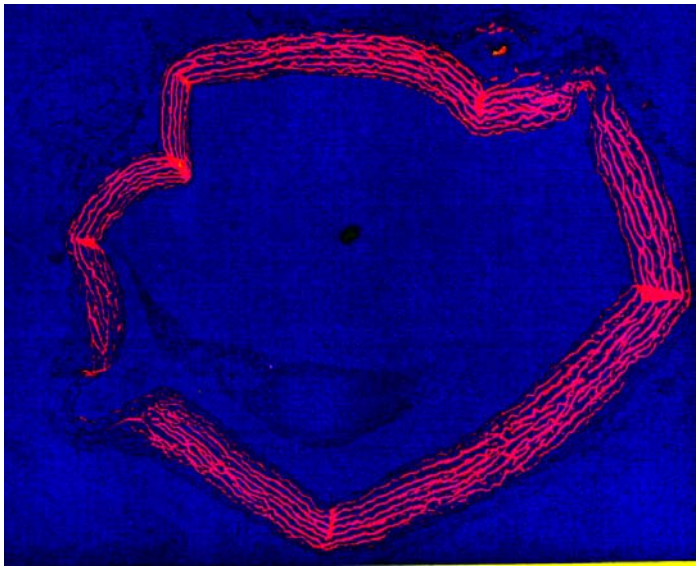
# *Fastin* ™

# ELASTIN

## Assay



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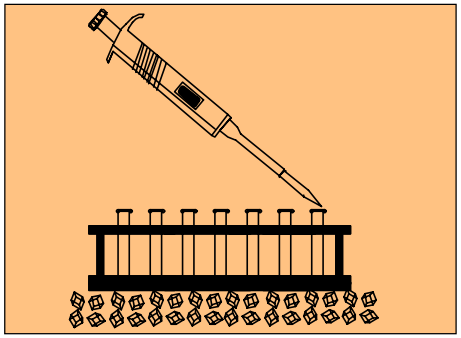
# *Fastin*

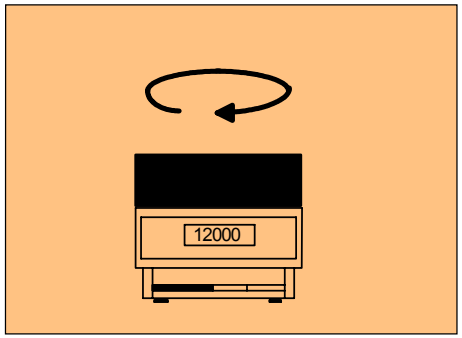
# ELASTIN

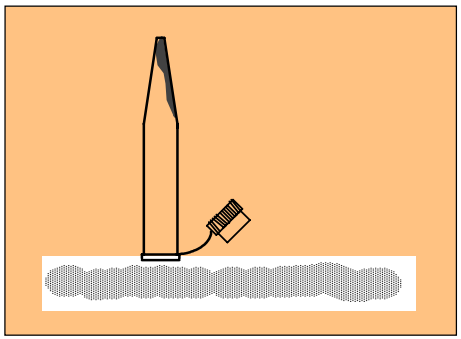
# *Assay*

**Assay Time:** **5 hours**

**Detection Limit:** **5 ug ***





## Set up assay:

Label a set of 1.5 ml microcentrifuge tubes. If sufficient material is available run duplicate samples.

Prepare;
[1] Reagent blanks; 100 ul of test sample buffer/salt/water solution, (pH ~7).

[2] a-elastin standard aliquots, (suggested 12.5, 25.0 and 50 ul volumes).

[3] Test samples, (oxalic acid extracts should be neutralised, prior to analysis).
Select aliquot volumes between 10 & 100 ul.

To all tubes add: 1.0 ml Elastin Precipitating Reagent, (stored cold, use cold).
Cap tubes and invert to mix contents.

## Elastin **COLD** precipitation:*

Place all tubes into an ice-water container and place into refrigerator, leave overnight.

## Recovery of cold precipitated elastin:

Centrifuge tubes, while still cold, at >10000 x g for 10 minute period.

Drain tubes and then, still inverted, remove any remaining fluid from lip of tubes by placing on an absorbent paper towel.

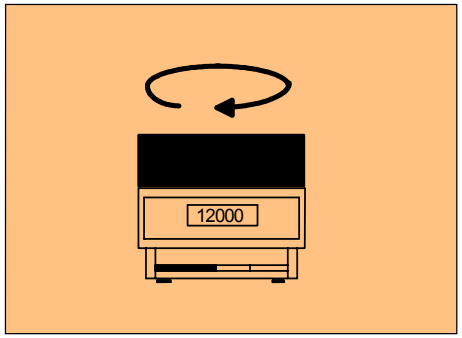
The elastin has a translucent gel like appearance, (see photo on outside back cover).

The isolated elastin, and the subsequent assay stages can now be performed at room temperature.

## Formation of elastin-dye complex:

To all tubes add 1.0 ml of Fastin Dye Reagent AND 100 ul 90% saturated ammonium sulfate, (within vials of Fastin Assay Kit).

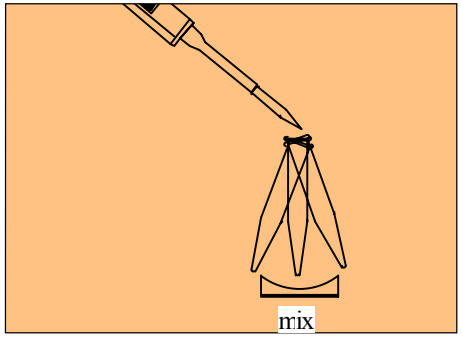
Cap tubes and mix contents by inverting. Using gentle mechanical agitation, allow 1 hour for the formation of the elastin-dye complex.
[When mechanical agitation is not available mix contents manually every 10 to 15 minutes].




## Recovery of the elastin-dye complex:

Centrifuge all tubes at >10000 x g for 10 minute period.

Drain tubes and then still inverted remove any remaining fluid from lip of tubes by placing on an absorbent paper towel.




## Release of elastin bound dye:

To all tubes add 1.0 ml of Fastin Dissociation Reagent. Cap tubes and release the dye into solution, using a vortex mixer to dislodge the pellets.

Mix gently for ~ 10 minutes so as to ensure full dye release.

Traces of insoluble ammonium sulfate, from an earlier step in the assay can cause slight turbidity. It is recommended that the tubes are briefly centrifuged.

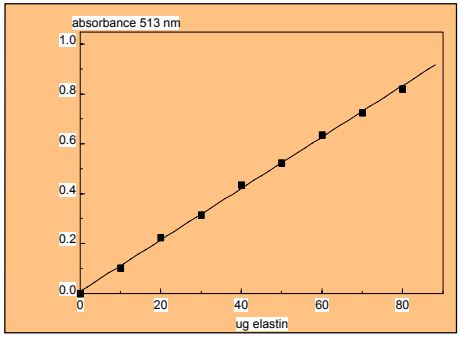
Avoid disturbing while pipetting tube contents into semi-micro cuvettes.

## Elastin measurement:

[1] Spectrophotometer; set wavelength at 513 nm. Using semi-micro, (1.0-1.5 ml capacity cuvettes).

[2] Colorimeter, select a blue-green filter * with semi-micro, (1.0-1.5 ml), cuvettes or tubes.

[3] Microplate Reader. Use wavelength or filter setting as in [1] or [2]. Transfer 250 ul aliquots to individual wells, (96 well plates *)




Prepare a standard curve, using the standard absorbances and known concentrations.

Plot elastin contents of test samples *.

Note: Where * occurs, see Fastin Manual for more detailed information.

# *Fastin* ™

# ELASTIN

# *Assay*

## TECHNICAL INFORMATION

### Contents

**The Fastin Assay has been designed**
**for**
***in-vitro* research work**
**only**

**Handle the Fastin Assay Kit**
**using**

**GOOD LABORATORY PRACTICE**

**Read Manual before use**

# Fastin Manual



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**Fastin**
**Elastin Assay**
**Manual**

**Intended Applications:**

The Fastin Elastin Assay is a quantitative dye-binding method for the analysis of elastins extracted from biological materials and of elastin released into cell culture medium..

The dye label employed is 5,10,15,20-tetraphenyl-21, 23-porphine sulfonate, (TPPS). For the structural form of the dye see Fig. 1, page 6.

**Test sample material:**

Tissue extracts, cell culture medium and protein chromatography fractions.
Elastinolytic enzymes recovered from cell extracts and tissue.

Elastin forms that can be measured by the Fastin Assay;
[i] soluble tropoelastins.
[ii] lathyrogenic elastins
[iii] insoluble elastins, as solubilized elastin polypeptides, [alpha-elastin; kappa-elastin]

It is also possible to use the assay to detect and/or measure;
[iv] elastase activity, using elastin as the enzyme substrate.

All mammalian elastins, so far examined, can be assayed. The dye reagent binds to the 'basic' and 'non-polar' amino acid sequences found in mammalian elastins.

Due to the difficultly of obtaining sufficient quantities of tropoelastin the assay development was carried out using a-elastin.

**Test sample quantities:**

A sample volume of between 5 and 100ul is required, containing not less than 5ug and not more than 75ug elastin.

Samples with elastin of >75ug/10ul should be diluted with water, dilute buffer or salt solution.

Samples with elastin of <5ug/100ul should be concentrated by freeze-drying or with the aid of an ultrafiltration membrane.

**Test sample composition:**

For analysis of soluble elastin, samplse should be free of any particulate material, (cell debris, insoluble extracellular matrix material). The presence of other soluble proteins or of complex carbohydrates, including collagens and proteoglycans does not interfere with the Fastin Assay.

Test solutions must have a pH between pH 6.0 and pH 8.0.
If the pH is outside this range, adjust pH using acetic acid or NaOH.

Test samples obtained by extracting elastin from tissues with oxalic acid, (see page 13), should be adjusted to ~ pH 7.0, using 1.0 M NaOH.

Cell culture medium, with foetal calf serum supplements of up to 5%, do not interfere with the Fastin Assay. When higher serum supplements have been used, the increasing bulk of serum proteins, relative to the microgram amounts of elastin present, can cause elastin-dye contact problems.

In samples where serum levels are high two options are available;

[i] reduce the serum supplement to 5%, either after cell attachment has occurred, or reduce the serum supplement in the medium that will be collected and used for assay.

[ii] selectively remove the major serum protein, albumin, from the test samples by using dye-affinity chromatography, (Blue-Sepharose CL-6B : Pharmacia Biotechnology)

**Fastin Assay Kit components:**

[1] The dye reagent contains 5,10,15,20-tetraphenyl-21,23-porphine sulfonate, (TPPS) in a citrate-phosphate buffer, also containing surfactants and anti-microbial agents.
The reagent has been formulated for specific binding to elastin under the assay conditions.
[2] Elastin precipitating reagent, contains trichloroacetic acid and arginine.
[3] Elastin standard is a high molecular weight fraction of a-elastin prepared from bovine neck ligament elastin.
The a-elastin standard is supplied as a sterile solution at a concentration of 1mg/ml.
[4] Elastin-dye complexing reagent is a 90% saturated solution of ammonium sulfate, supplied as a sterile solution.
[5] Fastin Dissociation reagent is prepared from guanidine HCl and propan-1-ol

**Recommended storage conditions for Assay Kit components:**

**Unopened;** All of the reagents have long term stability, (at least 6 months), when stored at room temperature.
Do not freeze as complete resolubilisation may not occur on thawing.
Avoid prolonged exposure to direct sunlight.

**Notes regarding the storage of OPENED Fastin Assay Reagents:**

***Reference Standard:*** When stored at $+4^0$C the a-elastin standard is a clear transparent solution. On holding at room temperature the solution may be observed to become opalescence. This is due to the characteristic coacervation property of soluble elastin. On cooling, the process is reversible and the elastin solution again becomes transparent.

As coacervation is a two-phase system, errors in sampling could occur if used in this form, *therefore*, always take sample aliquots from cold solutions, (both for standards and test materials).

The metal seal should not be removed from the vial, the contents can be sampled as follows:

[1] Remove the centre metal disc only from the vial top.

[2] Obtain aliquots from the vial by using a syringe fitted with a sterile hypodermic needle. The butyl rubber seal on the vial has a thin centre disc.

The a-elastin standard should be discarded if the solution contents develop turbidity.

***Fastin Dye Reagent::*** The pH of this reagent is pH 7.0 and the dye label, TPPS, is probably a carbon source for some forms of microbial organisms . Microbial contamination can be accidentally introduced to the Dye Reagent from using non-sterile pipette tips, or when unused aliquots of reagent are returned to the bottle.

To limit microbial growth, inhibitors have been added to the reagent. These agents; bromopol and sorbic acid are compatible with the Fastin Assay; but are not 'universal' microbial inhibitors. Good laboratory practice and the storage of an opened Fastin Dye Reagent bottle at $4^0$C can extend the shelf life of the reagent, DO NOT FREEZE.

The Fastin Dye Reagent active component is TPPS. In opened bottles this compound will gradually oxidise as the air space inside the bottle increases during usage. This does not alter elastin binding.. The oxidised form of TPPS, however, is less soluble and this is observed in higher 'Reagent Blank' values. This can be corrected, if desired, by filtering the Fastin Dye Reagent before use, so as to remove any insoluble material, (a general lab grade filter paper is suitable). This should not be necessary within three months of opening the reagent. The loss of soluble TPPS does not alter the linear Standard Curve. In old, repeated filtered, reagent a calibration check can be run to confirm that TPPS has not become substrate limiting at the higher elastin concentrations.

**Other components required, but not supplied:**

Capped conical, 1.5ml capacity microcentrifuge tubes and variable volume micropipettors with matching pipette tips.

A mechanical mixer for the microcentrifuge tubes. Any equipment that provides consistent shaking, rolling or rotation of the tubes is suitable.

A centrifuge, fitted with a 1.5ml microtube rotor head and capable of at least 10000 x g; so as to permit firm packing of the elastin-dye pellet.

A spectrophotometer, colorimeter or a microplate reader with suitable colour filters, (absorbance peaks at; 513 nm and 405 nm).

**Mode of action of the Fastin dye reagent with elastins:**

The Fastin Dye Reagent contains a synthetic porphrin; 5,10,15,20-tetraphenyl-21,23-porphrine in a water soluble form as the sulfonate. The majority of the TPPS molecules contain three or four sulfate groups.

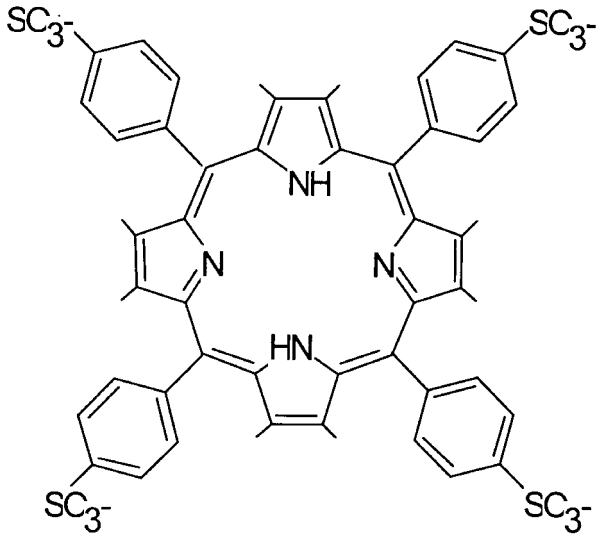


*Fig. 1. The structural form of 5,10,15,20-tetraphenyl-21,23-porphrine, tetrasulfonate*

The affinity of TPPS for elastin was first observed when used as a 'vital stain' on live animals. Most tissue tissues initially took up the dye, but with time only elastin retained the TPPS molecules. [Winkelman, J. (1962), Cancer Res. **22,** 589-596; Winkelman, J & Spicer, S.(1962), Stain Technol. **37,** 303-305].

The mode of action of TPPS with elastin remains uncertain. It may be due to shape-and-fit with the acidic dye being firmly retained by the basic amino acid side chain residues of elastin.

At pH 7.0 and $20^0$ C, the TPPS has been reported to occur as a dimer producing an out-of-plane type conformational change, [Schneider, W. (1975) Struct. Bond. **23**, 123].

**Set up assay:**

To duplicate labelled 1.5ml capacity conical microcentrifuge tubes are added aliquot volumes, between 5 and 100ul, of test samples, elastin standards and reagent blanks.

Adjust the fluid volume in all tubes to 100 ul, using water or with the buffer/salt solution as used for the test samples.

---

**Working standards:**

It is recommended that with each Fastin Assay Kit the elastin standard is initially run in duplicate at four concentrations; using 12.5, 25.0, 50.0 and 75.0ul aliquots.

The standards along with the reagent blank can then be used to produce a straight line calibration curve from a selected spectrophotometer, colorimeter or microplate reader.

In subsequent assay batches a minimum calibration requirement are duplicate 25 ul aliquots of the elastin standard and the reagent blank. This secondary standard should give absorption values to within $\pm$5% of that defined by the standard curve

When using a different measuring system, or following instrument servicing, a new standard curve should be prepared.

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**Test samples:**

With test samples where the approximate elastin concentrations is as yet unknown, initially try single 50ul aliquots for the first trial, ( also see notes at top of page 4).

If the absorbance readings, at 513nm, are found to be >1.5, take 100 or 200ul aliquots of the deep pink solution and dilute to 1000ul with dissociation reagent and again measure absorbance at 513nm..

If your initial sample trial aliquots produced absorbance values of <0.05 , at 513nm, after subtraction of the reagent blank value, the test sample contains less than 5ug elastin and will require concentration before being re-assayed.

A spin freezer concentrator (e.g. SpeedVac; Savant Instrument Inc.), can quickly dry 1ml of sample within a 1.5ml microcentrifuge tube, the sample can then be reconstituted in 5 to 25ul of water or buffer, (but be beware of drying samples containing excessive amounts of salts as these will also be concentrated, therefore prior desalting may also be required).

For reliable and accurate results all test samples should have their absorbance readings within the range of the Standards that were plotted on the calibration curve.

**START ASSAY:**

---

**Elastin isolation: [This is a Temperature Critical Step]**

The Precipitating Reagent has been developed to perform this fractionation; it should not be diluted with sample volumes greater than 1 to 10, or used at temperatures above $5^0$ C.

The reagent should be pre-cooled to $<5^0$C, (it is convenient to store this reagent in the refrigerator so that it is ready for use).

[1] To each tube add 1.0ml of the *cold* Fastin Precipitating Reagent..

[2] Tubes are capped and the contents mixed., then placed into an ice and water mixture, contained within a plastic box, and placed into a refrigerator overnight.

The following morning replacement ice should be added to the container and returned to the refrigerator for about 30 minutes. This will ensure that the tube contents are at $0^0$C, prior to centrifuging.

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**Recovery of elastin:**

[3] Following the precipitation of the elastin, the microcentrifuge tubes are centrifuged, *while the contents are still cold,* at >10000 x g for 10 minutes to pack the precipitated elastin, (a refrigerated centrifuge while useful is not essential; see [2]).

[4] Remove tubes from centrifuge, uncap and carefully invert and drain the liquid contents into a waste beaker. It is important to remove the acid solution from the tubes.

While inverted remove any remaining fluid from the top of the tubes by gently tapping the tube onto an absorbent paper towel. The tubes now contain only the precipitated elastin.

*This elastin is difficult to 'see' as it occurs as a weak translucent gel.. A photograph of this gel is shown on the outside back cover of this manual. To obtain this image required 1 mg of elastin, where a small pellet can just be seen through the wall of the white translucent microcentrifuge tube.*

---

***Subsequent assay steps can now be carried out at room temperature.***

**Reaction of the elastin with the Fastin dye:**

[5] To each tube add 1.0ml of the Fastin Dye Reagent; followed by 100ul of 90% saturated ammonium sulfate.

[6] Cap the tubes and use a vortex mixer to bring the elastin gel precipitate into solution with the dye reagent.

If high g forces were used during centrifugation, (>12000 x g), the precipitate will have been compacted and may require two or three short periods with the vortex mixer to bring the elastin into solution.

Low g forces, (<8000 x g), produce less well packed precipitates, but have the risk of causing partial loss of the precipitate during the removal of the supernatant).

[7] The elastin and the dye reagent are allowed to interact for 60 mins.

Gentle mechanical mixing is recommended during this period to ensure maximum elastin dye binding. Any form of mixer, to which the microcentrifuge tubes or rack can be attached is suitable. If no suitable mixer is available then mix the tube contents at ~10 min intervals either by manual inversion or by using the vortex mixer.

For optimum results the above conditions should be standardised.
Time periods of less than 1 hour for reacting the elastin with the TPPS are not recommended.

**Recovery of the elastin-dye complex::**

Following the dye binding step the elastin-dye complex formed becomes insoluble in the presence of the added ammonium sulfate.

[8] The elastin-dye complex is separated from the remaining soluble unbound dye by centrifuging the tubes, (>10000 x g for 10 minutes).

[9] The tubes are uncapped and the supernatants discarded. Any remaining fluid is removed by gentle tapping of the inverted tubes onto a paper towel.

Visual inspection should reveal a brown-red residue within the elastin standard tubes and, hopefully, also in the test sample tubes. The reagent blank tubes will also have a smaller deposit, *(see photograph on the outside back cover of this manual).*

***It is possible to stop the assay at this stage,* cap the tubes and store in the dark, *avoid allowing the residues to dry.***

**Release of the elastin bound dye:**

[10] To each tube add 1.0 ml Fastin Dissociation Reagent.

Cap the tubes and use a vortex mixer to bring the elastin bound dye into solution. Two short mixing periods are usually more effective than one long mixing period.

Traces of ammonium sulfate still present in the tubes may cause turbidity and this can be removed by centrifuging.

Tubes should not be uncapped until measurement of the absorbance. Dye colour is stable for several hours, but if reading are delayed store the tube rack in a light-proof box or cupboard.

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**Elastin measurement:**

The elastin content of the assayed samples is determined by the amount of bound dye that was released by the elastin.

The absorbance peak of TPPS occurs at 513nm and at 405 nm).

**[a]** ***Measurement using a spectrophotometer;***

Set wavelength to 513 nm. Adjust absorbance to zero using a semi-micro cuvette, (capacity 1.0 to 1.5ml), containing water.

Although the instrument can be set to zero using the reagent blank, it is usually better to determine the absorbance of the blank as a quality control check of the performance of the assay.

Duplicates; blanks, standards and test samples should not exceed $\pm$5% of their duplicate means. With practice the analyst can usually obtain this after processing one or two batches of assays.

When any set of duplicate test sample readings exceed $\pm$10% of their mean value the sample should be checked for clarity and re-assayed.

**[b]** ***Measurement using a colorimeter;***

Check the colour filter options that are available for the colorimeter, a blue green filter will probably be found to be suitable.

Initially also try the filters on either side of the selected filter, read the blank and standards with each of the three filters.

Plot standard curves for each of the filters and select the optimum curve based on that which give the highest absorbance readings of the standards *and also produced* a straight line fit for the standard readings.

**[c]** ***Measurement using a microwell plate reader, (colorimeter);***

The filter selection as described for a tube/curvette colorimeter also apply to the plate reader.
Aliquots, 100ul, of the Dissociation Reagent are added to the wells of a 96 well plate.

When elastin levels are low the amount of TPPS present in the 100ul aliquots can give unacceptably low absorbance readings when using some microwell plate readers.

In these cases, if a suitable filter is available, the aliquots can be read at 405 nm, some stronger coloured aliquots may require dilution with Fastin Dissociation Reagent.

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**Elastin concentration determination:**

The duplicate absorbance readings of the reagent blank and the three or more sets of elastin standards are used to produce a standard curve.

Subtract the reagent blank mean absorbance value from the absorbance means of each duplicate set of elastin standards.

Plot the elastin standard values; vertical axis 'Absorbance at 513 nm' and horizontal axis; 'Elastin concentration; ug'.

Obtain the best fit line through these plotted points.

The line should pass through zero, (0.0 absorbance, 0.0ug elastin).

Do not extend the line beyond the highest plotted point. All of the points should be within $\pm$5%, (Absorbance), of the fitted line.

The concentration of elastin present in the test samples can now be determined.

Take the mean of each duplicate test sample and subtract the reagent blank value.

The equivalent elastin concentration for the absorbance value obtained is then found on the standard curve.

The quantity of elastin in the aliquot taken for analysis is then multiplied to give the elastin concentration present either in 100ul or in 1ml of the test sample.

**Standard curve; obtained by measuring the amount of elastin bound dye.**

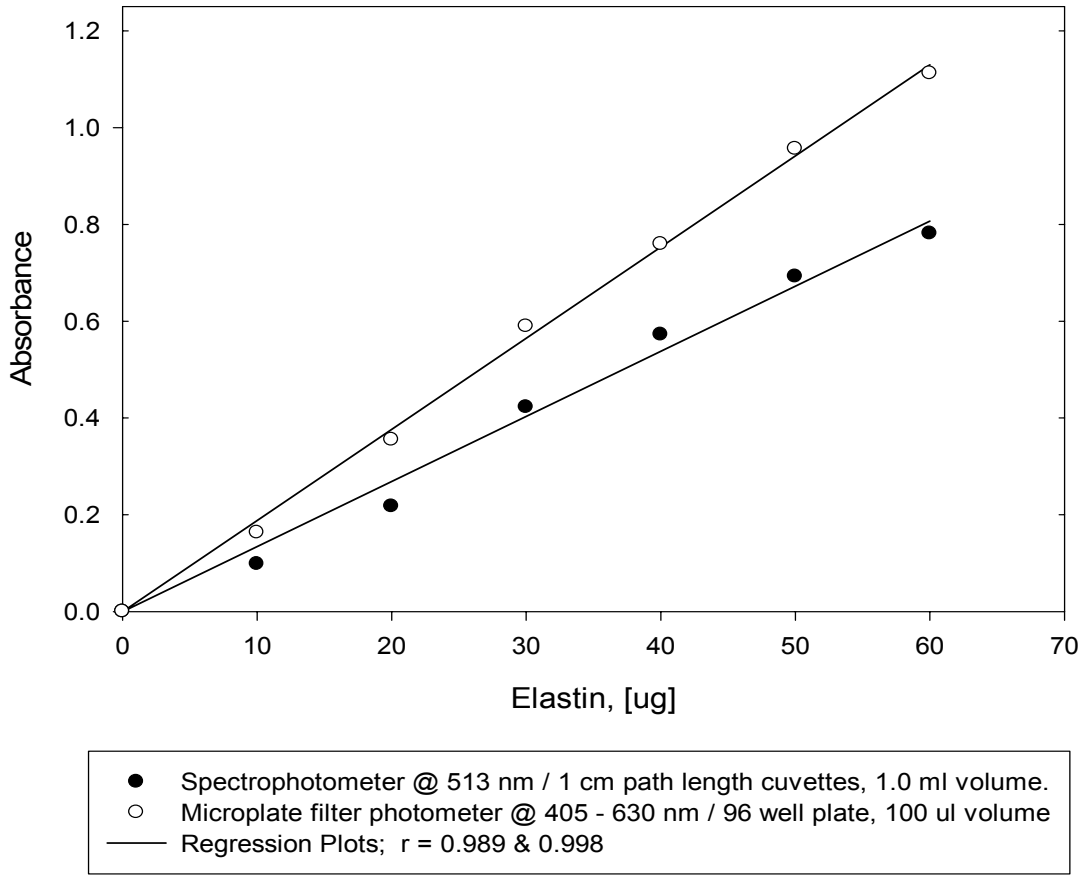


*Fig. 2. Elastin Standard Curve.*

**THE MEASUREMENT OF INSOLUBLE ELASTIN.**

The Fastin Elastin Assay can be used to measure insoluble, cross-linked elastin. The insoluble elastin is extracted from tissue in the form of high molecular weight cross-linked polypeptides.

The insoluble elastin is extracted in the form of a-elastin or k-elastin. After neutralisation, or removal, of the extraction acid or alkali, the samples can be assayed by the procedure previously described for soluble elastins, (pages 7 - 12).

**Conversion of insoluble elastin to soluble a-elastin**.

[a] The tissue samples are weighed, (decide if the elastin content is to be expressed as wet weight or as dry weight; ug elastin / mg tissue).
Place the weighed samples into glass centrifuge tubes, (~10 x 2 cm), and add ~20 volumes of 0.25M oxalic acid, (assuming tissue density is 1.00 $g/cm^3$).

[b] The tubes are then placed into a boiling water-bath, (or a metal heating block, with the electrical thermostat set at $95^0$ C), for 60 mins.

[c] Remove the tubes from heat and cool quickly to room temperature. Centrifuge, at low speed, ~3000 rpm for 10 mins. Pipette off the liquid, and retain this extract in labelled containers.

[d] To the residual tissue in the tubes add a further 20 volumes of 0.25M oxalic acid and again heat for 60 mins. Up to four heat extractions should be initially employed to ensure complete solubilisation of the tissue elastin.

[e] Some tissue material, such as from foetal or immature animals, can be fully solubilised after one or two extractions. Tissue from mature or old animals, including humans may require up to four extractions.

[f] Initially when using new test material retain each of the oxalic acid extracts separately and analyse each for elastin to establish that elastin extraction was quantitative. The last extract should contain no elastin. Elastin extracted from mature tissue will often produce yellow coloured extracts.

[g] The individual extracts from each tissue sample are pooled and placed in either dialysis tubing or in pressure filtration cells, fitted with low molecular weight cut-off membranes, (~15000 MW).

[h] For rapid processing, aliquots of pooled extracts can be quickly cleared of acid and concentrated by using a microcentrifuge tube fitted with a filter membrane and centrifuging at 10000 x g..

[i] The oxalic acid free extract contains the tissue elastin in the form of a-elastin which has an average molecular weight of ~80000 MW. The extract can now be assayed by the procedure described for soluble elastins. Retain a record of the extract volumes so as to permit calculation of the tissue elastin content.

Some of the elastin extracted by oxalic acid will be < 5000 MW. This form of solublised elastin is lost during removal of the oxalic acid.

**Conversion of insoluble elastin to soluble k-elastin.**

In this method, insoluble elastin is solubilised using ethanolic potassium hydroxide, (1.0M KOH in 80% ethanol).

The elastin solubilisation reaction is carried out at $37^0$ C for three or more one hour periods. The excess alkali in the pooled extracts is then neutralised by titration using concentrated perchloric acid and the alcohol removed by distillation, (using a rotary glass evaporator, under reduced pressure).

The solubilised elastin recovered has been termed Kappa-elastin, (k-elastin). This high molecular weight fraction is composed of elastin polypeptides that have 'similar' properties to that of a-elastin.

Further details on how to prepared k-elastin can be found in the following reference sources;

[a] Kornfeld-Poullain, N & Robert, L. (1968) *Bull. Soc. Chim. Biol.*, **50**, 759.

[b] Moczar, M., Moczar, E. & Robert, L. (1979), *Conn. Tiss. Res.* **6,** 207.

[c] Moczar, M., Moczar, E. & Robert, L. (1980), *Frontiers Matrix Biol.* 8, 174.

**ELASTASE ASSAY**

The identification and characterisation of a protease as an elastase is not without problems. None of the presently named 'elastases' have a unique specificity for elastin, rather some proteases can hydrolyse a range of proteins and peptides, *including* elastin. In addition many synthetic peptidyl and nonpeptidyl peptides, amides, amides and esters substrates have been prepared and are used for the detection and measurement of elastases. .

The Fastin Elastin Assay offers the opportunity of examining test material for elastases that cause elastolysis to elastin. A substrate that provides extended enzyme binding site(s).

Although various insoluble elastins, (dyed and undyed), have been used in elastase assays the particle size, and the particle size variations, tend to produce non-linear elastolysis curves. This is due to the action of the elastase being restricted to the outside surface of these particles.

**Preparation of the elastin substrate.**

[1] To a sterile microwell plate, add 50ul aliquots of a-elastin, (0.2 um filtered), per well. Substrate free blanks also need to be included in the assay so only 88 of the 96 wells should be filled, (50ul placed in wells in columns 1 to 11, rows A to H). Column 12, A-H remaining unfilled.

[2] The a-elastin can be prepared as described on page 13, or the Fastin Elastin Standard can be used.

[3] When the 50ul aliquots have been added to the wells, hold the plate horizontally and transfer to a $37^0$ C incubator, which contains a fan. Or place the microplate in a dessicator, over calcium chloride, and dry under mild vacuum.

[4] Leave overnight to permit the elastin to dry down to a thin film on the bottom surface of the microwells. The U-shaped well type provide a more uniform film than do flat bottom wells.

[5] The plastic surface of microwells, as used for tissue culture, will bind a monolayer of protein molecules. The additional layers of elastin are, however, unattached to either the plastic or to the plastic bound layer of elastin. To prevent non-enzymatic loss of substrate during subsequent experimental treatments it is necessary to partially cross-link the elastin within the wells.

[6] The dried elastin films in the microwells is exposed to formaldehyde vapour, within a sealed container for 1 to 2 hours at room temperature, (a dessicator with 50ml 40% formaldehyde replacing the desiccant is convenient). The plates are then removed and well 'aired' to remove any traces of absorbed formaldehyde.

[7] The elastin layers of molecules are now cross-linked and will remain firmly attached to the wells until exposed to elastases. These plates can be prepared in batches and if stored dry, (return to dessicator containing $CaCl_2$ ,under vacuum), can be held for up to 2 weeks at $20^0$C.

**Assay for Elastase.**

To the elastin films in the prepared microwell plate add test solutions, buffer blanks, reference enzymes, etc. Where enzyme activators or inhibitors are to be examined ensure adequate controls are also included.

Adjust all test volumes to a common value, e.g. 100ul. Seal the wells by covering the plate top with an adhesive vinyl sheet.

Incubate the microwell plate under the required conditions of temperature and time. Trial time periods will be required to obtain 25 to 50% digestion of the substrate by the test material.

When ample active elastase is available a 60 to 120 min incubation period would permit the assay to be completed in one working day.

When the test enzyme quantity is limited, consider reducing the amount of elastin in the wells or using an overnight incubation.

At the completion of the incubation period remove the seal from the plate. Drain the contents from the wells into a sink. The plate in then inverted and allow to drain on a paper towel. Tap the plate firmly onto the paper towel so as to dislodge any fluid remaining in the wells.

Place the plate, open well side up. Add 100ul Fastin Dye Reagent to each well.

With the plate on a smooth level surface place your hand above the plate so that your fingers can hold the side walls of the microwell plate. Gently mix the well contents by swivelling the plate, left and right, using wrist action only, do not let the plate lift off the bench surface.

Following mixing allow the TPPS dye to react with the residual elastin for 60 mins.

At the end of the dye binding incubation, drain the TPPS dye into the sink and again remove any remaining fluid from the wells with the aid of a paper towel, as described above.

Place the microwell plate on a light-box and examine the well staining pattern obtained. A map of the results can be made using a copy of the diagram on page 17..

When proteolysis has been extensive most of the residual elastin appears as a ring around the bottom edge of the well(s). A more accurate picture can then be obtained by adding 100ul of the Fastin Dissociation Reagent to each well. This brings the residual elastin bound TPPS into solution.

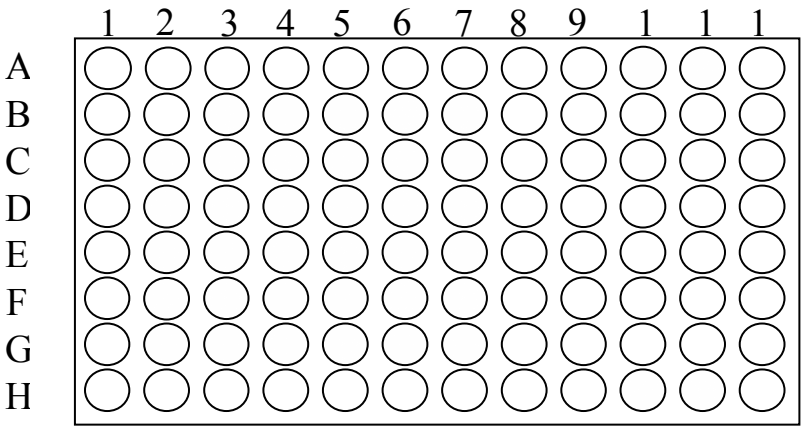


*Fig. 3: Visual inspection of elastase action.*

**Map key:**

| | | | |
|---|---|---|---|
| Pink coloured wells | | | fill in completely |
| Medium pink | .. | .. | half-filled circles |
| Pale pink | .. | .. | quarter-filled circles |
| Colourless wells | | | unfilled circles |

Two other options are available; (a) a photographic record of the microwell plate and (b) measurement of how much of the elastin remains in each wel, ( see next paragraph)l.

**Quantitative elastase assay.**

As soon as possible after adding the dissociation reagent to the above plate transfer to a microwell plate reader, (colorimeter).

Select a suitable colour filter; ~513nm, or for increased sensitivity a ~405nm filter.

Column 12, A-H provides the reagent blank value. Other wells will require known aliquots of the a-elastin standards to have been added, dried and fixed in a similar manner to that of the substrate a-elastin used for the test enzyme samples. The Fastin a-elastin standard aliquots should be incubated with the same buffer/salt solution as the test samples. Do not add enzyme to these standard wells. A standard curve is prepared from the standard readings.

With a-elastin aliquots containing 10 to 100ug measure absorbance at 513nm. For a-elastin aliquots containing 0.5 to 5.0ug measure absorbance at 405 nm.

## TROUBLE SHOOTING

[1] A concern to many new users of the Fastin Elastin Assay is what reference method(s) can the method be compared with? Until the introduction of the Fastin Assay there was no assay procedure for quickly measuring large numbers of elastin samples.

The Fastin Elastin Assay was calibrated using purified elastin, (free of microfibril protein and traces of collagens), as was defined from the elastin's amino acid profile. Where the user has pure elastin and access to an amino acid analyser a reference check could also be performed. Biocolor used a-elastin as the reference material for the Fastin Assay.

[2] The most common problem encountered by new users of the Fastin Assay is at the initial elastin precipitation step. Ensure your test sample buffer/salt solution does not interact with the Fastin Elastin Precipitating Reagent. Add aliquots of the Fastin a-elastin standard to this solution, with and without your native elastin, and check the recoveries obtained..

[3] Ensure that the elastin precipitation is carried out at temperatures between 0 and $5^0$C

[4] Take care to remove most of the Precipitating Reagent from the microcentrifuge tubes *before* adding the Elastin-Dye Reagent. Excess residual acid will turn the Dye Reagent *green* and cause dye precipitation.

[5] An explanation for high reagent blank values can be due to ageing Dye Reagent. The Dye Reagent can be filtered, (using conventional filter paper discs), to reduce high absorbance values..

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# *Fastin*

# Elastin

## *Assay Kit*

Options

***Standard Assay Kit*** **……Product Code. F2000**
***Components of the assay kit:,*** **(120 assays)**
Fastin Dye Reagent ..... 120 ml

Elastin Precipitating Reagent .....120 ml

Dye Complexing Reagent ..... 25 ml

Fastin Dissociation Reagent .....120ml

Elastin, [ a high MW $\alpha$-elastin preparation]..... 5ml
1mg / ml standard in water, sterile vial.
(bovine elastin, from USA disease free animals).

Fastin Assay Manual.....24 pages
(available to view, or as a print download from our website).

***Assay Large Ecomony Kit Pack.*****…… Product Code: F4000**
**Components of this assay kit, as F2000, [475 assays]**

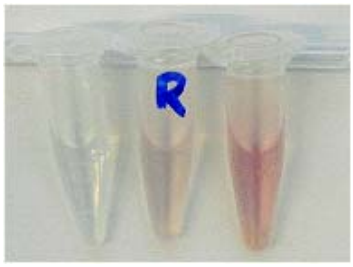
***Supplementary Unit Packs.***

**Fastin Dye Reagent only,..... [3 x 120 ml]**
***Product Code*** **…… F2300**

**Elastin Standard, .....[3 x 5 ml ampoules]**
***Product Code*** **…… F2010**
Sterile bovine Elastin, [ a high MW $\alpha$-elastin preparation, water and salt soluble]. Concentration: 1 mg/ml in water.

**[1] Cold precipitated elastin, ( 1 mg ), overnight, then centrifuged and drain dried**

**[2] Elastin- dye complex after centrifuging and removing unbound dye**
**Left: Reagent Blank**
**Right: Standard, 50 ug**




**[3] Recovery of elastin bound dye, after adding Dissociation Reagent and centrifuging to remove turbidity**

**Left: Solvent/ buffer Blank**
**Middle: Reagent Blank**
**Right: Standard, 50 ug**

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