Viscous Liquid Handling Automation using Opentrons OT-2

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Molecular assays require precise pipetting reagents and samples with a diverse set of properties — including viscous ones that can clog tips, distribute unevenly and complicate reproducibility. The Opentrons OT-2 is an automated liquid handling robot capable of pipetting these difficult liquids. The default parameters in the Opentrons Protocol Python API are optimized for efficient and accurate handling of aqueous reagents. Commonly used reagents differ in viscosity, evaporation rate and surface tension among other properties. These inherent liquid characteristics require changes of default protocol parameters to meet optimum handling for a given workflow. This application note focuses on best practices to automate handling of viscous liquids using the Opentrons OT-2 based on testing performed using computer vision-based methods and gravimetric methods.

VISCOUS LIQUIDS

Viscous liquids have different rheological properties such as varied density (e.g. low for oils but high for glycerol), varied surface tension (e.g. lower in soapy liquids but higher in mercury), stickiness etc. which directly affect their behavior when pipetting. We categorize viscous liquids as shown in **Table 1**. Liquids such as honey, glycerol, and oil exhibit a slower flow rate compared to water which may require specialized pipetting parameters.

Table 1: Characterization of the viscous liquids depending on their molecular structure, vapor pressure, and surface tension.

Viscous Liquid Characterization			
Glycated Liquids	Volatile Viscous Liquids	Surfactant viscous liquids	Oils
Honey, glycerol, PEGs etc.	Hand sanitizer etc.	Liquid soaps, Tween® 20, Triton® X – 100 etc.	Mineral oil, engine oil etc.

Glycated Liquids

Glycated liquids are composed of sugars and exhibit higher adhesion to the pipette tip wall and comparatively higher cohesive forces compared to other viscous liquid categories mentioned in **Table 1**. The viscosity of these liquids varies depending on the dilution. As these liquids stick to the tip, slow tip withdrawal rate after aspiration, and dispensing from liquids help in avoiding bubbles, droplet formation, and clean aspiration and dispensing.

Volatile Viscous Liquids

Volatile viscous liquids are a blend of glycerol/PEG and a volatile solvent such as ethanol or isopropanol prototypical. The glycerol/PEG addition increases viscosity whereas the volatile solvent increases vapor pressure. Thus, to pipette such liquids, users can use the default aspiration flow rate used for water. However, the dispensing flow rate needs to be reduced. To counteract dripping problems due to higher vapor pressure, adding an air gap after aspiration is necessary to ensure accurate pipetting.

Viscous Surfactant Liquids

Viscous surfactant liquids are a blend of a surfactant and a viscous glycated liquid. The most popular viscous surfactants are Tween® 20 and Triton® X – 100 with viscosity as high as 400 millipascal-seconds (mPa). These liquids exhibit higher adhesion to the pipette tip but lower cohesive forces. The viscous surfactants must be aspirated with slower aspiration flow rates and withdrawn with minimal withdrawal speed. The dispensing flow rate needs to be reduced such that ample time is available for the liquid to slide off the wall of the pipette tip.

One way to compensate for slow flow rate is using longer delays with two-step dispensing. As an example: if you are dispensing 200 uL of viscous surfactant with the Opentrons GEN2 P300 Single-Channel pipette, split the dispense step into two parts: first dispense 150 uL with ½ to ¾ of original flow rate and add a pause for the liquid to slide towards the orifice of the tip; then, dispense 50 uL with slower than ½ of original flow rate, being sure to add an additional blow out step and a slower touch tip step. As an additional step for faster dispensing, a touch dispense strategy can be implemented where the liquid is dispensed as the pipette tip is touched to the wall of the well.

Oils

Oils exhibit similar properties to viscous surfactants and can be handled with the process described above. Oils require longer time to slide off the wall completely, meaning it takes longer for the pipette to cleanly dispense. Slower withdrawal helps reduce loss of oil sticking to the exterior wall of the pipette tip, but since the adhesive forces of the oils to the tip are higher than the cohesive forces of the oil, clean dispensing of oil requires longer liquid settlement time causing delays. Depending on the dead volume loss estimation of oil reagents, a longer delay can be implemented after aspirating and dispensing steps. The touch tip dispense and touch tip functions are also good ways to mitigate losses by adding a faster dispensing speed.

Slower aspiration and dispense, slower tip withdrawal from the liquid, and slower blow out flow rate are the key parameters impacting the viscous liquid handling experience and clean pipetting of liquids.

METHODS

We developed the optimization strategy for viscous liquid handling using the OT-2 based on the rheological properties of the liquids, specifications of the pipettes and the profile of the labware used. The study is limited to viscous liquid handling capacity of Opentrons GEN2 Single-Channel pipettes. The liquid handling functions implemented are aspiration and dispense; the parameters may not work when complex liquids are mixed or formed and different workflows may require different parameters.

Materials

Different dilutions of glycerol were made by diluting Glycerol 99% (Sigma-Aldrich, G5516) with respective proportions of molecular grade water (Invitrogen, cat. no. 10977023).[1] Glycerol is characterized as a viscous liquid with varied viscosity ranging from 1 mPa.s to 1400 mPa.s differing with dilution. We therefore consider glycerol as a representative liquid of viscous class enabling a qualitative understanding of viscosity and comparing with liquids of similar viscosity. Due to the exponential rise in viscosity of glycerol, we have chosen glycerol 10%, 90% and 99% for parameter development. All the concentrations were made in a 15 ml falcon tube with 10 ml total mix of glycerol and water in two proportions: Glycerol 90%, Glycerol 10%. Opentrons 15 tube rack adapter was placed on rack 7. The testing was performed using Opentrons GEN2 Single-Channel V2.2 pipettes: P20, P300, and P1000. We also used Opentrons P20 Tips and Opentrons P300 Filter Tips and Opentrons P1000 Filter Tips. Temperature and humidity were monitored using integrated thermometer and hygrometer.

Gravimetric Testing Methodology

Gravimetric testing was performed on a custom-built rig with weighing scale attached to the OT-2. The scale was placed on a separate bench to isolate the vibrations generated by OT-2. Glycerol of 10%, 90%, and 99% concentrations with molecular grade water were tested on the gravimetric testing rig. Three volumes of 1uL, 10 µL, and 20 µL were tested with the Opentrons P20 GEN2 pipette with 4 repetitions per volume. For the Opentrons P300 GEN2 pipette 20 µL, 150 µL, and 300 µL were tested with 10 repetitions per volume. For the Opentrons P1000 GEN2 pipette 100 µL, 500 µL and 1000 µL were also tested with 10 repetitions per volume. The testing was performed with ambient room temperature ranging from 23 °C to 32 °C and relative humidity ranging from 35% to 45%.

The OT-2 was programmed with set parameters from appendix **Tables A1, A2, and A3** in Opentrons Python Protocol API 2.9 to perform aspiration, dispense and blow out at desired flow rates. The default parameters of aspiration, dispense and blowout were used for testing water.

OPTIMIZATION RESULTS AND DISCUSSION

The intended choice of three concentrations of glycerol 99%, 90%, and 10% was based on the dynamic viscosity of glycerol acting as a representative of viscous liquid classes. The dynamic viscosities are 1.3 mPa for glycerol 10%, 168 mPa for glycerol 90%, and 893 mPa for glycerol 99% concentration.[3]

The default Opentrons GEN2 Single-Channel pipettes were calibrated for water handling. **Figure 1** shows that optimized parameters for viscous liquids show greater correlation to water handling:

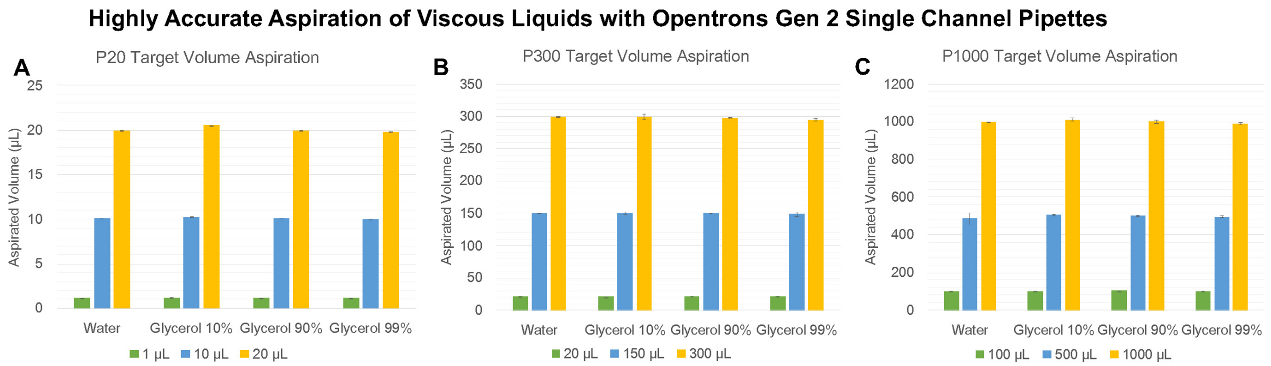


Figure 1: Highly accurate viscous handling using Opentrons GEN2 P20, P300, and P1000 pipettes. These results were achieved using a slower aspiration rate, introducing suitable settling time for the liquid to aspirate completely, and slower withdrawal speed leading to comparable results of water.

Results in **Figure 1** show highly accurate aspiration of viscous liquids using Opentrons GEN2 P20, P300 and P1000 pipettes and Opentrons Tips and Opentrons Filter Tips. The key to accurate aspiration is having a slower flow rate, sufficient settling time and slower withdrawal rate. Highly viscous liquids require slower time to aspirate — even when aspirated with a higher flow rate. However, the compensation is that inaccurate volume can be aspirated due to gravitational and adhesive forces on the tip.

These results were achieved by overcoming those adhesive forces by slowly aspirating and allowing sufficient settling time for the liquid to aspirate. The lag between the liquid aspiration movement of the plunger and the time taken for the liquid to be aspirated is therefore overcome by the settling time. While the liquid has high affinity to the tip, faster withdrawal resulted in formation of drops around the tip and spillage of liquid. The optimized parameter implemented slower tip withdrawal speed leading to clean tip withdrawal with high reproducibility of the aspirated volume, as shown in **Table 2**.

Table 2: Minimal Losses in Dispensing Viscous Liquids. The optimized parameters allowed <0.9% liquid loss for Opentrons GEN2 P20 Single-Channel pipette, and <0.2% liquid loss for Opentrons GEN2 P300 and Opentrons GEN2 P1000 Single-Channel pipettes after dispensing.

Opentrons Gen2 P20 Dispense Loss

	Water	Glycerol 10%	Glycerol 90%	Glycerol 99%
1 µL	0.099867	0.081619	0.015068	0.007467
10 µL	0.12	0.09827	0.100163	0.000373
20 µL	0.108667	0.128436	0.162276	0.001547

Opentrons Gen2 P300 Dispense Loss

	Water	Glycerol 10%	Glycerol 90%	Glycerol 99%
20 µL	0.212867	0.085341	0.001734	0.137013
150 µL	0.198133	0.173294	0.088238	0.140693
300 µL	0.282	0.309958	0.757832	0.093333

Opentrons Gen2 P1000 Dispense Loss

	Water	Glycerol 10%	Glycerol 90%	Glycerol 99%
100 µL	0.134467	0.304146	0.111545	0.05888
500 µL	0.525067	0.793013	0.098266	0.122507
1000 µL	0.490467	0.287692	0.066179	0.042667

The addition of a slower blow out removed this excess liquid by synchronizing the flow of the liquid in the narrow region of the tip, thus completely dispensing the liquid inside the tip. Further, slowing the tip withdrawal speed helped in avoiding droplet spilling and sticking. The parameters for tip withdrawal after aspirating and dispensing remain the same if the liquid is unmixed and the viscosity hasn't changed. As shown in **Table 2**, the whole optimized dispensing process significantly reduced the pipetting loss providing intended target volumes for the reactions thereby improving accuracy.

CONCLUSION

The results of viscous testing validate that the OT-2 with Opentrons GEN2 Single-Channel Pipettes can handle liquids of varied viscosity with accuracy comparable to the water handling capacity of OT-2 with high precision. The optimized recommended parameters and guidelines improve the liquid handling performance of OT-2 and help ensure a successful protocol run. The implementation of the viscous liquid class is summarized in **Figure 2** and **Figure 3**. The lower flow rates coupled with critical settling times allow viscous liquids to settle; therefore, successive steps such as slower blow out help in minimal loss of viscous liquid, enabling efficient usage of reagents and improved accuracy in results.

If the blow out volume is too low or too high for the liquid dispensing, an air gap can be implemented before aspirating viscous liquid with a slower dispense rate instead of a slower blow out for the desired air volume to push the viscous liquid out of the tip, but the trade-off is that less liquid can be aspirated than the maximum limits of the pipette. This may not be the best suited case for the Opentrons GEN2 P20 Single-Channel pipette. Finally, slower withdrawal speeds after aspirating and dispensing steps are highly recommended to prevent droplets sticking on the tip and reagents spilling in the labware.

In conclusion, the OT-2, Opentrons GEN2 Single-Channel pipettes, and Opentrons Tips and Opentrons Filter Tips can optimally handle viscous liquids and enhance protocol accuracy.

Viscous Handling Strategy: Aspiration

Step 1: Hover to Top

`move_to()` to top of labware at default protocol speed

Step 2: Move to bottom

`move_to()` to bottom of labware at 10 mm/s speed to aspirate

Step 3: Aspiration

- `aspirate()` at slower flowrate
- Add `delay()` after aspiration for the liquid to aspirate completely

Step 4: Tip Withdrawal

- `move_to()` from top of labware at 1 – 5 mm/s Use
- default speed to move to another well

Figure 2: Strategizing an aspiration cycle with Opentrons Python Protocol API.

Viscous Handling Strategy: Dispense

Step 1: Hover to Top

`move_to()` to top of labware at default protocol speed

Step 2: Dispense

- `move_to()` to bottom of labware at 10 mm/s speed to aspirate
- `dispense()` at slower flowrate
- Add `delay()` for the liquid to settle down and complete

Step 3: Blowout

- Residual liquidis removed with"slower" blowout
- `blowout()` at slower flowrate

Step 4: Tip Withdrawal

- `move_to()` from liquidto top of labware at 1 – 5 mm/s
- Set the blowout flowrate to default pipette flowrate

Figure 3: Strategizing a dispense cycle with Opentrons Python Protocol API.

REFERENCES

1. Calculate density and viscosity of glycerol/water mixtures. (n.d.). Retrieved June 24, 2021, from http://www.met.reading.ac.uk/~sws04cdw/viscosity_calc.html
2. https://opentrons.com/publications/OT-2-Pipette-White-Paper.pdf
3. Segur, J. B., & Oberstar, H. E. (1951). Viscosity of glycerol and its aqueous solutions. Industrial & Engineering Chemistry, 43(9), 2117-2120

APPENDIX

RECOMMENDED PARAMETERS FOR PIPETTING VISCOUS LIQUIDS

The listed parameters on **Tables A1, A2, and A3** are tested and verified for best accuracy and precise dispensing of viscous liquids using the Opentrons platform.

Table A1: Opentrons GEN2 P20 Single-Channel Pipette Optimized Parameters By Liquid

Pipette	Liquid	Aspiration Rate (µL/s)	Aspiration Delay (s)	Aspiration Withdrawal Rate (mm/s)	Dispense Rate (µL/s)	Dispense Delay (s)	Blowout Rate (µL/s)	Touch tip
P20	Glycerol 10%	6.804	2	5	6.804	2	0.5	No
	Glycerol 90%	5.292	7	2	5.292	7	0.5	No
	Glycerol 99%	3.78	10	2	3.78	10	0.5	No
	PEG 8000 50% w/v	6.048	7	5	6.048	7	0.5	No
	Sanitizer 62% Alcohol	1	2	20	3.78	2	0.5	Yes
	Tween 20	5.292	7	2	3.024	7	0.5	Yes
	Engine oil	6.048	7	1	6.048	7	0.5	Yes

Table A2: Opentrons GEN2 P300 Single-Channel Pipette Optimized Parameters By Liquid

Pipette	Liquid	Aspiration Rate (µL/s)	Aspiration Delay (s)	Aspiration Withdrawal Rate (mm/s)	Dispense Rate (µL/s)	Dispense Delay (s)	Blowout Rate (µL/s)	Touch tip
P300	Glycerol 10%	83.25	2	5	83.25	2	10	No
	Glycerol 90%	64.75	8	1	64.75	8	4	No
	Glycerol 99%	55.5	10	1	55.5	10	4	No
	PEG 8000 50% w/v	74	6	1	74	74	4	No
	Sanitizer 62% Alcohol	92.5	2	20	92.5	2	4	Yes
	Tween 20	13.9	10	1	13.9	11	7	Yes
	Engine oil	74	3	2	46.25	7	10	Yes

Table A3: Opentrons GEN2 P1000 Single-Channel Pipette Optimized Parameters By Liquid

Pipette	Liquid	Aspiration Rate (µL/s)	Aspiration Delay (s)	Aspiration Withdrawal Rate (mm/s)	Dispense Rate (µL/s)	Dispense Delay (s)	Blowout Rate (µL/s)	Touch tip
P1000	Glycerol 10%	247.05	2	30	247.05	2	75	No
	Glycerol 50%	247.05	3	30	247.05	3	75	No
	Glycerol 90%	164.7	10	3	109.8	10	15	No
	Glycerol 99%	41.175	20	1	19.215	20	5	No

PYTHON HELPER FILES

Python definition helper files speed your protocols and implement them directly by changing variables rather than the entire protocol. These variables can be tuned to your desired viscous liquids, but the general strategy remains similar. Following are the helper definitions for the strategy described in **Figure 1**.

Aspiration of viscous liquid

```
def aspirate_viscous(pipette, vol, well, asp_rate, asp_delay, asp_with):
    pipette.move_to(well.top())
    pipette.aspirate(vol, well.bottom(),rate = asp_rate)
    protocol.delay(asp_delay)
    pipette.move_to(well.top(),speed = asp_with)
```

Dispensing viscous liquid

```
def dispense_viscous(pipette, disp_well,vol,disp_rate,disp_delay,blowout_rate, asp_with)
    pipette.move_to(disp_well.top())
    #dispense in well at custom dispense flowrate
    pipette.dispense(vol,disp_well.bottom(),rate = disp_rate)
    #allow the excess liquid in tip to settle towards tip orifice
    protocol.delay(disp_delay)
    #lower the blow rate
    def_pipette = pipette.flow_rate.blow_out
    pipette.flow_rate.blow_out = blowout_rate
    pipette.blow_out()
    pipette.flow_rate.blow_out = def_pipette  #set to default blow out rate
    pipette.move_to(well.top(),speed = asp_with)
```

Sample Program: P300 as pipette to handle 50% Glycerol from centrifuge tube in rack ‘A1’ to cell culture plate in well ‘E5’

```
from opentrons import protocol_api
metadata = {
    'protocolName': '50% Glycerol Handling with P300',
    'author': 'Anurag Kanase <anurag.kanase@opentrons.com>',
    'description': 'Demo to handle 50% glycerol from centrifuge tube in rack ‘A1’ to flat bottom 96 well plate well ‘E5'',
    'apiLevel': '2.9'
}

def run(protocol:protocol_api.ProtocolContext):
    #200uL tip rack is placed on deck 4
    tip200 = protocol.load_labware('opentrons_96_tiprack_300ul', '4')
    #P300 attached to the left channel
    P300 = protocol.load_instrument('p300_single_gen2', 'left',tip_racks=[tip200])
    #1.5 ml tube as A1 on tube rack on deck 5
    tube = protocol.load_labware('opentrons_24_tuberack_eppendorf_1.5ml_safelock_snapcap','5')
    plate = protocol.load_labware('corning_96_wellplate_360ul_flat', '6')


#helper files
def aspirate_viscous(pipette,protocol, vol, well, asp_rate, asp_delay, asp_with):
    pipette.aspirate(vol, well.bottom(),rate = asp_rate)
    protocol.delay(asp_delay)
    pipette.move_to(well.top(),speed = asp_with)

def dispense_viscous(pipette,protocol, disp_well,vol,disp_rate,disp_delay,blowout_rate, asp_with)
    pipette.move_to(disp_well.top())
    #dispense in well at custom dispense flowrate
    pipette.dispense(vol,disp_well.bottom(),rate = disp_rate)
```

```
    #allow the excess liquid in tip to settle to brim
    protocol.delay(disp_delay)

    #lower the blow rate
    def_pipette = pipette.flow_rate.blow_out
    pipette.flow_rate.blow_out = blowout_rate
    pipette.blow_out()
    pipette.flow_rate.blow_out = def_pipette #set to default blow out rate
    pipette.move_to(well.top(),speed = asp_with)

#Initialize variables and set the parameters
source_well = tube['A1']
dest_well = plate['E5']
vol = 75 #ul
asp_rate = 0.9 #times default flow_rate
asp_delay = 2 #seconds
asp_with = 10 #mm/s
disp_rate = 0.9 #x default flow_rate
disp_delay = 2 #seconds
blowout_rate = 10 #ul/s
P300.pickup_tip()
#run the helpers
#aspirates at customized flow_rate from centrifuge tube A1
aspirate_viscous(pipette, protocol, vol, well, asp_rate, asp_delay, asp_with)
#dispenses at customized flow rate to 96 well plate well E5
dispense_viscous(pipette, protocol, disp_well, vol, disp_rate, disp_delay, blowout_rate, asp_with)
P300.drop_tip()
protocol.comment("Test Run Complete")
```

VISCOUS LIQUIDS HANDLING PARAMETERS

The critical parameter in handling viscous liquids is the dispense flow rate. If you are developing a protocol for an entirely new viscous liquid, the first step is to aspirate slowly and withdraw slowly so you have a clean aspiration cycle. Slower flow rates and speeds have resulted in lower failure with viscous liquid handling. Further, you can start dispensing at the rate where you observe the meniscus of the liquid is unbroken. Maintaining a steady meniscus results in clean dispensing and blowout. **Figure A1** illustrates problems associated with higher flow rate: namely, dead volume is generated if the excess liquid inside the tip is not removed after blow out. The higher flow rate forces the liquid to dispense from the center, where excess liquid sticks to the wall of the tip. If the blowout capacity of the pipette is lower than the excess volume inside the tip, it can result in dead volume. Thus maintaining a lower flow rate is essential.

P1000- Dispensing Viscous Liquid Meniscus at Higher Flow Rate

1. 1000 µL Liquid is aspirated at slower speed

2. Breaking meniscusis at top is seen at constant larger bore diameter

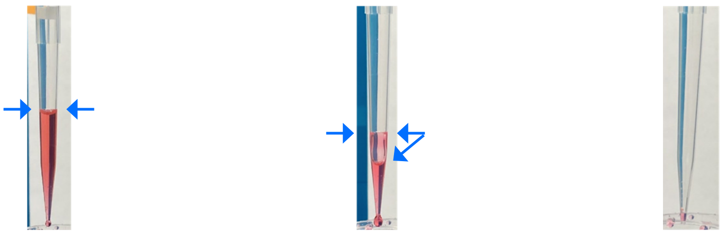
3. After complete dispense and before blowout

4. Excess liquid after blow out

Figure A1: The flowrate used is constant throughout the tip. At high flow rate this causes breaking of the meniscus in Glycerol 99% and excess fluid remains inside the tip after the blowout, creating a dead volume.

We can determine the correct flow rate by observing different flow rates where the tip changes shape. In the case of the Opentrons P1000 Tip, it is around 2 cm above the orifice. A clean dispense can be observed in **Figure A2**. As the flow rate is lowered, the meniscus is maintained until the tip changes shape and breaks down slowly. The delay after the dispense cycle helps allow the excess liquid in the interior region of the tip to slide to the brim. Thus, a slower blow out results in the tip dispensing cleanly.

P1000 Dispense Meniscus Maintaining at Slower Flow Rate



1. 1000 µL Liquid is aspirated at slower speed
2. Clean meniscusis seen at lower flow rate
3. After complete dispense and before blow out
4. After complete blow out

Figure A2: Slower dispense flow rate allows meniscus maintenance in Glycerol 99% and thus helps reduce dead volume after complete blow out.