



# PathoSense

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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 µL 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 µL	52 µL
Nonamer_TSO primer	1 µL	13 µL
dNTP	1 µL	13 µL
Total	6 µL	78 µL

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

### Protocol

- Take a PCR tube for the mix and add **4 µL/sample DNase/RNase free water**
- Vortex and quick spin the **dNTPs** and the **Nonamer TSO** primer
- Add **1 µL/sample dNTPs** and **1 µL/sample Nonamer\_TSO primer** to the mix
- Vortex and quick spin mix

### Primer binding ~ DNA area

- Add **6 µL of the primer/dNTP mix** to each sample
- Gently mix the suspension by tapping the tube with your fingers and quick spin
- 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 $\mu$ L	26 $\mu$ L
SuperScript IV buffer 5x	4 $\mu$ L	52 $\mu$ L
DTT (100 mM)	1 $\mu$ L	13 $\mu$ L
TSO_SS primer (10 $\mu$ M working stock)	1 $\mu$ L	13 $\mu$ L
SuperScript IV enzyme KEEP ON COOL RACK!	1 $\mu$ L	13 $\mu$ L
Total	9 $\mu$ L	117 $\mu$ 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  $\mu$ L/sample DNase/RNase free water**
12. Vortex and quick spin the SuperScript Buffer, DTT and TSO\_SS primer
13. Add **4  $\mu$ L/sample SuperScript Buffer**, **1  $\mu$ L/sample DTT** and **1  $\mu$ L/sample TSO\_SS primer** to the mix
14. Quick spin Superscript IV enzyme (DO NOT VORTEX!)
15. Add **1  $\mu$ 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

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#### 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.