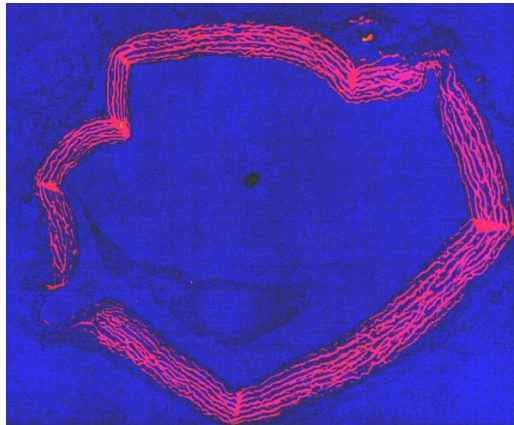


FastinTM

Elastin Assay



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Fastin™

Elastin Assay

The Fastin Assay has been designed for research work only.
Handle the Fastin Assay using Good Laboratory Practice.

TECHNICAL INFORMATION

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Assay Manual

Intended Applications

The Fastin Elastin Assay is a quantitative dye-binding method for the analysis of elastins extracted from mammalian sources.

The dye label employed is 5,10,15,20-tetraphenyl-21H,23H-porphine tetra-sulfonate (TPPS). For the structural form of the dye see Fig.2 (page 3).

Test Sample Material

Tissue extracts and cells during *in-vitro* culture.

Elastin forms that can be measured by the Fastin Assay as α -elastin:

- (i) soluble tropoelastins
- (ii) lathyrogenic elastins
- (iii) insoluble elastins, following solubilization to elastin polypeptides, [α -elastin; κ -elastin]

The dye reagent binds to the 'basic' and 'non-polar' amino acid sequences found in mammalian elastins.

Test sample quantities

A sample volume of between 50 and 500 μ l is required, containing not less than 5 μ g and not more than 70 μ g elastin.

Test sample composition

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

Fastin Kit Pack Size and Storage Conditions

Standard Assay Kit Product code: F2000 (110 assays)

Economy Pack Product Code: F4000 (440 assays)

Storage Unopened - All of the reagents have long term stability (one year), when stored at room temperature. Do not freeze as complete solubility may not occur on thawing.

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

Fastin Assay Kit Components

1. **Fastin Dye Reagent** contains 5,10,15,20-tetraphenyl-21H,23H-porphine tetrasulfonate (TPPS) in a citrate-phosphate buffer.
The pH of this reagent is pH 7.5 and, to limit microbial growth a compatible inhibitor has been added. This is not a 'universal' microbial inhibitor and so we recommend storing an opened bottle of Fastin Dye Reagent at 4°C to extend the shelf life.
2. **Elastin Precipitating Reagent** contains trichloroacetic and hydrochloric acids.
3. **Elastin Standard** is a high molecular weight fraction of α -elastin prepared from bovine neck ligament elastin. The α -elastin standard is supplied as a sterile solution; concentration 1.0 mg/ml, in 0.25M oxalic acid.
The full metal seal should not be removed from the vial.
 - (a) Remove the centre metal disc only from the vial top.
 - (b) 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.
 - (c) Do not return any unused aliquots to the vial.
 - (d) The α -elastin standard should be discarded if the solution becomes turbid.
4. **Dye Dissociation Reagent** contains guanidine HCl and propan-1-ol.
5. **Oxalic acid**, 1.0M. Dilute to 0.25M for tissue samples (see page 4).
6. **Assay Manual**. Additional free copies can be downloaded from Biocolor's website.

Equipment Required

Mechanical shaker to provide gentle mixing of the elastin and the Dye Reagent.

A centrifuge, fitted with a 1.5 ml microcentrifuge tube rotor head; capable of 13000 $\times g$.

A Microplate Reader, with a suitable colour filter (absorbance peak of dye occurs at 513 nm).

A metal heating block with the thermostat set between 95 and 100°C is convenient for extracting elastin from tissue samples. Otherwise a boiling water resistant glass beaker on a hotplate with thermostat set between 95 and 100°C, can be used.

Mode of Action of the Fastin Dye Reagent with Elastin

The Fastin Dye Reagent contains a synthetic porphyrin, 5,10,15,20-tetraphenyl-21H,23H-porphine, that is water soluble in the sulfonate form. The TPPS molecules contain four sulfate groups.

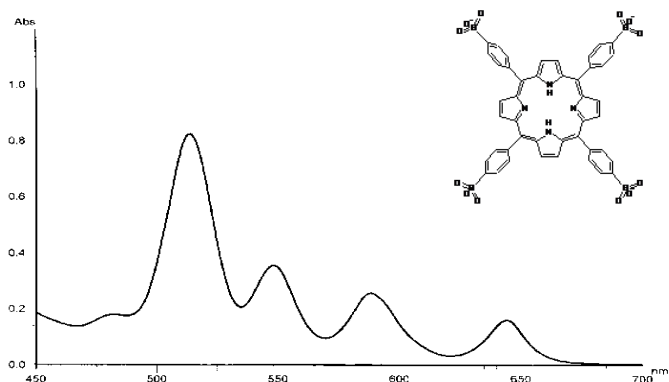


Fig. 1 The visible absorbance spectrum and structural form of 5,10,15,20-tetraphenyl-21H,23H-porphine, tetra-sulfonate.

'The Winkelman Reaction'

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

SAMPLE PREPARATION PRIOR TO ASSAY

Before the assay can be performed cell and tissue elastin must be converted into a water soluble derivative; α -elastin. Extraction of the elastin component of the elastic matrix is obtained by heating the test samples in hot (95 - 100°C) oxalic acid for one hour, converting the hydrophobic elastin into water soluble α -elastin.

The recommended extraction procedures are described in the following pages.

A sample preparation flowchart may be found on p12.

EXTRACTION OF INSOLUBLE ELASTIN FROM TISSUE SAMPLES

The Fastin Assay can be used to measure insoluble cross-linked elastin. Insoluble elastin is extracted from tissue in the form of soluble cross-linked polypeptide elastin fragments; α -elastin.

Following extraction and clarification the samples can be assayed.

Conversion of insoluble elastin to water soluble α -elastin

- a) Tissue samples should be weighed before use, we recommend using between **10 - 20mg** of tissue and keeping a record of the weight. This will enable you to express the elastin content as μg elastin/mg wet tissue or μg elastin/mg dry tissue.
Place the weighed samples into 1.5ml microcentrifuge tubes and add 750 μl of **0.25 M** oxalic acid.
(Stock 1.0M oxalic acid is supplied, dilute 1 in 4 with water for 0.25M).
- b) Place the tubes into a metal heating block with the thermostat set at 100°C for 60 minutes. Do not tighten tube caps.
- c) Remove the tubes from the heat and cool to room temperature.
Centrifuge at 13000 x *g* for 10 minutes. Pipette off the liquid and retain this extract in labeled containers for analysis.
- d) To the **residual tissue** in the tubes add a further 750 μl of 0.25M oxalic acid and heat again for 60 min. Up to three heat extractions should be initially used to check that complete solubilisation of the tissue elastin has occurred.
- e) Some tissue material, such as that from fetal or immature animals, can be solubilised after one or two extractions. Tissue from mature or old animals, including aged human tissues, may require up to three extractions.
- f) Initially when using new test material retain each of the oxalic acid extracts separately and analyze each for elastin to establish that elastin extraction was quantitative. The last extract should contain no elastin.
Elastin extracted from mature tissue, (elderly adult human tissue), will often produce yellow coloured extracts. This colour does not interfere with the assay.
- g) The tissue elastin in the form of α -elastin has a molecular weight range of 60-84 KDa. The extract can now be directly assayed using the procedure described for soluble elastin.
Retain a record of the extract volumes to permit calculation of the tissue elastin content.

(See Page 5 for worked examples of mouse tissue analysis).

TEST RESULTS: Extraction of Elastin from Mouse Tissue Samples

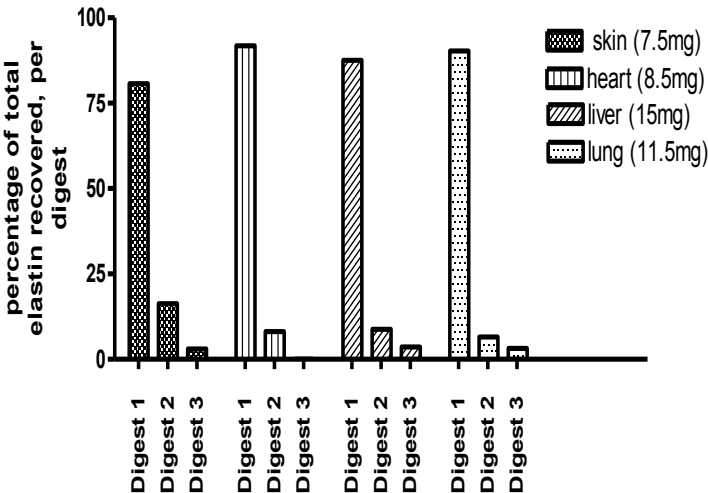


Fig. . a Extraction of elastin from mouse tissue by hot oxalic acid digestion. Elastin recovered from three 1 hour acid extractions. Samples were briefly shaken after 30 minutes to disrupt any solid residue.

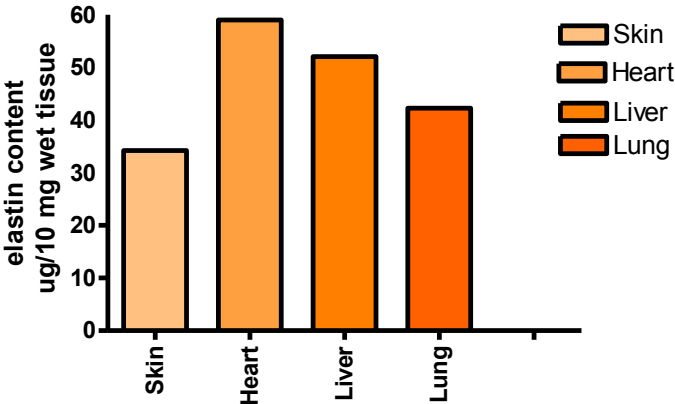


Fig. 2b Extraction of elastin from mouse tissue by hot oxalic acid digestion. The three extracts shown in Fig. 2a were pooled and expressed as μg elastin per 10mg wet tissue

EXTRACTION OF ELASTIN FROM CELLS GROWN IN CULTURE

Tropoelastin, the native monomer form of elastin as exported from mammalian cells, has a molecular weight between 62 – 72 KDa.

Tropoelastin binds avidly to a co-exported microfibril glycoprotein to form 'elastic matrix' or 'an elastic tissue raft'. This would appear to be linked, or tethered, to the external surface of the cell membrane.

Removal of Cells from Tissue Culture Flasks and from Microwell Plates

The release of cells from flasks or wells is required to permit subsequent treatment with hot oxalic acid. Cells can be removed using trypsin or a non-enzyme based Cell Dissociation Solution, (Sigma-Aldrich Product Code: C5914).

- a) Remove and discard cell culture medium. (Elastin has not been detected in cell spent medium using the Fastin Assay; with a detection limit of 5 µg).
- b) Pre-warm all reagents to 37°C before rinsing the cells twice with PBS. Add the Cell Dissociation Solution or trypsin release solution to the washed cells - 1ml for a T-25 flask and 250µl/well for 12 well plates.
- c) Incubate for 10 minutes at 37°C then sharply tap the flask or plate to release the cells into suspension.
- d) Transfer the cell suspension to 1.5ml microcentrifuge tubes and centrifuge gently, (max 3000 rpm). Retain the cell pellet in about 300µl of supernatant and discard rest of supernatant.

Conversion of Cell Bound Elastin to Water Soluble α -Elastin

- e) To the 300µl of cell suspension add 100µl 1.0M oxalic acid, (final concentration 0.25M oxalic acid). Place the loosely capped microcentrifuge tubes in the heating block at 100°C for one hour.
- f) The cell bound elastin is now in the form of α -elastin. The extracts can be directly assayed using the procedure described for soluble α -elastins.

See Page 7 for worked examples of fibroblast cell analysis.

TEST RESULTS: Extraction of Elastin from *In Vitro* Cultured Fibroblasts

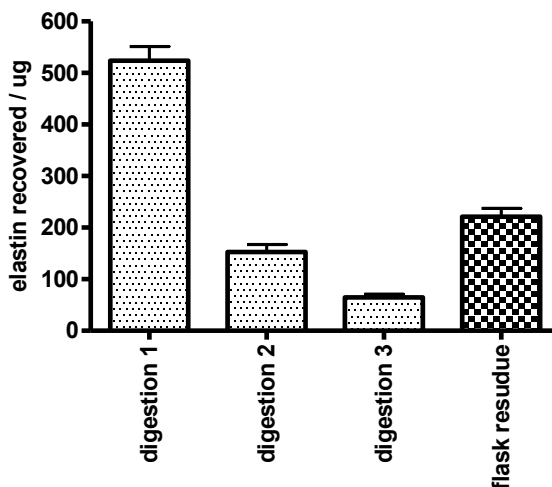


Fig. 3 Extraction of elastin from a CHO cell line using hot oxalic acid.

T25 cell culture flasks containing CHO cells were permitted to come to confluency. The flasks were then drained of cell medium and washed twice with PBS. Two ml of 0.25M oxalic acid was added to each flask. The flasks were placed in an oven, thermostat controlled, at 100°C for one hour. The digest extract was then removed and retained. To the residue 1.0ml of fresh oxalic acid was added and the extraction collected, and the process repeated to give a total of three extractions.

The flask was then washed with PBS and 2 ml of dye added for 90 mins. The unbound dye was removed and bound dye released. The finding indicated some accumulation of cells and elastin on the plastic surface of the flasks, (Fig. 4). Elastin was not detected in the spent medium, (detection limit; 5µg).

Each extract (200µl) was mixed with the Elastin Precipitating Reagent (200µl). After 10 minutes the samples were centrifuged. The drained elastin pellets were then mixed with 2ml of the Fastin Dye for 90 mins. Unbound dye was removed. Bound dye was released from the elastin-dye pellet using 1 ml of the Dye Dissociation Reagent.

The mean data from six T25 flasks with CHO cells is shown in Fig.4. The findings displayed an abundance of elastin produced by CHO cells after 48 hours in culture. A 100µl aliquot of the pooled extracts produced 30µg of elastin. This was well within the range of the Fastin Standard Curve.

Measurement of Cell Generated Elastin in T25 Flasks

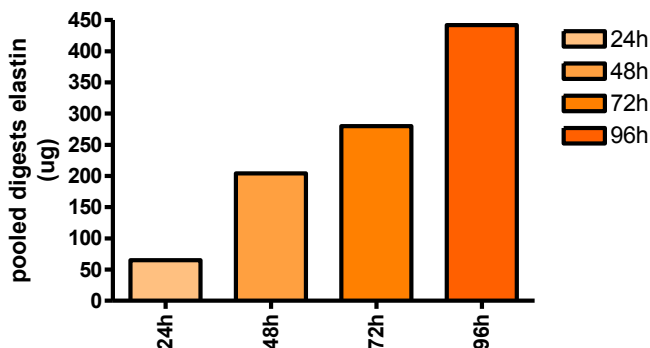


Fig. 4 Rate and quantity of elastin produced by CHO cells.

In this series of experiments using CHO cells, (seeding density 5×10^5), in T25 flasks the cells were removed from the flasks using 1 ml of a detergent based cell releasing agent, (Sigma-Aldrich Cell Dissociation Solution, Product Code: C1419). This permitted the acid digestion to be carried out in 1.5 ml microcentrifuge tubes. 1.0 M oxalic acid (100 μ l) was added to the cell suspensions (300 μ l) giving a final concentration of 0.25 M oxalic acid. This was digested at 100°C for 1 hour, with occasional vortexing. The transparent extract was treated with an equal volume of the Acid Precipitating Reagent and the elastin pellet recovered then mixed with the Fastin Dye (Fig. 5). Unlike the previous experiment where digestion and extraction was carried out in the T25 flask these T25 flasks were stripped of elastin and cells before acid digestion.

Measurement of Cell Generated Elastin in 12 Well Microplates.

The seeding density was 1×10^5 and grown to confluence by 48 hours. The cell medium was removed and cells washed twice with PBS. 300 μ l of the detergent based cell releasing agent was added and the plate incubated at 37°C for 10 mins. The wells were tapped and scraped and the cell suspension transferred to microcentrifuge tubes where 100 μ l of 1.0M oxalic acid was added, and extraction carried out as described above.

Elastin Recovered from CHO Cells after 48 Hours of *In-Vitro* Culture.

Format	Seeding density	Growth surface	Elastin Recovered
T25 Flask	5×10^5	25 cm ²	204 μ g
12 well plate	1×10^5	3.8 cm ²	42 μ g

SUPPLEMENTARY NOTES FOR THE MEASUREMENT OF SOLUBLE ELASTIN

(i) Test Samples

Try single 50 μ l aliquots of test sample, for the first trial, where the elastin concentration range is as yet unknown

If the absorbance readings are found to be more than 1.0 (after subtraction of the reagent blank value) repeat assay using a smaller test sample aliquot, or dilute.

If the absorbance readings are less than 0.05 the test sample contains less than 5 μ g elastin and will require a larger sample aliquot or concentration before being re-assayed.

For reliable and accurate results all test samples should have their absorbance readings within the range of the Elastin Standards that were plotted on the calibration curve. Fig.6 displays a typical standard curve.

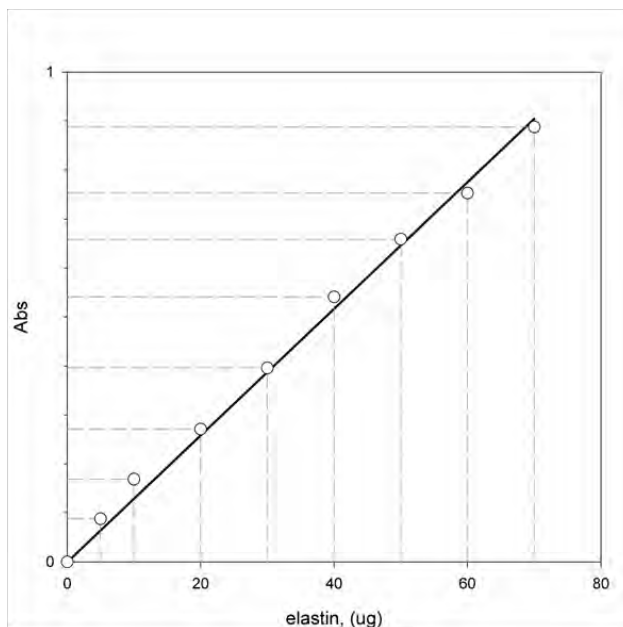


Fig. 5 Elastin Standard Curve. The curve was generated from samples on a 96 well microplate, 250 μ l/well.

(ii) Elastin Isolation

The **Precipitating Reagent** has been developed for elastin recovery. The reagent should not be diluted with sample volumes greater than in a ratio of 1:1. The reagent can be pre-cooled to $<5^{\circ}\text{C}$ (store this reagent in the refrigerator so that it is ready for use). To tubes, containing Standards or Test Samples, add an equal volume of the Reagent.

Following the precipitation of the elastin, the microcentrifuge tubes are centrifuged for $13000 \times g$ for 10 minutes, to pack the precipitated α -elastin. Remove tubes from the centrifuge, uncap and carefully invert, to drain the liquid contents into a waste beaker. While inverted remove any remaining fluid from the top of the tubes by tapping the tube onto an absorbent paper towel. A 'cotton bud', (or Q-tip), can be useful to remove any fluid droplets from the rim of the tube. On returning the tube to the upright position not more than $25\mu\text{l}$ of fluid should be found in the bottom of the tube.

Low concentrations of α -elastin can be difficult to 'see' as it occurs as a translucent gel. The photographed display of a precipitated protein pellet in Fig 6.a used a $100\mu\text{g}$ quantity of α -elastin.

(iii) Recovery of the Elastin-Dye Complex

Following the dye binding step the elastin-dye complex formed becomes insoluble in the presence of ammonium sulfate within the Fastin Dye Reagent.

The elastin-dye complex is separated from the remaining soluble unbound dye by centrifuging the tubes ($>13000 \times g$ for 10 minutes).

Visual inspection should reveal a red residue within the elastin standard tubes and, hopefully, also in the test sample tubes, (Fig.6b).

(iv) Release of the Elastin Bound Dye

To each tube add $250\mu\text{l}$ of **Dye Dissociation Reagent**. Cap the tubes and bring the elastin-bound dye into solution using a vortex mixer. Two brief mixing periods are usually more effective than one long mixing period.

Tubes should not be uncapped until transfer to the wells of a 96-well microplate for absorbance measurement.

The dye extract is stable for several hours, but if readings are to be delayed store the tube rack containing the microcentrifuge tubes in a light-proof container or cupboard.

(iv) Elastin Measurement

The elastin content of the assayed samples is determined by the amount of bound dye released from the α -elastin.

Transfer a volume of $100\mu\text{l}$ of labeled dye extract from microcentrifuge tubes to wells of a 96 well microplate, (with flat-bottom wells to reduce light scatter).

The absorbance peak of TPPS in the Dye Dissociation Reagent occurs at 513 nm.

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 assay.

Check the colour filter options that are available for the Microplate Reader; a blue green filter will probably be found to be suitable.

The fluorescent micrograph on the front cover shows a Fastin Dye labeled cross section of a primate thoracic aorta. The elastic laminas are labelled deep pink in colour.



Fig. 6 (a) The precipitation of α -elastin (0, 25 & 100 μ g) following the addition of the Elastin Precipitation Reagent.
(b) Dye bound α -elastin (0, 12.5 & 25 μ g) after removal of unbound dye from the tubes.

ELASTIN SOURCE REFERENCES

Biochemistry, biophysics; preparation, extraction & analysis

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***Biocolor's website has a list of research papers
that have cited the Fastin Assay.***

Preparation of Elastin standards

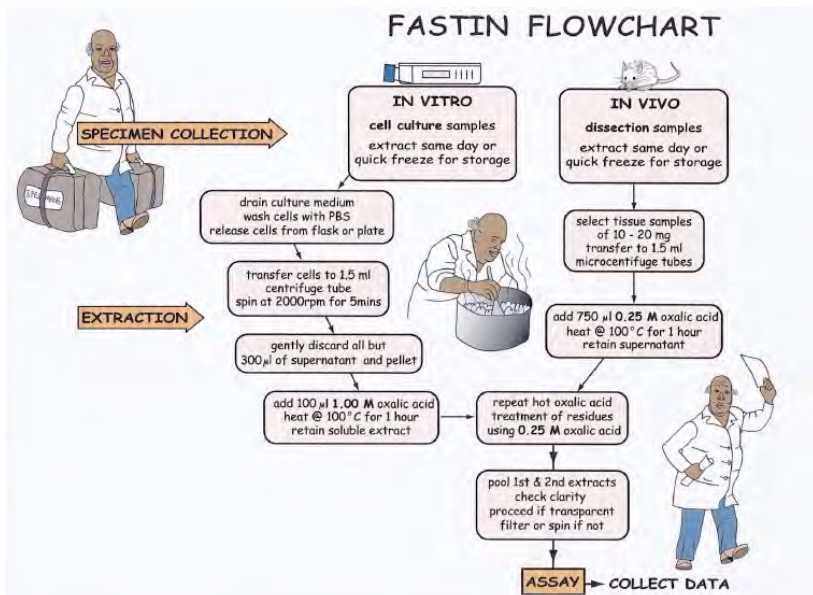
Elastin standards are used to prepare the assay standard curve. These are prepared via dilution of the provided Elastin Reference Standard (1.0mg/ml) according to Table.1. For convenience these can be prepared directly within the provided 1.5ml microcentrifuge tubes.

Table 1. Preparation of Fastin assay elastin standards

Elastin Concentration (µg/ml)	Elastin content (µg per tube)	Volume of Elastin reference standard (µl)	Volume of 0.25M oxalic acid (µl)
0	0	0	100
50	5.0	5	95
125	12.5	12.5	87.5
250	25.0	25	75
500	50.0	50	50
750	75.0	75	25

Sample Preparation Flowchart

The following flowchart may be used to select the most appropriate sample preparation method.



Fastin Elastin Assay

General Protocol

Detection Limit:

5µg/100ul (50µg/ml)

Extraction

All test samples require the conversion of the native hydrophobic elastin into a water soluble derivative, (α -elastin).

To extract as α -elastin the sample is heated at 100°C for two one hour periods with 0.25M oxalic acid.

For detailed information on extraction treatment of in-vivo and in-vitro samples see pages 4 to 8.

Isolation

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

[1] Prepare Reagent Blanks: 100µl of test solution solvent, (buffer / PBS / water / 0.25M oxalic acid).

[2] α -elastin standard: suggested volumes - 0.5, 12.5, 25, 50 & 75µl, duplicate aliquots. *See preparation guide on p13 of assay manual.*

[3] Test samples: in vivo derived tissue extracts or in vitro cell culture samples. Select volumes between 50µl and 250µl of α -elastin.

Assay

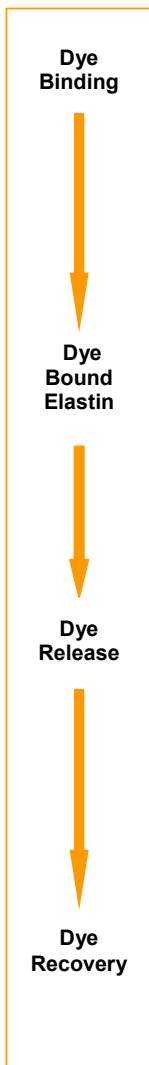
[4] To each tube add an equal volume of Elastin Precipitating Reagent (stored at 4°C).

[5] Cap tubes and briefly vortex to mix contents; leave for 15 minutes to complete precipitation of α -elastin.

[6] Centrifuge tubes @ >13000 x g for 10 minutes, (Fig.6a in manual).

[7] Drain tube's liquid contents into a beaker. While the tube is still inverted remove most of the remaining fluid from the tube by tapping the inverted tube onto a single thickness absorbent paper towel.





Formation of Elastin - Dye Complex

[8] To all tubes add 1.00ml of Dye Reagent.

Cap tubes and mix contents by inverting the tubes. Then disperse the elastin precipitate using a vortex mixer.

Place the rack of tubes on a mechanical shaker and allow reaction between the elastin and the dye to proceed for 90 minutes.

[9] Centrifuge the tubes @ $>13000 \times g$ for 10 minutes.

Recovery of Elastin-Dye Complex

[10] Drain the tubes of unbound dye. While the tube is still inverted remove most of the remaining fluid from the tube by FIRMLY tapping the lip of the tube onto a single thickness absorbent paper towel. A 'cotton bud', (or Q-tip), can be useful for removing any fluid droplets from the rim of the tube. On returning the tube to the upright position not more than 25 μ l of fluid should be found in the bottom of the tube.

[11] The elastin-dye complex can be observed as a reddish-brown deposit in the bottom and inside lower wall of the tube, (Fig. 6b in manual).

Release, and Recovery, of the Elastin Bound Dye

[12] To each tube add 250 μ l of Dye Dissociation Reagent. Cap tubes and release the dye into solution with the aid of a vortex mixer. Repeat the vortex mixing after 10 minutes to ensure that all bound dye has passed into solution.

[13] Transfer 100 μ l of dye extract from each tube to a well in a 96-well flat bottom microwell plate. Record which tube contents went into which well.

Elastin Measurement; (dye recovered)

[14] Place microwell plate into the Microplate Reader. Select wavelength or colour filter nearest to 513 nm, (blue-green colour). Plot Reference Standards and use this graph to determine the elastin content of the Test Samples.

