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Galectin-3-Binding and Metastasis

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i. Summary

Galectin-3 is a member of a family of carbohydrate-binding proteins. It is present in the nucleus, the cytoplasm and also extracellular matrix of many normal and neoplastic cell types. Arrays of reports show an upregulation of this protein in transformed and metastatic cell lines (1, 2). Moreover, in many human carcinomas, an increased expression of galectin-3 correlates with progressive tumor stages (3–6). Several lines of analysis have demonstrated that the galectins participate in cell-cell and cell-matrix interactions by recognizing and binding complimentary glycoconjugates and thereby play a crucial role in normal and pathological processes. Elevated expression of the protein is associated with an increased capacity for anchorage-independent growth, homotypic aggregation, and tumor cell lung colonization (7–9). In this chapter we describe the methods of purification of galectin-3 from transformed *E. coli* and some of the commonly used functional assays for analyzing galectin-3 binding.

Keywords

galectin-3; purification; homotypic aggregation; heterotypic aggregation; anchorage independent growth; wound healing

1. Introduction

Human galectin-3 is a ~30 kDa unique chimeric gene product belonging to the family of non-integrin -galactoside binding lectins with conserved amino acid sequences at the carbohydrate-binding motif (10). Clinical investigations have shown a correlation between expression of galectin-3 and malignant properties of several types of cancers (11), consequently, galectin-3 is thought to be a cancer-associated protein. It consists of three structural domains, each associated with at least one specific function: a) a N-terminal domain containing a serine phosphorylation site important to regulate its cellular signaling (12); b) a collagen- like sequence cleavable by matrix metalloproteinases (13); and c) a COOH terminal containing a single carbohydrate recognition domain (CRD) and the NWGR anti-death motif (14). Galectin-3 is mainly a cytosolic protein, but can easily traverse the intracellular and plasma membranes to translocate into the nucleus, mitochondria and be externalized, which suggests that galectin-3 is a shuttling protein and may have multiple functions accordingly (15–17). Galectin-3 lacks the classical secretion signal sequence and does not pass through the standard ER/Golgi pathway (18), still it can be transported into the extra cellular milieu via a non-classical pathway (19), where it can interact with a myriad of partners regulating a number of biological functions (11) (20, 21). Many of the functions of

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⁶After step 15, save the flow through and reload onto the column.

¹⁷It is important to siliconize the glass tubes in which aggregation is performed so cells adhere to the glass.

¹⁸The aggregates are usually very fragile and can be disrupted by harsh pipeting.

galectin-3 are dependent on its carbohydrate binding properties and therefore can be inhibited by a specific disaccharide inhibitor, namely lactose.

The analysis of galectin-3 properties often requires use of recombinant galectin-3. Currently, a few methods are available to purify galectin-3 either using affinity of galectin-3 to the substrate or by using affinity tags fused to galectin-3. Glutathione S-transferase (GST) is a popular affinity tag commonly used with recombinant proteins. The method is based on the affinity of GST to glutathione ligand coupled to a matrix. The binding of GST-tagged protein to matrix is reversible, and the fused protein can be eluted under mild, non-denaturing conditions by adding reduced glutathione to the elution buffer. If desired, cleavage of the protein from GST can be achieved using a site-specific protease whose recognition sequence is located immediately upstream of N-terminal of galectin-3.

GST-tagged galectin-3 is constructed by inserting cDNA sequence encoding full length or fragment of galectin-3 to multiple cloning site of one of the pGEX vectors or any other vector expressing GST protein under the regulation of bacterial or mammalian promoter. In case of pGEX vector the expression is under control of the *tac* promoter, which is induced by isopropyl -D thiogalactoside (IPTG). All pGEX vectors also contain *lacIq* gene, which produce repressor protein preventing expression until induction by IPTG. Although all pGEX vectors have a range of protease cleavage recognition sites we use pGEX-6P vectors that contain unique cleavage site that is recognized by PreScission Protease.

To analyze the binding of galectin-3 to its receptors the most commonly used protocol involves labeling the protein either by biotin or by ¹²⁵I (22, 23). We have discussed here the biotinylation protocol because of its relative simplicity and avoidance of radioactive reagents. After binding of biotin labeled protein to the cell surface receptors, the binding efficiency can be measured in terms of color development by using a substrate chromogen mixture.

Laminin, fibronectin, or collagen type IV are the ECM proteins with affinity for galectin-3 (24). In section 3.2a we describe the assay for binding of the cell surface proteins to ECM ligands. Since a number of surface proteins can bind to a ligand, this is generally performed as an indirect assay with a number of cell lines varying in their galectin-3 expression that are derived from the same origin.

It has been presumed that tumor cell surface lectins might play a role in cellular interactions *in vivo* that are important for the formation of emboli and for the arrest of circulating tumor cells (25). Homotypic aggregation is an assay that reflects on the formation of tumor cell emboli in circulation. This assay is performed using asialofetuin, which is a glycoprotein possessing several branched oligosaccharide side chains with terminal non-reducing galactosyl residues. It binds to the lectins present on the cell surface of tumor cells and induces homotypic aggregation by serving as a cross-linking bridge between adjacent cells (26). Heterotypic interactions between the tumor cells and endothelial cells can be measured by wound healing assay and the 3-dimensional co-cultures on Matrigel (27). These assays help in analyzing the interactive properties of different variants of galectin-3, thus signifying the role of various mutations or protein fragments.

One *in vitro* property of tumorigenic cells is their ability to grow progressively in semi-solid medium, which indicates an autonomy from growth regulatory mechanisms (28). Anchorage independent growth is an assay in which the cells are seeded on soft agar and allowed to grow. The cells, which divide and form colonies over a period of 10–15 days usually exhibit a higher metastatic potential in *in vivo* studies (5).

2. Materials

2.1.a Purification of galectin-3 using affinity column

1. LB broth: 1% tryptone, 0.5% yeast, 1% NaCl.
2. Galectin-3 expressing bacterial clone: E coli transformed with a suitable vector containing cDNA encoding the human galectin-3 transcript.
3. Ampicillin: 1% ampicillin. Ampicillin stock should be stored at -20°C
4. Lysis buffer: 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl, pH-8.0, 0.1% Leupeptin, 1 mM PMSF. Leupeptin and PMSF stocks should be stored at -20°C and added prior to use.
5. IPTG: 0.1M IPTG.
6. Phosphate buffer: 8 mM Na_2HPO_4 , 2 mM NaH_2PO_4 , 1 mM MgSO_4 , 1 mM PMSF, 0.2% NaN_3 , 5 mM DTT. PMSF and DTT should be added just prior to use.
7. Elution buffer: phosphate buffer containing 0.3 M lactose.
8. MOPS buffer: 0.5 M, pH-7.5
9. Asialofetuin column: Prepare as follows and store at 4°C

Preparation of asialofetuin

- a. Dissolve 100 mg fetuin in 40 mL of 0.025 N H_2SO_4 .
- b. Incubate the solution at 80°C for 1h.
- c. Dialyze against 40 liters of distilled H_2O to remove the SO_4 ions
- d. Lyophilize overnight and resuspend in 13 mL of water.
- e. To make sure that the fetuin is converted into asialofetuin, run it on a reducing SDS-polyacrylamide gel. Fetuin runs at ~66 kDa, whereas asialofetuin runs a little lower. (Notes 1).

Preparing Asialofetuin Column

- a. Dissolve asialofetuin in 0.5 M MOPS buffer at a concentration of 10 mg/mL.
- b. Take 5 mL of affigel-15 (Bio-Rad Cat#153-6051) slurry and wash with 15 volumes of cold deionized water, less than 20 minutes before the coupling reaction.
- c. For the coupling reaction combine 4 mL of 10 mg/mL cold asialofetuin/buffer solution with 4 mL of affigel-15. Mix at 4°C for 2 h.
- d. Centrifuge slurry, pour off supernatant and resuspended in deionized water. Add 1M ethanolamine pH-8.0 at 0.1 mL/mL of mixture to block the unreacted sites. Agitate gently at 4°C for 1 h.
- e. Pack a column with the slurry, leave overnight in a cold room, and wash with 0.1 M MOPS buffer until eluent is free of protein.
- f. Equilibrate column in 10 mM phosphate buffer containing 0.2% sodium azide. (Notes 2)

¹In the dialyzed form asialofetuin can be stored at 4°C for months.

²The binding efficiency of asialofetuin in the column can be determined by calculating the protein content in the flow through.

2.1.b Purification of GST-tagged galectin-3

1. 2xYT broth: 1.6% tryptone, 1% yeast, 0.5% NaCl
2. Galectin-3 expressing bacterial clone: E coli transformed with a suitable vector containing cDNA encoding the human galectin-3 transcript.
3. Ampicillin: 1% ampicillin. Ampicillin stock should be stored at -20°C
4. Lysis buffer: 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl, pH-8.0, 0.1% Leupeptin, 1 mM PMSF. Leupeptin and PMSF stocks should be stored at -20°C and added prior to use.
5. IPTG: 0.1M IPTG.
6. Phosphate buffer: 8 mM Na_2HPO_4 , 2 mM NaH_2PO_4 , 1 mM MgSO_4 , 1 mM PMSF, 0.2% NaN_3 , 5 mM DTT. PMSF and DTT should be added just prior to use.
7. Cleavage Buffer (CB) 100 ml: 50 mM Tris-HCl pH-7, 150 mM NaCl, 1 mM EDTA, 1 mM DTT
8. PreScission Protease (PP): For 1 ml bed volume, mix 80 μl (160 units) of PP with 920 μl of CB
9. Glutathione Sepharose 4B beads (GE Healthcare Bioscience)

2.2 Binding of Galectin-3

2.2a. To cell surface receptors

1. Recombinant galectin-3
2. EZ-link, Sufo-NHS-Biotinylation Kit (Pierce, IL, USA)
3. Substrate chromogen mixture (prepared immediately before use): Dissolve 0.5 mg/ml ABTS (2,2 -Azino-thiazoline sulfonic acid) in 0.1 M Citrate buffer, pH-4.2 containing 0.03% hydrogen peroxide. Alternatively, ABTS substrate kit for HRP (Zymed Laboratories, Inc. CA, USA) can be used.
4. Lactose: 1 M in ddH₂O
5. 96 well microtiter plates
6. Elisa plate reader

2.2b To soluble Extracellular Matrix Proteins

1. 96 well microtiter plates
2. EHS laminin: 100 $\mu\text{g}/\text{mL}$, store at -20°C
3. Collagen type IV: 100 $\mu\text{g}/\text{mL}$, store at -20°C
4. Fibronectin: 100 $\mu\text{g}/\text{mL}$, store at -20°C
5. PBS
6. BSA: 30% in distilled water, store at 4°C
7. Alamar blue (Biosource International)
8. Elisa Plate reader

2.3 Homotypic Aggregation

1. EDTA: 0.02% in CMF-PBS

2. Asialofetuin: as described in section 2.1
3. Formaldehyde
4. Trypan blue: 0.4% in normal saline
5. Glass tubes coated with sigmacote (Sigma Chemical Co. USA): pour 1ml sigmacote in the glass tube, rotate the tube, so that the entire area inside the tube is covered with sigmacote. Let it dry and wash the excess with distilled water.
6. Orbital shaker
7. Hemocytometer

2.4 Heterotypic aggregation

1. Matrigel
2. 8 chamber slide
3. Endothelial cells (HUVEC or any other)
4. Tumor cells (galectin-3 expressing)
5. Trypsin (0.25%)
6. Trypan blue: 0.4% in normal saline
7. Hemocytometer
8. Viable stains DiI and DiO (Invitrogen)
9. Tissue culture incubator

2.5 Wound Healing Assay

1. Cell Culture insert (ibidi GmbH)
2. Endothelial cells
3. Tumor cells (galectin-3 expressing)
4. Trypsin (0.025%)
5. Hemocytometer
6. Viable stains DiI and DiO (Invitrogen)
7. Tissue culture incubator

2.6 Anchorage independent growth

1. Complete medium: appropriate medium with 10% fetal bovine serum
2. 1% agar solution: Add 1 g of sea-plague agarose in 10 mL of distilled H₂O and autoclave. Cool down to 45°C and add 90 mL of complete medium and mix well. Agar solution in medium can be stored at 45°C for 2–3 days.
3. 2.5% glutaraldehyde
4. 6-well plates

3. Methods

3.1.a Isolation of recombinant galectin-3

- 1 Inoculate 10 mL of LB Broth containing 100 µg/mL of ampicillin with galectin-3 expressing bacterial clone.
- 3 Incubate overnight at 37°C with constant shaking at 225 rpm.
- 4 Use the overnight bacterial culture to inoculate fresh 10×200 mL of LB containing 100 µg/mL of ampicillin.
- 5 Incubate for 3 h at 37°C with shaking at 225 rpm. (Notes 3)
- 6 Add ampicillin again and 2.5 mL of IPTG stock to induce the protein synthesis. It is important to add more ampicillin to allow only the resistant bacteria to grow.
- 7 Incubate for 4 h at 37°C with shaking at 225 rpm.
- 8 Transfer the contents to centrifuge bottles and spin down for 15 min at 1,000 g at 4°C.
- 9 Discard the supernatant and resuspend pellet containing the bacteria in 20 mL PBS, combine into one bottle, centrifuge for 15 min at 1,000 g at 4°C.
- 10 Discard the supernatant and store pellet at –70°C overnight. The pellet can be stored for up to 1 week without losing the protein yield.
- 11 Resuspend pellet in 80 mL of ice-cold lysis buffer.
- 12 Disrupt the cells with a probe type sonicator using multiple short bursts at maximum intensity for 4 × 30 sec on ice. Do not sonicate for more than 30 sec in one time, otherwise the temperature of the lysate will increase and degrade some of the proteins. In-between the strokes cool the lysate on ice.
- 13 Centrifuge the lysate for 20 min at 18,000 g, and save the supernatant. Rest of the steps are performed in a cold room.
- 14 Equilibrate the asialofetuin column with 3 bed volumes of phosphate buffer.
- 15 Load the supernatant from step 13. Allow the supernatant to flow through the column at the rate of 10–12 drops per minute.
- 16 Wash the column with 3 bed volumes of phosphate buffer.
- 17 Elute the protein with 15 mL of elution buffer in 1 mL fractions.
- 18 Wash the column with 3 bed volumes of phosphate buffer. The column can be reused if stored properly at 4°C.
- 19 Measure the protein content of the samples collected from step 17 by using standard protein estimation methods. Pool samples containing the protein and dialyze against PBS till all lactose is removed from the samples. The purified protein can be stored at –70°C. (Notes 4–7)

³After step 5, determine the O.D. at 600 nm. It should be 0.5.

⁴The normal yield of galectin-3 from one liter of bacterial culture varies from 1–5 mg. If the yield is too low, we suggest going through notes 5–7

⁷At no stage let the column dry, because it will reduce the binding efficiency.

3.1.b Purification of galectin-3 using Glutathione S-transferase (GST) affinity tag

1. Grow BL21-CodonPlus (DE3)-RIPL (Notes 8) transformed with pGEX-6p-Galectin-3 in 25 mL 2xYT/AMP at 37°C overnight (ON) (100 mL flask).
2. Transfer the bacteria to 1 L 2xYT/AMP media (5 L flask) and grow at 37°C until OD₆₀₀ reaches 0.5–0.8 (usually after 5–6 h). Transfer the flask to 20°C and wait about 30 min.
3. Add IPTG to a final concentration of 0.5 mM and continue incubation 4–5 h or ON at 20°C.
4. Harvest bacteria by centrifugation at 6,000 rpm for 10 min, 4°C. You can freeze the bacterial pellet, or lyse the cells directly.
5. Cell lysis: resuspend the bacteria in (10 mL per 1 g of pellet) cold 1x PBS with protease inhibitors.
6. Sonicate the bacteria on ice for a total time of 2 min (10 sec pulse 50 sec off at 65 % amplitude) in 50 mL centrifuge tubes (not less than 30 mL total volume). Check the percent of intact bacteria under the phase contrast microscope. Save a sample of the supernatant. Immediately add Triton X-100 to a final concentration of 1% and incubate for 30 min with gentle agitation at 4°C.
7. Centrifuge the sample at 15,000 g for 10 min at 4°C and then transfer to new tubes. Save a sample of the supernatant.
8. Incubate the supernatant with 0.5 mL of Glutathione Sepharose (GS) 4B beads (Notes 9) (washed with 1 x PBS) per 10 mL lysate for 30–60 min at 4°C with gentle agitation. Spin at 500 g for 1 min, remove supernatant and wash the beads with 5 mL PBS three times.
9. Spin at 500 g for 10 min, remove supernatant and wash the beads with 5 mL of cleavage buffer
10. During the wash with cleavage buffer prepare the mixture of PreScission Protease (Notes 10) and cleavage buffer. Use 80 µL of PreScission Protease (160 units) and 920 µL of cleavage buffer.
11. Add the mixture to GS beads and incubate for 4 h to ON at 4°C.
12. Spin the beads (500 × g for 5 min) and collect the supernatant.
13. Wash the beads three times with 500 µL of PBS. Save aliquots for analysis and estimation of protein concentration. The eluate will contain galectin-3, while GST moiety and PreScission Protease (also GST-tagged) will remain bound to the beads.

3.2 Binding of galectin-3

3.2a To cell surface receptors

- 1 Isolate rgalectin-3 as in 3.1

⁸Although variety of *E. coli* host strains can be used for cloning and expression with pGEX vectors we recommend specially engineered strain that are more suitable for maximize expression of mammalian proteins: BL21-CodonPlus (DE3)-RIPL.

⁹For affinity purification of GST-galectin-3 can be used variety of glutathione sepharose. However based on our experience we would like to recommend Glutathione Sepharose 4B (GE Healthcare) as a first choice. Total amount of sepharose beads is dependent upon the final amount of the desired galectin-3 yield (Table 1).

¹⁰The choice of protease is based on few factors such as presence in galectin-3 recognition sequences of other proteases and specificity.

Biotinylation of galectin-3

- 2 Dissolve 2 mg protein in 1 mL of PBS solution and calculate the number of mmoles dissolved using the following formula

$$\frac{\text{mg protein}}{M.W. \text{ protein}} = \text{mmoles of protein}$$

- 3 Dissolve 2 mg sulfo-NHS-Biotin in 100 μ L distilled water and add 30 μ L of this solution to the protein solution to give 20-fold molar excess over galectin-3.
- 4 Incubate on ice for 2 h for biotinylation of galectin-3 to be completed.
- 5 To remove excess salt from the protein, equilibrate the 10 mL desalting column with 30 mL PBS and apply the protein sample. Allow the sample to permeate the gel. Add buffer to the column in 1 mL aliquots and collect 1mL fractions of the purified eluent protein in separate test tubes.
- 6 Monitor protein content in the tubes by absorbance at 280 nm, pool fractions containing protein. (Notes 11)

Binding of galectin-3 to the receptors

- 7 Plate cells in a 96 well plate at a density of 1×10^4 cells per well and incubate overnight.
- 8 Adjust the concentration of biotinylated galectin-3 ranging from 0–20 μ g/50 μ L.
- 9 Remove medium from the cells.
- 10 Add fresh medium containing 0.5% FCS and add 50 μ L biotin labeled galectin-3 at different concentrations. (Notes 12)
- 11 Incubate the plates for 2 h at 37°C
- 15 Wash 3 times with PBS to remove unbound proteins.
- 16 Add 1:1000 dilution of horseradish peroxidase conjugated streptavidin avidin complex to the wells. The HRP conjugated complex binds to biotin labeled galectin-3. Incubate for 30 min at room temperature.
- 17 Wash with PBS 3 times to remove unbound complex.
- 18 Color development is obtained by the addition of 100 μ L of substrate chromogen mixture. Incubate for 1 min.
- 19 Measure OD at 405 nm. (Notes 13)

3.2b To Extracellular matrix Proteins

- 1 Coat the 96 well microtiter plates with serially diluted (0–10 μ g) EHS laminin, collagen type IV, or fibronectin.
- 2 Incubate the plates for 1 h at 37°C or at 4°C overnight to dry the ECM protein. (Notes 14)

¹¹Store biotinylated protein at 4°C until ready for use. The protein can be stored under these conditions for 1 week.

¹²In some wells 50–100 mM lactose can be added along with the protein for specific inhibitory studies.

¹³The biotin streptavidin complex cleaves H₂O₂ which is coupled to the oxidation of substrate ABTS giving a green end product.

¹⁴Make sure to keep the plates horizontally, so that there is a uniform coating on the well.

- 3 Block the non-specific sites in the wells by incubating with sterile 1% BSA in PBS for 1 h at 37°C
- 4 Wash wells with sterile PBS three times to remove extra proteins.
- 5 Detach the cells from the plate using 0.02% EDTA. Count the viable cells using trypan blue exclusion method using hemocytometer and seed at 4×10^4 cells per well.
- 6 Allow the cells to adhere to the plates for 15 min to 24 h. This time can be varied according to the requirement of the experiment.
- 7 Wash off the non-adherent cells with medium. Repeat twice. (Notes 15)
- 8 To count the number of cells attached to the ECM proteins, add 200 μ L medium and a 1:10 dilution of Alamar blue. The live cells create a reducing environment, which changes the color of dye from blue to pink.
- 10 Incubate for 3–4 h at room temperature. 11. Read absorbance at 570 nm.

3.3 Homotypic Aggregation

1. Detach the cells from monolayer with 0.02% EDTA in CMF-PBS. Use of trypsin is avoided because it may interfere with the surface proteins.
2. Suspend at 1×10^6 cells per ml in CMF-PBS with or without 20 μ g/mL asialofetuin.
3. Place 0.5 mL aliquots in siliconized glass tubes. Agitate at 80 rpm for 60 minutes at 37°C.
4. Terminate the aggregation by fixing the cells with 1% formaldehyde in CMF-PBS.
5. Count the number of single cells by haemocytometer (Notes 16-18).
6. Calculate % aggregation by $(1 - N_t/N_c) \times 100$, where N_t is the number of single cells in control and N_c is the number of single cells in the presence of test compound.

3.4 Heterotypic Aggregation

1. Thaw Matrigel on ice (Notes 19)
2. Cool an 8 chamber slide on ice.
3. Pour 200 μ L Matrigel in each chamber of the 8 chamber slide, carefully remove air bubbles and place the slide in a 37°C tissue culture incubator for approximately 15 min till the Matrigel is solidified, forming a uniform layer.
4. Trypsinize cells, calculate the number of cells required for the experiment. You will need 50,000 cells per chamber for each cell line.
5. Suspend the cells at a concentration of 1×10^6 per mL complete medium in a tube and incubate with 5 μ L of DiI or DiO as desired at 37°C for 15 min in dark.
6. Centrifuge cells at 1000 rpm for 5 min and wash once with complete medium
7. Re-suspend the cells at a density of 1×10^6 /mL

¹⁵The wells should be washed thoroughly to remove the unbound protein. However, the washes should be gentle so the cells remain unaffected.

¹⁶Make sure that the cells are a single cell suspension after detaching from monolayer.

¹⁹Matrigel should be thawed at 4°C, because it has a tendency to gel at higher temperatures.

8. Mix 50,000 epithelial and endothelial each (each stained with different dye) in an eppendorf tube, centrifuge, remove medium and suspend in 250 μ L medium specific for endothelial cells (Notes 20).
9. Seed the cells on Matrigel (Notes 21)
10. Observe and photograph after 24 hr (Fig 1)

3.5 Wound Healing Assay

1. Trypsinize cells, calculate the number of cells required for the experiment.
2. Suspend the cells at a concentration of 1×10^6 per ml complete medium in a tube.
3. Incubate with 5 μ L of DiI or DiO as desired at 37°C for 15 min
4. Centrifuge cells at 1000 rpm for 5 min
5. Wash once with complete medium, suspend the cells at a density of 1×10^6 /mL
6. Seed 2.4×10^4 cells in each chamber of cell culture insert. If the migration of cells towards each other is to be analyzed, two different kinds of cells can be seeded. We perform this assay to study wound healing and cell migration of endothelial cells towards epithelial cells harboring various galectin-3 variants.
7. After 24 hr incubation in the tissue culture incubator, remove the insert carefully using sterile forceps, wash the chamber 2–3 times with endothelial cell medium to remove any floating cells. Add endothelial cell growth medium. Observe under light or fluorescent microscope at different time intervals to analyze migration (Notes 21). (Fig 2)

3.6 Anchorage independent growth

1. Pour 2 ml of 1% agar solution into 6-well plates.
2. Allow it to solidify at room temperature for 15–20 min. Plates can be wrapped with parafilm and stored at 4°C for about 2 weeks.
3. Suspend 500 or 1000 cells in 1mL of complete medium. Immediately add 1mL of 1% agar solution. Mix and pour gently on the plates. (Notes 16)
4. Let the cell-containing top layer solidify at room temperature for 15 min and 2 hr at 4°C.
4. Transfer to 37°C in a CO₂ incubator for overnight.
5. Next morning, add 1mL of complete medium, and allow the colonies to grow for 2 weeks. Medium should be changed two times per week. (Notes 22)
6. Fix colonies with 2.5% glutaraldehyde and compare the number and size of the colonies.

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²⁰In our experience, the endothelial cells are more sensitive to any change in medium composition, while the tumor cells can grow in endothelial cell specific medium. Therefore, we use endothelial cell medium for co-cultures.

²¹The cells should be added very carefully in the center of the chamber so as not to disturb the Matrigel layer.

²²The agarose layers should be allowed to gel completely to prevent the sliding of the top layer or passage of the cells to the bottom of the plates.

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Fig. 1. Cell migration using wound healing assay: Endothelial cells BAMEC and Galectin-3 variant H64 or P64 transfected BT-549 cells were prelabeled with DiO (green) and DiI (red) respectively and seeded in each chamber of cell culture insert. After 24 hr, the inserts were removed and cell migration was studied. a–a' : migration of BAMEC and BT-549-H64; b–b' : migration of BAMEC and BT-549-P64; a, b : 0 hr; a', b' : 24 hr.

Fig. 2. Endothelial cell morphogenesis and interactions with H64 or P64 transfected BT-549 cells. Endothelial cells BAMEC and Galectin-3 variant H64 or P64 transfected BT-549 cells were prelabeled with DiO (green) and DiI (red) respectively. 50,000 cells were seeded in each chamber on top of gelled Matrigel. The 3-dimensional cultures were observed after 24 hr. a: BAMEC alone; b: BAMEC and BT-549 cells; c: BAMEC and BT-549 H64; d: BAMEC and BT-549 P64.

Table 1

Tagged protein yield	50 mg	10 mg	1 mg	50 µg
Culture volume	20 l	4 l	400 ml	20 ml
Volume of extract	1 l	200 ml	20 ml	1 ml
Glutathione Sepharose bed volume	10 ml	2 ml	200 µl	10 µl
1× PBS ^I	100 ml	20 ml	2 ml	100 µl
Glutathione elution buffer	10 ml	2 ml	200 µl	10 µl

^IThis is volume required per wash. Three washes are recommended per sample.