Supplementary Materials: Three-stage Pooled-Plasma HCV RNA Testing for the Identification of Acute HCV Infections in At-Risk Populations

Methods

Simulation analysis

Let $N$ be the pooled size of the first stage. Let $X_i$ be the number of HCV RNA for a specimen, $i = 1, \ldots, N$. At the first stage, let $\phi_N = 1$ if $\sum_{i=1}^{N} X_i / N \geq c$, otherwise, 0. Here $c$ is the detectable limit. Also, let $k$ be the pooled size of each subgroup at the second stage, let $\phi_{k,j} = 1$ if

$$\sum_{i=(j-1)k+1}^{jk} X_i / k \geq c,$$

otherwise, 0, for $j = 1, \ldots, N/k$.

Then, the required tests for the proposed three-stage pooled testing was

$$T = 1 + \left[ \phi_{Se,N} \phi_N + (1-\phi_{Sp,N})(1-\phi_N) \right] \left\{ \frac{N}{k} + k \sum_{j=1}^{N/k} \left[ \phi_{Se,k,j} \phi_{k,j} + (1-\phi_{Sp,k,j})(1-\phi_{k,j}) \right] \right\},$$

where $\phi_{Se,N} = 1$ and $\phi_{Se,k,j} = 1$ with sensitivity, say $Se$, and $\phi_{Sp,N} = 1$ and $\phi_{Sp,k,j} = 1$ with specificity, say $Sp$.

It was noted that $\phi_N$ and $\phi_{k,j}$'s were not independent. For example, with $N=10, k=5$ and $\phi_{10} = 1$, we must have either $\phi_{5,1} = 1$ or $\phi_{5,2} = 1$. Also, if $\phi_{5,3} = 0$, we must have $\phi_{5,2} = 1$. Therefore, the dependence led to the difficulty of deriving the statistical characteristics. Alternatively, a simulation of the three-stage pooled testing was applied.
Simulation of the three-stage pooled testing

**Step 1:** Generate \( N \) indicators (detectable or undetectable) from a Bernoulli distribution with prevalence, say \( P \). If the indicator was 0, then the specimen was denoted by undetectable. In this case, we still assigned a value to this undetectable specimen. Else if the indicator was detectable, then we would randomly select a value from the real detectable database.

**Step 2:** The simulated three-stage pooled testing was performed; that is, the \( N \) specimens were pooled to test. The testing was continued to the second stage with probability of \( S_e \) if \[ \sum_{i=1}^{N} X_i / N \geq c ; \] otherwise, the testing was continued to the second stage with probability of \( 1 - S_p \); otherwise, the testing was stopped and the \( N \) specimens were considered as undetectable.

**Step 3:** At the second stage, every \( k \) specimens were pooled to test. The testing was continued to the third stage with probability of \( S_e \) for each subgroup if \[ \sum_{i=(j-1)k+1}^{jk} X_i / k \geq c \text{ for } j = 1, \ldots, N / k ; \] otherwise, the testing was continued to the third stage with probability of \( 1 - S_p \); otherwise, the subgroup was stopped to test and this \( k \) specimens were considered as undetectable.

**Step 4:** At the third stage, every specimen was tested individually. If the \( \bar{X} \) was greater than \( c \), then the specimen was denoted by detectable with probability of \( S_e \); else if the \( \bar{X} \) was less than \( c \), then the specimen was denoted by detectable with probability of \( 1 - S_p \); otherwise, undetectable.
The next section is the R code of the proposed 3-stage pooled strategy. This R code allows user to specify the prevalence, individual sensitivity, individual specificity, N, and k. An additional csv file is required to provide a sample of value of detectable responses. The first row in the csv file is the variable name (can be arbitrary). The following figure is an example of the content:

![Example Table]

While an example of the output is as following figure:

![Example Output]

**Supplementary R code**

```r
a <- read.csv("E:/AAA.csv")  #Pathway of the input data

UDTV <- 14  #Value of undetectable viral load
```
Se <- 0.9894 #Individual sensitivity
Sp <- 1.0000 #Individual specificity
N <- 20 #Size of the first stage
k <- 5 #Size of the second stage
p <- 0.018 #Prevalence rate

#============================================================================#

MyCfunc <- function(DT, UDTV, Se, Sp, N, k, p)
{
  Ng <- 100 #Number of procedures
  NSS <- Ng*N
  a1 <- rbinom(NSS, 1, p)
  a2 <- array(0, NSS)
  a3 <- a1
  for(i in 1:NSS)
  {
    if(a1[i]==1)
    {
      a2[i] <- sample(DT, 1, replace = TRUE)
    }else{
      a2[i] <- UDTV
    }
  }
  kg <- N/k
  c <- 0 #Number of Detection
  p15 <- 0 #Number of Positive in the 3rd Tier
}
mytemp <- NULL

for(i in 1:Ng){
    L20 <- 1+N*(i-1)
    U20 <- N*i
    c <- c + 1
    if((sum(a2[L20:U20])/N) >= 15){
        #First Tier
        tSe <- rbinom(1,1,Se)
        if(tSe==1){
            for(j in 1:kg){
                Lk <- L20+k*(j-1)
                Uk <- L20-1+k*j
                c <- c + 1
                if((sum(a2[Lk:Uk])/k) >= 15){
                    #Second Tier
                    tSe <- rbinom(1,1,Se)
                    if(tSe==1){
                        c <- c + k
                        for(l in Lk:Uk){
                            if(a2[l] >= 15){
                                #Third Tier
                                tSe <- rbinom(1,1,Se)
                            }
                        }
                    }
                }
            }
        }
    }
}


if(tSe==1){
    p15 <- p15 + 1
    a3[l] <- 1
} else {
    a3[l] <- 0
}
}

else{
    tSp <- rbinom(1,1,Sp)
    if(tSp==1){
        a3[l] <- 0
    } else {
        p15 <- p15 + 1
        a3[l] <- 1
    }
}

} else{
    a3[Lk:Uk] <- 0
}
}
}
}
}
}
else{
    #Second Tier
    tSp <- rbinom(1,1,Sp)
if(tSp==1){
    a3[Lk:Uk] <- 0
}else{
    c <- c + k
    for(l in Lk:Uk){
        if(a2[l] >= 15){
            #Third Tier
            tSe <- rbinom(1,1,Se)
            if(tSe==1){
                p15 <- p15 + 1
                a3[l] <- 1
            }else{
                a3[l] <- 0
            }
        }else{
            tSp <- rbinom(1,1,Sp)
            if(tSp==1){
                a3[l] <- 0
            }else{
                p15 <- p15 + 1
                a3[l] <- 1
            }
        }
    }
}
else{
  a3[L20:U20] <- 0
}
}
}else{
  #First Tier
  tSp <- rbinom(1,1,Sp)
  if(tSp==1){
    a3[L20:U20] <- 0
  }else{
    for(j in 1:kg){
      Lk <- L20+k*(j-1)
      Uk <- L20-1+k*j
      c <- c + 1
      if((sum(a2[Lk:Uk])/k) >= 15){
        #Second Tier
        tSe <- rbinom(1,1,Se)
        if(tSe==1){
          
        
      }else{
        
      }
    }
  }
}
else{
  
}

c <- c + k

for(l in Lk:Uk){
    if(a2[l] >= 15){
        # Third Tier
        tSe <- rbinom(1,1,Se)
        if(tSe==1){
            p15 <- p15 + 1
            a3[l] <- 1
        }else{
            a3[l] <- 0
        }
    }else{
        tSp <- rbinom(1,1,Sp)
        if(tSp==1){
            a3[l] <- 0
        }else{
            p15 <- p15 + 1
            a3[l] <- 1
        }
    }
}
}
a3[Lk:Uk] <- 0

}

}else{

#Second Tier

tSp <- rbinom(1,1,Sp)

if(tSp==1){

  a3[Lk:Uk] <- 0

}else{

c <- c + k

  for(l in Lk:Uk){

    if(a2[l] >= 15){

      #Third Tier

      tSe <- rbinom(1,1,Se)

      if(tSe==1){

        p15 <- p15 + 1

        a3[l] <- 1

      }else{

        a3[l] <- 0

      }

    }else{

      tSp <- rbinom(1,1,Sp)

      if(tSp==1){

        }else{
a3[l] <- 0

} else {

  p15 <- p15 + 1
  a3[l] <- 1

}

}

}

}

}

}

}

}

}

}

}

}

mytemp <- cbind(mytemp, c)

c <- 0

}

TP <- 0    # True Positive

FN <- 0    # False Negative

TN <- 0    # True Negative

FP <- 0    # False Positive

for(i in 1:NSS) {

  if(a3[i] < a1[i]) {

    FN <- FN + 1

  }
```r
else if(a3[i]>a1[i]){
    FP <- FP + 1
}
else if(a3[i]==1 & a1[i]==1){
    TP <- TP + 1
}
else if(a3[i]==0 & a1[i]==0){
    TN <- TN + 1
}
}

ESe <- TP/(TP+FN)  # Empirical Sensitivity
ESp <- TN/(TN+FP)  # Empirical Specificity
temp <- mean(mytemp)
c(temp, sd(mytemp), 1-temp/N, ESe, ESp, p15/NSS)
```
Mytemp <- c(p,N,k,round(mymean[1:2],1),round(mymean[3:6],4))

names(Mytemp) <- c("Prevalence","N","k","Mean","SD","Cost_Reduction",
   "Test_Sensitivity","Test_Specificity","Prevalence")

Mytemp2 <- list(Mytemp[1:3], Mytemp[4:5], Mytemp[6:9])

names(Mytemp2) <- c("Procedure Size",
   "Number of tests in one procedure",
   "Estimates")

Mytemp2

} else{print("ERROR!N must be exactly divisible by k.")}

}

#-------------------------------------------------------------#

DT <- a[,1]

MyCfunc2(DT,UDTV,Se,Sp,N,k,p)
**Table S1.** The analytical sensitivity of pooled samples. Twenty individual positive clinical samples with 25% of the positive samples near the limit of detection of the assay (5 samples with HCV RNA loads <300 IU/mL and 15 with loads between 300 to 1000 IU/mL) were selected to determine the analytical sensitivity of pooled samples. Each positive sample were pooled with other 19 negative samples as described in Methods. The determination of HCV RNA loads by pooled testing was repeated twice, and the results are shown in the following table. For the 5 samples with HCV RNA loads <300 IU/mL, the sensitivity of pooled testing ranges 0-50%. For the 15 samples with HCV RNA loads of 300-1000 IU/mL, the sensitivity of pooled testing is 86.7% (13/15). The two negative results came from samples of 313 and 367 IU/ml, respectively.

| Patient Number | Original HCV RNA loads by individual testing | The 1st HCV RNA loads after pooled testing | The 2nd HCV RNA loads after pooled testing |
|----------------|---------------------------------------------|------------------------------------------|-------------------------------------------|
|                | IU/mL | log_{10} IU/mL | IU/mL | log_{10} IU/mL | Differences* | IU/mL | log_{10} IU/mL | Differences* |
| 1              | 276   | 2.44           |       |               |              | 440   | 2.64           | 0.20         |
| 2              | 518   | 2.71           | 394   | 2.60          | -0.12        | 628   | 2.80           | 0.08         |
| 3              | 526   | 2.72           | 414   | 2.62          | -0.10        | 466   | 2.67           | -0.05        |
| 4              | 367   | 2.56           |       |               |              | 362   | 2.56           | 0.32         |
| 5              | 435   | 2.64           | 746   | 2.87          | 0.23         | 684   | 2.84           | 0.20         |
| 6              | 175   | 2.24           |       |               |              | 362   | 2.56           | 0.32         |
| 7              | 928   | 2.97           | 1716  | 3.23          | 0.27         | 900   | 2.95           | -0.01        |
| 8              | 556   | 2.75           | 534   | 2.73          | -0.02        | 614   | 2.79           | 0.04         |
| 9              | 313   | 2.50           |       |               |              | 313   | 2.50           | 0.00         |
| 10             | 356   | 2.55           | 408   | 2.61          | 0.06         | 548   | 2.74           | 0.19         |
| 11             | 265   | 2.42           |       |               |              | 265   | 2.42           | 0.00         |
| 12             | 317   | 2.50           | 428   | 2.63          | 0.13         | 460   | 2.66           | 0.16         |
|   |   |   |   |   |   |
|---|---|---|---|---|---|
| 13 | 139 | 2.14 | 808 | 2.91 | 0.76 |
| 14 | 181 | 2.26 |   |   | 422 |
| 15 | 595 | 2.77 | 878 | 2.94 | 0.17 |
| 16 | 964 | 2.98 | 1278 | 3.11 | 0.12 |
| 17 | 616 | 2.79 | 356 | 2.55 | -0.24 |
| 18 | 734 | 2.87 | 336 | 2.53 | -0.34 |
| 19 | 870 | 2.94 | 776 | 2.89 | -0.05 |
| 20 | 338 | 2.53 | 806 | 2.91 | 0.38 |

Footnote: The grey and black bars mean no detection of HCV RNA.
* Differences in HCV RAN loads (log_{10} IU/mL) by individual or pooled testing
Table S2. Clinical characteristics of 62 people living with HIV who tested positive for HCV viremia in the pooled HCV study

| Variables                                                        | Data                     |
|-----------------------------------------------------------------|--------------------------|
| Participant number                                              | 62                       |
| Participants with HCV viremia at the first determination, n (%) | 30 (48.4)                |
| Participants with documented acute HCV infection*               | 51 (82.3)                |
| Interval from negative to positive HCV viremia, median (IQR), days | 91 (81-105)              |
| Age, median (IQR), years                                        | 33.1 (29.6-40.0)         |
| Men, n (%)                                                      | 62 (100)                 |
| HIV transmission risk, n (%)                                     |                          |
| Men who have sex with men                                        | 61 (98.4)                |
| Heterosexuals                                                    | 0 (0)                    |
| Injection drug users                                             | 1 (1.6)                  |
| At enrollment, n/N (%)                                           |                          |
| CD4 count ≥200 cells/mm³                                         | 57/61 (93.4)             |
| Plasma HIV RNA load <50 copies/mL                                | 53/62 (85.5)             |
| Positive anti-HCV before enrollment                              | 27/61 (44.3)             |
| Having received HCV treatment (both IFN-based and DAA)           | 20/62 (32.3)             |
| NHI-reimbursed DAA treatment                                     | 12/20 (60.0)             |
| Reasons for enrollment, n (%)                                    |                          |
| Occurrence of sexually transmitted infections                   | 46 (74.2)                |
| Event                                                                 | Count (%) |
|----------------------------------------------------------------------|-----------|
| Achievement of sustained virologic response                          | 18 (29.0) |
| Development of spontaneous clearance                                 | 4 (6.5)   |
| Abnormal liver function                                              | 11 (17.7) |

At the time of positive pooled HCV RNA

| Description                                                                 | Count (%) |
|---------------------------------------------------------------------------|-----------|
| Plasma HCV RNA load, median (range), log\(_{10}\) IU/mL                   | 6.4 (1.3-8.5) |
| Participants with negative anti-HCV, n (%)                                | 15 (24.2) |
| Negative anti-HCV in participants with negative anti-HCV before enrolment | 15/34 (44.1) |

Participants initiating DAA treatment, n (%) 47 (75.8)

| Interval from diagnosis to treatment, median (range), days                | 56 (9-258) |
| DAA treatment reaching EOT, n (%)                                        | 47 (100)   |
| Detectable HCV RNA at EOT                                                 | 1/47 (2.1) |
| Undetectable HCV RNA at EOT                                               | 44/47 (93.6) |
| No data at EOT                                                            | 2/47 (4.3) |

Follow-up reaching 12 weeks after EOT, n (%) 47 (75.8)

| Undetectable HCV RNA 12 weeks after EOT (SVR12)                           | 42 (89.4)  |
| Positive HCV RNA 12 weeks after EOT                                       | 4** (8.5)  |
| Loss to follow-up 12 weeks after EOT                                      | 1 (2.1)    |

Participants not receiving DAAs, n (%) 15 (24.2)

| History of previous NHI-reimbursed DAA treatment                         | 12/15 (70.6) |
| Spontaneous clearance of HCV infection                                   | 1/15 (5.9)   |
|                          |       |
|--------------------------|-------|
| Loss to follow-up        | 1/15  |
|                          | (5.9) |
| Incarceration            | 1/15  |
|                          | (5.9) |

*Acute HCV infection was defined as HCV viraemia identified in individuals without HCV viremia within the past one year.

**Three participants with HCV viraemia 12 weeks after EOT experienced HCV genotype switch after off-DAA for 3 months, and the 4th PLWH was the one who had detectable HCV RNA at EOT.

Abbreviations: DAA, direct-acting antiviral; EOT, end of treatment; HCV, hepatitis C virus; IFN, interferon; SVR12, sustained virologic response 12 weeks off-treatment.
Table S3. Averaged results from 10,000 times simulation study with individual sensitivity of 0.9894 and individual specificity of 0.9999 in the setting of different HCV viremia prevalence, and pooled and mini-pooled sizes.

| Prevalence | N  | k | Number of tests Mean | SD | Cost reduction | Three-stage Sensitivity | Specificity |
|------------|----|---|----------------------|----|----------------|-------------------------|-------------|
| 0.001      | 20 | 2 | 1.2                  | 1.6| 0.9384         | 0.9502                 | 1.0000      |
|            | 4  | 1.2| 1.2                  | 0.9413| 0.9499           | 1.0000                  |
|            | 5  | 1.2| 1.2                  | 0.9413| 0.9500           | 1.0000                  |
| 0.001      | 30 | 3 | 1.4                  | 2.1| 0.9547         | 0.9136                 | 1.0000      |
|            | 5  | 1.3| 1.8                  | 0.9565| 0.9137           | 1.0000                  |
|            | 6  | 1.3| 1.8                  | 0.9565| 0.9138           | 1.0000                  |
|            | 10 | 1.4| 2.1                  | 0.9547| 0.9138           | 1.0000                  |
| 0.001      | 40 | 4 | 1.5                  | 2.6| 0.9621         | 0.9141                 | 1.0000      |
|            | 5  | 1.5| 2.5                  | 0.9630| 0.9141           | 1.0000                  |
|            | 8  | 1.5| 2.5                  | 0.9631| 0.9140           | 1.0000                  |
|            | 10 | 1.5| 2.7                  | 0.9621| 0.9144           | 1.0000                  |
| 0.005      | 20 | 2 | 2.1                  | 3.5| 0.8940         | 0.9500                 | 1.0000      |
|            | 4  | 1.8| 2.7                  | 0.9077| 0.9502           | 1.0000                  |
|            | 5  | 1.8| 2.7                  | 0.9077| 0.9503           | 1.0000                  |
| 0.005      | 30 | 3 | 2.7                  | 4.5| 0.9092         | 0.9178                 | 1.0000      |
|            | 5  | 2.5| 3.8                  | 0.9176| 0.9180           | 1.0000                  |
|            | 6  | 2.5| 3.9                  | 0.9174| 0.9180           | 1.0000                  |
|   |   |   |   |   |   |
|---|---|---|---|---|---|
| 10 | 2.8 | 4.6 | 0.9082 | 0.9184 | 1.0000 |
| 0.005 | 40 | 4 | 3.4 | 5.4 | 0.9140 | 0.9193 | 1.0000 |
| 5 | 3.3 | 5.1 | 0.9179 | 0.9195 | 1.0000 |
| 8 | 3.3 | 5.2 | 0.9172 | 0.9198 | 1.0000 |
| 10 | 3.5 | 5.6 | 0.9125 | 0.9199 | 1.0000 |
| 0.01 | 20 | 2 | 3.2 | 4.6 | 0.8423 | 0.9534 | 1.0000 |
| 4 | 2.6 | 3.6 | 0.8677 | 0.9537 | 1.0000 |
| 5 | 2.7 | 3.6 | 0.8675 | 0.9535 | 1.0000 |
| 0.01 | 30 | 3 | 4.4 | 5.9 | 0.8533 | 0.9540 | 1.0000 |
| 5 | 3.9 | 5.1 | 0.8687 | 0.9549 | 1.0000 |
| 6 | 4.0 | 5.2 | 0.8680 | 0.9550 | 1.0000 |
| 10 | 4.5 | 6.2 | 0.8493 | 0.9516 | 1.0000 |
| 0.01 | 40 | 4 | 5.7 | 7.0 | 0.8564 | 0.9561 | 1.0000 |
| 5 | 5.5 | 6.6 | 0.8630 | 0.9558 | 1.0000 |
| 8 | 5.6 | 6.9 | 0.8603 | 0.9516 | 1.0000 |
| 10 | 6.0 | 7.5 | 0.8511 | 0.9516 | 1.0000 |
| 0.02 | 20 | 2 | 5.0 | 5.8 | 0.7500 | 0.9559 | 1.0000 |
| 4 | 4.1 | 4.6 | 0.7942 | 0.9553 | 1.0000 |
| 5 | 4.1 | 4.7 | 0.7927 | 0.9555 | 1.0000 |
| 0.02 | 30 | 3 | 7.1 | 7.0 | 0.7621 | 0.9580 | 1.0000 |
| 5 | 6.4 | 6.3 | 0.7851 | 0.9579 | 1.0000 |
| 6 | 6.5 | 6.5 | 0.7826 | 0.9585 | 1.0000 |
|   |   |   |   |   |   |
|---|---|---|---|---|---|
| 10 | 7.6 | 8.0 | 0.7461 | 0.9528 | 1.0000 |
| 0.02 | 40 | 4 | 9.4 | 8.0 | 0.7644 | 0.9601 | 1.0000 |
| 5 | 9.1 | 7.8 | 0.7732 | 0.9596 | 1.0000 |
| 8 | 9.4 | 8.5 | 0.7638 | 0.9523 | 1.0000 |
| 10 | 10.2 | 9.4 | 0.7448 | 0.9525 | 1.0000 |
| 0.05 | 20 | 2 | 9.2 | 6.4 | 0.5419 | 0.9616 | 1.0000 |
| 4 | 7.7 | 5.6 | 0.6127 | 0.9613 | 1.0000 |
| 5 | 7.9 | 5.9 | 0.6051 | 0.9610 | 1.0000 |
| 0.05 | 30 | 3 | 12.9 | 6.9 | 0.5707 | 0.9642 | 1.0000 |
| 5 | 12.2 | 7.1 | 0.5923 | 0.9642 | 1.0000 |
| 6 | 12.6 | 7.6 | 0.5801 | 0.9640 | 1.0000 |
| 10 | 14.9 | 9.5 | 0.5032 | 0.9568 | 1.0000 |
| 0.05 | 40 | 4 | 16.8 | 7.6 | 0.5797 | 0.9657 | 1.0000 |
| 5 | 16.7 | 7.9 | 0.5823 | 0.9659 | 1.0000 |
| 8 | 18.3 | 9.6 | 0.5436 | 0.9555 | 1.0000 |
| 10 | 19.9 | 10.8 | 0.5021 | 0.9566 | 1.0000 |
| 0.07 | 20 | 2 | 11.1 | 6.0 | 0.4428 | 0.9636 | 1.0000 |
| 4 | 9.7 | 5.7 | 0.5175 | 0.9634 | 1.0000 |
| 5 | 9.9 | 6.0 | 0.5036 | 0.9634 | 1.0000 |
| 0.07 | 30 | 3 | 15.5 | 6.3 | 0.4848 | 0.9662 | 1.0000 |
| 5 | 15.1 | 7.1 | 0.4954 | 0.9660 | 1.0000 |
| 6 | 15.7 | 7.6 | 0.4762 | 0.9663 | 1.0000 |
|        |      |      |     |     |     |     |      |
|--------|------|------|-----|-----|-----|-----|------|
| 0.07   | 30   | 10   | 18.6| 9.4 | 0.3813| 0.9589| 1.0000|
| 4      | 20.2 | 7.1  | 0.4958| 0.9673| 1.0000|
| 5      | 20.4 | 7.8  | 0.4910| 0.9675| 1.0000|
| 8      | 22.7 | 9.7  | 0.4331| 0.9574| 1.0000|
| 10     | 24.6 | 10.7 | 0.3840| 0.9587| 1.0000|
|        |      |      |-----|-----|-----|-----|------|
| 0.1    | 20   | 2    | 13.3| 5.3 | 0.3328| 0.9658| 1.0000|
| 4      | 12.0 | 5.5  | 0.3985| 0.9658| 1.0000|
| 5      | 12.4 | 5.9  | 0.3776| 0.9657| 1.0000|
| 0.1    | 30   | 3    | 18.4| 5.6 | 0.3869| 0.9676| 1.0000|
| 5      | 18.7 | 6.9  | 0.3773| 0.9677| 1.0000|
| 6      | 19.5 | 7.4  | 0.3511| 0.9675| 1.0000|
| 10     | 22.8 | 8.9  | 0.2416| 0.9615| 0.9999|
| 0.1    | 40   | 4    | 24.2| 6.8 | 0.3951| 0.9681| 1.0000|
| 5      | 24.8 | 7.7  | 0.3795| 0.9681| 1.0000|
| 8      | 27.9 | 9.4  | 0.3028| 0.9598| 1.0000|
| 10     | 30.1 | 10.2 | 0.2470| 0.9613| 0.9999|
| 0.15   | 20   | 2    | 15.9| 4.3 | 0.2041| 0.9676| 1.0000|
| 4      | 15.1 | 5.2  | 0.2457| 0.9676| 1.0000|
| 5      | 15.7 | 5.6  | 0.2164| 0.9677| 0.9999|
| 0.15   | 30   | 3    | 22.1| 5.3 | 0.2621| 0.9684| 1.0000|
| 5      | 23.2 | 6.6  | 0.2260| 0.9682| 1.0000|
| 6      | 24.2 | 7.0  | 0.1934| 0.9685| 0.9999|
| 10 | 27.3 | 7.6 | 0.0888 | 0.9643 | 0.9999 |
|----|------|-----|--------|--------|--------|
| 0.15 | 40 | 4 | 29.6 | 7.0 | 0.2601 | 0.9685 | 1.0000 |
| 5 | 30.7 | 7.7 | 0.2333 | 0.9684 | 1.0000 |
| 8 | 34.1 | 8.7 | 0.1469 | 0.9626 | 0.9999 |
| 10 | 36.1 | 8.9 | 0.0965 | 0.9642 | 0.9999 |

| 0.2 | 20 | 2 | 17.8 | 3.9 | 0.1093 | 0.9680 | 1.0000 |
|----|----|----|------|-----|--------|--------|--------|
| 4 | 17.4 | 4.9 | 0.1281 | 0.9683 | 1.0000 |
| 5 | 18.1 | 5.2 | 0.0972 | 0.9680 | 0.9999 |
| 0.2 | 30 | 3 | 25.2 | 5.4 | 0.1597 | 0.9685 | 1.0000 |
|----|----|----|------|-----|--------|--------|--------|
| 5 | 26.7 | 6.3 | 0.1112 | 0.9683 | 0.9999 |
| 6 | 27.6 | 6.5 | 0.0797 | 0.9685 | 0.9999 |
| 10 | 30.0 | 6.4 | -0.0011 | 0.9661 | 0.9999 |

| 0.2 | 40 | 4 | 34.0 | 7.0 | 0.1495 | 0.9687 | 1.0000 |
|----|----|----|------|-----|--------|--------|--------|
| 5 | 35.2 | 7.5 | 0.1190 | 0.9684 | 0.9999 |
| 8 | 38.3 | 7.9 | 0.0433 | 0.9646 | 0.9999 |
| 10 | 39.7 | 7.6 | 0.0074 | 0.9660 | 0.9999 |
Figure S1. The process of 3-stage pooled-plasma HCV RNA testing
Stage 1
20 specimens

Plasma of 50 micro-liters from each specimen is pooled together.

If the result of stage-1 specimen is negative, all the 20 individual specimens are negative for HCV RNA.

Stage 2: If the result of stage-1 specimen is positive, 5 individual specimens would be pooled in each mini-pooled specimen.

Stage 3: Each of the 5 specimens were retested individually to identify the one with HCV RNA.
Footnote: The star sign indicates the individual specimen positive for HCV RNA.
Figure S2. Outcomes of 62 people living with HIV in whom HCV viremia were identified by three-stage pooled-plasma HCV testing in the present study

Abbreviations: EOT, end of treatment; HCV, hepatitis C virus; NHI, National Health Insurance; PLWH, people living with HIV; SVR12, sustained virologic response 12 weeks off-therapy
**Figure S3.** Pooled sensitivity against prevalence with individual sensitivity of 98.94\% and individual specificity of 99.99\% (Given the detection limit of 15 IU/mL, the value of the undetectable individual specimen is assigned to 0 IU/mL in the left panel, and that of the undetectable individual specimen is assigned to 14 IU/mL in the right panel). The lower the HCV prevalence or larger pooled size, the lower sensitivity of pooled HCV RNA testing. With the HCV viremia prevalence of 1\% and the undetectable values of 14 IU/mL, the pooled sensitivity decreased to around 97\% while the individual sensitivity was 99.98\%. If undetectable values were assigned to be 0 IU/mL, the pooled sensitivity decreased to only around 92\%. However, if undetectable values were set as 0 IU/mL, the pooled sensitivity for N=20 was much greater than those for N=30 and N=40, quite different from the conditions with the undetectable value of 14 IU/mL.
**Figure S4.** Pooled specificity against prevalence with individual sensitivity of 98.94% and individual specificity of 99.99% (Given the detection limit of 15 IU/mL, the value of the undetectable individual specimen is assigned to 0 IU/mL in the left panel, and that of the undetectable individual specimen is assigned to 14 IU/mL in the right panel). For the pooled specificity, all were better than the individual specificity regardless of any HCV viremia prevalence.
**Figure S5.** Cost reduction against prevalence with different lower individual sensitivities (I_Se) and individual specificities (I_Sp) in the setting of the combinations of pooled sizes of (pool size, N = 20, 30, or 40; mini-pool size, k=5) and the value of undetectable individual specimen of 14 IU/mL. The performance remained similar in all settings.
**Figure S6.** Pooled sensitivity against prevalence with different individual sensitivities ($I_\text{Se}$) and individual specificities ($I_\text{Sp}$) in the setting of the combinations of pooled sizes of (pool size, $N = 20, 30, 40$; mini-pool size, $k=5$) and the value of undetectable individual specimen of 14 IU/mL. When the individual sensitivity was 90%, the pooled sensitivity was only around 72%, which was considered poor performance.
**Figure S7.** Pooled specificity against prevalence with different individual sensitivities (I_\text{Se}) and individual specificities (I_\text{Sp}) in the setting of the combinations of pooled sizes of (pool size, N = 20, 30, or 40; mini-pool size, k=5) and the value of undetectable individual specimen of 14 IU/mL. The pooled specificity decreased with a decrease of individual specificity, but the decrease was acceptable since it was still around 94% even when the HCV viremia prevalence was 20%.