

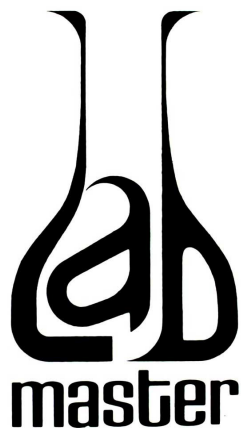
Labmaster TR-FIA tests:

1212-2001	Enterolactone kit
1212-2002	Equol kit
1212-2003	Genistein kit
1212-2004	Daidzein kit

Labmaster ELISA tests:

1212-1003	Anti-Gliadin IgG kit
1212-1004	Anti-Gliadin IgA kit
1212-1006	Troponin I kit

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LABMASTER

D I A G N O S T I C S

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1212-2002

Labmaster TR-FIA Research Reagents for the Measurement of

Equol

Reagents for 96 assays

For Research Use Only
Not for use in diagnostic procedures

LABMASTER TR-FIA RESEARCH REAGENTS

This set of TR-FIA (Time-Resolved FluoroImmunoAssay) research reagents has been put together for use in research, development and investigation work. Whilst there may have been preliminary clinical work carried out with these reagents, there are no clinical claims associated with this product. It is for the investigator to identify and establish the clinical utility of this product.

INTENDED USE

This kit is made for the measurements of the equol in heparin plasma and serum after sample treatment according to the instructions.

SUMMARY AND EXPLANATION OF THE ASSAY

Equol (*4',7-dihydroxyisoflavan*) is metabolite produced by intestinal bacteria from the isoflavone daidzein, which occur in soy beans. Recently it was shown that high excretion of equol is associated with a significant reduction in breast cancer risk. Equol showed to have a higher anti-carcinogenic potency, in vitro and in vivo, than daidzein itself.

Until now available methods for the detection and quantification of isoflavones in human biological fluids and in food samples have been based on gas-liquid chromatography (GC) or high performance liquid chromatography (HPLC). These methods are not only expensive and time-consuming but also not sensitive enough for the assay of phytoestrogens in small plasma samples. Radioimmunological methods for formononetin, daidzein and genistein have been published. Labmaster Research Reagents for the measurement of equol are based on time-resolved fluoroimmunoassay (TR-FIA) and they combine the advantages of non-radioisotopic assay with 10-100-fold increase in sensitivity.

PRINCIPLES OF THE ASSAY

The Labmaster Research Reagents for the measurement of equol are presented as a time-resolved fluorometric assay based on competition. Goat anti-rabbit IgG immobilised to the walls of low fluorescence microtiter plate will bind the anti-equol antibody. Europium-labelled equol and sample equol will compete for this antibody.

Enhancement solution dissociates europium ions from the labelled equol into solution, where they form highly fluorescent chelates with components in the enhancement solution. The fluorescence from each sample is inversely proportional to the concentration of equol in the sample.

RESEARCH REAGENTS CONTENTS

Each Equol package contains reagents for 96 assays.

Reagents

Component	Quantity
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Antibody-coated Microtitration Strips 8 x 12 wells coated with goat anti-rabbit IgG.	1 plate
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Store at +2- +8°C until expiry date stated on pack label.
Make sure that the plastic tray pack remains sealed prior to use.

Equol Standard	1 vial
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The vial contains lyophilised equol. Store at +2 - +8°C.

Equol-Eu tracer	1 vial
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The vial contains lyophilised equol Eu tracer. Store at +2- +8°C.

Anti-Equol antibody	1 vial
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The vial contains lyophilised anti-equol antibody. Store at +2- +8°C.

Assay Buffer	1 bottle	50 mL
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Ready for use Tris-HCl buffered (pH 7.8) salt solution with bovine serum albumin, bovine globulin, Tween 40, an inert red dye, and 0.1 % sodium azide as preservative. Store at +2-+8°C until expiry date stated on vial label.

Wash Concentrate (25X)	1 bottle	40 mL
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A 25-fold concentration of Tris-HCl buffered (pH 7.8) salt solution with Tween 20 and *Germall II as preservative. Wash solution is prepared by diluting wash concentrate 25-fold (i.e. 40 mL concentrate diluted to 1 litre) with distilled water. Store at +2-+8°C until expiry date stated on vial label.

Enhancement Solution	1 bottle	50 mL
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Ready for use with Triton X-100, acetic acid and chelators. Store at +2- +25 °C until expiry date. Direct light avoided.

* Germall is a registered trademark of Sutton Laboratories Inc., and Triton is a registered trademark of the Rohm and Haas Co.

MATERIALS REQUIRED BUT NOT SUPPLIED WITH THESE REAGENTS

Equipment

1. Time-resolved fluorometer
2. Automatic washer (e.g. DELFIA® Platemash prod. no. 1296-024 or 1296-026)
3. Automatic shaker (e.g. DELFIA® Plateshake prod. no. 1296-001 or 1296-003)
4. Pipettes for dispensing buffer, the diluted tracer solution and the diluted antibody solution (e.g. Eppendorf Multipette prod. no. 1296-014 with 5mL Combitips prod. no. 1296-016, or alternatively DELFIA® Plate Dispense with the DELFIA® Dispense Unit prod. nos. 1296-041 and 1296-043).
5. Pipette for dispensing the Enhancement Solution (e.g. Eppendorf Multipette prod. no. 1296-014 with 5 mL Combitips prod. no. 1296-016, or alternatively the DELFIA® Plate Dispense prod. no. 1296-041)

Reagents for sample treatment

1. β -glucuronidase (e.g. Boehringer Mannheim cat.no. 1585665)
2. Sulfatase (e.g. Sigma cat. no. S9626)
3. Diethyl ether
4. Acetate

In addition to the Labmaster equol kit the following are required:

- precision pipettes for dispensing microlitre volumes
- pipettes for dispensing the millilitre volumes of Assay Buffer required to prepare the tracer and antibody dilutions
- distilled water

SPECIMEN COLLECTION AND HANDLING

Collect the blood by venipuncture, allow clotting and separate the serum by centrifugation. Heparin plasma can be used. The samples should be frozen and stored at $-20\text{ }^{\circ}\text{C}$ until analysed.

* DELFIA is a registered trademark of Wallac Oy

For time-resolved fluorescence the plasma equol glucuronides and sulfates are hydrolysed and the equol is further extracted. For the hydrolysis 200 μL of acetate buffer 0.1 M, pH 5.0, containing 0.2 U/mL of β -glucuronidase and 2 U/mL of sulfatase is added to tubes containing 200 μL of plasma. After mixing, the samples are incubated o/n at $+37\text{ }^{\circ}\text{C}$. The following day the free-equol is extracted with 1.5 mL diethyl ether by careful mixing of two phases for 3 minutes. The water phase is frozen in dry ice-ethanol mixture, and the ether phase is transferred into a disposable glass tube. After thawing, the water phase is re-extracted with ether, and the ether phases are combined and evaporated to dryness at $+45\text{ }^{\circ}\text{C}$ water bath. Assay buffer (200 μL) is added to the tubes, and after careful mixing 20 μL of the solution corresponding to 20 μL of the original plasma sample is taken for TR-FIA.

If recovery calculations are made, 20 μL of (6,7- ^3H) estradiol-17 β -glucuronide (for example NEN™ Life Science Products) is added to each tube as hydrolysis takes place. After reconstituting the evaporated sample, 20 μL will be taken for liquid scintillation counting. Based on the results the final values are corrected for losses during the hydrolysis and extraction. Recovery = CPM after extraction / CPM added. If such equipment is not available, the recovery can be estimated as 80 %.

WARNINGS AND PRECAUTIONS

For research use only.

Not for use in diagnostic procedures.

Handle all specimens as potentially infectious. Please refer to the U.S. Department of Health and Human Services (Bethesda, Md., USA) publication No. (CDC) 88-8395 on laboratory safety procedures or any other local or national regulation.

Reagents contain **sodium azide** (NaN_3) as a preservative. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.

Disposal of all waste should be in accordance with local regulations.

ASSAY PROCEDURE

Perform each determination in duplicate for both standards and unknowns. A standard curve should be run with each assay. All reagents and samples must be brought to room temperature (+20 to +25°C) before use.

1. Preparation of reagents

Wash solution

Pour 40 mL of Wash Concentrate into a clean container and dilute 25-fold by adding 960 mL of distilled water to give a buffered wash solution (pH 7.8).

Stays stable 2 weeks at +2 - +25°C in a sealed container.

Equol Standards

Reconstitute the lyophilised sample by adding 500 µL of distilled water (⇒330 nmol/L). Mix gently and allow to stand for at least 30 min before use.

If some stock solution is left, store at +4 °C.

Preparation of the standard dilutions:

Prepare within one hour of use.

STD.	FINAL CONCENTRATION (nmol/l)	PIPETTE THE PREVIOUS DILUTION:	PIPETTE ASSAY BUFFER
A	330	500µL	-
B	132	120 µL of 330 nmol/L-dil.	180µl
C	33	75 µL of 132 nmol/L-dil.	225µl
D	13.2	120 µL of 33 nmol/L-dil.	180µl
E	3.3	75 µL of 13.2 nmol/L-dil.	225µl
F	0.66	60 µL of 3.3 nmol/L-dil.	240µl
G	0	-	200µl

Anti-equol antibody

Reconstitute lyophilised anti-equol antibody by adding 500 µL of distilled water. Mix antibody stock solution gently and allow to stand for at least 30 min before use. If some stock solution is left, store at +4 °C.

Prepare the needed volume of anti-equol antibody by mixing antibody stock solution with Assay Buffer 1:30. The working solution is to be used within one day.

Equol-Eu tracer solution

Reconstitute the lyophilised Eu-labelled equol by adding 500 µL of distilled water. Mix tracer stock solution gently and allow to stand for at least 30 min before use. If some stock solution is left, store at +4 °C.

Prepare the needed volume of tracer solution by mixing tracer stock solution with Assay Buffer 1:30. The working solution is to be used within one day.

It is important that the assay buffer does not come into contact with tracer stock solution not intended for immediate use.

We advise the use of a disposable plastic container to prepare the tracer working solution.

- Transfer the required number of microtitration strips to a strip frame (Return the remaining strips to the plastic tray pack and reseal.) and pre-wash them (x 1) with platewasher.
- Pipette 20 µL of diluted standard or sample into the appropriate antibody-coated wells as duplicates.
- Pipette 100 µL of diluted Anti-equol antibody into each well.
- Pipette 100 µl of diluted equol – Eu tracer solution to each well using the recommended Eppendorf Multipipette after discarding the first aliquot, or use the DELFIA® Dispense Unit. Avoid carry-over by holding the pipette tip slightly above the top of the well and avoid touching the plastic strip or the surface of the liquid.
- Shake for 90 minutes at room temperature with slow shaking using plateshaker.
- After the incubation step, wash each strip with the platewasher 4 times.
- Add 200 µl of Enhancement Solution directly from the reagent bottle to each well using the recommended Eppendorf Multipipette after flushing the Combitip once with Enhancement Solution, or use the DELFIA® Plate Dispense. Refill the

Combitip and discard the first aliquot. Avoid touching the edge of the well or its contents.

- Shake the frame for 5 minutes at RT. The fluorescence is stable for several hours if evaporation is prevented. However we recommend measurement within 1 hour as external factors may cause a decrease in signal with time, although it is extremely rare.
- Measure the fluorescence (e.g. by Victor² 1420 Multilabel Counter with the time-resolved fluorometry Parameters). Create a program as follows for automatic measurement and result calculation including recovery and dilution information:

Corrected concentration = $\text{CONC} / 0.8 (\text{recovery}) \times \text{DILUTION FACTOR}$

ASSAY TYPE:	FIA
FITTING METHOD:	SPLINESMOOTHED
X-AXIS:	LOGARITHMIC
Y-AXIS:	B/B _{max}
STANDARDS:	7
STANDARD REPLICATES:	2
STANDARD CONC	A
STANDARD CONC	B
STANDARD CONC	C
STANDARD CONC	D
STANDARD CONC	E
STANDARD CONC	F
STANDARD CONC	G
UNKNOWN REPLICATES	2
OUTPUT	

C_CONC = $\text{CONC} / 0.8$: (include C_CONC also in the Display, Printer and Results rows)

PROCEDURAL NOTES

- A thorough understanding of this package insert is necessary for successful use of these research reagents.
- Reagents should be allowed to reach room temperature (+20 to +25°C) prior to sample preparation.
- When washing the strips, ensure that each well is filled up completely to the top edge of the well. After washing the strips, check that the wells are dry. If there is moisture left, invert the plate and tap firmly against absorbent paper.

For detailed information on the cleaning and maintenance of the washing device, please refer to the manual of the platewasher.
- The avoidance of europium contamination and resulting high fluorescent background demands high standard pipetting and washing techniques. Thus it is extremely important to use the pipettes supplied with the DELFIA[®] system for the recommended purposes only.

The Enhancement Solution should be dispensed using only the recommended Eppendorf Multipette after the Combitip has first been flushed with Enhancement Solution according to the Directions for Use. The same Combitip **must not be** used for pipetting any other reagent. After use place the Eppendorf Multipette on the pipette stand, with the Combitip still attached.

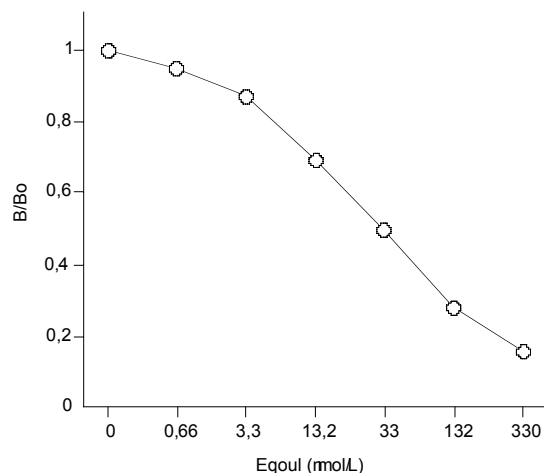
When using the DELFIA[®] Plate Dispense and DELFIA[®] Dispense Unit, please refer to the manual.

CALCULATION OF RESULTS

The DELFIA® system incorporates programs for data reduction, and the results are obtained as printouts of standard curves, unknown concentrations etc. (see Fluorometer instrument manual or MultiCalc™ manual for detailed information).

PERFORMANCE CHARACTERISTICS

A standard curve is shown below. Typically CV % is below 10 % over the standard curve range.



WARRANTY

Purchase of the research reagents gives the purchaser the right to use this material in his own research, development and investigational work. They are not to be administrated to humans or used for medical diagnostics. Labmaster Ltd. reserves the right to discontinue or refuse orders to any customer who plans to use these products for any other purposes. Products to be used for other purposes than research, development and investigation are only under licence from Labmaster Ltd. Purchase of this product implies agreement with these conditions of sale.

Labmaster Ltd. does not warrant or guarantee that its products are merchantable or satisfactory for any particular purpose, nor free from any claim of foreign or domestic patent infringement by a third part, and there are no warranties, expressed or implied, to such effect. Labmaster Ltd. will not be liable for any incidental, consequential or contingent damages involving their use.

All information supplied with the research reagents and technical assistance given is believed to be accurate, but it remains the responsibility of the investigator to confirm all technical aspects of the applications. We appreciate receiving any additions, corrections, or updates to information supplied to the customer.

REFERENCES

1. Soini, E. and Kojola, H. (1983): Time-resolved fluorometer for lanthanide chelates – A new generation of nonisotopic immunoassays. Clin. Chem. 29, 65-68.
2. Hemmilä, I., Dakabu, S., Mukkala, V-M., Siitari, H. and Lövgren, T. (1984): Europium as a label in time-resolved immunofluorometric assays. Anal. Biochem. 137, 335-343.

3. Lövgren, T., Hemmilä, I., Pettersson, K. and Halonen, P. (1985): Time-resolved fluorometry in immunoassays. In *Alternative Immunoassays*. Ed. W.P.Collins. John Wiley & Sons Ltd, England, pp. 203-217.
4. Mukkala, V-M., Mikola, H. and Hemmilä, I. (1989) *Anal. Biochem.* 176, 319-325.
5. Mikola, H. and Miettinen, P. (1991) *Steroids* 56, 17-21.
6. Hemmilä, I. A. (1991) Applications of fluorescence in immunoassay. In *Chemical Analysis* (Winefordner, J. D. and Kolthoff, I. M., Eds.) Wiley & Sons Inc., New York.

PATENTS

This test system is covered by the following patents:

Europe (Austria, Belgium, Italy, Switzerland, Holland, UK, France): 0064484, 0139675

Federal Republic of Germany: P32722605-08, P3462252.7

Sweden: 8102753-4

USA: 4,565,790, 4,808,541

Last revision April 1999

SAMPLE TREATMENT FOR LABMASTER EQUOL MEASUREMENTS

Collect the blood and separate the serum.



Add 200 μ L of acetate buffer 0.1 M, pH 5.0, containing 0.2 U/mL of β -glucuronidase and 2 U/mL of sulfatase to tubes containing 200 μ L of plasma.



Incubate o/n at + 37 °C.



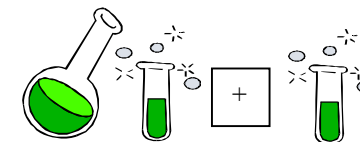
Extract the free-equol with 1.5 mL of diethyl ether by careful mixing for 3 minutes.



Freeze the water phase and transfer the ether phase into a disposable glass tube.



Re-extract the water phase and combine the ether phases. Evaporate to dryness at + 45 °C water bath.









Add 200 μ L of Assay Buffer to the tubes to achieve a concentration corresponding to the original equol concentration in plasma. Take 20 μ L of the solution for TR-FIA.





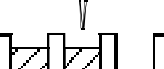
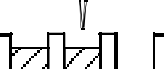



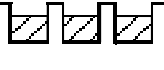
Labmaster Equol kit

Summary Protocol Sheet

ASSAY PREPARATION

Reconstitute standards		0.5 mL distilled water, 30 min.			
Dilute standards		Look for the assay procedure in page 6			
Reconstitute equol –Eu tracer solution		0.5 mL distilled water, 30 min			
Reconstitute anti-equol antibody		0.5 mL distilled water, 30 min			
Dilute anti-equol antibody and equol –Eu tracer solution (see table)	 	Strips	Anti- Equol antibo- dy (µL)	Tracer stock solution (µL)	Buffer (µL)
		1	120	120	3480
		2	150	150	4350
		3	200	200	5800
		4	240	240	6960
		5	270	270	7830
		6	310	310	8990
		7	345	345	10005
		8	380	380	11020

ASSAY PROCEDURE

Pre-wash		x 1
Add standards and unknowns		20 µL
Add anti-equol antibody dilution		100 µL
Add equol –Eu tracer dilution		100 µL
Incubate		90 min slow shaking at RT
Wash		x 4
Enhance		200 µL
Incubate		5 min slow shaking at RT
Count	