Standard Operating Procedure for:

Microcystin (ELISA)

University of Missouri Limnology Laboratory

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1 Identification of the method

1.1 Measurement of Microcystins-ADDA via Eurofins Abraxis immunoassay for quantitative and sensitive congener-independent detection.

2 Applicable matrix or matrices

2.1 This method is suitable for the analysis of environmental samples.

3 Detection limit

3.1 Method Detection Limit: 0.10 µg/L. [1]

3.2 This Method Detection Limit was determined by the manufacturer, Eurofins Abraxis.

4 Scope of the method

4.1 This standard operating procedure is intended to provide MU Limnology operators, technicians, and analysts with guidance on the collection and congener-independent analysis of water samples for Microcystins and Nodularins using the Eurofins ELISA test 520011.

5 Summary of the method

5.1 ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying soluble substances such as peptides, proteins, antibodies, and hormones.

5.2 Operating Range: 0.1 to 5 µg/L

6 Interferences

6.1 Buffer/preservative solution included with the anatoxin and saxitoxin kits can interfere with results. Use only unbuffered samples for microcystin analysis.

6.2 Non-glass fiber filters may produce falsely low sample results as Microcystins may bind to the filter material.

6.3 According to Eurofins, some glass fiber filters are manufactured using a process which could cause inaccurate (falsely high) results. If using a new filter brand (currently using Foxx Life Sciences or Whatman 0.45um 13mm), a greater volume of sample is required (10 ml) than usually collected. In this case, pass first 5 ml of sample through filter and discard, then retain final 5 ml of filtered sample.

7 Health and Safety

These analyses involve handling freshwater samples that may contain live microorganisms and therefore pose some threat of infection. Laboratory personnel who are routinely exposed to such water samples are encouraged to protect themselves from water borne illnesses by wearing clean disposable gloves and washing hands frequently.

7.1 Wear protective gloves, lab coats, and other appropriate PPE when handling all chemical substances used in this method. All operators and technicians preforming this method should review the MSDS for additional information and safety concerns regarding the chemical substances used throughout these procedures.

8 Personnel qualifications

8.1 Samples will be analyzed by MU Limnology staff who have been trained to the operators or technician level in this method and who are familiar with all of the MU Limnology sampling handling and labeling procedures and appropriate SOPs.

9 Equipment and supplies

9.1 0.5-meter sampling tube

9.2 0.5 L PETG container (e.g., Fisher #0992316B)

9.3 Amber glass vial with PTFE lined cap (e.g., Fisher #14955332)

9.4 Eurofins/Abraxis Microcystins-ADDA ELISA kit (product# 520011)

9.5 Stepping pipette, 50 µL pipette, associated tips

9.6 Syringe with Luer-lock tip, 0.45 µm glass fiber filters (e.g., Fisher # 501049931)

9.7 500 ml squirt bottle

9.8 96 well microplate reader, computer, software

10 Reagents and standards (included in kit)

All reagents and standards used in this method are included with the kit and do not require further preparation.

10.1 Microtiter plate (12 X 8 strips) coated with an analog of Microcystins conjugated to a protein.

10.2 Standards (6): 0, 0.15, 0.40, 1.0, 2.0, 5.0 ppb, 1.5 mL each.

10.3 Control: 0.75 ± 0.185 ppb, 1.5 mL, prepared from a secondary source, for use as a Quality Control Standard.

10.4 Sample Diluent, 25 mL, for use as a Laboratory Reagent Blank and for dilution of samples above the range of the standard curve.

10.5 Antibody Solution, 6 mL.

10.6 Anti-Sheep-HRP Conjugate Solution, 12 mL.

10.7 Wash Buffer (5X) Concentrate, 100 mL, must be diluted prior to use.

10.8 Substrate (Color) Solution (TMB), 12 mL.

10.9 Stop Solution, 6 mL.

11 Quality Control

11.1 Positive Control

A positive control with a known concentration is included with each run and analyzed.

12 Analysis

12.1 Freeze/thaw lysis

Prior to analysis, samples should undergo a total of 3 freeze/thaw cycles to lyse cells and release cell-bound toxins.

- Remove vials from freezer and place into test tube racks. Place test tube rack into plastic tub.
- Slowly begin to fill plastic tub with cool water. When water slightly covers bottom of vials, remove rack from water and inspect for cracked vials. Do this several times as vials begin to thaw. If there are no broken vials, fill plastic tub until approximately half of each vial is submerged. Allow to thaw, typically 15 minutes.

  If a crack is discovered (usually the bottom of the vial will break loose entirely), immediately remove the vial from the rack and place into a clean glass test tube of suitable size. Allow ice in broken vial to thaw inside test tube. After thawing, pour contents into new vial. Reuse the same cap as it will have identifying information.
- Once thawed, vials should be re-frozen, again on their sides to reduce the risk of breakage. A total of 3 freeze-thaw cycles need to be completed before samples are ready for analysis.

12.2 Filtering

- After lysing, samples should be filtered through a glass fiber filter to remove particulates that may interfere with the analysis.
- Remove syringe from packaging, remove plunger from syringe. Set both on clean paper towel to avoid contamination from lab surfaces.
- Screw inline syringe filter to syringe body.
- Pour sample from vial into syringe, tap empty vial on another clean paper towel to remove remaining water droplets. Put open end of filter into vial and insert plunger into syringe. Depress plunger until filtered sample is returned to vial. Recap vial and mark a line on the cap with a colored Sharpie marker. This mark indicates the sample has been filtered. Discard syringe and filter into temporary waste bin.

12.3 Sample analysis

- Samples and ELISA kit should be at room temperature prior to analysis.
- Add 50 µL of the standard solutions, control, or samples into the wells of the test strips according to the working scheme given. Samples are analyzed in duplicate unless project-specific instructions direct otherwise.

- Add 50 µL of the antibody solution to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for 90 minutes at room temperature.
- During incubation time, dilute concentrated wash buffer by pouring entire contents into clean 500 ml squirt bottle, fill with DI water to line on bottle. This represents a 5:1 dilution.
- Remove the covering, decant the contents of the wells into a sink, and blot the inverted plate on a stack of paper towels. Wash the strips three times using the diluted wash buffer. Please use at least a volume of 250 µL of 1X wash buffer for each well and each washing step. Blot the inverted plate after each wash step on a stack of paper towels. After the last wash/blot, check the wells for any remaining buffer in the wells, and if necessary, remove by additional blotting.
- Add 100 µL of the enzyme conjugate solution to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for 30 minutes at room temperature.
- Remove the covering, decant the contents of the wells into a sink, and blot the inverted plate on a stack of paper towels. Wash the strips three times using the diluted wash buffer. Please use at least a volume of 250 µL of 1X wash buffer for each well and each washing step. Blot the inverted plate after each wash step on a stack of paper towels. After the last wash/blot, check the wells for any remaining buffer in the wells, and if necessary, remove by additional blotting.
- Add 100 µL of substrate (color) solution to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for 20-30 minutes at room temperature. Protect the strips from sunlight.
- Add 50 µL of stop solution to the wells in the same sequence as for the substrate (color) solution using a multi-channel pipette or a stepping pipette.
- Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.
- Samples with concentrations exceeding the high standard should be diluted with the included diluent and reanalyzed. The typical dilution is 10:1.

13 Method performance

13.1 Desired Performance Criteria

- Method Detection Limit: 0.10 µg/L
- Precision: CV < 15% for ≤ 0.4 µg/L and CV < 10% for > 0.4 µg/L
- Calibration $r^2 > 0.99$

14 Data assessment, acceptable criteria for quality control measures, and Corrective actions for out-of-control or unacceptable data

14.1 Standard curve should have an r^2 value of at least 0.99.

14.2 Standard curve replicates should have an absorbance CV >10%. One standard may have an absorbance CV of up to 15%, if no other standard CV exceeds 10%.

14.3 The concentration of the positive control should be 0.75 ± 0.185 ug/L (between 0.565 and 0.935 ug/L).

14.4 Samples with concentrations ≤ 0.4 ug/l may have a CV up to 15%.

14.5 Samples with concentrations > 0.4 may have a CV up to 10%.

14.6 Samples not meeting the above criteria should be reanalyzed.

14.7 Samples with replicates having concentrations < 0.10 µg/L and > 0.15 µg/L should be reanalyzed.

14.8 Samples with concentrations > 5.0 µg/L should be diluted and reanalyzed.

14.9 Results from subsequent sample analyses will be combined with initial analysis to test for outliers and determine further actions.

14.10 Samples not meeting criteria outlined above should be reanalyzed.

15 Waste management

15.1 All waste generated is considered hazardous.

15.2 All analyzed standards, and reagents should be treated as waste upon completion of the run.

15.3 Waste should be kept in an approved container with proper labeling.

15.4 Waste will not be held for longer than 6 months and MU Environmental Health and Safety will be notified an appropriate time before this point so that waste can be collected and disposed of.

15.5 Empty temporary waste bin directly in dumpster, not into lab trash cans. Syringes (even without needles) discarded in the lab trash may pose a risk to custodial staff.

16 References

16.1 [1] Microcystin ELISA, Eurofins 520011 User Guide. Eurofins Abraxis.

17 Additional materials

17.1 Eurofins 520011 User Guide (downloaded November 2020)

Microcystins-ADDA ELISA (Microtiter Plate)

Enzyme-Linked Immunosorbent Assay for the Congener-Independent* Determination of Microcystins and Nodularins in Water Samples

Product No. 520011

1. General Description

The Eurofins Abraxis Microcystins-ADDA ELISA is an immunoassay for the quantitative and sensitive congener-independent* detection of Microcystins and Nodularins in water samples. This test is suitable for the quantitative and/or qualitative detection of Microcystins and Nodularins in water samples **[please refer to the appropriate technical bulletins for sample collection, handling, and treatment of drinking (treated and untreated) and recreational water samples]**. If necessary, positive samples can be confirmed by HPLC, protein phosphatase assay, or other conventional methods.

2. Safety Instructions

The standard solutions in the test kit contain small amounts of Microcystins. The substrate solution contains tetramethylbenzidine (TMB) and the stop solution contains diluted sulfuric acid. Avoid contact of the TMB and stopping solution with skin and mucous membranes. If these reagents come in contact with skin, wash with water.

3. Storage and Stability

The Microcystins-ADDA ELISA kit should be stored in the refrigerator (2-8°C). The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the last day of the month as indicated by the expiration date on the box. Consult state, local, and federal regulations for proper disposal of all reagents.

4. Test Principle

The test is an indirect competitive ELISA for the congener-independent detection of Microcystins and Nodularins. It is based on the recognition of Microcystins, Nodularins, and their congeners by specific antibodies. Toxin, when present in a sample, and a Microcystins-protein analogue immobilized on the plate compete for the binding sites of the anti-Microcystins/Nodularins antibodies in solution. The plate is then washed and a second antibody-HRP label is added. After a second washing step and addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of Microcystins present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the Microcystins-ADDA ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in water samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that may be found in water samples, test interferences caused by matrix effects cannot be completely excluded.

Samples containing methanol must be diluted to a concentration < 5% methanol to avoid matrix effects.

Seawater samples must be diluted to a concentration ≤ 2.5% to avoid matrix effects. Alternately, if a lower detection limit is required, interfering compounds can be removed from seawater or brackish water samples prior to analysis. Please see the Microcystins in Brackish Water or Seawater Sample Preparation for the Microcystins-ADDA ELISA Technical Bulletin (available upon request).

No matrix effects have been observed with samples which have been treated with sodium thiosulfate at concentrations ≤ 1 mg/mL.

Mistakes in handling the test can cause errors. Possible sources for such errors include: inadequate storage conditions of the test kit, incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, and extreme temperatures during the test performance (lower than 10°C or higher than 30°C). The assay procedure should be performed away from direct sunlight.

As with any analytical technique (GC, HPLC, etc.), positive results requiring regulatory action should be confirmed by an alternative method.

A. Materials Provided

1. Microtiter plate (12 X 8 strips) coated with an analog of Microcystins conjugated to a protein
2. Standards (6): 0, 0.15, 0.40, 1.0, 2.0, 5.0 ppb, 1.5 mL each
3. Control: 0.75 ± 0.185 ppb, 1.5 mL, prepared from a secondary source, for use as a Quality Control Standard (QCS)
4. Sample Diluent, 25 mL, for use as a Laboratory Reagent Blank (LRB) and for dilution of samples above the range of the standard curve
5. Antibody Solution, 6 mL
6. Anti-Sheep-HRP Conjugate Solution, 12 mL
7. Wash Buffer (5X) Concentrate, 100 mL, must be diluted prior to use, see Test Preparation (Section E)
8. Substrate (Color) Solution (TMB), 12 mL
9. Stop Solution, 6 mL

B. Additional Materials (not delivered with the test kit)

1. Micro-pipettes with disposable plastic tips (20-200 µL)
2. Multi-channel pipette (50-300 µL), stepper pipette (50-300 µL), or electronic repeating pipette with disposable plastic tips
3. Deionized or distilled water
4. Container with 500 mL capacity (for diluted 1X Wash Buffer, see Test Preparation, Section E)
5. Graduated cylinder
6. Paper towels or equivalent absorbent material
7. Timer
8. Tape or parafilm
9. Microtiter plate reader (wavelength 450 nm)
10. Microtiter plate washer (optional)

C. Sample Collection and Handling

Collect water samples in **glass or PETG** containers and test within 24 hours. Use of other types of plastic collection and/or storage containers may result in adsorptive loss of Microcystins, producing inaccurate (falsely low) results. Drinking water samples should be treated with sodium thiosulfate immediately after collection (refer to appropriate technical bulletin). If samples must be held for longer periods (up to 5 days), samples should be stored refrigerated. For storage periods greater than 5 days, samples should be stored frozen.

If total Microcystins concentration (free and cell bound) is required, an appropriate cell lysing procedure (freeze and thaw, QuikLyse™†, etc.) must be performed prior to analysis. *Note: The use of sonication in cell lysing can negatively affect toxin concentrations, producing falsely low sample results. Please see the appropriate sample preparation technical bulletin for additional information on cell lysis.*

Samples may be filtered prior to analysis using glass fiber filters. The use of alternate filter types (non-glass fiber filters) may produce falsely low sample results, as Microcystins may bind to the filter material, removing it from the sample. Also, please note that some glass fiber filters are manufactured using a process which may cause interference which would cause inaccurate (falsely high) results. To avoid this potential bias in sample results, a total volume of at least 10 mL should be passed through the glass fiber filter, with the first 5 mL of filtered sample being discarded and the second 5 mL collected for testing (please see the Microcystins sample filtration technical bulletin for additional information on sample filtration). If determining total Microcystins concentration, samples should be lysed prior to filtration to prevent the removal of cell-bound Microcystins, which would cause inaccurate (falsely low) results.

D. Notes and Precautions

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary.

The use of a multi-channel pipette, stepping pipette, or electronic repeating pipette is recommended for the addition of the antibody, enzyme conjugate, substrate, and stop solutions in order to equalize the incubation periods on the entire microtiter plate.

To avoid drift and obtain accurate results, the addition of the antibody, conjugate, color, and stop solutions should be performed in less than 2 minutes for each reagent. If additions to the entire microtiter plate cannot be completed in less than 2 minutes, run size should be decreased to the number of rows which can be pipetted in less than 2 minutes.

Please use only the reagents and standards from one kit lot in one test, as they have been adjusted in combination.

E. Test Preparation

1. Allow the reagents and samples to reach ambient temperature before use.
2. Remove the number of microtiter plate strips required from the resealable pouch. The remaining strips are stored in the pouch with the desiccant (tightly sealed).
3. The standards, control, sample diluent, antibody, enzyme conjugate, substrate, and stop solutions are ready to use and do not require any further dilutions.
4. Dilute the Wash Buffer (5X) Concentrate at a ratio of 1:5 with deionized or distilled water. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water and mix thoroughly.

F. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std5: Standards
Contr.: Control
Samp1, Samp2, etc: Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 0	Std 4	Samp2									
B	Std 0	Std 4	Samp2									
C	Std 1	Std 5	etc.									
D	Std 1	Std 5	etc.									
E	Std 2	Contr.										
F	Std 2	Contr.										
G	Std 3	Samp1										
H	Std 3	Samp1										

G. Assay Procedure

1. Add **50 µL of the standard solutions, control, or samples** into the wells of the test strips according to the working scheme given. Analysis in duplicate or triplicate is recommended.
2. Add **50 µL of the antibody solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for **90 minutes** at room temperature.
3. Remove the covering, decant the contents of the wells into a sink, and blot the inverted plate on a stack of paper towels. Wash the strips **three times** using the diluted wash buffer. Please use at least a volume of **250 µL of 1X wash buffer** for each well and each washing step. ***Blot the inverted plate after each wash step*** on a stack of paper towels. After the last wash/blot, check the wells for any remaining buffer in the wells, and if necessary, remove by additional blotting.
4. Add **100 µL of the enzyme conjugate solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for **30 minutes** at room temperature.
5. Remove the covering, decant the contents of the wells into a sink, and blot the inverted plate on a stack of paper towels. Wash the strips **three times** using the diluted wash buffer. Please use at least a volume of **250 µL of 1X wash buffer** for each well and each washing step. ***Blot the inverted plate after each wash step*** on a stack of paper towels. After the last wash/blot, check the wells for any remaining buffer in the wells, and if necessary, remove by additional blotting.
6. Add **100 µL of substrate (color) solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for **20-30 minutes** at room temperature. Protect the strips from sunlight.
7. Add **50 µL of stop solution** to the wells in the same sequence as for the substrate (color) solution using a multi-channel pipette or a stepping pipette.
8. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

H. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs such as 4-Parameter (preferred) or Logit/Log. For a manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the $\%B/B_0$ for each standard on the vertical linear (y) axis versus the corresponding Microcystins concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for the control and samples will then yield levels in ppb of Microcystins by interpolation using the standard curve. Results can also be determined using a spreadsheet macro available from Eurofins Abraxis upon request.

The concentrations of the samples are determined using the standard curve run with each test. Samples showing a lower concentration of Microcystins than standard 1 (0.15 ppb) should be reported as containing < 0.15 ppb of Microcystins. Samples showing a higher concentration than standard 5 (5.0 ppb) must be diluted to obtain accurate results. The concentration of the positive control provided should be 0.75 ± 0.185 ppb.

Semi-quantitative results can be derived by simple comparison of the sample absorbances to the absorbances of the standards. Samples with lower absorbances than a standard will have concentrations of Microcystins greater than that standard. Samples which have higher absorbances than a standard will have concentrations of Microcystins less than that standard.

Importance of Microcystins/Nodularins Determination

Most of the world's population relies on surface freshwaters as its primary source for drinking water. The drinking water industry is constantly challenged with surface water contaminants that must be removed to protect human health. Toxic cyanobacterial blooms are an emerging issue worldwide due to increased source water nutrient pollution caused by eutrophication. Microcystins and Nodularins are cyclic toxin peptides. Microcystins (of which there are many structural variants, or congeners) have been found in fresh water throughout the world. To date, approximately 80 variants of Microcystin have been isolated. The most common variant is Microcystin-LR. Other common Microcystin variants include YR, RR, and LW. These toxins are produced by many types of cyanobacteria (blue-green algae), including *Microcystis, Anabaena, Oscillatoria, Nostoc, Anabaenopsis*, and terrestrial *Hapalosiphon*. Nodularins are produced by the genus *Nodularia* and are found in marine and brackish water.

Acute poisoning of humans and animals constitutes the most obvious problem from toxic cyanobacterial blooms, and in several cases has lead to death. Human and animal exposure to these toxins occurs most frequently through ingestion of water, through drinking or during recreational activities in which water is swallowed. These toxins mediate their toxicity by inhibiting liver function and are potent inhibitors of the serine/threonine protein phosphatases, and therefore may act as tumor promoters.

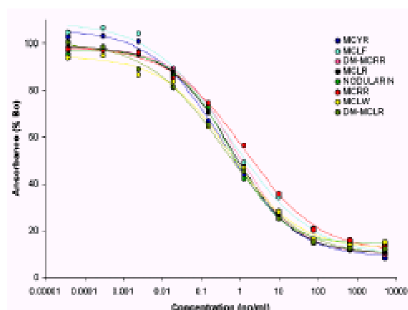
To protect consumers from adverse health effects caused by these toxins, the World Health Organization (WHO) has proposed a provisional upper limit for Microcystin-LR of 1.0 ppb (µg/L) in drinking water.

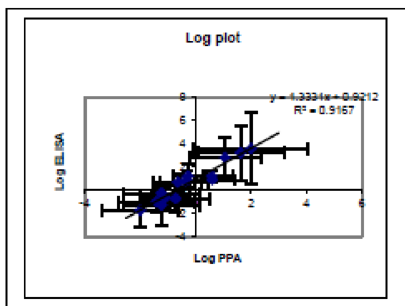
Performance Data

Test sensitivity: The detection limit for this assay, based on MC-LR, is 0.10 ppb (µg/L).

Test reproducibility: Coefficients of variation (CVs) for standards: <10%; for samples: <15%.

Selectivity*: The assay exhibits very good cross-reactivity with all cyanobacterial cyclic peptide toxin congeners tested to date (see cross-reactivity illustration below).





Samples: Sample correlation between HPLC, PPA, and ELISA methods showed a good correlation (see ELISA and PPA correlation above).

References

(1) W. J. Fischer, I. Garthwaite, C.O. Miles, K.M. Ross, J.B. Aggen, A.R. Chamberlin, N.A. Towers, and D.R. Dietrich, Congener-Independent Immunoassay for Microcystins and Nodularins. Environ. Sci. Technol. 35, 2001, 4849-4858.
(2) Worldwide Patenting PCT WO 01/18059 A2.
(3) U.S. Patent Number 6,967,240.
(4) U.S. Patent Number 9,739,777.

†QuikLyse™ reagents may be used in a method of U.S. Patent 9,739,777

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