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Wet lab flow diagnostics PathoSense

WET LAB III - PCR assay

cDNA generation and PCR amplification

Timing for 12 samples:

- **30 min manual work**
- **1h25 waiting time**
- **10 min manual work**
- **1h45 waiting time**



- All surfaces should be cleaned with Hibiscrub, 70% Ethanol, and 10% Bleach in this order of cleaning. Prevent contamination.
- Aerosol-barrier tips should be used throughout the entire procedure.
- Perform all steps in the dedicated area:
 - **For preparation of Master Mix > MM area***
 - **For working with samples > DNA area**

*Change gloves before working in MM area to prevent contaminating primers and enzymes

Overview

Denaturation

Preparation of the primer/dNTP Master Mix

Primer binding

Preparation of the SuperScript IV Master Mix

Reverse Transcription and Template Switching Reaction

Preparation of the KAPA Hifi Hotstart Master Mix

KAPA Hifi Hotstart Reaction

Denaturation ~ DNA area

Reagents and Materials

- DNA/RNA extract
- 0.2 mL PCR tube (1 per sample + 3)
- deoxynucleotide (dNTP) Solution Mix, N0447L NEB
- SuperScript IV, 18090200 Invitrogen
- KAPA Hifi Hotstart ReadyMix, KK2602 Roche
- Primers, provided by PathoSense
 - nonamer_TSO primer
 - TSO_SS primer
 - TSO_PCR primer
 - random hexamer

Protocol

1. **Label** a new 0.2 mL PCR tube (strip) for each sample and add **5 μ L of the DNA/RNA extracts**
2. **Quick spin** to collect all droplets
3. Incubate at **95°C for 2 minutes** in a PCR thermocycler

Immediately transfer to ice

DO NOT LET COOL TO 4°C IN THE THERMOCYCLER!

Preparation of the primer/dNTP Master Mix ~ MM area



Change gloves before preparation of the Master Mix

Reagents

REAGENT	1 RXN	13 RXN*
DNase/RNase free water	4 μ L	52 μ L
Nonamer_TSO primer	1 μ L	13 μ L
dNTP	1 μ L	13 μ L
Total	6 μ L	78 μ L

*For 12 samples prepare master mix for 13 RXN, to take account of loss

Protocol

4. Take a PCR tube for the mix and add **4 μ L/sample DNase/RNase free water**
5. Vortex and quick spin the **dNTPs** and the **Nonamer TSO** primer
6. Add **1 μ L/sample dNTPs** and **1 μ L/sample Nonamer_TSO primer** to the mix
7. Vortex and quick spin mix

Primer binding ~ DNA area

8. Add **6 μ L of the primer/dNTP mix** to each sample
9. Gently mix the suspension by tapping the tube with your fingers and quick spin
10. Incubate at **65°C for 5 minutes** in a PCR thermocycler to allow proper primer binding

Immediately transfer to ice
and keep for at least 1 minute

Preparation of the SuperScript IV Master Mix ~ MM area



Change gloves before preparation of the Master Mix

Reagents

REAGENT	1 RXN	13 RXN*
DNase/RNase free water	2 μ L	26 μ L
SuperScript IV buffer 5x	4 μ L	52 μ L
DTT (100 mM)	1 μ L	13 μ L
TSO_SS primer (10 μ M working stock)	1 μ L	13 μ L
SuperScript IV enzyme KEEP ON COOL RACK!	1 μ L	13 μ L
Total	9 μ L	117 μ L

*For 12 samples prepare master mix for 13 RXN, to take account of loss

Protocol

11. Take a PCR tube for the mix and add **2 μ L/sample DNase/RNase free water**
12. Vortex and quick spin the SuperScript Buffer, DTT and TSO_SS primer
13. Add **4 μ L/sample SuperScript Buffer, 1 μ L/sample DTT and 1 μ L/sample TSO_SS primer** to the mix
14. Quick spin Superscript IV enzyme (DO NOT VORTEX!)
15. Add **1 μ L/sample SuperScript IV Enzyme** to the mix
16. Gently mix the suspension by tapping the tube with your fingers and quick spin

Reverse Transcription and Template Switching Reaction ~ DNA area

17. **Quick spin** to collect all droplets
18. Add **9 µL of the SuperScript IV Master Mix** to each sample
19. Gently mix the suspension by tapping the tube with your fingers and quick spin
20. Incubate in a PCR thermocycler according to the program below:

50°C	42°C	80°C	4°C
60 min.	10 min.	10 min.	hold

Preparation of the KAPA Hifi Hotstart Master Mix ~ MM area



Change gloves before preparation of the Master Mix

REAGENT	1 RXN	13 RXN*
DNase/RNase free water	3.5 µL	45.5 µL
KAPA Hifi Hotstart	25 µL	325 µL
TSO_PCR primer (10 µM working stock)	0.5 µL	6.5 µL
random hexamer (10 µM working stock)	1 µL	13 µL
Total	30 µL	390 µL

*For 12 samples prepare master mix for 13 RXN, to take account of loss

PROTOCOL

21. Take a PCR tube for the mix and add **3.5 µL/sample DNase/RNase free water**
22. Add **0.5 µL/sample TSO_PCR primer** and **1 µL/sample Random Hexamer primer** to the mix
23. Quick spin the KAPA Hifi HotStart ReadyMix
24. Add **25 µL/sample KAPA Hifi Hotstart ReadyMix** to the mix and pipet up and down to mix the Master Mix
25. Quick spin the KAPA Hifi HotStart Master Mix

KAPA Hifi Hotstart Reaction ~ DNA area

26. **Quick spin** to collect all droplets
27. Add **30 µL of the KAPA Hifi Hotstart Master Mix** to each sample
28. Gently mix the suspension by tapping the tube with your fingers and quick spin
29. Incubate in a PCR thermocycler according to the program below:

Denaturation	Denaturation	Annealing	Elongation	Final elongation
98°C	98°C	67°C	72°C	72°C
3 min.	20 sec.	15 sec.	4 min.	5 min.
	18 cycles	18 cycles	18 cycles	

Take the samples from the PCR thermocycler, quick spin to collect all droplets, and keep on ice.

Proceed immediately with

WET LAB IV - DNA clean-up

Critical step

Do **not freeze** the DNA as this results in additional fragmentation. If the samples cannot be processed immediately, the DNA can be kept overnight at 4°C.