**IMPALA ESBL PCR Protocol**

**Purpose**

Method for conducting a multiplexed real time PCR on IMPALA isolates to detect ESBL resistance genes.

**Materials**

* Lo bind Eppendorf tubes
* 1X TE Buffer
* Sterile loops
* P2 pipette & tips
* P10 pipette & tips
* P200 pipette & tips
* P1000 pipette & tips
* Molecular grade water
* Promega Master Mix
* Primer mixes (200uM) for CTX A, CTX B, CMY, SHV, TEM
* Probe mixes (100uM) for CTX A, CTX B, CMY, SHV, TEM
* Applied Biosystems 96 well plate & film

**Procedure**

* Lysis Buffer Recipe

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **1X TE Buffer** |  |  |  |  |  |  |
|  |  |  | Final Volume: | | 50 | mL |
| Chemical | [Stock] | | [Final] | | Volume to Add | |
| Tris-HCl, pH 8.0 | 1000 | mM | 10 | mM | 0.500 | mL |
| EDTA | 500 | mM | 1 | mM | 0.100 | mL |
|  |  |  |  | Total: | 0.600 | mL |
|  |  |  |  | + ddH2O | 49.400 | mL |
|  |  |  | Final Volume: | | 50.000 | mL |

* Bacterial Cell Lysis
  1. If plated isolates are older than 2 weeks, subculture on blood agar and incubate for 24 hours at 37C.
  2. Add 5-10 colonies to 100uL lysis buffer.
  3. Briefly vortex samples for homogeneity.
  4. Incubate samples at 95C for 10 min.
  5. Freeze samples at -20C until ready for PCR.
* Real Time PCR
  1. 88 samples per plate (not completed in triplicate), 4 negative controls, 4 positive controls
     + CTX control = ARLG 7
     + CMY control = ARLG 16
     + SHV control = ARLG 3
     + TEM/SHV control = ARLG 1
       - *Note: we don’t have any ARLG samples that are only TEM positive.*
  2. While samples thaw, prepare mastermix solution using the following calculation:
     + *Note: new primers are premixed, so the forward primer and reverse primer for each target are in their own solution.*

**Master Mix Recipe:**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **1 Rxn** | **96 Rxn** | **+10%** |
| Master Mix (2X) | 10 | 960 | 1056 |
| Primer Mix (900 nM final concentration) |  |  |  |
| CTX A | 0.056 | 5.376 | 5.9136 |
| CTX B | 0.056 | 5.376 | 5.9136 |
| CMY | 0.056 | 5.376 | 5.9136 |
| SHV | 0.056 | 5.376 | 5.9136 |
| TEM | 0.056 | 5.376 | 5.9136 |
| Probe (250 nM final concentration) |  |  |  |
| CTX A (JUN) | 0.025 | 2.4 | 2.64 |
| CTX B (JUN) | 0.025 | 2.4 | 2.64 |
| CMY (VIC) | 0.025 | 2.4 | 2.64 |
| SHV (ABY) | 0.025 | 2.4 | 2.64 |
| TEM (6FAM) | 0.025 | 2.4 | 2.64 |
| Water | 7.095 | 681.12 | 749.232 |
|  |  |  |  |
| Total (17.5 uL) | 17.5 | 1680 | 1848 |
|  |  |  |  |
| Template | 2.5 | 2.5 | 2.5 |

**Thermocycling Settings:**

|  |  |  |  |
| --- | --- | --- | --- |
| **Thermal Cycling** |  |  |  |
| Activation | 1X | 95C | 2 min |
| Denaturation | 40X | 95C | 15 sec |
| Annealing/Extension | 40X | 60C | 1 min |
|  |  |  |  |
| **FAST Thermal Cycling** |  |  |  |
| Activation | 1X | 95C | 2 min |
| Denaturation | 40X | 95C | 3 sec |
| Annealing/Extension | 40X | 60C | 30 min |