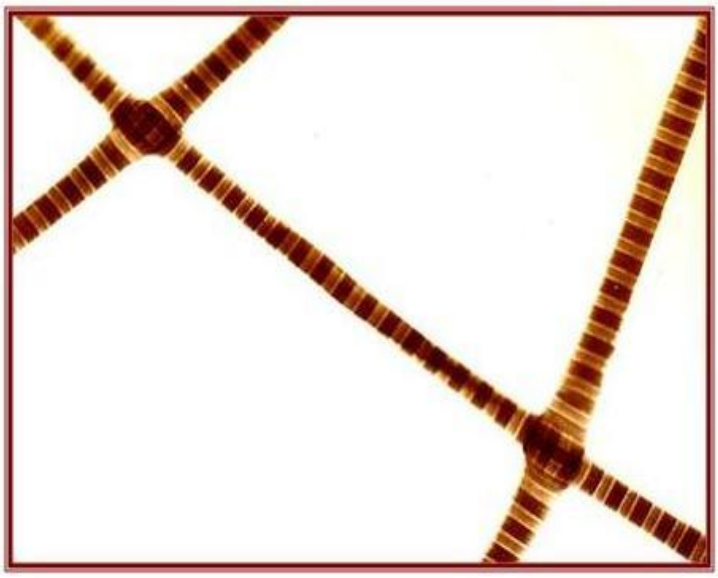
Sircol™

Soluble

COLLAGEN

Assay



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Sircol™
Soluble Collagen
Assay

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

TECHNICAL INFORMATION

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What is Sircol?

The Sircol range of dye-binding assays designed for user-friendly quantification of either soluble collagens or insoluble (crosslinked) collagens from in-vivo and in-vitro mammalian sources.

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.

What Sircol assay should I choose?

Use the **Sircol 2.0 (preferred)** or **S1000 Sircol Soluble Collagen assay** if you wish to quantify recently synthesised collagen.This is immature biochemical collagen which can be solubilised by acid-pepsin digestion.

Use the **S2000 Sircol Insoluble Collagen assay** if you wish to quantify crosslinked collagen.This is biochemically mature biophysical (structural) collagen and is resistant to acid-enzyme treatment.

Our **S4000 Sircol combination pack** can be used to measure the ratio of soluble:insoluble collagen in samples. This enables sequential analysis of both soluble and then insoluble collagen based on the same Sircol assay principle.

How does the assay work?

Addition of Sircol Dye reagent to samples containing soluble collagen results in dye-labeling and precipitation of the collagen. This is isolated via centrifugation and washed to remove any unbound dye. Collagen-bound dye is then eluted and measured spectrophotometrically (see Fig.1b for example absorption spectra of eluted dye). Unknown samples can be compared against a calibration curve to determine the amount of collagen present.

How does the Sircol dye bind collagen?

Sircol dye reagent contains Sirius Red - a linear anionic dye with sulphonic acid side chain groups (see Fig.1a. Under assay conditions the Sircol dye binds the basic groups of soluble collagen molecules. Maximal binding occurs in collagens possessing intact triple helix organisation as the highly ordered [Gly-X-Y]n helical structure of tropocollagen further contributes to dye binding. This results in a high degree of dye-collagen specificity. Affinity is progressively reduced during heat denaturation (>45ºC) due to the unwinding of the triple helix and formation of random chains.

Sample Material for Soluble Collagen Analysis

The Sircol soluble 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 tissues, cartilages and fluids.

in vitro - collagens, soluble in cold acid or pepsin, released into cell culture medium during cell growth and cell maintenance. Or 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) or 3D culture matrices.

Assay Kit Components

1. **Dye Reagent** contains Sirius Red, picric acid and surfactants. This has been formulated for specific binding to collagen under the conditions defined in the Sircol Manual.

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. **The contents of the vial must FIRST be diluted to a final volume of 100ml using 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.

7. **1.5ml microcentrifuge tubes** - for dye-labelling reaction

Components required for sample preparation - not supplied

8. **Acetic acid**, 0.5 M, store and use at 4^0C.

9. **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*. Prepare to a concentration of 0.1mg/ml in 0.5M acetic acid).

10. **Optional: Low-protein-binding microcentrifuge tubes (1.5ml)**.
    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: *EP0030108116-100EA*).

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 '***General Protocol'*** (supplied on separate sheet with each kit) 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 2.5µg/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. Samples from older organisms may require two enzyme extractions to fully recover pepsin soluble collagen.

COLLAGEN EXTRACTION USING COLD ACID-PEPSIN PROTOCOL

Incubation of samples overnight at 4°C with the enzyme Pepsin is an effective means of removing collagen's terminal non-helical telopeptides. This results in release of collagen into solution where it can be subsequently quantified by following the Sircol General Protocol.

Acid Pepsin digest protocol

1. Prepare Pepsin at a concentration of 0.1mg/ml in 0.5M acetic acid
2. Add the prepared Acid-Pepsin solution to sample in a ratio of 1:10 or 1:20. *For example, if the sample weighs 10mg, add 100 or 200 µl acid-pepsin solution.*
3. Incubate overnight at 4°C with mechanical shaking.
4. Centrifuge tubes at *3000 x g* for 10 minutes to move any residual tissue to the bottom of the tubes.
5. Transfer 100µl of supernatant into a new tube and continue with General Protocol. *Supernatant may be frozen (-20°C) at this stage.*

Having examined several pepsin preparations we found that Sigma-Aldrichs pepsin product, (E.C. 3.4.23.1 Product Code: *P7012*.) forms a suitable transparent colourless solution.

Digestion of In-vitro samples:

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°C.

This allows soluble collagen recovery from Extracellular Matrix formed on cell culture treated T-flask, microwell plates and 3D cell culture scaffolds. *See examples on p6.*

Further information for *in-vivo* derived samples:

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

Samples derived from older animals may require a second acid-pepsin extraction to release all acid-pepsin soluble collagen.

Soft tissues (muscle, liver, lung etc.)

Lung and liver samples can be difficult to 'clean-up' as they are often blood saturated. It is advisable to dice these samples into small cubes before rinsing in cold phosphate buffered saline. The acid-pepsin extraction can then be performed as described above.

Please note that Acid-pepsin collagen levels are often present at less than 2.5µg/100µl of extract. Such samples can be processed using 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.

Setup :

***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.

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

Collagen Concentration:

1. Add 1.0ml of sample to tube. Include a sample of unused cell medium and/or extract solvent as blank control.
2. Add 100µl **Acid Neutralising Reagent** to 1.0ml of acid extracts,(0.5M acetic acid ± pepsin), from tissues.
   This step is <u>not</u> required for culture medium samples
3. To each tube add cold **Isolation & Concentration Reagent** (200µl/ tube). Vortex to mix contents (3x5 seconds).
4. Incubate at 4°C for 15 minutes (we recommend using a floating microtube rack in a pre-cooled water bath).
5. Remove tubes and vortex each tube a further 3x 5-seconds.
6. Incubate at 4°C for a further 15 minutes.
7. Centrifuge tubes at *13000 x g* for 10 minutes.
8. Decant supernatant by gently inverting tube over a waste vessel. Keeping the tube inverted, gently place onto a folded paper towel with the opening in contact with the paper - allowing residual fluid to be removed by a wicking action. A cotton bud may be used to gently remove any fluid on the upper part of the tube and the tube cap and rim.

Washing of Collagen Precipitate

9. Add 1000ul of cold (4°C) diluted **Acid-Salt Wash Reagent** to each tube. Gently invert tubes 3x to ensure all interior surfaces are rinsed.
10. Spin *13000 x g* for 10 minutes
11. Decant and drain residual fluid onto paper towel as in step 8.

DO NOT ATTEMPT TO REMOVE FLUID BY TAPPING OR PIPETTING AS THIS CAN DISLODGE PRECIPITATED COLLAGEN.

Collagen Assay:

Tubes should now contain any precipitated and purified collagen. This may be labeled through the addition of Sircol Dye Reagent (1000ul) and continuing with the **'General Protocol'**.

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 2.5µg/100µl 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 following manual sections contain supplementary details that should be read through, alongside the supplied '***General Protocol' (supplied on separate sheet with each kit)***, 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. 2.

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. 1b 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. 1b.

The Collagen Reference Standard curves were obtained using a microplate reader and are presented in Figs. 2a & 2b to offer a guide for filter selection in other microplate readers.

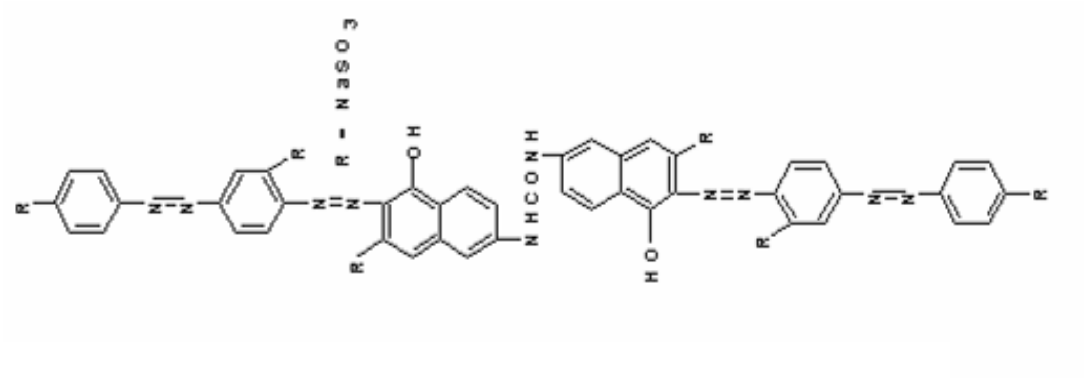

Fig. 1a: Molecular structure of Sircol Dye (Sirius Red).

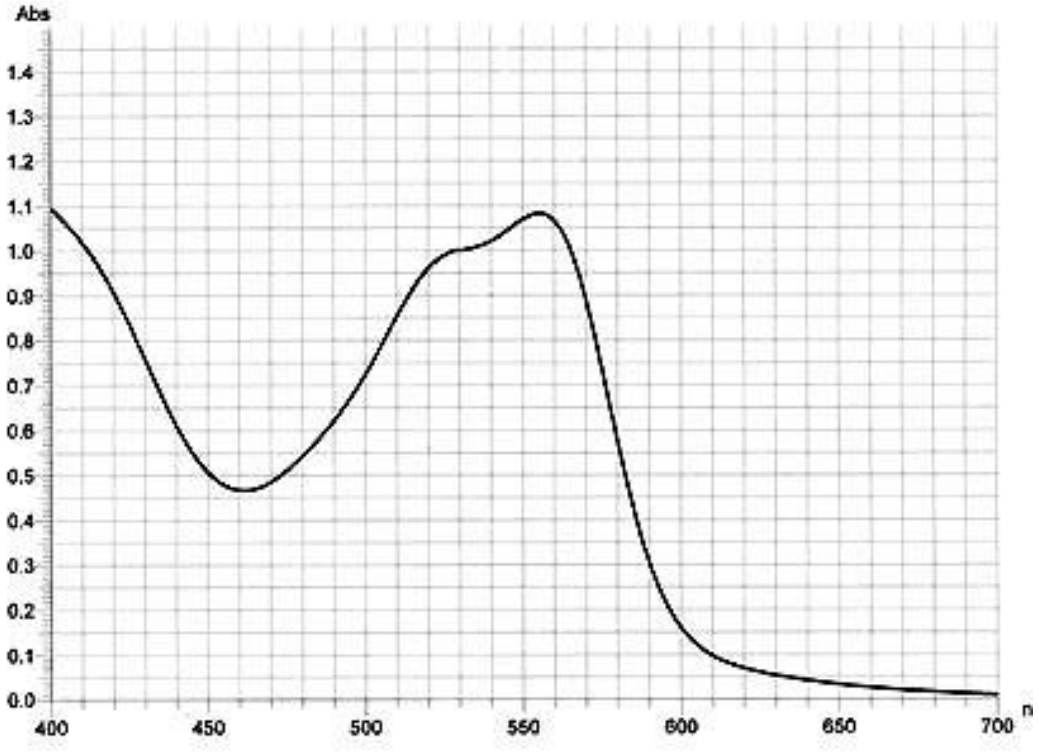

Fig. 1b: Absorption spectrum of the Sircol Dye in Alkali Reagent. Sirius Red is an anionic dye with sulfonic acid side chain groups. These groups react with side chain groups of the side chain present in collagen. The affinity of the dye for collagen is due to the elongated dye molecules becoming aligned parallel to the long rigid structure of native collagen.

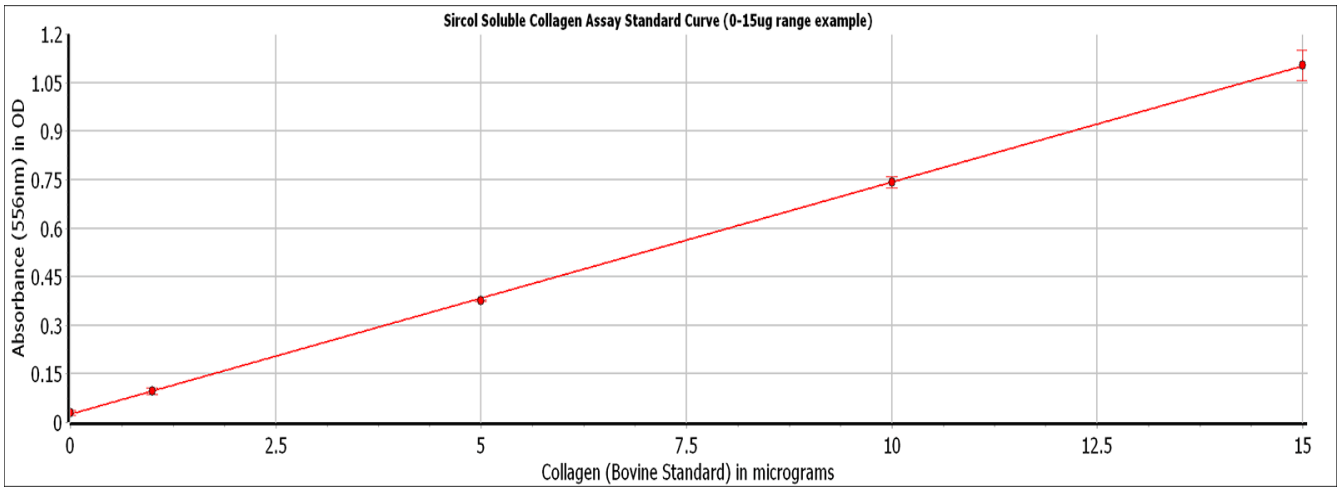

Fig. 2a: Collagen Reference Standards, 0 – 15 µg
using 250µl of Alkali Reagent to recover the collagen bound dye.

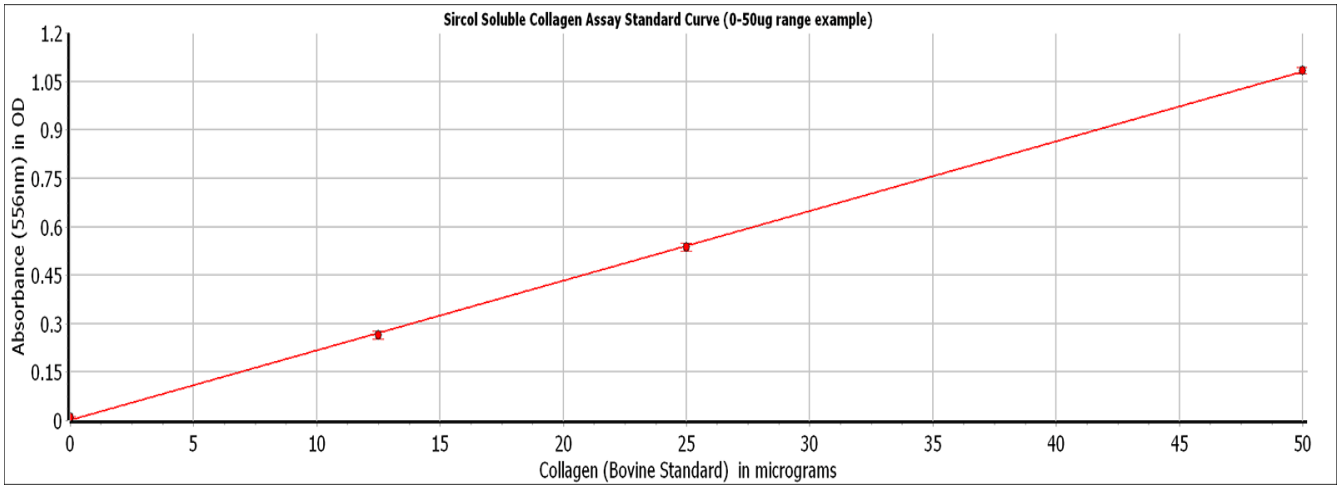

Fig. 2b: Collagen Reference Standards, 0 – 50.0 µg
using 1000µl of Alkali Reagent to recover the collagen bound dye.

Collagen Biography:

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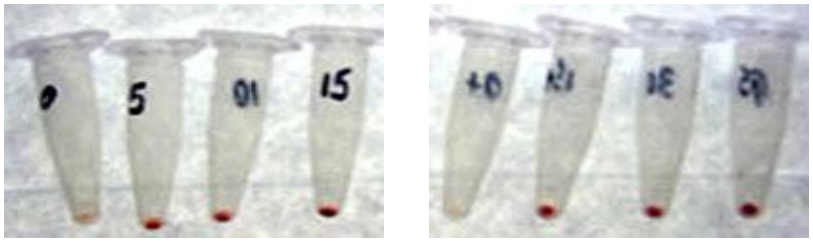
(a)



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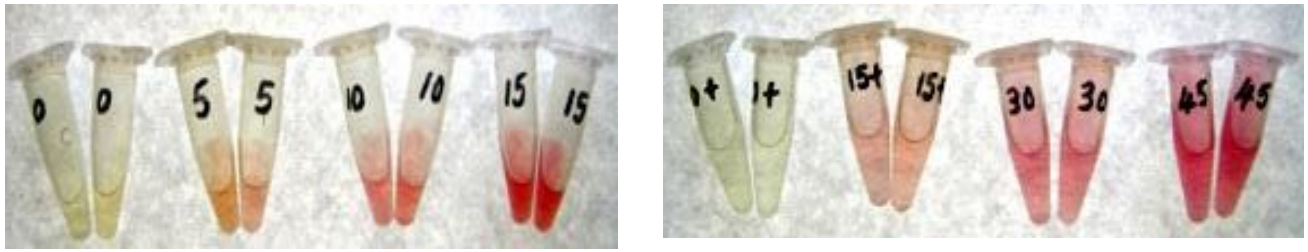

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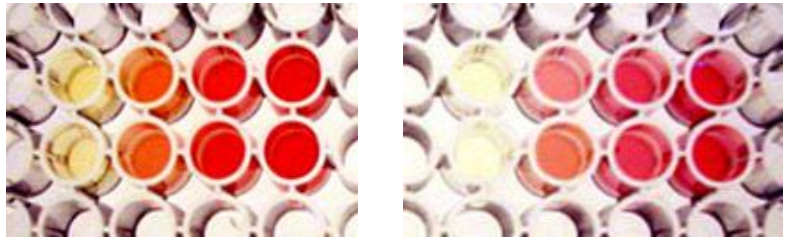

Fig. 3 (a) Sets of collagen standards, Low range 0, 5, 10 & 15 µg and High range 0, 15, 30 & 45 µg, following collagen-dye mixing, centrifuging and removal of unbound dye, (duplicates not shown).
(b) Low Standards after adding 250 µl of Alkali Reagent.
High Standards after adding 1000 µl of Alkali Reagent.
(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.

Sircol Soluble Collagen Assay
General Protocol

Detection Limit: **1.0 µg/100µl (10µg/ml)**

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 0.5 M acetic acid or fresh cell culture medium or extraction buffer.
- Collagen standards - Depending on the assay range required prepare aliquots containing (at least) the following:

a. 0,5, 10 and 15µg of Collagen Reference Standard.

b. 0,10, 25 and 50µg of 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 13000 *x g* 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. Longer centrifuge times can be used if required.

Washing and Draining

Gently layer on 750µl of 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. *NB: do not use the Acid-Salt Wash in it's concentrated form.*

Centrifuge at 13000 *x g* 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 Alkali Reagent to reagent blanks, standards and samples as follows:

a. Add 250µl Alkali Reagent if using Collagen Reference Standard in the range 0 - 15µg.

b. Add 1000µl Alkali Reagent if using Collagen Reference Standard in the range 0 - 50µg.

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

Measurement

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.

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 556nm, 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.