A robust bacterial high-throughput screening system to evaluate single nucleotide polymorphisms of human homogentisate 1,2-dioxygenase in the context of alkaptonuria

Created on: 09-12-2022 - Last modified on: 06-12-2023

Contact person

Sien Lequeue

Organisation

Name of the organisation Vrije Universiteit Brussel (VUB)

Department In Vitro Toxicology and Dermato-Cosmetology

Country Belgium

Partners and collaborations

RWTH Aachen

SCOPE OF THE METHOD

The Method relates to	Human health
The Method is situated in	Translational - Applied Research
Type of method	In vitro - Ex vivo

DESCRIPTION

Method keywords

High-throughput

Screening assay

Escherichia coli

Missense mutations
Pyomelanin
Robust system
Simple
Cost-effective

Scientific area keywords

Alkaptonuria
Humans
Homogentisate dioxygenase
Homogentisic acid
Rare disease
Tyrosine degradation pathway
Ochronosis
inborn error of metabolism

Method description

Alkaptonuria (AKU) is a rare inborn error of metabolism caused by a defective homogentisate 1,2-dioxygenase (HGD), an enzyme involved in the tyrosine degradation pathway. Loss of HGD function leads to the accumulation of homogentisic acid (HGA) in connective body tissues in a process called ochronosis, which results on the long term in an early-onset and severe osteoarthropathy. HGD's quaternary structure is known to be easily disrupted by missense mutations, which makes them an interesting target for novel treatment strategies that aim to rescue enzyme activity. However, only prediction models are available providing information on a structural basis. Therefore, an E. coli based whole-cell screening was developed to evaluate HGD missense variants in 96-well microtiter plates. The screening principle is based on HGD's ability to convert the oxidation sensitive HGA into maleylacetoacetate. More precisely, catalytic activity could be deduced from pyomelanin absorbance measurements, derived from the auto-oxidation of remaining HGA. Optimized screening conditions comprised several E. coli expression strains, varied expression temperatures and varied substrate concentrations. In addition, plate uniformity, signal variability and spatial uniformity were investigated and optimized. Finally, eight HGD missense variants were generated via site-directed

mutagenesis and evaluated with the developed high-throughput screening (HTS) assay. For the HTS assay, quality parameters passed the minimum acceptance criterion for Z' values > 0.4 and signal window values > 2. We found that activity percentages versus wildtype HGD were 70.37 ± 3.08% (for M368V), 68.78 ± 6.40% (for E42A), 58.15 ± 1.16% (for A122V), 69.07 ± 2.26% (for Y62C), 35.26 ± 1.90% (for G161R), 35.86 ± 1.14% (for P230S), 23.43 ± 4.63% (for G115R) and 19.57 ± 11.00% (for G361R). To conclude, a robust, simple, and cost-effective HTS system was developed to reliably evaluate and distinguish human HGD missense variants by their HGA consumption ability. This HGA quantification assay may lay the foundation for the development of novel treatment strategies for missense variants in AKU.

Lab equipment

- Biosafety cabinet,
- Plate reader,
- Shaking incubator.

Method status

Published in peer reviewed journal

PROS, CONS & FUTURE POTENTIAL

Advantages

In the first place, it provides a tool to fill the knowledge gap on the estimation of residual enzyme activities of AKU-causing missense variants based on their intrinsic activity to prevent the formation of pyomelanin. The latter is of high clinical relevance for AKU since its progression is characterized by the formation of ochronotic pigment leading to its disease outcome. Secondly, the assay may contribute to the identification and development of novel treatment strategies for selected variants in AKU. Finally, the method is fast, cost-effective, reliable and simple.

Challenges

The system is in the first place designed in bacteria and not in human cell lines and can therefore not be considered as the real-life situation. This indicates that the found residual enzyme activities can differ from what is estimated in AKU patients.

Moreover, higher HGA concentrations were used compared to what is found in AKU patients. Although, if lower HGA concentrations would be used, assay quality parameters would tremendously decrease which will eventually result in a poor assay quality. Additonally, the method is not suitable to estimate the effect of compound heterozygosity in AKU patients as in bacteria it is not possible to express and evaluate 'hybrid hexamers'.

Modifications

The method should not further be optimized and there are no future changes planned.

Future & Other applications

Our newly developed high-throughput screening assay provides a tool to study the effect of individual substitutions on the HGA consumption ability as well as the effect of enzyme activity rescuing molecules to develop optimized treatments for selected variants in AKU.

REFERENCES, ASSOCIATED DOCUMENTS AND OTHER INFORMATION

References

Lequeue, S., Neuckermans, J., Nulmans, I. et al. A robust bacterial high-throughput screening system to evaluate single nucleotide polymorphisms of human homogentisate 1,2-dioxygenase in the context of alkaptonuria. Sci Rep 12, 19452 (2022). https://doi.org/10.1038/s41598-022-23702-y

Associated documents

s41598-022-23702-y.pdf