Cancer Cell Technology and Immunology Principles

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Table of Contents

INTRODUCTION: 4
SCIENCE BACKGROUND: 4
Testicular Cancer 4
Cancer Vaccines 5
The Inhibins 5
Autoreactive T Cells 6
STUDENT OUTCOMES: 6
LEARNING OBJECTIVES: 6
NGSS (Next Generation Science Standards) 7
TIME REQUIREMENTS: 7
ADVANCE PREPARATION: 8
MATERIALS AND EQUIPMENT 12
Student Workstation Checklist 12
Cryopreservation of Cancer Cells 14
Handling Procedures for Frozen Cells 15
RNA Purification and cDNA Amplification 17
Possible Substitutes 18
DAILY UNIT PLANS 19
Thawing Procedure for Direct Cell Shipment from ATCC (Teacher) 19
Subculturing Cells 21
Cryopreservation of Cancer Cells 23
Handling Procedures for Frozen Cells 25
Cell Counting, Trypan Blue Exclusion 27
RNA Purification and cDNA Amplification 29
Hematoxylin & Eosin Staining 30
Immunohistochemistry 31

ABSTRACT:

Testicular cancer mainly affects men between the ages of 20 and 39, but it is the most common neoplasm in men between the ages of 15 and 34. The National Cancer Institute states that localized testicular cancer has a recurrence rate of 15-20%, and those tumors that are determined to be Sertoli or Leydig cell derived do not respond well to chemotherapy or radiation treatment. My summer research was centered on the effort to better characterize the immune response to a testicular tumor. This was accomplished through the use of different immunology techniques such as proliferation assays, immunohistochemistry, ELISA, and ELISPOT. My lab experience inspired me to design a unit curriculum which will help elucidate the various physiognomies of tumor growth. Students first learn and apply the essential techniques of cancer cell culture. The testicular cancer cell line I-10 (CCL-83, ATCC) is used to familiarize students with routine feeding and maintenance, subculturing, counting, and cryopreservation. I-10 cell RNA is converted to cDNA and qualitatively analyzed for inhibin-alpha gene expression, which serves as a tumor marker, using RT-PCR. Then, they analyze previously formalin-fixed, paraffin embedded murine testes (normal and cancerous) using H&E staining and immunohistochemical staining for CD3, a marker for T cells. Students then complete this unit by demonstrating these learned skills in a combined written and practical examination.

INTRODUCTION:

Testicular cancer mainly affects men between the ages of 20 and 39, but it is the most common neoplasm in men between the ages of 15 and 34[1,2]. The National Cancer Institute states that localized testicular cancer has a recurrence rate of 15-20%, and those tumors that are determined to be Sertoli or Leydig cell derived do not respond well to chemotherapy or radiation treatment[1]. It also states that following radical inguinal orchiectomy, or removal of the testicle through an incision in the scrotum, there is a 15% chance of relapse which can increase to 32% if the tumor is greater than 4 cm and invasion of the rete testis has occurred[1]. In an effort to improve therapy for testicular cancer and offer an immunotherapy option, researchers decided to work on the development of a testicular cancer vaccine. This therapeutic vaccine could strengthen the body's natural defenses against the cancer and may therefore identify local or systemic tumors. This vaccine could be administered as adjuvant therapy, meaning in conjunction with or after the main treatment.

Researchers immunized male mice with inhibin-α (Inα), a protein produced by Leydig cells that negatively regulates pituitary release of follicle stimulating hormone (FSH). This protein is capable of activating T cells and inducing experimental autoimmune orchitis. Mice immunized with inhibin-α had a 33% lighter mean testicular weight compared to the control group. Researchers concluded that targeting testicular stromal cells with T cell autoimmunity results in the reduction of tumor size in BALB/c mice. The data indicated a possible useful application of targeted T cell autoimmunity in vaccinating against an inhibin-α target that is overly expressed in these tumors.

SCIENCE BACKGROUND:

Testicular Cancer

Testicular cancer is a malignancy that forms in the tissues of one or both testicles[3]. More than 99% of these tumors develop in germ cells; these are biological cells that give rise to gametes[4]. There are two main types of germ cell tumors (GCTs); seminomas and non-seminomas. Seminomas arise from sperm-producing germ cells of the testicles and contain two subclasses known as classical seminoma and spermatocytic seminoma. More than 95% of classical seminomas are typical and generally occur in men between the ages of 30 and 50 while spermatocytic seminomas are slow-growing, infrequent, and tend to occur in older men[4]. Non-seminomas on average occur in males between their teens and early 40s and have four subclasses: embryonal carcinomas which have a high proliferation rate and when viewed under the microscope, resemble early embryonic tissues; endodermal sinus tumors which bear a resemblance to the embryonic yolk-sac and are common in children under 3; choriocarcinomas which tend to be very rare, aggressive, and mainly affect adults; and teratomas which resemble the primary embryonic germ layers and may be either invasive or noninvasive[4]. Testicular tumors may also develop in hormone-producing tissues called stroma and are referred to as gonadal stromal tumors. These can be Leydig or Sertoli cell derived and can affect both adults and children. Gonadal stromal tumor metastases usually have a poor prognosis due to their lack of response to chemotherapy or radiation therapy[5].

1. National Institutes of Health. (2014). Comprehensive Cancer Information. In *National Cancer Institute*. Retrieved June 9, 2014, from http://www.cancer.gov/cancertopics/types/testicular
2. *Diagnosis and Treatment of Testicular Cancer*. (2014). Retrieved June 9, 2014, from Stanford Medicine website: http://cancer.stanford.edu/male/testicular.html
3. *Testicular Cancer*. (2014). Retrieved June 9, 2014, from National Cancer Institute website: http://www.cancer.gov/cancertopics/types/testicular
4. *Testicular Cancer*. (2014, February 11). Retrieved April 9, 2012, from American Cancer Society website: http://www.cancer.org/Cancer/TesticularCancer/DetailedGuide/testicular-cancer-what-is-testicular-cancer
5. *Testicular Cancer Treatment*. (2014, April 2). Retrieved June 9, 2014, from National Cancer Institute website: http://www.cancer.gov/cancertopics/pdq/treatment/testicular/

Treatment options for testicular cancer include surgery involving radical inguinal orchiectomy, or removal of the testicle through an incision in the groin. Radiotherapy is a local therapy that employs the use of high energy rays to destroy cancer cells, but may also destroy normal cells. Chemotherapy is the most common systemic cytostatic that targets hyperproliferating cells found in tumors, and therefore, may also have deleterious effects on bone marrow, hair, and intestinal cells[5]. However, following radical inguinal orchiectomy, there is a 15% chance of relapse which can increase to 32% if the tumor is greater than 4cm and invasion of the rete testis has occurred[1].

Cancer Vaccines

Currently, there are two different concepts for vaccination that are followed; one involves the use of whole tumor homogenates, and the other defined antigens. Historic achievements in immunization, such as those obtained from the smallpox and polio vaccines, have inspired scientists to attempt the immunization of cancer patients to their own cancer cells as a means of therapy. This method involves the immunization with autologous or allogenic intact tumor cells or their extracts; however, the limited quantity of whole tumor cell surface antigens present has rendered this method inefficient[6]. Various attempts have been made at augmenting the immunogenicity of this style of vaccine including adjuvant emulsification and the transduction of cytokine genes for granulocyte-macrophage colony-stimulating factor, tumor necrosis factor, and interferon-γ. Despite such determination, it has been difficult to distinguish a specific immune response to the vaccine and gauge its efficacy using whole tumor cell homogenates[6].

Some promising results have been obtained with the use of melanoma antigen E-3 (MAGE-3), cancer/testis antigen 1B (CTAG1B or NY-ESO-1), melanocyte antigen/melanoma antigen recognized by T cells (Melan-A/MART-1), tyrosinase, and glycoprotein 100 (gp100) from malignant testicles. These antigens have been used alone or combined with cytokines such as interleukins 2 or 12 (IL-2, IL-12) or with an adjuvant (i.e. incomplete Freund's adjuvant) in order to increase their immunogenicity. These methods did produce measurable peptide-specific immune responses of CD8+ T cells coupled with the retrogression of metastasis[7,8]. Recombinant virus constructs, such as adenovirus and vaccinia virus, have also been employed but with poor results that are probably due to the presence of anti-viral proteins in the participating cancer patients. Dendritic cells transfected with recombinant viruses have delivered some promising results, but due to their copious antigen repertoire, the immunological responses have been difficult to gauge[7,8].

The Inhibins

The inhibin hormones are heterodimeric glycoproteins that contain a single α-chain and one of two β-chain isoforms. Thus, there are two inhibins, inhibin A (α:βA) and inhibin B (α:βB), that belong to the transforming growth factor-β (TGFβ) superfamily[9]. The inhibins are gonadal endocrine proteins that function as potent inhibitors of synthesis and secretion of pituitary follicle stimulating hormone (FSH)[10]. As such, the inhibins are important regulators of spermatogenesis. The inhibins are produced by testicular Sertoli and Leydig cells within and outside the seminiferous tubules of the testicles. Several inhibin-α peptides have been shown to be immunogenic and capable of inducing a CD4+ T cell response in female and male transgenic mice[11-13].

6. Rosenberg, S. A. (2001, May). Progress in human tumour immunology and immunotherapy. *Nature, 411*, 380-384.
7. Marchand, M., van Baren, N., Weynants, P., Brichard, V., Dréno, B., Tessier, M.-H., . . . Boon, T. (01, 1999). Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. International Journal of Cancer, 80(2), 219–230.
8. Jager, E., Gnjatic, S., Nagata, Y., Stockert, E., Jager, D., Karbach, J., & Knuth, A. (24, 2000, October). Induction of primary NY-ESO-1 immunity: CD81 T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-11 cancers. Proceedings of the National Academy of Sciences, 97(22), 12198-12203.
9. Kingsley DM. 1994. The TGF-β superfamily: new members, new receptors and new genetic tests of function in different organisms. Genes Dev. 8, 133-146

Autoreactive T Cells

T cells recognize antigens only when presented by molecules on the surface of antigen presenting cells such as macrophages and dendritic cells[14]. CD4+ T cells recognize peptide antigens via the T cell receptor (TCR) when the peptide is presented by class II molecules of the major histocompatibility complex (MHC). However, CD8+ T cells recognize peptide antigens when they are presented by class I molecules of the MHC. In most organ-specific autoimmune models, CD4+ responses predominate, but CD8+ T cells may be involved in the self-recognition process and in the pathogenesis of disease often by recognizing totally different peptide domains of self-proteins than those recognized by CD4+ T cells[15].

STUDENT OUTCOMES:

Students learn and apply the essential techniques of cancer cell culture. The testicular cancer cell line I-10 (CCL-83, ATCC) is used to familiarize students with routine feeding and maintenance, subculturing, counting, and cryopreservation. I-10 cell RNA is converted to cDNA and qualitatively analyzed for inhibin-alpha gene expression, which serves as a tumor marker, using RT-PCR. Then, they analyze previously formalin-fixed, paraffin embedded murine testes (normal and cancerous) using H&E staining and immunohistochemical staining for CD3 and inhibin-alpha. Finally, students complete this unit by demonstrating these learned skills in a combined written and practical examination. (Please see below.)

LEARNING OBJECTIVES:

Within this unit, students will be able to do the following:

- Students should be able to demonstrate proper cancer cell culture methodology including media preparation, aseptic technique, and subculturing.
- Students will demonstrate the proper use of a hemocytometer slide and the trypan blue exclusion method for counting Leydig cancer cells.
- Students will employ all necessary steps needed to preserve the Leydig cancer cells in liquid nitrogen.
- Students will successfully extract and purify RNA from Leydig cancer cells.
- Students will use RT-PCR to measure inhibin-α gene expression, which serves as a marker for Leydig cell tumors.
- Students will analyze formalin-fixed, paraffin embedded murine testes (normal and cancerous) using H&E staining and immunohistochemical staining for CD3.

NGSS (Next Generation Science Standards)
This unit aligns with the following NGSS:

- PS1A: Structure and Properties of Matter
- PS1B: Chemical Reactions
- PS3B: Conservation of Energy and Energy Transfer
- PS4C: Information Technologies and Instrumentation
- LS1A: Structure and Function
- LS1B: Growth and Development of Organisms

TIME REQUIREMENTS:

Task	**Week 1**				**Week 2**				**Week 3**			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Culture												
Counting												
Cryopreservation												
RNA/cDNA												
H&E Staining												
Immuno-histochemistry												
Lab Practical												

ADVANCE PREPARATION:

Equipment	Purchased	Cost	Substitute*
CO_2 Incubator	Donation	$7,000	CO_2 Independent Medium
Clinical Centrifuge (refrigerated)	Donation	$6,500	Unrefrigerated
Inverted Microscope	BioExpress	$2,500	Buy refurbished
Laminar Flow Hood w/HEPA filter	BioExpress	$2,200	Fume hood
Freezer -80 (optional)	BioExpress	$10,000	Discard cells
Liquid Nitrogen Storage Tank (optional)	BioExpress	$2,000	Discard cells
Water bath	Fisher Scientific	$700	Buy refurbished
Pipettor (cordless)	BioRad	$250	Pipet Pump
Mini-Sub Cell GT Electrophoresis Chamber (170-4487)	BioRad	$240	N/A
Power Supply (164-5050)	BioRad	$280	N/A
Micropipette (P10, P100, P1,000)	Pipette.com	$80	Plastic bulb pipette
Vacuum Pump	Carolina Biological	$500	Faucet Aspirator Vacuum Pump
Gel UV Hood w/digital camera	Carolina Biological	$500	Not needed if using white light
Upright Microscope			Buy refurbished
Thermal Cycler	Ward's Science	$2,000	Water baths
Transilluminator	Ward's Science	$1,000	White Light Transilluminator
Aroma 8-Cup Food Steamer	Walmart	$30	Optional
Lab Counter App (Zappy Lab)	App Store	Free	
Lab Timer App (0x0c)	App Store	Free	
Google Drive	App Store	Free	

*See "Possible Substitutes" section for more information.

Materials	Purchased	Cost	Substitute
I-10 Leydig Cancer Cells (CCL-83)	ATCC	$450	N/A
F-12K Medium (30-2004)	ATCC	$18	CO_2 Independent Medium
Horse Serum (30-2040)	ATCC	$50	N/A
Fetal Bovine Serum (SH3091002)	Fisher Scientific	$70	N/A
Penicillin/Streptomycin (BP2959-50)	Fisher Scientific	$50	N/A
Trypan Blue (SV3008401)	Fisher Scientific	$20	N/A
Vacuum Filtering Flask (10-182-50B)	Fisher Scientific	$30	N/A
Cell-VU Hemocytometer Slides (DRM700)	Fisher Scientific	$200	N/A
Permount	Fisher Scientific	$170 (for 6)	Clear nail polish
$25cm^2$ Culture Flasks (T-3400-25)	BioExpress	$400	Tissue culture dish
Bottle Top Filter (F-2629-3)	BioExpress	$190	N/A
Dimethyl sulfoxide (DMSO) (N182-5X10ML)	BioExpress	$70	
Trypsin EDTA (TE) (45000-664)	BioExpress	$50	N/A
50mL Conical Tubes (BX-C-3394-4)	BioExpress	$100	Glass tubes
15mL Conical Tube (BX-C-3394-2)	BioExpress	$90	Glass tubes
10mL Serological pipette (BX-P-2837-10)	BioExpress	$50	Glass pipettes
2mL Cryotubes (T-2889-2A)	BioExpress	$380	Optional
Hematoxylin (0701-25G)	BioExpress	$60	N/A
Eosin (0109-100G)	BioExpress	$40	N/A
Xylene (BDH2000-5GLMS)	BioExpress	$300	N/A
Ethanol 100% (E402-4L)	BioExpress	$80	N/A
Phosphate Buffered Saline (1X)	BioExpress	$140 for 12	PBS Tablets
Triton X-100 (AAA16046-AE)	BioExpress	$20	N/A
Tris Borate EDTA (MRGF-4215)	BioExpress	$45	N/A
Ethidium Bromide (161-0433)	BioRad	$40	Methylene Blue
DNA Loading Dye (166-0401)	BioRad	$20	N/A
1kb DNA Ladder (N3232S)	New England Bio Labs	$50	N/A
RNeasy Mini Kit (74104)	QIAGEN	$270	N/A
OneStep RT-PCR Kit (210210)	QIAGEN	$150	N/A
ImmunoCruz Rabbit Staining System (sc-2051)	Santa Cruz Biotechnology	$150	N/A
CD3 Antibody (MCA1477)	AbD Serotec	$440	N/A
Liquid Nitrogen refill (optional)	Praxair	$20.00/month	Optional
CO_2 Gas Tank	Praxair	$30.00/month	CO_2 Independent Medium

Sterile Materials

1. The following items should be sterilized by spraying with 70% ethanol and left permanently inside the cell culture hood (laminar flow hood):
    a. Permanent marker
    b. Test tube racks (Do not use wood!)
2. The following items should be sterilized by steaming and left permanently inside the cell culture hood (laminar flow hood):
    a. Pasteur pipettes (in pipet sterilizing box)

Horse Serum (HS)

1. *Time required: Thaw-1.0 hr., aliquot-30 min.*
2. Thaw HS by placing it in a 37ºC water bath.
3. Aliquot into 50mL conical tubes.
4. DO NOT decomplement!
5. Store at -20°C.

Fetal Bovine Serum (FBS)

1. *Time required: 1.5 hrs.*
2. Thaw FBS by placing it in a 37ºC water bath.
3. Decomplement serum by placing bottle in a 56ºC water bath for no more than 30 minutes.
4. Aliquot into 50mL conical tubes.
5. Store at -20°C.

F-12K Complete Medium

1. *Time required: 1.0 hr.*
2. Conduct the following procedures using aseptic technique inside a sterile laminar flow hood.
3. To make the complete growth medium, add the following components to the base medium F-12K:
    a. Fetal bovine serum to a final concentration of 2.5%
    b. Horse serum to a final concentration of 15%
    c. Pen/Strep to a final concentration of 5%
4. Make 500mL at a time just in case it gets contaminated.
5. Vacuum filter through a Bottle Top Filter.
6. Store at 4°C.

Trypsin EDTA (TE)

1. *Time required: 1.0 hr.*
2. Thaw TE by placing it in a 37ºC water bath.
3. Aliquot into 15mL conical tubes; 10mL in each.
4. Store at -20°C.

0.5X Tris Borate EDTA (TBE)

1. Dilute 10X stock solution with dH_2O.
2. Mix 50mL of 10X TBE with 950mL of dH_2O for a total volume of 1L.
3. Stir for at least 1 minute.

0.8% Agarose Gel

1. In a 150ml Erlenmeyer flask, pour enough 0.5X TBE to cover the bottom.
2. Weigh 0.8g of agarose and carefully pour it inside the flask.
3. Bring the volume up to 100mL with 0.5X TBE.
4. Now, bring to a boil for at least 3 times in a microwave or on a hot plate until the agarose is fully dissolved. It should be as clear as water.
5. Add 2µL of ethidium bromide and stir (1µL for every 50mL of agarose solution).
6. Let it cool to the point where you can hold the bottom of the flask with a gloved hand, comfortably for 10s.
7. Pour into gel cast.

DNA Ladder

1. Prepare loading mixture:
2. Distilled water - 4 µl
3. 6X Blue Loading Dye - 1 µl
4. DNA Ladder - 1 µl
   Total volume - 6 µl
5. Mix gently.
6. Load onto the agarose gel.

MATERIALS AND EQUIPMENT

Student Workstation Checklist

One workstation serves 2-3 students.

Thawing Procedure (Cell Culture Hood)			
Item	**Contents/Volume**	**Number**	**(☒)**
Inverted Microscope		1	☐
CO_2 Incubator		1	☐
Water Bath		1	☐
Clinical Centrifuge		1	☐
Gloves		2-3	☐
Beaker (for ice)	1L	1	☐
Ice	600mL	1	☐
Culture flask		2	☐
F-12K bottle	F-12K complete medium/500mL	1	☐
Pasteur pipette		5	☐
70% Ethanol	500mL	1	☐
Cryotube	Cancer cells/2mL	1	☐
Serological pipette	10mL	5	☐
Pipettor		1	☐
Conical tube	50mL	4	☐
Tube rack		1	☐
Paper Towels		1	☐
Marker (permanent)		1	☐
Biohazard Bin		1	☐

Cell Subculture (Cell Culture Hood)			
Item	**Contents/Volume**	**Number**	**(☒)**
Inverted Microscope		1	☐
CO_2 Incubator		1	☐
Water Bath		1	☐
Clinical Centrifuge		1	☐
Gloves		2-3	☐
Beaker (for ice)	1L	1	☐
Ice	600mL	1	☐
Culture flask		2	☐
F-12K bottle	F-12K complete medium/500mL	1	☐
Pasteur pipette		5	☐
70% Ethanol	500mL	1	☐
TE	5mL per $25cm^2$ flask (when subculturing)	1	☐
Serological pipette	10mL	5	☐
Pipettor		1	☐
Conical tube	50mL	4	☐
Tube rack		1	☐
Paper Towels		1	☐
Marker (permanent)		1	☐
Biohazard Bin		1	☐

Cryopreservation of Cancer Cells (Cell Culture Hood)			
Item	**Contents/Volume**	**Number**	**(☒)**
Inverted Microscope		1	☐
CO_2 Incubator		1	☐
-80 Freezer		1	☐
Liquid Nitrogen Storage Tank		1	☐
Water Bath		1	☐
Clinical Centrifuge		1	☐
Gloves		2-3	☐
Beaker (for ice)	1L	1	☐
Ice	600mL	1	☐
Culture flask		2	☐
F-12K bottle	F-12K complete medium/500mL	1	☐
Pasteur pipette		5	☐
70% Ethanol	500mL	1	☐
TE	5mL per $25cm^2$ flask	1	☐
DMSO	1mL	1	☐
Serological pipette	10mL	5	☐
Pipettor		1	☐
Conical tube	50mL	4	☐
Cryogenic tube	2mL	2	☐
Tube rack		1	☐
Paper Towels		1	☐
Marker (permanent)		1	☐
Biohazard Bin		1	☐

Handling Procedures for Frozen Cells (Cell Culture Hood)			
Item	**Contents/Volume**	**Number**	**(☒)**
Inverted Microscope		1	☐
CO_2 Incubator		1	☐
-80 Freezer		1	☐
Liquid Nitrogen Storage Tank		1	☐
Water Bath		1	☐
Clinical Centrifuge		1	☐
Gloves		2-3	☐
Beaker (for ice)	1L	1	☐
Ice	600mL	1	☐
Culture flask		2	☐
F-12K bottle	F-12K complete medium/500mL	1	☐
Pasteur pipette		5	☐
70% Ethanol	500mL	1	☐
Serological pipette	10mL	5	☐
Pipettor		1	☐
Conical tube	50mL	4	☐
Frozen Cryogenic tube	Cancer cells/1mL	2	☐
Tube rack		1	☐
Paper Towels		1	☐
Marker (permanent)		1	☐
Biohazard Bin		1	☐

Cell Counting **Trypan Blue Exclusion (at bench)**			
Item	**Contents/Volume**	**Number**	**(☒)**
Inverted Microscope		1	☐
CO_2 Incubator		1	☐
Water Bath		1	☐
Clinical Centrifuge		1	☐
Gloves		2-3	☐
Beaker (for ice)	1L	1	☐
Ice	600mL	1	☐
Culture flask		2	☐
F-12K bottle	F-12K complete medium/500mL	1	☐
Pasteur pipette		5	☐
70% Ethanol	500mL	1	☐
TE	5mL per 25cm^2 flask (when subculturing)	1	☐
Serological pipette	10mL	5	☐
Pipettor		1	☐
Conical tube	50mL	4	☐
Tube rack		1	☐
Paper Towels		1	☐
Marker (permanent)		1	☐
Biohazard Bin		1	☐
1.5mL microtube	Trypan blue/300µL	1	☐
Microscope		1	☐
Micropipette or plastic bulb pipette		1	☐
Hemocytometer slide & cover slip		1	☐
Lab Counter		1	☐

RNA Purification and cDNA Amplification (at bench)			
Item	**Contents/Volume**	**Number**	**(☒)**
For RNA purification of I-10 cells, follow handbook included with QIAGEN's RNeasy Mini Kit			
For cDNA amplification of I-10 cell RNA, follow handbook included with QIAGEN's OneStep RT-PCR Kit			
250mL Beaker	ice	1	☐
Micropipette or plastic bulb pipette		1	☐
Thermal cycler		1	☐
Water bath		1	☐
Power supply		1	☐
Gel cast		1	☐
Electrophoresis chamber		1	☐
Erlenmeyer flask	0.8% agarose/100mL	1	☐
Bottle	TBE/500mL	1	☐
Microtube	Loading Dye/1mL	1	☐
Microtube	DNA Ladder/12µL	1	☐

Possible Substitutes

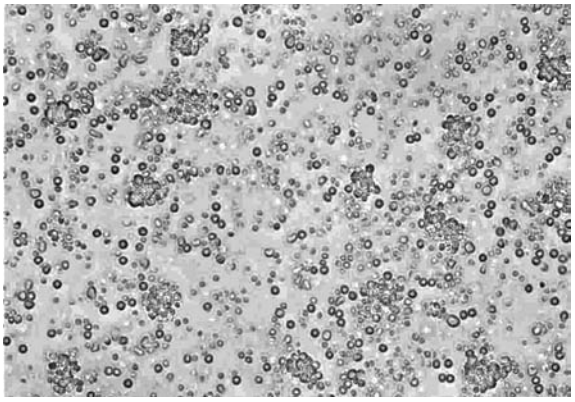
1. **CO_2 Incubator**- If you do not have access to a CO_2 incubator, you may consider using a CO_2 independent medium such as the one offered by Life Technologies for $32 (#18045-088).
2. **Laminar flow hood**- If you decide to use a fume hood, sterilize it using 10% bleach.
3. **Clinical Centrifuge (refrigerated)**- You may use a plain (unrefrigerated) clinical centrifuge that can accommodate 15mL conical tubes without a notable loss of cell viability. I've had the cells exposed to room temperature for a period of up to 1 hr. without any noticeable effects. **NOTE**: It does make it more difficult to make a single-cell suspension in the narrower 15mL conical tubes instead of the 50mL conical tubes.
4. **Inverted microscope**- You may buy a refurbished microscope from companies such as Angelus Medical & Optical Co. Inc. for about $950.
5. **Pipettor**- You may buy a manual version such as the one offered by Carolina for $20 #736872. **NOTE:** Students will have to be careful not to depress plunger with too much force since this may damage the cells or cause them to spray out of the conical tube when making the single-cell suspension.
6. **Micropipette**- plastic bulb pipettes
7. **Vacuum Pump**- A Faucet Aspirator Vacuum Pump will work just fine, but you must be close to a faucet (about 72cm). Amazon cells a Nalgene 6140-0010 Faucet Aspirator Vacuum Pump, Polypropylene, 3/8" NPT Threading for $14.
8. **Upright microscope**- You may buy a refurbished microscope from companies such as Angelus Medical & Optical Co. Inc. for about $350.
9. **Thermal Cycler**- Water baths or heat blocks set at three different temperatures (denaturation, annealing, and elongation) have been used instead, but they tend to be very time consuming.
10. **Water Bath**- I have seen some refurbished water baths on eBay for as low as $50.
11. **Serological pipette**- You may use glass pipettes instead of disposable plastic pipettes, but I have found that they are a lot of trouble to clean and sterilize.

DAILY UNIT PLANS

Thawing Procedure for Direct Cell Shipment from ATCC (Teacher)

1. This should be started at least **1 week** in advance in order to ensure that you have enough cells for all of your student workstations.
2. Under proper growth conditions, one week will render about three $75cm^2$ culture flasks with about 80% confluency. Each $75cm^2$ culture flask can then be subcultured into six $25cm^2$ culture flasks.
3. At least 30 minutes before you begin, turn on the 37°C water bath and place F-12K complete growth medium inside to warm it.
4. Sterilize your gloved hands by squirting 70% EtOH on them. Then, rub your gloved hands together as if you are washing them.
5. Once warm, take the F-12K complete bottle and squirt it with 70% EtOH. Then, place it inside the cell culture hood (laminar flow hood).
6. Prior to thawing cancer cells, prepare your cancer cell wash by labeling a 50mL conical tube "**RINSE**" inside the cell culture hood and pipette 10mL of F-12K complete medium into it using a 10mL serological pipette.
7. Thaw the cancer cell vial by gently agitating it in a 37°C water bath taking care not to submerse the O-ring and cap. This should take about 2 minutes.
8. Immediately remove the vial from the water bath as soon as the cells are partially thawed (once frozen cells are able to slide up and down the cryotube) and decontaminate the cryotube by quickly squirting it with 70% EtOH.
9. **NOTE:** All of the operations from this point on should be carried out under strict aseptic conditions.
10. Transfer the cells by inverting it into your 50ml **RINSE** tube containing 10ml of complete F-12K culture medium and spin @ 1,500 RPM, 4°C, for 5 minutes.
11. While the tubes are spinning, get 1 new $75cm^2$ culture flask and label it as "**I-10** and the **Date"** while inside the cell culture hood. For every cryotube, you will need 1 flask.
12. After the spin, the **RINSE** tube at this point can either be left in the centrifuge @ 4°C while you work or can be put back on ice. Be careful not to disturb the cell pellet while you are handling it!!! If you do, you must spin it down again for 5 minutes.
13. Squirt the outside of the **RINSE** tube with 70% EtOH to sterilize it, and then, place it in the cell culture hood. Be careful not to rub the marker label off the **RINSE** tube.
14. Using a sterile glass Pasteur pipette, aspirate the supernatant from the **RINSE** tube. Be careful not to suck up the cell pellet!!!
15. Cap the **RINSE** tube and gently tap it on the bottom with your index finger while you hold the top of the tube with the other hand in order to dislodge the cell pellet. Do this about 5 times.
16. Using a sterile 10mL serological pipette, pipette 10mL of new F-12K complete medium into the center of the **RINSE** tube in order to dislodge the cell pellet further.
17. Now, pipette up and down around five times in order to make a single-cell suspension. Each time you push the cells/F-12K medium out, angle the tip of your serological pipette on the side of the 50mL **RINSE** tube, so as to decrease the size of the pipette's opening. This will help break the pellet apart.
18. **NOTE:** You should not be able to see any visible clumps. These are easier to keep track of by looking through the serological pipette when it is filled.

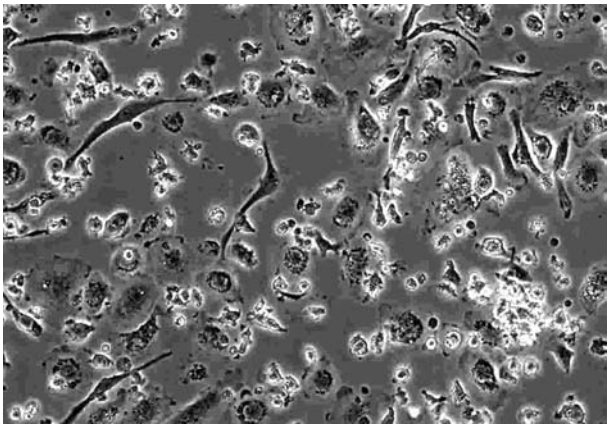
19. Once you have a single cell suspension, pipette 10mL of the **RINSE** tube into one culture flask.
20. Now using a new serological pipette, add 3mL of new F-12K complete medium to each flask in order to bring the volume up to a total of 13mL.
21. Take a look at the cell-filled culture flasks under the inverted microscope.
22. At this point all of the cells will be suspended in the medium and should look relatively round and dense. See below.



23. Now, say a short prayer, and carefully, place it in the CO_2 incubator.
24. Dispose of any used tubes or pipettes that have come into contact with the cells in the biohazard bin.
25. Wipe the floor of the cell culture hood with 70% EtOH to sterilize.
26. Make sure that there are no perishables such as cells or culture media still inside the cell culture hood.
27. Close the hatch, and turn on the UV light.

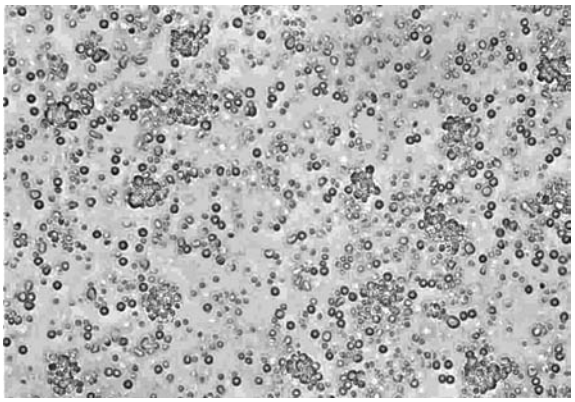
Subculturing Cells (Week 1, Days 1, 2, 4/Week 2, Days 1 & 4/Week 3, Day 1)

1. At least 30 minutes before you begin, turn on the 37°C water bath and place F-12K complete growth medium inside to warm it and the TE for thawing.
2. **NOTE:** You will need 5mL of TE for each culture flask.
3. Sterilize your gloved hands by squirting 70% EtOH on them. Then rub your gloved hands together as if you are washing them.
4. Obtain your cell culture flasks from the CO_2 incubator, and take a look at them under the inverted microscope. Healthy growing I-10 cells will have adhered to the bottom of the flask, and some should have a spindle shape. See below.



5. Estimate percent confluency by comparing the amount of space covered by the cells with the unoccupied spaces. The figure above is about 50% confluent. Once you have about 80% confluency and most cells are spindle-shaped, they need to be subcultured.
6. **NOTE:** In order to prevent contamination, never place the culture flask directly on the bench top. Instead, first place a clean paper towel underneath the flask.
7. Obtain a sterile 50 mL tube, and use the marker that is already in the cell culture hood to label it "**SUB**, **Station #and/or initials**" (one 50mL conical tube for each flask).
8. Now, unscrew the cap on the culture flask while inside the cell culture hood, and place the cap on the floor of the cell culture hood so that the open end faces up.
9. Using the supernatant from within the culture flask, rinse the "belly" of the flask by squirting it with the supernatant in a side to side motion. Do this about 5 times. This will help initiate the dislodging process.
10. **NOTE:** It is important not to let the cells sit dry (without growth medium) for too long otherwise their viability will be compromised.
11. Pipette loosened cells from flask into the corresponding **SUB** tube.
12. Place tube on ice.
13. **DO NOT** let the cells in the flask sit dry for too long!
14. Squirt the TE tube with 70% EtOH to sterilize it before placing it inside of the cell culture hood. Pipette 5 mL of TE into the 25cm2 flask (10 mL if using a 75cm^2 flask).
15. Carefully, place flask in CO_2 incubator for at least 3 minutes in order to dislodge cells from the flask. (Repeat for other flasks.)
16. Remove your TE/cells/flask from the CO_2 incubator and again pipette TE/cell solution from within the flask, and use it to rinse the "belly" of the flask by squirting it from side to side. Do this about 5 times.

17. Pipette the TE/cell solution from the flask into the corresponding **SUB** tube with cells that you had on ice. (Repeat for other flasks.)
18. Place **SUB** tubes in centrifuge and spin @ 1,500 rpm, 4°C, for 5 minutes.
19. While the tubes are spinning, get as many new culture flasks as you need and label them with "**Cell type, Station #,** and **Date** inside the cell culture hood. For every **SUB** tube, you will need 2 flasks.
20. After the spin, the **SUB** tubes at this point can either be left in the centrifuge @ 4°C while you work or can be put back on ice. Be careful not to disturb the cell pellet while you are handling it!!! If you do, you must spin it down again for 5 minutes.
21. Squirt the outside of the **SUB** tube with 70% EtOH to sterilize it, and then, place it in the cell culture hood.
22. Using a sterile glass Pasteur pipette, aspirate the supernatant from the **SUB** tube. Be careful not to suck up the cell pellet!!!
23. Cap the **SUB** tube, and gently tap it on the bottom with your index finger while you hold the top of the **SUB** tube with the other hand in order to dislodge the cell pellet. Do this about 5 times.
24. Using a sterile 10mL serological pipette, pipette 10mL of new F-12K complete medium into the center of the **SUB** tube in order to dislodge the cell pellet further.
25. Now, pipette up and down around five times in order to make a single-cell suspension. Each time you push the cells/F-12K medium out, angle the tip of your pipette on the side of the **SUB** tube so as to decrease the size of the serological pipette's opening. This will help break the pellet apart.
26. **NOTE:** You should not be able to see any visible clumps. These are easier to see through the serological pipette when it is filled.
27. Once you have a single cell suspension, pipette 5mL of the **SUB** tube into one culture flask and 5mL into the other culture flask.
28. Using a new serological pipette, add 2mL of F-12K complete medium to each flask in order to bring the volume up to a total of 7mL.
29. Take a look at the cell-filled culture flasks under the inverted microscope.
30. At this point, all of the cells will be suspended in the medium and should look relatively round and dense. See below.

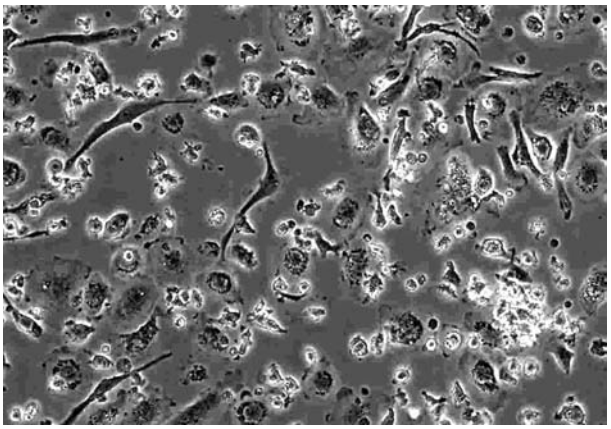


31. Now, say a short prayer, and carefully, place it in the CO_2 incubator. Culture flasks may be stacked.

32. Dispose of any used tubes, pipettes or flasks that have come into contact with the cells in the biohazard bin.
33. Wipe the floor of the cell culture hood with 70% EtOH to sterilize.
34. Make sure that there are no perishables such as cells or culture media still inside the cell culture hood.
35. Close the hatch and turn on the UV light.

Cryopreservation of Cancer Cells (Week 3, Day 1)

1. This procedure is for culture flasks that are at about 80% confluency. I generally do not let students go beyond 4 flasks.
2. At least 30 minutes before you begin, turn on the 37°C water bath and place F-12K complete growth medium inside to warm it and the TE for thawing.
3. **NOTE:** You will need 5mL of TE for each culture flask.
4. Sterilize your gloved hands by squirting 70% EtOH on them. Then, rub your gloved hands together as if you are washing them.
5. Obtain your cell culture flasks from the CO_2 incubator, and take a look at them under the inverted microscope. Healthy growing I-10 cells will have adhered to the bottom of the flask and some should have a spindle shape. See below.



6. Estimate percent confluency by comparing the amount of space covered by the cells with the unoccupied spaces. The figure above is about 50% confluent. Once you have about 80% confluency and most cells are spindle-shaped, they can be cryopreserved.
7. **NOTE:** In order to prevent contamination, never place the culture flask directly on the bench top. Instead, first place a clean paper towel underneath the flask.
8. Obtain a sterile 50 mL tube and use the marker that is already in the cell culture hood to label it "**FROZ**, **Station #and/or initials**" (one 50mL conical tube for each flask).
9. Now, unscrew the cap on the culture flask while inside the cell culture hood, and place the cap on the floor of the cell culture hood so that the open end faces up.
10. Using the supernatant from within the culture flask, rinse the "belly" of the flask by squirting it with the supernatant in a side to side motion. Do this about 5 times. This will help initiate the dislodging process.
11. **NOTE:** It is important not to let the cells sit dry (without growth medium) for too long otherwise their viability will be compromised.

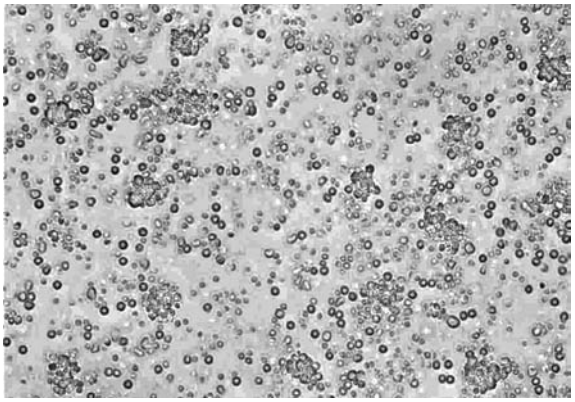
12. Pipette loosened cells from culture flask into the corresponding **FROZ** tube.
13. Place tube on ice.
14. **DO NOT** let the cells in the culture flask sit dry for too long!
15. Squirt the TE tube with 70% EtOH to sterilize it before placing it inside of the cell culture hood. Pipette 5 mL of TE into the 25cm2 flask (10 mL if using a 75cm^2 flask).
16. Carefully, place flask in CO_2 incubator for at least 3 minutes in order to dislodge cells from the flask. (Repeat for other flasks.)
17. Remove your TE/cells/flask from the CO_2 incubator and again pipette TE/cell solution from within the flask and use it to rinse the "belly" of the flask by squirting it from side to side. Do this about 5 times.
18. Pipette the TE/cell solution from the flask into the corresponding **FROZ** tube with cells that you had on ice. (Repeat for other flasks.)
19. Place **FROZ** tubes in centrifuge and spin @ 1,500 rpm, 4°C, for 5 minutes.
20. While the tubes are spinning, get as many new 2mL cryogenic tubes as you need and label them with "**Cell type, Station #,** and **Date** inside the cell culture hood. For every **FROZ** tube, you will need two 2mL cryogenic tubes.
21. At this time, you should also prepare the cryoprotectant medium by making a 5% DMSO/F-12K complete growth medium solution. You will need 2mL of cryoprotectant media for every 80% confluent 25cm^2 culture flask.
22. After the spin, the **FROZ** tubes at this point can either be left in the centrifuge @ 4°C while you work or can be put back on ice. Be careful not to disturb the cell pellet while you are handling it!!! If you do, you must spin it down again for 5 minutes.
23. Squirt the outside of the **FROZ** tube with 70% EtOH to sterilize it. Then, place it in the cell culture hood.
24. Using a sterile glass Pasteur pipette, aspirate the supernatant from the **FROZ** tube. Be careful not to suck up the cell pellet!!!
25. Cap the **FROZ** tube and gently tap it on the bottom with your index finger while you hold the top of the **FROZ** tube with the other hand in order to dislodge the cell pellet. Do this about 5 times.
26. Using a sterile 10mL serological pipette, pipette 2mL of cryoprotectant medium into the center of the **FROZ** tube in order to dislodge the cell pellet further.
27. Now, pipette up and down around five times in order to make a single-cell suspension. Each time you push the cells/cryoprotectant medium out, angle the tip of your pipette on the side of the **FROZ** tube, so as to decrease the size of the serological pipette's opening. This will help break the pellet apart.
28. **NOTE:** You should not be able to see any visible clumps. These are easier to see through the serological pipette when it is filled.
29. Once you have a single cell suspension, pipette 1mL of the **FROZ** tube into one 2mL cryogenic tube and 1mL into the other 2mL cryogenic tube.
30. Freeze them immediately at -20ºC for 2 hours. The DMSO is toxic to the cells, but it is necessary in order to prevent crystallization of the medium.
31. Dispose of any used tubes, pipettes or flasks that have come into contact with the cells in the biohazard bin.
32. Wipe the floor of the cell culture hood with 70% EtOH to sterilize.
33. Make sure that there are no perishables such as cells or culture media still inside the cell culture hood.

34. Close the hatch and turn on the UV light.
35. After 2 hours at -20ºC, transfer to -80ºC and let the cryogenic tubes sit overnight. They can be kept here for up to a week.
36. The next day, transfer it to liquid nitrogen for permanent storage.

Handling Procedures for Frozen Cells (Only if needed)

1. At least 30 minutes before you begin, turn on the 37°C water bath and place F-12K complete growth medium inside to warm it.
2. **NOTE:** Do not remove cells from storage until the water bath is at the proper temperature. The frozen cells should go from liquid nitrogen to the water bath.
3. Sterilize your gloved hands by squirting 70% EtOH on them. Then, rub your gloved hands together as if you are washing them.
4. Once warm, take the F-12K complete bottle and squirt it with 70% EtOH. Then, place it inside the cell culture hood (laminar flow hood).
5. Prior to thawing cancer cells, prepare your cancer cell wash by labeling a 50mL conical tube "**RINSE, Station #,** and/or **initials**" inside the cell culture hood and pipetting 10mL of F-12K complete medium into it using a 10mL serological pipette.
6. Thaw the cancer cells' vial by gently agitating them in a 37°C water bath taking care not to submerse the O-ring and cap. This should take about 2 minutes.
7. Immediately remove the vial from the water bath as soon as the cells are partially thawed (once frozen cells are able to slide up and down the cryogenic tube) and decontaminate the cryotube by quickly squirting it with 70% EtOH.
8. **NOTE:** All of the operations from this point on should be carried out under strict aseptic conditions.
9. Transfer the cells by inverting it into your 50ml **RINSE** tube containing 10ml of complete F-12K culture medium and spin @ 1,500 rpm, 4°C, for 5 minutes.
10. While the tubes are spinning, get 2 new 25cm^2 culture flasks and label them as "**Cell type, Station #,** and **Date** while inside the cell culture hood. For every cryotube, you will need 2 flasks.
11. After the spin, the **RINSE** tube at this point can either be left in the centrifuge @ 4°C while you work or can be put back on ice. Be careful not to disturb the cell pellet while you are handling it!!! If you do, you must spin it down again for 5 minutes.
12. Squirt the outside of the **RINSE** tube with 70% EtOH to sterilize it, and then, place it in the laminar flow hood. Be careful not to rub the marker label off the **RINSE** tube.
13. Using a sterile glass Pasteur pipette, aspirate the supernatant from the **RINSE** tube. Be careful not to suck up the cell pellet!!!
14. Cap the **RINSE** tube, and gently tap it on the bottom with your index finger while you hold the top of the tube with the other hand in order to dislodge the cell pellet. Do this about 5 times.
15. Using a sterile 10mL serological pipette, pipette 10mL of new F-12K complete medium into the center of the **RINSE** tube in order to dislodge the cell pellet further.
16. Now, pipette up and down around five times in order to make a single-cell suspension. Each time you push the cells/F-12K medium out, angle the tip of your serological pipette on the side of the 50mL tube so as to decrease the size of the pipette's opening. This will help break the pellet apart.

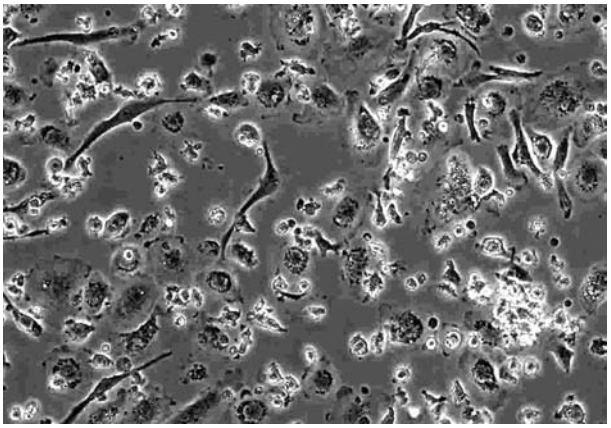
17. **NOTE:** You should not be able to see any visible clumps. These are easier to see through the serological pipette when it is filled.
18. Once you have a single cell suspension, pipette 5mL of the **RINSE** tube into one culture flask and 5mL into the other culture flask.
19. Using a new serological pipette, add 2mL of F-12K complete medium to each flask in order to bring the volume up to a total of 7mL.
20. Take a look at the cell-filled culture flasks under the inverted microscope.
21. At this point, all of the cells will be suspended in the medium and should look relatively round and dense. See below.



22. Now, say a short prayer, and carefully, place it in the CO_2 incubator. Culture flasks may be stacked.
23. Dispose of any used tubes or pipettes that have come into contact with the cells in the biohazard bin.
24. Wipe the floor of the cell culture hood with 70% EtOH to sterilize.
25. Make sure that there are no perishables such as cells or culture media still inside the cell culture hood.
26. Close the hatch and turn on the UV light.

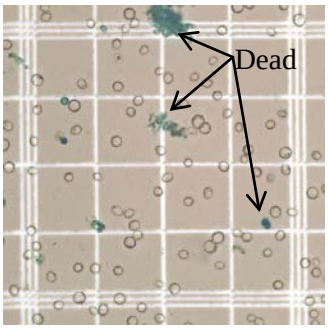
Cell Counting, Trypan Blue Exclusion (Week 2, Days 1 & 4)

1. At least 30 minutes before you begin, turn on the 37°C water bath and place F-12K complete growth medium inside to warm it and the TE for thawing.
2. **NOTE:** You will need 5mL of TE for each culture flask.
3. Sterilize your gloved hands by squirting 70% EtOH on them. Then, rub your gloved hands together as if you are washing them.
4. Obtain your cell culture flasks from the CO_2 incubator and take a look at them under the inverted microscope. Healthy growing I-10 cells will have adhered to the bottom of the flask and some should have a spindle shape. See below.



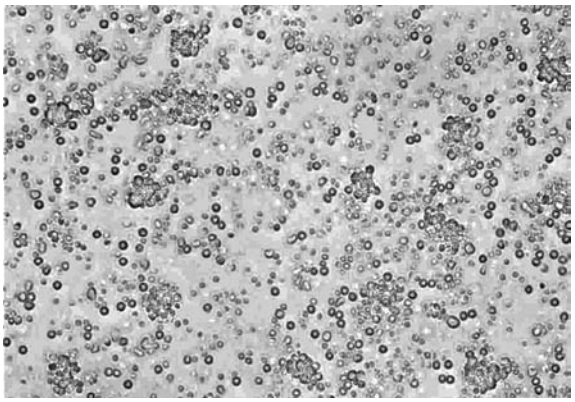
5. Estimate percent confluency by comparing the amount of space covered by the cells with the unoccupied spaces. The figure above is about 50% confluent. Once you have about 80% confluency and most cells are spindle-shaped, they need to be subcultuted.
6. **NOTE:** In order to prevent contamination, never place the culture flask directly on the bench top. Instead, first place a clean paper towel underneath the flask.
7. Obtain a sterile 50 mL tube and use the marker that is already in the cell culture hood to label it "**SUB**, **Station #and/or initials**" (one 50mL conical tube for each flask).
8. Now, unscrew the cap on the culture flask while inside the cell culture hood, and place the cap on the floor of the cell culture hood so that the open end faces up.
9. Using the supernatant from within the culture flask, rinse the "belly" of the flask by squirting it with the supernatant in a side to side motion. Do this about 5 times. This will help initiate the dislodging process.
10. **NOTE:** It is important not to let the cells sit dry (without growth medium) for too long otherwise their viability will be compromised.
11. Pipette loosened cells from flask into the corresponding **SUB** tube.
12. Place tube on ice.
13. **DO NOT** let the cells in the flask sit dry for too long!
14. Squirt the TE tube with 70% EtOH to sterilize it before placing it inside of the cell culture hood. Pipette 5 mL of TE into the 25cm2 flask (10 mL if using a $75cm^2$ flask).
15. Carefully, place flask in CO_2 incubator for at least 3 minutes in order to dislodge cells from the flask. (Repeat for other flasks.)
16. Remove your TE/cells/flask from the CO_2 incubator and again pipette TE/cell solution from within the flask and use it to rinse the "belly" of the flask by squirting it from side to side. Do this about 5 times.

17. Pipette the TE/cell solution from the flask into the corresponding **SUB** tube with cells that you had on ice. (Repeat for other flasks.)
18. Place **SUB** tubes in centrifuge and spin @ 1,500 rpm, 4°C, for 5 minutes.
19. While the tubes are spinning, get as many new culture flasks as you need and label them with "**Cell type, Station #,** and **Date** inside the cell culture hood. For every **SUB** tube, you will need 2 flasks.
20. After the spin, the **SUB** tubes at this point can either be left in the centrifuge @ 4°C while you work or can be put back on ice. Be careful not to disturb the cell pellet while you are handling it!!! If you do, you must spin it down again for 5 minutes.
21. Squirt the outside of the **SUB** tube with 70% EtOH to sterilize it, and then, place it in the cell culture hood.
22. Using a sterile glass Pasteur pipette, aspirate the supernatant from the **SUB** tube. Be careful not to suck up the cell pellet!!!
23. Cap the **SUB** tube and gently tap it on the bottom with your index finger while you hold the top of the **SUB** tube with the other hand in order to dislodge the cell pellet. Do this about 5 times.
24. Using a sterile 10mL serological pipette, pipette 10mL of new F-12K complete medium into the center of the **SUB** tube in order to dislodge the cell pellet further.
25. Now, pipette up and down around five times in order to make a single-cell suspension. Each time you push the cells/F-12K medium out, angle the tip of your pipette on the side of the **SUB** tube so as to decrease the size of the serological pipette's opening. This will help break the pellet apart.
26. **NOTE:** You should not be able to see any visible clumps. These are easier to see through the serological pipette when it is filled.
27. Once you have a single cell suspension, use a micropipette with a sterile tip to mix 180µL of Trypan Blue with 20µL of the single-cell suspension in a 1.5mL microtube.
28. Place the **SUB** tube on ice.
29. Place a small amount on hemocytometer (according to manufacturer's directions), enough so that it does not bulge out beyond the cover slip.
30. Load the hemocytometer onto the microscope stage and count the cells (clear) in all the squares between the triple lines that are not touching the lines. Again, this procedure depends on the type of hemocytometer you purchased. (You must count 4 sets of these.)
31. Live cells exclude trypan blue while dead cells cannot and therefore stain blue.



32. Calculate as follows:
    Total # cells counted ÷ 4 (4 sets) x 10^4 (constant) x 10 (diluted 10 times with Trypan Blue)
    e.g. 616/4 x 10^4 x 10 = 1.54 x 10^7 cells/ml

33. Keep track of your cell count using a Lab Counter App.
34. Pipette 5mL of the **SUB** tube into one culture flask and 5mL into the other culture flask.
35. Now, using a new serological pipette, add 2mL of F-12K complete medium to each flask in order to bring the volume up to a total of 7mL.
36. Take a look at the cell-filled culture flasks under the inverted microscope.
37. At this point, all of the cells will be suspended in the medium and should look relatively round and dense. See below.



38. Now, say a short prayer and carefully place it in the CO_2 incubator. Culture flasks may be stacked.
39. Dispose of any used tubes, pipettes or flasks that have come into contact with the cells in the biohazard bin.
40. Wipe the floor of the cell culture hood with 70% EtOH to sterilize.
41. Make sure that there are no perishables such as cells or culture media still inside the cell culture hood.
42. Close the hatch and turn on the UV light.

RNA Purification and cDNA Amplification (Week 2, Days 1 & 2)

1. For RNA purification of I-10 cells, follow handbook included with QIAGEN's RNeasy Mini Kit.
2. For cDNA amplification of I-10 cell RNA, follow handbook included with QIAGEN's OneStep RT-PCR Kit.

<u>Cast Agarose Gel</u>

3. Make a 0.8% agarose gel as described in "Advance Preparation."
4. Seal ends of gel-casting tray, and insert well-forming comb.
5. Place gel-casting tray out of the way on lab bench so that agarose poured in the next step can set undisturbed.
6. Carefully, pour enough agarose solution into casting tray to fill to a depth of about 3/4 the height of comb teeth. While gel is still liquid, a pipette tip can be used to move large bubbles or solid debris to sides or end of tray away from the comb.
7. Gel will become cloudy as it solidifies (about 10 minutes). Do not move or jar casting tray while agarose is solidifying.
8. When agarose has set, unseal ends of casting tray and place tray on platform of gel box so that comb is at negative (black) end.
9. Fill box with 0.5X TBE buffer to a level that just covers entire surface of gel.

10. Carefully, pull comb straight up without wiggling.
11. If you notice "dimples" from the gel in the buffer, slowly add more buffer until they are completely submerged.

Load Gel

12. Add 1µL of loading dye to your PCR reaction and mix by pipetting up and down.
13. Load a 1kbp DNA Ladder into the first well and then 20µL of your PCR reaction into each well after.
14. Draw a gel map that shows which wells were loaded with what sample.
15. Connect to the power supply and set it for 150V for 30 minutes.
16. Unplug chamber and view gel on Transilluminator.

Hematoxylin & Eosin Staining (Week 1, Day 3)

1. Tissue samples were obtained, embedded in paraffin wax, and sliced at the Cleveland Clinic.
2. Place slides containing paraffin sections in a slide holder (glass or metal).
3. The following steps should be completed under a fume hood.
4. Deparaffinize and rehydrate sections as follows:
    a. 3 x 3 min. 100% Xylene
    b. 3 x 3 min. 100% Ethanol
    c. 1 x 3 min. 95% Ethanol
    d. 1 x 3 min. 80% Ethanol
    e. 1 x 5 min. dH_2O
5. Hematoxylin staining:
    a. 1 x 3 min. Hematoxylin
    b. Rinse dH_2O
    c. 1 x 5 min. tap H_2O
    d. Dip 8-12x (fast) Acid Ethanol (to destain)
    e. Rinse 2 x 1 min. tap H_2O
    f. Rinse 1 x 2 min. dH_2O (can leave overnight at this stage)
6. Blot excess water from the slide holder before going into Eosin.
7. Eosin staining and dehydration:
    a. 1 x 30s. Eosin
    b. 3 x 5 min. 95% Ethanol
    c. 3 x 5 min. 100% Ethanol
    d. 3 x 15 min. Xylene
8. You can leave slides in xylene overnight to get good clearing of any water.
9. Coverslip slides using Permount (xylene based).
    a. Place a drop of Permount on the slide using a micropipette, taking care not to leave any bubbles directly over the tissue.
    b. Angle the coverslip, and let it fall gently onto the slide.
    c. Allow the Permount to spread beneath the coverslip, covering all the tissue.
    d. Dry overnight in the hood.
    e. In case any of the Permount spills over the slide, it's a good idea to set the finished slide on a plastic platform for drying and not a paper towel (pipette tips box works well). Otherwise, the paper towel will be permanently glued to the slide.

Immunohistochemistry (Week 2, Days 2 & 3)

Rehydrate Paraffin Embedded Tissue (30 min.)

All steps should be completed in a Coplin Jar on gentle shaker within a fume hood. Some of these steps will be completed using the ImmunoCruz Staining System.

1) Xylene 2 x 5 min @ R.T.
2) 100% EtOH 1 x 5 min @ R.T.
3) 95% EtOH 1 x 5 min @ R.T.
4) 70% EtOH 1 x 5 min @ R.T.
5) PBS 1 x 5 min @ R.T.

Unmask Antigens (2 hrs. 15 min.)

6) Place slides inside open plastic container and pipette 10 mM NaCHO buffer, pH 6.0 inside until the slides are covered, and then, steam for 1 hr. Fill steamer with dH_2O through side spout and turn the dial to 60 minutes **(can leave overnight)**.
7) Once the time is up (rings), leave for an extra 20 minutes in order to cool.
8) Close plastic container lid and then leave it inside the steamer for an extra 45 minutes.
9) Alternatively, 10 mM NaCHO buffer, pH 6.0 may be brought to a boil in the microwave and then the slides placed inside the hot buffer for 5 minutes. Let it cool for 20 minutes.
10) Wash 1 x with PBS for 5 minutes.

Humidified Chamber (1hr. 15 min)

11) Incubate slides at R.T. in 1% H_2O_2/5% Triton/PBS solution for 30 minutes.
12) Wash 2 x with PBS for 5 minutes.
13) To quench endogenous peroxidase activity, incubate specimens for 5 minutes in 1-3 drops of peroxidase block (white cap). Rinse with PBS and transfer to a PBS wash for 2 minutes on stir plate. Aspirate excess liquid from slides.
14) Incubate specimens for 20 minutes in 1-3 drops of serum block (blue cap). Aspirate serum from slides.
15) Dilute primary antibody to 1:500 (Rat anti-Human CD3) in serum block (blue cap).
16) Incubate overnight @ 4°C.
17) Wash 2 x with PBS for 5 minutes.
18) Dilute biotinylated secondary antibody to 1:1,000 (Goat anti-Rat) in serum block (blue cap).
19) Incubate specimens for 1 hour @ R.T.
20) Wash 2 x with PBS for 5 minutes.
21) Incubate specimens for 30 minutes in 1–3 drops of HRP-streptavidin complex (purple cap). Rinse with PBS. Then, wash in PBS twice for 2 minutes each on stir plate. Aspirate excess liquid from slides.
22) During the above incubation step, prepare HRP substrate in the substrate mixing bottle (yellow cap) as follows (sufficient for 15-20 slides): remove tip from mixing bottle and combine 1.6 ml deionized H_2O, 5 drops 10x substrate buffer (orange cap), 1 drop 50x DAB chromogen (yellow cap) and 1 drop 50x peroxidase substrate (yellow cap).
23) Add 1-3 drops of HRP substrate to each slide. Develop until light brown staining is visible, usually 30 seconds– 10 minutes, although up to 20 minutes may be required. The section may be checked for staining by rinsing with dH_2O and viewed under a microscope. If necessary, add additional HRP substrate and continue to incubate. Rinse with dH_2O and transfer to deionized H_2O wash for 2 minutes on stir plate.

24) Counterstain slides in Gill's formulation #2 hematoxylin for 5-10 seconds. Immediately, wash with several changes of deionized H_2O.
25) Dehydrate sections as follows: 2x 70% ethanol for 10 seconds each, 2x 95% ethanol for 10 seconds each, 2x 100% ethanol for 10 seconds each and 3x xylenes for 10 seconds each. Wipe off excess xylene from edges and bottom of slide.
26) Immediately, add 1–2 drops of permanent mounting medium directly on the tissue and cover with glass coverslip.
27) **DO NOT LET XYLENE DRY OR THE TISSUE WILL CRACK.**
28) Observe by light microscopy.

AP Biology Lab Practical

Lab Basics Version A

Directions:

- You may NOT help each other with the questions, only the lab experiment.
- You are allowed to work with your lab partner, but you may not help or seek help from any other group.
- Remember time is your biggest enemy, so make sure you divide all tasks equally.
- You may not ask me what to do next or how to do it; however, you may ask me where something is.
- DO NOT write on this lab practical, use the answer sheet.

Hands-on:

1) Complete the "Small-volume Micropipettor Exercise" on p. 327 of the "Measurements, Micropipetting, and Sterile Techniques" lab. (Make sure you share these tasks equally.
2) Please call me over when you are about to begin step 8.
3) Obtain a piece of crab meat and perform a DNA extraction.
4) Place your purified crab DNA in the designated microtube rack.

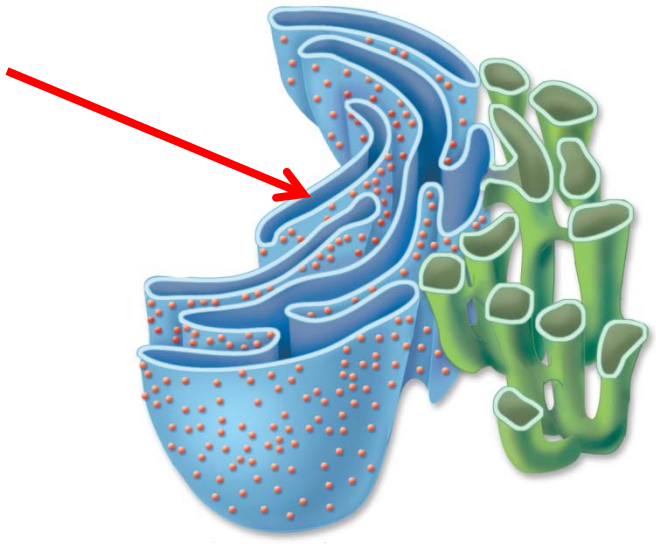
Answer the following questions by yourself:

Mystery Organelle

1) What is the name of this organelle in blue?

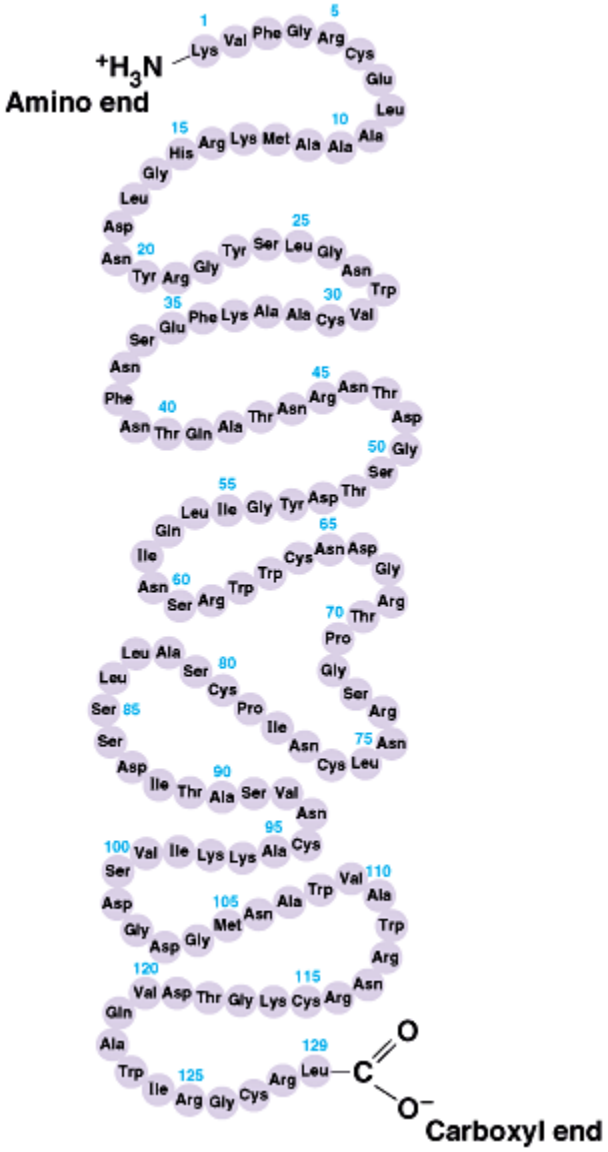
2) What is the membrane's structure (monomer)?

3) What is its primary function?

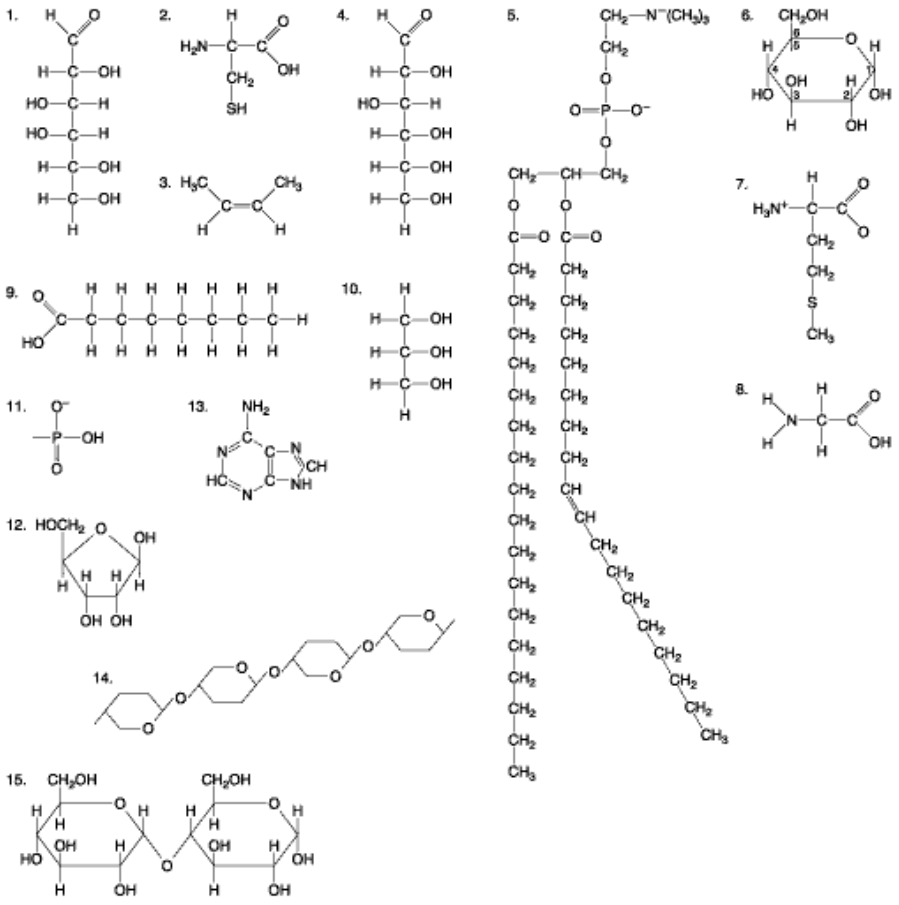


A protein is being translated.

4) In what part of the cell does translation first begin? Be specific!
5) If the protein is an antibody, what kind of a cell is producing it?
6) If an organism was running a high fever and the antibodies became denatured, what can be used in order to refold the protein correctly?
7) What level of protein structure is represented by this sequence below?



Molecules



8) Which molecule above is the monomer of the organelle in the previous question (#2)? Write down its number.
9) Write down all the numbers of the molecules above which are classified as carbohydrates.

AP Biology Lab Practical

Lab Basics Version B

Directions:

- You may NOT help each other with the questions, only the lab experiment.
- You are allowed to work with your lab partner, but you may not help or seek help from any other group.
- Remember time is your biggest enemy, so make sure you divide all tasks equally.
- You may not ask me what to do next or how to do it; however, you may ask me where something is.
- DO NOT write on this lab practical, use the answer sheet.

Hands-on:

5) Complete the "Small-volume Micropipettor Exercise" on p. 327 of the "Measurements, Micropipetting, and Sterile Techniques" lab. (Make sure you share these tasks equally.)
6) Please call me over when you are about to begin step 8.
7) Obtain a piece of crab meat and perform a DNA extraction.
8) Place your purified crab DNA in the designated microtube rack.

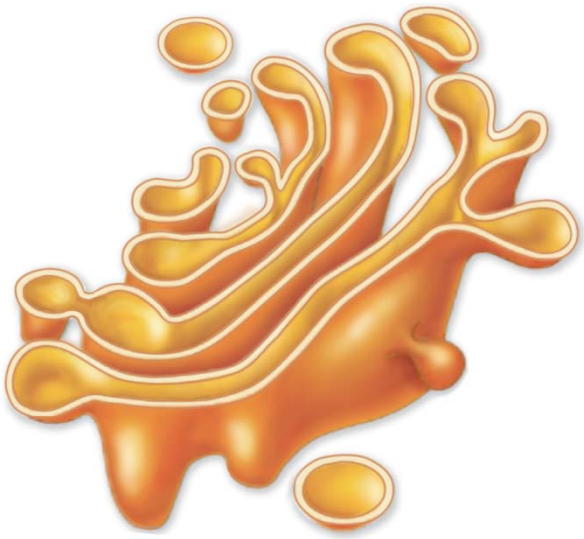
Answer the following questions by yourself:

Mystery Organelle

10) What is the name of this organelle?

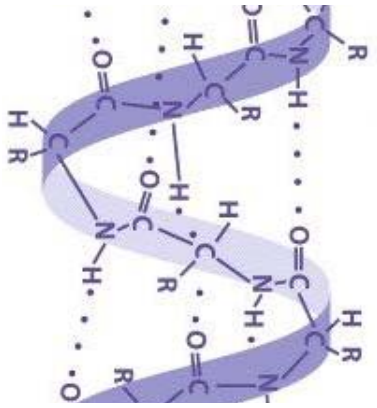
11) What is the membrane's structure (monomer)?

12) What is its primary function?

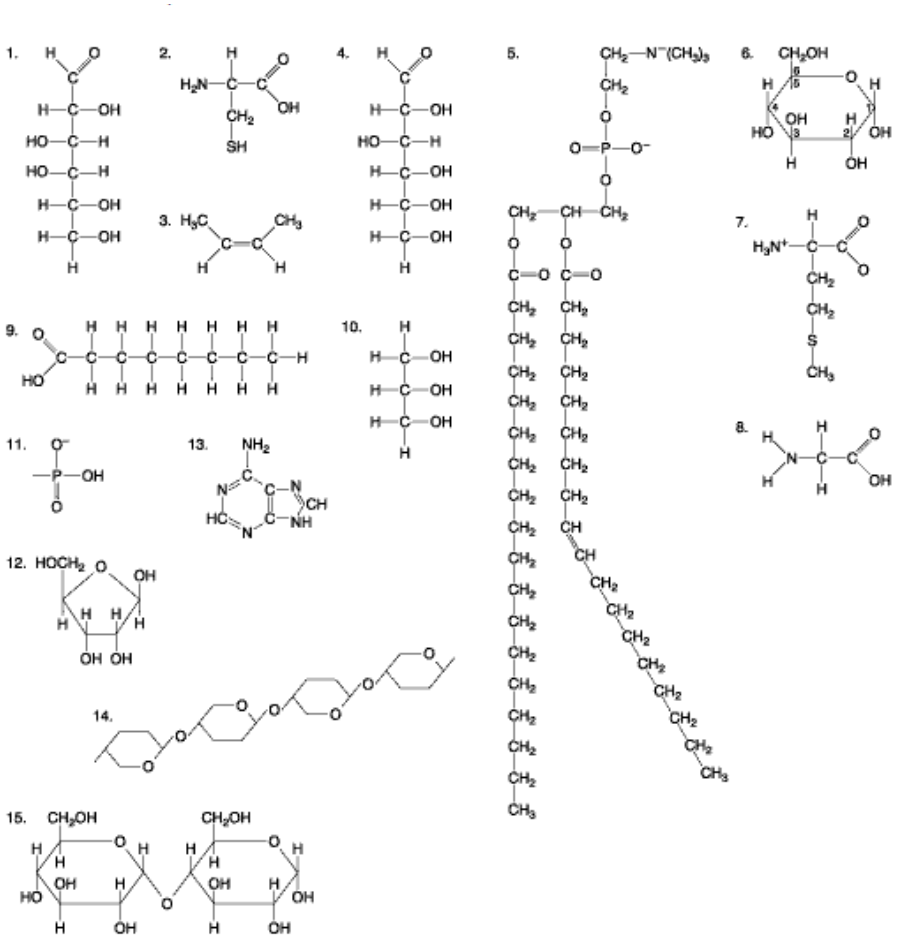


A protein is being translated.

13) In what part of the cell does translation first begin? Be specific!
14) If the protein is hemoglobin, what kind of a cell is producing it?
15) If a protein needed to be modified with a lipid and the lipid was being built, which organelle would the protein travel to?
16) What level of protein structure is represented by this sequence below?



Molecules



17) Which molecule above is the monomer of the organelle in the previous question (#2)? Write down its number.

18) Write down all the numbers of the molecules above which are classified as amino acids.

AP Biology Lab Practical

Cell Culture Techniques

- You may NOT help each other with the questions, only the lab experiment.
- You are allowed to work with your lab partner, but you may not help or seek help from any other group.
- Remember time is your biggest enemy, so make sure you divide all tasks equally.
- You may not ask me what to do next or how to do it; however, you may ask me where something is.
- DO NOT write on this lab practical, use the answer sheet.

Hands-on:

1) Remove two of your cancer cell flasks from the CO_2 incubator, and prepare one for counting and DNA extraction. The other flask will be subcultured.
2) Call me over once you have counted one grid square of cells so that I may verify your count. **NOTE:** Each student will prepare and perform a count.
3) Perform a DNA extraction on these cells, and then, verify your DNA extraction was successful by using the appropriate primers.
4) Do not forget to subculture the other flask. I will be grading your technique.

Answer the following questions by yourself:

1) What process do we use in order to count cells under the microscope? Briefly describe how it works. **NOTE:** Make sure you incorporate what you've learned about transport of materials across the membrane.

2) Which process do these cancer cells use in order to divide? Pick one phase and briefly describe it.

3) If you wanted to draw fluid out of these cancer cells through their membrane, what household chemical could you add? Explain why this would draw the fluid out of the cells.

4) What is CD3? Examine the prepared slide of the tumor infiltrating CD3+ T cells. Briefly describe how the immunoperoxidase stain is able to bind CD3+ cells specifically. Why did we use CD3 as a marker?