**Antioxidant Capacity Assay Optimization**

2023-01-12

Brian & Jack

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Reagent** | **Solvent** | **Catalog** | **Vendor** | **Quantity** | **Cost** |
| Dihydrorhodamine 123 | DMSO | D1054-2MG | Sigma | 2 mg | $97.62 |
| [HEPES (20 mM HEPES, 150 mM NaCl, pH 7.4)](http://protocol-place.com/basic-lab-techniques/stock-solutions/hepes-stock-solution-0-1-m-ph-7-4/) |  |  |  |  |  |
| HEPES (For buffer) | DI water | H3375-25G | Sigma | 25 g | $29.00 |
| Sodium hydroxide Pellets (For Increasing pH buffer) | DI water | S5881-500G | Sigma | 500 g | $41.53 |
| NaCl (We have this in lab) | DI water | NA | NA | NA | NA |
| pH-indicator strips pH 2.0 - 9.0 | N/A | 1.096E+09 | Sigma | 100 strips | $33.15 |
| Chelex 100 (Remove Iron from Buffer) | N/A | C7901-25G | Sigma | 25 g | $94.02 |
| 1 mM 2,2′-azobis-2-methyl-propanimidamide-dihydrochloride | DMSO/Water/ethanol | 440914-25G | Sigma | 25 g | $49.59 |
| DMSO | N/A | D8418-50ML | Sigma | 50 mL | $59.38 |
|  |  |  |  | **Total** | **$404.29** |

**Objective**: To determine whether the antioxidant assay modified from [Kelesidis et al. 2011](https://doi.org/10.1194/jlr.D018937) (also adopted in Marsche’s paper) is compatible with our **isolated HDL**, HDL isolated by sequential flotation ultracentrifugation followed by SEC using our FPLC. The assay is Dihydrorhodamine 123 (DHR)-based and cell-free to measure HDL function.

**Requirement:**

1. 96-well plate (Flat Bottom, black, or oval black bottom)
2. Microplate reader compatible with fluorescence and kinetics
3. Everything else on the above table

**Sample concentration to measure:**

1. HDL starting concentration - 3100 ug/ml or 3.1ug/ul
   1. 1ug, 5ug, 10ug
2. ApoB:
   1. 2%, 5%
3. Negative Control:
   1. No sample

**Method**

**We are going to make enough for 30 replicates, having extra aliquots is always better if possible.)**

**DHR Dilution and amount needed for assay (Make enough for 30 samples**

1. DHR diluted in DMSO to 5.8 mM stock
   1. 2mg of DHR (346.38 g/mol)
   2. Dilute 2mg DHR in 1 mL of DMSO
2. 5800\*x = 175\*10
   1. X = 0.3 uL DHR/sample
3. 0.3\*30 = 9 uL of DHR stock
4. 30 replicate\*25 uL= 750uL Total Volume

**Azobis Dilution and amount needed for assay**

1. **Azobis diluted in WATER to 500mM Stock**
   1. 271.19mg in 2mL WATER
   2. 500mM \* x = 175uL \* 1mM
      1. x = 0.35 uL
   3. Azobis needed: 0.35 \* 30samples = 10.5 uL

**Buffer needed to dilute Azobis and DHR**

1. **750 uL – 9 uL – 10.5 uL = 730.5 uL**

HDL (prepare 3.5 replicates, total volume 150 uL \* 3.5 = 525 uL Buffer/HDL mix

1. 1 ug HDL: = 3.1ug/uL \* x = 1 ug HDL protein
   1. X = 0.3 uL
   2. 3.5 replicate \* 0.3 uL = 1.05 uL HDL
   3. 525 uL– 1.05uL HDL = 523.95 Buffer
2. 5 ug HDL: = 3.1ug/uL \* x = 5 ug HDL protein
   1. X = 1.6 uL
   2. 3.5 replicate \* 1.6 uL = 5.6 uL HDL
   3. 525 uL– 5.6uL HDL = 519.4 uL Buffer
3. 10 ug HDL: = 3.1ug/uL \* x = 10 ug HDL protein
   1. 3.2 uL
   2. 3.5 replicate \* 3.2 uL = 11.2 uL HDL
   3. 525 uL– 11.2uL HDL = 513.8 uL Buffer
4. 20 ug HDL: 3.1ug/uL \* x = 20 ug HDL
   1. X = 6.45 uL HDL
   2. 3.5 replicate \* 6.25 uL = 21.9 uL HDL
   3. 525 uL– 21.9 uL HDL = 503.1 uL Buffer

**ApoB depleted**

1. 2%: 0.02\* 175 uL = 3.5 uL
   1. 3.5 replicate \* 3.5 uL = 12.25 uL apob-depleted
   2. 525uL – 12.25 = 512.75 uL Buffer
2. 5%: 0.05\*175 uL = 8.75 uL
   1. 3.5 replicate \* 8.75 uL = 30.63 uL apob-depleted
   2. 525 uL – 30.63 uL = 494.37 uL Buffer

**Recipe for Dilution**

|  |  |  |
| --- | --- | --- |
| Sample | Sample Volume (uL) | Buffer (uL) |
| HDL (1 ug) | 1.05 | 523.95 |
| HDL (5 ug) | 5.6 | 519.4 |
| HDL (10 ug) | 11.2 | 513.8 |
| HDL (20 ug) | 21.9 | 503.1 |
| ApoB-depleted (2%) | 12.25 | 512.75 |
| ApoB-depleted (5%) | 30.63 | 494.37 |
| Negative Control | - | 525 |

|  |  |
| --- | --- |
| Sample | Sample Volume (uL) |
| DHR | 9 uL |
| Azobis | 10.5 |
| Buffer | 730.5 |
| **Total Volume** | **750** |

**Plate Design**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | x | x | x | x | x | x | x | x | x | x | x | x |
| B | x | HDL\_1ug | HDL\_10ug | apob-dep\_2 | nc | nc | x | x | x | x | x | x |
| C | x | HDL\_1ug | HDL\_10ug | apob-dep\_2 | nc | nc | x | x | x | x | x | x |
| D | x | HDL\_1ug | HDL\_10ug | apob-dep\_2 | nc | nc | x | x | x | x | x | x |
| E | x | HDL\_5ug | HDL\_20ug | apob-dep\_5 | nc | x | x | x | x | x | x | x |
| F | x | HDL\_5ug | HDL\_20ug | apob-dep\_5 | nc | x | x | x | x | x | x | x |
| G | x | HDL\_5ug | HDL\_20ug | apob-dep\_5 | nc | x | x | x | x | x | x | x |
| H | x | x | x | x | x | x | x | x | x | x | x | x |

**Steps**

1. Prepare sample in above concentration to a total volume of 150 uL per replicate
2. Mix sample 10 times
3. Plate in the order according to plate design
4. Prepare DHR/Azobis/Buffer mix according to table above
5. Add 25 uL of DHR/Azobis/Buffer mix to each well
   1. **Total Volume is now 175 uL**
6. Read using Microplate reader a 485/538 nm excitation/emission on kinetic every 1 or 5min for 60 minutes