WORKSHOP:

Cancer Investigators

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Introduction

Cancer contributes to almost one in every four deaths in the United States. Fortunately, innovations in biomedical research have improved our understanding of the differences between normal and cancer cells. In this hands-on workshop, participants use microscopy and electrophoresis to explore the hallmarks of cancer.

Background Information

THE ROLE OF GENES IN CANCER

Familial cancers constitute a very small fraction of the total reported cancers and occur in dominant inherited patterns. Mutations that are directly inherited are referred to as germline mutations. Such mutations can be detected in familial pedigrees. A second type of mutation, known as somatic mutations, do not have direct genetic links and are acquired during the life of the individual. Patterns of typical hereditary and sporadically acquired nonhereditary pedigrees appear in Figure 1.

Tumor suppressor genes in most individuals will require two sequential mutations, one per allele, to initiate tumors. This model is referred to as the "Two-hit" hypothesis. However, in an individual with an inherited mutation to one allele a single somatic mutation can result in the inactivation of the remaining healthy allele. Historically, some of the first familial cancer genes identified include the retinoblastoma (RB) gene, Wilm's' tumor (WTI), neurofibromatosis type II gene and p53.

In recent years, the p53 tumor suppressor protein has become the center of many cancer biology studies. As an essential tumor suppressor there is great impetus to study how this gene functions in normal cells compared to cancer cells. The gene for the p53 protein is located on the short arm of chromosome 17. It encodes a normal 53,000 molecular weight nuclear phosphoprotein. Wild type p53 functions as a cell regulator. There is now well-documented evidence that normal p53 is a sequence-specific DNA-binding protein that is a transcriptional regulator. Upon introduction of mutations, p53 loses its ability to bind to DNA. By contrast, mutated versions of p53 can actually promote uncontrolled cell growth and therefore function as oncogenes. For a tumor suppressor gene such as p53 to play a role in transformation in cancer, both alleles need to be altered, as shown in Figure 1.

LI-FRAUMENI SYNDROME

Li-Fraumeni syndrome (LFS) typically affects young family individuals and results in high mortality rates. Two physicians, Li and Fraumeni first described the syndrome after examining death certificates of 648 childhood sarcomas. It was discovered in four families where siblings and cousins had childhood sarcomas. Further analysis showed more than 50% of the affected families had extended phenotypes that included brain cancer, breast cancer, and leukemia. Cells from individuals with LFS have only a single wild type p53 allele. Examination of their p53 genes has shown correlations of the cancers to mutations in "hotspot" regions. These hotspot regions represent locations in the gene that are routinely mutated in cancer patients, and are a major focus for cancer researchers.

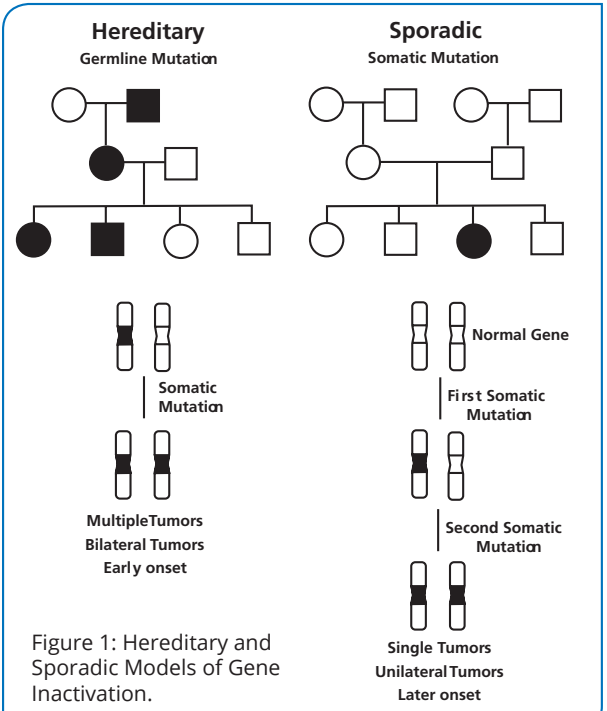
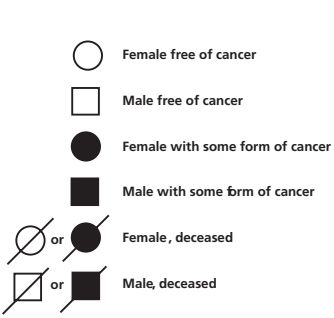
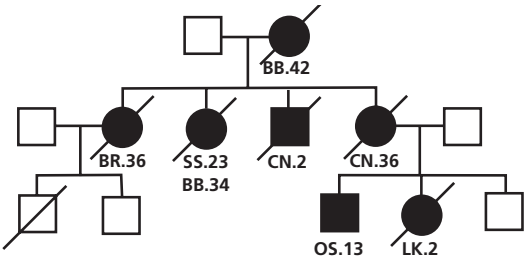


Figure 1: Hereditary and Sporadic Models of Gene Inactivation.





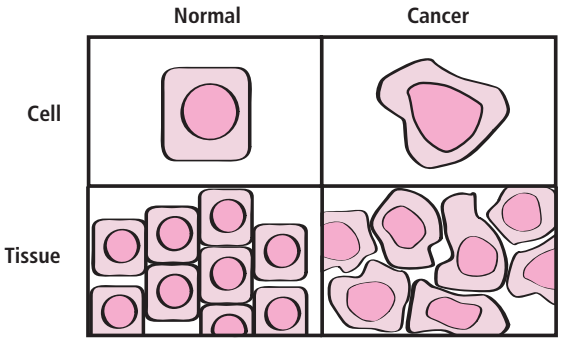
BB	Bilateral breast cancer
BR	Breast cancer
CN	Brain tumor
LK	Leukemia
OS	Osteosar coma
SS	Soft tissue sar coma

Figure 2: Example of a Li-Fraumeni family pedigree.

CANCER CELL MORPHOLOGY AND ANALYSIS

Along with genomic alterations and accelerated growth, physical characteristics can be used to classify cancer cells. The nuclear structure of cancer cells undergoes changes that result in a large, irregularly shaped nucleus, and modifications to the chromosomes. These morphological characteristics have been considered the "gold-standard" for diagnosing cancer. In general, normal cells have a regular and ellipsoid shape while cancer cells are often irregular and contoured. Decreased adherence in cancer cells can lead to disorganized cell spreading and fewer cell-cell contacts, as well as an overall chaotic appearance to the cell population. In contrast, normal cells will grow as a uniform layer of cells with many tight connections between neighboring cells. Structural changes to nuclear lamina proteins, which provide mechanical support to cells, can affect chromatin organization and alter gene expression. Cancer cells also often feature changes to cell structures. For example, the size of the endoplasmic reticulum and mitochondria often decreases, the Golgi apparatus is underdeveloped, and the number of peroxisomes increases (Figure 3).

For this activity, students will rehydrate and stain pre-fixed cells provided on a glass slide. They will observe and analyze the morphological differences between normal cells and cancer cells using a compound microscope. Upon completion, the students will be able to describe the physical differences between normal and cancer cells and will understand the functional significance of these structural changes.



Changes in Cancer Cells

- Loss of normal cell structures
- Changes in cell shape
- Changes in nucleus

- Large number of dividing cells
- Loss of monolayer
- Decreased cell-cell junctions
- Disorganized arrangement of cells

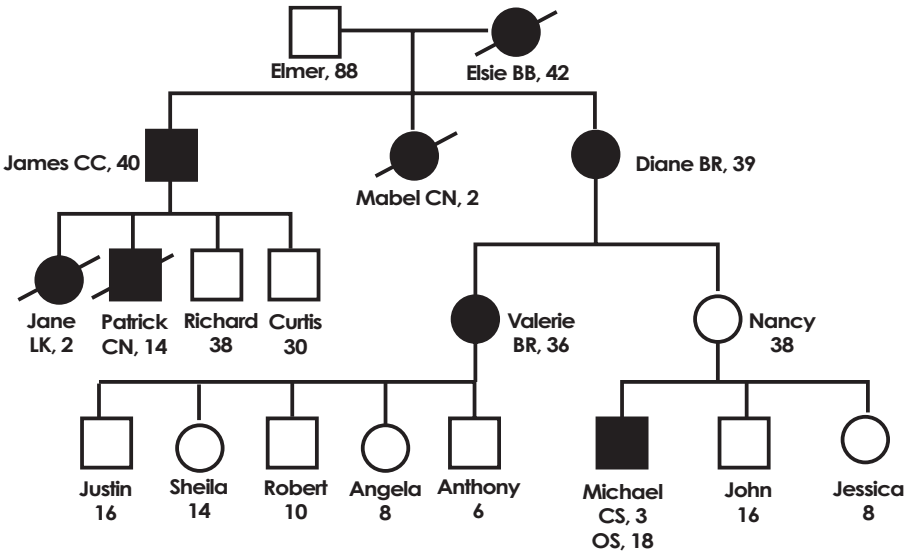
Figure 3: Distinguishing characteristics of normal and cancer cells.

In this workshop you will construct a pedigree for a family that is suspected to suffer from Li-Fraumeni syndrome. DNA samples which have been enzymatically digested will be separated by electrophoresis on an agarose gel as an independent diagnostic test. Next, slides containing prepared normal and cancer cells will be analyzed for morphology. Finally, DNA sequencing autorads simulating p53 hot spot sequences will be examined and mutations will be identified.

Module I: Construction a Family Pedigree

A first step in the search and diagnosis of Li-Fraumeni syndrome is to establish the family pedigree of the patient. The first part of the experiment is based on the information made available as part of a diagnosis by a family physician and an oncologist. The pedigree information that your students will develop is for a young woman who is suspected to have the Li-Fraumeni syndrome.

NOTE – for this workshop, the pedigree has been filled in advance.

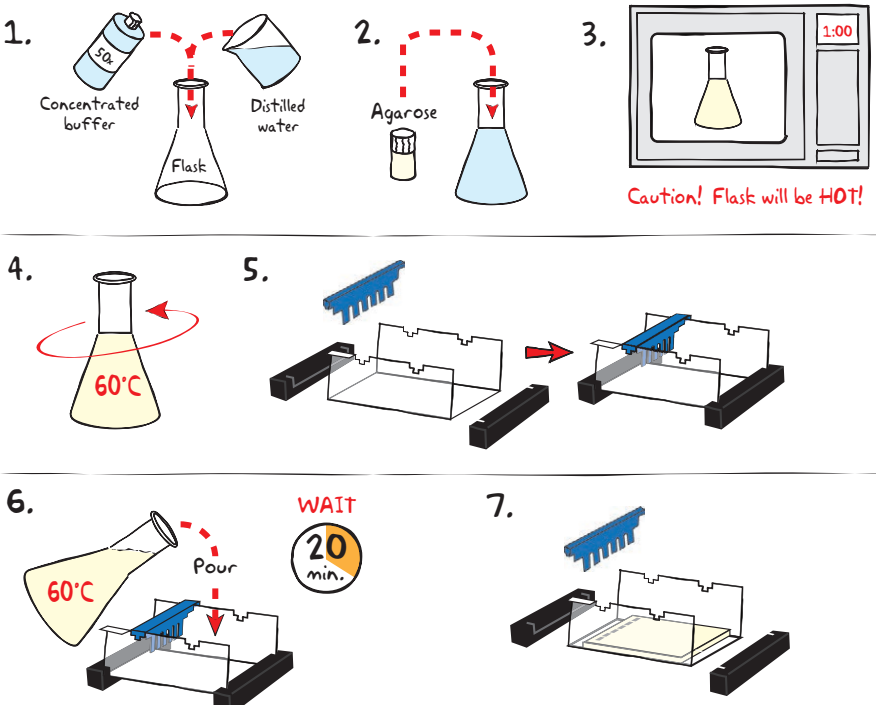


Module II: Agarose Gel Electrophoresis

The familial pedigree in Module I strongly suggests Li-Fraumeni syndrome. In such a case, a secondary diagnostic test is normally conducted. In this scenario, Valerie provides a sample of blood and tumor biopsy tissue to conduct DNA analysis on the p53 gene. Normally the procedure is to amplify the gene using polymerase chain reaction. This is followed by one of several methods to detect the presence of a point mutation at the hot spots.

In this simulated experiment, Valerie's DNA has already been digested with a restriction enzyme that recognizes the mutant sequence at the simulated hot spot site at nucleotide 165. In addition to Valerie's tumor and blood samples, the clinic has also isolated DNA from a normal control sample and histologically "normal" breast tissue. The predigested DNA samples with the control wild type and DNA markers will be separated by agarose gel electrophoresis, stained, and then analyzed.

Module II: Agarose Gel Electrophoresis



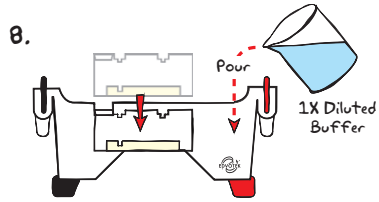
CASTING THE AGAROSE GEL

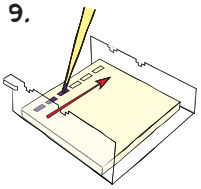
1. **DILUTE** concentrated 50X Electrophoresis buffer with distilled water (See Table A).
2. **MIX** agarose powder with 1X buffer in a 250 mL flask (refer to Table B, page 8).
3. **DISSOLVE** agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).
4. **COOL** agarose to 60° C with careful swirling to promote even dissipation of heat.
5. While agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the well template (comb) in the appropriate notch.
6. **POUR** the cooled agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.
7. **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

Table A — 1x Electrophoresis Buffer (Chamber Buffer)

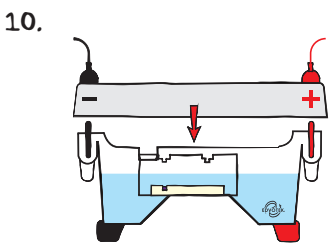
EDVOTEK Model #	Total Volume Required	Dilution: 50x Conc. Buffer	+ Distilled Water
M6+	300 ml	6 ml	294 ml
M12	400 ml	8 ml	392 ml
M36	1000 ml	20 ml	980 ml

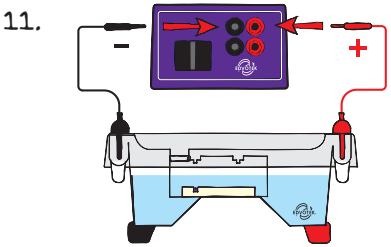
Module II: Agarose Gel Electrophoresis





REMINDER: Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.





RUNNING THE GEL

8. **PLACE** the gel (still on the tray) into the electrophoresis chamber. **COVER** the gel with 1X Electrophoresis Buffer (See Table A for recommended volumes). The gel should be completely submerged.
9. **LOAD** the entire sample (30 µL) into the well in the order indicated by the Table, below.
10. **PLACE** safety cover on the unit. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.
11. **CONNECT** leads to the power source and **PERFORM** electrophoresis (See Table C for time and voltage guidelines).
12. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber and proceed to instructions for **STAINING** the agarose gel.

Table 1: Gel Loading

Lane		
1	Tube A	DNA Standard Marker
2	Tube B	Control DNA
3	Tube C	Patient Peripheral Blood DNA
4	Tube D	Patient Breast Tumor DNA
5	Tube E	Patient Normal Breast Tissue DNA

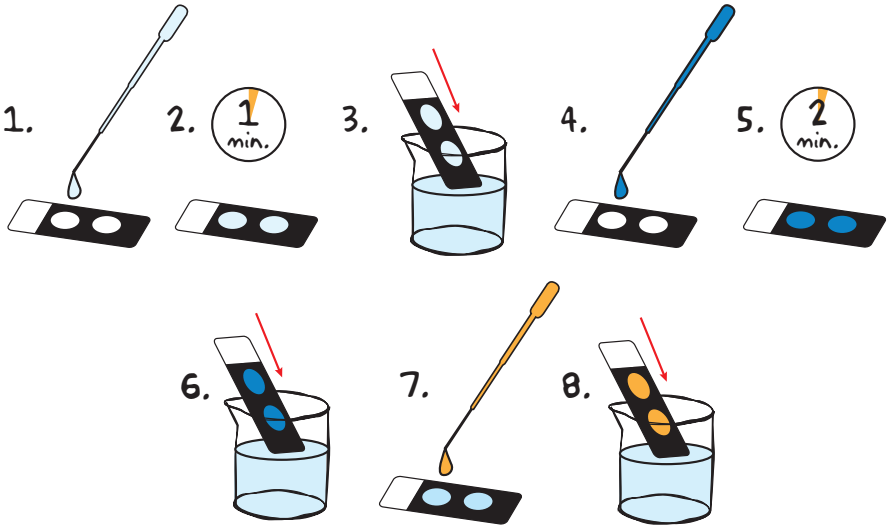
Table B Individual 2.0% UltraSpec-Agarose™ Gel

Size of Gel Casting tray	1X Diluted Buffer	+	Amt of Agarose
7 x 7 cm	25 ml		0.50 g
7 x 14 cm	50 ml		1.0 g

Table C Time and Voltage Guidelines (2.0% Agarose Gels)

Volts	Time: 7 x 7 cm gel ~4.0 cm migration	Time: 7 x 14 cm gel ~6.5 cm migration
125	30 min.	60 min.
70	60 min.	120 min.
50	90 min.	150 min.

Module III: Staining the Pre-fixed Cells



NOTE: Before beginning the experiment, ensure that the slide is facing upright.


Wear gloves and safety goggles

1. Using a transfer pipet, **COVER** each well with rehydration buffer.
2. **INCUBATE** the slide for 1 minute at room temperature.
3. **RINSE** the slide briefly by submerging in the beaker of distilled water. Gently tap the slide on a paper towel to remove excess water.
4. Using a fresh transfer pipet, **COVER** each well with methylene blue stain.
5. **INCUBATE** the slide for 2 minutes at room temperature.
6. **RINSE** the slide briefly by submerging in the beaker of distilled water. Gently tap the slide on a paper towel to remove excess water.
7. Using a fresh transfer pipet, **COVER** each well with eosin stain.
8. Immediately **RINSE** the slide by submerging in the beaker of distilled water. If residual stain remains, change water and repeat until the water no longer turns orange. Gently tap the slide on a paper towel to remove excess water. **PROCEED** to Module IV: Microscopic Observation.

OPTIONAL STOPPING POINT:
At this point, the stained slides can be stored at room temperature.

Module IV: Microscopic Observation

1. **LOCATE** cells in well #1 (see diagram, below) using the lowest magnification objective. Adjust the slide to find a random field of nicely stained cells that contains at least 15-20 cells.
2. **DESCRIBE** the overall morphology of the cells in the table on the following page. Features you might record include the number of cells, overall cell morphology, shape and size of the nucleus, and the intensity or color of staining.
3. Using the space provided, **DRAW** an image of the field of cells that you observe.
4. **MOVE** the slide and observe a second field of cells. Are your observations from the first area consistent with the second field?
5. **SWITCH** to a higher magnification and record your observations as in steps 2-3.
6. **CHANGE** the microscope back to the lower magnification objective and repeat steps 1-5 for the second cell type in well #2.
7. Based on your observations, **CLASSIFY** each cell type as normal or cancerous.

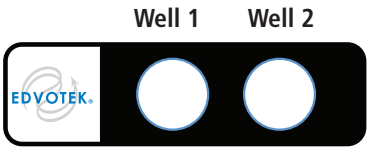


Diagram of ready-to-stain slide.

Module V: Analysis of Autorads to Search for p53 Mutagens

In this part of the experiment, x-ray results of the wild p53 and samples from Valerie's five children will be read to determine whether or not there are mutations.

Valerie's children	Ages	Autorad #
Justin	16	1
Sheila	14	2
Robert	10	3
Angela	8	4
Anthony	6	5

1. For each of Valerie's children, obtain the appropriate sample autoradiograph and place it on a light box to enhance visualization.
2. The sequencing reactions have all been loaded in order: G-A-T-C.
3. Begin analysis of the DNA sequence at the bottom of the autoradiograph with the circled band, which is an A.
4. Compare the deduced sequence to the wild type sequence shown in the box below.
5. Identify the location of the potential mutant nucleotide. What was the mutation? Is there more than one mutation?

Wild Type Sequence:

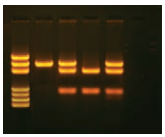
5'-AGCTTGGCTGCAGGTCGACGGATCCCCAGGAATTGTAAT-3'

6. Based on the information obtained from the x-rays, which of Valerie's children have a mutation in their DNA sequence?

NOTE: This is a simulation and the DNA sequence is not that of p53. The principles of reading DNA sequences and finding the point mutation is the same.

Experimental Results and Analysis

MODULE II - AGAROSE GEL ELECTROPHORESIS



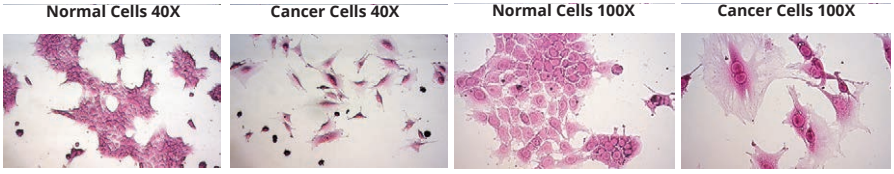
DNA obtained from tissue culture and normal patient cells (without an inherited p53 mutation in the PCR amplified DNA product) will not have the restriction enzyme site and therefore the amplified DNA will not be cut and will appear as one band (lane 2). The patient DNA from the tumor sample has a mutation in both p53 genes at this particular site and therefore the tumor PCR amplified DNA will be cut to yield two DNA fragments (Lane 4). The peripheral blood and non-tumor patient DNA will have the same pattern (lanes 3 and 5) where there are three bands due to the combined DNA patterns from the normal gene (lane 2) and the mutant gene (lane 4).

Lane	Tube	Sample	Molecular Weights (in bp)
1	A	DNA Standard Maker	---------
2	B	Control DNA	4282
3	C	Patient Peripheral Blood DNA	4282 3000 1282
4	D	Patient Tumor DNA	3000 1282
5	E	Patient Breast Normal DNA	4282 3000 1282

MODULE IV - MICROSCOPIC OBSERVATION

The cells in Well #1 (closer to the label) are normal cells, while the cells in Well #2 are a cancer cell line. Sample cell images are shown below for both types of cells. These images represent the typical results achieved from these cells; student results will vary due to slight fluctuations in cell preparation and the intensity of staining.

Students should be able to clearly observe the nuclei and cytoplasm of both cell types (see below for examples). In addition, differences in spreading, cell-cell contacts, and contact inhibition should be observed.

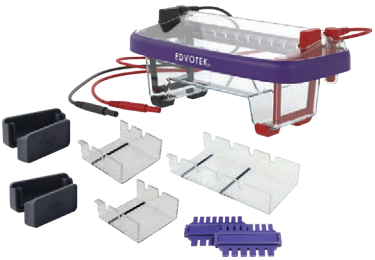


Cell type	Cell Shape	Spreading	Cell-Cell Contacts	Nuclei
Normal Epithelial Cells	Uniform, compact	Some spreading, but more compact. Monolayer.	Many cell-cell junctions	Uniform, small
Cancer Epithelial Cells	Random shapes, larger cells, disorganized arrangement.	Very spread cells, does not form uniform monolayer.	Fewer cell-cell junctions	Random, larger

MODULE V - ANALYSIS OF AUTORADS

Sheila (#2), Angela (#4), and Anthony (#5) have a normal DNA sequence for p53. Justin (#1) and Robert (#3) have a mutation in their DNA sequence, thus increasing their chance for developing a cancer at some point during their lifetimes.

Related Equipment



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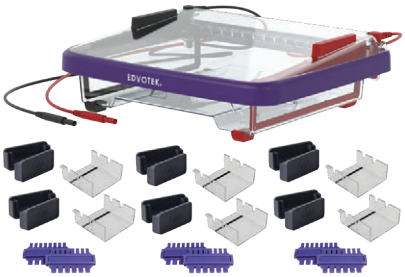

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