Dimethylmethylene Blue Assay (DMMB)

Vivien Jane Coulson- Thomas[1*] and Tarsis Ferreira Gesteira[2, 3]

[1]John van Geest Centre for Brain Repair, University of Cambridge, Cambridge, UK; [2]Department of Ophthalmology, University of Cincinnati, Cincinnati, USA; [3]Division of Developmental Biology, Cincinnati Children's Hospital Research Foundation, Cincinnati, USA
*For correspondence: vcoulsonthomas@gmail.com

[Abstract] Glycosaminoglycans (GAGs) are long unbranched polysaccharides consisting of repeating disaccharide units composed of a hexosamine (glucosamine or galactosamine) and a hexuronic acid (glucuronic or iduronic acid). Depending on the disaccharide unit the GAGs can be organized into five groups: chondroitin sulfate, dermatan sulfate, heparan sulfate, keratan sulfate and hyaluronic acid. The GAGs are heterogeneous molecules with great variability in molecular mass and both sulfation density and pattern. Spectrophotometric assays to measure the GAG content in biological fluids and tissue/cell extracts are valuable tools. The dye 1,9-dimethylmethylene is a thiazine chromotrope agent that presents a change in the absorption spectrum due to the induction of metachromasia when bound to sulfated GAGs enabling rapid detection of GAGs in solution (Whitley *et al.*, 1989; Chandrasekhar *et al.*, 1987; Farndale *et al.*, 1982). Moreover, there is a window in which a linear curve may be drawn (approximately between 0.5-5 µg of GAGs) enabling the quantification of GAGs in solution.

Materials and Reagents

1. Dimethylmethylene blue (DMMB) (Sigma-Aldrich, catalog number: 341088)
2. NaCl
3. Glycine (Sigma-Aldrich, catalog number: 410225)
4. Glacial Acetic acid (Sigma-Aldrich, catalog number: S7653)
5. Tris-Base (Merck KGaA, catalog number: 648310)
6. Bovine chondroitin 4-sulfate as standard (Sigma-Aldrich, catalog number: C9819)
7. DMMB reagent (see Recipes)

Equipment

1. Plate mixer (VWR International, catalog number: 89202-332)
2. Cover adhesive (R&D Systems, catalog number: DY992)
3. Microplate reader with 525 nm (BioTek Instruments, catalog number: 11-120-531)

4. 96 well microplate spectrophotometer with 525 nm filter set (Thermo Fisher Scientific, catalog number: 51119200)
5. Microplate shaker (VWR International, catalog number: 97043-608)

Procedure

1. Prepare DMMB reagent and paper filter using Whattman® 3MM. The pH of this solution is around 3.0. To prepare 1 L dye solution, dissolve 16 mg DMMB in 1 L water containing 3.04 g glycine, 1.6 g NaCl and 95 ml of 0.1 M Acetic Acid.
2. Prepare standard solution of chondroitin 4 sulfate (500 µg/ml in H_2O). Prepare standard curve as stated in the table bellow.
3. Pipet the standard stock solution and complete the volume to 20 µl with H_2O into the 96 well microplate.
4. Pipet 20 µl of each sample into the microplate.
5. Add 200 µl of DMMB to each sample and shake the plate of a plate shaker for 5 sec.
6. Read the absorbance using a plate reader at 525 nm immediately.

Std (µg/ml)	Vol (µl) of 500 µg/ml std	vol H_2O (µl)	vol DMMB (µl)
0	0	20	200
1.25	2.5	17.5	200
2.5	5	15	200
5	10	10	200
7.5	15	5	200
10	20	0	200

Notes

1. DMMB assay can normally be performed on samples with high detergent and salt concentrations; however the standard curve should be prepared in the same solution.
2. Avoid to performing the assay on samples in high albumin or serum concentrations which may interfere with the assay (Warren, 2000).
3. Some groups have reported the interference of DNA in the DMMB assay, however, decreasing the pH to approximately 3 and increasing salt concentrations makes the interference of DNA negligible.
4. DMMB requires the length of glycosaminoglycan chain be over a tetrasaccharide.

5. DMMB reacts with the sulfate group of the GAG chain and therefore will not work with unsulfated GAGs such as hyaluronic acid.
6. Further information can be acquired by utlilizing the carbazole reaction (sensitivity from 1 to 20 µg) to assay the carboxyl groups of the uronic acid for heparin sulfate, chondroitin sulfate and dermatan sulfate and/or anthrone reaction to assay the hexose group for keratan sulfate (Mort and Roughley, 2007).
7. The DMMB can also be very efficient when performing chromatography to rapidly assay for fractions containing GAGs (Burton-Wurster *et al.*, 2003).
8. The DMMB-GAG complex that is formed results in the immediate formation of turbidity, however this complex starts to precipitate within 10 min, therefore the absorbance measurement should be performed immediately.

Recipes

1. DMMB reagent
   Dissolve 16 mg DMMB, 3.04 g glycine, 1.6 g NaCl and 95 ml of 0.1 M acetic acid and complete the volume to 1 L
   Filter (0.45 µm)
   Protect from light
   Do not use if precipitate is present in the solution

Acknowledgments

This protocol was adapted or modified from Farndale *et al.* (1982) and Whitley *et al.* (1989).

References

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