**Annex E8**

**Nested PCR** **assay for genotyping measles virus**

**Equipment**

* Micropipettors with RNase free pipet tips. Use only pipet tips with filters.
* Bucket with ice and metal 96 well plate
* Thermocycler (e. g. AB GeneAmp PCR System 9700)
* Disposable lab coat
* Disposable gloves
* Class II biological safety cabinet with UV light (BSC)
* Vortex mixer
* Microcentrifuge, refrigerated to 4 C with rotor for 1.5 ml tubes and inserts for 0.2 ml tubes

*General reagents*

* Deionized water
* Invitrogen Platinum Taq High Fidelity Polymerase *(cat #* 11304-011 )
* (Autoclaved PCR tubes (0.2 ml, thin-walled)

***Primers***

|  |  |  |
| --- | --- | --- |
| Name | Size nt | Sequence |
| **MeV210** (forward) | 24 | 5’- gctatgccatgggagtrgga gtgg -3’ |
| **MeV217** (reverse) | 17 | 5’- caatgatggagggtagg -3’ |
| **MeN-Seq1** (forward) | 17 | 5’- CGATCTTACTTTGATCC -3’ |

**PCR product size (nested PCR):**

|  |  |  |
| --- | --- | --- |
|  | **Current** |  |
| **Primers** | MeV **210**, MeV **217** | **MeN-Seq1**, MeV **217** |
| **Product size** | 627bp | 568bp |

*Preparation of working stocks (20 µM) of PCR primers*

Concentration of primer stocks varies. Dilute primers to 20 uM with de-ionized water.

**Caution**: The risk for cross-contamination is high when performing nested PCR. It is very important to include a separate water control for the nested step (in addition to the water control for the first round of RT-PCR). Nested PCR should be set up in a PCR hood with UV light. It must be set up in a room designated for post –PCR procedures.

**Preparation of master mix (Invitrogen kit)**

* Always include a water control for the nested step.
* Label a 0.2 ml thin-walled PCR tube for each sample according to spreadsheet example below and the additional water control. Place tubes in pre-cooled metal 96 well plate on ice.
* Keep RT-PCR reaction tubes on ice. If they had been frozen, vortex briefly, spin to collect, place on ice.
* Use 2 µl unpurified RT-PCR reaction as template.
* Make more master mix than needed to prepare for pipetting losses, e.g. if you need 10 reactions, make enough master mix for 11 reactions.
* Thaw all reagents needed for the master mix at room temperature (except for the enzyme), vortex briefly, centrifuge to collect, place on ice. The enzyme must always be kept on ice. Vortex the enzyme, centrifuge briefly to collect, place on ice.
* Add the first 6 reagents for master mix in the order below. Close tube, vortex, centrifuge to collect.
* On ice add: enzyme
* Close tube, vortex, centrifuge to collect, place on ice
* Aliquot 48 ul of master mix into each tube. First add water control then add the RT-PCR reactions. Use a fresh pipet tip every time you pipet.
* Close tubes and vortex briefly. Spin to collect. Place tubes on ice.

**Reaction Mix/reaction**

Water 37.8 µl

10 x buffer 5.0 µl

MgSO4, 50 mM 2.0 µl

dNTP mix, 10 mM (not part of kit) 1.0 µl

MeV210 or MeN-Seq1, 20 µM 1.0 µl

MeV217, 20 µM 1.0 µl

HiFi Plat Taq 0.2 µl

Sample 2.0 µl

**Thermocyling**

Do not bring PCR reactions back into the clean area after the thermocycler program has finished.

If samples are not processed immediately, they must be stored at -20 C.

**PCR conditions**

1. 94 °C,30 sec

*2. 40 cycles of:*

 94 °C 15 sec

 50 °C 30 sec

 68 °C 1 min

3. 72 C, 5 min

4. 4 °C, hold

* Perform agarose gel analysis as described above. If any of the negative (water) controls have bands that are approximately the same size as the measles PCR, product, discard the entire run and repeat. PCR products should be purified and sequenced as described above.