**T7 Assay Protocol**

* Starting from cells plated in 24 well format
  + Aspirate media
  + Add 200 uL of Trypsin to each well, incubate for 3 minutes
  + Add 800 uL of DMEM, wash cells so they are floating in the media
  + Transfer 200 uL of media containing cells to labelled PCR tubes
  + Centrifuge tubes for 5 minutes at 4000 g
  + Remove media from tubes and be sure not to disturb the pellet (media has PCR inhibitor)
  + Add 50 uL of lysis buffer to resuspend cells. Mix aggressively until foamy
    - Lysis buffer: 95% Dilution Buffer, 5% DNA release
  + Denature DNA by putting tubes in thermal cycler at 95C for 5 minutes
    - Pause point: can store denatured DNA at -20C
* PCR to verify we have desired T7 amplicon
  + 50 uL final volume for PCR (1x reaction)
    - PCR MM: 25 uL
    - DNA: 1 uL
    - Primer mix: 2 uL
      * T7 primers, 10F + 10R + 80dw
    - Dw: 22 uL
  + Make sure each sample has its own control (i.e. sample without gRNA)
  + After PCR is set up, run colony PCR protocol
    - PCR Program
      * 98C: 2 min
      * 10 cycles
        + 98C: 5 seconds
        + 65C: 5 seconds (-0.5C per cycle)
        + 72C: 20 seconds
      * 25 cycles
        + 98C: 5 seconds
        + 60C: 5 seconds
        + 72C: 20 seconds
      * 72C: 1 min
      * 22C: Hold
  + After PCR is complete, run samples on gel to verify we have product
    - Band sizes should be expected amplicon length based on T7 primer design
    - 1% agarose gel, 150 volts, 50 minutes
  + If verification is successful, continue with T7 assay
* T7 Assay
  + 30 uL final volume for T7 (1x reaction)
    - PCR product: 10 uL
    - 10X T7 Buffer: 3 uL
    - Dw: 16 uL
    - T7E1 enzyme: 1uL
  + **IMPORTANT: DO NOT ADD T7E1 INITIALLY**
  + First combine PCR product, buffer, and dw
  + Put in thermocycler and run step down protocol
    - T7 step down Program

|  |  |  |  |
| --- | --- | --- | --- |
| **STEP** | **Temperature** | **Ramp Rate** | **Time** |
| Initial Denaturation | 95C |  | 5 minutes |
| Annealing | 95-85C | -2C/second |  |
| 85-25C | -0.1C/second |  |
| Hold | 4C |  | Hold |

* + After step down is complete, make a 2X dilution of the T7 enzyme (i.e. 15 uL T7, 15 uL dw)
  + Then add 1 uL of 2x diluted enzyme to each PCR tube
  + Place tubes in 37C water bath for 1 hour
  + Run samples on gel to observe fragmentation of bands