Sircol 2.0 Product Manual

Product code: SICR2 (96 tests)

This kit has been designed for research use only.

Always handle using Good Laboratory Practice.

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PART A – General Information

Kit introduction

What is Collagen?

Collagen is a major constituent of the extracellular matrix and the predominate protein found in animals, making up approximately 30% of all protein mass. Collagens are characterised by possessing at least one 'domain' of triple helix structure.

At least 28 different types of collagens have been identified, with Type I collagen being the most prevalent form, particularly in ligaments, tendons, skin, and bone tissue. The biophysical properties of mature, **'Insoluble collagen'** allow it to withstand high tensile forces as well as being resistant to stretch. These are essential properties that enable the locomotion of organisms.

Collagen also exhibits key biochemical properties, being involved in cell growth, proliferation, and differentiation. This 'biochemical' form of collagen is typically recently synthesised and due to minimal crosslinking, can be readily solubilised via cold acid / enzymatic digestion. This is commonly referred to as **'Soluble Collagen'**.

Aged collagen undergoes extensive cross-linking and therefore remains 'insoluble' under cold acid / enzymatic digestion.

The Sircol assay kit product range

The Sircol range of collagen assay kits have been designed for user-friendly quantification of **soluble collagens (Sircol 2.0 kit)** or **insoluble (cross-linked) collagens (S2000 kit)**.

NB: The Sircol range is primarily designed for use with in-vivo / in-vitro samples of mammalian origin. Collagens originating from other taxonomic groups and kingdoms may also be analysed. See note on p6 on for general guidance.

How does Sircol 2.0 detect collagen?

Under assay conditions, Sircol Dye reagent is formulated to bind specifically to the *(Gly-X-Y)n* helical structure of soluble collagen.

Step 1. Prepared samples are placed in the wells of the assay microplate, together with Sircol Dye Reagent. After 30 minutes mixing, any collagen-dye complexes will form as a precipitate. These are collected on the base of the microplate wells by centrifugation.

Step 2. Unbound dye is removed by gentle aspiration, followed by a rinse with Plate Wash Reagent.

Step 3. Following further centrifugation, collagen-bound dye is eluted by incubation with a Dye Release Reagent. Eluted dye is detected 'in-situ' by spectrophotometric analysis of the microplate at 556nm.

Step 4. The collagen content of unknown samples can be quantified by comparison against a calibration curve, prepared using the Collagen Reference Standard supplied with the kit.

How does Sircol 2.0 differ from previous dye-based collagen assays?

The Sircol 2.0 assay offers two significant improvements over previous assays on the market (including Biocolor's earlier S1000 Soluble Collagen assay kit):

- **It uses an enhanced dye formulation:**
  - This improves collagen specificity, owing to a ›10x decrease in interference from non-collagenous proteins such as albumin.
  - Challenging samples such as serum-containing culture media can be directly assayed without the need for time-consuming sample pre-treatment steps.

- **It uses a microwell plate format:**
  - This is more convenient when processing multiple samples at once.
  - Collagen is labelled and detected 'in-situ' within the plate. This improves intra-assay CV by reducing fluid losses during transfer.

NB: *Components from the Biocolor's S1000 and S2000 assay kit range are <u>not</u> compatible with the Sircol 2.0 assay.*

Sircol 2.0 kit specifications		
Limit of Detection	2 µg/ml. 0.25 µg/ml (using optional Collagen Concentration protocol, see p11)	*In-vitro samples containing added animal serum will exhibit a slightly reduced limit of detection, approx 2-10µg/ml, serum depending.*
Range	2 - 200µg/ml 0.25 - 20µg/ml (using optional Collagen Concentration protocol, see p11)	*Optional Collagen Concentration protocol requires a minimum of 1ml sample volume.*
Detection Method	Colorimetric, 556nm	*Wavelengths between 530 – 560nm are suitable for analysis.*
Measurements per kit	96 in total	*Allows a maximum of 41 samples to be run in duplicate alongside a standard curve.*
Suitable samples	Liquid extracts containing *soluble collagen. **In-vivo:** tissues, cartilages, and fluids. **In-vitro:** 2D/3D culture extracellular matrices/conditioned media.	**Prior salt/acid/acid-pepsin extraction may be necessary to release soluble collagen.*
Sample Volume	10 – 100µl	*If using the optional Collagen Concentration protocol, then 1000µl is required.*
Reactivity	Soluble collagens containing intact triple-helix structure. As supplied, Sircol 2.0 is calibrated for samples of *mammalian origin	*Sircol 2.0 does not distinguish between different types of the collagen family.* **See explanation below*

NB: *Applicability of Sircol 2.0 to different species
Customers are advised that the kit has been calibrated for samples of mammalian origin by virtue of the included mammalian-derived Collagen Reference Standard.
Analysis of collagens from other taxonomic groups or kingdoms is possible. However, the published limit of detection will be reduced since non-mammalian collagens generally bind less dye. For accurate quantification customers should replace the kit Collagen Reference Standard with a species-matched, purified preparation of collagen.

Kit contents & storage

Sircol 2.0 has an unopened, room temperature shelf life of 1-year. The kit expiry date is recorded on the Certificate of Analysis supplied with each kit.

Once opened, please follow the storage conditions in the **Table.1**.

Table.1 Sircol 2.0 storage conditions

Component	**Volume supplied**	**Storage Recommendations (once kit is opened)**
Dye Reagent	20ml	Room Temperature
Collagen Reference Standard (200µg/ml)	5ml	4°C
Plate Wash Reagent	28ml	4°C
Collagen Concentration Reagent	25ml	4°C
Neutralisation Reagent*	8ml	Room Temperature
Dye Release Reagent	25ml	Room Temperature
Assay Microplate	(1x 96-wells)	Room Temperature
Microplate Seals	6x	Room Temperature
Documentation	QuickStart Guide / Manual / Certificate of Analysis	NA

What else is required to use the kit?

The following reagents or equipment may be required, depending on sample type used.

Acetic acid (0.5 M). *Used to dilute standards and samples.*
Store and use at 4°C.

Pepsin (enzyme). *Used to release collagen in soluble form suitable for assay.*
Biocolor recommends Merck/Sigma-Aldrich Pepsin, Product Code: P7012, E.C. 3.4.23.1. This should be prepared to a concentration of 0.1 mg/ml in 0.5M acetic acid. Store at 4°C and use within two days.

Microplate reader. *Used to measure dye released from collagen samples.*
Should be capable of 96-well microplate absorbance analysis at wavelengths between 530 – 560nm (556nm is optimal).

Microplate Centrifuge. *Used to separate collagen-bound dye from unbound dye.*
As a minimum, we recommend that the centrifuge can centrifuge a 96-well microplate at 400 x *g* for 120 minutes. Higher speed centrifuges are recommended (up to a maximum of 2000 x *g*), allowing a reduction in centrifuge time.

Vortexer / Shaker. *Used to mix samples.*

Sodium Hydroxide (5.87 M). *Will be required if using the optional 'Collagen Concentration protocol'. This reagent is used in combination with the kit 'Neutralisation Reagent' to neutralise acid-pepsin extracted samples (pH ~2.5) prior to collagen concentration (see Fig 1, p12 for further instruction).*

Due to its caustic nature, IATA transport regulations disallow Biocolor from supplying this component within the kit.

Sodium Hydroxide Preparation instructions:
Dissolve 15.5 grams of Sodium Hydroxide in 50 ml of water and store in a suitably labelled, plastic, screw-cap bottle.

Danger:

- Causes severe skin burns and eye damage:
    - WEAR PROTECTIVE GLOVES/PROTECTIVE CLOTHING/EYE PROTECTION/FACE PROTECTION.
- Corrosive to metals, absorb any spillages to prevent material damage.
- Store in a corrosive resistant container with a resistant inner liner.

PART B – Assay protocols

The General Protocol

i. Setup of samples / standards / controls

Setup samples:
Use the flowchart on the back of the provided QuickStart guide to select an appropriate sample preparation protocol. Then process samples accordingly.

NB: Samples may be frozen (-20°C) for assay at a more convenient time.

- We recommend assaying prepared samples in duplicate (as a minimum).

- Where there is no prior knowledge of collagen content, we suggest performing a trial run using 20 µl & 100µl volumes of test material. This helps any possible high collagen content samples to remain within the range of the standard curve.

Setup any controls:
It is always good practice to run assay controls. As a minimum, we would advise running a 'plate blank', comprising 200µl of Dye Release Reagent. This should be subtracted from all other readings to correct for microwell plate absorbance. You may also wish to run a 'reagent control', comprising sample extraction reagent. This is typically 0.5M Acetic Acid, cell culture medium, extraction buffer or deionised water.

Setup standards:
Prepare the standards according to **Table.2**. These can be prepared directly within the microplate wells.

Table 2. Preparation of assay Standards

Standard Collagen Concentration (µg/ml)	Mass of Collagen per well (µg)	Volume of STANDARD to be added (µl)	Volume of DILUENT* to be added (µl)
0	0	0	100
10	1	5	95
50	5	25	75
100	10	50	50
150	15	75	25
200	20	100	0

**Diluent can be water or unused sample extraction reagent.*

ii. General Protocol

Dye-labelling of collagen.

1. Add samples directly to the microplate wells. The maximum recommended sample volume per well is 100µl. If using sample volumes between 10 and 100µl, then use H2O/buffer to make the sample up to a total volume of 100µl.

2. Then add 175µl of Sircol Dye Reagent to wells containing standard or sample (excluding the plate-blank). Apply a microwell plate seal (provided with the kit) to fully seal the wells of the plate, then reapply the lid. *This begins the collagen labelling process.*

3. Place microplate on a microplate shaker and shake for 30 min at 300 rpm. *During this time any dye-labelled collagen will form an insoluble precipitate.*

Collection and washing of dye-labelled collagen.

4. Transfer the microplate to a suitable centrifuge and spin at 1500 x *g* for 90 min. If using a lower speed class centrifuge (such as those used for PCR plates) then a 400 x *g* spin for 120 min is sufficient. *During this time any precipitated collagen is collected and retained on the plate base.*

   NB: To optimise collection of precipitated collagen and aid subsequent liquid removal, assay plate(s) should be placed in the centrifuge so that Row H is positioned nearest the rotor.

   Do not exceed a maximum force of 2000 x *g*. Always use an equivalently weighted balance plate during each centrifuge step.

5. Remove plate from centrifuge and carefully remove the microwell plate seal. The total liquid contents of each well should be removed using gentle(!) aspiration:

   NB: Dye-labelled collagen will be present as a loosely attached deposit on the base and sidewalls of the wells. Be careful not to accidently remove this by excessive suction or scratching with the aspiration tip or needle.

   Helpful tip: *We recommend manual aspiration via micropipette. The microwell plate should be securely held at a 45° angle and the pipette tip positioned against the base of each well, at the angle formed between microwell wall and base. The liquid may then be gently removed.*

   If using a 96-well Aspirator, please ensure it is configured to avoid removing any precipitated material from the base of the wells.

6. Add 250µl of Plate Wash Reagent to each well (excluding the plate-blank). Then apply a microwell plate seal. Centrifuge the plate at a force of 1500 x *g* for 30 min (alternatively 400 x *g* for 60 min can be used).

Release of dye-label from the collagen precipitate.

7. Carefully remove the liquid from all wells (using the same procedure as per point '5'). Then add 200µl of Dye Release Reagent to the appropriate sample/standard/blank microwells. Recover the plate with an adhesive Microwell plate seal.

8. Place the plate on the microplate shaker (700 rpm) for approximately 20-30 min until a uniform coloration is observed in the highest concentration assay standards.

9. The microwell plate seal should be carefully removed, the assay microplate is now ready for measurement.

iii. Assay Plate Measurement

10. Set the microplate reader to 556nm, (alternatively any wavelength between 530 – 560nm may be used) and measure the absorbance in 'endpoint' mode.

11. For graphing and data analysis proceed to Part C of this manual.

Collagen Concentration Protocol (optional)

What is this protocol used for?

We recommend that 'unknown' sample types are initially assayed via the General Protocol (unless specifically advised in a sample preparation protocol).

However, you may find that some samples measure towards the very low concentration side of the standard curve. For such samples (measuring between 0 - 20µg/ml) the Collagen Concentration Protocol may be a useful option.

This requires a 1 ml volume of the low-concentration, liquid sample. This undergoes a sample pre-concentration step which recovers and concentrates any collagen in the sample, prior to Sircol 2.0 analysis.

The result is that soluble collagens in the 2.5 - 20µg/ml range may be quantitatively analysed.

How is the sample concentrated?

Each Collagen Concentration should be performed in a 1.5ml microcentrifuge tube to which 1 ml of sample with low soluble collagen content (‹20µg/ml) is added. Addition of the provided '*Collagen Concentration Reagent*', combined with overnight incubation causes the selective precipitation of collagens. These are compacted via centrifugation and following removal of fluid, and redissolution of the precipitated collagen a 'concentrated' sample is achieved. This can then be processed through according to the General Protocol.

Collagen Concentration Protocol:

1. Identify the *approximate* pH of your extracted sample. This is typically:
    a. pH 'neutral', such as samples of culture media or extracted in neutral saline.
    b. pH ~2.5, such as samples from an Acid or Acid-pepsin extraction.

2. Prepare 1.5ml microcentrifuge tubes as shown in **Figure 1**, corresponding to the approximate pH of samples. Replicate tubes should be prepared if sample quantity permits.

NB: Customers who may have lower Acid-pepsin extract volumes (i.e., 0.5ml) should adjust these to a total volume of 1ml before proceeding with the protocol. This can be achieved by either 'pooling' replicate samples (the preferred approach), or adding 0.5M Acetic Acid, to a total volume of 1ml.

3. Vortex prepared tubes to mix contents (approx. 5 seconds per tube).

4. Incubate tubes overnight at 4°C. *Any collagens present will precipitate during this time.*

5. The next day, centrifuge tubes at 13000 x *g* for 20 mins. *Precipitated collagen will be retained on the inside tube surfaces.*

6. Use a micropipette to slowly remove the liquid contents of each tube. *Do not use*

excessive suction, and do not to scratch the sidewalls or base of the tube with pipette tip as this reduces collagen yield.

7. Add 80µl 0.5M acetic acid to each tube. Vortex each tube for 3-5 sec and leave for 15min. *This converts precipitated collagen into a soluble collagen sample.*

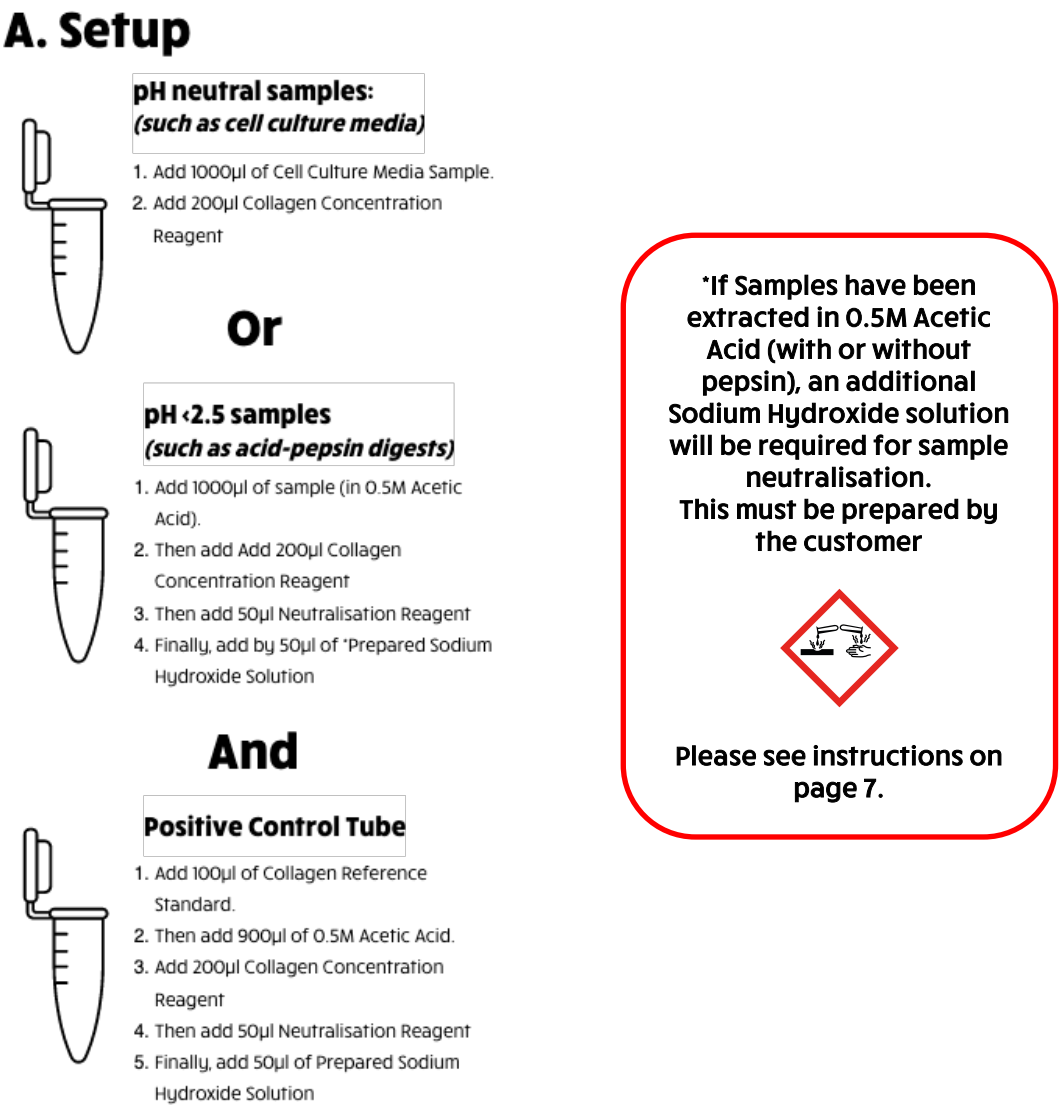
8. Vortex for a further 3-5 sec and either briefly centrifuge (10 seconds) or wait 15 min to allow all liquid to drain to the base of the tube.

9. Using a pipette (set to 100µl), pipette the liquid tube contents up and down 3x to ensure full mixing, then transfer all liquid to the appropriate well of the assay microplate.

10. Proceed with the General Protocol.

NB: *Samples processed via the Collagen Concentration Protocol will require the use of a correction factor when calculating the collagen content using the standard curve. See data analysis on p16.*

Figure 1. Setup of samples for Collagen Concentration



PART C – Data analysis

Data Analysis

1. Having collected the absorbance data, the value of any assay 'blank(s)' can be subtracted to generate a set of 'blank-corrected' data.

2. The average values and any desired statistics for standards or samples can then be calculated, preferably via suitable software.

3. Use these data to plot a graph of absorbance (*y*-axis) against collagen concentration (*x*-axis), as prepared from ***Table 2. 'Preparation of assay standards'***.

   A linear regression should be fitted to the curve as this best fits the dye-collagen binding characteristics of Sircol 2.0. This can then be used to calculate the concentration of collagen in your unknown samples.

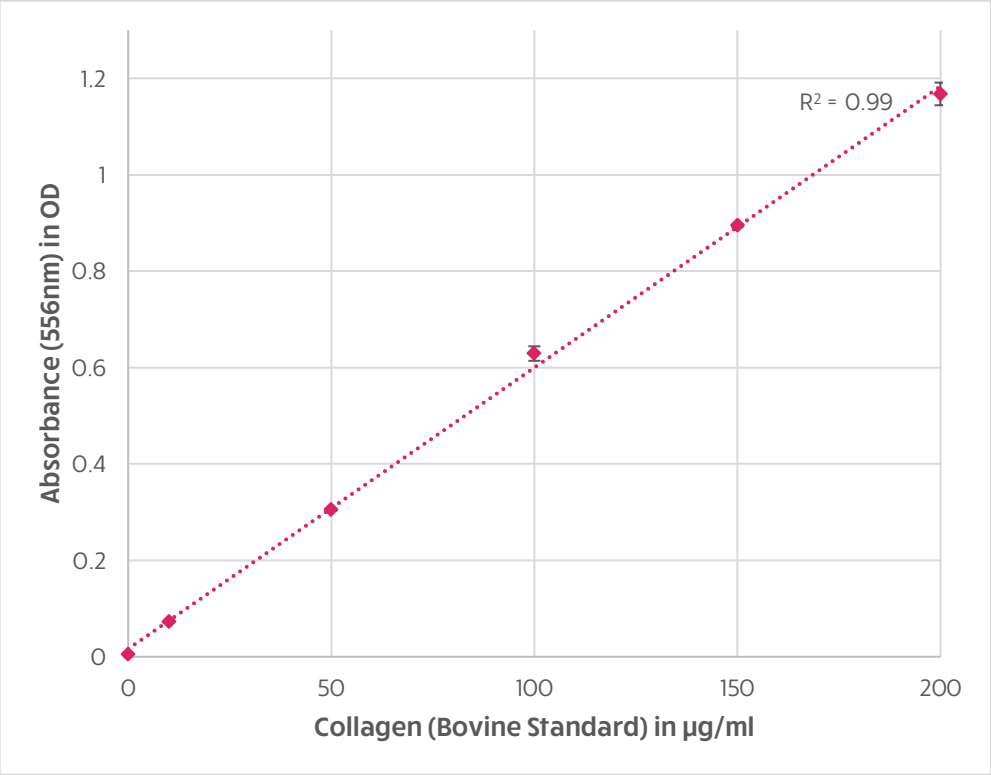
   Ideally your plot should exhibit the following:

   a. A linear plot, ideally with a R-squared value ≥0.95. An example is shown in Figure 2., below.

   b. The '0' collagen standard should exhibit an absorbance (556nm) of between 0 – 0.1, depending on sample composition.

   c. Duplicates should be close to ±5% of their main value.

Figure 2. Sircol 2.0 Standard Curve (example)



Calculating the concentration of soluble collagen in the liquid extract of assayed samples:

The concentration of soluble collagen in the assayed sample liquid extract(s) should be *calculated from the linear regression.

*If your samples were **diluted** prior to assay, then the collagen content, calculated from the standard curve should be multiplied by the sample dilution factor.*

NB: Accurate quantification can only be achieved for samples that fall within the range of the standard curve:

- **Are your sample absorbances too high?**
  Samples with absorbance values greater than the upper range of the assay standards should be *diluted* and rerun.

- **Are your sample absorbances too low?**
  If your samples have a Sircol 2.0 absorbance of ‹2.0µg/ml then consider using the Collagen Concentration Protocol (see p11 for details).

**Consult your plate-reader or statistical software manual for instructions on how to calculate the concentration of unknown samples from a linear regression line.*

Calculating the soluble collagen content of the pre-extraction sample.

Many samples used with the Sircol 2.0 assay will have been extracted from solid tissue. The calculation of collagen content from the Standard Curve only expresses the collagen concentration of the prepared liquid sample (in µg/ml). For comparison with other samples, it may be preferable to express the collagen content ***µg/mg of tissue*** or ***µg/growth area or volume*** (for some *in-vitro* samples). This can be achieved as follows:

1) Calculation of soluble collagen in a tissue sample (*µg/mg*) = C /1000*V / M

V is the total volume of Acid-pepsin (in µl) added to the original sample (when extracting soluble collagen).
C is the Sircol 2.0 calculated concentration of soluble collagen (in µg/ml).
M is the mass of original sample processed (in mg)

2) Calculation of soluble collagen per growth area (*µg/cm²*) = C /1000*V / A

V is the total volume of Acid-pepsin (in µl) added to the original sample (for extraction of soluble collagen).
C is the Sircol 2.0 calculated concentration of soluble collagen (in µg/ml).
A is the growth area that processed sample was derived from (in *cm²*)

Calculating the concentration of soluble collagen in samples processed via the Collagen Concentration protocol.

Step 1.
The absorbance values of any samples processed via the ***Collagen Concentration Protocol*** will require multiplication by a correction factor of '***1.15***. This accounts for real-world efficiency of collagen concentration. The corrected absorbance value can then be used to calculate the concentration of collagen (in the processed, concentrated sample) via the assay standard curve.

*For example, a sample processed by the Collagen Concentration Protocol had a measured absorbance of **0.33** (at 556nm. This should be multiplied by **1.15** as follows:*

0.33(measured absorbance) x 1.15 = 0.3795 (corrected absorbance)

In this example, the corrected absorbance was then used to calculate a collagen concentration from the standard curve of 62.6 µg/ml.

Step 2.
This calculated value should then be divided by 10x to calculate the amount of collagen in the pre-concentrated, 1ml sample.

62.6 µg/ml (concentrated sample)/ 10 = 6.26 µg/ml (original sample)

PART D – Sample Preparation protocols

General notes on Sample Preparation

Why do samples need preparation?

Sircol 2.0 is designed for analysis of liquid sample extracts containing collagen in soluble form. However, in samples such as solid tissues or cells the collagen is not initially soluble, being associated other proteins and biomolecules. Samples will therefore require preparation to convert and release all possible collagens to soluble form.

Samples such as fluids or cell culture media do not need extraction since the sample is already in 'liquid' form.

If you are new to Sircol analysis, we recommend you use the flowchart on the back of the provided QuickStart guide to guide you to the appropriate protocol for your sample type.

Please note that some protocols will require reagents or equipment not supplied with the kit. Customers should familiarise themselves with these requirements beforehand.

Modification or further optimisation may be required for some samples. If in any doubt, please feel free to contact us, we are always happy to help!

How is soluble collagen extracted from samples?

Soluble collagen is usually extracted by one of the following methods:

i. Recently synthesised soluble collagens in the sample can be extracted with neutral salt solution; however, the yield would typically be low. A greater extraction yield can be achieved by using substituting the neutral salt for 0.5M acetic acid.

   Both these extraction approaches result in what is termed 'telo-collagen' since the non-helical 'telopeptide' regions on either side of tropocollagen molecules are kept intact. These telopeptides are sites of natural crosslinking and contribute to collagen fibril formation.

ii. Unless you have specific reason to extract only telo-collagen, then Biocolor recommends digestion of sample with a combination of the enzyme – Pepsin, and acetic acid (referred to as Acid-pepsin digestion). This cleaves the collagen telopeptide regions which ensures the maximum yield of soluble tropocollagen.

   Collagen processed in this way is referred to as 'atelo-collagen', or pepsin-soluble collagen.

iii. Finally, collagen that resists acid-pepsin digestion is referred to as insoluble collagen. This can be quantified by our Sircol 'Insoluble Collagen Assay kit', code S2000.

Our protocols use acid-pepsin digestion with the aim of maximising the yield of measurable collagen from samples.

General guidance for prepared Samples

Following extraction, Sircol 2.0 samples should be:

- Free of any particulate material, as this can interfere with colorimetric analysis.
- Prepared in acetic acid (0.5M maximum), salt buffer, or tissue culture medium. Samples with a ‹pH 2.5 or › pH 10 should be neutralised prior to analysis.
- Some samples may contain added surfactant, detergents, or 'non-standard' chemistries, for example – crosslinking reagents from 3D-scaffold supports. For such samples we would recommend that a control sample of fresh (unused) buffer or sample is assayed, both with and without added collagen standard. This will allow customers to verify that these components do not interfere with dye binding.
- Prepared samples may be stored frozen (-20°C to -80°C) prior to analysis. This is convenient as samples may then be harvested over different time periods and then assayed together. Frozen samples should be thawed slowly at 4°C and then vortexed to mix prior to assay.

I. In-vivo samples

Step 1: Preparing tissue samples for Sircol 2.0 analysis.

The following protocol is suitable for extraction of soluble collagen from *in-vivo* tissue samples. These are usually obtained by biopsy or dissection and may be processed as 'wet' tissue or freeze-dried for processing as 'dry' samples.

NB: Guidelines on sample collection and preparation can be found after the extraction protocol.

The protocol utilises overnight acid-pepsin digestion at 4^{0}C - the method of choice for recovering the largest yield of recently synthesised collagen.

We recommend that initial trials are performed on non-essential tissue to determine optimal extraction conditions!

Example samples and extraction conditions are as follows:

Table 3. Tissue samples and extraction conditions

Tissue	Recommended Extraction conditions	Sample:Pepsin solution ratio (w/V)	Pepsin Concentration	Notes
Muscle, lung, liver, cartilage, tendons, aorta, valves, skin (Prepared as per guidelines on p22)	Acid-pepsin extraction. (18-24 hours) (Samples from older animals may require a second extraction)	1:10 – 1:20 (i.e., for a 10mg sample, add 100 - 200µl acid-pepsin solution)	0.1 mg/ml, in 0.5M acetic acid	Blood should be rinsed away using cold phosphate buffered saline. Fat should be removed by trimming. Hair should be removed from skin samples.

Step 2: Acid-Pepsin soluble collagen extraction

Biocolor recommends Merck/Sigma-Aldrich Pepsin, Product Code: P7012, [E.C. 3.4.23.1 as a suitable source of pepsin.

1. Prepare Acid-Pepsin solution by dissolving Pepsin into 0.5M acetic acid at a concentration of 0.1mg/mL. *Store at 4°C and use within two days.*

2. Harvest sample, paying attention to the Guidelines for Sample harvesting and preparation (see below).

3. Dice sample into small cubes of around 1 mg weight (2-3mm size). **Record the weight of the sample added to each tube (this will be required for data analysis)**. Alternatively, samples can be homogenised. *These increase surface area for pepsin digestion, increasing the efficiency of soluble collagen release.*

   NB: Care must be taken that the samples are not heated above room temperature during homogenisation as this can denature the collagen, reducing Sircol dye binding.

4. Add Acid-Pepsin solution to the sample in a ratio of 1:10 or 1:20. **Record the volume added to each tube (required for subsequent data analysis).**

   For example, if the sample weighs 10mg, add 100 - 200µl acid-pepsin solution.

5. Incubate overnight at 4°C with mechanical shaking. *This aids efficient digestion while protecting the collagen from any temperature-induced denaturation.*

6. Following incubation, centrifuge the tubes at 3000 x *g* for 10 minutes to compact any undigested material into a pellet at the tube base. The supernatant will contain any released soluble collagen. This should be carefully transferred by pipette into a labelled fresh tube.

   The supernatant samples can be assayed immediately, processed via the Collagen Concentration Protocol or frozen (at -20°C) for subsequent Sircol analysis.

NB: The pellet remaining at the bottom of the digestion tube will contain any insoluble collagen (which is pepsin resistant), as well as other undigested material. This is suitable for analysis using Biocolor's Sircol Insoluble Collagen Assay (S2000). These pellets may be frozen at ≤ 20°C until required.

For samples with a soluble collagen content of less than 20µg/ml we recommend using the **Collagen Concentration Protocol. (See Page 11)**

Guidelines for *in-vivo* sample harvesting and preparation:

Material sampled post-mortem should be collected as soon after death, in an aseptic a manner as possible.

1. Briefly wash the external surface with sterile water or saline to remove any debris and blood.

   NB: *Lung and liver samples can be difficult to 'clean-up' as they are usually blood saturated. It is advisable to dice these samples and briefly rinse in cold phosphate buffered saline, before proceeding with preparation.*

2. If the sample contains attached adipose (fat) tissue, it should be trimmed off using a scalpel.

3. If collagen extraction is to be carried out at a later date, then samples should be weighed, placed into labelled, sealable containers and frozen (≤ 20°C) as quickly as possible. When defrosting, the frozen samples should be thawed slowly (in a refrigerator at 4°C) before subsequent processing.

4. Decide if collagen content of the test samples is to be expressed as 'dry weight' or 'wet weight'.

   If using dry weight then it will be necessary to take a representative sample, first obtain its wet weight and then dry the sample in an un-heated drying cabinet containing drying granules. Weigh samples daily until a constant dry weight value is obtained (most tissues and cartilages are ~ 70% water).

5. For efficient collagen extraction the tissue samples 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 prepared samples into a suitable tube or container.

   NB: *Non-homogeneous collagen distribution within the tissue can be a cause of sample variation. Provided there is sufficient tissue, this can be mitigated by selecting larger samples, or by pooling multiple smaller samples.*

II. In-vitro samples

Sircol 2.0 can be used to measure collagen from *in-vitro* samples. These are generally obtained following culture of animal cells in either 2D (conventional) or 3D cell culture systems.

Suitable sample sources include:

- **Conditioned cell culture medium**.
  Many types of cells secrete collagen into the extracellular space when cultured in-vitro. This collagen is naturally soluble and accumulates in the growth medium that bathes the cells. For this reason, conditioned cell culture medium samples can be readily analysed by Sircol 2.0 assay.

- **Extracellular matrix (ECM).**
  This is deposited onto substrates over (extended) cell culture time. These include:
  - T-flasks or microwell plates as used in conventional 2D cell culture.
  - 3D culture matrices (provided they are not composed of collagen-derived materials)

When analysing deposited ECM collagens, they must first be solubilised. Maximal solubility can be achieved using an 18 – 24-hour digestion at 4°C using a combination of the enzyme – Pepsin, and acetic acid (Acid-pepsin digestion). This cleaves the collagen telopeptide regions, maximising the yield of soluble tropocollagen.

For samples with a soluble collagen content of less than 20µg/ml we recommend using the **Collagen Concentration Protocol. (See Page 11)**

Analysis of collagen secreted into the growth media (2D/3D cell culture)

1. Gently rock/swirl the cell culture plate or flask for 10 seconds to ensure media sample is homogenous. Take care not to dislodge any adherent cells or spill media.

2. Remove the media sample using a pipette or needle and transfer to a suitable tube. Remove aseptically if cell culture is being maintained after sampling.

   NB: The general protocol requires 100µl samples. If performing the Collagen Concentration Protocol, then 1000µl samples will be required.

3. Centrifuge the media sample(s) at 1500 x *g* for 5 mins to pellet any loose cells or insoluble material.

4. Transfer the now clarified supernatant (which contains any soluble collagen), to a fresh, labelled tube.

   This can be assayed directly, processed via the Collagen Concentration Protocol or frozen (at -20°C) for subsequent Sircol analysis.

NB: The original Biocolor S1000 Sircol soluble collagen assay exhibited non-specific binding when using samples containing animal serum-supplements. The improved Sircol 2.0 assay is not affected by this issue. Samples containing serum supplements may therefore be analysed without concern.

Analysis of collagen-deposited within 2D cell culture environments.

T-flasks, 6, 12 and even 24-well plates can be used for cultivation of cells for collagen analysis. We do not recommend smaller growth areas as it can be difficult to recover sufficient volume of sample for analysis.

Table.4 Suggested Acid-pepsin volumes for collagen extraction

Growth Environment	Volume of Acid-pepsin (ml)
6-well plate	2
12-well plate	1
24-well plate	0.5
T25 flask	5

Cell scraping method.

Seed cells in the appropriate vessel and grow under the defined experimental conditions.

1. Aspirate or decant growth media, taking care not to dislodge attached cells.

2. Add 0.1mg/ml Pepsin in 0.5M Acetic Acid (Acid-pepsin) to the monolayer (see Table.4 for guidance on required volumes) and then use a cell scraper to remove attached cells from the entire growth surface. Transfer the cells (in the acid-pepsin solution), into labelled tube(s) of suitable volume.

3. Tubes should be incubated with gentle shaking for 18-24 hours at 4°C. *This releases any acid or pepsin soluble collagens into solution.*

4. Centrifuge the tube(s) at 1500 x *g* for 5 mins to pellet any undigested material.

   NB: The pellet remaining at the bottom of the digestion tube will contain any insoluble collagen (which is pepsin resistant), as well as other undigested material. This is suitable for analysis using Biocolor's Sircol Insoluble Collagen Assay (S2000). These pellets may be frozen at ≤ 20°C until required.

5. Transfer the supernatant (which contains any soluble collagen), to a fresh, labelled tube. This can be assayed directly, processed via the Collagen Concentration Protocol or frozen (at -20°C) for subsequent Sircol analysis.

For samples with a soluble collagen content of less than 20µg/ml we recommend using the **Collagen Concentration Protocol. (See Page 11)**

'In-situ' digestion of monolayer method

Collagen may be digested 'in-situ', this avoids cell scraping and digestion in a tube.

1. Culture media should be removed by gentle aspiration. You may wish to keep this media for collagen analysis, if so, then transfer to a labelled tube and process as per the relevant protocol (p24).

2. Gently rinse the cell monolayer with phosphate-buffered saline (PBS) to remove traces of media, taking care not to dislodge attached cells. The PBS should then be removed by decanting / aspiration.

3. Add 0.1mg/ml Pepsin in 0.5M Acetic Acid to each well, (see **Table.4** for guidance on required volumes).

4. Incubate with gentle shaking for 18-24 hours at 4°C.

5. Acid-pepsin solution removed from each well and centrifuged 1500 x *g* for 5 mins to pellet any undigested material.

6. Supernatant containing possible soluble collagen, can be decanted, and then assayed directly, processed via the Collagen Concentration Protocol or frozen (at -20°C) for subsequent Sircol analysis.

Analysis of collagen-deposited within 3D cell culture environments.

A wide variety of 3D cell culture technologies are now available, each differing in composition and setup. As a result, we cannot offer a definitive sample preparation protocol. The following protocol should therefore be considered as a starting point and will require adaption to your experimental conditions.

NB: If in any doubt, please feel free to contact us, we are always happy to help!

Our recommended approach for Sircol 2.0 measurement of collagens in 3D-scaffold culture is based on the use of the enzyme, pepsin, and acetic acid (referred to as Acid-pepsin). This will cleave the collagen telopeptide regions and release soluble 'tropocollagen' from the scaffold/cell surfaces. This is suitable for assay.

Scaffold composition:

- Scaffolds or hydrogels constructed from collagen or collagen-derived products should be used with caution, and only with good controls in place. This is because scaffold-derived collagen may interfere with any cell-derived collagen measurements.

- It is possible that some scaffold components may bind or interfere with the Sircol 2.0 dye reagent. We recommend that a similar digestion is also performed on a sample of scaffold that has had media added without any cells (i.e., a 'mock' or 'sham' seeded scaffold). This will act as a negative control sample and will ideally exhibit a low absorbance value for released dye.

In-situ' digestion method

1. Culture media should be removed from around each scaffold by gentle aspiration. This can be reserved for later Sircol 2.0 analysis, if so, then transfer to a labelled tube and process as per the relevant protocol (p24).

2. The scaffold should be rinsed with 1 or 2x changes of PBS to remove traces of culture media. Any rinse PBS should be removed by gentle aspiration.

3. Transfer the scaffold(s) to a clean tube (suitable for a centrifuge) and add sufficient 0.1mg/ml Pepsin in 0.5M Acetic acid to cover the scaffold. *Tubes should then be extensively vortexed 3x 15-secs to ensure that the acid-pepsin fully contacts the surfaces and pores of the scaffold.*

4. If transfer of the scaffold to a tube is inconvenient or experimentally difficult, then it is also possible to directly add 0.1mg/ml Pepsin in 0.5M Acetic acid, sufficient to cover the scaffold 'in-situ'. Please ensure that all surfaces that were exposed to cell culture media are also contacted by the acid-pepsin solution.

5. Scaffolds should then be incubated in the Acid-pepsin for 18-24 hours at 4°C with gentle shaking. *This will release any collagen from the scaffold ECM.*

6. Following incubation, tubes should then be centrifuged at 1500 x *g* for 5 mins to pellet any insoluble material.

   NB: If Acid pepsin has been added to wells 'in-situ', then at this stage, transfer the acid-pepsin digest fluid to a labelled centrifuge tube before centrifuging as above.

7. The supernatant (containing any soluble collagen) can then be transferred to fresh, labelled tubes. This can be assayed directly, processed via the Collagen Concentration Protocol or frozen (at -20°C) for subsequent Sircol analysis.

For samples with a soluble collagen content of less than 20µg/ml we recommend using the **Collagen Concentration Protocol.** **(See Page 11)**