Inter-Laboratory Variation in Interpretation of DNA Mixtures Study:

Number of Contributors (NoC) Subtest Instructions

Contents

1 Overview

The *Number of Contributors (NoC) Subtest* is the third phase of the DNAmix 2021 study. In this subtest you will be assigned 12 *NoC Packets*, each containing a DNA mixture profile. All DNA mixture profiles will be provided as electropherograms (HID files). Each electropherogram you are assigned will be prepared using a specified combination of *Amp/CE Settings*[*] you select in *Mixture Configuration Selection,* which must be completed prior to the *NoC Subtest* (see Section 2 for additional details).

For each *NoC Packet*, participants will be asked to provide assessments of suitability and number of contributors.

The *Number of Contributors (NoC) Subtest* will be accessible via a link on the Participant Homepage of the DNAmix 2021 website. When the *NoC Subtest* initially becomes available, all registered participants will be notified via e-mail. Prior to the launch of the *NoC Subtest,* a "NoC Beta Test" will be temporarily available, which will consist of one comparison packet for review. Although the *NoC Beta Test* is not required, participants are highly encouraged to complete it as practice and are welcomed to provide feedback for improving the *NoC Subtest* (e.g., clarity of questions, functionality of software, etc.). Once the *NoC Subtest* becomes available, the *NoC Beta Test* will be removed from the website.

Conduct your assessments of each DNA mixture profile (HID file) and respond to each of these questions based upon the policies and validated procedures in your Standard Operating Procedures (SOPs), using the same considerations and diligence that you would employ for operational casework samples.

Please review the "DNAmix2021 — Glossary" prior to beginning the *NoC Subtest* for details about the acronyms and terminology as specifically used in this study.

The *P&P Questionnaire* and the *Casework Scenario Questionnaire* (Phases 1 and 2 of the DNAmix 2021 study) may be completed at any time throughout the study period and need not be submitted in order to complete the *NoC Subtest*. However, participating laboratories must complete and submit these questionnaires prior to the end of the study in order to retrieve their study results.

[*] *"Amp/CE Settings" refers to a specific combination of amplification kit, amplification cycles, volume of amplification reaction, CE instrument, and injection time and voltage*

2 Mixture Configuration Selection

Mixture Configuration Selection is a two-question online survey accessed from the DNAmix 2021 website conducted for participants to:

- Indicate if you will participate in the NoC and/or ICSA Subtests
- Select the Amp/CE settings used to prepare the mixtures that will be assigned to you in the NoC/ ICSA Subtests
- Indicate how the selected Amp/CE Settings compare to your SOPs

Before you begin the *NoC Subtest*, you must complete *Mixture Configuration Selection*. The study team will use this information to assign mixtures to participants, and the DNAmix website software will use the information to determine which questions and response options are presented in the NoC and ICSA Subtests.

Mixture Configuration Selection includes the following questions:

- *Select one of the following Amp/CE Settings that you will use to participate in the NoC and/or ICSA Subtests of this study:*
  - 6C29: Promega PowerPlex Fusion 6C@29 cycles; Amp volume 25µL; ABI 3500xl injection at 1.2kV for 24 seconds (equivalent to ABI 3500 for 15 seconds)
  - GF28: Applied Biosystems GlobalFiler@28 cycles; Amp volume 25µL; ABI 3500xl injection at 1.2kV for 24 seconds (equivalent to ABI 3500 for 15 seconds)
  - GF29: Applied Biosystems GlobalFiler@29 cycles; Amp volume 25µL; ABI 3500xl injection at 1.2kV for 24 seconds (equivalent to ABI 3500 for 15 seconds)
  - ID28: Applied Biosystems AmpFLSTR Identifiler Plus@28 cycles; Amp volume 15µL; ABI 3500xl injection at 1.2kV for 12 seconds (equivalent to ABI 3500 for 7.5 seconds)
  - None of the above (We will not participate in the NoC or ICSA Subtests)
- *[if not "none of the above"] Please indicate how the selected Amp/CE Settings compare to your SOPs:*
  - This corresponds exactly to our lab's validated settings—we can use these settings for NoC and ICSA
  - This is equivalent to our lab's settings; this differs in details we consider minor or inconsequential (such as injection time of 15 vs 16 seconds)—we can use these settings for NoC and ICSA
  - This differs from our lab's validated settings, but we are willing to participate using these settings in both NoC and ICSA (Note: these results will be analyzed separately during analysis)
  - This differs from our lab's validated settings; we are willing to participate using these settings in NoC, but not in ICSA (Note: these results will be analyzed separately during analysis)

3 NoC Packets: DNA Mixture Profiles

In the *NoC Subtest* you will be assigned a total of 12 *NoC Packets*. Each *NoC Packet* includes one DNA mixture profile, positive and negative controls, and allelic ladders. No case information will be provided. All DNA mixture profiles are electropherograms, provided to participants as HID files.

You will have access to only *NoC Packet* at a time: to avoid the possibility of administrative errors or misunderstandings, you must submit your responses for a DNA mixture profile before downloading the next DNA mixture profile.

3.1 Amp/CE Settings

In order to represent the SOPs of as many participating laboratories as feasible, electropherograms were prepared using four combinations of "Amp/CE Settings" (which refers to a specific combination of amplification kit, amplification cycles, volume of amplification reaction, CE instrument, and injection time and voltage). The combinations of Amp/CE Settings that have been implemented were the four most commonly-used Amp/CE

settings selected by registered participants,[†] using the abbreviations listed in *Section 2 (Mixture Configuration Selection)* [6C29, GF28, GF29, or ID28].

You will be assigned mixtures that were prepared using the Amp/CE setting option that you chose during *Mixture Configuration Selection*, prior to the *NoC Subtest*.

3.2 Preparation of DNA Mixture Profiles

The DNA used to create the mixture profiles for this study came from various sources, including buccal, blood, and tissue samples. There were no simulated/contrived profiles; all DNA profiles in this study are from real people. DNA samples were extracted prior to mixing.

Mixtures were quantified using ABI Quantifiler Trio on an ABI 7500 real-time PCR instrument. The mixture quantification results (including the total amount of DNA amplified, amount of male DNA, and degradation index) will be included with the mixtures in the *NoC Subtest*.

Various volumes of DNA were pipetted into a single tube to make a large mixture stock. That stock was then aliquoted and amplified in each of the four amp kits (see "Amp/CE settings" above for amplification volumes and cycles). The ABI 9700 thermocycler was used for amplification, using the specific Amp/CE settings and other standard manufacturer recommended settings. The ABI 3500xl was used for capillary electrophoresis (CE), using injection time and voltage settings specified in the Amp/CE Settings (see above); settings for run time, run voltage, capillary length, polymer type, etc. use the default settings specified for each amp kit.

GeneMapper (v1.5; incorporated into ABI 3500xl) was used to create HID files. We are not providing PDFs (images) of the electropherograms because creating such PDFs implements decisions regarding the analytical threshold (AT) value and the utilization of stutter filters, and we want all such decisions to be made by the participants.

Every effort was made with respect to quality assurance in creating these mixtures. Note that in some cases there may be artifacts (such as pull-up) present, as may be found in ordinary casework — please review the controls provided.

3.3 Data Provided

Each *NoC Packet* is numbered (NoC_01 through NoC_99, shown as "NoC_XX" in the table below). Participants are not necessarily assigned the same packets, and the order of assignments varies among participants.

Each *NoC Packet* is specific to the Amp/CE Settings previously selected by participants (shown as "YYYY" in the table below), using the abbreviations listed in *Section 2 (Mixture Configuration Selection)* [6C29, GF28, GF29, or ID28].

Each *NoC Packet* is contained in a Zip file, downloaded from the DNAmix 2021 website (https://dnamix.edgeaws.noblis.org/). Each *NoC Packet* includes the following files:

All Packets	1 DNA mixture profile (HID file)	NoC_XX_YYYY_Mixture.hid
	Amp/CE Settings used to create the electropherograms	AmpCESettings_YYYY.pdf
	Quantitation data for the mixture	NoC_XX_QuantResults.pdf
	2 Ladders	NoC_XX_YYYY_Ladder1.hid NoC_XX_YYYY_Ladder2.hid

[†] *Registered participants were contacted by email and were given a deadline of 23 August 2021 to indicate preferences for Amp/CE settings.*

	Positive and Negative controls	NoC_XX_YYYY_Pos.hid NoC_XX_YYYY_Neg.hid

In a few cases the positive or negative controls were re-injected, in which case they are in a subdirectory (named POS or NEG) with the associated ladders.

4 NoC Subtest Questions

On the *DNAmix 2021 website*, you will be asked to answer the following questions for ***each*** of the 12 DNA mixtures that you are assigned in the *NoC Subtest*. The *NoC Subtest* is completed online; this information is provided here as a reference.

As a quality assurance measure, the website will display an image of the electropherogram for the first several loci in the DNA mixture profile for the assigned *NoC Packet*. Please ensure that you are submitting your responses for the given mixture profile.

After downloading the packet, we recommend clicking HOME in the main menu bar (at the top of the DNAmix webpage) and returning to the NoC Subtest screen only when you are ready to enter your responses. (Some users have timeout issues if this window is left open.)

Please enter and submit all responses for the assigned *NoC Packet* in one session. You will not be able to save your responses and return to complete response entry within a single *NoC Packet*; if you exit prior to submitting, you will be required to re-enter all of your responses for the *NoC Packet*.

Note for each mixture profile assessed in the NoC Subtest, you will be asked to review and confirm your responses prior to submission. After submission, your responses are considered final and cannot be changed.

Packet Assignment Details

1. Please re-enter the Participant ID shown at the top of the page (Dxxxx):_____

   Note: this information will be used for quality assurance purposes only. The Participant ID (a 5 character alpha-numeric string starting with D) is located at the top right of the Number of Contributors (NoC) Page (right about the electropherogram preview image).

2. Please double-check the NoC Packet number: verify that the number of the HID mixture file you are assessing is the same as shown at the top of this page. Please enter that NoC Packet number here (For example, in the NoC Beta Test, you would enter the following NoC packet number: 99): _____

   The NoC Packet number is located in the HID filename, in the electropherogram preview image (located at the top of the NoC Subtest page of the DNAmix 2021 website), and embedded within the electropherogram data. You do not need to enter the "NoC_" portion; please only enter the two digit NoC Packet number.

Settings

3. Did you use an analytical threshold (AT) for this mixture?

   In other words, was there a minimum RFU value (either globally or per dye channel) to delineate signal (above the threshold) from noise (below the threshold)? This analytical threshold may have been utilized explicitly (by comparing measured RFU values to the threshold value(s)) or via general evaluation ("eye-balling" the data and comparing to the threshold value(s)).

   3.a Yes, I used a single AT (Please specify:_____)
   3.b Yes, but my ATs varied by dye channel

3.c No

4. Did you use a stochastic threshold (ST) for this mixture?

> *In other words, was there a minimum RFU value (either globally or per dye channel) to delineate peaks (above the threshold) from potential artifacts or stochastic effects (below the threshold)? This stochastic threshold may have been utilized explicitly (by comparing measured RFU values to the threshold value(s)) or via general evaluation ("eye-balling" the data and comparing to the threshold value(s)).*

4.a Yes, I used a single ST (Please specify:_____)
4.b Yes, but my STs varied by dye channel
4.c No

Replicate Amplifications

> *The next two questions both ask whether you would have conducted replicate amplifications if you had received this sample in actual casework, but please note the differences. Question 5 is regarding replicate amps based on the amount of DNA available: if only a small amount of DNA was available, would you have amplified all the DNA or divided it and done multiple amps? Question 6 is regarding replicate amps based on the review of the electropherogram: if additional DNA were available, after reviewing this EPG would you conduct another amp?*

5. If you received this DNA mixture sample in casework and the total quantity of DNA available was the amount specified in the quantitation data, would you have divided the sample and conducted multiple replicate amplifications?

> *In other words, given the DNA quantity alone (not based upon review of the electropherogram), would you have amplified the entire sample (as was done here), or would you have instead done 2 or more amplifications each using a part of the total sample? Assume that all DNA available in the entire (wet) sample was used here and there will not be additional DNA to permit a subsequent amplification after CE.*

5.a We do not ever conduct replicate amplifications in my laboratory (per our SOPs)
5.b No: we would not have done replicate amplifications in this case (but we do in some cases)
5.c Yes: we would do 2 replicate amplifications, each with 1/2 of the total amount, and increase sensitivity by adding 1 cycle
5.d Yes: we would do 3 replicate amplifications, each with 1/3 of the total amount, and increase sensitivity by adding 1 cycle
5.e Yes: we would do 3 replicate amplifications, each with 1/3 of the total amount, and increase sensitivity by adding 2 cycles
5.f Other (Please explain:_____)

6. If there was sufficient DNA remaining for an additional amplification, would you do another amplification (re-amp) after seeing this mixture profile?

> *In other words, if sufficient DNA remained would you conduct another amplification based upon the mixture profile/electropherogram provided in this NoC packet (e.g., to verify alleles, observe stochastic effects, etc.)?*

6.a No: we are not permitted to re-amp per our SOPs
6.b No: we would interpret this profile
6.c Yes: we would re-amp using more DNA
6.d Yes: we would re-amp using less DNA
6.e Yes: we would re-amp using the same amount of DNA

Suitability

7. Is this DNA mixture profile suitable for comparison and/or statistical analysis?

> *In other words, did you determine that this DNA mixture profile can appropriately be used to conduct comparisons (i.e., comparison of the mixture to reference profiles of POIs, victims, consensual partners, and/or expected contributors) and/or statistical analyses (i.e., compute an LR, RMP, or CPI/CPE with respect to a POI)?*

7.a Yes (for the entire mixture and all contributors)
7.b Yes, but only for a subset of the contributors (e.g., major(s))
7.c Yes, but only for a subset of loci
7.d Yes, but only for a subset of loci, and only for a subset of the contributors
7.e No

8. *[If the mixture is NOT suitable for comparison and/or statistical analysis]* Why is this profile unsuitable for comparison and statistical analysis? (check all that apply; check at least one)

> *In other words, if you indicated "No" in the previous question, what factor(s) informed your determination? Please select all factors that you considered in your determination that the DNA mixture profile was not suitable for comparison/statistical analysis.*

8.a Not enough alleles or loci suitable for analysis
8.b DNA template levels too low overall
8.c Sample too degraded
8.d Sample too inhibited
8.e Too many contributors
8.f Too much uncertainty in the number of contributors
8.g Mixture proportions/contributor ratios
8.h Other (Please specify:_____)

Number of Contributors *[Only shown if the mixture is suitable for comparison and/or statistical analysis]*

9. How would you report the number of contributors in this profile?

> *In other words, how would you report the number of contributors to this DNA mixture profile if you encountered this mixture in casework? Would you be able to assess the number of contributors given this DNA mixture profile? If so, would you report an exact/single estimate of number of contributors (e.g., 3 contributors) or would you report a range of possible numbers of contributors (e.g., 3-4 contributors or minimum of 3 contributors/maximum of 4 contributors) or would you report a minimum number of contributors (e.g., at least 3 contributors)?*

9.a I would report an exact number of contributors
9.b I would report a range of possible numbers of contributors
9.c I would report a minimum number of contributors
9.d The levels (overall quantity and/or peak heights) are not sufficient to determine the number of contributors [Go to Additional Comments]
9.e *The mixture is too complex to determine the number of contributors* [Go to Additional Comments]

9.1 Provide your estimate of NoC.

> *Note: you will only see the version of question 8.1 that is associated with your response to question 8 above. In other words, you will only see one of the three options in the question text below.*

– *[if selected 8a: Exact NoC]* Select your estimate of the number of contributors:

> *In other words, select your single estimate for the number of contributors to this DNA mixture profile.*

– *[if selected 8b: Range of NoC]* Select your estimate of the range of possible numbers of contributors. (For example, if you estimate that there are 3-5 possible contributors to this mixture profile, you must select 3, 4, and 5): (check all that apply; select at least two)

In other words, select all possible numbers of contributors included in your estimated range for this DNA mixture profile, which must include at least two options based upon your response to Q8 that you would report the NoC for this profile using a range of possible numbers of contributors.

– *[if selected 8c: Exact NoC]* Select your estimate of the minimum number of contributors:

In other words, select your single estimate for the minimum number of contributors to this DNA mixture profile.

9.1-a At least 1 contributor
9.1-b At least 2 contributors
9.1-c At least 3 contributors
9.1-d At least 4 contributors
9.1-e At least 5 contributors
9.1-f At least 6 contributors
9.1-g At least 7 contributors
9.1-h At least 8 or more contributors

10. What were the PRIMARY loci used as the basis for determining the number of contributors? In other words, indicate the loci that were most informative or most helpful. (check all that apply; select at least one)

Note: you will only see the set of loci included in the amplification kit that you previously selected in Mixture Configuration Selection.

Names of commercial manufacturers are included for the systems that are the most frequently used by registered participants; inclusion does not imply endorsement by the study team.

Applied Biosystems GlobalFiler (display order)	Promega PowerPlex Fusion 6C (display order)	Applied Biosystems AmpFLSTR Identifiler Plus (display order)
D3S1358	Amel	D8S1179
vWA	D3S1358	D21S11
D165539	D1S1656	D7S820
CSF1PO	D2S441	CSF1PO
TPOX	D10S1248	D3S1358
Y indel	D13S317	TH01
Amel	Penta E	D13S317
D8S1179	D16S539	D16S539
D21S11	D18S51	D2S1338
D18551	D2S1138	D19S433
DYS391	CSF1PO	vWA
D2S441	Penta D	TPOX
D19S433	TH01	D18S51
TH01	vWA	Amel
FGA	D21S11	D5S818
D22S1045	D7S820	FGA
D5S818	D5S818	
D13S317	TPOX	
D7S820	D8S1179	
SE33	D12S391	
D10S1248	D19S433	
D1S1656	SE33	
D12S391	D22S1045	
D2S1338	DYS391	
	FGA	
	DYS576	
	DYS570	

11. Which factors affected your assessment of number of contributors? (check all that apply; select at least one)

In other words, what factors did you consider when estimating the number of contributors for this DNA mixture sample? Please select all factors that informed your determination.

11.a Discriminating potential/variability of loci (or allele frequency)
11.b Expected stutter ratios
11.c Information below the analytical threshold
11.d Maximum Allele Count (MAC) per locus
11.e Overall level of data (peak heights in relation to laboratory validated thresholds)
11.f Peak heights (RFU)
11.g Peak morphology (e.g., CE resolution; unresolved microvariants; peak shouldering)
11.h Presence of degradation
11.i Presence of inhibition
11.j Quantitation data
11.k Relative peak heights (peak height ratios and possible shared/stacked alleles)
11.l Sex determining markers
11.m Total allele count in sample
11.n Other (Please specify:_____)

12. Are you able to identify any major contributors?

In other words, would you consider one (or more) contributors to be major contributors according to the criteria outlined in your SOPs (e.g., based upon peak height ratios or RFU percentages). This separation of

major contributor(s) may have been conducted explicitly (by computing peak height ratios/RFU percentages and comparing to a threshold, such as a 3:1 peak height ratio or 70% of the total RFUs) or via general evaluation (distinguished visually, without calculation). If your SOPs do not permit you to differentiate between major and minor contributors, please indicate as such.

12.a There are no contributors I would consider majors
12.b There is one major contributor
12.c There are two or more major contributors
12.d We do not differentiate between major and minor contributors

13. Did you use any software tool to assist in assessing the number of contributors?

In other words, please indicate how you assessed number of contributors for this DNA mixture profile. If you used a combination of manual assessment and software, please select the option which most informed your assessment.

13.a No, I assessed the number of contributors manually
13.b Yes, I used NOCIt
13.c Yes, I used PACE
13.d Yes, I used FaSTR/STRmix
13.e Yes, I used an internally developed tool
13.f Yes, I used another commercial or open-source tool (Please specify:_____)

Additional Comments

14. Additional comments: Please provide a comment ONLY if there is an issue or a limitation for this NoC packet that you could not adequately address using any of your responses above. (Please limit your responses to 75 words or less.)