Two-pot RPA-CRISPR/Cas12a assay protocols

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**Detailed Protocol**

1. All master mixes should be made in a sterile UV cabinet if possible to avoid contamination
2. UV sterilize all previously autoclaved plastic ware for the reactions needed. For each assy you will need to UV for approximately 15 min:
   1. 1.5 mL tube for RPA Mmix
   2. 1.5-2 mL tube for the RNP
   3. 1.5-2 mL tube for the reporter and buffer mix
   4. Any tubes required for diultions of primers, enzymes, reporter, etc.
   5. 1.5 mL tubes of molecular grade water
   6. Plate and plate caps or strip tubes and caps
   7. microAMP optical plate for CRISPR assay on qPCR machine
   8. Pipette tips
   9. Pipettors
   10. Racks (to hold strips of TwistDx)
3. Thaw the buffers and reagents needed on ice
   1. RPA
      1. rehydration buffer
      2. Forward primers (10 uM stock)
      3. Reverse primers (10 uM stock)
      4. TwistDx pellets in strip tubes
   2. CRISPR/Cas12a
      1. Cas12a enzyme (1:10 dilution - 6.4 uM)
      2. crRNA (10 uM)
      3. PBS
      4. NEBB 2.1 (2x - diluted from the 10x stock commercially ordered)
      5. Reporter (10 uM)
4. RPA step -
   1. Make the RPA Mmix based on the sheet - Concentrations here of Mmix per pellet - 5 reactions per pellet

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| --- | --- | --- |
| Reagent | Final Concentration |  |
| F RPA primer | 0.48 | uM |
| R RPA primer | 0.48 | uM |
| Primer Free Rehydration buffer | 1 | x |
| Water | To make 40 ul Mmix/pellet |  |

* 1. Apply 40 ul of RPA Mmix to each pellet - mix by pipetting up and down, then move the >40 uL of mix with rehydrated pellet back into the RPA mastermix tube with the residual mix
  2. Add 8 uL of Mix to each reaction tube
  3. Add 1 uL of starting DNA (~ 1 ng/ul or eDNA sample)
  4. Add 1 uL of MgOAc (1:2 dilution from stock - so 14 mM final concentration) to the caps.
  5. Place caps on tubes, centrifuge MgOAc down into mix to start reaction.
  6. Incubate at 37℃ for 20 minutes
  7. Can visualize on Bioanlyzer but can also directly move onto the adding the RPA product to the CRISPR?Cas12a reaction

1. CRISPR/Cas12a step -
   1. Make the RNP mastermix
      1. Here is the basic concentration based off the final concentration

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Reagent** | **Initial Concentration** |  | **Final Concentration** |  |
| As Cas12a nuclease\* 1:10 | 6.4 | uM | 2.52 | uM |
| crRNA 1:10 | 10 | uM | 3.20 | uM |
| PBS | 1 | x | 1 | x |

* + 1. Let that incubate at room temperature at high concentration for 20 minutes.
    2. Add the water necessary for final concentration
    3. Mix the reporter and NEBB 2.1 2x mix (0.05 uM reporter, 1x NEBB 2.1)
    4. Combine the RNP and the reporter/NEBB mixes
    5. Add 18 uL into each well of the microAmp optical plate
    6. Add 2 uL of RPA product to the wells and mix
    7. Seal with adhesive plate cover
    8. Incubate reactions for 60 min at 37 °C with fluorescence measurements taken every 30 sec (ƛex = 485 nm, ƛem = 535 nm).
       1. We ran on a qPCR light cycler - Applied Biosystems One-Step RT-PCR light cycler

1. Export the multicomponent data and the sample set up for data analysis of results.