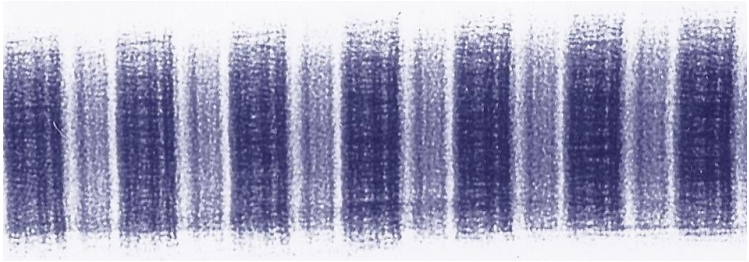
Sircol

INSOLUBLE
Collagen Assay



biocolor life science assays

www.biocolor.co.uk

Sircol INSOLUBLE Collagen Assay

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

TECHNICAL INFORMATION

Published by

Biocolor Ltd.

8 Meadowbank Road, Carrickfergus,

BT38 8YF, Northern Ireland, U.K.

www.biocolor.co.uk

BIO.INS.VER.250222-WEB

What is Sircol ?

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

Soluble and Insoluble Collagen measurement

Collagen is the most abundant protein found in animals. During a healthy life span the insoluble, covalent, cross-linked collagen fibres retain their biophysical functions and shapes. The collagen fibres remain isolated from most of the biochemical activities of the cells (Fig 1).

Trauma caused by metabolic internal events, external chemical agents, or physical injuries, quickly lead to dramatic activity, resulting in rapid collagen removal followed by a wound healing response (collagen regeneration and associated remodelling). Newly formed soluble collagen production and the residual insoluble collagen fibres can be monitored using the Sircol S1000 and S2000 Assays respectively.

Collagen proteins contain one or more domains with a triple helical structure. The three chains are described as alpha chains and 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 [$(Gly\text{-}XY)_n$].

Proline is frequently an occupant in the 'Y' location of the tri-peptide [$(Gly\text{-}X\text{-}Y)_n$]. Many of these residues are converted, post translation, into hydroxyproline residues prior to triple helix formation and the release of the tropocollagen into the ECM.

Until now, an investigator seeking to measure covalent cross-linked insoluble collagen required measurements based on free hydroxyproline content - a procedure that requires strong acid (6.0 M HCl), high temperatures (+95°C) and overnight cooking (18 to 24 hrs).

How does Sircol dye bind collagen?

Sircol dye reagent contains Sirius Red - a linear anionic dye with sulphonic acid side chain groups (see Fig.2). Under assay conditions the Sircol dye binds the basic groups of soluble collagen molecules.

The Sircol Insoluble Assay uses mild acid and temperature treatment for 2 to 3 hrs. The solubility conversion to denatured collagen and subsequent measurement can be completed within one working day. A further advantage of the Sircol Assay is that it permits the **ratio** of soluble and insoluble collagens to be measured using the same Sircol Dye Reagent *(by processing the samples using both Sircol S1000 and S2000 kits)*.

Intended Applications

S2000 Sircol Assay is a dye-binding method for analysis of **insoluble collagen fibres**. The assay can assess the rate of newly laid down collagen fibres produced during periods of rapid growth, development, tissue repair, remodelling and wound healing.

Pack Sizes and Storage Conditions:

Standard Assay Kit Product Code: **S2000 (100 assays)**
Economy Assay Pack Product Code: **S6000 (400 assays)**

All components are stable for one year when stored unopened at 10 – 25 0C. The glass vial containing denatured collagen standard and the diluted Acid-Salt Wash Reagent should both be stored at +4°C once opened (see page 4).

Assay Kit Components

Fragmentation Reagent for insoluble collagen fibres. Contains dilute acetic acid, antimicrobial agent, and surfactants.

Dye Reagent contains Sirius Red formulated for specific binding to collagen under the conditions defined in the Sircol Manual.

Alkali Reagent contains 0.5M sodium hydroxide and is used to recover Sircol Dye from the pellet of the collagen-dye complex.

Reference Standard: 1000 µg/ml. A sterile solution of water-soluble denatured collagen from bovine skin, stored in 0.1M acetic acid within a sealed vial. Once opened store at 4^0C, discard if the solution becomes turbid.

Acid-Salt Wash Reagent (Concentrate - contains acetic acid, sodium chloride and surfactants. **Before using this reagent dilute the contents of the vial to 100ml with deionised water.**

Screw capped round bottomed micro-centrifuge tubes, 2 ml capacity, used to contain the denaturising process for converting cross-linked collagen fibres to soluble, denatured collagen.

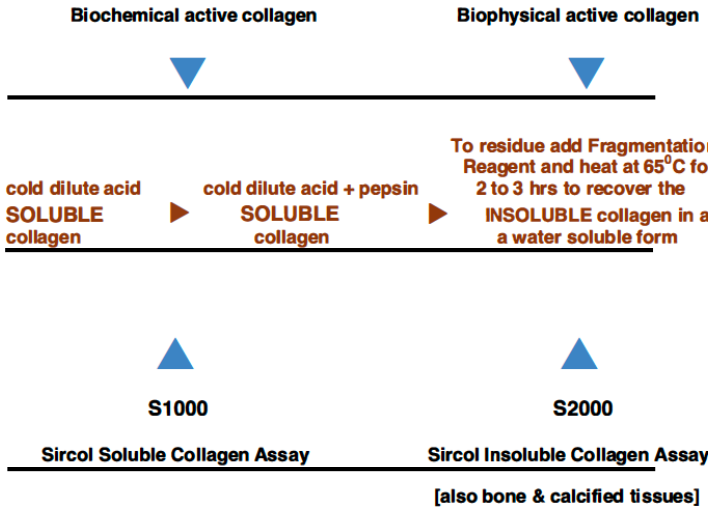
Assay Protocol

The General Assay protocol is found as a printed insert supplied with the kit. It may also be downloaded from our website.

The following sections contain further sample preparation information, together with supplementary details that should be read through, alongside the General Protocol, before the assay is carried out.

Sircol Assay selection and collagen processing

Fig.1 ***De-crystallisation of collagen, under laboratory conditions.***



Sample Processing for Insoluble collagens

This kit is designed for quantification of **insoluble collagens**. These are typically found as the left-over 'residues' in the tube/vessel following prior soluble collagen extraction by acid-pepsin digestion.

NB: If soluble collagens are not first extracted/removed by processing it is possible that these will further contribute to any insoluble collagen signal.

Selected Tissue samples, (20 to 30 mg wet weight), are exposed to dilute acid at **a temperature of 65°C for not less than 2 hours and for not more than 3 hours** (see General Protocol). Under these conditions, the insoluble collagen fibres are converted

to water-soluble denatured collagen, without generating the release of hydroxyproline residues.

The denatured collagen has an affinity of **45%** of dye-binding compared with native soluble collagen (Fig.3). Providing that the above temperature and heat conditions are employed, the absorbance can be expressed as micrograms equivalent of native collagen.

Where the S1000 Soluble Collagen and the S2000 Insoluble Collagen Standard Curves are being produced, both sets of Standards should be read using the same Microplate Reader and the same settings to confirm the reduced dye binding recovered from denatured collagen.

To adjust the denatured collagen dye binding to that of native collagen, a correlation factor of **2.2** is applied to the denatured absorbance reading at 556nm (see p7). This permits the Total Collagen in a tissue sample to be expressed as percentages of native soluble collagen and insoluble collagen fibre.

Sample requirements prior to assay

A metal heating block unit for 2 ml tubes with thermostat set to 65°C. A temperature-controlled water-bath is also suitable. Tube diameter is 10 mm.

The Sircol assay is a colorimetric procedure following centrifugation the sample must be transparent as turbidity will cause light absorption and scattering.

The presence of soluble protein in samples, including proteoglycans, tropoelastin and other soluble ECM material does not react with the assay dye.

Samples containing blood can interfere with the assay due to the large amount of albumin present in serum. Albumin forms an insoluble film on many plastic surfaces. The Sircol Dye, like most dyes, has an affinity for insoluble polymers, including plastic bound albumin.

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 at 556 nm. Increased absorbance values, due to traces of unbound dye, will be observed when the Acid-Salt Wash step is omitted.

The reagent blank's absorbance value should be subtracted from all standard and test sample 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. With some spare collagen standards, practice draining and drying the top of the microcentrifuge tubes to obtain a consistent mode of operation. Duplicate samples should read within ± 5% of their mean value.

Analysis using a computer spreadsheet programmed with graphical output. The denatured 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 vs Concentration). 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 0.10 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 1.0 ml of Sircol Dye Reagent cannot fully dye saturate these increased collagen levels.

Spectrum of the Sircol Dye in Alkali Reagent has a peak in the visible region at 556 nm. The absorbance profile is broad, and most microplate colour filter type readers will have a colour filter between 520 and 570nm. This should provide an absorbance profile similar to that in Fig.2b. The Collagen Reference Standard curves were obtained using a microplate spectrophotometer reader and are presented in Fig.3 as a guide.

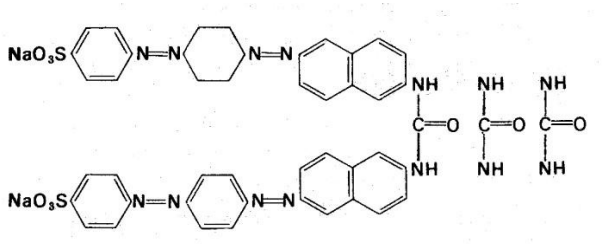


Fig. 2a: The molecular structure of the Sircol dye, (Sirius Red).

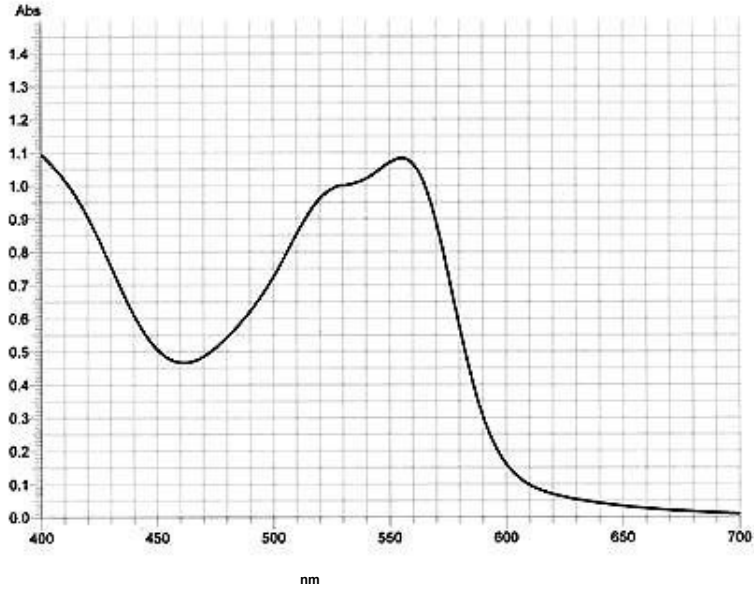


Fig. 2b: Absorption spectrum of the Sircol Dye in Alkali Reagent.

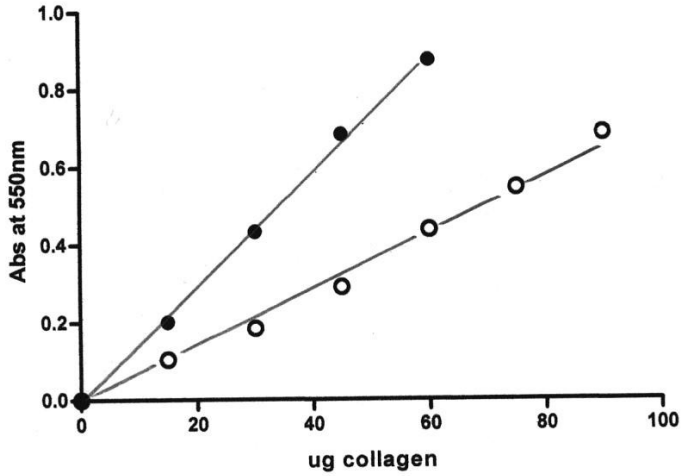


Fig. 3: Collagen Reference Standards:

Filled circles; cold native collagen, (r = 0.9991)
Unfilled circles; denatured collagen, (r = 0.9965)

Conversion of denatured collagen data to match native collagen data.

A x k = B

Where::

A is denatured collagen Absorbance at 556nm
B is native collagen Absorbance at 556nm
k s the conversion factor 2.2.
(dye binding data 100/45 = 2.2)

The denatured collagen Absorbance value at 556nm can be expressed as native collagen by using the Conversion Factor; 2.2. This permits the native collagen and denatured collagen to be expressed as g/mg tissue and as percentages of the Total Collagen within a tissue sample. For some applications for tissue analysis see page 8.

Assay Application worked examples

Test material: Black and white male rats, average body weight 335 grams, stored frozen until thawing and sampling for analysis. Rats were dissected: and the heart, kidneys, lungs, liver, skin and bone (femurs) were held at 4°C in 15 ml sterile screw capped plastic tubes. Samples were assayed within one week of dissection.

Soluble collagen was assayed using the S1000 Sircol Soluble Assay system. Insoluble collagen extractions using the S2000 Sircol Soluble Assay were applied to the insoluble residues remaining from the S1000 analysis.

	heart	kidney	liver	lung	skin	femur
Soluble collagen (µg/mg)	31.8	37.6	13.9	40.4	81.5	22
(as a % of total collagen)	68.7	58.4	21.5	67.6	22.3	12
Insoluble collagen (µg/mg)	14.5	26.8	50.9	19.4	284	162
(as a % of total collagen)	31.3	41.6	78.5	32.4	77.7	88
Total collagen (µg/mg)	46.3	64.4	64.8	59.8	365.5	184
Total collagen µg/mg (measured as OH-proline)					339	167
					93%	**91%**

Processing Bone and Calcified Cartilages

To 10 ml of the Fragmentation Reagent add 0.1 grams of EDTA sodium salt, forming a milky suspension. Use 1.0 ml of this acid-EDTA mix per test sample, (10 to 20 mg).

Cut the samples into small segments and part dry overnight at 4^0C, then grind into a paste using a mortar and pestle, before weighing. If the acid-EDTA suspension goes colourless after 24 hrs add more solid EDTA to ensure all the calcium deposits have been solubilised.

The insoluble calcium free residue can now be heated to 65°C for 2 - 3 hours to recover the denatured collagen. The samples are ready for the S2000 Sircol Assay.

Sircol Insoluble Collagen Assay
General Protocol

Detection Limit: **10.0 µg/100µl (100 µg/ml)**
Time required: **4 hours**

Conversion of native INSOLUBLE collagen to soluble denatured collagen

Take a weighed amount (typically 20 to 30 mg) of wet tissue or tissue residue remaining following cold acid-pepsin extraction, (S1000 Assay) and place into a screw capped round bottom 2 ml digestion tube (as supplied in the S2000 Assay Kit).

Add 50 ul of Fragmentation Reagent per 1 mg of tissue or residue. Close screw cap and incubate samples at 65 ^{0}C for 2 to 3 hrs. At approx 30 min intervals during incubation remove and shake, or vortex tube contents to aid tissue disintegration (see page 3 for further details).

Following incubation, remove tubes from heating block or water bath and centrifuge at 13000 *x g*.for 10 mins.

Setup Assay

To labelled 1.5 ml ***conical*** centrifuge tubes transfer;

Test samples - using volumes between 10 and 100 µl and make up to 100µl with deionised water. Where there is no previous knowledge of the collagen content use 50 ul and 100 µl of the transparent supernatant of the test material for a trial run.

Reagent blanks – 100 µl of deionised water.

Denatured collagen standards - use aliquots containing 0, 20, 40, and 60 µg of the standard. Make each standard up to 100 µl using water.

Commence Assay

Dye Labelling: To all tubes add 1.0 ml Sircol Dye Reagent. (1 ml of dye is required to fully saturate the collagen molecules within a 100 µl sample volume).
Cap tubes: mix by inverting contents and place tubes in a gentle mechanical mixer for 30 minutes (or manually mix at 10-minute intervals). During this time a collagen- dye complex will form and precipitate out from the soluble unbound dye. Transfer the tubes to a microcentrifuge and spin at 13000 *x g*. for 10 minutes. Carefully invert and drain excess dye from tubes *(Take care not to dislodge pellet!)*

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 13000 *x g*. for 10 minutes.
Drain the wash into a waste container and carefully remove fluid from the lip of the tubes using cotton wool buds.

Release and Recovery of Collagen Bound Dye

Add 1.0 ml Alkali Reagent to reagent blanks, standards, and samples.
Recap tubes and release the collagen bound dye into solution using a vortex mixer.

When the collagen 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 tubes capped until ready to measure Absorbance.

Measurement

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

Set the microplate reader to 556 nm, 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 after diluting the test sample(s) one in ten with deionised water before commencing the Sircol assay.

Calculation to express results as native collagen

Denatured collagen has an affinity of **45%** of dye-binding compared to native soluble collagen. Providing that the above temperature and heat conditions are employed, the collagen absorbance can be expressed as microgram equivalent of native collagen. For further details see pages 7 and 8.