Τμήμα Χημείας
Πανεπιστήμιο Κρήτης
Department of Chemistry
University of Crete

High-throughput screening assay

3rd Seminar

Πρωτεϊνική Μηχανική

Διδάσκων Καθηγητής: Ιωάννης Παυλίδης

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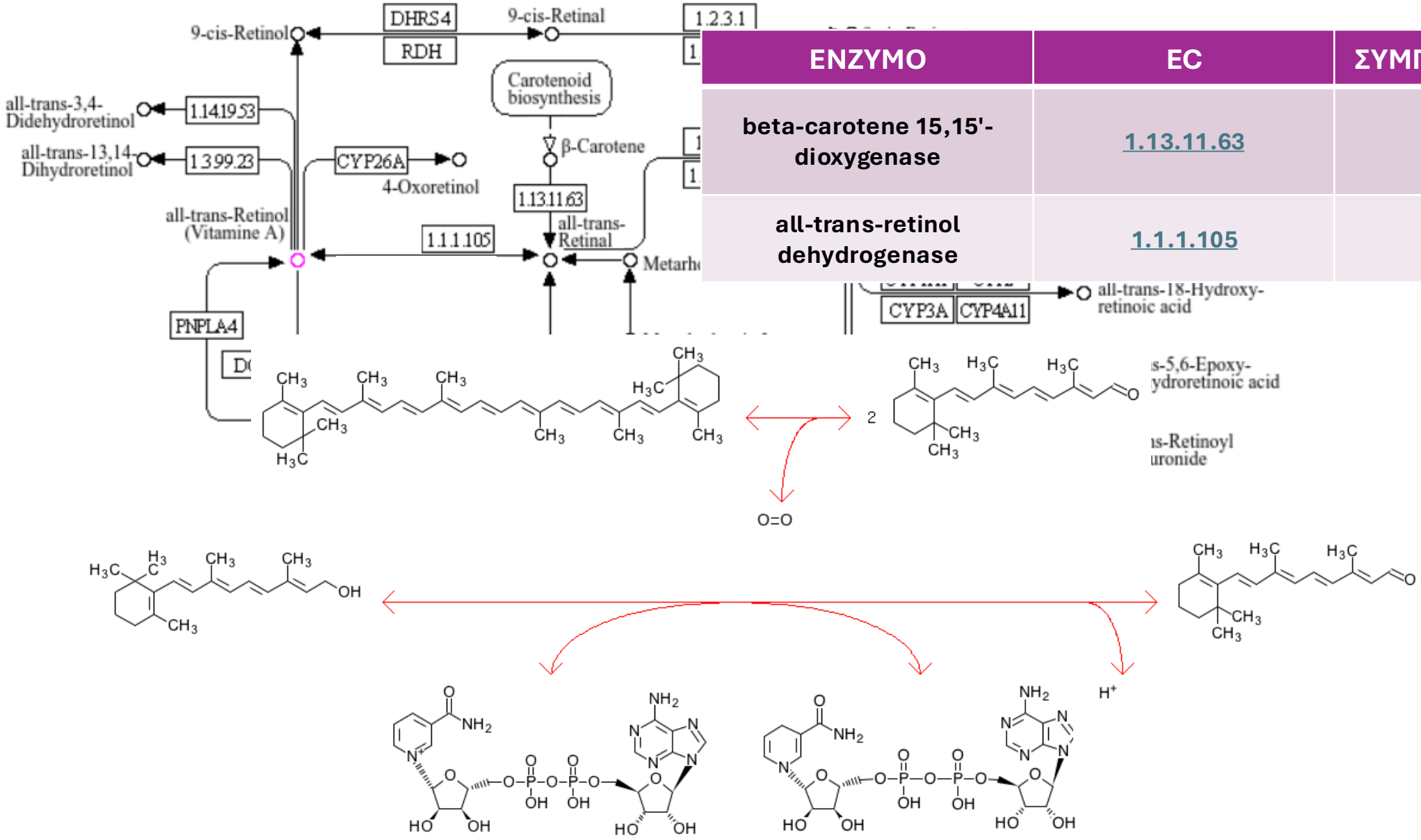
Ντόκο Αριστέα (ΑΜ:1321)

Μάιος 2026, Ηράκλειο

Μεταβολισμός Ρετινόλης



ΕΝΖΥΜΟ	EC	ΣΥΜΠΑΡΑΓΟΝΤΑΣ
beta-carotene 15,15'-dioxygenase	1.13.11.63	Fe^{2+}
all-trans-retinol dehydrogenase	1.1.1.105	NAD^{+}

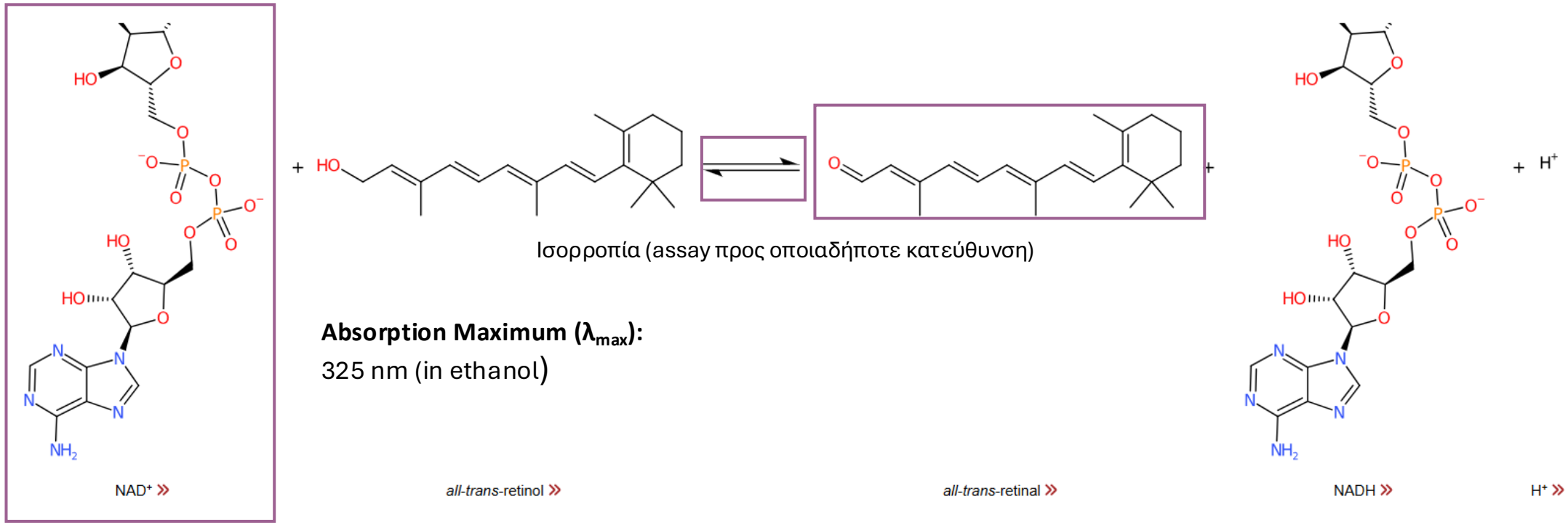
Μεταβολισμός Ρετινόλης - all-trans-retinol dehydrogenase

Επιλογή αντίδρασης του ενζύμου 1.1.1.105 καθώς υπάρχουν περισσότερα βιβλιογραφικά HTS assays

Kegg Reaction: R02124

RHEA: 21287

all-trans-retinol + NAD(+) <=> all-trans-retinal + NADH + H(+)



Ισορροπία (assay προς οποιαδήποτε κατεύθυνση)

Absorption Maximum (λ_{max}):

325 nm (in ethanol)

Συμπαράγοντας - ανίχνευση

λ_{max} ~ 380 nm (visible range – violet -> πολύ οριακό)

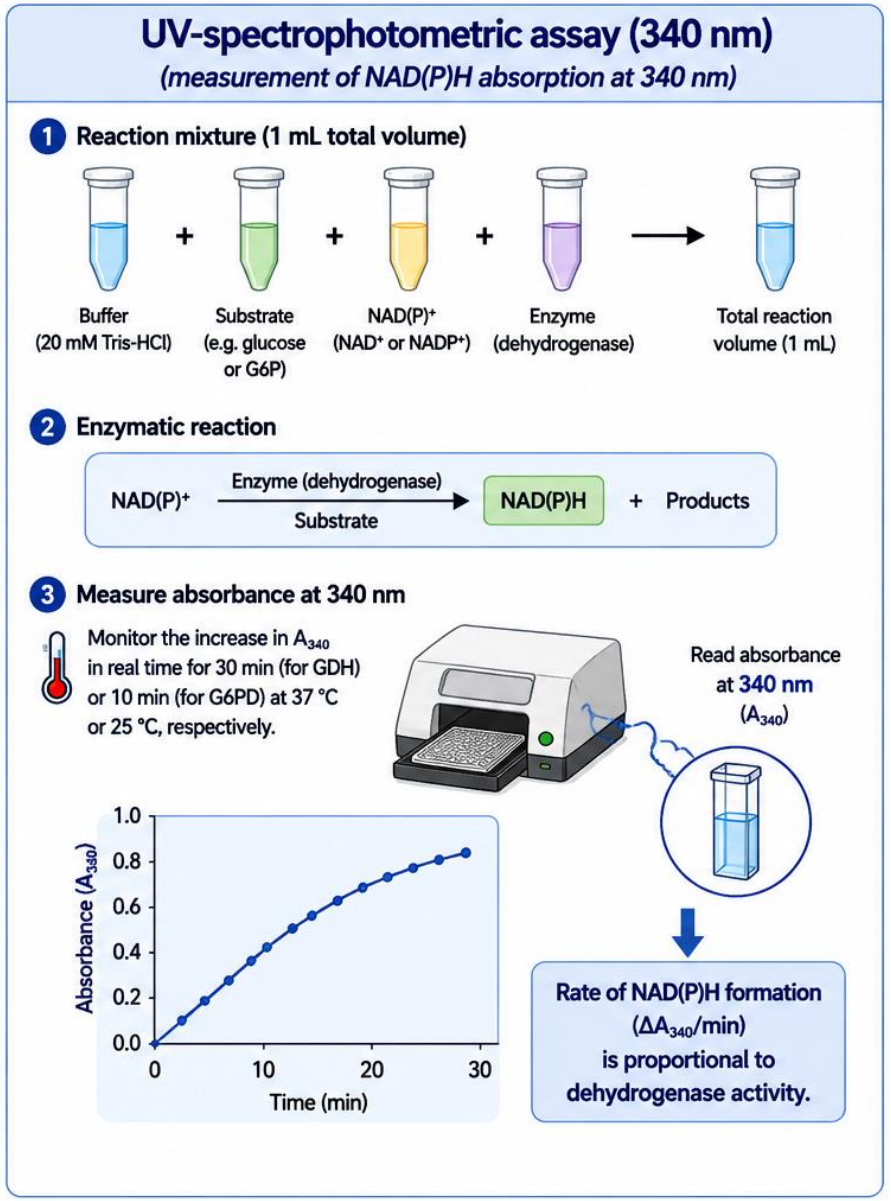
High Throughput Assay – Enzymatic Activity

Απορρόφηση του NADH/NADPH στα 340 nm (UV spectroscopy)

- ✓ Άμεση απορρόφηση NADH/NADPH στα 340 nm
- ✓ Linear range : 3.4–400 nmol
- ✓ Καλύτερο για μεγάλες ποσότητες χωρίς dilution.
- ✓ Real-time continuous

- Δεν είναι αρκετά ευαίσθητη σε μικρές ποσότητες NAD(P)H
- Δεν είναι ιδανική για high-throughput screening, αργή.
- Παρεμβολές (σε βιολογικά δείγματα hemoglobin, χολερυθρίνη , ανόργανες ενώσεις όπως φώσφορος, νικέλιο, χρώμιο)

Και οι δύο είναι αναπαραγώγιμες, αλλά το WST-8 θεωρείται εξαιρετικό για screening.

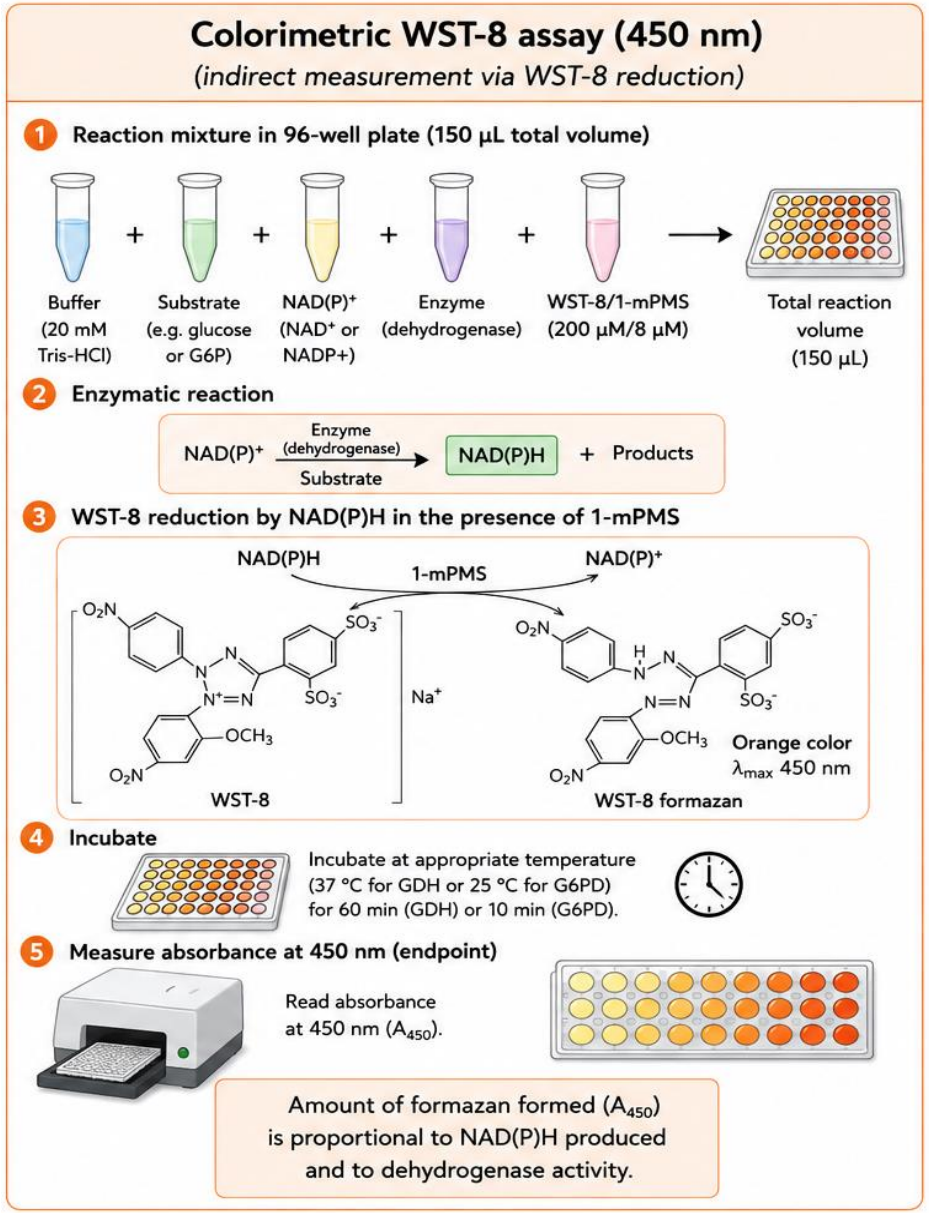


High Throughput Assay – Enzymatic Activity

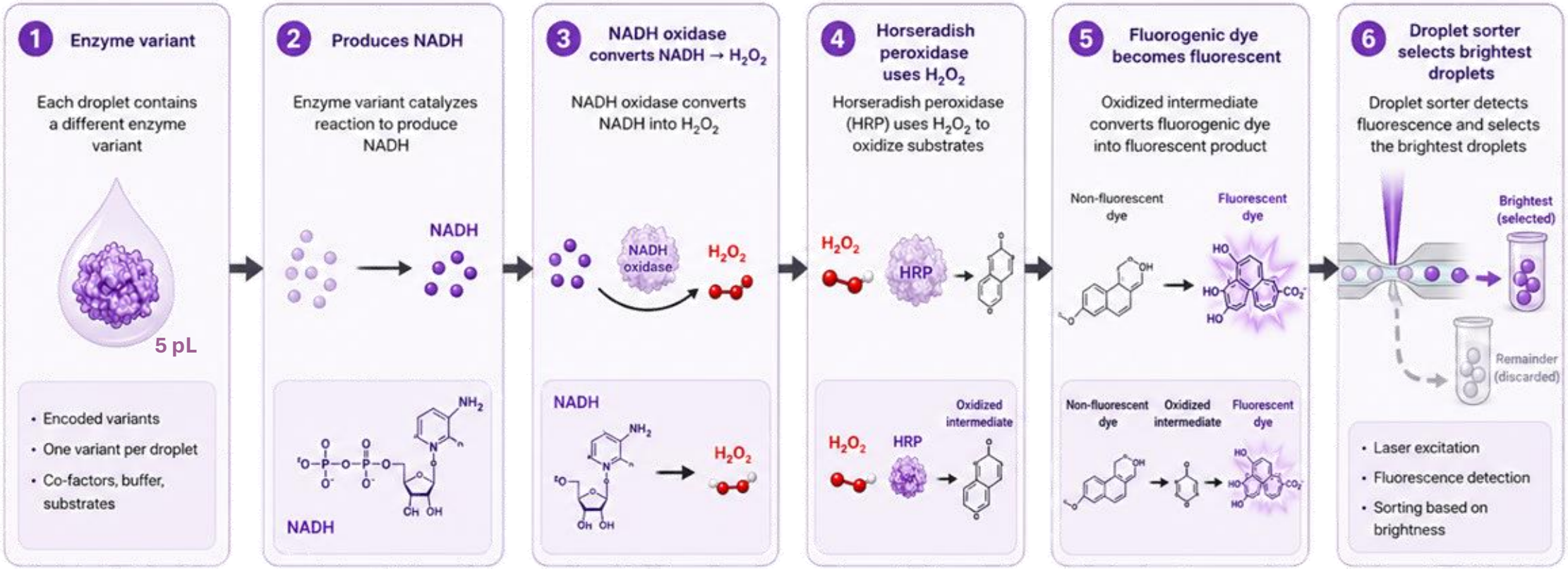
WST-8 colorimetric assay

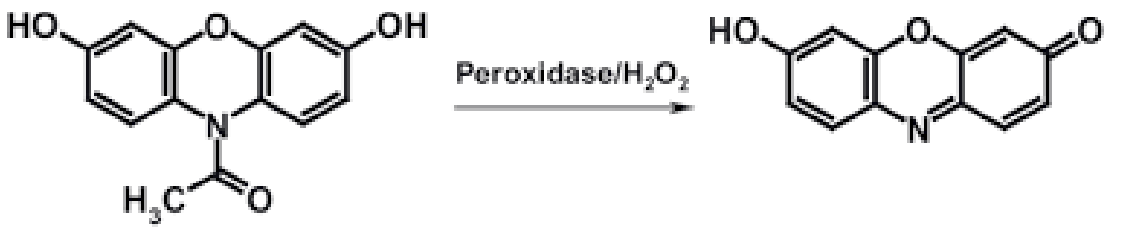
- ✓ Πολύ πιο ευαίσθητη μέθοδος
  Ανιχνεύει πολύ μικρές ποσότητες NADH
- LOD = 0.32 nmol (Colorimetric WST-8)
- LOD= 1.65 nmol (UV μέθοδος)
  5 φορές μεγαλύτερη ευαισθησία
- ✓ Πολύ καλό Z' factor (0.9–0.99)
- ✓ Κατάλληλη για High Throughput Screening
- ✓ Συμβατή με screening πολλών συνθηκών / υποστρωμάτων
- ✓ ~$0.35/reaction
- ✓ Το WST-8 μπορεί να αποθηκευτεί στους 4°C έως και 1 χρόνο.

- Μικρό linear range : 0.4–19 nmol NAD(P)H (Άρα σε υψηλές συγκεντρώσεις χρειάζονται dilutions)
- Endpoint assay
- Συχνά χρειάζεται incubation αρκετών λεπτών
- Παρεμβολές από reducing agents (DTT, β mercaptoethanol)
- Fructose interference (Background Signal)



NAD(P)H Detection Assay – Droplet Based Microfluidics





Amplex Red
10-acetyl-3,7-dihydroxyphenoxazine
(Non-fluorescent)

Resorufin
7-hydroxy-3H-phenoxazine-3-one
(Fluorescent)

Z-factor = 0.822
NADH oxidase: SmNOX
Cell lysate
Amplex UltraRed → Resorufin
(Ex/Em = 568 nm/ 581 nm)

FADS: Workflow Overview

From Cell Preparation to Sorted, Active Variants

3 Master Mix
(Dispersed Phase)

Component	Concentration
HRP	4 U mL^{-1}
Lysozyme	2 mg mL^{-1}
Polymyxin	4 mg mL^{-1}
Amplex UltraRed	0.4 mM
EGTA	0.01 mM
EDTA	0.02 mM
Potassium phosphate pH 7.8	50 mM
SmNOX	30 % (v/v)
Substrate	As needed
NAD	5 mM
H_2O	To desired volume

2,4-DNPH Colorimetric Assay for Ketone & Aldehyde Detection

Kozaeva et al., 2022 | Microbial Biotechnology

PRINCIPLE

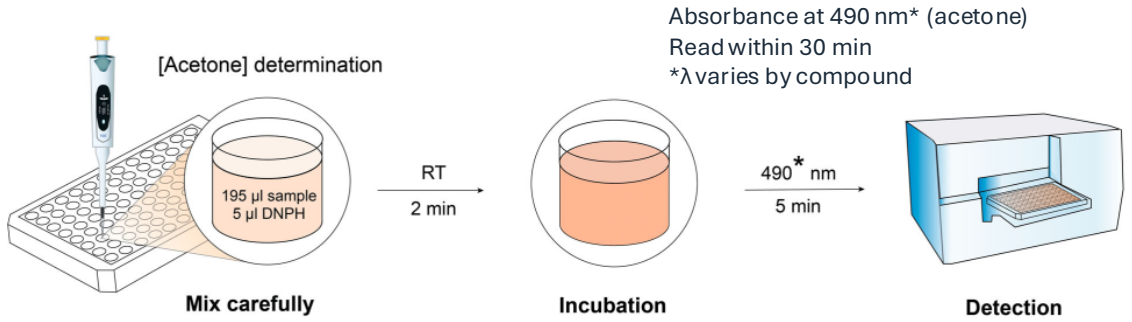
2,4-DNPH contains a benzene ring, two nitro groups, and a hydrazine group.
It reacts with the **carbonyl group (C=O)** of ketones and aldehydes under acidic conditions (H_3PO_4), forming a coloured **2,4-dinitrophenylhydrazone** adduct (yellow → orange → red).

Does NOT react with: amides, esters, carboxylic acids, or alcohols → selective in fermentation broth.

KEY CONDITIONS & REAGENTS

Reagent:	200 mM 2,4-DNPH in H_3PO_4 (Sigma-Aldrich)
Volume ratio:	195 µL sample : 5 µL reagent
Detection range:	25–500 mg/L (Milli-Q) | 10–200 mg/L (LB medium)
Solubility note:	Low solubility at neutral pH → read within 30 min to avoid precipitation
Matrix effect:	LB medium narrows range but does not prevent quantification

PROTOCOL (96-WELL FORMAT)



Bioproduction of acetone in Ecoli with biological or synthetic pathway

Cell Supernatant Preparation:

Transformed Ecoli Cultures with vectors (rhamnose inducible expression):

pSEVA4318 (control) pS4318MKc pS4318MKs

- 24h fermentation in 10 mL minimal M9 medium (10 g/L glucose, streptomycin 100 µg/mL, 1 mM rhamnose)/LB medium, 200 rpm
- Cool at 4 °C for 1h + centrifuge 5000 g (15 min)

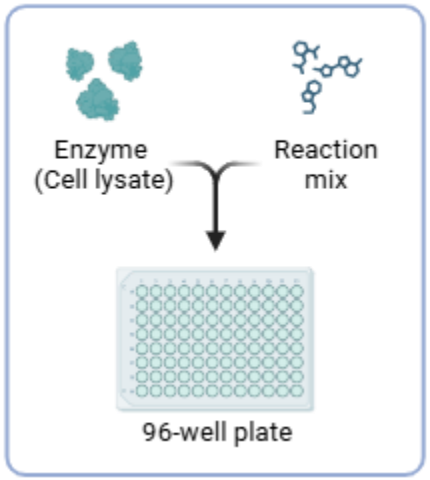
Cell density: OD_{600}= 0.05 (dilute in fresh medium)

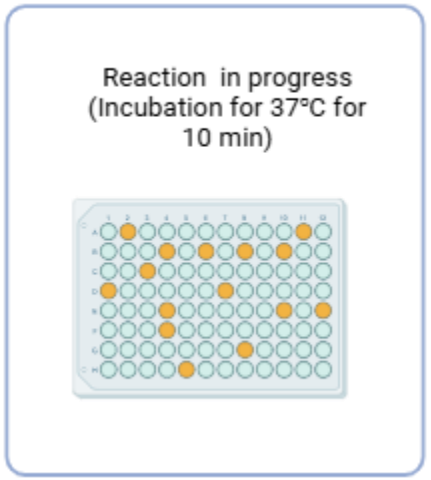
NAD(P)H Detection Assay for all-trans-retinol dehydrogenase

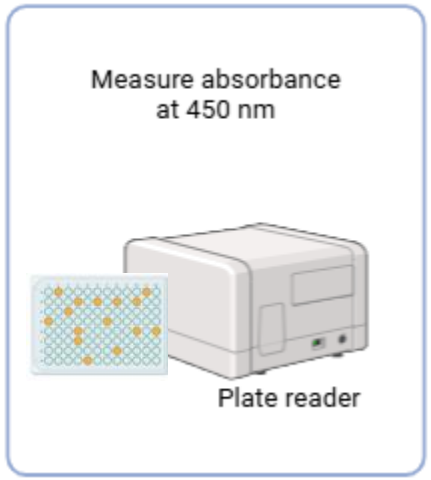
Conditions	Values
Buffer Tris-HCl or Sodium Phosphate	10-20 mM pH = 7.4
Substrate All-trans retinol	250 mM (10x km)
Cofactor NAD^+	1 mM
Enzyme all-trans-retinol dehydrogenase	• Crude cell lysate [E] = 20-50 µg/ well • Purified enzyme [E] = 0.2-1.0 µg/ well
WST-8 (Tetrazolium salt)	200 µM
1-mPMS (electron mediator)	8 µM
Total Volume (per well)	150 µL

Control	Purpose
(-) NAD^+	non-specific reduction
(-) enzyme	spontaneous retinol oxidation
(-) substrate	NADH-independent signal
Blank buffer	Plate background

- Incubation: at 32°C -37°C for 10-30 min
- Absorbance at 450 nm (measurement)
- 96-well plate
- Reaction/Storage under dark conditions
- All-trans retinol stock in DMSO (≤1%)
- Use of detergent: Triton-X (no interfernece)



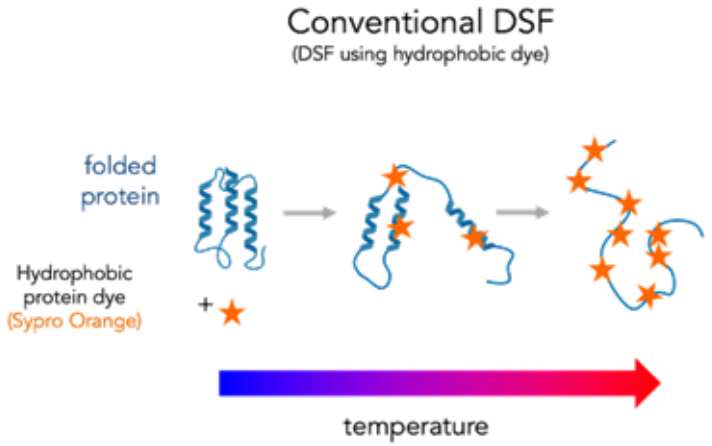


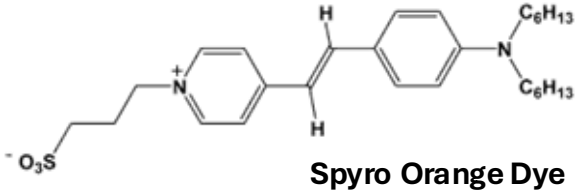


Thermofluor Assay - Differential Scanning Fluorimetry (DSF)

DSF Principle

Folded protein → Partial unfolding → Unfolded state
Hydrophobic regions become exposed
Sypro Orange binds exposed hydrophobic patches
Fluorescence increases during thermal denaturation
Tm = midpoint of protein unfolding transition

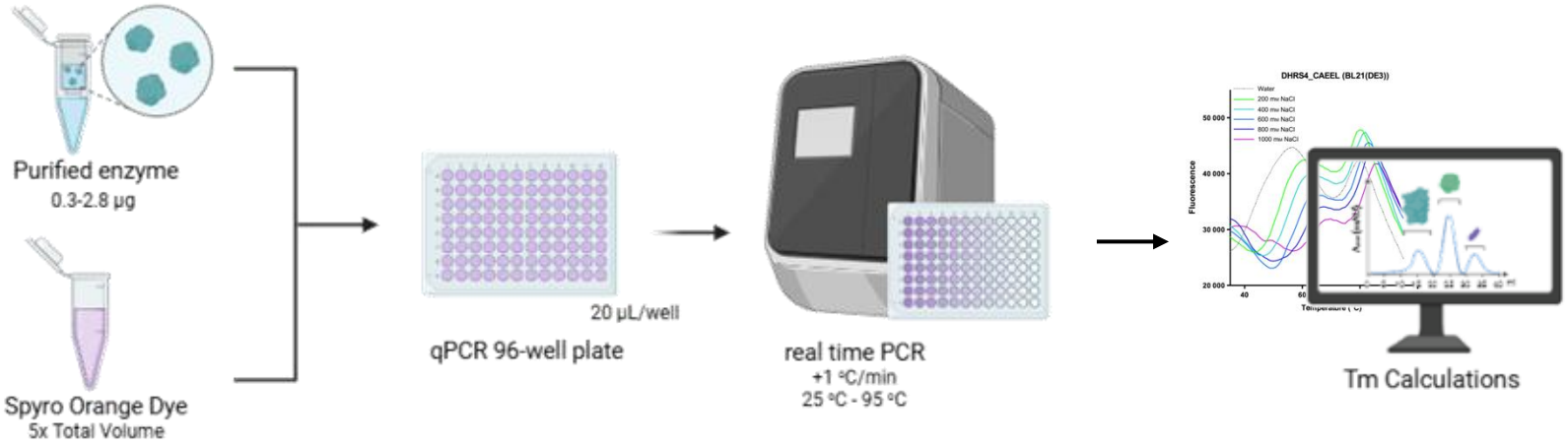




- Simple, fast screening method
- High-throughput assay

Experimental Setup

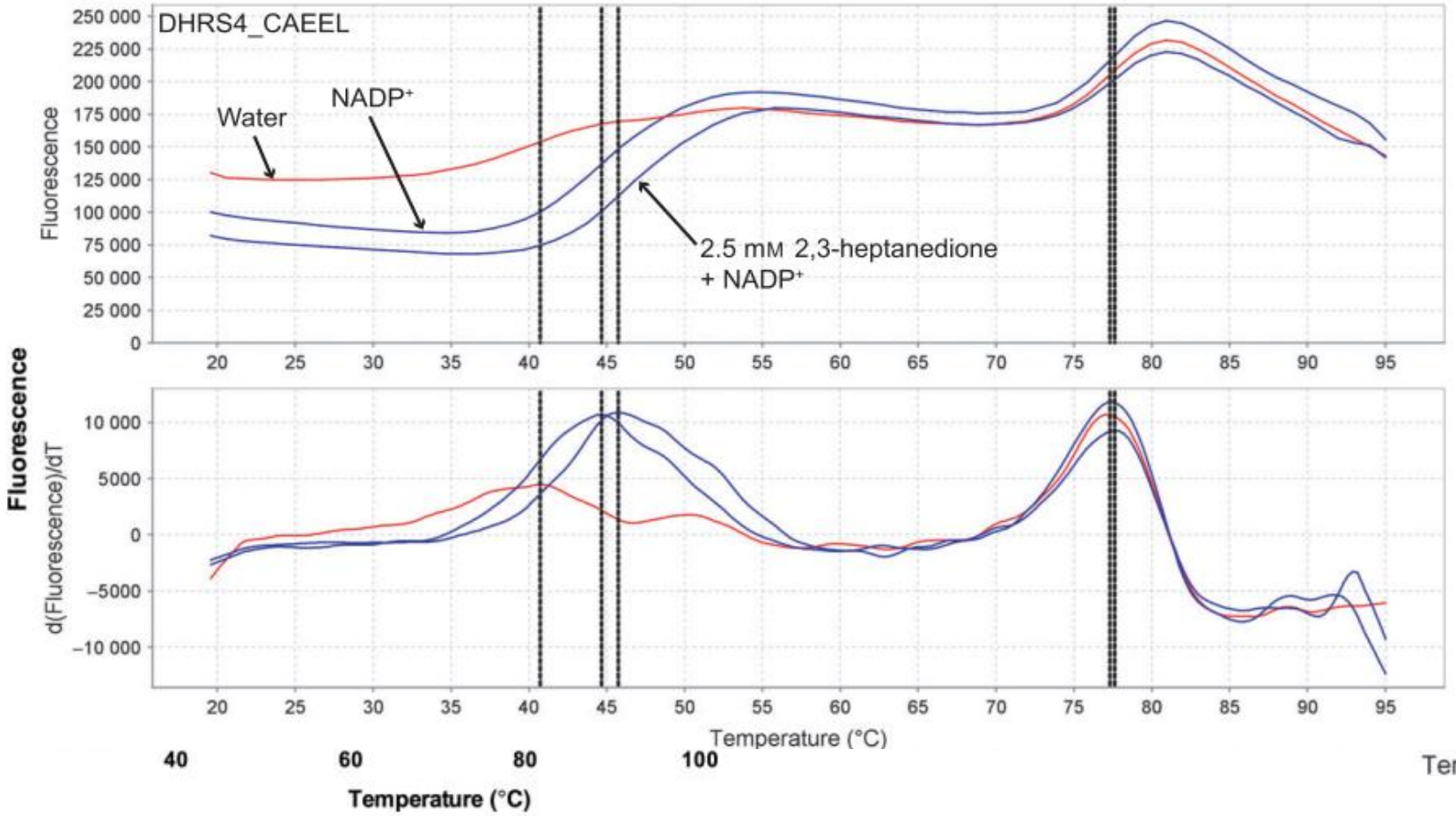
Protein: purified enzyme
Protein amount: 0.3–2.8 µg
Volume: 20 µL/well
Dye: 5× Sypro Orange
Plate: 96-well qPCR
Ramp: +1 °C/min
Range: 25–95 °C (apprx.)

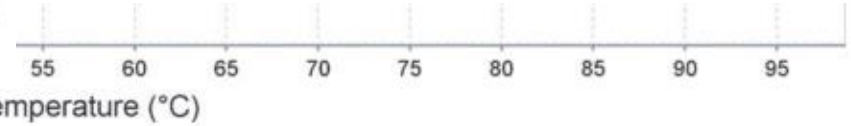


Simultaneous screening of (de)stabilizing buffer/salt conditions (shift thermal stability)
Application: identification of optimal conditions for: storage, crystallization experiments, purification

Kisiela, M., Faust, A., Ebert, B., Maser, E. and Scheidig, A.J. (2018), Crystal structure and catalytic characterization of the dehydrogenase/reductase SDR family member 4 (DHRS4) from Caenorhabditis elegans. FEBS J, 285: 275-293. https://doi.org/10.1111/febs.14337

Thermofluor Assay - Differential Scanning Fluorimetry (DSF)





Kisiela, M., Faust, A., Ebert, B., Maser, E. and Scheidig, A.J. (2018), Crystal structure and catalytic characterization of the dehydrogenase/reductase SDR family member 4 (DHRS4) from Caenorhabditis elegans. FEBS J, 285: 275-293. https://doi.org/10.1111/febs.14337

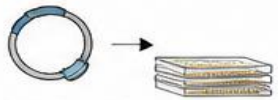
HIGH-THROUGHPUT DSF (THERMOFLUOR) WORKFLOW USED IN AALBERS et al., eLIFE 2020

1 Computational prescreening (FRESCO)

- All possible single mutations analyzed (4,503 total)
- Selection: $\Delta\Delta G_{fold} \leq -5$ kJ/mol
- MD simulations (YASARA) and manual filtering

177 mutations selected

2 Library construction & expression



Site-directed mutagenesis (QuikChange)

Expression in *E. coli*
NEB 10-beta
Vector: **pBAD**
N-terminal His_6-tag
Cultivation:
- 1 mL TB medium / well
- 5% (v/v) overnight preculture
- Induction: **arabinose**
- **38 h, 24 °C, 250 rpm**
- **4 × 96-deepwell plates**

3 Cell lysis

Resuspend cells in 50 mM Tris-HCl pH 7.5

(volume ~1 mL)

4 Purification

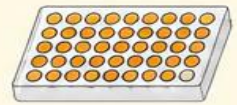
His-tag purification (Ni-NTA)

Wash buffer:
50 mM Tris-HCl pH 7.5,
5 mM imidazole

Elution buffer:
50 mM Tris-HCl pH 7.5,
300 mM imidazole

Desalting / buffer exchange **into 50 mM Tris-HCl pH 7.5**

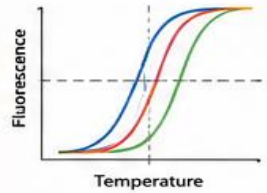
5 DSF / ThermoFluor screening



177 purified mutants screened in duplicate using DSF

Instrument:
BioRad CFX96 Touch Real-Time PCR system (FRET mode)

6 Data analysis



Determine apparent T_m (melting temperature) as midpoint of unfolding transition

Calculate ΔT_m relative to wild type (WT)

7 Hit selection & combination

Select stabilizing and active single mutants

Combine beneficial mutations stepwise (M2 → … → M9)

Final optimized mutant: M9* (ΔT_m = +45 °C)

DSF ASSAY (ThermoFluor) – Experimental details

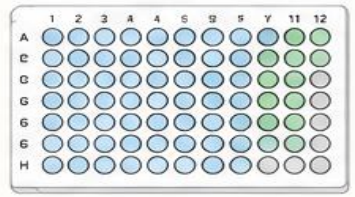
SAMPLE MIX PER WELL (total volume: 20 µL)

Component	Final conc.
Purified enzyme (mutant)	~0.2 mg/mL (≈ 2–4 µg)
SYPRO™ Orange dye (5,000× stock in DMSO)	1× (1 µL)
50 mM Tris-HCl pH 7.5	q.s. to 20 µL

Notes:
- SYPRO Orange working solution: dilute 5,000× stock 1:5,000 in buffer to obtain 1× dye solution.
- Protein concentration range typically 0.05–0.5 mg/mL for DSF.

PLATE SETUP

96-well PCR plate (e.g., BioRad hard-shell)



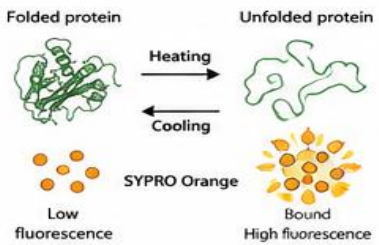
- Mutant (duplicate)
- WT control (duplicate)
- Buffer + dye (no protein)

THERMOCYCLER PROGRAM

Step		Condition
1	Initial hold	25 °C for 2 min
2	Temperature ramp	25 °C → 95 °C
	Ramp rate	1.0 °C/min (continuous acquisition)
3	Fluorescence read	FRET mode (excitation ~485 nm / emission ~530 nm)
4	Final hold	95 °C for 1 min

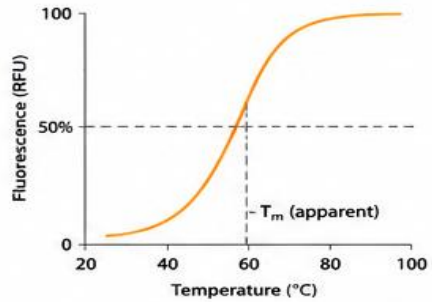
Instrument: BioRad CFX96 Touch Real-Time PCR system (FRET mode)

PRINCIPLE



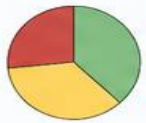
Binding of SYPRO Orange to exposed hydrophobic surfaces upon unfolding results in increased fluorescence.

OUTPUT



SCREENING RESULTS (from paper)

- 177 mutants screened in duplicate
- 26 gave no reliable **signal** (expression / low yield)
- **151** mutants **evaluated**



- 56 stabilizing (ΔT_m > +1 °C)
- 52 neutral (−1 ≤ ΔT_m ≤ +1 °C)
- 43 destabilizing (ΔT_m < −1 °C)

25 mutants with $\Delta T_m \geq +3$ °C → activity counter-screen → 10 beneficial + active

COMBINATORIAL ASSEMBLY

Stepwise combination of 10 mutations

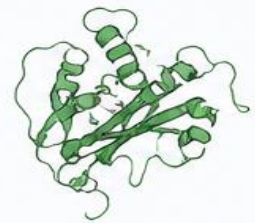
Additive increase in T_m

Theoretical: **+56.5 °C** Observed (M9): **+51.5 °C**

FINAL OUTCOME

M9* (without S197E)

- Apparent T_m = 88 °C (ΔT_m = +45 °C vs WT)
- Activity restored close to WT (k_{cat} and $K_{M,NADP+}$ ~ WT)
- Half-life at 37 °C:
  WT ~ 2 h | **M9* ~ 11 days**

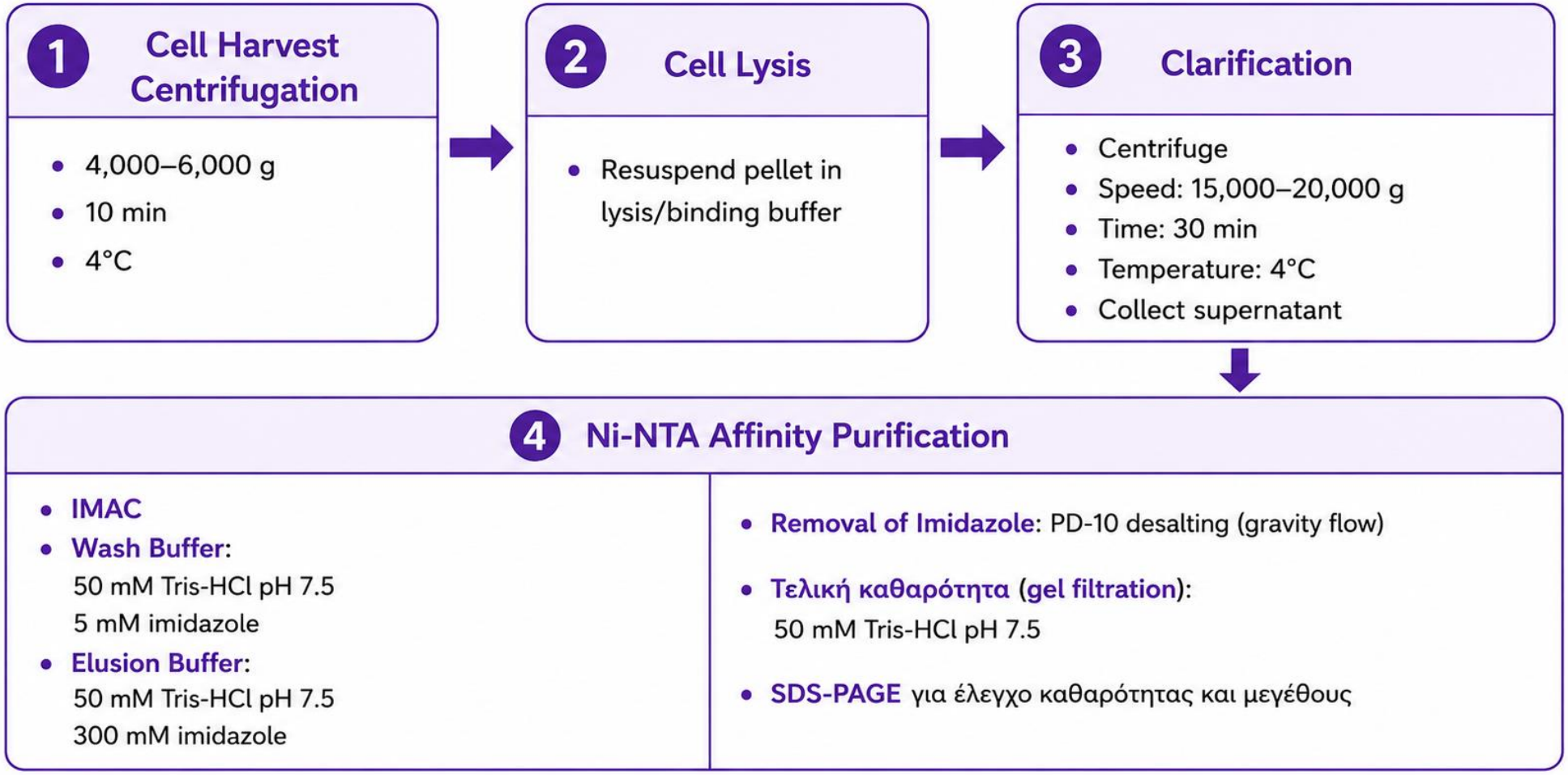


Buffers used throughout: 50 mM Tris-HCl, pH 7.5 | All DSF measurements in duplicate | Total protein per well: ~2–4 µg in 20 µL

Cell Lysis Buffer
50 mM Tris-HCl (pH 7.5)
1 mM DTT
5% έως 10% glycerol
1 mM έως 10 mM MgCl2
+ 0.25 mg/mL λυσοζύμης

Καθαρισμός Mutants πριν το HTS-DSF

EC 1.1.1.105 (all-trans-retinol dehydrogenase)



HT-DSF / ThermoFluor Assay Protocol - EC 1.1.1.105 (all-trans-retinol dehydrogenase)

THERMOCYCLER PROGRAM
(Real-Time PCR – FRET mode)

Instrument:
BioRad CFX96 Touch
Real-Time PCR System
(FRET mode)

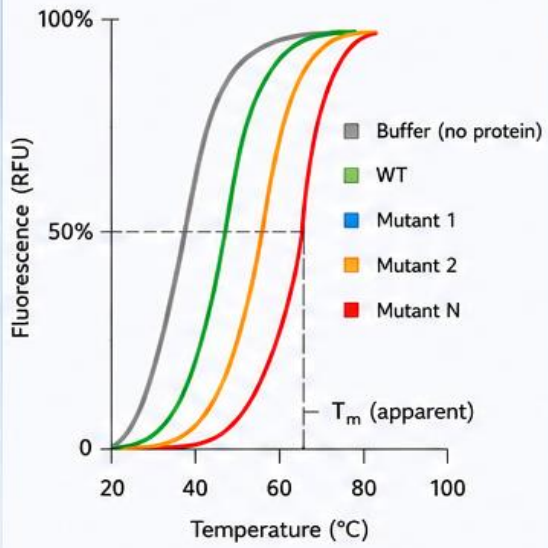
Step	Temperature (°C)	Time	Rate
1 Initial hold	25	2 min	–
2 Temperature ramp	25 → 95	Continuous	~1.0 °C/min
3 Fluorescence read (FRET mode) Ex 485 nm / Em 530 nm	–	–	–
4 Final hold	95	1 min	–

Lid temperature: 95 °C

Total run time: ~70–80 min

DATA ANALYSIS & OUTPUT

1. Raw fluorescence curves



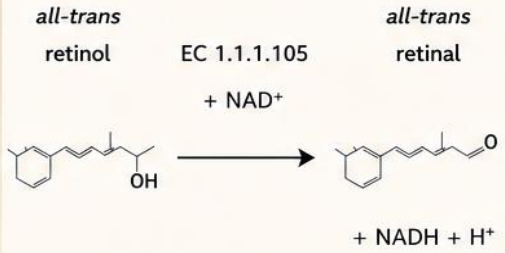
2. Determine T_m (apparent)

T_m = temperature at 50% unfolding (midpoint)

3. Calculate ΔT_m

$\Delta T_m = T_m(\text{mutant}) - T_m(\text{WT})$

ACTIVITY COUNTER-SCREEN
(Retinol dehydrogenase activity assay)



EC:1.1.1.105 :

- Χρησιμοποιεί NAD+/NADH (co-factor)
- Μεμβρανικής φύσης RDH isoform (μπορεί να παρουσιάζει aggregation χωρίς ήπιο detergent)
- Αλληλεπιδρά με retinoids που έχουν υψηλή ευαισθησία στο φως και στην οξείδωση

Buffer DSF
50 mM Tris-HCl pH 9
150 mM NaCl
5% glycerol
0.005% DDM

Cofactor
0.1–0.5 mM

Total volume :
20 μL

Enzyme Concentration
0.1–0.5 mg/mL
Ίσως : 0.2 mg/mL

Dye SYPRO Orange
Commercial stock:
5000×
Assay: 1x (1μl)

Τα plates καλύπτονται με foil για προστασία από φως και οξείδωση

Βιβλιογραφία

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https://doi.org/10.7554/eLife.54639

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Thank you for your attention!