GFP ELISA Assay Protocol Version 3

James P. O'Callaghan, PhD

Abstract

This is the protocol to be used in the quantification of glial fibrillary acidic protein (GFAP) in mouse brain tissue.

Citation: James P. O'Callaghan, PhD GFP ELISA Assay Protocol. **protocols.io**
dx.doi.org/10.17504/protocols.io.hsub6ew
Published: 22 Feb 2018

Guidelines

TRAINING REQUIREMENTS

Able to use a plate reader and to accurately dilute tissue samples.

REFERENCES

Paper*: Quantification of Glial Fibrillary Acidic Protein: Comparison of Slot-Immunobinding Assay with a Novel Sandwich ELISA*, O'Callaghan, J.P., Neurotoxicology and Teratology, Vol. 13, pp. 275-281, Pergamon Press plc. 1991

SoftMax User's Manual, Molecular Devices Corporation, Sunnyvale, CA

Materials

- Ultrasonic Cell Disruptor by Contributed by users
- Pipettes by Contributed by users
- Hot/Stir Plate by Contributed by users
- Plate Reader by Contributed by users
- 37˚C Incubator by Contributed by users

  Glial Fibrillary Acidic Protein DAKO Z0334 by Agilent Technologies
- NESTLE CARNATION Instant Non-fat Dry Milk by Contributed by users

Anti-Glial Fibrillary Acidic Protein (GFAP) Mouse mAb (G-A-5) Calbiochem IF0350 by Emd Millipore

Phosphate Buffered Saline 28374 by Thermo Fisher Scientific

Alkaline Phosphatase AffiniPure Rabbit Anti-Mouse IgG (H L) 315-055-003 by Jackson Immunoresearch

Alkaline Phosphatase Substrate Kit 1721063 by Bio-rad Laboratories

Triton X-100 1610407 by Bio-rad Laboratories

Sodium Hydroxide S320 by Thermo Fisher Scientific

Immulon 2 by Thermo Fisher Scientific

- 1.5 ml Plastic Tubes by Contributed by users
- 5 ml Plastic Tubes by Contributed by users

Protocol

STANDARD CURVE PREPARATION

Step 1.

1. Remove a tube of the Central Lab GFAP standard from the freezer, thaw at room temperature and vortex prior to aliquoting.

2. Preparation of the standard curve requires dilution of the Central Laboratory's GFAP standard. The standard has a known total protein concentration (11.5 mg/ml) and a known concentration of GFAP (2.4µg GFAP/ mg total protein). The total protein value is used to prepare a standard curve in PBS with 0.5% Triton X-100. The protein values for the standard curve should be between 0.25 to 10 µg/100µl (i.e. 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, 10µg/100µl).

GFAP Standard Curve Preparation

Tube #	µg of Protein/100µl/well (µg of GFAP)	µl of Rat Hippo Standard (11.5ug/ul)	Serial Dilution	µl of PBS+ 0.5% Triton X-100
1	10µg (.024µg)	26 µl		2974µl
2	7.5µg (.018µg)		1875µl from tube #1(10 µg)	625µl
3	5.0µg(.012µg)		1665µl from tube #2(7.5µg)	835µl
4	2.5µg(.006µg)		1000µl from tube #3(5µg)	1000µl
5	1.0µg(.0024µg)		800µl from tube #4(2.5µg)	1200µl
6	0.5µg(.0012µg)		1000µl from tube #5(1µg)	1000µl

7	0.25µg(.0006µg)	500µl from tube #6(0.5µg)	500µl

SAMPLE PREPARATION

Step 2.

1. Control and experimental animals are sacrificed, brain regions are dissected, weighed, and placed in the appropriately labeled tube.

2. At this point, the samples are either placed on dry ice or hot 1% SDS (85-95°C) is added (10X volume the weight of the tissue e. g. tissue weight = 0.045 grams 10X volume= 450µl 1%SDS) and then sonified. In both cases, the samples are stored at -70°C.

3. Before the frozen tissue can be used in the GFAP ELISA Assay, they are removed from the freezer, placed on dry ice, then sonified in hot 1% SDS (85-95°C) 10X volume (step2). Continue with step 4.

4. Experimental samples already sonified in 1%SDS are removed from the freezer, allowed to thaw at room temperature, and diluted in PBS with 0.5% Triton X-100 to a concentration of 10µg total protein/ 100µl of PBS with 0.5% Triton X-100. Samples high in GFAP (e. g. cerebellum) may need to be diluted to a concentration of 5µg total protein/ 100µl of PBS with 0.5% Triton X-100. Samples low in GFAP (e.g. striatum) may need to be diluted to 20µg total protein/ 100µl of PBS with 0.5% Triton X-100. The dilution of the samples is critical because the optical density readings for each samples needs

to fall on the linear portion of the standard curve.

5. Samples are diluted according to the total protein value of the sample (e. g. Sample = 11.5mg/ml total protein. The dilution for this sample will be 8.70 µl 991.3 µl of PBS with 0.5% Triton X-100).

6. If samples have to be diluted to a lower concentration, a 1:1 dilution of the 10µg/100µl dilution results in the 5µg/100µl (200µl of the 10µg/100µl dilution plus 200µl of the PBS with 0.5% Triton X-100).

REAGENT PREPARATION

Step 3.

1. **Volumes Required-** The volumes of reagents are 100µl/well (10ml per plate) and the volume of wash is 200µl/well (20ml per plate).

2. **Phosphate Buffered Saline (PBS)**- One packet of PBS is mixed thoroughly with 500ml of deionized water.

3. **PBS+0.5% Triton X-100**- 2.5ml of Triton X-100 is added to 500ml of PBS.

4. **Blotto- PBS + 5% powdered milk**. Step 4 uses BLOTTO without Triton X-100, in steps 7 and 9, BLOTTO contains 0.5% Triton X-100. Before the assay is started, the total volume of BLOTTO needed for the entire assay should be determined (total volume should be made fresh on the day of the assay). The larger volume allows a good mix for the addition of the Triton X-100 detergent. PBS may be warmed slightly; this will facilitate getting the powdered milk into solution but use at room temperature.

5. **Polyclonal anti-GFAP** (Dako)- 25µl/10ml of PBS

6. **Monoclonal anti-GFAP** (Calbiochem)- 1:250 40µl/10ml Blotto+0.5% Triton X-100

7. **Alkaline Phosphatase**- 1:3000 3.3µl/10ml Blotto+0.5% Triton X-100

8. **P-nitrophenylphosphate substrate**- 2ml of diethanolamine buffer, 2 p-nitrophenylphosphate tablets in 8ml of deionized water.

ASSAY PROCEDURE

Step 4.

1. Coat Immulon-2 flat bottom plates with aGFAP, 1.0µg total immunoglobulin protein / 100µl PBS / well. (25µl of aGFAP, Dako Z0334, in 10ml of PBS is the quantity needed per plate).

2. Incubate the coated plate at 37°C for 1 hour. This step may be done at the beginning of the assay or it may be done the night before and refrigerated overnight at 4°C.

3. Empty plate, tap on absorbent paper to remove excess (this procedure is important to eliminate the possibility of any reagent carry-over between steps). Wash plates 4X with PBS (200µl per well), tapping and blotting between each wash.

4. Block 1 hour 30 min at room temperature with BLOTTO (5% w/v non-fat powdered milk in PBS) 100µl per well.

5. Empty plate, tap on absorbent paper to remove excess, load diluted standard curve and samples in a volume of 100µl per well. Incubate for 1 hour at room temperature. The template below is an example of a typical plate for this lab's GFAP Assay.

Microtiter Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	Blk	? 1	? 1	? 9	? 9	? 17	? 17	? 25	? 25	? 33	? 33
B	Std 1	Std 1	? 2	? 2	? 10	? 10	? 18	? 18	? 26	? 26	? 34	? 34
C	Std 2	Std 2	? 3	? 3	? 11	? 11	? 19	? 19	? 27	? 27	? 35	? 35
D	Std 3	Std 3	? 4	? 4	? 12	? 12	? 20	? 20	? 28	? 28	? 36	? 36
E	Std 4	Std 4	? 5	? 5	? 13	? 13	? 21	? 21	? 29	? 29	? 37	? 37
F	Std 5	Std 5	? 6	? 6	? 14	? 14	? 22	? 22	? 30	? 30	? 38	? 38
G	Std 6	Std 6	? 7	? 7	? 15	? 15	? 23	? 23	? 31	? 31	? 39	? 39
H	Std 7	Std 7	? 8	? 8	? 16	? 16	? 24	? 24	? 32	? 32	? 40	? 40

6. Wash 4X with PBS + 0.5% Triton X-100, 200µl/well.

7. Incubate for 1 hour at room temperature in monoclonal aGFAP (Calbiochem) in a dilution of 1:250 and alkaline phosphatase conjugated anti-mouse IgG (Jackson ImmunoResearch) in a dilution of 1:3000 made up in BLOTTO plus 0.5% Triton X-100, 100µl/well.

8. Wash 4X with PBS + 0.5% Triton X-100, 200µl/well.

9. Add P-nitrophenylphosphate substrate (Bio-Rad) in a volume of 100µl/well and incubate for 20 minutes at room temperature.

10. Stop reaction with 0.4N NaOH 100µl/well.

11. Check wells and pop any bubbles, read plate at 405nm.

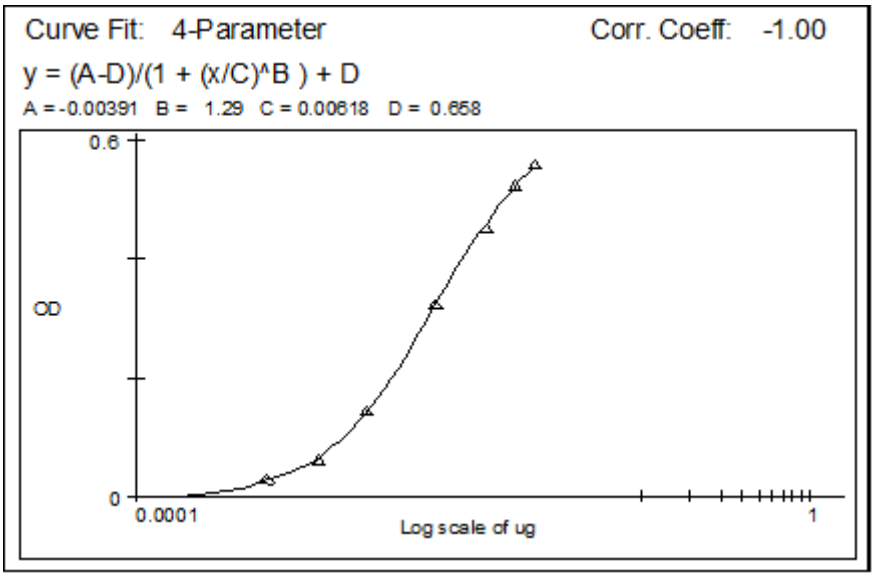
CALCULATIONS

Step 5.

1. Plates are read on a Molecular Devices UV Max or Thermo Max microplate reader. The plate reader is coupled to a Macintosh computer running the SoftMax (Molecular Devices) program.

2. A template is set up for each plate in the assay. The template includes the position of the standard curve and value for each point, and the name, number, and dilution factor of the unknown samples. The dilution factor for the samples is determined by the amount of total protein loaded into the wells (e.g. 10µg of total protein=100x dilution factor , 5µg of total protein=200x dilution factor, etc. The dilution factor is the multiplier needed to bring the total protein value up to 1mg of total protein in the final calculations.)

GFAP Standard Curve



y=((A-D)/1 + (x/C)^B)) + D

PLATE BLANK	Well	OD	Mean	Std Dev	CV
BL	A1	0.096	0.095	0.001	1.489
	A2	0.094			

STANDARDS	Value	Well	OD	Mean	Std Dev	CV
STD01	6.00e-4ug	B1	0.033	0.03	0.004	14.14
		B2	0.027			
STD02	0.0012ug	C1	0.065	0.065	*****	*****
		C2	0.065			
STD03	**0.0024ug**	D1	**0.146**	**0.145**	**7.07E-04**	**0.486**
		D2	**0.145**			
STD04	**0.006ug**	E1	**0.326**	**0.327**	**0.001**	**0.432**
		E2	**0.328**			
STD05	**0.012ug**	F1	**0.463**	**0.454**	**0.013**	**2.963**
		F2	**0.444**			
STD06	0.018ug	G1	0.53	0.526	0.006	1.075
		G2	0.522			
STD07	0.024ug	H1	0.546	0.561	0.022	3.904
		H2	0.577			

3. The above standard curve is a typical curve generated by the rat hippocampus standard. The OD values for unknown samples need to fall on the straight portion of the curve, between STD 3 and STD 5.

4. The SoftMax program automatically subtracts the blank from each OD reading (plate blank information is given in the table above), means the two readings for each sample, and multiplies the value obtained from the curve for this OD by the dilution factor for the unknown sample.

5. The report generated by the SoftMax program may be imported into an Excel spreadsheet or data may be entered manually. The chart below is an example of the report generated in Softmax for 10 unknown hippo samples that has been imported into an Excel spreadsheet. These samples were diluted to 2.5µg of total protein/100µl, therefore the dilution factor for all ten samples is 400(this factor may be included as another column on the spreadsheet).

UNKNOWNS	Mean	Std Dev	CV	Well	OD	Value
HIP01	2.633	0.114	4.323	A3	0.334	2.552
				A4	0.347	2.713
HIP02	2.482	0.099	3.984	B3	0.334	2.552
				B4	0.322	2.412
HIP03	3.099	*****	*****	C3	0.375	3.099
				C4	0.375	3.099
HIP04	2.792	0.093	3.34	D3	0.358	2.858
				D4	0.348	2.726
HIP05	2.206	0.074	3.338	E3	0.298	2.154
				E4	0.308	2.259
HIP06	2.681	0.027	0.999	F3	0.343	2.662
				F4	0.346	2.7
HIP07	3.077	0.094	3.048	G3	0.369	3.011
				G4	0.378	3.144
HIP08	2.631	0.044	1.663	H3	0.338	2.601
				H4	0.343	2.662
HIP09	2.934	0.108	3.697	A5	0.369	3.011
				A6	0.358	2.858
HIP10	2.626	0.087	3.325	B5	0.345	2.688
				B6	0.335	2.564

6. If sample dilutions were prepared by pipetting the exact amount of total protein, the value for GFAP has been calculated and no further calculations need to be done.

7. If sample dilutions were prepared by pipetting 10µl volumes for all samples, the exact amount of total protein needs to be included in the final calculations. This may be done on the Excel spreadsheet using the formula below:

GFAP value ¸ 1/10 Total protein value=µg GFAP/mg Total Protein

Unadjusted GFAP Value for Hippo #3 = 3.099

Protein Value for Hippo #3 = 9.883

3.099 ¸ 9.883 x 10 = 3.136 µg GFAP/mg Total Protein

Warnings

CAUTIONARY NOTES/SPECIAL CONSIDERATIONS

1. Powdered milk should be purchased from a grocery store and used within six months. Old powdered milk gives a yellow color in all samples and masks any GFAP results.
2. When preparing the alkaline phosphatase substrate (p-nitrophenylphosphate), observe the

color. The color should be a faint yellow, if it is darker a new kit should be used.

3. All Reagents should be at room temperature for use.