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Some methods in fish immunology and protein chemistry

Bergljót Magnadóttir 2012

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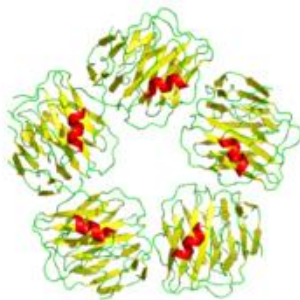

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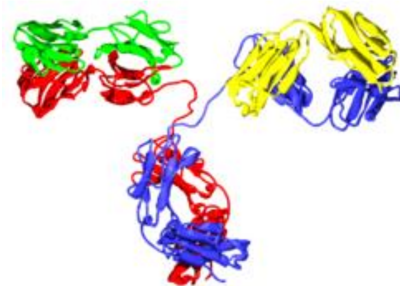

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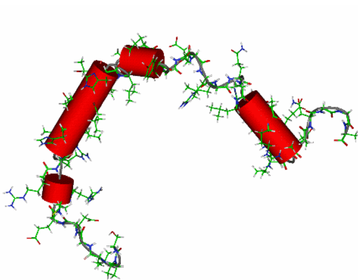

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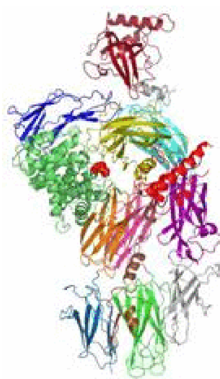

C3

These methods have been routinely used by me for many years. Some mistakes may have been introduced in this write-up without my noticing and this method book is published here without liability.

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1. Standard ELISA: The antibody activity of cod serum

This protocol was developed and used at Keldur, see Magnadottir et al. 1999, CBP Part B, 122, 173-180 and Magnadottir et al. 1998 in Methodology in Fish Diseases Research.

MATERIALS

- MaxiSorp 96 well microtrays from Nunc (442404)
- Coating buffer (made fresh once a week), from tablets (Sigma C3041), 0.05M carbonate-bicarbonate buffer, pH 9.6.
- Diluting buffer, PBS-tween (made fresh once a week) from tablets (Sigma P4417), containing 0.05% tween 20 (50 µl/100 ml).
- Block solution (made fresh): 0.1% semi skimmed milk powder in coating buffer (10 mg/10 ml).
- ELISA washing buffer, PBS-tween 20 hand made. 400 ml 5x PBS Keldur stock solution, 1600 ml H_2O, 1 ml tween 20.
    - Stock solution: 250g NaCl, 6.25g KCl, 89.5g $Na_2HPO_412H_2O$, 6.25g KH_2PO_4, in 5 l H_2O.
- Ethanolamine buffer, 0.1M, pH 9: 610µl ethanolamine, 250 µl conc. HCl in 100 ml H_2O.
- Alkaline phosphatase conjugated antibody (from DAKO), e.g. goat anti-mouse Ig's AP D0486.
- AP substrate solution (made fresh). Use 5 mg tablets of p-nitrophenyl phosphate (Sigma 104):
    - For 10 ml (1 tray): 2 tablets (10 mg), 10 ml ethanolamine buffer, 10 µl of 1M $MgCl_2$ (2g $MgCl_26H_2O$ in 10ml H_2O). Kept in the dark until used.
- Stop solution, 3M NaOH: 60g NaOH in 500 ml H_2O.

METHOD

Coating of trays

- MaxiSorp microtray is coated with 10 µg/ml of antigen in coating buffer, 100 µl/well.
- Cover with tape and incubated overnight at 4°C. Can be stored for about 1 – 2 weeks at 4°C. Can be stored for several months at –20°C but may then show slightly reduced results compared to the original unfrozen trays.
- The coating concentration of the antigen can vary and has to be checkerboard tested for each antigen.
- Bacterial or other protein antigens are usually coated in the concentration 10 µg/ml, TNP-BSA is usually coated in the concentration 5 µg/ml and whole bacteria are commonly coated in the concentration $5x10^6$ of sonicated cells/ml.

The ELISA

- The liquid is tipped out of the tray (after thawing if frozen) and (without washing) blocked in blocking solution, 100 µl/well for 1 h at room temperature (RT) (washing before blocking is ok, makes no difference).
- Wash the tray 2x in ELISA washing buffer: 1x wash = emptying and filling the wells 3x and then allow to sit for at least 5 min before next wash.

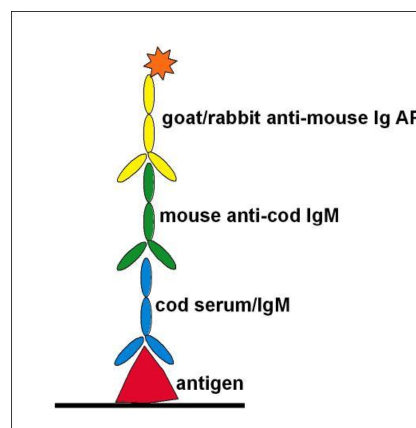
- Test serum is kept on ice while diluting. Diluted in fresh PBS-tween, vortex and then put 50 µl/well in duplicates. Buffer in place of serum is blank. Cover and incubate overnight at 4°C.
- Wash 3x
- Add (mouse) anti-cod IgM antibody diluted in fresh PBS-tween in predetermined (checkerboard titration) dilution, commonly 1/2000 – 1/5000, 50 µl/well.
- Incubate for 1 h at 37°C. Wash 3x.
- Add goat anti mouse, alkaline phosphatase conjugated, in fresh PBS-tween in predetermined dilution, commonly 1/1000 or 1/2000, 50µl/well.
- Incubate for 1 h at 37°C. Wash 3x.
- Add freshly prepared AP substrate solution, 100µl/well.
- Incubate for 30 min exactly at room temperature (22°C).
- Add stop solution, 3M NaOH, 50µl/well.
- Read optical density at 405 nm.

Expression of activity

- After subtracting the blank (should be $\leq$ 0.12) the antibody activity is expressed as the mean OD value of serum diluted 1/100.
- Serial dilution of the serum and expression of the antibody activity as titre is usually not of value when measuring cod serum antibody activity except for the natural (anti-TNP) antibody activity when $OD_{405\ nm} > 1.0$.
- When measuring antibody activity of the same fish over a period of extended time it is a good practise to include a set of standard sera with known OD values for comparison

Notes

- Peroxidase conjugated anti-mouse/rabbit antibody can be used in place of alkaline phosphatse conjugated antibody with an OPD (H_2O_2/1,2-phenylendiamin, dihydroclorid, Dako, Dk) substrate, H_2SO_4 stop solution and OD read at 492 nm.
- Overnight incubation of serum is needed for cod serum. Shorter incubation e.g. 1 – 2 h at room temperature will work for other fish serum (like salmonids or halibut).



Summary of Method 1

2. Competitive ELISA for quantitative analysis of IgM in serum

Magnadottir & Guðmundsdottir 1992, VII, 127, 1525 and Magnadottir, 1998, Icel.Agr.Sci. 12, 47

MATERIALS

- Purified cod IgM (or any other fish IgM)
- Mouse anti-cod IgM (or any other anti-IgM) antibody
- MaxiSorp 96 well microtrays from Nunc (442404)
- Coating buffer (made fresh once a week), from tablets (Sigma C3041), 0.05M carbonate-bicarbonate buffer, pH 9.6.
- Diluting buffer, PBS-tween (PBST) made fresh once a week) from tablets (Sigma P4417), containing 0.05% tween 20 (50 µl/100 ml).
- Block solution (made fresh): 2% bovine serum albumin in coating buffer (0.2 g/10 ml).
- ELISA washing buffer, PBST 20 hand made. 400 ml 5x PBS Keldur stock solution, 1600 ml H_2O, 1 ml tween 20 (see Method 1)
- Ethanolamine buffer, 0.1M, pH 9: 610µl ethanolamine, 250 µl conc. HCl in 100 ml H_2O.
- Alkaline phosphatase conjugated antibody (from DAKO), e.g. goat anti-mouse Ig's AP D0486.
- AP substrate solution (made fresh). Use 5 mg tablets of p-nitrophenyl phosphate (Sigma 104), see Method 1.
- Stop solution, 3M NaOH: 60g NaOH in 500 ml H_2O.

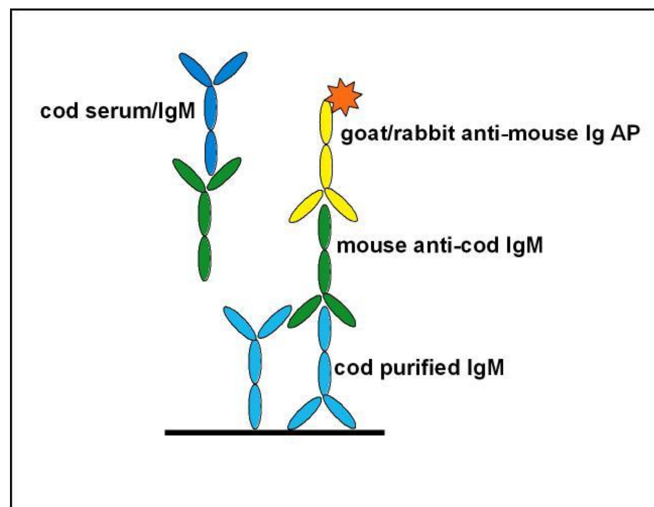
METHOD

- Coat the tray with purified cod (fish) IgM: 10 µg/ml in coating buffer, 100 µl/well
- Incubate overnight at 4°C, wash 3x with ELISA wash buffer
- Block residual sites with blocking solution, 100 µl/well, for 1 h at room temp. Wash 3x
    - Each tray should contain 6 twofold serial dilutions purified cod (fish) IgM e.g.: 40, 20, 10, 5, 2.5 and 1.25 µg/ml (competing IgM), in duplicates, 50 µl/well for plotting of a standard graph
    - The test serum is diluted in PBST, (cod serum is normally tested 1/100), add in duplicates add 50 µl/well
    - The tray should also include, in duplicates: a blank – PBST replacing the competitive IgM/serum and the anti-IgM antibody and a 100% reference: PBST replacing competitive IgM/serum but keeping the anti-IgM antibody.
- In duplicates add 50 µl/well of the standard IgM dilution and 50 µl/well of serum dilutions
- To all wells (except blank) add 50 µl/well of mouse-anti-cod (fish) IgM diluted in PBST to give optimum reaction (see Method 5 for anti-IgM titrations)
- Incubate for 1 h at 37°C. Wash 3x
- Prepare anti-mouse-AP conjugated antibody (1/2000), add 50µl/well.
- Incubate for 1 h at 37°C. Wash 3x
- Add freshly prepared AP substrate solution, 100µl/well, incubate for 30 min exactly at room temperature (22°C).
- Add stop solution, 3M NaOH, 50µl/well.
- Read optical density at 405 nm

- Plott a standard graph for each tray and extrapolate the IgM concentration of the serum from this graph

Notes

- This is a competitive ELISA, hence, the higher the reading the lower IgM concentration in the serum.
- Generally the “Pilström” method (see Method 3) is for measuring **cod serum** using two types of anti-IgM antibodies: mouse anti-cod IgM and rabbit-anti-cod IgM. This is, however, a useful method when only one type of anti-fish IgM antibody is available.
- Cod serum has relatively high IgM content (>2 mg/ml) while other fish sera like salmon has low IgM content (<1 mg/ml) and the serum dilution used has to take this into account.
- Peroxidase conjugated anti-mouse/rabbit antibody can be used in place of alkaline phosphatse conjugated antibody with an OPD (H_2O_2/1,2-phenylendiamin, dihydroclorid, Dako, Dk) substrate, H_2SO_4 stop solution and OD read at 492 nm.



Summary of Method 2

3. ELISA for quantitative analysis of IgM in serum (Pilstöm's method)

Pilström & Peterson 1991, DCI 15:143-152, Magnadottir et al. 1999, CBP, 122B:173-180

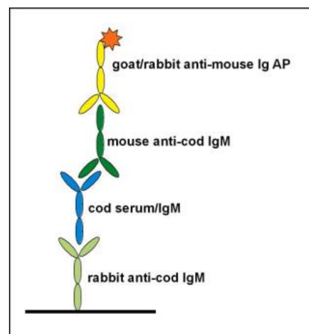
MATERIALS

- MaxiSorp 96 well microtrays from Nunc (442404), buffers, blocking solution etc. as in Method 2.
- Alkaline phosphatase conjugated antibody (from DAKO), e.g. goat anti-mouse Ig's AP D0486.
- Purified cod IgM for a standard
- Rabbit anti-cod IgM purified immunoglobulin stock solution from L.P., stored at -80°C.
- Mouse anti-cod IgM polyclonal antibody prepared at Keldur (or monoclonal from L.P.), stored at -80°C.

METHOD

- Using rabbit anti-cod IgM Ig solution from L.P. for coating: Stock solution is diluted to give 2.5 – 5.0 µg/ml* in coating buffer, coating with 100 µl/well
- Incubated overnight at 4°C, wash 3x
- Prepare IgM standard solutions in PBS-T (see note below), add 50µl/well in duplicates.
- Prepare test serum dilutions in PBS-T, add 50µl/well in duplicates
- Incubate at 37°C for 2 hours, wash 3x
- Dilute mouse anti-cod IgM in PBS-T, 1/2000*, add 50µl/well
- Incubate at 37°C for 1 hour, wash 3x
- Dilute goat-anti-mouse AP conjugate in PBS-T, 1/2000, add 50µl/well
- Incubate at 37°C for 1 h, wash 3x
- Prepare the AP substrate solution and add 100µl/well, incubate for exactly 30 min at room temperature
- Add 3M NaOH, 50µl/well to stop the reaction
- Read OD at 405 nm
- Calculate mean values, draw a standard graph of IgM standards and extrapolate the serum IgM concentration from this, choosing dilutions that give OD values that fall on the best standard line.

*checkerboard titration is needed for exact dilutions



Summary of Method 3

Standard IgM dilutions

Normally standard IgM is diluted to give about 10 – 6000 ng/ml:

Example, stock IgM solution 120 µg/ml:

Standard	Stock solution + PBS-T	Dilution	ng IgM/ml
A	30 µl stock + 570 µl buffer	1/20	6000
B	200 µl A + 600 µl buffer	1/4	1500
C	200 µl B + 600 µl buffer	1/4	375
D	300 µl C + 300 µl buffer	1/2	187,5
E	300 µl D + 300 µl buffer	1/2	93,7
F	300 µl E + 300 µl buffer	1/2	46,8
G	300 µl F + 300 µl buffer	1/2	23,4
H	300 µl G + 300 µl buffer	1/2	11.7

Serum dilutions

Normally 3 serum dilutions are tested: 1/1000, 1/10.000 and 1/50.000:

Prepare the following 5 dilutions

Serum	Serum + buffer	Dilution	Final dilution
A	10 µl serum + 90 µl buffer	1/10	1/10
B	10 µl A + 90 µl buffer	1/10	1/100
C*	20 µl B + 180 µl buffer	1/10	1/1000*
D*	20 µl C + 180 µl buffer	1/10	1/10.000*
E*	40 µl D + 160 µl buffer	1/5	1/50.000*

*The last 3 dilutions are used in the ELISA and the reading that falls on the standard line used

4. SCN - ELISA: Antibody affinity

Nieto et al., 1984, Mol. Immunol, 21, 537-543, Magnadottir et al. 2009 CBP 154, 309-316

MATERIALS

- Standard ELISA trays (MaxiSorp, Nunc) and other material (see Method 1)
- Ammonium thiocyanide, 6M solution: 45.7 g/100 ml of 0.1M phosphate buffer, pH 6.0 (see buffer tables, Method 38)

METHOD

- Prepare double serial dilutions of the coating antigen (here TNP-BSA) e.g. 5.0, 2.5, 1.25, 0.625 and 0.315 µg/ml in coating buffer
- Put 100 µl per well per column as shown below and incubate overnight at 4°C. For blank coat 3 wells of each dilution in columns 11 and 12 as shown.
- Tip out the buffer and block with 100 µl/well of 0.1% semi skimmed milk powder in coating buffer for 1 h at room temperature
- To each set of coating dilutions (total 40 wells) add 50 µl/well of serum (antibody) diluted in PBS-T to give OD (with 5 µg/ml coating antigen) of about 1.0 – 1.5 (see Method 1). Omit serum from the blank columns.
- Incubate overnight at 4°C. Wash the tray
- Prepare ammonium-SCN dilutions in 0.1M phosphate buffer, pH 6.0: 0.05, 0.1, 0.5, 1.0, 2.0, 4.0 and 6.0 M
- Add 50 µl/well as shown 1 row per each dilution
- Incubate for 15 min at rt.
- Wash the tray and add mouse anti cod –IgM, diluted 1/2000 in PBS-T, 50 µl/well
- Incubate for 1 h at 37°C
- Wash the tray and add anti-mouse Ig-AP conjugate (1/2000) in PBS-T, 50 µl/well
- Incubate for 1 h at 37°C
- Develop colour with substrate solution, 100 µl/well for 30 min and stop the reaction using 50 µl/well of 3 M NaOH.
- Read OD at 405 nm

Calculating affinity

Blank should be about ≤ 0.1 for all coatings.

The affinity constant K is worked out by plotting SCN molarity versus absorbance and K is the molaritiy of SCN that elutes 50% of the antibody for each Ag coating concentration

The % of maximum binding in row A is calculated:e.g. $100[(Ab_{2.5}-Ab_{1.25})/Ab_5]$.

An affinity distribution patterns are then constructed by plotting the K (x-axis) and the % binding for, in the set up below, 4 values.

The experimental set up for 2 serum samples per 1 tray

Antigen / NH_4SCN	1 0,3	2 0,6	3 1,25	4 2,5	5 5,0	6 0,3	7 0,6	8 1,25	9 2,5	10 5,0	11 Anti-	12 gen
A 0											0,3	0,6
B 0,05											0,3	0,6
C 0,1											0,3	0,6
D 0,5											1,25	2,5
E 1,0											1,25	2,5
F 2,0											1,25	2,5
G 4,0											5	5
H 6,0											5	5
		SERUM 1						SERUM 2			BLANK	

5. ELISA: Antibody (anti-cod IgM) titration

MATERIALS

- MaxiSorp 96 well microtrays from Nunc (442404)
- Purified fish (cod) IgM about 0.5 – 1 mg/ml
- Coating buffer (made fresh once a week), from tablets (Sigma C3041), 0.05M carbonate-bicarbonate buffer, pH 9.6.
- Diluting buffer, PBS-T (made fresh once a week) from tablets (Sigma P4417), containing 0.05% tween 20 (50 µl/100 ml).
- Block solution (made fresh): 2% bovine serum albumin in coating buffer (0.2g /10 ml).
- ELISA washing buffer, PBS-tween 20 hand made. 400 ml 5x PBS Keldur stock solution, 1600 ml H_2O, 1 ml tween 20, see Method 1.
- Ethanolamine buffer, 0.1M, pH 9: 610µl ethanolamine, 250 µl conc. HCl in 100 ml H_2O.
- Alkaline phosphatase conjugated antibody (from DAKO), e.g. goat anti-mouse Ig's AP D0486.
- AP substrate solution (made fresh). Use 5 mg tablets of p-nitrophenyl phosphate (Sigma 104), see Method 1.
- Stop solution, 3M NaOH: 60g NaOH in 500 ml H_2O.

METHOD

- Coat the tray with purified IgM: 10 µg/ml in coating buffer, 100 µl/well
- Incubate overnight at 4°C, wash 3x with ELISA wash buffer
- Block residual sites with blocking solution, 100 µl/well, for 1 h at room temp. Wash 3x
- Prepare serial dilutions of the antibody (serum/ascites) in PBS-T
- In duplicates add 50 µl/well of each dilution
- Incubate for 1 h at 37°C. Wash 3x
- Prepare anti-mouse/rabbit-AP conjugated antibody (1/2000), add 50µl/well and incubate for 1 h at 37°C. Wash 3x
- Add freshly prepared AP substrate solution, 100µl/well, incubate for 30 min exactly at room temperature (22°C).
- Add stop solution, 3M NaOH, 50µl/well.
- Read optical density at 405 nm
- After subtracting the blank (buffer in place of serum) a titration graph is plotted of OD values *versus* dilutions. Titre is the dilution that gives approximately double - treble the blank value (see example).

Notes

- 0.1% semi-skimmed milkpowder in coating buffer can also be used for blocking
- Peroxidase conjugated anti-mouse/rabbit antibody can be used in place of alkaline phosphatse conjugated antibody with an OPD (H_2O_2/1,2-phenylendiamin, dihydroclorid, Dako, Dk) substrate, H_2SO_4 stop solution and OD read at 492 nm.

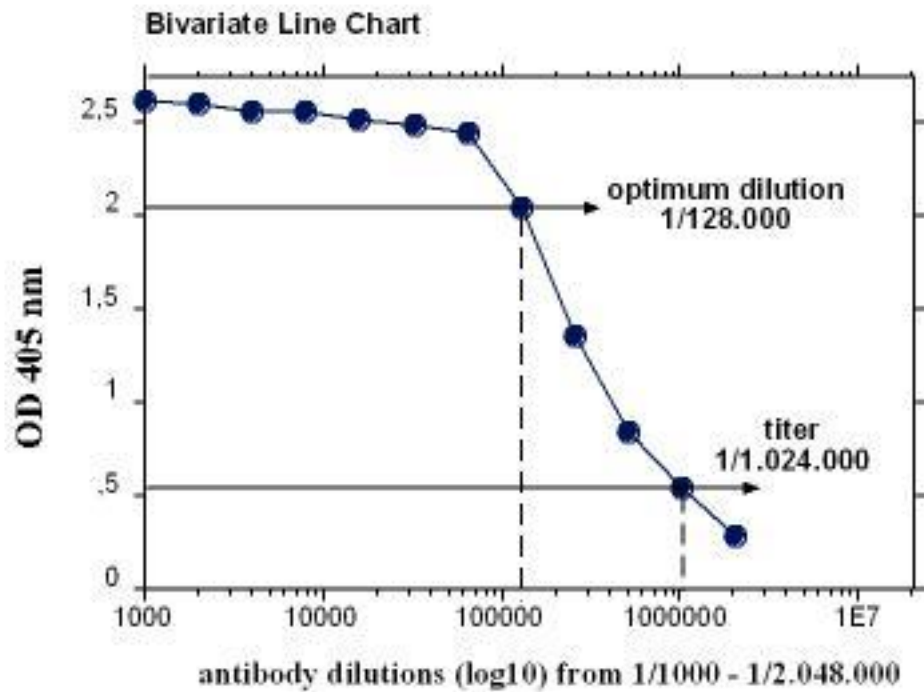
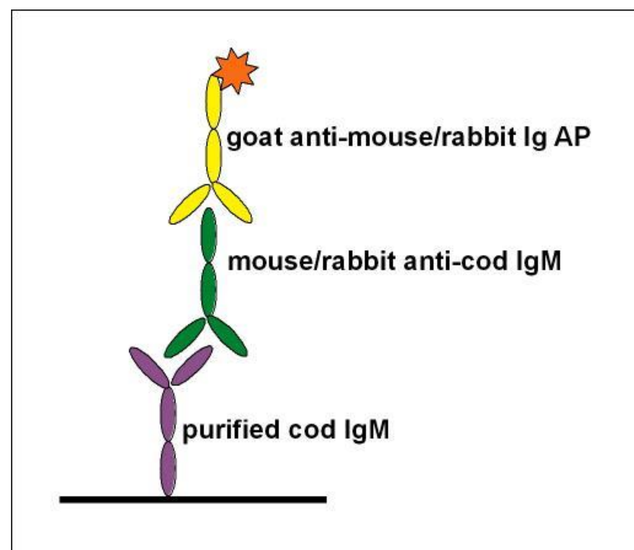


Figure: The titration curve of mouse-anti-cod IgM ascites, showing the optimum dilution for e.g. competitive ELISA (Method 2) and the titre



Summary of Method 5

6. Capture ELISA for CRP PI & PII quantity measurements

Magnadottir et al., Icel.Agric.Sci. 2010. 23, 23-35.

MATERIALS

- MaxiSorp 96 well microtrays from Nunc (442404)
- Coating buffer (made fresh once a week), from tablets (Sigma C3041), 0.05M carbonate-bicarbonate buffer, pH 9.6.
- Diluting buffer, PBS-tween (made fresh once a week) from tablets (Sigma P4417), containing 0.05% tween 20 (50 µl/100 ml).
- Block solution (made fresh): 0.1% semi skimmed milk powder in coating buffer (10 mg/10 ml).
- ELISA washing buffer, PBS-tween 20 hand made. 400 ml 5x PBS Keldur stock solution, 1600 ml H_2O, 1 ml tween 20, see Method 1.
- Ethanolamine buffer, 0.1M, pH 9: 610µl ethanolamine, 250 µl conc. HCl in 100 ml H_2O.
- AP substrate solution (made fresh), see Method 1.
- Mouse anti CRP-PI and CRP-PII immunoglobulins (Ig's) (see Method 16), used for coating the tray.
- The same as above biotinylated for the detection (see Method 17 for the biotinylation).
- Streptavidin-alkaline phosphatase (AP) labelled (from DAKO, Dk)
- Purified CRP-PI and CRP-PII proteins for standards (see Method 11)

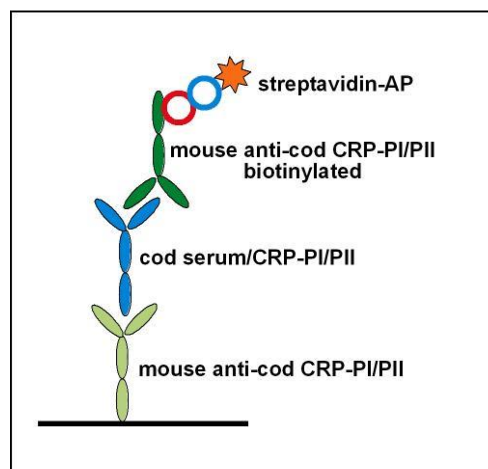
METHOD

- Coat 96 well microtray, MaxiSopr, with 10 µg/ml, 100 µl per well of
  - a) mouse anti CRP-PI Ig's (e.g. 569 µg CRP-PI/ml: 26.4 µl + 14.97 ml coating buffer)
  - b) mouse anti CRP-PII Ig's (e.g. 340 µg CRP-PII/ml: 440 µl + 14.56 ml coating buffer)
- Incubate overnight at 4°C
- Tip out the coating solution and block with 100 µl per well of 0.1% semi skimmed milk powder in coating buffer and leave for 1 h at room temperature
- Wash 3x in PBS-t (phosphate buffered saline containing 0.05% tween 20)
- Prepare standard solutions and serum dilutions:
  - Standard:
    - CRP-PI purified, 240 µg/ml prepare 8 five fold dilutions starting with 30 µl + 120 µl PBS-t, mix and transfer 30 µl of this to 120 µl PBS-t etc. total 8 tubes
    - CRP-PII purified, 960 µg/ml, as above but starting with 7.5 µl of this + 142.5 (1/20) µl PBS-t, then transfer 30 µl of this to 120 µl PBS-t etc. total 8 tubes
  - Serum: Prepare 2 dilutions: 1/500 and 1/2000:
    - 2 µl serum in 1000 µl and transfer from this 200 µl + 600 µl PBS-t

- On each coated, blocked and washed tray put 1 column blank (PBS-t), 2 columns 8 standard solutions and the rest serum samples diluted 1/2000 in duplicates, in all cases 50 µl per well and incubate for 2 h at room temperature.
- Wash 3 x in PBS-t
- Prepare anti-CRP PI and PII biotinylated:
    - anti-CRP-PI-biotin: 515 µg/ml diluted 1/100 (from 23.4.08): 80 µl + 7.92 ml PBS-t
    - anti-CRP-PII-biotin: 343 µg/ml diluted 1/100: 80 µl + 7.92 ml PBS-t
- On the whole tray put 50 µl per well, of a) or b) above (depending on the parameter to be measured), and incubate for 1 h at 37°C
- Wash 3 x in PBS-t
- Prepare Streptavidin-AP 1/1000: 8 µl + 8 ml PBS-t for each set of tests (1 ½ tray), 50 µl per well and incubate for 1 h at 37°C.
- Wash 3 x in PBS-t
- Add AP substrate solution (made fresh), 100 µl per well and incubate for 30 min at room temperature
- Add stop solution, 3M NaOH, 50µl/well and read optical density at 405 nm.
- Plot a standard graph and interpolate the serum values and calculate the CRP-PI and CRP-PII concentration in serum.

Comments:

This is not a very "robust" method, preferably 3 serum dilutions should be tested in duplicates or/and all the serum samples to be tested in an experiment should be tested in one go. However, this is an "expensive" method so normally serum is tested 1/2000 and if the value does not fall on the standard line the test is repeated for this serum either more or less diluted.



Summary of Method 6

7. ELISA: Cortisol in serum – a kit from Neogen

Neogen Cortisol ELISA kit, Magnadottir et al. 2010, Icel Agric Sci 23, 23-35

MATERIALS

- A kit from Neogen Corp.Ky, USA
- HCl
- Ethyl ether
- Liq. nitrogen
- Speed-Vac
- Vortex

METHOD

Preparation of sample

- In a glass test tube (or an eppendorf 1.5 ml test tube) mix together 100 µl of serum/plasma and 1 ml ethylene ether (FUME CUBOARD)
- Vortex for 30 sec and allow phases to separate (can be speeded up by centrifugation at 400 rpm for 2 min
- Collect the upper organic phase into a clean eppendorf tube, cool on ice or freeze in liq. nitrogen
- Evaporate in the SpeedVac until dry
- Dissolve the residue in 100 µl of diluted extraction buffer from the kit
- Take 10 µl of this and add to 990 µl of diluted extraction buffer (1/100)
- Assay in duplicates 50 µl of this sample

Preparation of standards (from the kit)

- A: Stock Cortisol solution 1 µg/ml
- B: 20 µl of A and 0.980 µl of EIA buffer from the kit, mix (=20 ng/ml)
- C: 200 µl of B and 1.8 ml EIA buffer, mix (=2 ng/ml)
- D: 200 µl of C and 1.8 ml EIA buffer, mix (=0.2ng/ml)

Standards for the tray:

Standard preparation	Cortisol concentration ng/ml	% S/S0
S0: EIA buffer	0	100
S1: 800 µl EIA + 200 µl D	0,04	80
S2: 500 µl EIA + 500 µl D	0,1	66
S3: D	0,2	52
S4: 800 µl EIA + 200 µl C	0,4	39
S5: 500 µl EIA + 500 µl C	1	26
S6: C	2	18
S7: 500 µl EIA + 500 µl B	10	6

Competitive ELISA

- One tray from the kit will take 40 samples and the standards in duplicates
- The kit's tray is already coated with anti-cortisol antibody.
  Add 50 µl per well of standards and samples in duplicates as shown in Figure below.
- Prepare the cortisol enzyme conjugate from the kit: Add 110 µl of enzyme conjugate to 5.5 ml of EIA buffer for one plate. Mix well.
- Add 50 µl per well of this solution to the tray, cover the tray and incubate at room temperature for 1 h
- Dilute the conc. wash buffer from the kit, 20 ml in 180 ml distilled water.
- After incubation tip out the content of the tray and fill with 300 µl per well with wash buffer, repeat 3 x.
- Add 150 µl per well of substrate from the kit and incubate for 30 min at room temperature.
- Add 50 µl per well of 1N HCl and read at 450 nm. The higher the reading the less cortisol is in the sample.

Calculating the cortisol concentration (see also instructions with the kit)

- Average all duplicates and plot a standard graph of % maximum binding *versus* ng cortisol/ml: S1 – 7 values divided by the mean S0 value (the maximum) x 100 (y-axis) plotted *versus* ng cortisol/ml (x-axis).*
- Do the same with the test samples i.e. (average/S0) x 100. Read the ng/ml off the standard curve. Multiply by 100 to get the final ng/ml conc. in serum.

Figure showing a typical experimental setup: S: standards, T: test sera

	1	2	3	4	5	6	7	8	9	10	11	12
A	S0	S0	T1	T1	T9	T9	T17	T17	T25	T25	T33	T33
B	S1	S1	T2	T2	T10	T10	T18	T18	T26	T26	T34	T34
C	S2	S2	T3	T3	T11	T11	T19	T19	T27	T27	T35	T35
D	S3	S3	T4	T4	T12	T12	T20	T20	T28	T28	T36	T36
E	S4	S4	T5	T5	T13	T13	T21	T21	T29	T29	T37	T37
F	S5	S5	T6	T6	T14	T14	T22	T22	T30	T30	T38	T38
G	S6	S6	T7	T7	T15	T15	T23	T23	T31	T31	T39	T39
H	S7	S7	T8	T8	T16	T16	T24	T24	T32	T32	T40	T40

Note:

The principle of this method is the same as in Method 2, i.e. competitive ELISA.

The preparation of the serum sample is critical. Other samples, e.g. mucus may not need extraction procedure (see the kits's instructions). Kits may be available for serum and other extractions.

8. Fish serum or plasma collection

MATERIALS

- Monovette syringes Serum or Plasma (EDTA or heparin coated): 1.2 ml, 4.5 ml or 9.5 ml, from Sarstedt, Germany
- Needles from Sarstedt, Germany for Monovette syringes, 20G or 21G

METHOD

Serum collection

- Using Monovette Serum syringe, blood is usually collected from the caudal vessel but in some cases (e.g. halibut) it is easier to collect blood from an aorta in the gills.
- Blood is allowed to clot at room temperature for 2 hours (optional) and then overnight at 4°C and serum collected by centrifugation at 2000 rpm for 10 min. Generally stored at -20°C.

Plasma collection

- Using Monovette Plasma syringe, blood is collected as above
- Centrifuged at once at 7000 xg for 10 min (can be stored on ice for a while before centrifugation) and the plasma collected and stored at -80°C.

Notes

Clots may form in the fish serum samples during storage, these can be removed with a pincer or just ignored.

Precipitates (fat?) may also form in serum during prolonged storage. This does not normally affect antibody activity.

9. Fish IgM purification

Magnadottir, B., Icel.Agr.Sci.4, 1990, 49-54, Magnadottir, B., Icel.Agr.Sci.12, 1998, 47-59

This is a three step column isolation:
a) "Clean-up" using CM-Affi-Gel Blue and SAS precipitation,

b) Gel filtration (isolation based on size) and

c) Anion exchange chromatography (isolation based on charge).

The method has worked well for the isolation of IgM from several fish species (e.g. cod, salmon, rainbow trout, arctic char, haddock, halibut, sea-bass, sturgeon, turbot and wolf fish)

MATERIALS

- Fish serum
- CM Affi-Gel Blue (BioRad) 100 ml column
- Superose 6 column (HR10/30, Pharmacia)
- MonoQ anion exchange column (HR5/5)
- FPLC system
- Buffers – filtered and de-gassed before use (see buffer preparations, Method 38):
    - For CM Affi-Gel Blue: 10mM Potassium phosphate buffer pH7.25, 0.15M NaCl
    - For the Superose 6 column: 0.1M Tris-HCl buffer, pH 8.0, 0.15M NaCl
    - For MonoQ column: 0.02M Tris-HCl, pH 7.5,
      a) containing 0.15M NaCl for equilibrating the MonoQ column and
      b) containing 1M NaCl for the gradient elution from the MonoQ column

METHOD

- About 20 ml of fish serum is passed through the CM Affi-Gel Blue column and the unbound, fall through material collected (the column should retain albumin and other major non-Ig serum proteins)
- The fall through fractions are pooled and protein precipitated by 50% saturation with ammonium sulphate (0.313 g/ml – see Method 31). Stirred gently for 2 h at room temp.
- Collect the precipitate by centrifugation at 10 - 15.000 rpm for 30 min and dissolve in minimum volume of saline (0.15M NaCl).
- Dialyse in saline until free of sulphate (see Method 35), finally in the Superose 6 buffer.
- Measure the protein using Bradford's method or the extinction coefficient E=13.7 (see Methods 29/30) and aliquote suitable amounts for Superose separation.
- Put about 250 µl of crude IgM on the column containing about 0.5 – 1 mg crude IgM.
- Fractions are tested in dot blot or Western blot with anti-fish IgM and to obtain the purest isolation check also by SDS-PAGE and pool fractions that contain IgM only.

- Further separation on anion exchange MonoQ column is generally advisable and will also show possible isotypes of IgM if present (e.g. salmon, haddock and sturgeon IgM)
- Sample of about 250 µg IgM in 250 µl of MonoQ equilibration buffer is put on the MonoQ column and eluted with a gradient which upper limit is 1M NaCl.
- The final IgM preparation is best kept in PBS in aliquots at -20°C or -80°C, 20 – 50% glycerol or sucrose can be added to improve stability.

10. Crude preparation of (fish) IgM

N.M.S. dos Santos et al. FSI 1997, 7, 176

MATERIALS

- Dextran sulphate: 10% solution in H_2O
- $CaCl_2H_2O$ 1M: 0.147 g in 10 ml
- TBS: 20 mM Tris, 50 mM NaCl, pH adjusted to pH 7.6 (with HCl)
- Ammonium sulphate

METHOD

- To 5 ml of fish serum add 100 µl of the dextran solution and 500 µl of the calcium chloride solution
- Stirr on ice for 30 min
- Centrifuge for 15 min at 4°C at 10.000 rpm (Sorval)
- Collect the supernatant and dialyse in TBS at 4°C overnight (2 – 3 changes)
- Measure the volume and add 0.243 g ammonium sulphate per ml (about 40% saturation, see Method 31)
- Stirr on ice for 4 h
- Centrifuge at 10.000 rpm (Sorval) for 30 min at 4°C
- Collect the precipitate and dissolve in TBS
- Dialyse in TBS until free of sulphate (see Method 35)
- Check purity in SDS-PAGE

Note

This method saves time if you want to isolate IgM from a great quantity of serum but it will need further purification e.g. on anti-IgM coupled column (or see Method 18).

11. Purification of cod pentraxins, CRP-PI and CRP-PII

From Lund & Olafsen DCI 1998, 22, 185-194 and Gisladottir et al. FSI 2009, 26, 210-219

MATERIALS

Columns

- Phosphoryl- agarose (PC) from Pierce Chemicals, Thermo Fisher Scientific, IL, USA. About 10 ml are required and will be packed into XK16 column from Amersham.
- MonoQ column HR5/5 from Amersham Pharmacia Biotech, GE Healthcare, Dk.
- PD buffer exchange column from Amersham Pharmacia Biotech, GE Healthcare, Dk.

Buffers (see also Method 38)

- PBS from tablets (Sigma P4417), containing 0.05% sodium azide.
- PC equilibrium buffer, Tris buffered saline + Ca (TBS-Ca): 50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl (TBS) and 2 mM $CaCl_2$.
- PC elution buffers:
  1) 50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl (TBS) and 10 mM EDTA

  2) 50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl (TBS) and 30 mM phosphorylcholine.

- MonoQ start buffer 1: 20 mM Tris-HCl, pH 7.5
- MonoQ elution/gradient buffer 2: 20 mM Tris-HCl, pH 7.5 containing 1 M NaCl

METHOD

Affinity Chromatography

- Approximately 10 ml of Phosphorylcholine (PC)-agarose from Pierce Chemicals (U.S.A.) is used. This is stored in a 50 ml centrifuge tube at 4°C in PBS containing sodium azide.
- The PC-agarose is equilibrated with several changes of excess TBS-Ca buffer (PC equilibrium buffer) in the centrifuge tube.
- The gel is mixed with 10 ml of TBS-Ca buffer and 10 ml of pooled and filtered cod serum and gently rotated overnight at 4°C.
- The gel is allowed to settle and the supernatant discarded (can be kept for IgM isolation) and the gel washed twice with the TBS-Ca buffer.
- The gel is packed onto a chromatographic column (XK16, Amersham Pharmacia Biotech, Sweden), connected to the ÄKTA FPLC system from Amersham Pharmacia Biotech and washed in TBS-Ca buffer until zero optical density is obtained.
- Bound protein is eluted with 10 mM EDTA in TBS buffer (PC elution buffer 1) and collected. This contains crude cod pentraxins.
- The column is then cleaned with 30 mM PC in TBS (PC elution buffer 2), this elutes some as yet unidentified proteins.

Ion Exchange Chromatography – separation of CRP-PI and CRP-PII

- MonoQ anion ion exchange column is used. The column is equilibrated in MonoQ start buffer.

- The crude pentraxin is equilibrated in the start buffer using PD buffer exchange column or dialysis and the protein concentration measured using Bradford's method (see Method 29).
- A sample is loaded onto the MonoQ column and eluted first with start buffer and then followed by a salt gradient of 0 to 1 M NaCl in the start buffer (the MonoQ elution buffer).
- The salt elution should distinguish 2 peaks, the first is CRP-PI and the second CRP-PII.
- The quantity is measured using the Bradford method and the purity is verified by SDS-PAGE and the specificity by Western blotting using specific anti-CRP-PI and CRP-PII antibodies.

The tris buffer recipes:

- Stock-Tris buffer: 6g Tris and 8,7g NaCl in 600 ml distilled water, pH adjusted to 8,0 með HCl, volume adjusted to 1 l
- This stock-Tris buffer is divided into 3 parts:
    - PC equilibrium buffer: 600 ml + 0,176g $CaCl_2$
    - PC elution buffer 1: 200 ml + 0.74 g EDTA
    - PC elution buffer 2: 200 ml + 1,5 g phosphorylcholine chloride (PC)

12. Apolipoprotein A1 isolation from cod serum

Amthauer et al., Comp. Biochem. Physio. 1989, 92B, 787-793.

MATERIALS

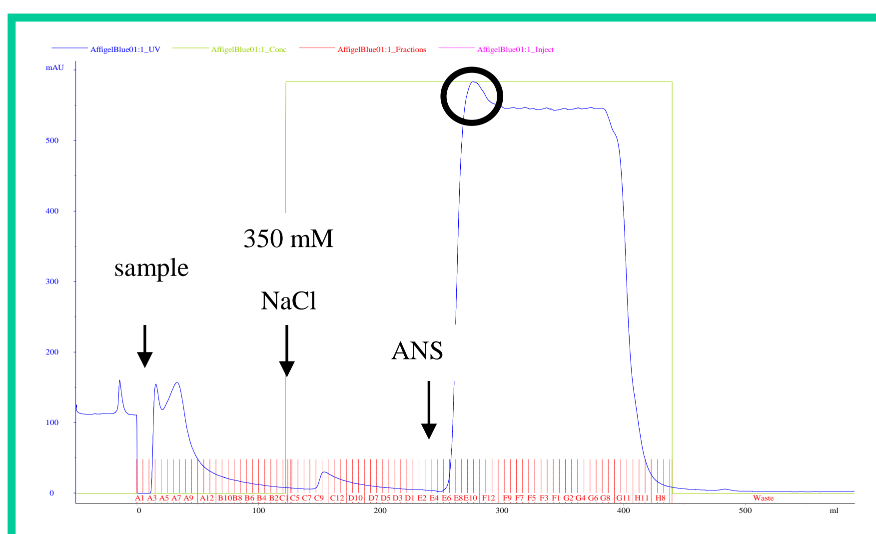
- AffiGel Blue 50 ml column
- Equilibration buffer: Sodium citrate buffer, 50 mM, pH 6.5 containing 50 mM NaCl
- Elution buffer 1: Sodium citrate buffer, 50 mM, pH 6.5 containing 350 mM NaCl
- Elution buffer 2: Sodium citrate buffer, 50 mM, pH 6.5 containing 50 mM NaCl and containing 2 mM 8-anilino-1-naphthalene sulfonate (ANS)
- UV lamp in a darkroom
- Anti-cod IgM-HiTrap column (see Method 18)

METHOD

- About 2 ml of cod serum is put on the AffiGelBlue column with the equilibration buffer.
- The column is washed with Elution buffer 1.
- Apolipoprotein is then eluted with the Elution buffer 2 containing ANS.
- Fractions are examined under a UV light and those with a high fluorescence pooled
- Pooled fractions dialysed in equilibration buffer at 4°C, concentrated in centrifugal filter and tested in SDS-PAGE and Western blotting using anti-ApoLP A1 antibody (also anti-IgM and anti-C3 antibodies, all available at Keldur).
- ApoLpA1 is the major 30 kDa band observed. However, this preparation may be contaminated with IgM (72 kDa + 26 kDa). To remove this the ApoLP A1 preparation, after a buffer change, is passed through an anti-cod IgM-HiTrap column (see Method 18).

Notes

Mouse-anti-cod IgM antibody columns are available at Keldur
Contamination with C3 may also be seen and an anti-cod C3 column can then be prepared from rabbit anti-cod C3 antibody available at Keldur



Typical elution of serum on AffiGel Blue column with ANS, circle shows the fractions collected

13. Isolation of fish C3 complement component

Lange et al., FSI 2004, 16, 227-239; Dodds et al. DCI, 1998,22, 207 - 216

MATERIALS

- Fish plasma* isolated by centrifugation at 700 xg for 10 min. from blood, collected into EDTA coated syringes from Monovette, kept on ice. Will need about 100 ml of serum. Make 5 mM with EDTA and store at 4°C until used.
- Polyethylene glycol (PEG 3350)
- Start buffer: 20 mM Tris-HCl, pH 7.4, 50 mM EACA (aminocaproic acid), 5 mM EDTA, 50 mM NaCl and 0.1mM protease inhibitor (Boehringer-Mannheim pefablock or others)
- Start buffer made 500 mM with NaCl for the gradient elution
- Q-Sepharose HP column (1.6 x 20 cm, Amersham)
- Superose 6 column (HR10/30)
- Superose 6 elution buffer: 20mM Tris-HCl, pH 6.8, 150 mM NaCl, 5mM EDTA, 0.1M pefablock
- MonoQ column 0.5 x 5 cm)
- MonoQ elution buffer: gradient from 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl and 0.1mM protease inhibitor (Boehringer-Mannheim pefablock) to the same buffer with 500 mM NaCl.

METHOD

- Add PEG slowly to the plasma solution to give 5% PEG, using 15% PEG in the starting buffer, stir gently at 4°C for 45 min.
- Centrifuge at 20.000 xg for 20 min.
- Collect the supernatant.
- Put on the Q-Sepharose column in the start buffer and elute bound proteins with the gradient elution buffer
- Collect fractions and test in Western blotting using anti-fish C3 antibody (if not available see original references for detecting C3)**
- Further purification on Superose 6 and MonoQ will be needed to obtain pure C3
- Test the final purity in SDS-PAGE***

Notes

*Serum can also be used but plasma is better.
**Monospecific rabbit anti-cod C3 is available at Keldur

***Two bands should appear under reducing conditions: the approx. 115 kDa α-chain and the 74 kDa β-chain.

14. C3 isolation for immunization using Macrogard

Jarl Bögwald personal communication

MATERIALS

- Macrogard (MG, dietary yeast beta-1.3/1.6-glucan) from Biotec Pharmacon, Norway
- Phosphate buffered saline (PBS)
- Gelatin-Veronal buffer (GVB) from Sigma (G6514) containing Ca^{2+} and Mg^{2+}
- Sodium dodecyl sulphate (SDS)

METHOD

- Approximately 2 ml of 2.5% suspension of MG is washed twice in 20 ml of PBS, centrifuge between the washing at 400 xg (1350 rpm) for 10 min and discard the supernatant
- The pellet is resuspended in 15 ml of the GVB buffer, hence, containing approximately 3.33 mg MG ml^{-1}
- Add 10 ml of fish serum and rotate at 14°C for 60 min
- Centrifuge at 400 xg for 10 min
- Wash the pellet 3x in PBS containing 0.1% SDS
- Wash the pellet 3x in PBS
- Resuspend the pellet in 2.5 ml PBS, hence, containing 20 mg coated MG ml^{-1}
- This suspension is used to immunize rabbits or mice with about 5 mg MG per rabbit (less per mouse (acites))

Comment
Macrogard is expected to bind C3b/C3bi.

This has been tested on cod and halibut serum for producing mouse anti-C3 antibody in ascites (see Method 15). The resulting antibody was not mono-specific against C3 but was still useful for detecting C3 in column fractions by Western blotting etc.

15. Production of polyclonal antibody in mouse ascites

Overkamp et al., J.Immunoassay 1988, 9, 51-68

MATERIALS

- Use 2 – 3 mice for each antigen, Balb/c mice have normally been used at Keldur but other types can be used
- Freund's complete and incomplete adjuvant (FCA/FIA)
- Pristane (Sigma)

METHOD

- The protein antigen (e.g. fish IgM) has to be as pure as possible. The purity of approximately 1 – 10 µg has to be checked in SDS-PAGE using silver staining (coomassie blue is not sensitive enough)
- 100 µl of the protein containing approximately 1 mg/ml is mixed with 100 µl PBS and 1.8 ml FCA (or FIA) and homogenized (using an syringe and as fine a needle as possible)
- 200 µl of this is injected intra-peritoneal into 2 – 3 mice on day 0 (approx. 10 µg protein/mouse).
- On day 14 this injection is repeated with a fresh protein/adjuvant preparation and the mice receive 500 µl of pristane i.p. as well
- On day 21 and day 28 the injection of antigen is repeated as required. Ascites fluid often starts to form before day 28 in which case the last injection is omitted
- When the swelling of the abdomen is suitable the mouse is sacrificed and the ascites collected with a bulb pipette.
- The ascites is centrifuged, aliquoted and stored at -20 or -80°C.

Notes

Many labs do not allow this method due to the pain induced in the mice, hence, use as few mice as possible and collect the ascites as soon as it forms
FCI is usually needed, at least in the first injection
The antibody response is dose related but higher dosage does not necessarily result in higher antibody titre, 5 – 50 µg of fish IgM per mouse per injection is usually used

Make sure the antigen solution does not contain Tris or any other harmful component (e.g. azide) – this has to be dialysed out before injection

16. Immunoglobulin isolation from mouse ascites/rabbit serum

Based on Midi and Mini Proteus kit from Pro-Chem Inc., GA, USA (see leaflet for more informations)

MATERIALS

- Proteus Mini or Midi kit from Pro-Chem Inc.
- Binding buffer A: 0.1M sodium phosphate, 0.15M, pH 7.4 (see buffers, Method 38)
- Elution buffer B2: 0.2M Glycine/HCl pH 2.5
- Neutralization buffer C: 1M Tris-HCl, pH 9.0

METHOD

1. Mini plug

- Load the Mini plug into the barrel of the Proteus spin column.
- Equilibrate the column with 650 µl of binding buffer A by centrifuging the spin column at 1800 g (4 – 5000 rpm) for 1 min and repeat once.
- Filter 1 ml of sample through 0.2 µm syringe filter and then dilute 1:1 in binding buffer A (or dilute before filtration if too viscous).
- Add 650 µl of sample to the spin column
- Centrifuge at 640 g (2.6 – 3000 rpm) for 6 min (increase the time if any sample remains above the plug.
- Wash the spin column 3 x with 650 µl of binding buffer A, spin for 1 min at 1800 g.
- Elute bound Ig's with 500 µl of elution buffer B2 directly into a fresh centrifuge tube containing 65 µl of neutralization buffer C, spin for 1 min at 1800 g. Mix the eluate immediately.
- Repeat the elution step and pool the two eluates (total about 1.1 ml).
- Change the buffer of the sample from glycine/tris to PBS using PD column and then concentrate in a centrifugation filter.
- Estimate protein by measuring OD at 280 nm using the coefficient $E^{1\%}$=13.7 i.e. 1 mg/ml will give a reading of 1.37 (see Method 30).
- Make 0.05 – 0.2% with sodium azide and store at 4°C or make 10 – 50% with glycerol and store in aliquots at –20°C.

Regenerate the column

- Wash the Mini plug twice with 650 µl of elution buffer B2 and then twice with 650 µl of binding buffer A, centrifuging at 1800 g for 1 min between each wash.
- Store in binding buffer A with 0.1% sodium azide at 2 – 8°C until further use.

2. Midi plug

- Load the Midi plug into the barrel of the Proteus spin column.
- Equilibrate the column with 10 ml of binding buffer A by centrifuging the spin column at 500 g for 3 min.
- Filter 12 - 15 ml of sample through 1.2 µm syringe filter and then through 0.2 µm filter, dilute 1:1 in binding buffer A (or dilute before filtration if too viscous).
- Add 20 ml of sample to the spin column
- Centrifuge at 150 g for 30 min (increase the time if any sample remains above the plug.
- Wash the spin column with 10 ml of binding buffer A for 3 min at 500 g.
- Elute bound Ig's with 10 ml of elution buffer B2 directly into a fresh centrifuge tube containing 1.3 ml of neutralization buffer C, for 3 min at 500 g. Mix the eluate immediately.
- Repeat the elution step and pool the two eluates (total about 22 ml).
- Change the buffer of the sample from glycine/tris to PBS using centrifugation filter, spinning and adding PBS to the concentrated top layer 1 – 2 x.
- Estimate protein by measuring OD at 280 nm using the coefficient $E^{1\%}$=13.7 i.e. 1 mg/ml will give a reading of 1.37 (see Method 30).
- Make 0.05 – 0.2% with sodium azide and store at 4°C or make 10 – 50% with glycerol and store in aliquots at –20°C.

Regenerate the column

- Wash the Midi plug with 10 ml of elution buffer B2 and then 10 ml of binding buffer A, centrifuging at 500 g for 3 min between each wash.
- Store in binding buffer A with 0.1% sodium azide at 2 – 8°C until further use.

17. Biotinylation of Antibodies (Ig's) and other proteins

Based on a protocol from the kit's producer (Amersham, ECL RPN 2202/03)

MATERIALS

- Biotinylation reagent from the kit (Amersham, ECL RPN 2202/03) – equilibrated at room temperature before use
- Bicarbonate buffer from the kit diluted 1/20: 40 mM, pH 8.6
- Sephadex G-25 column from the kit equilibrated with 5 ml PBS containing 1% BSA and then with 20 ml PBS
- Purified immunoglobulin from the antibody solution (e.g. mouse ascites or rabbit serum) using the Proteus kit (see Method 16).

METHOD

- Estimate the Ig concentration using e.g. the OD_{280} absorbance method (see Method 30) and prepare a 1 mg/ml solution in the bicarbonate buffer, 2 – 2.5 ml is a suitable volume
- For each mg of Ig in the antibody solution add 40 µl of the biotinylation reagent and incubate for 1 h at room temperature with constant agitation.
- At the same time prepare the G-25 column as described above
- After 1 h apply the biotinylation solution to the column, let it enter the column and then elute with 5 ml of PBS. Collect 0.5 - 1 ml fractions and monitor at 280 nm and pool the peak fractions.
- The biotinylated antibody is ready for use. Best to store at 4°C in the presence of 0.1% sodium azide.

Note

More than one biotinylation kits have been tested and this kit from Amersham has worked best.

18. Protein coupling to NHS-activated columns, ligand binding and elution

Based on protocol that comes with the columns (Amersham)

These columns have mainly been used for coupling mouse anti- cod IgM Ig's (isolated using the Proteus kit (see Method 16)) and then used for the purification of fish IgM from serum or crude preparations; also for the decontamination of other samples (e.g. cod ApoLP A1).

MATERIALS

Columns

- NHS- activated HiTrap columns, 1 or 5 ml, from Amersham Biosciences

Buffers

- Coupling buffer, 0.2M $NaHCO_3$, 0.5M NaCl, pH 8.3: 8.4 g $NHCO_3$ and 14.6 g NaCl in 3-400 ml H_2O, pH adjusted to 8.3, volume adjusted to 500 ml
- Washing and deactivating buffers, 0.5 M ethanolamine, 0.5M NaCl, pH 8.3: 15 ml ethanolamine in 3 – 400 ml H_2O add 14.6 g NaCl, adjust pH to 8.3, adjust volume to 500 ml.
- Acetate buffer, 0.1 M acetate, 0.5M NaCl, pH 4.0 (see Method 37)
- Storage buffer, 0.05 M Na phosphate buffer, pH 7, 0.05% sodium azide (Buffers ÐÐÐ)
- Sample and column buffer, PBS, prepared from tablets from Sigma
- Elution buffer, 0.1M glycine, pH 2.3: 3.75 g glycine in 250 ml H_2O, pH adjusted to 2.3 with HCl, volume adjusted to 500 ml
- Neutralizing buffer, 1M Tris: 1.12 g Tris in 10 ml H_2O
- 1 mM HCl: 10 µl conc. HCl in 100 ml

METHOD

A) Coupling proteins to a 1 ml NHS-activated HiTrap column

- The protein is equilibrated in the coupling buffer e.g. by dialysis og by buffer change on PD-10 or NPD column. The concentration should be 0.5 – 10 mg/ml in 1 ml
- Remove the cap of the column and add a drop of ice cold 1 mM HCl.
- Remove the bottom end when ready
- Using a 1 or 2 ml syringe wash the column with 3x2 ml of 1 mM HCl – do not exceed flow rate of ca. 2 drops/sec
- Inject 1 ml of the protein
- Seal the column and let stand for 15 – 20 min at room temperature or 4 h at 4°C
- Wash and deactivate the column as follows:
    - 3x2 ml ethanolamine buffer
    - 3x2 ml acetate buffer
    - 3x2 ml ethanolamine buffer, let stand for 15 – 30 min
    - 3x2 ml acetate buffer
    - 3x2 ml ethanolamine buffer
    - 3x2 ml acetate buffer
    - Finally inject storing buffer or PBS

B) Coupling proteins to a 5 ml NHS-activated HiTrap column

- Same reagents and general protocols as above but using a 5 ml column
- The protein is equilibrated in the coupling buffer e.g. by dialysis og by buffer change on PD-10 or NPD column. The concentration should be 0.5 – 10 mg/ml in 5 ml
- Remove the cap of the column and add a drop of ice cold 1 mM HCl.
- Remove the bottom end when ready
- Using a 5 or 10 ml syringe wash the column with 3x10 ml of 1 mM HCl – do not exceed flow rate of ca. 2 drops/sec
- Inject 5 ml of the protein
- Seal the column and let stand for 15 – 20 min at room temperature or 4 h at 4°C
- Wash and deactivate the column as follows:
    - 3x10 ml ethanolamine buffer
    - 3x10 ml acetate buffer
    - 3x10 ml ethanolamine buffer, let stand for 15 – 30 min
    - 3x10 ml acetate buffer
    - 3x10 ml ethanolamine buffer
    - 3x10 ml acetate buffer
    - Finally inject storing buffer or PBS

C) Binding to ligand and elution of affinity bound protein

- The FPLC system is used and the programme for 1 or 5 ml HiTrap NHS affinity column.
- Equilibrium buffer is PBS, elution buffer 0.1M glycine pH 2.3, neutralizing eluted bound fractions with a suitable amount of 1M Tris.
  Test the amount of Tris needed to neutralize the fraction size of glycine-HCl using pH strips is the safest but potassium chromate* titration (yellow to orange) can also be used.

Note

Five 5 ml columns coupled with **mouse anti-cod IgM immunoglobulins** are available at Keldur, stored in the lab refrigerator.

*Potassium chromate:

19. Proteolytic digestion of Ig's (fish IgM)

Magnadottir et al. 1996 FSI 6:185-198, protocols from Sterogen Biochemicals

MATERIALS

- Trypsin-actigel from Sterogen Biochemicals, USA
- Trypsin buffer: 0.1N Tris-HCl buffer, pH 8.0, 10mM $CaCl_2$
- Pepsin-actigel from Sterogen Biochemicals, USA
- Pepsin buffer: 20mM sodium acetate buffer, pH 5.5 – 6.0
- Papain-actigel from Sterogen Biochemicals, USA
- Papain buffer: 20 mM phosphate buffer, pH 6.2 containing 1mM dithiothreitol and 5 mM EDTA
- Glass filter connected to a water suction pump

METHOD

1. **Trypsin digestion**
    - On a glass filter trypsin-gel containing about 1 mg enzyme per ml gel was washed several times with the trypsin buffer and finally suction dried, 1 ml of suction dry trypsin actigel weighs about 570 mg
    - The immunoglobulin solution, approximately 1 mg/ml, is equilibrated by dialysis or PD column in the trypsin buffer
    - 50 mg of the suction dry gel (= 85 µg trypsin) are mixed with 75 µl of the trypsin buffer and 50µl of the Ig solution
    - Control omitting the trypsin gel is included
    - Incubated overnight (> 16 h) at 45°C with gentle or occasional shaking
    - Centrifuge the sample at 200xg for 10 min and collect the supernatant
    - Check digestion by SDS-PAGE

2. **Pepsin digestion**
    - On a glass filter pepsin-gel containing about 3 - 4 mg enzyme per ml gel was washed several times with the pepsin buffer and finally suction dried
    - The immunoglobulin solution, approximately 1 mg/ml, is equilibrated by dialysis or PD column in the pepsin buffer
    - 10 mg of the suction dry gel (= 50 - 70 µg pepsin) are mixed with 75 µl of the trypsin buffer and 50µl of the Ig solution
    - Control omitting the pepsin gel is included
    - Incubated for 1 h at 37°C with gentle or occasional shaking
    - Centrifuge the sample at 200xg for 10 min and collect the supernatant
    - Check digestion by SDS-PAGE

3. **Papain digestion**
    - On a glass filter pepsin-gel containing about 2 mg enzyme per ml gel was washed several times with the papain buffer and finally suction dried
    - The immunoglobulin solution, approximately 1 mg/ml, is equilibrated by dialysis or PD column in the papain buffer
    - 10 mg of the suction dry gel (= 34 µg papain) are mixed with 75 µl of the trypsin buffer and 50µl of the Ig solution
    - Control omitting the papain gel is included
    - Incubated for 1 h at 37°C with gentle or occasional shaking
    - Centrifuge the sample at 200xg for 10 min and collect the supernatant
    - Check digestion by SDS-PAGE

Note

Digestion conditions i.e. pH, enzyme concentration and incubation temperature will vary depending on the Ig-species (or protein) being studied

20. Preparation of organ/larvae protein suspension

Magnadottir et al., Comparative Biochemistry and Physiolog PartB 2004, 139:217-224

MATERIALS

- Triton X-100 lysis buffer prepared by adding 0.5 ml of Triton X-100 to 100 ml of Tris-saline buffer (ice cold):
- Tris saline buffer, 50 mM Tris-HCl, pH 7.6: 0.6 g tris in <100 ml, pH adjusted with HCl, add to this 1.75 g NaCl (0.3M) and adjust the volume to 100 ml.
- As needed add 100 µl protease inhibitor cocktail (from Sigma) per ml of lysis buffer

METHOD

- Organ or larvae samples are pressed few times through a tea strainer with ca. 1 ml of the lysis buffer containing the protease inhibitor using syringe piston and a petri dish **(done on ice)**,.
- The suspension was frozen at - 80°C
- Thawed out again and centrifuged at 15.000 rpm for 5 min (at 4°C)
- Supernatant collected
- Protein estimated at OD 280 nm and/or using Bradford's method
- Used at once or stored in aliquots at -80°C.

Note

If lipids are a problem this may work:

- In a glass tube or separation funnel:
- To 1 ml of supernatant add 3.75 ml of methanol:chloroform (2:1)
- Shake well, let stand for 5 min, shake again.
- Centrifuge for few min or let stand until separated into layers.
- Collect the upper water/methanol phase which should contain proteins free of lipids.

Also available is Lipoclean lipid removal kit from Behring.

Extra step to reduce protein – protein bonds (used befor e.g. immunoprecipitaion):

Add 10% Na-DOC to give a final concentration of 0.2% Na-DOC and 10% SDS to give final concentration of 0.2%, all kept at room temperature before mixing.

21. Sponatneaous haemolytic assay

(the alternative pathway)

Magnadottir 2000, FSI 10: 731-735, Lange et al. 2001, FSI 11:523-535.

MATERIALS

- Gelatin-Veronal buffer (GVB) from Sigma (G6514) containing Ca^{2+} and Mg^{2+} (expensive!)
- Sheep blood stored 1:1 in Alsever‘s solution (from Eggert)
- Saline, 0.85% NaCl in H_2O
- EDTA (ethylene diamine tetra acetic acid)
- EGTA (ethylene glycol tetra scetic acid)
- 96 well round bottom micro tray from Nunc (CFT-trays)
- 96 well flat bottom, non-absorbent tray from Nunc (269620)

METHOD

- Sheep red blood cells (RBC) are washed 3x in saline and then diluted in GVB to give 1% suspension.
- The RBC suspension is standardised as follows: Add 100 µl of the 1% suspension to 3.4 ml H_2O and check OD at 414 nm using H_2O as a blank. The OD should be about 0.720. Add to the 1% suspension more washed RBC or more GVB to obtain this value (approx.).
- The standardized 1% RBC suspension is diluted 1:1 in GVB to give 0.5% RBC suspension
- Prepare twofold serial dilutions of the fish serum in GVB and put 100 µl per well in duplicates for each dilution on the CFT-tray. Include on the tray (in duplicates) 1) 100% lyses control of 100 µl distilled water and 2) Blank or 0% lyses control of 100 µl GVB.
- Add 50 µl of the 0.5% RBS suspension to each well.
- Cover the tray and incubate with gentle shaking at room temperature* for 60 min. Knock the tray gently from time to time to suspend the RBC.
- Centrifuge the tray for 10 min at 750 xg. Remove carefully 125 µl of the supernatant and transfer to a 96 well flat bottom tray and read OD at 405 nm.
- Subtract the blank value and calculate the % haemolysis with reference to the 100% lyses control. Plot a graph of % haemolyses versus log dilution and calculate the SH_{50} (ACH_{50}) value i.e. the serum dilution that gives 50% lyses,

Note

Further analysis of the HA activity:

EDTA blocks all HA activity i.e. by the alternative, classical and lectin pathway. Use 1 – 10 mM EDTA in the GVB.

EGTA blocks the HA activity of the classical pathway only. Use 1 – 10 mM EGTA in GVB.

*Check also the effects of variable incubation temperature for each fish species

22. Bactericidal activity

Budino et al. 2006 CBP 145, 108-113

MATERIALS

- Bacterial culture of choice
- Flat-bottomed, non-absorbent 96 well microtiter plates (Nunc nr. 269620)
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma M5655)

METHOD

- Bacteria were cultivated to approx. 10^9 CFU/ml, washed twice in saline and diluted to 10^7, $5x10^7$ and 10^8 CFU/ml in culture broth.
- Using flat-bottomed, non-absorbent 96 well microtiter plates 4 wells were used per bacterial dilution.
- Add to each well 33 µl of fresh serum, 133 µl of each bacterial dilution, mix
- Use PBS instead of serum for control
- Incubate for 6.5 h at 20°C
- Add to each well 86 µl MTT (2 mg/ml), mixed and incubated for 15 min at 20°C.
- MTT reduction (by live bacteria) was measured at 630 nm.
- Bactericidal index was determined as the mean absorbance of 4 wells *versus* absorbance of control.

Note

Have tested this method once with the results that cod serum seemed to feed the bacteria rather than kill!

. 23. Anti-trypsin activity of fish serum

Ellis, Tech.Fish Immunol. 1999 p. 95-96, Magnadottir et al. 1999, CPB122B: 173 - 180

MATERIALS

- Phosphate buffer, 0.1M, pH 7.0: 5.44 g KH_2PO_4, 43 g Na_2HPO_4,$12H_2O$ (or 17 g Na_2HPO_4) in 1 L. Store at 4°C.
- Azocasein, 2%: 1 g azocasein (Sigma A-2765) in 50 ml of H_2O. Divide into 8 ml aliquotes and store at -20°C.
- TCA, 10%: 5 g trichloro acetic acid in 50 ml of H_2O. Store at 4°C (fume cupboard)
- NaOH, 1N: 4 g NaOH in 100 ml of H_2O. Store at 4°C (fume cupboard).
- Trypsin: 5 mg trypsin (Sigma T-7409, 1000 – 2000 BAEE per mg solid) per ml H_2O (ice cold). Estimate how much is needed for the whole experiment (or more) make up this amount and divide into 1 ml aliquotes and store at -80°C.
- Non-absorbent microtrays (Nunc nr. 269620).

METHOD

- Suitable test size for one 96 well microtray: 29 serum samples (tested singly), 2 standards and 1 blank all in triplicates and this needs 1 ml trypsin solution and 8 ml of azocasein solution.
- Take all reagents to room temperature but keep the trypsin solution on ice until needed.
- Serum sample: In an 1.5 ml eppendorf test tube mix 20 µl of serum and 20 µl of trypsin (make 2 sets if there is enough serum)
  100% control: Mix 20 µl of phosphate buffer and 20 µl of trypsin solution (make 2 sets)
  Blank: 40 µl phosphate buffer.
- Once the trypsin has been added allow to stand at room temperature for 10 min
- Add 200 µl phosphate buffer and 250 µl azocasein to each tube, vortex and let stand at room temperature for 60 min.
- Add 500 µl TCA (in fume cupboard), vortex and let stand at room temperature for 30 min.
- Centrifuge at 8000 rpm for 5 – 10 min.
- Using 96 well non-absorbent microtray, add 100 µl of NaOH to each well and to this add 100 µl of the supernatant in duplicates or triplicates
- Read OD at 450nm
- Express the anti-trypsin activity: Calculate the mean values and for each serum sample calculate the % of the 100% control value. The higher the serum anti-trypsin activity the lower this value.
  To express the anti-trypsin activity in reverse i.e. % inhibition: Subtract this value from 100 i.e. the higher the % value the stronger the anti-trypsin activity.

24. Lysozyme assay

Ellis 1990, Techniques in Fish Immunology, Lange et al. 2001 FSI 11, 523-535

MATERIALS

- 96 well non-absorbent microtray (Nunc nr. 269620)
- Phosphate buffer, 0.05M, pH 6.2*
- *Micrococcus lysodeikticus* (Sigma, USA), 0.4 mg ml^{-1}, fresh suspension
- Hen Egg White lysozyme (ICN, USA)

METHOD

- Test serum is diluted 1/10** in the phosphate buffer and 100 µl per well in duplicates put on the microtray
- For a positive control replace serum with hen egg white lysozyme in serial dilutions starting with 1.6 µg ml^{-1} in phosphate buffer
- For a negative control replace the serum with phosphate buffer
- Add 100 µl of the micrococcus suspension and incubate at room temperature***
- Read the OD at 540 nm at 0 and 15 min (and up to 60 min if required) and plot the decrease in OD versus time
- The reduction by 0.001 min^{-1} is 1 lysozyme unit.

Notes:

*The optimum pH has to be checked for each species (here sea bass)

**Suitable serum dilution has to be pre-determined (here sea bass)

***The optimum incubation temperature has to be checked for each species (here sea bass)

Unlike many fish serum cod serum shows no or very low lysozyme activity while cod mucus shows lysozyme activity.

25. Dot blot

From Schleicher & Schuell & various testing of cod serum

MATERIALS

- 96 well Dot-blot instrument from Schleicher & Schuell (Germany)
- NC paper (water mixable e.d. Hybond ECL from Amersham, (**not PDV film**)
- Filterpaper
- TBS-T buffer: 0.5M Tris, 150 mM NaCl pH 7.2, 0.1% tween 20
- Blocking solution: 1% semi skimmed milk powder in TBS-T
- Primary & Secondary antibody diluted in TBS-T
- ECL developing kit (Amersham Pharmacia Biotech)

METHOD

a) Using the Schleicher & Schuell instrument

- Prepare standard solutions of the protein of interest e.g. CRP and of the test material (e.g. fish serum) in TBS-T
- Wet the NC paper and the filter paper in TBS-T, place the filter paper on the platform and the NC paper on top, close the cassette and connect to the suction pump. Put 30 µl of TBS-T per well and suck dry, then disconnect the pump
- Dot 40 µl of standard or test samples per well in duplicates, suck dry, then disconnect the pump
- Remove the NC paper from the cassette and allow to dry before placing in the blocking solution overnight at 4°C or at room temperature for 1 h
- Incubate the NC paper in the primary antibody solution e.g. mouse-anti-CRP antibody for 1 h at room temperature
- Wash in TBS-T for 3 x 10 min at room temperature
- Incubate in the secondary antibody e.g. goat anti-mouse Ig's peroxidase labelled for 1 h at room temperature
- Wash in TBS-T as before
- Develop using the ECL kit and analyse the film using a density scanner
- Plot a graph of density versus standard concentration and extrapolate the concentration of the test sample from this

Note

This can be problematic method e.g. the dots are often not of uniform density and hence difficult to scan the density accurately. Careful direct dotting on NC paper often gives better results.

b) Direct dotting using strips of NC paper

For testing the presence of a particular protein 0.5 – 2 µl of the protein solution can be dotted directly onto the NC paper, allow to dry before blocking and incubating in the primary and secondary antibodies as described above

26. Enzyme SDS-PAGE for cod larvae lysate

Magnadottir et al., 2004, CBP B 139, 217-224

MATERIALS

- 1.5M Tris buffer pH 8.8
- 0.05M Tris, pH 8.0
- 0.005M Ca Cl2 in 0.05M Tris buffer, pH 8.0
- Triton X-100
- Acrylamide-bis (Sigma)
- 10% gelatine, heat to dissolve
- 10% casein
- 10% collagen
- 10% sodium dodecyl sulphate (SDS)
- 10% ammonium persulphate (AMPS)
- Temed (Sigma)
- Useful sample buffer: 5% SDS, 2% sucrose, pheol red mixed 1:1 with the sample

1. **Gelatine gel to analyse gelatinase activity**
   - H_2O 3.8 ml
   - 1.5M pH 8.8 Tris buffer 2.5 ml
   - Acrylamide-bis 3.5 ml
   - 10% gelatine, heat to dissolve 50 µl
   - SDS 100 µl
   - AMPS 50 µl
   - Temed 5 µl

2. **Casein gel to analyse caseinase activity**
   - H_2O 3.8 ml
   - 1.5M pH 8.8 Tris buffer 2.5 ml
   - Acrylamide-bis 3.5 ml
   - 10% casein 50 µl
   - SDS 100 µl
   - AMPS 50 µl
   - Temed 5 µl

3. **Collagen gel to analyse collagenase activity**
   - H_2O 3.8 ml
   - 1.5M pH 8.8 Tris buffer 2.5 ml
   - Acrylamide-bis 3.5 ml
   - 10% collagen 0.4 ml
   - SDS 100 µl
   - AMPS 50 µl
   - Temed 5 µl

METHOD

- N.B. sample is **neither heated nor reduced** before being loaded onto the gel
- Standard SDS PAGE is carried out in MINI PROTEAN II SYSTEM from Bip-Rad (USA) using 4.5% stacking gel and 14% resolving substrate gel (see above) according to the manufacturer's procedure
- Following the electrophoresis SDS is eluted from the gel by washing in 2% Triton X-100 in 0.05M Tris, pH 8.0 for 30 min at room temperature
- This is followed by an incubation in the same tris buffer containing 0.005M Ca Cl_2 overnight at room temperature or for 3 h at 37°C.
- Standard staining with coomassie blue and carefull destaining to detect clear bands of enzyme activity

27. Thin (3.5%) SDS-PAGE

Avtalion & Mor 1992, Israeli Journal of Aquaculture 44, 93-98, Magnadottir 1998, Icel. Agr. Sci. 12, 47 - 59

MATERIALS & METHOD

- All ingredients and tools for standard SDS-PAGE
- Set up the gel casette and keep it and the bulb pipette and other pipettes to be used and the sample comb warm (at 37 - 40°C) until used.
- 2% agarose in SDS-PAGE running buffer, dissolved in a boiling waterbath and keep at 50°C
- Make up the acrylamide solution:
  - 3.5 ml H_2O
  - 2.5 ml 1.5 M tris-HCl pH 8.8
  - 0.875 ml of 40% Acrylamide
  - Warm at 40 – 50°C
- Add to this:
  - 3 ml of 2% agarose,
  - 100 µl 10% SDS,
  - 50 µl 10% ammonium persulphate
  - 5µl Temed
- Put into the warmed cassette with the sample comb in place (no sample gel)
- Allow to set and then run the samples within the same day
- Silver or coomassie blue staining as for normal gels.

Note

This gel works well for analysis of large proteins or about 150 kDa - 1000 kDa

28. Destaining silverstained SDS-PAGE gel

MATERIALS

- Solution A: 18.5 g NaCl and 18.5 g $CuSO_4$ anhydr. in 425 ml H_2O, put on a stirrer and add conc. Ammonium hydroxide until no precipitation forms or remains
- Solution B: 218 g sodium thiosulphate $5H_2O$ in 500 ml H_2O
- 10% acetic acid, 10 ml of conc. acetic acid in 100 ml H_2O
- Silver staining kit from BioRad

METHOD

- Following standard silver staining the gel is washed in H_2O for 30 min
- Mix 20 ml of Solution A and 20 ml of Solution B and suspend the gel in this for few seconds
- Wash few times with H_2O
- Stop the destaining with 10% acetic acid for 15 min
- Wash with several changes of H_2O for 60 min
- Restain with the silver staining kit omitting the first step (Fixative Enhancer)

Note

This method greatly enhances the results of standard silver staining of acrylamide gels and gives clearer background. It should be carried out if the results are to be published.

29. Bradford method for protein assay

Bradford, Anal.Biochem 1976. 72 ,248-254

MATERIALS

- Coomassie Plus Protein Assay Reagent from Pierce or Thermo Scientific stored at 4°C but kept at room temperature before the assay is carried out.
- Bovine Serum Albumin 2 mg/ml (from Pierce) is used as a standard for most assays (Bovine Gamma Globulin can also be used if measuring the concentration of immunoglobulins)
- Use a non-absorbent 96 well microtray (Nunc nr. 269620).

METHOD

Prepare the following set of standards and mix well (can be stored at –20°C):

BSA standard (2 mg/ml) µl	PBS or H_2O µl	µg protein/sample (i.e. in 10 µl)	µg protein/ml
0	100	0	0
5	95	1	100
10	90	2	200
20	80	4	400
40	60	8	800
60	40	12	1200
80	20	16	1600

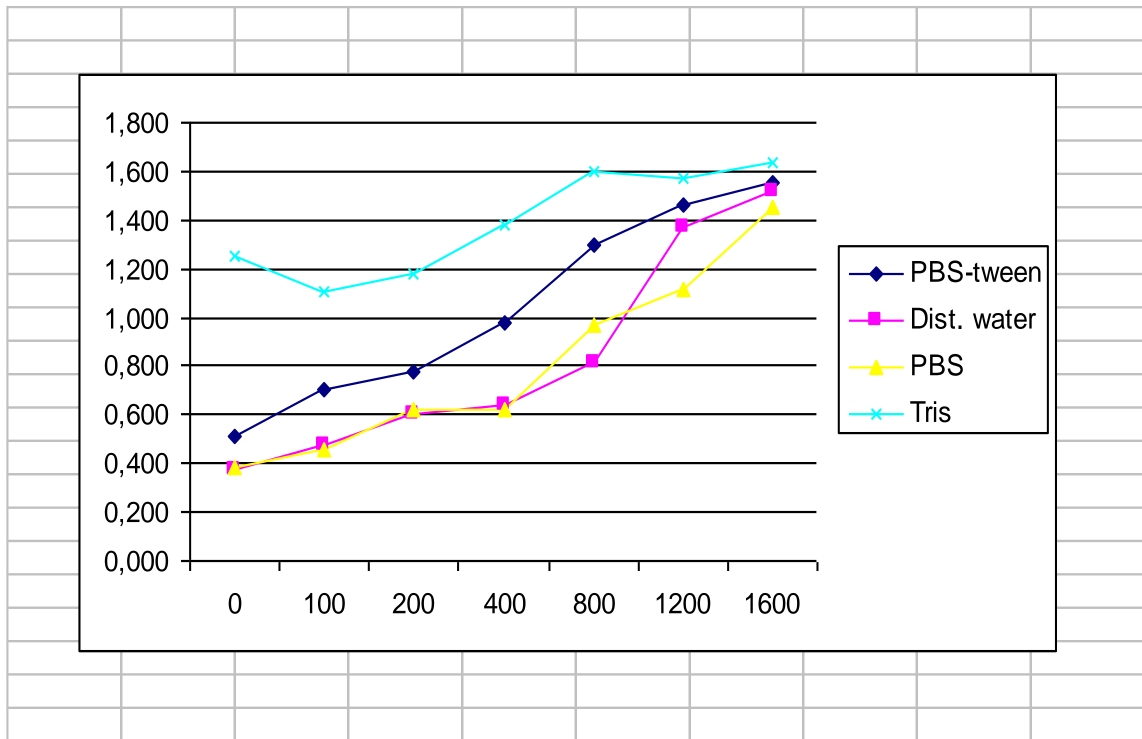
The assay

- 10 µl of standard per well in duplicates or triplicates
  10 µl test sample per well (fish serum normally diluted 1/100), in duplicates or triplicates
- Add 300 µl (2x 150 µl) of the reagent per well* and incubate at room temperature for 30 min. Read OD at 600 nm (or 590 nm)
- Draw up a graph of mean OD values versus standard protein quantity and extrapolate the test sample from this.
- ***NB: Calculate how much Bradford reagent is needed for the assay and remove this quantity from the bottle before the analysis – discard the leftovers i.e. do not pour the remains back into the reagent bottle**

Notes

- The buffer that the protein sample is in can affect the results (see figure). Tris buffer and Triton-X-100 lysis buffer interfere seriously – see graph below!
- Other methods less sensitive to Tris or lysis buffers are available as kits (e.g.from BioRad) based on the Lowry's method (Lowry et al. 1951, J. Biol. Chem. 193, 265) or the Bramhall

method (Bramhall et al, Anal. Biochem. 1969, 31.146) and can be used instead of the Bradford kit.



Standard protein (bovine serum albumin) in distilled water, PBS, PBS-tween and tris buffer

30. Protein concentration at 280 mµ

Methods in Immunology and Immunochemistry vol II, appendix

MATERIALS

- Protein solution, the purer the protein the more accurate is the method
- Spectrophotometer Perkin-Elmer 550 S, UV-VIS and **quartz cuvettes**

METHOD

- Reading is taken at 280 mµ subtracting a blank H_2O or the buffer the protein is dissolved in
- Concentration is calculated from the E [0.1% /1cm] standard for each protein: A solution of 1 mg/ml (i.e. 0.1% solutions) has an extension coefficient E = the reading at 280 mµ shown below

Protein	Optimum Mµ	E [0.1% /1cm]	Buffer
Fish IgM	280	1.37*	PBS
Cod IgM	280	1.115**	saline
BSA	279	0.667	H_2O
Human IgG	280	1.43	PBS
Human IgM	280	1.185	PBS
Rabbit IgG	278 - 280	1.38 - 1.46	PBS
Chicken IgG	280	1.35	PBS
Shark IgM	280	1.279 – 1.375	saline
Pepsin	280	1.43	H_2O
Trypsin	280	1.44	H_2O
Papain	278	2.50	H_2O

*The value commonly uses for a rough estimation of Ig concentration

**The value calculated for cod IgM according to L. Pilström (DCI, 1991, 15, 143)

31. Ammonium sulphate & organic solvent protein precipitation

Old QUB methods

1. 50% saturated ammonium sulphate ($(NH_4)_2SO_4$) precipitation

MATERIALS

- Ammonkum sulphate (sulphate) ($(NH_4)_2SO_4$) solid
- Saline: 0.85% NaCl in distilled water: 8.5 g NaCl in 1 l distilled water
- 2N NaOH
- 1% $BaCl_2$: 0.5 g $BaCl_2$ in 50 ml distilled water
- Dialysis tubes

METHOD

- Measure the volume of the solution* (e.g. serum) containing the protein and multiply by 0.313, e.g. 50 ml solution x 0.313 = 15.65 g ammonium sulphate to obtain 50% saturation (see Table below)
- Add this amount of ammonium sulphate gradually to the solution with mixing.
- pH may be checked and adjusted to pH 7.4 with 2N NaOH.
- Put on a magnetic mixer and stir gently for 2 h at room temperature or overnight at 4°C.
- Centrifuge the solution at 1000 xg for 15 – 30 min, discard the supernatant, wash the precipitate once with 50% ammonium sulphate saturated saline (see table) and repeat the centrifugation.
- Dissolve the protein precipitate in saline, approximately 1/20** of the original solution (in the example above: 2.5 ml, see notes)
- Dialyse the protein in saline until free of sulphate, generally for 24 - 48 h at 4°C with frequent changes of saline.
- Test the dialyses liquid for sulphate with the BaCl precipitation test (see Method 35). If a cloud of precipitation is seen continue the dialysis.
- When free of sulphate the sample is collected from the dialysis bag and the protein concentration e.g. crude Ig's is measured using Bradford's method (Method 29).

Notes

*Applies to any liquid, see Table below for other % ammonium sulphate saturations
Ig precipitation is commonly done at 33% ammonium sulphate saturation (see Table)

**If precipitating e.g. from serum the crude Ig solution needs to be dissolved in approx. 1/5 of the original volume (in the example above in 10 ml)

Table: How to make solution with different saturation of ammonium sulphate

Final concentration of ammonium sulphate % saturation																
10	20	25	30	33	35	40	45	50	55	60	65	70	75	80	90	100
Grams solid ammonium sulphate to be added to 1 L of solution																
56	114	144	176	196	209	243	277	313	351	390	430	472	516	561	662	767

Highlighted are the quantities used for 33% and 50% saturation

2. Protein precipitated with organic solvents

MATERIALS

- Protein solutions: Should contain approx. 5 – 30 mg/ml and the salt content should not be too high (0.05 – 0.2M)
- Solvent: Ethanol or acetone

METHOD

- In an ice bath (the lower the temp. the better precipitation) add the solvent slowly to the protein solution to give final content of 20 – 30% solvent e.g. 5 ml to 10 ml of protein solution. More solvent can be added when the solution has cooled (commonly up to 50% solvent)
- Centrifuge at ≤ 4°C at 10.000 rpm
- Dissolve the precipitated in buffer, store as before

32. Protein isolation from gel for amino acid sequence analysis or immunization

Magnadottir et al., 2004, CBP 139, 217-224, Lange PhD thesis 2005

MATERIALS

- Standard SDS-PAGE equipment (MINI PROTEAN II system from Bio-Rad or equivalent) and material for gel preparation
- Standard Western blotting equipment and materials and PVDF (polyvinylidene difluoride) transfer paper (nitrocellulos paper is also ok).
- Coomassie blue stain (Sigma)
- Red poreceau S: 0.1% (w/v) Red Ponceau S in 5% acetic acid (Amido black stain can also be used)

METHOD

1. **Protein isolated for amino acid sequence analysis**
   - Following standard SDS-PAGE separation and standard Coomassie blue staining (see below) the protein band of interest is excised from the gel, placed in an eppendorf tube (can be stored at 0 - 4°C) and sent for mass spectrometric analysis as soon as possible to:
   - Kevin Bailey, School of Biomedical Sciences, Queens Medical Centre, Nottingham, UK, e-mail: Kevin.Bailey@nottingham.ac.uk

2. **Protein isolated for N-terminal sequence analysis**
   - Following standard SDS-PAGE separation and western blot transfer on PVDF membrane the membrane is stained using red ponceau S stain* (or amido black stain) and the protein band of interest is excised from the membrane and sent for N-terminal amino acid sequencing using Edman's degradation method to:
   - Dr. J. d'Alayer, Protein Microsequencing and Analyses, Institut Pasteur, Paris, France, e-mail: jdalayer@pasteur.fr

*Red Ponceau S stain (but not amido black) can be completely de-stained by washing in H_2O or buffer

3. **Coomassie blue staining for proteomics**

- Stain the gel in 0.1% CBB in 45% methanol, 10% acetic acid for 30 min at room temp.
- Destain in 45% methanol, 10% acetic acid, approx. 30 min at room temp.
- Cut out the gel slice and cut at least 1 mm around the visible band if possible

4. Protein isolated for antibody production

- Following standard SDS-PAGE separation and weak (0.01%) Coomassie blue staining the gel is washed in distilled water for 1 h
- The protein band of interest is excised from the gel and emulsified by passing cut up gel pieces through a 1 ml syringes, starting with 18G needle, then 19G, then 20G and finally 21G
- This can be used directly for injection of mice for polyclonal antibody production or mixed with Freund's adjuvant (complete or incomplete, see polyclonal antibody production in mice, Method 15, p. 23)

33. Preparation of haptenated protein

Pilström & Petersson, DCI 1991, 15, 143 o.fl

MATERIALS

- Picric acid, the chemical compound formally called 2,4,6-trinitrophenol (TNP)
- Protein e.g. bovine serum albumin (BSA)
- Cacodylate (Na(CH3)2 AsO2 • 3H2O) buffer, 0.28M, pH 6.9
- Glycylglycine

METHOD

- 5 mg of 2,4,6-trinitrobenzene (TNP) picric acid in 1 ml dist.water is added to10 mg BSA in 1 ml of 0.28M cacodylate buffer, pH 6.9
- Leave at room temp. for 1 h
- Add 10 mg glycylglycine to each ml (i.e. here 20 mg).
- Dialyse against PBS with several changes at 4°C (can instead be passed through a PD column).
- Read OD at 280 mμ (protein) and 340 mμ (TNP)
- Should give about 5.25 mg/ml protein containing 16.3 TNP residues per BSA molecule
- Stored in aliquots at -20°C.
- Used in ELISA, coating with 5 – 10 μg/ml (protein).

Note

Haptenated proteins like TNP-LPH are also available from e.g. Sigma

34. Titration of NaCl concentration

Old Queen's method

MATERIALS

- 5% potassium chromate* pH indicator, end-point yellow-orange
- 0.01M $AgNO_3$

METHOD

- To 0.1 ml sample add a drop of the indicator
- Using a titration column (or pipett) add $AgNO_3$ until end-point has been reached
- Calculate the M NaCl:

  ml $AgNO_3$ x 0.1 = M NaCl

*potassium chromate:

35. Detection of sulphate in dialysis

Old Queen's method

MATERIALS

- 1% $BaCl_2$ solution: 0.5 g $BaCl_2$ in 50 ml H_2O

METHOD

- Take 2 ml of the dialysis buffer and add 2 ml of the BaCl2 solution.
- If a cloud of precitpitate forms SO_4 ions are still present and dialysis is continued

Notes

This method is used when dialysing out sulphate ions after ammonium sulphate precipitation (see Method 31).

36. Immunohistochemistry using a kit from Dako

Dako REAL Detection system K5005, AP/Red Rabbit/Mouse

MATERIALS

All reagents to be prepare fresh

- TBS buffer, pH 7.2, 0.5M Tris, 150 mM NaCl (TBS) pH 7.2: 60 g Tris, 8.7 g NaCl in ca. 500 ml H_2O, pH adjusted to 7.2 with 5 N HCl, volume adjusted to 1 L
- Sodium borohydrate, 1% $NaBH_4$ in 0.1M phosphate buffer, pH 8.0: 1 g $NaBH_4$ in 0.1 M phosphate buffer pH 8.0 (see Section on buffers)
- Blocking solution, 20% semiskimmed milk powder (smp), 3% BSA, 0.3% tween 20, 500 IU/ml heparin in TBS:
    - 2 g smp,
    - 0.3 g BSA,
    - 30 µl tween 20 and
    - 1 ml 5000 IU/ml heparin
    - All in 10 ml TBS total volume
- Primary antibody prepared just before use. Diluted in blocking solution e.g. 1/100, 1/200 or 1/500.
- Substrate prepared just before use:
    - 750 µl AP substrate – bottle F
    - 30 µl Chromogen Red 1 (bottle C)
    - 30 µl Chromogen Red 2 (bottle D)
    - 30 µl Chromogen Red 3 (bottle E)

METHOD

- Tissue (formalin fixed and paraffin wax embedded) cut sections on Super-Frost*/plus slides incubated at 60°C for 45 min
- Deparaffinise:
    - Tissue clear 2 x 7 min,
    - Dip in 2 alcohol baths,
    - Incubate in the 3rd for 5 min,
    - Dip in the 4th
- Rehydrating:
    - Rinse in distilled water for 2 x 5 min,
    - In TBS for 5 min
- Incubate in 1% NaBH4 in 0.1M phosphate buffer pH 8.0 for 20 min
- Wash in TBS for 5 min
- Blocked in SMP/BSA blocking solution for 20 min
- Biotin-avidin blocker (Biotin Blocking System, x0590):
    - Avidin 10 min,
    - Biotin 10 min
- Primary antibody (and control) diluted in TBS (or in block solution): Incubated in humidity at 4°C overnight

- Wash in TBS 5 min
- Incubate in secondary biotynilated antibodies:
    - Solution A from the kit, ready to use, in humidity chamber at rt for 30 min
- Wash in TBS 5 min
- Incubate in streptavidine alkaline phosphatase
    - Solution B from the kit, ready to use, in humidity chamber at rt for 30 min
- Wash in TBS 5 min
- Colour detection using Substrate solution from the kit (see above), rt for 5 – 10 (watch the red colour develop)
- Rinse in distilled water
- Counter stain with 1% methylene green for 2 min (methyl blue may work better).
- Rinse and mount with Crystal Mount Aqueous mounting medium from Sigma (C0612).
- Showing red stain = antibody reaction

37. Two dimensional (2D) electrophoresis

From Berglind Gísladóttir's notes from a course in proteomics in Aberdeen & MSc thesis p. 22

The method involves
1) the first dimension, isoelectrophocusing of the sample on strips with pre-determined pH range and
2) the second dimension, standard electrophoresis of the strip on 12 – 15% SDS-PAGE gel

MATERIALS

For the first dimension

- Multiphor II Electrophoresis unit (GE Healthcare BioSciences) and anode/cathode electrode unit
- DryStrip Aligner platform with a lid
- Cellophane sheets with grooves, 100 per pk. From Web Scientific, cat.no. E3545-100
- IPG Strips, Bio-Rad ready strips, 7 cm, pH 3 – 10, 12 per packet (cat.no. 163-2001)
  [Other pH ranges are available and strips are also sold by other firms like GE Healthcare BioSciences cat. No. 17-6001-10 (formerly Amersham)].
- IEF, electrode wax strips (100 per pk.) from GE Healthcare BioSciences 18-1004-40
- Ampholyte buffer: Bio-Lyte 3/10 Ampholyte, cat. No. 163-1112 (10 ml) from BioRad
- IPG Reswelling solution (pH 4 – 7/3 – 10):
  Urea 2.01g
  Thiourea 0.76 g
  CHAPS (3-((3-cholamidopropyl)-dimethylammonio)-1-propane-sulphonate) 0.2 g
  DTT (dithiothreitol) 0.015 g
  Add 3 ml of dH_2O and stir until dissolved (makes about 5 ml), add 25 µl Ampholyte buffer (0.2%) and a drop of bromophenol blue. Allocate 5 x 1 ml and store at -20°C.
- DryStrip Cover Fluid, GE Healthcare BioSciences 17-1335-01
- DTT - Equilibrium buffer, 7 ml/strip:
  Stock solution:
  Urea 72 g
  0.5M Tris (pH 6.8) 20 ml
  Glycerol 60 ml
  10% SDS 40 ml
  Volume made up to 200 ml, allocate and store at -20°C.
  Working solution, need 7 ml per strip: add 10 mg/ml DTT, stir until dissolved.
- IODO-Equilibrium buffer, 7 ml/strip using the same stock solution as above: 25 mg/ml of iodoacetamide
- IPG buffer, pH 4- 7 from GE Healthcare BioSciences, 1 ml concentrate cat.no. 17-6000-86
- IPG BUFFER, Ph 3 – 10, from GE Healthcare BioSciences, 1 ml concentrate cat.no. 17-6000-88

For the second dimensions

- Standard SDS-PAGE equipment and gels, using a preparative sample comb and standard running buffer.

METHOD

Sample preparation

Here: Serum (kits for protein isolation from organ samples are available, see notes)
Mix 5 µl serum with 135 µl of IPG reswelling solution and incubate for 10 min at room temperature, centrifuge at 13.000 rpm for 5 min, use 125 µl of the supernatant.

Preparation of strips

- On a Immobiline DryStrip Reswelling Tray add 125 µl of the sample to a groove. Take an IPG strip, remove the plastic film and lay the strip with that side (which is the gel side) down on the sample, taking care that no air bubbles remain, (the + on the strip turned in the same direction).
- Cover with DryStrip cover fluid oil (start at one end to about half way down the strip and then add the cover fluid from the other side).
- When all the samples/strips are in place, put the lid on the tray and incubate at room temperature overnight.

First dimension electrophoresis

- Oil poured over the middle of the platform of the Multiphor II unit, place the cathode/anode unit on the platform so that the oil lies underneath and there are no air bubbles. Connect the cathode and the anode.
- Pour oil (can use used oil) on the electrode platform and put a plastic strip- tray on the platform. To be ready, cut out two 11 cm wax strips, place on a glass surface and wet with H_2O and remove excess water with a paper towel.
- Using a pincette, remove one strip at a time from the re-swelling tray, allow most of the oil to run off onto a paper towel and then place in the strip tray on the Multiphor II unit so that gel side turns up and the + sign towards the cathode.
- Place the wetted wax strip over both ends of the strips, just touching the gel. Put the electrodes on the wax strips, red towards the cathode, black towards the anode. Pour oil over the strips and adjacent empty wells (use new oil but this can be re-used for underlay).
- Connect the platform to running cold water for cooling.
- Put the lid on and run the electrophorsis: BioRad:
  200v for 1 min;
  3500 v for 90 min,
  3500 v for 3 h (or other settings depending on experience etc.).

  Check that the blue dye is carried from the anode end to the cathode end.

- When finished each strip is removed and put into a 15 ml covered test tube with the plastic side leaning towards the side of the test tube. Can be stored at -20°C for few days or used at once in standard SDS-PAGE following equilibration.

Equilibration of the strips

- In the first step the strips are covered with 7 ml of the DTT equilibrium buffer and incubated for 30 min on a rocking platform at room temperature. Pour the buffer off carefully holding the strip or removing with a pincette while emptying the tube.
- In the second step this buffer is replaced with the IODO equilibration buffer and incubated for 30 min on a rocking platform at room temperature.
- Both solutions should be discarded into "chemical waste" bottles

Second dimension electrophoresis

By this time standard SDS-PAGE gels, home made or commercial, should be ready with a stacking gel containing a standard well (may be optional) and a preparative well. Put a protein marker in the standard well (if used). Dip the strip into dH_2O and then put it sideways onto the gel in the preparative well, with the plastic side towards the glass and overlay with running buffer, etc.

Notes

1. Cleaning the trays and equipment is important.

- The reswelling tray is put on its end into a polythene bag and the oil allowed to run off and discarded. The tray is then washed well in soap and dH_2O.
- The oil in the strip tray on the Multiphor II unit is poured into a bottle for reuse. The rest of the oil is then wiped off and the tray and platform cleaned with soapy water and dH_2O.

2. Kits for cleaning up serum samples (removing albumin and immunoglobulins) are available from GE Healthcare BioSciences, VivaScience and Qiagen (Depletion columns 6), all expensive. Using these kits has the disadvantage that one may loose important proteins and add some artefacts.
   However, fish (i.e. cod) serum is relatively "clean".

3. Kit for preparation of organ samples: GE Healthcare BioSciences (Amersham) Cat. No. 80-6483-37

4. So far the best results using the 2D system have been obtained by transferring the proteins following the 2nd dimention to a NC paper (Western blotting) and immunostain with a relevant antigen (e.g. serum vs. anti-cod CRP-PI or PII antibodies)

38. Glycoprotein detected by Western blotting

Cristina Columinas pers. comm.. + kit from Glycoscience

MATERIALS

- Standard SDS-PAGE & Western blottin materials and equipments
- NC paper
- Sodium metaperiodate
- Acetate buffer, 0.1M, pH 5 (see buffers, Method 38)
- Biotin hydrazide
- Streptavidin-HP
- PBS

METHOD

- Carry out normal SDS-PAGE separation of the proteins
- Cool the transfer instrument, transfer buffer and NC and filter papers to 4°C
- Normal transfer carried out at 4°C
- Wash the blot in PBS for 10 min
- In the dark, put the blot in 10 mM sodium metaperiodat in the acetate buffer for 20 min
- Rinse twice in PBS and then wash for 3 x 10 min in PBS
- Incubate the blott in 40 µl of 0.125 mM biotin hydrazide in 20 ml of 100 mM acetate buffer for 1 h
- Wash as above
- Incubate in 20 l of streptavidin-HP in 20 ml PBS for 30 min
- Wash as above
- Develop with the ECL developing kit

Notes

Various kits are available for glycoanalysis of blotts e.g. from Glycoscience and Amersham

39. Routine bath challenge test for cod larvae/fry

FISHAID Report III, p.27 & Magnadottir et al., JFD 2006, 29, 147.

MATERIALS

- Cod fry approx. 2 – 6 g
- Two sets of 12 plastic 10 L buckets with lid, both bucket and lid labelled 1 – 12, mark also the 5 and 10 L level in the buckets.
- Sea water and aerating equipment
- Bacterial suspension of predetermined dosage (cfu/ml)

METHOD

- The challenge is carried out in triplicates as shown in Figure 1.
- The cod fry has been allowed to acclimatise in tanks of sea water at 8 – 10°C for few days
- The cod fry are divided between 12 buckets, 20 fish per bucket, containing 5 L of sea water
- Asa bacterial stock solution (e.g. 1.9 x 10^9 cfu/ml) is prepared and dilutions in PBS prepared and the same volume (different dosage) added to the buckets as shown (the exact dosage may vary). Control bucket receives PBS in place of bacterial suspension. Incubated for 1 h.
- Add 5 L of sea water to each bucket and incubate for further 1 h
- Prepare another set of 12 buckets with fresh 10 L of sea water and transfer the fish to these.
- The experiment should last about 28 days and death is monitored twice daily, bucket number noted, dead fish measured, condition of fish noted and infection verified by bacterial isolation from the kidney on blood agar containing salt (see Fiskjúkdómadeild!).
- The fish are fed with few grains of the appropriate fish feed once a day, 2 teaspoons should do for the 12 buckets.

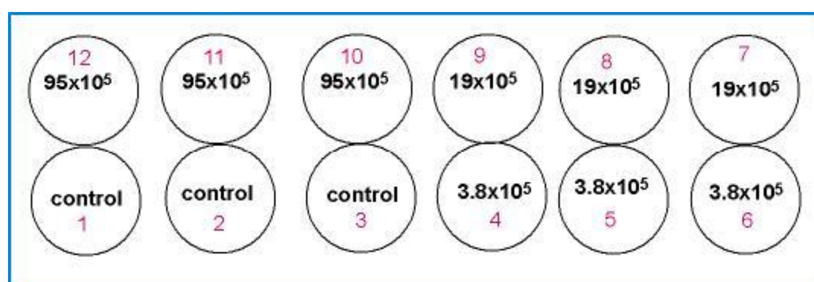


Figure 1. Arrangement of buckets: The bacterial dosage is shown as cfu/ml

Routine procedure during the challenge experiment:

- Each morning, starting with buckets 1 – 3 (control) and ending with buckets 10 – 12 (highest dosage), the fish is transferred to a corresponding set of buckets containing fresh sea water (10 L) taking care not to expose the fish to air.
- While this is being done the aerating equipment is placed in 500 mL flasks containing fresh sea water, replacing old “stones” if needed.

- The aerating equipment is put in its place, taking care this goes into the same bucket number as before. Corresponding lid put on loosely (n.b. the cod fry may jump out!).
- Used buckets and lids are washed carefully, starting with 1, ending with 12, using disinfecting washing powder and rinsing thoroughly. They are then arranged in the right order with lids, ready for the next day. The 500 mL flasks and all other equipment used are also thoroughly cleaned.

40. Dehert's stress test on cod larvae

Dhert P., Lavens P. Sorgeloos P. (1992). Aquaculture Europe, 17: 6 – 10.

The stress test involves testing the survival of cod larvae at high salt concentrations and can be used to evaluate and compare the conditions and the general status of different groups of larvae, the higher survival the lower the stress index.
The test is in two parts: a) the critical salt concentration is calculated for cod larvae of a particular size/age and b) this concentration is used in the test proper on the same set of larvae subjected to different treatments.

MATERIALS

- 100 ml glass beakers
- Sea water, um 32‰
- Marine salt (aquaculture quality)
- Cod larvae/fry's maintained in well aerated seawater at 7°C

METHOD

- Use 100 ml beakers, 20 larvae per beaker, 3 beakers for control and 3 for each additional salt concentration to be tested. The test is normally carried out at 7°C.
- Put ca. 25 ml of well aerated seawater (32 ‰) in each container.
- Fish out 20 larvae so that they are not exposed to air and avoid stressing, use white weighing containers. Adjust the volume to 50 ml with sea water.
- Allow to equilibrate for 15 min.
- Prepare the double salt solutions in sea water, 200 ml of each. For example testing 35, 45, 55, 65, 75 ‰ see table below

	Marine salt added to ml sea water (32 ‰)
2 x 35 ‰	2 x 0.3 = 0.6 g / 100 ml = 1.2 g/ 200 ml
2 x 45 ‰	2 x 1.3 = 2.6 g / 100 ml = 5.2 g/ 200 ml
2 x 55 ‰	2 x 2.3 = 4.6 g / 100 ml = 9.2 g/ 200 ml
2 x 65 ‰	2 x 3.3 = 6.6 g/ 100 ml = 13.2 g/ 200 ml
2 x 75 ‰	2 x 4.3 = 8.6 g/ 100 ml = 17.2 g/ 200 ml

- Add 50 ml of each salt concentration to the relevant beaker resulting in the correct final salt concentration. Mix gently e.g. with a glass rod, trying not to disturb the larvae.
- Incubate for 1 h and then count dead larvae.

- Determine the suitable salinity for the test proper i.e. the % death has to be about 60 – 80% within the test time, this may vary depending on the age/size of the larvae but around 70 ‰ has commonly been used

Test proper:

- Cod larvae have been subjected to some treatment e.g. immune stimulus, temperature change, handling, change in the feeding regimes etc.
- Set up (at least) 2 x 3 100 ml beakers with sea water and 2 x 3 beakers with e.g. 70 ‰ sea water.
- Count 20 larvae from the control group into each of the sea water beakers and into each of the 70 ‰ beakers. Do the same with the treated larvae.
- Incubate for 1 h and then count the dead larvae.
- The higher the survival in 70 ‰ sea water the less stressed and better health status of the larvae.

41. Buffer preparations

Comprehensive description of various buffer preparations is published in Williams & Chase: Methods in Immunology and Immunochemistry Volume II, Appendix II, p. 365 – 408.

1. For various columns and FPLC separations.

All buffers are filtered and deaerated before use. Azide is only used for storage or if the running buffer is kept on the column for some length of time (3 – 4 days)

For washing and storing (applies to most columns):

20% ethanol: 100 ml ethanol + 400 ml H_2O or

0.02% azide: 500 ml H_2O + 1 ml 10% sodium azide (NaN_3)

Superose 6 and Superose 12 columns

0.1M Tris-HCl, pH 8.0, 0.15M NaCl (with or without 0.01% azide):

Tris 12.1 g

NaCl 8.5 g in 500 ml H_2O,

pH adjusted to 8.0 with 5N HCl

(add 1 ml of 10% NaN_3 if used)

adjust the final volume to 1000 ml with H_2O.

MonoQ column

Basic buffer, 20 mM Tris-HCl, pH 7.5 (0.01% azide if used):

Tris 2.42 g in 500 ml H_2O

pH adjusted to 8.0 with 5N HCl

(add 1 ml of 10% NaN_3 if used)

adjust the final volume to 1000 ml with H_2O

Final elution buffer: 1M NaCl in basic buffer (i.e. elution from 0 – 1M NaCl)

NaCl 5.84 g in 100 ml of basic buffer

Basic buffer for IgM analysis: 0.15M NaCl in basic buffer (i.e. elution from 0.15M – 1M NaCl)

NaCl 4.4 g in 500 ml basic buffer

Protein G column

Basic buffer, 20 mM sodium phosphate buffer, pH 7.0 (0.02% azide if used)

For stock phosphate buffer solution and relevant pH and molarity see table below

Mix 19.5 ml of stock A and 30.5 ml stock B

(add 0.5 ml 10% azide if used)

Adjust volume to 500 ml with H_2O

Elution buffer, 0.1M glycine-HCl

Glycine 3.75 g in 250 ml H_2O.

Adjust pH to 2.2 – 2.4 with 1N HCl

Adjust volume to 500 with H_2O.

Neutralizing 1M Tris, approx. 60µl of this solution will neutralize 1 ml of the elution buffer

Tris 1.21 g in 10 ml H_2O

CM AffiGel Blue column

Stock solution A: 0.5M KH_2PO_4 (68.04 g/L)

Stock solution B: 0.5 M K_2HPO_4 (114.1 g/L)

Equilibrium buffer: 10 mM potassium (K) phosphate buffer, pH 7.25, 0.15M NaCl

Stock solution A: 5.2 ml + stock solution B: 14.8 ml + 8.5 g NaCl in 1L H_2O

Elution buffers:

For gradient elution: 1.5 M NaCl in equilibrium buffer buffer (43.8 g NaCl in 500 ml)

For complete elution: 2M guanidine-HCl in H_2O (95.53 g guandine-HCl in 500 ml)

2. Na - phosphate buffers

0.1M NaH_2PO_4 / Na_2HPO_4 phosphate buffers, pH 5.7 – 8.0

Solution A: 0.2M NaH_2PO_4 (27.6 g/L of $NaH_2PO_4 H_2O$)

Solution B: 0.2M Na_2HPO_4 (53.65 g/L of Na_2HPO_4 $7H_2O$ or 71.64 g/L of Na_2HPO_4 $12H_2O$)

pH	Solution A ml/L*	Solution B ml/L*
5.7	467.5	32.5
5.8	460	40
5.9	450	50
6.0	438.5	61.5
6.1	425	75
6.2	407.5	92.5
6.3	387.5	112.5
6.4	367.5	132.5
6.5	342.5	157.5
6.6	312.5	187.5
6.7	282.5	217.5
6.8	255	245
6.9	225	275
7.0	195	305
7.1	165	335
7.2	140	360
7.3	115	385
7.4	95	405
7.5	80	420
7.6	65	435
7.7	52.5	447.5
7.8	42.5	457.5
7.9	35	465
8.0	26.5	473.5

*i.e. add 500 ml H_2O for 0.1 M buffer

3. PBS

1. Made from tablets from Sigma
2. 0.01M phosphate buffer, pH 7.0 + 8.5 g NaCl per L:
   - See table above: 19.5 ml Stock solution A + 30.5 ml Stock solution B and 8.5 g NaCl made up to 1L with H_2O
3. ELISA washing buffer, PBS-tween 20 hand made. 400 ml 5x PBS Keldur stock solution, 1600 ml H_2O, 1 ml tween 20:
   - Stock solution: 250g NaCl, 6.25g KCl, 89.5g $Na_2HPO_4 12H_2O$, 6.25g KH_2PO_4, in 5 l H_2O.

4. K - phosphate buffer

0.1M KH_2PO_4 / K_2HPO_4 0.1M phosphate buffers, pH 5.8 – 7.7 (more soluble at 4°C than the Na phosphate buffers

Solution A: 0.5M KH_2PO_4 (68.04 g/L) and Solution B: 0.5M K_2HPO_4 (87.09 g/L)

	0.1 M	
pH	Solution A ml/L*	Solution B ml/L*
5.8	184	16
5.9	180	20
6.0	175.4	24.6
6.1	170	30
6.2	163	37
6.3	155	45
6.4	147	53
6.5	137	63
6.6	125	75
6.7	113	87
6.8	102	98
6.9	90	110
7.0	78	122
7.1	66	134
7.2	56	144
7.3	46	154
7.4	38	162

7.5	32	168
7.6	26	174
7.7	21	179

*i.e. and add 800 ml H2O for 0.1M buffer

5. Acetate buffers

0.1M Acetate buffer, pH 3.6 – 5.6

Stock solution A: 0.2M acetic acid (11.55 ml glacial acetic acid/L) and Stock solution B: 0.2M sodium acetate (27.2 g/L of Na acetate $3H_2O$)

pH	Stock solution A ml/L*	Stock solution B ml/L*
3.6	436	37
3.8	440	60
4.0	410	90
4.2	368	132
4.4	305	195
4.6	255	245
4.8	200	300
5.0	148	352
5.2	105	395
5.4	88	412
5.5	68	432
5.6	48	542

*i.e. add 500 ml H_2O for 0.1M buffer