Tau aggregation protocol—for combined seeding and chaperoning on one plate (MOK)

Materials:

* 0.2 um syringe filters for sterilizing (MCE membranes)
* 384 well low volume black plate (Corning)🡪round bottom, REF 4511
* 30 ul matrix tips
* 25 ml divided reservoirs
* adhesive sealing films for plates
* optically clear sealing films (VWR cat# 89134-428 (CS))
* D-PBS pH7.4, no Calcium or Magnesium (Life technologies cat# 14190)
* Magnesium chloride hexahydrate
* Thioflavin T (Sigma)
* Heparin, MW 6000-25000 (Santa Cruz, sc 203075)
* DTT
* Tau and chaperone protein buffer exchanged into the assay buffer

Preparation of working solutions:

(**Filter sterilize all solutions with a 0.2 um filter**)

* **1M MgCl2**: Dissolve Magnesium chloride hexahydrate in ddH2O. Store at room temp.
* **Assay buffer**: Add MgCl solution to D-PBS at a final concentration of 2 mM.
* **Thioflavin T (ThT) stock solution (0.5 mg/ml):** Dissolve Thioflavin T in assay buffer. Incubate at 37C for 20 min in dark of until ThT goes into solution. Dispense into single use aliquots and store at -20C
* **Heparin stock solution (0.22 mg/ml \* See notes ):** Measure out approx. 2 mg of heparin in an eppendorf. Dissolve in 1 ml assay buffer. Dilute the stock to make a solution of 0.22 mg/ml in assay buffer. Filter sterilize immediately. Store at 4C for up to 1 day.
* **100 mM DTT solution**: Dissolve DTT at 100 mM in assay buffer.
* **Triton coating solution**: Make a 0.01% solution of Triton X-100 in sterile ddH2O

Assay conditions:

1. **General overview for chaperoning**: Tau is mixed with chaperones and pre-incubated for 30 min at 37C. Thioflavin T is added to each sample followed by heparin (inducer) or assay buffer (control)

**General overview for seeding**: Tau solution is aliquoted into wells. Thioflavin T is added to each sample followed by heparin (inducer) or assay buffer (control). Tau fibril seeds are at a final concentration of 3% w/w (monomer)

1. **Precoat plates \* See notes**:Add 25 uL of Triton coating solution to each well. Immediately dump out solution and remove as much liquid as possible by forcefully tapping plate on stack of paper towels. Allow wells to completely dry. Dry coated plates can be sealed and kept in the fridge for several days.
2. Set up the plate reader:
   * + - Remove plate adaptor if in use
       - Set temperature to 37C
       - Fluorescence top read, kinetic
       - Corning 384 well low volume
       - Read entire plate
       - Excitation 444 nm, Emission 485 nm, auto cutoff (480 nm)
       - Medium PMT, 15 reads per well
       - 24 h with reads every 5 min.
       - Shake between each read for 190 sec
       - Include 2 plates; 1 for a time zero endpoint read and 1 for the 24 hour kinetic assay
3. **For chaperoning:** The total volume per well is 20 uL. All concentrations are listed as final concentration in assay. Each group is carried out in triplicate. Each group also has matching controls without inducer (also in triplicate)

-component 1: 10 uM Tau, 1mM DTT in assay buffer 6.5 uL

-component 2: 5-20 uM chaperone in assay buffer 6.5 uL

-component 3: 10 uM ThT 3.0 uL

-component 4: 22 ug/ml heparin or assay buffer 4.0 uL

Total volume 20 uL

Make Tau solution: Calculate the total volume of tau (10 uM final) and DTT (1 mM final) required for the assay. Bring up the volume so that the tau solution can be aliquoted for 6.5 uL per well.

* 1. E.g. If using 6.5 ul of tau solution per well this means that the tau is at (20ul/6.5 ul\*10 uM=30.8 uM) and DTT is at 3.07 mM
  2. I usually make enough for 8 wells per group.

**For seeding:** The total volume per well is 20 uL. All concentrations are listed as final concentration in assay. Each group is carried out in triplicate. Each group also has matching controls without inducer (also in triplicate)

-component 1: 10 uM Tau, 1mM DTT in assay buffer 6.0 uL

-component 2: 10 uM ThT 3.0 uL

-component 3: 44 ug/ml heparin or assay buffer 3.0 uL

-component 4: 3% w/w tau fibril seed 8. 0 uL

Total volume 20 uL

Make Tau solution: Calculate the total volume of tau (10 uM final) and DTT (1 mM final) required for the assay. Bring up the volume so that the tau solution can be aliquoted for 6.0 uL per well.

1. E.g. If using 6 ul of tau solution per well this means that the tau is at (20ul/6 ul\*10 uM=**33.3 uM**) and DTT is at **3.3 mM**
2. I usually make enough for wells + at least 2 extra wells per group.
3. Make chaperone solution: Dilute chaperones in assay buffer to a final volume of 6.5 ul per well. I usually make enough for 8 wells per group
4. **For chaperoning:** Pipette 6.5 ul per well of Tau solution into each well with matrix pipettor. Add 6.5 ul per well of chaperone solution. **Change tips between groups to avoid cross-contamination.**

**For seeding:** Pipette 6 ul per well of Tau solution into each well with matrix pipettor. **Change tips between groups e.g. different tau variants to avoid cross-contamination.**

1. Cover plates with a sealing film to prevent evaporation and incubate for 30 min at 37C.
2. Prepare fibrils to be incubated with chaperones in Eppendorf tubes, and also incubate for 30 min at 37C.
3. During the 30 min incubation, prepare the remaining tau seed solutions. It is best to prepare them in a 96 well plate to allow for quick delivery to wells in the assay. I usually make enough for wells plus at least 2 extra wells per group. Since the concentration of tau monomer in the assay is 10 uM (0.43 mg/ml for 0N4R tau). The final concentration of the seed in the assay is 0.43 mg/ml\*0.03= 0.0129 mg/ml. The stock concentration you should prepare is 0.0129 mg/ml X 20 uL/8 uL= **0.03225 mg/ml.**
4. Dilute ThT stock solution 66.66 uM in assay buffer (1 part ThT stock : 23.5 total dilution). Put in a reservoir
5. After the 30 min incubation is complete, add 3 uL of ThT to each well. Change tips between groups and do not redip tips into reservoir.
6. **For chaperoning:** For uninduced samples : Put assay buffer in a reservoir. Add 4 ul of assay buffer to 3 wells for each group. Change tips between groups and do not redip tips into reservoir.

**For seeding:** For uninduced samples : Put assay buffer in a reservoir. Add 3 ul of assay buffer to 3 wells for each group. **Change tips between groups and do not redip tips into reservoir.**

1. It may help to bring all the solutions, pipettes, tips to the plate reader and carry out the following steps of the assay there.

**For chaperoning:** For induced samples: Put heparin solution in a reservoir. Add 4 ul of assay buffer to remaining 3 wells for each group. Change tips between groups and do not redip tips into reservoir.

**For seeding:** For induced samples: Put heparin solution in a reservoir. Add 3 ul of assay buffer to remaining 3 wells for each group. Change tips between groups and do not redip tips into reservoir. **Work quickly** to move through the next steps.

1. Take a reading of the entire plate. This will be your t= 0
2. **Quickly** but carefully add the tau fibril seeds. **Important: first, quickly mix the samples in the 96 well plate by performing a quick loading and complete ejection using the matrix pipettor.** Then load the matrix again with the same tips and eject into your sample wells. The volumes used are large enough that you don’t need to eject the first volume. **Change tips between groups and do not redip tips into the 96 well plate.**
3. Quickly seal plate with optically clear film. Use scraper to ensure seal around each well. Place plate in plate reader and start assay.

**For chaperoning:**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Initial conc. | Final conc. in assay(20 ul total) | Volume per well (20 ul total) | For 6 wells with extra for pipetting |
| **Tau solution** | | | | |
| Tau |  | 10 uM |  |  |
| DTT | 100 mM | 1 mM | 0.2 ul | 1.6 ul |
| Assay buffer |  |  |  |  |
|  |  | Total volume | 6.5 uL | 52 ul |
|  |  |  |  |  |
| **Chaperone solution** | | | | |
| Chaperone |  | 1-20 uM |  |  |
| Assay buffer |  |  |  |  |
|  |  | Total volume | 6.5 ul | 52 ul |
|  |  |  |  |  |
| ThT solution | 66.66 uM | 10 uM | 3 ul |  |
|  |  |  |  |  |
| Heparin or assay buffer | 0.11 mg/ml | 0.022 mg/ml | 4 ul |  |

**For seeding:**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Initial conc. | Final conc. in assay(20 ul total) | Volume per well (20 ul total) | For 6 wells with extra for pipetting |
| **Tau solution** | | | | |
| Tau |  | 10 uM |  |  |
| DTT | 100 mM | 1 mM | 0.2 ul | 1.6 ul |
| Assay buffer |  |  |  |  |
|  |  | Total volume | 6 uL | 48 ul |
|  |  |  |  |  |
|  |  |  |  |  |
| ThT solution | 66.66 uM | 10 uM | 3 ul |  |
|  |  |  |  |  |
| Heparin or assay buffer | 0.147 mg/ml | 0.022 mg/ml | 3 ul |  |
| seeds | 0.03225 mg/ml | 3% w/w (0.0129 mg/ml) | 8 uL |  |

Other notes:

* Assay is very sensitive to concentrations of tau and heparin. Minimizing pipetting error is very important. When repeat pippeting using matrix, the first dispense is the least accurate. I set pipettor to draw up at least enough for 1 extra dispense then I expel the 1st dispense back into my stock solutions before dispensing into my wells. I have found the pipettor is not accurate when dispensing smaller volumes (2 uL).
* Assay buffer should be uniform throughout assay. Buffering agent, salt, metals etc. can affect aggregation of tau or chaperone stability/function. If you use different buffers in your assay make sure you have tau only controls containing the same volume of buffer.
  + **All proteins must be dialyzed into the assay buffer. Even dilution of concentrated stock solutions of proteins can affect the aggregation kinetics if the buffers are not matched**
  + HEPES, and Tris buffers are not ideal because their pH changes greatly with temperature.
  + 140 mM salt is usually used. Higher salt conc. 300 mM completely block tau aggregation
  + The DTT in the buffer inhibits intramolecular disulfide bond formation in the tau molecule that impede intermolecular interactions.
  + Hsp70s and J-proteins may require Magnesium in assay for stability.
* The optimal ratio for heparin to tau to promote aggregation is 1:4. Since the heparin comes as a range of MW (6000-25000), this is difficult to calculate. For each batch of heparin I test a range of concentrations and pick a concentration where the kinetics do not vary over at least a 4-fold range in either direction.
* ThT and heparin can not be premixed prior to addition to the assay. This greatly increasing the lag time for tau aggregation.
* We have found variation in the plates with respect to non-specific binding of proteins to the wells. Tau will not aggregate in commercial non-binding plates in our hands. A quick 0.01% triton wash reduces non-specific binding of chaperones tested.