

MEGACULT™-C**MegaCult™-C Staining Kit - Erythroid**

Catalog # 04966

PRODUCT DESCRIPTION:**Table 1: Components of MegaCult™-C Staining Kit - Erythroid**

Component Number	Description	Unit Size	Storage Conditions
04808	Primary Antibody Anti-human Glycophorin A	0.4 mL/vial	Store at 2-8°C. Stable for at least two years.
04804	Negative Control Antibody Anti-TNP	0.1 mL / vial	Store at 2-8°C. Stable for at least two years.
04905	Avidin-Alkaline Phosphatase Conjugate	0.2 mL/tube	Store at 2-8°C. Do not freeze. Stable for at least one year.
04906	Biotin Conjugated Goat Anti- Mouse IgG	0.125 mL/tube	Store at -20°C. Stable for a least one year.
04807	Human Serum	6 mL	Store at -20°C. Stable for at least one year. Stable for 3 months at 2-8°C if kept sterile.
04809	Alkaline Phosphatase Substrate tablets	30 gold 30 silver	Store at -20°C. Stable for two years.
04913	Evans Blue counterstain	3 mL	Store at 2-8°C. Stable for at least two years.
04915	10% BSA Solution	6 mL	Store at -20°C. Stable for 6 months at 2-8°C if kept sterile.

HANDLING INSTRUCTIONS AND DIRECTIONS FOR USE:

For information on the set up, culture conditions, dehydration and fixation of the assays for erythroid progenitors in serum-free collagen gels, refer to **Appendix I: Protocol for CFC Assays in Serum-Free Collagen gels.**

STAINING PROCEDURE: REAGENTS

All solutions must be freshly prepared and used within eight hours. The only exception is the alkaline phosphatase substrate solution, which must be used within one hour. The amount prepared should be based on the number of slides to be stained. Refer to the **Table 2, Calculation of Staining Volumes.**

1. 0.05 M Tris/NaCl buffer, pH 7.6, (not provided) is prepared as follows:

A. 0.15 M isotonic saline

8.766 g NaCl dissolved in 1 L deionized, distilled water
(stable for one month at room temperature)

B. 0.5 M Tris/HCl, pH 7.6

78.8 g Tris/HCl (Sigma #T-3253) dissolved in 1 L deionized, distilled water
Adjust pH to 7.6 using 5M NaOH
(stable for one month at room temperature)

On day of use, mix nine parts solution A plus one part solution B. One liter of this buffer is enough to stain 48 slides. Discard remaining buffer at the end of the day.

Phosphate buffers should not be used, as the staining reaction may be inhibited.

2. 04807: Human serum
Prepared from male clotted blood. Tested negative for antibodies to HIV-1 and HBsAg. Filter sterilized.
This product is derived from human blood and should be handled using universal handling precautions as required when using any potentially infectious materials. See MSDS for additional information.

Dilute human serum to a concentration of 5% in 0.05 M Tris/NaCl buffer.
The human sera is a source of human immunoglobulin to block nonspecific binding of the anti-glycophorin A antibody.
3. 04808: Primary antibody: Anti-human Glycophorin A antibody
The mouse anti-human Glycophorin A antibody (isotype IgG₁) is supplied at 1.0 mg/mL in phosphate buffered saline.
Dilute 1/100 in 5% human serum for use at 10 µg/mL.
4. 04804: Control antibody: anti-TNP antibody
The mouse anti-TriNitroPhenol antibody (isotype IgG_{2a}) is supplied at 0.5 mg/mL in phosphate buffered saline. Dilute 1/100 in 5% human serum for use at 5 µg/mL.
Anti-TNP is used as negative control antibody for immunocytochemical staining.
5. 04915: 10% BSA Solution. BSA solution is buffered to pH 7.2-7.6 with NaHCO₃.
Dilute 10% BSA 1/10 in 0.05 M Tris/NaCl buffer to a final concentration of 1% BSA.
6. 04906: Biotin conjugated goat anti-mouse IgG antibody (0.125 mL/vial)
Supplied at concentration of 3 mg/mL in saline, pH 6.2. Contains 15 mM sodium azide (see MSDS) as a preservative. **Store at -20°C.**
Working aliquots should be prepared by diluting 04906 1/10 with sterile 0.05 M Tris/NaCl buffer pH 7.6 containing 1% BSA. Diluted aliquots are stable for one month at 2°C to 8°C.

Dilute 1/300 in 0.05 M Tris/NaCl buffer, pH 7.6, with 1% BSA.
If using 1/10 diluted working solutions, dilute 1/30 to achieve a final 1/300 (10 µg/mL).
7. 04905: Avidin-Alkaline Phosphatase Conjugate (0.2 mL per vial)
Store at 2°C to 8°C. **Do not freeze.** Provided at 2.5-3.0 mg/mL in solution of 0.05 M Tris-HCl, pH 8.0, containing 1 mM MgCl₂, 1% BSA and 15 mM sodium azide (see MSDS) as a preservative.
Working aliquots should be prepared by diluting avidin-alkaline phosphatase conjugate 1/10 with sterile 0.05 M Tris/NaCl buffer pH 7.6 containing 1% BSA. Diluted aliquots are stable for one month at 2°C to 8°C.

Dilute 1/150 in 0.05 M Tris/NaCl buffer, pH 7.6, with 1% BSA.
If using 1/10 diluted working solutions dilute 1/15 to achieve a final 1/150 (approximately 18 µg/mL).
8. 04809: Alkaline phosphatase substrate tablets (1 mL; 30 gold, 30 silver)
Silver tablet - Fast Red TR/Naphthol AS-MX Phosphate (4-Chloro-2-methylbenzenediazonium/3-Hydro-2-naphthoic acid 2,4-dimethylanilide).
Gold tablet - Tris buffer tablet
Substrate tablets are toxic. Do not touch with bare hands. Wear gloves at all times. Causes irritation to eyes, respiratory system and skin (refer to MSDS).

The alkaline phosphatase substrate solution is prepared in several steps:

- a) Determine the number of tablets required to make the appropriate volume of substrate (0.5 mL per slide). Each tablet set makes 1 mL of substrate solution. Allow tablets to come to room temperature (one to two hours).
 - b) Add the tablet(s) marked "tris buffer" (in gold foil) to the required volume of water. Vortex until fully dissolved.
 - c) Next add the tablet(s) marked "naphthol" (in silver foil) and vortex until dissolved.
The solution should be pale pink in color.
 - d) Use the prepared substrate solution within one hour.
9. 04913: Evans Blue 0.5% (w/v) in Phosphate Buffered Saline with 0.1% sodium azide. See MSDS for additional information.

Dilute stock 1/6 in methanol (three to four drops Evans Blue/mL methanol).

STAINING PROTOCOL

Allow slides to come to room temperature before staining (approximately half an hour). All steps of the staining procedure should be carried out with the slides in a horizontal position at room temperature.

Nalgene polypropylene pipet/instrument sterilizing tray with cover (Nalgene #13-361-5) works well for this staining procedure. Tape two plastic pipettes ~3 cm apart to bottom of container to allow slides to rest firmly on pipettes during staining.

0.05 M Tris/NaCl buffer, pH 7.6 for wash step can be placed in clean plastic wash bottles. The buffer can then be easily applied to slides for wash steps.

1. Prepare all staining solutions except the alkaline phosphatase substrate.
2. Rehydrate the cultures on the slides by applying approximately 1.5 mL of 0.05 M Tris/NaCl buffer, pH 7.6 and incubate at room temperature for 20 minutes. The buffer can be gently applied using a wash bottle.
Ensure that the cultures are completely covered with each solution used. The use of covered containers is recommended to prevent the cultures from drying out at any stage.
3. Remove buffer, then apply 0.5 mL of 5% human serum in Tris/NaCl buffer to each slide for 20 minutes. Make sure entire surface of the culture is covered with this solution.
Do not omit this step. It is necessary to block non-specific antibody binding.
At each step of the staining procedure, as much of the staining solution as possible should be removed by gently tipping the slide over a waste container, then carefully touching the edge of the slide to an absorbent paper.
4. Apply 0.5 mL of either the primary or the control antibody and incubate for 30 minutes.
One slide per batch should be stained with the negative control antibody rather than the primary antibody. All other steps of the staining procedure are the same.
5. Gently rinse three times (three minutes each) with Tris/NaCl buffer.
6. Apply 0.5 mL of biotin-conjugated goat anti-mouse IgG antibody to each slide and incubate for 30 minutes.
7. Gently rinse three times (three minutes each) with Tris/NaCl buffer.
8. Apply 0.5 mL of the avidin alkaline phosphatase conjugate and incubate for 30 minutes.

9. Prepare the alkaline phosphatase substrate solution.
10. Gently rinse slides three times (three minutes each) with Tris/NaCl buffer.
11. Apply 0.5 mL of alkaline phosphatase substrate solution to each slide for 15 minutes.
12. Gently rinse three times (three minutes each) with Tris/NaCl buffer.
13. Apply 0.5 mL of Evans Blue counterstain for each slide for a maximum of 10 minutes.
Increasing the incubation time to greater than 10 minutes may result in a decrease in staining intensity. Alkaline phosphatase substrates are soluble in organic solvents.
14. While holding the slide over a waste container, use a wash bottle containing distilled water to gently rinse off excess Evans Blue.
15. Allow the slides to air dry. They can now be stored in covered containers at room temperature or at 2°C to 8°C for prolonged storage.
If the slides are to be coverslipped, an aqueous mounting medium should be used. Most alkaline phosphatase complexes are soluble in organic solvents.

For additional details refer to the MegaCult™-C Technical Manual available at our website at www.stemcell.com

Table 2: Calculation of Staining Volumes

Solution	Components	Number of Test Slides (Test and Negative Control)				
		2(3)	4(5)	6(7)	8(9)	12(13)
		Volume of Components (in mL)				
Total Buffer Required	Tris/NaCl Buffer pH 7.6	150	250	350	450	650
5% Human Serum in Tris/NaCl buffer (Blocking and Antibody Dilution)	Human Serum (HS)	0.25	0.35	0.5	0.6	1.0
	Tris/NaCl buffer	4.75	6.65	9.5	11.4	19.0
Primary Antibody; Anti-human Glycophorin A (For test slides)	Antibody	0.015	0.025	0.035	0.045	0.065
	5% HS in Tris/NaCl buffer	1.49	2.48	3.47	4.46	6.44
Negative Control Antibody; anti-TNP (For negative control)	Antibody	0.01	0.01	0.01	0.01	0.01
	5% HS in Tris/NaCl buffer	0.99	0.99	0.99	0.99	0.99
1% BSA in Tris/NaCl Buffer (For antibody dilutions)	10% BSA	0.5	0.7	0.9	1.1	1.5
	5% HS in Tris/NaCl buffer	4.5	6.3	8.1	10.9	13.5
Biotin Anti-mouse IgG (For all slides)	Antibody (1/10 dilution)	0.067	0.100	0.133	0.167	0.233
	5% HS in Tris/NaCl buffer	1.93	2.90	3.87	4.84	6.77
Avidin-Alkaline Phosphatase Conjugate (For all slides)	Conjugate (1/10 dilution)	0.133	0.2	0.267	0.333	0.467
	5% HS in Tris/NaCl buffer	1.87	2.8	3.74	4.67	6.53
Alkaline Phosphatase Solution (For all slides)	Water	2	3	4	5	7
	Number of tablet sets	2 sets	3 sets	4 sets	5 sets	7 sets
Counterstain: (For all slides)	Drops of Evans Blue	8 dps	12 dps	16 dps	20 dps	28 dps
	Methanol	2 mL	3 mL	4 mL	6 mL	7 mL

Notes:

1. Each calculation allows sufficient reagents for one negative control slide per staining experiment.
2. Calculated volumes allow for approximately 0.5 mL of each solution per slide with 0.5 mL extra volume.
3. Biotin anti-mouse IgG antibody and avidin-alkaline phosphatase conjugate volumes are calculated using 1/10 working aliquots.

4. Definitions:

Tris/NaCl Buffer	0.05 M Tris/NaCl buffer, pH 7.6
Primary antibody	mouse anti-human Glycophorin A (1.0 µg/mL)
Negative control antibody	mouse anti-TNP (0.5 µg/mL)
10% BSA	10% bovine serum albumin in 0.05 M Tris/NaCl buffer
Biotin anti-mouse IgG	Biotin-conjugated goat anti-mouse IgG (3.0 mg/mL) Use at final dilution of 1/300
Avidin-alkaline phosphatase conjugate	Stock solution 2.8 mg/mL Working aliquot 0.28 mg/mL Final concentration ~18 µg/mL

Helpful Hints

- Slides should be brought to room temperature before staining.
- All solutions must be added gently to surface of slide.
- Do not allow slides to dry out at any point during staining procedure.
- Prepare buffers as outlined in manual and ensure buffer is the correct pH. Prepare fresh buffer daily.
- Phosphate buffers should not be used, as they can interfere with the alkaline phosphatase staining reaction.
- Do not skip blocking step.
- Ensure human serum or BSA are added at the correct concentration where indicated.
- It is critical that substrate and buffer tablets are stored correctly. They should be allowed to come to room temperature prior to use (one to two hours).
- Dissolve buffer tablet(s) completely before adding substrate tablet(s). Solution should be pale pink in color. To ensure solution is completely dissolved, leave for 10 minutes in the dark prior to use. Use within one hour.
- Evans Blue counterstain **cannot** be added to substrate staining solution. The alkaline phosphatase reaction is inhibiting.
- Do not leave slide in Evans Blue counterstain for longer than 10 minutes.
- If coverslipping is desired, only aqueous mounting media should be used. The alkaline phosphatase complex is soluble in organic solvents.
- Store stained slides in covered slide boxes in cool place.

**THIS REAGENT IS FOR RESEARCH USE ONLY.
IT IS NOT TO BE ADMINISTERED TO HUMANS.**

Appendix I: Protocol for CFC Assays in Serum-Free Collagen gels

1.0 Reagents

Product	Catalog number
MegaCult™-C Collagen and Serum-Free Medium without Cytokines	04960, 04964
OR	
MegaCult™-C Collagen and Serum-Free Medium with lipids without Cytokines	04974
Double Chamber Slides or Culture Dishes and Slides Kit	04963 or 04863
Iscove's MDM	36150
Suggested Cytokines for the growth of CFU-E and BFU-E colonies	
rh SCF	02630, 02830
rh Erythropoietin	02625

StemCell Technologies offers MegaCult™-C Serum-Free Medium without Cytokines in two different formulations - with and without lipids. Either product may be used for the culture of erythroid progenitors. Each individual researcher should choose the appropriate product for their application.

StemCell Technologies Inc. offers a number of different recombinant cytokines for the growth of other colony types. Please see our catalog for additional cytokines.

Final Formulation:

Component	Final Concentration
Collagen	1.1 mg/mL
Bovine Serum Albumin	1%
Human Transferrin	200 • g/mL
rh Insulin	10• g/mL
L-glutamine	2 mM
2-Mercaptoethanol	10 ⁻⁴ M
Suggested Cytokines for the growth of CFU-E and BFU-E colonies	
rh SCF	50 ng/mL
rh Erythropoietin	3 U/mL

1. Thaw MegaCult™-C Serum-Free Medium without Cytokines, StemCell 04974 (with lipids) or 04964 (without lipids) at room temperature or overnight at 4°C. Aliquot 1.7mL per tube. *Aliquotting is not necessary if cat no. 04960 is purchased as it is already aliquotted at 1.7mL per tube.*
2. Prepare a mixture of recombinant human cytokines at 11x the desired final concentration in Iscove's MDM.
3. Prepare a cell suspension at 33x the desired final concentration in Iscove's MDM.

Cells	Cell Suspension (cells/mL)	Cells per slide (1.5mL volume)
Human Bone marrow, Cord Blood, Mobilized Peripheral Blood		
Mononuclear preparation	6.6 – 16.5 x 10 ⁵ cells/mL	2 - 5 x 10 ⁴ cells/slide
CD34+ enriched	3.3 – 9.9 x 10 ⁴ cells/mL	1000-3000 cells/slide

Suitable cell density is dependent on the cytokine combination used. Each individual researcher will need to establish the appropriate cell concentration for each application.

4. Add 0.3mL of cytokines at 11x the desired final concentration to each 1.7mL of MegaCult™-C Serum-Free Medium. Mix well.
5. Add 0.1mL of cells at 33x the desired final concentration to each tube. Mix well.
6. Add 1.2mL of bovine collagen (StemCell, 04902) to each tube, gently vortex to mix well.
7. Dispense 0.75mL per well of a double chamber slide or 1.5mL per 35mm dish.

8. Place chamber slides or 35mm dishes in 100mm petri dishes or square bioassay dishes containing open 35mm dishes filled with 3 to 4 mL of sterile water.
9. Place dishes into a humidified incubator at 37°C with 5% CO₂ in air for 14-16 days.
10. Cultures should be visually assessed for overall colony growth and morphology using an inverted microscope prior to fixation and staining.

2.0 Dehydrating and Fixing Cultures:

Materials required for fixing the cultures:

1. Methanol – Methanol ACS (e.g. BDH #ACS531, 4L/bottle)
 2. Acetone – Acetone optima (e.g. Fisher #A929-4, 4L/bottle)
 3. Filter cards
 4. Polypropylene spacers
 5. Plastic 2.5L container with a tight fitting lid
1. Prepare 200mL of a 1:3 methanol: acetone solution. Place in a 2.5L plastic container and tightly seal the lid to prevent evaporation of the fixative.
It is important to use a high quality grade of methanol and acetone.
 2. Leave the cultures at 37°C until just prior to dehydration as the collagen gel may become unstable at lower temperatures. Remove slides from the incubator singly or in small batches.
Ensure that each slide is correctly labeled with a pencil or diamond point pen. Ink labeling will become illegible when the slide is immersed in the fixative solution.
 3. **If chamber slides are used:**
Carefully remove the plastic walls and rubber seal of the chamber slide without damaging the culture using the following procedure:
 - Partially remove the plastic chamber by pulling it up and away from the slide (use forceps to hold the slide down) starting at one corner near the labeled end of the slide.
 - When the rubber seal surrounding the collagen gel is visible under the plastic chamber, use forceps to grasp one corner of the rubber seal and gently stretch it over the corner of the plastic chamber so that it remains hooked there.
 - Use forceps to hook the rubber seal over the other corner of the plastic chamber at the same end of the slide.
 - The plastic chamber and rubber seal can now be easily removed in one motion by continuing to slowly pull them up and away from the slide. Gel and associated liquid will remain on the slide.**If 35mm dishes are used:**
 - Remove lid and release the gel from the walls of petri dish by rimming with a thin plastic pipette tip or microspatula.
 - Cover the petri dish with a pre-labeled glass slide.
 - While holding the petri dish firmly onto the glass slide invert the dish and slide.
 - Gently shake and let the gel slip out of the petri dish and spread out evenly onto the glass slide. Remove the petri dish.
 - Gently remove folds in the gel using the tapered end of a microspatula or pipette tip.
 4. Gently place a polypropylene spacer onto the chamber slide. Place a thick white filter card on top and allow the liquid to soak the card.
 5. Remove the thick white card, leaving the polypropylene spacer in place.
 6. Place the slides into the 2.5L plastic container filled with 200mL of the fixative solution. To ensure that the slides are completely covered with fixative, no more than 12 slides should be placed in the container at one time. Tightly seal the lid of the container. The polypropylene spacer will float off, leaving two squares of gel on the chamber slide or one circle of gel on the large slides used with petri dishes.
 7. Leave the slides in the methanol:acetone solution for 20 minutes at room temperature. Do not agitate the fixative solution.
 8. Remove the slides from the fixative and allow to air dry for 15 minutes.
 9. Cultures can then be stained immediately or stored at 4°C in the dark for up to one month until immunocytochemical staining can be performed.