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🌐 n-PCR for a single sample

📖 PLOS One

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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.

GUIDELINES

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

MATERIALS

MATERIALS

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

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 careful.

Protocol status: Working

BEFORE START INSTRUCTIONS

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- 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.

Keywords: nPCR, HTLV

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.

PCR summary protocol

4

- 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 Conc entrat ion	Final Conc entrat ion	For 1 sampl e
	PCR Buffer	10x	1 X	2,5 ul
	Q soluti on	5x	1X	5 ul
	Mgl2	25m M	2,5m M	2,5 ul
	dNTP s	2mM	0,2m M	2,5 ul
	PolEF	10m M	0,3m M	0,75 ul
	PolER	10m M	0,3m M	0,75 ul
	Taq polym erase		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
Coral Load PCR Buffer	10x	1 X	2,5 ul
Q solution	5x	1X	5 ul
MgI2	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	TTTA GGTG CCCA AACT GGAG
Nest-1 PCR	PolER	GCAG GATA TTGG AAGC CTCA G3
Nest-2 PCR	PolIF	GCCC TCAT GCCA GTGT TTAC
Nest-2 PCR	PolIR	CCTG GAGA TGGG ATCA GGTA G
Nest-2 PCR	Actin F	ATCG AGCA CGGC ATCG TCAC CAAC
Nest-2 PCR	Actin R	GTTG AAGG TCTC AAAC ATGA TCTG

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

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