#  *Bordetella* Reagent and Process Control Preparation

**PURPOSE**

* This procedure provides instructions for preparation of reagents and procedural controls

**ABBREVIATIONS**

1. BOR: *Bordetella*
2. BORDP: *Bordetella* PCR
3. Bp: *Bordetella pertussis*
4. Bpp: *Bordetella parapertussis*
5. BSC: biosafety cabinet
6. Ct: crossing threshold
7. F/T: freeze/thaw
8. IC: internal control
9. MM: master mix
10. NEGC: negative control
11. NFW: nuclease free water
12. PCR: polymerase chain reaction
13. PCTL: process control
14. PP: primer – pair
15. PPE: personal protective equipment
16. SEAC: Simplexa extraction and amplification control
17. TE buffer: Tris – EDTA buffer

## SAFETY CONSIDERATIONS

1. Standard precautions. Refer to [MB002.2](file:///G%3A%5CLAB%5CMolecular%20Biology%5CA.%20Molecular%20Procedure%20Manual%5CMB002%20Safety%5CMB%20002.2%20v4%20Biohazard%20Containment.docx) Biohazard Containment
2. Use of engineering controls: Refer to [MB003.1](file:///%5C%5Ckidsnet.childrenshc.org%5Cchcdfs%5Cdept%5CLAB%5CMolecular%20Biology%5CA.%20Molecular%20Procedure%20Manual%5CMB003%20Engineering%20Controls%5CMB%20003.1%20Engineering%20Controls%20to%20Prevent%20Contamination.doc) Engineering Controls to Prevent Nucleic Acid Contamination

#### MATERIALS REQUIRED

|  |  |  |
| --- | --- | --- |
| **Equipment** | **Reagents** | **Supplies** |
| Room 1: Clean room* Laminar-flow hood, Clean rm 1
* Freezer, -10 to -30⁰ C
* Refrigerator, 2 to 8⁰ C
* Microcentrifuge
* Nalgene cooling block
* Vortex
* Eppendorf Repeater pipette
* Dedicated set of pipettes: 2 µl, 10 µl, 20 μl, 100 μl, 200 μl, and 1000 μl pipettes
* Pipet-Aid

Room 2: Processing * BSC, Process rm 2
* Refrigerator, 2 to 8⁰ C
* Freezer, ≥ - 70⁰C
* Nalgene cooling block
* Vortex
* Microcentrifuge
* Dedicated set of pipettes: 2 µl, 10 µl, 20 μl, 100 μl, 200 μl, and 1000 μl pipettes

Room 3: Amplification and detection* Focus Simplexa Integrated Cycler

Location: Microbiology* McFarland densitometer
 | TE buffer | Micro tube racks |
| Nuclease Free Water (NFW) | 2 ml cryovials |
| SEAC* Internal control PP
* Internal control DNA
 | Sterile filtered pipette tips for 10 µl, 20 µl, 100 μl, 200 µl, 1000 µl pipettes |
| Bp PP | Micro tubes 1.5 ml, RNase/DNase free |
| Bpp PP | Nitrile gloves (powder-free) |
| Bordetella Molecular Control (POSC) | Sharps disposal container  |
| Bordetella process control (PCTL) | Gripper rack, rm 2 |
| TA MasterMix | Orange barrier wipes |
| Sani-Cloth Bleach wipes | 12X75 sterile plastic test tubes |
| 70% alcohol | Sterile Q – Tipped applicator swabs |
| 5% Extran | 50 ml sterile conical tube |
| *Bordetella pertussis* ATCC 8467 | Eppendorf 5 ml tips |
|  | Serological pipettes, 5 and 10 ml |
|  |  |
|  |  |
|  |  |

**PROCEDURE A:** Follow the activities in the table below for Process Control preparation

Preparing Process Control Suspension

| **Activity** | **Step** | **Action** | **Related Doc** |
| --- | --- | --- | --- |
| **Prepare** | 1 | Pool approximately 10 ml of nasal wash matrix in a 50 ml sterile conical tube  |  |
| **Matrix** | 2 | Dilute matrix in NFW to achieve a 30 – 35 ml suspension |  |
| Room 2 | 3 | Vortex well |  |
|  | 4 | Test suspension in duplicate according to the BORDP assay procedure to ensure that it is target free | [BOR 005](BOR%20005%20Simplexa%20Bordetella%20pertussis%2C%20parapertussis%20Assay.docx) Bordetella PCR Assay |
| **BORD 0.5 McFarland****Micro** | 5 | Prepare a 0.5 McFarland suspension of *Bordetella pertussis* ATCC 8467

|  |  |
| --- | --- |
| Step | Action |
| a | Pick isolated colonies (3 – 4 d growth) with sterile CultureSwab |
| b | Suspend in saline; vortex |
| c | Adjust suspension to 0.5 McFarland(~1.5 X 108 CFU/mL) using densitometer  |
| d | **Dilution 1**: make a 1:100 dilution of this suspension in NFW (~1.5 X 106 CFU/mL)* Pipette 10 µl into 990 µl NFW
* Vortex well
 |
| e | **Dilution 2:** make a 1:10 dilution from dilution 1 in NFW (~1.5 X 105 CFU/mL)* Pipette 100 µl into 900 µl NFW
* Vortex well
 |

 | 0.5 McFarland Standard turbidity range = 0.5 – 0.63 |
| **Working suspension****Room 2** | 6 | **Dilution 3** (working suspension): Make a 1:100 dilution from dilution 2 (final concentration ~1.5 X 103 CFU/mL)

|  |  |
| --- | --- |
| Step | Action |
| a | Pipette 300 µl from BORDP dilution 2 into 30 ml of matrix |
| b | Mix well by inversion/vortexing  |
| c | Test suspension prior to freezing (3 µl Bp suspension into 7 µl BORD mm) |
| d | Target control range: Ct values 30 – 32 |
| e | I f necessary, adjust suspension to obtain specified range with NFW; retest |

 | 1 log = ~ 3 Ct |
| **Aliquot** | 7 | Label 1.5 ml micro-centrifuge tubes with contents and date of preparation (approx. 60 tubes) |  |
|  | 8 | Dispense 0.5 ml of working suspension into tubes |  |
| **Freeze**  | 9 | Freeze aliquots at –70° C |  |
| **Decontaminate Hood** | 10 | Wipe down BSC with 5% bleach followed by water and 70% alcohol |  |
| **Room 2** | 11 | UV hood for 15 min  |  |
| **Test aliquots** | 12 | Before use, thaw one BORDP PCTL aliquot, vortex and perform direct testing 5 times to determine average Ct value |  |
| **Before use** | 13  | * Document Ct values on BORDP PCTL New Reagent Worksheet [MB005.8.F3](file:///%5C%5Ckidsnet.childrenshc.org%5Cchcdfs%5Cdept%5CLAB%5CMolecular%20Biology%5CA.%20Molecular%20Procedure%20Manual%5CMolecular%20Resources%5CQC%20forms%5CSimplexa%5CSimplexa%20Bordetella%5CMB005.8.F3%20BOR%20PCTL%20New%20Reagent%20QC%20Worksheet.docx)
 |  |
|  | 14 | Place worksheet and BORDP Segment report including graphs in *New Lot Inventory and QC* manual |  |
| **Stability** | 15 | Once thawed, process control is stable for 7 days at refrigerated temperature |  |
|  | 16 | Do not refreeze (only 1 F/T cycle) |  |

**PROCEDURE B:** Follow the activities in the table below for aliquoting TE buffer (sample buffer tubes) and Nuclease Free Water (NFW) used for NEGC and MM

Preparing TE buffer and NFW

| **Activity** | **Step** | **Action** | **Related Doc** |
| --- | --- | --- | --- |
| **PPE** | 1 | Wear lab coat and gloves dedicated to the Clean room 1 |  |
| **TE buffer and NFW** | 2 | Label 1.5 ml micro-centrifuge tubes with contents and date of preparation |  |
| Room 1 | 3 | Aliquot the following amounts into appropriately labeled tubes* Elution buffer: 200 µl of TE buffer into 1.5 ml tube
* NEGC: 500 µl of NFW into 1.5 ml tube
 |  |
|  | 4 | Place in cryoboxes labeled with contents and date of preparation |  |
| **Storage** | 5 | Refrigerate aliquots in room 1 |  |
|  | 6 | Keep working supply in room 2 |  |

**PROCEDURE C:** Follow the activity below for preparing master mix (MM)

Preparing RT-PCR Master Mix (MM)

| Activity | Step | **Action** |
| --- | --- | --- |
|  | 1 | ***MM must be used within 30 min of preparation****.* |
|  | 2 | Wear lab coat and gloves dedicated to the Clean room 1 |
| **Warm reagents to RmTemp** | 3 | Thaw Primer Probe mix, IC and the Master Mix at room temperature* Protect from light
* Use within 1 hour
 |
| Room 1 | 4 | Gently mix each component

|  |  |
| --- | --- |
| Component | Mixing action |
| TA mm | Vortex 2 – 3 sec, setting 8 |
| Bp PP | Gently flick  |
| Bpp PP | Gently flick  |
| IC DNA | Vortex 2 – 3 sec, setting 8 |
| IC PP | Gently flick  |

 |
|  | 5 | Quick spin reagents |
|  | 6 | Prepare MM in a 1.5 mL micro-centrifuge tube by combining the reagents according to **Table 1** |
|  | 7 | Gently vortex MM 2 – 3 sec to mix; vortex setting 8 ***Note:***Adjust mixing time according to volume.  |
|  | 8 | Quick spin MM |
| **Refrigerate reagents** | 9 | Do not refreeze reagents; store in refrigerator up to 30 days***Note:*** Refer to procedure BOR 003 for storage conditions and expiry dates |
| **Transport** | 10 | Transport to room 2 |
| Room 2 | 11 | Keep the MM in refrigerator or cooling block protected from light until PCR reaction set-up. |

**Table 1: BORDP Master Mix Table**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **No. of samples** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| **TA Master Mix (µl)** | 6 | 10 | 14 | 18 | 24 | 28 | 32 | 36 | 40 | 44 | 48 | 52 |
| **Bp Primer Mix (µl)** | 0.6 | 1 | 1.4 | 1.8 | 2.4 | 2.8 | 3.2 | 3.6 | 4 | 4.4 | 4.8 | 5.2 |
| **Bpp Primer Mix ( µl)** | 0.6 | 1 | 1.4 | 1.8 | 2.4 | 2.8 | 3.2 | 3.6 | 4 | 4.4 | 4.8 | 5.2 |
| **IC DNA (µl)** | 0.3 | 0.5 | 0.7 | 0.9 | 1.2 | 1.4 | 1.6 | 1.8 | 2 | 2.2 | 2.4 | 2.6 |
| **IC Primer Mix (µl)** | 0.3 | .05 | 0.7 | 0.9 | 1.2 | 1.4 | 1.6 | 1.8 | 2 | 2.2 | 2.4 | 2.6 |
| **NFW (µl)** | 2.7 | 4.5 | 6.3 | 8.1 | 10.8 | 12.6 | 14.4 | 16.2 | 18 | 19.8 | 21.6 | 23.4 |
| **Total volume (µl)** | 10.5 | 17.5 | 24.5 | 31.5 | 42 | 49 | 56 | 63 | 70 | 77 | 84 | 91 |

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **No. of samples** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| **TA Master Mix (µl)** | 56 | 60 | 66 | 70 | 74 | 78 | 82 | 86 | 90 | 94 | 98 | 102 |
| **Bp Primer Mix (µl)** | 5.6 | 6 | 6.6 | 7 | 7.4 | 7.8 | 8.2 | 8.6 | 9 | 9.4 | 9.8 | 10.2 |
| **Bpp Primer Mix ( µl)** | 5.6 | 6 | 6.6 | 7 | 7.4 | 7.8 | 8.2 | 8.6 | 9 | 9.4 | 9.8 | 10.2 |
| **IC DNA (µl)** | 2.8 | 3 | 3.3 | 3.5 | 3.7 | 3.9 | 4.1 | 4.3 | 4.5 | 4.7 | 4.9 | 5.1 |
| **IC Primer Mix (µl)** | 2.8 | 3 | 3.3 | 3.5 | 3.7 | 3.9 | 4.1 | 4.3 | 4.5 | 4.7 | 4.9 | 5.1 |
| **NFW (µl)** | 25.2 | 27 | 29.7 | 31.5 | 33.3 | 35.1 | 36.9 | 38.7 | 40.5 | 42.3 | 44.1 | 45.9 |
| **Total volume (µl)** | 98 | 105 | 115.5 | 122.5 | 129.5 | 136.5 | 143.5 | 150.5 | 157.5 | 164.5 | 171.5 | 178.5 |

**PROCEDURE D:** Follow the activity below for preparing miscellaneous reagents

Preparing miscellaneous reagents

| Reagent | Step | **Action** |
| --- | --- | --- |
| 5% Extran Working solution | 1 | Prepare in amplification room. *Caution: Protective eyewear must be worn when working with concentrated Extran* |
| Room 2 | 2 | Make working solution as follows:

|  |  |  |
| --- | --- | --- |
| Working Volume | Conc. Extran | Water |
| 2000 ml | 100 ml | 1900 ml |
| 3000 ml | 150 ml | 2850 ml |
| 4000 ml | 200 ml | 3800 ml |

 |
| **70% alcohol** | 1 | Prepare from 100% Dehydrant alcohol located in the Flammable cabinet in the Recycling room. |
| Room 3 or Recycling room | 2 | Make working solution as follows:

|  |  |  |
| --- | --- | --- |
| Working Volume | 100% Dehydrant | Water |
| 1000 ml | 700 ml | 300 ml |

 |

**REFERENCES**

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4. *Bordetella parapertussis* Primer Pair (50 µl) ASR, Circular PI.MOL9007 Rev. B, 07-February-2011, Focus Diagnostics, Cypress, CA 90630
5. Simplexa™ *Bordetella* Molecular Control, Circular PI.MOL8006 Rev. A, 06-Feb-2013, Focus Diagnostics, Cypress, CA 90630
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