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Qualitative paper ELONA test

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Abstract

The aim of this protocol is to test the best method that identifies the efficiency of the detection system that will be present in the strips. 3 methods are tested and compared.
This is a dot blot performed with streptavidin and aptamers, also named paper ELONA.
The expected result is a good binding between the streptavidin and the aptamers (knowing already that the streptavidin binds correctly to the nitrocellulose membrane thanks to previous experiments), great hybridization between both aptamers and a clear visible result when yielding with the ABTS.
The method that works best from this assay will be then repeated in a quantitative experiment.

Guidelines

After washing the membranes with BSA or PBS, is convinient to let the membrane dry off for a little bit. Although it is not necessary to have it completely dry, if it's too wet, the drop of the next reagent added will probably elute out of the wax dot, which is inconvenient. If it's dry enough, the drop added will have enough surface tension to stay within the wax circle, which is the expected.

On the other hand, when adding the antibody make sure the membrane will remain wet during the whole incubation time. Same with the ABTS. A poor control of these conditions will lead to less reproducible results.

Materials

MATERIALS

- Hot/Stir Plate
- Pipette Tips
- Glass Petri dishes 90 × 15 cm
- Streptavidin, 1mg **Promega** **Catalog #**Z7041
- BSA
- Anti-Digoxigenin-AP, Fab fragments **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**11093274910
- PBS
- Eppendorf tubes 1.5 mL uncolored **Eppendorf** **Catalog #**022363204
- nitrocellulose membrane sheets size 210 m × 297 mm thickness 200 µm **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**Whatman® FF170HP Din A
- Wax crayons (non water-soluble)
- Ultrospec 1100 pro
- ABTS solution **Roche** **Catalog #**11684302001

- Various pipettes in a range from 2 µL to 1000 µL.
- Wet paper to set the humid chamber.
- Ice.
- Tweezers.
- Pen with permanent ink to mark the membranes.

Troubleshooting

Safety warnings

Lab coat and gloves should be worn throughout the whole experiment. All working surfaces must be clean and all the reactives should be treated following manufacturer's instructions.

Preparing the nitrocellulose strip

1 Set the hot plate to 100ºC or at least warm enough to melt the wax. Cut a small amount from one of the wax pencils and place it on a Petri dish. Set the Petri dish on the hot plate and wait for the wax to melt.

Cut a strip from the nitrocellulose sheet with the desired size.

Once the wax is melt, grab a 200µL pipette tip from the pointy side and place the broad end in the wax. Check the whole circle contains enough wax and carefully place it on the nitrocellulose strip. Apply a little pressure and remove the pipette tip. You should be able to see the wax circle on the strip. Repeat as many times as necessary to set all the dots for the assay.

Cover the nitrocellulose strip with paper (one sheet on top, another underneath). Place the sandwich on the hot place with a Petri dish on top (this is just to apply some pressure, any temperature-resistant flat object is valid here). Allow the strip to warm for 5 minutes. This will allow the wax to penetrate all through the strip.
Remove the strip from the hot plate and let it cool down again. You should see a circle of wax but the center must be clear, otherwise the protein will not have anywhere to bind. If the circle is too thick, consider repeating these steps.

Hybridization of the aptamers and addition of the streptavidin

2 This will be the **test method number 1.**

2.1 In an Eppendorf tube, mix the two aptamers selected: the aptamer Apta-Eco which has a biotin tag, and the reverse complementary aptamer to Apta-Eco marked with a digoxigenin tag (RC-dig). Check the concentrations are the same for both aptamers. We worked with the resuspended stock, which concentration was 100 µM. Then, add Aptamer Buffer (PBS, 1,4 mM Mg^{2+}) in proportion 1:1:3 with the aptamers.

2.2 Set the hot plate to 95ºC and place the tube with the mix for 5 minutes. After, put the tube back in ice to allow it to cool down again.

When the mix is cooled, mix it in proportion 1:1 with streptavidin 0,2 µg/µL (18µM). Let the protein and the aptamer bond for a few minutes in ice.

2.3 Finally, pipette 5 µL from the mix tube in each one of the wax dots previously prepared on the nitrocellulose membrane, and allow it to dry completely. It is recommended to set

at least 2 or 3 dots for each concentration, to increment the total n and have average data for each concentration.

Attachment of the streptavidin to the membrane and addition of the aptamers

3 This will be the **test method number 2**.

3.1 Pipette 5 µL of streptavidin 0,1 µg/mL (10µM) on the dots reserved for this part. Allow it to dry completely (the membrane inside the wax circle should have the same color as outside the dot)

3.2 Pipette 5 µL of the aptamer Apta-Eco. Allow it to dry. Once the membrane is dried up (patience!), pipette 5 µL of the RC-dig aptamer. Let it dry again

Hybridization of the streptavidin with Apta-Eco and addition of the RC-dig

4 This will be the t**est method number 3.**

4.1 In an Eppendorf tube, mix 5 µL of 10 µM streptavidin with 5µL of 10 µM Apta-Eco (if more stock is needed, just be sure to follow 1:1 proportion).

4.2 Add 5µL from the mix to each one of the dots reserved for this section. Let the dots dry.

4.3 Add 5 µL of RC-dig 10 µM and let it dry again.

Setting the control dots

5 Prepare four different controls:

5.1 **NEGATIVE 1**. Pipette 5 µL of streptavidin 0,1 µg/mL on the dots reserved for this section. Allow it to dry. Without aptamers, this strip should not give a positive result. This one will not have antibody.

5.2 **NEGATIVE 2**. Pipette 5 µL of streptavidin 0,1 µg/mL on the dots reserved for this section. Allow it to dry. Similar to the negative 1, without aptamers, this strip should not give a positive result. But in this case, antibody will be added in further steps, to check if the presence of the aptamers is necessary for the reaction to happen.

5.3 **POSITIVE 3**. Pipette 5 µL of streptavidin 0,1 µg/mL on the dots reserved for this section. Allow it to dry. Add 5 µL of the antibody (Anti-Dig-POD 1:100 in PBS 1,4mM Mg^{2+} BSA 0,2%). This control will reveal if the antibody by itself works when the free pores in the membrane are not blocked and the antibody binds non-specifically.

5.4 **NEGATIVE 4**. Pipette 5 µL of streptavidin 0,1 µg/mL on the dots reserved for this section. Allow it to dry. Then, pipette 5 µL of the aptamer Apta-Eco. Allow it to dry. Once the membrane is dried up (patience again!), pipette 5 µL of the RC-dig aptamer. Let it dry again. This is the same construction as method number 2 but this control will not have antibody.

Washing and blocking with BSA

6 Set the strip in a Petri dish and pipette BSA 5% (in PBS). Pipette excess of it and with tweezers, move the strip around gently, to let it wash and block the free spaces in the membrane. Take the strip out of the Petri dish and allow it to dry for a bit. It is not necessary that the strip is perfectly dried up, but it must not be soaked. The more dry, the more chances for the antibody not to spread out of the wax dot (if it's too wet, there won't be enough surface tension to hold the next reagent within the wax circle).
Do it for all three methods and controls negative 1 and negative 2 but NOT with the positive 3. With control positive 3, the antibody is added without the BSA blocking.

Addition of the antibody

7 Once is dry enough, pipette 5µL of the antibody (Anti-Dig-POD 1:100 in PBS 1,4 mM Mg^{2+} BSA 0,2%). The controls negative 1 and negative 4 skip this step and have no antibody addition.

Incubate for one hour: set a humid chamber. This can be easily done by adding wet paper in a Petri dish. This will prevent the antibody from drying completely while incubating.

7.1 After one hour, remove the strips from the chamber, and wash them again with PBS (pipetting excessive liquid and moving gently with the help of tweezers. Allow it to dry enough to proceed to the next step (just like before, there is no need to completely dry the membrane). Since the controls negative 1 and negative 4 had no antibody addition, they are therefore not washed with PBSand also skip this step.

Reveal with ABTS

8 Set again the humid chamber and pipette 10 µL of ABTS on each dot. Incubate in humid conditions for 30 minutes. If no color is developed, 15 more minutes can be added. On the other hand, if the reaction happens fast and you are able to see a strong change in color, this period can be shortened to 15 minutes. Done in all methods and all controls.

8.1 Dry the strips on the hot plate. 5 minutes at 95ºC should be enough. This will stop the reaction in all the strips at the same time, to make the results comparable. Independently of the total incubation duration, it is crucial that all the strips stop the reaction at the same time (otherwise, the results are not comparable).

Results

9 To see the results with higher quality, they can be scanned. The density of each dot can be analysed with software tools.