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C-SOP-101: Bacterial Genomic DNA Isolation using the Nexttec 1-step Kit (96-plate format)

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1 *Works for me* This protocol is published without a DOI.

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ABSTRACT

This protocol describes an easy, convenient and rapid method of genomic DNA isolation and purification from Gram (+) and (-) bacterial broth suspensions. The workflow, dubbed as a 1-step, single buffer system is a reversal of the traditional silica membrane and magnetic bead-based DNA extraction protocols. Proteins, cellular debris and other low-molecular weight contaminants are retained by the nexttec™ sorbent while pure dsDNA is pulled through as an eluate in a single wash step.

The protocol described is suitable for extraction of DNA from pure bacterial isolates of the following organisms:
1. *Acinetobacter baumannii*
2. *Escherichia coli*
3. *Klebsiella pneumoniae*
4. *Staphylococcus aureus*
5. *Neisseria gonorrhoea*
6. *Salmonella typhi and typhimurium*
7. *Non-typhoidal Salmonella spp.*
8. *Pseudomonas aeruginosa*
9. *Streptococcus pneumoniae*
10. *Vibrio cholerae*

This protocol has been adapted from nucleic acid extraction methods developed by nexttec Biotechnologie GmbH.

PROTOCOL CITATION

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KEYWORDS

null, DNA, gDNA, DNA isolation, DNA extraction, Bacterial DNA isolation, Bacterial DNA extraction

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GUIDELINES

Storage (Nexttec 1-step):
During shipment (10-15 days in transit), all kit components are stable at room temperature.
On arrival, store:
1. Prep Solution, Buffer B, EDTA, RNase A and Proteinase K at +2 °C to +8 °C.
2. DTT and Lysozyme/Lysostaphin at -18 °C to -25 °C.
3. SDS Solution, the nexttecTM cleanPlates96 and plate seals at room temperature (+20 °C to +25 °C).
If properly stored, the expiration date on the kit will apply.

MATERIALS TEXT

Equipment:
1. Centrifuge, at least up to 4000 rpm, with deepwell plate buckets (Eppendorf 5810R/5910R, Cat no. 5811000010 or equivalent)

2. Dry incubator-shaker, at least up to 300 rpm and 75°C (New Brunswick Innova 42, Cat no. M1335-0012 or equivalent)

3. Single- and multi-channel (8) pipettes (Eppendorf Research Plus series or equivalent)

4. McFarland Nephelometer

5. Tabletop tube spinner (Sigma-Aldrich MyFuge, combi-rotor, Cat no. Z681725 or equivalent)

Optional:
6. Repeat dispense pipettor (Eppendorf Multipette M4, Cat no. 4982000012)

Labware:
7. Filtered pipette tips, sterile (Compatible with the chosen pipettes)

8. 96-deepwell plate, 2.0 ml, round bottom, sterile (StarLab, Cat no. E2896-2110 or equivalent)

9. Breathable plate seal (Sigma-Aldrich AeraSeal film, Cat no. A9224 or equivalent)

10. 15 & 50 mL Falcon tubes (BD Falcon, Cat nos. 352096 and 352070 or equivalent)

Optional:
11. Repeat dispense pipettor tips (Eppendorf Combitips Advanced, 2.5 ml, 1.0 ml, 0.5 ml, 0.2 ml)

12. Universal containers, 30 ml (StarLab, Cat no. E1412-3021 or equivalent)

Reagents:
13. Nuclease-free water, molecular biology grade (Local supplier)

14. Lysostaphin (Sigma-Aldrich, Cat no. L9043)

Optional (based on organism):
15. Blood Agar

16. Tryptic Soy Broth (TSB)

17. Chocolate Agar

18. Alkaline Peptone Water (APW)

19. Sheep Blood Agar (SBA)

20. Normal Saline Solution (NSS)

Extraction Kit:
21. Nexttec 1-step Bacteria cleanPlate 96 (Cat no. 20N.901 / 20N.902 / 20N.904 / 20N.924)

i. This protocol has been performed successfully under test conditions with the following equipment, labware and reagent concentrations. Although they serve as strong recommendations when performing DNA isolation using this kit, there is no strict requirement to adhere to this choice of items. There are several equivalent alternatives available, however, please be aware that results may vary.

ii. For storage of kit components and auxiliary reagents, refer to the **Guidelines Section**.

SAFETY WARNINGS

Safety Information (Nexttec 1-step):

1. Proteinase K (Danger) H334 P304+P341, P342+P311
2. DTT (Warning) H315, H319 P280, P305+P351+P338, P321, P362, P332+P313, P337+P313

Hazard Statements (Nexttec 1-step)

H315 Causes skin irritation
H319 Causes serious eye irritation
H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled

Precautionary Statements (Nexttec 1-step)

P280: Wear protective gloves/protective clothing/eye protection/ face protection

P342+P311: If experiencing respiratory symptoms: Call a Poison Center or doctor/physician

P305+P351+ P338:
IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing

P304+P341:
IF INHALED: if breathing is difficult, remove victim to fresh air and keep at rest in a position comfortable for breathing

P321: Specific treatment (see on this label)

P362: Take off contaminated clothing and wash before reuse

P332+P313: If skin irritation occurs: Get medical advice/attention

P337+P313: If eye irritation persists: Get medical advice/attention When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information please consult the appropriate material safety data sheets (MSDS).

DISCLAIMER:

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ABSTRACT

This protocol describes an easy, convenient and rapid method of genomic DNA isolation and purification from Gram (+) and (-) bacterial broth suspensions. The workflow, dubbed as a 1-step, single buffer system is a reversal of the traditional silica membrane and magnetic bead-based DNA extraction protocols. Proteins, cellular debris and other low-molecular weight contaminants are retained by the nexttec™ sorbent while pure dsDNA is pulled through as an eluate in a single wash step.

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8. *Pseudomonas aeruginosa*
9. *Streptococcus pneumoniae*
10. *Vibrio cholerae*

This protocol has been adapted from nucleic acid extraction methods developed by nexttec Biotechnologie GmbH.

Before Starting

1 Prior to initiating the protocol, ensure that all active workbenches are cleaned with 80% ethanol, all relevant personal protective clothing is worn and the work area is prepared for DNA extraction according to local GLP guidelines.

2 Create an organised bench space by clearing away all clutter in order to maximize work efficiency. Avoid movements that will expose sterile materials to airborne contaminants.

3 ⚠

Treat all isolates and cultures as infectious. Local laboratory safety practices should be followed at all times.

Preparation of the Overnight Bacterial Suspension

4

Refer **step 5** for *Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Salmonella Typhi, Non-typhoidal Salmonella spp., Pseudomonas aeruginosa,* and *Vibrio cholerae.*

Refer **step 6** for *Neisseria gonorrhoea.*

Refer **step 7** for *Streptococcus pneumoniae.*

5 Broth suspension preparation for: ***Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Salmonella Typhi, Non-typhoidal Salmonella spp., Pseudomonas aeruginosa,* and *Vibrio cholerae.***

5.1 23h

Grow the bacterial culture on Blood Agar medium and incubate the plates for **23:00:00** at **37 °C** .

5.2 After incubation, examine each plate for contamination to ensure purity of isolates/colonies.

5.3 18h

Pick a pure, single bacterial colony off the agar plate and passage to grow in Tryptic Soy Broth (TSB) at **35 °C** in dry incubator for **18:00:00** .

Next, proceed to pelleting (step 8).

6 Broth suspension preparation for: ***Neisseria gonorrhoea***.

6.1 1d

Grow the bacterial culture on Chocolate Agar medium and incubate the plates for **24:00:00** at **35 °C** in a CO_2 controlled incubator or shift candle jar.

6.2 After incubation, examine each plate for contamination to ensure purity of isolates/colonies.

6.3 For each sample to be extracted, dispense **2.5 mL** of Alkaline Peptone Water (APW) in a sterile 5 mL tube.

6.4 Using a sterile, autoclaved toothpick, pick a pure, isolated colony of *N. gonorrhoea.* Immediately suspend and emulsify the colony scoop on the inner wall of the tube containing APW in order to break the cell clumps.

Mix properly until a homogeneous bacterial suspension is obtained.

6.5 Measure this suspension using a McFarland nephelometer. Adjust and remeasure until the homogenous bacterial suspension standard (turbidity) is 3.0.

Next, proceed to pelleting (step 8).

7 Broth suspension preparation for: ***Streptococcus pneumoniae***.

7.1 18h

Grow the bacterial culture on Sheep Blood Agar medium and incubate the plates for **18:00:00** at **36 °C** in a CO_2 controlled incubator or shift candle jar.

7.2 After incubation, examine each plate for contamination to ensure purity of isolates/colonies.

7.3 For each sample to be extracted, dispense **2.5 mL** of 0.85% Normal Saline Solution (NSS) in a sterile 5 mL tube.

7.4 Using a sterile, autoclaved toothpick, pick a pure, isolated colony of *S. pneumoniae.* Immediately

suspend and emulsify the colony scoop on the inner wall of the tube containing NSS in order to break the cell clumps.

Mix properly until a homogeneous bacterial suspension is obtained.

7.5 Measure this suspension using a McFarland nephelometer. Adjust and remeasure until the homogenous bacterial suspension standard (turbidity) is 3.0.

Next, proceed to pelleting (step 8).

Pelleting the Bacterial Culture

8 ⚠

Carefully transfer **1.5 mL** of each broth suspension culture (with a minimum OD_{600} = 1.5) grown overnight to an empty well of a clean, sterile round-bottom 96-deepwell plate.

Ensure that pipette tips are lowered to the bottom of each well when dispensing broth into it. This will avoid any cross-well droplet spillage and, in turn, sample contamination.

9 Repeat step 8 for each of the isolates from which DNA needs to be isolated and note down the positional layout of all samples on the deepwell plate.

The 96-deepwell plate has wells labelled A1 to H12. It is recommended to fill only up to 95 positions (i.e. A1 to G12) leaving H12 empty as a control well.

10

Seal the plate tight with an 'Alu' seal (provided with the Nexttec kit) and centrifuge the suspensions at **3000 rpm, 00:04:00** .

11 ⚠

Carefully remove the plate from the centrifuge and discard the supernatant from each well by aspirating the liquid using a multichannel pipette.

Take extra care not to dislodge any pellets.

12 Invert the plate onto a clean piece of tissue to absorb any excess supernatant drain-off. Give the plate a few gentle taps to ensure all of the liquid has drained onto the paper.

Cellular Lysis of the Bacterial Pellet 4h 40m

13 Pre-heat the incubator-shaker to **56 °C** and set the rotation speed at **200 rpm** .

14 Defrost all of the frozen reagents, and place them along with the chilled components in a large bucket of ice.

For best results, ensure the larger volume contents are fully submerged in the ice.

15 Gather all of the lyophilised Lysozyme powder at the bottom of the tube by centrifuging the vial in the tabletop tube spinner.

To prepare **25 mg/ml** of Lysozyme solution, add the relevant volume of nuclease-free water to the lyophilised lysozyme vial provided.

Kit 20N.901 – add **1.5 mL** of water

Kit 20N.902 – add **4.5 mL** of water

Kit 20N.904 – add **6.0 mL** of water

Kit 20N.924 – add **30.0 mL** of water

16 ⚠

Using Table 1 below, in a 50 mL Falcon tube, prepare Lysis Mix 1 (LM1) mixing together the required volumes of: Buffer B, Lysozyme or Lysostaphin solution and RNase A.

Preparation of GRAM -VE LM1 pre-mixes

No. of samples	1	4	8	16	24	48	96
Buffer B	90	450	810	1530	2250	4410	8730
Lysozyme	15	75	135	255	375	735	1455
RNase A	20	100	180	340	500	980	1940

Mix 15 times by gentle inversion ONLY, DO NOT vortex.
Add 120µl of LM1 to each sample.

(All volumes in µl)

Preparation of GRAM +VE LM1 pre-mixes

No. of samples	1	4	8	16	24	48	96
Buffer B	90	450	810	1530	2250	4410	8730
Lysozyme	15	75	135	255	375	735	1455
Lysostaphin	15	75	135	255	375	735	1455
RNase A	20	100	180	340	500	980	1940

Mix 15 times by gentle inversion ONLY, DO NOT vortex.
Add 135µl of LM1 to each sample.

(All volumes in µl)

Table 1

i. Always prepare fresh LM1 for each experiment; Do NOT store it overnight.

ii. Prepare a **separate LM1** for Gram (-) and Gram (+) samples.

17 Using a P200 pipette (single or multi-channel), add:

- **120 µl** to every well containing a **Gram (-)** organism.

- **135 µl** to every well containing a **Gram (+)** organism.

18

Gently re-suspend the pelleted cells by pipetting the LM1 up and down.

> Pipette **extremely slowly** to avoid frothing of the mixture.

19

Carefully raise the plate above eye level (without tilting) and check that to confirm that the pelleted clumps at the well bottom have fully dissolved in LM1.

20 Seal the deepwell plate tightly using an 'Alu' seal.

> Ensure the seal is fully affixed by flattening the center and edges with a roller.

40m

21

Place the plate in the incubator-shaker at **56 °C** , **200 rpm** for **00:40:00** .

22

When the incubation is complete, remove the plate and briefly centrifuge at **500 rpm, 00:00:10** to gather any condensed droplets from underneath the seal.

Now, preset the incubator-shaker to **68 °C** and **230 rpm .**

23 Back at the bench, gripping the plate tightly with one hand, carefully peel back the 'Alu' seal and discard it.

24

Using Table 2 below, in a 50 mL Falcon tube, prepare Lysis Mix 2 (LM2) mixing together the required volumes of: SDS solution, Proteinase K, DTT and EDTA.

Table 2: Preparation of LM2 pre-mixes							
No. of samples	1	4	8	16	24	48	96
SDS solution	100	500	900	1700	2500	4900	9700
Proteinase K	15	75	135	255	375	735	1455
DTT	3	15	27	51	75	147	291
EDTA	2	10	18	34	50	98	194
Mix thoroughly 25 times by gentle inversion ONLY, DO NOT vortex. **Add 120µl of LM1** to each sample.							

(All volumes in µl)

Table 2

i. Always prepare fresh LM2 for each experiment; Do NOT store it overnight.

ii. There are no separate LM2 for Gram (-) and Gram (+) samples.

25

Using a P200 pipette (single or multi-channel), add **120 µl of LM2** to every sample well of the deepwell plate.

Mix the solution repeatedly by pipetting up and down until homogenous.

Pipette **extremely slowly** to avoid frothing of the mixture.

26

4h

Reseal the plate and return it to the incubator-shaker at now set to **68 °C** , **230 rpm** for **04:00:00** .

i. **Longer incubation times** lead to more efficient cellular lysis. Lysates can be incubated up to 6 hours at this temperature (with a mandatory minimum of 3.5 hours). If the starting suspensions were 'stringy and goopy' due to the presence of thick capsular formations or biofilms, increase the lysis time by 1-2 hours. Clumpy final lysates can clog the extraction column and drastically reduce DNA yield.

ii. **Shaking at 230 rpm** is critical to homogenising the lysis mix through dissolution of course cellular debris.

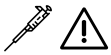
iii. The protocol can be **paused** on completion of the 4 hour incubation period. Pauses should only be taken if absolutely necessary as results can vary slightly.
The deepwell plate with lysates needs to be centrifuged briefly to gather condensation, resealed with a fresh Alu seal and stored at **4 °C** **Overnight** .

27

20 minutes before the incubation is due to finish, start equilibrating the 96-column plate as described in the next section (steps 28-32).

Equilibration of the cleanPlate96 10m

28 Place a cleanPlate 96 with fresh, unused columns flat on the workbench (with a waste plate set up underneath it).

29 

Pour out Prep Solution into a reservoir.

Using the P300/P1000 8-channel pipette (with filtered tips), **add 350 µl of ice-cold Prep Solution** into each column that is to be used for DNA isolation.

Equilibrate only those wells/columns corresponding to occupied sample wells on the 96-deepwell (i.e. those that will be needed for DNA isolation)

Do NOT engage the entire plate if you wish to use less than 96 sample wells.

When expurgating the Prep solution into each well, ensure that:

i. the Prep Solution is ice cold.
ii. the pipette tips are held exactly perpendicular and upright above the centre of the column.
iii. they are just above the surface of the white resin WITHOUT touching it. If the tips come in contact with the resin, it may rupture.

30  10m

Incubate for **00:10:00** at **Room temperature** .

31 

Centrifuge the plate setup at **1300 rpm, 00:01:00** .

Once complete, remove the plate setup from the centrifuge and discard the waste-plate below.

i. You can use either a previously spent or a fresh, unwrapped cleanPlate96 to balance inside the centrifuge.

ii. For batches less than 96 samples (or 96 occupied wells), the waste plate can be reused for this step until every column on the cleanPlate 96 has been utilised.

32 

Place the cleanPlate96 onto a new, fresh deep-well plate (provided with the kit).

Label this the **DNA collection plate**.

Continue with step 33 **soon after** completing the above.

If the columns wetted with Prep solution are allowed to dry out for too long, **DNA yield will reduce** drastically.

DNA Purification and Elution 12m

33 Remove the lysate 96-plate (from step 26) from the incubator. Extra care should be taken when handling it as the plate will be hot.

When resuming a protocol pause, the lysates (stored at 4°C overnight) should be left to warm up to **68 °C** for **00:30:00** prior to step 34.

34 Briefly centrifuge the plate to gather any condensation on the seal.

Slowly and carefully peel off the seal avoiding any droplet spillage.

Gently pipette up and down 10 times to homogenise the lysates.

Pipette **extremely slowly** to avoid frothing of the mixture.

35 Using a P300/P1000 8-channel pipette (with filtered tips), **transfer 120 µl of the lysate** to the corresponding positions on the cleanPlate96.

Reseal the deepwell plate and store the remaining lysate at **4 °C** .

i. Using a permanent marker, outline the wells whose columns you wish to use for this batch of extractions.

ii. The remaining lysate is only viable for up to 36 hours at 4°C. Results may vary from fresh lysate.

iii. When expurgating the lysates into the well, ensure that:
a) the pipette tips are held exactly perpendicular and upright above the centre of the column.
b) the pipette tips are just above the surface of the white resin without touching it.
c) the entire lysate from the tip is deposited onto the column without bubbles.

36 12m

Incubate for **00:12:00** at **Room temperature** .

37

Centrifuge the cleanPlate96 at **1900 rpm, 4°C, 00:01:30** .

Do NOT exceed the spin speed specified for this step. Operating at >1900rpm will cause proteins and cellular debris to elute through and contaminate the DNA.

38 The DNA collection plate now contains purified double-stranded genomic DNA.

Store the plate setup (including the used cleanPlate 96) at 4°C until quantification assays have shown adequate amounts in solution.

In case there is insufficient DNA, try either of the following remedial measures:
1. Add a further **120 µL** of Prep solution to the same column wells and repeat steps 36-37.
OR
2. Repeat steps 35-37 on a fresh set of cleanPlate 96 column positions with the remaining lysate from step 35.

Additional Information & Troubleshooting

39 Base Protocol and User Manual