature , 00:05:00**
4. Discard supernatant

3.9 1m
1. Add **500 µl** PBS with 0.5% BSA
2. Mix/Vortex

3.10 Samples are now ready to be run on the flow cytometer.

Run samples on the Flow Cytometer 22m

4 5m **Prepare FACS-tubes for beads:**
1. Label 3 tubes for beads and add 500 µl PBS to each.
2. Add 1 drop of beads to each tube (Calibration beads, Blank beads, and rainbow beads).

Calibration beads and blank beads are part of a calibration kit from Agilent (#K011011-2)
Rainbow beads are puchased separately (BD Biosciences, #556298)

5 2m **Setup flow cytometer (Canto II):**
1. Set it to LOG-mode for FSC, SSC, and FL1
2. Apply a threshold of 5000 for the FSC.

6 15m **For each patient sample:**
1. Start running the Calibration beads and adjust the voltage of the PMT in such a way that the fluorescence FL1 is approximately 50,000 (or as close hereto as possible). Acquire 15,000 events.
2. Run Blank beads and acquire 15,000 events.
3. Run Rainbow beads and acquire 15,000 events.
4. Run Control samples and acquire 15,000 events.
5. Run Patient sample and acquire 15,000 events.

Analyze results 5m

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7 The analysis of the results can be done differently. 5m
Step 7 includes a Step case.
Beads + CF
Beads only
Controls only

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