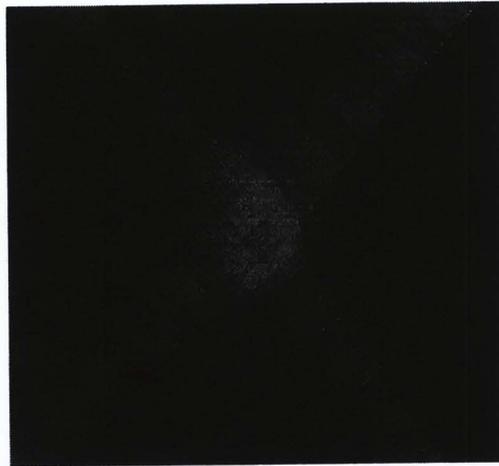


เอกสารแนบที่ 2

Sircol™
Soluble
Collagen
Assay

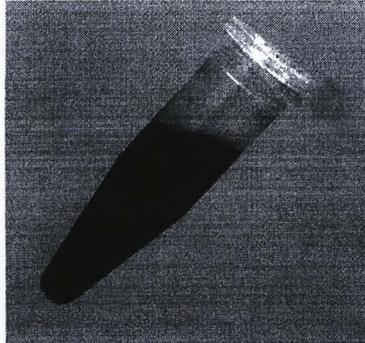


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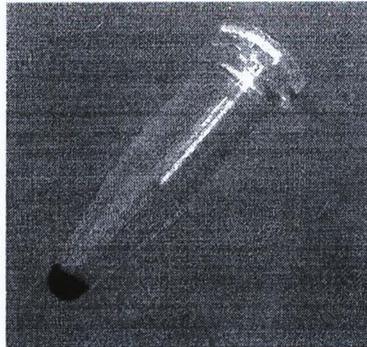
Assay Reference Images

+30min



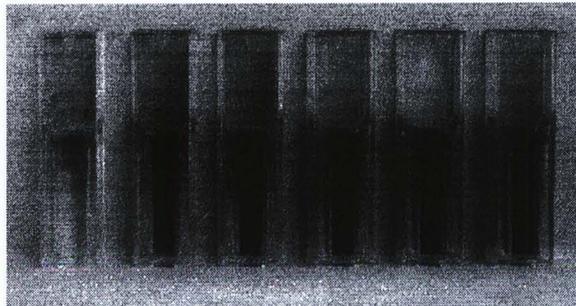
Mix Sircol reagent and collagen reference standard

+40min



Centrifuge and remove supernatant carefully

+50min



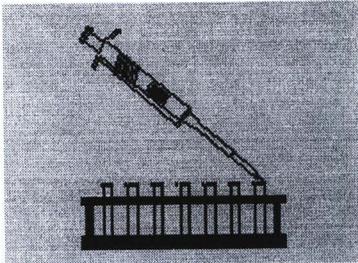
Add alkali reagent
(picture obtained using 0-50 μ g collagen reference standard)

email: info@biocolor.co.uk

Sircol Soluble Collagen Assay

Time Req: 1 hour

Detection Limit: 2.5 μ g*



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 μ l of distilled water or the test sample buffer).
- [2] Collagen standards: (aliquots containing 5, 10, 25, 50 μ g).
- [3] Test samples, (volumes: 10 to 100 μ l *).
Adjust the contents of all tubes to 100 μ l with distilled water or appropriate buffer.

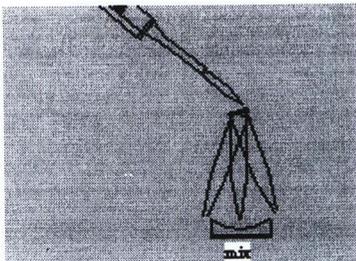
To each tube add 1 ml Sircol Dye reagent and cap all of the tubes; mix contents by inverting.

Mixing:

Place tubes in a mechanical shaker for 30 minutes, (or manually shake at 5 minute intervals).

During this time period the Sircol Dye will bind to soluble collagens.

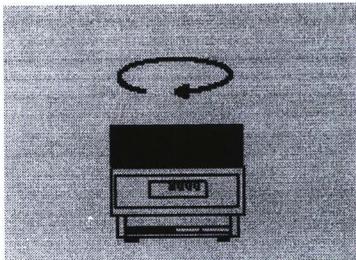
The dye reagent is designed so that the collagen-dye complex will precipitate out of solution.



Centrifuging:

Transfer the tubes to a micro centrifuge and spin the tubes at >10,000 x g for a 10 minute period *.

It is important to firmly pack the insoluble pellet of the collagen-dye complex at the bottom of the tubes, so as to avoid any loss during draining.



Draining:

The unbound dye solution is removed by carefully inverting and draining the tubes.

Any remaining droplets can be removed from the tubes by *gently* tapping the inverted tube on a paper tissue or a cotton wool bud can be used for removing droplets of dye from the rim of the tubes.

Do not attempt to physically remove any fluid that is in close contact to the deposit.

Release of bound dye:

To each tube add 1 ml of the Alkali reagent.

Re-cap the tubes and release the bound dye into solution. A vortex mixer is suitable.

When the bound dye has been dissolved, usually within 10 minutes, the samples are ready for measurement.

The colour is light stable, but should be read within 2 to 3 hours.

Keep the tubes capped until ready for measurement.

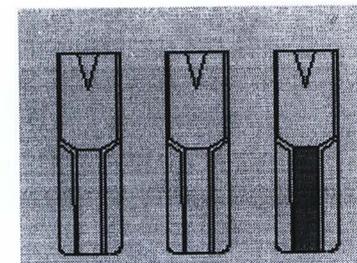
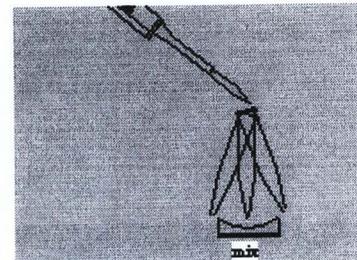
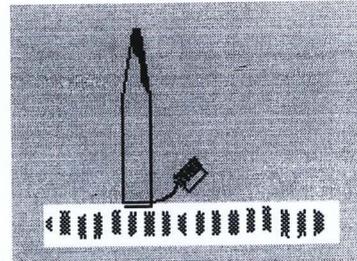
Measurement:

- (a) Spectrophotometer, set wavelength to 540 nm.
Use semi-micro glass, or plastic disposable cuvettes.
- (b) Colorimeter, set using a blue-green filter.
Use semi-micro cuvettes or tubes.
- (c) Multiwell plate reader, set using a blue-green. Transfer 200 μ l aliquots of samples from tubes to the wells of a 96 well, multiwell plate.

Set the above instruments to zero using water. Measure absorbance of reagent blanks, collagen standards and the test samples.

Subtract the reagent blank reading from the standard and test sample readings. Check duplicates are within $\pm 10\%$.

Plot standards on graph and use the graph to calculate the collagen content of the test samples. (see manual)



***Sircol*[™]**

Soluble Collagen

Assay

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

**Handle the
Sircol Assay Kit
using**

GOOD LABORATORY PRACTICE

Read Manual Before Use

5th Edition 2007

***Sircol* Manual**

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Sircol Assay

Manual

Intended Applications:

The Sircol Collagen Assay is a quantitative dye-binding method designed for the analysis of acid-soluble collagens extracted from mammalian tissues and collagens released into culture medium by mammalian cells during *in vitro* culture.

Collagen forms that can be measured:

- [i] salt-soluble collagens (1 M NaCl in 0.05M Tris, pH 7.5.)
- [ii] acid-soluble collagens (0.01 to 1.0 M acetic acid)
- [iii] pepsin-soluble collagens (0.5 M acetic acid + pepsin)
- [iv] total soluble collagens (composed of [i],[ii] & [iii])

Collagen Types that can be measured:

- [1] Mammalian collagens, Types I to V, can be measured
- [2] Collagen Types VI to XIV can be assayed, but have not been calibrated due to insufficient purified material available for the preparation of standard curves
- [3] Sircol dye binding does not discriminate between collagen types
- [4] The dye reagent binds specifically to the [Gly-X-Y]_n helical structure found in all collagens
- [5] Sircol dye binding decreases, gradually with the thermal denaturation of collagen (collagen to gelatin)
- [6] Non-vertebrate collagens bind less dye, due in part to lower denaturation temperature and less hydroxyproline residues, and would require species matched purified preparations for standard calibration curves

Sircol Assay Kit components:

- [1] The dye reagent contains Sirius Red in picric acid and has been formulated for specific binding to collagen under the conditions defined in the Sircol Assay Manual.
- [2] Alkali reagent contains 0.5 M sodium hydroxide, for releasing Sircol Dye from the collagen-dye complex.
- [3] Salt soluble collagen precipitating reagent; contains L-lysine monohydrochloride.



- [4] Collagen standard, acid soluble Type I, supplied as a sterile solution in 0.5 M acetic acid within a sealed vial. Concentration: 1 mg/ml.
This is sterile bovine skin collagen that has been imported from the USA, and was obtained from disease free animals.
In countries that forbid the importation of bovine derived material, a rat tail collagen standard, acid soluble Type I, is supplied as a sterile solution in 0.5 M acetic acid within a sealed vial. Concentration: 0.5 mg/ml.
- [5] This Sircol Assay Manual; (also available as a pdf, see www.biocolor.co.uk)

Other components required, but not supplied:

- [a] Capped 1.5ml capacity microcentrifuge tubes
- [b] Variable volume micropipettors and pipette tips
- [c] A mechanical mixer for the microcentrifuge tubes. Any equipment that provides consistent shaking, rolling or rotation of the tubes is suitable
- [d] A centrifuge with a 1.5ml tube rotor head and capable of at least 10,000 x g, to firmly pack the collagen-dye pellet.
- [e] A spectrophotometer, a colorimeter or a microwell plate reader with a blue-green filter.

Recommended storage conditions for components:

Unopened;

All components have been prepared for long term stability (at least 12 months), when stored at room temperature.

Do not freeze as complete re-solubilisation may not occur.

Opened;

The assay components will retain their shelf-life, providing the glass vials of collagen standard and lysine solution are:

- (i) stored at +4°C when not in use
- (ii) the metal seal is not removed

The contents of both vials are best sampled as follows:

Remove the centre metal disc only from the vial tops. Obtain aliquots from the vials, when required, using a plastic syringe fitted with a sterile hypodermic needle. The butyl rubber seal on the vial has a thin centre disc, suitable for needle insertion into the vial. Discard vial if solution contents become turbid.

Mode of action of the Sircol dye reagent with soluble collagens:

The Sircol dye reagent contains Sirius Red. The Colour Commission name is Direct Red 80. The molecular structure of the dye is shown below (Fig. 1).

Mechanism by which the dye reacts with collagen:

Sirius Red is an anionic dye with sulphonic acid side chain groups. These groups react with the side chain groups of the basic amino acids present in collagen.

The specific affinity of the dye for collagen, under the assay conditions is due to the elongated dye molecules becoming aligned parallel to the long, rigid structure of native collagens that have intact triple helix organisation.

Dye affinity is much reduced when collagen is heat denatured (>45°C to the form of random chains of gelatin) and the triple helix unwinds.

Dye binding is gradually lost when collagen (and gelatin) are exposed to bacterial collagenases.

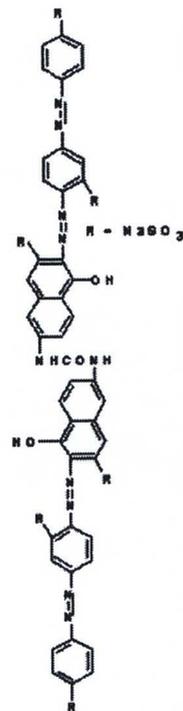


Fig. 1

The molecular structure of the Sircol Dye

Test sample compositional requirements:

As the Sircol Assay is a colorimetric procedure, samples for analysis should be free of any particulate material (cell debris and insoluble extracellular matrix).

- [1] The test sample can be in a salt buffer solution, acetic acid or culture medium.
- [2] If a surfactant has been used during tissue extraction, it is recommended that this extraction solution is Sircol tested with the Reference Collagen Standard to check that it has no adverse effects on collagen-dye binding.
- [3] The presence of soluble proteins in samples, including proteoglycans, tropoelastin and other soluble ECM materials, does not interfere with the Sircol Assay.
- [4] Cell culture medium with foetal calf serum supplements of up to 5% does not interfere with the collagen assay. When higher serum supplements have been used, the increasing bulk of serum proteins, relative to the amount of collagen present, can cause problems.

In this latter case the following options could be examined:

- [a] reduce the serum supplement to 5%, either after cell attachment has occurred, or reduce serum to 5% for the culture medium that will be collected and used for assay;
- [b] selectively remove the bulk of the serum albumin from the test sample by affinity chromatography (Blue-Sepharose CL-6B);
- [c] precipitate the collagen out of solution, adding NaCl to a concentration of 4 M for samples with neutral pH; or 2 M NaCl for samples in acetic acid. Centrifuge, drain well and re-solubilize the collagen pellet in 0.5 M acetic acid.

Assay of test solutions containing < 25 µg/ml collagen, without prior concentration:

Method

The Sircol Dye Reagent contains sufficient dye to permit test sample volumes to be increased up to 200 µl, before adding 1.0ml of Dye Reagent.

Note 1

It is not recommended that more than 200 µl of sample volumes be used with 1.0 ml of Dye Reagent. Excessive dye dilution, by increasing sample volumes above 200 µl, can result in loss of collagen dye saturation.

Note 2

For test samples with collagen levels less than 25 µg/ml, but with more than 5 µg/ml, it is possible to directly obtain assay results without sample concentration by *increasing* both sample and dye volumes, using larger volume conical centrifuge tubes.

Add 1000 µl of test solution, followed by 5.00ml of Dye Reagent, contained in a 15 ml capacity conical centrifuge tube. The collagen bound dye complex, recovered after centrifugation, is then solubilised in 1.00 ml of the Alkali Reagent.

No multiplication factor is required, when using 1.00 ml test samples/5.00 ml dye volumes, to express the results as µg/ml.

Set up assay:

To duplicate 1.5 ml microcentrifuge tubes, add sample volumes of between 10 and 100 μ l:

- (a) collagen standards
- (b) test samples and reagent blanks (100 μ l of 0.5 M acetic acid, extraction buffer or fresh unused tissue culture medium).
Adjust the volume in all tubes to 100 μ l.

Working Standards:

It is recommended that the collagen standard is initially run, in duplicate, at three concentrations; using 12.5, 25 and 50 μ l aliquots of the supplied Reference Collagen Standard.

The standards, with the reagent blanks, are used to produce a calibration curve with the selected spectrophotometer, colorimeter or microwell plate reader.

In subsequent assay batches a minimum requirement is duplicates of a mid-range collagen standard and reagent blanks.

In repeated assays these secondary standard and reagent blanks should give absorption values, at 540 nm, to within \pm 5% of that defined by the initial standard curve.

Test samples:

With test samples, where the approximate collagen concentrations are as yet unknown (collagen absorption at 280 nm is very low due to the limited number of aromatic amino acids and, therefore, cannot be used as a guide), 50 μ l aliquots are suggested for the first run.

If required use more or less, in the next batch of assays to bring all test sample readings within the concentration range covered by the standard curve.

Dye reagent and mixing:

Add 1.0 ml Sircol dye reagent to all tubes. Cap tubes and mix contents. First by inversion, as the density/viscosity difference between the sample and the dye reagent can differ considerably. Then gently mix tube contents at room temperature for 30 ± 5 minutes.

The same mechanical mixer, at the same setting, should if possible be used for all assay batches to minimise experimental variations.

Centrifuge to recover collagen-dye complex:

Transfer tubes to a microcentrifuge and centrifuge at 10,000 x g for 10 minutes. This operation is to pack the collagen-dye pellet at the bottom of the tubes.

A minimum RCF of 10,000 x g is required; higher values, if available, can be used as this will force more unbound dye out of the pellet. This also reduces the risk of any pelleted material being lost when the tube contents are decanted.

Removal of unbound dye:

Remove tubes from centrifuge and uncap. The supernatants are drained off and discarded. The collagen bound dye remains as a pellet at the bottom of the tubes.

While the tube is still inverted, use an absorbent paper tissue or a cotton bud to remove any dye solution from the top end of the tube wall. Do not remove any beads of fluid close to the collagen-dye precipitate on the side wall of the tube.

The Sircol dye reagent contains a surfactant to aid draining from the non-wettable plastic tubes. A wash step is **not** recommended, but may be considered necessary for low concentration test samples. Wash with 500 μ l of ethanol (99% pure; and methanol free).

Do not wash with water. An ethanol wash step can reduce reagent blank readings close to zero, but increases the experimental variation between duplicate samples to above that obtainable without the wash step. This is due to the dye precipitate not packing as firmly after the ethanol wash, which causes a loss of some bound dye during the second draining step.

Recovery of collagen bound dye:

To the collagen-dye pellet add 1.0 ml of the Alkali reagent and then cap the tubes. Bring the collagen bound dye back into solution; a vortex tube mixer is most convenient, but holding the top of the tube in one hand while flicking the bottom of the tube with a finger of the other hand is also effective.

The dye should be in solution within 5 minutes. Pellets that are centrifuged at a high RCF may take a little longer. Gelatin samples can require several mixing operations to fully release gelatin-bound dye.

Measurement of collagen bound dye:

The alkaline dye solution is stable to indoor light, but should be measured within 3 hours. Ensure tubes remain capped, to avoid loss due to evaporation, until ready for reading.

Spectrophotometer;

Use semi-micro glass, or plastic disposable cuvettes. Set the instrument wavelength to 540 nm and use water to set the absorbance reading to zero. Read and record the absorbance values of assay blanks, standards and test solutions.

Colorimeter;

The instrument should have a sample cell or cuvette suitable for reading test volumes within 1 ml, and a 1 cm light path length.

The range of filters in colorimeters varies. A blue-green filter, often labelled 500, 510 or 550 nm, will usually be found suitable. To confirm that the filter selected is suitable, use the three concentrations of collagen standards. Ensure that these readings produce a straight line standard curve, that passes through zero.

Microwell plate colorimeter;

Transfer 200 μ l aliquots of the alkali dye solutions from the assay tubes to the wells of a microwell plate.

The selection and testing of a suitable colour filter should follow the recommendations given for the colorimeter above.

Calculation of collagen concentration in test samples:

Subtract the reagent blank reading from all the standard and test readings. The reagent blank value should be between 0.15 and 0.18 (a guide range only – based on the equipment used). Higher values (>0.25) indicate that the draining and removal of unbound dye technique could be improved.

When low reagent blank values are obtained consistently, it may be more convenient to set the measuring instrument to zero with the reagent blank; thus avoiding the need to subtract the background value from the test samples.

Monitor the variation in absorbance readings between duplicate samples. Initially some wide variations may occur. Assuming that this is not due to a pipetting error, the most likely source of error is the draining step. A little practice with draining and drying of the top of the tubes leads to a consistent mode of practice, and the ability to bring experimental error of duplicate samples to within $\pm 5\%$ of the mean.

Using a computer spreadsheet with graphical output, plot the three Collagen Reference Standard absorbance means against their known collagen concentrations. On joining the points, these should produce a straight line graph which can then be extended to pass through zero (absorbance and concentration).

Test sample absorbance values can now be read off the graph to determine their collagen concentration. Readings below 0.05 and higher than 1.00 are unreliable and should be re-assayed – after either concentration or dilution of the test material.

Where test material contains uncommonly used salts, detergents or biological molecules, it may be necessary to initially evaluate their suitability with the assay. This can be performed by adding known amounts of the Collagen Reference Standard to the test samples and checking collagen recovery, following completion of the assay.

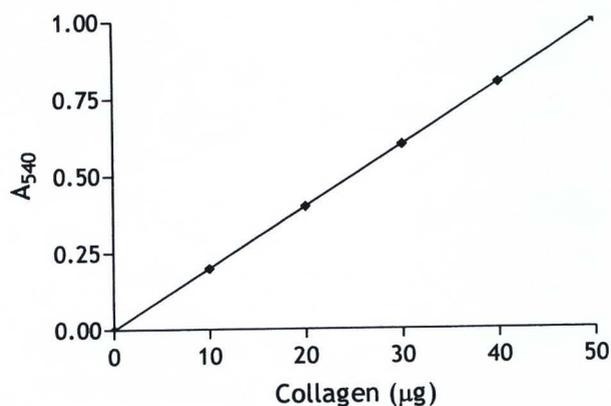


Fig. 2 A typical straight line calibration curve for dye bound by acid soluble collagen.

The above calibration graph was prepared using aliquots of the collagen standard solution; according to the assay procedure outlined on the inside cover.

TEST SAMPLE PREPARATION

General comments

Collagen is ubiquitous to all animals and is found within, or surrounding, all tissues and organs. Collagen is the most abundant animal protein, accounting for about one third of the total protein of mammals.

The range of collagen types and their functional roles ensures that they play a key role in all aspects of growth, maturity, pregnancy and ageing. Because of their widespread occurrence, changes to collagens occur in most chronic and some acute diseases.

Given the diversity of animal species and tissue material that are used to study the above processes, there can be no single universal sample preparation procedure. Some of the more commonly used preparations and extractions are described below. A further valuable source of information is in published research papers (see coloured insert for recent research papers that have used the Sircol Assay).

IN VITRO STUDIES

Application for low collagen concentrations in cell culture medium:

Common practice during cell culture is to employ the culture medium at a volume of ~0.25 ml/cm² surface area of the selected culture flask, dish or plate. This culture medium ratio has been found to provide an acceptable balance between providing sufficient medium to meet the cells nutritional requirement, while avoiding excessive concentrations of cellular metabolites that accumulate during culture. The 0.25 ml/cm² ratio also ensures that culture medium volume, above anchorage dependent cells, does not unduly restrict exchange of CO₂ and O₂.

When monitoring the secretion of collagen the 0.25 ml/cm² culture medium ratio can frequently produce collagen concentrations of ~ 5 µg/100 µl (50 µg/ml) in the culture medium.

The amount and rate of production of collagen can vary considerable from this 'average' value under various test conditions; collagen synthesis is often found to increase as the cell population nears confluence.

Examination of the 'standard curve' in the Sircol manual reveals that for test samples with collagen concentrations of ~5 µg/100 µl the absorbance reading is too close to the reagent blank absorbance value to provide confidence in the collagen value obtained.

The need to concentrate test samples, prior to the assay, would cause a substantial delay and additional work before results can be obtained. A delay that detracts from a major benefit of using the Sircol Assay – the convenience of obtaining results in one hour.

For direct procedures to measure test samples with collagen concentrations below 25 µg/ml, and more than 5 µg/ml, see page 4.

IN VIVO STUDIES

Extraction of soluble collagens from tissue, cartilage and organs:

Samples for analysis should be collected under aseptic conditions, where possible. Material sampled post-mortem should be collected as soon after death as possible. Briefly wash the external surface with sterile water or saline to remove any debris and blood stains. If the sample contains attached adipose tissue this should also be trimmed off using a scalpel.

If extraction is not to be carried out immediately, then the samples should be placed into labelled, sealable, plastic envelopes and frozen as quickly as possible (weigh prior to freezing). Do not store at 0 to 5°C, even if extraction is to be performed the following day. The major risk at this early stage of preparation is proteases; these are released by dead cells and by contaminating bacterial enzymes (many active at low temperatures).

Stored frozen samples are best 'thawed-out', in the plastic envelope, within a refrigerator at 5°C. Decide if collagen content of the test samples is to be expressed as 'dry weight' or 'wet weight'. If dry weight, it will be necessary to take a representative sample, obtain its wet weight and then dry the sample in a heated, or un-heated, drying cabinet containing drying granules. The samples are weighed daily until a constant dry weight value is obtained; most tissues and cartilages are ~ 70% water.

To optimise collagen extraction the tissue sample should be 'diced' into small cubes, using a sharp scalpel. Avoid producing cubes of less than 2 to 3 mm as the 'squeezing' of these small tissue particles can result in fluid being lost from the cut surfaces. Weigh the prepared samples into sterile flasks or beakers. Use as large a weight sample as possible. You cannot have too much collagen and larger sample sizes also reduce variation, due to non-homogeneous collagen distribution within the tissue.

[1] Salt soluble collagens

The salt soluble collagen fraction represents the most recent collagen secreted by the cell. Within a few hours in the extracellular matrix (or in the cell culture medium) this salt soluble collagen (tropocollagen monomers) will 'crystallise' into collagen fibrils and become salt insoluble. The salt soluble collagen fraction will be small, needing a large sample weight to produce Sircol detectable amounts of collagen (>2.5 µg). The salt soluble collagen fraction is also the most vulnerable to protease degradation.

Extraction: Salt soluble collagen solvent is a 0.05 M Tris buffer, pH 7.5, containing 1.0 M sodium chloride. This solvent should also include a 'Protease Inhibitor Cocktail' (ready to use cocktail mixtures are available from Sigma-Aldrich).

Beware if preparing a DIY cocktail from dry components, as many of these agents are **toxic**.

Extraction solvent volumes will depend on the material being extracted. A 10 volumes of solvent to wet tissue weight ratio is suggested. The sample should be stirred overnight at 0 to 5°C. To obtain a transparent solution, containing the salt soluble collagen, centrifuge at 15000 x g for 60 minutes. As the Sircol Assay is a colorimetric assay, turbid or translucent extracts are not suitable for analysis. If centrifugation does not produce a transparent supernatant, consider filtering this solution through a 0.4 or 0.8 µm filter unit. Initial trials should be performed on non-essential tissue; to determine test sample weights, weight to solvent ratio and whether a second extraction of the residue is required for quantitative extraction.

[2] Acid soluble collagens

Dilute acetic acid (0.5 M) solubilises non-cross linked, and some cross linked forms of collagen. The pH of 0.5 M acetic acid is ~3.0, so as with salt extraction a protease inhibitor cocktail is recommended. The solvent to tissue ratio and extraction times are also similar to salt extraction.

This method of extraction represents the quickest and simplest procedure for recovering the recently synthesised collagen pool from tissues. The yield of acid soluble collagen recovered will be dependent on the age of the animal (more collagen is synthesised during early growth).

In adult animals most of the collagens have long term stability, usually exceeding the life span of the animal. Increases in acid soluble collagens, however, are found in various disease processes; where the extracellular matrix is being destroyed or collagen is being laid down to replace cell loss in tissues and organs.

[3] Pepsin soluble collagens

This extraction procedure is usually the 'method of choice' for recovering the recently synthesised collagen pool from tissues. It produces a larger yield of collagen than when 0.5 M acetic acid is used alone.

This is due to pepsin cleaving off part of the C- terminal, non-helical region of the alpha-chains that make up the triple helix of tropocollagen. This C- terminal non-helical region contains the initial covalent cross link between the alpha-chains. This cross-link aligns the three tightly wound left handed helices, permitting them to form the right handed super helix of tropocollagen. In the ECM further cross-linking occurs between adjacent alpha-chains and other tropocollagen molecules that are packed into the forming collagen fibrils.

The pepsin (EC 3.4.23.1) should have good activity, and is dissolved in 0.5 M acetic acid. As a general rule, use about a 1:10 ratio of pepsin: tissue wet weight. Aseptic conditions should apply during overnight extraction at room temperature. Stir vigorously during this time period.

Treatment of Extracts prior to Assay:

The Sircol Assay is a colorimetric assay and it is essential that test samples are transparent. Opalescence or turbidity will result in the non-specific attachment of Sircol Dye to suspended material. Coloured transparent solutions are suitable. It is not uncommon to have reddish-brown extracts due to the presence of haemoglobin and/or myoglobin. These soluble proteins do not cause interference with the Sircol Assay.

When turbidity occurs, it should be removed prior to assay. High speed centrifugation is often effective. Filtering a small representative aliquot of the test solution through a 0.4 or 0.8 μm filter unit (attached to a 2 or 5 ml syringe) is also usually effective.

Final Note: Beware of microbial protease activity; assay samples as soon as possible following extraction.

Cross-linked insoluble collagen:

The fraction of collagen remaining in the tissue residue after salt, acid and pepsin extraction is insoluble, covalently cross-linked collagen. Extraction treatments that can solubilise this collagen do so by either causing peptide cleavage into fragments or by denaturation of the collagen to gelatin.

Gelatin can be measured by the Sircol assay, but the degree of protein denaturation effects how much dye will be bound. If heat is used to solubilise the insoluble collagen, then providing the temperature/time are standardized, and similarly treated insoluble collagen standards are included as controls, the assay can be used to measure insoluble collagen.

Extraction procedure:

Insoluble collagen when suspended in water and heated at temperatures above 60°C will be gradually converted into water soluble gelatin. The time required will depend, in part, on the nature and frequency of cross-links and the collagen: water ratio. However, the major factor that the time will depend on is the temperature used for extraction. The higher the temperature the shorter the time required, but also the more collagen that is denatured. The more collagen that is denatured the less Sircol dye that can be bound by the gelatin.

The time-temperature effect on dye binding by gelatin is shown below. A temperature of 80°C is recommended, as this permits accurate temperature control by using a water-bath with a thermostat and a lid to reduce evaporation loss. It is important to ensure that all of the insoluble collagen has been solubilised from the test samples. Use small samples of test material and run similar weights of insoluble collagen standards as controls. Insoluble collagens are readily available from biochemical suppliers at low cost.

The heat extraction procedure, although prolonged, can after the initial calibrations be performed with limited supervision.

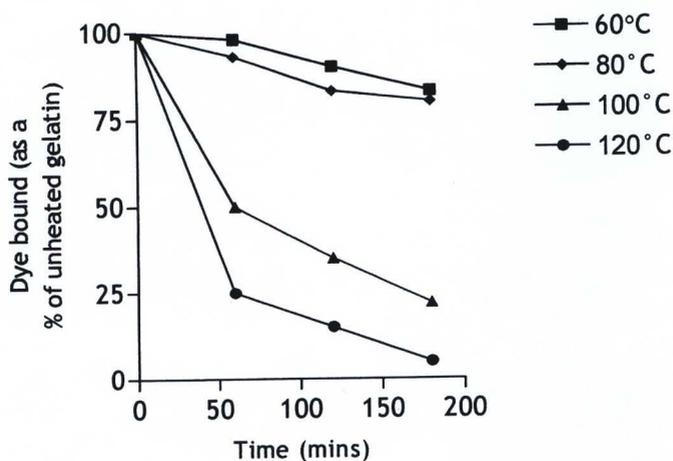


Fig. 3

The effect of temperature/time on gelatin, and the subsequent dye-binding of the Sircol Dye

Collagenase assays:

These assays aim to examine test material for the presence of collagenase activity or the presence of activators/inhibitors of collagenase. Studies to determine the amount of collagen remaining at known time intervals, or at a fixed time periods with increasing concentrations of the test agent, can usually be completed within one working day.

The collagen standard supplied with the Sircol Assay Kit can be used as a collagenase substrate. If larger quantities are required, a convenient source is rat tail tendons. Some commercial sources of 'acid-soluble' collagens may be found to be only ~40% acid-soluble; this appears to be due to the acid-soluble collagen 'ageing' (in the presence of a trace of moisture) during storage. Freshly prepared acid-soluble collagen is a preferable substrate.

Collagenase activity assay:

To dried, formaldehyde vapour fixed, collagen and/or gelatin films on microwell plates add test solutions, buffer blanks, reference enzymes etc. Adjust all test volumes to a common value e.g. 100 μ l. Seal the wells by covering the plate in 'cling film' or cover the top surface with clear plastic adhesive tape strips.

Incubate at the optimum temperature for the enzyme(s) under investigation. The time required for 20-50% digestion of the substrate(s) will need to be determined. With bacterial collagenase of ~10 U, a 3 hour period at 20°C, aided by the use of a rocking type mixer, can digest ~40% of the collagen film (this data is offered as a starting guide, results can vary considerably due to differences in enzyme purity and activity). On completion of the incubation period remove the plastic film from the plate.

Drain the contents from the wells into a sink. The plate is then inverted and allowed to drain dry on a paper towel. Tap the plate firmly on to the towel so as to dislodge any fluid remaining within the wells. Replace the plate, open-well side up, and add 200 μ l Sircol dye reagent to each well. Carefully cover the fluid-full wells and leave for 30 minutes, then drain the dye from the plate.

To measure the amount of bound dye present, and thus the amount of collagen remaining, add 100 μ l Sircol Alkali reagent to each well. If possible place the plate on a mechanical rocker; otherwise periodically move the plate in a rotational manner, without lifting it of the bench surface. A 15 minute extraction period is sufficient to bring the bound dye into solution. Do not drain the fluid contents from the wells. The samples are now ready for inspection and/or measurement.

Visual inspection:

Place the microwell plate on a light-box and examine the well staining pattern. A map of the results can be made for permanent record (a blank map is provided) or photographed.

Quantitative Measurement:

Place the plate into a multiwell plate colorimeter. Select a suitable colour filter (see collagen assay for advice on filter selection). Read the absorbance of each well and obtain a printout of the absorbance readings.

The collagen plus water/buffer samples will have the highest absorbance readings as no collagen was removed/digested.

The wells in column 12 will have the lowest reading as no collagen was present.

The test wells values can be expressed as a percentage of the 100% standard or if a range of collagen concentrations were used the quantity (mg or moles) of collagen digested by the test samples can be calculated.

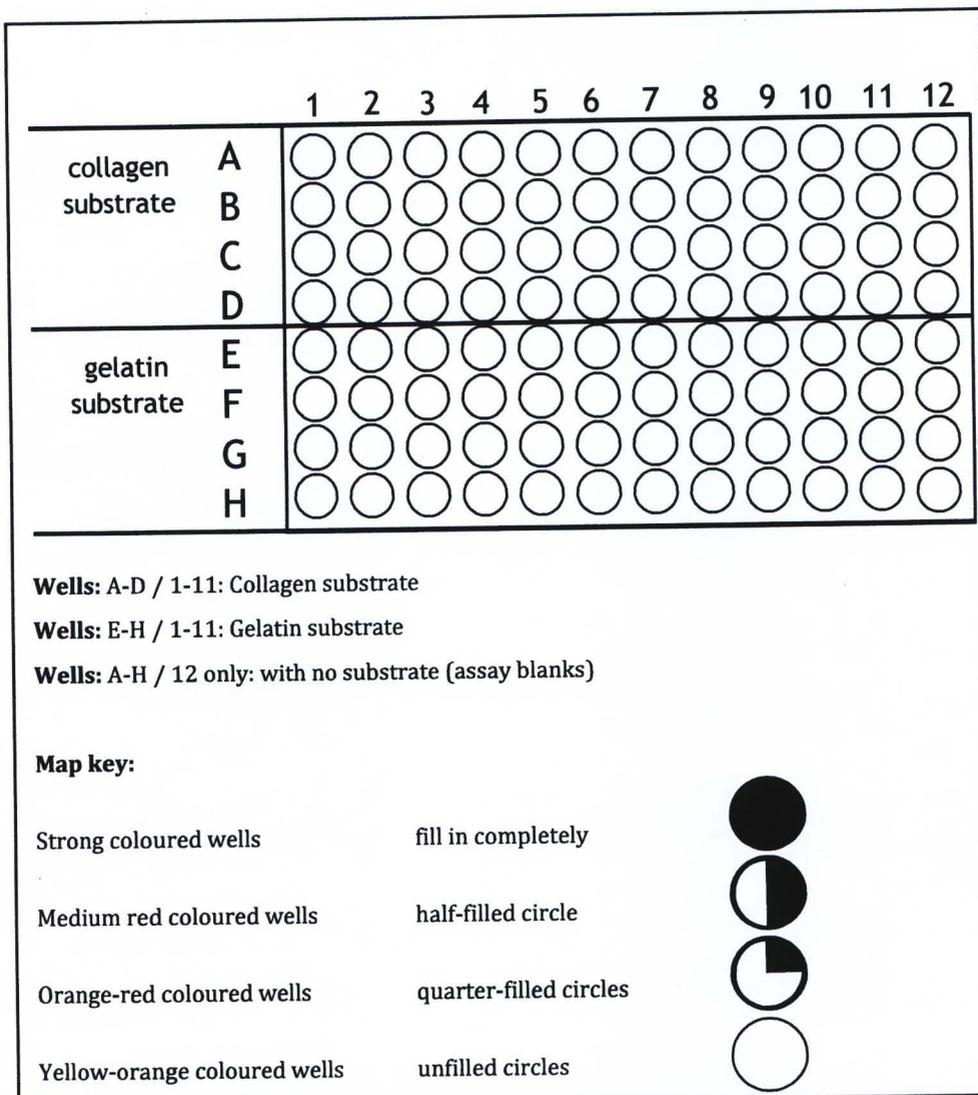


Fig. 4

Design of a microplate assay for protease detection and quantification

Other methods for the measurement of collagen:

Where the analyst has been using other, indirect, methods for measuring collagen it is useful to run comparison duplicates with the Sircol dye-binding assay. The results from earlier work will then be comparable with planned future work.

The collagen standards used in the previous method and the collagen standard supplied in the Sircol assay kit should be measured by both methods. In case the test material contains any interfering substance(s), a selected number of test samples (with and without added collagen) should also be assayed by both methods; with as small a time difference between the two assay methods as is practical.

Likewise method comparisons should be obtained, where necessary, to be able to compare collagen assayed by the Sircol dye-binding method with earlier published work that has used amino acid analysis, hydroxyproline determinations or dpm measurements of H^3/C^{14} labelled proline.

The initial effort required in running these correlation trials will be rewarded by the substantial savings in time, labour and cost upon subsequent conversion to the Sircol collagen assay.

A major advantage of the Sircol assay is that it can be carried out in a 'non-biochemical' laboratory. The correlation of the Sircol collagen dye-binding assay with the hydroxyproline method is shown in Fig. 5.

Collagen measurement by hydroxyproline determination:

Many methods and modifications for the determination of hydroxyproline have been published. The procedure can be both difficult and unreliable.

The assay procedure can be subdivided into four steps:

- [1] The collagen containing sample is placed into a glass ampoule, and sufficient concentrated HCl added to provide a final acid concentration of 6 M. The ampoules are sealed by melting the glass neck in a hot flame.
The sealed ampoules, including ampoules containing known amounts of collagen and hydroxyproline standards, are placed in a heating block, or a sand bath and maintained at 110°C for 18 hours.
- [2] Remove and allow cooling, before opening the ampoules. All the protein that had been present, including collagen, has been digested to a mixture of free amino acids. The excess HCl needs to be removed, either by titrating with strong NaOH (add a drop of methyl red) or by evaporation of the contents to dryness.

The former method produces a high concentration of NaCl which may interfere in the subsequent steps. The drying method requires a fan extracted fume cupboard and heat/time to drive off the HCl vapour. Some of the residues formed can be difficult to redissolve. Where the test material contained much carbohydrate or glycoprotein a brown/black residue can form that interferes in subsequent steps.

- [3] Aliquots of the hydrolysed protein, containing an estimated hydroxyproline concentration of between 1 and 5 mg, are added to labelled glass stoppered tubes (~15 x 150mm).

Standards, collagen digests, hydroxyproline standards (hydrolysed treated and untreated) and reagent blanks are similarly treated.

To all tubes add 1ml of a freshly prepared solution of Chloramine-T (0.05 M) [the Chloramine-T should be of analytical quality and the solid sealed and stored from moisture/light]. Stopper all tubes, mix and leave for 10 minutes.

To all tubes add 1 ml *p*-dimethylaminobenzaldehyde (20%) cap tubes and ensure good mixing of this dense addition with the tube contents.

Transfer to a 60°C water bath and maintain at this temperature for 20 minutes, then remove and allow cooling to room temperature.

Measure absorbance at 560 nm within one hour.

Plot standard curve and determine the hydroxyproline content of the test samples, within the linear range of the curve. Test samples below 1 mg, or above 5 mg, will require either concentration or dilution before being re-assayed.

- [4] Conversion of hydroxyproline concentration to collagen concentration:

Collagens are frequently cited to contain 14% hydroxyproline by weight; this value is based on mammalian type I collagens, other collagens will contain more or less hydroxyproline. Foetal collagens can contain much less hydroxyproline, as indeed do many non-mammalian collagens.

The 14% hydroxyproline value should therefore be used as an estimate for comparison with the actual value obtained from the test standard collagen; which should be the same species and type as that present in the test samples.

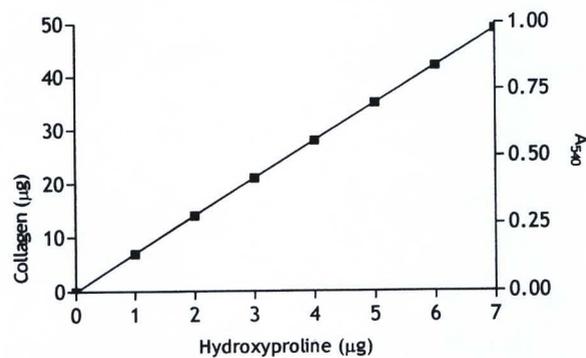


Fig. 5

Assay correlation;

Sircol dye binding versus hydroxyproline. (bovine skin collagen)

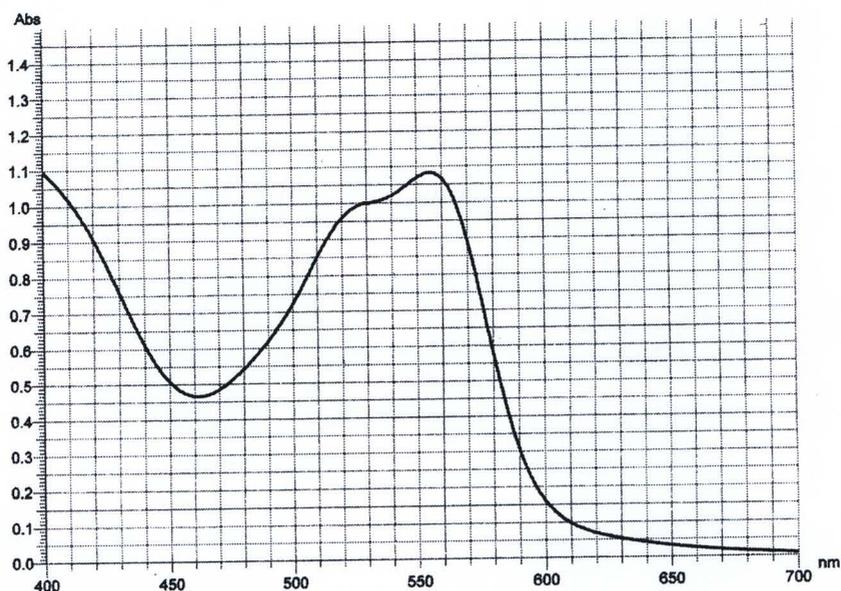


Fig. 6

Absorption spectrum for the Sircol Dye in Alkali Reagent

Abs max @ 555nm

COLLAGEN SOURCE REFERENCES

Biochemistry, biophysics; preparation, extraction & analysis

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Ramachandran, G.N., (Ed). 1967 'Treatise on Collagen: Volume 1; Chemistry of Collagen.'
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Sircol™
Collagen
Assay Kit
Options

Standard Assay Kit **Product Code: S1000**

Components of the assay kit:	(120 assays)
Sircol Dye Reagent	120 ml
Alkali Reagent	120 ml
Salt-soluble collagen Precipitating Reagent	15 ml
Collagen [Type I]	5ml
1mg/ml standard in 0.5 M acetic acid, (Bovine; produced from USA disease free animals)	
Sircol Assay Manual	24 pages
(also available to download from our website)	

Assay Large Economy Pack **Product Code: S5000**

Components of this assay kit, as S1000 (475 assays)

Supplementary Unit Packs

Sircol Dye Reagent only [3 x 120 ml]

Product Code: S1005

Collagen Standard [3 x 5 ml ampoules]

Product Code: S1010

Sterile bovine acid-soluble collagen; [Type I]

Concentration: 1 mg/ml in 0.5M acetic acid.

Collagen Standard [3 x 5 ml ampoules]

Product Code: S1011

Sterile rat tail acid-soluble collagen; [Type I]

Concentration: 0.5 mg/ml in 0.5 M acetic acid.



Other Assays available from Biocolor

APOPercentage Apoptosis Assay

For mammalian cells that have a conventional phospholipid composition membrane. Assay is not recommended for non-mammalian cells and is not suitable for neural cells. Requires an inverted microscope, (magn. x 100), for detection and a microplate reader for measurement. Digital microphotograph analysis option using Adobe Photoshop.

Assay sensitivity; single apoptotic cell Assay run time; 1 hour

Fastin Elastin Assay

Suitable for *in-situ* tissue insoluble cross-linked elastin as found in elastic cartilages and in soft tissues, such as the aorta and skin. Elastin produced during *in-vitro* cell culture. Hot oxalic acid extraction is required prior to analysis to bring the elastin into solution.

Assay sensitivity; 5ug Assay run time; 4 hours

Blyscan Sulfated Glycosaminoglycans Assay

For analysis of sulfated glycosaminoglycan components of proteoglycans including decorin, biglycan, fibromodulin, aggrecan, syndecan, betaglycan. **Galactosaminoglycans:** chondroitin sulfates, dermatan sulfate. **Glucosaminoglycans:** heparan sulfate, heparin, keratan sulfate. Test material; cell culture medium, amniotic fluid, urine, synovial fluid and tissue extracts.

Assay sensitivity; 0.5ug Assay run time; 1 hour

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