Title Page

Title

Rapid Onsite Evaluation uses a very small proportion of total material aspirated at Endobronchial or Endoscopic Ultrasound in the investigation of suspected thoracic malignancy

Short Running Title

Rapid Onsite Evaluation uses a small proportion of EBUS material

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AM – Idea, ROSE, initial data analysis and manuscript; WT - specimen processing and triage and manuscript; CP, CK and SB – specimen processing and triage; JW – data recording; MC and NS – statistical analysis; AB and RM – specimen acquisition. All reviewed the manuscript.

Data availability

Original data is available from the corresponding author on reasonable request.

Abstract

Objective

1) To measure the proportion of aspirated material used to make direct slides for ROSE at EBUS and EUS in suspected thoracic malignancy
2) To correlate pass weights with ROSE category and needle size.

Method

All EBUS and EUS cases for possible thoracic malignancy October 2018 - May 2019 were included. All material from each pass was expelled into a Petri dish. One drop of material was placed on each of two slides; one used for ROSE, the other fixed and remaining material processed to cell block. Dish and slides were weighed before and after this procedure on a sensitive balance and weight of aspirate and slide

material calculated. When ROSE identified malignancy, slide production ceased but target sampling for ancillary studies continued.

Results

ROSE accuracy was 96.8%. Mean percentage by target of aspirated material used to make direct slides for ROSE was 1.9% in malignant cases and 3.6% in non-malignant cases (p=0.027 for difference). Mean percentage by pass was 5.9%. Mean weight of a single aspirate was 128.8mg. Mean weight of aspirates insufficient on ROSE (175.7mg) was significantly higher than the mean weight of benign or malignant aspirates (117.1 and 114.0mg respectively). Mean weight of aspirates using 22G needles (132.6mg) was significantly higher than that for 25G needles (87.1mg).

Conclusion

Material made into direct slides at EBUS and EUS and used in part for ROSE uses a tiny proportion of aspirated material with over 98% processed to cell block and available for ancillary testing in malignant cases.

Keywords

rapid on-site evaluation
rapid onsite evaluation
endobronchial ultrasound
sample weight
thoracic neoplasms
specimen handling

Introduction

Endobronchial ultrasound (EBUS) has revolutionised the investigation of lung cancer as it enables needle sampling of mediastinal and hilar lymph nodes as well as central lung masses under direct ultrasonographic visualisation[1,2]. Endoscopic ultrasound (EUS) may also be used to sample potential distant metastatic sites such as the left adrenal gland[3]. It is therefore possible to both diagnose and stage lung cancers in the same procedure.

Local policies result in these fine needle aspirates being processed in a wide variety of ways in different institutions. This ranges from placing the entire sample directly into a liquid fixative such as formalin or alcohol to making direct microscopic slides at the time of the procedure, in addition to fixation[4].

The latter approach facilitates Rapid Onsite Evaluation (ROSE), whereby direct slides are rapidly stained and evaluated in real time in the endoscopy suite for adequacy and the presence of diagnostic material[5-7].

There is good evidence that ROSE enables a reduction in sites needing targeting because, once a site has been established as malignant, the site can be confidently 'harvested' for material for diagnostic and molecular predictive evaluation with no need for assessing further sites[8-12]. It also enables appropriate triage at the time of the procedure for other investigations including microbiology, in granulomatous disease, or flow cytometry, in the case of potential lymphoma[4,13]. Recent guidelines and meta-analyses state that, when performing EBUS-TBNA, ROSE should be used, if the service is available[4,14,15].

The major disadvantage of ROSE is that it necessitates the presence of trained biomedical scientists (BMS) or a cytopathologist or both, although some argue that this is outweighed by the more efficient targeting[8,9,16,17]. The effect on diagnostic yield and accuracy is also controversial with some authors arguing for an improvement[18] with others finding no significant effect[19-21]. However, the practice of ROSE is far from standardised and some of this variation may be due to local differences in technique.

A further objection that has been raised is that a "considerable proportion" that could be formalin-fixed and used for immunohistochemical and molecular studies is "wasted" making direct slides[14,22].

The philosophy for ROSE in our institution is that slides are made from each pass, according to the method below, and are assessed for adequacy and the presence of diagnostic malignant cells. If malignant cells are seen, no more slides are made, but the node continues to be sampled to maximise cell block material for subsequent immunocytochemistry or molecular analysis and sampling only ceases when it is judged that there is ample material in the fixative for these investigations.

If malignant cells are not seen, slides continue to be made from each pass until the team judge that the site has been adequately sampled and is benign.

The primary aim of this study was to measure the proportion of material used to make slides for ROSE in EBUS and EUS cases, both by individual pass and by target site. As part of this process, the weight of material aspirated at each pass was quantified.

Materials and methods

All EBUS and EUS cases performed by the respiratory physicians in our institution from October 2018 to May 2019 that had a potential malignant diagnosis were included in the study. This mainly comprised staging and diagnostic procedures for possible primary lung cancer but also included occasional non-thoracic malignancies and radiologically indeterminate mediastinal masses.

Specimen preparation – standard procedure

Before the EBUS procedure, target sites were identified via PET or CT scans. The EBUS bronchoscope used was a Pentax EB-1970UK. The patient was intubated and the scope positioned at a target site. Most samples were obtained using a Cook (Cook Ireland Ltd, Limerick, Ireland) 22G standard needle but 25G standard needles were also used as well as Procore versions of both these gauges. The needle was advanced out of the protective sheath, the stylet removed, suction applied and the target punctured 10-20 times. The needle was returned to the protective sheath and removed from the scope.

A formalin pot and universal container with 5ml of sterile saline were labelled with the patient details. Three standard slides were labelled with the patient's surname, the target site and pass number.

Aspirated material was expelled into a Petri dish using the stylet followed by air from a 10ml syringe. The lid of the Petri dish was held over the base to protect the BMS from splashing and aerosol generation.

A pipette was used to transfer a drop of the liquid material onto two of the labelled slides. The third slide was used to spread the material on the slides. One was fixed with 99% alcohol for later Papanicolaou (Pap) staining. The spreader slide was air-dried for later staining with May-Grunwald Giemsa (MGG). Both the Pap and MGG slides were stained on return to the laboratory on a Thermo Gemini AS stainer.

The other prepared slide, used for ROSE, was air-dried and stained on site using a rapid Romanowsky stain (TCS Biosciences, Milton Keynes, UK). The rapid Romanowsky stain is a kit containing 3 solutions. Solution A is a fixative and the slide was fixed for 30 seconds. The slide was then dipped 3 times into each of solution B and C successively. Any excess stain was washed off with Sorenson's buffer (pH6.8).

The ROSE slide was examined on a microscope by a cytopathologist (AM) and a judgment made as to whether the material on the slide was adequate and whether it contained a malignant cell population. The material was categorised as

- Insufficient
- Adequate and benign
- Equivocal, probably benign
- Suggestive of malignancy
- Malignant

Additional detail (eg tumour type) was also recorded as well as these broad categories.

Immediately after preparing the slides, any solid material was transferred with forceps into a formalin pot for subsequent paraffin embedding, facilitating further investigations including immunocytochemistry and molecular testing. Any remaining sample in the Petri dish was transferred into a universal container using a pipette. To ensure that a maximal amount of residual material was recovered, it was necessary to pipette saline into the Petri dish and reaspirate for transfer to the universal.

All the above steps are illustrated in Figure 1.

The material in saline was made into a cell block. The specimen was transferred into a centrifuge tube and spun at 1500rpm for 5 minutes in a Sorvall ST16 centrifuge which is calibrated according to UKAS ISO15189. The supernatant was decanted back into the original container leaving only a cell pellet. 2 drops of plasma (National Health Service Blood and Transplant service) and 4 drops of thrombin (Werfen UK, Warrington, UK) were added to the cell pellet to form a clot. The clot was then transferred to a second labelled formalin pot for processing and paraffin embedding.

For each target site, fresh consumables were used to avoid any cross contamination from different target sites.

<u>Weight of samples and ROSE material</u>

The above standard method was modified slightly to accommodate weighing of samples. The balance used was an Avery Berkel FA64, which is capable of weighing to subdivisions of 0.0001g. The balance was calibrated before use.

At the beginning of the procedure for a particular site, a clean, dry Petri dish and the two labelled slides which were to receive a drop of aspirated material were weighed.

These parameters were recorded on a proforma together with patient details, the date and personnel involved. A dedicated member of staff was used for the recording of the weights to allow the BMS and pathologist to attend to their roles.

After the material from the first pass was expelled into the Petri dish, it was immediately weighed and the weight recorded, then passed back to the BMS. A drop was then placed on each of the two labelled slides and their weight recorded. The slides were passed back to the BMS, who spread the material with the third slide. This is illustrated in Figure 2.

From these measurements, the weight of the whole aspirate and the weight of the material used to make direct slides, one of which is used for ROSE, can be calculated. The remainder of the material was appropriately distributed between formalin and saline for later cell block preparation.

For the weight of the next pass, the Petri dish, with as little residual material as possible, was placed on the balance and the balance doors shut. This is because, with such a sensitive balance, the weight of the Petri dish drops significantly over a short period of time as the saline evaporates and thus, to obtain an accurate weight of the dish before the next sample, the weight should be recorded immediately before the sample is expelled into it.

If stained slides were to be made from the next pass, the slides were labelled and the two that would have material placed on them were weighed.

Once malignant cells were identified, no further slides were made but material continued to be harvested and weighed as above.

The recorded weights from the printed proforma for each site were entered into an Excel spreadsheet (Microsoft Corporation, Redmond, USA) containing formulae which calculated the weight of aspirate and slide material per pass as well as percentage of material per pass and per target used on slides.

Statistical analysis was undertaken using SPSS, version 26 (IBM Corporation, Armonk, New York, USA)

Results

Number of targets, passes and exclusions

53 target sites from 31 procedures were initially sampled, yielding 244 passes, of which 154 were assessed with ROSE. 22 passes from 18 target sites had anomalies in the data collection.

In 16 passes, either the “after” weight was less than the “before” weight for an aspirate or the initial weight of the Petri dish was greater than at least one “before” weight for a subsequent aspirate (the dry weight of the Petri dish must be its minimum weight for the target) or, in one pass, the slide weight was greater than the weight for the pass. In one further pass, all three slides were weighed (including the spreader) rather than the two destined to have material placed on them.

In 6 passes, either the “before” or “after” weight (or, occasionally, both) was not noted down (omission errors).

Although the measurements for the majority of passes from these 18 target sites appeared individually safe, a single unreliable pass weight from a target means that the total weight and the proportion used for slides cannot be calculated. Since this was the primary outcome for the study, it was decided to exclude from analysis all of the data from these targets. A table summarising data for all initial 53 targets is available as supporting information.

There are three possible reasons for these anomalies in weight measurement. Firstly, the balance is very sensitive and has three sliding doors, two on each side and one on top, to shield the sample from draughts. If any one of these doors is not completely closed, there is the possibility of an unreliable weight.

Secondly, once dilute liquid (substantially saline) material is present in the Petri dish, there is a substantial risk of loss of weight due to evaporation. This was tested by mixing 2ml of saline with a proteinaceous wetting agent and recording the weight with the Petri dish lid alternatively on and off for two-minute cycles, thus showing that it is possible to lose 200mg to evaporation in approximately 25 minutes (Figure 3). Thus, any time gap between the weighing of the Petri dish (for the “before” weight) and the arrival of the aspirate will affect the calculated weight of the aspirate. This is illustrated schematically in Figure 4.

Thirdly, there may have been simple transcription errors.

Since each pass and slide weight requires two measurements (before and after), the total number of measurements needed for the pass weights is 488 (2 x 244) and the total number of measurements needed for the slide weights is 308 (2 x 154). The error rate in measurement is 2.0% (16/796) for weight errors and 0.8% (6/796) for omission errors.

The remaining data thus comprises **144** individual passes (of which **93** had ROSE performed) from **35** targets in **21** procedures.

Demographics, targets and outcome for ROSE

The age and sex distribution is shown in Table 1 and targeted sites in Table 2.

The outcome for ROSE is shown in Table 3. Note that “NSCC” (non-small cell carcinoma) is used purely as a descriptive term at ROSE and does not imply lung origin and can therefore include metastatic breast carcinoma, for example. There were four cases that were insufficient on ROSE. One of these showed a few fragments of thyroid gland on cell block and was deemed to represent upper mediastinal thyroid in a 2R position. The other three showed scanty lymphocytes only on ROSE and all showed benign lymphoid fragments on cell block. One further

case showed mildly atypical epithelioid cells on ROSE favoured for reactive macrophages and the cell block confirmed a benign population.

There was one discrepant target showing a benign lymphoid population on ROSE with the cell block revealing occasional fragments of adenocarcinoma. This was an 11R node in a procedure in which ROSE correctly predicted malignancy in a 4R node and the primary right upper lobe lung lesion.

For this dataset, assuming “equivocal, probably benign” is categorised as benign, the sensitivity for ROSE is 93.8%, the specificity 100%, the positive predictive value (PPV) 100% and the negative predictive value (NPV) 93.8%. The accuracy is 96.8%

Molecular analysis

Eight patients (nine targets) with a final diagnosis of adenocarcinoma or non-small cell carcinoma, favour adenocarcinoma were eligible for molecular testing.

Two patients were not tested; one because the procedure was undertaken for the possibility of recurrence and had a known molecular status (and had not had therapy with tyrosine kinase inhibitors) and one because the patient was deemed palliative shortly after the procedure.

Four patients had a successful analysis (three EGFR wild type, one L858R mutation) on four of the 35 targets with a complete weight profile.

Two patients (three targets) had successful analysis (EGFR wild type) on a target taken contemporaneously at the same procedure but with an incomplete weight profile and therefore not part of the final 35 target dataset, but with material acquired using the described ROSE technique.

Weight of aspirates

The overall distribution of aspirate weights is shown in Figure 5. The mean is 0.1288g (128.8mg) and the median is 0.1390g (139mg). 98.6% of aspirates weighed less than 0.25g (250mg). There is a suggestion of some clustering at around 0.18g (180mg). Multivariate analysis[23] was performed to ascertain if this correlated with initial ROSE assessment (insufficient, benign or malignant – note that 3 passes with ROSE assessments of equivocal ?benign or suggestive of malignancy were excluded as they formed groups too small for analysis), needle type and gauge (standard or Procore, 22 or 25) and pass number.

The outcome is shown in Table 4 and demonstrates that the mean weight of aspirates deemed insufficient on ROSE is greater than the mean of aspirates assessed as benign or malignant ($p=0.003$). In addition, the mean weight of aspirates using a 22G needle is greater than those using a 25G needle ($p=0.003$). There was insufficient evidence for a significant difference in mean weights for

aspirates performed with standard or ProCore needles and a plot of the weights against pass number (1 to 7) showed no evidence of a relationship (data not shown)

Percentage of aspirate used for direct slides and ROSE by pass and by target

The distribution of percentages used for direct slides by pass in 93 aspirates is shown in Figure 6. The mean is 5.9% and the median is 3.3%. In 95.7% of passes, 20% or less of the material was used and in 87.1% of passes, 10% or less was used.

The distribution of percentages used for direct slides by target in 35 targets is shown in Figure 7, broken down by overall ROSE assessment for the target (non-malignant and malignant). The mean percentage used for malignant cases is 1.9% (median 1.2%) and the mean used in non-malignant cases is 3.6% (median 2.7%). This difference between malignant and non-malignant targets is significant with a p-value of 0.027.

Discussion

In the last decade, EBUS has revolutionised the investigation of lung cancer as it can both diagnose and stage the disease at the same procedure[1,2].The provision of a ROSE service allows for real-time sample triage into cell block, flow cytometry and microbiological investigation, in addition to direct slides. It also enables node harvesting (for accurate subtyping and potential molecular analysis[11,12]) to be concentrated on those nodes known to be positive for malignancy, thus reducing the total number of sites targeted[8-10,16]. Recent guidelines and meta-analyses state that, in EBUS-TBNA, ROSE should be offered, if available[4,14,15].

On the other hand, it has been argued that ROSE is labour-intensive, does not increase diagnostic yield and may reduce the amount of material available for mutational analysis[20,21,24]. This may occur, it is argued, if the procedure is terminated when diagnostic material is first seen but the objection has also been raised that material is “wasted” on slides that could have been usefully placed in cell block[14,22].

We agree that ROSE is labour intensive and that the balance of evidence is that it does not increase diagnostic yield, though would argue that it allows for control and triage of the specimen and that the reduction in sites targeted allows for a more efficient procedure. We also agree that terminating the procedure on first appearance of malignant diagnostic material may lead to suboptimal material for mutational analysis. Our practice, once ROSE has established malignancy in a site, is to cease making slides but to continue harvesting the site in order to maximise material for analysis.

As to the idea that material is wasted on slides; firstly, the current guidelines for molecular analysis in lung cancer now allow for mutational analysis on stained or unstained material scraped off microscopic slides[25] and secondly, it was our impression that the actual amount of material on slides was, in any case, probably small. This is what led us to the current study.

As far as we aware, this is the first attempt to quantify the amount of material used for ROSE, though previous studies have weighed Fine Needle Aspirate (FNA) specimens. One weighed the amount of material recovered in a hand-held, suction-assisted FNA of animal liver[26] and found, using a standardised technique of 10 traverses of the needle, each of 1.5cm, that the mean weight of material recovered by “old residents” was 11.6mg (0.0116g), two other groups of clinicians having lower, but statistically insignificant values . The method involved blocking the needle hub with gauze to prevent material being lost in the syringe and the needles were weighed before and after aspiration.

A more recent study using material from EBUS procedures[27] weighed the samples from 19G and 22G needles and found a mean weight per pass of 10.2mg (0.0102g) in the 22G group and 20.0mg (0.02g) in the 19G group. The nodes were sampled with 3 to 5 passes, each with 10-15 needle thrusts. It was not stated whether suction was applied. The study was primarily concerned with investigating the amount of diagnostic material removed using the two different needle gauges. The samples were weighed in formalin pots when sampling for the site was finished and the average weight per pass calculated. A correction was subsequently made for blood content (using a semi-quantitative scale) and the cell content measured on microscope slides.

A previous study by the same group[28], primarily to assess methods of mutational analysis, also weighed EBUS samples, which were obtained with a 22G needle, without suction, using 2 or more passes. The samples were split; formalin-fixed for pathological examination and, for mutational analysis, freshly frozen in 1ml of saline, then stored at -80^{0}C. These latter samples were weighed and had an average weight of 0.048g (range 0.012 – 0.230g).

In terms of the sample weights, our study has shown much higher values than either of first two studies, though closer to the third. This may be because our clinicians appear to use many more needle thrusts or agitations per pass, frequently more than 20, and always use suction. The needle gauges are similar as the most frequent size used in our practice is 22G, whether standard or ProCore.

Multivariate analysis of the distribution of the weights revealed the mean weight of aspirates deemed insufficient on ROSE was significantly greater than the mean weights of aspirates deemed either benign or malignant. This is most likely to be due to a bloody tap, in which the needle fills with blood and contains no diagnostic material, and the weight simply reflects the weight of that volume of blood. The weight of aspirates performed with a 22G needle is also significantly more than those performed with a 25G needle.

However, the key finding from our study is that, in our institution, a small amount of material per target is used for direct slides and ROSE and that significantly less is used in malignant cases (malignant: mean 1.9%, benign: mean 3.6%) as a result of

our practice of ceasing ROSE when malignancy is established but continuing to harvest material. Thus, in malignant cases, a mean of 98.1% of the material is processed to cell block.

The sample that is expelled from the needle in an EBUS or EUS procedure is a mixture of solid material, which can be picked up with forceps, and bloody material that we recover with saline and a pipette. This is true for this kind of target (lymph nodes or malignant lung masses) sampled by multiple needle traverses whatever the needle size or type and there is no threshold which differentiates a “cytological” from a “histological” sample.

Many centres, even if they do not offer ROSE, treat these two fractions (solid and bloody material) differently (personal communications) and most would agree that the solid fraction is the mainstay of subsequent immunocytochemistry and/or mutational analysis. In general, when the bloody fraction is cell blocked, it often appears poorly cellular, yet this is the fraction that provides the material for ROSE – as stated, we place all solid material directly into formalin.

Our view is that the reason that the bloody fraction is informative for ROSE and has a high concordance with the final diagnosis whereas a cell block prepared from the same material is often paucicellular is that a histological section of the cell block material is looking at a small fraction of the material whereas a drop of the same material on a slide is spread such that it is all available for examination. It is clear that this fraction contains material composed of small groups of cells and single cells which cannot be separated with forceps and the naked eye but are nonetheless diagnostic.

We intended to attempt to quantify the amount used for direct slides as a proportion of these fractions and, particularly, the bloody component, in addition to the proportion of the whole amount. However, it became apparent that the amount lost to evaporation precluded this. This, therefore, raises the issue of whether the weights of the Petri dish and slides, before and after sampling, are reliable.

Both Petri dish and slides were weighed within seconds of the samples being place in or on them respectively as the balance was within 70cm of the preparation area. Therefore, apart from possible transcription errors, the “after” weights can be assumed to be accurate. The weight of the slides before the sample is placed on them is stable, the slides being dry.

The weight of the dry Petri dish at the beginning of the procedure is stable but, during the procedure, the “before” weight for each pass will involve weighing a Petri dish that has contained some saline (though with as much as possible removed by pipette) and will be subject to evaporation. This was appreciated early in the study and we attempted to control for this by keeping the dish inside the balance and noting the final weight just before the next sample arrived at the preparation area.

However, it is worth examining what the outcome would be if weighing took place some time before the sample and significant evaporation occurred before the next sample were placed in the dish (as shown in figure 4). This would lead to the recording of an erroneously high “before” weight. In extreme cases, with a very scanty aspirate, it may be that the weight of the aspirate does not make up the weight lost since the “before” measurement and thus leads to a negative weight for the aspirate. As stated above, a few of our passes may have been affected in this way and the data for these targets was excluded.

It could be argued that this issue is still a risk, even in those cases with positive weights. However, this would have led to an erroneously low sample weight and, therefore, an incorrectly high percentage used for slides. Thus, if this error occurred significantly, the percentages used for ROSE would be even lower than we found.

Conclusion

We have shown that, in cases of suspected thoracic malignancy in our institution, the mean overall percentage of the target material aspirated at EBUS and EUS that is used to make direct slides from which ROSE is performed is 1.9% in malignant cases and 3.6% in non-malignant cases, a difference that is statistically significant. Nonetheless, ROSE correctly predicted the final outcome (in this dataset) with an accuracy of 96.8% despite using the non-solid fraction of the aspirate that is frequently collected but rarely used for molecular analysis and that this material is not, therefore, wasted when used for ROSE.

We have also shown that the weight of material aspirated at EBUS or EUS in our institution has a mean of 0.1288g and that 98.6% weighed less than 0.25g. The weight of aspirates judged to be insufficient on ROSE is significantly higher than for cases judged benign or malignant, presumably because these represent aspirates composed of blood only.

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Table 1 Age and sex distribution for final dataset

Age range	Male	Female	Total
51-60	1	2	3
61-70	5	3	8
71-80	2	5	7
>80	3	0	3
Total	**11**	**10**	**21**

Table 2 Targets in final dataset

Target/site	
Lymph nodes	
Station 7	8
Station 4R	8
Station 11L	4
Station 11R	5
Station 11Ri	1
Station 11Rs	2
Station 2R	3
Lung	
Right upper lobe	1
Left adrenal	
	3
Total	**35**

Table 3 Outcomes for ROSE

	Final outcome for target/site									
	Benign		**Malignant**							
ROSE	?Ectopic thyroid	Benign NOS	Adenoca	NSCLC, favour adenoca	SCC	NSCLC NOS	SCLC	Metastatic breast carcinoma	DLBCL	**Total**
Insufficient - Scanty lymphocytes only		3								**3**
Blood and macrophages only	1									**1**
Benign NOS		14	1							**15**
Equivocal, probably benign		1								**1**
Malignant ?SCLC ?lymphoma									1	**1**
Adenoca			1							**1**
NSCC, favour adenoca				1						**1**
NSCC			4	2	2			1		**9**
SCLC							3			**3**
Grand Total	**1**	**18**	**6**	**3**	**2**		**3**	**1**	**1**	**35**

Abbreviations: NOS, not otherwise specified; Adenoca, adenocarcinoma; NSCLC, non-small cell lung carcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung carcinoma; DLBCL, diffuse large B-cell lymphoma; NSCC, non-small cell carcinoma (morphological assessment at ROSE ie not necessarily of lung origin).

Table 4

	Number of aspirates	Mean weights (mg)	95% confidence interval (mg)	Difference assessed	Method of assessment	p-value	95% confidence interval for difference in means (mg)
ROSE							
Benign	42	117.1	97.0-137.2	Benign v malignant	Pooled variances t-test	0.865	-33.2-39.4
Malignant	20	114.0	80.7-147.4	Benign v insufficient	Bootstrapping (10,000 samples)	**<0.001**	33.3-83.0
Insufficient	28	175.7	159.2-192.1	Malignant v insufficient	Bootstrapping (10,000 samples)	**0.003**	27.0-97.0
Needle size							
22G	132	132.6	121.3-143.9	22G v 25G	Separate variances t-test	**0.003**	18.2-72.9
25G	12	87.1	61.5-112.6				
Needle type							
Standard	97	133.4	120.0-146.8	Standard v Procore	Pooled variances t-test	0.227	-36.9-8.8
Procore	47	119.4	101.1-137.6				

Legends for Figures

Figure 1 (A) Aspirated material expressed into Petri dish. (B) Single drop of aspirated material placed on each of two slides – one will be stained for ROSE. (C) Solid material placed directly into formalin. (D) Remainder of material transferred to saline.

Figure 2 (A) Weight of Petri dish before aspirate. (B) Weight of Petri dish with aspirate. (C) Weight of slides before aspirate. (D) Weight of slides, each with one drop of aspirated material.

Figure 3 Effect of evaporation on idealised “sample” (2ml of saline mixed with proteinaceous material) and simulated process. Petri dish weighed every two minutes with lid alternatively on and off.

Figure 4 Schematic showing effect of evaporation between passes with potential for inaccurate sample weights.

Key:	
	Correct overall pass weight
	Weight of material used for ROSE
	Weight of material processed to cell block
	Saline/blood lost to evaporation between passes
	Incorrectly low overall pass weight due to incorrectly high “before” weight
	Apparently negative overall pass weight due to incorrectly high “before” weight and scanty aspirate

Figure 5 Distribution of weights of 144 EBUS passes. Frequency and cumulative percentage.

Figure 6 Percentage of aspirate per pass used for ROSE in 93 passes. Frequency and cumulative percentage.

Figure 7 Percentage of aspirate by target used for ROSE in 35 targets.

Figure 1

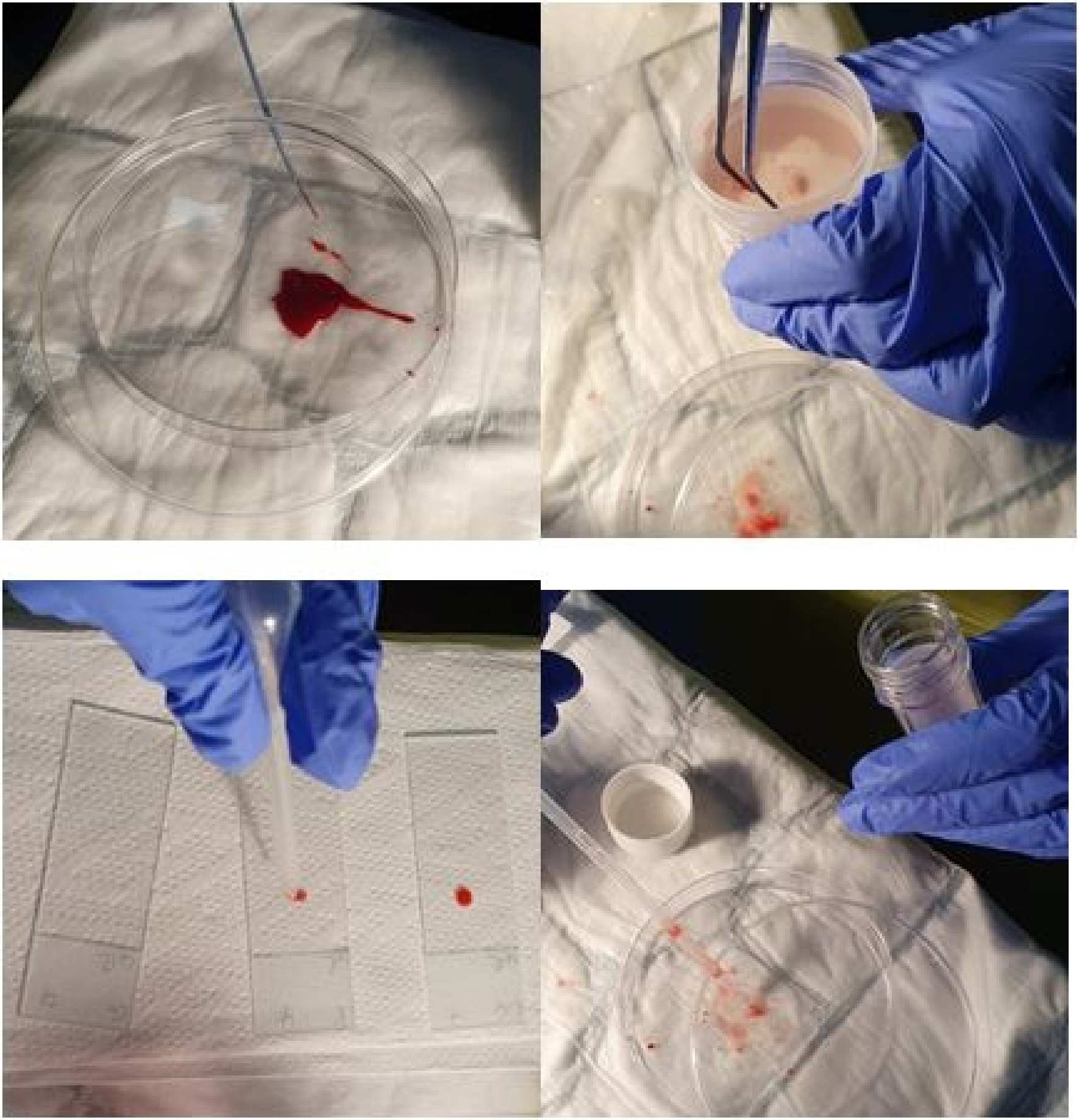
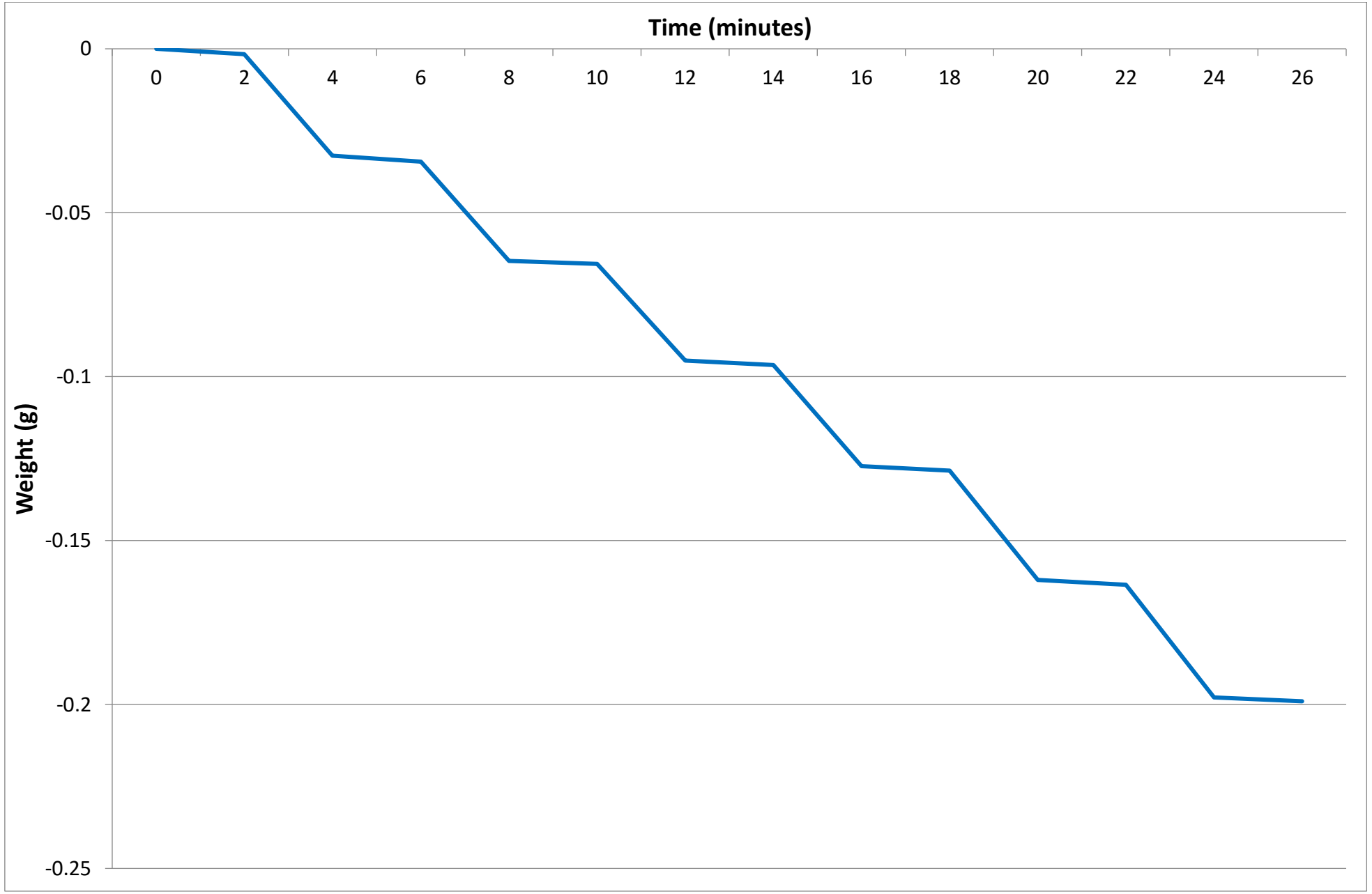
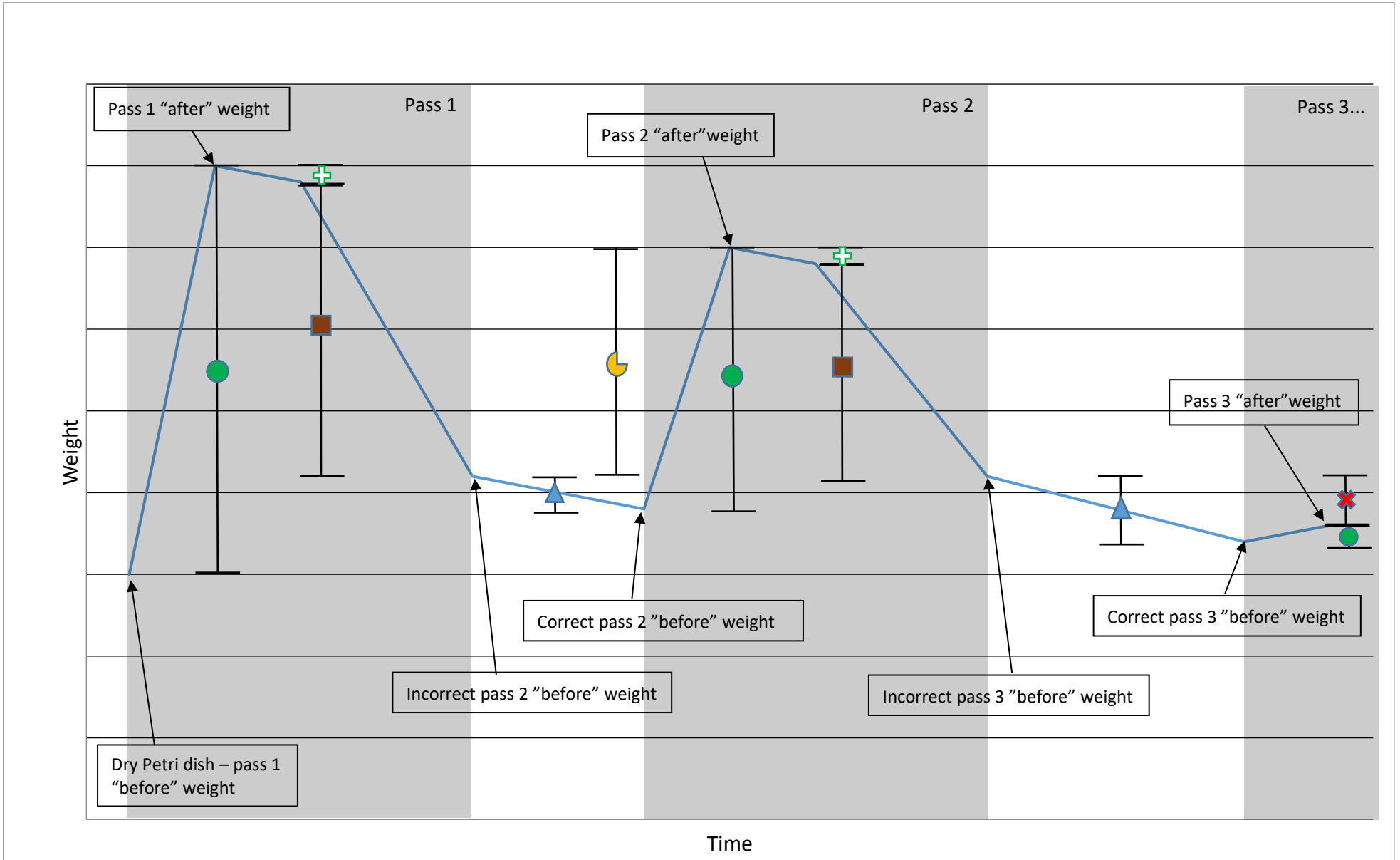
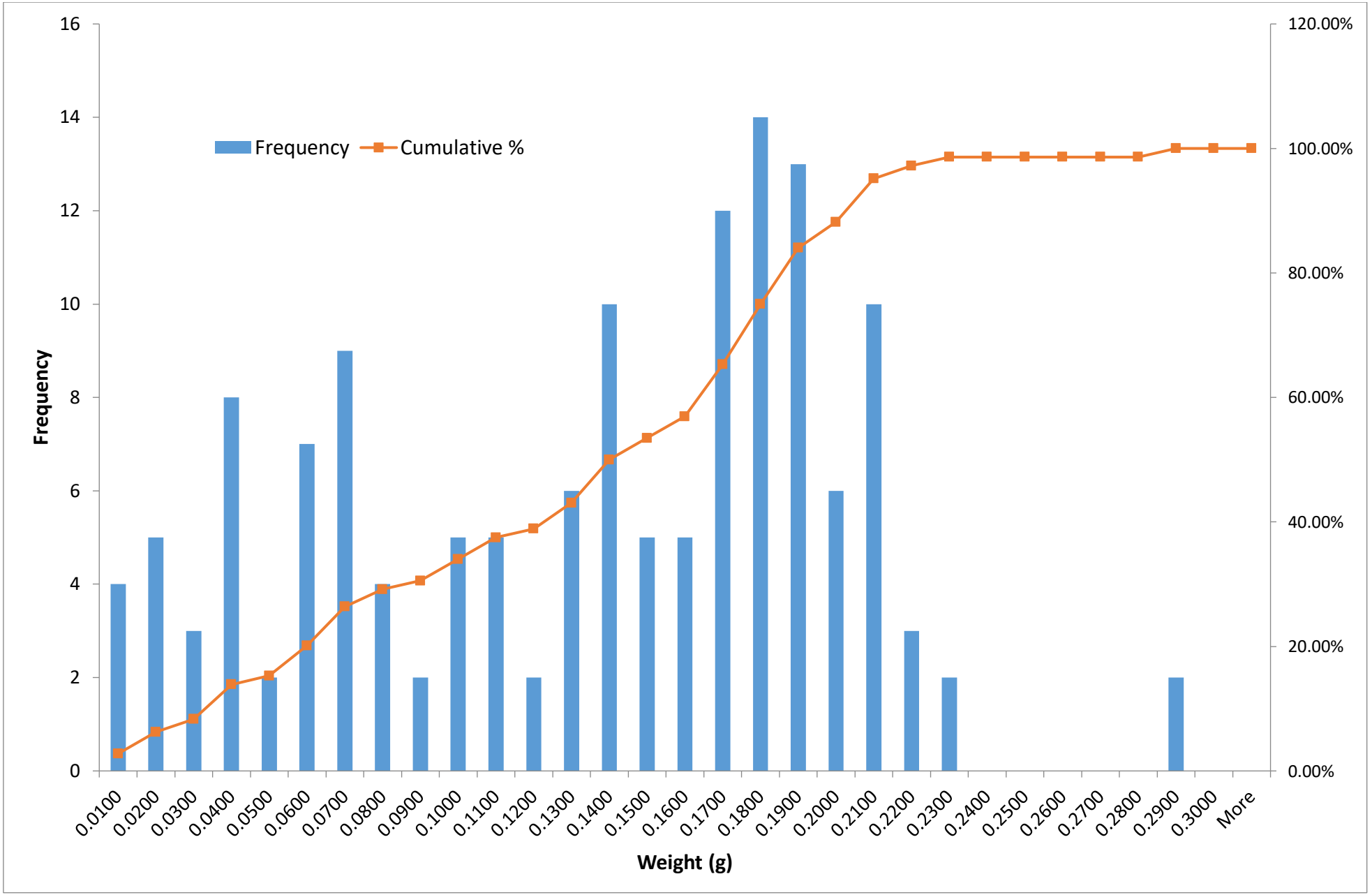
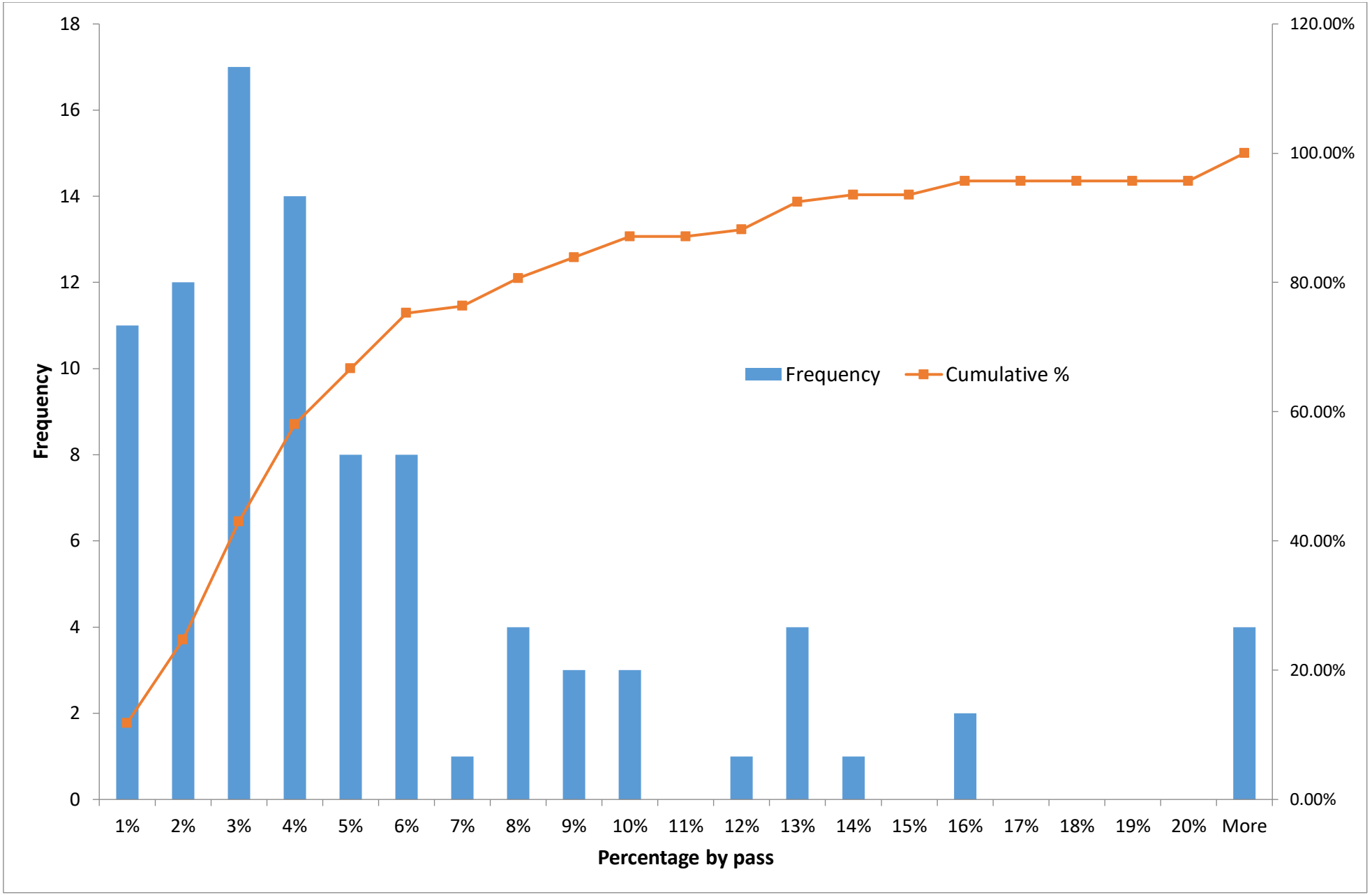


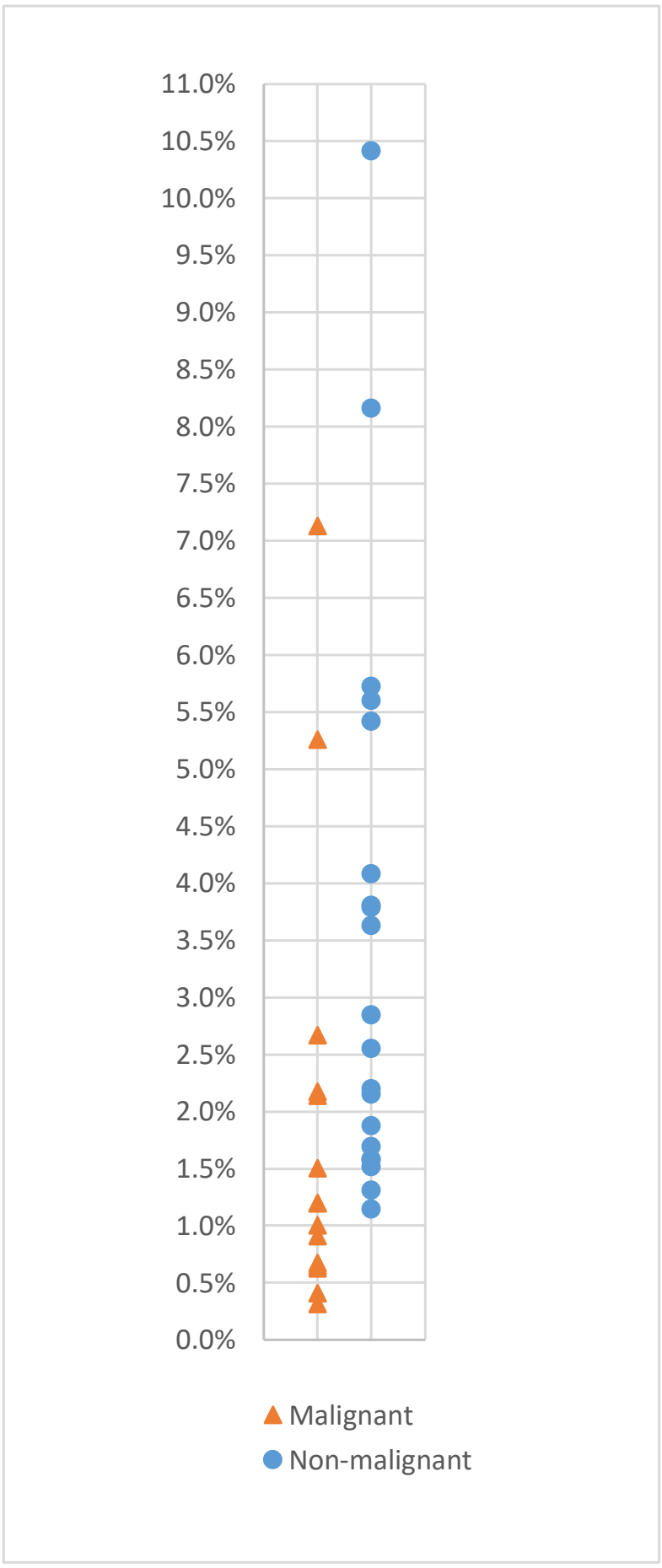
Figure 2


Time (minutes)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
0
-0.05
-0.1
-0.15
-0.2
-0.25
Weight (g)


Pass 1 “after” weight
Pass 1
Pass 2
Pass 3...
Pass 2 “after”weight
Pass 3 “after”weight
Weight
Correct pass 2 ”before” weight
Correct pass 3 ”before” weight
Incorrect pass 2 ”before” weight
Incorrect pass 3 ”before” weight
Dry Petri dish – pass 1
“before” weight
Time


Frequency
Cumulative %
16
14
12
10
8
6
4
2
0
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
Frequency
0.0100
0.0200
0.0300
0.0400
0.0500
0.0600
0.0700
0.0800
0.0900
0.1000
0.1100
0.1200
0.1300
0.1400
0.1500
0.1600
0.1700
0.1800
0.1900
0.2000
0.2100
0.2200
0.2300
0.2400
0.2500
0.2600
0.2700
0.2800
0.2900
0.3000
More
Weight (g)


18
16
14
12
10
8
6
4
2
0
Frequency
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
Frequency
Cumulative %
1%
2%
3%
4%
5%
6%
7%
8%
9%
10%
11%
12%
13%
14%
15%
16%
17%
18%
19%
20%
More
Percentage by pass


11.0%
10.5%
10.0%
9.5%
9.0%
8.5%
8.0%
7.5%
7.0%
6.5%
6.0%
5.5%
5.0%
4.5%
4.0%
3.5%
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
Malignant
Non-malignant