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Working

n-PCR for a single sample

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ABSTRACT

HTLV-1 diagnosis using PCR is based on the amplification of the viral DNA sequences. This is usually conducted using a nested PCR which involves two rounds of DNA amplification reactions targeting the viral polymerase gene. In this case, we also add in the second round primers to amplify the human actin gene.

EXTERNAL LINK

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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GUIDELINES

n-PCR with *pol*/HTLV-1/actin co-amplification

MATERIALS

NAME	CATALOG #	VENDOR
GeneRuler 50 bp DNA Ladder	SM0371	Thermo Fisher Scientific
Taq DNA Polymerase (1000 U)	Cat No./ID: 201205	Qiagen
MT-2 cells	237	
Thermo Fisher Scientific Germany	10416014	Invitrogen - Thermo Fisher
100 bp DNA Ladder	15628019	Invitrogen - Thermo Fisher
dNTP Set 100 mM Solutions	R0182	Thermo Fisher Scientific

SAFETY WARNINGS

- Gloves should be changed regularly, particularly after preparation and before the dispensing of master mixes into PCR plates or strips to avoid cross contamination.
- Be aware that virus genetic material is being use, so be carful.

BEFORE STARTING

- To avoid contamination risk, DNA extraction, Pre PCR set-up (mastermix preparation), and post-PCR procedures should be performed at least in separate workplaces.
- Benches, work stations, centrifuges, vortexes, and pipettes must be cleaned with 70% ethanol before and after every PCR set-up.

1 Equipment and Materials

1. Pipettes
2. 10µl presterilized filter tips
3. 20µl presterilized filter tips
4. 200µl presterilized filter tips
5. 1000µl presterilized filter tips
6. Thermal Cycle (we used TProfessional TRIO)
7. Electrophoresis equipment

2 Reagents and Chemicals

- Taq DNA Polymerase kit (1000 U), by Qiagen, Catalog #: Cat No./ID: 201205

1. Buffers (PCR Buffer and CoralLoad PCR Buffer)
2. MgCl2
3. Q solution
4. Taq polymerase

- dNTPs
- Nuclease free H2O
- Forward (F) and reverse (R) primers
- Agarose
- Markers: 50pb and/or 100pb (Thermo Fisher Scientific, *Germany)*

3 Preparation and storage of reagents

- Stock reagents for PCR (10X buffer, dNTPs, MgCl2, Q solution and primers) and enzymes should be stored at -20°C.
- Stock reagents should be diluted into working concentrations and aliquoted at -20°C into separate tubes.
- Working aliquots of sample and control DNA as well as primary PCR (Nest-1) products must be kept at -20°C for long-term storage but can be kept in the fridge for daily use.

4 PCR summary protocol

- Primary PCR/ Nest-1 PCR should be done using PolEF and PolER primers.
- For each group of samples include positive control (DNA from MT2 cell line) and a negative control of PCR mix without a DNA template.
- Nest-2 PCR should be done using the primers PolIF and PolIR. Also the primers Actin F and Actin R are necessary to amplify human Actin gene.
- Once the PCR run is complete, remove tubes and briefly centrifuge to spin down products.
- Store PCR tubes in the fridge or freezer
- Run the samples on 2% Agarose gel electrophoresis using gel red nucleic acid stain.
- Score results as presence or absence of bands comparing with control bands.

5 PCR Protocol

- For each run, at least two controls (1 positive and 1 negative) should be included in addition to the samples.
- Prepare the PCR master mix calculating enough volume for number of the samples +8%, to account for pipetting loss and dead volume. E.g. for 22 samples, one positive control and one negative control (total of 24 tubes), make a master mix for 26 reactions.
- Master Mix for Nest-1 PCR (**10X PCR buffer**, dNTPs, MgCl2, primers, Taq polymerase and water)
- Master Mix for Nest-2 PCR (**10X CoralLoad PCR Buffer**, dNTPs, MgCl2, primers, Taq polymerase and water)

6 eparation of PCR (Nest-1 PCR)

	Initial Concentration	Final Concentration	For 1 sample
PCR Buffer	10x	1 X	2,5 ul
Q solution	5x	1X	5 ul
Mgl2	25mM	2,5mM	2,5 ul
dNTPs	2mM	0,2mM	2,5 ul
PolEF	10mM	0,3mM	0,75 ul
PolER	10mM	0,3mM	0,75 ul
Taq polymerase		0,5 units	0,1 ul
DNA sample			5 ul (20 ng/µl)
Water			add to a final concentration of 25 ul

Preparation of PCR (Nest-2 PCR)

	Initial Concentration	Final Concentration	For 1 sample
CoralLoad PCR Buffer	10x	1 X	2,5 ul
Q solution	5x	1X	5 ul
Mgl2	25mM	2,5mM	2,5 ul
dNTPs	2mM	0,2mM	2,5 ul
PolIF	10mM	0,3mM	0,75 ul
PolIR	10mM	0,3mM	0,75 ul
Actin F	10mM	0,3mM	0,75 ul
Actin R	10mM	0,3mM	0,75 ul
Taq polymerase		0,5 units	0,1 ul
First reaction product			2 ul
Water			add to a final concentration of 25 ul

7 Primers

Round	Name	5'-3' oligonucleotide sequence
Nest-1 PCR	PolEF	TTTAGGTGCCCAAACTGGAG
Nest-1 PCR	PolER	GCAGGATATTGGAAGCCTCAG3
Nest-2 PCR	PolIF	GCCCTCATGCCAGTGTTTAC
Nest-2 PCR	PolIR	CCTGGAGATGGGATCAGGTAG
Nest-2 PCR	Actin F	ATCGAGCACGGCATCGTCACCAAC
Nest-2 PCR	Actin R	GTTGAAGGTCTCAAACATGATCTG

8 PCR Cycling conditions

Nest-1:
Pol EF + Pol ER

Temperature (°C)	Time	
94	3 min	
94	10 sec	40 cycles
54	20 sec	
70	45 sec	

70	5 min	
4	Hold	

Nest-2:
Pol IF + Pol IR + Act F + Acr R

Temperature (°C)	Time	
94	3 min	
94	10 sec	40 cycles
52	20 sec	
70	45 sec	
70	5 min	
4	Hold	