Random mutagenesis is effective because it is possible, in a simple procedure, to generate a significant fraction of the total spectrum of simple (i.e. single site) mutations.

Selection refers to the procedures used to pick out the few useful mutations from this population of billions of irrelevant alterations.

Selection: procedures that act on a whole population so that an undesired subset can be eliminated or a desired subset can be isolated. Selection techniques are generally based on selective growth or selective killing of subpopulations.

Screening: monitoring properties of individuals in a population that bear signs of the desired genetic change.

A labour intensive screening test is ineffective if it is only possible to test a few samples from the population; if $10^9$ mutants are present but only a few hundred can be tested, most of the mutant population is wasted.

If $10^9$ mutants are first subject to selection that eliminates most of the cells containing irrelevant changes, the same screening test might be quite effective in finding the desired phenotype from a reduced population of the order of $10^4$ variants.

Antimetabolite selection: selective growth of the desired population

Antimetabolites are substances that closely resemble a required biochemical growth factor

- amino acid analogs, e.g. fluorophenylalanine
- nucleotide analogs, e.g. 5-fluorouracil, 8-azaguanine, 3’-deoxyadenosine
- coenzyme analogs e.g. aminopterin, analog of tetrahydrofolate

The antimetabolite is sufficiently similar to interfere with enzymes utilizing the normal compound, but sufficiently different not to function properly in its place, so is toxic to normal cells.

Antimetabolite resistance acquired by mutation has been correlated with induced overproduction of the normal metabolite in certain cases.
The antimetabolite aminoethylcysteine (AEC) is an analog of lysine.

**Toxicity of AEC:**

If incorporated into protein, AEC may render protein non-functional.
It can bind to **regulatory sites** in enzymes controlling the lysine synthesis pathway, down-regulating synthesis.
Since no real lysine is made, the cells starve for lysine.

**Resistance to AEC toxicity** may arise by the following mechanisms:

- Loss of Lys/AEC binding sites in regulatory components of enzymes, making them lysine insensitive.
- General up-regulation of the lysine pathway so that higher cellular concentration of lysine dilutes out the antimetabolite.

**Mutation and selection protocol:**

Start with *lexA*—cells (SOS constitutive) that have normal AEC susceptibility.

A **killing curve** is a plot of the % survival of cells cultured at increasing concentrations of AEC:
This tells you the limit of tolerance in the original population.

A fresh culture is exposed to UV for 10% mutation rate.

UV survivors are subcultured in in $10^{-5}$ M AEC; only cells which have acquired resistance as a result of mutation survive. This is the **selection** step.

The surviving subpopulation is identified as $\text{AEC}^R$, for AEC Resistance phenotype. The lysine pathway has more than one lysine regulated step, and more than one regulatory process may act at each step e.g. direct enzyme inhibition as well as repression of gene expression. Since the mutation level used should not yield more than one mutation per cell, there is scope for introducing additional mutations into lysine regulation by repeating the whole process.
A new killing curve is plotted, and should show that the mutants have tolerance to a higher level of AEC.

First round $AEC^R$ cells are exposed to UV once more, and then subcultured into $10^{-4}$ M AEC. Only cells which have additional AEC resistance as a result of mutation in the second round now survive.

The whole process may be repeated for a third round, or until no further significant gain in resistance can be detected.

At this point, the population of cells should have been reduced from billions of possible mutants to a few hundred, and they may now be screened for their ability to overproduce lysine.

A smarter approach (large number easily testable) would be to use the medium to supplement growth of a tester microorganism that requires lysine for growth. A good tester strain might also incorporate a distinctive colour for easy detection.

**Auxotrophs**

An auxotrophic mutant is one which has lost the capability of synthesizing a metabolite required for growth. In order to grow, the medium must be supplemented with the missing compound. For example, Biotin$^-$ mutants require biotin to be added to the medium to grow, or they can be supplied with fatty acids, since biotin is a required factor in fatty acid biosynthesis.

The lysine overproducing strains of *Corynebacterium glutamicum* are homoserine auxotrophs, not converting ASA to homoserine so that substrate won't be diverted to the Met, Thr, or Ile branch of the synthetic pathway.
Selecting for auxotrophs - the antibiotic selection method

Autotrophs won’t grow if the missing factor is absent from the medium.

Penicillin blocks a step in cell wall peptidoglycan synthesis, so penicillin or a related antibiotic only kills growing cells (nystatin for yeast).

Start by exposing a wild type cell culture to mutagen, e.g. UV exposure.

Grow cells in minimal medium, in presence of penicillin:

Unmutated cells grow, and are killed
Non-growing mutants don’t proliferate but survive as quiescent cells- these are auxotrophic for a variety of factors, not necessarily what is desired.

Subculture in fresh medium without penicillin, supplemented only with the intended auxotrophic factor, in this case homoserine.

Auxotrophs for other factors fail to grow. 
Homoserine auxotrophs grow due to the supplement.

The defect must lie after ASA in the pathway, or the strain would also be auxotrophic for Lys, hence homoserine dehydrogenase is the enzyme that’s defective (Δ). It should be possible to knock out the enzyme by a single mutation, hence repeated rounds of mutation are unnecessary.

\[
\text{Met} \quad \Delta \quad \uparrow \\
\text{Asp} \rightarrow \text{AspP} \rightarrow \text{ASA} \rightarrow \text{Hom} \rightarrow \text{HomP} \rightarrow \text{Thr} \\
\downarrow \\
\text{DHP} \rightarrow \text{Lys}
\]

Threonine-supersensitive cells were obtained by growing mutated cells (starting from wild type) in presence of a moderate concentration of Thr + Penicillin. Thr sensitivity blocks synthesis of Met as well as Thr, hence Thr supersensitive cells behave as Met auxotrophs during the penicillin selection step. Once removed from penicillin medium, the desired cells grow in the absence of added Thr.