# Role of EGFR in the Proliferation of Human Lung Cancer Cells

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## Abstract

The gene for the epidermal growth factor receptor (EGFR) is often mutated in many cancers, including lung cancer. The EGFR gene directs the formation of epidermal growth factor receptor proteins, which when activated causes rapid cellular reproduction. Mutations to this gene produce proteins that are constitutively active and are often associated with lung cancers. Research is being conducted by inserting a mutant form of the EGFR gene along with a sequence for puromycin resistance into a plasmid for expression in cancer cells. The plasmid will then be introduced into lung cancer cells to assess transient expression and cell viability when combined with chemotherapy treatments. This research utilized two lines of lung cancer cells, H1299 and A549, both of which were harvested, suspended, and assayed under similar conditions. Lipofectamine transfection reagent is a non-viral gene insertion reagent that was used to introduce the plasmid into these cell lines. Techniques such as PCR, qPCR, Western blotting, and DNA sequencing were utilized to amplify and quantify expression of this introduced gene. Cotreatment with mutant EGFR and standard-of-care chemotherapeutics Paclitaxel, Cisplatin, and Doxorubicin in cancer cells will ideally aid in understanding the role of this mutation in drug resistant tumors.

## Introduction

The epidermal growth factor receptor(EGFR) gene is a commonly mutated gene in lung cancer. Its purpose is to promote cell proliferation by tyrosine autophosphorylation via the tyrosine kinase domain on the C-terminal domain of the gene sequence (Mitsudomi, 2010). To better understand the role of mutant EGFR in lung cancer, a mutant form of this gene known to be common in lung cancer is being inserted into a plasmid for the purpose of overexpression in lung cancer cells. The mutant EGFR gene is inserted along with puromycin which will allow for selection of cells containing the overexpression construct.. Puromycin inhibits protein synthesis by interfering with the elongation of a gene's genomic sequence which causes early termination of translation, and it is most commonly used as a resistance transgene cell marker for tracking the regulation of protein synthesis (Aviner 2020). The primary techniques used in this experiment included polymerase chain reaction (PCR) for amplifying the sequence of a gene to identify its size with primers and polymerases, Western blots for separating proteins based on size using primary and secondary antibodies (Gwozdz, 2017), and cloning which is a method of inserting a foreign piece of DNA into another plasmid.

## Materials and Methods

**Plasmid Isolation of Puromycin and EGFR**: Plasmids containing the coding sequence for mutant EGFR and an expression contruct containing the resistace cassette for puromycin resistance (pPuro) were isolated.

**PCR of EGFR**: Restriction sites were added to the ends of the EGFR PCR product for cloning into pPuro.

**Clean and Concentrate of PCR**: EGFR PCR was cleaned to remove residual proteins present in the PCR reaction.

**Digest of NheI and NotI**: PCR and pPuro cut with NheI and NotI enzymes to create compatible overhangs for ligation.

**Clean and Concentrate of Digests**: Digest reactions were purified prior to ligation.

**Ligation**: EGFR was ligated into pPuro using the compatible overhangs created by the double digest.

**Patched Putative Transformants**: Putative pPuro-EGFR transformants were transferred to a second plate.

**Plasmid Preparations of Transformants**: Putative transformants E1-E6 were grown in LB broth with ampicillin and plasmids isolated from each.

**PCR Conformation**: For putative transformants that were larger than the empty plasmid, the same primers used to amplify the sequence originally were used to try to attempt to amplify EGFR from putative transformants.

**Sequencing** : The putative pPuro-EGFR plasmid was sent to an industry lab to be sequenced to confirm that the EGFR sequence had been successfully inserted.

**Western Blot**: Total lysates of A549 and H1299 cells were screened for background level of EGFR. Both cell lines have WT copies of the EGFR gene.

**MTT Survival Assay**: IC50 was identified by testing concentrations of puromycin cells in MTT assay to determine cellular proliferation with puromycin present.

## Literature Cited

Oncogenic Transformation by Inhibitor-Sensitive and -Resistant EGFR Mutants. Greulich H, Chen TH, Feng W, Janne PA, Alvarez JV, Zappaterra M, Bulmer SE, Frank DA, Hahn WC, Sellers WR, Meyerson M. PLoS Med. 2005 Oct 4. 2(11):e313. 10.1371/journal.pmed.0020313

pcDNA3.1-(-)-Pur was a gift from Bertrand Collet (Addgene plasmid # 200458 ; http://n2t.net/addgene:200458 ; RRID:Addgene_200458)

Mitsudomi, T. and Y. Yatabe, *Epidermal growth factor receptor in relation to tumor development: EGFR gene and cancer.* The FEBS journal, 2010. **277**(2): p. 301-308.

Gwozdz, T. and K. Dorey, *Western blot*, in *Basic science methods for clinical researchers.* 2017, Elsevier. p. 99-117.

Aviner, R., *The science of puromycin: From studies of ribosome function to applications in biotechnology.* Comput Struct Biotechnol J, 2020. **18**: p. 1074-1083.

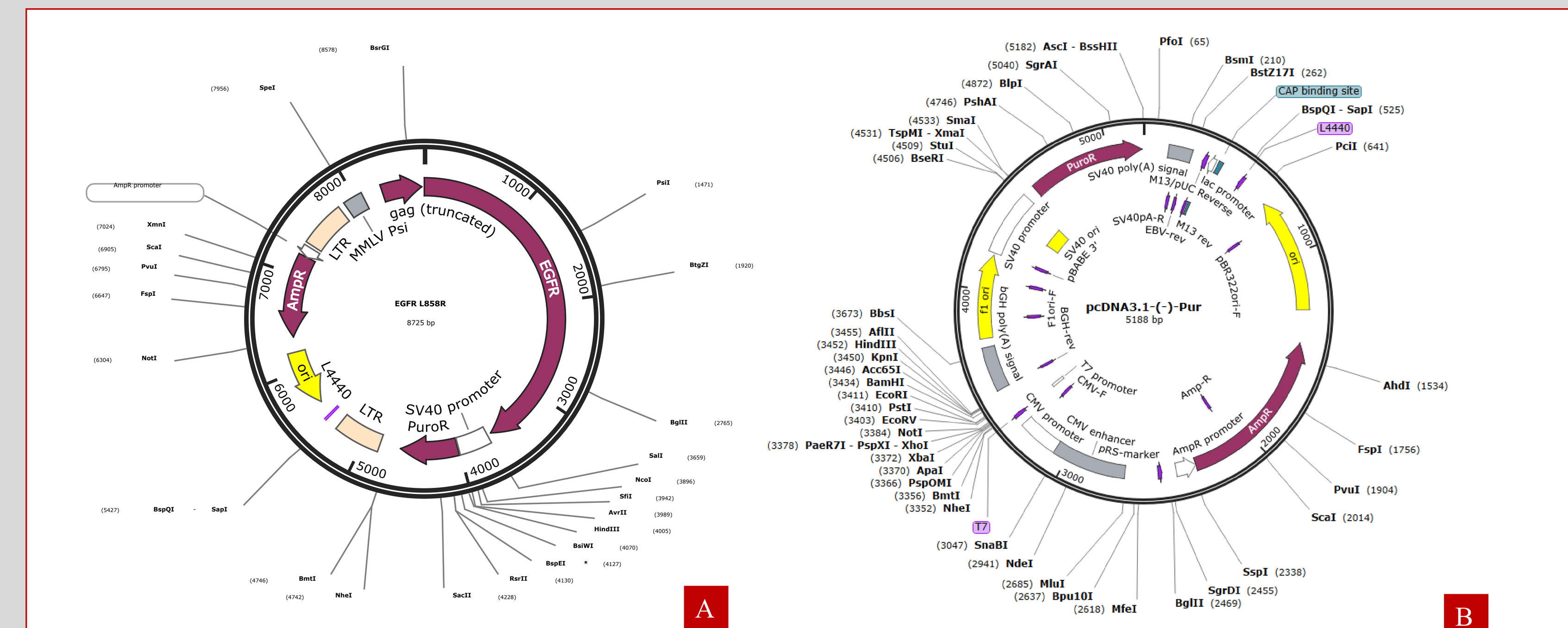


**Figure 1. Plasmids of EGFR gene.** Plasmid A shows the EGFR plasmid that the EGFR sequence was taken from to be introduced into Plasmid B which contained the puromycin sequence. The EGFR gene which is about 3500 base pairs long. Restriction enzymes NotI and NheI were used for cutting Plasmids A and B for insertion of the gene into Plasmid B.

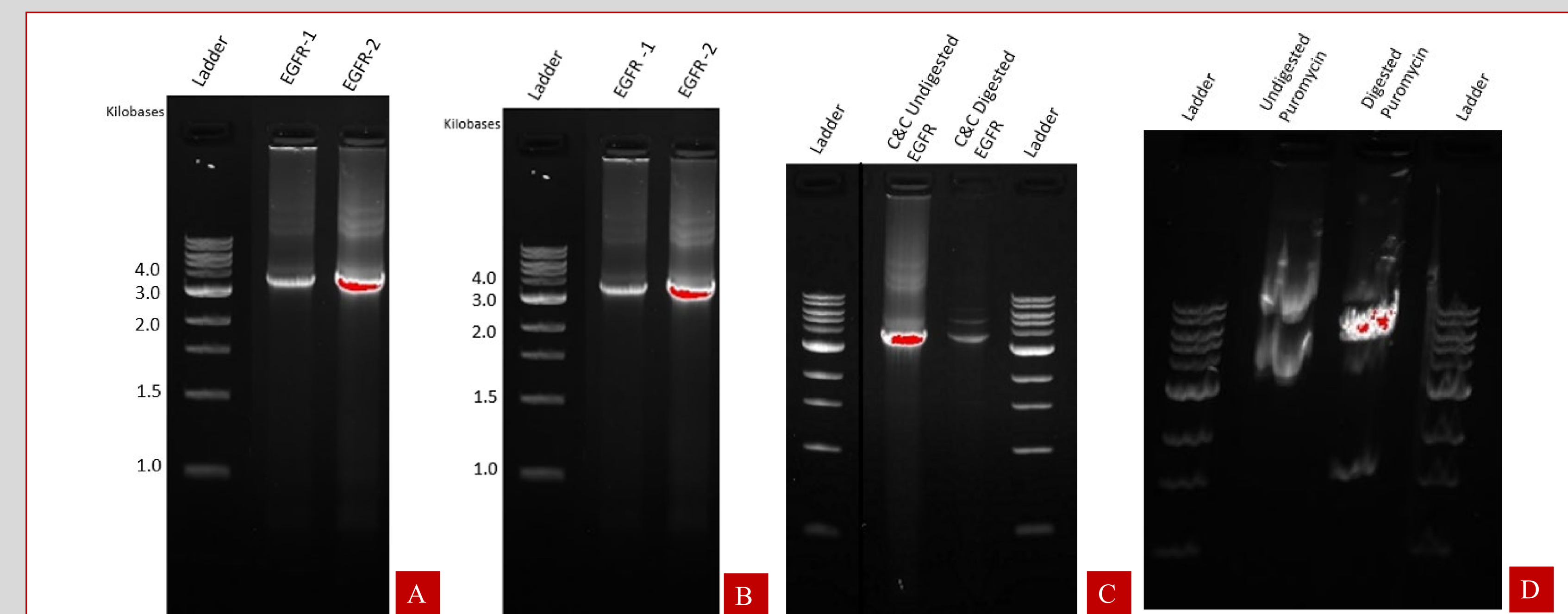


**Figure 2. PCR Gels.** EGFR is a large gene showing to be around 3.5 kilobases long which is large in comparison to most genes. PCR A is the original plasmid screen to determine the size of the EGFR gene. PCR B shows EGFR after the clean and concentrate that was performed to ensure that the plasmid was clean of any nutrients in the media used for the original PCR. PCR C shows EGFR once Not1 and Nhe1 had been introduced into the plasmid and the PCR was taken to verify that none of the EGFR had been degraded during the plasmid splicing. PCR D shows puromycin without EGFR after the digest. PCR D was heated to much while the gel ran which caused the abnormal shapes of the gel lines.

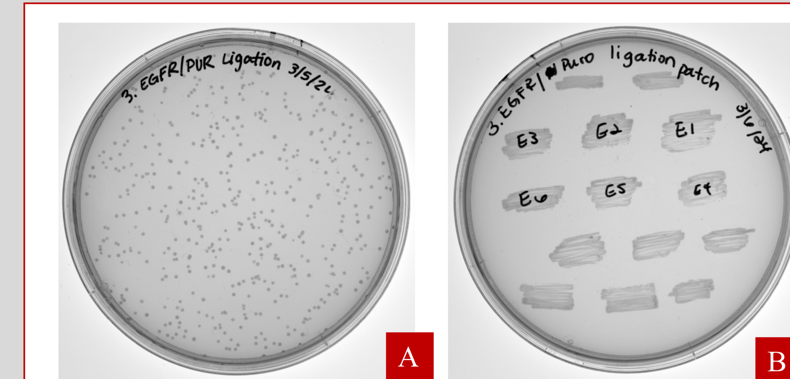


**Figure 3. Puromycin Transformation and Patch Plates.** Image B shows subcultures in E.coli of the transformant colonies in Image A containing putative pPuro-EGFR.

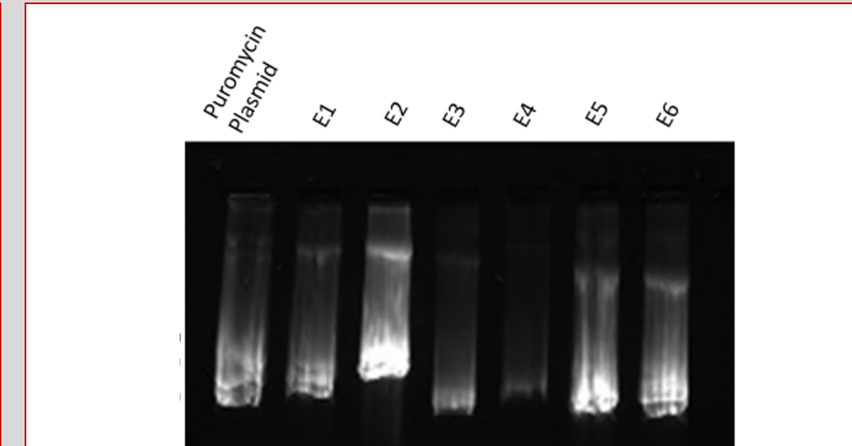


**Figure 4. EGFR Puromycin Plasmid**
Paying attention to wells #1 and #3, once EGFR had been inserted into the genome that it was a much larger plasmid.

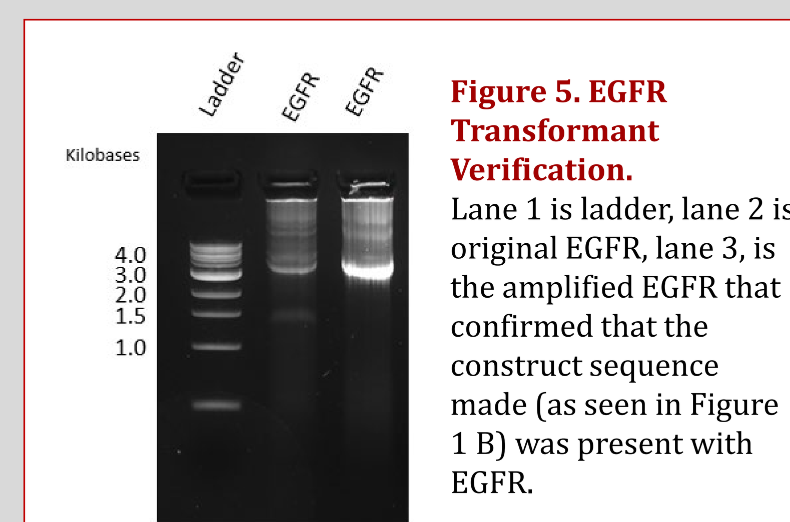


**Figure 5. EGFR Transformant Verification.** Lane 1 is ladder, lane 2 is original EGFR, lane 3, is the amplified EGFR that confirmed that the construct sequence made (as seen in Figure 1 B) was present with EGFR.

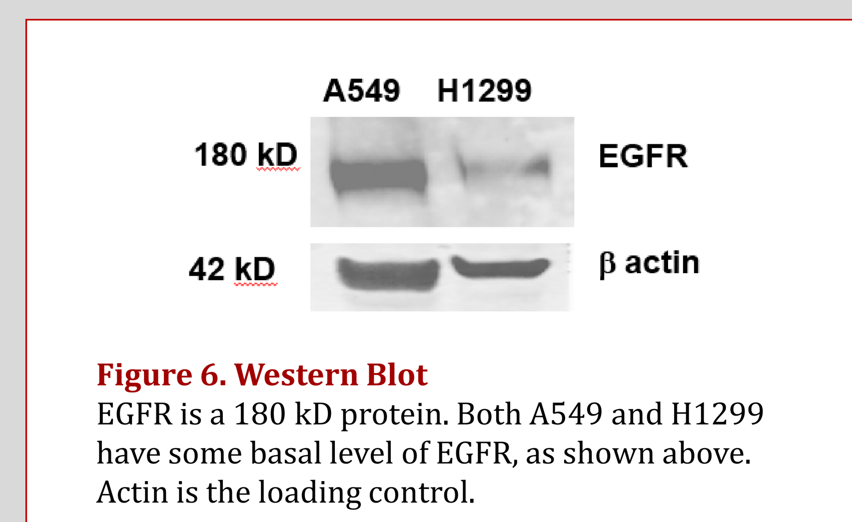


**Figure 6. Western Blot**
EGFR is a 180 kD protein. Both A549 and H1299 have some basal level of EGFR, as shown above. Actin is the loading control.

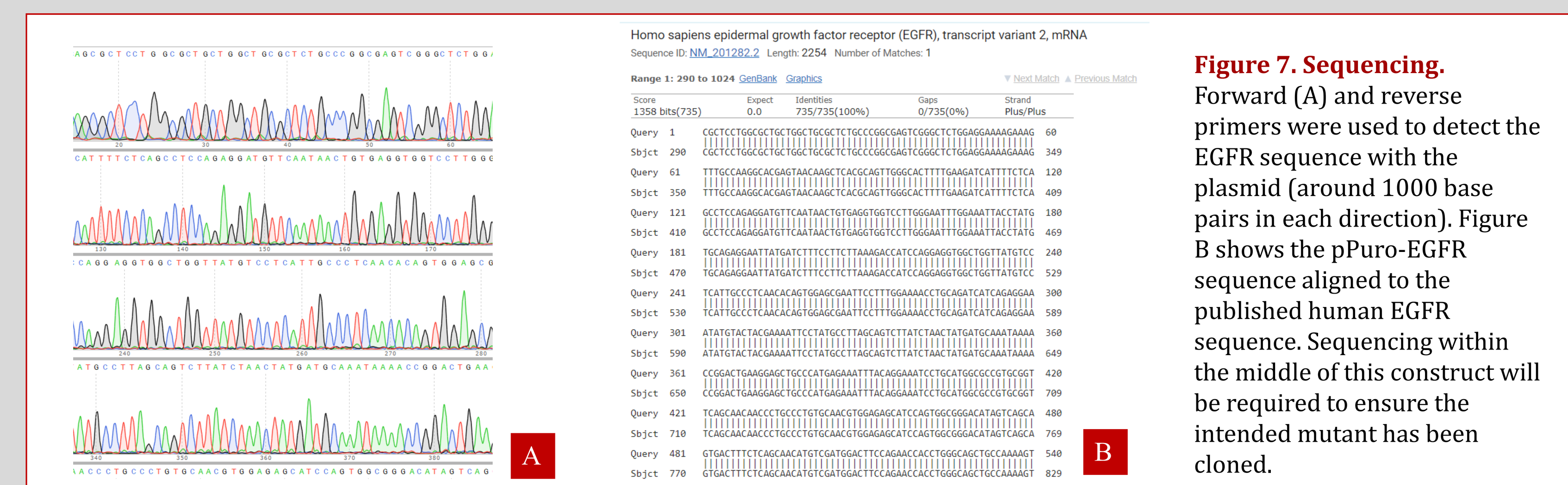


**Figure 7. Sequencing.** Forward (A) and reverse primers were used to detect the EGFR sequence with the plasmid (around 1000 base pairs in each direction). Figure B shows the pPuro-EGFR sequence aligned to the published human EGFR sequence. Sequencing within the middle of this construct will be required to ensure the intended mutant has been cloned.

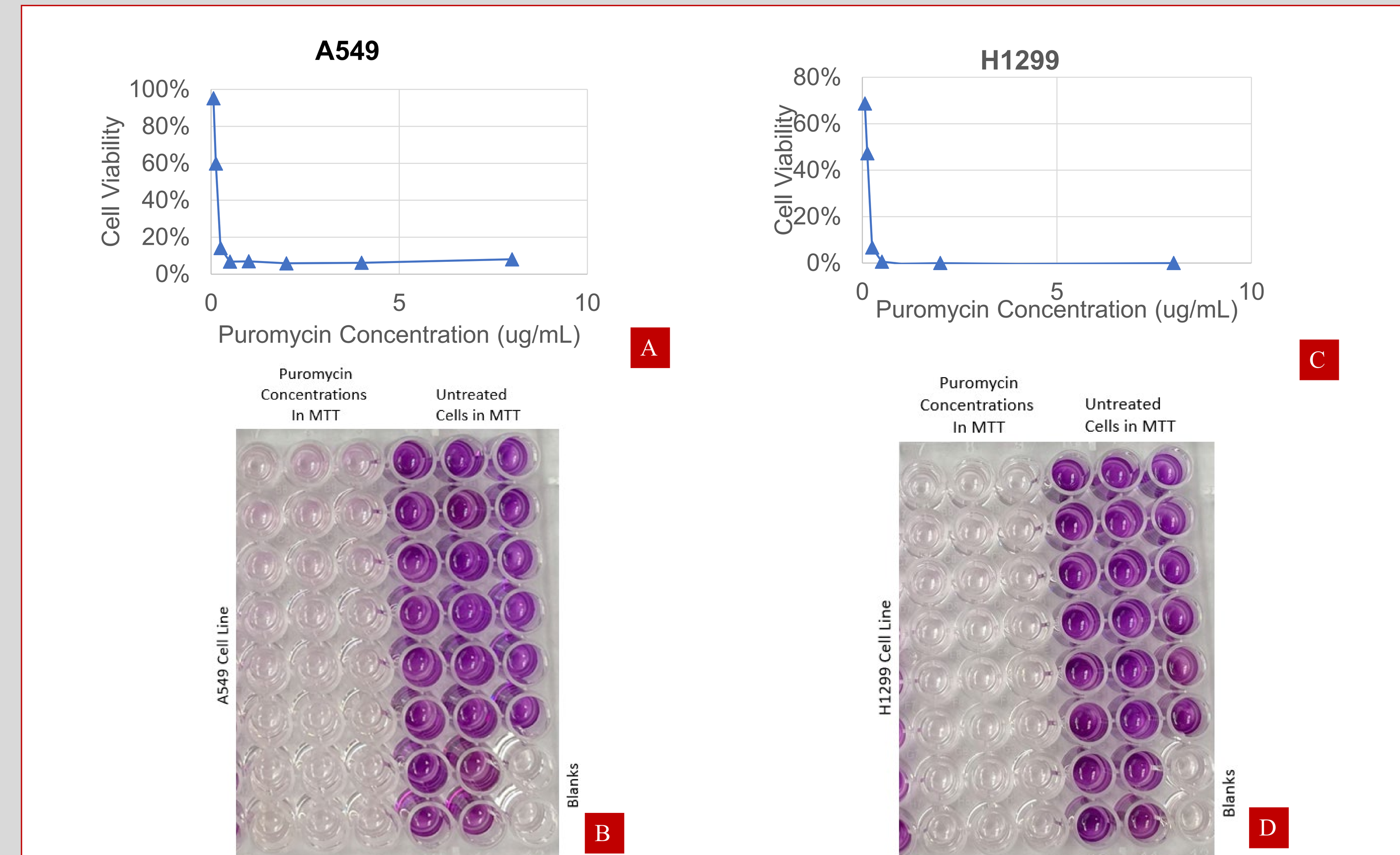


**Figure 8. MTT Survival Assay**
Figures A and B represent the A549 cell line, while Figures C and D represent the H1299 cell line with increasing concentrations of puromycin in the respective lung cancer cells in the first three columns, cancer cells without puromycin in the last three columns, and the final two wells only containing water to serve as a control.
Cells introduced with the EGFR gene were tested for cell viability by finding IC50, inhibitory concentration of 50% of the cells. The MTT Assay tests the mitochondrial activity of cells and loses its purple pigment as cells die. The puromycin antibiotic will serve as a selective pressure for stable expression in later experiments.

## Discussion

Using cloning and classic cloning techniuces, a mutant version of EGFR was cloned into an expression construct containing the resistance cassette for puromycin. The cloning of the EGFR sequence into pPuro vector was verified by size comparison, PCR and sequencing. Ultimately to produce transient cell lines, pPuro-EGFR will be introduced into cancer cell lines. Determining the inhibitory concentration of puromycin will allow for determination of appropriate concentrations to use for selection. Both H1299 and A549 have some background level of EGFR expression, although not a mutant version. When the mutant form of EGFR is introduced by the plasmid, cells are expected to have higher expression levels of EGFR.

## Future Directions

- Introduce EGFR transiently into H1299 and A549 using liposome-based transfection
- Produce puromycin resistant mutant EGFR stably expressing cell lines.
- Determine if overexpression EGFR mutant makes each cell line more or less susceptible to standard chemotherapeutic drugs Paclitaxel, Cisplatin, and Doxorubicin

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