ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA
INSTITUTO POLITÉCNICO DE LISBOA

IPL

Workshop de Pipetagem

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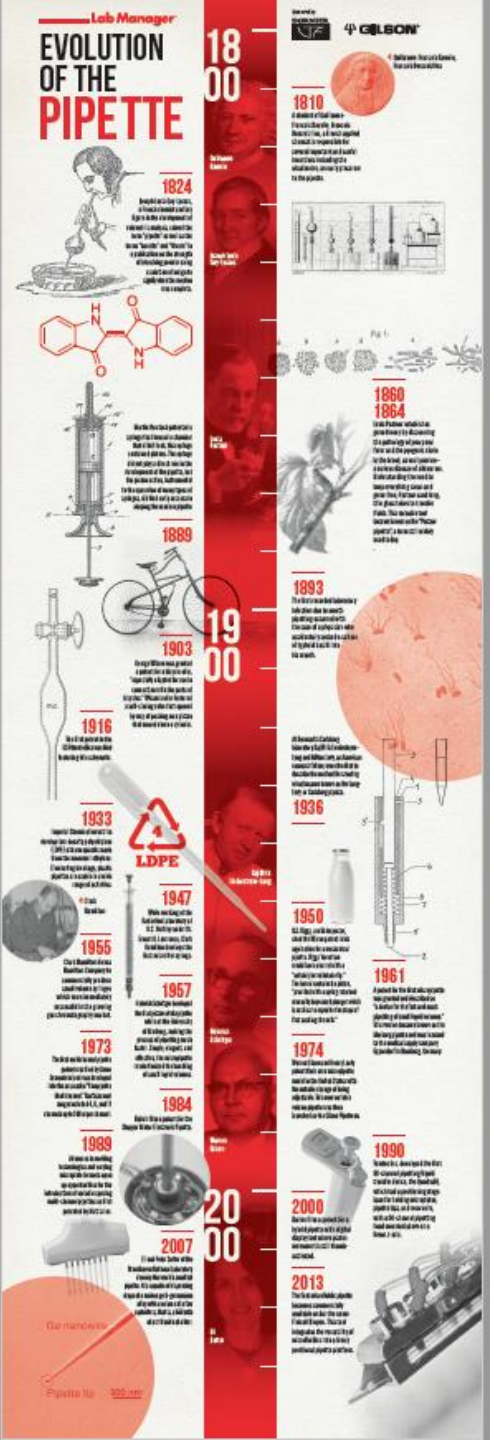
25 de fevereiro de 2019

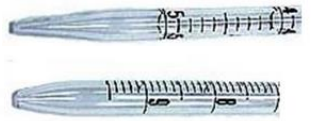


Instrumento que permite a medição e transferência precisa e rigorosa de líquidos

Tipos de Pipetas

- **Pipetas de pasteur**

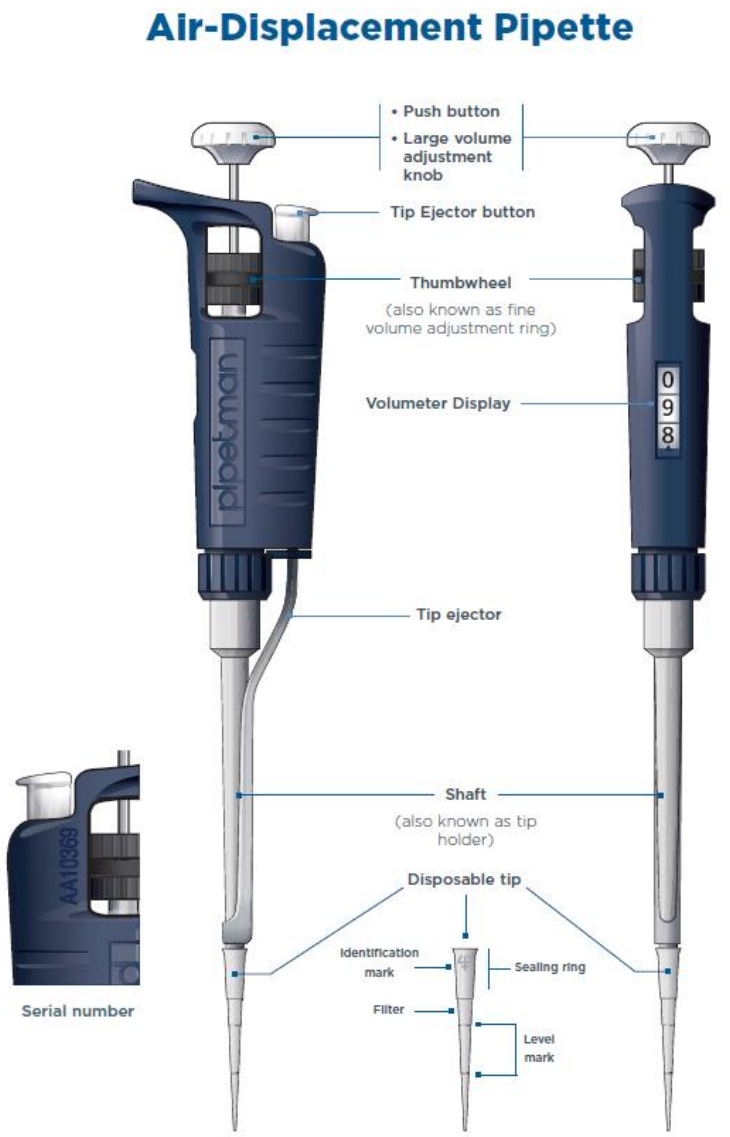
- **Pipeta volumétrica**



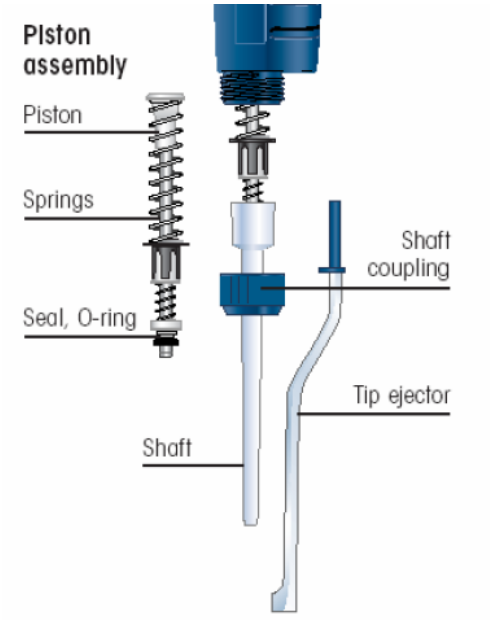
- **Pipeta graduada**

- **Micropipeta**

Tipos de micropipetas



- almofada de ar que é deslocada dentro da pipeta por um pistão durante a aspiração



Tipos de micropipetas

- Funciona como seringa para aspirar e dispensar a amostra

- Líquidos viscosos e líquidos voláteis.

Técnica pipetagem

pipeta deslocamento de ar

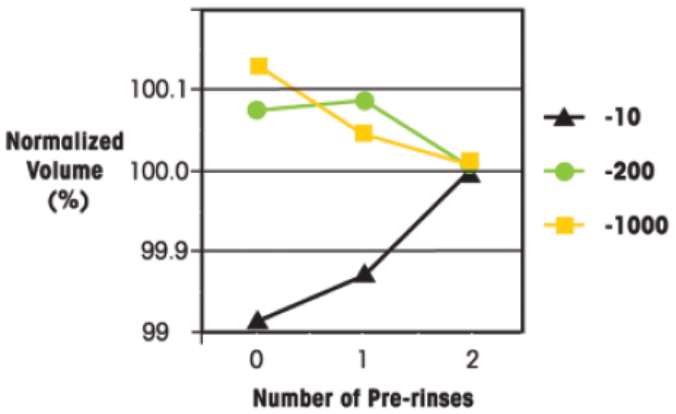
Preparação pipetagem

1. **Agarrar a pipeta gentilmente**

2. **Selecionar volume:**

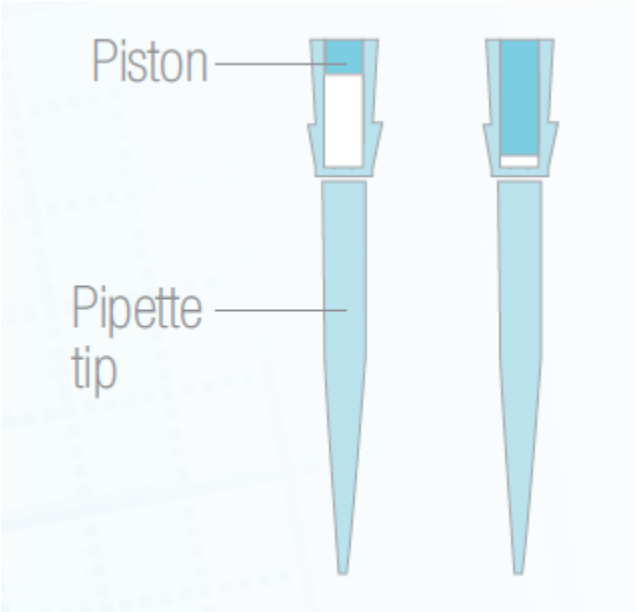
Rodar 180ºC acima do Vpretendido e rodar novamente posição correcta

3. Colocar ponta

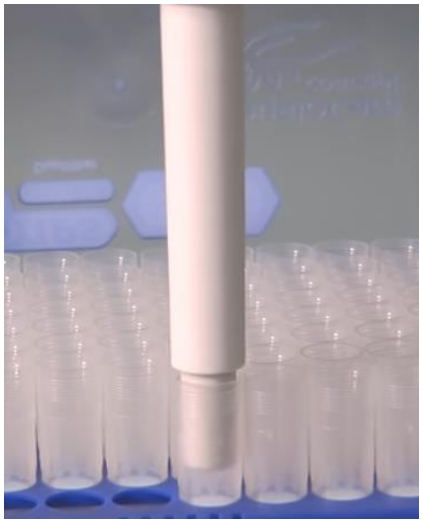


4. Pré-equilibrar a ponta

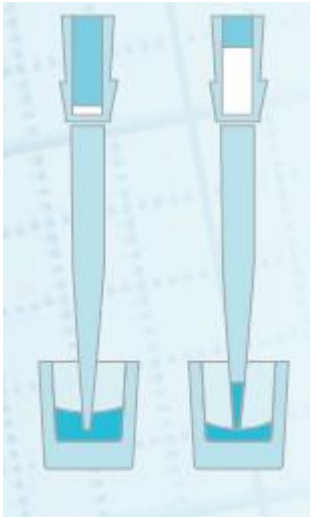
3 etapas principais



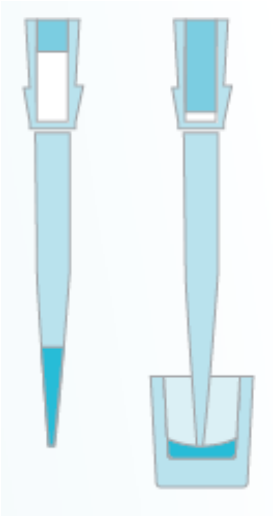
1- Colocar ponta



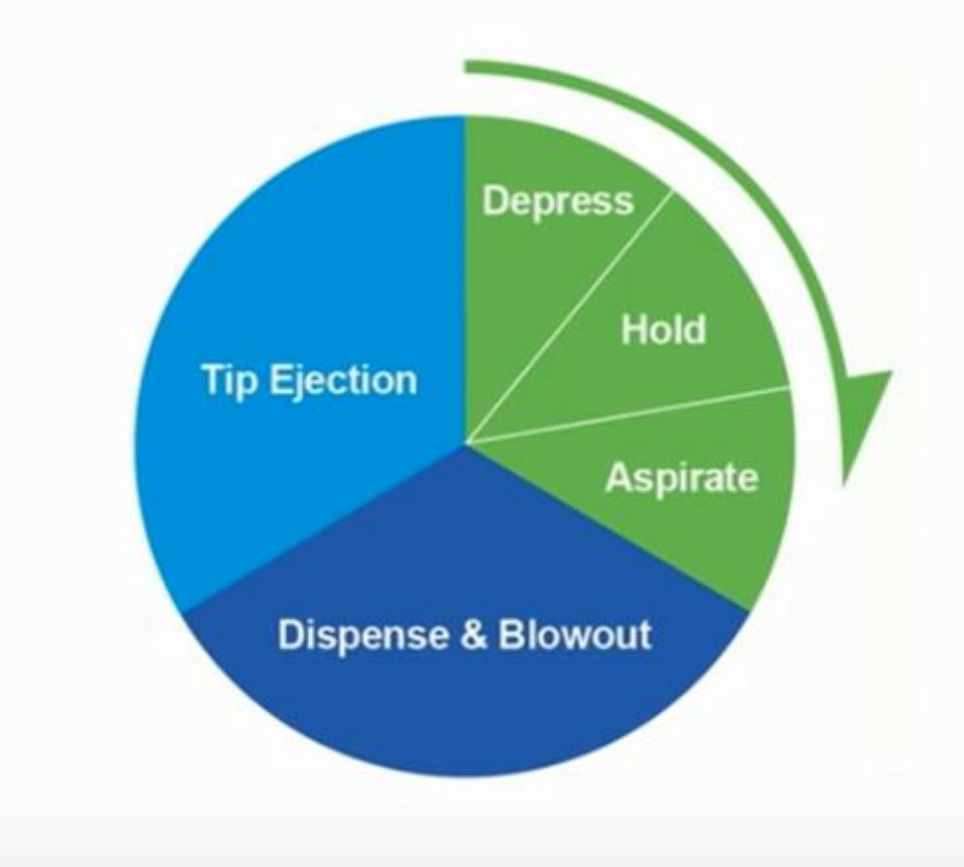
2- Aspirar



3- Dispensar

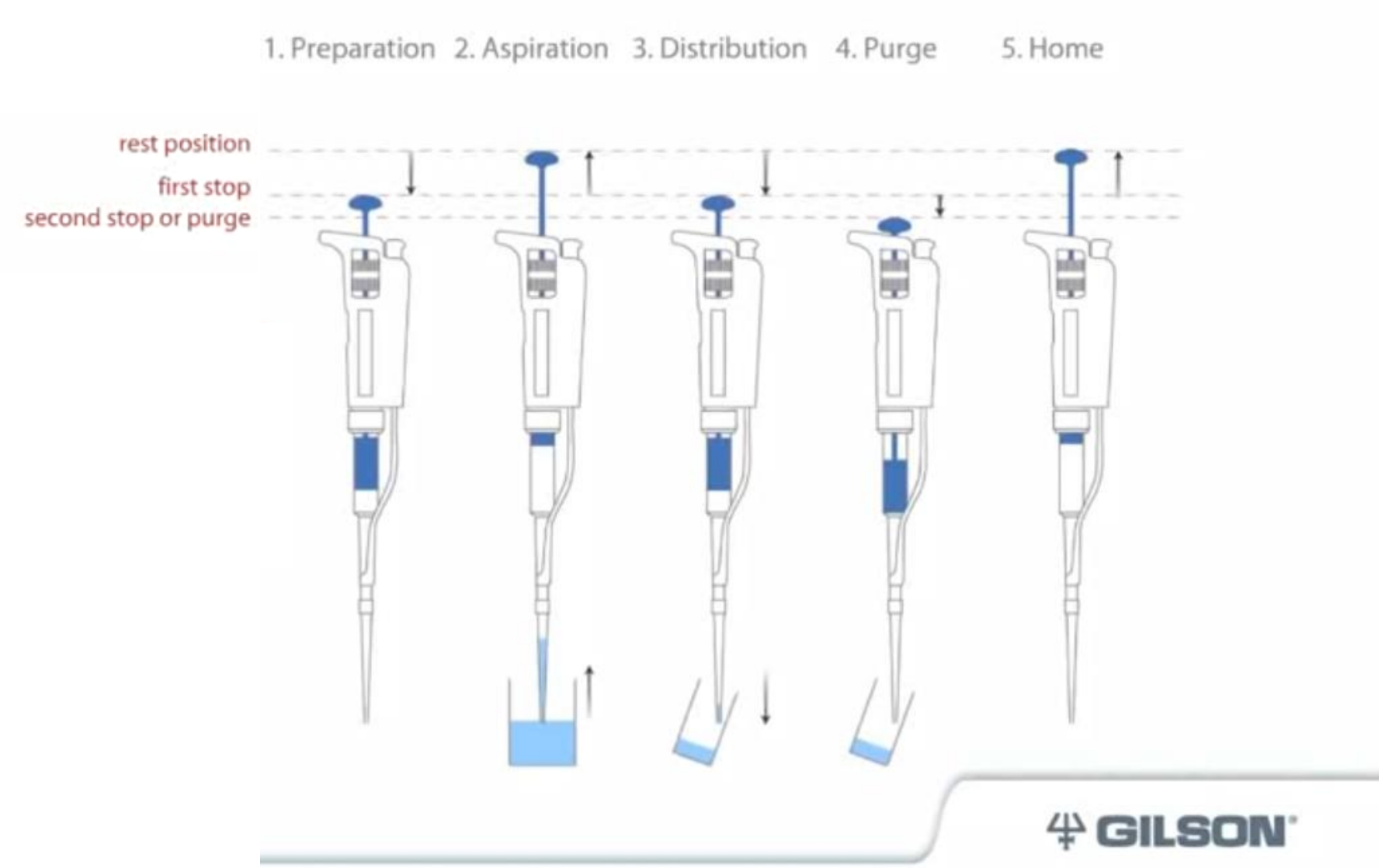


Pipetagem directa - Ciclo de Pipetagem

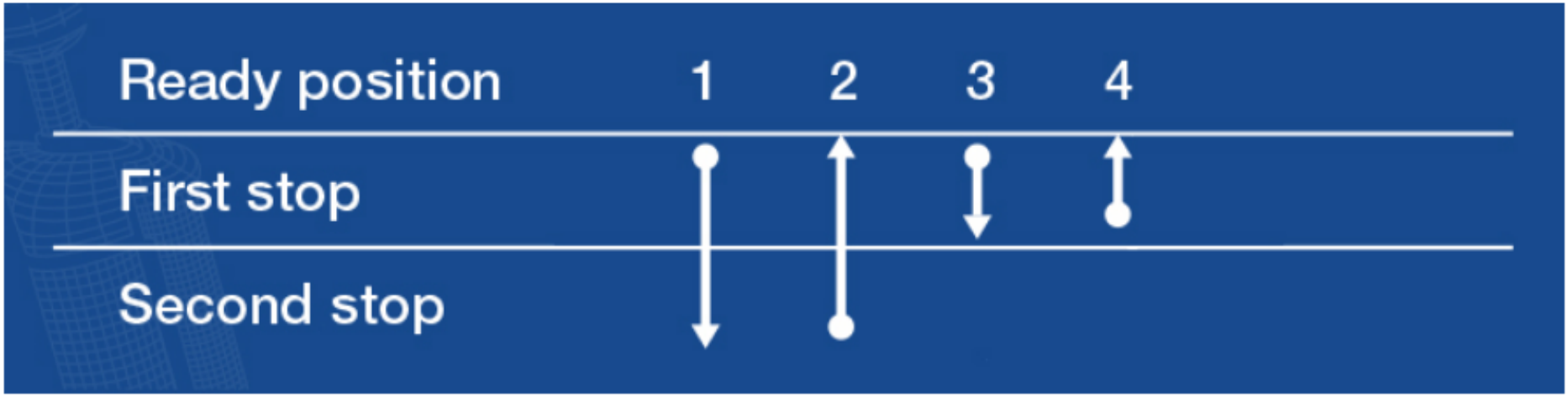


Pipetagem Directa

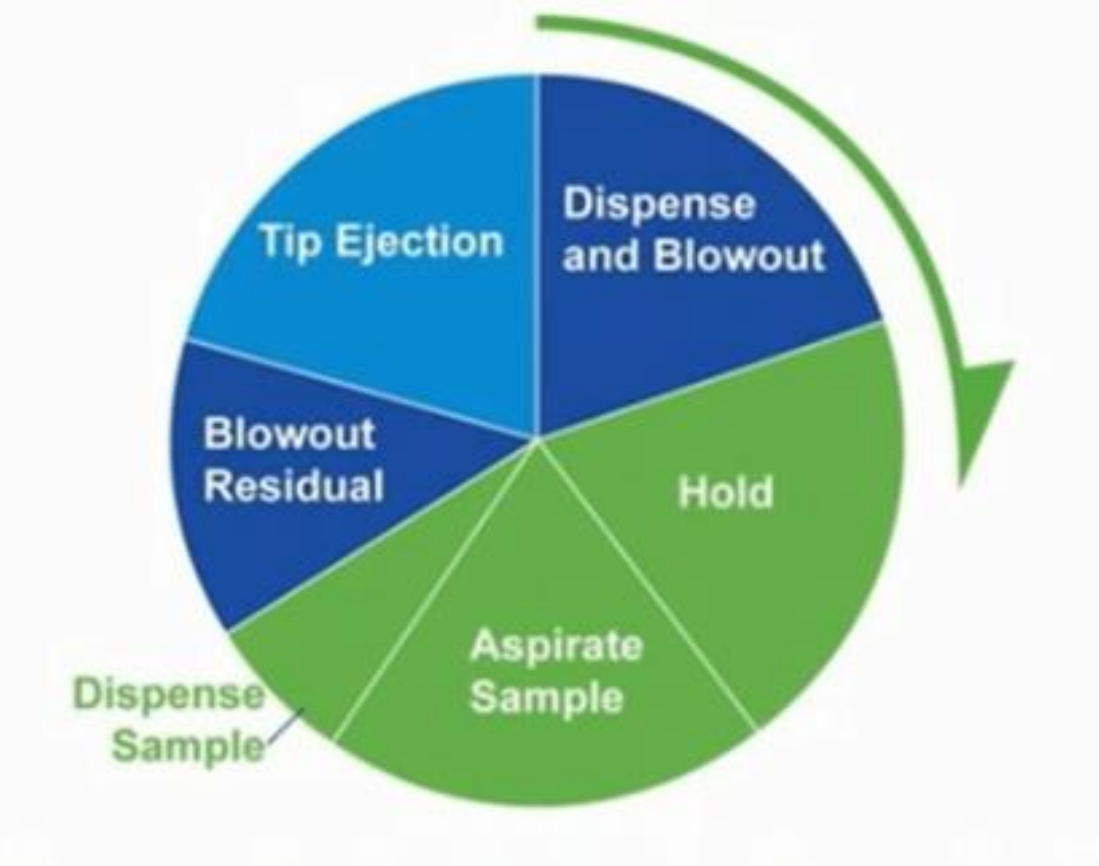
- Soluções aquosas (tampões, ácidos, bases)



Pipetagem Repetitiva

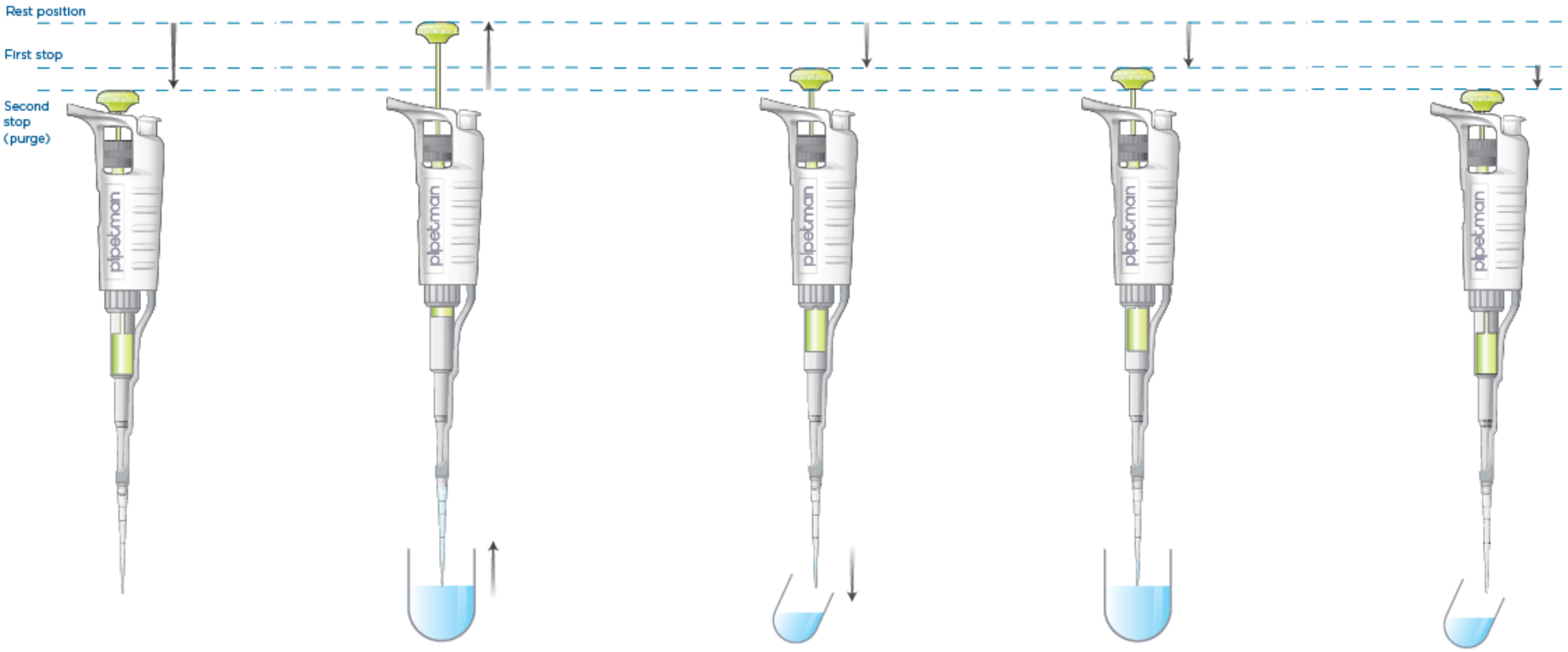


Pipetagem reversa- Ciclo de Pipetagem



Pipetagem Reversa

- Soluções viscosas, densas, voláteis.



Pipetagem de soluções viscosas

Pipetas e volumes

- P2: 0.2 µL – 2 µL
- P10: 1 µL – 10 µL
- P20: 2 µL – 20 µL
- P100: 10 µL – 100 µL
- P200: 20 µL – 200 µL
- P1000: 100 µL – 1000 µL

Pipetas e volumes

1,52 µL

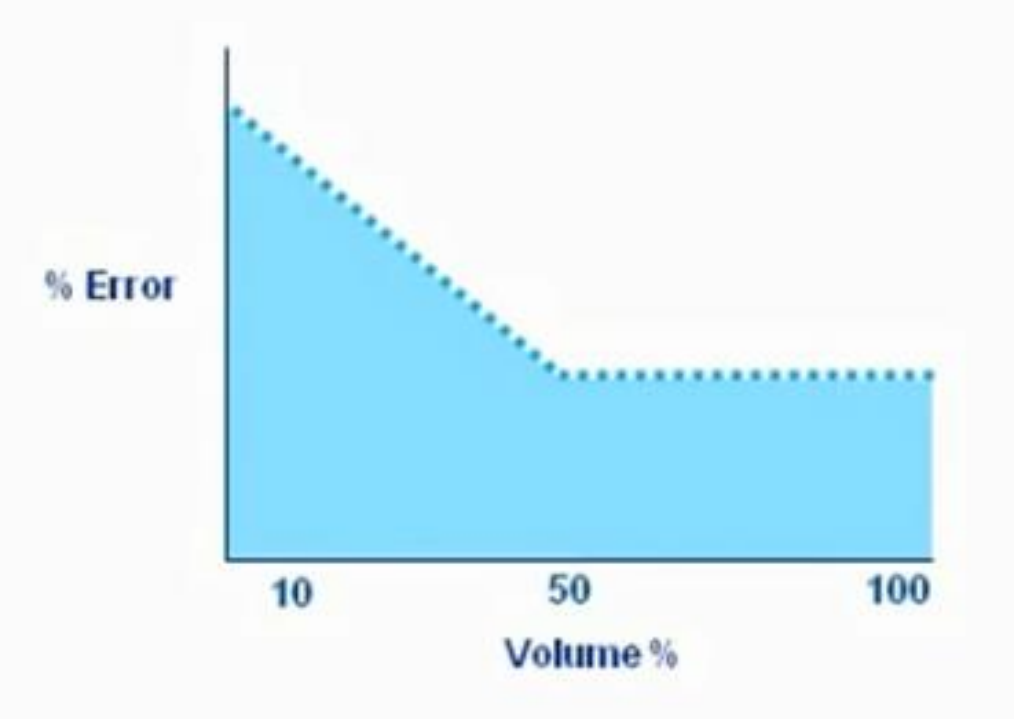
15,2 µL

152 µL

520 µL

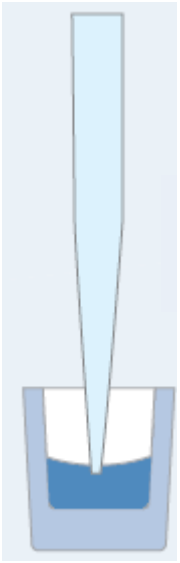
Volume apropriado

- Pipetar entre 10 a 100% do volume nominal (idealmente 35-100%)

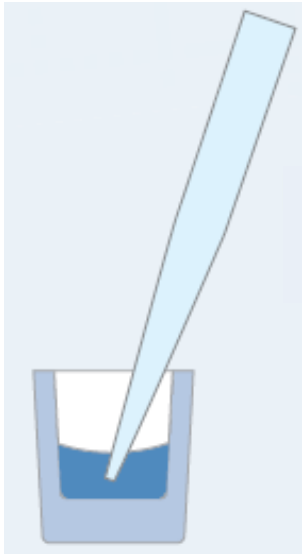


Ângulo de pipetagem

Aspirar



vs



Inaccuracy
0.2–0.4%

Inaccuracy
1–1.2%

Manter pipeta vertical – aumenta precisão e exactidão até 2.5%

Dispensar

10º- 45º parede reservatório

Profundidade de imersão da ponta

Ponta deve ultrapassar a superfície do líquido

Taxa de aspiração

- Amostra contacta com o eixo, danificando o pistão
- Introdução de aerossóis
- Contaminação entre amostras

Pipetas macro-volume:

- Aguardar 1 seg antes de remover a ponta

Dispensa do líquido

Recomendações para pipetar líquidos

1. Verificar se pipeta se encontra limpa
2. Utilizar pontas uma única vez
3. Não virar a pipeta quando ponta contém líquido
4. Evitar contaminações: ejector ponta
5. Manter as pipetas na vertical

Solution/ compound	Examples	Pipette	Pipette Tip	Pipetting technique	Comments
Aqueous solution	Buffers, diluted salt solutions	Air displacement	Standard	Forward	
Viscous solution	Protein and nucleic acid solutions, glycerol, Tween 20/40/60/80	Air displacement Positive displacement	Standard or wide orifice, Low Retention Positive displacement	Reverse	Pipette slowly to avoid bubble formation.
Volatile compounds	Methanol, hexane	Air displacement Positive displacement	Filter/Barrier Positive displacement	Reverse	Pipette rapidly to reduce the effect of evaporation. Carbon filter tips protect the integrity of the pipette by eliminating exposure to harmful vapors.
Body fluids	Whole blood, serum	Air displacement	Standard or wide orifice tip	Heterogeneous*	Residual liquid can be found on the outer surface of the tip. Wipe the tip against the edge of the vessel to remove this liquid before dispensing.
Nucleotide solutions	Genomic DNA, PCR products	Air displacement Positive displacement	Filter/Barrier or wide orifice Positive displacement	Forward	For genomic DNA wide orifice tips can be used to eliminate mechanical shearing.
Radioactive compounds	^{14}Carbonate, ^{3}H-thymidine	Air displacement Positive displacement	Filter/Barrier Positive displacement	Forward	
Acids/alkalis	H_2SO_4, HCl, NaOH	Air displacement	Filter/Barrier	Forward	
Toxic samples		Air displacement Positive displacement	Filter/Barrier Positive displacement	Forward or reverse	

Escolha da Ponta adequada

1. **Ponta standard sem filtro:** pipetagem comum

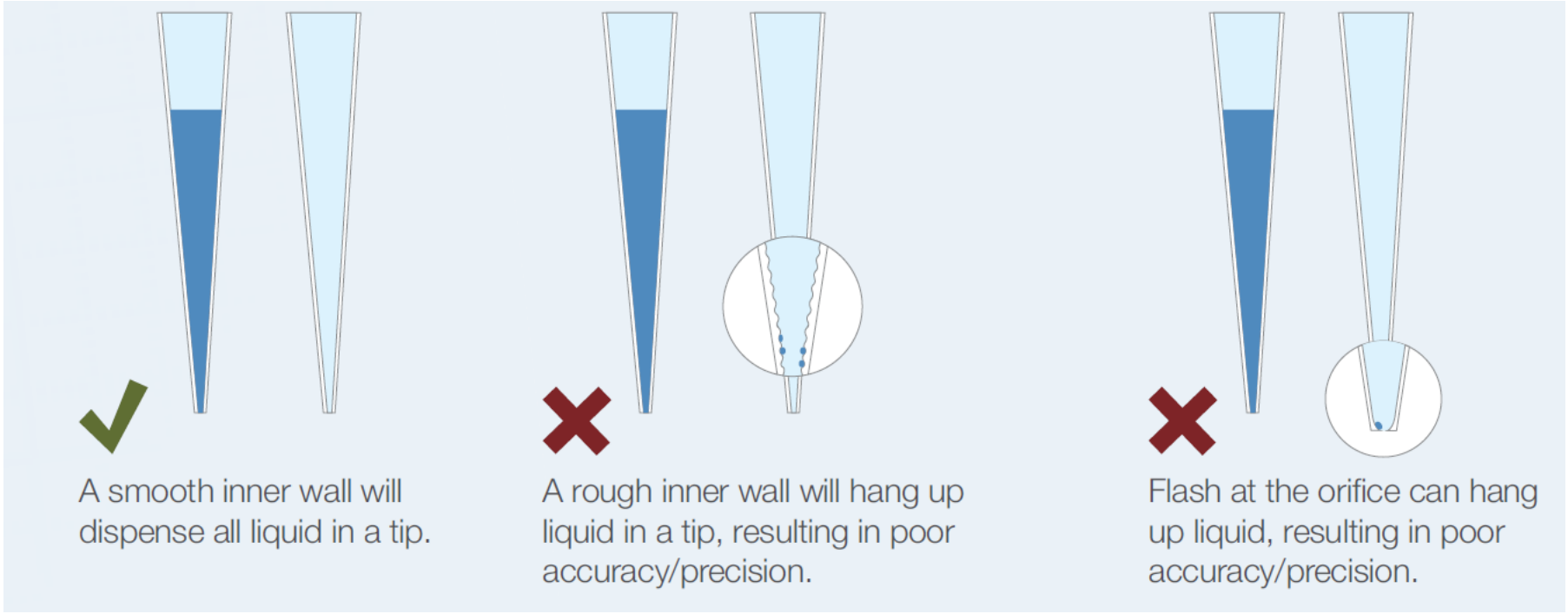
2. **Ponta com filtro:** PCR, estudos forenses e amostras com radioisotopos.

3. **Ponta alongada:** evita contaminação entre amostras e da pipeta.

4. **Ponta de aplicação de amostras em gel**

5. **Ponta *solvent-safe:*** soluções caústicas e corrosivas

Escolha da Ponta adequada



A smooth inner wall will dispense all liquid in a tip.

A rough inner wall will hang up liquid in a tip, resulting in poor accuracy/precision.

Flash at the orifice can hang up liquid, resulting in poor accuracy/precision.

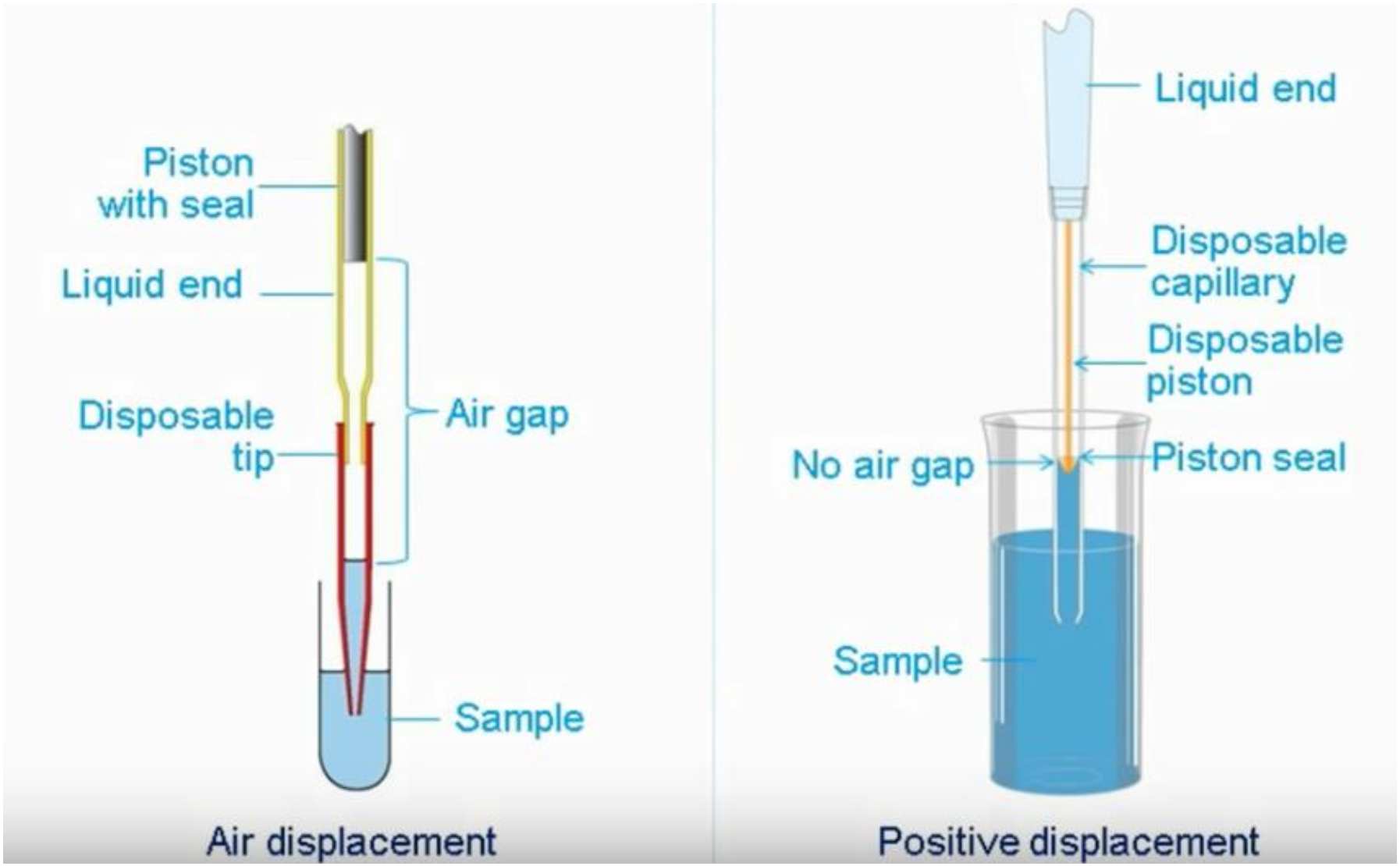
Dicas para aumentar a precisão

1. Pré-equilibrar a ponta antes de pipetar
2. Equilibrar o líquido e a pipeta à TA
3. Verificar se a ponta tem gotas
4. Adequar a técnica de pipetagem ao tipo de líquido
5. Fazer pausas consistentes

Dicas para aumentar a precisão

6. Manter a pipeta na vertical durante a aspiração
7. Minimizar a manipulação da pipeta e da ponta
8. Mergulhar a ponta de forma adequada na amostra
9. Utilizar a ponta adequada
10. Premir o botão de pipetagem com força e rapidez consistentes

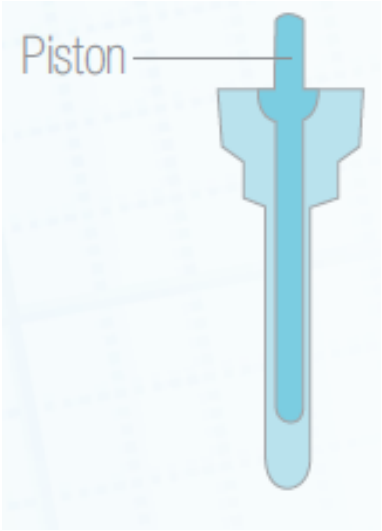
Pipeta de deslocamento de ar *vs* pipeta de deslocamento positivo



Pipeta de Deslocamento positivo

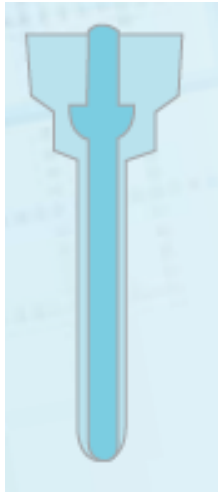
- ✓ **Para amostras viscosas, densas e voláteis.**
- ✓ **Não depende da TA, pressão atmosférica ou humidade.**
- ✓ **Exactidão e precisão mais facilmente alcançáveis**
- ✓ **Não contaminação ou dano do sistema capilar descartável**
- X **Sistema capilar + pistão caros**
- X **Requer força ergonómica superior**

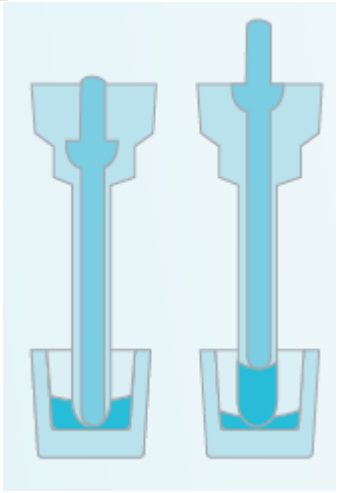
Pipeta de Deslocamento positivo



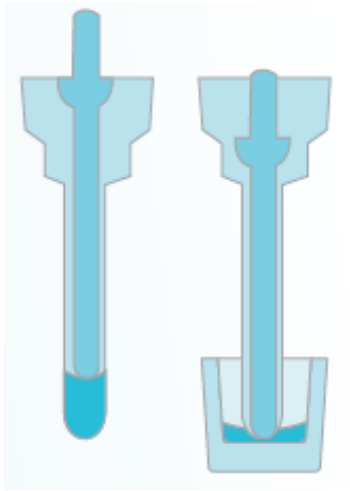
1- Colocar pistão

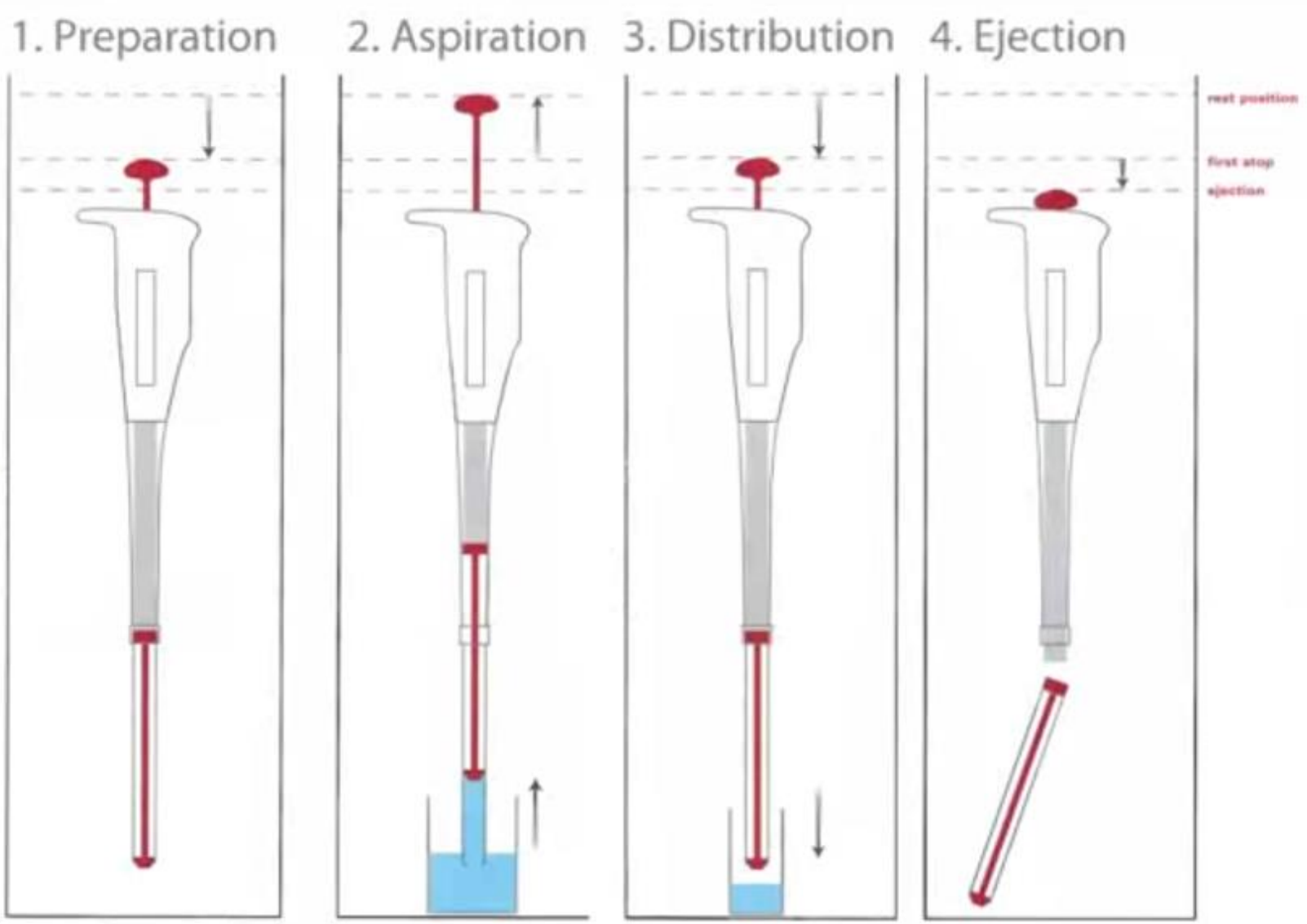
2- Aspirar





3- Dispensar




1. Preparation
2. Aspiration
3. Distribution
4. Ejection
rest position
first stop
ejection

GILSON

Escolha da Pipeta

SAMPLE TYPES	EXAMPLES	RECOMMENDED PIPETTES
Aqueous	Water, sucrose, Tris, buffers with a pH of 7	Air-displacement.
Biological	DNA, RNA, proteins	Air-displacement with filter tips
Viscous	Glycerol, surfactants, oil	
Volatile	Ethanol, hexane, formaldehyde	
Hazardous	Radioactive isotopes, blood, infectious bacteria or viruses	
Corrosive	Acids such as hydrochloric acid or sulfuric acid, bases such as ammonium hydroxide, salts such as sodium chloride	Positive-displacement

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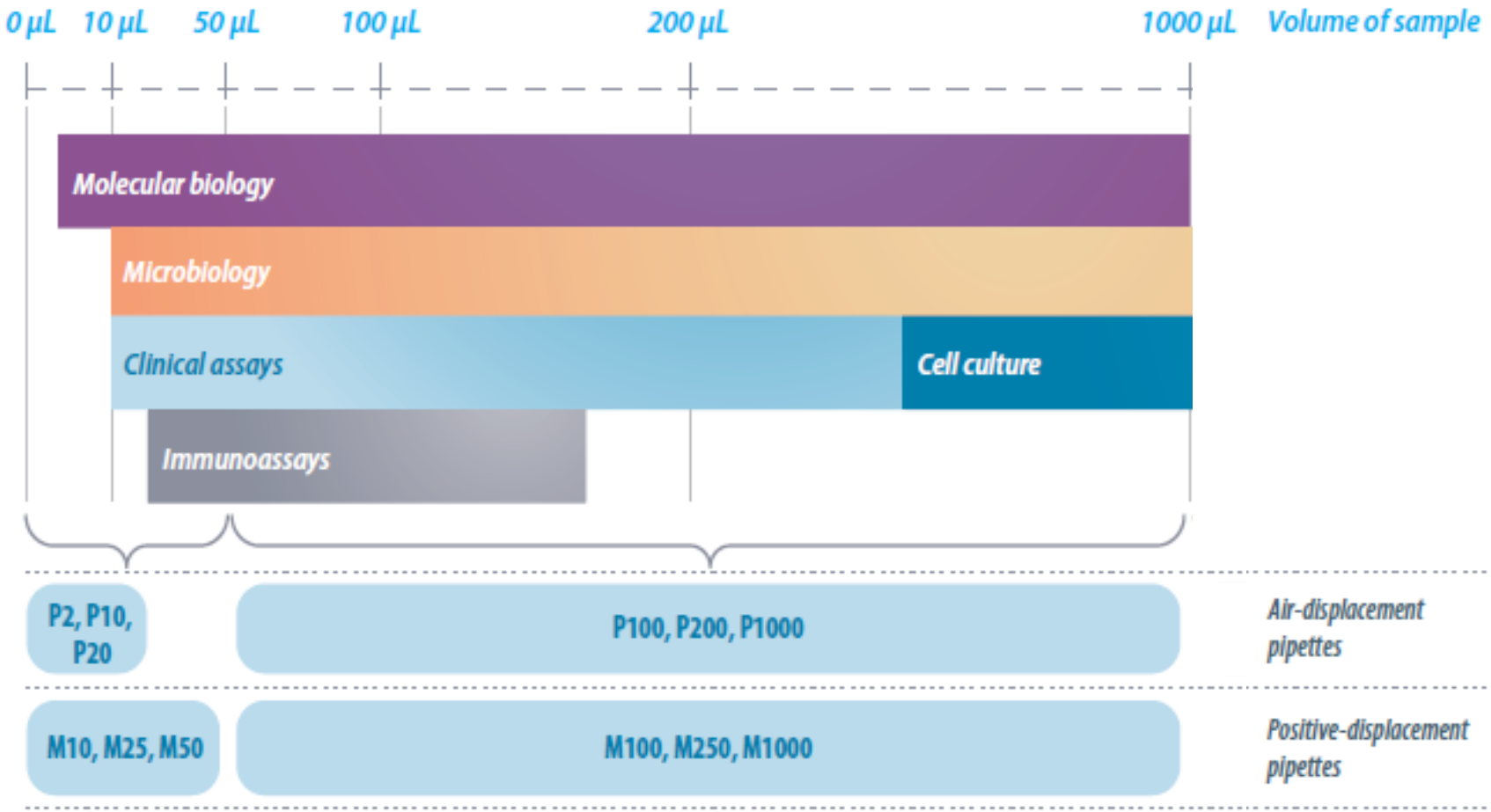
Escolha da Pipeta



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Fontes de erro comuns

1. Bolhas de ar
2. Líquido no exterior da pipeta
3. Contaminação amostras
4. Aquecimento da pipeta

Redução dos factores de risco da pipetagem

- **Ambiente:** posição do equipamento circundante que permita postura correcta.

- **Postura:** correcto posicionamento do corpo.

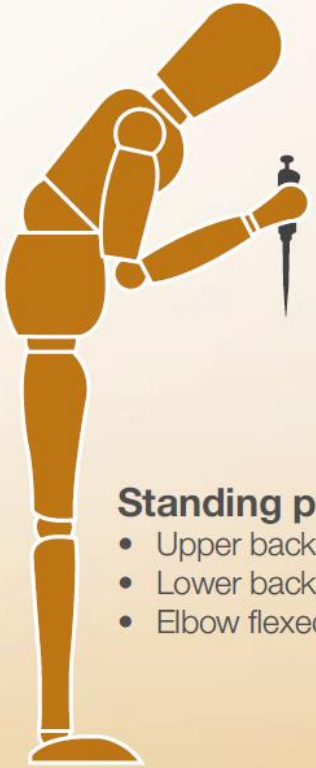
- **Força:** redução da força utilizada para colocar e ejectar a ponta e pipetar.

- **Repetição:** Número limite de movimentos de pipetagem.

Bad Posture

Seated posture:

- Shoulders elevated
- Upper arm elevated
- Elbow extended
- Wrist in deviation



Standing posture:

- Upper back and neck stooped
- Lower back and trunk stooped
- Elbow flexed

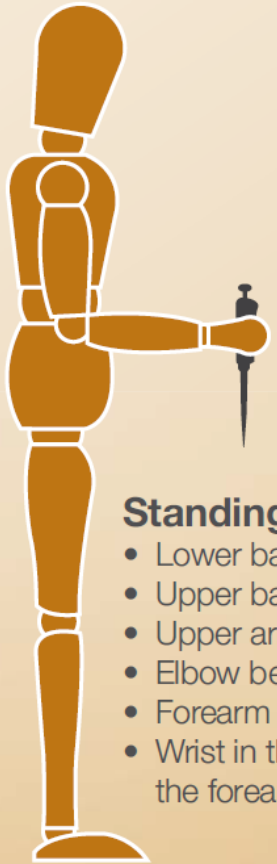
Wrist posture:

- Upper arm flexed
- Elbow extended
- Wrist deviated downward

Good Posture

Seated posture:

- Lower back supported by chair
- Upper back and neck upright
- Upper arm vertical
- Wrist in the same plane as the forearm



Standing posture:

- Lower back and trunk upright
- Upper back and neck upright
- Upper arm vertical
- Elbow bent at 90°
- Forearm parallel to the floor
- Wrist in the same plane as the forearm

Wrist posture:

- Forearm parallel to the floor
- Wrist and forearm in the same plane

Limpeza das pipetas

- Seguir instruções do fabricante
- Limpar com etanol 70% diariamente
- Limpar o interior
- Limpar solventes orgânicos com detergentes
- Eliminar DNA: imergir partes pipeta em 3% (p/v) hipoclorito de sódio , 15 min. Lavar bem com água destilada e deixar secar.
- Autoclavagem, de acordo com instruções fabricante

Calibração

Diferença entre volume dispensado e volume selecionado

Definição dos limites de erro pela ISSO 8655

Calibração

1. Verificar o volume nominal
2. Verificar o volume mínimo ou 10% do Volume nominal
3. Utilizar uma ponta pré-equilibrada (3-5x)
4. Realizar 10 pipetagens de ambos os volumes
5. Verificar se o volume dispensado está dentro dos limites definidos

Calibração pelo método gravimétrico

Baseada na determinação do peso de amostras de água

- Temperatura de 21.5 ± 1.5ºC
- Humidade relativa 50%-75%

Equipamento:

- Termómetro calibrado (erro máx 0.2ºC)
- Higrómetro (erro máx 10%)
- Barómetro (erro máx 0.5 KPa)
- Água destilada

Calibração pelo método gravimétrico

- Balanças

SELECTED VOLUME (V) OF APPARATUS UNDER TEST	BALANCE RESOLUTION MG	REPEATABILITY AND LINEARITY MG	STANDARD UNCERTAINTY OF MEASUREMENT MG
1 µL < V ≤ 10 µL	0.001	0.002	0.002
10 µL < V ≤ 100 µL	0.01	0.02	0.02
100 µL < V ≤ 1000 µL	0.1	0.2	0.2
1 mL < V ≤ 10 mL	0.1	0.2	0.2

- Tubos/frascos

EXAMPLES OF INSTRUMENTS	VOLUMES	SAMPLE RESERVOIR	WEIGHING VESSEL	BALANCE RESOLUTION	OTHER EQUIP.
P2 - P20 PM x20 F2 - F10 M10 to M25 M100	0.1 to 20 µL	Ø 35 mm H 50 mm	Ø 10.5 mm H 13 mm	0.001 mg	Lid Tweezers Filters
P100 - P200 F25 - F200 PM x300 M50 - M250	> 20 to 200 µL	Ø 35 mm H 50 mm	Ø 21 mm H 50 mm	0.01 mg	Lid
P1000 - P5000 F250 - F5000 M1000	> 200 to 5000 µL	Ø 50 mm H 70 mm	Ø 35 mm H 50 mm	0.1 mg	Lid
P10 mL	> 5 to 10 mL	250 mL beaker	Ø 40 mm H 100 mm	0.1 mg	Lid

Calibração pelo método gravimétrico

1. Peso para volume

$$V_i = (W_i + \bar{e})\, Z$$

W_i is the weight as read on the balance

$\bar{e}$ is the mean evaporating loss during the cycle time.

Z expressed in µL/mg, is a conversion factor incorporating density of water buoyed in air, at test temperature and barometric pressure.

$$\bar{W} = \frac{1}{n} \sum_{i=1}^{n} W_i$$

n: number of weighings

$\bar{W}_i$ weighing results

$$\bar{e} = \frac{1}{m} \sum_{i=1}^{m} e_i$$

m : number of weighings

Z

The reference calculation equation is: $Z = [1/(P_W - P_A)]\,[1-(P_A/P_B)]$

Where: P_A = density of air at t°C.
P_W = density of the test liquid at t°C.
P_B = density of the balance weights. Use 8 g/cc for PB

NOTE Weights conforming to International Recommendation No. 33 of OIML have been adjusted to give results when weighing in air as if the density of the weights were 8.0 g/mL.

Values of the conversion factor Z (µL/mg) as a function of temperature and pressure for distilled water.

Temperature °C	AIR PRESSURE HPA					
	800	853	907	960	1013	1067
15	1.0018	1.0018	1.0019	1.0019	1.0020	1.0020
15.5	1.0018	1.0019	1.0019	1.0020	1.0020	1.0021
16	1.0019	1.0020	1.0020	1.0021	1.0021	1.0022
16.5	1.0020	1.0020	1.0021	1.0022	1.0022	1.0023
17	1.0021	1.0021	1.0022	1.0022	1.0023	1.0023
17.5	1.0022	1.0022	1.0023	1.0023	1.0024	1.0024
18	1.0022	1.0023	1.0024	1.0024	1.0025	1.0025
18.5	1.0023	1.0024	1.0025	1.0025	1.0026	1.0026
19	1.0024	1.0025	1.0025	1.0026	1.0027	1.0027
19.5	1.0025	1.0026	1.0026	1.0027	1.0028	1.0028
20	1.0026	1.0027	1.0027	1.0028	1.0029	1.0029
20.5	1.0027	1.0028	1.0028	1.0029	1.0030	1.0030
21	1.0028	1.0029	1.0030	1.0030	1.0031	1.0031
21.5	1.0030	1.0030	1.0031	1.0031	1.0032	1.0032
22	1.0031	1.0031	1.0032	1.0032	1.0033	1.0033
22.5	1.0032	1.0032	1.0033	1.0033	1.0034	1.0035
23	1.0033	1.0033	1.0034	1.0035	1.0035	1.0036
23.5	1.0034	1.0035	1.0035	1.0036	1.0036	1.0037
24	1.0035	1.0036	1.0036	1.0037	1.0038	1.0038
24.5	1.0037	1.0037	1.0038	1.0038	1.0039	1.0039
25	1.0038	1.0038	1.0039	1.0039	1.0040	1.0041
25.5	1.0039	1.0040	1.0040	1.0041	1.0041	1.0042
26	1.0040	1.0041	1.0042	1.0042	1.0043	1.0043
26.5	1.0042	1.0042	1.0043	1.0043	1.0044	1.0045
27	1.0043	1.0044	1.0044	1.0045	1.0045	1.0046
27.5	1.0044	1.0045	1.0046	1.0046	1.0047	1.0047
28	1.0046	1.0046	1.0047	1.0048	1.0048	1.0049
28.5	1.0047	1.0048	1.0048	1.0049	1.0050	1.0050
29	1.0049	1.0049	1.0050	1.0050	1.0051	1.0052
29.5	1.0050	1.0051	1.0051	1.0052	1.0052	1.0053
30	1.0052	1.0052	1.0053	1.0053	1.0054	1.0055

Procedimento

1. Colocar água destilada num recipiente de pesagem
2. Registar condições (TA, T água, humidade relativa, pressão barométrica)
3. Selecionar o volume em teste na pipeta
4. Colocar ponta
5. Pré-equilibrar ponta
6. Pipetar água
7. Abrir porta balança, retirar recipiente de pesagem, colocar amostra e colocar na balança. Fechar a porta.

Procedimento

8. Depois de estabilizar – registar peso.
9. Repetir 9x
10. Para amostras ≤ 50 µL, estimar a perda por evaporação, ao repetir passos 6-7, sem adicionar a amostra. Registar valores (e_i) e repetir (m).
11. Registar condições
12. Determinar Z
13. Calcular exactidão e precisão e comparar com especificações fabricante ou ISSO 8655-2

Cálculo da exactidão e precisão

Exactidão

$$E = \bar{V} - V_0$$

E systematic error

V_0 nominal volume

$\bar{V}$ mean volume

$$\bar{V} = \frac{\sum_{i=1}^{n} V_i}{n}$$

V_i individually measured volume

n number of measurements

The accuracy of a pipette can be expressed as a percentage of the nominal volume:

$$E\% = \frac{\bar{V} - V_0}{V_0} \times 100$$

Precisão

$$SD = \sqrt{\frac{\sum_{i=1}^{n} (V_i - \bar{V})^2}{n - 1}}$$

$\bar{V}$ mean volume

$$CV = \frac{SD}{\bar{V}} \times 100$$

Below is an example of how to evaluate the performance of PIPETMAN P10 at 1 µL.

1. Determine the mean value ē of the evaporation loss e_l that occurs during your pipetting cycles.
Proceed as described in appendix III to determine e_i

$$\bar{e} = \frac{1}{m} \sum_{i=1}^{m} e_i$$

m : number of weighings

$e_1 = 0.016$ mg $\quad e_3 = 0.021$ mg
$e_2 = 0.018$ mg $\quad e_4 = 0.017$ mg

$\bar{e} = (e_1+e_2+e_3+e_4) / 4$

$\bar{e} = (0.016+0.018+0.021+0.017) / 4$

$\bar{e} =$ **0.018 mg/per cycle**

2. Change the pipette tip and perform the first weighing. Then, keep a regular cycle and perform the 10 following measurements.

$W_r = 0.957$ mg

$W_1 = 0.968$ mg $\quad W_6 = 0.966$ mg
$W_2 = 0.960$ mg $\quad W_7 = 0.955$ mg
$W_3 = 0.984$ mg $\quad W_8 = 0.972$ mg
$W_4 = 0.942$ mg $\quad W_9 = 0.958$ mg
$W_5 = 0.969$ mg $\quad W_{10} = 0.967$ mg

W_r rinsing measurement which is disregarded for the calculation

3. Calculate the mean weight

$$\bar{W} = \frac{1}{n} \sum_{i=1}^{n} W_i$$

n: number of weighings

$\bar{W}_i$ weighing results

$\bar{W} = (0.968 + 0.960 + 0.984 + 0.942 + 0.969 + 0.966 + 0.955 + 0.972 + 0.958 + 0.967) / 10$

$\bar{W} =$ **0.964 mg**

4. Calculate the mean volume
For a temperature of 21.5°C and an air pressure of 1013 hPa, the Z factor is equal to 1.0032 µL/mg (see table in Appendix II).

$$\bar{V} = (\bar{W} + \bar{e}) \times Z$$

5. Evaluate accuracy
Systematic error (**E**):

$$E = \bar{V} - V_0$$

V_0 true value on the instrument
$E = 0.985 - 1 =$ **0.015µL**

Relative error (E%):

$$E\% = (\bar{V} - V_0) \times 100 / V_0$$

$E\% = (\bar{V} - V_0) \times 100 / V_0$
$E\% = (-0.015 \times 100)/1 =$ **-1.50%**

6. Evaluate precision (repeatability)
Standard Deviation (SD_w)

$$SD_w = \sqrt{\sum_{i=1}^{n} \frac{(W_i - \bar{W})^2}{n-1}}$$

$$SD_w^2 = \frac{1}{n-1} \sum_{i=1}^{n} (W_i - \bar{W})^2$$

$$SD_w^2 = \frac{1}{9} \left[\begin{array}{l} (0.968-0.964)^2 + (0.960-0.964)^2 + (0.984-0.964)^2 + \\ (0.942-0.964)^2 + (0.969-0.964)^2 + (0.966-0.964)^2 + \\ (0.955-0.964)^2 + (0.972-0.964)^2 + (0.958-0.964)^2 + \\ (0.967-0.964)^2 \end{array} \right]$$

Random error (**SDv**):

$SD_w =$ **0.011 mg**
$SD_v = SD_w \times Z$
$SD_v = 0.011 \times 1.0032 =$ **0.011 µL**

Procedure for the Determination of Evaporation Loss.

Use the same distilled water, weighing vessel, and balance as you will be using for the gravimetric check.

1. Half fill the weighing vessel with distilled water.
2. Cover the weighing vessel with its lid and place it on the balance using a pair of tweezers.
3. Aspirate a sample.
4. Tare the balance and take the weighing vessel out of the balance.
5. Take off the lid with tweezers.
6. Dispense the sample into a dummy vessel.
7. Replace the lid on the weighing vessel and, using tweezers, replace the vessel on the balance.
8. Read the negative result e1 (record the absolute value).
9. Repeat steps 3 to 8, three times to obtain e2, e3, and e4.
10. Calculate the evaporation loss ee using the formula:

$$\bar{e} = \frac{1}{4}\ (e_1 + e_2 + e_3 + e_4)$$

Under normal conditions, this value is usually between 0.01 mg and 0.03 mg.

Manutenção

Diária

- Verificar se tem sujidades
- Manter posição vertical

Periódica

- Limpeza a cada 3 meses: desmontar pipeta e verificar calibração
- Pipetagem de solventes orgânicos: abrir parte inferior da pipeta e deixar ao ar ON
- Pipetagem frequente de ácidos ou bases: lubrificar pistão
- Utilizar pontas com filtro

Verificação em 2 minutos

Prevenção de contaminação

EPIs

Bata, luvas, óculos, máscara.

Pipeta-para-amostra

- Utilização de pontas com filtro.
- Mudança de ponta depois de pipetar cada amostra.
- Limpeza da pipeta regular.

Prevenção de contaminação

Amostra-para-pipeta

- Manter a pipeta na vertical durante a pipetagem.
- Libertar o botão de pipetagem suavemente.
- Para evitar contaminação por aerossois: pontas com filtro ou pipeta de deslocamento positivo.

Amostra-para-amostra

- Mudar a ponta em cada amostra.
- Suspeita de contaminação da pipeta: limpar pipeta e autoclavar.

Descontaminação

Pipetted liquids	Cleaning guidelines
Aqueous solutions and buffers	Open the pipette, rinse the contaminated parts thoroughly with distilled water, and allow to dry.
Acids and alkalis	It is advisable to clean the tip cone and lower part of the tip ejector with distilled water more frequently if acids or alkalis are handled. Clean as described in "Aqueous solutions and buffers."
Organic solvents	Immerse the contaminated parts in a detergent solution such as Deconex® 12 Basic. Rinse thoroughly with distilled water and allow to dry.
Radioactive solutions	Open the pipette and place the contaminated parts in a strong detergent or cleaning solution. Rinse several times with distilled water and allow to try. Decontamination should always be followed by confirming that radioactivity has been reduced to an acceptable level. All used cleaning materials are radioactive waste and must be disposed of according to regulations.
Proteins	Open the pipette, immerse the parts in a detergent solution, such as Deconex® 12 Basic. Rinse well with distilled water and allow to dry.
DNA, RNA	• DNA can be eliminated by immersing pipette parts in at least 3% (w/v) sodium hypochlorite for at least 15 minutes (2, 3). Rinse well with distilled water and allow to dry. • Treat the pipette parts with Thermo Scientific DNA AWAY Surface Decontaminates according to instructions. • Exposure to ultraviolet (UV) light for 30–60 minutes will further reduce but not completely eliminate DNA contamination on the pipette surface (4). • No special treatment is required to remove RNA because it degrades rapidly and is sensitive to ubiquitous RNases.
DNase, RNase	• RNase can be removed by first cleaning the pipette with a detergent solution, followed by thoroughly rinsing with water and then 95% ethanol to speed the drying process. Pipette parts are then soaked in a 3% hydrogen peroxide solution for 10 minutes. Finally, the parts are rinsed thoroughly with DEPC-treated water (5) and allowed to dry. • Treat the pipette parts with Thermo Scientific RNase AWAY Surface Decontaminates according to instructions. • DNase can be destroyed by autoclaving (15 min., 121 °C/250 °F).
Viruses, mycoplasma, bacteria, and fungi	Ultraviolet (UV) radiation is a practical method for inactivating viruses, mycoplasma, bacteria, and fungi. If the inner parts of the pipette are exposed to UV light, make sure the piston and O-rings are sufficiently lubricated.

Before assembling the pipette, wipe the piston with 70% ethanol and lubricate with the lubricant that is provided with the pipette. When removing RNase, use a freshly opened ethanol bottle and prepare 70% ethanol in DEPC treated water.

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Esterilização e desinfecção

- Autoclavagem: 20min a 121ºC

- Desinfectantes ou esterilizantes químicos

	Disinfection time (at 20°C/68°F)	Sterilization time (at 20°C/68°F)
Hydrogen peroxide (7.5%)	30 minutes	6 hours
Glutaraldehyde (2.5%)	20–90 minutes	10 hours
Sodium hypochlorite (5%)	20 minutes	N/A
Ethanol (70%)	10–30 minutes	N/A

Pipetas Serológicas

- Etapas da Pipetagem com pipetas serológicas:

1. Preparação do pompete + pipeta
2. Aspiração da solução
3. Dispensa da solução

"...a small mistake in pipetting can cause a large error in the final result. It is, therefore, of great importance to evaluate and to reduce, wherever possible, both random and systematic errors in liquid sample handling." Sari Ylätupa, PhD, "Choosing a Pipetting Technique Affects the Results of Your Analysis", European Clinical Laboratory [1]


That's all Folks!