**Schedule for TOPO cloning of Sirt3(118-399) into pET151/D-TOPO**

1. Determine strategy for PCR of the gene insert: **Time =** **2-3 days**

* Most critical step. Need to design the forward and reverse PCR primers to amplify the DNA sequence encoding the 118-399 protein.
* Since we have the 102-399 clone, I will use that as the template to amplify the 118-399 region.
* To enable directional cloning into the pET151/D-TOPO vector, with the His-tag at the N-terminus of the 118-399 protein, the sequence CACC will be incorporated into the 5’ region of the forward PCR primer. The CACC sequence will base pair with the GTGG overhang sequence in the pET151/D-TOPO vector.
* General guidelines for primer design will be followed and the final forward and reverse primers will be verified by the OligoAnalyzer tool from IDT.
* Upon verification, the primers will be ordered from Genscript.

1. Produce blunt-end PCR products: **Time = variable**

* Materials required:

1. Thermocycler and thermostable, proofreading polymerase
2. 10X PCR buffer
3. dNTPs, adjusted to pH 8 (provided in the kit)
4. DNA template and primers for PCR product

* The PCR reaction will have to be optimized to produce a single, discreet PCR product.
* The PCR product will be checked and quantified by agarose gel electrophoresis.

1. Setting up the TOPO cloning reaction: **Time = 2-3 days**

* The reaction will be set up following the manufacturer’s instructions.
* Part of the cloning reaction will be analyzed by agarose gel electrophoresis and the rest will be used for transformation into Top10 competent cells (next step).

1. Transformation of the cloning reaction into Top10 competent cells: **Time = 2 days**

* The transformation reaction will be done following manufacturer’s instructions.
* Colonies obtained from transformation will be screened in the next step.

1. Analyzing transformants: **Time = variable**

* Transformants will be analyzed by colony PCR using the forward and reverse sequencing primers provided in the kit and an additional mid-sequencing primer that will be ordered from Genscript.
* Positive clones obtained from colony PCR analysis will be selected and grown in liquid culture for plasmid prep.
* The isolated plasmids will be sent for sequencing at Genewiz and will also undergo restriction digestion analysis to verify the integrity of the insert and also the correct orientation in the vector.
* The constructs that come back positive from both sequencing and restriction analysis will be stored in -20C and a glycerol stock will be made for long term storage.
* A positive construct will then be used for transformation into the expression cell line (next step).

1. Expression and purification of Sirt3(118-399): **Time = variable**

* The construct from the previous step will be transformed into BL21 Star (DE3) One Shot competent cells following manufacturer’s instructions.
* Transformants will be selected and screened for expression of Sirt3(118-399) upon induction with IPTG.
* Clones which show strong expression will be selected and be used for large scale (200 ml) cell culture and overexpression.
* The previously used truncated purification protocol will be used for trial purifications using 1 ml His-Gravitrap columns.
* Upon successful elution of the Sirt3(118-399) protein, the protocol will be optimized for maximum purity.