Annex C 16: CDC Protocol for amplification and sequencing of measles virus MF-NCR from patient samples

This protocol is intended for laboratories that have experience with the amplification and sequencing of the measles N-450 region. It is not a detailed description of all steps but lists important details and differences to the N-450 protocol.

Primers

Name	Sequence
MeV 4869R-H1	5'-CTT GGC CCT CAG TTT TGT TTA G -3'
MeV 4869R	5'-CTT GGC CCT TAG TTT TGT TTA G -3'
MeV G22F	5'-CAC AAG CGA CCG AGG TGA CC -3'
MeV 4801F	5'-CAC AAG CGA CCG AGG TGA C -3'
MeV 5609R	5'-CGA GTC ATA ACT TTG TAG CTT GC -3'
MeV 5145R-B3	5'-GGT TGC CGT GGT GGT GTG TG -3'
MeV 5145R	5'-GGT TGC CGT GGT CGT GTG TG -3'
MeV 4331F	5'-CAG ATG CAA GAT AGT AAG AAT CCA G -3'

Note: For two primers (highlighted in blue and green), there are 2 versions to cover sequence differences between genotypes. We order each version separately and mix them at a 1:1 ratio. The kit only contains the tubes containing the mixtures.

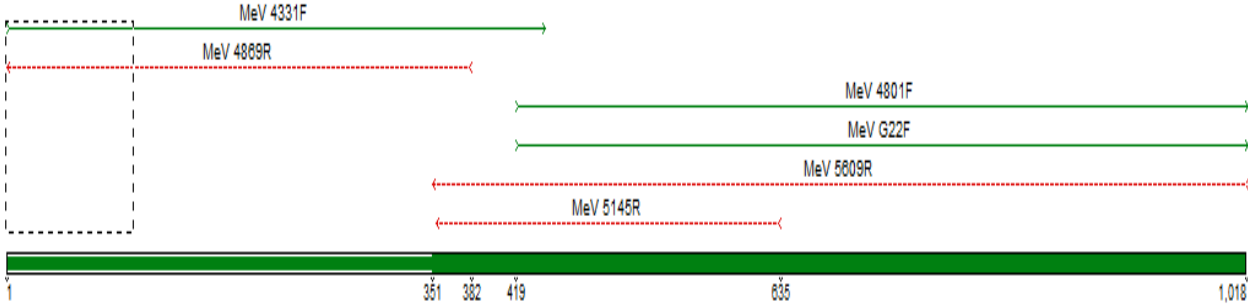
RT-PCR strategies

There are two options for amplification of the MF-NCR:

1. Amplification of the whole region in one fragment (4331F/5609R) which is a 1279 nt amplicon.
2. Two primer sets used to amplify the MF-NCR in two fragments:
    - Fragment 1: (4331F/4869R) which is a 566 nt fragment
    - Fragment 2: (4801F/5609R) which is an 809 nt fragment **OR**
    - Fragment 2: (G22F/5609R) which is an 810 nt fragment

MeV MF-NCR RT-PCR and sequencing strategies

Example alignment of chromatograms generated from sequencing the RT-PCR amplicons using the various primers plus the additional sequencing primer.



RT-PCR assay

Invitrogen SuperScript III One-step RT-PCR with Platinum Tag DNA Polymerase Catalog #12574-026 or similar. The RNA is the same extracted RNA used in the real time assay and/or genotyping assay. Extracted RNA from low-passage cell culture can also be used.

Kit components and final concentrations

Invitrogen Components	Vol/Rxn (µl)	Final Conc
Nuclease-free water	4.5	
Betaine solution	5.0	10%
2x Invitrogen One-step buffer	25	1x
5 mM $MgSO_4$	8	0.8 mM
Forward primer (20 µM)	0.5	0.2 µM
Reverse primer (20 µM)	0.5	0.2 µM
RNase inhibitor	0.5	
Invitrogen enzyme mix	1	
RNA	5	
Total	50	

Cycling conditions

Step	Temperature (°C)	Duration	Number of Cycles
Reverse transcription	55	30 min	1
Denaturation	94	2 min	1
Denaturation	94	15 sec	40
Annealing	56	30 sec	
Extension	68	1 min	
Final extension	68	7 min	1
Hold	4	hold	1

Sequencing

An additional sequencing primer (5145R) can also be used for better sequence coverage for fragment 2 if sequences generated with primer 4801F or 5609R are not long enough to overlap with sequences of the first fragment.

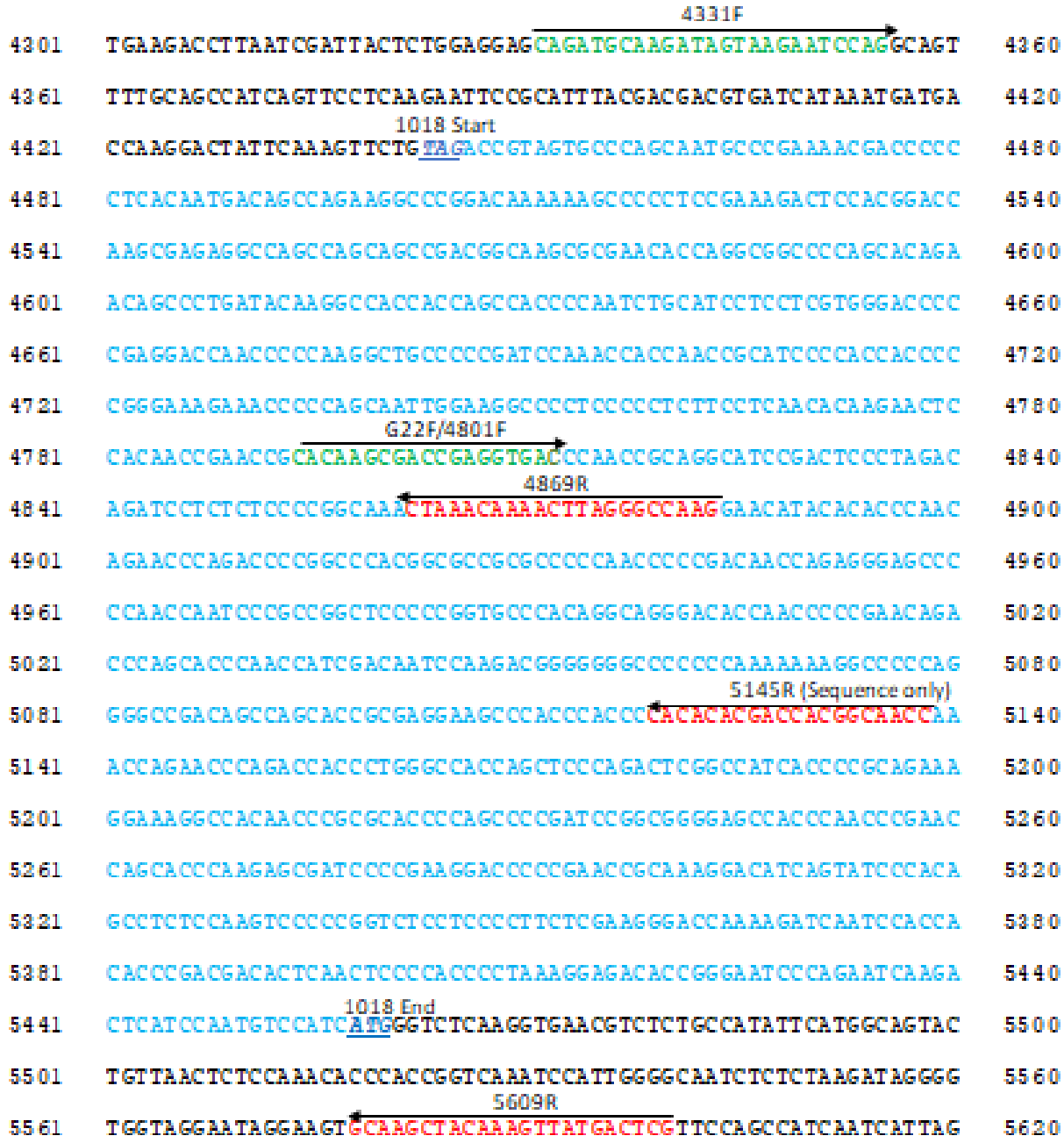
All listed primers have been used successfully for sequencing PCR amplicons. If the gel images indicate faint bands or RNA concentration is low, the RT-PCR can be repeated with higher volumes of RNA to increase the likelihood of successful amplification. Additionally, extra DNA could be added to the sequencing reactions if the PCR products still produce faint bands.

Size of the MF-NCR

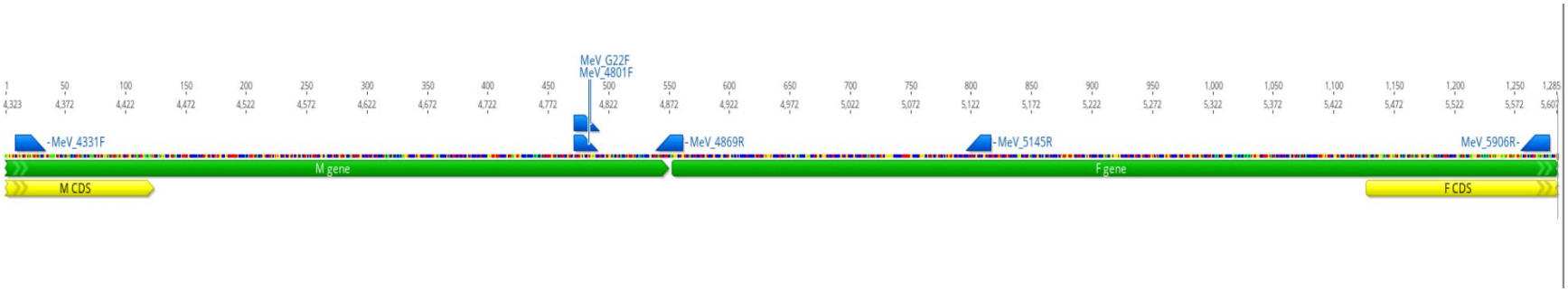
The sequence below shows the complete MF-NCR, starting with the stop codon of the M ORF (nt 4443 in a full-length genome) and including the start codon of the F ORF (nt 5460). In most viruses, this region is 1018 nt long. It should be noted that in many but not all measles viruses there are two in-frame start codons for the F ORF. The correct start codon is nt 5458-5460.

This numbering is for a standard genome of 15,894 nt. We have found several MF-NCRs with indels, which may affect the size of the MF-NCR and the genome. Measles virus follows the rule of six, which requires that the size of the full genome is a multiple of six. Viruses that do not follow this rule are not viable. Most indels we have found so far maintain the rule of six within the MF-NCR (i.e. a one nt insertion and a one nt deletion in the same MF-NCR). While it is theoretically possible that indels in the MF-NCR that violate this rule (e.g. an insertion of one nt with no corresponding deletion) could be corrected elsewhere in the genome, such changes should be viewed with suspicion and may require sequencing the whole genome.

>MF sequencing window is smaller than the combined PCR amplicons



Genome map for MF region:
Blue: primers
Yellow: coding sequence
Green: genes



Numbers are nucleotide positions in the whole genome.