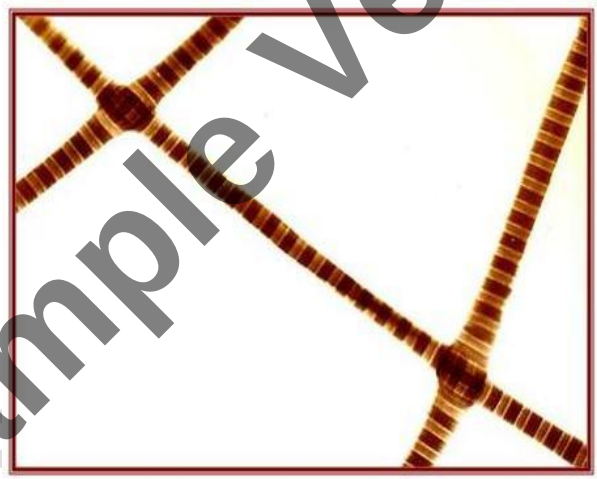
Sircol™

Soluble

COLLAGEN

Assay



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life science assays

Sircol Collagen Assay

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General Protocol

Detection Limit: **1.0 µg**

Time required: **1.5 hours**

Set Up Assay

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

Prepare:
Reagent blanks - 100µl of deionised water or 0.5M acetic acid or fresh cell culture medium or extraction buffer.

Collagen standards - use aliquots containing 5, 10 and 15µg of the Collagen Reference Standard. Make each standard up to 100µl using the same solvent as the Reagent blanks.

Test samples - use volumes between 10 and 100µl and make up to 100µl. Where there is no previous knowledge of the collagen content 50 or 100µl of the test material is suggested for a trial run.

Commence Assay

To each tube add Sircol Dye Reagent (1.0ml).
(1 ml of dye is required to fully saturate the collagen molecules within a 100µl sample volume).

Cap tubes; mix by inverting contents and place tubes in a gentle mechanical shaker for 30 minutes, (or manually mix at 5 minute intervals).

During this time period a collagen-dye complex will form and precipitate out from the soluble unbound dye.

Centrifuge

Transfer the tubes to a microcentrifuge and spin at 12,000 r.p.m. for 10 minutes. Carefully invert and drain tubes.

Important: firmly packing the collagen-dye complex at the bottom of the tubes is required to avoid any pellet loss during draining of unbound dye.

ASSAY PROTOCOL CONTINUED ON INSIDE BACK COVER
PROTOCOL CONTINUED FROM INSIDE FRONT COVER

Washing and Draining

Gently layer on 750µl ice-cold Acid-Salt Wash Reagent to the collagen-dye pellet to remove unbound dye from the surface of the pellet and the inside surface of the microcentrifuge tube.

Centrifuge at 12,000 r.p.m. for 10 minutes. Drain the wash into a waste container and carefully remove any fluid from the lip of the tubes using cotton wool buds.

Release and Recovery of Collagen Bound Dye

Add 250µl Alkali Reagent to reagent blanks, standards and samples.

Recap tubes and release the collagen bound dye into solution. A vortex mixer is suitable.

When all of the bound dye has been dissolved, usually within 5 minutes, the samples are ready for measurement. The colour is light stable, but should be read within 2 to 3 hours. Keep tubes capped until ready to measure Absorbance.

Measurement

Transfer 200µl of each sample to individual wells of a 96 micro well plate, (keep a record map of the contents of each well; A1 to H12).

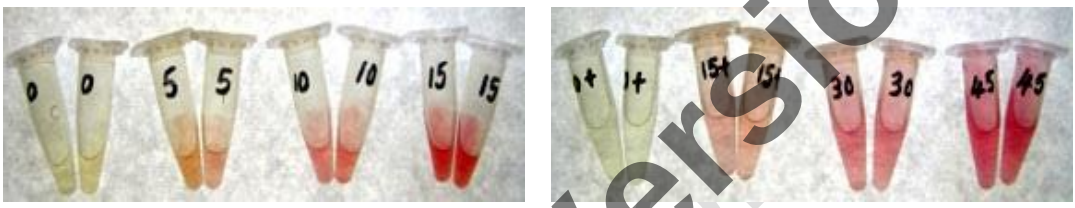
Set the microplate reader to 555nm, or the closest matching blue-green colour filter.

Measure absorbance against water for the reagent blanks, standards and test samples.
Obtain collagen concentrations from the Standard Curve. Duplicates should be close to ± 5% of their mean value.

If sample absorbance values are at the top end of the standard curve the assay should be repeated and a set of higher concentration standards (15, 30 and 50µg) used, together with 1000µl of alkali reagent rather than 250µl. The assay is completed as above.

READ SIRCOL MANUAL FOR SAMPLE PREPARATION DETAILS

(a)



(b)

(c)

Fig. 1 (a) Sets of collagen standards, Low range 0, 5, 10 & 15 µg and High range 0, 15, 30 & 45 µg, following collagen-dye mixing, centrifuging and removal of unbound dye, (duplicates not shown).
(b) Low Standards after adding 250 µl of Alkali Reagent.
High Standards after adding 1000 µl of Alkali Reagent.
(c) Tube aliquots (200µl) transferred to 96 well microplate.

The Reagent Blanks are coloured without an Acid-Salt wash, notably when using 250µl Alkali volumes.

SEE INSIDE COVER OF MANUAL FOR ASSAY DETAILS

Sircol™
Soluble Collagen
Assay

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

TECHNICAL INFORMATION

Published by

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Sircol
Assay Manual

Intended Applications

The Sircol Assay is a dye-binding method designed for the analysis of acid and pepsin-soluble collagens. The assay can assess the rate of newly synthesised collagen produced during periods of rapid growth and development. New collagen is also generated during inflammation, wound healing and tumour development.

Assay Sample Material

The Sircol Assay is suitable for monitoring collagen produced *in situ* or during *in-vitro* cell culture and *in-vitro* extracellular matrix, (ECM), formation.

in vivo
- collagens, soluble in cold acid or pepsin, recovered from mammalian soft tissues, cartilages and fluids.

in vitro
- collagens, soluble in cold acid or pepsin, released into cell culture medium during cell growth and cell maintenance.
- collagens, soluble in cold acid or pepsin, recovered from newly formed extracellular matrix that has been deposited onto cell culture treated plastic surfaces, (T-flasks and microwell plates).

Mammalian collagens: Types I to V can be measured.

Sircol Dye reagent does not discriminate between collagen types, binding to the [Gly-X-Y]n helical structure as found in collagen.

Thermal denaturation (> 40^0C) of collagen will decrease dye binding.

Non-mammalian collagens bind less dye, due in part to lower denaturation temperatures and the reduced occurrence of hydroxyproline residues.

Not suitable for covalent cross-linked collagen. This insoluble collagen can be estimated, following acid hydrolysis, (HCl (6M), 110^0C, overnight), with an amino acid analyser to measure the collagen marker - hydroxyproline.

Sircol Kit Pack Sizes and Storage Conditions

Standard Assay Kit Product Code: S1000 (110 assays)
Economy Pack Product Code: S5000 (440 assays)

All components are stable for one year, (from Invoice Date), when stored at 15-25^0C. The glass vial containing collagen standard should be stored at +4^0C once opened.

Assay Kit Components

1. **Dye Reagent** contains Sirius Red in picric acid and has been formulated for specific binding to collagen under the conditions defined in the Sircol Manual. **[The composition of the Sircol dye in the current kit remains unaltered from the original composition and is fully compatible with any previous Sircol based studies].**

2. **Alkali Reagent** contains 0.5M sodium hydroxide and is used to release Sircol dye from the collagen-dye complex.

3. **Reference Standard** - a sterile solution of cold acid-soluble collagen Type I, in 0.5M acetic acid within a sealed vial. (This is bovine skin collagen imported from the USA. In countries that forbid the importation of bovine derived material a rat collagen standard is also available, Product Code S1111).

   Concentration: 500 µg /ml for bovine and rat collagen.
   Store at 4^0C, remove aliquots using a sterile needle and discard if the clear solution becomes turbid.

4. **Acid-Salt Wash Reagent (Concentrate)** - contains acetic acid, sodium chloride and surfactants. **Dilute the contents of the vial, before use, to 100ml with deionised water.**

5. **Acid Neutralising Reagent** - contains TRIS-HCl and NaOH.

6. **Collagen Isolation & Concentration Reagent** - contains polyethylene glycol in a TRIS-HCl buffer, pH 7.6.

Components required for sample preparation - not supplied

7. **Acetic acid**, 0.5 M, store and use at 4^0C. (Sigma-Aldrich supply 2.0 M acetic acid in a 100ml pack. Product Code: A8976).

8. **Pepsin** is required that produces a clear transparent solution with effective non-collagen protease activity at 4^0C. (Recommended - Sigma-Aldrich Pepsin, E.C. 3.4.23.1 Product Code: P7012, at a concentration of 0.1mg/ml in 0.5M acetic acid).

9. **Capped conical microcentrifuge tubes, 1.5ml capacity**. For *in-vitro* samples with serum supplemented cell culture medium a low protein binding microcentrifuge tube is recommended. This is due to albumin from blood serum forming an insoluble film on the inside surface of most plastic microcentrifuge tubes, to which many dyes, including Sircol are absorbed. (Eppendorf produce a suitable low protein binding product; these Protein LoBind Tubes are also available from Sigma-Aldrich, Product Code: Z666505/ 100 tubes).

SAMPLE PREPARATION PRIOR TO ASSAY

Test Material Requirements for Compatibility with the Sircol Assay

The Sircol assay is a colorimetric procedure therefore test materials for analysis must be free from particulate material, such as cell debris and insoluble ECM fragments. The sample must also be transparent as turbidity will cause light absorption and scattering.

The test sample can be solubilised in a low molarity salt buffer solution, dilute acetic or hydrochloric acid or cell culture medium. The same solvent should be used to prepare the Collagen Standard curve.

If a surfactant has been used during tissue extraction it is recommended that this extraction solution be Sircol tested with the Collagen Standard to check that it has no adverse effects on collagen-dye binding.

The presence of other soluble protein in samples, including proteoglycans, tropoelastin and other soluble ECM material does not interfere with the assay. However cell culture medium containing blood serum supplement can interfere with the assay due to the albumin present in serum. Albumin forms an insoluble film on many plastic surfaces. Sircol Dye, like most dyes, has an affinity for insoluble polymers (see page 2 item 9).

To use the standard assay format as described on the inside cover of this manual the sample must be a clear solution in phosphate buffered saline (PBS), weak acid or serum free cell culture medium.

Cell Culture Samples (in vitro)

Sample - cell culture medium**.** Where the collagen concentration is less than 5µg/100µl a **Collagen Isolation & Concentration** step (page 5) is recommended. This step requires overnight incubation at 4^{o}C. Low protein binding microcentrifuge tubes should be used when the culture medium contains a serum supplement.

Sample - cell cultured extracellular matrix**.** The recovery and measurement of ECM from cell culture plastic ware may require the collagen **Isolation & Concentration** step (page 5) for 1 to 3 day cell cultures. **Acid-Pepsin Extraction** (page 4) may be required for extended time cultured cell samples, before the isolation and concentration step.

Tissue Samples (in vivo)

Sample - tissue/cartilages**.** For tissue samples the **Acid-Pepsin Extraction procedure** (page 4) requiring over-night incubation at 4^{0}C must be used. Some older age samples may require two enzyme extractions to fully recover pepsin soluble collagen.

COLLAGEN EXTRACTION USING COLD ACID-PEPSIN PROTOCOL

In-vitro samples

Extracellular Matrix formed on cell culture treated T-flasks and microwell plates

Where cell culture time has been extended to more than four days, a proportion of the ECM collagen can become acid insoluble but remains acid-pepsin soluble. Pepsin soluble ECM collagen can be solubilised by overnight incubation at 4^0C.

At a pepsin concentration of 0.1 mg/ ml 0.5M acetic acid the enzyme activity, at 4^0C, is effective in removing the terminal non-helical telopeptides to release the collagen into solution.

Having examined several pepsin preparations we found that Sigma-Aldrich's pepsin product, (Pepsin E.C. 3.4.23.1, Product Code : P7012), forms a transparent colourless solution.

We have no experience of collagen coated constructs, where fibroblasts are used to form a three dimensional ECM shape on a preformed inert scaffold. The pepsin soluble protocol should permit the collagen composition of such constructs to be recovered and analysed.

In-vivo derived samples

Hard tissues (aorta, cartilages, skin, tendons & valves)

At a pepsin concentration of 0.1 mg/ ml of 0.5M acetic acid the enzyme activity, at 4^0C, is effective in removing the terminal non-helical telopeptides to release the collagen into solution. A second acid-pepsin extraction should be considered for older animals.

Soft tissues (muscle, liver, lung etc.)

Lung and liver samples can be difficult to 'clean-up' as they are usually blood saturated. It is advisable to dice these samples and wash in cold phosphate buffered saline, before using the acid-pepsin extraction as described above.

Acid-pepsin collagen levels are often present at less than 5µg/ml of extract and will require the application of the **Collagen Isolation & Concentration Protocol** (page 5).

COLLAGEN ISOLATION & CONCENTRATION PROTOCOL

Adapted from Ramshaw, J.A.M., Bateman, J.F. & Cole, W.G. (1984), Anal.Biochem. **141**, 361-365.

Set Up Procedure:

***In-vitro* samples**
Remove spent medium from T-flask or multiwell plate and record total volumes of medium.

***In-vivo* samples**
Recover clarified extract from tissue residue and record total volume and/or the initial weight of sample.

Collagen Isolation & Concentration:

Use low protein binding 1.5ml conical microcentrifuge tubes, (see page 2 Item 9).

Add samples (1.0ml), including test sample of unused cell medium and/or extract solvent as blank controls.

Add 100µl **Acid Neutralising Reagent** to 1.0ml acid extracts, (0.5M acetic acid ± pepsin), from tissues.
This step is not required for culture medium samples.

To each tube add cold **Isolation & Concentration Reagent** (200µl/ tube). Mix tube contents by tube inversions.

Place the microcentrifuge tube filled rack into a container half filled with an ice-water mix. Incubate overnight at 0 to 4^{0}C. Remove tubes from rack without shaking their contents.

Centrifuge tubes at 12,000 r.p.m. for 10 minutes, without delay.
The pellet of hydrated transparent collagen is invisible.

To avoid loss of the pellet do not invert tubes to remove supernatant. Use a micropipette to slowly remove 1000µl of supernatant from each tube.

A cotton bud can be used to remove any fluid on the upper part of the tube and the tube cap and rim.

Collagen Assay:

Add **Sircol Dye Reagent** (1.0ml) and proceed with the assay as described on the inside covers of this Manual.

(The collagen will become visible, following the dye labelling step. 1µg of collagen-dye pellet can be observed after centrifugation).

Possible Applications for Collagen Isolation & Concentration Protocol

In-vitro samples

Cell culture medium containing blood serum supplement

Fresh and spent medium containing serum require both purification and concentration. Typical collagen values, found after 48 hours of culture in spent medium, range from 1 to 3 µg/ml from the cell population in 12 well plates. These values are too low to measure without the use of the **Collagen Isolation & Concentration Protocol.** With this protocol, collagen at a concentration of 1.0µg/ml of culture medium, can be recovered, concentrated and subsequently measured.

The albumin content of blood serum forms an insoluble film on the inside wall of the microcentrifuge tube, unless low-protein binding microcentrifuge tubes are used (see page 2 Item 9). The insoluble albumin film absorbs many dyes, including Sircol Dye.

Extracellular Matrix formed on cell culture treated T-flasks and microwell plates

After removing spent medium, add either cold acetic acid (0.5M) or, where cell culture exceeded five days, cold acetic acid (0.5M) with pepsin (0.1mg/ml) in the following volumes;

T-25 flask 5.0 ml 12 well plate 1.0 ml / well

Harvest these extracts and where collagen concentration is less than 5µg/ml process using the **Collagen Isolation & Concentration Protocol** (page 5).

Trial Sample data from CHO cells cultured in a T-25 flask

Duration of Growth (hours)	Confluence	Collagen Concentration (µg/flask)	Collagen Concentration (µg/ml)
24	60%	44.4	11.1
48	100%	72.4	18.1
72	over-confluent	96.0	24.0

In-vivo samples

Cartilage and Tissue samples obtained post-mortem from laboratory animals

Skin samples may not require the **Collagen Isolation & Concentration Protocol.** However, soft tissues, such as lung, are likely to require this protocol.

Trial Sample data from mouse samples

Tissue Sample	Extraction Method	Collagen Concentration
skin 4mm diameter disc	acid acid-pepsin	2.8 µg/mm^2 4.7 µg/mm^2
lung, pre-wash in PBS	acid acid-pepsin	0.70 µg/mg wet weight 1.73 µg/mg wet weight

ASSAY PROTOCOL

The general Sircol protocol is found on the inside/rear cover of this manual. The following manual sections contain supplementary details that should be read through, alongside the cover protocol, before the assay is carried out.

Collagen Concentration in Test Samples

The absorbance values of the reagent blank, reference standards and test samples are measured against water. The reagent blank value should be less than 0.10 absorbance units. Higher absorbance values, due to traces of unbound dye, will be obtained when the Acid-Salt Wash step is omitted.

The reagent blank's absorbance value is subtracted from all of the standards and test samples absorbance readings. It can be more convenient to set the microplate reader to zero using the reagent blank when low reagent blank values are consistently being obtained.

Variations in absorbance values between duplicate samples should be monitored. Initially some wide variations may occur. If this is not due to inaccurate pipetting the most likely source of error is in the drainage step. Practice with draining and drying the top of the microcentrifuge tubes will lead to a consistent mode of practice. Duplicate samples should read within ± 5% of their mean value.

Using a computer spreadsheet programmed with graphical output the three collagen reference standard absorbance means should be plotted against their known collagen concentrations. Joining the points should produce a straight line graph that can be extended downwards to pass close to or through zero (Absorbance v Concentration). Example calibration curves are shown in Fig. 3.

Test sample concentration values can be read off the graph or calculated from the degree of the slope. Absorbance readings less than 0.05 and greater than 0.80 are unreliable and samples should be re-assayed after either concentration or dilution of the test material. Values above 0.80 should not be further diluted with the Alkali Reagent as the 1.00ml of Sircol Dye Reagent cannot fully dye saturate these increased collagen levels.

The spectrum chart in Fig. 2b of the Sircol Dye in Alkali Reagent has a peak maximum in the visible region of 555 nm. The absorbance peak is broad and most microplate colour filter type readers will have a colour filter between 520 and 570nm. This should provide an absorbance slope similar to, but not necessarily matching, that of the 550nm filter as in Fig. 2b.

The Collagen Reference Standard curves were obtained using a microplate reader and are presented in Figs. 3a & 3b to offer a guide for filter selection in other microplate readers.

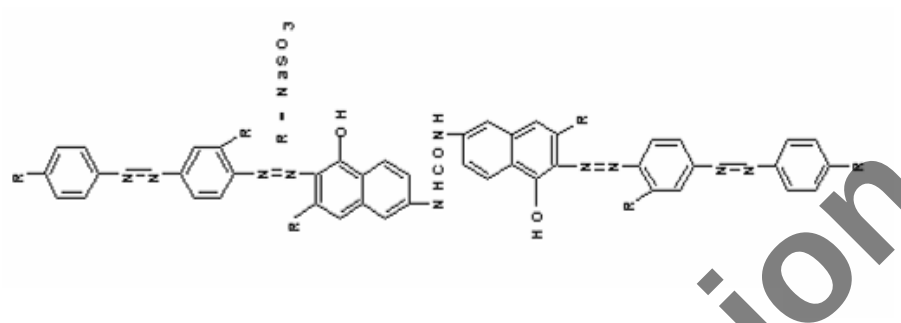

Fig. 2a: Molecular structure of Sircol Dye (Sirius Red).

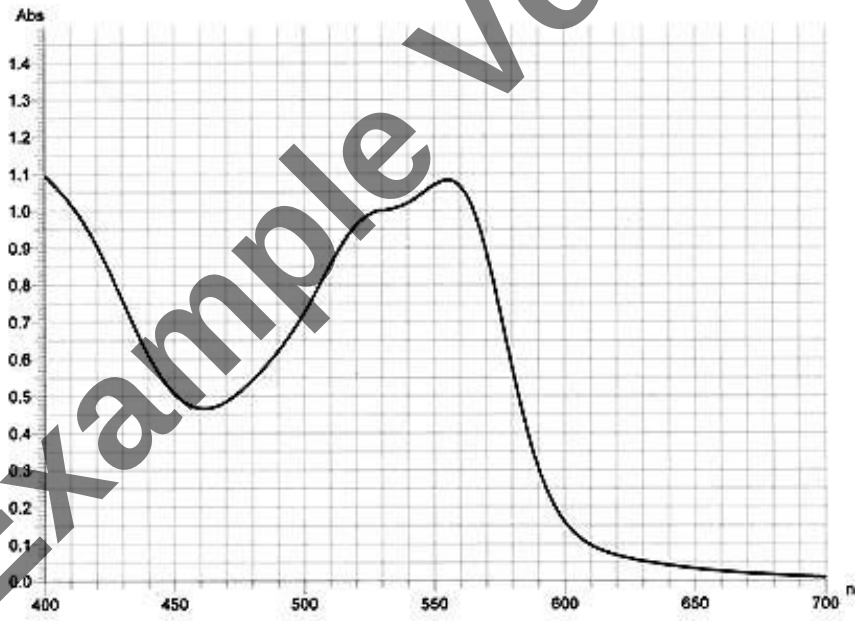

Fig. 2b: Absorption spectrum of the Sircol Dye in Alkali Reagent. Sirius Red is an anionic dye with sulfonic acid side chain groups. These groups react with side chain groups of the side chain present in collagen. The affinity of the dye for collagen is due to the elongated dye molecules becoming aligned parallel to the long rigid structure of native collagen.

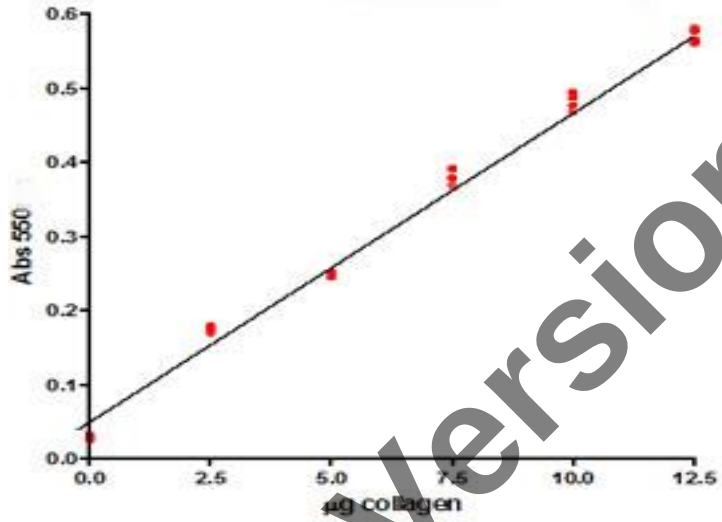

Fig. 3a: Collagen Reference Standards, 0 - 12.5 µg
using 250µl of Alkali Reagent to recover the collagen bound dye.

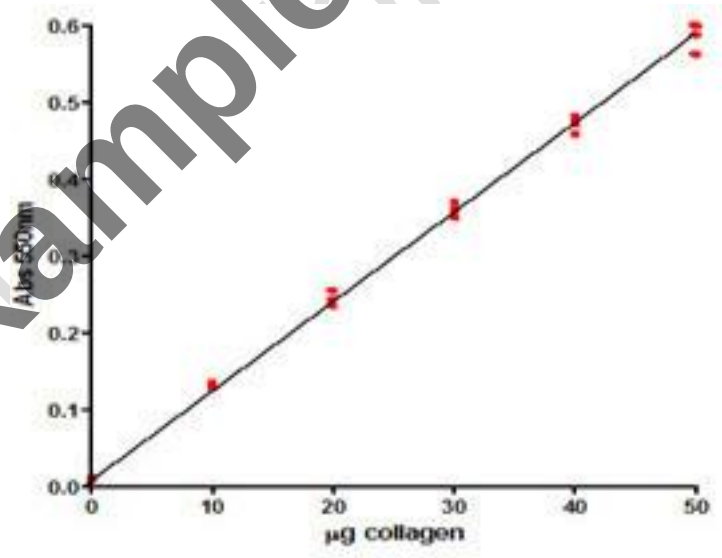

Fig. 3b: Collagen Reference Standards, 0 – 50.0 µg
using 1000µl of Alkali Reagent to recover the collagen bound dye.

MACROMOLECULAR COLLAGEN

Collagen is the predominant protein found in animals. During a healthy life span the insoluble covalent cross linked collagens retain their biophysical functions and shapes; isolated from biochemical activities.

Trauma caused by metabolic internal events, external agents or physical injuries can however quickly lead to dramatic activity, resulting in rapid collagen removal followed by a wound healing response (collagen regeneration and associated remodeling). This newly formed collagen production can be monitored using the Sircol Assay.

Collagen proteins contain one or more domains with a triple helical structure. The three chains, described as alpha chains should not be confused with the alpha helixes found in other proteins. The fibrillar collagens (Types I, II, III, V & XI) have most of their alpha chain structure composed of a continuous repeating tri-peptide sequence made up of glycine in every third amino acid residue, $[(gly\text{-}X\text{-}Y)_n]$. It is to this sequence that the Sircol Dye binds.

INSOLUBLE COLLAGEN MEASUREMENT

Proline is frequently an occupant in the 'Y' location of the tri-peptide sequence $[(gly\text{-}X\text{-}Y)_n]$ and many of these residues are converted post translationally into hydroxyproline residues. Prolyl hydroxylase performs this function within the smooth endoplasmic reticulum, prior to triple helix formation and the release of the tropocollagen into the ECM. Collagen is not stored within the cell.

Should an investigator seek to measure the total collagen content of a test sample that is composed of recently synthesized soluble collagen and aged covalent cross-linked insoluble collagen then the method of choice is to carry out collagen measurement by hydroxyproline determination.

Collagens are frequently cited to contain 14% hydroxyproline by weight. This value is based on mammalian type I collagens. Other species collagens can contain more or less hydroxyproline.

Many methods and modifications for the determination of hydroxyproline have been published. First the samples are placed into glass ampoules and concentrated HCl added to provide a final acid concentration of 6 N. The sealed ampoules, including ampoules containing known amounts of collagen and hydroxyproline standards, are placed in a heating block or sand bath and maintained at 110ºC for 18 hours. All the protein present, including collagen, is digested to a mixture of free amino acids, permitting the hydroxyproline to be isolated and measured by ion-exchange chromatography / ninhydrin.

Collagen Biography; Textbooks, Monographs & Practical Accounts

Short reviews suggested for initial introduction to collagen for young researchers.

Collagen: The Anatomy of a Protein, [**1980**], J. Woodhead-Galloway, **60** pages. Publisher: Edward Arnold, London.

Collagen in the Physiology and Pathology of Connective Tissue, [**1978**], S. Gay & E.J. Miller, **110** pages. Publisher: Gustav Fischer Verlas, Stuttgart.

The following collection (including the contributors within the edited volumes) were authored by many of the pioneers of collagen research.

Collagen. Structure and Mechanics, [**2008**], Editor: P. Fratzl. Publisher: Springer, New York.

Collagen. Primer in Structure, Processing and Assembly (Topics in Current Chemistry, Volume 247), [**2005**], Editors: J. Brinckmann, H. Notbohm & P.K. Muller. Publisher: Springer, Berlin.

Fibrous Proteins: Coiled-Coils, Collagen and Elastomers (Advances in Protein Chemistry, Volume 70), [**2005**], Editors: D.A.D. Parry & J.M. Squire. Publisher: Elsevier Academic Press, San Diego.

Posttranslational Modifications of Proteins (Methods in Molecular Biology, Volume 194), [**2002**], Editor: C. Kannicht. Publisher: Humana Press, New Jersey.

Extracellular Matrix Protocols, (Methods in Molecular Biology, Volume 139), [**2000**], Editors: C.H. Streuli & M.E. Grant. Publisher**:** Humana Press, New Jersey.

Extracellular Matrix, Volume 2, [**1996**], Editor: W.D. Comper. Publisher: Harwood Academic The Netherlands.

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The Extracellular Matrix Facts Book, [**1994**], Editors: Shirley Ayad, R. Boot-Handford, M.J. Humphries, K.E. Kadler & A. Shuttleworth. Publisher: Academic Press, London.

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Collagen Genes: Extracellular Matrix Genes, [**1990**], Editors: L.J. Sandell & C.D. Boyd. Publisher: Academic Press, New York.

Collagen: Biochemistry, Biotechnology and Molecular Biology, [**1989**], Editors: B.R. Olsen & M.E. Nimni. Publisher: CRC Press, Boca Raton.

Structure and Function of Collagen Types, [**1987**], Editors: R. Mayne & R.E. Burgeson. Publisher: Academic Press, Orlando.

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SIRCOL FLOWCHART