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A high throughput screening assay to quantify, visualize, and standardize biological activities: Enzyme-Linked Immuno-Culture Assay (ELICA)

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Abstract

A high throughput screening assay, termed enzyme-linked immuno-culture assay (ELICA), was originally developed to identify, visualize, and purify biological activities of unknown compounds, e.g., human recombinant proteins and morphogenetic proteins. The ELICA was based on concepts inherent to an ELISA for the identification and characterization of antibodies binding to antigens. The ELISA is performed by absorbing either antigens or antibodies to a nylon membrane, probing with either antibody or antigen with an attached HRP, then incubating with HRP substrate. The ELICA was based on a 96-well plate format. Cultured cells (or unknown compounds) are fixed to the plate surface using the ELICA fixative. The wells are then incubated in succession with an antibody to a phenotypic expression marker (PEM), the primary probe; followed by antibody against species of primary probe with an attached biotin, the secondary probe; followed by avidin with attached horseradish peroxidase enzyme, the tertiary probe. Then the wells are incubated with a soluble HRP substrate. The soluble HRP substrate is removed and scanned in a spectrophotometer to quantify amount of phenotypic expression marker, based on a titration curve. The wells are then incubated with an insoluble HRP which precipitates at sites of antibody binding, allowing visualization of labeled cells/material. The wells are photographed and then processed for DNA quantification. Each of the above procedures were performed in each well of 96-well plates. Results are reported as quantity of PEM standardized to quantity of DNA.

Keywords: Healing Cells; Telomerase Positive; Embryonic; Adult; Stem Cells; Assay

1. Introduction

The ability to identify and quantify biological activity is paramount if the goal is to test purified compounds, or compounds generated from gene sequences (e.g., recombinant proteins), or to isolate and purify compounds from other sources (e.g., morphogenetic proteins isolated from demineralized bone matrix; exosomes generated by differentiated cells) [1-9]. A high throughput screening assay, termed enzyme-linked immuno-culture assay (ELICA) [10], was based on concepts inherent to an ELISA (enzyme-linked immuno-sorbent assay) for the identification and characterization of antibodies binding to antigens [11-28]. The differences between the two technologies are that the ELISA absorbs an antigen or antibody to a membrane, usually nylon, and incubates the membrane with its counterpart, either antigen bound to membrane and incubated with an antibody with an attached HRP enzyme, or vice versa, an antibody bound to

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the nylon membrane and incubated with an antigen with an attached HRP enzyme. The ELISA is associated with hybridoma technologies, detection of bacterial infections, detection of viral infections to elucidate antigen – antibody pairing [1-8, 11-28]. The antigen or antibody needs to be suspended in a solution, to be absorbed onto a membrane for subsequent processing. Two additional procedures were incorporated into the ELICA to increase sensitivity to antigenic sites. These two procedures were amplification using a biotin-avidin-HRP complex and the generation of high titer ascites for a more sensitive assay using fewer cells.

The ELICA procedure can be performed with tissues incubated in the ELICA fixative [9,10,29,30], either vibratome or cryosection, for use with immunocytochemistry to locate antigenic sites within the tissues [31-40]. It can be performed with substances dissolved in solution and fixed to the plate surface with the ELICA fixative [9]. It can be performed with cultured cells incubated with the ELICA fixative [30-40]. And it can be performed with cultured cells challenged with biological agents to assess resultant phenotypic expression markers and quantify DNA content [40-45].

Four hypotheses were tested

- Hypothesis 1. A fixative could be developed for fast penetration, retention of excellent cellular morphology, prevention of apoptosis leading to self-destruction, with a physiological osmolarity of 1.0, and used with any antibody;
- Hypothesis 2. The signal could be amplified using biotin-avidin-HRP for phenotypic expression markers;
- Hypothesis 3. High titer ascites would increase sensitivity to the antigenic sites; and
- Hypothesis 4. Quantitation of PEM/DNA sensitivity could be increased by several orders of magnitude.

2. Materials and methods

2.1. Assay Reagents: [9,10,29-46]

- Species-specific buffers:
  - Amphibians & Reptiles – 10% Holtfreter's solution [46]
  - Avians – Tyrode's balanced salt solution, #T-2145 (Sigma)
  - Non-human mammals – Phosphate Buffered Saline, PBS (Sigma)
  - Humans – Dulbecco's Phosphate-Buffered Saline (10X) #310-4080AJ (GIBCO)
- ELICA Fixative Reagents
  - Species-Specific Buffers
  - Paraformaldehyde, #P6146, Sigma
  - Glutaraldehyde, #G5882, Sigma
  - Sodium Azide, #S2002, Sigma
  - D-Glucose, # G-6138, Sigma
- Inhibition and Exhaustion of Endogenous Peroxidases
  - 5% Sodium Azide, #S2002 Sigma
  - 30% Hydrogen Peroxide, #H-1009, Sigma
- Blocking Agents for ELICA
  - Horse serum, #H7889, Sigma
  - Goat serum, #200-6210-AG, DSHB
  - Porcine Serum, #P9783 Sigma
  - Bovine serum albumin, #A-7906, Sigma
  - Neonate Bovine Serum, #2002, BioCell
  - Fetal Calf Serum, #F0392, Sigma
  - Human Serum, #H4522, Sigma
  - Tween 20 (polyoxyethylenesorbitan), ChemPure, Curtain Matheson Scientific, Houston, TX
  - Nonidet P-40, P-40, #56007, BDH
  - Triton-X-100, #T-6878 (Sigma)
  - Powdered non-fat dry milk, Kroger's
  - Gelatin (cold water fish soluble collagen), #935425, Sigma
- Positive Standards
  - Calf thymus DNA type-I, #D-1501, Sigma
  - Myosin from chicken muscle, #M-7266, Sigma
  - Rabbit anti-myosin (skeletal-smooth), #M-7648, Sigma
- Primary Probes

- 12/101, Developmental Studies Hybridoma Bank (DSHB)
- 31-2, DSHB
- 5D2-27, DSHB
- MF-1, DSHB
- MF-5, DSHB
- C3/1, DSHB
- M3F7, DSHB
- 22/18, DSHB
- ALD-58, DSHB
- MF-20, DSHB
- B3/D6, DSHB
- M-38, DSHB
- D76, DSHB
- ALD-66, DSHB
- JLA-20, DSHB
- SP1.D8, DSHB
- CHI, DSHB
- 5C6, DSHB
- 33-2, DSHB
- 2E8, DSHB
- D3, DSHB
- ID4B, DSHB
- MF-30, DSHB
- ABL-93, DSHB
- 5-D-4, #69-625, ICN
- Anti-myosin, #65-790-1, ICN
- Anti-desmin, #65-793-1, ICN
- Anti-vimentin, #69-127-2, ICN
- Anti-type IV collagen, #69-108-1, ICN
- Anti-type II collagen, #1320-01, Southern Biotechnology
- Anti-heparan sulfate proteoglycan, #MAB459, Chemicon
- CEA-CAM-1, DH (D. Hixson, Providence, RI)
- HCEA, RB
- CEA, #111518, Sigma
- CD66e, BD (Beckton-Dickinson)
- Dh-TuAg1, DH
- MC-480 (SSEA-1), DSHB
- MC-631 (SSEA-3), DSHB
- MC-813 (SSEA-4), DSHB
- CD10, BD
- CD90, BD
- Thy-1, DSHB
- CD56, BD
- Pax-6, DSHB
- FORSE-1, DSHB
- Vimentin, DSHB
- Nestin, DSHB
- R401, DSHB
- HNES, R&D
- MAB353, Sigma
- RT-97, DSHB
- NF68, Prospec
- S-100, DSHB
- NF-145, R&D
- N-200, R&D
- 8A2, DSHB
- NG2, DSHB
- TH, DSHB
- SV2, DSHB

- DOPA, Thermo-Fisher
- T8660, Sigma
- Tuj1, DSHB
- GFAP,
- CNPase, Abcam
- Rip, DSHB
- MOSP, Sigma
- MAB, DSHB
- 40E-C, DSHB
- VM-1, DSHB
- CD13, BD,
- OP-137, Antibodies Inc
- F5D, DSHB
- MF-20, DSHB
- ALD-58, DSHB
- A4.74, DSHB
- IA4, Thermo-Fisher
- Calp, Abcam
- MAB-3252,
- MAB1548,
- WV1D1,
- MP111,
- CIIC1, DSHB
- II-4CII, DSHB
- HC-II, Abcam
- D1-9, DSHB
- 9/30, DSHB
- 12/21, DSHB
- 12C5, DSHB
- H-CD34, BD
- CD31, BD
- P1H12, DSHB
- P2B1, DSHB
- P2H3, DSHB
- H-endo, Fisher Scientific
- H5A4, DSHB
- H4C4, DSHB
- Hermes-1, DSHB
- CD45, BD
- CD63, BD
- CD95, BD
- H5A5, DSHB
- H5C6, DSHB
- HFSP, DSHB
- 1B10, DSHB
- H-AFP, R&D Systems
- R-AFP, Abcam
- DESMO, Abcam
- LAP, DSHB
- 151-Ig, DSHB
- HA4c19, DSHB
- OC2, DH
- OC3, DH
- OC4, DH
- OC5, DH
- OC10, DH
- H.4, DH
- H.1, DH
- DPPIV, DH

    - OV6, DH
    - HESA, DSHB
    - YM-PS087, Sigma
    - INS, Sigma
    - YM-PS5088, Sigma
    - SOMA, Sigma
    - 11180, Sigma
    - Telom, Abcam
    - PI, Abcam
    - DAPI, Abcam
    - Gal-19, Abcam
- Histochemical Stains
    - Von Kossa, Chroma-Gesellschaft
    - AlkPhos, Sigma
    - Mallory Heidenhain One-Step reagents, Chroma-Gesellschaft
    - Alcian blue, Chroma-Gesellschaft
        - pH 1.0
        - pH 2.5
    - Alcec blue, Chroma-Gesellschaft
        - pH 1.0
        - 2.5
    - Safranin-O, Chroma-Gesellschaft
        - pH 1.0
        - pH 2.5
    - Sudan Black-B, 199664, Sigma
    - Oil Red-O, 00625, Sigma
- Verification of Histochemical Staining
    - Toluene, removes hydrocarbons from fat cells, Sigma
    - Hyaluronidase, Sigma
    - Chondroitinase-AC, Sigma
    - Chondroitinase-ABC, Sigma
    - Keratanase, Sigma
    - Heparanase, Sigma
    - EDTA, divalent cations (Ca^{+2}, Mg^{+2}, Zn^{+2}, Ba^{+2}), Sigma
    - EGTA, univalent cation (Ca^{+2} only), Sigma
- Secondary Probes
    - Goat anti-rat osteocalcin, #BT-413, Biomedical Technology
    - Biotin, goat anti-mouse IgG, #OB1126-21, Fisher
    - Biotin, goat anti-mouse IgG (fab specific), #B-7151, Sigma
    - Biotin, goat anti-rabbit IgG, #OB1316-21, Sigma
    - Biotin-SP, goat anti-mouse IgG, #JGM-065003, Accurate
    - Biotin-SP, goat anti-mouse fab-specific, #JGM-066003, Accurate
    - Biotinylated affinity purified, rat adsorbed anti-mouse IgG (H + L) (BA-2001, Vector Laboratories)
- Tertiary Probes
    - Streptavidin-peroxidase, #JSA-030084, Accurate
    - Avidin-peroxidase, #JSA-030083, Accurate
    - Monoclonal anti-goat IgG clone GT-34 biotin, #B-3148, Sigma
    - Horseradish peroxidase conjugated Avidin-D, #A-2004, Vector
    - Peroxidase Standard PK-4000 Vecstatin ABC Reagent Kit, Vector Laboratories
- Reagents to Quantify or Visualize Tertiary Probes
    - ABTS kit, #506201, Kirkegaard & Perry
    - 4-Cloro-1-napthol, #C-8890, Sigma
    - 3,5-Diaminobenzoic acid dihydrochloride, #11-383-2, Aldrich
    - Vecstatin ABC Reagent Kit, Vector Laboratories Inc., Burlingame, CA
- Miscellaneous Supplies
    - Parafilm, #13-374-12, Diagger
    - Aqua-Mount (mounting coverslips), Vector Laboratories
    - Ethanol, Sigma
    - Isopropyl alcohol, Sigma

  - Nitrile Gloves, Diagger
- Suppliers
  - Sigma, Sigma Chemical Co., St Louis, MO
  - Aldrich, Aldrich Chemical Co, Milwaukee, WI
  - GIBCO, GIBCO, Grand Island, NY
  - DSHB, Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained at the University of Iowa, Department of Biological Sciences, Iowa City, IA
  - ICN, ICN Biomedicals, Costa Mesa, CA
  - SBA, Southern Biotechnology Associates, Birmingham, AL
  - Chem, Chemicon, El Segundo, CA
  - BTH, Biomedical Technology Incorporated, Cambridge, MA
  - Fisher, Fisher Scientific Co., Norcross, GA
  - TF, Thermo-Fisher Scientific, Waltham, MA
  - Accurate, Accurate Chemical & Scientific Corporation, Westbury, NY
  - Vector, Vector Laboratories, Burlingame, CA
  - KP, Kirkegaard & Perry Laboratories, Gaithersburg, MD
  - BDH, BDH Chemicals Ltd, Poole, England
  - Kroger's, Kroger's, Macon, GA
  - DFRD, Dragonfly Foundation for Research and Development, Macon, GA
  - ChemPure, Curtain Matheson Scientific, Houston, TX
  - DH, Douglas Hixson, Department of Medicine, Brown University, Providence, RI
  - Morton Thiokol, Morton International, Rohm and Haas, Philadelphia, PA
  - Kodak, Kodak, Rochester, NY
  - Aldrich, Aldrich Chemical Co., Sigma-Aldrich, St. Louis, MO
  - Jackson, Jackson Laboratories, Bar Harbor, ME
  - AirGas, AirGas, Local Sore
  - Atlas, Atlas Biologicals, Fort Collins, CO
  - Fisher-Biotech, Waltham, MA
  - 96-well plate reader, R & D Systems, Minneapolis, MI
  - Vortex Mixer, Thermo-Fisher, Waltham, MA
  - Diagger Scientific, Vernon Hills, IL
  - RB, Ray Biotech, Atlanta, GA
  - R&D, R&D Systems, Minneapolis, MN
  - Abcam, Cambridge, MA
  - Prospec, Prospec, Rehovot, Israel
  - Antibodies Inc, Davis, CA
  - Chroma-Gesellschaft, Roboz Surgical Co, Washington, DC

2.2. Procedure

2.2.1. Elica Fixative [29,30]

The ELICA fixative is composed of 55-ml of DPBS (Sigma), 12.5-ml of 37% w/v powdered paraformaldehyde (Fisher Scientific) in DPBS, 2.0-ml of 50% v/v glutaraldehyde (Sigma), 30.0-ml of 5% w/v sodium azide (Sigma), and sufficient amount of tissue culture grade D-glucose (Sigma) to reduce osmolarity of 1.0, then pH to 7.4. The fixative was stored at room temperature when not in use. When using the ELICA fixative for extracellular matrix glycoconjugate histochemisty,10% w/v cetylpyridinium chloride was added to the fixative [47-58], the osmolarity returned to 1.0 with D-glucose, and pH to 7.4. The ELICA fixative was utilized for ALL microscopy in studies with endogenous adult telomerase stem cells (aTPSCs), e.g., TEM, SEM, SEM-Energy dispersive microanalysis, brightfield, brightfield with microspectrophotometry, fluorescence microscopy; immunocytochemistry and glycoconjugate histochemistry [30-45,47-58].

2.2.2. Biotin-Avidin Amplification Incorporated into the ELICA Procedure 96-Well Plate ELICA

The 96-well plate ELICA procedure utilizes multiple wash and blocking steps to prevent non-specific binding of reagents to cultured cells and/or tissue sections. Reagents used are a primary (1^{o}) probe, which is an antibody to a cell-specific phenotypic expression marker (PEM) (Table 1); a secondary (2^{o}) probe, which is an antibody directed against the species of the 1^{o} probe with an attached biotin; a tertiary (3^{o}) probe, consisting of an avidin molecule with attached horseradish peroxidase enzyme; and HRP substrates (Fig. 1) [30,33,40,59].

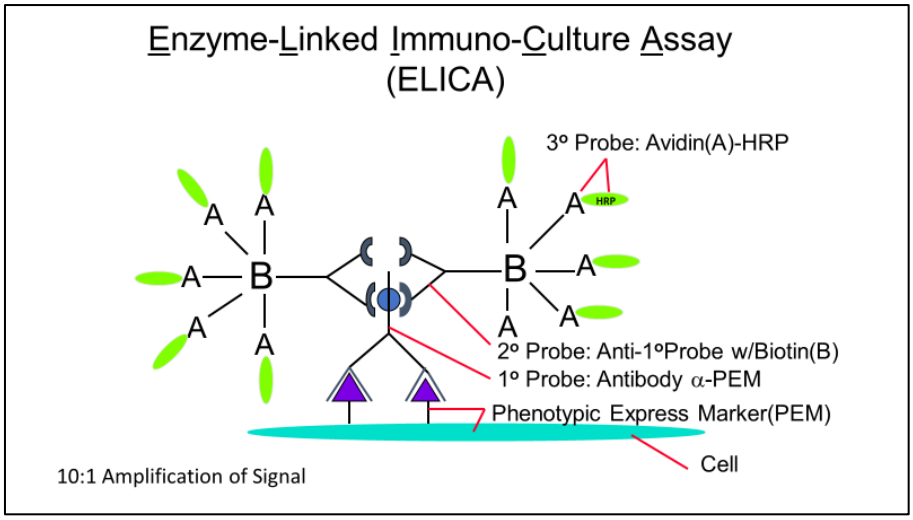

Figure 1 Enzyme-Linked Immuno-Culture Assay (ELICA), a high throughput immunocytochemical procedure was developed to amplify signal expression to quantify antibody binding using a soluble HRP substrate, followed by visualizing antibody binding using an insoluble HRP substrate (AEC or DAB), followed by quantification of DNA, all within the same well of a 96-well plate or on a single tissue section. This procedure utilizes multiple wash and blocking steps to prevent non-specific binding of reagents to cultured cells and/or tissue sections, visualized by brightfield microscopy. Reagents used are a primary (1°) probe, which is an antibody to a phenotypic expression marker (PEM); a secondary (2°) probe, which is an antibody directed against the species of the 1° probe with an attached biotin; a tertiary (3°) probe, consisting of an avidin molecule with an attached horseradish peroxidase (HRP) enzyme; and HRP substrates. Hydrogen peroxide and sodium azide were used in the initial blocking steps because all cells contain an endogenous peroxidase that would react with the exogenous HRP substrate, giving a false positive. Positive and negative controls were run simultaneously to verify that the staining was real and not an artifact of technique. Four negative controls were included in each staining run, either during staining of cryosectioned tissues or within cultured cells using the ELICA fixative with the ELICA procedure. The negative controls consisted of 1. no primary probe only, with all other steps remaining intact; 2. no secondary probe only, with all other steps remaining intact; 3. no tertiary probe only, with all other steps remaining intact; and 4. no substrate, with all other steps remaining intact [30,33,40,59]. Reprinted with permission from Young HE, Speight MO. Characterization of endogenous telomerase-positive stem cells for regenerative medicine, a review. Stem Cell Regen Med 2020; 4(2):1-14 [59]

2.3. Inhibition and Exhaustion of Endogenous Peroxidases

Five percent aqueous sodium azide v/v in HPLC water (Sigma) and 30% hydrogen peroxide (Sigma) were used in separate initial blocking steps prior to antibody binding. Sodium azide inhibits extracellular and cell surface endogenous peroxidases, while 30% hydrogen peroxide exhausts endogenous intracellular peroxidases in the cells that would react with the exogenous HRP substrate, giving a false positive [9].

2.4. Positive and Negative Controls

Positive and negative controls were run simultaneously to verify that the staining was real and not an artifact of technique.

For sectioned tissues, the positive procedural control consisted of using IA4, an antibody to smooth muscle alpha-actin, which is present in the muscular wall of blood vessel(s) within the tissue sections [30,33]. Six negative procedural controls were included in each staining run of cryosectioned or vibratome sectioned tissues with or without using the ELICA fixative with the ELICA procedure. The negative controls consisted of 1. Species-specific buffer only; 2. HRP substrate only; 3. no primary probe, with all other steps remaining intact; 4. no secondary probe, with all other steps remaining intact; 5. no tertiary probe, with all other steps remaining intact; and 6. no substrate, with all other steps remaining intact [30,33].

For cultured cells, the positive procedural control consisted of using CEA-CAM-1 (D Hixson), an antibody to telomerase positive totipotent stem cells, a pure clonal population generated by repetitive single cell clonogenic analysis [31]. The same negative controls consisted of 1. Species-specific buffer only; 2. HRP substrate only; 3. no primary probe, with all

other steps remaining intact; 4. no secondary probe, with all other steps remaining intact; 5. no tertiary probe, with all other steps remaining intact; and 6. no substrate, with all other steps remaining intact [30,33].

2.5. Quantitation of Phenotypic Expression

The following procedure is used for 96-well tissue culture plates [9,10]. Remove culture medium from each well

- Wash each culture well with 0.2-ml species-specific buffer 2 x 1 min each
    - Avian cells – Tyrode's buffer, pH 7.4
    - Non-Human Mammalian Cells – PBS with 0.2% glucose, pH 7.4
    - Human Cells – Dulbecco's Phosphate Buffered Saline, pH 7.4
- Remove ELICA buffer and fix cells for 5 min with ambient temperature ELICA Fixative (a combination of species-specific buffer, aldehydes, sodium azide, and glucose, see below). The ELICA fixative was far superior to most other common fixatives, e.g., methanol, ethanol, formalin, glutaraldehyde, paraformaldehyde, etc., with respect to morphology, inhibition of autophagy, and physiological osmolarity, with similar or better antigen-antibody binding properties, depending on the primary antibody used.
- Remove fixative and wash each well with 0.2-ml of species-specific buffer, 2 x 1 min each
- For intracellular phenotypic expression markers, cell membranes need to be solubilized for antibody to gain entrance into the cell.
    - Remove species-specific buffer and solubilize cell layers with 0.1-ml per well of 1% Triton X-100 (1:99) in species-specific buffer for 1 min. Time will vary depending on density of cells on the plate. Optimum time for Triton X-100 incubation is reflected by solubilized cell membranes, but with intracellular components still attached to plate surface. Remove Triton X-100 and wash with 0.2-ml of species-specific buffer, 4 x 2.5 min
- If phenotypic expression markers are either cell surface or extracellular, disregard above Step iv.
- Remove species-specific buffer and incubate with 0.2-ml per well of 0.03% sodium azide (in species-specific buffer) for 1 min to irreversibly inhibit endogenous peroxidases. Time will vary dependent on the particular content of endogenous peroxidases for each specific cell type. Sodium azide in the ELICA fixative negates peroxidase activity on cell surface and in extracellular space, but does not inhibit intracellular peroxidases. Optimum incubation time with sodium azide is determined empirically by testing a range of times from 0 to 5 min, using 5-sec intervals. *Optimum azide incubation time is the respective time point at which the ABTS substrate remains clear, plus 5 sec.
- After azide incubation for designated time for each cell type, wash the cells with 0.2-ml of species-specific buffer twice for 2.5 min.
- Remove species-specific buffer and incubate with 0.2-ml 30% hydrogen peroxide for 1 min to exhaust intracellular peroxidases. Time will vary dependent on the particular content of endogenous peroxidases for each specific cell type. Optimum incubation time with hydrogen peroxide is determined empirically by testing a range of times from 0 to 5 min, using 5-sec intervals. *Optimum hydrogen peroxide incubation time" is the respective time point at which the ABTS substrate remains clear, plus 5 sec.
- After hydrogen peroxide incubation for designated time for each cell type, wash the cells with 0.2-ml of species-specific buffer twice for 2.5 min.
- Residual azide and hydrogen peroxide can negate activity of exogenously added HRP and therefore must be removed from the system to prevent erroneous results. This is accomplished by washing 3 times for 5 min each with 0.2-ml species-specific buffer containing primary blocking agent (Table 1). The primary, secondary, and tertiary blocking agents used are dependent on the non-specific binding kinetics of the primary probe (anti-PEM), secondary probe (anti-species of primary), tertiary probe (anti-species of secondary with attached biotin) and therefore must be determined empirically for each probe. determine the non-specific binding kinetics.

Within each staining run, one plate within the humidified chamber is designated as the control plate and run at same time as "experimental" plates. There are 6 negative controls to determine the non-specific binding kinetics of the particular probes used in the procedure, to ensure sensitivity of the assay. The optimum background value should be less than 5% of maximal spectrophotometric reading of the plate reader / spectrophotometer: 1. species-specific buffer only, 2. No substrate, but all other steps intact; 3. No primary probe, but all other steps intact; 4. No secondary probe, but all other steps intact; 5. No tertiary probe, but all other steps intact; And 6. No exogenous horseradish enzyme, but all other steps intact. There is also the addition of one procedural positive control for each phenotypic expression marker assayed by various antibodies.

- Remove primary blocker and incubate with 0.2-ml per well of primary probe directed against particular phenotypic expression marker (Table 1), for 1 hour at 37°C in a humidified environment. The probe is diluted with species-specific buffer (Note: addition of blocking agent at this step will decrease binding efficiency of primary probe). The humidified environment used consisted of a glass baking dish containing water-soaked paper towels, the dish covered with Saran wrap, and placed into a 37°C incubator. One positive (per primary probe) and six negative procedural controls should be run simultaneously with experiment to make sure there are no mistakes with the procedures. Positive control – antibody to known phenotypic expression marker. Negative procedural controls: 1. Species-specific buffer only; 2, No substrate, but all other steps intact; 3. no primary probe, but all other steps intact; 4. no secondary probe, but with all other steps intact; 5. No tertiary probe, but all other steps intact; 6. No avidin with attached HRP.
- Remove the primary probe and wash 8 times 2.5 min with 0.2-ml per of species-specific buffer containing the secondary blocking agent to prevent nonspecific attachment of secondary probe to the culture dish or cells or both. This is determined empirically before the assay.
- Remove the secondary blocker and incubate with 0.1-ml per well of biotinylated secondary probe for 30 min at 37°C in a humidified environment.
- Remove the secondary probe and wash 4 times doe 2.5 min with 0.2-ml per well of species-specific buffer with tertiary blocking agent.
- Remove the of species-specific buffer with tertiary blocking agent and wash 4 times for 2.5 min with 0.2-ml of species-specific buffer without the blocking agent.
- Remove the species-specific buffer and incubate with 0.1-ml per well of tertiary probe, e.g., 0.5 micrograms of horseradish peroxidase conjugated avidin-D/0.2-ml of species-specific buffer for 30 min at 37°C in a humidified environment.
- Remove the tertiary probe and wash 8 times for 2.5 min with 0.2-ml per well of species-specific buffer.
- Make peroxidase substrate by mixing equal volumes of 4°C 2,2-azino-di[3-ethyl benzothiazoline sulfonate] and hydrogen peroxide (ABTS kit).
- Test the reactivity of the substrate by placing 0.5-ml of ABTS solution into a clean test tube and then adding 0.1-ml of tertiary probe. The solution should change color from clear to dark blue-green within a matter of seconds to minutes. Test ABTS solution first before proceeding. If solution does not change color 10 minutes after adding tertiary probe, discard and make up fresh ABTS solution (should be water clear).
- Remove the species-specific buffer and incubate with 0.1-ml of ABTS solution per well in the dark (within a closed drawer) for a standard amount of time, such as 15 min.
- Place 10 microliters of 0.03% sodium azide in species-specific buffer in each well of a 96-well plate to stop reaction. It is essential to use only a 1:20 azide to substrate ratio to stop the HRP/ABTS reaction. Too little azide and the reaction will continue in a non-linear fashion once the plate is placed in the light even without the HRP enzyme. Too much azide in the solution and the blue-green color will bleach in the light toward a clear color in a non-linear fashion within a matter of minutes.
- After a predetermined standardized time period, transfer 0.2-ml of reacted substrate from each well into separate wells of an untreated 96-well ELICA plate.
- Read the absorbance of the reacted substrates in each well of the 96-well ELICA plate in a spectrophotometric plate reader using a 405-nm filter.

Table 1 Antibodies, Immunocytochemistry and Histochemistry for Phenotypic Expression Markers

Antibody	**Antigen**	**Embryological Origin**
CEA-CAM-1	Carcinoembryonic antigen-cell adhesion molecule-1	Totipotent
HCEA	Human Carcinoembryonic antigen	Totipotent
CEA	Carcinoembryonic antigen	Totipotent
CD66e	Carcinoembryonic antigen	Totipotent
DH-TuAg1	Spermatogonia	Totipotent Gamete
MC-480	SSEA-1	Pluripotent
MC-631	SSEA-3	Pluripotent
MC-813	SSEA-4	Pluripotent
CD10	Neutral endopeptidase	Pluripotent

AlkPhos	Alkaline Phosphatase	Pluripotent
CD56	Neural cell adhesion molecule	Ectoderm
Pax-6	Neurogenic lineage	Ectoderm
FORSE-1	Neuronal precursor cells	Ectoderm
Vimentin	Cells of neurogenic lineage	Ectoderm
Nestin	Cells of neurogenic lineage	Ectoderm
R401	Nestin-neuronal lineage	Ectoderm
HNES	Nestin-neuronal lineage	Ectoderm
MAB353	Nestin-neuronal lineage	Ectoderm
RT-97	Neurofilaments = neurons	Ectoderm
NF68	Neurofilament-68 = neurons	Ectoderm
S-100	Neurofilaments-100 = neurons	Ectoderm
NF-145	Neurofilaments-145 = neurons	Ectoderm
N-200	Neurofilaments-200 = neurons	Ectoderm
8A2	Neurons	Ectoderm
NG2	Neurons	Ectoderm
TH	Tyrosine hydroxylase, precursor to neural transmitters	Ectoderm
SV2	Synaptic vesicles	Ectoderm
DOPA	Dopamine, transmitter of dopaminergic neurons	Ectoderm
T8660	Beta-tubulin-III	Ectoderm
Tuj1	Beta-tubulin	Ectoderm
GFAP	Glial-fibrillary acidic protein	Ectoderm
CNPase	Glial cells = oligodendrocytes & astrocytes	Ectoderm
Rip	Oligodendrocytes	Ectoderm
MOSP	Oligodendrocyte specific proteins	Ectoderm
MAB	Oligodendrocyte marker	Ectoderm
40E-C	Radial cells and radial glial cells	Ectoderm
VM-1	Keratinocytes	Ectoderm
M3F7	Type-IV collagen, basement membrane	Ectoderm & Mesoderm
31-2	Laminin, basement membrane	Ectoderm & Mesoderm
5D2-27	Cell adhesion molecule	Ectoderm & Mesoderm
B3/D6	Fibronectin, basement membrane	Ectoderm & Mesoderm
5C6	Type-IV collagen, basemen membrane	Ectoderm & Mesoderm
Anti-type IV	Type-IV collagen	Ectoderm & Mesoderm
33-2	Heparan sulphate proteoglycan	Ectoderm & Mesoderm
Anti-HSPG	Heparan Sulphate proteoglycan	Ectoderm & Mesoderm
5D4	Keratan sulphate proteoglycan	Ectoderm & Mesoderm

2E8	Laminin, basement membrane	Ectoderm & Mesoderm
D3	Desmin, in all 3 muscle groups	Ectoderm & Mesoderm
Anti-vimentin	Vimentin, lens of the eye	Ectoderm & Mesoderm
D76	Desmin, in all 3 muscle groups	Ectoderm & Mesoderm
CD13	Amino endopeptidase	Mesoderm
12/101	Skeletal Muscle	Mesoderm
C3/1	Glycoprotein of myoblast plasma membrane	Mesoderm
OP-137	MyoD	Mesoderm
F5D	Myogenin = skeletal muscle	Mesoderm
ALD-66	Slow twitch muscle fibers	Mesoderm
MF-1	Fast twitch muscle fibers	Mesoderm
MF-5	Myosin light chain-2 of fast muscle	Mesoderm
MF-20	Sarcomeric myosin = skeletal muscle	Mesoderm
MF-30	Neonatal and adult myosin	Mesoderm
ALD58	Myosin heavy chain	Mesoderm
CH1	Myosin tropomyosin	Mesoderm
A4.74	Myosin fast chain	Mesoderm
JLA-20	Actin	Mesoderm
Anti-Myosin	Skeletal muscle myosin	Mesoderm
IA4	Smooth muscle alpha actin = smooth muscle	Mesoderm
Calp	Calponin	Mesoderm
MAB-3252	Cardiotin = cardiac myocytes	Mesoderm
MAB1548	Myosin heavy chain of cardiac muscle	Mesoderm
M-38	Type 1 collagen	Mesoderm
SP1.D8	Procollagen type-III	Mesoderm
Anti-type-II	Type-II collagen	Mesoderm
WV1D1	Bone sialoprotein II = bone	Mesoderm
Anti-OsteC	Osteocalcin / Bone Gla-protein	Mesoderm
MP111	Osteopontine = bone	Mesoderm
EGTA	Leaches Calcium from bone, negative control	Mesoderm
CIIC1	Type-II collagen	Mesoderm
II-4CII	Type-II collagen	Mesoderm
Anti-type2	Type-II collagen	Mesoderm
HC-II	Human type-II collagen	Mesoderm
D1-9	Type-IX collagen = cartilage	Mesoderm
9/30	Cartilage link protein	Mesoderm

12/21	Cartilage proteoglycan hyaluronate binding region	Mesoderm
12C5	Versican hyaluronate binding region	Mesoderm
H-DC34	Sialomucin-containing hematopoietic/endothelial cells	Mesoderm
CD31	PECAM, Peripheral endothelial cell adhesion molecule	Mesoderm
P1H12	Human endothelial cell surface marker	Mesoderm
P2B1	Peripheral endothelial adhesion molecule	Mesoderm
P8B1	VCAM, vascular cell adhesion molecule	Mesoderm
P2H3	CD62e, E-selectin (vasculature)	Mesoderm
H-endo	CD146, Endothelial cells	Mesoderm
H5A4	CD11b, granulocytes, monocytes, NK-cells	Mesoderm
H4C4	CD44, hyaluronate receptor	Mesoderm
Hermes-1	CD44, hyaluronate receptor	Mesoderm
H5A5	CD45, all hematopoietic cells except RBCs	Mesoderm
H5C6	CD63, macrophages, monocytes, platelets	Mesoderm
HFSP	Human fibroblast specific protein	Mesoderm
1B10	Fibroblast-specific protein	Mesoderm
Sudan Black-B	Stains fat (adipocytes)	Mesoderm
Oil Red-O	Stains fat (adipocytes)	Mesoderm
H-AFP	Human alpha-fetoprotein = fetal liver	Endoderm
R-AFP	Rat alpha-fetoprotein = fetal liver	Endoderm
DESMO	Endodermal epithelial marker of liver	Endoderm
LAP	Canalicular cell surface protein of liver	Endoderm
151-Ig	Liver epithelial growth factor	Endoderm
HA4c19	Bile canalicular cells of liver	Endoderm
OC2	Progenitor cells, oval cell, & biliary cells of liver	Endoderm
OC3	Progenitor cells & biliary cells of liver	Endoderm
OC4	Progenitor cells & biliary cells of liver	Endoderm
OC5	Progenitor cells & biliary cells of liver	Endoderm
OC10	Progenitor cells & biliary cells of liver	Endoderm
H.4	Intracellular staining of liver hepatocytes	Endoderm
H.1	Liver hepatocytes cell surface marker	Endoderm
DPPIV	Progenitor, canalicular, and biliary cells of the liver	Endoderm
OV6	Biliary and oval cells of liver; biliary cells of liver	Endoderm
HESA	Human GI (Gastrointestinal) Epithelium	Endoderm
YM-PS087	Glucagon-secreting cells of endocrine pancreas	Endoderm
YM-PS5088	Insulin-secreting cells of endocrine pancreas	Endoderm
11180	Somatostatin-secreting cells of the endocrine pancreas	Endoderm

CK-19	Ductal cells of the exocrine pancreas	Endoderm
ABL-93	Lysosomal membrane glycoprotein	Ectoderm, Mesoderm, Endoderm
22/18	Regeneration cells	Ectoderm, Mesoderm, Endoderm
Telom	Telomerase positive cells	aTPSCs
CD90	Glycosylphosphatidylinositol anchoring membrane protein (Thy-1)	Transition: EctoSCs, MesoSCs, and EndoSCs to Progenitor Cells
Thy-1	Glycosylphosphatidylinositol anchoring membrane protein (CD90)	Transition: EctoSCs, MesoSCs, and EndoSCs to Progenitor Cells
CD95	Cells undergoing apoptosis	Dead Cells
PI	Propidium Iodide, measure of live cells, flow cytometry	Live Cells
DAPI	Fluorescent marker to visualize living and fixed DNA	Live & Dead Cells
Gal-19	Insect beta-galactosidase genomic marker	Cell tracking marker
Alcian Blue	Stains anions on carbohydrate groups	Extracellular Matrix
AB 1.0	Alcian Blue, pH 1.0 stains sulphate groups on GAGs	Extracellular Matrix
AB 2.5	Alcian Blue, pH 2.5 stains carboxyl groups on GAGs	Extracellular Matrix
Alcec Blue	Stains anions on carbohydrate groups	Extracellular Matrix
AcB 1.0	Alcec Blue, pH 1.0 stains sulphate groups on GAGs	Extracellular Matrix
AcB 2.5	Alcec Blue, pH 2.5 stains carboxyl groups on GAGs	Extracellular Matrix
Safranin-O	Stains anions on carbohydrate groups	Extracellular Matrix
SO 1.0	Safranin-O, pH 1.0 stains sulphate groups on GAGs	Extracellular Matrix
SO 2.5	Safranin-O, pH 2.5 stains carboxyl groups on GAGs	Extracellular Matrix
Von Kossa	Stains calcium in bone matrix	Extracellular Matrix
EDTA	Removes divalent cations, +2 charges Ca^{+2}, Mg^{+2}, Fe^{+2}, Zn^{+2}, Ba^{+2}	Extracellular Matrix
EGTA	Removes Ca^{+2} from tissues / bones	Extracellular Matrix
Enzyme	Streptomyces Hyaluronidase, negative staining control to verify presence of hyaluronic acid	Extracellular Matrix
Enzyme	Chondroitinase-AC, negative staining control to verify presence of non-sulphated chondroitin proteoglycans	Extracellular Matrix
Enzyme	Chondroitinase-ABC, negative control to verify presence of chondroitin sulphate proteoglycans	Extracellular Matrix
Enzyme	Keratanase, negative control to verify presence of keratan sulphate proteoglycans	Extracellular Matrix
Enzyme	Heparanase, to verify presence of heparan sulphate proteoglycans	Basement Membranes
PAS	Periodic Acid Schiff reaction for glycoproteins with vicinal hydroxyl groups	Extracellular Matrix

Table 1. Antibodies (left column) used to identify specific phenotypic expression markers (center column) for particular cell types (right column) [9,28,29]. Reprinted with permission from Young HE, Sippel J, Putnam LS, Lucas PA, Morrison DC. Enzyme-linked immuno-culture assay. Journal of Tissue Culture Methods, 14:31-36, 1992 [10]; Young HE, Black AC. Pluripotent Stem Cells, Endogenous versus Reprogrammed, a Review. MOJ Orthop Rheumatol 1(4): 00019, 2014 [60];

Young HE. Carcinoembryonic antigen-cell adhesion molecule-1 and stage-specific embryonic antigen-4 are present in the reproductive organs of adult mammals. GSC Advanced Research and Reviews. 2025; 23(03): 149-157 [30].

2.6. Visualization of Phenotypic Expression

2.6.1. Immunocytochemical Staining of Sectioned Tissue [30,33]

Vibratome sections were 25 microns in thickness. Cryostat sections were 7 microns in thickness. The vibratome and cryostat tissue sections were incubated with 5.0% (w/v) sodium azide in species-specific buffer for 60 minutes to irreversibly inhibit endogenous cell surface and extracellular peroxidases. They were washed in running water for five minutes, and then incubated with 30% hydrogen peroxide (Sigma) for 60 minutes, to exhaust residual intracellular endogenous peroxidases. Tissue sections were rinsed with running water for five minutes and then incubated for 60 minutes with blocking agent (Vecstatin ABC Reagent Kit) in DPBS. The blocking agent was removed and the sections rinsed with running water for five minutes. The tissue sections were then incubated with primary antibody for 60 minutes. The primary antibodies to identify telomerase positive totipotent stem cells (TSCs) consisted of 0.005% (v/v) carcinoembryonic antigen cell adhesion molecule-1 (CEA-CAM-1) in species-specific, dependent on species; to identify telomerase positive pluripotent stem cells (PSCs) 1-microgram per ml of stage-specific embryonic antigen-4 (SSEA-4) was diluted in species-specific buffer; and to identify smooth muscle alpha-actin present within adjacent blood vessels in the tissue sections, 1-microgram per ml of IA4 was diluted in species-specific buffer as the positive procedural control. The primary antibody was removed. The sections were rinsed with running water for five minutes, and incubated with secondary antibody for 60 minutes. The secondary antibody consisted of 0.005% (v/v) biotinylated affinity purified, rat adsorbed anti-mouse immunoglobulin G (H + L) (BA-2001, Vector) in species-specific buffer. The secondary antibody was removed. The sections were rinsed with running water for five minutes, and then incubated with avidin-HRP for 60 minutes. The avidin-HRP consisted of 10 ml of 0.1% (v/v) Tween-20 (ChemPure) containing 2 drops reagent-A and 2 drops reagent-B (Peroxidase Standard PK-4000) in species-specific buffer. The avidin-HRP was removed. The sections were rinsed with running water for five minutes, and incubated with AEC substrate (Sigma) for 60 minutes. The AEC substrate was prepared as directed by the manufacturer. The substrate solution was removed. The sections were rinsed with running water for 10 minutes. If double staining was required for experiment, such as phenotypic expression marker and beta-Gal genomic label [32], sections were then incubated with DAB substrate (Vector) for 60 min. The DAB substrate was prepared as directed by the manufacturer. The substrate solution was removed. The sections were rinsed with water for 10 min and then cover-slipped with Aqua-mount (Vector) [10,19].

2.6.2. Immunocytochemical Staining of Cultured Cells [9,10]

Each well of 96-well plates were processed. The wells were incubated with 5.0% (w/v) sodium azide in species-specific buffer for 60 minutes to irreversibly inhibit endogenous peroxidases. The wells were rinsed with water, and then incubated with 30% hydrogen peroxide for 60 minutes, also to exhaust intracellular endogenous peroxidases. Wells were rinsed with water and incubated for 60 minutes with blocking agent (Vecstatin ABC Reagent Kit, Vector) in species-specific buffer. The blocking agent was removed and the wells rinsed with water. The wells were then incubated with primary antibody for 60 minutes in species-specific buffer. [Each time a new lot of primary antibodies was obtained, the antibody was titrated to a dose response curve to its specific phenotypic expression markers to determine optimal concentration of antibody to use.] The primary antibodies to identify telomerase positive totipotent stem cells (TSCs) consisted of 0.005% (v/v) carcinoembryonic antigen cell adhesion molecule-1 (CEA-CAM-1, D. Hixson) in species-specific buffer [30,33]. To identify telomerase positive pluripotent stem cells (PSCs) 1-microgram per ml stage-specific embryonic antigen-4 (SSEA-4, DSHB) was diluted in species-specific buffer, also as a positive procedural control [30,33]. The primary antibody was removed. The wells were rinsed with water, and incubated with secondary antibody for 60 minutes. The secondary antibody consisted of 0.005% (v/v) biotinylated affinity purified, rat adsorbed anti-mouse immunoglobulin G (H + L) with an attached biotin molecule (BA-2001, Vector) in species-specific buffer. The secondary antibody was removed. The wells were rinsed with water, and then incubated with avidin-HRP for 60 minutes. The avidin-HRP consisted of 10 ml of 0.1% (v/v) Tween-20 (ChemPure) containing 2 drops reagent-A and 2 drops reagent-B (Peroxidase Standard PK-4000 Vecstatin ABC Reagent Kit, Vector) in species-specific buffer. The avidin-HRP was removed. The wells rinsed with running water for five minutes, and incubated with an insoluble HRP substrate, precipitating at the site of antibody binding. The insoluble substrates consisted of either AEC substrate (Sigma) of DAB substrate (Sigma) for 60 minutes. The AEC and DAB substrates were prepared as directed by the manufacturer. The substrate solution was removed. The sections were rinsed with multiple changes of water. If double staining was required for an experiment [9], an antibody/substrate for phenotypic expression marker (AEC), was followed by an antibody/substrate for beta-Gal genomic label (DAB). Wells were then incubated first with AEC substrate, rinsed in water and then incubated with DAB substrate. The substrate solution was removed. The wells were rinsed with water for 10 min and then processed for DNA quantification [9,10].

2.7. DNA Quantification [9,10,61,61]

DNA analysis in the same culture wells can be determined using the in situ diaminobenzoic acid (DABA, Sigma) procedure [61] with modifications [62]. Calf thymus DNA (Sigma) is used to prepare the DNA standards as described [62], and DABA is use as the fluorescent agent. Spectrophotometric analysis is performed using an excitation of 420 and an emission wavelength of 510 nm. Remove ELICA buffer and wash each well twice for 5 min with 70% Ethanol (Sigma). Remove 2nd 70% ethanol change and incubate each well for a minimum of 1 hour in 70% ethanol. Remove ethanol and dry plates at either ambient temperature or in a drying oven (Thermo-Fisher) at 37°C. Set water bath temperature to 60°C [9,10].

2.7.1. Stock DABA Solution [10,62]

Determine the quantity of DABA to be used by multiplying the number of wells to be assayed by 0.1-ml DABA/well. Always include an additional 2-ml to the final volume of DABA solution to account for possible pipetting errors. Determine the amount of DABA needed for working solution by multiplying number of ml times 0.8 g DABA / ml of double-distilled water or HPLC water. The clearer the DABA solution, the better. If the solution of DABA appears yellowish to straw-colored, decolorize the DABA stock solution with activated charcoal as described [62].

Add 0.1-ml DABA solution to each well. Cover plates. Seal plates with precut Parafilm (Diagger). Float plates in 60°C water bath for 45 minutes. [An alternative procedure is to use a 60°C microbead bath, at which point plates are placed onto leveled surface of beads.] Turn on fluorometer and set excitation wavelength to 420 nm and emission wavelength to 510 nm. Remove plates from 60°C water bath or bead bath. Add 6N HCl (Sigma) to each well for a final concentration of 1N HCl. Bring the volume in each well to 0.2-ml by adding 0.1-ml of double-distilled or HPLC water. Transfer 0.15-ml of the DABA/HCL solution to clean 0.2-ml glass test tubes (Thermo-Fisher), one tube for each well [*Caution: handle the test tubes at the top only, because the fluorometer will read fingerprints.*] Adjust the final volume for your particular fluorometer by adding additional 1N HCl as necessary. Slowly vortex each tube with lowest setting on Vortex mixer (Thermo-Fisher), slow enough to prevent splashing. Wipe the test tube clean with a Kimwipe and then read tube in fluorometer. Make sure the excitation wavelength is set at 420-nm and the emission wavelength is set at 510-nm. (If the 0.2-ml tube is too small to be analyzed in your fluorometer, the 0.2-ml glass test tube can be placed into a larger / higher volume clean glass test tube) [10]. Record values.

2.7.2. Standard Curve for Quantifying Phenotypic Expression

Commercially available phenotypic expression markers are used to generate standard curves to determine the quantity of material present within the cell cultures. This is accomplished using linear regression analysis [63].

2.7.3. Preparing Concentration Gradient of Material to be Examined

Weigh 2000-micrograms, 1500-micrograms, 1000-micrograms, and 500-micrograms of material to be examined, and place each into separate 15-ml pre-labeled polypropylene tubes (Thermo-Fisher). Add 2.0-ml of species-specific buffer and dissolve the testing solutions. Remove 1-ml from each tube and place into separate 15-ml polypropylene tubes. Dilute the testing solutions (labeled 1000-micrograms, 750-micrograms, 500-micrograms, and 250-micrograms) 1:10 with species-specific buffer. Concentrations after 1st dilution will be 100-micrograms, 75-micrograms, 50-micrograms, and 25-micrograms. Remove 1-ml from each tube and place into separate 15-ml polypropylene tubes. Dilute each tube 1:10 with species-specific buffer. Concentrations after 2nd dilution will be 10-micrograms, 7.5 micrograms, 5 micrograms, and 2.5 micrograms. Remove 1-ml from each tube and place into separate 15-ml polypropylene tubes. Dilute each tube 1:10 with species-specific buffer. Concentrations after 3rd dilution will be 1000-nanograms, 750-nanograms, 500-nanograms, and 250 nanograms. Remove 1-ml from each tube and place into separate 15-ml polypropylene tubes. Dilute each tube 1:10 with species-specific buffer. Concentrations after 4th dilution will be 100-nanograms, 75 nanograms, 50 nanograms, and 25 nanograms. Remove 1-ml from each tube and place into separate 15-ml polypropylene tubes. Dilute each tube 1:10 with species-specific buffer. Concentrations after 5th dilution will be 10-nanograms, 7.5-nanograms, 5-nanograms, and 2.5-nanograms. Remove 1-ml from each tube and place into separate 15-ml polypropylene tubes. Dilute each tube 1:10 with species-specific buffer. Concentrations after 6th dilution will be 1000-picograms, 750-picograms, 500-picograms, and 250-picograms. Remove 1-ml from each tube and place into separate 15-ml polypropylene tubes. Dilute each tube 1:10 with species-specific buffer. Concentrations after 7th dilution will be 100-picograms, 75-picograms, 50-picograms, and 25-picograms. Remove 1-ml from each tube and place into separate 15-ml polypropylene tubes. Dilute each tube 1:10 with species-specific buffer. Concentrations after 8th dilution will be 10-picograms, 7.5-picograms, 5.0-picograms, and 2.5-picograms. Remove 1-ml from each tube and place into separate 15-ml polypropylene tubes. Dilute each tube 1:10 with species-specific buffer. Concentrations after 9th dilution will be 1000-femtograms, 750-femtograms, 500-femtograms, and 250-femtograms. Remove 1-ml from each tube and place into separate 15-ml polypropylene tubes. Dilute each tube 1:10 with species-specific buffer. Concentrations after

10th dilution will be 100-femtograms, 75-femtograms, 50-femtograms, and 25-femtograms. Remove 1-ml from each tube and place into separate 15-ml polypropylene tubes. Dilute each tube 1:10 with species-specific buffer. Concentrations after 11th dilution will be 10-femtograms, 7.5-femtograms, 5.0-femtograms, and 2.5-femtograms [9,10].

Prepare 20 x 96-well plates for standard curves. Removing 0.2-ml from each tube and placing material in each well of 96-well plate as discussed below. Based on the above serial dilutions there should be 12 tubes of 4 sets of dilutions, for a total of 48 dilutions. Based on a 96-well plate format, two concentration gradients should be on each plate, giving a n=2 for each dilution. Once plates have attached material, they can be covered, the edges sealed with Parafilm, and stored at 4°C until used [9,10].

2.7.4. Attaching Material to Plates

A concentration gradient of commercially available material is either absorbed directly to a 50% ethanol pretreated 96-well plate, a polyclonal antibody technique is used to bind the material to the 96-well plate, or the ELICA fixative is used to bind the material to the 96-well plate. The procedures for attaching material to a 96-well plate is as follows [9,10].

Binding to a 50% Ethanol Pretreated 96-Well Plate

Incubate a 96-well plate with 0.2-ml of 50% ethanol for 30 min and then allow to air dry at ambient temperature. Use 0.02M sodium carbonate/bicarbonate buffer, pH 9.2, as the diluent for the concentration gradient of known standards. Make a concentration gradient in the binding buffer fresh each time because the 0.02M sodium carbonate/bicarbonate buffer, pH 9.2, will degrade proteins when stored for periods longer than 24 hours. To attach standards (or a constant amount of a polyclonal antibody) to a 96-well plate, incubate 0.2-ml of the material / binding buffer solution in each well of 50% ethanol pre-treated plate for 1 hour at 37°C in a humidified environment. Remove the binding solution, wash 4 times for 2.5 min with species-specific buffer, and then 4 times for 2.5 min with species-specific buffer containing the blocking agent [9,10].

Antibody Capture Technique

Incubate a 50% ethanol pretreated antibody-containing plate with a concentration gradient of phenotypic expression marker antigen for 1 hour at 37°C in a humidified environment. Remove solutions and wash 4 times for 2.5 min with species-specific buffer containing the blocking agent. If using direct absorbing material to plate, disregard this step. Process the 96-well plate with primary, secondary, and tertiary blockers and probes as listed for ELICA analysis [9,10].

ELICA-Fixative Binding Material to 96-Well Plate

Incubate a 96-well plate with 0.2-ml of 50% ethanol for 30 min and then allow to air dry at ambient temperature. Suspend material to be bound into an aqueous-based solution. Remove of 0.2-ml of material and allow to air dry at ambient temperature. Once dried, add 0.2-ml of ELICA fixative to each well and incubate for 1 hour. Remove ELICA fixative and rinse once with 0.2-ml 50% ethanol. Remove 50% ethanol and rinse with 70% ethanol. Prepare for in situ DNA analysis [9,10].

2.7.5. High Titer Ascites Amplification

Medium

RPMI Medium 1640 with L-Glutamine (cat # 320-1875, GIBCO) – 450-mls: remove 50-ml from original 500-ml bottle, pH to 7.4, place into sterile 50-ml centrifuge tube and store at 4°C. The incomplete RPMI medium was used to wash ultra-pure DMSO (Morton-Thiokol) from frozen hybridoma cells; Fetal Bovine Serum (# 200-6140, GIBCO) – 100-ml; L-Glutamine (# 320-1350, GIBCO) – 5-ml. HT Supplement (# 320-1067, GIBCO) – 5-ml; Penicillin / Streptomycin (# 600-5140, GIBCO); total volume 615-ml.

Cell Plating Procedure

Use nitrile gloves (Diagger), spray gloved hands with 70% isopropyl alcohol (Sigma) [spraying gloves with 70% isopropyl alcohol is performed prior to every time gloved hands are inserted into Class-2 biosafety cabinet]. Using sterilized gauze or sterilized Kimwipe, wipe down inside of HEPA-filtered Class-2 biosafety cabinet (Lab Conco, Thermo-Fisher), floor and sides, with 70% isopropyl alcohol. Turn on UV light for five minutes, then turn on blowers of unit and allow air to circulate for a minimum of 45 minutes. Remove hybridoma cell vial from liquid nitrogen and place on dry ice to transport to tissue culture room. Place vial into a 37°C water bath or 37°C bead unit to JUST thaw. Using sterile technique and universal precautions, remove cell/DMSO suspension to a 15-ml polypropylene centrifuge tube (Falcon, Thermo-Fisher) and add to 4°C incomplete RPMI medium at a 1:10 ratio. Immediately centrifuge at 1,000 RCF (relative

centrifugal force) for 5-minutes. Decant and discard supernatant to bleach. Resuspend cells in ambient temperature complete RPMI hybridoma medium in twice original volume of cell suspension.

Counting Cells

Remove 50-microliters of cell suspension and place into small polypropylene tube. Add 50-microliters of 0.4% Trypan blue (Kodak) dissolved in species-specific buffer. Triturate (ingress / egress of cell suspension into and out of a sterile pipet) to disperse cells. Count number of viable (Trypan blue-excluded) cells and total number of cells to determine % cell recovery viability. Remember to multiply cell number by 2 because of dilution 1:1 with Trypan Blue [9].

Initial Plating Cell Density

Initial plating density is 400,000 cells/ml. Determine amount of medium necessary to make proper dilution of cells. Using smallest culture flask possible (10-ml maximum volume), add medium to flask first, and then add cell suspension. Preferably, use flasks with filters within their caps. If not, screw cap on tight, then unscrew cap ½ turn for adequate gas exchange. Place flask(s) in a 37°C 95% air/5% CO_2 incubator. Place flasks horizontal if volume of medium is 5-10-mls, or place flasks vertical if volume is 2-5-mls. [Sterilized rods (#2 pencils) can be used to prop up flasks to 45° angle to maintain all cells covered with medium.] Next day (18-24 hours after initial plating) triturate cells in flask with sterile pipet, remove 50-microliters to tube, add 50-microliters of 0.4% Trypan blue in species-specific buffer, and count cells as above. Determine total number of viable cells per total volume of medium and number of viable cells per ml of medium [9].

If cell number approximates 200,000 cells per ml of medium and medium looks pink in color, it indicates death of some or all of the cells. Remove medium, place in polypropylene centrifuge tube, spin at 600 RCF for 5 minutes, discard supernatant to bleach, add fresh complete RPMI medium to a final initial plating density of 400,000 cells per ml and place in a new fresh flask and return to incubator [9].

If cell number is at or above 300,000 cells per milliliter, add fresh medium to dilute to propagating density of 200,000 cells per milliliter and return flask to incubator. DO NOT THROW OUT OLD FLASK. Rather, add 10-ml of fresh complete RPMI medium and return to incubator. Cells adhering to flask will divide and increase cell population. Continue propagating cells under log phase growth conditions (propagating density) until desired cell numbers are reached. Generate at least 2 aliquots of cells, one aliquot for freezing and one aliquot for ascites injection times 5 x 10^6 cells times number of animals to be injected [9].

Cryopreservation [9]

Always freeze a minimum of 2-3 vials of cells (5 X 10^6 cells/ml) when growing hybridoma monoclonal cells for ascites fluid production. Using a Class-2 Biosafety Cabinet, pool cells in a 50-ml centrifuge tube. When completed, centrifuge at 1,000 RCF for 5 minutes, remove and discard supernatant to bleach. Very slowly, resuspend cells in complete RPMI medium at 5 x 10^6 cells per ml. Make up a 10% solution of dimethyl sulfoxide (99-100% ultra-pure DMSO) (Sigma) at a ratio of 1:10 v/v with complete RPMI medium. Vortex solution to mix, 10 x 1-sec bursts. Slowly add the 10% DMSO/RPMI dropwise to the cells and invert to mix. Aliquot 1.0-ml of cell/DMSO/RPMI suspension into pre-labeled polypropylene cryovials. Freeze cells for 2 hours in a -20°C freezer, followed by freezing cryovials in a -70°C Revco for 2 hours, and finally storage of cryovials in liquid nitrogen [9].

Transfer Cells to Lag Growth Phase Prior to Injection for Ascites Production [9]

To switch cells from log phase growth to lag phase growth necessitates slowing down cell growth by plating cells at higher and higher densities of cells per ml of RPMI complete medium. First day after split, plate cells at 400,000 cells per ml. Second day, plate cells at 600,000 cells per ml. Third day, plate cells at 800,000 cells per ml. Continue, increasing daily concentration of cells per ml by 200,000 cells per ml. Need to perform cell viability counts each day. For procedure to work properly, cell viability cannot drop below 80%. One needs 10 x 10^6 cells per mouse or 20 x 10^6 cells per rat for injection. Optimum growth rate prior to injection is 0-10% [9].

Pristane Prime Animals Before Ascites Injection [9]

Pristane (2,6,10,14-tetramethylpentadecane, 96%, Aldrich #T2,280-2, 100 g) prime female Balb/c retired breeders (Jackson) at 1.0-mililiters per mouse by intraperitoneal injection 24 hours prior to injecting cells. Need to use a stainless-steel syringes and needles (Diagger), because Pristane will dissolve plastic syringes and plastic couplings between syringe and needle.

Injecting Hybridoma Cells for Ascites Production [9]

Remove cell suspensions from flasks and place into centrifuge tubes. Spin tubes at 600 RCF for 5 minutes. Remove and discard supernatant to bleach. Count cells. Resuspend viable cells at a density of 10^7 cells per milliliter of sterile 0.1M species-specific buffer, pH 7.4. Take up 1.0-milliliter of cells in stainless-steel syringe and inject mouse with a single bolus intraperitoneally. Check mice the next day to see if all survived the injection procedure. Wait one week, then check mice regularly for harvesting ascites fluid. Mice injected with lag phase cells do not look sickly or have diarrhea compared to their log phase cell counterparts. Lag phase injected mice have the appearance of giant balloons with paws, head, and tail. Their abdomen will feel "spongy" but not "squishy", as with injected log phase cells [9].

Harvesting Mouse Generated High Titer Ascites Fluid [9]

Euthanize mice with CO_2 (AirGas) inhalation, then cervically dislocate them. Swab abdomen with 70% ethanol. Make a small incision through the skin and abdominal musculature. With a plastic pipette withdraw ascites fluid, which is the consistency of thick syrup. The average amount of ascites fluid per mouse is 10-15-milliliters. Place ascites fluid in centrifuge tube on wet (crushed) ice. Wash peritoneal cavity of mouse with sterile species-specific buffer until fluid appears clear using multiple rinses equaling 10-15-milliliters of saline, place ascites fluid into separate tubes and place on wet crushed ice. Usually, the wash ascites has an antibody titer 2-3 orders of magnitude less than pure ascites [9].

One can either pool pure ascites fluid with wash ascites fluid or process them separately. Spin tubes at 2800 RCF for 10 minutes, decant and save supernatant, discard pellet. Store at 4^{o}C for 24 hours to precipitate any residual fibrin. If any fibrin material remains, spin tube(s) at 2800 RCF, save supernatant and discard pellet [9].

Prepare 4% stock solution of sodium azide with HPLC water. Filter through a 0.2-micron bottle-top filter (Diagger) before use. [CAUTION: In the dry powdered form, inhalation of sodium azide will KILL you. A 4% or higher aqueous solution of sodium azide absorbed through the skin will KILL you.] Therefore, it is imperative that weighing powdered sodium azide and dissolving it in HPLC water be performed in an operational chemical fume hood, wearing nitrile gloves, a respirator, and a chemical resistant apron. Suggest making up a stock solution of 20% sodium azide in HPLC water and then diluting it to desired concentration with HPLC water when needed [9].

Add 4% sodium azide* to a final volume of ascites for a final concentration of 0.05% sodium azide = 1/80th dilution (divide total ascites volume by 80, then add appropriate volume of 4% sodium azide for a 1:80 dilution).

*Sodium azide inhibits the peroxidase enzyme. Therefore, must be washed from system before using added HRP and HRP substrates for visualization.

Perform a serial dilution titer analysis to determine titer of ascites generated. Add a spot of decreasing concentration of ascites stepping by 1:10 dilutions at each step. Starting at 1.0 and diluting to pure water is necessary. Spot titer concentrations will be at 1:10^0, 1:10^1, 1:10^2, 1:10^3, 1:10^4, 1:10^5, 1:10^6, 1:10^7, 1:10^8, 1:10^9, 1:10^10, 1:10^11, and a1:10^12 to nylon membrane and allow it to be absorbed. Probe the nylon membrane with the antigen that the monoclonal antibody is directed against. One is looking for the least concentration of ascites that will bind to the antigen. It was routine to obtain mouse ascites antigen titers in the millions [9].

High Titer Ascites Generation in Rats [9]

If one substitutes 400 g Sprague-Dawley retired breeders for Blab/c retired breeders, the absolute numbers increase. Primed post-breeder rats require 5-ml of Pristane for priming. Resuspend cells at a density of 10^9 cells per ml of sterile 0.1M species-specific buffer, pH 7.4. Take up 2.0-ml of cells in a stainless-steel syringe and inject rats with a single bolus intraperitoneally. Check rats the next day to see if all survived the injection procedure. Wait one week, then check rats regularly for harvesting ascites fluid. Rats injected with lag phase cells do not look sickly or have diarrhea compared to their log phase cell counterparts. Lag phase injected rats have the appearance of giant balloons with paws, head, and tail. Their abdomen will feel "spongy" but not "squishy", as with injected log phase cells.

Harvesting Rat Generated Ascites Fluid

Differing from mice, rats do not need to be euthanized and/or cervically dislocated to harvest their ascites fluid. Rather they can be anesthetized (Chloroform inhalation, circa 1980's; Isoflurane inhalation, circa 2020's, F. Lochner, DVM, personal communication) and harvested multiple times with the insertion of a peritoneal drain tube in the abdomen. Swab abdomen with 70% ethanol. Make a small incision through the skin and abdominal musculature and insert a peritoneal drain tube. With a sterile syringe, insert into peritoneal drain tube and add 5-ml of sterile PBS to abdomen, triturate, and remove ascites fluid, which is the original consistency of toothpaste. Continue process of adding 5-ml of

sterile PBS, triturating, and removal of ascites, until wash fluid appears clear, utilizing multiple 5-ml washes. The average amount of "pure" ascites fluid per rat is 30-50-ml, with an average amount of wash solutions in the range of 75-100-ml per rat. Place ascites fluid in centrifuge tube on wet (crushed) ice. Usually, wash ascites has an antibody titer 2-3 orders of magnitude less than pure ascites. The rats are allowed to waken, placed in individual housing, and fed and watered ad libitum [9].

Process rat-generated ascites as described for mouse-generated ascites, as described above. Perform a serial dilution titer analysis. Routine titers for rat-generated ascites were in the billions.

Experiment: Myosin ELICA of Day 8 Myogenic Lineage-Committed Progenitor Cells

Embryonic chick myogenic lineage-committed progenitor cells were thawed and suspended at 5 x 10^3 cells per well in 1% collagen-coated 24-well plates in plating medium [10]. The plating medium consisted of OptiMEM + GlutaMax (GIBCO), 10% Heath Inactivated Serum (Atlas), 1% Penicillin/ Streptomycin (Sigma), pH 7.4 [Y-27]. Twenty-four hours after plating, the plating medium was removed, rinsed with species-specific buffer, and fed with maintenance medium for the remaining 7 days. Maintenance medium consisted of plating medium with the addition of 2 micrograms Insulin (Sigma) per ml, pH 7.4. [Insulin is a progression agent that will accelerate phenotypic expression in lineage-committed progenitor cells.] The maintenance medium is changed to fresh medium when there is a color (pH) change. Original color of maintenance medium is salmon. It is time to remove spent medium and replace with fresh medium when the medium color changes to orange/yellow. [The gradient of colors (pH) ranges from salmon, to salmon/orange, to orange, to orange/yellow, to yellow as lactic acid builds up in the cultures. If medium is yellow and murky, the cells are dead.] [9]

A myosin-ELICA was performed as described. The 96-well plate was equally divided into two groups: In species-specific buffer only AND with primary antibody. Blockers for primary, secondary, and tertiary probes consisted of 2% powdered non-fat dry milk in species-specific buffer (Tyrode's buffer for avians), at pH 7.4. Primary probe consisted on MF-20 (anti-sarcomeric myosin antibody) diluted 1:100 in species-specific buffer, pH 7.4; secondary probe consisted of Biotin-conjugated goat anti-mouse IgG (Fisher-Biotech) diluted 1:10,000 in species-specific buffer, pH 7.4; and tertiary probe consisted of avidin-horseradish peroxidase (Vector) diluted to 1:10,000 with species-specific buffer, pH 7.4. For quantification of myosin, all wells within the 96-well plate were incubated with ABTS substrate for 15 min in the dark. The reacted substrate was removed and read in a spectrophotometric 96-well plate reader (Titer Tek), using a 405-nm excitation filter. For visualization/localization of myosin, the wells were rinsed with species-specific buffer and incubated with 4-chloro-1-napthol for 30 min in the dark. The substrate was removed, the wells rinsed with species-specific buffer, and photographed using 10X magnification with brightfield optics on a Nikon TMS inverted phase contrast microscope. The plate was then processed with 70% ethanol for in situ DNA analysis.

3. Results

Table 2 Myosin and DNA analysis (ELICA) of Day Eight Myogenic Lineage-Committed Progenitor Cells

Assay	Without Primary Antibody	With Primary Antibody
Myosin content ng/ml $\pm$ SD	0.056 $\pm$ 0.138	21.204 $\pm$ 6.110
DNA content micrograms/well $\pm$ SD	0.360 $\pm$ 0.264	0.355 $\pm$ 0.090
ng Myosin per microgram DNA	0.16	59.73

4. Discussion

The original ELICA was developed based on the ELISA technologies to quantify, visualize, and standardize biological activities of unknown human recombinant proteins, unknown morphogenetic proteins, and differentiated cell generated exosomes. The ELISA is performed by absorbing either antigens or antibodies to a nylon membrane, and then probing with either antibody or antigen. The ELICA was based on a 24-, 48-, 96-, 384-, or -1536 well plate format. Cultured cells (or product to be tested) are fixed to the plate surface using the ELICA fixative. The cultures are washed to remove the fixative, each well or series of wells are incubated with one or more antibodies to a particular phenotypic expression marker (PEM), termed the primary probe. The primary probe is removed, the culture wells incubated with an antibody directed against the primary probe (= secondary probe) with attached horseradish peroxidase (HRP = tertiary probe). Excess tertiary probe is removed and wells incubated with soluble HRP substrate. The soluble HRP

substrate is removed and scanned in a spectrophotometer to quantify material present, based on a titration curve. The cultures are then incubated with an insoluble HRP which precipitates at sites of antibody binding, allow visualization of cells/material, the cultures photographed, and then processed for DNA quantification. Each of the above procedures were performed in each well of 96-well plate. Results are given as quantity of PEM standardized to quantity of DNA.

Initially, direct labeled-antibody to antigen binding was performed. It allowed for the measure of microgram quantities of PEM per microgram quantities of DNA. By adding the biotin-avidin amplification procedure, the ELICA became more sensitive to the appearance of PEM markers, in the realm of nanogram quantities of PEM per microgram quantities of DNA. Lastly, by the addition of high titer ascites to the ELICA procedure, fewer cells need to be tested to distinguish phenotypic expression markers, in the realm of femtogram quantities of PEM per microgram quantities of DNA. The increased sensitivity utilizing biotin-avidin amplification coupled with high titer ascites amplification allowed the use of plates with 384-wells, and in some instances, 1536-wells as the platform for high throughput screening [9].

5. Conclusions

Four hypotheses were tested

- **Hypothesis 1**. A fixative could be developed for fast penetration, retention of excellent cellular morphology, prevention of apoptosis leading to self-destruction, with a physiological osmolarity of 1.0, and used with any antibody. Yes, the ELICA Fixative.
- **Hypothesis 2**. The signal could be amplified using biotin-avidin-HRP for phenotypic expression markers. Yes, by three orders of magnitude.
- **Hypothesis 3.** High titer ascites would increase sensitivity to the antigenic sites; Yes, by an additional three orders of magnitude above biotin-avid HRP.
- **Hypothesis 4.** Quantitation of PEM/DNA sensitivity could be increased by several orders of magnitude. Yes, amplified by biotin-avidin-physiological osmolarity and high titer ascites.

In addition, it was demonstrated that n = number of participants per experiment and is not limited to the number of wells in a plate, such as 24-well, 96-well, 384-well, and 1,536-well plates. While the techniques are the same, the volumes needed for incubation need to be adjusted to the size/volume of the wells being analyzed.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest was disclosed.

Statement of ethical approval

Animal Use The use of animals in this study complied with the guidelines of Mercer University's Institutional Animal Care and Use Committee (ACUC). These guidelines reflect the criteria for humane animal care of the National Research

Council as outlined in "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health [64].

Human Use The use of human biopsy specimens in this study complied with the guidelines of Mercer University's Institutional Review Board (IRB). These guidelines reflect the Federal Regulations for Protection of Human Research Subjects, HHS Office for Human Research Protections – 45 and 46 CFR: 46.102(I) [65].

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