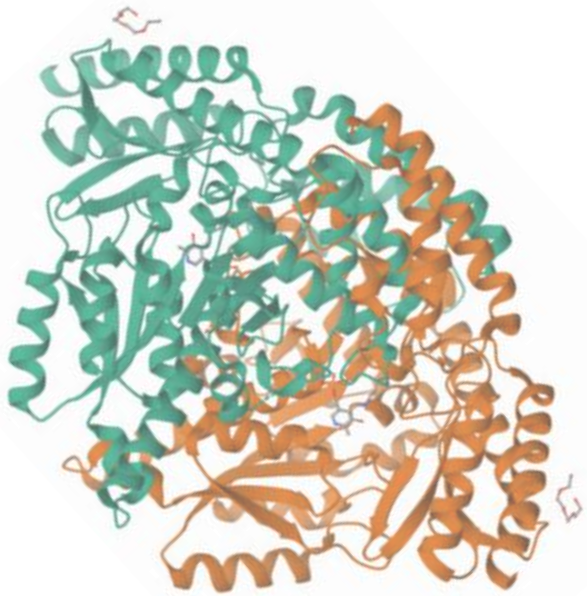
ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
UNIVERSITY OF CRETE

# Description/Creation of a HTS assay for L-Aromatic Amino Acid Decarboxylase



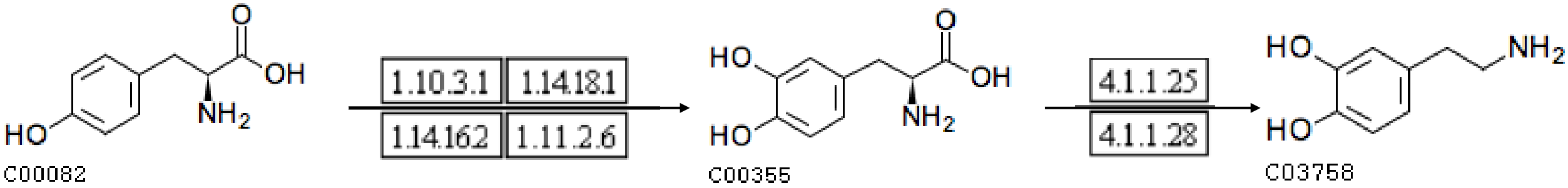
Πρωτεϊνική Μηχανική-3ο Σεμινάριο

29.05.2026

Διβάρης Παναγιώτης (Α.Μ. 1281)

Πατούνη Τατιάνα (Α.Μ. 1295)

# Tyrosine metabolism



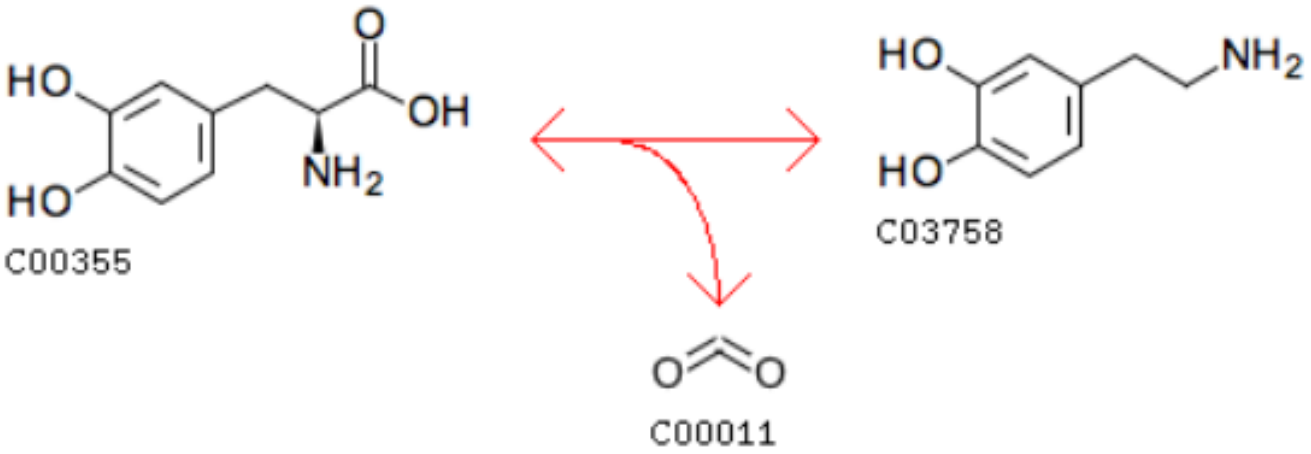

L-Tyrosine

L-DOPA

Dopamine

# Aromatic-L-amino-acid decarboxylase

EC:4.1.1.28




**4.** - Λυάσες

4.**1** -Άνθρακα-άνθρακα λυάσες

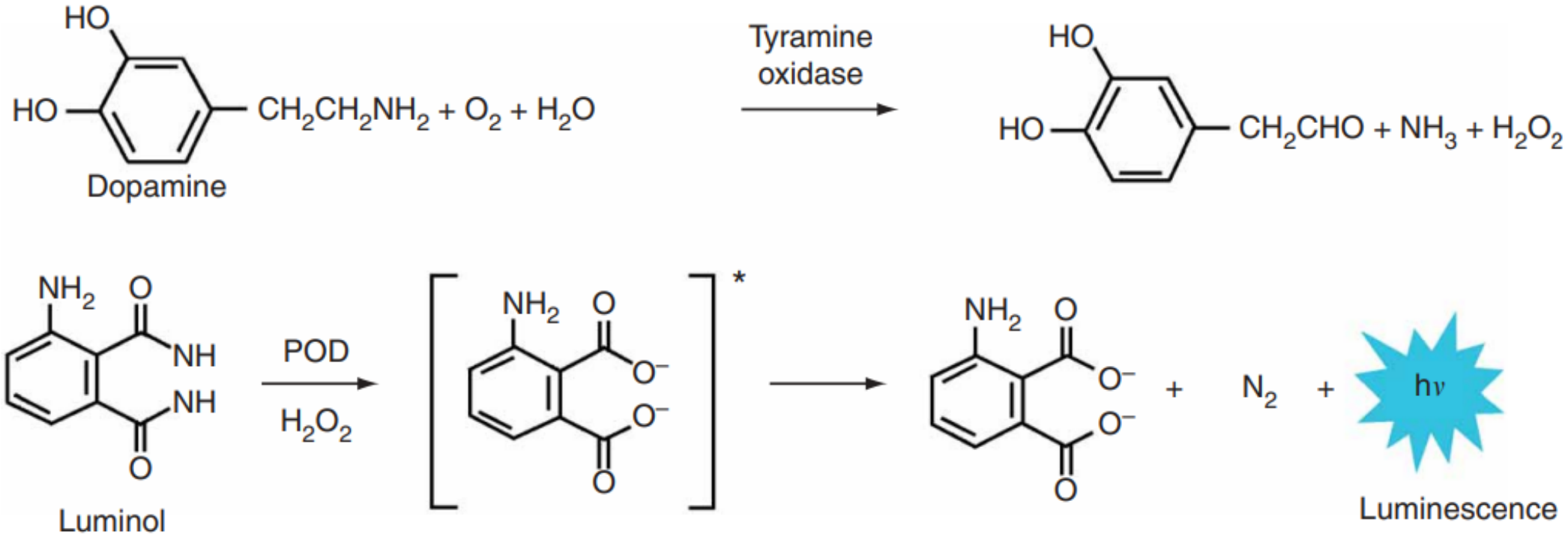
4.1.**1** -Καρβόξυ-λυάσες

4.1.1.**28** -Αποκαρβοξυλάση αρωματικών L-αμινοξέων

- **Συμπαράγοντες:** Φωσφορική πυριδοξάλη (PLP)
- Δρα και σε αρωματικά L-αμινοξέα (τρυπτοφάνη, φαινυλαλανίνη, τυροσίνη)

# Product formation-based detection

Dopamine release is measured using a luminescence detectable plate reader, FLUOstar OPTIMA (BMG LABTECH, Germany) at the maximum gain.



# Dopamine standard curve preparation

- In a 96-well plate: 200 ml Locke's solution, 30 ml tyramine oxidase -TOD (4.6 U/ml), 30 ml horseradish peroxidase -POD (64 U/ml) and 30 ml luminol (1 mM) were added to each well
- Incubation of the plate at 37 °C for 10 min
- Inject 10 ml of standard dopamine solutions (0–1 mM) into each well. It is important that the dopamine solution is added at the same time to all wells
- Observe the plate reader output for luminescence intensity of each well

# Dopamine assay protocol

1. One day before dopamine release assay, inoculate PC12 cells (i.e., 0.5–1 x $10^{-6}$ cells/ml) into a 96-well plate (200 ml each well)

2. Incubate overnight at 37 °C , 5% CO2, to promote cell adhesion

3. Discard the medium and wash the wells with warmed (37 °C ) sterile Locke's buffer twice

4. Count the number of cells in two wells using a hemicytometer

5. Add the measurement solution into each well, put the plates back into cell culture incubator and incubate for at least 10 min

6. Transfer the 96-well plate into a luminescence plate reader.

7. Stimulation is done by autoinjection of 10 µl of stimulant (stock solution of ACh or BK) in each well to induce dopamine release

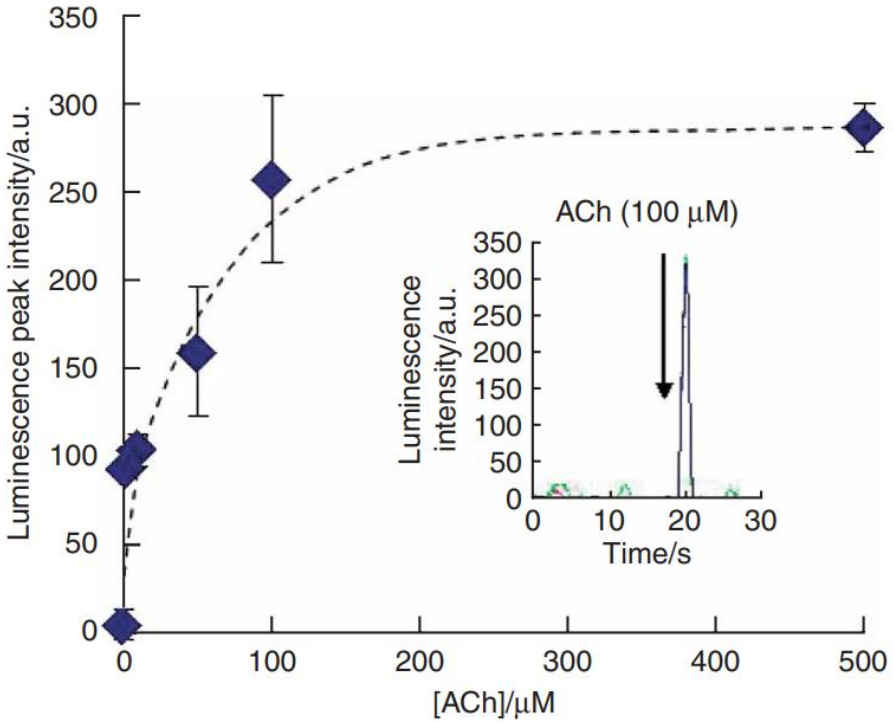
8. Observe the time course of luminescence intensity (a.u.) for each well using the luminescence plate reader equipped with a photomultiplier

# Conditions necessary for the assay

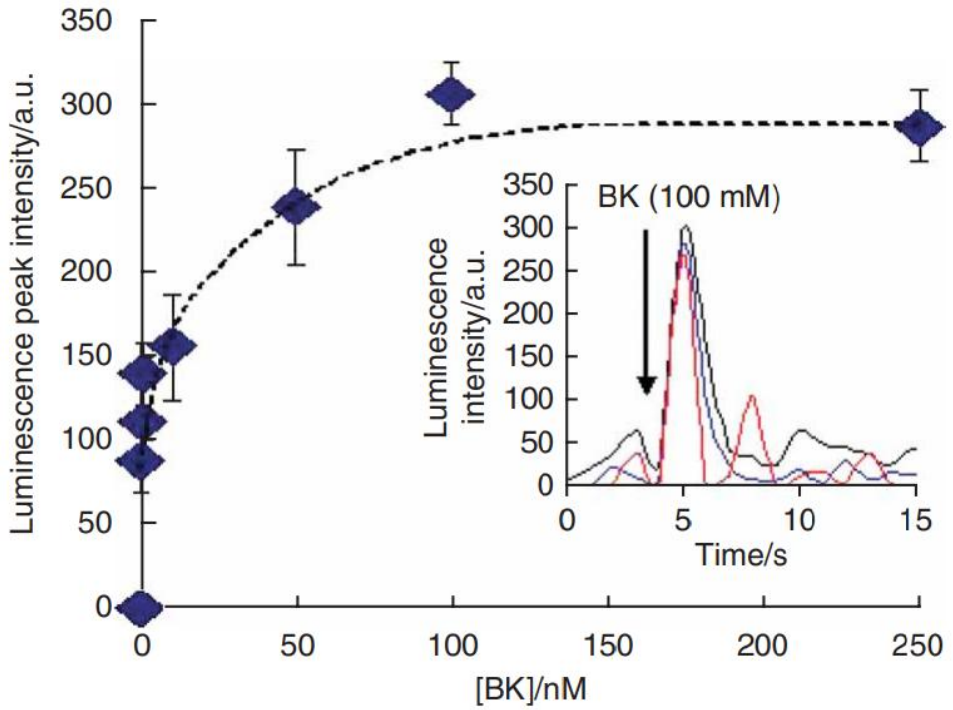
- Acetylcholine (ACh) , bradykinin (BK) both for inducing dopamine release
- The stimulation of the cells was done by autoinjection of 10 µl solution of drugs
- Locke's solution (pH 7.4, 10 µl)

| Stimulation conditions | Measurement solution supplemented with | Purpose |
|---|---|---|
| Stimulation with either ACh or BK in the presence of cells | 200 µl Locke's, 30 µl TOD (4.6 U $ml^{-1}$), 30 µl POD (64 U $ml^{-1}$) and 30 µl luminol (1 mM) for each well | Role of different activators in the release of dopamine from PC12 cells |
| Stimulation with Locke's solution only in the presence of cells | 200 µl Locke's, 30 µl TOD (4.6 U $ml^{-1}$), 30 µl POD (64 U $ml^{-1}$) and 30 µl luminol (1 mM) for each well | Negative control experiment |
| Stimulation with ACh in the absence of cells | 200 µl Locke's, 30 µl TOD (4.6 U $ml^{-1}$), 30 µl POD (64 U $ml^{-1}$) and 30 µl luminol (1 mM) for each well | Confirmation of dopamine release from PC12 cells |
| Stimulation with ACh in the presence of cells | 230 µl Locke's, 30 µl POD (64 U $ml^{-1}$) and 30 µl luminol (1 mM) for each well. Do not add TOD | Specificity of dopamine oxidation by TOD |

# Dose-dependent effects on dopamine release




- $EC_{50}$: 66 μM
- Triggers dopamine release
- Quick luminescence peak intensity detection




- $EC_{50}$: 20 μM
- More potent in inducing dopamine release than ACh

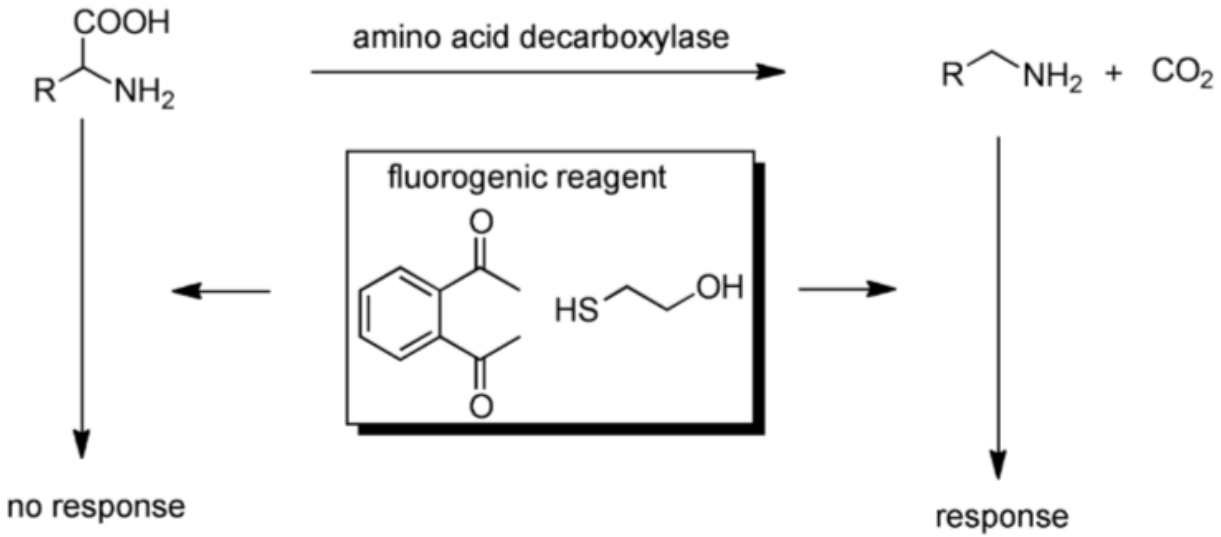
# Positive-negative

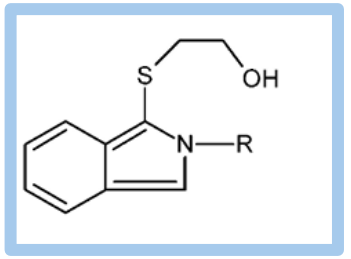
- High sensitivity - dopamine detection limit around 10 nM
- Dopamine release can be monitored within seconds after stimulation)
- Non-lethal method as the exposure to measurement solution is short and $H_2O_2$ is oxidized quickly

- Experiments need to be carried out entirely in a dark room to avoid high background signal interference
- Method is not specific to dopamine - could also detect other catecholamines such as norepinephrine
- Enzyme/drug solutions should be prepared on the day of the experiment to avoid deterioration
- Dependence on cell number/health for accurate measurements

# Coupled colorimetric assay

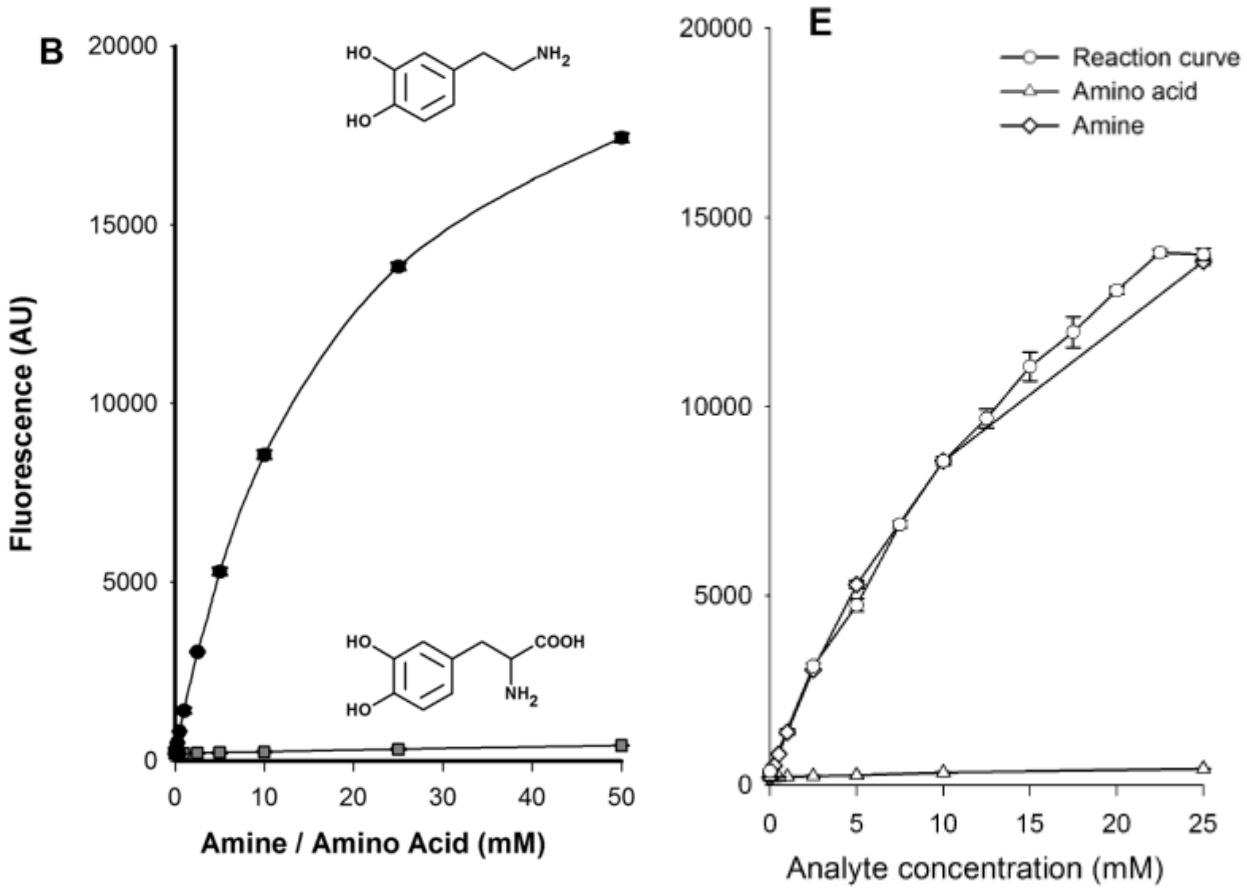
- Selective fluorometric detection of primary amines using **o-diacetylbenzene (DAB)** and **mercaptoethanol**




$\lambda_{ex}$= 355 nm
$\lambda_{em}$= 445 nm

# Assay and application to Aromatic Amino Acids




- Reaction volume: 155 uL of a solution containing o-diacetylbenzene (0.98 mM), b-mercaptoethanol (1.16 mM) and sodium borate buffer (0.2M, pH 9.5)
- Addition of 2.5 uL of the sample (purified enzyme or cell extracts)
- Record fluorescence every 10 min up to 2 hours

Fluorescence development time: ~100 min

# Determination of z and z’ values

- Statistical parameters used to evaluate the quality, reliability, and suitability of an assay

z’ factor value calculated by using a solution of amino acid or amine as negative and positive controls

$$z' = 1 - \frac{3\,SD_{c+} + 3\,SD_{c-}}{\left| mean_{c+} - mean_{c-} \right|}$$

L-DOPA/Dopamine = 0.92

z factor value calculated by using the fluorescent intensities of L-serine decarboxylation catalysed by *E. coli* cell-free extracts

$$z = 1 - \frac{3\,SD_{\text{amino acid}} + 3\,SD_{\text{amine}}}{\left| mean_{\text{amino acid}} - mean_{\text{amine}} \right|}$$

z = 0.70

Confirms the suitability of the assay for future directed evolution experiments

# Advantages and Disadvantages

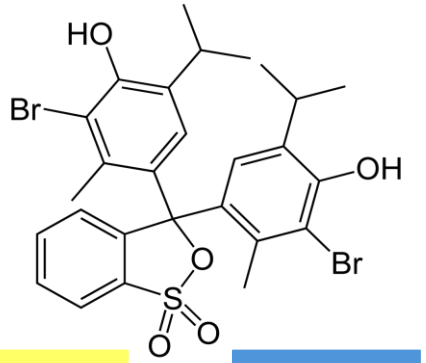
- **High throughput:** Suitable for screening large enzyme libraries
- **Specificity:** Discriminates amines from amino acids effectively, even in complex mixtures
- **Sensitivity:** Low detection limits sufficient for enzymatic activity screening
- Compatible with crude cell extracts and mixtures

- **Slow Reaction Kinetics:** fluorescence measurements over up to 2 hours
- **No selectivity** for some amino acid substrates
- **Detection Limit:** 13 mM ethanolamine in 50 mM L-serine
- **Interference from Complex Samples / Crude Extracts:** The presence of other fluorescent or reactive could affect assay accuracy
- **pH and Buffer Constraints:** Alkaline borate buffer at pH 9.5 may not be compatible with all enzymatic conditions
- **Requirement for Specialized Reagents and Equipment**

# pH-indicator microplate assay

$$RCH(NH_3^+)COO^- \rightarrow RCH_2NH_2 + CO_2$$

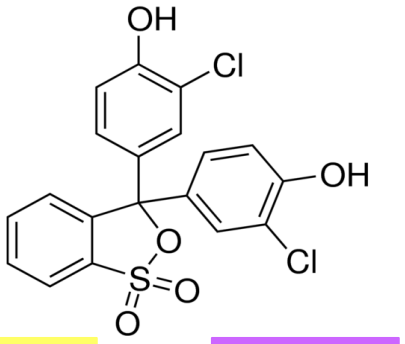
***Bromothymol blue***




**pH 6.0** ⇌ **pH 7.6**

**pKa= 7.1**

***Chlorophenol red***

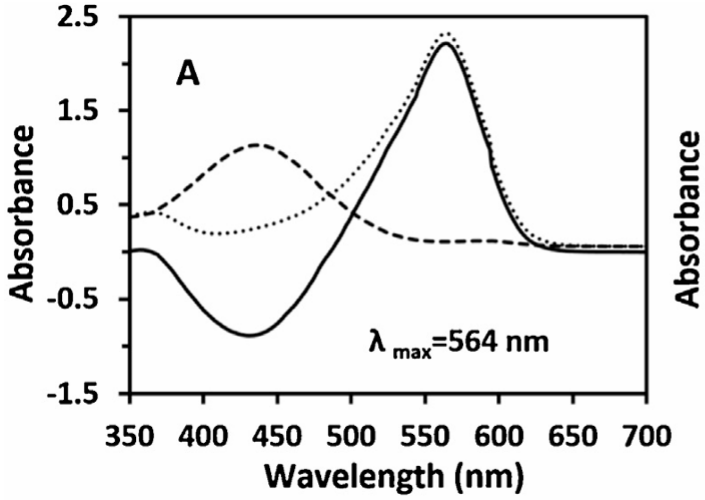



**pH 5.2** ⇌ **pH 6.7**

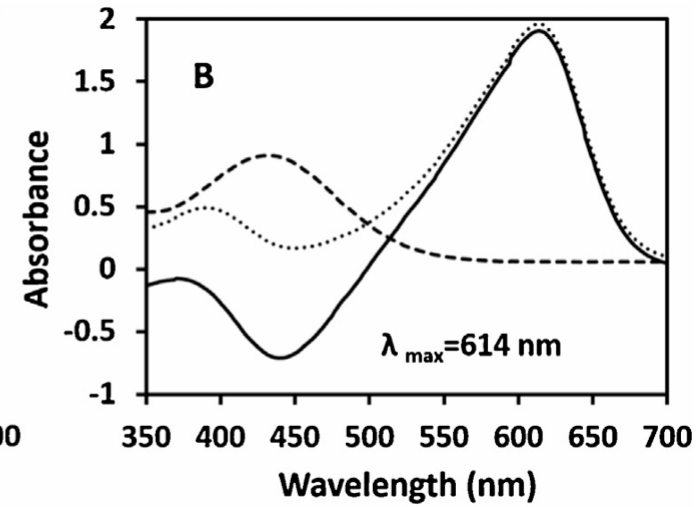
**pKa= 6.0**

***Absorption spectra***

*Chlorophenol red*




*Bromothymol blue*

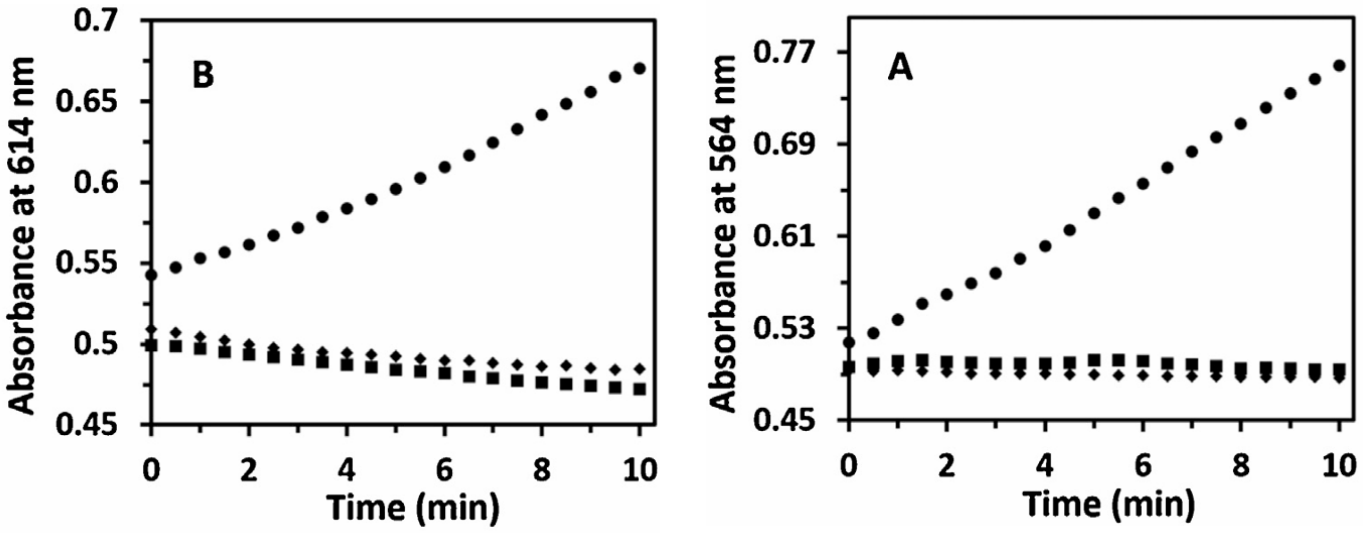



- Protonated (dashed line)
- Deprotonated (dotted line)
- Differential spectra (solid line)

# High-Throuput Assay Workflow

- Optimization with NaOH titration mimics proton consumption → color changes tracked by absorbance
- Optimal conditions found: 5 mM buffer and 50-75 µM indicator

1. Express Lys decarboxylase enzyme in E. coli BL21
2. Lyse cells to prepare crude extract
3. Reaction (V=200 uL): 10 mM amino acid substrate, 5 mM MES or MOPS buffer, 50–75 µM pH indicator 10 mM, 0.1 mM PLP
4. Add 10–50 µL of crude cell extract to initiate the reaction
5. Monitor absorbance with shaking at 37°C (every 30 seconds for 10 minutes)
6. Calculate enzymatic activity normalized to cell biomass ($OD_{600}$)



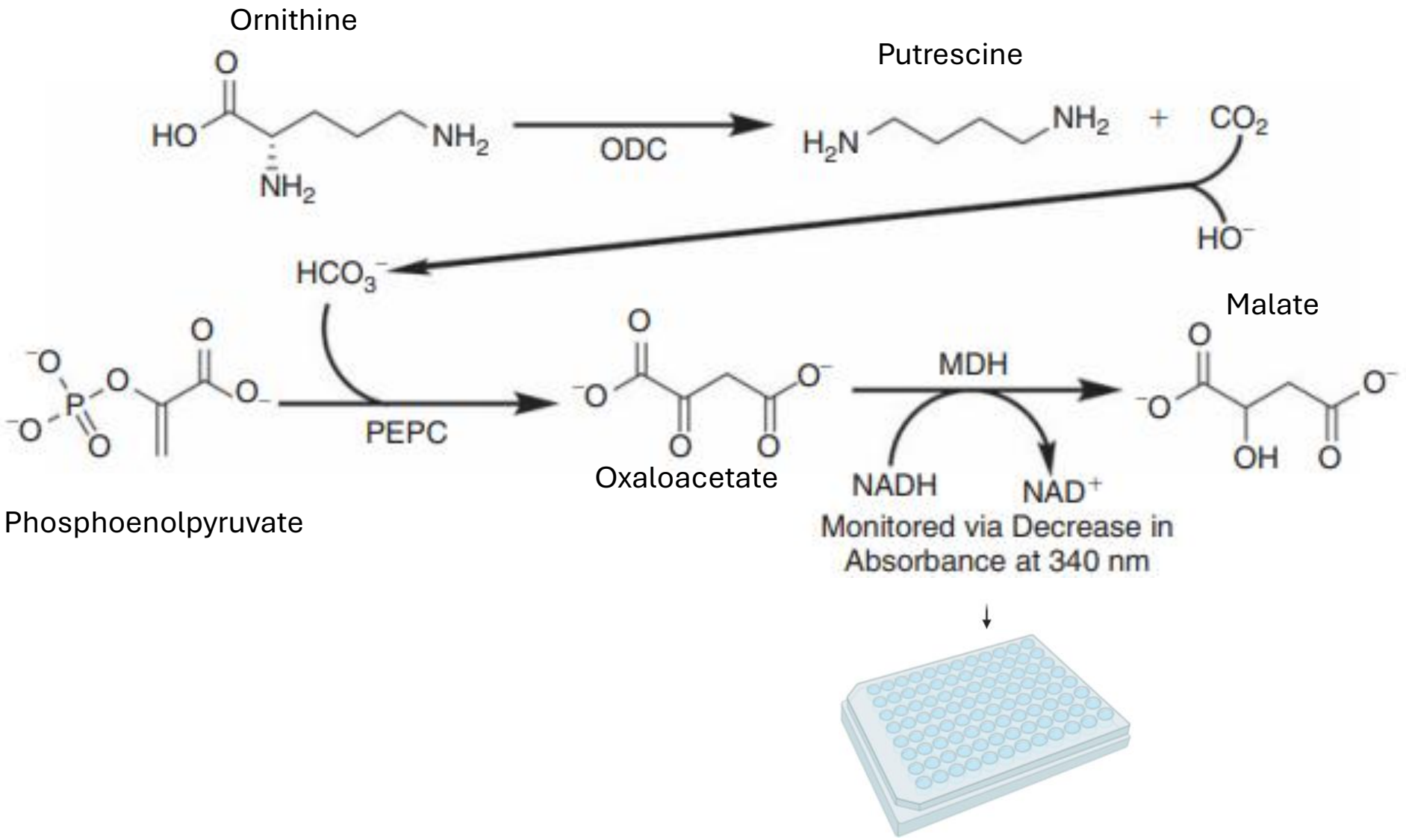

(●) Cells harboring pET30a-cadA
(◆) Cells without vector pET-30a
(■) Cell lysis buffer as blank controls

# Positives-negatives

- **Rapid and continuous monitoring** of enzymatic reactions
- **Does not require** derivatization or separation steps
- **Cost-effective**: uses common reagents, no specialized equipment besides plate reader
- **Flexible**: adaptable to different pH and substrate types by buffer/indicator selection
- **Suitable for high-throughput** screening of mutant libraries

- **pH Buffer and Indicator Dependency:** The acidic or basic nature of substrates can affect assay performance
- **Limited Dynamic Range:** Too much buffer reduces sensitivity, too little reduces linearity
- **Interference from Other pH-altering Compounds:** Any substances in crude extracts may interfere with the colorimetric signal
- **Not Suitable for All Decarboxylases Without Modification:** due to substrate differences

# $CO_2$ production-based detection



# $CO_2$ production-based detection

## Infinity™ Carbon Dioxide Liquid Stable Reagent

### SYSTEM PARAMETERS

| | |
|---|---|
| Temperature | 30/37°C |
| Primary Wavelength | 380 nm(375-380nm) |
| Assay Type | End Point |
| Direction | Decrease |
| Sample:Reagent ratio | 1:100 |
| e.g. Sample vol | 3 µL |
| Reagent vol | 300 µL |
| Incubation Time | 300 seconds |
| Reagent Blank Limits | Low 1.0 AU |
| (380nm, 1cm lightpath) | High 2.0 AU |
| Linearity | 3 - 50 mmol/L (3 - 50 mEq/L) |
| (refer to Linearity section) | |
| Analytical Sensitivity | 0.01 ΔA per mmol/L |
| (380 nm, 1 cm lightpath) | (0.01 ΔA per mEq/L) |

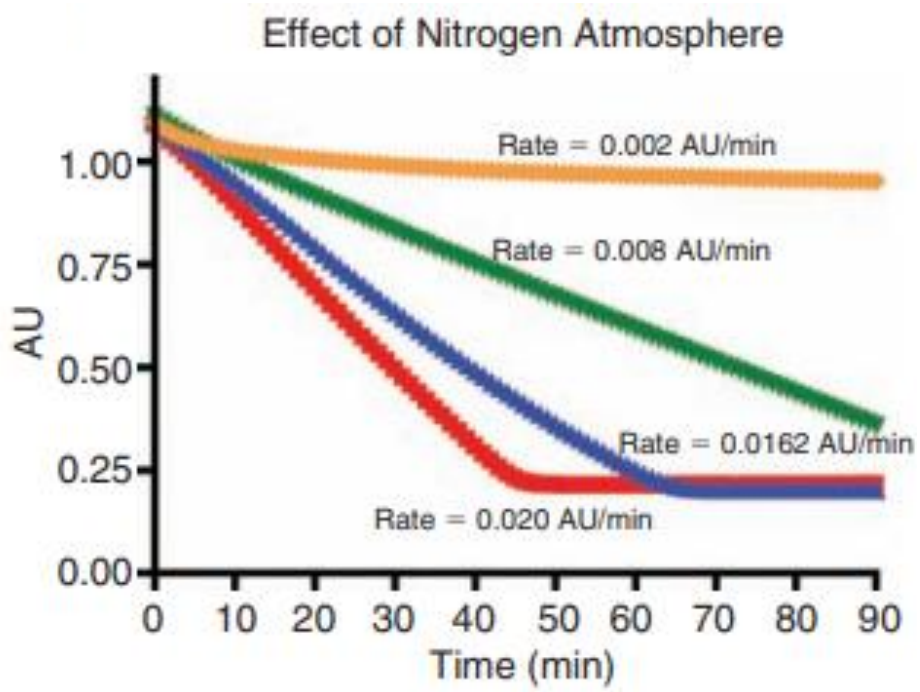
If a shorter light path is used the assay may be run at 340nm.

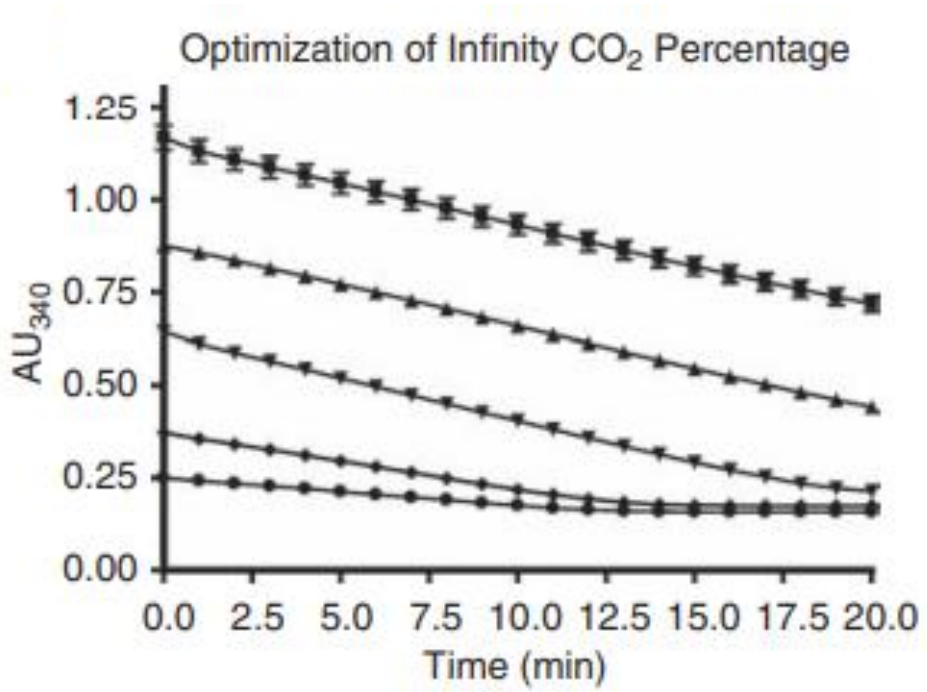
### REAGENT COMPOSITION

| Active Ingredients | Concentration |
|---|---|
| Phosphoenolypyruvate | 8.0 mmol/L |
| NADH | 1.6 mmol/L |
| Phosphoenolpyruvate carboxylase (microbial) | >1000 U/L |
| MDH (microbial) | > 200 U/L |
| Buffer | 66 mmol/L |

Also contains non-reactive stabilizers
pH 8.05 ± 0.1 at 20°C

# $CO_2$ production-based detection

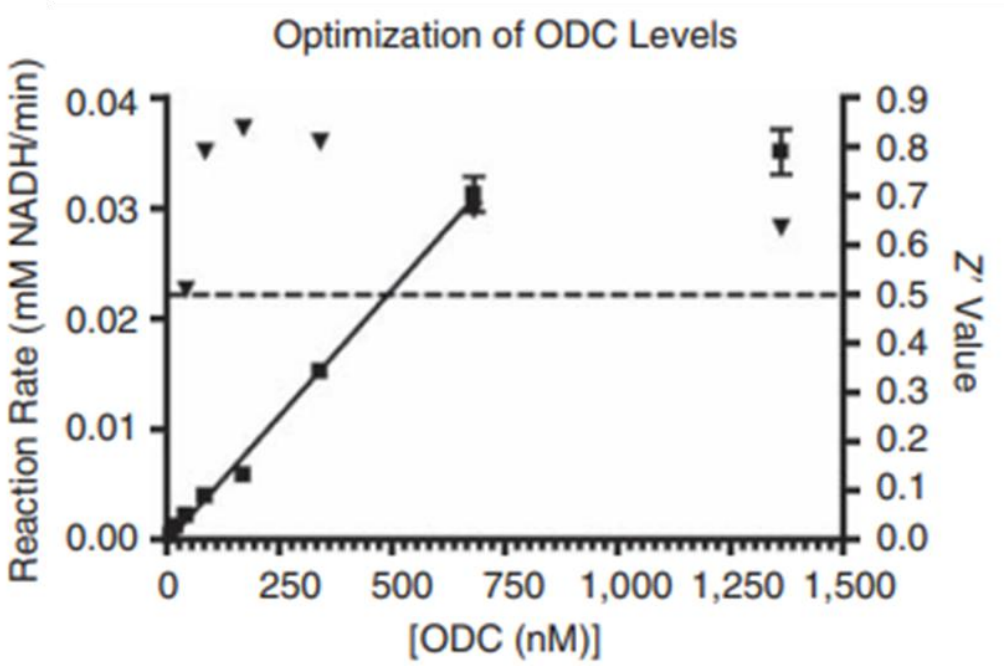


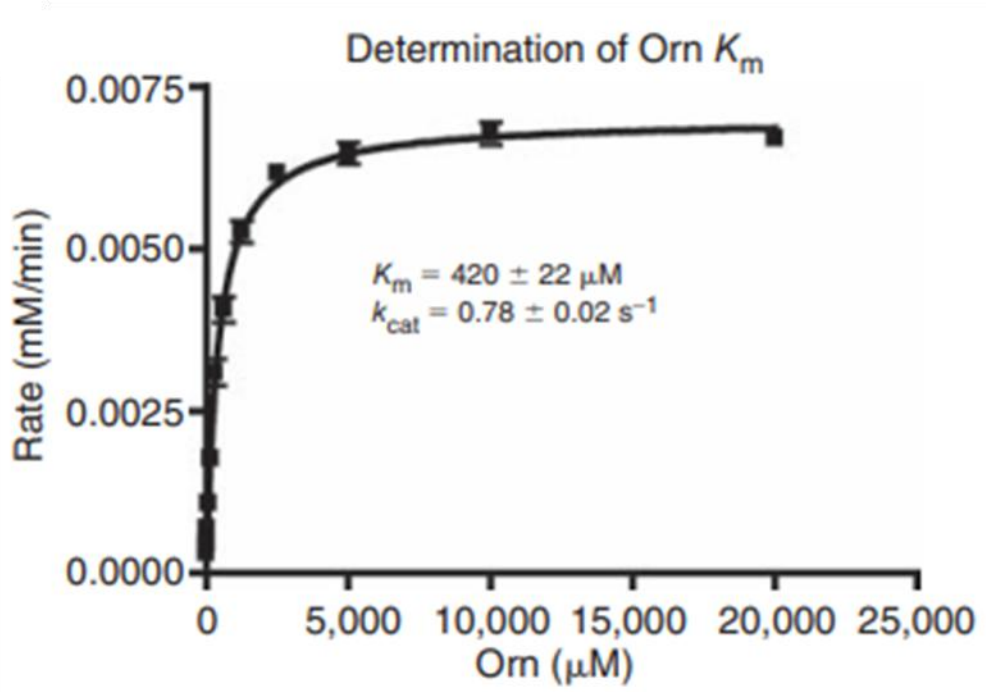




- Orange: nitrogen atmosphere, no ornithine
- Green: standard atmosphere, no ornithine
- Blue: nitrogen atmosphere, 625 µM ornithine
- Red: standard atmosphere, 625 µM ornithine

- Chosen final concentration: 60%
- Good linearity/ signal window

# $CO_2$ production-based detection




- Optimal ODC concentration: 150 nM
- 5-fold signal window
- Z' > 0.5 → HTS-suitable
- Z' Values were calculated using 8 positive (1 mM DFMO) and 8 negative (DMSO) controls

- 150 nM ODC & 60 µM PLP
- Assay buffer same as linking buffer

# $CO_2$ production-based detection

Endogenous E. coli metabolism can add CO2 background:

- Cell debris/proteins increase A340 scatter.
- NADH-consuming enzymes can mimic activity.
- Variable expression makes activity look like altered catalytic efficiency.
- L-DOPA oxidation and pH drift distort the slope

- The assay was performed using purified enzyme and most likely crude lysate would require further purification
- Inclusion of lysate-only controls (without substrate) to assess background.
- Partial purification steps (e.g., centrifugation, filtration) to reduce interfering substances
- Testing linking enzyme activities in the presence of lysate to confirm coupling system remains functional

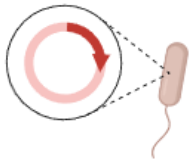
| Well type | Purpose |
|---|---|
| Lysate + L-DOPA | Measures apparent AADC-dependent $CO_2$ signal |
| Lysate, no L-DOPA | Measures endogenous/background $CO_2$ signal |
| Empty-vector lysate + L-DOPA | Measures E. coli lysate/substrate background |
| Heat-inactivated lysate + L-DOPA | Tests non-enzymatic/background signal |
| AADC lysate + no PLP | Tests PLP-dependent activity |
| Purified/semi-purified AADC positive control | Confirms assay is working |

# Positives-negatives

- Higher specificity due to the 1:1 stoichiometry between CO2 release and NADH oxidation
- Minimized background by perfoming assay under nitrogen atmosphere (improved signal-to-noise from 2.5 to 8.1)
- Good repeatability – consistency
- Good sensitivity – allows usage of 20-600 nM concentrations

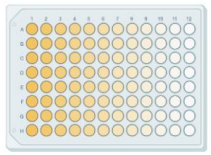
- Very important to be conducted under nitrogen atmosphere as atmospheric CO2 significantly increases noise
- Buffer composition need to remain similar to the commercial linking system formulation to maintain performance of the linking enzymes
- Other redox reactions can give false-positives by interfering with NADH

# Catalytic activity assay

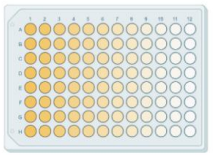


1. **Transformation**

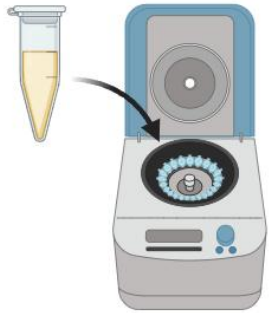
2. **Colony Selection**/
Antibiotic



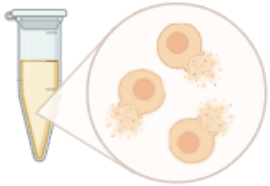
3. **Preculture**
200 uL LB, Antiobiotic,
30°C O/N



4. **Culture**
200 uL LB, Antiobiotic,
30°C for 3h,
IPTG 0.1 mM



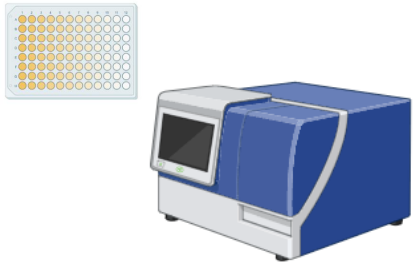
5. **Centrifuge**,
3500 rpm, 4°C for 35 min



6. **Cell lysis**,
0.1 mM of PLP, 1 g/L
lysozyme, 0.1 g/L of DNAse I,
Incubation at 37°C for 1h,
freeze/thaw cycles

7. **Colorimetric**,
50 mM Potassium phosphate,
0.1 mM of PLP, lysate, HRP 64
U/mL, Luminol 1 mM, AMO
0.5 U/mL

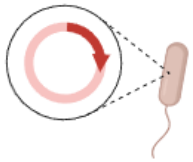
Incubation at 37°C
for 15 min



8. **Absorbance** at **425 nm**

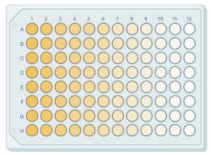
Higher absorbance = higher dopamine production.

# Thermostability assay

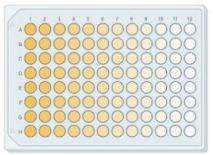


1. **Transformation**

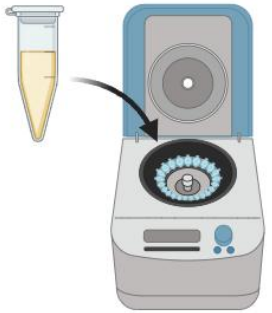
2. **Colony Selection**/
Antibiotic



3. **Preculture**
200 uL LB, Antiobiotic,
30°C O/N



4. **Culture**
200 uL LB, Antiobiotic,
30°C for 3h,
IPTG 0.1 mM



5. **Centrifuge**,
3500 rpm, 4°C for 35 min

6. **Cell lysis**,
0.1 mM of PLP, 1 g/L
lysozyme, 0.1 g/L of DNAse I,
Incubation at 37°C for 1h,
freeze/thaw cycles

Before thermostability screening:
measure baseline activity

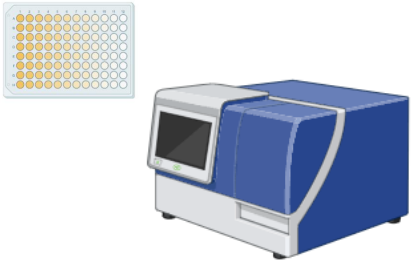
7. **Incubation**

- At 45°C for 30 min
- At 55°C for 30 min
- At 65°C for 30 min

8. **Ice bath**,
for preventing aggregation and denaturation

7. **Colorimetric**,
50 mM Potassium phosphate,
0.1 mM of PLP, lysate, HRP 64 U/mL, Luminol 1 mM, AMO 0.5 U/mL

Incubation at 37°C
for 15 min



8. **Absorbance** at **425 nm**

# Limitations of protocols

- Possibility of dopamine autooxidation
- Heat often causes apoenzyme formation because of PLP dissociation
- False positives because of protein expression level
- Light sensitivity of Amplex Red
- Validate hits with purified enzyme

# References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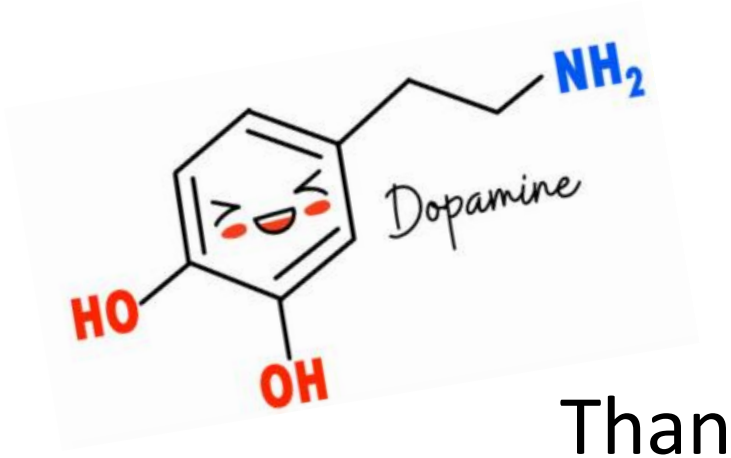


Thank you for your attention!